IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

Amber H. Koiri **.... Petitioner**

Versus

State of Maharashtra & Ors. **...Respondents**

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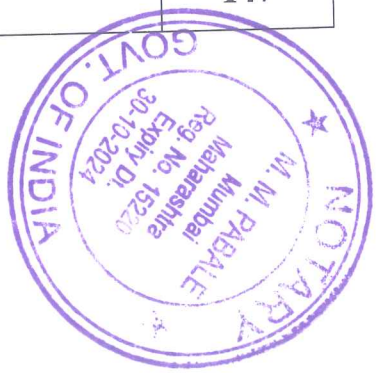

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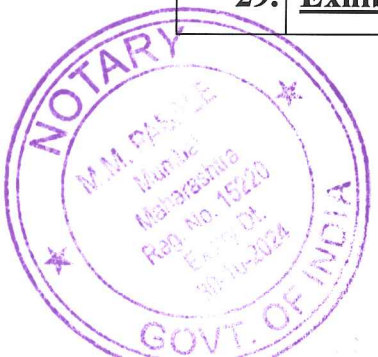

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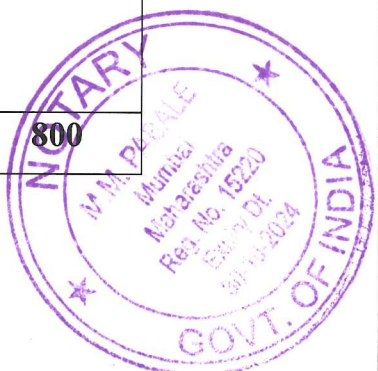

	A copy of article published in 'Hindustan Times' titled as "As cases drop, number of covid tests halved in Mumbai" dated 18 May, 2021	
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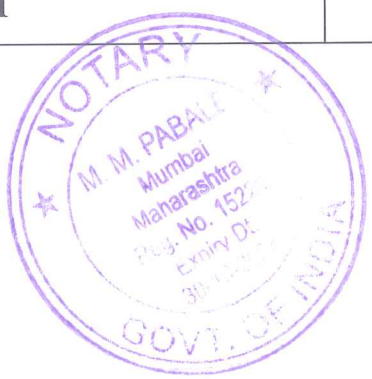

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	A copy of article published in **'india.com'** titled as **"Maharashtra Lockdown Big Update: Deputy CM Warns of Shutting Down Everything if Covid Third Wave Hits"** dated **3 September, 2021**	
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A

IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

Amber H. Koiri **.... Petitioner**

Versus

State of Maharashtra & Ors. **...Respondents**

Advocate for the Petitioner

Office Notes: Memorandum of Coram. Appearance, Court's Orders of direction and Prothonotary's orders	Court's or Judge's Orders

NOTARY
M. M. PABA
Mumbai
Maharashtra
Reg. No. 152
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B

Office Notes: Memorandum of Coram. Appearance, Court's Orders of direction and Prothonotary's orders	Court's or Judge's Orders

C

Office Notes: Memorandum of Coram. Appearance, Court's Orders of direction and Prothonotary's orders	Court's or Judge's Orders

I

IN THE HIGH COURT OF JUDICATURE AT BOMBAY
ORDINARY ORIGINAL CIVIL JURISDICTION
WRIT PETITION NO. OF 2021

Amber H. Koiri **.... Petitioner**

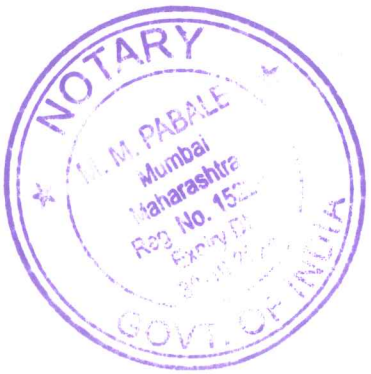
Versus

State of Maharashtra & Ors. **...Respondents**

SYNOPSIS

The Petitioner has filed the present writ petition invoking extra ordinary jurisdiction of this Hon'ble Court under Article 226 and 227 of the Constitution of India seeking direction to Respondent Maharashtra Government & Municipal bodies to stop testing asymptomatic healthy people in malls, railway stations, bus stands, markets, etc.

Sr No	Date	Events
1		Despite suggestions by experts and directions by the Central Government authorities, the State of Maharashtra is unnecessarily doing the Covid-19 testing of the asymptomatic & healthy people.
2	27.11.2021	The orders passed by the State authorities are arbitrary, unlawful and actuated with malafides and ulterior purposes.



II

3	27.11.2021	The decisions of State Authority is giving wrongful profit of thousands of crores to the private companies who are manufacturing the testing kits and it is an offence of misappropriation of public money and property, punishable under **Section 409, 52, 120(B), 34, 109 etc. of IPC.**
4	04.10.2021	The corruptions & frauds of state authorities is already exposed by Shri. Kirit Somaiya of BJP.
5	27.11.2021	The mandates by the state are unconstitutional violating fundamental rights of the citizen and therefore liable to be quashed.
6	09.05.2011	Hon'ble Supreme Court in **Noida Entrepreneurs Assn. vs. Noida, (2011) 6 SCC 508** has ruled that in such cases the investigation through CBI be ordered.
7	08.02.2022	Hence this petition.

CASE LAW TO BE REFFERED :-

As mentioned in the petition and other applicable authorities pertaining to the subject matter of the present petition.

ACTS TO BE REFFERED :-

1. CONSTITUTION OF INDIA, 1950

NOTARY M.M. PABALE Mumbai Maharashtra Regn. No. 15220 Expiry Dt. 30-6-2024 GOVT. OF INDIA

Date : **08.01.2022**

ADVOCATE FOR PETITONER

PETITIONER

IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

IN THE MATTER OF ARTICLE 226 AND 227 OF THE CONSTITUTION OF INDIA;

AMBAR H. KOIRI]

]

]

]...**PETITIONER**

Versus

1. State of Maharashtra]

Through Chief Secretary & Chairman]

State Disaster Management Committee]

The Government of Maharashtra,]

Mantralaya, Mumbai- 400 023]

2. Shri. Iqbal Chahal]

The Muncipal Commissioner,]

M.C.G.M. Annex Building,]

Mahapalika Marg,]

C.S.T, Mumbai, 400001.]

3. Suresh Kakani]

Addl. Municipal Commissioner]

Mahapalika Marg,]

C.S.T, Mumbai, 400001.]

4. Ministry of Health And Family]

Through Chief Secretary]

Government of India]

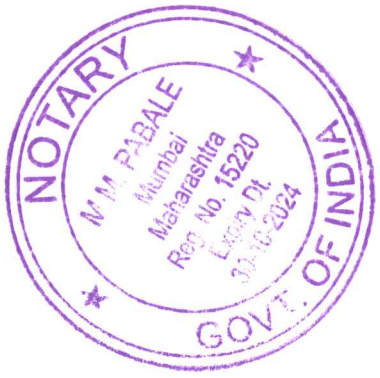
5. National Disaster Management Authority,]

Through its Chairperson,]

Safdarjung Enclave, NDMA Bhawan,]

A-1, Block A-1, Nauroji Nagar,]

New Delhi, Delhi - 110029.]



6. State Disaster Management Authority,]

Through it's chairperson,]

Revenue and Forest Department,]

Maharashtra State Disaster Management Authority,]

Mantralaya, Mumbai - 400032.]

7. Shri Manish Joshi]

Dy. Commissioner, Health]

New Administrative Building, Chandan Wadi,]

Pachpakhadi, Mahapalika Bhavan Rd,]

Thane West, Thane, Maharashtra 400602] ....**Respondents**

TO

THE HON'BLE CHIEF JUSTICE AND OTHER PUISNE JUDGES OF THIS HON'BLE COURT OF JUDICATURE AT BOMBAY

NOTARY M. M. PABALE Mumbai Maharashtra Reg. No. 1522 Expiry Dt GOVT. OF INDIA

THE HUMBLE PETITION OF THE PETITIONER ABOVENAMED

MOST RESPECTFULLY SHEWETH:

1. The Petitioner has filed the present writ petition invoking extra ordinary jurisdiction of this Hon'ble Court under Article 226 and 227 of the Constitution of India seeking direction to Respondent Maharashtra Government & Municipal bodies to stop testing asymptomatic healthy people in malls, railway stations, bus stands, markets, etc. as done earlier in the second wave. Petitioner is further seeking direction to the officer of Respondent state government to not to do contact tracing of asymptomatic contacts of a symptomatic case and further direction to concerned authorities to only test symptomatics, and in those with covid symptoms, an RAT test or an RT-PCR test should be conducted, but the RT-PCR cycle threshold should be reduced to 24. No retest should be conducted with a PCR, if someone tests negative with the RAT. Petitioner is also seeking direction to all private bodies, including societies, companies, schools, universities, banks etc & all government officials/bodies to not mandatorily demand negative test reports from asymptomatic healthy people.

2. Respondent No.1 is state of Maharashtra. The Respondent No. 2 is Municipal Corporation of Greater Mumbai. The Respondent No. 3 is Ministry Of Health and Family.

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3. The petitioner by way of this petition challenging the guidelines issued by BMC Respondent **No. 2** dated **27th November, 2021** and Thane Municipal Corporation Respondent **No.7** dated 6th January, 2022 about mandatory RT PCR or RAT test on people including Asymptomatic healthy person.

(A copy of **Guidelines issued by Brihanmumbai Municipal Corporation BMC & Thane Municipal Corporation, TMC dated 27.11.2021 & 6th January, 2022** marked and annexed herewith respectively **Exhibit "EEEE" "FFFF")**

4. The said guidelines is unconstitutional, illogical, unlawful, and violated the fundamental right of the petitioner and other citizen to the extent of mandating the Covid test on Asymptomatic healthy person since as per the medical experts and research it is found that "People Without Covid-19 symptoms Do Not Need Testing".

The petitioner being aggrieved by the said guidelines, approach this Hon'ble Court on the basis of following facts and Myths of Asymptomatic Transmission & Unscientific Use of RT-PCR/RAT Tests which are as follows;

SUMMARY OF DATA RELIED BY PETITIONER

NOTARY
M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
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5. The Expert Testimonies on Asymptomatic Transmission.

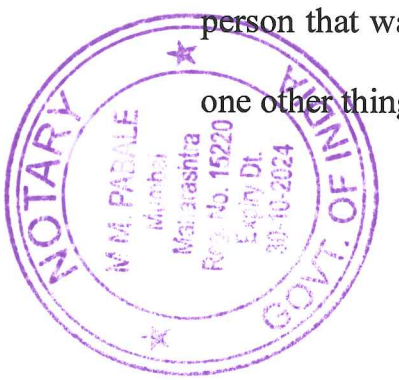
5.1. As Dr Anthony Fauci of the US National Institute of Allergy and Infectious Diseases stated in March 2020:

> "*In all the history of respiratory-borne viruses of any type, asymptomatic transmission has never been the driver of outbreaks. The driver of outbreaks is always a symptomatic person*".

Source: https://www.youtube.com/watch?v=vrAvjU2LBkg&t=2s

NOTARY M.M. PABALE Nagpur Maharashtra Regd. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

5.2. According to Dr Mike Yeadon, Ex Vice President of Pfizer: "In order to be a good source of infectious virus, say its me, I have to have a lot of virus in my airway. I can't infect you at a distance if I've got a tiny amount, that's because the worlds full of pathogens all the time, and you're able to fight them off routinely minute by minute, throughout your whole life. You have to have an amount- over an amount- such that it becomes an infective dose. That only happens when you're in close contact with someone that's emitting lots of virus, and in order to be emitting lots of virus, you have to have a lot of them in your body. If you have a lot of viruses in your body, you will have symptoms. Its simply not possible for you to have a high viral load, and for that virus to be attacking you when you have no symptoms; and for your immune system to be fighting back and protecting you, and for you to have no symptoms. Its simply not possible. There might be a brief period of a few hours, when the virus is growing quickly, the body is just starting to respond, you might not notice, you might not feel a 100 percent ideal yet, that's called pre-symptomatic and yeah, I guess it's possible that a few people will infect it that way. But the idea that transmission & a major contributor to epidemic spreading occurred in a person that was full of virus and had no symptoms - it's just bunk. & just one other thing that I think will chime with people, that we are very good at



noticing whether someone's health threat to us, usually a respiratory health threat to us. When you come up to a person, a relative, or a stranger without trying, you scan them, and you're aware if their gaze is normal, if their head is normal, are they looking at you clear eyed, or do they look hunched and a little bit ill, and without thinking about it, if you think they're ill, you'll skirt around them, in essence, socially distance unconsciously. & so the two things I've just said there, in order to be a good infectious source you have to be full of virus and you will be symptomatic. The only chance you will encounter someone like that in your community would be I think still averted because if you saw someone stumbling around full of flu or a cold you'd think "oh my god, Ive got to get around this person". So, it's my contention, that there was almost no transmission in the community because there weren't symptomatic people they would be feeling ill if they were in that situation so there are hardly any infectious contacts in the general community, and you know what, that explains why lockdowns systematically across the world have'nt done anything. & that's because the places where you do encounter symptomatic infectious people are where they have no choice to be there either hospitals, care homes or occasionally your own domestic environment. Everywhere else you simply wont find infectious sources so when you lock down and smash the economy and civil

society, of course you don't lower transmission - it wasn't taking place there obviously not. So that's where I'm at on asymptomatic transmission.

5.3. According to Professor Beda M Stadler (Emeritus Professor of Immunology) - If you say, somebody without symptoms can infect, that means this person must throw a whole bunch of viruses out when he speaks; in his droplets there must be a lot of load, viral load which must be significant, else you cannot. But how do you make these viruses? These Viruses are made by your body cells that have been destroyed. Then the virus comes out. Now if you do this, the body will immediately recognize this and immunologically react against it, and that's called an inflammation. If you have inflamed regions, there are 5 cardinal symptoms but its not only that you get fever and all this, No, its pain. So if somebody says, i have not felt anything, no scratch nothing, no symptoms but i have it, that is a ridiculous assumption. Imagine I could walk around and say i had aids for two weeks and i didn't have symptoms, and now im fine again. If you can all of a sudden have viral diseases without symptoms and transmit the disease constantly, this is a huge problem for our health system because any school pupil who doesn't wanna go to school he can tell the teacher look i have influenza for 3 weeks , no symptoms but i have influenza so i have to stay home. So its a

ridiculous assumption. And I am just amazed at how everybody is repeating it.

Source: https://www.bitchute.com/video/lIj22KttYq7z/

6. Studies on Asymptomatic Transmission;

6.1. The Petitioner states that there are many and various problems with the studies done to prove that asymptomatic transmission exists which we will highlight below, but the main reason that we cannot rely on these studies is that all of them use the PCR test to measure whether the infection is spreading or not, and we will prove below in this petition that the PCR test cannot be used to find infectious viruses in people. Despite using RT-PCR, many studies still show that asymptomatic transmission is rare & the studies are summarised below.

6.2. As far as the scientific literature goes, the evidence is clear: truly asymptomatic transmission (when separated from pre-symptomatic transmission) is very rare. This position is supported by a large study from the city in China where the SARS-CoV-2 outbreak originated. Published in Nature.

6.3. Communications on November 20, the study is titled "Post- lockdown SARS-CoV-2 nucleic acid screening in nearly ten million residents of Wuhan, China". Researchers in Wuhan did a city-wide screening between May 14 and June 1 using reverse transcription polymerase chain reaction (RT-PCR) assays to detect viral RNA fragments in residents. Among eligible residents, which was those aged six years or older, 92.9 percent participated, which amounted to 9,899,828 people. With this intensive screening program, there were positive test results for 300 individuals who were asymptomatic. Among these, 63 percent also tested positive for antibodies to SARS-CoV-2, offering additional evidence that they had indeed been infected. Nevertheless, contact tracing of 1,174 close contacts of asymptomatic individuals with evidence of infection revealed none who also tested positive. The researchers also tried to culture virus from asymptomatic individuals who tested positive, but the results indicated that there was "no 'viable virus' in positive cases detected in this study".

Consequently, despite testing positive for viral RNA, none of these individuals appeared capable of transmitting the virus to others. As the authors stated, "there was no evidence of transmission from asymptomatic positive persons to traced close contacts."

6.4. Following this study, An oped was published in the British Medical Journal titled : "**Evidence of asymptomatic spread is insufficient to justify mass testing for Covid-19**". It is published in full below;

"*Dear Editor,*

Whilst we would take issue with Lateral Flow tests being the main culprit, Mike Gill is absolutely correct to criticise mass testing programmes.

His ire should really be directed, though, at PCR testing. Data from PCR testing – for which there is no proper determination of an end-to-end operational false positive rate – has almost exclusively dictated tier restrictions and lockdown policy in the UK.

PCR's fingerprints can in fact be found all over the entire global response to this pandemic. Testing with Lateral Flow, other antigen tests and bedside PCR tests are all finding far fewer cases than diagnosed by PCR testing. Even a low sensitivity for all these other tests could not account for the size of the discrepancy.

Mass testing and accompanying harmful lockdown policies are justified on the assumption that asymptomatic transmission is a genuine risk. Given the harmful collateral effects of such policies, precautionary principle should result in a very high evidential bar for asymptomatic transmission being set. However, the only word which can be used to describe the quality of evidence for this is woeful.

It is important to carefully distinguish purely asymptomatic (individuals who never develop any symptoms) from pre-symptomatic transmission (where individuals do eventually develop symptoms). To the extent that the latter phenomenon - which has in fact happened only very rarely - is deemed worthy of public health action, appropriate strategies to manage it (in the absence of significant asymptomatic transmission) would be entirely different and much less disruptive than those actually adopted.

Many early studies which purported to demonstrate the phenomenon of asymptomatic transmission were from China, yet the fact that Chinese studies are only published following government approval must bring into question their reliability

(1). Nevertheless, the high volume of these studies spawned significant salience of the issue within the medical community, and an assumption of the likelihood of asymptomatic transmission being an important contributory factor. There then followed a number of meta-analyses examining the issue of asymptomatic transmission which tended to aggregate and give equal weight to studies regardless of origin or quality. In this way, these meta-analyses, given undue credibility by their association with reputable universities, amplified minimal evidence of asymptomatic spread to an importance the data did not warrant.

As reported in a manuscript submitted to this journal and also to medRvix on 16 Dec 2020 (the latter available for download shortly), we examined the papers most frequently cited in support of the existence of asymptomatic transmission. Even despite our criticisms of the sources of the data above, we did in fact find only 6 case reports of viral transmission by people who throughout remained asymptomatic, and this was to a total of 7 other individuals, however all of these were in studies with questionable methodology.

Moreover in all these studies, confirmation of "cases" was made via PCR testing without regard to the possibility that any of the cases found might be false positives. The case numbers found, are, in any event extremely small and certainly not sufficient to conclusively determine that asymptomatic transmission is a major component of spread.

It is also notable that, in what would seem to represent an abrupt volte face by the CCP, a further (presumably government-approved) study from China was recently published (2) which entirely contradicts the earlier conclusions regarding the phenomenon of asymptomatic transmission, which had been driven by Chinese data in particular, early in the pandemic.

Some might conclude that that study lacks the credibility one might expect for a paper published in Nature; it is claimed, for example, that they PCR-tested 92% of Wuhan's population (~10m individuals) over a 19-day period at the end of May, and found just 300 positive PCR tests, implying a FPR of no greater than 0.003%. Further, it is claimed that while 100% of the 300 PCR positive cases were asymptomatic, there were zero

symptomatic PCR positive cases out of ~10m tested during a period only a few weeks after the epidemic had peaked in Wuhan.

If this seems incredulous, then surely that has serious implications for the way in which earlier studies from China - data from which formed a significant part of the worldwide evidence base for asymptomatic transmission - should be regarded.

Jonathan Engler MBChB LLB

Source: **https://www.bmj.com/content/371/bmj.m4436/rr-10**

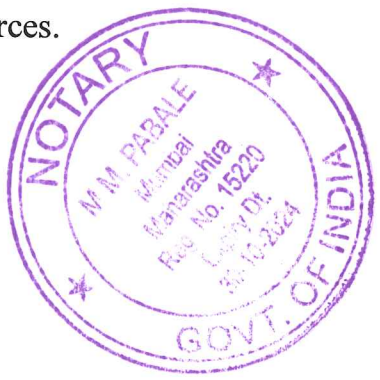
(A copy of an article was published in the British Medical Journal titled: **"Evidence of asymptomatic spread is insufficient to justify mass testing for Covid-19"**, dated 16 November, 2020 marked and annexed herewith **Exhibit "A")**

6.5. Three studies following up on 17, 91, and 455 close contacts of asymptomatic cases, respectively, found no evidence for asymptomatic transmission—an attack rate of "0%". A fourth study following up on 305

contacts of 8 asymptomatic cases identified one secondary case, for an attack rate of "0.3%". A fifth study following up on 119 contacts of 12 asymptomatic cases likewise identified one secondary case, for an attack rate of "0.8%". a sixth and seventh study respectively "indicated an asymptomatic secondary attack rate of 1% and 1.9%". An eighth followed up on 106 contacts of 3 asymptomatic cases and found 3 secondary cases, for an attack rate of "2.8%". The ninth and largest study followed up on 753 contacts of asymptomatic index cases and identified one secondary case, for a secondary attack rate of "0.13%".Together, the nine studies reported secondary attack rates of "zero to 2.8%", which compared with secondary attack rates for symptomatic cases of "0.7% to 16.2%", which suggests that people who are infected with SARS-CoV-2 but never develop COVID-19 "are responsible for fewer secondary infections than symptomatic and pre-symptomatic cases."

In other words, just because a person receives a positive RT-PCR test does not mean that they should be considered infectious, and pursuing policies based on the opposite assumption—as public health officials in India and other countries have been doing—is a waste of precious resources.

Sources:



https://www.nature.com/articles/s41467-020-19802-w

(A copy of an article was published in Nature Communication titled as **"Post-lockdown SARS-CoV-2 nucleic acid screening in nearly ten million residents of Wuhan, China",** dated 20 November, 2020 marked and annexed herewith **Exhibit "B"**)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7392450/

(A copy of study titled as "Coronavirus Disease Outbreak in Call Center, South Korea", **dated 26 August, 2020** marked and annexed herewith **Exhibit "C")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7195694/

(A copy of study titled as "Contact Tracing Assessment of COVID-19 Transmission Dynamics in Taiwan and Risk at Different Exposure Periods Before and After Symptom Onset", dated 1st May, 2020 marked and annexed herewith **Exhibit "D"**)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7219423/

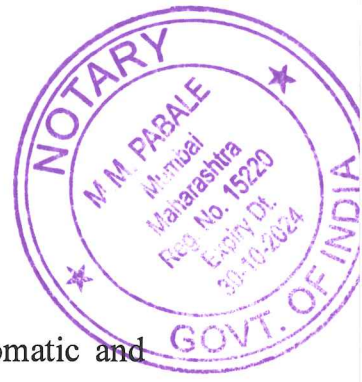
(A copy of study titled as "A study on infectivity of asymptomatic SARS - CoV-2 carriers", dated **13 May, 2020** marked and annexed herewith **Exhibit "E")**

https://papers.ssrn.com/sol3/papers.cfm?abstract_id=3566149

(A copy of study titled as "Modes of Contact and Risk of Transmission in COVID-19: A Prospective Cohort Study 4950 Close Contact Persons in Guangzhou of China", dated **9 April, 2020** marked and annexed herewith **Exhibit "F")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7392433/

(A copy of study titled as "Secondary Transmission of Coronavirus Disease from Pre symptomatic Persons, China", dated 26 August, 2020 marked and annexed herewith **Exhibit "G")**



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7188140/

A copy of study titled as "Transmission potential of asymptomatic and paucisymptomatic SARS-CoV-2 infections: a three-family cluster study in China", dated **22 April, 2020** marked and annexed herewith **Exhibit "H")**

https://www.medrxiv.org/content/10.1101/2020.05.03.20082818v1

A copy of study titled as "Contact tracing and isolation of asymptomatic spreaders to successfully control the COVID-19 epidemic among healthcare workers in Milan (Italy)", dated **08 May, 2020** marked and annexed herewith **Exhibit "I")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7588541/

A copy of study titled as "Analysis of SARS-CoV-2 Transmission in Different Settings, Brunei", dated **26 November, 2020** marked and annexed herewith **Exhibit "J")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7906723/

(A copy of study titled as "Transmission of COVID-19 in 282 clusters in Catalonia, Spain: a cohort study", dated **21 May, 2021** marked and annexed herewith **Exhibit "K")**

7. Misinformation & Pseudoscience on Asymptomatic & Presymptomatic Transmission spread by CDC: -

NOTARY
M.M. PARALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

7.1. A pre-symptomatic case of COVID-19 is an individual infected with SARS-CoV-2, who has not exhibited symptoms at the time of testing, but who later exhibits symptoms during the course of the infection. An asymptomatic case is an individual infected with SARS-CoV-2, who does not exhibit symptoms during the course of infection. there are studies that estimate that individuals who are pre-symptomatic, meaning that they do go on to develop disease symptoms, are responsible for a large proportion of community spread. The estimates reported matter-of-factly by the media come from modelling studies that have serious methodological flaws and limitations biasing results artificially toward a higher proportion of pre-symptomatic spread.

Model outputs are dependent upon the input assumptions. One key lesson from the pandemic is that findings from models may have little bearing on reality. Estimates from modelling studies do not represent real life pre-symptomatic transmission events.

7.2. Take, for instance, the modelling study from the CDC titled: "SARS-CoV-2 Transmission from People Without COVID-19 Symptoms" published in JAMA Network Open in January 2021. This study has been used by the authorities & mainstream media to support the purposefully false claim that "approximately 50% of transmission" is "from asymptomatic

persons". As already noted, that proportion mostly referred to pre-symptomatic transmission. Furthermore, that estimate depended on the assumption that before the person developed symptoms, there was a highly infectious virus incubation period. The incubation period is the time from infection until the development of symptoms. The reference cited as the basis for that assumption is the Nature Medicine modelling study titled "Temporal dynamics in viral shedding and transmissibility of COVID-19" was published in April 2020, but that study has numerous methodological flaws and limitations that give reasonable cause for questioning that assumption. The first thing to note about it is that the study authors, as they point out, "did not have data on viral shedding before symptom onset". They only had "viral load" data from patients who were already in the hospital and after those patients' symptoms had already developed. This introduced the problem of patient "recall bias" as to when their symptoms actually started. This was an issue with data from other studies estimating the incubation, as well. (In simple terms, instead of the researchers themselves knowing when the patients' symptoms started, they had to rely on the patient's memory for when they started.) The authors acknowledged that recall bias would likely tend toward overestimation of the incubation period, which would in turn

bias their findings toward an estimated proportion of pre- symptomatic transmission that is "artificially inflated."

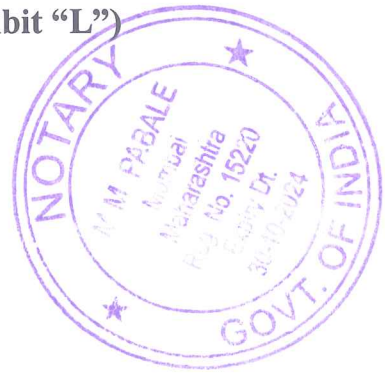
7.3. In addition to an estimated mean incubation period, their calculations also depended on an estimate from another study of the mean serial interval, which is the time from symptom onset in a person who transmits the virus until symptom onset in the person to whom the virus was transmitted. If the mean serial interval is shorter than the mean incubation period, it "indicates that a significant portion of transmission may have occurred before infected persons have developed symptoms." Their data on the serial interval was based on "settings with substantial household clustering" while lockdown measures were in place in China. As the corresponding author, Eric Lau, acknowledged, more frequent and intensive contact within households "results in shorter serial intervals". This in turn results in a greater proportion of estimated pre symptomatic transmission and limits the generalizability of their findings to the broader community setting in the absence of "stay-at-home" orders and other lockdown measures. (In simple words, these findings are based on families that have to cluster together in their houses for a long period of time during lockdowns, & hence their results cannot be applied to the general population which is not under movement restrictions. The irony here is that estimates of pre- symptomatic transmission are used

in order to justify lockdowns & movement restrictions, yet it is the same lockdowns & movement restrictions which make the estimate of pre-symptomatic spread higher in these studies!) Consequently, as noted in a systematic review of estimates on asymptomatic and pre-symptomatic transmission published on the preprint server medRxiv on June 17, it is "not possible to ascertain if the difference between calculated serial interval and incubation period are true differences, or an artefact of rounding error." It's also important to note with respect to their data on "viral loads" that when the authors of the modelling study use the term "viral shedding", they don't mean that patients were shown to be expelling infectious virus into the environment around them which was measured via a Gold Standard viral culture test. They mean that RT-PCR tests were used to detect SARS-CoV-2 RNA in patients' nasal cavity or throat. We know through the evidence discussed earlier in the article that at RT-PCR CT>30, the likelihood of being able to culture a virus goes down to 20% (80 percent false positives).

Source:

https://jamanetwork.com/journals/jamanetworkopen/fullarticle/2774707

(A copy of article published in Jama Network titled as "SARS-CoV-2 Transmission from People Without COVID-19 Symptoms", dated 7 **January, 2021** marked and annexed herewith **Exhibit "L")**



https://www.nature.com/articles/s41591-020-0869-5

(A copy of article published in nature.com titled as "Temporal dynamics in viral shedding and transmissibility of COVID-19 ", dated **15 April, 2020** marked and annexed herewith **Exhibit "M")**

https://www.medrxiv.org/content/10.1101/2020.06.11.20129072v2

(A copy of article published in Jama Network titled as **"Asymptomatic and presymptomatic transmission of SARS-CoV-2: A systematic review "**, dated 17 June, 2020 marked and annexed herewith **Exhibit "N")**

8. WHO's Statement on Asymptomatic Transmission;

8.1. The WHO observed in a guidance document about modes of SARS-CoV-2 transmission published on July 9, 2020 titled "Transmission of SARS-CoV-2: implications for infection prevention precautions": "individuals without symptoms are less likely to transmit the virus than those who develop symptoms." (Note that this statement includes pre-symptomatic as well as asymptomatic individuals.)

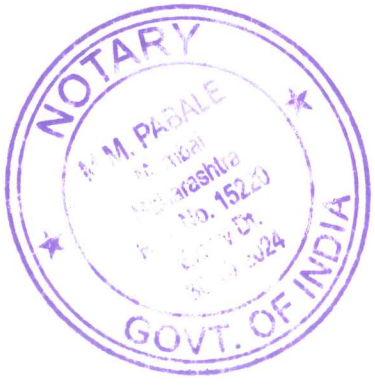
Source:

https://www.who.int/news-room/commentaries/detail/transmission-of-sars-cov-2-implications-for-infection-prevention-precautions

(A copy of scientific brief published on WHO website titled as "Transmission of SARS-CoV-2: implications for infection prevention precautions", dated 9 July, 2020 marked and annexed herewith **Exhibit "O")**

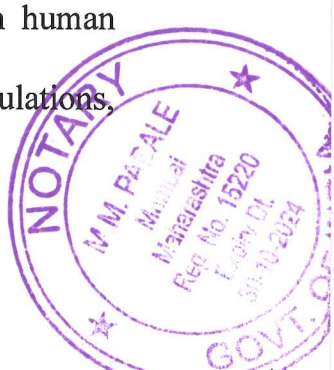
9. Study and reasoning on Dangerous Viruses Found in Healthy People;

9.1. We know from past studies, that many healthy asymptomatic humans harbour multiple viruses associated with diseases in them. For example, in a study titled "Blood DNA virome in 8000 humans" published in Plos Pathogens by A Moustafa et al., March 2017, in 8240 healthy individuals, none of whom were ascertained for any infectious disease, the researchers found that with a lower bound of 2 viral copies per 1,00,00 cells, 42% of healthy individuals had sequences of 94 different viruses, including sequences from 19 human DNA viruses, proviruses and RNA viruses (herpesviruses, anello viruses, papillomaviruses, three polyomaviruses, adenovirus, HIV, HTLV, hepatitis B, hepatitis C, parvovirus B19, and influenza virus.) HIV was found to be 5 times more prevalent than Hepatitis C & Influenza in this healthy cohort of 8200 people. If this study group is



representative of the human population, there would be around 432 million healthy people with HIV in their bloodstream worldwide. Another study published in the journal BioMed Central Biology, titled:

9.2. "Metagenomic analysis of double-stranded DNA viruses in healthy adults" by KM Wylie et al., in September 2014, scientists found that in 102 healthy adults aged 18 to 40, at least one virus was detected in 92 percent of the people sampled, and some individuals harboured 10 to 15 viruses. Herpesvirus 6 or 7 was found in 98 percent of individuals, & certain strains of Papillomavirus were found in about 75 percent of samples. Adenoviruses which are associated with the common cold & pneumonia were also very common. This study was also referenced in an Economic Times article from **2014** titled "Healthy Humans carry viruses too". Another experiment conducted by researchers at the University of Pennsylvania found that healthy human lungs are a home to a family of 19 newfound viruses – which are present at higher levels in the lungs of critically ill people. This study is titled "Redondo viridae, a Family of Small, Circular DNA Viruses of the Human Oro- Respiratory Tract Associated with Periodontitis & Critical Illness" published in Cell Host & Microbe in May 2019 by AA Abbas et al. These Redondo viruses found are known to be associated with human diseases. This paper also admits a crucial fact: "Global virome populations,



I.e., "the virome" are still mostly uncharacterized", meaning that scientists haven't yet done adequate research on many people to figure out what kinds of viruses are present in healthy people' bodies.

Source:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5378407/

(A copy of study titled as **"The blood DNA virome in 8,000 humans"**, dated 22 March, 2017 marked and annexed herewith **Exhibit "P")**

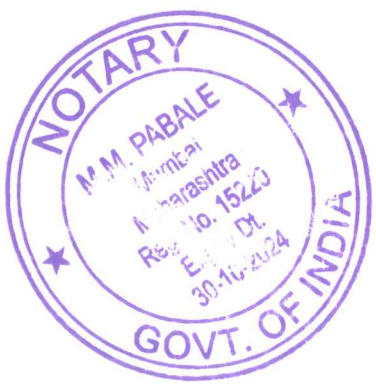
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4177058/

(A copy of study titled as "Metagenomic analysis of double-stranded DNA viruses in healthy adults", dated 10 September, 2014 marked and annexed herewith **Exhibit "Q")**

https://economictimes.indiatimes.com/magazines/panache/healthy-humans-carry-viruses-too/articleshow/42716248.cms

(A copy of article published in Economic Times titled as "Healthy humans carry viruses too!", dated 17 September, 2014 marked and annexed herewith **Exhibit "R")**

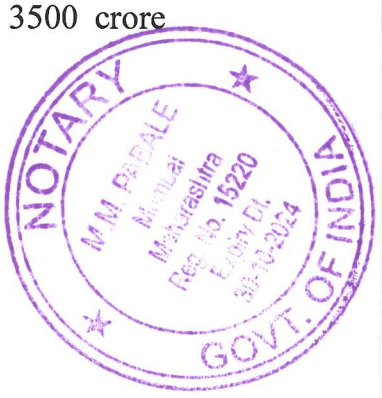
https://www.sciencedirect.com/science/article/pii/S1931312819301714



(A copy of study titled as "Redondoviridae, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness", dated 8th May, 2019 marked and annexed herewith **Exhibit "S")**

10. Illogical & Unscientific Use of the PCR Test;

10.1. <u>How the RT-PCR Test Works :</u> The RT-PCR test takes genetic material from the throat sample that is collected on the swab, runs it through an enzyme called Reverse Transcriptase to convert the RNA from the virus into DNA, & then multiplies the DNA exponentially to find if fragments of the Sars-Cov-2 virus are present in the person or not. Since complete live viruses are necessary for transmission & not their fragments, the RT-PCR test is not designed to tell us whether someone has an active Sars-Cov-2 infection or not. When the genetic material is being amplified, it is being done via cycles, which makes the quantity double after every cycle. For e.g. If 35 cycles of the RT-PCR are run, the first cycle will multiply the material from 1 to 2, the next one will take it from 2 to 4, & so on, until 35 cycles are completed. To put this into perspective, if the RT-PCR starts with a quantity of 2 virus fragments, at the end of 35 cycles it will create 3500 crore fragments.



Source:https://www.medicinenet.com/pcr_polymerase_chain_reaction/article.htm

(A copy of article published in **Medicine Net** titled as "PCR (Polymerase Chain Reaction)", marked and annexed herewith **Exhibit "T")**

https://www.youtube.com/watch?v=V_ Zx0qS7uI

https://theinfectiousmyth.com/coronavirus/RT-PCR_Test_Issues.php

(A copy of study titled as "Issues with the RT-PCR Coronavirus Test", dated 23 April, 2020 marked and annexed herewith **Exhibit "U")**

11. Inventor of RT-PCR (Kary Mullis) view on the test;

11.1. Karry Mullis, an American Biochemist who got the Nobel Prize for his invention of the RT-PCR technique, said the following about the RT-PCR test: "With RT-PCR, if you do it well, you can find almost anything in anybody. It doesn't tell you that you're sick, & it doesn't tell you that the thing you ended up with really was going to hurt you. I'm skeptical that any RT-PCR test is ever true."

Source : https://www.youtube.com/watch?v=V_ Zx0qS7uI

12. Facts about the RT-PCR Test;

12.1. A document published by FDA (U.S Food and Drug Administration) regarding the efficacy of RT-PCR test released in the beginning of the so-called pandemic released on 04th February,20202 (Please refer to page 38) The document clearly states that the RT-PCR test is only capable of checking the presence of genetic material of coronavirus in one's body. As cited in the document, CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, "Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms." Above evidence clears that RTPCR Test cannot detect any infectious virus (2019-nCov) in a person (detecting viral RNA is not same as detecting the Virus) The document further points out that, "This test cannot rule out diseases caused by other bacterial or viral pathogens" In other words, FDA document clears that RTPCR Test cannot diagnose the cause of sickness or death.

Source : https://www.fda.gov/media/134922/download

(A copy of study titled as "CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel", dated 21 July, 2021 marked and annexed herewith **Exhibit "V")**

13. Understanding how the Gold Standard Test for detecting infectious virus (i.e. viral culture) works;

13.1. The Gold Standard for testing infectious disease is known as bacteria or virus culture, where viruses are injected in laboratory cell lines to see if they cause cell damage & death, thus releasing a whole new set of viruses that can go on to infect other cells. This has always been the gold standard in other viruses & bacteria as well, like Ebola, Whooping Cough, etc. In a sick person with symptoms, if scientists are able to culture a virus or bacteria, it means he possesses sufficient quantities of it in his body which shows that he is infected. In the case of Sars-Cov-2 as well, this is the gold standard that the RT-PCR & other quick diagnostic tests like the Rapid Antigen Tests should be compared to. A paper published by Indian scientists in 2020 titled "COVID diagnostics: Do we have sufficient armamentarium for the present and the unforeseen?", published in the Indian Journal of Medical Specialties, the authors admit that viral culture is the gold standard for Sars-Cov-2.

Source: **https://www.ijms.in/article.asp?issn=0976-2884;year=2020;volume=11;issue=3;spage=117;epage=123;aulast=Kashyap**

(A copy of article published in **ijms.in** titled as "COVID diagnostics: Do we have sufficient armamentarium for the present and the unforeseen?", marked and annexed herewith **Exhibit "W")**

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https://www.cebm.net/covid-19/infectious-positive-pcr-test-result-covid-19/

(A copy of article published in ijms.in **titled as** "Are you infectious if you have a positive PCR test result for COVID-19?", dated 5 August 2020 marked and annexed herewith **Exhibit "X")**

https://www.ijms.in/article.asp?issn=0976-2884;year=2020;volume=11;issue=3;spage=117;epage=123;aulast=Kashyap

(A copy of study titled as **"COVID diagnostics: Do we have sufficient armamentarium for the present and the unforeseen?",** dated 10 September, 2020 marked and annexed herewith **Exhibit "Y")**

14. Studies comparing RT-PCR to the Gold Standard.

14.1. In a study titled "Correlation between 3790 per positive samples & positive cell cultures including 1941 Sars-Cov-2" published in the peer-reviewed scientific journal "Clinical Infectious Diseases", by R Jafaar et al., in September 2020, when scientists compared the RT-PCR against the gold standard (I.e., viral culture), this is what they found:

Ct = 25, up to 70% of patients have a positive viral culture. (meaning that in 30 percent of samples where RT-PCR was positive, the virus could not be

cultured from those people, hence they were not infectious. Thus, at this level the false positive rate of the RT-PCR = 30%)

Ct = 30, up to 20% of patients had a positive viral culture

Ct= 35, less than 3 percent had a positive viral culture

Hence at 25-30 cycles, false positive rate is 30%-80% (10% increase at every cycle)

30-35 cycles, false positive rate is 80% - 97%

35 cycles & above, false positive rate is 97%-99.9%

14.2. In a study titled: "Predicting Infectious Severe Acute Respiratory Syndrome Coronavirus 2 From Diagnostic Samples" published in the journal of Clinical Infectious Diseases in December 2020, the authors took 90 RT-PCR positive Sars-cov-2 samples and performed a viral culture test on them. They found that there was no viral growth in samples where the CT value of the RT-PCR was greater than 24. They also found that there was no viral growth in culture 8 days after symptoms began. Hence they concluded: "SARS-CoV-2 Vero cell infectivity was only observed for RT-PCR Ct < 24 and STT < 8 days. Infectivity of patients with Ct > 24 and duration of symptoms > 8 days may be low."

14.3. According to a Meta-Analysis of 29 studies, titled: "Viral cultures for Covid-19 infectivity assessment – a systematic review" published in "Clinical Infectious Diseases" by T Jefferson et al., in September 2020 in medRxiv : "Twelve studies reported that Ct values were significantly lower & log copies higher in samples producing live virusculture. Five studies reported no growth in samples based on a CT cut- off value, which ranged from CT>24 for no growth to Ct ≥ to 34. Two studies report a strong relationship between Ct value & ability to recover infectious virus & that the odds of live virus culture reduced by 33% for every 1 unit increase in Ct. Cut-off of RT-PCR greater than 30 was associated with non-infectious samples" Conclusion of this study: "A binary Yes/No approach to the interpretation RT-PCR unvalidated against viral culture will result in false positives with possible segregation of large numbers of people who are no longer infectious & hence not a threat to public health"

Basically, in this paper they are saying that after analysing 29 studies, higher CT values are not associated with active infection of Sars-Cov-2, & that with each cycle increase of the RT-PCR, the chances of someone being infected reduce by 33%. The authors concluded by saying that RT-PCR results should be tested against viral culture, or else a large number of healthy

people will be wrongly quarantined & have other restrictions imposed on them.

Source:

https://pubmed.ncbi.nlm.nih.gov/32986798/

(A copy of article published titled as ''Correlation Between 3790 Quantitative Polymerase Chain Reaction-Positives Samples and Positive Cell Cultures, Including 1941 Severe Acute Respiratory Syndrome Coronavirus 2 Isolates'' dated 1 June, 2021 marked and annexed herewith **Exhibit "Z")**

https://pubmed.ncbi.nlm.nih.gov/32442256/

(A copy of article published titled as ''**Predicting Infectious Severe Acute Respiratory Syndrome Coronavirus 2 From Diagnostic Samples**'' dated 17 December, 2020 marked and annexed herewith **Exhibit "AA")**

https://www.medrxiv.org/content/medrxiv/early/2020/09/29/2020.08.04.20167932.full.pdf

(A copy of article published titled as ''Viral cultures for COVID-19 infectivity assessment – a systematic review'' dated 29 September,2020 marked and annexed herewith **Exhibit "BB")**

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15. Practical issues with the RT-PCR.

15.1. The above has been seen in the scientific literature as well. A paper from China by Li Y et al. Titled "Stability issues of RT-PCR testing of SARS-CoV-2 for hospitalized patients clinically diagnosed with COVID-19." published in the Journal of Medical Virology on Mar 26 2020. [14] reported on consecutive testing results, defined as either Negative (N), Positive (P) or Dubious (D, presumably intermediate). Results for 29 people with contradictory results out of about 600 patients were: 1 DDPDD, 2 NNPN, 3 NNNPN, 4 DNPN, 5 NNDP, 6 NDP, 7 DNP, 8 NDDPN, 9 NNNDPN, 10 NNPD, 11 DNP, 12 NNNP, 13 PPNDPN, 14 PNPPP, 15 DPNPNN, 16 PNNP, 17 NPNPN, 18 PNP, 19 NPNP, 20 PNPN, 21 PNP, 22 PNP, 23 PNP, 24 PNDDP, 25 PNPNN, 26 PNPP, 27 PNP, 28 PNPN, 29 PNP, A study from Singapore did tests almost daily on 18 patients and the majority went from Positive to Negative back to Positive at least once, and up to four times in one patient.

Testing data collected from Massachusetts, New York, Nevada and elsewhere show that upwards of 90 percent of people who test "positive" with a RT-PCR test are perfectly normal and disease- free.

Source:https://jamanetwork.com/journals/jama/fullarticle/2762688

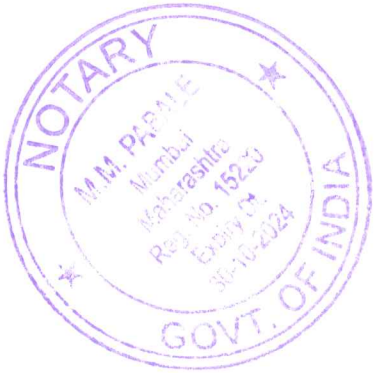
A copy of Article published tilted as "Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore" dated 3 March, 2020 marked and annexed herewith **Exhibit "CC")**

https://www.nytimes.com/2020/08/29/health/coronavirus-testing.html

A copy of Article published tilted as "Your Coronavirus Test Is Positive. Maybe It Shouldn't Be." dated 29 August, 2020 marked and annexed herewith **Exhibit "DD")**

16. Why the RT-PCR Can Test Positive Long After Symptom Onset.

16.1. The RT-PCR is so sensitive that it can pick up non-infectious viral fragments in those who have already dealt with the virus and are not contagious anymore. We have seen the same phenomena in the past, where measles virus cannot be grown in cell culture but is detected as RT-PCR positive 3 months after infection. According to Sergio Santos & Matteo Chiesa, of Department of Physics and Technology, The Artic University of Norway, who wrote an article titled: "RT- PCR positives: What do they mean?" for the Center for Evidence Based Medicine.



"This detection problem is ubiquitous for RNA virus's detection. SARS- CoV, MERS, Influenza Ebola and Zika viral RNA can be detected long after the disappearance of the infectious virus. ...because inactivated RNA degrades slowly over time it may still be detected many weeks after infectiousness has dissipated." The same thing is taking place with Sars-Cov-2 as well, where people are testing positive weeks & months after the infection. But instead of questioning the validity & interpretation of the test, most people think that they have got a re-infection."

Source: **https://www.cebm.net/covid-19/pcr-positives-what-do-they-mean/**

(A copy of article published titled as "PCR positives: what do they mean?" dated 17 September,2020 marked and annexed herewith **Exhibit "EE")**

16.2. This is the reason why the MOHFW's guidelines on discharge from the hospital don't require patients to get retested, as the RNA of the virus takes weeks/months' time to degrade in the body.

Source:https://www.mohfw.gov.in/pdf/ReviseddischargePolicyforCOVID19.pdf

(A copy of Revised Discharge Policy for COVID-19 marked and annexed herewith **Exhibit "FF")**

ICMR's latest advisory dated 04/05/2021 clearly states the following:

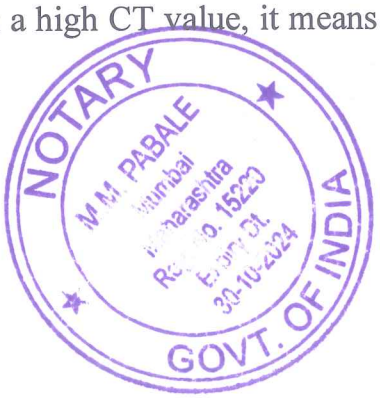
> RTPCR test must not be repeated in any individual who has tested positive once either by RAT or RTPCR. The need for RTPCR test in healthy individuals undertaking inter-state domestic travel may be completely removed to reduce the load on laboratories.

Source:https://www.icmr.gov.in/pdf/covid/strategy/Advisory_COVID_Testing_in_Second_Wave_04052021.pdf

(A copy of ''Advisory for COVID-19 testing during the second wave of the pandemic'' dated **04.05.2021** marked and annexed herewith **Exhibit "GG")**

17. WHO's Position on the RT-PCR Test

17.1. In a notice written on January 13, 2021 and published on January 20, 2021, the WHO warned that high cycle thresholds on RT-PCR tests will result in false positives. To quote their own words: The design principle of RT-PCR means that for patients with high levels of circulating virus (viral load), relatively few cycles will be needed to detect virus and so the CT value will be low. Conversely, when specimens return a high CT value, it means



that many cycles were required to detect virus. In some circumstances, the distinction between background noise and actual presence of the target virus is difficult to ascertain.

17.2. The WHO confirmed that RT-PCR tests should not be used as the sole method of diagnosing COVID-19; they should only be used where clinical signs and symptoms are present, and they can yield false positive results at high amplification cycles. The package inserts accompanying RT-PCR test kits, state that the test should be administered only to patients with signs and symptoms suggestive of COVID-19.

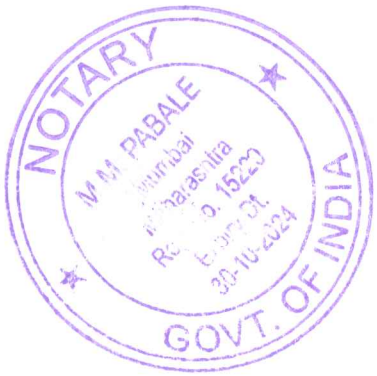
Source: **https://www.who.int/news/item/20-01-2021-who-information-notice-for-ivd-users-2020-05**

(A copy of article titled as ''Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2''dated **13.01.2021** marked and annexed herewith **Exhibit "HH")**

18. Fake Epidemics Created in the Past due to RT-PCR Misuse

18.1. We have had many episodes in the past where, based on wrong use of the RT-PCR, false epidemics of diseases have been created. A striking case of this has been highlighted in a New York Times article from 2007, titled

"Faith in Quick Test Leads to Epidemic that Wasn't", [20] explaining how a fake whooping cough (also known as pertussis) epidemic was created in 2006. A lady called Dr. Brooke Herndon started coughing nonstop for 2 weeks in Mid-April of 2006. Because of this, an infectious disease expert at the hospital called Dr. Kathryn Kirkland, thought that could be the start of a whooping cough epidemic. By the end of April, few others at the hospital started coughing. Based on this fear that a whooping cough epidemic had started, the hospital tested nearly 1000 healthcare workers with the RT-PCR test, out of that 142 people were told they had the disease. These people were given antibiotics & vaccines (1445 health care workers took antibiotics & 4524 health care workers took the vaccine). Many beds at the hospital including ICU beds, were reserved solely for whooping cough patients. (Similar to what is happening now) After 8 months, healthcare workers were shocked to receive an email saying that this whole episode was a false alarm. Epidemiologists at the hospital decided to take extra steps to confirm if what they were seeing really was pertussis. Doctors sent 27 samples from patients they thought had pertussis to the American CDC. There scientists tried to grow the bacteria, & they concluded that there was no pertussis in any of the samples. They also tested 39 samples from patients who had tested positive



and had not got themselves vaccinated, but only one of those cases showed an increase in antibody levels indicative of pertussis.

Source: https://www.nytimes.com/2007/01/22/health/22whoop.html

(A copy of article published in **'The New York Times'** titled as ''Faith in Quick Test Leads to Epidemic That Wasn't'' dated 22.01.2007 marked and annexed herewith **Exhibit "II"**)

19. How the Rapid Antigen Test (RAT) works:

19.1. Now that we have thoroughly dissected the RT-PCR test & its limitations / incorrect use, let us turn to the Rapid Antigen Test. Instead of detecting the genetic fragments of the Sars-Cov-2 virus, it detects the proteins on the surface of the virus which are specific to it. Here is how this test works: "A typical antigen test starts with a health-care professional swabbing the back of a person's nose or throat. The sample is then mixed with a solution that breaks the virus open and frees specific viral proteins. The mix is added to a paper strip that contains an antibody tailored to bind to these proteins, if they're present in the solution. A positive test result can be detected either as a fluorescent glow or as a dark band on the paper strip."

19.2. This test now makes up 50% of the testing done in Mumbai, according to the ICMR as well as PM Narendra Modi, the RT-PCR test should make

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up 70% of India's testing, while the remaining 30% can be done via the Rapid Antigen Test. The current mindset among people in our country is fully biased against false negatives, (I.e., if the test tests negative but the person actually has a Sars-Cov-2 infection). Hence the current guidelines in India state that if a person has symptoms & amp; he tests negative on the RAT, then he needs to retest with the RT-PCR. The reasoning according to many is that since for an antigen test to test positive one would need to have many viral particles in their body, the test could miss out on someone who has low levels of viral particles in the body. But as we have mentioned, viral culture is the gold standard for detecting viral agents, & studies have shown that the RAT correlates much better with virus culture than the RT-PCR does.

Source:

https://www.nature.com/articles/d41586-020-02661-2

(A copy of article published in **'Nature'** titled as ''Fast coronavirus tests: what they can and can't do'' dated 16 September, 2020 marked and annexed herewith **Exhibit "JJ")**

https://www.ndtv.com/india-news/50-covid-tests-in-mumbai-are-less-reliable-antigen-tests-data-shows-2402588

(A copy of article published in **'NDTV'** titled as ''50% Covid Tests In Mumbai Are Less Reliable Antigen Tests, Data Shows'' dated 30 March, 2021 marked and annexed herewith **Exhibit "KK")**

https://www.livemint.com/news/india/need-to-stop-second-covid-19-peak-70-rt-pcr-test-must-for-states-pm-modi-11615972632349.html

(A copy of article published in **'mint'** titled as ''Need to stop second COVID-19 peak; 70% RT-PCR test must for states: PM Modi'' dated 17 March, 2021 marked and annexed herewith **Exhibit "LL")**

20. Studies Comparing RAT to the Gold Standard.

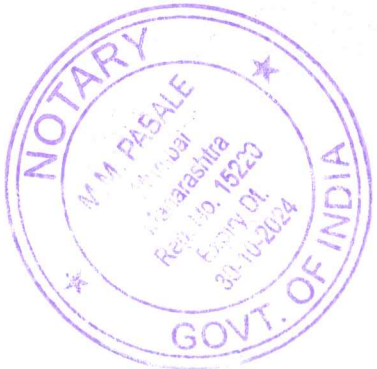
20.1. The following studies demonstrate that Rapid Antigen Tests correlate better with the Gold Standard (viral culture) than the RT-PCR.

Title: "Antigen-based testing but not real-time RT-PCR correlates with SARS-CoV-2 virus culture" by A Pekosz et al., in 2020. In this study 38 samples with evidence of SARS-CoV-2 by RT-PCR were collected from individuals symptomatic for COVID-19 with onset of symptoms. Samples were tested by rapid antigen test and in laboratory- based cell culture (Gold Standard) to assess infectivity. Of 38 RT-PCR- derived positive samples, 28

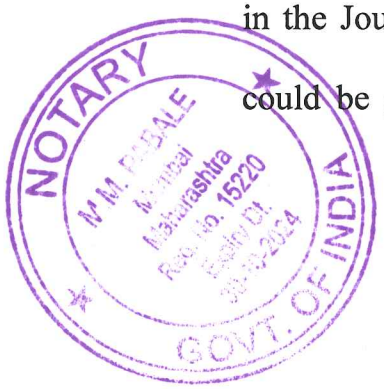
were positive, and 10 were negative in virus culture testing. This means that the RT-PCR had 10 false positive results (rate of 26.3%). By comparing antigen-based test results, the scientists observed that all samples except one that were positive in both the RT-PCR-based and culture-based tests, were also positive in the antigen-based test. (Only one false negative, rate of 3.5%) Of 10 samples that were positive in RT-PCR but negative in vira culture, two were positive in the antigen-based testing. (0 out of 10 RT-PCR tests matched with viral culture here, whereas 8 out of 10 rapid antigen tests matched with viral culture.)

These findings indicate the antigen tests perform better in detecting the presence of the infectious virus in patients' samples compared to RT-PCR-based tests.

20.2. Another study titled: "Evaluation of Abbott BinaxNOW Rapid Antigen Test for SARS-CoV-2 Infection at Two Community-Based Testing Sites — Pima County, Arizona, November 3–17, 2020" by JL Prince-Guerra et al., in Jan 2021, published in Morbidity & Mortality Weekly Report, BinaxNOW rapid antigen test was used along with real-time reverse transcription-polymerase chain reaction (RT-PCR) testing to analyze 3,419 samples. 274 of these samples that either had a RT-PCR positive or an antigen positive were sent for viral culture. Out of these 124 were RT-PCR



positive only, 147 were RT-PCR & antigen positive, & only 3 were antigen positive & RT-PCR negative. Using viral culture to compare against RT-PCR results, it was found that out of the 124 RT-PCR only positive tests, only 11 could be cultured. This indicates a 91 percent false positive rate for the RT-PCR (with a median CT value of 33.9). Out of the 147 samples that tested positive for both the RT-PCR & RAT, 85 of them could be cultured (giving the RAT a false positive rate of 42%). Using samples which tested positive using the RAT got down the false positive rate to 42%, a marginal improvement over using RT-PCR only positive samples. Further, it was found that the median CT value goes down to 22, indicating higher viral load on samples which test positive on the RAT. No virus could be cultured from the 3 samples that were RAT positive & amp; RT-PCR negative. This study confirms that the RT-PCR has a much higher rate of false positives than the RAT, that lower RT-PCR CT values correlate with higher viral load, & that the RAT correlates better with the gold standard of viral culture than the RT-PCR. And finally, a study titled: "Evaluation of a SARS-CoV-2 rapid antigen test: Potential to help reduce community spread?" by T Toptan et al., published in December 2020 in the Journal of Clinical Virology, out of 32 RT-PCR samples, only 19 could be grown via cell culture, whereas out of those 32 only 27 were



Antigen Test Positive.

20.3. All of these studies indicate that the RT-PCR test produces way more false positives than the antigen test, & that the antigen tests correlate with the gold standard better than the RT-PCR. Hence the worry about false negatives with the Antigen test is misleading as that is based on treating the RT-PCR as the gold standard, whereas what we have demonstrated here is that the reliability of the RT-PCR test is too low to depend on, & therefore the viral culture must be taken as the true gold standard.

Source:

https://www.medrxiv.org/content/10.1101/2020.10.02.20205708v1

(A copy of research study titled as ''**Antigen-based testing but not real-time PCR correlates with SARS-CoV-2 virus culture**'' dated October 05, 2020 marked and annexed herewith **Exhibit "MM")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7821766/

(A copy of research study titled as ''Evaluation of Abbott BinaxNOW Rapid Antigen Test for SARS-CoV-2 Infection at Two Community-Based Testing

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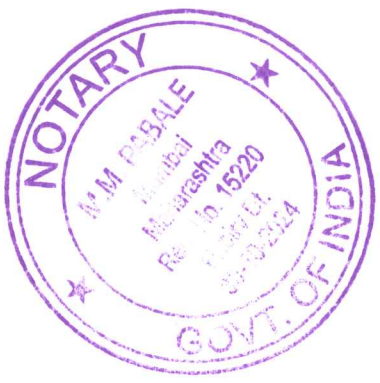
Sites — Pima County, Arizona, November 3–17, 2020'' dated 22 January, 2021 marked and annexed herewith **Exhibit "NN")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7832367/

(A copy of research study titled as ''Evaluation of a SARS-CoV-2 rapid antigen test: Potential to help reduce community spread?'' dated 05 December, 2020 marked and annexed herewith **Exhibit "OO")**

21. Practical Issues with the RAT;

21.1. Just like the RT-PCR, we have seen the same practical results with the antigen test as well, where people are getting different test results from different labs with the same sample. Tesla's CEO Elon Musk, claimed that he was tested positive twice and tested negative twice on the same day: "Something extremely bogus is going on," Musk tweeted. "Was tested for covid four times today. Two tests came back negative, two came back positive. Same machine, same test, same nurse. Rapid Antigen test from BD."

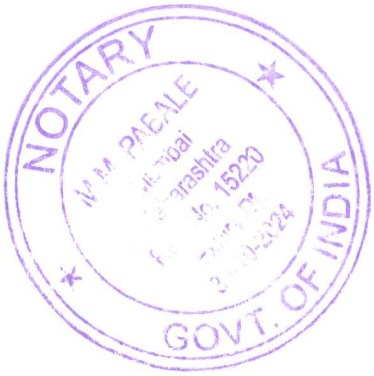


21.2. In the USA, when the health care workers in Nevada and Vermont reported false positives with the RAT, US's HHS (Department of Health & Human Services) defended the Rapid Antigen Tests and threatened Nevada with unspecified sanctions until state officials agreed to continue using them in nursing homes. It took several more weeks for the U.S. Food and Drug Administration to issue an alert on Nov. 3 that confirmed what Nevada had experienced: Antigen tests were prone to giving false positives, the FDA warned in a report.

21.3. The FDA laid out various guidelines to reduce the risk of false positives from the Antigen tests, after it was found that this test was producing many false positive in nursing homes. They can be found in an article titled: "Potential for False Positive Results with Antigen Tests for Rapid Detection of SARS-CoV-2 - Letter to Clinical Laboratory Staff and Health Care Providers". These guidelines must be implemented in India as well.

A paper titled: "Challenges and Controversies to Testing for COVID- 19", found that if a quarter of American school kids were tested three times a week with an antigen test that's 98% specific, it would produce 800,000 false positives a week.

Source:



https://www.fda.gov/medical-devices/letters-health-care-providers/potential-false-positive-results-antigen-tests-rapid-detection-sars-cov-2-letter-clinical-laboratory

(A copy of article titled as ''Potential for False Positive Results with Antigen Tests for Rapid Detection of SARS-CoV-2 - Letter to Clinical Laboratory Staff and Health Care Providers' 'dated 11.03.2020 marked and annexed herewith **Exhibit "PP")**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7587118/

(A copy of research study titled as "Challenges and Controversies to Testing for COVID-19" dated 21 October, 2020 marked and annexed herewith **Exhibit "QQ")**

22. Court Rulings Against the RT-PCR Worldwide;

22.1. Multiple courts around the world have given judgements against the RT-PCR test. A Portuguese court issued the following ruling: "Given how much scientific doubt exists – as voiced by experts, i.e., those who matter – about the reliability of the RT-PCR tests, given the lack of information concerning the tests' analytical parameters, and in the absence of a physician's diagnosis supporting the existence of infection or risk, there is

no way this court would ever be able to determine whether C was indeed a carrier of the SARS-CoV-2 virus, or whether A, B and D had been at a high risk of exposure to it," "Most importantly, the judges ruled that a single positive RT-PCR test cannot be used as an effective diagnosis of infection." "In their ruling, judges Margarida Ramos de Almeida and Ana Paramés referred to several scientific studies. Most notably [a study by Jaafar et al], which found that – when running RT-PCR tests with 35 cycles or more – the accuracy dropped to 3%, meaning up to 97% of positive results could be false positives." "The ruling goes on to conclude that, based on the science they read, any RT-PCR test using over 25 cycles is totally unreliable. The Court was declaring the RT-PCR test alone could not be sufficient for a diagnosis of disease, and it was outrageous to believe it could.

A "case of COVID disease" without a medical assessment of clinical symptoms in the patient is no case at all. It is a misnomer, and, the Court stated, represents a serious breach of the law. Not surprisingly, this decision received a total blackout in the mainstream media.

22.2. On December 31, anti-coronavirus activists won a court case against the Dutch state to ensure a family can return from holiday in Tanzania without having to produce negative coronavirus tests.

The court in The Hague ruled that the family can return from the high- risk country on January 3 without a negative test and ordered the state to pay the legal costs.

The judge said the family have the right to protest about being forced to undergo a RT-PCR test against their will. 'Introducing such a requirement for citizens of the Netherlands who want to return home requires legal grounding, and this is not covered by article 53 or 54 of the public health act,' the judge is quoted as saying.

The fact that further spreading of the virus needs to be tackled urgently is not up for discussion, the judge said. 'But such a far-reaching obligation as this, which concerns physical integrity, requires a concrete legal basis.

22.3. Following the Portuguese and Dutch rulings, now the Austrian court has ruled that RT-PCR tests are not suitable for COVID-19 diagnosis and that lockdowns have no legal or scientific basis. The Vienna Administrative Court granted a complaint by the FPÖ against the prohibition of its meeting registered for January 31 in Vienna.

> "The prohibition was wrong," the court said said in the ruling. The court stated on the basis of scientific studies that the grounds for the prohibition put forward by the Vienna State Police Department are

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completely unfounded. It is expressly pointed out that, even according to the World Health Organization, "a RT-PCR test is not suitable for diagnosis and therefore does not in itself say anything about the disease or infection of a person".

Source:

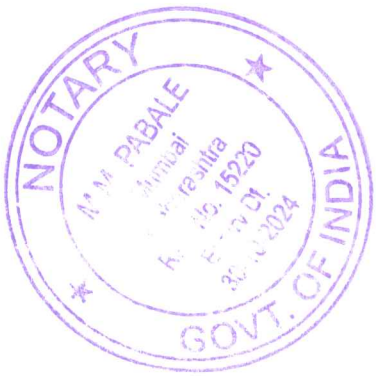
https://translate.google.com/translate?hl=&sl=pt&tl=en&u=http%3A%2F%2Fwww.dgsi.pt%2Fjtrl.nsf%2F33182fc732316039802565fa00497eec%2F79d6ba338dcbe5e28025861f003e7b30

(A copy of Judgment Passed by Hon'ble Lisbon Court of Appeal "MARGARIDA RAMOS DE ALMEIDA" dated 11 November, 2020 marked and annexed herewith **Exhibit "RR")**

https://off-guardian.org/2020/11/20/portuguese-court-rules-pcr-tests-unreliable-quarantines-unlawful/

(A copy of article titled as ''Portuguese Court Rules PCR Tests "Unreliable" & Quarantines "Unlawful"." dated 20.11.2020 marked and annexed herewith **Exhibit "SS")**

https://www.thehagueonline.com/news/2021/01/04/negative-test-mandatory-for-entry-to-nl



(A copy of article titled as ''Negative Test Mandatory For Entry To Nl" dated 04 January, 2021 marked and annexed herewith **Exhibit "TT")**

https://principia-scientific.com/austrian-court-rules-pcr-unsuited-for-covid-lockdowns-unlawful/

(A copy of article titled as ''Austrian Court Rules PCR Unsuited For COVID, Lockdowns Unlawful" dated 15 April, 2021 marked and annexed herewith **Exhibit "UU")**

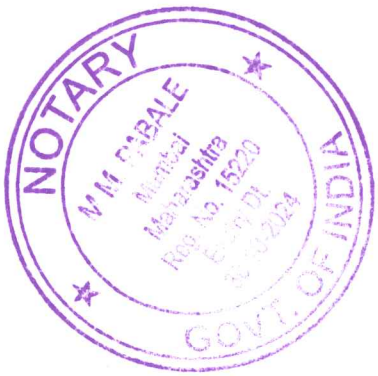
23. Asymptomatic Cases in the First & Second Wave, & Asymptomatic Cases Across India.

23.1. Now that we have come to see the real meaning of asymptomatic cases & how they don't cause infections in other people, let us take a look at how much of our case burden has come from asymptomatic people.

23.2. That, the letter dated 5th January, 2022 it is admitted by the Government of India's Ministry of Health & Family Welfare (Respondent No. 4) that the majority of the cases are asymptomatic.

The first para reads thus;

" ***1. Background***



Over the past two years, it has been seen globally as well as in India that majority of cases of COVID-19 are either asymptomatic or have very mild symptoms. Such cases usually recover with minimal interventions and accordingly may be managed at home under proper medical guidance and monitoring.

Ministry of Health & FW has thus issued and updated guidelines for home isolation from time to time to clarify selection criteria, precautions that need to be followed by such patients and their families, signs that require monitoring and prompt reporting to health facilities.

The present guidelines are applicable to COVID-19 patients who have been clinically assessed and assigned as ***mild /asymptomatic cases of COVID-19"***

The last **para 9** reads thus;

9. When to discontinue home isolation

Patient under home isolation will stand discharged and end isolation after at least 7 days have passed from testing positive and no fever for 3 successive days and they shall continue

wearing masks. There is no need for re-testing after the home isolation period is over.

Asymptomatic contacts of infected individuals need not undergo Covid test & monitor health in home quarantine.

Source:
https://www.mohfw.gov.in/pdf/RevisedHomeIsolationGuidelines05012022.pdf

(A copy of guidelines titled as "Revised guidelines for Home Isolation of mild /asymptomatic COVID-19 cases", published by Ministry of Health & Family Welfare dated **5th January, 2022** marked and annexed herewith **Exhibit "GGGG")**

We will first look at last year's data, 2020

23.3. In an article by the Print titled: "80% Covid patients in India are asymptomatic, health ministry analysis finds", Analysis of cases across India until 23 August 2020 shows about 25.93% of the of the symptomatic patients reported with fever and 17.18% with cough.

23.4. In an article titled "Over 70% children with Covid-19 are asymptomatic: AIIMS data" by the Hindustan Times, as of November 2020, the author wrote: "With 73.5%, the highest proportion of asymptomatic cases was reported among children below the age of 12. The proportion

decreased linearly with age, with only 38.4% of the cases among those above the age of 80 being asymptomatic.

23.5. In an article by Hindustan Times from December 2020, titled: "71 percent active covid cases in Mumbai asymptomatic",the author wrote: "Of the 12,926 active Covid-19 patients in Mumbai, 9,155 (71%) are asymptomatic, displaying no symptoms before undergoing tests for the presence of Sars-CoV-2."

Now let's review the data of the Second Wave (2021).

23.6. In an article by NDTV from March 30 2021 titled "85,000 Covid Cases In Second Wave, Most Asymptomatic: Mumbai Civic Body", the author wrote: "The second wave of coronavirus in Maharashtra started on February 10 and till March 20, Mumbai logged 85,000 cases, said Iqbal Chahal, the Commissioner of Brihanmumbai corporation, the civic body of Mumbai. Of the total number of cases, 69,500 are asymptomatic, he added. The remaining 8,000 patients reached hospitals with mild symptoms."

23.7. In an article from Deccan Herald, titled: "Majority of Bengaluru's In an article from Deccan Herald, titled: "Majority of Bengaluru's Covid-19 patients are asymptomatic" from 19th April 2021, the author wrote: "reports show that 95.9 per cent of the state's cases are 'asymptomatic'. The

percentage of asymptomatic cases in the state capital of Bengaluru is even higher – 99.4 per cent, according to a report by Bangalore Mirror quoting data from the state's Covid-19 war room.

23.8. According to Dr. Balram Bhargava himself, Covid-19 symptoms in this wave are much less than last year, & that there is no difference in the percent of death between the first wave and second wave. He further said that only a marginally high proportion of COVID-19 patients are of younger age and that the average age of patients in the first wave was 50 years and, in this wave, it is 49 years. A higher number of asymptomatic individuals got admitted this year, than a higher proportion of patients admitted with breathlessness.

23.9. Yet, the Indian Medical Research Council (ICMR) warned about asymptomatic patients who can be hidden super-spreaders of the Coronavirus in the country. This is despite all the evidence which shows us that the opposite is true.

23.10. Most importantly, currently Navi Mumbai has started testing people randomly on the streets, even if they're asymptomatic. Healthy asymptomatic children who have just started going to school are being tested, & they're also coming positive. Despite ICMR's guidelines last

guidelines stating that asymptomatics should not be tested during travel, they are still being tested.

Given all that we have learnt in this document until now, this data should make us pause & think very deeply about all the unnecessary harm to society that has been caused because of our irrational fear of asymptomatic cases.

Source:

https://theprint.in/health/80-covid-patients-in-india-are-asymptomatic-health-ministry-analysis-finds/487761/

(A copy of article titled as ''80% Covid patients in India are asymptomatic, health ministry analysis finds" dated 24 August, 2020 marked and annexed herewith **Exhibit "VV")**

https://www.hindustantimes.com/mumbai-news/71-active-covid-cases-in-mumbai-asymptomatic/story-r5x39rSpDHRBpYjazpUnDK.html

(A copy of article published in 'Hindustan Times' titled as ''71% active Covid cases in Mumbai asymptomatic" dated 7 December, 2020 marked and annexed herewith **Exhibit "WW")**

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https://www.ndtv.com/india-news/85-000-covid-cases-in-second-wave-most-asymptomatic-mumbai-civic-body-2402202

(A copy of article published in 'NDTV' titled as ''85,000 Covid Cases In Second Wave, Most Asymptomatic: Mumbai Civic Body" dated 30 March, 2021marked and annexed herewith **Exhibit "XX")**

https://www.deccanherald.com/city/top-bengaluru-stories/majority-of-bengalurus-covid-19-patients-are-asymptomatic-976071.html

(A copy of article published in 'DECCAN HERALD' titled as ''Majority of Bengaluru's Covid-19 patients are asymptomatic" dated 19 April, 2021 marked and annexed herewith **Exhibit "YY")**

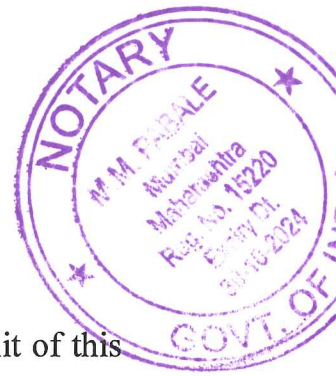
https://www.punekarnews.in/need-to-focus-on-asymptomatic-covid-patients-icmr/

(A copy of article published in 'PUNEKAR NEWS' titled as ''Need To Focus On Asymptomatic COVID Patients: ICMR" dated 04 April, 2021 marked and annexed herewith **Exhibit "ZZ")**

Asymptomatic testing across India

Question

Request count of Covid-19 tests done all over India. Request split of this data between the different kinds of tests and between the symptomatic status.



Answer

Symptomatic status count of people who tested Covid-19 positive –Data

Link: https://drive.google.com/file/d/139Cxcsu-gQwHaG87zR8ZXnFMQqby3X_d/view?usp=sharing

(A copy of **RTI Reply on RTPCR Asymptomatic by ICMR** dated 11 August, 2021 marked and annexed herewith **Exhibit "AAA")**

Conclusion: 97.2% of the people who tested positive were asymptomatic (that is, they were healthy).

24. Covid Deaths in India and Infection Fatality Rate.

24.1. Total Covid Deaths in India are presently at **4,82,876.** Compared to the size of the population of 140 crore, that amounts to a mortality rate of **0.00033%.**

Source: https://www.worldometers.info/coronavirus/country/india/

(A copy of statistic of deaths in India Compared to the size of the population of 140 crore marked and annexed herewith **Exhibit "BBB")**

24.2. India's CFR & IFR.

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The Case Fatality Rate in India (which is the number of deaths divided by the number of cases which tested positive) has fallen from over 3% in the beginning of the pandemic to less than 1.5 percent now.

24.3. India's Infection Fatality Rate is 0.1%.

https://www.hindustantimes.com/india-news/one-year-of-covid-19-how-india-fought-the-virus-101614553310402.html

(A copy of article published in 'Hindustan Times' titled as "One year of Covid-19: How India fought the virus" dated 01 March, 2021 marked and annexed herewith **Exhibit "CCC")**

"Combining waves 1 and 2, as of May 15, while India reported a total of nearly 25 million cases and 270 thousand deaths, the estimated number of infections and deaths stand at 491 million (36% of the population) and 1.21 million respectively, yielding an estimated (combined) infection fatality rate of **0.25%.**"

Source: https://bmcresnotes.biomedcentral.com/articles/10.1186/s13104-021-05652-2

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(A copy of study titled as "Estimating the wave 1 and wave 2 infection fatality rates from SARS-CoV-2 in India" dated 8 July, 2021 marked and annexed herewith **Exhibit "DDD")**

24.4. Global Data

The latest Infection Fatality Rate (the number of deaths divided by the number of actual infections) was calculated by John Ioannidis for Covid 19 in his peer-reviewed study titled: "Infection fatality rate of COVID-19 inferred from seroprevalence data".

Source: https://www.who.int/bulletin/volumes/99/1/20-265892.pdf

(A copy of study titled as "**Infection fatality rate of COVID-19 inferred from seroprevalence data**" dated 14 October, 2020 marked and annexed herewith **Exhibit "EEE")**

24.5. Here he calculated the IFR for COVID-19 in a review of 61 seroprevalence studies, which was a median of 0.23%, and 0.05% in people younger than 70. There's a 1,000 times difference in mortality among those younger than 19 and those older than 70 — something that should have been taken into account in the pandemic response. The CEBM (Centre for Evidence Based Medicine) at the University of Oxford, currently estimates the CFR (which is the number of deaths

divided by the number of cases which tested positive) globally at 0.51%, and the IFR to be at 0.1%-0.26%.

Source: https://microbiologyjournal.org/covid-19-case-fatality-rate-misapprehended-calculations/

(A copy of study titled as "**COVID-19 Case Fatality Rate: Misapprehended Calculations**" dated 21 August, 2020 marked and annexed herewith **Exhibit "FFF")**

24.6. The CFR for Influenza is about 3%, & the IFR for Influenza is 0.1%.

Source: https://wattsupwiththat.com/2020/09/08/covid-19-cfr-and-ifr-confused/

(A copy of study titled as "Covid-19 CFR and IFR Confused" dated 08 September, 2021 marked and annexed herewith **Exhibit "GGG")**

CFR = Total number of Covid deaths / Number of Covid Cases
IFR = Total number of Covid deaths / Number of actual infections as determined by serosurveys.

24.7. The CFR only looks at those who have been tested via RT-PCR or RAT, whereas the IFR considers all actual infections by the data generated

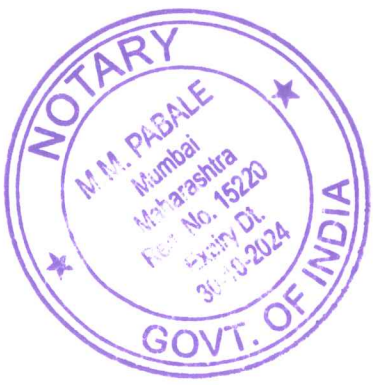
from serosurveys which tell us about how many people have had the infection and developed antibodies.

25. Practical Ground Reality of Testing Situation in Mumbai, & its implications for the Third Wave;

25.1. Right at the onset of the second wave, BMC commissioner took the decision to massively ramp up Covid-19 testing in Mumbai. From March 20 2021 article in India today: "The latest order by the BMC in this regard says Covid-19 tests will be conducted at malls, railway stations (for inbound trains), MSRTC Bus Depots, khau gullies, hawkers, market places, tourist spots, and various government offices. The BMC has also clarified that the Covid-19 tests will be carried out without the consent of citizens who are present at these crowded places.

"If the citizen refuses the test, it would amount to an offense under the Epidemic Act, 1897. Hence, the action shall be initiated under the Epidemic Act, 1897 against the offender," the BMC has said.

The civic body intends to test at least 400 people visiting malls each day. For the 27 malls in Mumbai, the BMC will conduct 10,800 random tests each day.



25.2. Each ward given Covid-19 testing target

Apart from malls, the BMC will bear the cost of Covid-19 testing at other crowded places.

Similarly, at least 1,000 passengers of inbound trains will be tested for Covid-19 at each railway station in Mumbai. A similar pattern will be followed at bus depots operated by the Maharashtra State Road Transport Corporation (MSRTC).

In addition to crowded places, the BMC has also instructed each ward to carry out at least 1,000 Rapid Antigen Tests at restaurants, market places, government offices and beaches among other crowded places.

The BMC has set a target of 47,000 random Covid-19 tests in crowded places across Mumbai every day. Each of Mumbai's 24 wards has also been given a testing target."

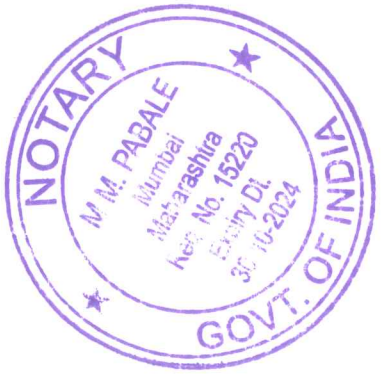
Source : https://www.indiatoday.in/cities/mumbai/story/mumbai-random-covid-corona-testing-crowded-places-bmc-rules-cost-punishment-1781633-2021-03-20

(A copy of article published in 'INDIA TODAY' titled as "Mumbai: BMC to conduct 47,000 random Covid tests everyday, refusing one can land you in trouble" dated 20 March, 2021 marked and annexed herewith **Exhibit "HHH")**

25.3. With this massive number of testing's being conducted on asymptomatic people, & the false positives from the RT-PCR & RAT test, the second wave had upwards of 80% cases in Mumbai that were asymptomatic. Alot of economic damage & loss of individual liberties could have been avoided if policymakers understood that asymptomatic people don't spread Sarscov2, & the huge number of false positives in both the PCR & RAT tests.

25.4. In February in Mumbai, nearly 20000 tests were being conducted daily, and in March this was nearly doubled to 40000.

Source: https://theprint.in/india/why-mumbai-is-relying-more-on-antigen-tests-than-rt-pcr-as-it-doubles-covid-testing/632919/



(A copy of article published in 'The Print' titled as "Why Mumbai is relying more on antigen tests than RT-PCR as it doubles Covid testing" dated 2nd April, 2021 marked and annexed herewith **Exhibit "III")**

25.5. In May, BMC head Iqbal Chahal issued a statement saying that testing had gone down since the second wave and that it should increase back again. "He said the number of tests being conducted per day fell from over 50,000 earlier to 38000 on May 1 and further to 28000 on May 2nd. He said BMC has been conducting maximum tests to "flush out coronavirus from Mumbai's environment". Firstly, if they increased the number of tests back higher, that would definitely increase the number of cases again. This is why his latter reasoning of doing more tests to flush out the virus is wrong, as the tests have a huge number of false positives. The longer we continue to test, the longer we will find cases.

Source : https://timesofindia.indiatimes.com/city/mumbai/daily-covid-19-tests-must-be-raised-to-40000-in-mumbai-iqbal-singh-chahal/articleshow/82372451.cms

(A copy of article published in 'The Times of India' titled as "Daily Covid-19 tests must be raised to 40,000 in Mumbai: Iqbal Singh Chahal" dated 3rd May, 2021 marked and annexed **Exhibit "JJJ")**

25.6. The number of tests conducted vastly influence how many cases come up, due to the huge number of false positives, & asymptomatic people who have dead viral fragments in them after infection. This is acknowledged by the authorities themselves in the following articles:

> "Almost a month after Mumbai registered its worst single-day tally of 11206 Covid-19 cases, the graph of the second wave dipped sharply on Monday, with 2624 cases detedcted in a 24 hr period. While the 23542 tests done to detect these cases were lower than the city's daily average of 44000 (in April) officials said the worst appears to be over as far as the second wave is concerned."

25.7. In the second half of the article, they acknowledge the effect of the number of tests conducted: Officials said daily detection's were down owing to fewer tests over the weekend and the fatalities had dipped due to delay in uploading data. The state conducted 2.1 lakh tests in the last 24 hrs, a dip from the **2.5-2.7 lakh** tests it conducts during the weekdays"

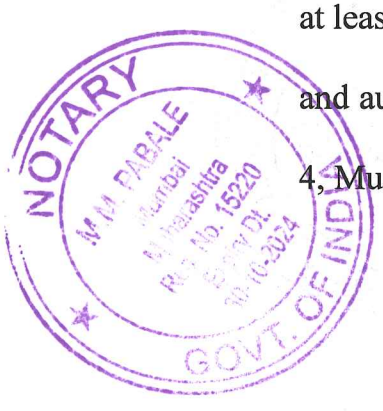
Source : https://timesofindia.indiatimes.com/city/mumbai/covid-19-big-dip-in-mumbai-cases-the-worst-may-be-over/articleshow/82379993.cms

(A copy of article published in 'The Times of India' titled as "Covid-19: Big dip in Mumbai cases, the worst may be over" dated 4th May, 2021 marked and annexed herewith **Exhibit "KKK")**

25.8. 18th May article from Hindustan Times : "With a constant decrease in the number of daily Covid-19 cases reported in Mumbai for the past two weeks, the tests conducted in the city have also almost halved. While an average of 50,000 tests was conducted per day in Mumbai during mid-April, they have now come down to an average of 20,000-25,000 daily.

Officials have attributed the decrease in the number to fewer people coming forward for a test.

25.9. On Sunday, Mumbai conducted only 17,640 tests, the results of which were revealed on Monday. By mid-March, Mumbai had started conducting at least 50,000 tests a day and on some occasions up to 55,000 tests. Experts and authorities believe the second wave started around March 11. On April 4, Mumbai reported the highest number of Covid-19 cases in a single day –



11,204 – of 51,319 tests conducted, revealed BMC data. By April 20, the daily tests dropped to below 40,000. For the past five days, Mumbai has been conducting lower than 30,000 tests per day."

Source: https://www.hindustantimes.com/cities/mumbai-news/as-cases-drop-number-of-covid-tests-halved-in-mumbai-101621278073321.html

(A copy of article published in 'Hindustan Times' titled as "As cases drop, number of covid tests halved in Mumbai" dated 18th May, 2021 marked and annexed herewith **Exhibit "LLL")**

25.10. On June 29 2021, an article in the Hindustan times attributes the rise in cases in Maharashtra to an increase in testing. "Maharashtra sees spike in single-daily Covid-19 cases as testing increases

Tuesday's daily spike marks an increase over the 6,727 positive Covid-19 cases Maharashtra had recorded on Monday.

The state tested 190,140 samples for coronavirus marking an increase from the 166,163 samples tested a day before.

Source: **https://www.hindustantimes.com/cities/mumbai-news/maharashtra-sees-spike-in-single-daily-covid-19-cases-as-testing-increases-101624983236317.html**

(A copy of article published in 'Hindustan Times' titled as "Maharashtra sees spike in single-daily Covid-19 cases as testing increases" dated 29th June, 2021 marked and annexed herewith **Exhibit "MMM")**

25.11. Cases to a reduction in testing. "Maharashtra & Mumbai reported a significant dip in daily detections in Covid cases on Monday. Officials attributed the dip to a drop in testing over the weekend. Maharashtra conducted only 1.5 lakh tests on Sundays, as against over 2 lakh on weekdays."

Source: **https://timesofindia.indiatimes.com/city/mumbai/mumbai-with-a-drop-in-testing-over-the-weekend-fewer-covid-19-detections-in-state-city/articleshow/85193228.cms**

(A copy of article published in 'The Times of India' titled as "With a drop in testing over the weekend, fewer Covid-19 detections in Maharashtra & Mumbai" dated 10th August, 2021 marked and annexed herewith **Exhibit "NNN")**

25.12. As on Aug 19 another article admits: "As tests increase, Covid cases tick up in Mumbai".

Source: **https://timesofindia.indiatimes.com/city/mumbai/mumbai-as-tests-increase-cases-tick-up-in-city-ity-still-under1/articleshow/85440355.cms**

(A copy of article published in 'The Times of India' titled as "As tests increase, Covid cases tick up in Mumbai, positivity still under 1%" dated 19th August, 2021 marked and annexed herewith **Exhibit "OOO")**

25.13. As on Aug 30 2021, another article admits: Maharashtra: Covid dip likely due to weekend effect on tests

Source:

http://timesofindia.indiatimes.com/articleshow/85751885.cms?utm_source=contentofinterest&utm_medium=text&utm_campaign=cppst

(A copy of article published in 'The Times of India' titled as "Maharashtra: Covid dip likely due to weekend effect on tests" dated 30th August, 2021 marked and annexed herewith **Exhibit "PPP")**

These articles fully confirms our earlier assertion.

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25.14. On 16th June, 2021, the Navi Mumbai Municipal Corporation decided to make RAT tests mandatory for all who visit shopping malls during the weekends, Friday to Sunday.

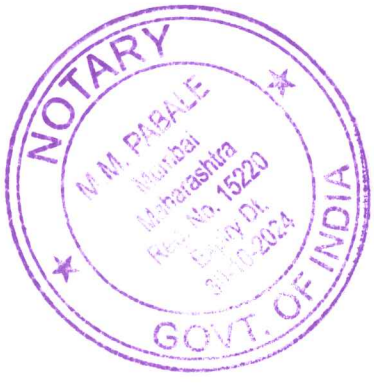
Source:

https://timesofindia.indiatimes.com/city/navi-mumbai/covid-test-must-for-visitors-to-navi-mumbai-malls/articleshow/83556738.cms

(A copy of article published in 'The Times of India' titled as "Covid test must for visitors to Navi Mumbai malls" dated 16th June, 2021 marked and annexed herewith **Exhibit "QQQ")**

25.15. On **August 11th**, the Brihanmumbai Municipal Corporation (BMC) *said they will expand testing and contact tracing, as well as increase additional medical resources, In order to prepare for a possible third wave of Covid-19 in Mumbai.*

"Given the third wave of the pandemic in various countries, essential planning and preparations are being undertaken to deal with similar circumstances more effectively, BMC said on Wednesday.



These primarily involve expanding hospitals, smaller treatment centres, beds and tests, besides implementing a more effective containment strategy, BMC said.

More testing kits:

25.16. Authorities to provide more testing kits, both RT-PCR as well as Rapid Antigen. They will also ensure the supply of the requisite number of kits to testing facilities.

Expanding contact tracing:

25.17. Contact tracing is carried out on people who have come into close contact with patients to avoid the spread of the virus. Previously, contact tracing of 15 persons was done for each patient. This number will now be 20."

Source: **https://www.indiatoday.in/cities/mumbai/story/bmc-braces-for-covid-third-wave-in-mumbai-here-how-1839757-2021-08-11**

(A copy of article published in 'India Today' titled as "BMC braces for Covid's third wave in Mumbai. Here's how" dated 11 August, 2021 marked and annexed herewith **Exhibit "RRR")**

25.18. As of Aug 19 2021,: "Eye on 3rd wave govt to spend 22.5 crore on 25lakh rat test kits."

Source: **https://timesofindia.indiatimes.com/city/mumbai/mumbai-as-tests-increase-cases-tick-up-in-city-ity-still-under-1/articleshow/85440355.cms**

(A copy of article published in 'The Times of India' titled as "As tests increase, Covid cases tick up in Mumbai, positivity still under 1%" dated 19 August, 2021 marked and annexed herewith **Exhibit "SSS")**

25.19. As on August 27: "With the gradual rise in the daily count of Covid-19 cases, the doubling rate has plunged by 232 days in the past 10 days — from 2,057 on August 18 to 1,825 on August 27 — indicating the start of a third wave. Against this backdrop, the Brihanmumbai Municipal Corporation (BMC) has changed its contact tracing policy. Ward officers have been instructed to mandatorily test all close contacts of Covid positive patients immediately to avoid transmission of the infection amid relaxations in Covid-19 restrictions.

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25.20. As HT reported on August 11, municipal commissioner Iqbal Singh Chahal along with Kakani, in a review meeting of the health department, decided to increase their contact tracing from 10 to 20 per infected patient. However, this week, the target of contact tracing was increased to 25 per infected patient.

> "So far, we used to test close contacts after 4-5 days if they develop any symptoms. Till then, they were being instructed to get home quarantined. But now, we have instructed ward officers to test them immediately, without any delay," said Suresh Kakani, additional municipal commissioner, BMC. "The movement of the people has increased with the relaxation. So, we don't want any asymptomatic patient to infect others in the crowd," he added.

Source:

https://www.hindustantimes.com/cities/mumbai-news/third-wave-mandatory-testing-of-all-close-contacts-of-covid-patients-in-mumbai-101630079669711.html

(A copy of article published in 'Hindustan Times' titled as "Third wave: Mandatory testing of all close contacts of Covid patients in Mumbai" dated

27 August, 2021 marked and annexed herewith **Exhibit "TTT")**

25.21. As of September 5 2021: "Ahead of Ganeshotsav, the Navi Mumbai Municipal Corporation has increased random testing in Navi Mumbai especially at the railway stations. Currently, around 500 random antigen tests are being conducted at railway stations. The corporation plans to conduct similar random tests at busy markets, pandals and immersion sites as well.

Pramod Patil, medical health officer, NMMC, said, "A total of 7,000 Rapid Antigen Tests and around 2,000 RT-PCR tests are conducted daily." **Source:**

https://www.hindustantimes.com/cities/mumbai-news/navi-mumbai-to-increase-covid-19-testing-ahead-of-festive-season-101630789285245.html

(A copy of article published in 'Hindustan Times' titled as "Navi Mumbai to increase Covid-19 testing ahead of festive season" dated 5 September, 2021 marked and annexed herewith **Exhibit "UUU")**

25.22. As of 6 September 2021: "Members of the task force stressed the importance of early testing. Dr Oak stated that family doctors have an

important role in encouraging a covid test ideally through an RT-PCR." Source: https://www.mumbailive.com/en/civic/state-covid-task-force-cautions-towards-new-symptoms-of-covid-19-68158

(A copy of article published in 'Mumbai Live' titled as "State COVID Task Force Cautions Towards New Symptoms Of COVID-19" in the month of October, marked and annexed herewith **Exhibit "VVV")**

25.23. All of the articles linked above show that the authorities in Mumbai & Maharashtra lowered testing rates, and are now starting to increase them again, as well as scaling up contact tracing from 5 contacts to 25 contacts! Forced testing of asymptomatic has also started in Navi Mumbai & could begin soon by the BMC as well. Since the tests have a high number of false positives, & asymptomatic cases are included in the case numbers as well, we would expect that there would be a rise in cases again, & this would warrant another unnecessary & deadly third lockdown, which will devastate the livelihoods of many more than the last two.

25.24. Given all these factors, officials have already started hinting that the third wave has begun:

"Third wave of coronavirus has arrived in Nagpur, thus fresh restrictions will be reimposed in the city soon', said State Energy Minister Nitin Raut after holding a review meeting with senior administrative officers from various state departments. Speaking to reporters, Raut said that third wave, which was predicted to hit the country anytime in September-October, has reached here (Nagpur) as the city has logged COVID cases in double digits."

Source : https://www.india.com/maharashtra/maharashtra-lockdown-news-today-7-september-2021-third-wave-arrived-in-nagpur-fresh-restrictions-to-be-announced-soon-in-mumbai-nagpur-pune-uddhav-thackeray-big-announcement-awaited-4939878/

(A copy of article published in 'india.com' titled as "Maharashtra Lockdown Update: Third Wave of Corona Hits Nagpur, Fresh Restrictions to be Announced Soon, Says Minister" dated 7th September, 2021 marked and annexed herewith **Exhibit "WWW")**

25.25. "Covid Third Wave Here Already," says Mumbai Mayor.

The third wave of Covid is already upon Mumbai, the city's Mayor has said, pointing to a sudden spike in cases. "The third-wave of Covid-19 is not

coming, it is here," said Kishori Pednekar, adding that an announcement regarding the matter had already been made in Nagpur

Source : https://www.ndtv.com/india-news/covid-third-wave-here-already-says-mumbai-mayor-2532865

(A copy of article published in 'NDTV' titled as ""Covid Third Wave Here Already," Says Mumbai Mayor" dated 7th September, 2021 marked and annexed herewith **Exhibit "XXX")**

25.26. Maharashtra Lockdown Big Update: Deputy CM Warns of Shutting Down Everything if Covid Third Wave Hits

Speaking to reporters, Pawar said the Centre has already cautioned all the states, citing that Kerala and Maharashtra have been reporting the highest number of cases.

"Maharashtra Deputy chief minister Ajit Pawar on Friday asked citizens to follow all the covid protocols and guidelines set by the government to contain the spread of the virus in the state. Expressing concerns over people letting their guard down against Covid-19 in rural areas Pawar urged citizens

not to put the state government in a position where it has to shut everything in the event of a third wave of the pandemic.

Source: https://www.india.com/news/india/maharashtra-lockdown-big-update-deputy-cm-ajit-pawar-warns-of-shutting-down-everything-if-covid-third-wave-hits-uddhav-thackeray-rajesh-tope-shopping-malls-temples-markets-coronavirus-4932761/

(A copy of article published in 'india.com' titled as "Maharashtra Lockdown Big Update: Deputy CM Warns of Shutting Down Everything if Covid Third Wave Hits" dated 3rd September, 2021 marked and annexed herewith **Exhibit "YYY")**

25.27. These unscientific testing guidelines are also leading to an increase in cases among children, as theyre being tested even when symptomless : Odisha issues guidelines for stricter implementation of COVID norms in schools amid surge in infection among childrenThe fresh guideline was issued by the School and Mass Education Department on Tuesday after some children and teachers tested positive for the infection in Dhenkanal and Bargarh districts.

Source: **https://www.moneycontrol.com/news/trends/current-affairs-trends/odisha-issues-guidelines-for-stricter-implementation-of-covid-norms-in-schools-amid-surge-in-infection-among-children-7445271.html**

(A copy of article titled as "Odisha issues guidelines for stricter implementation of COVID norms in schools amid surge in infection among children" dated **8th September, 2021** marked and annexed herewith **Exhibit "ZZZ"**)

26. Conclusion On Theory Of Infection By Asymptomatic Infectious People & Unscientific Testing :-

26.1. Harm Caused Due to Unscientific Testing Guidelines.

Because of improper use of the RT-PCR & Antigen Tests, & testing being done on asymptomatic people, we are seeing an explosion of cases as well as deaths, because a case is defined as a positive RT-PCR regardless of symptoms, & death certificates also can list someone as a Covid death just based on a RT-PCR positive and/or broad symptoms. Quick diagnostic tests should never be considered as confirmed markers of evidence, based on

which strategic decisions such as isolation, lockdowns & vaccines need to be implemented. They are only temporary tests that need confirmation with the gold standard of viral culture.

26.2. Due to this, many healthy people who are not infectious or a threat to anyone have had their fundamental rights taken away from them, have had to pay a lot of money to finance their institutional quarantines, have had to miss out on income because they were wrongly quarantined, have had to be quarantined with people in a room who are true positives (big risk for the elderly & immune compromised), have had to face societal stigma, & have taken wrong medications because of an incorrect diagnosis, which comes with many side effects. Elderly, Immunocompromised people & those with Co-morbidities, if falsely diagnosed, can die due to medicines given to them like Remdesivir, Favipiravir, etc that have now shown to not be effective & at the same time come with toxic side effects. People who suffer from Covid related symptoms but actually have influenza or the common cold, are put on wrong medications that damage their body unnecessarily. More hospital & ICU beds get occupied as well, as people wrongly think they have Covid.

26.3. False positives are not an acceptable price to pay in order to minimize false negatives. Throwing in false positive cases in isolation wards & exposing them to actual infectious disease carriers is no less than throwing

innocent people in jail to live among murderers & rapists. Our whole judicial system works on the principle of innocent until proven guilty, hence we must apply the same to healthy asymptomatic people and see them as such, until proven otherwise through the evidence-based methods described above.

A "case" is defined in medicine as an active, symptomatic and diagnosed infection. Not any more: Any "positive" in the faulty RT-PCR "test" or RAT is now counted as a "case". The mass RT-PCR testing & RAT campaign of the general asymptomatic population, which has no clinical or epidemiological utility, thereby feeds media propaganda of fear, and disastrous consequences: RT-PCR/RAT → meaningless- "cases" → propaganda → arbitrary-measures/great-harm.

27. Searching for people who are asymptomatic yet infectious is like searching for needles that appear and reappear transiently in haystacks, particularly when rates are falling.

27.1. Mass testing risks the harmful diversion of scarce resources. A further concern is the use of inadequately evaluated tests as screening tools in healthy populations. The absence of strong evidence that asymptomatic people are a driver of transmission is another good reason for pausing the roll out of mass testing in schools, universities, and communities."

27.2. This is not only causing trouble to a common man, but it is also an offence of misappropriation of thousand of crores of public money. Which an offence punishable under section 409, 120(B), 34 etc. of IPC.

27.3. That, as per section 52 of IPC the act of public servant without taking due care and caution is said to be done in bad faith and not in good faith.

28. ICMR Testing Guidelines

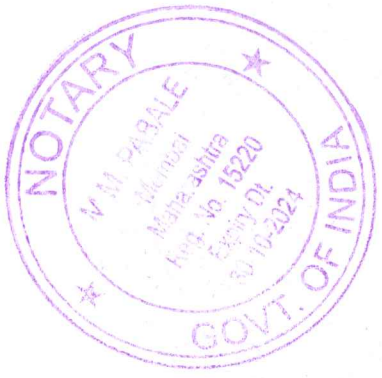
Source :

https://www.icmr.gov.in/pdf/covid/strategy/Advisory_COVID_Testing_in_Second_Wave_04052021.pdf

(A copy of Guidelines by ICMR titled as "Advisory for COVID-19 testing during the second wave of the pandemic" **dated 4th May, 2021** marked and annexed herewith **Exhibit "AAAA")**

The last ICMR guideline which was issued in May 2021 and is still valid, clearly states;

"The need for RTPCR test in healthy individuals undertaking inter-state domestic travel may be completely removed to reduce the load on laboratories."



29. Center warns States should not make rules against Central Guidelines

29.1. Needless to point out that, recently, **Rajesh Bhushan**, the Secretary of Health Ministry, Union of India has sent a letter to **Dr. Pradeep Kumar Vyas**, Additional Chief Secretary, Department of Health & family Welfare, Government of Maharashtra asking him to not to frame any policies against the policies framed by the Union of India.

The relevant Para reads thus;

> "***D.O.No.01/S(HFW)/Omicron/Maha/2021***
>
> ***01 December, 2021***
>
> *Dear Dr. Vyas*
>
> *This is with reference to the Govt. Of Maharashtra Order No. DMU/2020/CR.92/DisM 1 dated 30th Nov. 2020, vide which the following restrictions have been imposed:*
>
> *i. Mandatory RTPCR testing of all international travellers at the Mumbai airport, irrespective of country of origin*

ii. Mandatory 14-day home quarantine for all international passengers, despite being tested RTPCR Negative upon arrival

iii. Mandatory RTPCR test for passengers planning to undertake connecting flights after disembarking at Mumbai and further travel subject to a negative RTPCR result

iv. Requirement of negative RTPCR test 48 hours prior to date of journey, for domestic passengers travelling from other States to Maharashtra

2. ***<u>This is in divergence with the SoPs & Guidelines issued by Ministry of Health & Family Welfare, Govt. of India.</u>*** *I would, therefore, urge you to align the Orders issued by the State with the Guidelines issued by the Ministry of Health & Family Welfare, Govt. Of India, so that uniform implementation of the guidelines may be ensured across all States/UTS. I would also advise that such modified orders of the State Government are given wide publicity to obviate any inconvenience to travellers.*

Warm Regards.

Yours sincerely

(Rajesh Bhushan)"

Source: https://drive.google.com/file/d/1bsnHjMU9_DB-2q12NmRsMNP-ca2r4EeU/view?usp=sharing

(A copy of letter dated **1.12.2021** being outward No. **D.O.No.01/S(HFW) Omicron/ Maha/2021** by Rajesh Bhushan, Ministry of Health & Family Welfare, Govt. of India marked and annexed herewith **Exhibit "BBBB")**

30. Scientific and logical approach adopted by director General Health of Republic of South Africa.

30.1. That, after considering all scientific studies and also considering new variants like **OMICRON**, the Director General Health of Republic of South Africa has formulated policies on **23.12.2021.**

The relevant excerpts from the order dated **23.12.2021** us as under;

1. *The following revisions have been accepted based on the - COVID-19 MAC advisories of 16.12.2021*

1.1 Proportion of people with some immunity from infection and/or vaccination is high

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

• *past infection in 60-80% in several sero-surveys*

1.2 Containment strategies are no longer appropriate – mitigation is the only viable strategy • Especially true of the newer, more infectious/transmissible variants like OMICRON 1.3 New knowledge about the virus:

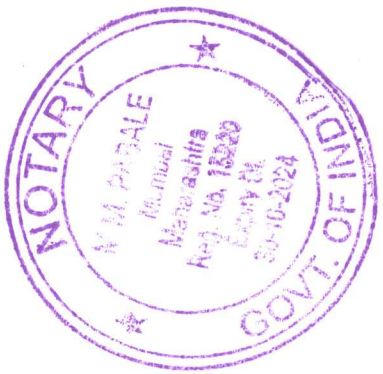
a) *high proportion of asymptomatic disease,*
b) *high degree of asymptomatic and pre-symptomatic spread,*
c) *aerosol spread.*
d) *Only a small proportion of cases are diagnosed.*

1.5 *We never identify most high risk patients*

a) *Testing skewed towards symptomatic (minority)*
b) *Not all symptomatic people test*
c) *Not all negative tests are true negatives*

1.6 *"High risk" definition probably isn't meaningful anymore*

a) *Doesn't take into account aerosol spread*
b) *Doesn't take into account the newer variants (increased transmissibility)*
c) *Doesn't take into account pre-existing immunity*



1.7 *Quarantine has been costly to essential services and society as many people stay away from their work and thus lose their income and children miss on their schooling.*

Thus, the following is applicable with immediate effect :

2. Contact Tracing

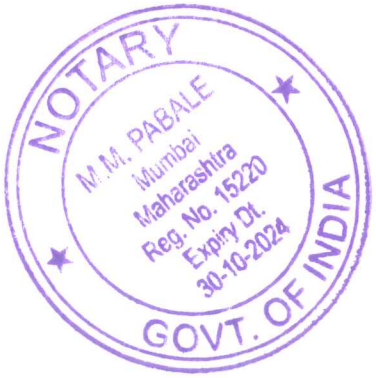
2.1 All contact tracing be stopped with immediate effect except in congregate settings and cluster outbreak situations or self-contained settings.

2.2 All contacts must continue with their normal duties with heightened monitoring (daily temperature testing, symptom screening) of any early signs. If they develop symptoms then they should be tested and be managed according to the severity of the symptoms

2.3 All contacts must not be tested unless if they develop symptoms

3. Quarantining for contacts of confirmed cases of Covid -19

3.1 All quarantine is to be stopped with immediate effect



3.2 This applies to both vaccinated and unvaccinated contacts

3.3 No testing for Covid -19 is required irrespective of the risk exposure unless the contact becomes symptomatic

4. Isolation

- *Isolation rules are applicable to both vaccinated and unvaccinated individuals*
- *Isolation rules are applicable to high and low risk individuals*
- *Return to work from Day 10 onwards must as always take into consideration the individual's clinical status. Only those patients well enough to work should do so.*

4.1 Asymptomatic Individuals

a) *No isolation period required*

b) *To do self-observation for 5-7 days for development of any symptoms with enhanced precautions including avoiding attending settings where many people gather, mask wearing and social distancing.*

4.2 Mild disease

Mild diseases refers to persons who have symptoms and have tested positive but who do not require hospitalization. do not have shortness of breath, dyspnoea or abnormal chest imaging.

Mild disease symptoms and signs include but are not limited to the following: fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhoea, loss of taste and smell.

a) *Isolation period is maintained at 8 days.*

b) *The person in this category must wear a mask at all times (even at home, work and all public spaces) for the duration of the 8 days period of isolation.*

c) *Where a health care worker returns to work after Day 8 such a worker must wear a N95 mask at all times and must at all times avoid contacts with extremely high risk patients (especially severely immune-compromised patients).*

d) *There is no need for Covid-19 test (either PCR or antigen test) be performed prior to returning to work after 8 days isolation period.*

e) *For mild cases, isolation beyond 8 days must be supported by the medical report*

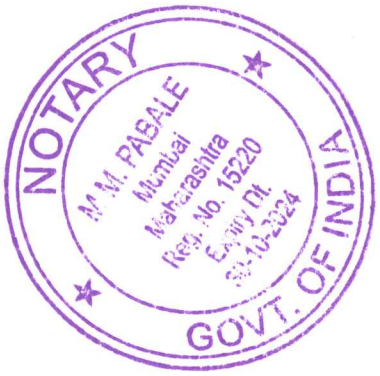
4.3 Severe Disease:

a) *Severe disease refers to persons who test positive and have exacerbated symptoms i.e shortness of breath, dyspnoea, chest pain and abnormal chest imaging and who require hospitalisation to manage the clinical presentation.*

b) *Isolation period is maintained at 10 days after clinical stability is achieved*

c) *The person in this category must wear a mask at all times (even at home, work and all public spaces) for the duration of the 10 days period of isolation.*

d) *Where a health care worker returns to work after Day 10, such a worker must wear a N95 mask at all times and must at all times avoid contacts with extremely high risk patients (especially severely immune-compromised patients).*



e) *There is no need for Covid-19 test (either PCR or antigen test) be performed prior to returning to work after 10-day isolation period*

f) *For severe cases, isolation beyond 10 days must be supported by the medical report*

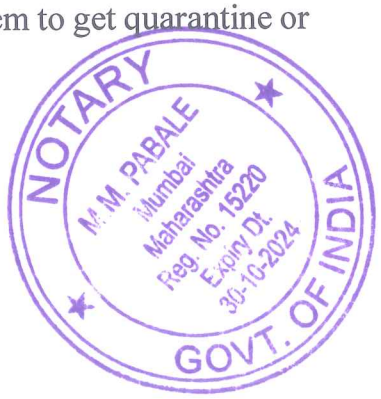
5. Return to work

All people that have been infected and have been in isolation, must be ready to return to work after completing mandatory period of isolation as above and no further testing is required after either 8 or 10 days of isolation."

(A copy of the said letter dated **23.12.2021** annexed herewith at **Exhibit "CCCC")**

31. Directions for regular testing of asymptomatic people is an offence under Section 409, 120(B) & 34, 52 of IPC as done with malafide intention to give undue profit of thousands of crores to the test kit manufacturing companies and wrongful loss and misappropriation of public property & money.

31.1. That, from the material available on record it is clear that, the decision of testing asymptomatic healthy people and asking them to get quarantine or



impose restrictions like lockdown was nothing but an unlawful, arbitrary and malafide act done to achieve ulterior purposes.

31.2. The ultimate beneficiary of the decision are the companies who are manufacturing the Testing Kit. The ultimate loss will be of the common man as thousands of crores are wasted for unauthorized, unlawful purposes, wasting public money & resources.

31.3. It is a punishable offence under **Section 52, 409, 120(B), 34, 109, 511 etc. of IPC.**

31.4. In **Noida Entrepreneurs Association and Ors. Vs. Noida and Ors. (2011) 6 SCC 508** it is ruled as under

*"**28.** While dealing with the issue of haste, this Court in Bahadursinh Lakhubhai Gohil v. Jagdishbhai M. Kamalia [(2004) 2 SCC 65] , referred to S.P. Kapoor (Dr.) v. State of H.P. [(1981) 4 SCC 716 : 1982 SCC (L&S) 14 : AIR 1981 SC 2181] and held that: (Jagdishbhai M. Kamalia case [(2004) 2 SCC 65] , SCC p. 75, para 25)*

*"25. ...**when a thing is done in a post-haste manner, mala fides would be presumed**...."*

***29.** In Zenit Mataplast (P) Ltd. v. State of Maharashtra [(2009) 10 SCC 388] this Court held: (SCC p. 399, para 39)*

*"39. **Anything done in undue haste can also be termed as arbitrary and cannot be condoned in law**...."*

***30.** Thus, in case an authority proceeds in undue haste, the Court may draw an adverse inference from such conduct. It further creates a doubt that if there was no sufficient reason of urgency, what was the occasion for Respondent 4 to proceed in such haste and why fresh tenders had not been invited.*

***38.** The State or the public authority which holds the property for the public or which has been assigned the duty of grant of largesse, etc. acts as a trustee and, therefore, has to act fairly and reasonably. Every holder of a public office by virtue of which he acts on behalf of the State or public body is ultimately accountable to the people in whom the sovereignty vests. As such, all*

powers so vested in him are meant to be exercised for public good and promoting the public interest. Every holder of a public office is a trustee.

39. *State actions are required to be non-arbitrary and justified on the touchstone of Article 14 of the Constitution. Action of the State or its instrumentality must be in conformity with some principle which meets the test of reason and relevance. Functioning of a "democratic form of Government demands equality and absence of arbitrariness and discrimination". The rule of law prohibits arbitrary action and commands the authority concerned to act in accordance with law. Every action of the State or its instrumentalities should neither be suggestive of discrimination, nor even apparently give an impression of bias, favouritism and nepotism. If a decision is taken without any principle or without any rule, it is unpredictable and such a decision is antithesis to the decision taken in accordance with the rule of law.*

40. *The public trust doctrine is a part of the law of the land. The doctrine has grown from Article 21 of the*

Constitution. In essence, the action/order of the State or State instrumentality would stand vitiated if it lacks bona fides, as it would only be a case of colourable exercise of power. The rule of law is the foundation of a democratic society. ***41.*** *Power vested by the State in a public authority should be viewed as a trust coupled with duty to be exercised in larger public and social interest. Power is to be exercised strictly adhering to the statutory provisions and fact situation of a case. "Public authorities cannot play fast and loose with the powers vested in them." A decision taken in an arbitrary manner contradicts the principle of legitimate expectation. An authority is under a legal obligation to exercise the power reasonably and in good faith to effectuate the purpose for which power stood conferred. In this context, "in good faith" means "for legitimate reasons". It must be exercised bona fide for the purpose and for none other.*

42. *In view of the above, we are of the considered opinion that these allegations being of a very serious*

nature and as alleged, Respondent 4 had passed orders in colourable exercise of power favouring himself and certain contractors, require investigation. Thus, in view of the above, we direct CBI to have preliminary enquiry and in case the allegations are found having some substance warranting further proceeding with criminal prosecution, may proceed in accordance with law. It may be pertinent to mention that any observation made herein against Respondent 4 would be treated necessary to decide the present controversy. CBI shall investigate the matter without being influenced by any observation made in this judgment."

31.5. Relevant Sections of IPC reads thus;

Section 52 in The Indian Penal Code

52. "Good faith".—Nothing is said to be done or believed in "good faith" which is done or believed without due care and attention.

Section 409 in The Indian Penal Code

409. Criminal breach of trust by public servant, or by banker, merchant or agent.—Whoever, being in any

manner entrusted with property, or with any dominion over property in his capacity of a public servant or in the way of his business as a banker, merchant, factor, broker, attorney or agent, commits criminal breach of trust in respect of that property, shall be punished with 1[imprisonment for life], or with imprisonment of either description for a term which may extend to ten years, and shall also be liable to fine.

Section 120B in The Indian Penal Code

1[120B. Punishment of criminal conspiracy.—

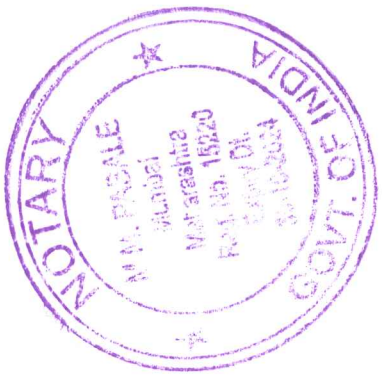
(1) Whoever is a party to a criminal conspiracy to commit an offence punishable with death, 2[imprisonment for life] or rigorous imprisonment for a term of two years or upwards, shall, where no express provision is made in this Code for the punishment of such a conspiracy, be punished in the same manner as if he had abetted such offence.

(2) Whoever is a party to a criminal conspiracy other than a criminal conspiracy to commit an offence punishable as aforesaid shall be punished with

imprisonment of either description for a term not exceeding six months, or with fine or with both.]

Section 34 in The Indian Penal Code

[37] [34. Acts done by several persons in furtherance of common intention.—When a criminal act is done by several persons in furtherance of the common intention of all, each of such persons is liable for that act in the same manner as if it were done by him alone.]



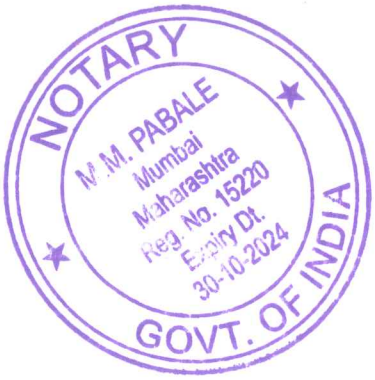
Section 109 in The Indian Penal Code

109. Punishment of abetment if the act abetted is committed in consequence and where no express provision is made for its punishment.—Whoever abets any offence shall, if the act abetted is committed in consequence of the abetment, and no express provision is made by this Code for the punishment of such abetment, be punished with the punishment provided for the offence. Explanation.—An act or offence is said to be committed in consequence of abetment, when it is

committed in consequence of the instigation, or in pursuance of the conspiracy, or with the aid which constitutes the abetment.

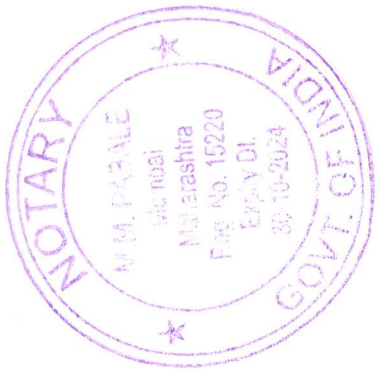
Section 511 in The Indian Penal Code

511. Punishment for attempting to commit offences punishable with imprisonment for life or other imprisonment.—Whoever attempts to commit an offence punishable by this Code with 1[imprisonment for life] or imprisonment, or to cause such an offence to be committed, and in such attempt does any act towards the commission of the offence, shall, where no express provision is made by this Code for the punishment of such attempt, be punished with 2[imprisonment of any description provided for the offence, for a term which may extend to one-half of the imprisonment for life or, as the case may be, one-half of the longest term of imprisonment provided for that offence], or with such fine as is provided for the offence, or with both.



31.6. Hon'ble Supreme Court in the Case of **Raman Lal Vs. State of Rajasthan 2000 SCC OnLine Raj 226** it is ruled as under;

> ***"Conspiracy – I.P.C. Sec. 120 (B)*** *– Apex court made it clear that an inference of conspiracy has to be drawn on the basis of circumstantial evidence only because it becomes difficult to get direct evidence on such issue – The offence can only be proved largely from the inference drawn from acts or illegal ommission committed by them in furtherance of a common design – Once such a conspiracy is proved, act of one conspirator becomes the act of the others – A Co-conspirator who joins subsequently and commits overt acts in furtherance of the conspiracy must also be held liable – Proceeding against accused cannot be quashed."*



31.7. Hon'ble Bombay High Court in the case of **CBI Vs. Bhupendra Champaklal Dalal 2019 SCC OnLine Bom 140,** ruled as under;

> ***"CHARGE FOR THE OFFENCE OF CRIMINAL BREACH OF TRUST:-***

Hon'ble Apex Court in the case of Ram Narain Poply Vs. Central Bureau of Investigation, AIR 2003 SC 2748, wherein the Hon'ble Apex Court has, at length, dealt with the charge of criminal conspiracy, in the backdrop of the similar allegations, in a case arising out of the decision of this Court in the matter of Harshad Mehta and others. While dealing with the essential ingredients of the offence of criminal conspiracy, punishable u/s. 120 B IPC, the Hon'ble Court was, in paragraph No.349 of its Judgment, pleased to hold that, "349. Privacy and secrecy are more characteristics of a conspiracy, than of a loud discussion in an elevated place open to public view. Direct evidence in proof of a conspiracy is seldom available, offence of conspiracy can be proved by either direct or circumstantial evidence. It is not always possible to give affirmative evidence about the date of the formation of the criminal conspiracy, about the persons who took part in the formation of the conspiracy, about the object, which the objectors set before themselves as the object of conspiracy, and about the manner in which

the object of conspiracy is to be carried out, all this is necessarily a matter of inference."

[Emphasis Supplied]

177. This Court can also place reliance on another landmark decision of the Hon'ble Apex Court in the case of State of Maharashtra Vs. SomNathThapa, (1996) 4 SCC 659, wherein the Hon'ble Apex Court was pleased to observe as follows :-

"24. The aforesaid decisions, weighty as they are, lead us to conclude that to establish a charge of conspiracy knowledge about indulgence in either an illegal act or a legal act by illegal means is necessary. In some cases, intent of unlawful use being made of the goods or services in question may be inferred from the knowledge itself. This apart, the prosecution has not to establish that a particular unlawful use was intended, so long as the goods or service in question could not be put to any lawful use. Finally, when the ultimate offence consists of a chain of actions, it would not be necessary for the

prosecution to establish, to bring home the charge of conspiracy, that each of the conspirators had the knowledge of what the collaborator would do, so long as it is known that the collaborator would put the goods or service to an unlawful use." [See State of Kerala v. P. Sugathan, (2000) 8 SCC 203, SCC p. 212, para 14]"."

[Emphasis Supplied]

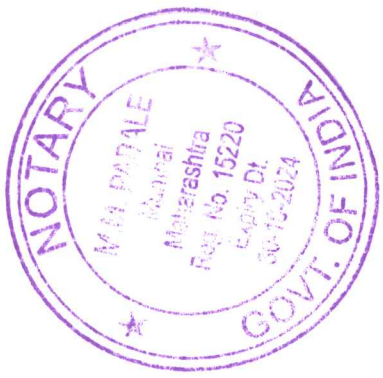
178. While dealing with the offence of criminal conspiracy in respect of the financial frauds, the Hon'ble Apex Court in the case of Ram NarainPoply (supra), in paragraph No.344, was pleased to observe that,

"344. ..... The law making conspiracy a crime, is designed to curb immoderate power to do mischief, which is gained by a combination of the means. The encouragement and support which co-conspirators give to one another rendering enterprises possible which, if left to individual effort, would have been impossible, furnish the ground for visiting conspirators and abettors with condign punishment. The conspiracy is held to be

continued and renewed as to all its members wherever and whenever any member of the conspiracy acts in furtherance of the common design."

[Emphasis Supplied]

179. In the context of Section 10 of the Indian Evidence Act, it was held by the Hon'ble Apex Court, in paragraph No.348, that, the expression "in furtherance to their common intention" in Section 10 is very comprehensive and appears to have been designedly used to give it a wider scope than the words "in furtherance of" used in the English Law : with the result anything said, done or written by co- conspirator after the conspiracy was formed, will be evidence against the other before he entered the field of conspiracy or after he left it. Anything said, done or written is a relevant fact only.



186. The Hon'ble Apex Court has further quoted with approval in paragraph No.101, the observations made in the case of State (NCT of Delhi) Vs. Navjot Sandhu @ Afsan Guru, (2005) 11 SCC 600, wherein it was held

that, "The cumulative effect of the proved circumstances should be taken into account in determining the guilt of the accused rather than adopting an isolated approach to each of the circumstances."

32. Proofs of earlier fraud and corruption done by Respondent No. 3 Iqbal Chahal in conspiracy with other accused.

32.1. That, frauds and corruption committed by the State Authorities **is ranging in Thousands of Crores in Covid management under the leadership of Chief Minister Shri Udhhav Thackery** as has been exposed by Shri Kirit Somaiya, Former, Minister of Parliamentary.

32.2. That, the summary of frauds related with covid-19 pandemic exposed by Bhartiya Janta Party's former M.P. Dr. Kirit Somaiyya are as under:

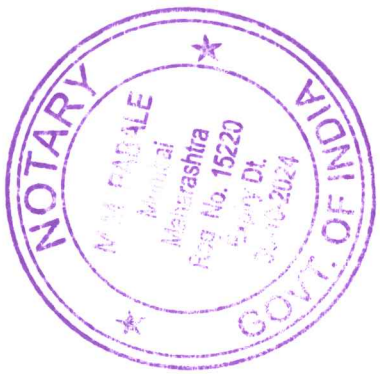
32.2.1. Fraud of around Rs. 2,100 crores in Covid-19 hospital. The land was sold by the government to a builder for Rs. 62 crores. However, in few hours the MCGM shown its willingness to purchase it for around Rs. 2,100 crores to build covid hospital. After intervention by Dr. Kirit Somaiyya said deal was cancelled.

32.2.2. MCGM invited tender for purchase of 1 Crore vaccine doses. All the 11 tenders were suspicious. Due to intervention by Dr. Kirit Somaiyya said process was cancelled.

32.2.3. The MCGM purchased 72,000 Remdesivir injections for covid treatment at the rate of Rs. 1668 per injection. While, at the same time the same injections were purchased by the Haffkine Institute for Rs. 668 means MCGM paid Rs. 1000 extra per injection for a purchase of 72,000 injection.

(A copy of fraud exposed by Mr Shir Kirit Somaiyya titled as **"Summary of frauds related with Covid-19 pandemic exposed by Bhartiya Janta Party's former M.P. Dr. Kirit Somaiyya"** marked and annexed herewith Exhibit **"DDDD"**

32. Now, having gone through all the facts stated above, The petitioner pleads to this Hon'ble Court to consider above mentioned concerns with a sense of urgency to pause the Illogical & Unscientific Use of the RT-PCR or RAT Test on asymptomatic people.



33. The Petitioner states that considering all the above facts and circumstances, there is need to stop testing asymptomatic healthy people.

34. In view of the above circumstances, the petitioners are left with no choice but to approach this Hon'ble Court to seeking direction to Respondent Maharashtra Government & Municipal bodies to stop testing asymptomatic healthy people in malls, railway stations, bus stands, markets, etc. as done earlier in the second wave and also to not to do contact tracing of asymptomatic contacts of a symptomatic case and further direction to concerned authorities to only test symptomatic.

35. That, the petitioner have no other remedy, none equally efficacious and as such are constrained to approach this Hon'ble High Court in their extra-ordinary jurisdiction under Article 226 and 227of the Constitution of India.

36. The petitioner have not moved either this Hon'ble Court or Supreme Court in the instant matter hereinbefore.

37. The petitioner state that the petitioner is approaching this Hon'ble High Court in reasonable time and hence the petition is not suffering from any delay or latches.

38. The petitioner state that the petitioners did not receive any caveat.

37. The petitioner crave leave of this Hon'ble High Court to add to, amend or delete any of the aforesaid para if so required by the interest of the Justice.

39. The petitioners are paying the necessary court fees.

40. PRAYERS: The petitioner, therefore, prays that, this Hon'ble Court may pleased to;

a) Direct CBI to investigate and to submit the report about misappropriation of public funds of hundreds of Crores in malafidely and deliberately testing asymptomatic people and then creating fear on the basis of faulty data with ulterior purpose, which is a serious offence punishable under section 409 r/w 120(B), 34, 52 etc. of Indian Penal Code;

b) Direct Respondent, Maharashtra Government its municipal bodies & all authorities within Maharashtra to stop wasting of public money and testing asymptomatic healthy people in malls, railway stations, bus stands, markets, etc., as done by them earlier in the second wave;

c) Direct labs & anyone conducting RTPCR or RAT tests, to only process those samples on the basis of doctors prescription which shows that the patient is symptomatic;

d) Direct Maharashtra Government to not to insist for negative test of asymptomatic people to enter into the state or cities in Maharashtra, for vaccinated as well as unvaccinated people.

e) Direct state officials to not to do contact tracing of asymptomatic contacts of a symptomatic case and follow this rule throughout as done earlier;

f) Direct authorities to only test symptomatics, and in those with Covid symptoms, an RAT test or an RT-PCR test should be conducted, but the RT-PCR cycle threshold should be reduced to 24. No retest should be conducted with a PCR, if someone tests negative with the RAT.

g) Direct all private bodies, including societies, companies, schools, universities, banks etc., & all government officials/bodies to not mandatorily

demand negative test reports from asymptomatic healthy people.

h) Direct authorities to implement body temperature testing instead of RT-PCR or RAT Test of persons entering Maharashtra state, railway stations, malls, etc., as for symptomatic people suffering from an acute infection, the temperature of the body will increase. As being done by Holkar Airport in Indore, MP.

i) Any other relief in the interest of Justice may be granted

Dated this _____ day of January, 2022 at Mumbai

ADV. Mangesh B. Dongre (O.S. 18213)
Address: 2 & 3, Kothari House, 5/7 Oak

Lane, A R Allana Marg, Near Burma
Burma Restaurant, Fort, Mumbai 400 023
Email: mangeshdongre4@gmail.com
Mob: +91- 720845690

Petitioner
Amber H. Koiri

VERIFICATION

I, **Amber Koiri**, the Petitioner abovenamed residing at [redacted] do hereby state on solemn affirmation that whatever stated in the foregoing **Paragraph No. 1 to 40** is true and correct to the best of my knowledge and belief and **Para 40** are my humble prayers before this Hon'ble High Court.

Solemnly affirmed at Mumbai)

This 10 day of January, 2022) Before me,

NOTARY M. M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

Interpreted and explained by me

Advocate for the Petitioner.

Petitioner

NOTARY M. M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 NOTARIAL

NOTARY M. M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 NOTARIAL

BEFORE ME

MANISH M. PABALE
B.Sc.LL.M.
ADVOCATE & NOTARY (GOVT. OF INDIA)
104, Natwar Chambers,
94 Nagindas Master Road,
Fort, Mumbai - 400 001.

NOTED & REGISTERED
Page No. 93/24 Sr. No. 636
Date 10 JAN 2022
ID / Aadhar / PAN / DL: 534203880642
Seen Org. / POA / Board Resol.: .....

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IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

AMBAR H. KOIRI]

]

]

]...**PETITIONER**

Versus

1. State of Maharashtra]
Through Chief Secretary & Chairman]
State Disaster Management Committee]
The Government of Maharashtra,]
Mantralaya, Mumbai- 400 023]

2. Shri. Iqbal Chahal]
The Muncipal Commissioner,]
M.C.G.M. Annex Building,]
Mahapalika Marg,]
C.S.T, Mumbai, 400001.]

3. Suresh Kakani]

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

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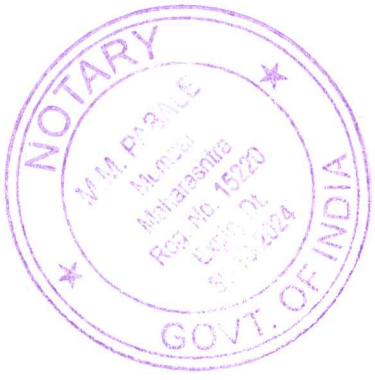
Addl. Municipal Commissioner]
Mahapalika Marg,]
C.S.T, Mumbai, 400001.]

4. Ministry of Health And Family]
Through Chief Secretary]
Government of India]

5. National Disaster Management Authority,]
Through its Chairperson,]
Safdarjung Enclave, NDMA Bhawan,]
A-1, Block A-1, Nauroji Nagar,]
New Delhi, Delhi - 110029.]

6. State Disaster Management Authority,]
Through it's chairperson,]
Revenue and Forest Department,]
Maharashtra State Disaster Management]
Authority, Mantralaya, Mumbai - 400032.]



7. Shri Manish Joshi]
Dy. Commissioner, Health]
New Administrative Building, Chandan Wadi,]
Pachpakhadi, Mahapalika Bhavan Rd,]
Thane West, Thane, Maharashtra 400602] ....**Respondents**

(122)

To,

Prothonotary and Sr. Master,

Ordinary Original Civil Jurisdiction

High Court, Bombay.

VAKALATNAMA

Sir,

I, **Amber H. Koiri,** the petitioner abovenamed do hereby appoint Mr. **Mangesh B. Dongre,** Advocate High Court to act, plead and appear for me in the above matter.

IN WITNESS WHERE OF, we have set and subscribed our hands to this writing at mumbai.

Date this day of January, 2022

I accepted:

Advocate for Petitioner

ADV. MANGESH DONGRE (O.S. 18213)

Address: 2 & 3, Kothari House, 5/7 Oak Lane,

A R Allana Marg, Near Burma Burma Restaurant,

Fort, Mumbai 400 023

Email: mangeshdongre4@gmail.com

Mob: +91- 9664815212

Petitioner

Amber H. Koiri

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30.10.2024 GOVT. OF INDIA

IN THE HIGH COURT OF JUDICATURE AT BOMBAY
ORDINARY ORIGINAL CIVIL JURISDICTION
WRIT PETITION NO. OF 2022

Amber H. Koiri .... Petitioner

Versus

State of Maharashtra & Ors. .... Respondents

VAKALATNAMA

Dated this _____day of January, 2021

NOTARY
M.M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
GOVT. OF INDIA

ADV. MANGESH DONGRE (O.S. 18213)
Address: 2 & 3,Kothari House, 5/7 Oak Lane,
A R Allana Marg, Near Burma Burma Restaurant,
Fort, Mumbai 400 023
Email: mangeshdongre4@gmail.com
Mob: +91- 9664815212

(123)

IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

Amber H. Koiri **.... Petitioner**

Versus

State of Maharashtra & Ors. **...Respondents**

MEMORANDUM OF REGD. ADDRESS

AMBAR H. KOIRI

NOTARY
M.M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

Petitioner

(Amber H. Koiri)

(124)

IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

Amber H. Koiri .... Petitioner

Versus

State of Maharashtra & Ors. ...Respondents

LIST OF DOCUMENTS

Sr No.	Particular
1.	**Exhibit "A"** A copy of an article was published in the British Medical Journal titled: **"Evidence of asymptomatic spread is insufficient to justify mass testing for Covid-19"**, dated **16 November, 2020.**
2.	**Exhibit "B"** A copy of an article was published in Nature Communication titled as **"Post-lockdown SARS-CoV-2 nucleic acid screening in nearly ten million residents of Wuhan, China"**, dated **20 November, 2020.**
3.	**Exhibit "C"** A copy of study titled as **"Coronavirus Disease Outbreak in Call Center, South Korea"** dated **26 August, 2020.**
4.	**Exhibit "D"** A copy of study titled as **"Contact Tracing Assessment of COVID-19 Transmission Dynamics in Taiwan and Risk at Different Exposure Periods Before and After Symptom Onset"** dated **1st May, 2020.**
5.	**Exhibit "E"**

NOTARY M.M. PABALE Mumbai Maharashtra Regn No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

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	A copy of study titled as **"A study on infectivity of asymptomatic SARS - CoV-2 carriers"** dated **13 May, 2020.**
6.	**Exhibit "F"** A copy of study titled as **"Modes of Contact and Risk of Transmission in COVID-19: A Prospective Cohort Study 4950 Close Contact Persons in Guangzhou of China"** dated **9 April, 2020.**
7.	**Exhibit "G"** (A copy of study titled as **"Secondary Transmission of Coronavirus Disease from Pre symptomatic Persons, China"** dated **26 August, 2020.**
8.	**Exhibit "H"** A copy of study titled as **"Transmission potential of asymptomatic and paucisymptomatic SARS-CoV-2 infections: a three-family cluster study in China"**, dated **22 April, 2020.**
9.	**Exhibit "I"** A copy of study titled as **"Contact tracing and isolation of asymptomatic spreaders to successfully control the COVID-19 epidemic among healthcare workers in Milan (Italy)"**, dated **08 May, 2020.**
10.	**Exhibit "J"** A copy of study titled as **"Analysis of SARS-CoV-2 Transmission in Different Settings, Brunei"**, dated **26 November, 2020.**
11.	**Exhibit "K"**

NOTARY M.N. PABALE Mumbai Maharashtra Regd. No. 15220 GOVT. OF INDIA

	A copy of study titled as **"Transmission of COVID-19 in 282 clusters in Catalonia, Spain: a cohort study"**, dated 21 May, 2021.
12.	**Exhibit "L"** A copy of article published in Jama Network titled as **"SARS-CoV-2 Transmission from People Without COVID-19 Symptoms"**, dated **7 January, 2021.**
13.	**Exhibit "M"** A copy of article published in nature.com titled as **"Temporal dynamics in viral shedding and transmissibility of COVID-19 "** dated **15 April, 2020.**
14.	**Exhibit "N"** A copy of article published in Jama Network titled as **"Asymptomatic and presymptomatic transmission of SARS-CoV-2: A systematic review "** dated **17 June, 2020.**
15.	**Exhibit "O"** A copy of scientific brief published on WHO website titled as **"Transmission of SARS-CoV-2: implications for infection prevention precautions"** dated **9 July, 2020.**
16.	**Exhibit "P"** A copy of study titled as **"The blood DNA virome in 8,000 humans"** dated **22 March, 2017.**
17.	**Exhibit "Q"** A copy of study titled as **"Metagenomic analysis of double-stranded DNA viruses in healthy adults"** dated **10 September, 2014.**
18.	**Exhibit "R"**

NOTARY M.M. PABALE Mumbai Maharashtra Regd No. 15220 Exp. On 30-10-2024 GOVT. OF INDIA

	A copy of article published in Economic Times titled as **"Healthy humans carry viruses too!"**, dated **17 September, 2014.**
19.	**Exhibit "S"** A copy of study titled as **"*Redondoviridae*, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness"** dated **8 May, 2019.**
20.	**Exhibit "T"** A copy of article published in Medicine Net titled as **"PCR (Polymerase Chain Reaction)"**
21.	**Exhibit "U"** A copy of study titled as **"Issues with the RT-PCR Coronavirus Test"**, dated **23 April, 2020.**
22.	**Exhibit "V"** A copy of study titled as **"CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel"** dated **21 July, 2021.**
23.	**Exhibit "W"** A copy of article published in **ijms.in** titled as **"COVID diagnostics: Do we have sufficient armamentarium for the present and the unforeseen?".**
24.	**Exhibit "X"** A copy of article published in ijms.in titled as **"Are you infectious if you have a positive PCR test result for COVID-19?"** dated **5 August, 2020.**
25.	**Exhibit "Y"**

NOTARY N.M. PABALE Mumbai Maharashtra Regd. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

	A copy of study titled as **"COVID diagnostics: Do we have sufficient armamentarium for the present and the unforeseen?"** dated **10 September, 2020.**
26.	**Exhibit "Z"** A copy of article published titled as **"Correlation Between 3790 Quantitative Polymerase Chain Reaction-Positives Samples and Positive Cell Cultures, Including 1941 Severe Acute Respiratory Syndrome Coronavirus 2 Isolates''** dated **1 June, 2021.**
27.	**Exhibit "AA"** A copy of article published titled as **"Predicting Infectious Severe Acute Respiratory Syndrome Coronavirus 2 From Diagnostic Samples''** dated **17 December, 2020.**
28.	**Exhibit "BB"** A copy of article published titled as **"Viral cultures for COVID-19 infectivity assessment – a systematic review''** dated **29 September, 2020.**
29.	**Exhibit "CC"** A copy of Article published tilted as **"Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore"** dated **3 March, 2020.**
30.	**Exhibit "DD"** A copy of Article published tilted as **"Your Coronavirus Test Is Positive. Maybe It Shouldn't Be."** dated **29 August, 2020.**
31.	**Exhibit "EE"** A copy of article published titled as **"PCR positives: what do they mean?''** dated **17 September, 2020.**
32.	**Exhibit "FF"**

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

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	A copy of Revised Discharge Policy for COVID-19
33.	**Exhibit "GG"** A copy of **''Advisory for COVID-19 testing during the second wave of the pandemic''** dated **04.05.2021.**
34.	**Exhibit "HH"** A copy of article titled as **''Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2''**dated **13.01.2021.**
35.	**Exhibit "II"** A copy of article published in **'The New York Times'** titled as **''Faith in Quick Test Leads to Epedemic That Wasn't''** dated **22.01.2007.**
36.	**Exhibit "JJ"** A copy of article published in **'Nature'** titled as **''Fast coronavirus tests: what they can and can't do''** dated **16 September, 2020.**
37.	**Exhibit "KK"** A copy of article published in **'NDTV'** titled as **''50% Covid Tests In Mumbai Are Less Reliable Antigen Tests, Data Shows''** dated **30 March, 2021.**
38.	**Exhibit "LL"** A copy of article published in **'mint'** titled as **''Need to stop second COVID-19 peak; 70% RT-PCR test must for states: PM Modi''** dated **17 March, 2021.**
39.	**Exhibit "MM"** A copy of research study titled as **''Antigen-based testing but not real-time PCR correlates with SARS-CoV-2 virus culture''** dated **October 05, 2020.**

NOTARY M.M. PABALE Mumbai Maharashtra Regd. No. 15220 Expiry Dt. 30-09-2024 GOVT. OF INDIA

40.	**Exhibit "NN"** A copy of research study titled as **''Evaluation of Abbott BinaxNOW Rapid Antigen Test for SARS-CoV-2 Infection at Two Community-Based Testing Sites — Pima County, Arizona, November 3–17, 2020''** dated **22 January, 2021.**
41.	**Exhibit "OO"** A copy of research study titled as **''Evaluation of a SARS-CoV-2 rapid antigen test: Potential to help reduce community spread?''** dated **05 December, 2020.**
42.	**Exhibit "PP"** A copy of article titled as **''Potential for False Positive Results with Antigen Tests for Rapid Detection of SARS-CoV-2 - Letter to Clinical Laboratory Staff and Health Care Providers''** dated **11.03.2020.**
43.	**Exhibit "QQ"** A copy of research study titled as **"Challenges and Controversies to Testing for COVID-19"** dated **21 October, 2020.**
44.	**Exhibit "RR"** A copy of Judgment Passed by Hon'ble Lisbon Court of Appeal **"MARGARIDA RAMOS DE ALMEIDA"** dated **11 November, 2020.**
45.	**Exhibit "SS"** A copy of article titled as **''Portuguese Court Rules PCR Tests "Unreliable" & Quarantines "Unlawful"** dated **20.11.2020.**
46.	**Exhibit "TT"**

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

(13:1)

	A copy of article titled as **"NEGATIVE TEST MANDATORY FOR ENTRY TO NL"** dated **04 January, 2021.**
47.	**Exhibit "UU"** A copy of article titled as **"Austrian Court Rules PCR Unsuited For COVID, Lockdowns Unlawful"** dated **15 April, 2021.**
48.	**Exhibit "VV"** A copy of article titled as **"80% Covid patients in India are asymptomatic, health ministry analysis finds"** dated **24 August, 2020.**
49.	**Exhibit "WW"** A copy of article published in **'Hindustan Times'** titled as **"71% active Covid cases in Mumbai asymptomatic"** dated **7 December, 2020.**
50.	**Exhibit "XX"** A copy of article published in **'NDTV'** titled as **"85,000 Covid Cases In Second Wave, Most Asymptomatic: Mumbai Civic Body"** dated **30 March, 2021.**
51.	**Exhibit "YY"** A copy of article published in **'DECCAN HERALD'** titled as **"Majority of Bengaluru's Covid-19 patients are asymptomatic"** dated **19 April, 2021.**
52.	**Exhibit "ZZ"** A copy of article published in **'PUNEKAR NEWS'** titled as **"Need To Focus On Asymptomatic COVID Patients: ICMR"** dated **04 April, 2021.**
53.	**Exhibit "AAA"**

NOTARY M. M. PABALE Mumbai Maharashtra Regn No. 15220 Expiry Dt. 30-10-2021 GOVT OF INDIA

	A copy of **RTI Reply on RTPCR Asymptomatic by ICMR** dated **11 August, 2021.**
54.	**Exhibit "BBB"** A copy of statistic of deaths in India Compared to the size of the population of 140 crore.
55.	**Exhibit "CCC"** A copy of article published in **'Hindustan Times'** titled as **"One year of Covid-19: How India fought the virus"** dated **01 March, 2021.**
56.	**Exhibit "DDD"** A copy of study titled as **"Estimating the wave 1 and wave 2 infection fatality rates from SARS-CoV-2 in India"** dated **8 July, 2021.**
57.	**Exhibit "EEE"** A copy of study titled as **"Infection fatality rate of COVID-19 inferred from seroprevalence data"** dated **14 October, 2020.**
58.	**Exhibit "FFF"** **A copy of study titled as "COVID-19 Case Fatality Rate: Misapprehended Calculations"** dated **21 August, 2020.**
59.	**Exhibit "GGG"** A copy of study titled as **"Covid-19 CFR and IFR Confused"** dated **08 September, 2021.**
60.	**Exhibit "HHH"** A copy of article published in **'INDIA TODAY'** titled as **"Mumbai: BMC to conduct 47,000 random Covid tests every day, refusing one can land you in trouble"** dated **20 March, 2021.**

NOTARY M.M. PABALE Mumbai Maharashtra Reg No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

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61.	**Exhibit "III"** A copy of article published in **'The Print'** titled as **"Why Mumbai is relying more on antigen tests than RT-PCR as it doubles Covid testing"** dated **2 April, 2021.**
62.	**Exhibit "JJJ"** A copy of article published in **'The Times of India' titled as "Daily Covid-19 tests must be raised to 40,000 in Mumbai: Iqbal Singh Chahal"** dated **3 May, 2021.**
63.	**Exhibit "KKK"** **A copy of article published in 'The Times of India' titled as "Covid-19: Big dip in Mumbai cases, the worst may be over" dated 4 May, 2021.**
64.	**Exhibit "LLL"** **A copy of article published in 'Hindustan Times' titled as "As cases drop, number of covid tests halved in Mumbai" dated 18 May, 2021.**
65.	**Exhibit "MMM"** **A copy of article published in 'Hindustan Times' titled as "Maharashtra sees spike in single-daily Covid-19 cases as testing increases" dated 29 June, 2021.**
66.	**Exhibit "NNN"** A copy of article published in **'The Times of India' titled as "With a drop in testing over the weekend, fewer Covid-19 detetections in Maharashtra & Mumbai"** dated **10 August, 2021**
67.	**Exhibit "OOO"**

NOTARY
M.M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

	A copy of article published in **'The Times of India'** titled as **"As tests increase, Covid cases tick up in Mumbai, positivity still under 1%"** dated **19 August, 2021.**
68.	**Exhibit "PPP"** A copy of article published in **'The Times of India'** titled as **"Maharashtra: Covid dip likely due to weekend effect on tests"** dated **30 August, 2021.**
69.	**Exhibit "QQQ"** A copy of article published in **'The Times of India'** titled as **"Covid test must for visitors to Navi Mumbai malls"** dated **16th June, 2021.**
70.	**Exhibit "RRR"** A copy of article published in **'India Today'** titled as **"BMC braces for Covid's third wave in Mumbai. Here's how"** dated **11 August, 2021.**
71.	**Exhibit "SSS"** A copy of article published in **'The Times of India'** titled as **"As tests increase, Covid cases tick up in Mumbai, positivity still under 1%"** dated **19 August, 2021.**
72.	**Exhibit "TTT"** A copy of article published in **'Hindustan Times'** titled as **"Third wave: Mandatory testing of all close contacts of Covid patients in Mumbai"** dated **27 August, 2021.**
73.	**Exhibit "UUU"** A copy of article published in **'Hindustan Times'** titled as **"Navi Mumbai to increase Covid-19 testing ahead of festive season"** dated **5 September, 2021.**
74.	**Exhibit "VVV"**

NOTARY M.M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

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	A copy of article published in **'Mumbai Live'** titled as **"State COVID Task Force Cautions Towards New Symptoms Of COVID-19"** in the month of October.
75.	**Exhibit "WWW"** A copy of article published in **'india.com'** titled as **"Maharashtra Lockdown Update: Third Wave of Corona Hits Nagpur, Fresh Restrictions to be Announced Soon, Says Minister"** dated **7 September, 2021.**
76.	**Exhibit "XXX"** A copy of article published in **'NDTV'** titled as **"Covid Third Wave Here Already," Says Mumbai Mayor"** dated **7th September, 2021.**
77.	**Exhibit "YYY"** A copy of article published in **'india.com'** titled as **"Maharashtra Lockdown Big Update: Deputy CM Warns of Shutting Down Everything if Covid Third Wave Hits"** dated **3 September, 2021.**
78.	**Exhibit "ZZZ"** A copy of article titled as **"Odisha issues guidelines for stricter implementation of COVID norms in schools amid surge in infection among children"** dated **8th September, 2021.**
79.	**Exhibit "AAAA"** A copy of Guidelines by ICMR titled as **"Advisory for COVID-19 testing during the second wave of the pandemic"** dated **4th May, 2021.**
80.	**Exhibit "BBBB"**

NOTARY M.M. PABALE Mumbai Maharashtra Regd. No. 15220 Expiry Dt. 30-10-2024 GOVT. OF INDIA

	A copy of letter dated **1.12.2021** being outward No. **D.O.No.01/S(HFW) Omicron/ Maha/2021** by Rajesh Bhushan, Ministry of Health & Family Welfare, Govt. of India.
81.	**Exhibit "CCCC"** A copy of **scientific studies by Director General Health of Republic of South Africa policy** dated **23.12.2021.**
82.	**Exhibit "DDDD"** A copy of fraud exposed by Mr Shir Kirit Somaiyya titled as **"Summary of frauds related with Covid-19 pandemic exposed by Bhartiya Janta Party's former M.P. Dr. Kirit Somaiyya"**
83.	**Exhibit "EEEE"** A copy of Guidelines issued by Brihanmumbai Municipal Corporation BMC dated **27.11.2021.**
84.	**Exhibit "FFFF"** A copy of Guidelines issued by **Thane Municipal Corporation, TMC** dated **06.01.2022.**
85.	**Exhibit "GGGG"** A copy of guidelines titled as **"Revised guidelines for Home Isolation of mild /asymptomatic COVID-19 cases"**, published by Ministry of Health & Family Welfare dated **5th January, 2022.**

NOTARY
N. M. PAREKH
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

EXHIBIT "A"

Mass Testing for Covid-19 in the UK

Source: The bmj

Link: https://www.bmj.com/content/371/bmj.m4436/rr-10

Published on: 16 November 2020

Rapid Response:

Evidence of asymptomatic spread is insufficient to justify mass testing for Covid-19

Dear Editor,

Whilst we would take issue with Lateral Flow tests being the main culprit, Mike Gill is absolutely correct to criticise mass testing programmes.

His ire should really be directed, though, at PCR testing. Data from PCR testing – for which there is no proper determination of an end-to-end operational false positive rate – has almost exclusively dictated tier restrictions and lockdown policy in the UK.

PCR's fingerprints can in fact be found all over the entire global response to this pandemic. Testing with Lateral Flow, other antigen tests and bedside PCR tests are all finding far fewer cases than diagnosed by PCR testing. Even a low sensitivity for all these other tests could not account for the size of the discrepancy.

Mass testing and accompanying harmful lockdown policies are justified on the assumption that asymptomatic transmission is a genuine risk. Given the harmful

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NOTARY A. M. PABALE Mumbai Maharashtra Regd. No. 15220 Expiry On 31-10-2024 GOVT. OF INDIA

collateral effects of such policies, precautionary principle should result in a very high evidential bar for asymptomatic transmission being set. However, the only word which can be used to describe the quality of evidence for this is woeful.

It is important to carefully distinguish purely asymptomatic (individuals who never develop any symptoms) from pre-symptomatic transmission (where individuals do eventually develop symptoms). To the extent that the latter phenomenon - which has in fact happened only very rarely - is deemed worthy of public health action, appropriate strategies to manage it (in the absence of significant asymptomatic transmission) would be entirely different and much less disruptive than those actually adopted.

Many early studies which purported to demonstrate the phenomenon of asymptomatic transmission were from China, yet the fact that Chinese studies are only published following government approval must bring into question their reliability (1). Nevertheless, the high volume of these studies spawned significant salience of the issue within the medical community, and an assumption of the likelihood of asymptomatic transmission being an important contributory factor. There then followed a number of meta-analyses examining the issue of asymptomatic transmission which tended to aggregate and give equal weight to studies regardless of origin or quality. In this way, these meta-analyses, given undue credibility by their association with reputable universities, amplified minimal evidence of asymptomatic spread to an importance the data did not warrant.

As reported in a manuscript submitted to this journal and also to medRvix on 16 Dec 2020 (the latter available for download shortly), we examined the papers most frequently cited in support of the existence of asymptomatic transmission. Even despite our criticisms of the sources of the data above, we did in fact find only 6 case reports of viral transmission by people who throughout remained

asymptomatic, and this was to a total of 7 other individuals, however all of these were in studies with questionable methodology.

Moreover in all these studies, confirmation of "cases" was made via PCR testing without regard to the possibility that any of the cases found might be false positives. The case numbers found, are, in any event extremely small and certainly not sufficient to conclusively determine that asymptomatic transmission is a major component of spread.

It is also notable that, in what would seem to represent an abrupt volte face by the CCP, a further (presumably government-approved) study from China was recently published (2) which entirely contradicts the earlier conclusions regarding the phenomenon of asymptomatic transmission, which had been driven by Chinese data in particular, early in the pandemic.

Some might conclude that that study lacks the credibility one might expect for a paper published in Nature; it is claimed, for example, that they PCR-tested 92% of Wuhan's population (~10m individuals) over a 19-day period at the end of May, and found just 300 positive PCR tests, implying a FPR of no greater than 0.003%. Further, it is claimed that while 100% of the 300 PCR positive cases were asymptomatic, there were zero symptomatic PCR positive cases out of ~10m tested during a period only a few weeks after the epidemic had peaked in Wuhan.

If this seems incredulous, then surely that has serious implications for the way in which earlier studies from China - data from which formed a significant part of the worldwide evidence base for asymptomatic transmission - should be regarded.

Jonathan Engler MBChB LLB

EXHIBIT "B"

140 nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-020-19802-w OPEN

Post-lockdown SARS-CoV-2 nucleic acid screening in nearly ten million residents of Wuhan, China

Shiyi Cao[1,11], Yong Gan[1,11], Chao Wang[1,11], Max Bachmann[2], Shanbo Wei[3], Jie Gong[4], Yuchai Huang[1], Tiantian Wang[1], Liqing Li[5], Kai Lu[6], Heng Jiang[7,8], Yanhong Gong[1], Hongbin Xu[1], Xin Shen[1], Qingfeng Tian[9], Chuanzhu Lv[10]✉, Fujian Song[2]✉, Xiaoxv Yin[1]✉ & Zuxun Lu[1]✉

Stringent COVID-19 control measures were imposed in Wuhan between January 23 and April 8, 2020. Estimates of the prevalence of infection following the release of restrictions could inform post-lockdown pandemic management. Here, we describe a city-wide SARS-CoV-2 nucleic acid screening programme between May 14 and June 1, 2020 in Wuhan. All city residents aged six years or older were eligible and 9,899,828 (92.9%) participated. No new symptomatic cases and 300 asymptomatic cases (detection rate 0.303/10,000, 95% CI 0.270–0.339/10,000) were identified. There were no positive tests amongst 1,174 close contacts of asymptomatic cases. 107 of 34,424 previously recovered COVID-19 patients tested positive again (re-positive rate 0.31%, 95% CI 0.423–0.574%). The prevalence of SARS-CoV-2 infection in Wuhan was therefore very low five to eight weeks after the end of lockdown.

[1] Department of Social Medicine and Health Management, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. [2] Norwich Medical School, Faculty of Medicine and Health Science, University of East Anglia, Norwich, UK. [3] Wuhan Municipal Health Commission, Wuhan, Hubei, China. [4] Wuhan Centre for Clinical Laboratory, Wuhan, Hubei, China. [5] Department of Management Science and Engineering, School of Economics and Management, Jiangxi Science and Technology Normal University, Nanchang, Jiangxi, China. [6] Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, China. [7] Centre for Alcohol Policy Research, School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia. [8] Melbourne School of Population and Global Health, University of Melbourne, Melbourne, VIC, Australia. [9] School of Public Health, Zhengzhou University, Zhengzhou, Henan, China. [10] Department of Emergency, Hainan Clinical Research Centre for Acute and Critical Diseases, The Second Affiliated Hospital of Hainan Medical University, Haikou, Hainan, China. [11] These authors contributed equally: Shiyi Cao, Yong Gan, Chao Wang. ✉email: lyuchuanzhu@hainmc.edu.cn; Fujian.song@uea.ac.uk; yxx@hust.edu.cn; zuxunlu@yahoo.com

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The Coronavirus Disease 2019 (COVID-19) was first reported in December 2019, and was classified as a pandemic by the World Health Organization on March 11, 2020[1]. Following strict lockdown measures, the COVID-19 epidemic was generally under control in China, and the whole country has progressed into a post-lockdown phase. In this phase, countries face new problems and challenges, including how to accurately assess the post-lockdown risk of the COVID-19 epidemic, how to avoid new waves of COVID-19 outbreaks, and how to facilitate the resumption of economy and normal social life. As the city most severely affected by COVID-19 in China, Wuhan had been under lockdown measures from January 23 until April 8, 2020. During the first 2 months after city's reopening, there were only a few sporadic COVID-19 cases in Wuhan (six newly confirmed cases from April 8 to May 10, 2020[2]). However, there was still concern about the risk of COVID-19 in Wuhan, which seriously affected the resumption of industrial production and social services, and hampered the normal lives of residents. In order to ascertain the current status of the COVID-19 epidemic, the city government of Wuhan carried out a comprehensive citywide nucleic acid screening of SARS-CoV-2 infection from May 14, 2020 to June 1, 2020.

The citywide screening of SARS-CoV-2 infection in Wuhan is a mass screening programme in post-lockdown settings, and provided invaluable experiences or lessons with international relevance as more countries and cities around the world entering the post-lockdown phase. In this study, we report the organisation process, detailed technical methods used, and results of this citywide nucleic acid screening.

Results

There were 10,652,513 eligible people aged ≥6 years in Wuhan (94.1% of the total population). The nucleic acid screening was completed in 19 days (from May 14, 2020 to Jun 1, 2020), and tested a total of 9,899,828 persons from the 10,652,513 eligible people (participation rate, 92.9%). Of the 9899,828 participants, 9,865,404 had no previous diagnosis of COVID-19, and 34,424 were recovered COVID-19 patients.

The screening of the 9,865,404 participants without a history of COVID-19 found no newly confirmed COVID-19 cases, and identified 300 asymptomatic positive cases with a detection rate of 0.303 (95% CI 0.270–0.339)/10,000. The median age-stratified CI-values of the asymptomatic cases were shown in Supplementary Table 1. Of the 300 asymptomatic positive cases, two cases came from one family and another two were from another family. There were no previously confirmed COVID-19 patients in these two families. A total of 1174 close contacts of the asymptomatic positive cases were traced, and they all tested negative for the COVID-19. There were 34,424 previously recovered COVID-19 cases who participated in the screening. Of the 34,424 participants with a history of COVID-19, 107 tested positive again, giving a repositive rate of 0.310% (95% CI 0.423–0.574%).

Virus cultures were negative for all asymptomatic positive and repositive cases, indicating no "viable virus" in positive cases detected in this study.

All asymptomatic positive cases, repositive cases and their close contacts were isolated for at least 2 weeks until the results of nucleic acid testing were negative. None of detected positive cases or their close contacts became symptomatic or newly confirmed with COVID-19 during the isolation period. In this screening programme, single and mixed testing was performed, respectively, for 76.7% and 23.3% of the collected samples. The asymptomatic positive rates were 0.321 (95% CI 0.282–0.364)/10,000 and 0.243 (95% CI 0.183–0.315)/10,000, respectively.

The 300 asymptomatic positive persons aged from 10 to 89 years, included 132 males (0.256/10,000) and 168 females (0.355/10,000). The asymptomatic positive rate was the lowest in children or adolescents aged 17 and below (0.124/10,000), and the highest among the elderly aged 60 years and above (0.442/10,000) (Table 1). The asymptomatic positive rate in females (0.355/10,000) was higher than that in males (0.256/10,000).

The asymptomatic positive cases were mainly domestic and unemployed residents (24.3%), retired older adults (21.3%), and public service workers (11.7%) (Fig. 1).

The asymptomatic positive rate in urban districts was on average 0.456/10,000, ranging from 0.317/10,000 in Hongshan to 0.807/10,000 in Wuchang district. A lower rate of asymptomatic positive cases was found in suburban districts (0.132/10,000), ranging from 0.047/10,000 in Xinzhou to 0.237/10,000 in Jiangan district (Fig. 2).

Among the 7280 residential communities in Wuhan, asymptomatic positive cases were identified in 265 (3.6%) communities (only one case detected in 246 communities), while no asymptomatic positive cases were found in other 96.4% communities.

Testing of antibody against SARS-CoV-2 virus was positive IgG (+) in 190 of the 300 asymptomatic cases, indicating that 63.3% (95% CI 57.6–68.8%) of asymptomatic positive cases were actually infected. The proportion of asymptomatic positive cases with both IgM (−) and IgG (−) was 36.7% (95% CI: 31.2–42.4%; $n = 110$), indicating the possibility of infection window or false positive results of the nucleic acid testing (Table 2).

Higher detection rates of asymptomatic infected persons were in Wucharg, Qingshan and Qiaokou districts, and the prevalence of previously confirmed COVID-19 cases were 68.243/10,000, 53.767/10,000, and 100.047/10,000, respectively, in the three districts. Figure 3 shows that districts with a high detection rate of asymptomatic positive persons generally had a high prevalence of confirmed COVID-19 cases ($r_s = 0.729$, $P = 0.002$).

Discussion

The citywide nucleic acid screening of SARS-CoV-2 infection in Wuhan recruited nearly 10 million people, and found no newly confirmed cases with COVID-19. The detection rate of asymptomatic positive cases was very low, and there was no evidence of transmission from asymptomatic positive persons to traced close contacts. There were no asymptomatic positive cases in 96.4% of the residential communities.

Previous studies have shown that asymptomatic individuals infected with SARS-CoV-2 virus were infectious[3], and might subsequently become symptomatic[4]. Compared with symptomatic patients, asymptomatic infected persons generally have low quantity of viral loads and a short duration of viral shedding, which decrease the transmission risk of SARS-CoV-2[5]. In the present study, virus culture was carried out on samples from asymptomatic positive cases, and found no viable SARS-CoV-2 virus. All close contacts of the asymptomatic positive cases tested negative, indicating that the asymptomatic positive cases detected in this study were unlikely to be infectious.

There was a low repositive rate in recovered COVID-19 patients in Wuhan. Results of virus culturing and contract tracing found no evidence that repositive cases in recovered COVID-19 patients were infectious, which is consistent with evidence from other sources. A study in Korea found no confirmed COVID-19 cases by monitoring 790 contacts of 285 repositive cases[6]. The official surveillance of recovered COVID-19 patients in China also revealed no evidence on the infectiousness of repositive cases[7]. Considering the strong force of infection of COVID-19[8–10], it is expected that the number of confirmed cases is associated with the risk of being infected in communities. We

Table 1 Characteristics of asymptomatic positive individuals.

	Total (%)	Asymptomatic positive persons (%)	Detection rate per 10,000 (95% CI)	P value
Total	9,899,828 (100.0)	300 (100.0)	0.303 (0.270–0.339)	
Sex				
Male	5,162,960 (52.2)	132 (44.0)	0.256 (0.214–0.303)	0.005
Female	4,736,868 (47.8)	168 (56.0)	0.355 (0.303–0.413)	
Age (years old)				
≤17	969,014 (9.8)	12 (4.0)	0.124 (0.064–0.216)	<0.001
18–44	4,448,230 (44.9)	104 (34.7)	0.234 (0.191–0.283)	
45–59	2,492,943 (25.2)	96 (32.0)	0.385 (0.312–0.470)	
≥60	1,989,641 (20.1)	88 (29.3)	0.442 (0.355–0.545)	
Administrative Districts in Wuhan				
Wuchang	904,636 (9.1)	73 (24.3)	0.807 (0.633–1.015)	<0.001
Qingshan	414,312 (4.2)	23 (7.7)	0.555 (0.352–0.833)	
Qiaokou	583,440 (5.9)	32 (10.7)	0.548 (0.375–0.774)	
Hanyang	717,429 (7.2)	29 (9.7)	0.404 (0.271–0.581)	
Jiangtan	524,224 (5.3)	19 (6.3)	0.362 (0.218–0.566)	
Hongshan	1,103,079 (11.1)	35 (11.7)	0.317 (0.221–0.441)	
East Lake High-tech Development Area	782,987 (7.9)	19 (6.3)	0.243 (0.146–0.379)	
Jiangan	800,440 (8.1)	19 (6.3)	0.237 (0.143–0.371)	
Caidian	503,595 (5.1)	11 (3.7)	0.218 (0.109–0.391)	
Jiangxia	671,248 (6.8)	14 (4.7)	0.209 (0.114–0.350)	
Huangpi	979,920 (9.9)	14 (4.7)	0.143 (0.078–0.240)	
Hannan	417,022 (4.2)	4 (1.3)	0.096 (0.026–0.246)	
Dongxihu	777,204 (7.9)	5 (1.7)	0.064 (0.021–0.150)	
Xinzhou	634,408 (6.4)	3 (1.0)	0.047 (0.010–0.138)	
East Lake Scenic Area of Wuhan	85,884 (0.9)	0 (0.0)	0.000 (0.000–0.430)	

χ^2 test was used to assess the association between the detection rate of asymptomatic cases increased and sex and age. Urban districts of Wuhan includes Wuchang, Qingshan, Qiaokou, Hanyang, Jiangan, Jianghan, and Hongshan; Suburban districts of Wuhan includes Hannan, Caidian, Dongxihu, Xinzhou, Jiangxia, Huangpi, East Lake High-tech Development Area and East Lake Scenic Area of Wuhan.

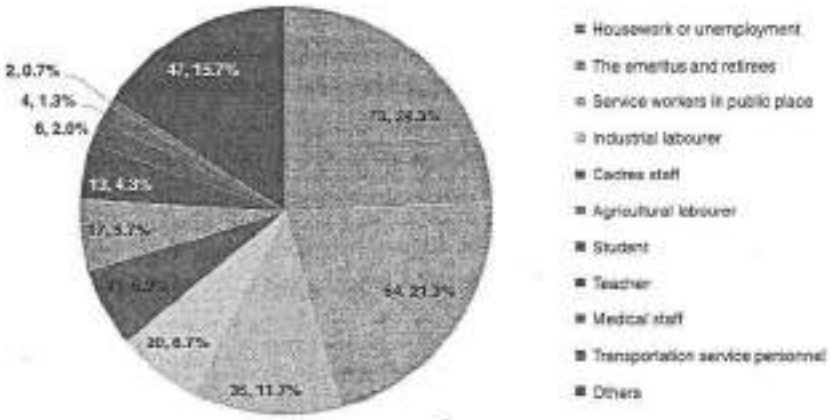

Fig. 1 The occupation distribution of asymptomatic positive cases (%). Note: Others included the self-employed, military personnel, and so on. (Source data are provided as a Source Data file.).

found that asymptomatic positive rates in different districts of Wuhan were correlated with the prevalence of previously confirmed cases. This is in line with the temporal and spatial evolution (especially the long-tailed characteristic) of infectious diseases[11].

Existing laboratory virus culture and genetic studies[9,10] showed that the virulence of SARS-CoV-2 virus may be weakening over time, and the newly infected persons were more likely to be asymptomatic and with a lower viral load than earlier infected cases. With the centralized isolation and treatment of all COVID-19 cases during the lockdown period in Wuhan, the risk of residents being infected in the community has been greatly reduced. When susceptible residents are exposed to a low dose of virus, they may tend to be asymptomatic as a result of their own immunity. Serological antibody testing in the current study found that at least 63% of asymptomatic positive cases were actually infected with SARS-CoV-2 virus. Nonetheless, it is too early to be complacent, because of the existence of asymptomatic positive cases and high level of susceptibility in residents in Wuhan. Public health measures for the prevention and control of COVID-19 epidemic, including wearing masks, keeping safe social distancing in Wuhan should be sustained. Especially, vulnerable populations with weakened immunity or co-morbidities, or both, should continue to be appropriately shielded.

Findings from this study show that COVID-19 was well controlled in Wuhan at the time of the screening programme. After two months since the screening programme (by August 9, 2020), there were no newly confirmed COVID-19 cases in Wuhan.

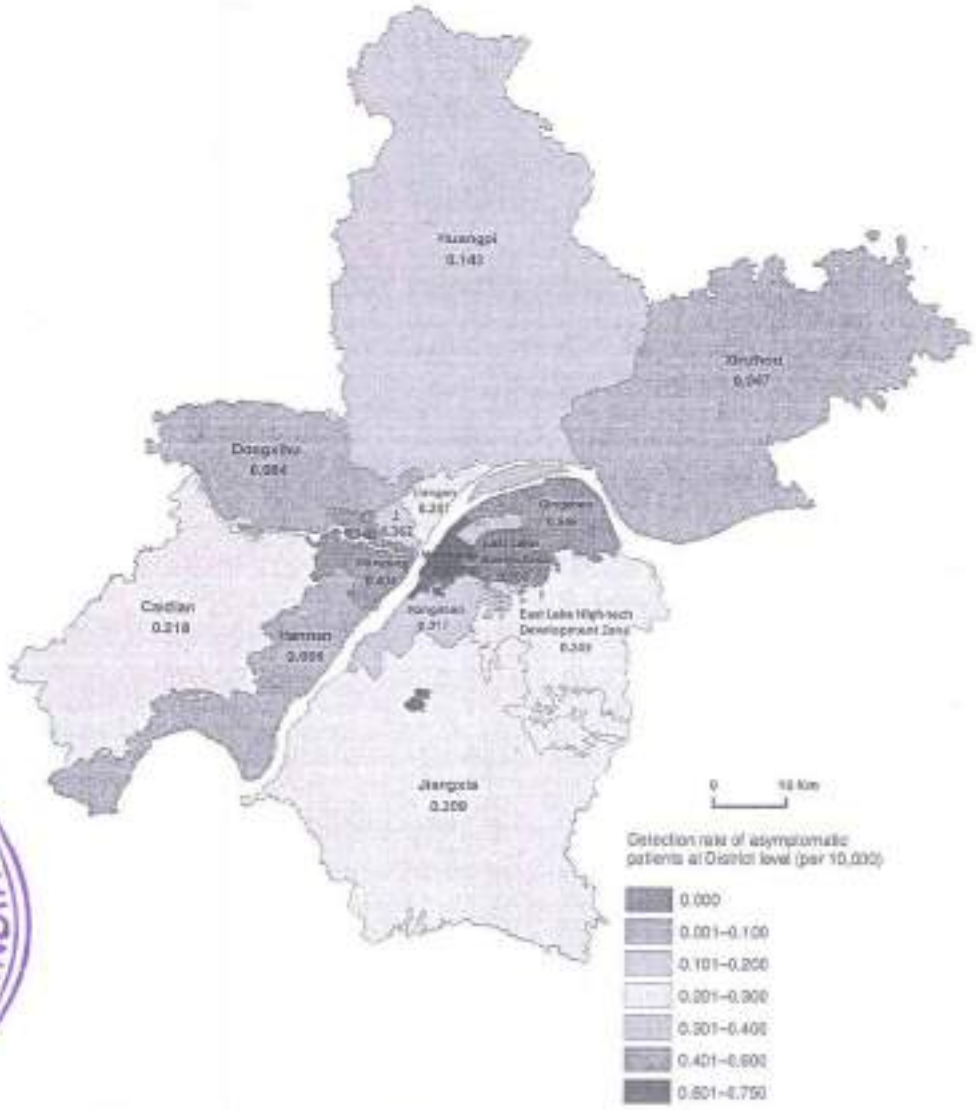

Fig. 2 The geographic distribution of the detection rate of asymptomatic positive cases. Note: 1 represents Jianghan district; 2 represents Qiaokou district. (Source data are provided as a Source Data file.)

Table 2 Results of the detection of antibody in 300 asymptomatic positive persons.

IgM Results	IgG	Asymptomatic positive persons	% (95% CI)
−	+	161	53.7 (47.8–59.4)
−	−	110	36.7 (31.2–42.4)
+	+	29	9.7 (6.6–13.6)
+	−	0	0.0 (0.0–1.2)

"−" indicates negative, "+" indicates positive.

Further testing of SARS-CoV-2 in samples collected from market environment settings in Wuhan were conducted, and found no positive results after checking a total of 52,312 samples from 1795 market setting during June 13 to July 2, 2020[12].

This study has several limitations that need to be discussed. First, this was a cross-sectional screening programme, and we are unable to assess the changes over time in asymptomatic positive and reoperative results. Second, although a positive result of nucleic acid testing reveals the existence of the viral RNAs, some false negative results were likely to have occurred, in particular due to the relatively low level of virus loads in asymptomatic infected individuals, inadequate collection of samples, and limited accuracy of the testing technology[13]. Although the screening programme provided no direct evidence on the sensitivity and specificity of the testing method used, a meta-analysis reported a

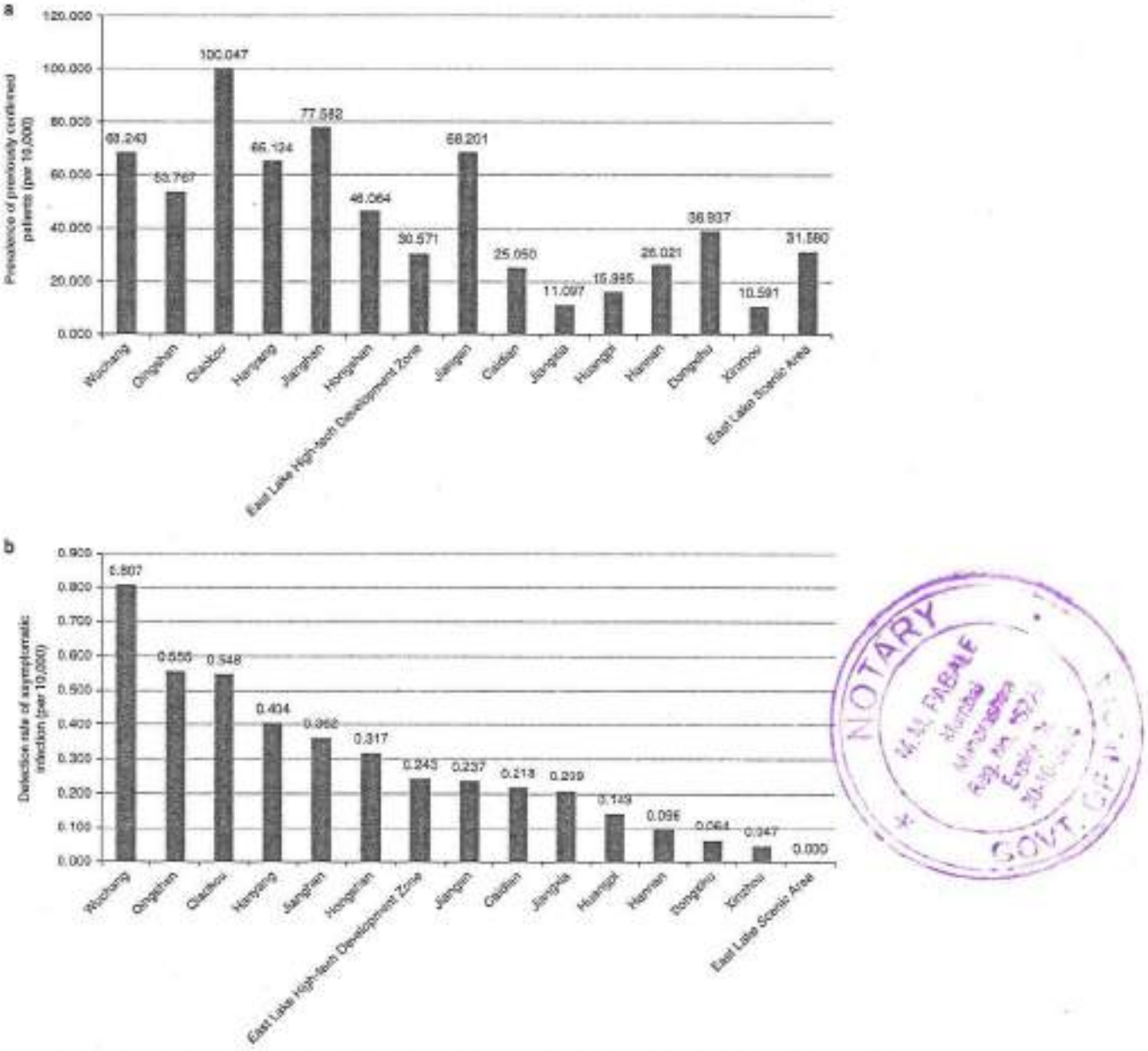

Fig. 3 The prevalence of previously confirmed patients and the detection rate of asymptomatic positive cases of COVID-19 in each district in Wuhan. **a** The prevalence of previously confirmed patients of COVID-19 in each district in Wuhan. **b** The detection rate of asymptomatic positive cases of COVID-19 in each district in Wuhan. (Source data are provided as a Source Data file.).

pooled sensitivity of 73% (95% CI 68–78%) for nasopharyngeal and throat swab testing of COVID-19[14]. Testing kits used in the screening programme were publicly purchased by the government and these kits have been widely used in China and other countries. Multiple measures were taken to possibly minimise false negative results in the screening programme. For example, standard training was provided to health works for sample collection to ensure the sample quality. The experiment procedures, including specimen collection, extraction, PCR, were according to official guidelines (Supplementary Note 1). For the real-time RT-PCR assay, two target genes were simultaneously tested. Even so, false negative results remained possible, particularly in any mass screening programmes. However, even if test sensitivity was as low as 50%, then the actual prevalence would be twice as high as reported in this study, but would still be very low. Around 7.1% of eligible residents did not participate in the citywide nucleic acid screening and the screening programme did not collect detailed data on reasons for nonparticipation, which is a limitation of this

study. Although there were no official statistics, a large number of migrant workers and university students left Wuhan before the lockdown, joining their families in other cities or provinces for traditional Chinese New Year. Therefore, it is likely that most nonparticipants were not in Wuhan at the time of the screening. The main objective of the screening programme was to assess the risk of COVID-19 epidemic in residents who were actually living in the post-lockdown Wuhan. Therefore, the estimated positive rates are unlikely to be materially influenced by nonparticipation of residents who were not in Wuhan or some residents who did not participate in the screening for other reasons. Moreover, people who left Wuhan were the target population for monitoring in other provinces and cities and were required to take nucleic acid testing. Although there was no official statistics showing the positive rate of nucleic acid testing in this population, there was no report that shown a higher positive rate of nucleic acid testing than our findings.

In summary, the detection rate of asymptomatic positive cases in the post-lockdown Wuhan was very low (0.303/10,000), and there was no evidence that the identified asymptomatic positive cases were infectious. These findings enabled decision makers to adjust prevention and control strategies in the post-lockdown period. Further studies are required to fully evaluate the impacts and cost-effectiveness of the citywide screening of SARS-CoV-2 infections on population's health, health behaviours, economy, and society.

Methods

Study population and ethical approvals. Wuhan has about 11 million residents in total, with seven urban and eight suburban districts. Residents are living in 7280 residential communities (or residential enclosures, "xiao-qu" in Chinese), and each residential community could be physically isolated from other communities for preventing transmission of COVID-19.

The screening programme recruited residents (including recovered COVID-19 patients) currently living in Wuhan who were aged ≥6 years (5,162,960 males, 52.2%). All participants provided written or verbal informed consent after reading a statement that explained the purpose of the testing. For participants who aged 6–17 years old, consent was obtained from their parents or guardians. The study protocol for an evaluation of the programme based on anonymized screening data was approved by the Ethics Committee of the Tongji Medical College Institutional Review Board, Huazhong University of Science and Technology, Wuhan, China (No. IRB00003571).

Organizational guarantee and community mobilization. A citywide nucleic acid screening group was formed, with specialized task teams contributing to comprehensive coordination, technical guidance, quality control, participation invitation, information management, communication, and supervision of the screening. The city government invested 900 million yuan (RMB) in the testing programme. From 14 May to 1 June 2020, in the peak time, up to 2927 sample collection sites were functioning at the same time in Wuhan. Each sample collection site had an assigned sample collection group, including several health professionals (staffed according to the number of communities' residents), 2–4 community managers, 1–2 police officers, and 1–2 inspectors. The sampling sites were set up based on the number and accessibility of local residents. Local community workers were responsible for a safe and orderly sampling process to minimise the waiting time. In addition, mobile sampling teams were formed by primary health care professionals and volunteers to conduct door-to-door sampling for residents who had physical difficulties or were unable to walk.

About 50,000 health professionals (mainly doctors and nurses from community health centers) and more than 280,000 person-times of community workers and volunteers contributed to sample collection, transport of equipment and samples collected, arrangement of participation process, and maintaining order of sampling sites. Public information communication and participant invitation were implemented through mass media, mobile messages, WeChat groups, and residential community broadcasts, so as to increase residents' awareness and the participation.

Acquisition, preservation, and transport of samples. All sampling personnel received standard training for the collection of oropharyngeal swab samples. To minimize the risk of cross-infection, the sampling process strictly followed a disinfection process and environmental ventilation were ensured. The collected samples were stored in a virus preservation solution or immersed in isotonic saline, tissue culture solution, or phosphate buffer (Supplementary note 1). Then, all samples were sent to testing institutions within 4 h using delivery boxes for biological samples refrigerated with dry ice to guarantee the stability of nucleic acid samples.

Technical methods for laboratory testing of collected samples. A total of 62 nucleic acid testing laboratories, 1451 laboratory workers and 701 testing equipment were involved in the nucleic acid testing. Received samples were stored at 4 °C and tested within 24 h of collection. Any samples that could not be tested within 24 h were stored at −70 °C or below (Supplementary note 1). In addition to "single testing" (i.e., separate testing of a single sample), "mixed testing" was also performed for 23% of the collected samples to increase efficiency, in which five samples were mixed in equal amounts, and tested in the same test tube. If a mixed testing was positive for COVID-19, all individual samples were separately retested within 24 h[15].

Details regarding technical methods for sequencing and virus culture were provided in Supplementary note 1. Real-time reverse transcription-polymerase chain reaction (RT-PCR) assay method was used for the nucleic acid testing. We simultaneously amplified and tested the two target genes: open reading frame 1ab (ORF1ab) and nucleocapsid protein (N) (Supplementary Note 1). A cycle threshold value (Ct-value) less than 37 was defined as a positive result, and no Ct-value or a Ct-value of 40 or more was defined as a negative result. For Ct-values ranging from 37 to 40, the sample was retested. If the retest result remained less than 40 and the amplification curve had obvious peak, the sample was classified as positive; otherwise, it was reported as being negative. These diagnostic criteria were based on China's official recommendations[16].

For asymptomatic positive cases, virus culture was carried out in biosafety level-3 laboratories. The colloidal gold antibody test was also performed for asymptomatic positive cases (Supplementary note 1). All testing results were double entered into a specifically designed database, and managed by the Big Data and Investigation Group of the COVID-19 Prevention and Control Centre in Wuhan, which was established to collect and manage data relevant to the COVID-19 epidemic.

Participant data collection and management. Before sample collection, residents electronically (using a specifically designed smartphone application) self-uploaded their personal information, including ID number, name, sex, age, and place of residence. Then, the electronic machine system generated a unique personal barcode and stuck it on the sample tube to ensure the match between the sample and the participant. Then trained staff interviewed each individual regarding the history of COVID-19 and previous nucleic acid testing. There was a database of confirmed COVID-19 cases in Wuhan, which can be used to validate the self-reported previous COVID-19 infection. All information was entered into a central database. The testing results were continually uploaded to the central database by testing institutions. Contact tracing investigations were conducted on participants who tested positive for SARS-CoV-2, to track and manage their close contacts. The pre-existing unique identification code for each resident was used as the programme's identification number, to ensure information accuracy during the whole process of screening, from sampling, nucleic acid testing, result reporting, the isolation of detected positive cases, and tracing of close contacts of positive cases. All screening information was kept strictly confidential and was not allowed to be disclosed or used for other purposes other than clinical and public health management. Personal information of asymptomatic positive cases was only disclosed to designated medical institution and community health centres for the purpose of medical isolation and identification of close contacts. Researcher was blind to the study hypothesis during data collection.

Biological security guarantee. Nucleic acid testing was performed in biosafety level-2 (BSL-2) laboratories, and virus culture was conducted in biosafety level-3 laboratories. Sampling and testing personnel adopted the personal protective measures according to the standard of biosafety level-3 laboratories. Participating laboratories implemented control measures to guarantee biological safety in accordance with relevant regulations[17].

Result query and feedback. Two to three days after sample collection, participants could inquire about their test results using WeChat or Alipay application by their unique ID numbers. The results included text descriptions of nucleic acid testing and coloured health codes. A green coloured health code refers to a negative result, and a red coloured health code indicates a positive result.

Definition and management of identified confirmed cases and close contacts. In this study, all confirmed COVID-19 cases were diagnosed by designated medical institutions according to National Guidelines for the Prevention and Control of COVID-19 (Supplementary Note 2). Asymptomatic positive cases referred to individuals who had a positive result during screening, and they had neither a history of COVID-19 diagnosis, nor any clinical symptoms at the time of the nucleic acid testing. Close contacts were individuals who closely contacted with an asymptomatic positive person since 2 days before the nucleic acid sampling[18]. Repositive cases refer to individuals who recovered from previously confirmed COVID-19 disease and had a positive testing again in the screening programme. All repositive cases, asymptomatic positive persons, and their close contacts were

isolated for at least 2 weeks in designated hotels managed by primary health care professionals, and they were released from isolation only if two consecutive nucleic acid tests were negative.

Statistical analysis. Detection rate of asymptomatic positive or repositive cases was calculated by dividing the number of individuals with a positive result of nucleic acid testing by the number of participants tested. Because of extremely low detection rates, we calculated 95% confidence intervals of estimated proportions using Pearson–Klopper exact method, implemented through R package "binom" version 1.1-1[18]. SPSS version 22.0 was used for other statistical analyses. We analyzed the distribution of asymptomatic positive cases and assessed the Spearman correlation between the asymptomatic positive rate and the prevalence of previously confirmed COVID-19 cases in different districts of Wuhan. Differences in asymptomatic positive rates by sex and age groups were assessed using the χ^2 test. ArcGIS 10.0 was used to draw a geographic distribution map of asymptomatic positive cases. A value of $P < 0.05$ (two-tailed) was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Detailed data directly used to generate each figure or table of this study are available within the article, Supplementary Information and source data are provided with this paper.

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Author contributions

S.Y.C., C.W., X.X.Y., and Z.X.L. conceived the study. C.W., Y.C.H., T.T.W., K.L., H.B.X., and Z.S. participated in the acquisition of data. S.B.W. and J.G. were responsible for the on-site specimen collection, laboratory testing quality evaluation, and control. Y.C.H., T.T.W., and L.Q.Z. analyzed the data. H.J., Y.H.G., and F.J.S. gave advice on methodology. Q.F.T. and C.Z.L. investigated the responses to the citywide nucleic acid testing among residents lived in outside of Wuhan city. S.Y.C., Y.G., C.W., and X.X.Y. drafted the manuscript. Y.G., M.S., and F.J.S. revised the manuscript, and M.B., C.Z.L., and F.J.S. critically commented and edited the manuscript. All authors read and approved the final manuscript. Z.X.L. is the guarantor of this study.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to C.L., F.S., X.Y. or Z.L.

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Coronavirus Disease Outbreak in Call Center, South Korea

Shin Young Park, Young-Man Kim, Seonju Yi, Sangeun Lee, Baeg-Ju Na, Chang Bo Kim, Jung-il Kim, Hea Sock Kim, Young Bok Kim, Yoojin Park, In Sil Huh, Hye Kyung Kim, Hyung Jun Yoon, Hanaram Jang, Kyungnam Kim, Yeonhwa Chang, Inhye Kim, Hyeyoung Lee, Jin Gwack, Seong Sun Kim, Miyoung Kim, Sanghui Kweon, Young June Choe, Ok Park, Young Joon Park, Eun Kyeong Jeong

We describe the epidemiology of a coronavirus disease (COVID-19) outbreak in a call center in South Korea. We obtained information on demographic characteristics by using standardized epidemiologic investigation forms. We performed descriptive analyses and reported the results as frequencies and proportions for categoric variables. Of 1,143 persons who were tested for COVID-19, a total of 97 (8.5%, 95% CI 7.0%–10.3%) had confirmed cases. Of these, 94 were working in an 11th-floor call center with 216 employees, translating to an attack rate of 43.5% (95% CI 36.9%–50.4%). The household secondary attack rate among symptomatic case-patients was 16.2% (95% CI 11.6%–22.0%). Of the 97 persons with confirmed COVID-19, only 4 (1.9%) remained asymptomatic within 14 days of quarantine, and none of their household contacts acquired secondary infections. Extensive contact tracing, testing all contacts, and early quarantine blocked further transmission and might be effective for containing rapid outbreaks in crowded work settings.

Since the first imported case of coronavirus disease (COVID-19) was confirmed in South Korea on January 20, 2020, a sharp increase in the number of COVID-19 cases has been observed, with most infections being reported from specific clusters (1). Outbreaks of COVID-19 related to mass gathering, religious activities, workplaces, and hospitals have accounted for the largest portion cases in the national outbreak (1).

In March 2020, the Korea Centers for Disease Control and Prevention (KCDC), South Korea's national-level public health authority, was informed about a cluster of cases of COVID-19 in a call center located in a commercial-residential mixed-use building (building X) in the capital city of Seoul. We describe the epidemiology of this COVID-19 outbreak and detail the containment efforts to limit the spread of the disease.

Materials and Methods

Setting

On March 8, the Seoul Metropolitan Government was notified of a confirmed case of COVID-19 in a person who worked in building X; the case reportedly was associated with a possible cluster of cases. On March 9, KCDC and local governments (in Seoul, the city of Incheon, and Gyeonggi Province) formed a joint response team and launched an epidemiologic investigation with contact tracing. Building X is a 19-story floor in one of the busiest urban area of Seoul. Commercial offices are located on the 1st through 11th floors, and residential apartments are located on the 13th through 19th floors. We identified and investigated 922 employees who worked in the commercial offices, 203 residents who lived in the residential apartments, and 20 visitors. The call center is located on the 7th through 9th floors and the 11th floor; it has a total of 811 employees. Employees do not generally go between floors, and they do not have an in-house restaurant for meals.

Case Definition

We defined a patient under investigation (PUI) as one who worked at, lived at, or visited building X during February 21–March 8, 2020. We defined a confirmed

Author affiliations: Korea Centers for Disease Control and Prevention, Cheongju, South Korea (S.Y. Park, Y.-M. Kim, S. Yi, S. Lee, H. Lee, J. Gwack, S.S. Kim, M. Kim, S. Kweon, O. Park, Y.J. Park, E.K. Jeong); Seoul Metropolitan Government, Seoul, South Korea (B.-J. Na, J.-I. Kim, H.S. Kim, Y.B. Kim); Seoul Health Foundation, Seoul (C.B. Kim); Seoul Center for Infectious Disease Control and Prevention, Seoul (Y. Park, I.S. Huh); Incheon Metropolitan City, Incheon, South Korea (H.K. Kim, H.J. Yoon, H. Jang); Gyeonggi Provincial Office, Suwon, South Korea (K. Kim, Y. Chang, I. Kim); Hallym University College of Medicine, Chuncheon, South Korea (Y.J. Choe)

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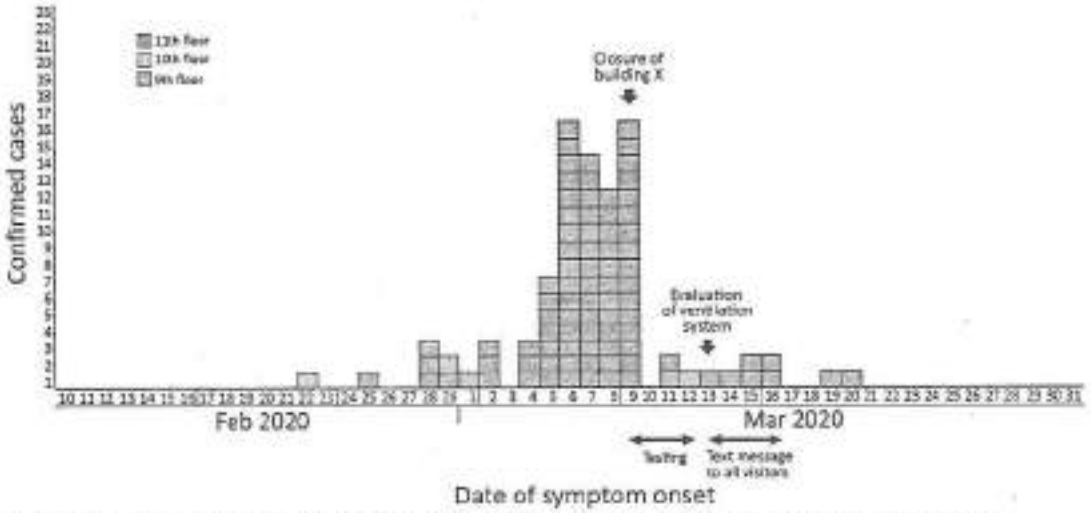

Figure 1. Epidemic curve of a coronavirus disease outbreak in a call center, by date of symptom onset, Seoul, Korea, 2020. Asymptomatic cases are excluded.

case-patient as a PUI with a positive COVID-19 laboratory test. We confirmed the diagnosis of COVID-19 by using real-time reverse transcription PCR assays. We defined a symptomatic case-patient as a confirmed case-patient with symptoms at the time of positive testing, a presymptomatic case-patient as a confirmed case-patient who was asymptomatic at the time of positive testing but later had symptoms during the 14 days of monitoring, and an asymptomatic case-patient as confirmed a case-patient with a positive COVID-19 test result who remained asymptomatic during the entire 14-day period.

Response Measures

Building X was closed on March 9, 2020, immediately after the outbreak was reported. We offered testing to all occupants (office workers and apartment residents) during March 9–12. We collected nasopharyngeal and oropharyngeal swab specimens from PUIs for immediate real-time reverse transcription PCR testing; the average turnaround time was 12–24 hours. Confirmed case-patients were isolated, and negative case-patients were mandated to stay quarantined for 14 days. We followed and retested all test-negative case-patients until the end of quarantine. We also investigated, tested, and monitored household contacts of all confirmed case-patients for 14 days after discovery, regardless of symptoms. During March 13–16, we sent a total of 16,628 text messages to persons who stayed >5 minutes near the building X; we tracked these persons by using cell phone location data. The messages instructed the recipients to avoid contact with others and go to the nearest COVID-19 screening center to get tested.

Data Collection and Analysis

We obtained information on demographic characteristics and presence of symptoms through face-to-face interviews with case-patients, using standardized epidemiologic investigation forms. We performed

Table 1. Attack rate by location during a coronavirus disease outbreak in a call center, Seoul, South Korea, 2020

Location type and floor	Potentially exposed, no. (%)	Confirmed cases, no. (%)	Attack rate, % (95% CI)
Commercial			
1st–6th	84 (7.3)	0	0
7th (call center)	182 (15.9)	0	0
8th (call center)	207 (18.1)	0	0
9th (call center)	206 (18.0)	1 (1.0)	0.5 (0.0–3.1)
10th	27 (2.4)	2 (2.1)	7.4 (1.3–25.8)
11th (call center)	216 (18.9)	94 (96.9)	43.5 (36.9–50.4)
Residential			
13th–19th	201 (17.6)	0	0
Other	20 (1.7)	0	0
Total	1,143	97	8.5 (7.0–10.3)

descriptive analyses reported the results as frequencies and proportions for categoric variables. The investigation was a part of public health response and was not considered research subject to institutional review board approval; therefore, written informed consent by participants was not required.

Results

Of 1,145 PUIs, we tested 1,143 (99.8%) for COVID-19 (922 employees, 201 residents, and 20 visitors) and identified 97 (8.5%, 95% CI 7.0–10.3) confirmed case-patients (Figure 1). Of 857 patients for whom demographic information was available, 620 (72.3%) were women; mean age was 38 years (range 20–80 years). Most (94 [96.9%]) of the confirmed case-patients were working on the 11th-floor call center, which had a total of 216 employees, resulting in an attack rate of 43.5% (95% CI 36.9%–50.4%) (Table 1; Figure 2). Most of the case-patients on the 11th floor were on the same side of the building. Among the 97

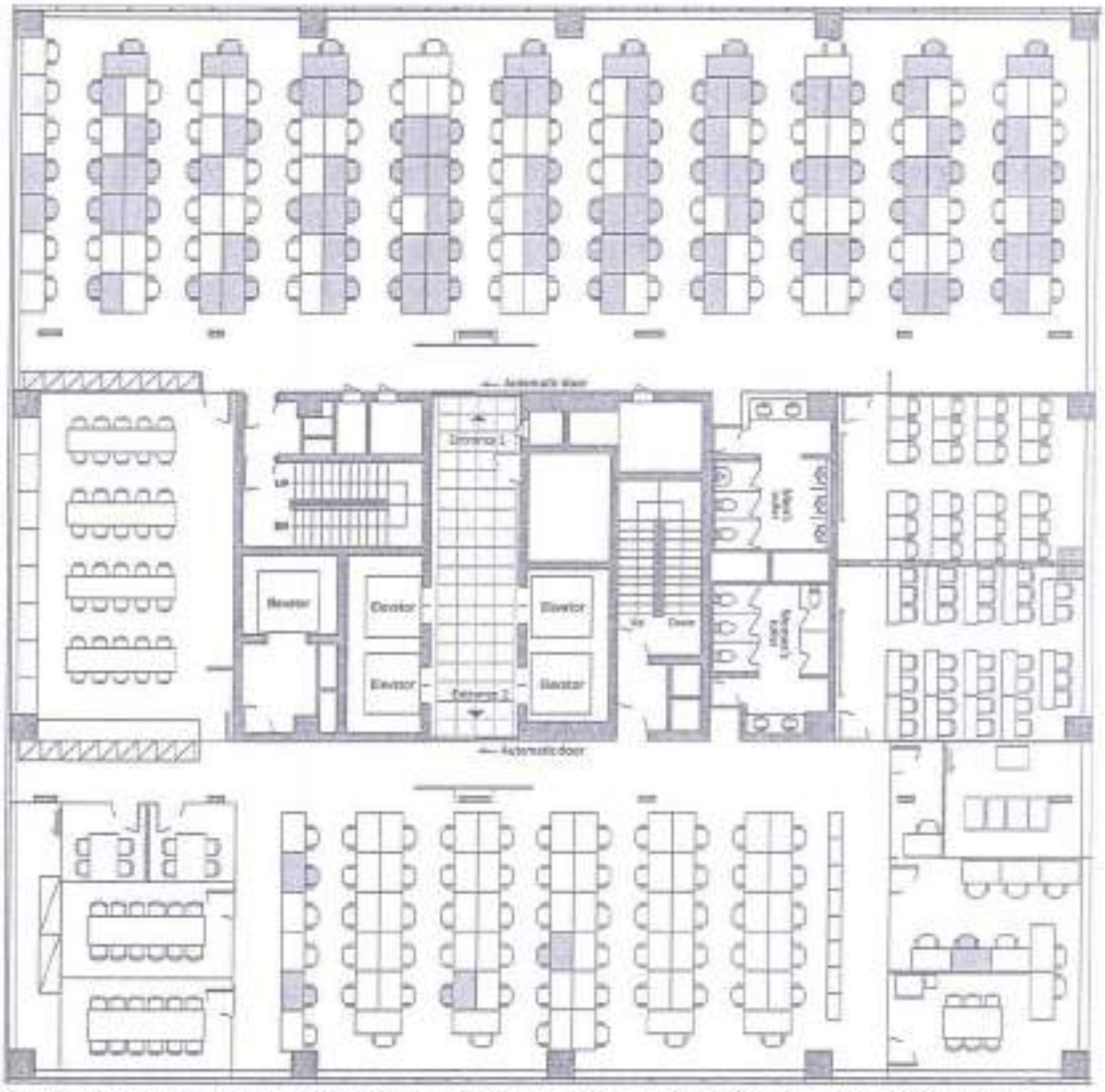

Figure 2. Floor plan of the 11th floor of building X, site of a coronavirus disease outbreak, Seoul, South Korea, 2020. Blue indicates the seating places of persons with confirmed cases.

confirmed case-patients, 89 (91.7%) were symptomatic at the time of investigation and 4 (4.1%) were presymptomatic during the time of investigation but later had onset of symptoms within 14 days of monitoring; 4 (4.1%) case-patients remained asymptomatic after 14 days of isolation.

The first case-patient with symptom onset, who worked in an office on the 10th floor (and reportedly never went to 11th floor), had onset of symptoms on February 22. The second case-patient with symptom onset, who worked at the call center on the 11th floor, had onset of symptoms on February 25. Residents and employees in building X had frequent contact in the lobby or elevators. We were not able to trace back the index case-patient to another cluster or an imported case.

We followed up on a total of 225 household contacts of confirmed COVID-19 case-patients (average 2.3 household members per confirmed case-patient). COVID-19 had occurred in 34 household members who had contact with symptomatic case-patients, translating to a secondary attack rate of 16.2% (Table 2). Among 11 household members of presymptomatic case-patients and 4 household members of asymptomatic case-patients, none had COVID-19 symptoms nor tested positive after 14 days of quarantine.

Discussion

We described the epidemiologic characteristics of a COVID-19 outbreak centered in a call center in South Korea. We identified 97 confirmed COVID-19 case-patients in building X, indicating an attack rate of 8.5%. However, if we restrict our results to the 11th floor, the attack rate was as high as 43.5%. This outbreak shows alarmingly that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can be exceptionally contagious in crowded office settings such as a call center. The magnitude of the outbreak illustrates how a high-density work environment can become a high-risk site for the spread of COVID-19 and potentially a source of further transmission. Nearly all the case-patients were on one side of the building on 11th floor. Severe acute respiratory syndrome coronavirus, the predecessor of SARS-CoV-2, exhibited multiple superspreading events in 2002 and 2003, in which a few persons infected others, resulting in many secondary cases. Despite considerable interaction between workers on different floors of building X in the elevators and lobby, spread of COVID-19 was limited almost exclusively to the 11th floor, which indicates that the duration of interaction (or contact) was likely the main facilitator for further spreading of SARS-CoV-2.

Unique features of this outbreak investigation include a complete 14-day follow-up of close contacts of case-patients after containment measures were implemented. Close contact with an infected person is a well-recognized risk factor for acquiring SARS-CoV-2 (*2*). In a recent US study, the symptomatic secondary attack rate among 445 close contacts of COVID-19 case-patients was 10.5% among household members (*3*). In this outbreak in South Korea, we found that the secondary attack rate within the household was 16.5% among symptomatic index case-patients, which is consistent with other reports.

The role of asymptomatic COVID-19 case-patients in spreading the disease is of great concern. Among 97 confirmed COVID-19 case-patients in this study, 4 (4.1%) remained asymptomatic during the 14-days of monitoring. This rate is lower than the 30.8% rate estimated in previous modeling (*4*). A case-patient series from Beijing, China, indicated that asymptomatic case-patients accounted for 5% (13/262) of patients transferred to a designated COVID-19 hospital (*5*). Our data might represent the likely proportion of asymptomatic COVID-19 infections in the community setting. We also found that, among 17 household contacts of asymptomatic case-patients, none had secondary infections. Previous reports have postulated that SARS-CoV-2 in asymptomatic (or presymptomatic) case-patients might become transmissible to others (*6*); however, given the high degree of self-quarantine and isolation measures that were instituted after March 8 among this cohort, our analyses might have not detected the actual transmissibility in asymptomatic COVID-19 case-patients. Robust mass testing of all suspected case-patients might have prevented asymptomatic transmission because asymptomatic persons were given information about their possible infection and therefore might have self-isolated from their household members.

Table 2. Household secondary attack rate, by presence of symptoms, during a coronavirus disease outbreak in in a call center, Seoul, South Korea, 2020

Symptom status of index patients	Exposed, no. (%)	Confirmed cases, no. (%)	Secondary attack rate, % (95% CI)
Symptomatic	210 (93.3)	34 (100.0)	16.2 (11.6–22.0)
Presymptomatic	11 (4.8)	0	0
Asymptomatic	4 (1.8)	0	0
Total	225	34	15.1 (10.8–20.6)

This outbreak investigation has several limitations. First, we could not track these cases to another cluster, making it difficult to identify the actual index case-patient. Second, not all clinical information was available for all confirmed cases, prohibiting detailed description of clinical syndromes. Date of symptom onset by office seat would be informative in understanding SARS-CoV-2 transmission in close contact area. However, our findings demonstrate the power of screening all potentially exposed persons and show that early containment can be implemented and used in the middle of national COVID-19 outbreak. By testing all potentially exposed persons and their contacts to facilitate the isolation of symptomatic and asymptomatic COVID-19 case-patients, we might have helped interrupt transmission chains. In light of the shift to a global pandemic, we recommend that public health authorities conduct active surveillance and epidemiologic investigation in this rapidly evolving landscape of COVID-19.

In summary, this outbreak exemplifies the threat posed by SARS-CoV-2 with its propensity to cause large outbreaks among persons in office workplaces. Targeted preventive strategies might help mitigate the risk for SARS-CoV-2 infection in these vulnerable group.

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About the Author

Ms. Park is a public health officer at the Korea Centers for Disease Control and Prevention. Her main interest is the epidemiologic investigation of infectious diseases.

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Address for correspondence: Eun Kyeong Jeong, Korea Centers for Disease Control and Prevention, Osong Health Technology Administration Complex, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, 28159, South Korea; email: jeonge8@korea.kr

EXHIBIT D

JAMA Internal Medicine | Original Investigation

Contact Tracing Assessment of COVID-19 Transmission Dynamics in Taiwan and Risk at Different Exposure Periods Before and After Symptom Onset

Hao-Yuan Cheng, MD, MSc; Shu-Wan Jian, DVM, MPH; Ding-Ping Liu, PhD; Ta-Chou Ng, BSc; Wan-Ting Huang, MD; Hsien-Ho Lin, ND, ScD; for the Taiwan COVID-19 Outbreak Investigation Team

Editor's Note page 1163
Author Audio Interview
Supplemental content

IMPORTANCE The dynamics of coronavirus disease 2019 (COVID-19) transmissibility are yet to be fully understood. Better understanding of the transmission dynamics is important for the development and evaluation of effective control policies.

OBJECTIVE To delineate the transmission dynamics of COVID-19 and evaluate the transmission risk at different exposure window periods before and after symptom onset.

DESIGN, SETTING, AND PARTICIPANTS This prospective case-ascertained study in Taiwan included laboratory-confirmed cases of COVID-19 and their contacts. The study period was from January 15 to March 18, 2020. All close contacts were quarantined at home for 14 days after their last exposure to the index case. During the quarantine period, any relevant symptoms (fever, cough, or other respiratory symptoms) of contacts triggered a COVID-19 test. The final follow-up date was April 2, 2020.

MAIN OUTCOMES AND MEASURES Secondary clinical attack rate (considering symptomatic cases only) for different exposure time windows of the index cases and for different exposure settings (such as household, family, and health care).

RESULTS We enrolled 100 confirmed patients, with a median age of 44 years (range, 11-88 years), including 44 men and 56 women. Among their 2761 close contacts, there were 22 paired index-secondary cases. The overall secondary clinical attack rate was 0.7% (95% CI, 0.4%-1.0%). The attack rate was higher among the 1818 contacts whose exposure to index cases started within 5 days of symptom onset (1.0% [95% CI, 0.6%-1.6%]) compared with those who were exposed later (0 cases from 852 contacts; 95% CI, 0%-0.4%). The 299 contacts with exclusive presymptomatic exposures were also at risk (attack rate, 0.7% [95% CI, 0.2%-2.4%]). The attack rate was higher among household (4.6% [95% CI, 2.3%-9.3%]) and nonhousehold (5.3% [95% CI, 2.1%-12.8%]) family contacts than that in health care or other settings. The attack rates were higher among those aged 40 to 59 years (1.7% [95% CI, 0.6%-2.1%]) and those aged 60 years and older (0.9% [95% CI, 0.3%-2.6%]).

CONCLUSIONS AND RELEVANCE In this study, high transmissibility of COVID-19 before and immediately after symptom onset suggests that finding and isolating symptomatic patients alone may not suffice to contain the epidemic, and more generalized measures may be required, such as social distancing.

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Author Affiliations: Epidemic Intelligence Center, Taiwan Centers for Disease Control, Taipei, Taiwan (Cheng, Jian, Liu); Institute of Epidemiology and Preventive Medicine, National Taiwan University College of Public Health, Taipei, Taiwan (Ng, Lin); Office of Preventive Medicine, Taiwan Centers for Disease Control, Taipei, Taiwan (Huang); Global Health Program, National Taiwan University College of Public Health, Taipei, Taiwan (Lin).

Group Information: A complete list of the members of the Taiwan COVID-19 Outbreak Investigation Team appears at the end of this article.

Corresponding Author: Hsien-Ho Lin, MD, ScD, National Taiwan University, 17 Xuzhou Rd, Taipei 100, Taiwan (hsienho@ntu.edu.tw).

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ADVOCATE FOR

The coronavirus disease 2019 (COVID-19) outbreak that originated in Wuhan, China, spread to more than 100 countries within 2 months of when the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in January 2020.[1,2] Following the Wuhan lockdown and other extreme social-distancing measures conducted by the Chinese government, several countries with widespread outbreaks implemented similar measures, including shutting down entire cities or communities, banning international or domestic travel, conducting border control with symptom screening, and implementing isolation and quarantine.

The unknown epidemiologic characteristics and transmission dynamics of a novel pathogen, such as SARS-CoV-2, complicate the development and evaluation of effective control policies.[3] The serial interval of COVID-19, defined as the interval between the symptom onset of the index case and that of the secondary case, was found to be short (4-5 days) and was similar to the estimated incubation period.[4] The short serial interval of COVID-19 and results from viral shedding studies suggested that most transmission occurred near or even before the time of symptom onset.[5,6] On the other hand, prolonged viral shedding raised concerns about prolonged infectiousness of patients and the need for extended isolation. A few preliminary contact-tracing studies showed that the highest-risk exposure setting of COVID-19 transmission was the household.[7-9] Nevertheless, it is not known when and how long a patient with COVID-19 should be isolated or whether close contacts should be quarantined. Additional information is needed about the transmission risk at different time points before and after symptom onset and with different types of exposures, such as through the household or a health care facility.

In Taiwan, the first COVID-19 case was confirmed on January 21, 2020.[10] With proactive containment efforts and comprehensive contact tracing, the number of COVID-19 cases remained low, as compared with other countries that had widespread outbreaks.[11,12] Using the contact tracing data in Taiwan, we aimed to delineate the transmission dynamics of COVID-19, evaluate the infection risk at different exposure windows, and estimate the infectious period.

Key Points

Question What is the transmissibility of coronavirus disease 2019 (COVID-19) to close contacts?

Findings In this case-ascertained study of 100 cases of confirmed COVID-19 and 2761 close contacts, the overall secondary clinical attack rate was 0.7%. The attack rate was higher among contacts whose exposure to the index case started within 5 days of symptom onset than those who were exposed later.

Meaning High transmissibility of COVID-19 before and immediately after symptom onset suggests that finding and isolating symptomatic patients alone may not suffice to interrupt transmission, and that more generalized measures might be required, such as social distancing.

Methods

Study Population

On January 15, 2020, in response to the outbreak in Wuhan, the Taiwan Centers for Disease Control (Taiwan CDC) made COVID-19 a notifiable disease. We conducted a prospective case-ascertained study that enrolled all the initial 100 confirmed cases in Taiwan between January 15 and March 18, 2020, and their close contacts. All contacts were followed up until 14 days after the last exposure to the index case. The last follow-up date was April 2, 2020.

The study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.[13] Information was collected according to the pronouncement of the Central Epidemic Command Center and in accordance with Article 17 of the Communicable Disease Control Act.[14] As part of the public health response functions of the Central Epidemic Command Center for surveillance purposes, institutional review board approval of this study and informed consent were waived. Prior to analysis, the data were deidentified.

Ascertainment of Cases

A confirmed case met the criteria of notification for COVID-19 in Taiwan and tested positive by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) test.[15] Detailed information, including demographic and clinical data, was reported to the National Notifiable Disease Surveillance System.[16] The investigation team determined the clinical severity of the confirmed patients following the World Health Organization (WHO) interim guidance.[17]

Contact Tracing for COVID-19

When a patient was laboratory-confirmed to have SARS-CoV-2 infection, a thorough epidemiological investigation, including contact tracing, was implemented by the outbreak investigation team of the Taiwan CDC and local health authorities. The period of investigation started at the date at symptom onset (and could be extended to up to 4 days before symptom onset when epidemiologically indicated) and ended at the date at COVID-19 confirmation. For asymptomatic confirmed cases, the period of investigation was based on the date at confirmation (instead of date at onset) and was determined according to epidemiological investigation. The definition of a close contact was a person who did not wear appropriate personal protection equipment (PPE) while having face-to-face contact with a confirmed case for more than 15 minutes during the investigation period. A contact was listed as a household contact if he or she lived in the same household with the index case. Those listed as family contacts were family members not living in the same household.

For health care settings, medical staff, hospital workers, and other patients in the same setting were included; close contact was defined by contacting an index case within 2 m without appropriate PPE and without a minimal requirement of exposure time. Whether the PPE was regarded as "appropriate" depended on the exposure setting and the procedures performed. For example, for physicians who performed aerosol-generating procedures, such as intubation, an N95 respirator

Table 1. Characteristics of the 2761 Close Contacts by Different Exposure Settings

	Exposure, No. (%)			
	Household (n = 151)	Family (n = 76)	Health care (n = 698)	Others (n = 1836)[a]
Age, median (range), y	33 (1-95)	45 (0-88)	39 (0-92)	35 (0-89)
Age group, y				
0-19	24 (16)	14 (18)	29 (4)	214 (12)
20-39	55 (36)	16 (21)	281 (40)	809 (44)
40-59	38 (25)	24 (32)	175 (25)	557 (30)
≥60	26 (17)	11 (14)	119 (17)	175 (10)
Unknown	8 (5)	11 (14)	94 (13)	81 (4)
Sex				
Female	70 (46)	41 (54)	454 (65)	872 (47)
Male	81 (54)	30 (39)	228 (33)	816 (44)
Unknown	0	5 (7)	16 (2)	148 (8)
Time from onset to exposure, median (range), d[b]	−4 (−4 to 9)	6 (−4 to 26)	1 (−4 to 23)	2 (−4 to 26)
Time from onset to exposure, d[b]				
<0	103 (68)	10 (13)	236 (34)	389 (21)
0-3	39 (26)	15 (20)	150 (21)	663 (36)
4-5	6 (4)	6 (8)	38 (5)	166 (9)
6-7	4 (3)	10 (13)	17 (2)	69 (3)
8-9	2 (1)	3 (4)	110 (16)	334 (18)
>9	0	24 (32)	146 (21)	114 (6)
Unknown	0	8 (11)	1 (0.1)	82 (5)

[a] Others include friends, airline crew members and passengers, and other casual contacts.

[b] Defined as the elapsed time between the date at symptom onset of the index case and the first date at exposure. For example, people from the group "<0 days" had their first contact with the index case before the case had any symptoms.

was required. For such procedures, a surgical mask would not be appropriate PPE. Accordingly, the medical staff would be listed as a close contact.

All close contacts were quarantined at home for 14 days after their last exposure to the index case. During the quarantine period, any relevant symptoms (fever, cough, or other respiratory symptoms) of close contacts would trigger RT-PCR testing for COVID-19. For high-risk populations, including household and hospital contacts, RT-PCR was performed regardless of symptoms. Essentially, these high-risk contacts were tested once when they were listed as a close contact. If the initial COVID-19 test result was negative, further testing would only be performed if a close contact developed symptoms during quarantine. The Taiwan CDC used an electronic tracing system (Infectious Disease Contact Tracing Platform and Management System) to follow and record the daily health status of those quarantined contacts.[18] The information collected included age, sex, the index case, date at exposure, and the exposure setting.

Data Processing and Analysis

Paired data of index case and close contacts were extracted from the contact tracing database and outbreak investigation reports. For a family cluster, the index case was determined based on the temporality of symptom onset and review of the epidemiological link. A secondary case was excluded from the paired data if the beginning of exposure was after symptom onset of the secondary case (only applied when the secondary case was symptomatic). For health care contacts, the date at exposure would be the date at admission of the case if the exact date at exposure was not recorded.

Incubation period and serial interval were estimated using the contact tracing data in Taiwan and publicly available data sets globally (eMethods in the Supplement). We used the Bayesian hierarchical model to increase the stability in small-sample estimation. The exposure window period was defined as the period between the first and last day of reported exposure to the index case based on contact investigation. Following the WHO, we defined the secondary clinical attack rate as the ratio of symptomatic confirmed cases among the close contacts.[19] We analyzed the dynamic change of secondary clinical attack rate in relation to symptom onset of the index case (days <0, 0-3, 4-5, 6-7, 8-9, or >9).

The percentage of missing information was small (7.0% for age, 6.1% for sex, and 3.3% for time from onset to exposure; Table 1). In the univariable analysis of secondary clinical attack rate by different exposure characteristics (eg, age), close contacts with missing information in that particular exposure attribute were excluded. All statistical tests were 2-sided with an α level of .05. All confidence intervals (CIs) were 95%. R software (R Foundation for Statistical Computing) and RStan (Stan Development Team) were used for data management and analysis.

Results

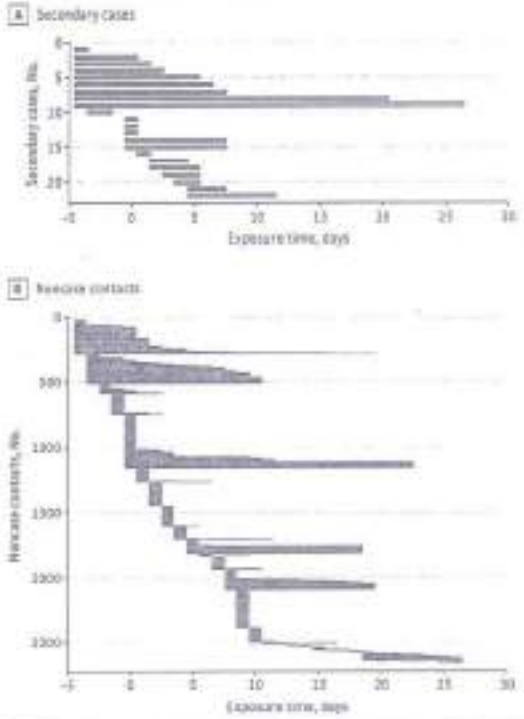
As of March 18, 2020, there were 100 patients with laboratory-confirmed COVID-19 in Taiwan, including 10 clusters of patients and 9 asymptomatic patients. The median age of the 100 patients was 44 years old (range, 11-88 years); 44 were men and 56 were women. Of the 2761 close contacts that were identi-

fied, 5.5% were household contacts, 2.8% were nonhousehold family contacts, and 25.3% were health care contacts (Table 1). Through contact tracing, 23 secondary cases were found. One of the 23 cases was excluded from subsequent transmission-pair analysis because the documented day at exposure occurred after symptom onset of the secondary case. None of the 9 asymptomatic case patients transmitted a secondary case. Using the data on the 22 paired cases, we estimated that the median incubation period was 4.1 days (95% credible interval [CrI], 0.4-15.8), and the median serial interval was 4.1 days (95% CrI, 0.1-27.8) (eTables 1-5 and eFigures 1-3 in the Supplement).

Among the 2761 close contacts, 22 secondary cases of COVID-19 infection (including 4 asymptomatic infections) were detected, with an infection risk of 0.8% (95% CI, 0.5%-1.2%). The secondary clinical attack rate was 18 of 2761, or 0.7% (95% CI, 0.4%-1.0%). Figure 1 shows the exposure window of all contacts. All of the 22 secondary cases had their first exposure before the sixth day of the index case's symptom onset. By comparison, only 68% of noncase contacts had their first exposure before day 6 (Table 1). The secondary clinical attack rate was higher among those whose initial exposure to the index case was within 5 days of symptom onset than those who were exposed after day 6 (zero transmission of 852 contacts [95% CI, 0%-0.4%]) (Table 2 and Figure 2A). The 735 contacts whose initial exposure occurred before symptom onset of the index case were also at risk, with a secondary clinical attack rate of 1.0% (95% CI, 0.5%-2.0%). A subgroup of 299 contacts with exclusive presymptomatic exposures were also at risk (secondary clinical attack rate, 0.7% [95% CI, 0.2%-2.4%]).

The secondary clinical attack rate was 4.6% (95% CI, 2.3%-9.3%) among 131 household contacts and 5.3% (95% CI, 2.1%-12.8%) in 76 nonhousehold family contacts (Table 2). The high attack rate from early exposure remained when the analysis was restricted to household and nonhousehold family contacts (Table 3 and Figure 2B). The attack rates were higher among those aged 40 to 59 years (1.3% [95% CI, 0.6%-2.1%]) and those aged 60 years and older (0.9% [95% CI, 0.3%-2.6%]). The 786 close contacts of the 6 confirmed cases presenting with severe disease were at a higher risk compared with the 1097 close contacts of the 56 cases presenting with mild disease (risk ratio, 3.76 [95% CI, 1.10-12.76] and 3.99 [95% CI, 1.00-15.84] for severe pneumonia and acute respiratory distress syndrome/sepsis, respectively). Among the 91 close contacts of the 9 asymptomatic cases, no secondary transmission was observed. The secondary attack rate among contacts of cases with infection acquired in Taiwan was higher than that among contacts of cases with infection acquired outside of Taiwan (Table 2).

Figure 1. Exposure Window Period Among Secondary Cases and Noncase Contacts



The exposure time was defined as the period from the first day of exposure to the index case to the last day of exposure. Time zero indicates the day of symptom onset of the index case.

Discussion

Our analysis of close contacts to confirmed COVID-19 cases revealed a relatively short infectious period of COVID-19 and a higher transmission risk around the time of symptom onset of the index case, followed by a lower transmission risk at the later stage of disease. The observed decreasing transmission risk over time for COVID-19 was in striking contrast to the transmission pattern of severe acute respiratory syndrome (SARS), in which the transmission risk remained low until after day 5 of symptom onset in the index cases.[20] Our study and the study by Nishiura et al[4] revealed a short serial interval of COVID-19, with a median of 4 to 5 days. In contrast, the mean serial interval of SARS was estimated to be 8.4 days in Singapore.[20] The present contact tracing analysis suggested that the shorter serial interval of COVID-19 was due to the combination of early-stage transmission and a short period of infectiousness.

The observed pattern of the secondary clinical attack rate over time was also consistent with the quantitative data of the SARS-CoV-2 viral shedding in upper respiratory specimens, which has been found in China to be a high viral load around the time of symptom onset, followed by a gradual decrease in viral shedding to a low level after 10 days.[5] The viral load was similar among asymptomatic, minimally symptomatic, and symptomatic patients. Another virological study in patients with COVID-19 in Germany also found no viable isolates of the virus after the first week of symptoms.[21] Our findings agree with the virological data on high transmissibility of COVID-19 in the first week after the onset of symptoms and decreased risk afterwards.[21] We also documented and quantified the

Table 2. Secondary Clinical Attack Rate for COVID-19 Among the 2761 Close Contacts by Different Exposure Settings, Times, and Characteristics

	No. of secondary cases (asymptomatic case)	No. of contacts	Secondary clinical attack rate, % (95% CI)	Risk ratio (95% CI)
Exposure setting				
Household	10 (3)	191	4.6 (2.3-9.1)	1 [Reference]
Nonhousehold family	5 (1)	76	5.3 (2.1-12.8)	1.14 (0.34-3.76)
Health care	6 (0)	898	0.9 (0.4-1.9)	0.19 (0.06-0.54)
Others[a]	1 (0)	1836	0.1 (0-0.3)	0.01 (0-0.09)
Time from onset to exposure, d[b]				
<0	10 (3)	735	1.0 (0.5-2.0)	1 [Reference]
0-3	9 (1)	867	0.9 (0.5-1.8)	0.97 (0.35-2.66)
4-5	3 (0)	216	1.4 (0.5-4.0)	1.46 (0.38-5.59)
6-7	0	119	0 (0-3.1)	0
8-9	0	449	0 (0-0.8)	0
>9	0	384	0 (0-1.0)	0
Exclusively presymptomatic exposure[c]				
No	20 (4)	2371	0.7 (0.4-1.1)	1 [Reference]
Yes	2 (0)	390	0.7 (0.2-2.4)	0.99 (0.23-4.29)
Age of close contacts, y				
0-19	1 (1)	281	0 (0-1.4)	0
20-39	8 (2)	1161	0.5 (0.2-1.1)	1 [Reference]
40-59	10 (1)	794	1.1 (0.5-2.1)	2.19 (0.78-6.14)
≥60	3 (0)	331	0.8 (0.3-2.6)	1.75 (0.44-6.97)
Source of index case				
Local	18 (3)	967	1.6 (1.0-2.5)	1 [Reference]
Imported	4 (1)	1794	0.2 (0.1-0.5)	0.11 (0.03-0.37)
Clinical severity of index case				
Asymptomatic	0	91	0 (0-4.1)	0
Mild illness	4 (0)	1397	0.4 (0.1-0.9)	1 [Reference]
Pneumonia				
Mild	5 (2)	791	0.4 (0.1-1.2)	1.08 (0.24-4.82)
Severe	7 (0)	511	1.4 (0.7-2.8)	3.76 (1.10-12.76)
ARDS/sepsis	6 (2)	275	1.5 (0.6-3.7)	3.99 (1.00-15.84)

Abbreviations: ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019.

[a] Others include friends, airline crew members and passengers, and other casual contacts.

[b] Defined as the elapsed time between the date at symptom onset of the index case and the first date at exposure. For example, people from the group "<0 days" had their first contact with the index case before the case had any symptoms.

[c] All the reported exposures occurred during the presymptomatic period of the index case.

transmission potential of COVID-19 in a subgroup of contacts whose exposure occurred exclusively during the presymptomatic period of the index case. Our analysis revealed a similar clinical attack rate between the contacts who only had presymptomatic exposure and those who had postsymptomatic exposure.

To summarize the evidence, the decreasing risk for secondary infection over time in our study, the observed short serial interval, and the trend of decreasing viral shedding and viability after symptom onset strongly suggested high transmissibility of the disease near or even before the day of symptom onset. Because the onset of overt clinical symptoms, such as fever, dyspnea, and signs of pneumonia, usually occurred 5 to 7 days after initial symptom onset, the infection might well have been transmitted at or before the time of detection.[22,23] This characteristic makes containment efforts challenging. In a modeling study, Hellewell et al[24] found that the possibility of controlling COVID-19 through isolation and contact tracing decreased with increasing proportion of transmission that occurred before symptom onset. The findings of this modeling study, when viewed in the context of our findings, might help to explain the difficult situation in such areas and countries as China, South Korea, Iran, and Italy. Aggressive social distancing and proactive contact tracing might be necessary to block the transmission chain of COVID-19 and to keep presumptive patients away from susceptible populations with a high risk for severe disease.

The observed short duration of infectiousness with lower risk of transmission 1 week after symptom onset has important implications for redirecting the efforts to control COVID-19. Given the nonspecific and mostly mild symptoms of COVID-19 at presentation, patients are often identified and hospitalized at a later stage of disease when the transmissibility of infection has started to decrease. In this case, hospitalization would not be helpful for isolation and reducing transmission, and should be only for patients whose clinical course is sufficiently severe. When the number of confirmed cases rapidly increases, home care for patients with mild illness may be

Figure 2. Number of Contacts, Secondary Cases, and Secondary Clinical Attack Rate by the Time of First Exposure

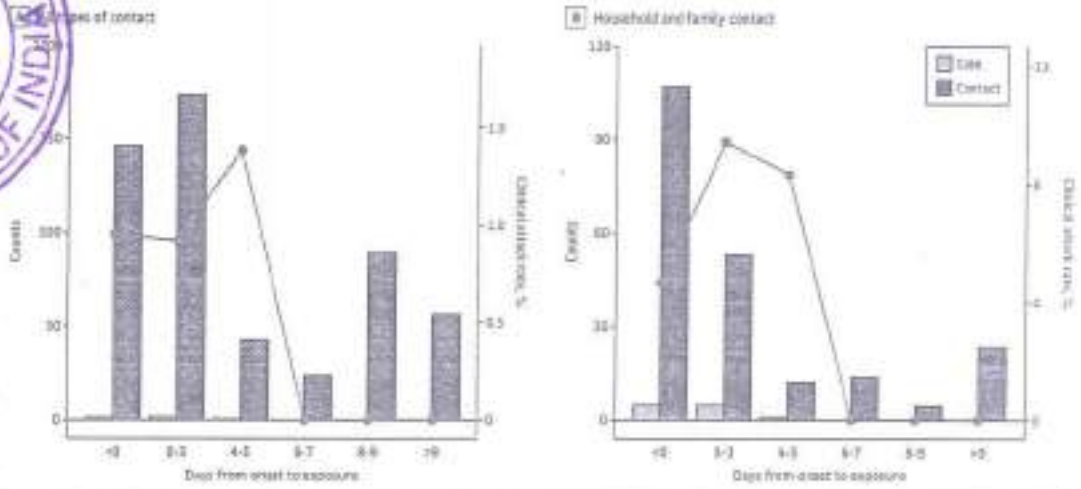


Table 3. Risk for Symptomatic COVID-19 Infection Among the 2761 Close Contacts, Simultaneously Stratified by Exposure Setting and Time From Symptom Onset of the Index Case to First Day of Exposure

First day of exposure, d	Household		Nonhousehold family		Health care		Others[a]	
	Case/contact, No.	Attack rate, % (95% CI)[b]	Case/contact, No.	Attack rate, % (95% CI)[b]	Case/contact, No.	Attack rate, % (95% CI)[b]	Case/contact, No.	Attack rate, % (95% CI)[b]
<0	4/150	4.8 (1.4-9.8)	1/10	10.0 (1.8-40.4)	2/236	4.8 (0.2-5.0)	0/389	0 (0-1.0)
0-3	2/39	5.1 (1.4-16.9)	3/15	20.0 (7.0-45.2)	3/150	2.0 (0.7-5.7)	0/683	0 (0-0.6)
4-5	1/6	16.7 (3.0-56.4)	0/6	0 (0-19.0)	1/38	2.6 (0.5-13.5)	1/186	0.6 (0.1-3.3)
6-7	0/4	0 (0-49.0)	0/10	0 (0-27.8)	0/17	0 (0-18.4)	0/88	0 (0-4.2)
8-9	0/2	0 (0-65.7)	0/3	0 (0-56.1)	0/110	0 (0-3.3)	0/354	0 (0-1.1)
>9	0/0	NC	0/24	0 (0-13.8)	0/146	0 (0-2.6)	0/114	0 (0-3.3)

Abbreviations: COVID-19, coronavirus disease 2019; NC, not calculable.

[a] Others include friends, airline crew members and passengers, and other casual contacts.

[b] Secondary clinical attack rate.

preferred.[28] In Taiwan (where patients with COVID-19 have been routinely hospitalized), the most prolonged duration of hospital isolation for the 100 confirmed cases was more than 2 months. If every patient with mild illness is to be isolated in the hospital or other isolation facilities for such a prolonged period during a large epidemic, the health care system would soon be overwhelmed, and the case-fatality rate may increase, as observed in Wuhan.[26,27] Similarly, better understanding of the potential duration of transmission could help direct containment strategies. For example, contact tracing could focus on the contacts near or even before symptom onset of the index cases when the number of index cases or contacts is too large for all contacts to be traced, given the available resources.

Several patients in our study were initially considered to have pneumonia of unknown etiology and had multiple contacts in the health care setting before being diagnosed. However, the number of health care contacts that led to nosocomial transmission was low. Besides the basic PPE used by medical staffs, this finding might be due to the late admissions of these patients and their lower risk of transmitting COVID-19 by the time of hospitalization. This pattern is compatible with the observations in China and Hong Kong. In China, the number of nosocomial infections might be lower than reported because some health care workers acquired infections in their households rather than in the health care facility.[3] In Hong Kong, most hospitalization was also delayed to at least 5 days after disease onset.[29] In closed settings such as a hospital or a cruise ship,[29,30] fomite transmission might play an important role, amplifying the risk of transmission and making the temporality of transmission less identifiable.[30-32] Better understanding of the dynamic change of transmissibility of COVID-19 over time and how health care workers are most likely to be infected could allow for better targeting of control measures, including the use of appropriate PPE.

In the contact tracing cohort, we observed a relatively low transmission rate of COVID-19. During the study period (January to early March 2020), the major containment measures in Taiwan were travel alerts with restriction to affected countries (principally China), home quarantine for travelers entering Taiwan, and comprehensive contact tracing for confirmed cases.[12] In response to a possible shortage of face masks,

the government proactively initiated a name-based rationing system for mask purchase and boosted the production of face masks to ensure the availability for both N95 respirators and face masks to both health care professionals and the general public. A general recommendation on social distancing from the government was not in place, but spontaneous behavioral changes that reduced community mobility were observed.[19]

Limitations

Our study has limitations. First, we did not completely examine contacts before the symptom onset of the index cases. Therefore, we might have underestimated the importance of early transmission. Thus, the actual contribution of early transmission to new infections could be greater than our estimates suggests. Our findings agree with the recommendation from the WHO to use 4 days before symptom onset as the starting date for contact tracing.[19] This modification may help to further understand the pattern of early transmission in COVID-19. Second, we could not completely separate out the effect of close household contact and early contact given the strong correlation of the 2. The increased transmissibility in the early stage of COVID-19 may be partially attributed to the effect of household and nonhousehold family contacts rather than increased infectiousness at the early stage. When we stratified by type of exposure, however, the pattern of early transmission remained.

Conclusions

In summary, the findings of this study suggest that most transmission of COVID-19 occurred at the very early stage of the disease or even before the onset of symptoms, and the secondary clinical attack rate among contacts decreased over time as symptoms developed and progressed. The pattern of high transmissibility near and before symptom onset and the likely short infectious period of the virus could inform control strategies for COVID-19, as well as additional studies to fully elucidate the transmission dynamics of the virus.

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Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Cheng, Jian, Ng, Lin.
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Group Information: The Taiwan COVID-19 Outbreak Investigation Team members include the following: Taiwan CDC: Office of Preventive Medicine and Taiwan Field Epidemiology Training Program: Wan-Ting Huang, Wan-Chin Chen, Angela Song-En Huang, Chia-Ping Su, Pin-Hui Lee, Pei-Chun Chan, Hao-Hsin Wu, Shih-Tse Huang, Tsung-Pei Tsou, Ying-Shih Su, and Yang Li. Taipei Regional Control Center: Hsin-Yi Wei, Meng-Yu Chen, Shiao-Ping Tung, Yu-Fang Tsai, Xiang-Ting Huang, and Chien-Yu Chou. North Regional Control Center: Pei-Yuan Wu, Fang-Tzu Chang, Chia-Ying Yen, Hsueh-Nei Chiang, Ju-Hui Lin, and Ming-Chu Tai. Central Regional Control Center: Kung-Chin Wang, Ching-Fan Ko, Pei-Fang Lai, Du-Ling Lin, Min-Tsung Lin, and Zhi-Jie Ding. South Regional Control Center: Huai-Te Tsai, Ping-Jung Liu, Pei-Yi Lin, Shu-Chen Chang, and Yi-Ying Lin. Kao-Ping Regional Control Center: Hsin-Chun Lee, Chi-Nan Hung, Ching-Li Lin, Chi-Mei Lai, and Hsiao-Mei Liu.

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Editor's Note

Contact Tracing, Testing, and Control of COVID-19—Learning From Taiwan

Robert Steinbrook, MD

Taiwan is a country of about 24 million people, 81 miles off the coast of mainland China. As of late April 2020, Taiwan had about 330 confirmed cases of coronavirus disease 2019 (COVID-19) and 6 deaths. By comparison, the US had about 1 million confirmed cases of COVID-19, and 60 000 deaths.

In this issue of *JAMA Internal Medicine*, there is a remarkable report from Taiwan on the use of contact tracing and virologic polymerase chain reaction testing to assess the transmission dynamics of COVID-19 in the country's initial 100 confirmed cases.[1] Among 2761 close contacts of the 100 cases, confirmed between January 15 and March 18, 2020, Cheng et al report that there were 22 paired-index secondary cases and an overall secondary clinical attack rate of 0.7% (95% CI, 0.4%-1.0%).[1]

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The study has important messages for the control of COVID-19 throughout the world. First, people with COVID-19 were found to be most infectious to others before and within 5 days of symptom onset. Within 5 days of symptom onset, the attack rate was 1.0% (95% CI, 0.6%-1.5%). With exclusive presymptomatic exposures, the attack rate was 0.7% (95% CI, 0.2%-2.4%), and with exposures 6 days or more after symptom onset, there were 0 cases from 852 contacts (95% CI, 0%-0.4%).[1]

These findings underscore the pressing public health need for accurate and comprehensive contact tracing and testing. Testing only those people who are symptomatic will miss many infections and render contact tracing less effective. The finding that asymptomatic people and those with minimal or fewer symptoms early in infection are those most likely to transmit COVID-19 strongly argues for maintaining social distancing and

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

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A study on infectivity of asymptomatic SARS-CoV-2 carriers

Ming Gao [a,1], Lihui Yang [b,1], Xuefu Chen [c], Yiyu Deng [d], Shifang Yang [e], Hanyi Xu [e], Zixing Chen [a], Xinglin Gao [a,*]

[a] The Second School of Clinical Medicine, Southern Medical University, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, 510515, China
[b] Hospital Infection Control Division, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, 510080, China
[c] Department of Infectious Disease, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, 510080, China
[d] Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology, Guangzhou, 510080, China
[e] Department of Respiratory and Critical Care Medicine, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangdong Provincial Geriatrics Institute, Guangzhou, 510080, China

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ABSTRACT

Background: An ongoing outbreak of coronavirus disease 2019 (COVID-19) has spread around the world. It is debatable whether asymptomatic COVID-19 virus carriers are contagious. We report here a case of the asymptomatic patient and present clinical characteristics of 455 contacts, which aims to study the infectivity of asymptomatic carriers.

Material and methods: 455 contacts who were exposed to the asymptomatic COVID-19 virus carrier became the subjects of our research. They were divided into three groups: 35 patients, 196 family members and 224 hospital staffs. We extracted their epidemiological information, clinical records, auxiliary examination results and therapeutic schedules.

Results: The median contact time for patients was four days and that for family members was five days. Cardiovascular disease accounted for 25% among original diseases of patients. Apart from hospital staffs, both patients and family members were isolated medically. During the quarantine, seven patients plus one family member appeared new respiratory symptoms, where fever was the most common one. The blood exams in most contacts were within a normal range. All CT images showed no sign of COVID-19 infection. No severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections was detected in 455 contacts by nucleic acid test.

Conclusion: In summary, all the 455 contacts were excluded from SARS-CoV-2 infection and we conclude that the infectivity of some asymptomatic SARS-CoV-2 carriers might be weak.

1. Introduction

The emergence of the coronavirus disease 2019 (COVID-19) since early December 2019, has spread to many countries recently and sparked world pandemic via mass gathering [1–3]. As of March 24, 2020, there have been 334981 confirmed cases and 14652 deaths globally [4].

It has been proved that the pathogen of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has high homology with SARS-CoV [5]. Similar to SARS-CoV, the dominant SARS-CoV-2 transmission mode is human-to-human transmission [6]. Differently, the reproductive number (R0) (the expected number of

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CHD, congenital heart disease; EDOU, emergency department observation unit; ED, emergency department; CT, computed tomography; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; GCDC, Guangzhou Center for Disease Control and Prevention; CDC, Chinese Center for Disease Control and Prevention; IQR, interquartile ranges; PPE, personal protective equipment.

* Corresponding author. Department of Respiratory and Critical Care Medicine, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangdong Provincial Geriatrics Institute, 106 Zhongshan Er Road, Guangzhou, 510080, China.
E-mail addresses: 741621766@qq.com (M. Gao), smylh007@163.com (L. Yang), chenxuefu1992@163.com (X. Chen), yiyudeng66@163.com (Y. Deng), huayiyang82@163.com (S. Yang), yixu2468@126.com (H. Xu), 1308643244@qq.com (Z. Chen), xinglingao@hotmail.com (X. Gao).
[1] Ming Gao and Lihui Yang contributed equally to this manuscript.

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secondary cases produced by a single infected person in a susceptible population) is estimated between two and three, which is higher than SARS [6,7]. Furthermore, the transmission of SARS occurs during the symptomatic period [8]. For COVID-19, numerous asymptomatic infections were found among close contacts of confirmed patients, like the report on "Diamond Princess" [9]. However, the epidemiological significance of asymptomatic infections is unclear until now. Recent studies indicated that transmission of COVID-19 could also occur from these individuals with no symptoms [10,11]. However, for now, whether asymptomatic SARS-CoV-2 carriers are contagious still remain controversial.

Here, we report a case of an asymptomatic SARS-CoV-2 carrier with nosocomial infection, as shown below, and describe the clinical characteristics of 455 contacts. Our purpose is to analyze the infectivity of asymptomatic carriers.

Case A was a 22-year-old female patient who had a medical history of congenital heart disease (CHD) presented to the emergency room of Guangdong Provincial People's Hospital (Guangzhou, Guangdong province, China) on January 13, 2020. She complained of shortness of breath for 16 years, and the symptom worsened for one month. The accompanied symptom was chest distress, without cough, sputum production and fever. Apart from CHD, she had no other diseases and had no smoking habit. Her temperature was normal, and laboratory measurements showed no apparent abnormalities (Table 1). Echocardiography displayed atrial septal defect and severe pulmonary hypertension. The diagnosis was congenital heart disease, atrial septal defect and pulmonary hypertension.

Case A was mainly given to oxygen therapy, diuretic treatment, plus pharmacotherapy of pulmonary hypertension. On January 16, as Case A's condition improved and vital signs became stable, she was transferred to emergency department observation unit (EDOU). Owing to the Spring Festival and COVID-19 outbreak, she had been hospitalized in EDOU along with her brother until February 11. Before admission, she underwent a serious of examinations according to hospital-formulated guidance during epidemic period. Nevertheless, the patient's nasopharyngeal swab tested positive for SARS-CoV-2 by real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). She was immediately admitted to quarantine ward in infectious department.

Case A and her brother both denied visiting Wuhan (the epidemic area in China) and any contact with COVID-19 patients. They wore masks all the time except at meals and drinking. How she became infected was unknown.

Notably, in isolation, the patient had never fever, sore throat, myalgia or other symptoms associated with virus infection [12]. Shortness of breath and chest distress, without further aggravation than before, were thought to be caused by CHD. Laboratory measurements reflected that white blood cells, lymphocytes, C-reactive protein and procalcitonin were within a normal range (Table 1). Upon admission, chest computed tomography (CT) scan showed non-COVID-19 imaging feature (Fig. 1. a, c). In the time of hospitalization, the patient received antiviral and interferon therapy. 11 days after the treatment (February 22), chest CT scan indicated no significant differences in comparison with before (Fig. 1. b, d).

Meanwhile, nucleic acid tests by real-time RT-PCR assay were performed repeatedly. Nucleic acid tests were positive for five consecutive days from February 11 to 15. The results of the test on quarantine day 16 (February 26) turned negative, subsequently, on quarantine day 18 and 20, which were also negative. She was then released from quarantine on day 21 (March 2, 2020). Due to only laboratory-confirmed positive, case A was diagnosed as an asymptomatic carrier, manifested by Guangzhou Center for Disease Control and Prevention (GCDC).

Table 1
Laboratory measurements of Case A.

	Jan, 13	Feb, 12	Feb, 19	Feb, 27	Normal range
Blood count, × 10^9/L					
White blood cell count	7.70	7.78	7.41	7.92	3.50–9.50
Neutrophil count	4.97	4.14	4.16	4.44	1.80–6.30
Lymphocyte count	2.13	2.93	2.13	2.63	1.10–3.20
Serological variables					
C-reactive protein, mg/L	0.6	5.89	NT	NT	< 5.0
Procalcitonin, ng/ml	Normal	Normal	NT	NT	< 0.05

NT = not tested.

2. Materials and methods

2.1. Study design and subjects

In our study, asymptomatic COVID-19 carrier was defined as a patient without related clinical symptoms, but whose SARS-CoV-2 test was positive. Since Case A was diagnosed, all contacts, including hospital staffs, family members and patients, were routinely screened. Amounting to 455 contacts – 224 hospital staffs, 196 family members and 35 patients – who had been exposed to case A in EDOU or circulated area of it became the subjects of study. Contacts were defined as individuals in the same ward with Case A. EDOU plus the circulated area of it covers 60 square meters where there are 14 patients' beds placed at least 1.2 m apart. The study was approved by the Medical Ethical Committee of Guangdong Provincial People's Hospital.

2.2. Data collection

Isolated individuals' detailed information during the emergency department (ED) and hospitalization were retrospectively collected from electronic medical records, while other data were reviewed from documents recorded by related departments. Emphatically, we gathered their contact history, consisting of the date when they went to the emergency, the reason why they came to the emergency, and the time how long they were exposed to the case A. Demographics characteristics were also collected.

Any new symptoms on each person were taken down, both in ED and quarantine, including fever, cough, sputum production, sore throat, etc.

Meanwhile, we obtained imaging and laboratory data from hospital staffs and patients, which of family members were not noted. Each of patients had undergone a CT scan prior to admission because of the outbreak. It had been reviewed after the medical isolation began. Hospital staffs were examined one time. For laboratory test, a complete blood count was focus of attention. Nasopharyngeal swab specimens had been collected at least one time in whole people. All samples were processed at clinical laboratory of hospital and sent to GCDC simultaneously.

Patients and family members were quarantined for medical observation and hospital staffs were not quarantined because of standard protection, in principle.

2.3. Laboratory nucleic acid test

In accordance with the protocol announced previously by the WHO, RT-PCR can be considered in the assessment of individuals who have had contact with a COVID-19 case [13]. Consequently, whether the patients were infected with the SARS-CoV-2 was detected by real-time RT-PCR. Chinese Center for Disease Control and Prevention (CDC) published the sequences of primers and probe targeted to envelope gene of SARS-CoV-2, in January 21, 2020, which were as follows: forward primer 5'-TCAGAATGCCAATCTCCCCAAC-3'; reverse primer 5'-AAAG GTCCACCCGATACATTGA-3'; and probe 5'CY5CTAGTTAGACTAGC-1CATCCTTACTGC-3'BHQ1.

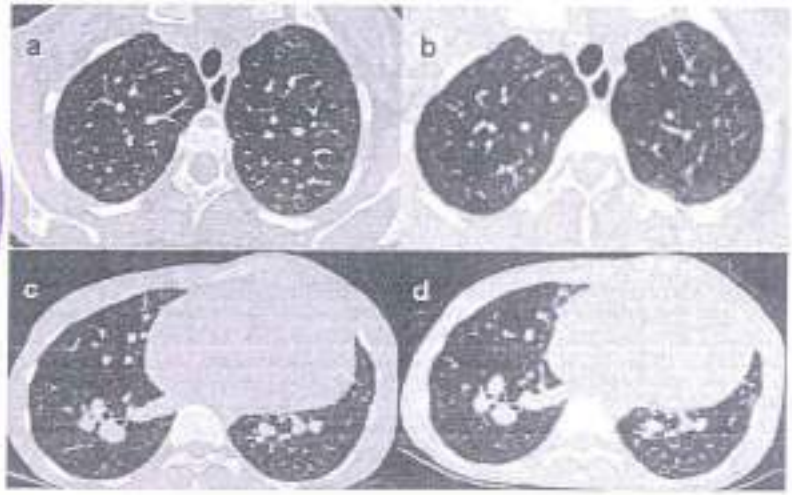

Fig. 1. Chest CT images of Case A. Figure a and c were taken on February 11, which showed non- COVID-19 imaging feature. Case A underwent a CT scan again on February 22. Figure b and d, the follow-up images, showed no significant differences than before.

2.4. Statistical analysis

All research objects employed fundamental descriptive analysis. Continuous variables were expressed as the medians and interquartile ranges (IQR). Categorical variables in each category were summarized as counts and percentages. All statistical analyses were conducted with IBM SPSS statistics 24.0.

3. Results

3.1. Patients

The statistics of 35 patients are displayed in Table 2. All patients also wore masks except for eating or drinking and were admitted to infectious department for medical isolation. The median contact time was four days (interquartile range, 1.0 to 8.0), whereas the longest among them reached 21 days. The median age of the patients was 62 years (interquartile range, 50.0 to 64.0). A total of 57.1% were males. All individuals had been to ED of our hospital for various diseases. Undoubtedly, acute cardiovascular event and digestive diseases were common, accounting for 25.7% and 22.9%, respectively.

In terms of clinical symptoms, 16 (45.7%) patients were free of respiratory symptoms, which more than half of them (19 [54.3%]) appeared inversely. The respiratory symptoms involved fever (11 [31.4%]), cough (8 [22.9%]), dyspnea (7 [20.0%]) and sputum (4 [11.4%]). Among patients with fever, hyperthermia (body temperature >39 °C) occurred in one patient with chills after chemotherapy. Three individuals presented slight fever (body temperature between 37.3 °C and 38.0 °C), of whom temperature could return to normal without therapy. Two individuals were diagnosed with infective endocarditis and acute pancreatitis severally with the cause of their symptoms including fever plus dyspnea and stomachache. Another five patients developed fever was consider as complication of the original disease, mostly pulmonary infection, whether the symptom was new or persistent. Last but not least, all dyspnea was associated with heart failure.

The blood counts in most patients were within normal range. Lymphocytopenia (<1.5 × 10^9 cells/L) had appeared in 34.3% of the patients and leukopenia (<4.0 × 10^9 cells/L) in 8.6%. One patient was also accompanied with neutropenia (<1.8 × 10^9 cells/L) on account of myelosuppression after chemotherapy. On the contrary, lymphocytosis (>3.5 × 109 cells/L) was observed in a patient with acute lymphoblastic leukemia. Besides one patient, died on quarantine 4-day, 34 patients underwent multiple nucleic acid tests of SARS-CoV-2. All the results were negative, including first time of the dead. Of 35 patients that had more than once chest CT scan, CT abnormalities were reported in 68.6%. The most common manifestations were inflammatory change (34.3%) and pleural effusion (45.7%). The interstitial change was detected less. Pulmonary edema and nodule were seen in persons with heart failure and lung cancer, respectively. Of particular concern, all CT findings were non-viral infection discussed professionally by radiologists. Normal CT image was found in 11 of 35 patients (31.4%).

According to evaluation from physicians, there were 19 infected patients (54.3%). Of these 19 patients, 15 (42.9%) had pneumonia, which was bacterial (37.1%), followed by pendulous (2.9%) and aspiration (2.9%). Mediastina inflammatory and abdominal infection arose from two patients with fistula. Other two patients had suffered from infection due to protopathy on admission. Above infected patients were administered for empirical antibiotic treatment. Four (11.4%) patients needed emergency surgery for their condition. The rest of them (34.3%) were received to heteropathy.

It totally took 14 days from the last contact with Case A to the end of medical observation. As of Feb 26, 2020, none of 35 patients was diagnosed with SARS-CoV-2 infection. Among whom eight (22.9%) were discharged from the hospital while 25 (71.4%) were transferred to the specialized department for further treatments. The remaining two individuals both died for severe heart failure judged by the clinical expert panel during the period of quarantine.

3.2. Family members

In total, 196 family members were enrolled. The situation on wearing masks was the same as that in patients. Local CDC took charge of following up 172 among them, who were ruled out SARS-CoV-2 infection after 14-days medical isolation, as far as we know. The rest of the escorts were placed in designated locations by GCDC for quarantine. Of these 24 escorts, 11 were male and 13 were female. They aged from 24 to 86 years old and the median age was 47.5 years old (interquartile range, 34.0 to 57.0). The median contact time was five days (interquartile range, 1.0 to 11.0). Regarding examinations, at least two nucleic acid tests were negative. All the attendants except a family member had no respiratory symptoms in the time of quarantine. Details as below.

Table 2
Clinical characteristics of 35 patients.

Clinical characteristics	Patients (n = 35)
Demographics characteristics	
Age, median (IQR), years	42 (36.0–64.0)
Male, sex, No. (%)	20 (57.1)
Times of contact with Case A, median (IQR), days	4 (1.0–6.0)
Pronopathy, No. (%)	
Cardiovascular disease	9 (25.7)
Digestive diseases	8 (22.9)
Cerebrovascular disease	5 (14.3)
Orthopedic disease	4 (11.4)
Hematological disease	2 (5.7)
Renal disease	2 (5.7)
Cancer	3 (8.6)
Others[1]	2 (5.7)
Respiratory symptoms	
None, No. (%)	16 (45.7)
Any, No. (%)	19 (54.3)
Fever	11 (31.4)
Newly emerged fever	7 (20.0)
Cough	8 (22.9)
Sputum	4 (11.4)
Dyspnea	7 (20.0)
Newly emerged dyspnea	1 (2.9)
Laboratory findings	
Leukocyte count ($\times 10^9$/L, normal range 4.0–10.0), No. (%)	
Increased	14 (40.0)
Decreased	3 (8.6)
Normal	18 (51.4)
Lymphocyte count ($\times 10^9$/L, normal range 1.5–3.5), No. (%)	
Increased	1 (2.9)
Decreased	12 (34.3)
Normal	22 (62.9)
Nucleic acid test negative, No. (%)	35 (100.0)
Radiologic findings	
Normal on chest CT, No. (%)	11 (31.4)
Abnormalities on chest CT, No. (%)	24 (68.6)
Inflammatory change	12 (34.3)
Pleural effusion	16 (45.7)
Interstitial change	2 (5.7)
Pulmonary edema	1 (2.9)
Pulmonary nodule	1 (2.9)
Clinical outcome	
Transfer to specialized department, No. (%)	25 (71.4)
Discharge from hospital, No. (%)	8 (22.9)
Death, No. (%)	2 (5.7)

[1] Others include urologic diseases and rheumatic disease.

A 37-year-old woman, who had stayed in EDOU (February 7) for 10 h as relative, developed slight fever (the highest temperature was 37.5 °C), dry cough and sore throat after six days (February 13). Then she was presented to our hospital immediately. According to the investigation, she had worn a mask during the hospital visit and had a history of recurrent acute tonsillitis. After admission, her CT images and laboratory measurements were normal. Additionally, she experienced four SARS-CoV-2 nucleic acid tests successively, all of which were negative. The case was given empirical antibiotic treatment since admission, and her symptoms disappeared afterwards. Based on clinical symptoms as well as auxiliary examination results, she received a diagnosis of acute tonsillitis finally. On February 25, she recovered and was discharged from the hospital.

3.3. Hospital staffs

To ensure safety, 224 hospital staffs who had come into contact with Case A were screened. As is clearly shown in Table 3, their identities were mainly composed of doctors (59 [26.3%]) and nurses (101 [45.1%]). Others coexisted in the same room with Case A for work, such as security guards, cleaners, transportation personnel, etc. Of these 224 staffs, 103 were male and 121 were female, with ages ranging 21–60 years old. The median age was 35 years old (interquartile range, 28.0 to 42.0). Most of them were emergency workers so that they had long exposure to confirmed case. Oppositely, some doctors in other departments had a brief stay, approximately one to 2 h, because of emergency consultation. It is noteworthy that all personnel working in ED must take medical protection – namely, using N95 mask, putting on isolation gown and wearing goggle.

Table 3
Clinical characteristics of 224 hospital staffs.

Clinical characteristic	Hospital staffs (n = 224)
Demographics characteristics	
Identity, No. (%)	
Doctor	59 (26.3)
Nurse	101 (45.1)
Others[1]	64 (28.6)
Age, median (IQR), yr.	35 (28.0–42.0)
Male, sex, No. (%)	103 (46.0)
Respiratory symptoms	
None, No. (%)	224 (100.0)
Laboratory findings	
Leukocyte count ($\times 10^9$/L, normal range 4.0–10.0), No. (%)	
Increased	29 (12.9)
Decreased	0 (0.0)
Normal	195 (87.1)
Lymphocyte count ($\times 10^9$/L, normal range 1.5–3.5), No. (%)	
Increased	5 (2.2)
Decreased	3 (1.3)
Normal	216 (96.5)
Nucleic acid test negative, No. (%)	224 (100.0)
Radiologic findings	
Normal on chest CT, No./total No. (%)	171/223 (76.7)
Abnormalities on chest CT, No./total No. (%)	52/223 (23.3)
Pulmonary nodule	29/223 (13.0)
Pulmonary fibrosis focus	18/223 (8.1)
Pulmonary emphysema	5/223 (2.2)
Quarantine personnel, No. (%)	0 (0.0)

[1] Others include security guards, cleaners, transportation personnel, support crew.

No respiratory symptoms were observed, either existing or emerging. Blood test results revealed normal leukocyte count in 87.1% and lymphocyte count in 96.5%. Lymphocyte counts were decreased in three staffs (1.3%) without leukopenia ($<4.0 \times 10^9$ cells/L). A pregnant nurse failed to accept a CT scan. A minority of hospital staffs (52 [23.3%]) found abnormalities in CT, where pulmonary nodule, fibrosis focus and emphysema were seen. All hospital staffs were tested twice for SARS-CoV-2 nucleic acid, and the results were negative, which was identical with the two groups mentioned above. In contrast to them, none of 224 hospital staffs required medical observation.

4. Discussion

In this study, we recorded in detail the hospitalized situation, diagnostic procedure, inspection results, treatment plans and clinical outcome of an asymptomatic SARS-CoV-2 carrier who was laboratory confirmation by RT-PCR assay, but without related symptoms and imaging changes in concert with previous reports [12,14]. Also, we analyzed epidemiological and clinical data from 455 contacts who had been exposed to the asymptomatic patient. All the 455 contacts were excluded from SARS-CoV-2 infection. Of the 231 quarantined people (196 family members and 35 patients), 229 were removed from medical observation successfully and two died for severe heart failure. New or existing respiratory symptoms were almost appeared in patients, which were considered to be associated with their original disease or complications. A family member complaining of fever was diagnosed as acute tonsillitis ultimately. Unlike COVID-19, normal blood count was found in most contacts [12,15]. All CT images showed no sign of COVID-19

infection. Unquestionably, all cases tested negative for SARS-CoV-2 nucleic acid. This fact illustrated that there had been no cases of infection in a relatively dense space.

Since the outbreak, our hospital has taken a series of effective prevention and control measures, which made a considerable effect on preventing the spread in this case. Above all, medical staffs abide by the principle of graded protection strictly. For patients and attendants, each patient can only be accompanied by one attendant, and both need to wear personal protective equipment (PPE). Nevertheless, there is still a risk of transmission of COVID-19 under stringent measures. Primarily, shortages of PPE were common in the early stages. Medical resources were supplied to healthcare workers priorly. Due to these factors, patients and attendants can only wear one mask for a long time, resulting in its ineffective. Besides, we noticed that some patients and relatives wore PPE incorrectly due to the lack of adequate training, which was also possible for hospital staffs. Last but not least, it is unavoidable to take off mask while eating or drinking, which provides an opportunity to spread the virus.

Considering all the mentioned factors, we suggest that there are more important reasons for achieving "Zero infection". As is well-known, person-to-person transmission through respiratory droplets is the main route of COVID-19 transmission [6]. Earlier research revealed that the viral load of respiratory tract samples in an asymptomatic patient was similar to that in the symptomatic patients [11]. However, a single sample is difficult to be representative. In the light of "Zero infection" for this case, we venture to guess that the viral load of respiratory tract samples in the asymptomatic patient might not be high. Moreover, although pathogenic nucleic acids can be detected in respiratory tract samples from asymptomatic carriers, the opportunity of transmission is less than that in symptomatic patient owing to the absence of the way expelling pathogen via cough and sneezing.

Based on the foregoing discussion, we conclude that the infectivity of some asymptomatic SARS-CoV-2 carriers might be weak. This finding implicates that there is not needful to worry unduly for asymptomatic or mild patients during the ongoing COVID-19 pandemic. Furthermore, excessive virus nucleic acid detection is unnecessary, which can relieve the pressure on public health resources. Under the development of epidemic circumstance, more and more public concerns on the increasing number of asymptomatic or mild patients hid in the community. However, combined with our results and the defense measures currently completed, we hope such worries are misplaced and we also believe the world will win this battle certainly.

The limitation of our study is that there is only one case and lack of detailed information on family members quarantined locally. Large-scale multicenter studies are needed to verify our conclusion. However, both asymptomatic carrier and 455 contacts were admitted to and treated in designated places. Hence, the study results are representative to some extent.

5. Conclusions

Infectivity of some asymptomatic SARS-CoV-2 carriers might be weak. Effective prevention and control measures are helpful to prevent COVID-19 spread of asymptomatic carriers. The result of this study may alleviate parts of the public concern about asymptomatic infected people.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ming Gao: Methodology, Data curation, Writing - original draft, Writing - review & editing. Lihui Yang: Investigation, Data curation, Formal analysis, Writing - review & editing. Xuefu Chen: Resources. Yiyu Deng: Writing - review & editing. Shifang Yang: Writing - review & editing. Hanyi Xu: Resources. Zixing Chen: Resources. Xinglin Gao: Conceptualization, Project administration, Supervision, Validation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rmed.2020.106026.

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EXHIBIT "F"

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Modes of contact and risk of transmission in COVID-19: a prospective cohort study 4 950 close contact persons in Guangzhou of China

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Corresponding Author:	Chen Mao Guangzhou, CHINA
First Author:	Lei Luo
Order of Authors:	Lei Luo
	Dan Liu
	Xin-long Liao
	Xian-bo Wu
	Qin-long Jing
	Jia-zhen Zheng
	Fang-hua Liu
	Shi-gui Yang
	Bi Bi
	Zhi-hao Li
	Jian-ping Liu
	Wei-qi Song
	Wei Zhu
	Zheng-he Wang
	Xi-ru Zhang
	Pei-liang Chen
	Hua-min Liu
	Xin Cheng
	Miao-chun Cai
	Qing-mei Huang
	Pei Yang
	Xing-fen Yang
	Zhi-gang Han
	Jin-ling Tang
	Yu Ma
	Chen Mao
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Abstract:	Background: Rapid spread of SARS-CoV-2 in Wuhan prompted heightened surveillance in Guangzhou and elsewhere in China. Modes of contact and risk of transmission among close contacts have not been well estimated.

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Methods: We included 4950 closes contacts from Guangzhou, and extracted data including modes of contact, laboratory testing, clinical characteristics of confirmed cases and source cases. We used logistic regression analysis to explore the risk factors associated with infection of close contacts.
Findings: Among 4950 closes contacts, the median age was 38.0 years, and males accounted for 50.2% (2484). During quarantine period, 129 cases (2.6%) were diagnosed, with 8 asymptomatic (6.2%), 49 mild (38.0%), and 5 (3.9%) severe to critical cases. The sensitivity of throat swab was 71.32% and 92.19% at first to second PCR test. Among different modes of contact, household contacts were the most dangerous in catching with infection of COVID-19, with an incidence of 10.2%. As the increase of age for close contacts and severity of source cases, the incidence of COVID-19 presented an increasing trend from 1.8% (0-17 years) to 4.2% (60 or over years), and from 0.33% for asymptomatic, 3.3% for mild, to 6.2% for severe and critical source cases, respectively. Manifestation of expectoration in source cases was also highly associated with an increased risk of infection in their close contacts (13.6%). Secondary cases were in general clinically milder and were less likely to have common symptoms than those of source cases.
Interpretation: The proportion of asymptomatic and mild infections account for almost half of the confirmed cases among close contacts. The household contacts were the main transmission mode, and clinically more severe cases were more likely to pass the infection to their close contacts. Generally, the secondary cases were clinically milder than those of source cases.

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Modes of contact and risk of transmission in COVID-19: a prospective cohort study 4 950 close contact persons in Guangzhou of China

Lei Luo[1]#, Dan Liu[2]#, Xin-long Liao[1]#, Xian-bo Wu[2]#, Qin-long Jing[1], Jia-zhen Zheng[2], Fang-hua Liu[1], Shi-gui Yang[3], Bi Bi[1], Zhi-hao Li[2], Jian-ping Liu[1], Wei-qi Song[2], Wei Zhu[1], Zheng-he Wang[2], Xi-ru Zhang[2], Pei-liang Chen[2], Hua-min Liu[2], Xin Cheng[2], Miao-chun Cai[2], Qing-mei Huang[2], Pei Yang[1], Xing-fen Yang[4], Zhi-gang Han[1]*, Jin-ling Tang[5]*, Yu Ma[1]*, Chen Mao[2]*.

[1] Guangzhou Center for Disease Control and Prevention

[2] Department of Epidemiology, School of Public Health, Southern Medical University

[3] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital College of Medicine, Zhejiang University

[4] Food Safety and Health Research Center, School of Public Health, Southern Medical University

[5] Guangzhou Women and Children's Medical Center

 # Lei Luo, Dan Liu, Xin-long Liao, and Xian-bo Wu Contributed equally to this article.

*Correspondence to:

Chen Mao, Ph.D.; Department of Epidemiology, School of Public Health, Southern Medical University, Guangzhou 510515, Guangdong, China; Email: maochen9@smu.edu.cn;

Yu Ma, master; Guangzhou Center for Disease Control and Prevention, Guangzhou 510440, Guangdong, China; Email: freedomsky_220@163.com;

Jin-ling Tang, Ph.D.; Guangzhou Women and Children's Medical Center, Guangzhou 510623, China; Email: jltang@cuhk.edu.hk;

Zhi-gang Han, master; Guangzhou Center for Disease Control and Prevention, Guangzhou 510440, Guangdong, China; Email: zhiganghan616@163.com.

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Abstract

Background: Rapid spread of SARS-CoV-2 in Wuhan prompted heightened surveillance in Guangzhou and elsewhere in China. Modes of contact and risk of transmission among close contacts have not been well estimated.

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Findings: Among 4950 closes contacts, the median age was 38.0 years, and males accounted for 50.2% (2484). During quarantine period, 129 cases (2.6%) were diagnosed, with 8 asymptomatic (6.2%), 49 mild (38.0%), and 5 (3.9%) severe to critical cases. The sensitivity of throat swab was 71.32% and 92.19% at first to second PCR test. Among different modes of contact, household contacts were the most dangerous in catching with infection of COVID-19, with an incidence of 10.2%. As the increase of age for close contacts and severity of source cases, the incidence of COVID-19 presented an increasing trend from 1.8% (0-17 years) to 4.2% (60 or over years), and from 0.33% for asymptomatic, 3.3% for mild, to 6.2% for severe and critical source cases, respectively.

Manifestation of expectoration in source cases was also highly associated with an increased risk of infection in their close contacts (13.6%). Secondary cases were in general clinically milder and were less likely to have common symptoms than those of source cases.

Interpretation: The proportion of asymptomatic and mild infections account for almost half of the confirmed cases among close contacts. The household contacts were the main transmission mode, and clinically more severe cases were more likely to pass the infection to their close contacts. Generally, the secondary cases were clinically milder than those of source cases.

Keywords: COVID-19, close contacts, modes of contact, risk of transmission

Introduction

In December 2019, the outbreak of Coronavirus Disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) emerged in Wuhan, Hubei Province, China, and has now developed into a global pandemic[1]. As of 15 March, worldwide a total of 153,517 people have been infected including 5,735 deaths, with 81,048 cases and 3,204 deaths in China[2].

The viral, epidemiological, and clinical characteristics of the disease have been documented[3-11]. However, some questions important for control of the epidemic remain outstanding[10]. For example, what is the transmissibility of the virus? What patients are more likely to spread the virus? What mode of contacts is most likely to cause transmission? What is the incidence of complete asymptomatic infection?

These questions are addressed in this follow-up study of 4,950 persons with close contact with confirmed COVID-2019 patients in Guangzhou, China.

Methods

Study Oversight

This is a prospective cohort study of all 4,950 persons who had a close

contact (or close contacts in short) with confirmed COVID-2019 patients (or source cases in short) identified between January 13 and March 6, 2020, in Guangzhou, Guangdong Province, China. A total of 129 cases were diagnosed with 42 before quarantine and 87 during the quarantine.

Data Sources

Close contacts include such unprotected contacts as living in the same household, face-to-face working together, sharing the same classroom, visit or stay in the same hospital ward, taking the same car or aeroplane, sharing neighbouring seats in the same train or ship as a diagnosed COVID-19 patient. It also includes giving direct care to a diagnosed patient. The full definition and whole list of forms of close contacts were showed in Appendix 1. When a COVID-19 patient was diagnosed then his or her close contacts were traced, and his or her close contacts may be locals or non-locals, if he or she had a history of travel or business. Thus, the source cases of close contacts included both local and non-local patients.

Between January 13, and March 6, 2020, 347 cases[12] were diagnosed in Guangzhou and their 4,950 close contacts were identified and enrolled in the study. Standard questionnaires were used to collect data at the time of enrollment, which was also the start of quarantine[13]. The registration

form (Appendix 2-Table a) was completed for each close contact. All close contacts were put under quarantine for 14 days from the last contact or longer for some cases if collection of samples for PCR testing was delayed. We recorded the last date of contact, the date of the start of quarantine, the date symptoms appeared, the date of each sampling, and the date of first positive PCR result. Temperature and symptoms monitoring were conducted every day and recorded in a standard form (Appendix 2-Table b).

Throat swab samples were collected and a real time RT-PCR testing performed once every two days. In one patient, the PCR testing was performed ten times as previous tests were consistently negative and has not released from quarantine. A close contact was released from quarantine if he had no symptoms and PCR testing was e negative for two consecutive samples. For those who were diagnosed with COVID-19, treatments followed and quarantine continued till recovery.

Data on demographic factors, risk factors, exposure history, mode of contact, symptoms, radiological and laboratory findings, severity of disease, treatments, and prognoses were collected on all close contacts (data form in Appendix 2). The information of source cases was also obtained through monitoring data from Guangzhou CDC. Close contacts confirmed COVID-19 (or secondary cases in short) and their source cases

are individually linked (details in Appendix 2) and their relations and contact modes were determined accordingly. For the 161 source cases who did not live in Guangzhou, so we could not know their severity of COVID-19, and could not linked their information with secondary cases.

Definitions

A source case is a person diagnosed with COVID-2019 a close contact person has made close contact with. Close contacts may have made contact with one or more patients.

The diagnosis of SARS-CoV-2 infection was made, according to the 6th National Criteria for Diagnosis of COVID-2019 in China[14]. As the study participants were all close contacts, a COVID-2019 case was referred to a person who had both a positive result for the virus' nucleic acid and symptoms and/or abnormal radiological/laboratory findings before, during or even after the 14 days of quarantine. Asymptomatic infection must have not clinical symptoms, must be positive for the virus' nucleic acid, and have or be free of radiological and/or laboratory alterations that indicate viral infection.

Fever was defined as an axillary temperature of 37.5°C or above. Severity of the disease includes 5 categories: asymptomatic, mild, moderate, severe and critical. Mild cases were those who had mild

149 symptoms but no radiological alterations. Moderate cases are those who
150 had both symptoms and radiological alterations. Severe cases were those
151 who had any of the following: breathing rate ≥30/min, or oxygen
152 saturation level ≤93% at rest, or oxygen concentration level PaO^2/FiO^2 ≤
153 300mmHg (1mmHg=0.133kPa), or lung infiltrates >50% within 24 to 48
154 hours. Critical cases are those who had respiratory failure requiring
155 mechanical ventilation, septic shock, or multiple organ
156 dysfunction/failure.

157 The mode of contact was classified into 5 categories: public
158 transport vehicles, healthcare settings, households, multiple, and others.
159 Tourists in the ship cruise were put in a special exposure group called
160 "Dream Cruise". The multiple contact includes those who were exposed
161 to more than one mode of contact (e.g. household and public transport
162 vehicles).

163 **Diagnosis of RT-PCR test, radiological and blood examination**

164 Throat swab samples were collected by trained CDC staff and transported
165 and stored in -70 °C refrigerators in biological safety level 2 laboratories.
166 Samples of cluster cases were also sent to China CDC for re-examination.
167 RT-PCR testing was performed by qualified staff and results were
168 identified through open reading frame 1ab (ORF1ab) and nucleocapsid

169 protein (N) in accordance with the protocol established by China CDC[13].
170 Details on laboratory processes are provided in Appendix 3. Radiological
171 and blood examinations were conducted in tertiary hospitals designated
172 for treating COVID-19 patients according to national standards[14].

173 **Statistical analysis**

174 The infection rate was estimated by dividing the number of diagnosed
175 cases with the number of close contact persons and compared among
176 different contact groups. Categorical variables were described in number
177 and percentage (%), and continuous variables in median and interquartile
178 range (IQR). Differences in proportions were tested by using the χ^2 test.
179 Univariate and multivariable logistic regressions were performed to
180 adjust for potential factors that may affect the risk of developing
181 COVID-19, and odds ratio (OR) and 95% confidence interval (95% CI)
182 were estimated.

183 Analyses were all performed with the SAS software (version 9.4 for
184 Windows, SAS Institute, Inc., Cary, NC, USA). Statistical tests were
185 two-sided, and P values of less than 0.05 were considered to indicate
186 statistical significance.

187 **Ethics Approval**

Ethics approval was obtained from the Ethics Committee of Southern Medical University. Data collection and analysis of close contacts and source cases were also required by the National Health Commission of the People s Republic of China to be part of a continuing public health outbreak investigation. Written informed consent was waived in light of the urgent need to collect data.

Results

Baseline characteristics of close contacts

By the end of the Mar 6, 2020, all the 4950 close contacts were enrolled. Males accounted for 2484 (50.2%). The median (IQR) age was 38.0 years and 783 (15.8%) were under 18 years (Table 1). Exposure in public transports was the commonest type of close contact. On average, 2.4 PCR tests were performed for each person. 129 (2.6%) cases were identified with 8 (6.2%) being asymptomatic throughout and 5 (3.9%) being clinically severe or critical.

The 4950 close contacts were quarantined for an average of 4.0 days, with 2.0 days for cases and 4.0 days for non-cases (Table 1 and Figure 1). In 20 persons, quarantine was unnecessary as they last contacted a patient 14 days ago and were free of symptoms and PCR test negative at the time they were identified. In 340 persons, quarantine was longer than 14.0

days because the PCR tests were delayed (Figure 1). There was on average 1.0 day from the start of quarantine to the first PCR testing, suggesting a slight delay in collecting samples for laboratory diagnosis (Table 1). PCR diagnosis was made within 14 days of quarantine for all 129 cases but two for whom it was on the 16th day; all the 8 asymptomatic cases were diagnosed within 10 days of quarantine (Figure 1).

There were on average 11.0 days from the last contact to the start of quarantine (Table 1), suggesting quarantine could in theory start 11.0 days earlier than it actually did. The delay from the last contact to quarantine was on average 1.0 day with over 3 days for 11 cases (Table 1 and Figure 2). In symptomatic cases, there was on average 1.0 day from the last contact to symptoms at onset, with 31 cases having already developed symptoms before the last contact and 22 cases over 3 days after the last contact (Table 1 and Figure 2). In 33 cases for whom the date of symptoms at onset was clear and the first PCR test was negative, we estimated that there were on average a delay of 2.0 days from symptoms to first PCR positivity and in 22 (66.7%) cases symptoms appeared 7.0 days prior to PCR positivity (Figure 2).

Mode of contact and risk of transmission

The age of close contacts was linearly associated with an increasing risk of getting infected after close contact with source patients (Table 2). The incidence was 1.8%, 2.2%, 2.9%, and 4.2% respectively for 0-17, 18-44, 45-59, and 60 or above age-groups (P=0.0016 for trend). Females seemed as likely as males to catch the infection after close contacts with patients (P=0.1202).

Among different modes of contact, household contacts and multiple contacts (with 70% including household contacts) were most dangerous in catching the infection and associated with an incidence of COVID-19 10.2% and 13.0%, respectively (Table 2). Healthcare settings contacts and public transport vehicles, the other two common forms of contacts, were associated with a risk of 1.0% and 0.1%, which were only about 10% and 1%, respectively, of the risk of household contacts (P<0.0001).

Furthermore, clinically more severe patients were more likely to pass the infection to their close contacts than less severe ones (P<0.0001 for trend). Asymptomatic infection is least likely to pass on the infection, with a chance of 33 per 100,000 contacts. Mild and moderate infections could increase the risk to 3.3% to 5.6%, and severe and critical infections to 6.2%. Manifestation of some symptoms in source patients was also associated with an increased risk of infection in their close contacts. For example, fever could increase the risk by over 100% (P=0.0103) and

expectoration by 400% (P<0.0001), whereas cough, fatigue and myalgia did not statistically significantly increase the risk (P>0.3700). In addition, a higher frequency of contact and greater number of patients contacted were highly associated with household contacts and thus were not separately assessed (Table 1S).

The above conclusions remained unchanged and statistically significant in multiple regression analyses which included age, sex, mode of contact, severity of source patients and expectoration included in the models (Table 2).

Comparison of source cases and secondary cases

We compared the characteristics between secondary cases and source cases they contacted with to see whether they may differ in the severity of the infection. Among 129 secondary cases, source cases were identifiable only for 121 cases. As compared with their 69 source cases, the 121 secondary cases were in general clinically milder and were less likely to have such common symptoms as fever, cough, expectoration, fatigue, myalgia and diarrhea (P<0.05). Secondary cases are also less likely than source cases to demonstrate radiological and laboratory alterations related to the infection (P<0.001). Most of the differences between them were both clinically important and statistically significant.

The clinical differences between source and secondary patients might be due to the fact that secondary cases were diagnosed earlier and the disease is milder at the early stage than source cases. To exclude this possibility, we also compared source cases with secondary cases who were diagnosed before the time of quarantine and not supposed not be early-stage patients. The conclusion remained unchanged (Table 2S).

Validity of PCR for Diagnosis

Among 4950 close contacts, 4653 completed at least one RT-PCR testing. If a person has no symptoms and the PCR test was negative, further testing continued to be arranged within 48 hours till he was diagnosed with the infection or released free of the infection from quarantine. The series of testing in the same persons allowed us to estimate the sensitivity and specificity of the PCT testing. The results were shown in Table 3S. In brief, the sensitivity was only 71.9% for the first testing and increased to 92.2% by the second testing, to 96.9% by the third testing, and to 100.0% by the sixth testing. In contrast, the first testing achieved a specificity 99.96%, which was reduced by less than 0.1% by further testing.

Discussion

Between January 13, and March 6, 2020, 4950 close contacts of confirmed cases were enrolled in Guangzhou, which is a city with large

confirmed cases of COVID-19 outside Hubei province in China. Here we evaluated the modes of contact and risk of transmission among close contacts, provided insights into transmission and control of COVID-19. To our knowledge, this study is the largest prospective cohort data of close contacts with COVID-19 so far.

Our study provided further evidence that the older aged contacts and household contacts were more likely to be infected, on the basis of previous researches[3,4,7,15]. The incidence of asymptomatic and mild infections was high (57/129), and the risk of transmission increased as the symptoms of source cases worsen with range from 0.33% (asymptomatic) to 6.2% (severe and critical). The symptomatic cases with expectoration symptom had a higher transmission capacity. The results provided the evidentiary foundation for evaluating control measures, and guiding the global response.

Household contacts characteristics have been discussed in previous studies[3,7], which were the source of person-to-person transmission evidence. And our study further confirmed that due to contact frequently of household, it was considered as a high-risk factor for COVID-19 transmission. The incidence of household contacts was estimated 10.2% in our study, and in other study was 14.9% out of Hubei Province[16], which is consistent with current understanding of COVID-19

310 transmission. However, other modes of contact have been less reported
311 for guiding persons self-protection and government for strengthening
312 control measures. In current study, 1540 close contacts were Dream
313 Cruise passengers, and almost of them had occasional contacts with
314 source cases, and their infected incidence was 0.1% (2/1540), which was
315 estimated low. This result was consistent with previous research. Nishiura
316 H[17] estimated the incidence of infection with COVID-19 on a cruise ship,
317 called Diamond Princess, and the risk of infection among passengers
318 contact occasionally was considered to be very limited. However, another
319 study reported that the food service workers had a high infected incidence
320 in the cruise ship[18] largely due to their frequent contact to others
321 including COVID-19 cases and had high chance to inhale droplet spread
322 of SARS-CoV-2.

323 Transportation was believed to be the main reason for the
324 SARS-CoV-2 spread from Wuhan to other cities in early stage of the
325 outbreak[19]. Most of our data were collected after seal off Wuhan, the risk
326 transmission of public transport vehicles was estimated low, which were
327 only about 1% and 10% of the risk of household contacts (P<0.0001).
328 Although, the public transport vehicles were at low risk to infected
329 COVID-19 from others, it still needed to be evaluated cautiously when
330 workers returning to work or students returning to school. Because that

we might have unknowingly come into contact with the source cases. With same reason, we recommend telemedicine to healthy people.

The proportion of close contacts who were diagnosed COVID-19 with asymptomatic and mild were high at 44.2% (57/129). Chowell[20] estimated asymptomatic proportion was at 17.9% among 700 infected individuals on Diamond Princess, and Miyama T[21] estimated at 30.8% among 13 Japanese evacuees from Wuhan City. Taking the results from several studies into account, Chowell[20] thought that asymptomatic or mild cases combined represented about 40% to 50% of all infections. It was consistent with the results of this study.

Given the large proportion of asymptomatic and mild infections, we are concerned about the risk at which they infect others. Wendtner[22] showed that patients with COVID-19 had high levels of the virus in throat swabs early in their illness, when their symptoms were mild. But no study had reported the infection risk of asymptomatic and mild cases to others, and asymptomatic infections might be seeding new outbreaks[23]. Our study showed that as severity increases, the risk of transmission increases in COVID-19 patients. Only 1 (1/305, 0.33%) and 19 (19/576, 3.3%) close contact was infected by asymptomatic and mild source cases, and it suggested the limited transmission capacity in asymptomatic and mild cases. The symptomatic cases with expectoration symptom had a higher

352 transmission capacity. These might be associated with more viral load of
353 SARS-CoV-2 in patients with severe symptoms[24].

354 Given the current evidence, due to asymptomatic cases have limited
355 transmission capacity, so the primary surveillance and control measures
356 should focus on symptomatic contacts. However, we should also be alert
357 for incubation transmission[25]. Asymptomatic and mild patients might not
358 aware of their infection and therefore not isolated themselves or seek
359 treatment, or they might be overlooked by health-care professionals and
360 thus unknowingly transmit the virus to others. Due to the imperfect
361 sensitivity of the PCR test (Table 3S), some asymptomatic contacts may
362 be missed[26,27]. Thus, based on our evidence, two times or more PCR tests
363 were recommended to ensure that almost all patients could to be
364 diagnosed.

365 Previous studies suggested that compared with patients initially
366 infected with SARS-Cov-2 in Wuhan City, the symptoms of patients in
367 out of Wuhan are relatively mild[28,29]. And a research reported that the
368 symptoms of imported cases (n=15) were severe than those of secondary
369 cases (n=17)[30], but due to the small sample size, it might be necessary to
370 verify the phenomenon. Thus, our study compared the severity of
371 symptom between sources cases and their secondary cases. And the
372 severity of clinical symptoms at onset was more severe to source cases

than that of secondary cases (P<0.001). It might be related to the higher Hubei exposure history of source cases (20/33 vs. 21/37) than that of secondary cases. This phenomenon was also apparent during the transmission of MERS-CoV[31].

Our study has some notable limitations. Firstly, we have not the data to show the prognosis of disease. Because many patients remained in the hospital and the outcomes were unknown at the time of data cutoff, and thus entire course of the disease cannot be fully demonstrated. Secondly, we used logistic regression analysis instead of cox proportional hazards model, because of the low incidence (2.6%) of COVID-19 among close contacts. In addition, by the end of the cohort, there were 245 close contacts remaining quarantines, but they were not likely to become COVID-19 cases, thus there was no censored data. Thirdly, there may be a recall bias of the symptoms at onset among source cases and secondary cases.

In conclusion, our cohort study showed that the proportion of asymptomatic and mild infections accounted for almost half of the confirmed cases among close contacts. The household contacts were the main transmission mode, and clinically more severe cases were more likely to pass the infection to their close contacts. In general, the secondary cases were clinically milder than those of source cases. The

results provide the evidentiary foundation for evaluating control measures, and guiding the global response.

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Legend of Tables:

Table 1. Baseline characteristics of 4 950 Persons with Close Contact with Confirmed COVID-2019 Cases, Guangzhou, China;

Table 2. Modes of contact and risk of transmission among 4 950 Close Contact Persons;

Table 3. Comparison of clinical, radiological and laboratory characteristics of COVID-2019 infection between 69 source cases and 121 secondary cases.

Supplementary Material:

Table 1S. Modes of contact and risk of transmission among 4 950 Close Contact Persons;

Table 2S. Comparison of clinical, radiological and laboratory characteristics of COVID-2019 infection between 36 source cases and 49 secondary cases;

Table 3S. Sensitivity, specificity, and positive and negative predictive values of sequential nucleic acid tests of throat swabs (n=4653);

Figure 1S. Distribution of 1540 Dream Cruises close contact persons by the number of days from start of quarantine to PCR diagnosis or release from quarantine and infection status (day 0 is the day when quarantine starts);

Appendix 1: The full detail of close contacts;

Appendix 2: COVID-19 Confirmed Case Investigation Form;

Appendix 3: Details regarding laboratory confirmation processes.

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1 **Table 1. Baseline characteristics of 4 950 Persons with Close Contact with Confirmed COVID-**

2 **2019 Cases, Guangzhou, China**

Characteristics	Number	%
Age (median (IQR))	4942	38.0 (25.0, 52.0)
0-17	783/4942	15.8
18-44	2338/4942	47.3
45-59	997/4942	20.2
60 or over	824/4942	16.7
Males	2484/4950	50.2
Modes of contact		
The Dream Cruise passengers	1540/4950	31.1
Other public transport vehicles	818/4950	16.5
Healthcare settings	679/4950	13.7
Households	946/4950	19.1
Multiple locations	92/4950	1.9
Others	875/4950	17.7
Real time RT-PCR testing test		
Persons with at least one test	4654/4950	94.0
Mean no. of test (mean (SD))	2.4	2.0
Confirmed COVID-2019 cases	129/4950	2.6
Symptoms of confirmed COVID-2019 cases		
Symptomatic	121/129	93.8

Asymptomatic throughout	8/129	6.2
Severity of confirmed COVID-2019 cases		
Asymptomatic	8/129	6.2
Mild	49/129	38.0
Moderate	67/129	51.9
Severe and critical	5/129	3.9
Days from last contact to start of quarantine (median (IQR))	4950	11.0 (1.0, 12.0)
Duration of quarantine (median (IQR)), days	4950	4.0 (3.0, 13.0)
Confirmed COVID-19	129	2.0 (1.0, 5.0)
Non-confirmed COVID-19	4533	4.0 (3.0, 13.0)
Days from last contact to symptoms onset (median (IQR))	121	1.0 (-1.0, 3.0)
Days from last contact to first test (median (IQR))	4654	11.0 (4.0, 13.0)
Confirmed COVID-19	129	2.0 (1.0, 3.0)
Non-confirmed COVID-19	4533	11.0 (5.0, 13.0)
Days from quarantine to first test (median (IQR))	4654	1.0 (0.0, 1.0)
Days from symptoms onset to PCR diagnosis (median (IQR))[a]	33	2.0 (0.0, 9.0)

3 a: excluding 88 cases with the first PCR diagnosis positive.

4

Table 2. Modes of contact and risk of transmission among 4 950 Close Contact Persons

Modes of contact	Number	COVID-2019 Events	Incidence (%)	Unadjusted		Adjusted*	
				OR (95% CI)	P	OR (95% CI)	P
Age (years)†							
0-17	783/4942	14	1.8	0.82 (0.45, 1.48)	0.5054	0.66 (0.35, 1.24)	0.9184
18-44	2338/4942	51	2.2	1.00	-	1.00	-
45-59	997/4942	29	2.9	1.34 (0.85, 2.13)	0.2104	1.03 (0.63, 1.68)	0.1966
60 or over	824/4942	35	4.2	1.99 (1.28, 3.08)	0.0021	2.17 (1.35, 3.50)	0.0014
Sex							
Males	2484/4950	56	2.3	1.00	-	1.00	-
Females	2466/4950	73	3.0	1.32 (0.93, 1.88)	0.1202	1.22 (0.84, 1.77)	0.3027
Modes of Contact							
The Dream Cruises	1540/4950	2	0.1	0.01 (0.00, 0.05)	<0.0001	0.03 (0.01, 0.14)	<0.0001

3

Other public transport vehicles	818/4950	1	0.1	0.01 (0.00, 0.08)	<0.0001	0.01 (0.00, 0.10)	<0.0001
Healthcare settings	679/4950	7	1.0	0.09 (0.04, 0.20)	<0.0001	0.11 (0.05, 0.25)	<0.0001
Households	946/4950	96	10.2	1.00	-	1.00	-
Multiple modes	92/4950	12	13.0	1.33 (0.70, 2.53)	0.3466	1.45 (0.74, 2.83)	0.3600
Others	875/4950	11	1.3	0.11 (0.06, 0.21)	<0.0001	0.13 (0.07, 0.25)	<0.0001
Severity of source cases*							
Asymptomatic	305/2610	1	0.33	0.06 (0.01, 0.40)	0.0042	0.29 (0.04, 2.22)	0.2340
Mild	576/2610	19	3.3	0.58 (0.35, 0.96)	0.0341	0.48 (0.28, 0.82)	0.0068
Moderate	1469/2610	82	5.6	1.00	-	1.00	-
Severe and critical	260/2610	16	6.2	1.11 (0.64, 1.93)	0.7133	1.19 (0.66, 2.15)	0.5611
Symptoms of source cases							
Fever							

	No	430/1813	14	3.3	1.00	-	1.00	-
	Yes	1383/1813	92	6.7	2.12 (1.19, 3.76)	0.0103	1.77 (0.96, 3.26)	0.0691
Dry cough								
	No	726/1813	39	5.4	1.00	-	1.00	-
	Yes	1087/1813	67	6.2	1.16 (0.77, 1.74)	0.4817	1.03 (0.66, 1.59)	0.9136
Expectoration								
	No	1329/1813	40	3.0	1.00	-	1.00	-
	Yes	484/1813	66	13.6	5.09 (3.38, 7.65)	<0.0001	5.22 (3.39, 8.05)	<0.0001
Fatigue								
	No	1366/1813	76	5.6	1.00	-	1.00	-
	Yes	447/1813	30	6.7	1.22 (0.79, 1.89)	0.3700	1.10 (0.68, 1.79)	0.6881
Myalgia								
	No	1517/1813	88	5.8	1.00	-	1.00	-

Yes	296/1813	18	6.1	1.05 (0.62, 1.77)	0.8510	1.00 (0.56, 1.78)	0.9945

a: Age, sex, mode of contact, severity of source cases, symptoms of source cases were included in multivariable logistic regression analysis.

P for trend: b was 0.0016 and 0.0007, c was <0.0001 and 0.0006 for unadjusted and adjusted.

Table 3. Comparison of clinical, radiological and laboratory characteristics of COVID-2019 infection between 69 source cases and 121 secondary cases

Characteristics	Events/total (%)		*P*
	Source cases	Secondary cases	
Hubei Exposure history, yes	48/59 (81.4)	46/87 (52.9)	0.0016
Severity			0.0125
Asymptomatic	1/65 (1.5)	8/121 (6.6)	
Mild	12/65 (18.5)	46/121 (38.0)	
Moderate	45/65 (69.2)	63/121 (52.1)	
Severe and critical	7/65 (10.8)	4/121 (3.3)	
Highest temperature (℃)			<0.0001
<37.5	4/52 (7.7)	8/45 (17.8)	
37.5-38	27/52 (51.9)	23/45 (51.1)	
38.1-39	15/52 (28.9)	14/45 (31.1)	
>39	6/52 (11.5)	0/45 (0.0)	
Symptoms at onset, yes			
Fever	51/61 (83.6)	43/103 (41.7)	<0.0001
Dry cough	38/61 (62.3)	34/103 (33.0)	0.0007
Expectoration	36/61 (59.0)	16/103 (15.5)	<0.0001
Fatigue	16/61 (26.2)	13/103 (12.6)	0.0233
Myalgia	11/61 (18.0)	5/103 (4.9)	0.0067
Diarrhea	9/61 (14.8)	1/103 (1.0)	0.0006

Other lung diseases	15/40 (37.5)	5/35 (14.3)	<0.0001
CT double lung abnormalities	45/61 (73.8)	27/103 (26.2)	<0.0001
Blood biochemical index			
(median (IQR))			
WBC (10^9/L) [a]	5.5 (4.3, 7.0)	5.3 (4.4, 6.4)	0.0148
Ne % [a]	70.1 (58.8, 77.8)	56.8 (49.8, 65.4)	0.0044
Ly % [b]	21.7 (13.8, 29.6)	32.2 (23.4, 40.9)	0.0004

WBC: White blood cell count; Ne%: lymphocyte percentage; Ly%: neutrophilic granulocyte percentage;

Number of participants with missing values: a=76, b=77.

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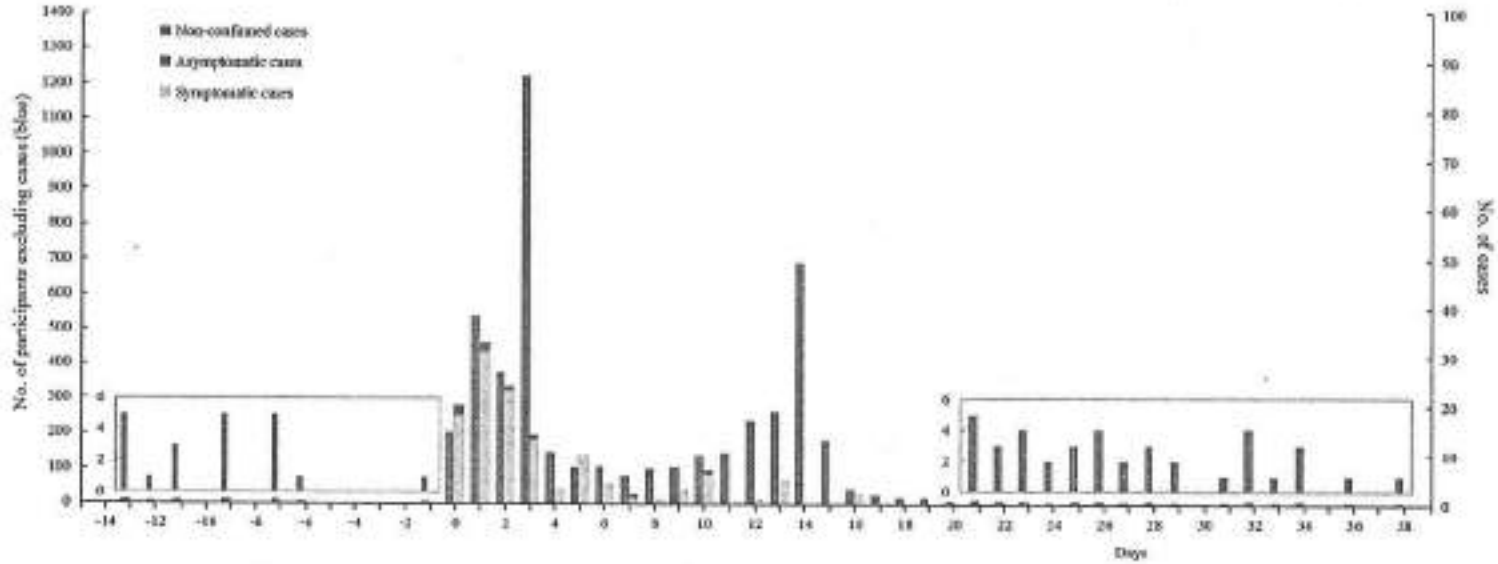

Fig 1. Distribution of 4950 close contact persons by the number of days from start of quarantine to PCR diagnosis or release from quarantine and infection status (day 0 is the day quarantine starts)

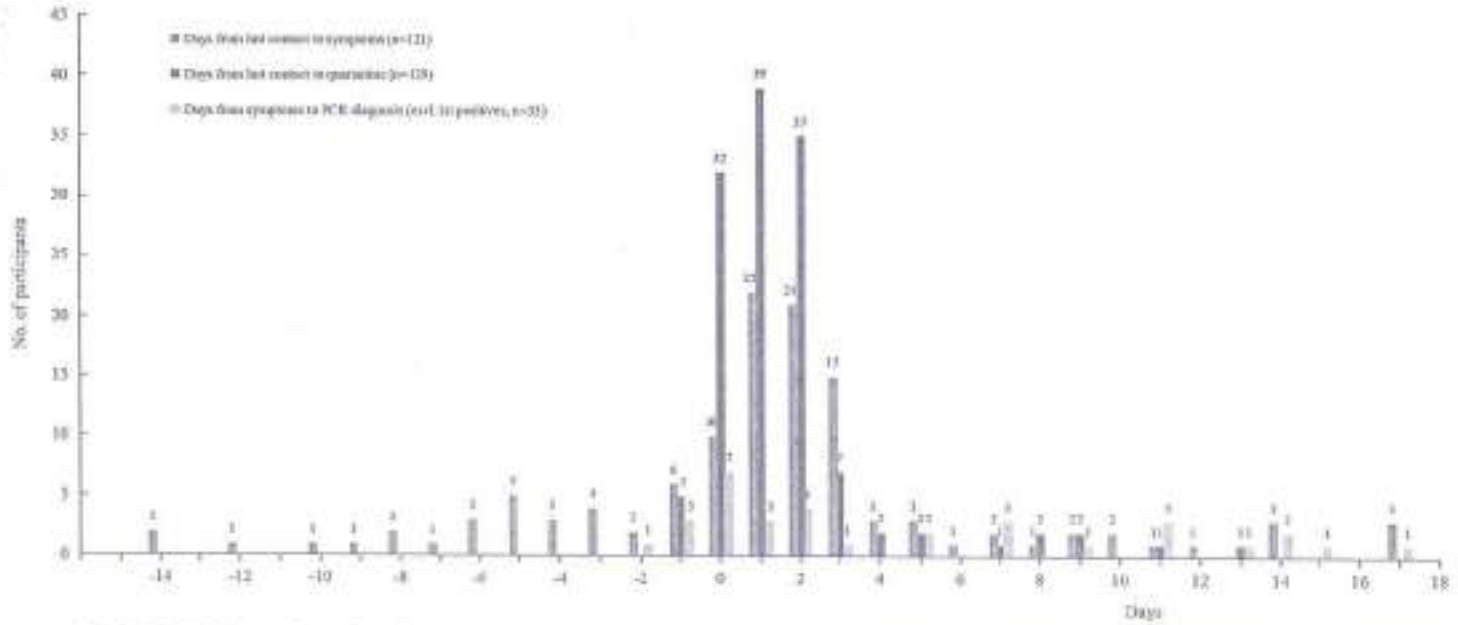

Fig 2. Distribution of days from last contact to symptoms onset and to start of quarantine and days from symptoms onset to PCR diagnosis

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Supplementary Material
supplementary table and figure.docx

EXHIBIT" G

Secondary Transmission of Coronavirus Disease from Presymptomatic Persons, China

Weiwei Zhang,[1] Weibin Cheng,[1] Lei Luo,[1] Yu Ma, Conghui Xu, Pengzhe Qin, Zhoubin Zhang

Author affiliations: Guangzhou Center for Disease Control and Prevention, Guangzhou, China (W. Zhang, L. Luo, Y. Ma, C. Xu, P. Qin, Z. Zhang); Guangdong Second Provincial General Hospital, Guangzhou (W. Cheng)

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We explored the secondary attack rate in different types of contact with persons presymptomatic for coronavirus disease (COVID-19). Close contacts who lived with or had frequent contact with an index case-patient had a higher risk for COVID-19. Our findings provide population-based evidence for transmission from persons with presymptomatic COVID-19 infections.

Coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is rapidly spreading across the globe. Some case reports and modeling studies suggest asymptomatic carriage of SARS-CoV-2 plays a role in transmission (1–3). Studies have shown that 30%–59% of SARS-CoV-2 infections are asymptomatic (3,4), which poses tremendous infection control challenges. To control asymptomatic infections, China implemented active case surveillance and enhanced social distancing measures, which include contact tracing, quarantine for key populations, medical observation, and curtailed social activities (5). However, additional information on the characteristics of presymptomatic transmission is needed to develop targeted control and prevention guidance.

We analyzed contact-tracing surveillance data collected during January 28–March 15, 2020, to explore the secondary attack rate from different types of contact with persons presymptomatic for COVID-19 in Guangzhou, China. Asymptomatic COVID-19 cases were found mainly through close contact screening, clustered epidemic investigations, follow-up investigation of infection sources, and active surveillance of key populations with travel or residence history in areas with continuous transmission of COVID-19 in China and abroad. We developed a case definition for presymptomatic COVID-19, criteria for close contact, and contact investigation and management guidelines (Appendix, https://wwwnc.cdc.gov/EID/article/26/8/20-1142.pdf). We estimated secondary attack rate (SAR) and 95% CI based on the proportion of COVID-19 incidence among close contacts. We calculated the mean reproductive number (R_t) from the number of secondary infections observed among close contacts of each index case. The study was approved by the ethics committee of Guangzhou Center for Disease Control and Prevention, which granted a waiver for informed consent. Data collection was conducted under the authority of the China Center for Disease Control and Prevention.

As of March 15, a total of 359 COVID-19 cases were confirmed in Guangzhou. Among them, 83 (23%) persons were asymptomatic at diagnosis; 71 (86%) of whom later developed symptoms. Among presymptomatic cases, 38 had ≥1 (range 1–90, median 4) close contact. We identified and included 369 close contacts in this study. Median age of close contacts was 35 years (range 0–93 years), 23.8% were family members of an index case, and 12 were confirmed to be infected via nucleic acid testing. Among them, 8 close contacts developed symptoms, and 4 were asymptomatic at the time of this study (Appendix Table).

The overall SAR was 3.3% (95% CI 1.9%–5.6%). The SAR among household contacts was 16.1% and was 1.1% for social contacts, and 0 for workplace contacts. Older close contacts had the highest SAR compared with other age groups; 8.0% in persons ≥60 years of age compared with 1.4%–5.6% in persons <60 years of age. Close contacts of asymptomatic index case-patients had the lowest SAR, 0.8%, but the SAR was 3.5% for those with mild symptoms, 5.7% for those with moderate symptoms, and 4.5% for those with severe symptoms. Close contacts that lived with an index case-patient had 12 times the risk for infection and those who had frequent contact with an index case-patient, >5 contacts during 2 days before the index case was confirmed, had 29 times the risk for infection (Table).

Our findings substantiate previous reports from China and Germany (1,2,6) and show that SARS-CoV-2 can be transmitted during asymptomatic COVID-19 infection period. The probability of infection increased substantially among close contacts who shared living environments or had frequent contact with an index case-patient, which underlines the need for prompt contact-based surveillance and social distancing (7). Our results also showed most secondary infections occurred in confined familial clusters and that persons ≥60 years of age appear to be more vulnerable to being infected. These results are consistent with previous reports on epidemiologic

[1]These first authors contributed equally to this article.

Table. Characteristics of and secondary attack rates among 365 close contacts of persons with presymptomatic coronavirus disease 2019, China*

Variable	No. contacts	No. infected	Attack rate, % (95% CI)	Relative risk (95% CI)
Sex				
M	217	5	2.3 (0.1–5.2)	Referent
F	152	7	4.6 (2.2–6.9)	2.1 (0.6–6.6)
Age				
≤17	46	2	4.3 (1.2–14.5)	Referent
18–30	104	3	2.9 (1.0–8.1)	0.7 (0.1–4.1)
31–40	72	1	1.4 (0.2–7.4)	0.4 (0.03–3.5)
41–50	68	1	1.5 (0.3–7.9)	0.4 (0.03–3.7)
51–60	54	3	5.6 (1.9–15.1)	1.3 (0.2–8.1)
≥61	25	2	8.0 (1.4–27.5)	1.9 (0.3–14.5)
Index case-patient status*				
Asymptomatic	119	1	0.8 (0.2–5.6)	Referent
Mild symptoms	141	5	3.5 (1.5–8.0)	4.3 (0.5–37.7)
Moderate symptoms	87	5	5.7 (2.5–12.8)	7.2 (0.8–62.7)
Severe symptoms	22	1	4.5 (0.8–21.8)	5.6 (0.3–93.4)
Contact mode				
Social interaction with friends or relatives	66	1	1.5 (0.3–8.1)	Referent
Lived together	62	10	16.1 (9.0–27.2)	12.5 (1.6–106.8)
Worked together	115	0	0	0
Social interaction with strangers	122	1	0.8 (0.2–4.9)	0.5 (0.03–8.7)
Contact frequency†				
Rare	145	1	0.7 (0.1–3.7)	Referent
Moderate	156	1	0.6 (0.1–3.5)	0.9 (0.1–15.1)
Frequent	61	10	16.4 (9.2–27.6)	25.0 (3.6–232.3)

*Status as of March 30, 2020, based on the person's clinical course assessed by a physician. Moderate symptoms included fever, respiratory symptoms, and radiographic evidence of pneumonia. Severe symptoms included breathing rate ≥30/min; oxygen saturation level ≤93% at rest; oxygen concentration level PaO_2/FIO_2 ≤300 mmHg (1 mmHg = 0.133kPa); lung infiltrates >50% within the past 24–48 h; respiratory failure requiring mechanical ventilation; septic shock; or multiple organ dysfunction or failure. All other symptomatic cases were classified as mild.
†Rare contact was defined as contacted with index cases <2 times during 2 days preceding confirmation of infection. Moderate contact was defined as contacted with index cases 3–5 times during 2 days preceding confirmation of infection. Frequent contact was defined as contacted with index cases ≥5 times during 2 days preceding confirmation of infection.

characteristics of 72,314 COVID-19 cases in China (*9*) and suggest that household-based isolation should be cautiously implemented for persons with asymptomatic suspected cases. We also noted that persons with asymptomatic infections appeared to be less effective in transmitting the virus. However, this finding should not discourage isolation and surveillance efforts. The R_0 in this cohort was 0.3 (95% CI 0.2–0.5), which was far smaller than the overall R_0 of 2.2 reported previously (*9*). This low transmission level could be the result of active surveillance, centralized quarantine, and forceful social-distancing strategies in Guangzhou.

Interpretation of the findings should be taken with caution, and several limitations influence our estimation of the SAR. First, the number of close contacts was limited because we only included those who had been reached, and asymptomatic infections might have been missed. Second, we excluded close contacts who were exposed to ≥2 confirmed COVID-19 case-patients. Third, the presymptomatic transmission period is not well defined.

Despite these limitations, our analysis provides valuable information on secondary transmission of SARS-CoV-2 in different types of contact with presymptomatic COVID-19 case-patients. Further evidence is needed to define the population characteristics, communicable period, and the volume and duration of viral shedding from persons with asymptomatic infections.

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About the Author

Dr. W. Zhang is a senior public health physician at the Guangzhou Center for Disease Control and Prevention. Her research interests are health promotion and infectious disease epidemic modeling.

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Addresses for correspondence: Zhoubin Zhang or Pengzhe Qin, Guangzhou Center for Disease Control and Prevention, No.1 Qide road, Guangzhou, 510440, China; email gzdczzb@gzcdc.org.cn or petgzy@gmail.com

Abdominal Visceral Infarction in 3 Patients with COVID-19

Giulia Besutti, Riccardo Bonacini, Valentina Iotti, Giulia Marini, Nicoletta Riva, Giovanni Dolci, Mariarosa Maiorana, Lucia Spaggiari, Filippo Moneli, Guido Ligabue, Giovanni Guaraldi, Paolo Giorgi Rossi, Pierpaolo Pattacini, Marco Massari

Author affiliations: Azienda Unità Sanitaria Locale di Reggio Emilia–Istituto di Ricovero e Cura a Carattere Scientifico di Reggio Emilia (AUSL–IRCCS), Reggio Emilia, Italy (G. Besutti, R. Bonacini, V. Iotti, G. Marini, N. Riva, G. Dolci, M. Maiorana, L. Spaggiari, P.G. Rossi, P. Pattacini, M. Massari); University of Modena and Reggio Emilia Clinical and Experimental Medicine PhD Program, Modena, Italy (G. Besutti, M. Maiorana); University of Modena and Reggio Emilia, Modena (R. Bonacini, G. Dolci, F. Moneli, G. Ligabue, G. Guaraldi)

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A high incidence of thrombotic events has been reported in patients with coronavirus disease (COVID-19), which is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. We report 3 clinical cases of patients in Italy with COVID-19 who developed abdominal viscera infarction, demonstrated by computed tomography.

Frequent thrombotic events, mostly pulmonary embolisms, have been reported in patients with coronavirus disease (COVID-19) (*1*–*4*). We describe 3 cases of COVID-19 complicated by abdominal visceral infarction that occurred in inhabitants of the Emilia Romagna region in northern Italy.

Patient 1, a 54-year-old male former smoker with a history of asthma and quiescent ulcerative colitis not receiving any treatment, was admitted to the emergency department (ED) on February 28, 2020, for syncope. He was discharged after undergoing chest radiography and brain computed tomography (CT), the results of which were unremarkable. He returned to the ED after 5 days for treatment of dyspnea, fatigue, and fever. Blood tests revealed decreased oxygen saturation (94%), increased C-reactive protein (CRP) level (5.38 mg/dL; reference <0.5 mg/dL), and lymphopenia (0.69 × 10^3 cells/mm^3; reference range 0.8–4 × 10^3 cells/mm^3). Chest CT scan demonstrated bilateral viral pneumonia, and nasopharyngeal and oropharyngeal swab specimens were positive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). He was hospitalized and treated with lopinavir/ritonavir (400/100 mg orally 2×/d), and hydroxychloroquine (200 mg orally 2×/d). He was discharged to home after 3 hospital days, on therapy; no anticoagulant prophylaxis was suggested. He was rehospitalized 6 days after discharge when he developed sharp right flank and lumbar pain, fever, and dysuria. Blood and urine tests revealed neutrophilia (9.9 × 10^3 cells/mm^3; reference range 1.6–7.5 × 10^3 cells/mm^3), increased lactate dehydrogenase (LDH) (1,507 U/L; reference range 28–378 U/L), increased CRP (1.43 mg/dL), and proteinuria (50 mg/dL). CT scan demonstrated a large right kidney arterial infarction (Figure, panel A). He was treated with low molecular weight

EXHIBIT "H"

Transmission potential of asymptomatic and paucisymptomatic SARS-CoV-2 infections: a three-family cluster study in China

Xiao-Lin Jiang[1,a], Xiao-Li Zhang[2,a], Xiang-Na Zhao[3,a], Cun-Bao Li[4], Jie Lei[1], Zeng-Qiang Kou[1], Wen-Kui Sun[1], Yang Hang[1], Feng Gao[5], Sheng-Xiang Ji[2], Can-Fang Lin[4], Bo Pang[1], Ming-Xiao Yao[1], Benjamin D. Anderson[6], Guo-Lin Wang[7], Lin Yao[7], Li-Jun Duan[7], Dian-Ming Kang[1,b], Mai-Juan Ma[7,b]

1. Shandong Provincial Center for Disease Control and Prevention, Jinan, China
2. Linyi Center for Disease Control and Prevention, Linyi, China
3. Institute of Disease Control and Prevention, Chinese People's Liberation Army, Beijing, China
4. Lanshan District Center for Disease Control and Prevention, Linyi, China
5. Linyi People's Hospital, Linyi, China.
6. Global Health Research Center, Duke Kunshan University, Kunshan, Jiangsu, China
7. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China.

[a]X.-L. J., X.-L. Z., and X.-N. Z. are joint first authors.

[b]D.-M. K. and M.-J. M are senior authors and contributed equally to this article

Corresponding author: Mai-Juan Ma, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China (mjma@163.com)

Alternate corresponding author: Dian-Min Kang, Dian-Ming Kang, Department of viral infectious disease control and prevention, Shandong Provincial Center for Disease Control and Prevention, Jinan 250014, China (dmkang66@163.com)

Brief summary

We report a unique three-family cluster of infection with SARS-CoV-2. The transmission of SARS-CoV-2 by individuals with asymptomatic or paucisymptomatic infections is likely occurred. SARS-CoV-2 was detected in contaminated environments of one household.

Abstract

Data concerning the transmission of SARS-CoV-2 in asymptomatic and paucisymptomatic patients are lacking. We report a three-family cluster of infections involving asymptomatic and paucisymptomatic transmission. Eight (53%) of 15 members from three families were confirmed with SARS-CoV-2 infection. Of eight patients, three were asymptomatic and one was paucisymptomatic. An asymptomatic mother transmitted the virus to her son, and a paucisymptomatic father transmitted the virus to his three-month-old daughter. SARS-CoV-2 was detected in the environment of one household. The complete genomes of SARS-CoV-2 from the patients were >99.9% identical and were clustered with other SARS-CoV-2 sequences reported from China and other countries.

Keywords. SARS-CoV-2; COVID-2019; asymptomatic; paucisymptomatic; transmission

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that causes coronavirus disease 2019 (COVID-19), emerged in December 2019 in Wuhan, China [1]. It has since been declared a global pandemic with over 1,000,000 cases reported as of April 3, 2020 [2]. Person-to-person transmission has been established [3-8], and asymptomatic transmission of SARS-CoV-2 has been reported [9]. However, studies on the potential transmission of SARS-CoV-2 by asymptomatic persons and those with mild illness have been limited [10]. Herein, we report a 3-family cluster study of eight patients associated with asymptomatic and pauciasymptomatic (one mild symptom only) SARS-CoV-2 transmission in Shandong Province, China.

METHODS

Epidemiological Investigation

The first positive SARS-CoV-2 patients in this cluster were identified on January 21, 2020 triggering an epidemiological investigation by the local center for disease control and prevention. To identify the possible infective source, the epidemiological investigation focused on exposure history before the onset of illness, such as travel history to Wuhan or Hubei Province, visiting live animal markets, and contact history with febrile persons. Medical records were also closely reviewed to verify the timelines of events and clarify clinical progressions.

To examine possible environmental contamination of SARS-CoV-2 in households, select surfaces that may be frequently touched by family members were sampled in the bedroom (door handle, bedside light switch, and sliding of wardrobe door), kitchen (door handle, faucet switch, light switch, rice cooker plug), and bathroom (door handle, handrail, the surface of the toilet bowl, sink). One swab per site (room) with multiple surfaces was collected.

All close contacts of SARS-CoV-2 positive patients were traced, including family members who lived with the patients and individuals who had contact with the patients within 1 meter without wearing proper personal protection. Close contacts were quarantined at home and monitored for fever (≥38°C) and symptoms. In addition, nasopharyngeal swabs of close contacts were collected every 24

hours from day 1 to 14 to detect SARS-CoV-2 by molecular assay. If any close contact had positive detection of SARS-CoV-2, they were sent to a hospital for isolation and treatment.

SARS-CoV-2 Molecular Detection, Sequencing, and Phylogenetic Analysis

All collected environmental and patient samples were stored at −80°C before being transported using cold chain to a biosafety level 2 enhanced laboratory to perform molecular detection of SARS-CoV-2. A real-time reverse transcriptase PCR (rRT-PCR) Test Kit (GZ-D2RM, Shanghai GeneoDx Biotech Co., Ltd) targeting the *ORF*1ab and *N* genes of SARS-CoV-2 was used. A cycle threshold (Ct) value less than 37 was interpreted as positive for SARS-CoV-2 RNA and a Ct value of 40 or more was defined as a negative test. A medium load (weakly positive), defined as a Ct value of 37 to less than 40, required confirmation by retesting. Positive samples were sequenced directly from the original specimens as previously described [11]. The maximum likelihood phylogenetic tree of the complete genomes was conducted by using RAxML software (version 8.2.9) [12] with 1000 bootstrap replicates, employing the general time-reversible nucleotide substitution model.

RESULTS

Description of SARS-CoV-2 Positive Patients

Patients 1 (62-year-old woman) and 2 (65-year-old man) were a couple who lived with their son (Patient 3), daughter-in-law (Patient 4), and two grandchildren. Patient 1 presented with cough, rhinorrhea, and sputum on January 12, 2020 (Figure 1). On January 15, she visited a health clinic and was diagnosed with a common cold. She was prescribed intravenous infusions of ampicillin and sulbactam, ribavirin, and traditional medicine for five days. On January 16, she developed a fever (38°C). On January 17, Patient 2 reported symptoms of fever (37.8°C), cough, sputum, earache, and upset stomach. He was also diagnosed with a common cold at the health clinic and received the same prescription as Patient 1 for three days. However, their symptoms did not resolve at the conclusion of the treatment regimen leading both to seek care at a local hospital on January 21. Nasopharyngeal swabs were collected from both patients at the hospital and confirmed positive for

SARS-CoV-2 by rRT-PCR. Thereafter, they were admitted to the isolation ward of the hospital for treatment. Major symptoms during hospitalization for both patients included fever, cough, and fatigue (Figure 1).

Patients 3 (37-year-old male) and 4 (35-year-old female), a young couple, were close contacts of Patients 1 and 2. They were self-quarantined at home for 14 days starting on the day of the hospital admission of Patients 1 and 2. Their nasopharyngeal swabs were collected on January 23 for SARS-CoV-2 testing. Patient 3 was confirmed positive that same day for SARS-CoV-2 by rRT-PCR, but had no symptoms. Patient 4 tested negative for SARS-CoV-2 but was later confirmed to be positive for SARS-CoV-2 on January 25 when a repeat pharyngeal swab was collected and tested. Upon hospital admission, Patient 3 developed a slightly dry and itchy throat. The cough was a major symptom during hospitalization, and two days fever and four days fatigue of the total hospital stay was also recorded. Patient 4 had no identified clinical symptoms (Figure 1).

Patient 5, a 53-year-old female, lived with her son (Patient 6) and parents, and was a close contact of Patients 3 and 4, being the mother-in-law of Patient 3 and the mother of Patient 4. She was self-quarantined at home beginning the day of Patient 3's confirmation (January 23). On January 24, her nasopharyngeal swab was collected to test for SARS-CoV-2 infection, which was later confirmed positive by rRT-PCR, despite her lack of symptoms. She also did not show any clinical symptoms of infection during hospitalization (Figure 1). Patient 6, a 28-year-old male, was identified as the close contact of his mother (Patient 5). He was self-quarantined at home beginning the day of his mother's (Patient 5) confirmed infection (January 24). His nasopharyngeal swab was collected on January 25 and confirmed SARS-CoV-2 positive by rRT-PCR. On admission in the afternoon on January 25, he developed a fever (37.5°C). Major symptoms during hospitalization included fever and cough, and the symptoms persisted more than two weeks.

Patient 7, a 35-year-old man, lived with his wife and three children, and was identified as the close contact of Patient 3. He was self-quarantined at home beginning the day of Patient 3's confirmed infection (January 23). On January 25, his nasopharyngeal swab was collected and tested positive for SARS-CoV-2 by rRT-PCR. During hospitalization, he was paucisymptomatic, with only an occasional cough. Patient 8, a 3-month-old female infant, was the close contact of Patient 7, her father. On January 27, her nasopharyngeal swab was collected and was a weak positive for SARS-

CoV-2. A repeat pharyngeal swab was collected on January 29 and was positive for SARS-CoV-2. The infant had no clinical symptoms before, during, or after hospitalization.

The chest CT images on admission or hospitalization showed that Patients 1-6 had ground-glass opacities. However, no significant abnormalities were observed for Patients 7 and 8 (Supplemental Figure 1). As of February 17, 2020, all patients recovered and were discharged to home isolation for 14 days.

Exposure Histories

Patients 1 and 2 traveled to their hometown in Xiaogan December 29, 2019 to January 15, 2020 (Figure 1). Xiaogan is a city adjacent to the epidemic center of Wuhan where the first case was identified on January 1, 2020. Moreover, they had changed trains at the Hankou railway station in Wuhan. Patients 3 and 4 had not traveled to Wuhan. They and their parents live together, eat together, and have frequent face-to-face interactions. Facemasks or other personal protective equipment (PPE) were not used at home. Patient 5 had contact with Patients 3 and 4 several times at a factory that they jointly operated. Patient 5 also visited the home of Patients 1-4 on the evening of January 21 and stayed one night. On the morning of January 22, Patient 5 was driven home by Patient 3. During these contacts, no facemasks or other PPE were used. Patient 6 did not report close contact with known COVID-19 cases except for his mother. Patient 7 reported that he had a frequency of 2 to 3 times daily contact with Patient 3 at the factory from January 15 to 18 and dined with Patient 3 and other colleagues on January 18. He did not report close contact with any known COVID-19 cases except for Patient 3. Except for contact with her father, patient 8 had no known contact with COVID-19 patients.

SARS-CoV-2 in Samples from Patients and Environments and Phylogenetic Analysis

SARS-CoV-2 was detected in nasopharyngeal swabs of all patients during hospitalization, including asymptomatic and paucisymptomatic patients (Figure 1). A total of 15 (5 per household) surface swab samples were collected from the bedrooms (n=9, three per household), kitchens (n=3, one per household), and bathrooms (n=3, one per household) of patient homes. Two (13.3%) of 15 swabs (one from the bedroom of Patient 3 and another one from his family kitchen) were positive for SARS-CoV-2 by rRT-PCR.

The full-genome sequences for eight patients were obtained and have been deposited in GISAID (accession numbers EPI_ISL_414934–414941). The full-genome for the two environmental swabs positive for SARS-CoV-2 were not obtained due to low-coverage genomes. The full-genomes of eight patients were >99.9% identical across the whole genome. Phylogenetic analysis revealed that the viruses from patients were clustered in the same clade and were genetically similar to other SARS-CoV-2 sequences reported from China and other countries (Figure 2). No significant mutation site was identified in the eight SARS-CoV-2 viral sequences compared with previous strains in China and other countries.

Close contacts

Fifteen contacts of either Patient 1 or Patient 2 were identified, and two contacts (Patients 3 and 4) were confirmed with COVID-19. A total of 88 contacts of Patient 3 were identified, and two contacts (Patients 5 and 7) tested positive for SARS-CoV-2. Seventy-three close contacts of Patient 5 were identified, and one contact (Patient 6) tested positive for SARS-CoV-2. Two contacts were identified for Patient 6 and all tested negative. Twenty-one contacts of Patient 7 were identified and one contact (Patient 8) tested positive. Of 101 close contacts identified for Patient 8, all tested negative. No other close contacts of the patients were identified during the 14-day follow-up. Thus, a crude estimation of the attack rate is 3.8% (4/105) for symptomatic and 1% (2/195) for asymptomatic and paucisymptomatic.

DISCUSSION

We report a unique three-family cluster of infection with SARS-CoV-2, in which eight of 15 members were confirmed with SARS-CoV-2 infection. Particularly interesting is that of 6 secondary patients, two were asymptomatic, one was pauciymptomatic, and three were symptomatic. Our findings show that the transmission of SARS-CoV-2 by individuals with asymptomatic or pauciymptomatic infections is possible. Patients 1 and 2 were likely first exposed to SARS-CoV-2 after visiting their hometown in Xiaogan Hubei Province, China. Their son (Patient 3) and daughter-in-law (Patient 4, asymptomatic), whom they live with, were later found to be infected with SARS-CoV-2. Patient 5 (asymptomatic) was identified to be infected with SARS-CoV-2 after frequent contact with Patients 3 and 4 during work and home visits. She transmitted the virus to her son (symptomatic) whom she lives with. Patient 7 (pauciymptomatic) was found to be infected with SARS-CoV-2 after frequent contact with Patient 3 during work. He likely transmitted the virus to his daughter (Patient 8, asymptomatic). In addition, consistent with previous studies [5-8], the transmission of SARS-CoV-2 during the incubation period of Patient 3 likely occurred. Patients 5 and 7 were infected after their exposures to a presymptomatic Patient 3 during working or home visits. These findings may help explain the rapid spread of SARS-CoV-2 between person-to-person.

The currently available evidence shows that SARS-CoV-2 is transmitted between people through droplets and close contact [13]. A recent study showed extensive environmental contamination by a SARS-CoV-2 patient [14], suggesting the contaminated environment as a potential medium of transmission. In this study, we detected SARS-CoV-2 in two environmental swabs from the household of Patient 3. Such detection of SARS-CoV-2 in contaminated environments of the household may provide an additional contribution to virus transmission among family members as the virus can remain viable and infectious on the surface up to seven days [15]. However, the direct research-based evidence describing exactly how SARS-CoV-2 is transmitted is limited, and further studies are required.

We cannot rule out the possibility of unknown COVID-19 patients (e.g., asymptomatic carriers) transmitting the virus. However, according to screening protocols implemented by the provincial, municipal, and county-level Center for Disease Control and Prevention, all close contacts were

traced, and all patients with positive rRT-PCR results in this study were confirmed by whole-genome sequencing, including those who were asymptomatic or pauciasymptomatic (Patients 4, 5, 7, and 8).

CONCLUSION

The transmission potential by individuals with asymptomatic and paucisymptomatic infection and the detection of SARS-CoV-2 in contaminated environments create challenges in control and prevention for the disease. Further studies are needed to investigate the contribution of persons with asymptomatic or pauciasymptomatic SARS-CoV-2 infection and the relationship with transmission of the virus in the household, occupational, and community settings.

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Potential conflicts of interest

All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Figure legends

Figure 1. Timeline of relevant exposures and clinical symptoms of eight patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. rRT-PCR: real-time reverse transcriptase-polymerase chain reaction.

Figure 2. Phylogenetic analysis of full-length genomes of SARS-CoV-2 in eight patients. Red text indicates SARS-CoV-2 detected in the patients in the present study.

Figure 1

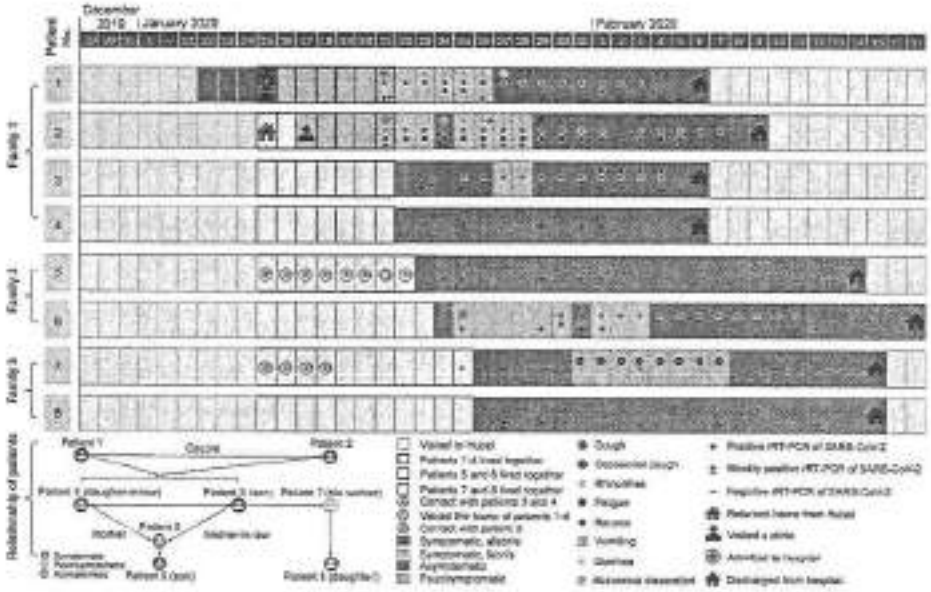

Figure 2

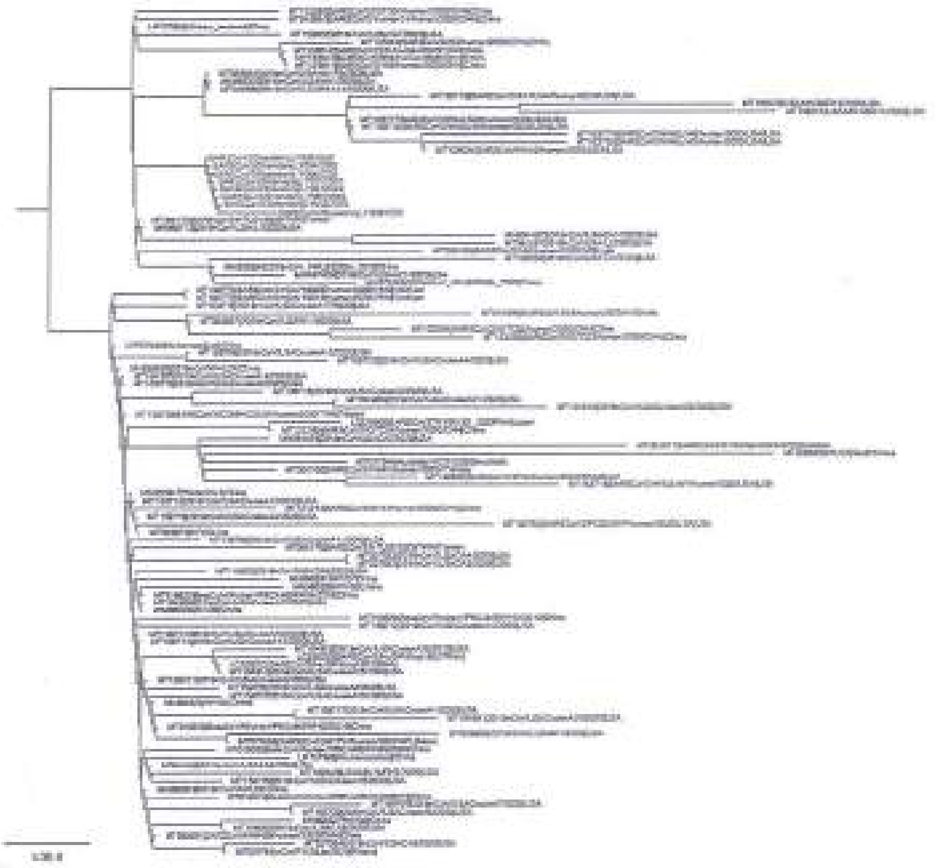

EXHIBIT I

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Contact tracing and isolation of asymptomatic spreaders to successfully control the COVID-19 epidemic among healthcare workers in Milan (Italy)

Stefan Mandić-Rajčević[†,*], *Federica Masci*[†], *Eleonora Crespi, Sara Franchetti, Anna Longo, Ilaria Bollina, Serena Velocci, Alessandro Amorosi, Riccardo Baldelli, Luisa Boselli, Lucia Negroni, Alessandro Zà, Nicola Vincenzo Orfeo, Giuseppe Ortisi, Claudio Colosio*[*]

Department of Health Sciences, University of Milan, Occupational Health Unit, International Centre for Rural Health and Central Health Care Management of the Saints Paolo and Carlo Hospitals, Milan, Italy.

[†] Equal contribution authors

*Corresponding Authors

Professor Claudio Colosio and

Dr. Stefan Mandić-Rajčević

Department of Health Sciences, University of Milan, Occupational Health Unit, International Centre for Rural Health and Central Health Care Management of the Saints Paolo and Carlo Hospitals, Milan, Italy

Via San Vigilio 43

20142 Milan

Italy

claudio.colosio@unimi.it

stefan.mandic-rajcevic@unimi.it

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Abstract

Objective

To study the source, symptoms, and duration of infection, preventive measures, contact tracing and their effects on SARS-CoV-2 epidemic among healthcare workers (HCW) in 2 large hospitals and 40 external healthcare services in Milan (Italy) to propose effective measures to control the COVID-19 epidemic among healthcare workers.

Design

Epidemiological observational study.

Setting

Two large hospitals and 40 territorial healthcare units, with a total of 5700 workers.

Participants

143 HCWs with a SARS-CoV-2 positive nasopharyngeal (NF) swab in a population made of 5,700 HCWs.

Main outcome measures

Clinical data on the history of exposure, contacts inside and outside of the hospital, NF swab dates and results. A daily online self-reported case report form consisting of the morning and evening body temperature and 11 other symptoms (cough, dyspnoea, discomfort, muscle pain, headache, sore throat, vomiting, diarrhoea, anosmia, dysgeusia, conjunctival hyperaemia).

Results

Most workers were tested and found positive due to a close contact with a positive colleague (49%), followed by worker-initiated testing due to symptoms (and unknown contact, 28%), and a SARS-CoV-2 positive member of the family (9.8%). 10% of NF swabs performed in the framework of contact tracing resulted positive, compared to only 2.6% through random testing. The first (index) case caused a cluster of 7 positive HCWs discovered through contact tracing and testing of 250 asymptomatic HCWs. HCWs rarely reported symptoms of a respiratory infection, and up to 90% were asymptomatic or with mild symptoms in the days surrounding the positive NF swab. During the 15-day follow-up period, up to 40% of HCWs reported anosmia and dysgeusia/ageusia as moderate or heavy, more frequently than any other symptom. The time necessary for 95% of HCWs to be considered cured (between the positive and two negative NF swabs) was 30 days.

Conclusion

HCWs represent the main source of infection in healthcare institutions, 90% are asymptomatic or with symptoms not common in a respiratory infection. The time needed to overcome the infection in 95% of workers was 30 days. Contact tracing allows identifying asymptomatic workers which would spread SARS-CoV-2 in the hospital and is a more successful strategy than random testing.

Keywords: SARS-CoV-2; healthcare personnel; infection prevention and control; occupational health;

What is already known on this topic?

There are more than 3 million SARS-CoV-2 positive cases and more than 200,000 deaths attributed to coronavirus disease (COVID-19) worldwide.

Commonly reported symptoms of COVID-19 include fever, cough, dyspnea, sore throat, muscle pain, discomfort, and many prevention strategies are based on identifying these symptoms of infection.

The virus can be spread even by asymptomatic patients or patients with mild symptoms, and healthcare workers (HCWs) represent 10% of overall cases and often more than 10% of hospital personnel are commonly infected.

HCWs represent both a vulnerable population and an irreplaceable resource in the fight against this epidemic and further analysis is needed to show how and why they get infected and introduce successful prevention measures.

What this study adds?

The first (index) case in our study was infected by a family member, but due to close contacts with colleagues managed to infect other 7 HCWs. Contrary to a common expectation that HCWs get infected from patients, they regularly get infected by other HCWs.

Up to 90% of HCWs were asymptomatic or had only mild symptoms. Random testing for SARS-CoV-2 was not efficient. Active search for suspect cases through contact tracing is the strategy of choice to identify most of the positive HCWs.

Most HCWs remained asymptomatic during the 15-day follow-up period, and even in the days prior to the positive NF swab. Anosmia and ageusia/dysgeusia were reported more commonly than classic symptoms of a respiratory infection.

Contrary to the recommended quarantine of 14 days, 30 days were necessary for 95% of the workers to be declared cured (two negative NF swabs)

Introduction

On the 30th of January 2020, the World Health Organization (WHO) declared the outbreak of the novel coronavirus disease 2019 (COVID-19) as a Public Health Emergency of International Concern. At the time of the writing of this paper, almost 3 million cases and almost 200,000 deaths have been reported worldwide (1). Most prominent symptoms include fever, dry cough, headache, sore throat and sneezing, although a growing number of reports underline asymptomatic and patients with mild symptoms having the same viral load as symptomatic patients and spreading the infection in the general population and among healthcare workers (HCW) (2–5). Most published reports on COVID-19 patients underline that HCWs get infected regularly and they represent one of the most vulnerable groups during this pandemic (6,7). The safety of HCWs is key not only to fight this international biological threat through their care for the critically ill patients, but also to prevent them from transmitting the virus.

WHO and European Centre for Disease Control (ECDC) recommendations for the rational use of personal protective equipment agree on the use of: a) a medical (surgical) mask, eye protection, long-sleeved water-resistant gown, gloves, and keeping 1 m distance when dealing with suspected or confirmed COVID-19 cases; b) a respirator (N95 or FFP3), eye protection, water-resistant gown and gloves during aerosol generating procedures; c) a medical (surgical) mask to be worn by suspected or confirmed COVID-19 patients, or any patient with respiratory symptoms. Good hand hygiene should be kept using 70% alcohol based disinfectants or soap and water (washing at least 20 seconds) (8–10). WHO definitions of suspect cases require at least mild symptoms, although contact tracing and adjustment of suspect case definition is encouraged (11). Inclusion of contact tracing and testing of asymptomatic patients can help identify potential spreaders of SARS-CoV-2 in hospitals, but with a high logistic burden (12). Having in mind that around 10% of infected persons are HCWs, and that most hospitals report 10% of staff getting infected (13), the additional burden might be worth it. Additional challenges are posed by the removal from workplace and return-to-work procedures which depend on the expected duration of the disease and a negative nasopharyngeal swab, which can be false negative (14).

In this frame, we developed our own experience in the Territorial Socio-Sanitary public company of the Saints Paolo and Carlo of Milano, Italy (TSSC). The first SARS-CoV-2 infected

HCW in our structure was confirmed on February 27th, 2020. Since that moment, we applied a protocol based on five main steps:

1. To identify a case (or cases) which could be diagnosed by any of our hospitals or reported by any other affordable source (patients and workers);
2. To identify symptomatic workers and to verify the existence of an infection;
3. To conduct an internal epidemiological survey addressed at identifying all close contacts of the infected subjects;
4. To "biologically" isolate these contacts, in the hospital and in the private life, and immediately perform a nasopharyngeal swab;
5. To decide, based on clinical and laboratory data, the return-to-work policy safe for the HCWs and their colleagues.

The aims of this paper are to analyse the effects of preventive measures taken in our TSSC to tackle the epidemic, the onset and follow-up of COVID-19 symptoms in the typical working age population of HCWs, the sources of infection in HCWs and the duration of the infection to propose adequate, evidence-based, and effective measures to control the COVID-19 epidemic in healthcare institutions and establish an appropriate quarantine period.

Material and Methods

The setting

Our TSSC is composed of two public hospitals of Milan and 40 territorial healthcare institutions (providing various services such as vaccination, preventive care, administration), which are part of the Region of Lombardy (Northern Italy) public healthcare system. The two hospitals employ a total of 4142 workers (healthcare workers and non-healthcare workers in a healthcare setting, all referred to as HCWs in our report). There are around 1500 workers in 40 territorial healthcare institutions. Out of 5,700 workers, 70% are female, with a mean age of 46 years. It is estimated that around 150,000 patients are examined in our emergency rooms of the two hospitals every year, with an average of 50 admissions per day in around 800 hospital beds available. The hospitals provide all diagnostic and medical procedures covering most existing medical specialties. San Paolo hospital is also the home of the Department of Health Sciences of the University of Milano, which integrates it into the University of Milan academic system. Workers of the whole groups of structures are provided with occupational health services in the healthcare setting.

Since at the beginning of the epidemic, most administrative workers started smart working, with only a few exceptions. During our routine occupational health surveillance of workers, we transferred the most vulnerable subjects (the elderly, those affected by chronic diseases) from the most risky departments (i.e. emergency room) to "safer" departments. Most vulnerable workers got the possibility to remain home, without any impact on their earnings. Our study is addressed at the health care personnel who continued their activities during the crisis.

Ethical approval

All data presented in this paper were extracted from the health surveillance files, and no experimental activity has been carried out. Nevertheless, all our workers are informed about our health surveillance procedures and have signed an informed consent regarding the data collection and analysis. No ethical approval was deemed necessary by the Ethical Committee of the Saints Paolo and Carlo Hospitals.

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research

Management of HCWs in the frame of SARS-CoV-2 pandemic

Figure 1 outlines the procedure and treatment of close contacts, quarantine and return-to-work procedure.

FIGURE 1 HERE

A "close contact" was defined as a person who had a face-to-face dialogue or who spent at least 15 minutes in an indoor environment with a COVID-19 patient, without wearing a personal protective device (PPD, e.g. surgical mask). The epidemiological survey was performed by the Health Care Management of the structure and close contacts were reported to our Occupational Health Unit. Symptomatic workers were those with any respiratory symptoms (defined at the beginning as fever, sore throat, cough, difficulty breathing, or diarrhoea). Asymptomatic workers were asked to adopt "source control" and isolation measures to reduce the viral charge and risk of infecting another colleague or a family member. Viral charge reduction or "source control" was performed by asking the worker, even if asymptomatic, to wear a surgical mask both while working, traveling and in the private life. The worker was to take meals separately from the family, live in a separate room and use a dedicated bathroom or at least carefully wash the only bathroom available after use.

Each close contact was required to do a NF swab. Workers absent from work due to respiratory symptoms were asked to come to the hospital for a NF swab when their symptoms would allow it (fever below 37.5°C and other symptoms not preventing to travel). If the swab was positive, the affected worker was placed in mandatory quarantine for a 14-day period. For symptomatic workers the quarantine period lasted for at least 14 days from the full termination of all symptoms, even if the symptoms were reported later during the disease (after the swab). Workers with symptoms were asked to immediately leave the workplace and to go home, adopting the same rules of source control and isolation given to the asymptomatic workers. In case of a negative NF swab, the worker would come back to work after the cessation of symptoms. To reduce the potential impact of false negative NF swabs, all close contacts, even if the swab

resulted negative, maintained precautionary source control measures (surgical mask) for a 14-day period after the contact.

During their absence or isolation (negative NF swab but confirmed close contact) all workers were asked to fill in and submit a daily symptoms report which was collected by phone and in paper for the first 5 workers, and then transformed into an online questionnaire to allow an overview and tracking of the whole population of HCWs under observation. This collection was performed in order to point out any sign or symptom indicative of a possible worsening of the health conditions of the worker.

Return-to-work procedure

We set up a specific procedure to manage safe return to work of the workers absent for COVID-19 or suspected of COVID-19. In particular, a symptomatic COVID-19 worker was considered cured 14 days after the resolution of respiratory infection symptoms and two consecutive negative tests for SARS-CoV-2 at least at 24 hour distance one from the other (15). The definition of "clearance" of the virus indicates the disappearance of detectable SARS-CoV-2 RNA in nasopharyngeal swabs. In case of a positive swab after the 14-day period, another 7 days was added to the quarantine, and then the two swabs were repeated.

We faced an additional problem of readmitting to work HCWs who were absent from work but did not undergo a NF swab during this period. If the worker was absent for 14 days or more (same as COVID-19 positive workers) and/or showed typical symptoms, she or he was treated as a COVID-19 patient. If the period of absence was shorter than 14 days, or the presence of typical symptoms was not recorded, the worker was treated as "close contacts" (followed the "source control" procedure, underwent a NF swab, and symptoms were followed).

Random testing of workers

Since the 25th of March, a new regulation recommended random testing of HCWs (16). We also report the results of HCWs found positive using this approach.

Symptoms report

Our study presents the results and analysis of 143 HCWs with a positive NF swab who filled the online symptoms report in the period from the 9th of March until the 8th of April 2020.

HCWs with a positive NF swab, symptomatic HCWs, as well as those under surveillance for a close contact with a positive HCW or a family member were asked to fill in a daily symptoms report. The symptoms report has evolved to improve tracking and adjust for clinical findings during the period in four phases. These phases are described shortly to better explain what data was collected and when:

1. Initially, from the 27th of February until the 8th of March, the daily symptoms report was in paper form and was given to (a few initial) HCWs to fill and submit in writing after the quarantine;
2. From the 9th of March, the symptoms report was put online, and all workers were given a link to an electronic sheet where they would fill and submit the daily report, and their data became available right away. The report included the morning and evening body temperature and there was a free- text field available to add any other symptoms;
3. From the 12th of March, the free-text field for symptoms was converted into specific questions regarding the symptoms (cough, dyspnoea, sore throat, headache, muscle pain, discomfort, vomiting), based on the current knowledge of COVID-19 symptoms;
4. Finally, based on clinical experience, additional symptoms (anosmia, dysgeusia, and conjunctival hyperaemia) were added.

Nasopharyngeal swab

Nasopharyngeal specimen is the gold standard for swab-based SARS-CoV-2 testing. Nasopharyngeal swab was performed by inserting the flexible wire shaft minitip swab through the nares parallel to the palate until resistance was encountered or the distance is equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx. Then the person performing the swab would gently rub and roll the swab and leave it in place for several seconds to absorb secretions. The swab was then slowly removed while rotating it. All HCWs in charge of taking this sample did a standardized course which allowed all samples to be taken in the same way.

Nasopharyngeal specimen analysis

Real-time reverse transcription–polymerase chain reaction (rRT-PCR) assay is currently the most reliable and the only available direct method to detect SARS-CoV-2 virus; it is the gold standard method for laboratory diagnosis of COVID19 (17,18).

SARS-CoV-2 is a large positive-sense single-stranded ribonucleic acid (RNA) virus that comprises of four structural proteins, i.e., nucleocapsid protein (NP) that holds the viral RNA, spike protein (SP), envelope protein (EP), and membrane protein (MP), that create the viral envelope. RNA-dependent RNA polymerase gene (RdRp), envelope (E), and nucleocapsid (N) have become key diagnostic targets for SARS-CoV-2 identification.

In the Clinical Laboratory of the Department of Diagnostic Sciences of the San Paolo hospital the NF swab analysis was performed with two kits: GeneFinderTM COVID-19 PLUS RealAmp Kit by ELITech Group and Roche Modular Wuhan CoV N, RdRP and E gene Kit.

Test was Positive if RdRp and E or N or both genes were detected; repeated to confirm Positive if only RdRP or N were detected.

Statistical analyses

We present a case series of 143 out of 185 SARS-CoV-2 positive HCWs in the study period. Each HCW was connected to their daily reports. The reports were centred around the date of the positive swab, making that day's report the follow-up day 0 (zero). Days following the positive swab are denoted with positive integers (from 1 to 15), and days leading to the positive swab are denoted with negative integers (from -5 to -1).

The morning and evening temperature were collected as numeric variables. The absence or intensity of each symptom was collected as: absent, light, moderate, and heavy. Categorical variables (i.e. symptom intensity) are presented in tables as the absolute count (N) and proportion among the grouping variable (e.g. number and percentage of HCWs reporting absent, light, moderate, and heavy for each symptom).

Data management, processing, analysis and visualization were done using R Language and Environment for Statistical Computing (19).

Results

From the 27th of February until the 8th of April, 2485 NF swabs (some of which repeated) were performed? for HCWs in our two hospitals and the territory, of which 460 NF swabs were done at random. These NF swabs resulted in a total of 185 SARS-CoV-2-positive workers, of which 12 were found through random swabs. The positive rate in non-random samples was around 10%, while the positive rate among randomly sampled HCWs was around 2.6%.

Table 1 shows the characteristics of the HCWs included in our study. The study group was made of 143 COVID-19 positive HCWs employed in the different healthcare structures of our TSSC. The data for San Paolo hospital also include the territorial healthcare institutions since their occupational health surveillance is done by the San Paolo hospital. The majority of HCWs were female (57.3%), and the most prevalent job title was nurse (48.3%), followed by medical doctors (26.6%), and assistant nurses (9.8%). Most workers were tested due to a close contact with a positive colleague (49%), followed by worker-initiated testing due to symptoms and unknown contact (total 28%), and a SARS-CoV-2 positive member of the family (9.8%). Close contact with COVID-19 positive patients was the reason for testing in 7.7% of cases HCWs, while 5.6% were tested on random bases. The HCWs under study submitted an average of 9 daily symptoms reports during their quarantine, ranging from 1 to 26.

TABLE 1 HERE

FIGURE 2 HERE

Figure 2 shows timeline of the positive swabs found in HCWs in our study. The first (index) case's swab was taken on the 25th of February and the result came back on the 27th of February. At the beginning, most individual cases were connected to this case. In the middle weeks of March 2020 the majority of cases (denoted dark red) were connected to other HCWs. The proportion of HCWs infected from close unprotected contacts with colleagues started to decline after the week of 20th of March, and weas replaced by symptomatic HCWs without identified close contact, family-related contacts, and randomly selected for testing HCW.

FIGURE 3 HERE

Figure 3 shows the results of contact tracing which started with the first case of a SARS-CoV-2 positive doctor in our study. The doctor had been infected by a family member which later

proved to be positive for SARS-CoV-2. Initial contact tracing resulted in 53 "close contacts" inside the hospital, and these 53 swabs resulted in one positive case. From this second positive case other 134 contacts were traced resulting in additional 2 positive HCWs. For each of the new cases another 29 and 20 swabs were performed, resulting in another 2 positive cases on one side, and no positive cases on the other side. This first cluster of cases grew to 7 known cases connected to the index case (data not shown).

FIGURE 4 HERE

Most HCWs started submitting daily symptoms reports on the day following the NF swab (66%). The highest percentage of filled reports was noted in the first week, going from 73% on the 2nd day, down to 58% on the 7th day. The gradual drop of daily reports submitted continued in the second week, going from 48% down to 33%. ***Figure 4*** shows the steep rise and gradual decline in the percentage of HCWs submitting the daily health report.

TABLE 2 HERE

Table 2 shows the respiratory and other commonly reported symptoms in the day prior to the positive NF swab (***Day -1***), on the day of the swab (***Day 0***), and in the following day (***Day 1***). On average, the SARS-CoV-2-infected HCWs were afebrile, with no or light respiratory symptoms. Cough and dyspnoea were absent or light in around 90% of the workers, while general symptoms, such as discomfort, muscle pain, and headache were reported more often, although by no more than 30% of the workers. Most symptoms were absent in more than 50% of the HCWs in the first week of the disease. Anosmia and dysgeusia were the only specific symptoms which were commonly reported, in light, moderate or heavy form by almost 50% of the workers.

Figure 5 shows the body temperature of HCWs on the day of the positive NF swab (day 0) and in the 15 days following the swab. ***Figure 6*** shows commonly reported symptoms associated with COVID-19, their intensity, from absent to heavy, as reported by the HCWs during the 15 days following the NF swab. The most common symptoms of a respiratory infection, such as cough, dyspnoea, or sore throat, were present in a heavy or moderate form in less than 15% of the study group in the first days of the infection. Between 70% and 90% of HCWs reported no or mild respiratory symptoms. The only two symptoms reported more commonly as moderate and heavy by between 30% and 40% of HCWs were anosmia and ageusia/dysgeusia. There was a gradual

reduction of most symptoms in the second week, with persisting anosmia and dysgeusia in around 30% of HCWs.

FIGURE 5 HERE

FIGURE 6 HERE

For a smaller group of workers who reported their symptoms also in the 5 days leading to the positive NF swab, ***Figure 7*** shows the body temperature and ***Figure 8*** shows other reported symptoms (days -5 to 0). These workers were defined as close contact but were unable to perform a NF swab, so they submitted symptoms reports until the moment of the swab. Although based on a smaller number of reports (see ***Table 2***), most HCWs report normal body temperature or light fever, and those with fevers above 37.5°C or 38°C can be considered outliers. HCWs reported cough (in light and moderate forms), and non-specific symptoms such as discomfort, muscle pain, sore throat, and headache most commonly in the days leading to the positive NF swab. We noted a gradual increase in the number of HCWs reporting anosmia, as well as the gradual worsening of this symptom from light and moderate forms to the heavy form.

FIGURE 7 HERE

FIGURE 8 HERE

The median time elapsed from the positive swab to two consecutive negative swabs was 21 days (minimum: 14 days; maximum: 34 days) for San Carlo hospital, and 25 days (minimum: 15 days; maximum: 46 days) for San Paolo hospital (including territorial healthcare institutions). Since the data was not normally distributed, we also report the median values of 20 and 25 in San Carlo and San Paolo hospitals, respectively. 95% of HCWs was considered cured and was able to return to work after 27 days and 36 days in San Carlo and San Paolo hospitals, respectively.

Discussion

According to the latest data of the Italian Institute of Health (Istituto Superiore Sanita', April 23rd 2020), there were more than 177 thousand persons positive for SARS-CoV-2 and more than 23 thousand COVID-19 related deaths in Italy. Healthcare workers represent more than 10% of cases (just below 20,000 infected), with a median age of 48 years and 33% male (20). Our results represent one of the first reports on the development of the SARS-CoV-2 epidemic among healthcare workers in large hospital. Our data has shown the number of newly diagnosed SARS-CoV-2-positive HCWs from only one (index case). At the peak of the epidemic in our hospitals and territorial units, up to 12 workers were being diagnosed positive every day, and the source of the infection were other HCWs. Most HCWs had no or only light symptoms which at the beginning never led them or others to doubt they were infective. Common respiratory symptoms, such as fever, cough, dyspnoea, or sore throat were much less reported than recently discovered symptoms such as anosmia and ageusia/dysgeusia. Finally, the time which passes from the positive NF swab to the cessation of symptoms and two consecutive negative NF swabs is more than double of the 14 days initially proposed, which should be taken into account when deciding the duration of the quarantine, even for asymptomatic workers.

The epidemic in our hospitals started with one HCW who was infected outside of the hospital setting. In the first 7 days of the epidemic, we traced more than 250 contacts among HCWs related to this first positive case, which finally resulted in at least 7 positive HCWs. This has been the largest cluster of cases found in our hospital. During the period of around 40 days presented in this report, the Occupational Health Unit of Saints Palo and Carlo hospital performed around 2500 swabs, resulting in 185 positive workers (of which 143 were included in this report), with a much higher percentage of positive cases found among workers identified through contact tracing (~10%) than among randomly sampled (2.6%). The 3rd and 4th weeks of the epidemic in our hospitals were characterized by 5 to 10 cases/day, of which the majority were related to other positive colleagues. Transfer and removal of high-risk workers from high-risk hospital environments, reduction of visiting hours, and avoiding close contacts among patients and colleagues (distance, use of PPD) reduced the potential of SARS-CoV-2 transmission. During the 5th and 6th week of the epidemic in our hospitals, the number of positive HCWs connected to other colleagues declined rapidly, reducing the both the overall number of daily new cases and

the size of the clusters surrounding each positive HCW. In the 6th week of tracking, most HCWs who were found positive were tested because they had a positive family member, noticed symptoms, or were tested at random.

The shape of the epidemic curve in our hospital follows the sharp increase of the number of COVID-19 cases in Italy, the Lombardy Region, and Milan. The sharp decline which has followed afterwards in our hospitals does not follow that of Italy as a whole, which was observed several weeks later. We attribute this difference to organizational and protective measures (distancing, use of PPDs for source control) and contact tracing performed by our Occupational Health Unit. Although the number of infected persons in Lombardy has surpassed 60.000, there were only 185 positive HCWs out of 5700 HCWs in our hospitals and the territory, representing just over 3% of workers.

A preliminary report on the epidemic in a large hospital in Madrid, where workers were tested only if presenting at least with mild symptoms, found 791 SARS-CoV-2-positive HCW among around 6800 workers (~11%). The authors found no relation with so-called "high-risk" areas of the hospital, and connect the dynamic of transmission in their hospital to that of the general population (13). Our approach which included contact tracing, source control and testing of asymptomatic workers if they fall under the definition of "close contact" resulted in almost 4 times lower incidence of infection in a setting with more positive cases in the general population. Non-pharmacological measures were underlined as leading to a decline in the effective reproductive number in Wuhan from 3.8 to 0.3, ultimately stopping the epidemic in China (21–23). Similar to the experience in China, our experience underlines the role of asymptomatic patients in driving the epidemic in the hospital but adds workplace transmission between colleagues as a driver of the epidemic among HCWs.

Most HCWs in our study reported no or only mild symptoms of a respiratory infection. In fact, on the day of the positive swab, the median body temperature measured by HCWs was 36.6°C, while cough and dyspnoea were reported as moderate or heavy by only 12.5% and 0% of HCWs, respectively. Unspecific symptoms, such as discomfort, muscle pain, and headache were reported more commonly, but only in around 30% of HCWs. A recent review article on asymptomatic COVID-19 transmission underlined this risk in healthcare setting, although concentrating on the risk arising from asymptomatic patients (24). Our results underline the risk of transmission

among asymptomatic HCWs. One of the measures implemented in Lombardy was a mandatory check of the body temperature before the beginning of the work shift for HCWs, and, in case of a temperature above 37.3°C a NF swab is performed and the worker suspended until the results are back (25). Our results show this measure, as any other "symptom-centred" measure, does not guarantee protection of HCWs and patients, and could even result in a false sense of safety in a scenario where up to 90% of cases could be asymptomatic. Most other experiences and opinions regarding the protection of HCWs underline the re-organization of work (e.g. moving triage and pharmacy outside of the hospital), reduction of the density of people (e.g. reducing visiting hours and numbers of visitors), adequate training and use of PPE as the main solutions to protecting HCWs (26–28).

Three additional symptoms drew our attention while working with suspected SARS-CoV-2 positive workers: anosmia, ageusia/dysgeusia, and conjunctival hyperaemia. In fact, since the moment they were added to our daily report in the middle of March, anosmia and ageusia became symptoms most commonly reported as moderate or heavy in SARS-CoV-2 positive HCWs, reported in more than 40% and 30% of cases, respectively. They also represented the most persistent symptoms, as most other symptoms typically connected to respiratory infections exhibited a reduction during the 15-day follow-up. Conjunctival hyperaemia, although noted by our doctors at the hospitals while performing the medical examination and NF swab, was not often reported by HCWs in the daily symptoms report, which might be connected to the fact that it requires an "outside observer" to notice it. It is also important to note that the duration of the infection (time from the positive NF swab to two negative NF swabs) was between 20 and 30 days with 30 days needed for 95% of workers to be considered cured. This information should be considered when deciding whether the quarantine should last for 28 days instead of 14.

A much smaller sample of workers (N=10), commonly found among close contacts but absent from the hospital for other reasons, reported their daily symptoms even in the days leading to the positive NF swab. In the days leading to a positive NF swab, the symptoms and their intensity were similar to those reported during the follow-up period. Cough and non-specific symptoms were reported more commonly, fever and other respiratory symptoms rarely, and we noted a gradual increase in anosmia and ageusia leading to the day of the positive NF swab. This is the first report of symptoms in the pre-swab period of SARS-CoV-2 positive patients.

Our study was based on the data collected to monitor workers health status while quarantined, the filling of the daily symptoms report was voluntary, and the report itself was adapted several times to answer the needs and field situation, which introduce bias in the presented results. Nevertheless, our report is based on a relatively high response rate of around 70% in the first week of follow-up. A limitation is the fact that 42 HCWs never filled in even one daily symptoms report. In a telephone survey currently in progress, the most common reasons for not filling the online symptoms report was the lack of a smartphone, computer or internet at home, lack of experience with online forms ("not being technological enough"), being diagnosed in the week prior to the implementation of the online report, and taking care of a sick family member ("lack of time for the reports"). Five HCWs did not fill the report because they were hospitalized. Their reports would certainly differ from the rest of our workers with a mild clinical picture, but heir percentage (less than 5% of 185), similar to that in Madrid where 29 out of 791 required hospitalization, leads us to believe that the data presented are representative of the majority SARS-CoV-2-positive HCWs and the selection bias in our report is negligible.

Future studies should analyse in more detail the circumstances surrounding the infection of HCWs, symptoms, and the overall outcome of their disease. New symptoms, such as anosmia and dysgeusia which were frequently reported by our HCWs could help clinicians arrive to a diagnosis sooner and reduce the time available for worker-to-worker and worker-to-patient transfer of SARS-CoV-2. Another step forward will be understanding whether HCWs have developed specific immunity, even among those with negative swab results. This could help us understand whether the presence of specific IgG could be an expression of effective immunity, as well as whether it is temporary or permanent. Finally, the lessons learned from controlling the SARS-CoV-2 epidemic among HCWs could be applied to other occupations/sectors at risk, such as transport workers, services and sales workers, and public safety workers.

Conclusions

HCWs represent one of the most important resources in the fight against COVID-19, but they are also one of the most vulnerable groups which is commonly infected. Our study has shown that at the peak of the epidemic in Italy the reorganization of work, physical distancing, use of PPDs (even for source control), and contact tracing and testing of asymptomatic HCWs were able to stop the worker-to-worker infection in the hospital and reduce the overall incidence of infection

among HCWs. Most HCWs were asymptomatic or with mild symptoms, which means they would most likely go undetected by symptom-centred preventive strategies. Most reported specific symptoms were anosmia and ageusia, and their role in arriving to a clinical diagnosis sooner should be further confirmed. Finally, our data suggest that the duration of the infection is longer than previously anticipated, and that a patient should not be considered recovered only 14 days after the positive swab. Readmitting such a patient or worker into the social or work life without a further swab assessment might create additional risk.

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Conflict of interest/Competing interest

Authors declare no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Authors Contributions

SMR: literature search, figures, study design, data collection, data analysis, data interpretation, original manuscript writing, manuscript revision; FM: literature search, figures, study design, data interpretation, original manuscript writing, manuscript revision; EC: study design, data collection, manuscript revision; SF: study design, data collection, manuscript revision; AL: study design, data collection, manuscript revision; IB: study design, data collection, manuscript revision; SV study design, data collection, manuscript revision; AA: study design, data collection, manuscript revision; RB: study design, data collection, manuscript revision; LB: study design, data collection, manuscript revision; LN: study design, data collection, manuscript revision; AZ: study design, data collection, manuscript revision; VO: study design, data collection, manuscript revision; GO: study design, data collection, manuscript revision,

laboratory analyses; *CC*: study design, data collection, manuscript revision, supervision, data interpretation, manuscript revision.

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The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit the publication.

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Table 1. Characteristics of the study population

	[ALL] *N=143*	San Carlo *N=58*	San Paolo *N=85*
Gender:			
Female	82 (57.3%)	33 (56.9%)	49 (57.6%)
Male	61 (42.7%)	25 (43.1%)	36 (42.4%)
Age	44.0 (26.0-66.0)	45.5 (27.0-66.0)	37.5 (26.0-63.0)
Job title:			
Nurse	69 (48.3%)	36 (62.1%)	33 (38.8%)
Doctor	38 (26.6%)	14 (24.1%)	24 (28.2%)
Social Assistant	14 (9.8%)	4 (6.9%)	10 (11.8%)
Intern	8 (5.6%)	1 (1.7%)	7 (8.2%)
Administrative	5 (3.5%)	0 (0.0%)	5 (5.9%)
Psychologist	5 (3.5%)	0 (0.0%)	5 (5.9%)
Laboratory	2 (1.4%)	1 (1.7%)	1 (1.2%)
Worker	2 (1.4%)	2 (3.4%)	0 (0.0%)
Contact or reason for NF swab:			
Symptomatic	20 (14.0%)	0 (0.0%)	20 (23.5%)
Unknown	20 (14.0%)	15 (25.9%)	5 (5.9%)
Family	14 (9.8%)	2 (3.4%)	12 (14.1%)
Random	8 (5.6%)	0 (0.0%)	8 (9.4%)
Patients	11 (7.7%)	0 (0.0%)	11 (12.9%)
Colleagues	70 (49.0%)	41 (70.7%)	29 (34.1%)
Number of reports per healthcare worker	9.0 (1.0-26.0)	13.5 (1.0-24.0)	7.0 (1.0-26.0)

Table 2. Symptoms of HCWs in the days surrounding the positive NF swab.

	-1 Day before + NF swab *N=10*	0 Day of the + NF swab *N=58*	1 1st day after + NF swab *N=94*
Morning temperature (°C)	36.4 (36.0-36.9)	36.6 (36.0-38.3)	36.3 (35.2-38.5)
Evening temperature (°C)	36.6 (36.0-37.0)	36.6 (35.0-38.5)	36.6 (35.0-38.6)
Cough:			
Absent	4 (40.0%)	24 (42.9%)	43 (45.7%)
Light	3 (30.0%)	25 (44.6%)	35 (37.2%)
Moderate	3 (30.0%)	6 (10.7%)	14 (14.9%)
Heavy	0 (0.0%)	1 (1.8%)	2 (2.1%)
Dyspnea:			
Absent	9 (90.0%)	54 (96.4%)	83 (88.3%)
Light	1 (10.0%)	2 (3.6%)	10 (10.6%)
Moderate	0 (0.0%)	0 (0.0%)	1 (1.1%)
Discomfort:			
Absent	5 (50.0%)	23 (41.1%)	44 (46.8%)
Light	5 (50.0%)	17 (30.4%)	30 (31.9%)
Moderate	0 (0.0%)	16 (28.6%)	19 (20.2%)
Heavy	0 (0.0%)	0 (0.0%)	1 (1.1%)
Muscle pain:			
Absent	6 (60.0%)	27 (48.2%)	46 (48.9%)
Light	3 (30.0%)	11 (19.6%)	23 (24.5%)
Moderate	1 (10.0%)	16 (28.6%)	24 (25.5%)
Heavy	0 (0.0%)	2 (3.6%)	1 (1.1%)
Headache:			
Absent	6 (60.0%)	33 (58.9%)	56 (59.6%)
Light	3 (30.0%)	14 (25.0%)	23 (24.5%)
Moderate	1 (10.0%)	8 (14.3%)	12 (12.8%)
Heavy	0 (0.0%)	1 (1.8%)	3 (3.2%)
Sore throat:			
Absent	7 (70.0%)	34 (60.7%)	66 (70.2%)
Light	3 (30.0%)	17 (30.4%)	19 (20.2%)
Moderate	0 (0.0%)	5 (8.9%)	9 (9.6%)
Vomiting:			
Absent	10 (100.0%)	54 (96.4%)	93 (98.9%)
Light	0 (0.0%)	2 (3.6%)	1 (1.1%)
Anosmia:			
Absent	4 (66.7%)	11 (50.0%)	24 (55.8%)
Light	1 (16.7%)	2 (9.1%)	3 (7.0%)
Moderate	1 (16.7%)	2 (9.1%)	7 (16.3%)
Heavy	0 (0.0%)	7 (31.8%)	9 (20.9%)
Ageusia:			
Absent	5 (83.3%)	12 (54.5%)	23 (51.5%)
Light	0 (0.0%)	3 (13.6%)	6 (14.0%)
Moderate	1 (16.7%)	2 (9.1%)	7 (16.3%)
Heavy	0 (0.0%)	5 (22.7%)	7 (16.3%)
Conjunctival hyperemia:			
Absent	6 (100.0%)	18 (81.8%)	38 (88.4%)
Light	0 (0.0%)	3 (13.6%)	2 (4.7%)
Moderate	0 (0.0%)	1 (4.5%)	3 (7.0%)

Figure legends

Figure 1. Outline of the HCW procedure for case identification, personal protection, removal and return to work procedure.

Figure 2. The number of positive swabs since the first (index) case in the hospital.

Figure 3. Index case and contact tracing in one of the hospitals.

Figure 4. Percentage of HCWs submitting the daily symptoms report relative to the date of the positive NF swab.

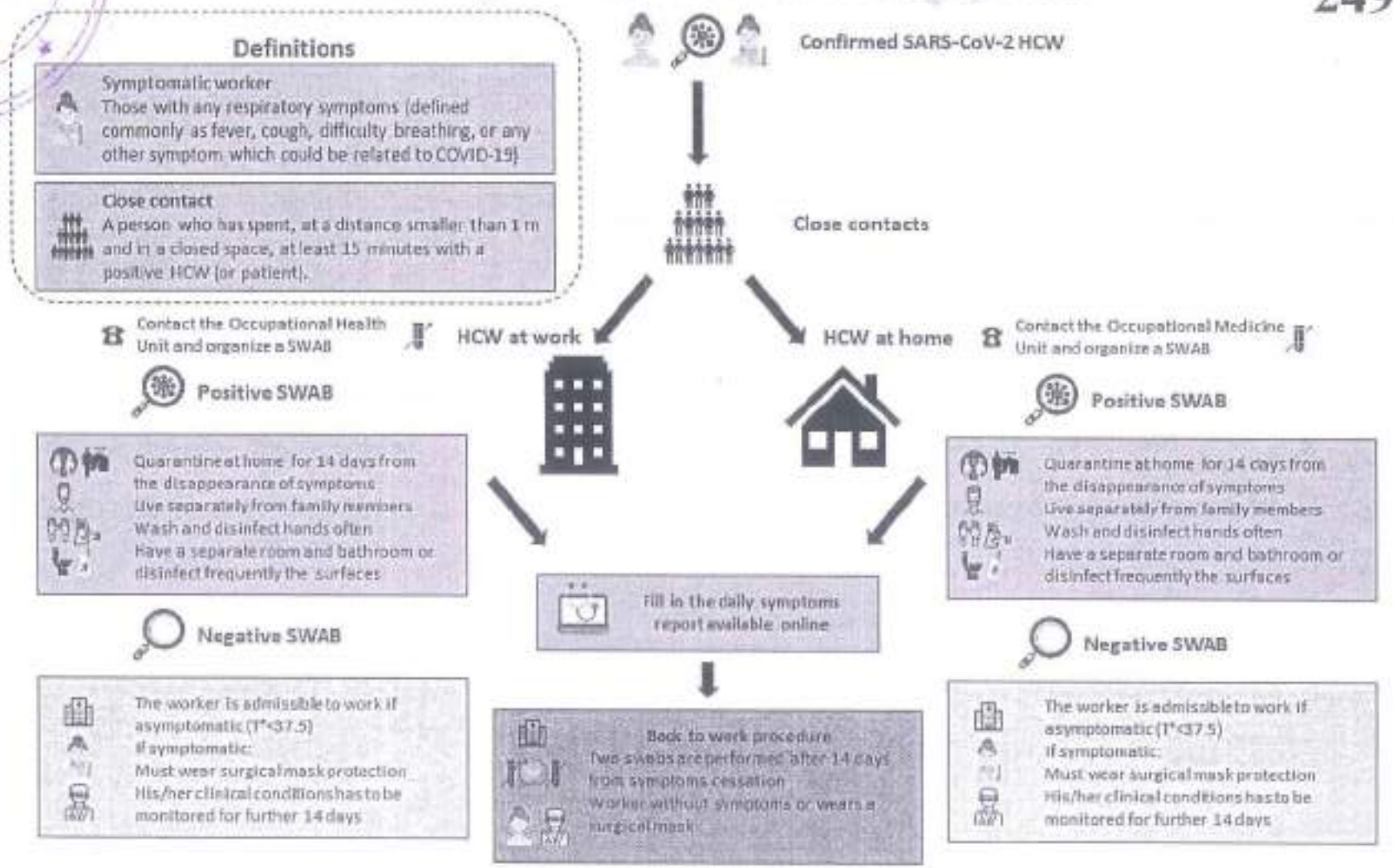
Figure 5. Morning and evening temperature of the SARS-CoV-2 positive HCWs in the first week following the NF swab.

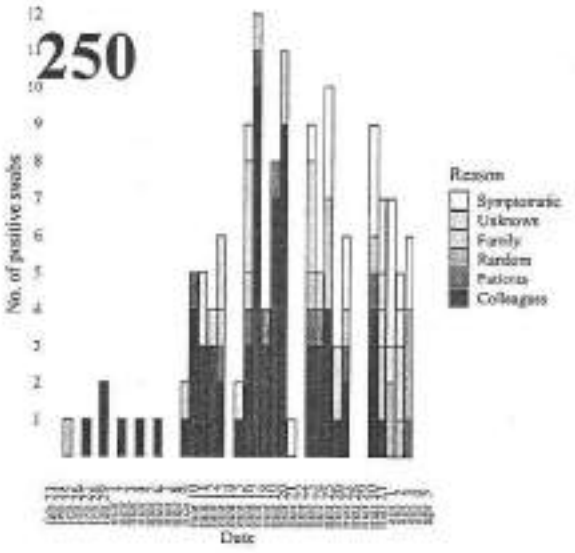
Figure 6. Commonly reported symptoms and signs by HCWs during the 15-day follow up.

Figure 7. Morning and evening temperature of the SARS-CoV-2 positive HCWs in the 5 days leading to the positive NF swab.

Figure 8. Common symptoms and signs reported by HCWs in the 5 days leading to the positive NF swab.

Contact tracing and management of SARS-CoV-2 positive HCWs




250
No. of positive swabs
Reason
Symptomatic
Unknown
Family
Random
Patients
Colleagues
Date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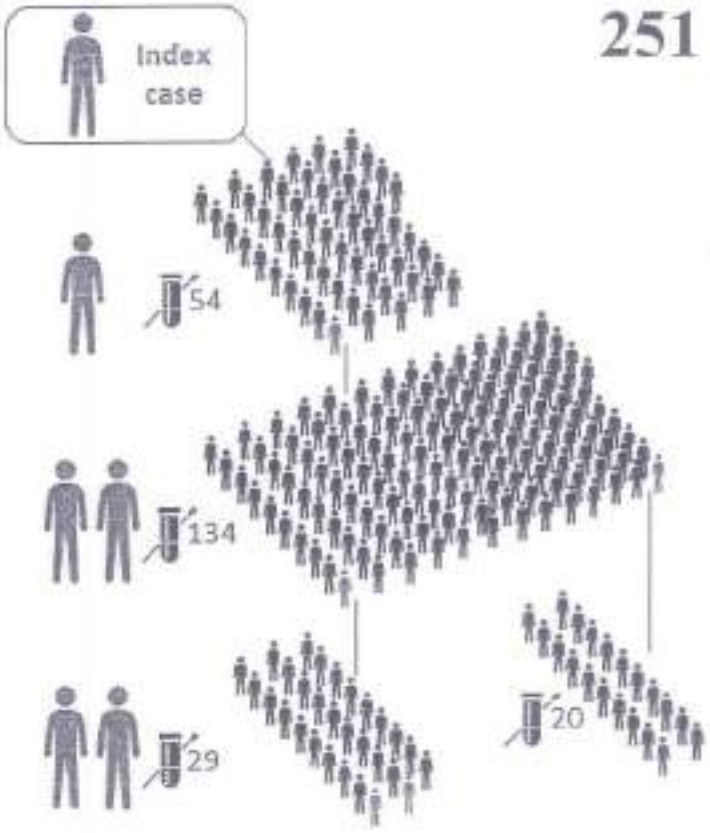
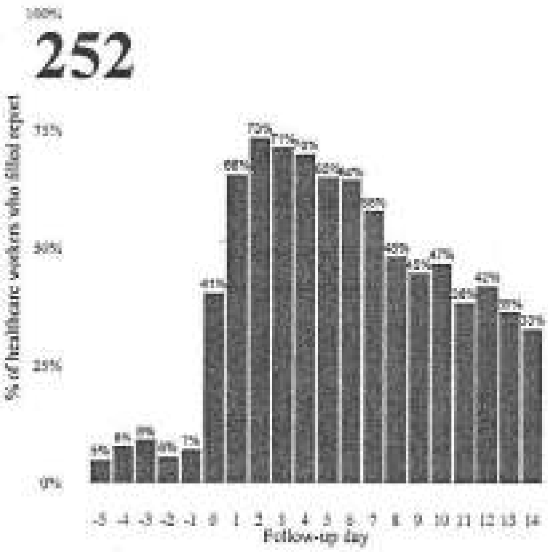
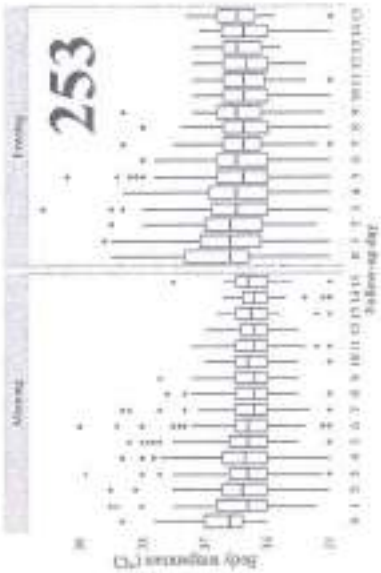

Index case
251
54
134
29
20

252




253
Morning
Evening
Body temperature (°C)
Before-up-day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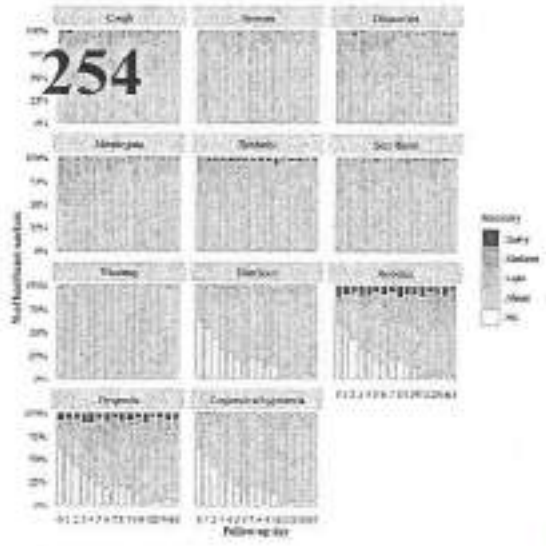
254


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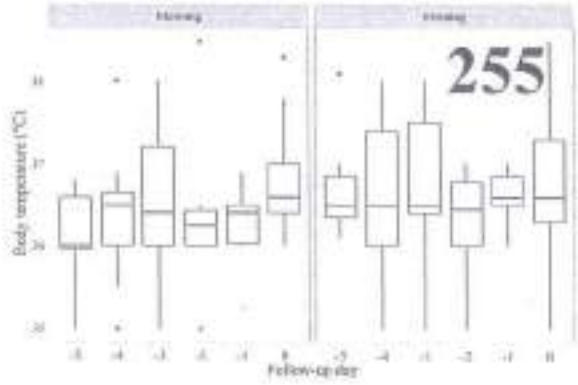

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Analysis of SARS-CoV-2 Transmission in Different Settings, Brunei

Liling Chaw, Wee Chian Koh, Sirajul Adli Jamaludin, Lin Naing, Mohammad Fathi Alikhan, Justin Wong

We report the transmission dynamics of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) across different settings in Brunei. An initial cluster of SARS-CoV-2 cases arose from 19 persons who had attended the Tablighi Jama'at gathering in Malaysia, resulting in 52 locally transmitted cases. The highest nonprimary attack rates (14.8%) were observed from a subsequent religious gathering in Brunei and in households of attendees (10.6%). Household attack rates from symptomatic case-patients were higher (14.4%) than from asymptomatic (4.4%) or presymptomatic (8.1%) case-patients. Workplace and social settings had attack rates of <1%. Our analyses highlight that transmission of SARS-CoV-2 varies depending on environmental, behavioral, and host factors. We identify red flags for potential superspreading events, specifically densely populated gatherings with prolonged exposure in enclosed settings, persons with recent travel history to areas with active SARS-CoV-2 infections, and group behaviors. We propose differentiated testing strategies to account for differing transmission risk.

Cases of coronavirus disease (COVID-19) have escalated since the disease was initially reported on December 31, 2019. A rapid response by the global scientific community has described many aspects of the causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Estimates suggest a basic reproduction number of 2–3 in the early stages of the outbreak (*1*), which can be valuable in assessing the spread of the virus but obscures individual heterogeneity in the level of infectivity among persons and in different settings (*2*,*3*). Early reports suggest that superspreading events (SSEs) might play a role in the explosive propagation of SARS-CoV-2 (*4*). Targeted approaches that reduce the likelihood of SSEs are contingent on the environmental, behavioral, and host factors that drive transmission and the most effective interventions to control those factors. To address these factors, we report an analysis of a transmission chain in Brunei that resulted from an international SSE.

Brunei is a small, well-connected country in Southeast Asia with a population of 459,500 (*5*). Brunei has multiple land borders and limited state capacity to manage large-scale outbreaks (*6*). Multigeneration households are common and social interactions center on strong family and religious relationships (*7*,*8*). These characteristics make Brunei particularly vulnerable to outbreaks and the rapid progression of clusters to widespread community transmission (*9*).

A COVID-19 case was detected in Brunei on March 9, after a 4-day religious gathering, Tablighi Jama'at, in neighboring Kuala Lumpur, Malaysia. The Tablighi Jama'at gathering in Malaysia has been recognized as an SSE and had >16,000 attendees, including international participants (*10*). Tablighi is an apolitical Islamic movement with adherents from >200 countries. Tablighi adherents usually gather at annual international events lasting several days where they participate in communal prayers, meals, and speeches. In Malaysia, the participants stayed and slept at the mosque, and several of them were deputized to cook and clean. Seventy-five persons from Brunei attended this event. Of the 135 confirmed cases in Brunei reported by the first week of April, 71 (52.6%) cases had an epidemiologic link to this event (Figure 1).

Because SARS-CoV-2 is a novel infection in a naive population, an outbreak investigation of this event can provide insights into its transmission dynamics and the effectiveness of outbreak control measures. Brunei's thorough contact tracing provides a rare opportunity to study the epidemiologic and transmission characteristics of SARS-CoV-2 in different community settings.

Author affiliations: Universiti Brunei Darussalam, Jalan Tungku Link, Brunei (L. Chaw, L. Naing); Centre for Strategic and Policy Studies, Jalan Pasar Baru Gadong, Brunei (W.C. Koh); Ministry of Health, Bandar Seri Begawan, Brunei (S.A. Jamaludin, M.F. Alikhan, J. Wong)

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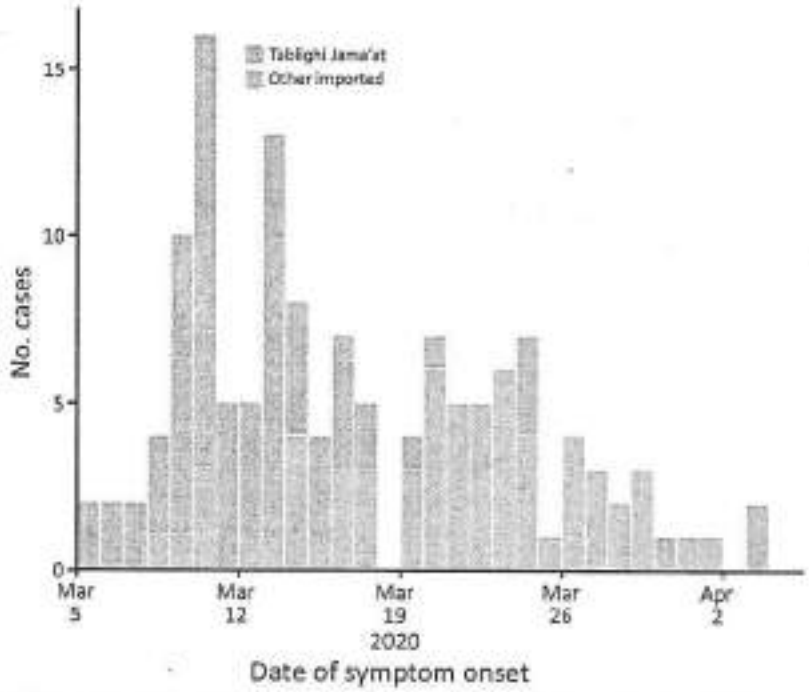

Figure 1. Epidemic curve for the first 135 cases of coronavirus disease (COVID-19) in Brunei Darussalam by cluster groups. Tablighi Jama'at cases were related to a religious gathering in Kuala Lumpur, Malaysia, during February 28–March 1, 2020.

Methods

Surveillance and Case Identification

Brunei's Ministry of Health (MoH) is responsible for communicable disease surveillance and implemented testing criteria for suspected COVID-19 cases on January 23, 2020. Initially, only persons with acute respiratory symptoms and history of travel to a high-risk area were tested for SARS-CoV-2. Over the next several weeks, the program expanded to include contacts of a confirmed case, regardless of symptoms; persons with pneumonia admitted to an inpatient healthcare facility; and persons with acute respiratory illness treated at a health facility for the second time within 14 days. On March 21, MoH started testing and isolating all travelers and returning residents. On March 25, MoH introduced SARS-CoV-2 sampling at selected sentinel health centers to test persons with influenza-like symptoms, and on April 7, MoH implemented mandatory random screening for selected groups of foreign workers.

MoH defined a confirmed COVID-19 case as a person who tested positive for SARS-CoV-2 through real-time reverse transcription PCR (RT-PCR) testing on a nasopharyngeal (NP) swab specimen (*11*). The first positive case in Brunei was detected on March 9 in a person who met the testing criteria by having a fever and cough and having recently traveled to Kuala Lumpur.

Epidemiologic Investigation

Under the Infectious Disease Act, MoH conducted epidemiologic investigations and collected data for each case and close contact by using the World Health Organization's first few cases protocol (*12*). The first identified case-patient was interviewed for demographic characteristics, clinical symptoms, travel history, activity mapping, and contact history. Once MoH identified the case-patient's participation at the Tablighi event in Malaysia, they identified several other persons from Brunei who also had participated at the event. We subsequently obtained the details of all participants from Brunei.

NP swabs were collected from all identified participants and tested with RT-PCR. Persons who tested positive were admitted to the National Isolation Centre (NIC). Persons who tested negative were quarantined for 14 days after their return to Brunei at a designated community quarantine facility, where they were screened for symptoms and body temperature daily. Persons who had symptoms develop at the NIC were retested. Activity mapping of confirmed cases was conducted, and contact tracing was initiated.

We defined a close contact as any person living in the same household as a confirmed case-patient or someone who had been within 1 m of a confirmed case-patient in an enclosed space for >15 minutes (13). We identified secondary cases through interviews and checked cellular phone data when information on contacts was uncertain. NP swabs from all close contacts of confirmed case-patients were tested by using RT-PCR. Persons who tested positive were admitted to the NIC and persons who tested negative were placed under home quarantine for 14 days from their last exposure to the confirmed case-patient. Public health workers monitored the compliance and health status of persons under home quarantine daily through video calls or face-to-face assessments. Persons who had symptoms develop during home quarantine were retested.

Clinical Management

All confirmed case-patients were treated and isolated at the NIC and monitored until recovery. We obtained clinical information on case histories, including any prior treatment by health services, clinical examination, and laboratory and radiological results, from digital inpatient records on the national health information system database. In addition, we collected information on each case-patient's oral history to ascertain whether they had symptoms ≤14 days before diagnosis. Case-patients were discharged from the NIC after 2 consecutive negative specimens collected ≥24 hours apart.

Case-Patients

We categorized cases into 2 groups: primary cases were in persons presumably infected at the Tablighi event in Malaysia and nonprimary cases were in persons who had an epidemiologic link to a primary case but did not attend the Tablighi event in Malaysia. For each case-patient, we recorded symptom status and classified them as follows: symptomatic patients reported having symptoms during or before NP swab collection; presymptomatic patients reported having symptoms after NP sampling but during admission to the NIC; and asymptomatic patients reported no symptoms during NP swab collection or admission to the NIC.

Close Contacts

We classified close contacts into 5 groups or settings: household, relatives, workplace, social, and a local religious gathering. We defined household contacts as persons living in the same household and further classified them by their relationship to a case-patient (spouse, child, or other, which included other familial relationships or housekeepers living in the household). We defined relatives as persons related to a case-patient who lived outside the household, workplace contacts as persons encountered at a workplace or school, and social contacts as those encountered during travel or at social events. We defined contacts from a local religious gathering as persons who attended a local Tablighi event in Brunei on March 5; the event ran throughout the night, and participants stayed all night. Such small local weekly gatherings usually take place among Tablighi adherents in their home countries.

Data Analysis

We used χ^2, Fisher exact, or Mann-Whitney tests to compare groups of primary and nonprimary cases, as appropriate. We calculated the incubation period from dates of exposure and symptom onset, when these were clear. We calculated serial interval by subtracting the date of symptom onset of an infectee (secondary case) from the date of symptom onset of the infector (primary case); we only included symptomatic and presymptomatic infector–infectee pairs for which epidemiologic links were clear.

We calculated the attack rate for each setting by dividing the number of positive contacts by the total number of close contacts. To identify risk factors for infection, we applied a log-binomial regression analysis to estimate the risk ratio for gender, age, and setting. We performed further stratification to assess differences in the symptom status of infectors across settings. We estimated the 95% CI by using the normal-approximation method, or the binomial method if the count was <5.

We calculated the mean observed reproductive number (R) and distribution of personal reproductive numbers in each setting by using the number of close contacts infected by each primary case-patient. We estimated the 95% CI by using a Poisson distribution (14).

We conducted all analyses by using Excel (Microsoft, https://www.microsoft.com) and R version 3.6.3 (15). We considered $p<0.05$ statistically significant. We obtained ethical approval from the University Research Ethics Committee, Universiti Brunei Darussalam (approval no. UBD/OAVCR/UREC/Apr2020-05).

Results

Epidemiologic Characteristics

Among 75 persons from Brunei who attended the Tablighi event in Malaysia, 19 tested positive for SARS-CoV-2; 52 local close contacts also tested positive, bringing the total cluster size to 71. We analyzed

the epidemiologic links in this cluster by generation in the transmission chain and case-patient symptom status. We noted 32 (45.1%) cases in generation 1, 15 (21.1%) in generation 2, and 5 (7.0%) in generation 3 (Figure 2).

We also analyzed the demographic and clinical characteristics of case-patients in the cluster (Table 1). The median age was 33.0 years (interquartile range [IQR] 21–50 years), 46 (64.8%) case-patients were male and 25 (35.2%) female, and 5 (7.1%) had preexisting chronic conditions. Compared with nonprimary case-patients, primary case-patients were much older, and most were men. Most (55/71; 77.4%) persons with diagnosed COVID-19 were immediately admitted to the NIC within 3 days of symptom onset or NP swab collection (data not shown).

Many case-patients were presymptomatic (22/71; 31.0%) or asymptomatic (9/71; 12.7%) and 40 (56.3%) case-patients reported symptoms during contact tracing investigation. The most reported symptoms were fever, cough, and sore throat. Only 1 (1.4%) case was critical and 2 (2.8%) were severe.

We calculated the incubation period from 8 case-patients who had confirmed epidemiologic links and had attended the March 5 religious gathering in Brunei. By using March 5 as the exposure date, the median incubation period was 4.5 days (range 1–11 days; IQR 2.75–5.5 days). Based on 35 symptomatic infector–infectee pairs, the serial interval was 4.26 days (SD ±4.27 days; range −4 to 17 days). Among the 35 symptomatic infector–infectee pairs, 4 (11.4%) had negative serial interval values. We noted that the serial interval distribution resembled a normal distribution (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/11/20-2263-App1.pdf).

Transmission Characteristics

Among 1,755 close contacts of the COVID-19 cluster among Tablighi members in Brunei, 52 local transmissions were detected, giving an overall nonprimary attack rate of 2.9% (95% CI 2.2%–3.8%). We excluded case 121 (Figure 2) from the analysis because the case-patient was not detected during contact tracing. The highest attack rates were among spouses (41.9%

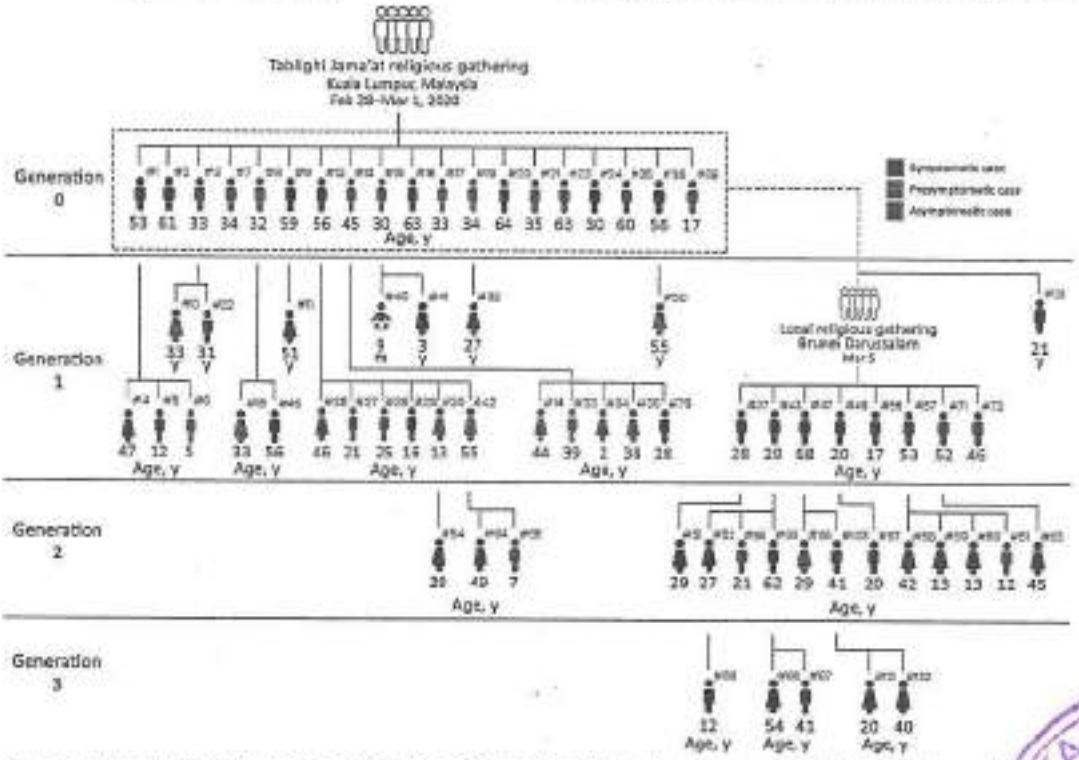

Figure 2. A cluster of coronavirus disease cases in Brunei Darussalam. Epidemiologic links are illustrated by generation and symptomatic status. Generation 0 occurred among attendees of a Tablighi Jama'at gathering in Kuala Lumpur, Malaysia, during February 28–March 1, 2020. Generations 1, 2, and 3 occurred in Brunei. #, case number.

Table 1. Demographic and clinical characteristics of cases in a cluster of coronavirus disease among a Tablighi Jama'at community, Brunei

Characteristics	Overall, n = 71	Primary cases, n = 19	Nonprimary cases, n = 52	p value
Median age, y (IQR)	33.0 (21–50)	35.0 (33–59.5)	29.0 (20–46)	0.009
Range	0.75–68	17–64	0.75–68	
Age group, y				
0–9	4 (5.6)	0 (0.0)	4 (5.6)	0.002
10–19	9 (12.7)	1 (1.4)	8 (11.3)	
20–29	16 (22.5)	1 (1.4)	15 (21.1)	
30–39	14 (19.7)	8 (11.3)	6 (8.5)	
40–49	10 (14.1)	1 (1.4)	9 (12.7)	
50–59	11 (15.5)	3 (4.2)	8 (11.3)	
60–69	7 (9.9)	5 (7.0)	2 (2.8)	
Sex				
F	25 (35.2)	0 (0.0)	25 (48.1)	<0.001
M	46 (64.8)	19 (100)	27 (51.9)	
Underlying conditions				
Obesity	4 (5.6)	2 (10.5)	2 (3.8)	0.289
Heart disease	4 (5.6)	3 (15.8)	1 (1.9)	0.056
Respiratory disease	5 (7.0)	2 (10.5)	3 (5.8)	0.605
Cancer	1 (1.4)	1 (5.3)	0 (0.0)	0.268
Diabetes mellitus	5 (7.0)	3 (15.8)	2 (3.8)	0.115
Symptom status				
Symptomatic	40 (56.3)	8 (42.1)	32 (61.5)	0.265
Median time from symptom onset to diagnosis, d (range; IQR)	4.0 (0–15; 2–6)	3.5 (0–7; 2.75–6)	4.0 (1–15; 2–6.25)	0.746
Presymptomatic	22 (31.0)	7 (36.8)	15 (28.8)	
Median time from symptom to and NP swab collection, d (range; IQR)	0 (–7 to –1; –2.5 to 0)	0 (–1 to 1; 0–1)	0 (–7 to 1; –3 to 0)	0.034
Asymptomatic	9 (12.7)	4 (21.1)	5 (9.5)	
Symptoms ever reported				
Fever	42 (59.2)	9 (47.4)	33 (63.5)	0.343
Cough	42 (59.2)	14 (73.7)	28 (53.8)	0.218
Runny nose	25 (35.2)	7 (36.8)	18 (34.6)	1.000
Sore throat	42 (59.2)	9 (47.4)	33 (63.5)	0.342
Disease severity				
Asymptomatic	9 (12.7)	4 (21.1)	5 (9.6)	0.278
Mild	52 (73.2)	12 (63.2)	40 (76.9)	
Moderate	7 (9.9)	2 (10.5)	5 (9.6)	
Severe or critical	3 (4.2)	1 (5.3)	2 (3.9)	

*Values are no. (%) except as indicated. IQR, interquartile range; NP, nasopharyngeal.

[95% CI 24.1%–60.7%]), attendees of a local religious gathering (14.8% [95% CI 7.1%–27.7%]), and children (14.1% [95% CI 7.8%–23.8%]). The overall household attack rate was 10.6% (95% CI 7.3%–15.1%).

Multiple log-binomial regression analyses revealed that the type of close contact was the only statistically significant variable (p<0.001; Table 2). Compared with social contacts, spouses of positive case-patients had the highest adjusted risk ratio for infection (45.2 [95% CI 16.8–156.1]), their children had a risk ratio of 14.1 (95% CI 4.8–51.5), and attendees of the local religious gathering had a risk ratio of 15.6 (95% CI 4.8–59.9).

Attack rates also differed by symptom status of the infector (Table 3; Appendix Table). In households where the infectors were symptomatic, attack rates were higher (14.4%) than in households in which the infectors were asymptomatic (4.4%) or presymptomatic (6.1%). We could not calculate the attack rate for attendees of the local religious gathering because the 3 primary cases at the event had different symptom statuses and we could not ascertain how transmission occurred. In the household setting, symptomatic case-patients had 2.7 times the risk of transmitting SARS-CoV-2 to their close contacts, compared with asymptomatic and presymptomatic case-patients (crude risk ratio 2.66 [95% CI 1.12–6.34]; Table 3).

The mean observed R was highest (2.67) among attendees of the local religious gathering. Observed R was 0.67 (95% CI 0.44–0.96) for household members (Table 4). The observed R distribution for the household setting was skewed toward 0 (Appendix Figure 2), and 71.4% (20/28 positive contacts) of household infections were from 16.7% (7/42) of possible links to primary cases.

Discussion

We characterized a cluster of COVID-19 cases in Brunei among attendees of the Tablighi Jama'at in Malaysia, an SSE that led to an epidemic in Brunei. Our

Table 2. Risk factors for severe acute respiratory syndrome coronavirus 2 infection among close contacts, Brunei*

Characteristics	Total, n = 1,755	Positive, n = 51	Attack rate, % (95% CI)	Crude risk ratio (95% CI)†	Adjusted risk ratio (95% CI)‡
Sex					
M	913	24	2.6 (1.7–3.9)	Referent	Referent
F	842	27	3.2 (2.2–4.7)	1.22 (0.71–2.11)	1.23 (0.69–2.27)
Age group					
0–9	267	4	1.5 (0.4–3.8)§	Referent	Referent
10–19	163	8	4.9 (2.3–9.8)	3.28 (1.05–12.12)	1.92 (0.53–7.03)
20–29	364	13	3.6 (2.0–6.2)	2.38 (0.85–8.39)	1.91 (0.70–6.51)
30–39	441	6	1.4 (0.6–3.1)	0.91 (0.26–3.53)	0.85 (0.24–3.38)
40–49	255	9	3.5 (1.7–6.8)	2.36 (0.78–8.81)	1.96 (0.63–7.32)
50–59	174	8	4.6 (2.2–9.2)	3.07 (0.96–11.36)	1.84 (0.58–7.05)
≥60	83	3	3.6 (0.8–10.2)§	2.41 (0.48–10.74)	1.00 (0.20–4.51)
Types of close contact					
Social	445	4	0.9 (0.2–2.3)§	Referent	Referent
Relatives	144	5	3.5 (1.3–8.3)	3.86 (1.04, 15.43)	**4.13 (1.10–16.51)**
Local religious gathering	54	8	14.8 (7.1–27.7)	16.48 (5.38–60.13)	**16.60 (4.81–59.87)**
Workplace or school	845	6	0.7 (0.3–1.6)	0.79 (0.23–3.07)	0.79 (0.23–3.10)
Household					
Child	85	12	14.1 (7.8–23.8)	15.71 (5.62–55.16)	**14.09 (4.79–51.54)**
Spouse	31	13	41.9 (24.1–60.7)	46.65 (17.77–158.39)	**45.20 (15.76–156.12)**
Others¶	145	3	2.0 (0.4–5.8)§	2.26 (0.45–10.2)	2.23 (0.44–10.0)

*Bold text indicates statistically significant value.
†Calculated by using simple log-binomial regression.
‡Calculated by using multiple log-binomial regression (sex, p = 0.485; age group, p = 0.339; types of close contact, p<0.001).
§For counts <5, calculated by using binomial 95% CI.
¶Others include siblings, parents, housekeepers, or relatives, such as grandparents and grandchildren.

analysis revealed several key findings. First, SSEs play a major role in SARS-CoV-2 transmission. Second, transmission variability is high across different settings. Third, transmission varies between symptomatic, asymptomatic, and presymptomatic persons. Our findings highlight the potential for silent chains of transmission.

Within this cluster, 38% of all cases were among participants at an SSE: 19 (26.7%) from the Tablighi event in Malaysia and 8 (11.3%) from a local religious gathering. Of note, 19/75 persons from Brunei who attended the Tablighi event in Malaysia tested positive for SARS-CoV-2. Assuming a representative sample, this suggests an attack rate of 25% and implies that >4,000 of the ≈16,000 participants at the event in Malaysia might have been infected. Moreover, we found that the highest overall nonprimary attack rate (14.8%) and mean observed R (2.67) were from a local religious gathering, which were higher than the attack rate (10.6%) and mean observed R (0.67) for the household setting. These observations suggest that mass gatherings facilitate SARS-CoV-2 transmission.

During this investigation, we identified several common characteristics at both the local religious gathering and the event in Malaysia (*10*). First, large numbers of attendees gathered in an enclosed area for a prolonged time. Second, some attendees had a history of recent travel; the Tablighi event in Malaysia drew participants from across the world and ≥3 attendees of the local religious gathering had recently returned from Malaysia. Third, the gatherings included communal sleeping areas, sharing of toilet facilities, and shared dining. We propose that these 3 characteristics are hallmarks for an SSE for

Table 3. Attack rates in different settings stratified by symptom status of the primary case of severe acute respiratory syndrome coronavirus 2, Brunei*

Setting and symptom status	Total, n = 1,701	Positive, n = 43	Attack rate, % (95% CI)	Crude risk ratio (95% CI)†	p value
Household					
Asymptomatic or presymptomatic	111	6	5.4 (1.2–9.6)	Referent	
Symptomatic	153	22	14.4 (8.8–19.9)	**2.66 (1.12–6.34)**	0.027
Nonhousehold‡					
Asymptomatic or presymptomatic	580	9	1.6 (0.5–2.6)	Referent	
Symptomatic	857	6	0.7 (0.1–1.3)	0.45 (0.16–1.26)	0.129
Overall					
Asymptomatic or presymptomatic	691	15	2.2 (1.1–3.3)	Referent	
Symptomatic	1,010	28	2.8 (1.8–3.8)	1.28 (0.69–2.37)	0.439

*Bold text indicates statistically significant value.
† Calculated by using simple log-binomial regression.
‡Includes visits to relatives, workplace, and social settings. The local religious gathering is excluded because 3 primary cases at the event had varying symptom status and we could not ascertain how transmission occurred.

Table 4. Characteristics and mean observed reproductive number for each setting in which infection of severe acute respiratory syndrome coronavirus 2 occurred, Brunei

Setting	No. nonprimary cases	Proportion of links with nonzero infections	Total no. of close contacts	Contacts traced per case	Range of setting size	Mean observed reproductive number (95% CI)
Household	28	0.36	294	9.4	1–13	0.67 (0.44–0.96)
Relatives	5	0.11	144	28.8	1–26	0.26 (0.09–0.61)
Workplace	6	0.20	848	141.3	1–202	0.24 (0.09–0.52)
Social	4	0.16	445	111.3	1–179	0.16 (0.04–0.41)
Local religious gathering	8	1.00	54	6.8	54*	2.67 (NA)*
Overall	51	0.37	1,755	34.4	1–220	0.94 (0.70–1.24)

*Indicates only a single event, so no range for setting size or calculated 95% CI are available. NA, not applicable.

SARS-CoV-2 transmission. Health authorities can use these characteristics as red flags in their risk assessment and mitigation strategies for preventing and detecting high-risk activities, including mass gatherings, and in other institutional settings, such as care homes, prisons, and dormitories.

To a lesser degree, our observations on the within-household transmission are similar to those observed for the 2 religious gatherings. Among 16 household contacts who subsequently became first generation cases, 10 (62.5%) were from just 3 primary cases. However, even within similar settings, we can expect wide variability in transmission patterns. This observation supports our finding of a moderately high household attack rate but an observed R of <1, suggesting that transmission is driven by a relatively small number of cases (*2*). High attack rates in spouses and children reflect intimate relationships with a high degree of interaction, close proximity, and in the case of the spouse, sleeping in the same room. Concordant with our SSE findings, we suggest that encounters among groups that involve close proximity in enclosed settings for prolonged times (≥1 night) are a main driver of SARS-CoV-2 transmission.

Our overall nonprimary attack rate result of 10.6% in the household setting is comparable to other studies that used contract tracing datasets (*16*; H.-Y. Cheng et al. unpub. data, https://www.medrxiv.org/content/10.1101/2020.03.18.20034561v1; L. Luo et al., unpub. data, https://www.medrxiv.org/content/10.1101/2020.03.24.20042606v1; Q. Bi et al. unpub. data, https://www.medrxiv.org/content/10.1101/2020.05.03.20028423v3). A study near Wuhan, China (*17*), reported a higher attack rate of 16.3%, but they detected 56.2% of cases >5 days after persons began having symptoms. By contrast, 77.4% of cases in our study were detected and patients were isolated ≤3 days of symptom onset, suggesting that early case isolation can reduce the attack rate. The Brunei MoH's strategy of aggressive testing of contacts might have contributed to reduced attack rates among household members.

We noted a low nonprimary attack rate (<1%) and mean observed R (<0.3) for workplace and social settings. Moderate physical distancing was implemented in Brunei following the identification of this cluster, but community quarantine and lockdown were not implemented. Public services and businesses remained open and no internal movement restrictions were imposed in the country.

Combined with our observations on the role of SSEs in driving SARS-CoV-2 transmission, we suggest that areas with limited community transmission can avoid full lockdown measures that adopt a blunt approach of restricting all movement. Instead, such areas can use a more targeted approach that combines case isolation, contact tracing, and moderate levels of physical distancing and takes into account the red flags for mass gatherings we identified. However, this approach is resource intensive and only feasible in communities with sufficient public health capacity. The high proportion of asymptomatic persons suggests that even with best efforts at contact tracing, the potential for widespread community transmission is clear. Once SARS-CoV-2 is established in a location, its suppression requires implementation of broader physical distancing measures (*18,19*). Nonetheless, effective contact tracing and case isolation approaches have been shown to control COVID-19 during the early stage of outbreaks (*20*). In addition, modeling studies using data from South Korea showed that less extreme physical distancing measures can help suppress an outbreak (*21*).

We identified several environmental settings and behavioral factors that potentially account for higher attack rates observed in mass gatherings and households. To assess the effect of host factors in driving transmission, we compared the nonprimary attack rate in symptomatic, asymptomatic, and presymptomatic persons, considering the high proportion of asymptomatic (12.7%) and presymptomatic (31.0%) case-patients. Case reports of presumptive asymptomatic and presymptomatic SARS-CoV-2 transmission have been published (*22,23*), but few observational studies quantify such transmissions.

A study from Ningbo, China, analyzed the overall attack rates between symptomatic and asymptomatic COVID-19 case-patients and did not find major differences between the 2 groups (24). Another study reinterpreted the same data and theorized that SARS-CoV-2 could be more transmissible from symptomatic than asymptomatic persons under certain conditions (25). In fact, our overall crude risk ratio for symptomatic case-patients showed no statistically significant difference compared with asymptomatic or presymptomatic case-patients (Table 3; Appendix Table). However, we suggest this finding masks the true picture in transmissibility when different settings are taken into account.

We did not find statistically significant differences in the attack rate for nonhousehold settings, which usually practice some form of nonpharmaceutical interventions (NPI), such as taking medical leave for persons with moderate or severe symptoms. In addition, some physical distancing likely would be practiced by contacts of persons with visible symptoms. However, our findings suggest that transmission occurs more frequently at the household level where such physical distancing and control measures are less practical. We observed that the household attack rate for symptomatic persons (14.4%) is higher than that of asymptomatic (4.1%) or presymptomatic (6.1%) persons, suggesting that presence of symptoms is a host factor in driving transmission.

The higher household attack rate observed among symptomatic case-patients suggests that testing for contacts of symptomatic persons should be prioritized, especially in low resource areas. Nonetheless, the attack rates we observed for asymptomatic (4.4%) and presymptomatic (6.1%) case-patients were not negligible and our findings have several implications for high resource areas with greater testing capacity. First, it strengthens the argument for testing household contacts in the absence of symptoms. Second, some flexibility should be permitted in the surveillance system because the high proportion of asymptomatic case-patients poses challenges for rapid detection and isolation. Thus, we recommend that moderate levels of physical distancing should be implemented even in countries with highly developed testing and tracing capacities. Third, proactive testing of travelers, attendees of red flag events, and persons housed in institutional settings might be necessary to contain COVID-19 spread.

This study has several limitations. First, because we conducted a retrospective study based on a contact tracing dataset, determination of the index case and direction of transmission could be uncertain, particularly because a substantial proportion of case-patients were asymptomatic. Moreover, we did not account for outside sources of infection, so setting-specific attack rates could have been overestimated even though no community transmission has been detected in Brunei. Viral sequencing can confirm homology between the strains infecting index and secondary cases across the various settings but was not conducted for all cases. Second, we have not accounted for other potential environmental factors, such as the relative household size, time spent at home with others, air ventilation, and transmission from fomites. Third, we do not have information on NPIs practiced by close contacts; presumably, persons would take precautions during an outbreak. Fourth, case-patients reported their symptom status during NP swab collection, which we assumed to be reflective of their condition when their close contacts were exposed; however, this might not be true for all cases. Finally, the generalizability of our results is limited because there was no community transmission, the small number of cases, and the lack of cases in communal settings, such as residential care facilities and dormitories.

The main strength of our study is the availability of a complete contact tracing dataset at the national level. Because all case-contacts were tested, we believe our study more accurately describes SARS-CoV-2 transmission than studies in which only symptomatic case-contacts were tested.

In conclusion, our analysis highlights the variability of SARS-CoV-2 transmission across different settings and the particular role of SSEs. We identify red flags for potential SSEs and describe environmental, behavioral, and host factors that drive transmission. Overall, we provide evidence that a combination of case isolation, contact tracing, and moderate physical distancing measures can be an effective approach for SARS-CoV-2 containment.

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L.C. and J.W. conceptualized and designed the study. J.W., S.J., and F.A. acquired data. L.C., W.C.K., and L.N. analyzed data. L.C., W.C.K., L.N., and J.W. interpreted data. L.C., J.W., and W.C.K. provided critical revisions of the manuscript for important intellectual content. All authors contributed to the writing of the first and final draft.

About the Author

Dr. Chaw is a lecturer at the Pengiran Anak Puteri Rashidah Sa'adatul Bolkiah Institute of Health Sciences, Universiti Brunei Darussalam, Bandar Seri Begawan. Her primary research interests include the burden and transmission dynamics of infectious diseases, particularly for acute respiratory diseases.

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Address for correspondence: Liling Chaw, PhD, PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link Gadong BE1410, Brunei Darussalam; email: liling.chaw@ubd.edu.bn

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Transmission of COVID-19 in 282 clusters in Catalonia, Spain: a cohort study

Michael Marks, Pere Millat-Martinez, Dan Ouchi, Chrissy h Roberts, Andrea Alemany, Marc Corbacho-Monné, Maria Ubals, Aurelio Tobias, Cristian Tebé, Ester Ballana, Quique Bassat, Bàrbara Baro, Martí Vall-Mayans, Camila G-Beiras, Nuria Prat, Jordi Ara, Bonaventura Clotet, Oriol Mitjà

Summary

Background Scarce data are available on what variables affect the risk of transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the development of symptomatic COVID-19, and, particularly, the relationship with viral load. We aimed to analyse data from linked index cases of COVID-19 and their contacts to explore factors associated with transmission of SARS-CoV-2.

Methods In this cohort study, patients were recruited as part of a randomised controlled trial done between March 17 and April 28, 2020, that aimed to assess if hydroxychloroquine reduced transmission of SARS-CoV-2. Patients with COVID-19 and their contacts were identified by use of the electronic registry of the Epidemiological Surveillance Emergency Service of Catalonia (Spain). Patients with COVID-19 included in our analysis were aged 18 years or older, not hospitalised, had quantitative PCR results available at baseline, had mild symptom onset within 5 days before enrolment, and had no reported symptoms of SARS-CoV-2 infections in their accommodation or workplace within the 14 days before enrolment. Contacts included were adults with a recent history of exposure and absence of COVID-19-like symptoms within the 7 days preceding enrolment. Viral load of contacts, measured by quantitative PCR from a nasopharyngeal swab, was assessed at enrolment, at day 14, and whenever the participant reported COVID-19-like symptoms. We assessed risk of transmission and developing symptomatic disease and incubation dynamics using regression analysis. We assessed the relationship of viral load and characteristics of cases (age, sex, number of days from reported symptom onset, and presence or absence of fever, cough, dyspnoea, rhinitis, and anosmia) and associations between risk of transmission and characteristics of the index case and contacts.

Findings We identified 314 patients with COVID-19, with 282 (90%) having at least one contact (753 contacts in total), resulting in 282 clusters. 90 (32%) of 282 clusters had at least one transmission event. The secondary attack rate was 17% (125 of 753 contacts), with a variation from 12% when the index case had a viral load lower than 1×10^{6} copies per mL to 24% when the index case had a viral load of 1×10^{10} copies per mL or higher (adjusted odds ratio per $\log_{10}$ increase in viral load 1·3, 95% CI 1·1–1·5). Increased risk of transmission was also associated with household contact (3·0, 1·59–5·65) and age of the contact (per year: 1·02, 1·01–1·04). 449 contacts had a positive PCR result at baseline. 28 (6%) of 449 contacts had symptoms at the first visit. Of 421 contacts who were asymptomatic at the first visit, 181 (43%) developed symptomatic COVID-19, with a variation from approximately 38% in contacts with an initial viral load lower than 1×10^{7} copies per mL to greater than 66% for those with an initial viral load of 1×10^{10} copies per mL or higher (hazard ratio per $\log_{10}$ increase in viral load 1·12, 95% CI 1·05–1·20; p=0·0006). Time to onset of symptomatic disease decreased from a median of 7 days (IQR 5–10) for individuals with an initial viral load lower than 1×10^{7} copies per mL to 6 days (4–8) for those with an initial viral load between 1×10^{7} and 1×10^{9} copies per mL, and 5 days (3–8) for those with an initial viral load higher than 1×10^{9} copies per mL.

Interpretation In our study, the viral load of index cases was a leading driver of SARS-CoV-2 transmission. The risk of symptomatic COVID-19 was strongly associated with the viral load of contacts at baseline and shortened the incubation time of COVID-19 in a dose-dependent manner.

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For the Catalan translation of the abstract see Online for appendix 1

Clinical Research Department, Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK (M Marks PhD, C h Roberts PhD); Hospital for Tropical Diseases, University College London Hospital, London, UK (M Marks); Barcelona Institute for Global Health, Hospital Clinic, University of Barcelona, Barcelona, Spain (P Millat-Martinez MD, Prof Q Bassat PhD, B Baro PhD); IrsiCaixa AIDS Research Institute, Badalona, Spain (D Ouchi MSc, E Ballana PhD, B Clotet PhD); Fight AIDS and Infectious Diseases Foundation, Badalona, Spain (A Alemany BM, M Corbacho-Monné BM, M Vall-Mayans PhD, C G-Beiras PhD, B Clotet, O Mitjà PhD); Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí I3PT, Sabadell, Spain (M Corbacho-Monné); Catalan Institution for Research and Advanced Studies, Barcelona, Spain (Prof Q Bassat); Infectious Diseases Department, Hospital Universitari Germans Trias i Pujol, Badalona, Spain (M Ubals MD, E Ballana, M Vall-Mayans, N Prat MSc, J Ara PhD, B Clotet, O Mitjà); Institute of Environmental Assessment and Water Research, Spanish Council for Scientific Research, Barcelona, Spain (A Tobias PhD); Biostatistics Unit, Institut

Introduction

According to current evidence, COVID-19 is primarily transmitted from person to person through respiratory droplets, as well as indirect contact through transfer of the virus from contaminated fomites to the mouth, nose, or eyes.[1,2] As with most respiratory viral infections, some transmission through smaller aerosols is likely to occur, but their relative contribution compared with droplets remains unclear. Several outbreak investigation reports have shown that COVID-19 transmission can be particularly effective in confined indoor spaces such as workplaces, including factories, churches, restaurants, shopping centres, and health-care settings.[3–6] In Spain and many other countries, health-care workers have had a high rate of COVID-19 infection.[7]

d'Investigació Biomèdica de Bellvitge, L'Hospitalet de Llobregat, Barcelona, Spain (C Tebé PhD); Department of Clinical Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain (C Tebé, O Mitjà); Càtedra de Malalties Infeccioses i Immunitat, Universitat de Vic, Universitat Central de Catalunya, Vic, Spain (B Clotet, O Mitjà); Lihir Medical Centre, International SOS, Lihir Island, Papua New Guinea (O Mitjà)

Correspondence to: Dr Michael Marks, Clinical Research Department, London School of Hygiene & Tropical Medicine, London WC1E 7HT, UK michael.marks@lshtm.ac.uk

Research in context

Evidence before this study

On Sept 20, 2020, we searched PubMed for articles reporting on factors influencing transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the risk of developing symptomatic COVID-19. Search terms included "COVID-19", "SARS-CoV-2", "transmission", "incubation time", and "risk", with no language restrictions. Various authors had reported on retrospective analyses of clusters of index cases and their corresponding contacts, as well as series of patients who developed symptomatic COVID-19 after a positive PCR result. Besides describing the secondary attack rate, these studies identified risk factors for transmission associated with the place and duration of exposure and not using personal protective equipment. A single study suggested that individuals who were symptomatic might be more likely to transmit than those without symptoms, but we found no clear evidence regarding the influence of viral load of the index case on transmission risk. Similarly, although various retrospective series of patients with positive PCR results had reported incubation times elsewhere, the characteristics of index case and contacts that might influence the risk of developing symptomatic COVID-19 and the time to this event had been barely addressed.

Added value of this study

We analysed data from a large cluster-randomised clinical trial on post-exposure therapy for COVID-19 that provided new information on SARS-CoV-2 transmission dynamics. Several design components add value to this dataset. Notably, quantitative PCR was available for the index cases to estimate risk of transmission. Additionally, quantitative PCR was also done on asymptomatic contacts at the time of enrolment allowing us to investigate the dynamics of symptomatic disease onset among them. We found that the viral load of the index case was the leading determinant of the risk of SARS-CoV-2 PCR positivity among contacts. Among contacts who were SARS-CoV-2 PCR positive at baseline, viral load significantly influenced the risk of developing the symptomatic disease in a dose-dependent manner. This influence also became apparent in the incubation time, which shortened with increasing baseline viral loads.

Implications of all the available evidence

Our results provide important insights into the knowledge regarding the risk of SARS-CoV-2 transmission and COVID-19 development. The fact that the transmission risk was primarily driven by the viral load of index cases, more than other factors such as their symptoms or age, suggests that all cases should be considered potential transmitters irrespective of their presentation and encourages the assessment of viral load in patients with a larger number of close contacts. Similarly, our results regarding the risk and expected time to developing symptomatic COVID-19 encourage risk stratification of newly diagnosed SARS-CoV-2 infections on the basis of the initial viral load.

The availability of data regarding the factors that might enhance transmission is essential for designing interventions to control the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Available data provide information on the risk of transmission related to the place and duration of exposure and the use of respiratory and eye protection, but not on other factors related to the characteristics of index cases and their contacts. Over the course of infection, the virus has been identified in respiratory tract specimens 1–2 days before the onset of symptoms, and it can persist for prolonged periods, over several weeks after the onset of symptoms in mild cases. However, the detection of viral RNA by PCR does not necessarily equate with infectivity, and the exact relationship between viral load and risk of transmission from a case is still not clear. Studies investigating case-contact pairs have reported highly variable secondary attack rates (ie, ranging from 0·7% to 75%), depending on the type of exposure—duration, place, pre-symptomatic or post-symptomatic.

Another challenge for public health interventions is the risk stratification of individuals who are infected for developing symptomatic illness. A living systematic review estimated that the proportion of PCR-positive infected contacts that progress to symptomatic disease is approximately 70–80%. Mean or median incubation period has been consistently estimated to be between 5 to 7 days. Although studies have suggested that the viral load of cases might be associated with risk of disease or transmission, no published data so far have directly addressed this question, and little is known about factors that might contribute to variation on the risk of developing COVID-19 symptoms or the incubation periods among individuals who are infected.

The overall aim of this study was to evaluate transmission dynamics of SARS-CoV-2 in the context of a trial of post-exposure prophylaxis. Specifically, the objectives of our study were threefold: to investigate the association between clinical and demographic features of cases and viral load, to evaluate the effect of viral load on SARS-CoV-2 transmission to close contacts, and to determine the influence of viral load in the exposed individuals on development of symptoms and on the incubation period.

Methods

Study design

This study was a post-hoc analysis of data collected in the BCN PEP CoV-2 Study (NCT04304053), a cluster-randomised trial that included individuals with PCR-confirmed COVID-19 and their close contacts. The trial occurred between March 17 and April 28, 2020, during the initial wave of the SARS-CoV-2 outbreak, in three of

nine health-care areas in Catalonia (northeast Spain)—*Catalunya Central*, *Àmbit Metropolità Nord*, and *Barcelona Ciutat*—with a total target population of 4 206 440 people. The study protocol of the BCN PEP CoV-2 Study was approved by the ethics committee of Hospital Germans Trias Pujol (Badalona, Spain). Written informed consent was obtained from all participants. Full details of the original study are reported elsewhere.[8]

COVID-19 cases were identified by use of the electronic registry of the Epidemiological Surveillance Emergency Service of Catalonia of the Department of Health.[10] Following government ordinance, the surveillance service registered all new COVID-19 diagnoses that occurred from March 16, 2020 onwards. The surveillance system included active tracing of all contacts with recent history of exposure, defined as being in contact with a case of SARS-CoV-2 confirmed by PCR within 2 m of distance for more than 15 min.

Study participants

All patients with COVID-19 included in our analysis were adults (aged 18 years or older) who were not hospitalised and had quantitative PCR results available at baseline, mild symptom onset within 5 days before enrolment, and no reported symptoms of SARS-CoV-2 infections in their accommodation (ie, household or nursing home) or workplace within the 14 days before enrolment. Contacts selected for the analysis were adults with a recent history of exposure and absence of COVID-19-like symptoms within the 7 days preceding enrolment. Contacts were exposed to the index case as either a health-care worker, a household contact, a nursing home worker, or a nursing home resident.

We selected all eligible individuals within the original trial population for each of the three analyses done in this study. As in the original trial, we found no evidence of an effect of hydroxychloroquine on either transmission or development of symptomatic disease; therefore, we included individuals from both groups of the trial in this study. First, all COVID-19 cases with quantitative PCR data were included in an analysis of the association between clinical and demographic features of cases and viral load. Second, we identified factors associated with transmission using all clusters of an index case (ie, a COVID-19 case with at least one close contact) and their corresponding contacts for which quantitative viral load was available for the index case. Finally, we assessed the risk of developing symptomatic disease and the variation in the incubation period among all contacts with a positive PCR result at baseline, irrespective of available data of their index case.

Study procedures and data collection

A dedicated outbreak field team visited cases and contacts at home or nursing home on days 1 (enrolment) and 14. At the first clinical assessment on day 1, the team did a baseline assessment, including a questionnaire for symptoms of COVID-19, and collected relevant epidemiological information by use of a structured interview: time of first exposure to the index case, place of contact (hospital, home, or nursing care facility), routine use of a mask by the contact when in close proximity to the index case and sleep location concerning the index case (eg, same room or same house). Symptom surveillance consisted of active monitoring by phone on days 3 and 7, a home visit on day 14, and passive monitoring whenever the participants developed symptoms. Participants who developed symptoms were visited the same day they notified the symptom onset (unscheduled visits) by the field team, who recorded the date of symptom onset, type of symptoms from a prespecified checklist, and symptom severity, graded on a scale of 1 to 4.

All participants underwent serial SARS-CoV-2 PCR test and viral load titration from nasopharyngeal swabs on day 1 and day 14 and on any unscheduled visit when the participant notified the onset of COVID-19 symptoms (appendix 2 p 1). The detection of SARS-CoV-2 virus was done by PCR from nasopharyngeal swabs at SYNLAB Diagnostics (Barcelona, Spain) by use of the TaqMan 2019-nCoV assay kit according to the manufacturer's protocol (Thermo Fischer Scientific, Waltham, MA, USA). Viral load was quantified from nasopharyngeal swabs at IrsiCaixa laboratory (Badalona, Spain) by PCR amplification, on the basis of the 2019 novel coronavirus real-time RT-PCR diagnostic panel guidelines and protocol developed by the US Centers for Disease Control and Prevention.[11] For absolute quantification, a standard curve was built using 1/5 serial dilutions of a SARS-CoV-2 plasmid (2019-nCoV_N_Positive Control, 2×10^5 copies

See Online for appendix 2

	Value
Cluster size	3 (2–3)
Index cases (n=282)	
Age, years	42 (13)
Sex	
Men	80 (28%)
Women	202 (72%)
Log viral load	8 (6–9)
Contacts (n=712)	
Age, years	42 (15)
Sex	
Men	295 (41%)
Women	385 (51%)
Missing	63 (8%)
Baseline positive PCR of contact case	82 (12%)
Form of contact	
Health-care worker	254 (34%)
Household	383 (51%)
Nursing home	11 (3%)
Unknown	96 (13%)

Data are n (%), mean (SD), or median (IQR).

Table 1: Baseline characteristics of linked transmission clusters

	Log_{10} viral load per mL	Unadjusted β coefficient (95% CI)	p value	Adjusted β coefficient (95% CI)	p value
Case age	NA	0·00 (−0·02 to 0·02)	0·78	0·01 (−0·01 to 0·02)	0·18
Case sex					
Men	8·15 (7·54 to 8·77)	Reference	–	Reference	–
Women	8·04 (7·47 to 8·60)	−0·24 (−0·72 to 2·48)	0·33	−0·21 (−0·61 to 0·34)	0·53
Days from symptom onset	NA	−0·17 (−0·26 to −0·08)	0·0002	−0·16 (−0·24 to −0·07)	0·0004
Symptoms					
Cough					
Absent	7·83 (7·24 to 8·43)	Reference	–	Reference	–
Present	8·07 (7·78 to 8·35)	0·46 (0·22 to 1·10)	0·0012	0·41 (−0·02 to 0·84)	0·055
Dyspnoea					
Absent	7·97 (7·50 to 8·43)	Reference	–	Reference	–
Present	8·22 (7·45 to 8·99)	0·17 (−0·40 to 0·94)	0·42	0·28 (−0·25 to 0·81)	0·28
Fever					
Absent	7·77 (7·16 to 8·38)	Reference	–	Reference	–
Present	8·42 (7·86 to 8·98)	0·80 (0·36 to 1·24)	0·0004	0·43 (0·02 to 0·87)	0·054
Anosmia					
Absent	8·32 (7·76 to 8·88)	Reference	–	Reference	–
Present	7·87 (7·25 to 8·49)	−0·57 (−1·00 to −0·09)	0·015	−0·52 (−1·00 to 0·09)	0·019
Rhinitis					
Absent	7·60 (7·23 to 7·98)	Reference	–	Reference	–
Present	8·53 (7·65 to 9·52)	0·88 (−0·05 to 1·81)	0·064	0·77 (−0·11 to 1·66)	0·087

NA=not applicable.

Table 2: Univariable and multivariable linear regression of association between index case variables and log_{10} viral load

per µL, Integrated DNA Technologies, Coralville, IA, USA) and run in parallel to all PCR determinations.

Outcomes and definitions

Transmission was characterised by examining the number of individuals infected and uninfected among close contacts of an index case. We defined transmission events as PCR positivity at any timepoint (ie, days 1, 14, or at any other unscheduled PCR testing when participants referred symptoms) of a contact in the same household or workplace within 14 days after enrolment. We defined secondary attack rate of viral transmission as the ratio of individuals with a positive PCR test among close contacts, according to WHO guidelines.

Development to symptomatic disease was defined as presence of at least one of the following symptoms: fever, cough, difficulty breathing, myalgia, headache, sore throat, new olfactory and taste disorder, or diarrhoea) and a positive SARS-CoV-2 RT-PCR test. The incubation period was defined as time from first exposure to symptom onset, with later confirmation of infection by PCR.[24] The earliest possible exposure with the symptomatic index case was determined for each contact individually.

Statistical analysis

We used log-transformed viral loads that were approximately normally distributed and that also aligned with common reporting norms. The relationship between characteristics of cases and viral load was assessed by use of linear regression considering age (in years), sex, number of days from reported symptom onset, and presence or absence of five key clinical features: fever, cough, dyspnoea, rhinitis, and anosmia. To identify risk factors for transmission, we used logistic regression modelling for the risk of transmission using a random-effect model to allow for within-cluster variation in the risk of transmission. Factors with potential influence on the risk of transmission included characteristics of the potential transmitter (ie, age, sex, viral load, and the presence or absence of respiratory symptoms) and contacts (ie, age, sex, and the type of contact they had with the index case). Finally, the risk of developing symptomatic COVID-19 was assessed by fitting a Cox-regression model considering the age (in years) and sex of the individual, the presence or absence of cardiovascular disease and chronic respiratory disease, and the initial viral load relative to the time to development of symptomatic disease. Data at 14 days after the first study visit were censored, in line with the follow-up done in the original trial. All analyses were done in R, version 4.0.

Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between March 17 and April 28, 2020, we identified 314 patients with COVID-19 in whom the viral load was tested. Of these, 220 (70%) were women, 94 (30%) were men, and the median age was 41 years (IQR 31–52). Of the 314 patients, 282 (90%) had at least one close contact, resulting in 282 corresponding clusters with a total of 753 contacts. Clusters had a median of two contacts (IQR 1–3) and a maximum of 19 contacts. Most index cases of the clusters were women (202 [72%]), with an average age of 42 years (SD 13; table 1).

The first study visit was done a median of 4 days (IQR 3–5) after symptom onset. At the first study visit, the median viral load among patients with COVID-19 was 1×10^8 copies per mL (IQR 1×10^4 to 1×10^{10}). In multivariable linear regression, the viral load among cases was positively associated with the presence of fever and negatively associated with the presence of anosmia (table 2), but no association was observed with age or sex of the COVID-19 case nor with the presence of reported dyspnoea or cough. As anticipated, viral load was negatively associated with the number of days from symptom onset.

For our risk factor analysis on SARS-CoV-2 transmission, we used linked case and contact data of 282 clusters with 753 contacts. At the cluster level,

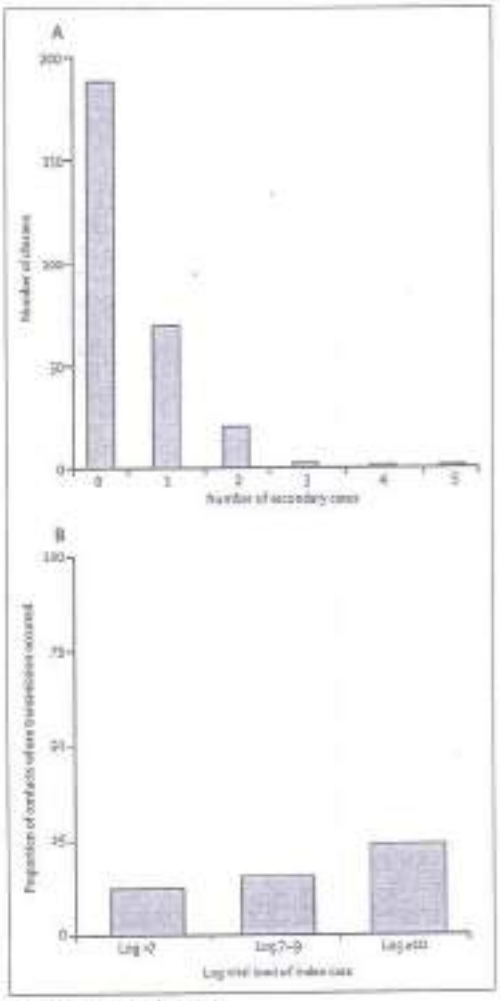

Figure 1: Transmission in a cluster
(A) Number of secondary cases per cluster. (B) Relationship between viral load of the index case and the proportion of contacts developing COVID-19: 16 of 284 contacts in group <1 × 10^7 copies per mL, 73 of 398 in group 1 × 10^7 to <1 × 10^9, and 17 of 71 in group ≥1 × 10^9.

	Unadjusted odds ratio (95% CI)	p value	Adjusted odds ratio (95% CI)	p value
Index case				
Age, per year	1·01 (0·99–1·03)	0·089	1·01 (0·99–1·03)	0·46
Sex				
Men	1 (ref)	NA	1 (ref)	NA
Women	0·74 (0·40–1·36)	0·33	0·71 (0·37–1·33)	0·28
Viral load, per $\log_{10}$ change	1·37 (1·09–1·68)	0·0030	1·29 (1·09–1·50)	0·0013
Cough	1·00 (0·55–1·82)	0·98	1·13 (0·64–2·00)	0·66
Dyspnoea	0·80 (0·31–2·07)	0·64	0·75 (0·30–1·89)	0·55
Rhinitis	1·46 (0·46–4·63)	0·52	1·31 (0·42–4·11)	0·64
Contact				
Age, per year	1·03 (1·01–1·05)	0·0085	1·02 (1·01–1·04)	0·0008
Sex				
Men	1 (ref)	NA	1 (ref)	NA
Women	0·93 (0·58–1·49)	0·77	1·33 (0·79–2·23)	0·28
Mask use by the contact				
Never	1 (ref)	NA	1 (ref)	NA
Always	0·83 (0·47–1·47)	0·84	1·55 (0·76–3·18)	0·23
Unknown	1·18 (0·59–2·36)	0·47	1·43 (0·74–2·80)	0·28
Contact type				
Health-care work	1 (ref)	NA	1 (ref)	NA
Household	3·07 (1·68–5·62)	0·0003	3·00 (1·59–5·65)	0·0008
Nursing home	1·75 (0·39–16·31)	0·62	1·90 (0·39–9·31)	0·43
Other	0·32 (0·03–3·05)	0·31	1·19 (0·10–4·33)	0·89

NA=not applicable. SARS-CoV-1=severe acute respiratory syndrome coronavirus 1.

Table 3: Risk factors for transmission of SARS-CoV-2

90 (32%) of 282 clusters had at least one transmission event, with a highly skewed distribution of the number of transmission events per cluster (figure 1A). The first visit for contacts took place a median of 5 days (IQR 4–7) after their first possible exposure to the index case. 125 (17%) of 753 contacts had a PCR positive result over the study period. The proportion of contacts who tested positive for SARS-CoV-2 within a cluster (secondary attack rate) progressively increased with the viral load of the index case: from 12% when the index case had a viral load lower than 1×10^6 copies per mL to 24% when the index case had a viral load of 1×10^{10} copies per mL or higher (figure 1B). According to the multivariate analysis, the viral load of the index case was strongly associated with the risk of onward transmission (adjusted odds ratio per $\log_{10}$ increase in viral load 1·3, 95% CI 1·1–1·5; table 3). 114 (90%) of 125 transmission events occurred in clusters where the index case had a viral load of 5·1 $\log_{10}$ copies per mL or higher, and 61 (50%) occurred in clusters where the index case had a viral load of 8·8 $\log_{10}$ copies per mL or higher. Other factors associated with an increased risk of transmission were household contact (adjusted OR 3·0, 95% CI 1·59–5·65) and age of the contact (1·02, 1·01–1·04). We observed no association of risk of transmission with reported mask usage by contacts, with the age or sex of the index case, or with the presence of respiratory symptoms in the index case at the initial study visit (table 3).

We did not find any evidence of an association between the viral load of the index cases and the first viral load of incident positive results among contacts (p=0·10, appendix 2 p 2), and this remained true when adjusting for both the day of illness on which the baseline viral load of the index case was measured and the number of days until the contact was enrolled (p=0·18). Additionally, after excluding contacts who were PCR positive at the first study visit, we found no association between the viral load of the index case and

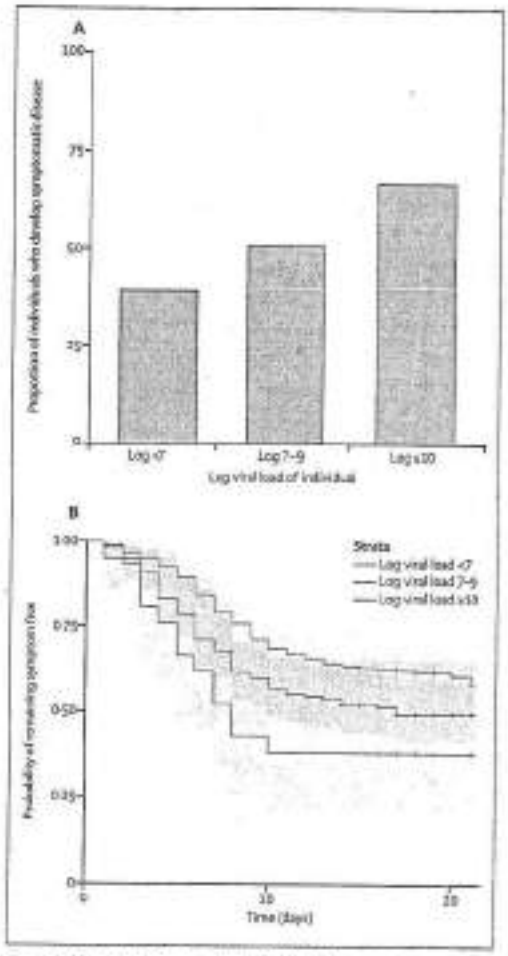

Figure 2: Risk of developing symptomatic COVID-19 according to characteristics of the contact at enrolment
(A) Probability of symptomatic disease by viral load. (B) Time to symptomatic disease by viral load.

the time to onset of incident SARS-CoV-2 infection (hazard ratio [HR] 1·01, 95% CI 0·83–1·23).

Overall, 449 contacts had a positive PCR result at first visit, whether viral load data of their index case was available (n=125) or not (n=324). 28 (6%) of 449 contacts had symptoms at the first visit. Of 421 contacts who were asymptomatic at the first visit, 181 (43%) developed symptomatic COVID-19 within the follow-up period. The multivariable cox-regression analysis, after adjusting for age and sex, showed that increasing viral load levels of the contact at day 1 were associated with an increased risk of developing symptomatic disease. Among contacts not already symptomatic at baseline, the risk of symptomatic disease was approximately 38% among individuals with an initial viral load lower than 1×10^7 copies per mL compared with a risk greater than 66% among those with an initial viral load of 1×10^{10} copies per mL or higher (HR per $\log_{10}$ increase in viral load 1·12, 95% CI 1·05–1·20; p=0·0006; figure 2A). In the multivariable analysis, no association was found between sex or age of individuals, the presence of diabetes, or presence of cardiovascular or respiratory disease and the risk of or time to developing symptomatic COVID-19.

The median time from exposure to symptom onset was 7 days (IQR 5–9). The time to onset of symptomatic disease decreased from a median of 7 days (5–10) for individuals with an initial viral load lower than 1×10^7 copies per mL to 6 days (4–8) for those with an initial viral load between 1×10^7 and 1×10^9 copies per mL, and 5 days (3–8) for those with an initial viral load of 1×10^{10} copies per mL or higher (figure 2B). Overall, 110 (61%) of 181 participants who developed symptoms did so before day 8, 45 (25%) between days 8–10, and 22 (12%) between days 11–14.

Discussion

In our study, we found that increasing viral load values in nasopharyngeal swabs of patients with COVID-19 were associated with the greater risk of transmission, measured by SARS-CoV-2 PCR positivity among contacts, and with a higher risk of transmission in a household environment compared with that in other indoor situations. Additionally, we found that higher viral loads in swabs of asymptomatic contacts were associated with higher risk of developing symptomatic COVID-19, and that these contacts had shorter incubation periods than those with a lower viral load. Relationships between viral load and infectivity have been described for other respiratory viruses, and our study shows that the same is true for SARS-CoV-2.

To our knowledge, this is the largest study that evaluated the relationship of viral load in patients with COVID-19 and risk of transmission. In our cohort, a high proportion (192 [68%] of 282) of index cases did not cause secondary infections. However, we identified 90 (32%) clusters with transmission events, and the multivariate analysis revealed that clusters centred on index cases with high viral load were significantly more likely to result in transmission. In line with previous analyses of case-contact clusters,[3,13,14] we also found that household exposure to an index case was associated with a higher risk of transmission than other types of contact, presumably reflecting duration and proximity of exposure. Increasing age of the contact was also identified in our multivariate analysis as a significant–albeit modest–determinant of transmission risk. This factor has shown uneven influence across results reported elsewhere but seems to play a secondary role among adults.[13,14] Finally, unlike previous analyses that reported a relationship between coughing and transmission,[13] we did not find any association. This finding suggests that

the absence of cough does not preclude significant onward transmission, particularly if the viral load is high. Taken together, our results indicate that the viral load, rather than symptoms, might be the predominant driver of transmission.

Importantly, we report that high viral load shortly after exposure in asymptomatic contacts was strongly associated with the risk of developing symptomatic COVID-19 disease. We found an approximately 40% risk of developing symptomatic disease among individuals with an initial viral load lower than 1×10^7 copies per mL, compared with a risk higher than 66% among individuals with a viral load of 1×11^{10} copies per mL or higher. These data might provide rationale for risk stratification for developing illness. Moreover, the initial viral load significantly shifted the incubation time, which ranged from 5 days in participants with a high viral load to 7 days in participants with a low viral load. To our knowledge, our study was the first analysis of prospective data that investigated the association between initial viral load and incubation time.

The study has several limitations. First, asymptomatic people were not enrolled as index cases, affecting our ability to fully characterise all types of transmission chain. Second, we did not find any evidence of decreased risk of transmission in individuals who reported mask use. Although this finding collides with the evidence reported elsewhere,[8] we did not have fine-grained data on type of mask (surgical vs FFP2) or use of other measures of personal protective equipment (PPE) or other infection control practices, thus limiting our ability to make clear inferences about the effect of PPE on transmission risk. Mask use is probably correlated with type of exposure, which might further confound associations, but we did not note any association between mask use and risk either in our unadjusted analysis (table 3) or in a multivariable model excluding type of exposure (data not shown). Third, we used time to symptom onset (with later confirmation of infection) rather than time to positive PCR test based on serial testing. Nonetheless, accurate calculation of the incubation period was feasible because of the prospective nature of the study, accurate identification of exposure by face-to-face interview, and intensive active and passive monitoring of exposed contacts. We followed up participants over 14-day periods, thus incubation periods longer than 14 days might not have been detected. Within each cluster, we cannot be completely certain about the directionality of transmission, but our inclusion criteria including the absence of COVID-19-like symptoms in the 2 weeks preceding enrolment is consistent with transmission from a case to a contact. We also cannot exclude that some individuals might have been infected by individuals outside of study clusters but, as per national guidelines, all contacts were quarantined after exposure to index cases, reducing the chance of transmission from elsewhere. Samples were available from index cases a median of 4 days after symptom onset, and the initial sample in contacts was taken on average 5 days after exposure, which might limit our ability to detect associations with peak viral load. Despite this, we still showed clear dose effects in relation to both risk of transmission and time to symptom onset. Finally, our study population is reflective of the trial from which the study sample was drawn and is therefore, biased towards female participants and participants with few comorbidities and predominantly mild to moderate infection; additional data are needed on the risk of transmission in other populations.

In summary, our results provide evidence regarding the determinants of SARS-CoV-2 transmission, particularly on the role of the viral load. The higher risk of transmission among individuals with higher viral loads adds to existing evidence and encourages the assessment of the viral load in patients with a large number of close contacts. When a patient with high viral load is identified, the implementation of reinforced contact tracing measures and quarantines might be crucial to reduce onward transmission. Similarly, our results regarding the risk and expected time to developing symptomatic COVID-19 encourage risk stratification of newly diagnosed SARS-CoV-2 infections on the basis of initial viral load.

Contributors

MM, DO, and OM accessed and verified the data. MM, DO, CHR, and OM conceived of the study. MM did the analysis. PM-M, AA, MC-M, MU, MV-M, CG-B, NP, JA, BC, and OM led the randomised controlled trial from which study data are derived. MM and OM wrote the first draft of the manuscript. All authors gave critical input into interpretation and revised the manuscript.

Declaration of interests

CT reports personal fees from Roche/ingelheim and Amgen, outside the submitted work. All other authors declare no competing interests.

Data sharing

A complete de-identified patient dataset, accompanied by the original trial protocol, will be made available to researchers on request. Individuals wishing to access the data should send a request to mcorbacho@fundeng.org. The dataset will be available between Dec 15, 2020, and Dec 15, 2021.

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For YoMeCorono see https://www.yomecorono.com/

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EXHIBIT

JAMA Network | Open

Original Investigation | Infectious Diseases

SARS-CoV-2 Transmission From People Without COVID-19 Symptoms

Michael A. Johansson, PhD; Talia M. Quandelacy, PhD, MPH; Sarah Kada, PhD; Pragati Venkata Prasad, MPH; Molly Steele, PhD, MPH; John T. Brooks, MD; Rachel B. Slayton, PhD, MPH; Matthew Biggerstaff, ScD, MPH; Jay C. Butler, MD

Abstract

IMPORTANCE Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiology of coronavirus disease 2019 (COVID-19), is readily transmitted person to person. Optimal control of COVID-19 depends on directing resources and health messaging to mitigation efforts that are most likely to prevent transmission, but the relative importance of such measures has been disputed.

OBJECTIVE To assess the proportion of SARS-CoV-2 transmissions in the community that likely occur from persons without symptoms.

DESIGN, SETTING, AND PARTICIPANTS This decision analytical model assessed the relative amount of transmission from presymptomatic, never symptomatic, and symptomatic individuals across a range of scenarios in which the proportion of transmission from people who never develop symptoms (ie, remain asymptomatic) and the infectious period were varied according to published best estimates. For all estimates, data from a meta-analysis was used to set the incubation period at a median of 5 days. The infectious period duration was maintained at 10 days, and peak infectiousness was varied between 3 and 7 days (−2 and +2 days relative to the median incubation period). The overall proportion of SARS-CoV-2 was varied between 0% and 70% to assess a wide range of possible proportions.

MAIN OUTCOMES AND MEASURES Level of transmission of SARS-CoV-2 from presymptomatic, never symptomatic, and symptomatic individuals.

RESULTS The baseline assumptions for the model were that peak infectiousness occurred at the median of symptom onset and that 30% of individuals with infection never develop symptoms and are 75% as infectious as those who do develop symptoms. Combined, these baseline assumptions imply that persons with infection who never develop symptoms may account for approximately 24% of all transmission. In this base case, 59% of all transmission came from asymptomatic transmission, comprising 35% from presymptomatic individuals and 24% from individuals who never develop symptoms. Under a broad range of values for each of these assumptions, at least 50% of new SARS-CoV-2 infections was estimated to have originated from exposure to individuals with infection but without symptoms.

CONCLUSIONS AND RELEVANCE In this decision analytical model of multiple scenarios of proportions of asymptomatic individuals with COVID-19 and infectious periods, transmission from asymptomatic individuals was estimated to account for more than half of all transmissions. In addition to identification and isolation of persons with symptomatic COVID-19, effective control of spread will require reducing the risk of transmission from people with infection who do not have symptoms. These findings suggest that measures such as wearing masks, hand hygiene, social distancing, and strategic testing of people who are not ill will be foundational to slowing the spread

Key Points

Question What proportion of coronavirus disease 2019 (COVID-19) spread is associated with transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from persons with no symptoms?

Findings In this decision analytical model assessing multiple scenarios for the infectious period and the proportion of transmission from individuals who never have COVID-19 symptoms, transmission from asymptomatic individuals was estimated to account for more than half of all transmission.

Meaning The findings of this study suggest that the identification and isolation of persons with symptomatic COVID-19 alone will not control the ongoing spread of SARS-CoV-2.

+ Multimedia

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

Abstract (continued)

of COVID-19 until safe and effective vaccines are available and widely used.

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Introduction

As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the novel coronavirus that causes coronavirus disease 2019 (COVID-19), began to spread globally, it became apparent that the virus, unlike the closely related SARS-CoV in the 2003 outbreak, could not be contained by symptom-based screening alone. Asymptomatic and clinically mild infections were uncommon during the 2003 SARS-CoV outbreak, and there were no reported instances of transmission from persons before the onset of symptoms.[1] SARS-CoV-2 spread faster than SARS-CoV, and accumulating evidence showed that SARS-CoV-2, unlike SARS-CoV, is transmitted from persons without symptoms. However, measures to reduce transmission from individuals who do not have COVID-19 symptoms have become controversial and politicized and have likely had negative effects on the economy and many societal activities. Optimal control of COVID-19 depends on directing resources and health messaging to mitigation efforts that are most likely to prevent transmission. The relative importance of mitigation measures that prevent transmission from persons without symptoms has been disputed. Determining the proportion of SARS-CoV-2 transmission that occurs from persons without symptoms is foundational to prioritizing control practices and policies.

Transmission by persons who are infected but do not have any symptoms can arise from 2 different infection states: presymptomatic individuals (who are infectious before developing symptoms) and individuals who never experience symptoms (asymptomatic infections, which we will refer to as never symptomatic). Early modeling studies of COVID-19 case data found that the generation interval of SARS-CoV-2 was shorter than the serial interval, indicating that the average time between 1 person being infected and that person infecting someone else was shorter than the average time between 1 person developing symptoms and the person they infected developing symptoms.[2-5] This finding meant that the epidemic was growing faster than would be expected if transmission were limited to the period of illness during which individuals were symptomatic. By the time a second generation of individuals was developing symptoms, a third generation was already being infected. Epidemiological data from early in the pandemic also suggested the possibility of presymptomatic transmission,[6,7] and laboratory studies confirmed that levels of viral RNA in respiratory secretions were already high at the time of symptom onset.[8-10]

Asymptomatic SARS-CoV-2 transmission also occurs because of individuals with infection who are never symptomatic (or who experience very mild or almost unrecognizable symptoms). The proportion of individuals with infection who never have apparent symptoms is difficult to quantify because it requires intensive prospective clinical sampling and symptom screening from a representative sample of individuals with and without infection. Nonetheless, evidence from household contact studies indicates that asymptomatic or very mild symptomatic infections occur,[11-14] and laboratory and epidemiological evidence suggests that individuals who never develop symptoms may be as likely as individuals with symptoms to transmit SARS-CoV-2 to others.[9,15,16]

Methods

The Centers for Disease Control and Prevention determined that this decision analytical study, which involved no enrollment of human subjects, did not require institutional review board approval. We used a simple model to assess the proportion of transmission from presymptomatic (ie, infectious before symptom onset), never symptomatic, and symptomatic individuals across a range of

scenarios in which we varied the timing of the infectious period to assess different contributions of presymptomatic transmission and the proportion of transmission from individuals who never develop symptoms (ie, remain asymptomatic).

For all estimates we used data from a meta-analysis of 8 studies from China to set the incubation period at a median of 5 days with 95% of symptomatic individuals developing symptoms by day 12.[17] Therefore the daily (t) probability of symptom onset (p_{so}) for individuals who develop symptoms was:

$$p_{so}(t) = F_{Log\text{-}Normal}(t; \text{logmean} = 1.63, \text{logsd} = 0.5).$$

To approximate a distribution of the infectious period, we made a baseline assumption that peak infectiousness occurs on average at the same time as the median incubation period, such that infectiousness begins prior to symptom onset (Table).[9,10,14-16,18,20] We then assumed that infectiousness (I) over time can be approximated by a γ density function and that the average person is infectious for as long as approximately 10 days (ie, 98% of transmission happens within a 10-day period)[11]:

$$I(t) = f_{\gamma}(t, \text{mode} = 5, \text{interval} = 10).$$

For all estimates, we maintained the infectious period duration as 10 days, but varied the mode between 3 and 7 days (−2 and +2 days relative to the median incubation period).

Uncertainty also remains about the proportion of individuals with infection who are never symptomatic (p_{ns}) and the relative contribution of these infections to transmission (r_{ns}). Estimates of p_{ns} range from single digits to more than 50%, many with potential biases related to the study population (eg, age, prevalence of comorbidities) and the extent of long-term follow-up[12-14,19,20] (Table). We made a baseline assumption that 30% of individuals with infection are never symptomatic and then assessed higher or lower assumptions. We also made a baseline assumption that individuals with asymptomatic infections are on average 75% as infectious as those with symptomatic infections.[9,15,16] Combined, these baseline assumptions imply that persons with infection who never develop symptoms may account for approximately 24% of all transmission (T):

$$T_{ns} = p_{ns} \times r_{ns} / (p_{ns} \times r_{ns} + [1 - p_{ns}]).$$

Table. Key Assumptions and Evidence Informing Those Assumptions

Source	Evidence base	Estimate or assumption
Assumptions for presymptomatic transmission		
Peak infectiousness relative to onset, d		
Cuiey et al, 2020[17]	Range, 17 studies	−3 to 1.2 d
Assumed baseline	NA	0 d
Assumed range	NA	−2 to 2 d
Assumptions for never symptomatic transmission		
Proportion never symptomatic		
Oran et al, 2020[12]	Inferred range, 18 studies	30% to 45%
Buitrago-Garcia et al, 2020[19]	Meta-estimate, 7 studies	20% to 37%
Davies et al, 2020[20]	Age-dependent estimate, 6 studies	30% to 70%
Assumed baseline	NA	30%
Relative infectiousness of individuals who never have symptoms		
Lee et al, 2020[9]	303 patients, assessment of viral shedding	Approximately 100%
Chaw et al, 2020[15]	1701 secondary contacts	40% to 140%
Mc Evoy et al, 2020[14]	Inferred range, 8 studies	40% to 70%
Assumed baseline		75%
Overall proportion of individuals who never have symptoms transmission		
Assumed range	NA	0% to 70%

Abbreviation: NA, not applicable.

We varied this overall proportion, T_{no}, between 0% and 70% to assess a wide range of possible proportions. The daily proportion of transmission from individuals after symptom onset (T_s) was therefore:

$$T_s(t) = (1 - T_{no}) \times p_{ss}(t) \times I(t),$$

and the daily proportion of transmission from presymptomatic (T_{ps}) individuals, ie, those who develop symptoms but become infectious prior to symptom onset, is:

$$T_{ps}(t) = 1 - T_s(t) - T_{no}.$$

We modified baseline assumptions to consider the relative importance of different levels of never symptomatic and presymptomatic transmission. Code is available in the eAppendix in the Supplement.

Statistical Analysis

All analyses were conducted in R version 4.0.3 (R Project for Statistical Computing). No statistical testing was conducted, so no prespecified level of significance was set.

Results

Under baseline assumptions, approximately 59% of all transmission came from asymptomatic transmission: 35% from presymptomatic individuals and 24% from individuals who are never symptomatic (**Figure 1**). Because each component is uncertain, we assessed different timings of peak infectiousness relative to illness onset and different proportions of transmission from individuals who never have symptoms. Maintaining the 24% of transmission from individuals who never have symptoms, but shifting peak infectiousness 1 day earlier (to day 4) increased presymptomatic transmission to 43% and all asymptomatic transmission to 67% (Figure 1A). A later peak (ie, day 6) decreased presymptomatic to 27% and all asymptomatic transmission to 51% (Figure 1C).

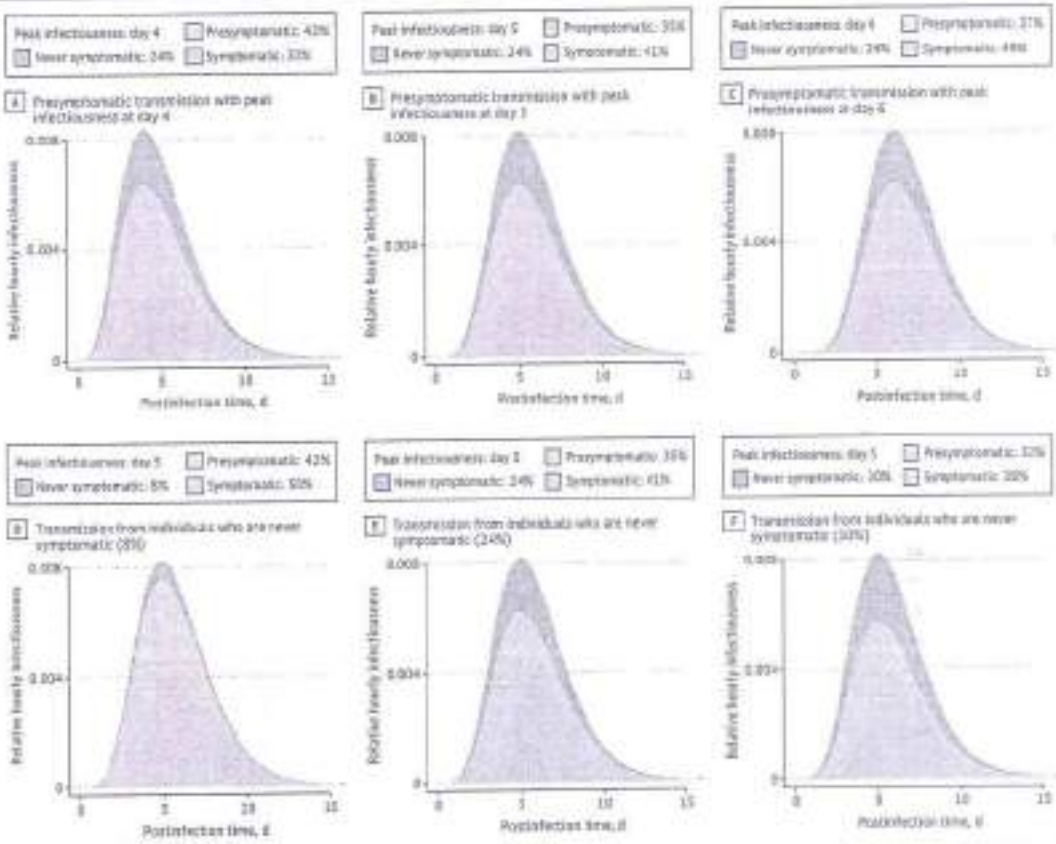
Holding the day of peak infectiousness constant at day 5 and decreasing the proportion of transmission from individuals who are never symptomatic to 10% with a relative infectiousness of 75% (baseline assumption), the proportion of all transmission from those who are never symptomatic decreased to 8%, presymptomatic transmission increased to 42%, and combined asymptomatic transmission was 50% of all transmission (Figure 1D). In contrast, if the proportion of those who ever develop symptoms was 30% and their relative infectiousness increased to 100%, they contributed 30% of all transmission, presymptomatic transmission was 32%, and combined asymptomatic transmission was 62% of all transmission (Figure 1F).

Uncertainty remains regarding the magnitude of both presymptomatic and never symptomatic transmission. Therefore, we analyzed a wider range of each of these components, with peak infectiousness varying between 2 days before (more presymptomatic transmission) to 2 days after (less presymptomatic transmission) median symptom onset and with never symptomatic transmission ranging from 0% to 70% (**Figure 2**). Under this broader range of scenarios, most combined assumptions of peak infectiousness timing and transmission from individuals who never have symptoms indicated that at least 50% of new SARS-CoV-2 infections likely originated from individuals without symptoms at the time of transmission. If more than 30% of transmission was from individuals who never have symptoms, total asymptomatic transmission was higher than 50% with any value of peak infectiousness, up to 2 days after the median time of symptom onset. If peak infectiousness was at any point approximately 6 hours before median symptom onset time, more than 50% of transmission was from individuals without symptoms, regardless of the proportion from those who never have symptoms. Even a very conservative assumption of peak infectiousness 2 days post–median onset and 0% never symptomatic transmission still resulted in more than 25% of transmission from asymptomatic individuals.

Discussion

The findings presented here complement an earlier assessment[31] and reinforce the importance of asymptomatic transmission: across a range of plausible scenarios, at least 50% of transmission was estimated to have occurred from persons without symptoms. This overall proportion of transmission from presymptomatic and never symptomatic individuals is key to identifying mitigation measures that may be able to control SARS-CoV-2. For example, if the reproduction number (R) in a given setting is 2.0, then at least a 50% reduction in transmission is needed to drive the reproductive number below 1.0. Given that in some settings R is likely much greater than 2 and more than half of transmissions may come from individuals who are asymptomatic at the time of transmission, effective control must mitigate transmission risk from people without symptoms.

Figure 1. The Contribution of Asymptomatic Transmission Under Different Infection Profiles



The top curve in each panel represents the average relative hourly infectiousness, such that while the lower curves change under different assumptions, the total hourly infectiousness equals 1 in all cases. Within each curve, the colored area indicates the proportion of transmission from each class of individuals. The portion attributed to individuals with symptoms (light blue) can also be interpreted as the maximum proportion of transmission that can be controlled by immediate isolation of all symptomatic cases. Panels A, B, and C show different levels of presymptomatic transmission. We calibrated infectiousness to peak at day 4 (A), 5 (B), median incubation period), or 6 (C) days. Panels D, E, and F show different proportions of transmission from individuals who are never symptomatic: 5% (D; eg, 10% never symptomatic and 75% relative infectivity), 24% (E; baseline, 30% never symptomatic and 75% relative infectivity), and 30% (F; eg, 30% never symptomatic and 100% relative infectivity).

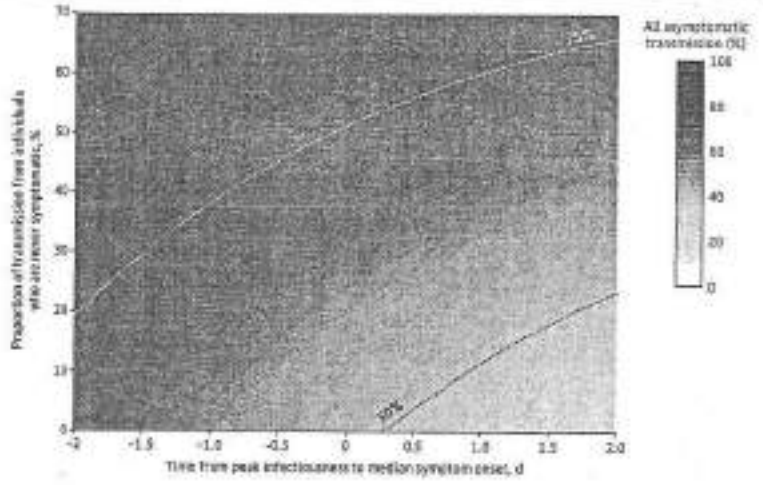
Limitations

This study has limitations. First, we used a simplistic model to represent a complex phenomenon, ie, the average infectiousness of SARS-CoV-2 infections over time. We used this model deliberately to test assumptions about the timing of peak infectiousness and transmission among asymptomatic individuals so that we could vary only these 2 critical parameters and assess their relative effects. Therefore, these results lack quantitative precision, but they demonstrate the qualitative roles of these 2 components and show that across broad ranges of possible assumptions, the finding that asymptomatic transmission is a critical component of SARS-CoV-2 transmission dynamics remains constant.

As discussed here, the exact proportions of presymptomatic and never symptomatic transmission are not known. This also applies to the incubation period estimates, which are based on individual exposure and onset windows that are difficult to observe with precision and therefore include substantial uncertainty even when leveraging estimates across multiple studies. Moreover, they likely vary substantially in different populations. For example, older individuals are more likely than younger persons to experience symptoms,[20] so in populations of older individuals, never asymptomatic transmission may be less important. However, specific age groups are rarely exclusively isolated from other age groups, so asymptomatic transmission risk is still important in those groups and even more so in younger age groups, in which transmission may be even more dominated by asymptomatic transmission.[20]

Real-world transmission dynamics are also not entirely dependent on the individual-level dynamics of infectiousness over time. Now that COVID-19 is widely recognized, individuals with COVID-19 symptoms are more likely to isolate themselves and further reduce the proportion of transmission from symptomatic individuals, shifting a greater proportion of transmission to those who do not have symptoms. In this sense, the estimates here represent the lower end of the proportion of asymptomatic transmission in the presence of interventions to reduce symptomatic transmission.

Figure 2. Combined Transmission From Individuals Who Are Presymptomatic and Those Who Never Have Symptoms



Colors indicate the proportion of transmission due to all individuals without symptoms at the time of transmission, including presymptomatic transmission (x-axis, the timing of peak infectiousness relative to symptom onset) and transmission from individuals who are never symptomatic (y-axis). For example, peak infectiousness at the same time as median symptom onset (0 days difference) with 10% of transmission from individuals who never have symptoms would mean that approximately 51% of transmission is from asymptomatic individuals.

Conclusions

Under a range of assumptions of presymptomatic transmission and transmission from individuals with infection who never develop symptoms, the model presented here estimated that more than half of transmission comes from asymptomatic individuals. In the absence of effective and widespread use of therapeutics or vaccines that can shorten or eliminate infectivity, successful control of SARS-CoV-2 cannot rely solely on identifying and isolating symptomatic cases; even if implemented effectively, this strategy would be insufficient. These findings suggest that effective control also requires reducing the risk of transmission from people with infection who do not have symptoms. Measures such as mask wearing and social distancing empower individuals to protect themselves and, if infected, to reduce risk to their communities.[21] These measures can also be supplemented by strategic testing of people who are not ill, such as those who have exposures to known cases (eg, contact tracing) or are at high risk of exposing others (eg, congregate facility staff, those with frequent contact with the public). Multiple measures that effectively address transmission risk in the absence of symptoms are imperative to control SARS-CoV-2.

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Corresponding Author: Jay C. Butler, MD, Office of the Deputy Director for Infectious Diseases, US Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop H24-12, Atlanta, GA 30329 (jcb3@cdc.gov).

Author Affiliations: COVID-19 Response, US Centers for Disease Control and Prevention, Atlanta, Georgia (Johansson, Quandelacy, Kada, Prasad, Steele, Brooks, Slayton, Biggerstaff); Office of the Deputy Directory for Infectious Diseases, US Centers for Disease Control and Prevention, Atlanta, Georgia (Johansson, Slayton, Biggerstaff, Butler).

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Acquisition, analysis, or interpretation of data: All authors.

Drafting of the manuscript: Johansson, Quandelacy, Brooks, Biggerstaff, Butler.

Critical revision of the manuscript for important intellectual content: Johansson, Kada, Prasad, Steele, Brooks, Slayton, Biggerstaff, Butler.

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SUPPLEMENT.
eAppendix. Code for Analysis

BRIEF COMMUNICATION
https://doi.org/10.1038/s41591-020-0869-5
nature medicine 283

Temporal dynamics in viral shedding and transmissibility of COVID-19

Xi He[1,3], Eric H. Y. Lau[2,3]✉, Peng Wu[2], Xilong Deng[1], Jian Wang[1], Xinxin Hao[2], Yiu Chung Lau[2], Jessica Y. Wong[2], Yujuan Guan[1], Xinghua Tan[1], Xiaoneng Mo[1], Yanqing Chen[1], Baolin Liao[1], Weilie Chen[1], Fengyu Hu[1], Qing Zhang[1], Mingqiu Zhong[1], Yanrong Wu[1], Lingzhai Zhao[1], Fuchun Zhang[1], Benjamin J. Cowling[2,4], Fang Li[1,4] and Gabriel M. Leung[2,4]

We report temporal patterns of viral shedding in 94 patients with laboratory-confirmed COVID-19 and modeled COVID-19 infectiousness profiles from a separate sample of 77 infector–infectee transmission pairs. We observed the highest viral load in throat swabs at the time of symptom onset, and inferred that infectiousness peaked on or before symptom onset. We estimated that 44% (95% confidence interval, 30–57%) of secondary cases were infected during the index cases' presymptomatic stage, in settings with substantial household clustering, active case finding and quarantine outside the home. Disease control measures should be adjusted to account for probable substantial presymptomatic transmission.

SARS-CoV-2, the causative agent of COVID-19, spreads efficiently, with a basic reproductive number of 2.2 to 2.5 determined in Wuhan[1,2]. The effectiveness of control measures depends on several key epidemiological parameters (Fig. 1a), including the serial interval (duration between symptom onsets of successive cases in a transmission chain) and the incubation period (time between infection and onset of symptoms). Variation between individuals and transmission chains is summarized by the incubation period distribution and the serial interval distribution, respectively. If the observed mean serial interval is shorter than the observed mean incubation period, this indicates that a significant portion of transmission may have occurred before infected persons have developed symptoms. Significant presymptomatic transmission would probably reduce the effectiveness of control measures that are initiated by symptom onset, such as isolation, contact tracing and enhanced hygiene or use of face masks for symptomatic persons.

SARS (severe acute respiratory syndrome) was notable, because infectiousness increased around 7–10 days after symptom onset[3,4]. Onward transmission can be substantially reduced by containment measures such as isolation and quarantine (Fig. 1a)[5]. In contrast, influenza is characterized by increased infectiousness shortly around or even before symptom onset[6].

In this study, we compared clinical data on virus shedding with separate epidemiologic data on incubation periods and serial intervals between cases in transmission chains, to draw inferences on infectiousness profiles.

Among 94 patients with laboratory-confirmed COVID-19 admitted to Guangzhou Eighth People's Hospital, 47/94 (50%) were male, the median age was 47 years and 61/93 (66%) were moderately ill (with fever and/or respiratory symptoms and radiographic evidence of pneumonia), but none were classified as 'severe' or 'critical' on hospital admission (Supplementary Table 1).

A total of 414 throat swabs were collected from these 94 patients, from symptom onset up to 32 days after onset. We detected high viral loads soon after symptom onset, which then gradually decreased towards the detection limit at about day 21. There was no obvious difference in viral loads across sex, age groups and disease severity (Fig. 2).

Separately, based on 77 transmission pairs obtained from publicly available sources within and outside mainland China (Fig. 1b and Supplementary Table 2), the serial interval was estimated to have a mean of 5.8 days (95% confidence interval (CI), 4.8–6.8 days) and a median of 5.2 days (95% CI, 4.1–6.4 days) based on a fitted gamma distribution, with 7.6% negative serial intervals (Fig. 1c). Assuming an incubation period distribution of mean 5.2 days from a separate study of early COVID-19 cases[7], we inferred that infectiousness started from 12.3 days (95% CI, 5.9–17.0 days) before symptom onset and peaked at symptom onset (95% CI, −0.9–0.9 days) (Fig. 1c). We further observed that only <0.1% of transmission would occur before 7 days, 1% of transmission would occur before 5 days and 9% of transmission would occur before 3 days prior to symptom onset. The estimated proportion of presymptomatic transmission (area under the curve) was 44% (95% CI, 30–57%). Infectiousness was estimated to decline quickly within 7 days. Viral load data were not used in the estimation but showed a similar monotonic decreasing pattern.

In sensitivity analysis, using the same estimating procedure but holding constant the start of infectiousness from 3, 8 and 11 days before symptom onset, infectiousness was shown to peak at 2 days before to 1 day after symptom onset, and the proportion of presymptomatic transmission ranged from 37% to 48% (Extended Data Fig. 1).

Finally, simulation showed that the proportion of short serial intervals (for example, <2 days) would be larger if infectiousness were assumed to start before symptom onset (Extended Data Fig. 2). Given the 7.6% negative serial intervals estimated from the infector–infectee paired data, start of infectiousness at least 2 days before onset and peak infectiousness at 2 days before to 1 day after onset would be most consistent with this observed proportion (Extended Data Fig. 3).

Here, we used detailed information on the timing of symptom onsets in transmission pairs to infer the infectiousness profile of

[1]Guangzhou Eighth People's Hospital, Guangzhou Medical University, Guangzhou, China. [2]World Health Organization Collaborating Centre for Infectious Disease Epidemiology and Control, School of Public Health, University of Hong Kong, Hong Kong, SAR, China. [3]These authors contributed equally: Xi He, Eric H. Y. Lau. [4]These authors jointly supervised this work: Benjamin J. Cowling, Fang Li, Gabriel M. Leung. ✉e-mail: ehylau@hku.hk

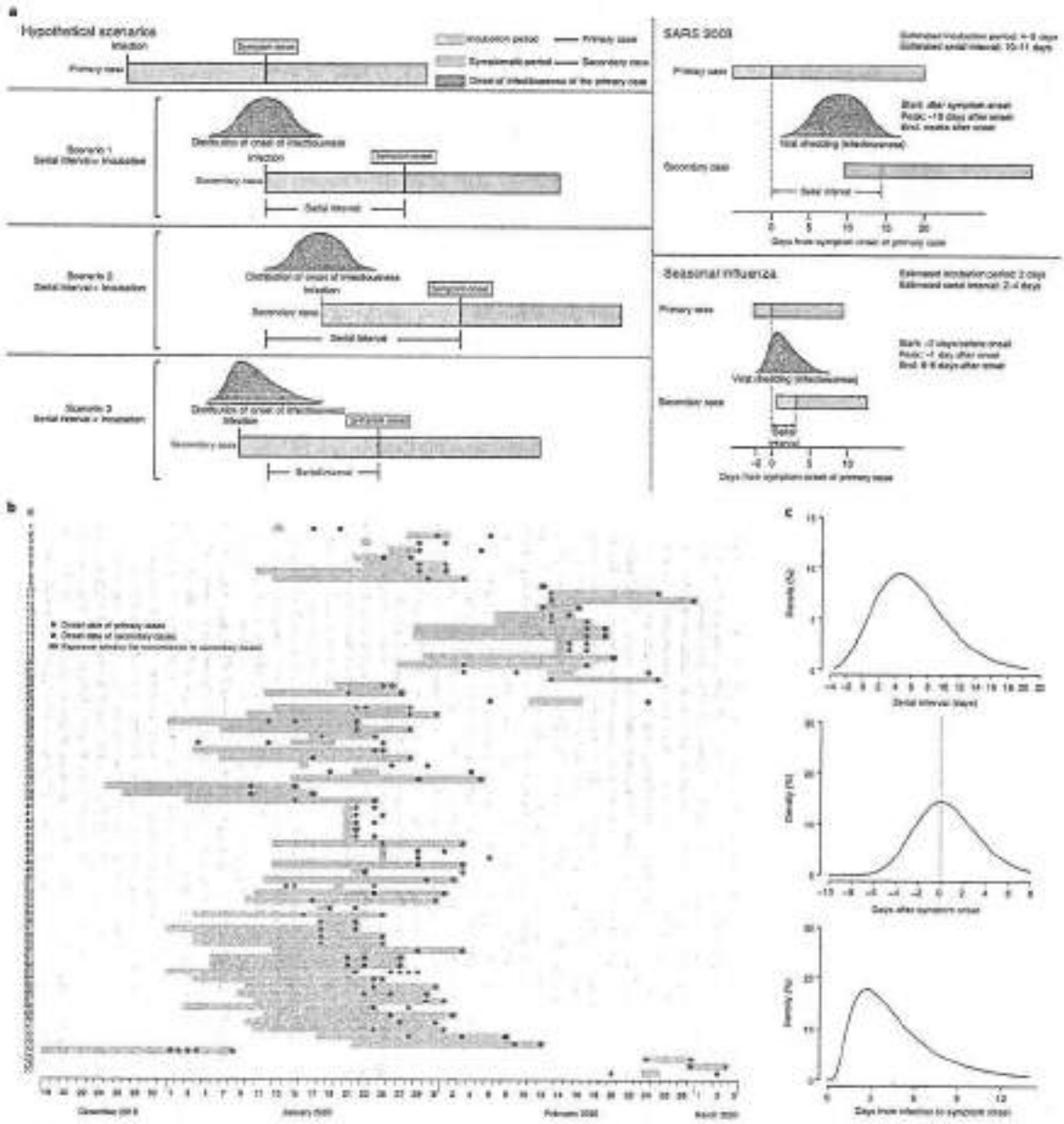

Fig. 1| Transmission of infectious diseases. a, Schematic of the relation between different time periods in the transmission of infectious disease. **b**, Human-to-human transmission pairs of SAR-CoV-2 virus ($N=77$). We assumed a maximum exposure window of 21 days prior to symptom onset of the secondary cases. Detailed information on the transmission pairs and the source of information is summarized in Supplementary Tables 2 and 3. **c**, Estimated serial interval distribution (top), inferred infectiousness profile (middle) and assumed incubation period (bottom) of COVID-19.

COVID-19. We showed substantial transmission potential before symptom onset. Of note, most cases were isolated after symptom onset, preventing some post-symptomatic transmission. Even higher proportions of presymptomatic transmission of 48% and 62% have been estimated for Singapore and Tianjin, where active case finding was implemented[7]. Places with active case finding would tend to have a higher proportion of presymptomatic transmission, mainly due to quick quarantine of close contacts and isolation, thus reducing the probability of secondary spread later on in the course of illness. In a rapidly expanding epidemic wherein contact tracing/quarantine and perhaps even isolation are no longer feasible, or in locations where cases are not isolated outside the

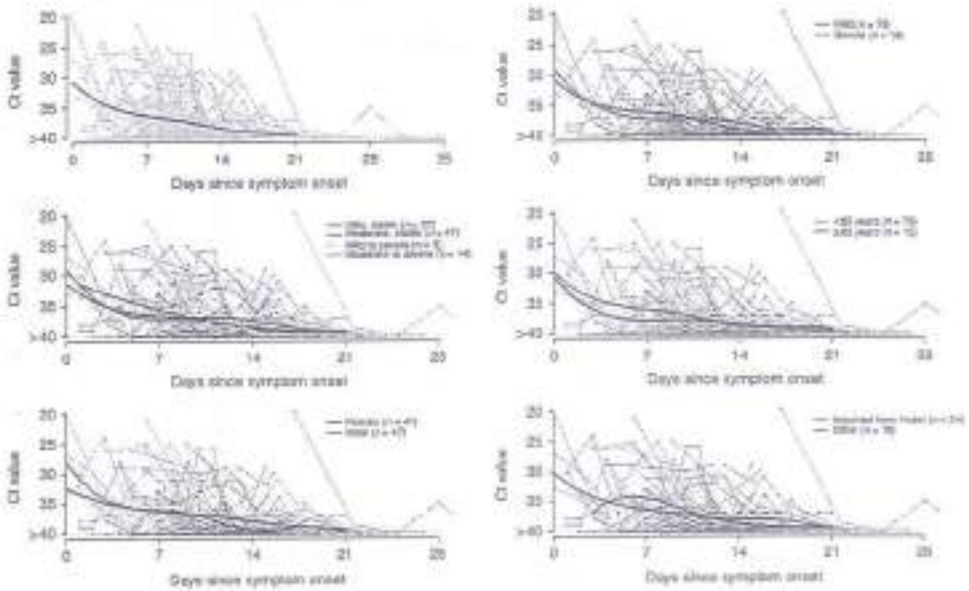

Fig. 2 | Temporal patterns of viral shedding. Viral load (threshold cycle (Ct) values) detected by RT-PCR (PCR with reverse transcription) in throat swabs from patients infected with SARS-CoV-2 ($N = 94$), overall and stratified by disease severity, sex, age group and link to Hubei province. The detection limit was Ct = 40, which was used to indicate negative samples. The thick lines show the trend in viral load, using smoothing splines. We added some noise to the data points to avoid overlaps.

home, we should therefore observe a lower proportion of presymptomatic transmission.

Our analysis suggests that viral shedding may begin 5 to 6 days before the appearance of the first symptoms. After symptom onset, viral loads decreased monotonically, consistent with two recent studies[5,6]. Another study from Wuhan reported that virus was detected for a median of 20 days (up to 37 days among survivors) after symptom onset[5], but infectiousness may decline significantly 8 days after symptom onset, as live virus could no longer be cultured (according to Wölfel and colleagues[7]). Together, these results support our findings that the infectiousness profile may more closely resemble that of influenza than of SARS (Fig. 1a), although we did not have data on viral shedding before symptom onset[1,2]. Our results are also supported by reports of asymptomatic and presymptomatic transmission[11,12].

For a reproductive number of 2.5 (ref. [2]), contact tracing and isolation alone are less likely to be successful if more than 30% of transmission occurred before symptom onset, unless >90% of the contacts can be traced[13]. This is more likely achievable if the definition of contacts covers 2 to 3 days prior to symptom onset of the index case, as has been done in Hong Kong and mainland China since late February. Even when the control strategy is shifting away from containment to mitigation, contact tracing would still be an important measure, such as when there are super-spreading events that may occur in high-risk settings including nursing homes or hospitals. With a substantial proportion of presymptomatic transmission, measures such as enhanced personal hygiene and social distancing for all would likely be the key instruments for community disease control.

Our study has several limitations. First, symptom onset relies on patient recall after confirmation of COVID-19. The potential recall bias would probably have tended toward the direction of under-ascertainment, that is, delay in recognizing first symptoms. As long as these biases did not differ systematically between infector and infectee, the serial interval estimate would not be substantially affected. However, the incubation period would have been overestimated, and thus the proportion of presymptomatic transmission artifactually inflated. Second, shorter serial intervals than those reported here have been reported, but such estimates lengthened when restricted to infector–infectee pairs with more certain transmission links[14]. Finally, the viral shedding dynamics were based on data for patients who received treatment according to nationally promulgated protocols, including combinations of antivirals, antibiotics, corticosteroids, immunomodulatory agents and Chinese medicine preparations, which could have modified the shedding dynamical patterns.

In conclusion, we have estimated that viral shedding of patients with laboratory-confirmed COVID-19 peaked on or before symptom onset, and a substantial proportion of transmission probably occurred before first symptoms in the index case. More inclusive criteria for contact tracing to capture potential transmission events 2 to 3 days before symptom onset should be urgently considered for effective control of the outbreak.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-020-0869-5.

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Methods

Sources of data. Guangzhou Eighth People's Hospital in Guangdong, China was designated as one of the specialized hospitals for treating patients with COVID-19 at both city and provincial levels on 20 January 2020. After that, many people with COVID-19 were admitted via fever clinics, the hospital emergency room or after confirmation of cases from community epidemiological investigation carried out by the Guangzhou Center for Disease Control and Prevention, or transferred from other hospitals. The first confirmed patient with COVID-19 was admitted on 21 January 2020, but in the initial phase, patients suspected to have COVID-19 were also admitted. We identified all suspected and confirmed COVID-19 cases admitted from 21 January 2020 to 14 February 2020 and collected throat swabs in each case. Patients included those who traveled from Wuhan or Hubei to Guangzhou as well as locals, with cases ranging from asymptomatic, mild to moderate at admission.

The samples were tested by N-gene-specific quantitative RT–PCR assay as previously described[17]. To understand the temporal dynamics of viral shedding and exclude non-confirmed COVID-19 cases, we selected 94 patients who had at least one positive result (cycle threshold (Ct) value < 40) in their throat samples. Serial samples were collected from some but not all patients for clinical monitoring purposes.

We collected information reported on possible human-to-human transmission pairs of patients with laboratory-confirmed COVID-19 from publicly available sources, including announcements made by government health agencies and media reports in mainland China and countries/regions outside China. A transmission pair was defined as two confirmed COVID-19 cases identified in the epidemiologic investigation by showing a clear epidemiologic link with each other, such that one case (infectee) was highly likely to have been infected by the other (infector), by fulfilling the following criteria: (1) the infectee did not report a travel history to an area affected by COVID-19 or any contact with other confirmed or suspected COVID-19 cases except for the infector within 14 days before symptom onset; (2) the infector and infectee were not identified in a patient cluster where other COVID-19 cases had also been confirmed; and (3) the infector and infectee pair did not share a common source of exposure to a COVID-19 case or a place where there were COVID-19 case(s) reported. We excluded possible transmission pairs without a clear exposure history reported prior to symptom onset. Data of possible transmission pairs of COVID-19 were extracted, including age, sex, location, date of symptom onset, type of relationship between the pair cases and time of contact of the cases.

Statistical analysis. We analyzed two separate data sets—clinical and epidemiologic—to assess presymptomatic infectiousness. First, we assessed longitudinal viral shedding data from patients with laboratory-confirmed COVID-19 starting from symptom onset, where viral shedding during the first few days after illness onset could be compared with the inferred infectiousness. Second, the serial intervals from clear transmission chains, combined with information on the incubation period distribution, were used to infer the infectiousness profile, as described in the following.

We present SARS-CoV-2 viral loads in the throat swabs of each patient by day of symptom onset. To aid visualization, a smoothing spline was fitted to the Ct values to summarize the overall trend. Specifically, a generalized additive model, $E(Y) = \beta_0 + s(t)$, with an identity link was fitted, where Y are the Ct values, β_0 is the intercept and $s(t)$ is a cubic spline evaluated at t days after symptom onset. We also compared the viral load by disease severity, age, sex and travel history from Hubei.

We fitted a gamma distribution to the transmission pair data to estimate the serial interval distribution. We used a published estimate of the incubation period distribution to infer infectiousness with respect to symptom onset from the first 425 patients with COVID-19 in Wuhan with detailed exposure history[1]. We considered that infected cases would become infectious at a certain time point before or after illness onset (t_1). Infectiousness—that is, transmission probability to a secondary case—would then increase until reaching its peak (Fig. 1). The transmission event would occur at time t_i with a probability described by the infectiousness profile $\beta_c(t_i - t_1)$ relative to the illness onset date, assuming a gamma distribution $\beta(t)$ with a time shift c to allow for start of infectiousness c days prior to symptom onset; that is, $\beta_c(t) = \beta(t + c)$. The secondary case would then show symptoms at time t_2, after the incubation period that is assumed to follow a lognormal distribution $g(t_2 - t_i)$. Hence the observed serial intervals distribution $f(t_2 - t_1)$ would be the convolution between the infectiousness profile and incubation period distribution. We constructed a likelihood function based on the convolution, which was fitted to the observed serial intervals, allowing for the start of infectiousness around symptom onset and window of symptom onset (t_{1a}, t_{1b}), given by

$$L(t_{1a}, t_{1b}, t_2|\theta) = \int_{t_{1a}}^{t_{1b}} \int_{-\infty}^{t_2} \beta_c(t_i - t_1) g(t_2 - t_i) dt_i dt_1$$

A normalization factor can be added to account for the uncertainty in the symptom-onset dates of the index cases. Assuming a uniform distribution, the likelihood would differ only by a multiplicative constant and give the same estimates.

Parameters θ, including the gamma distribution parameters and the start of infectiousness, were estimated using maximum likelihood. The 95% CIs were obtained by bootstrapping with 1,000 replications. We also performed sensitivity analyses by fixing the start of infectiousness from days 5, 8 and 11 before symptom onset and inferred the infectiousness profile.

As an additional check, we simulated the expected serial intervals assuming the same aforementioned incubation period but two different infectiousness profiles, where infectiousness started on the same day and from 2 days before symptom onset, respectively. A recent study isolated live infectious SARS-CoV-2 virus from patients with COVID-19 up to 8 days after symptom onset[4], thus we assumed the same duration of infectiousness. We also assumed that infectiousness peaked on the day of symptom onset. The timing of transmission to secondary cases was simulated according to the infectiousness profile using a lognormal and exponential distribution, respectively, where the serial intervals were estimated as the sum of the onset to transmission interval and the incubation period. We drew random samples for the transmission time relative to symptom onset of the infector $T_1 \sim \beta_c(t)$, and also the incubation period $T_{inc} \sim f(t)$, then the simulated serial interval was $T_1 + T_{inc}$. We also performed simulation considering combinations of different infectiousness profiles, with start of infectiousness 7 days before to 3 days after symptom onset, and peak infectiousness also 7 days before to 3 days after symptom onset. We present the distribution of the serial intervals and proportion of negative serial intervals over 10,000 simulations.

All statistical analyses were conducted in R version 3.6.3 (R Development Core Team).

Ethics approval. Data collection and analysis were required by the National Health Commission of the People's Republic of China to be part of a continuing public health outbreak investigation.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Detailed transmission pairs data in this study are provided in the Supplementary Information and viral shedding data will be available upon request and approval by a data access committee. The data access committee comprises leadership of the Guangzhou Eighth People's Hospital and the Guangzhou Health Commission. There is no restriction to data access.

Code availability

We provided the code for generating Fig. 1c in the Supplementary Information and at https://github.com/ehylau/COVID-19. Other codes are available upon request to the corresponding author.

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Author contributions

X. He, E.H.Y.L., P.W., B.J.C., F.L. and G.M.L. conceived and designed the study. X. He, X.D., J.W., Y.G., X.T., X.M., Y.C. and B.L. were responsible for clinical care and collected all biomaterials. W.C. and F.H. carried out laboratory testing. Q.Z., M.Z. and Y.W. collected and collated linked clinical–epidemiologic data. L.Z., F.Z. and F.L. supervised and coordinated all aspects of the study at Guangzhou Eighth People's Hospital. P.W., X. Hao, Y.C.L. and J.Y.W. collected and verified all infector–infectee transmission data. E.H.Y.L., B.J.C. and G.M.L. wrote the first draft. All authors contributed to data interpretation, critical revision of the manuscript and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-020-0869-5.

Supplementary information is available for this paper at https://doi.org/10.1038/s41591-020-0869-5.

Correspondence and requests for materials should be addressed to E.H.Y.L.

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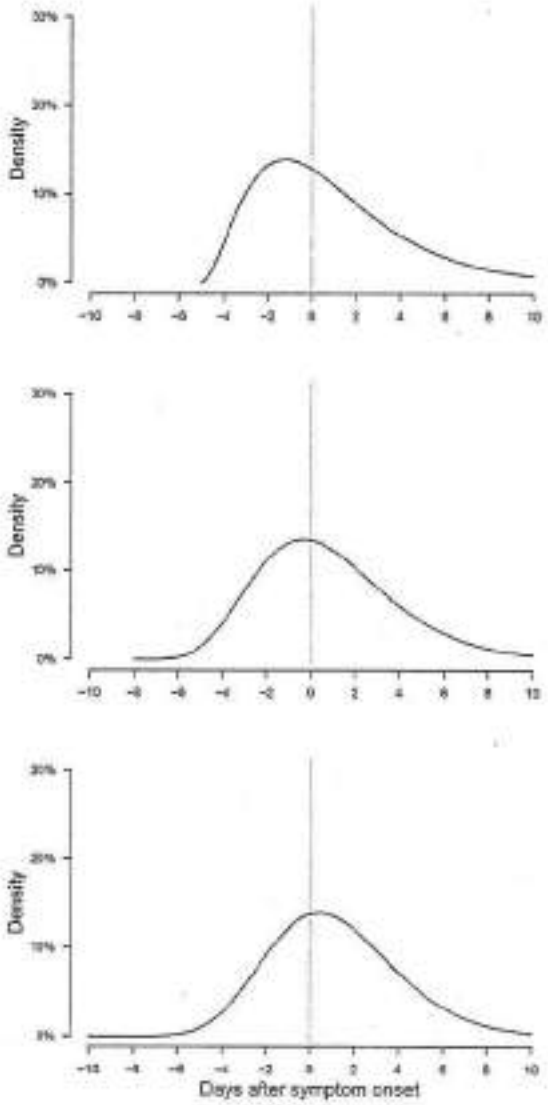
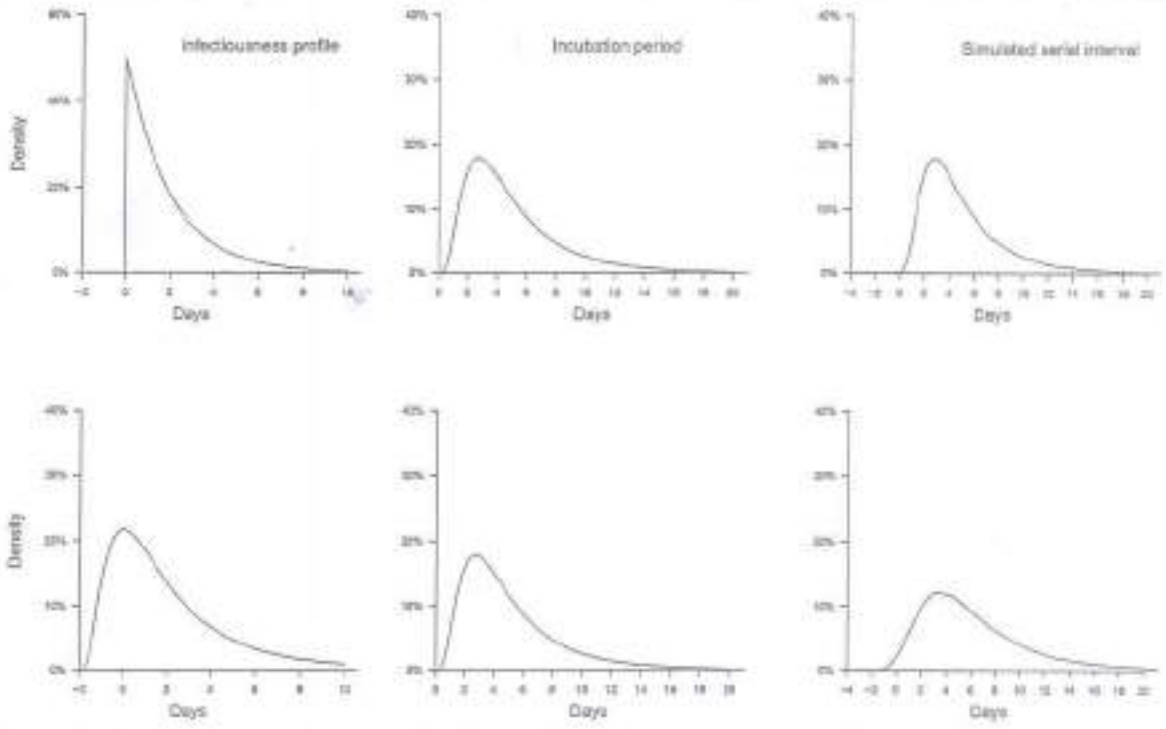
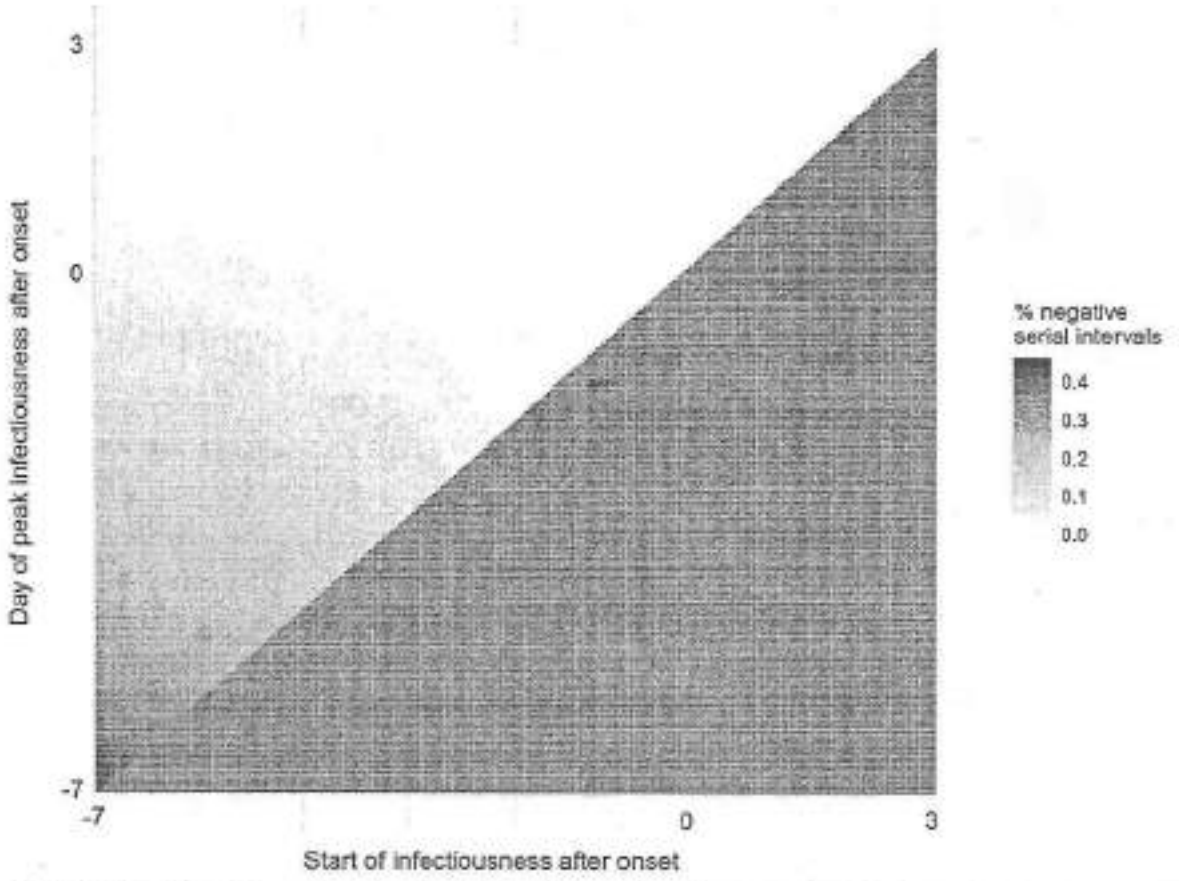


Extended Data Fig. 1 | Inferred infectiousness profile. Infectiousness was assumed to start from 5 days (top) to 8 days (middle) and 11 days (bottom) before symptom onset.



Extended Data Fig. 2 | Simulated serial intervals. Simulated serial intervals assuming infectiousness started on the same day of symptom onset (top panel) and from 2 days before symptom onset (bottom panel) to about 10 days after symptom onset. Both scenarios assumed that infectiousness peaked on the first day of symptom onset.



Extended Data Fig. 3 | Simulated proportions of negative serial intervals. Simulated proportions of negative serial intervals assuming start of infectiousness and peak infectiousness from 7 days before symptom onset to 3 days after symptom onset. From the estimated serial interval distribution based on infector-infectee pairs, 7.6% of the serial intervals were negative. Grey area represents the implausible range where the peak infectious is earlier than the start of infectiousness.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	MS Excel 2013.
Data analysis	All statistical analyses were conducted in R version 3.6.2 (R Development Core Team, Vienna, Austria).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	94 COVID-19 patients who had at least one positive results (Cycle threshold value < 40) by in their throat samples, tested by N-gene-specific quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay; 77 infector–infectee pairs from publicly available data
Data exclusions	No data was excluded
Replication	No replication
Randomization	Observational study, no randomization
Blinding	Observational study, no blinding

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☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Human research participants

Policy information about studies involving human research participants

Population characteristics	COVID-19 patients admitted to Guangzhou Eighth People's Hospital
Recruitment	Samples were collected from patients for clinical monitoring purposes.
Ethics oversight	Data collection and analysis were required by the National Health Commission of the People's Republic of China to be part of a continuing public health outbreak investigation.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

EXHIBIT "N"

Asymptomatic and presymptomatic transmission of SARS-CoV-2: A systematic review

Christina Savvides[1*], Robert Siegel[2]

1. Department of Biomedical Informatics, Stanford University, Stanford, California, USA

2. Department of Microbiology and Immunology, Stanford University, Stanford, California, USA

*Corresponding author

Email: css19@stanford.edu

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Abstract

Background and Purpose

Many of the statutes comprising the shelter-in-place and phased-reopening orders are centered around minimizing asymptomatic and presymptomatic transmission. Assumptions about the presence and relative importance of asymptomatic and presymptomatic transmission are based on case reports, the failing of quarantine measures aimed at sequestering ill patients, viral dynamic studies suggesting SARS-CoV-2 production peaks before symptoms appear, and modeling evidence that calculates serial interval between successive generations of infection. In aggregate, these data offer compelling evidence of asymptomatic and presymptomatic transmission, but individually these studies have notable shortcomings that undermine their conclusions. The purpose of this review is to discuss the literature of asymptomatic and presymptomatic transmission, highlight limitations of recent studies, and propose experiments that, if conducted, would provide a more definitive analysis of the relative role of asymptomatic and presymptomatic transmission in the ongoing SARS-CoV-2 pandemic.

Methods

We conducted a systematic review of literature on PubMed using search filters that relate to asymptomatic and presymptomatic transmission as well as serial interval and viral dynamics. We focused on studies that provided primary clinical data.

Results

34 studies were eligible for inclusion in this systematic review: 11 case reports pertaining to asymptomatic transmission, 9 viral kinetic studies, 13 serial interval studies, and 1 study with viral kinetics and serial interval.

Conclusion

Different approaches to determining the presence and prevalence of asymptomatic and presymptomatic SARS-CoV-2 transmission have notable shortcomings, which were highlighted in this review and limit our ability to draw definitive conclusions. Conducting high quality studies with the aim of understanding the relative role of asymptomatic and presymptomatic transmission is instrumental to developing the most informed policies on reopening our cities, states, and countries.

Introduction

Understanding how SARS-CoV-2 is transmitted is a question that has been at the forefront of efforts to curtail the pandemic. On January 14, 2020, the World Health Organization announced that "there is no clear evidence of human-to-human transmission" of SARS-CoV-2.[1] Six days later, when WHO announced evidence of human to human transmission, countries were left scrambling to enact policy to identify and isolate the ill.[2] Only after these efforts failed, were the more comprehensive quarantine and isolation policies enacted in cities like Wuhan, China. In the absence of definitive evidence of asymptomatic transmission, these intervention policies were made out of an abundance of caution. Understanding the temporal dynamics of SARS-CoV-2 transmissibility is key to safely and successfully reopening our cities, states, and countries until the development of an effective vaccine. Unfortunately, with nearly 8 million confirmed cases and over 434,000 deaths, there is still confusion and a dearth of adequate research around the dynamics of transmissibility of SARS-CoV-2 in the general population.[3] On June 8th, 2020, WHO official Maria Van Kerkhove said that asymptomatic transmission of the coronavirus was "very rare." However, she later clarified this statement saying, "the available evidence from contact tracing reported by Member States [of WHO] suggests that asymptomatically-infected individuals are much less likely to transmit the virus than those who develop symptoms."[4] Given the absence of definitive information, and because of the importance of this question, there is an urgent need to direct high quality studies towards examining asymptomatic and presymptomatic transmission of SARS-CoV-2.

Asymptomatic individuals are defined as individuals who test RT-PCR positive, but lack symptoms that would indicate SARS-CoV-2 infection. While some individuals may go the entire course of infection and never experience symptoms, other individuals who initially present as asymptomatic may go on to develop symptoms days or weeks later. The individuals who will later develop symptoms are defined as being presymptomatic.

The first large scale reporting of asymptomatic SARS-CoV-2 infection occurred on the Diamond Princess cruise ship, where an estimated 17.9% of cases on board were asymptomatic.[5] The phenomenon of asymptomatic SARS-CoV-2 infection has since been established in multiple studies, including a UCSF study that found that 53% of individuals who tested positive were not experiencing symptoms at the time of the test.[6] While the existence of asymptomatic cases is well understood, the link between asymptomatic/presymptomatic cases and transmissibility is more tenuous. RT-PCR testing can tell us whether there is detectable virus present, but it does not accurately tell us whether an individual is

most effective interventions are fundamentally rooted in an understanding of asymptomatic and presymptomatic transmission.

The basis for asymptomatic and presymptomatic transmission in other viral infections

Viral illnesses have varying transmission profiles. Seasonal influenza is characterized by having peak viral load one day after symptom onset, and individuals generally have detectable levels of RNA from two days before clinical symptoms appear to eight days afterward.[12] Although asymptomatic and presymptomatic individuals may shed influenza virus, studies have not determined if such people effectively transmit influenza.[13]

Other viral illnesses like MERS, SARS and Ebola are notable because infectivity appears to increase later in course of illness. MERS-CoV concentrations peaked during the second week of illness.[14] Ebola virus does not appear to have presymptomatic transmission, though individuals can remain infectious for long periods of time after symptoms resolve.[15] Notably, in the case of SARS-CoV, infectiousness peaked 7-10 days after symptom onset.[16]

Understanding the viral dynamics and transmission profile of a virus is critically important because it informs the most effective outbreak curtailment strategies. In the case of SARS-CoV and Ebola viruses, efforts aimed at sequestering the ill and contact tracing are highly effective. In the case of influenza virus, contact tracing must extend to the presymptomatic phase, and more aggressive prophylactic containment strategies are necessary. Efforts to curtail SARS-CoV-2 virus will rely on successful contact tracing to halt further transmission. Decisions on how far back to trace contacts and if/when to test asymptomatic contacts will rely on a comprehensive understanding of asymptomatic and presymptomatic transmission.

The aim of this review is to summarize the literature that informs the current understanding of the presence and prevalence of asymptomatic and presymptomatic transmission through studying viral kinetics, case reports, and calculation of serial interval.

Methods

Search Strategy

All efforts were taken to comply with PRISMA standards (see Supplement Fig. 1). However, due to the rapidly changing vocabulary and information regarding the SARS-CoV-2 pandemic, this study was not

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preregistered. Articles for this review were extracted from a PubMed search conducted on June 10, 2020. Articles had to either contain the phrase SARS-CoV-2 or COVID-19 as well as one of the following phrases: presymptomatic transmission, asymptomatic transmission, viral dynamics, viral kinetics, virological analysis, or serial interval. The exact search phrase was: (("SARS-CoV-2" or "COVID-19") AND ("presymptomatic transmission" OR "asymptomatic transmission" OR "viral dynamics" OR "viral kinetics" OR "virological analysis" OR "serial interval")). No year restrictions were applied during search, but due to the fact that SARS-CoV-2 is a novel coronavirus, all studies were from 2020.

Study Selection

We assessed the eligibility of the studies retrieved during the PubMed search through a two-stage screening process. We first screened the titles and abstracts of all articles. Reviews, correspondence, duplicate references, articles written in languages besides English, and studies that did not mention data collection were excluded.

For all studies that appeared relevant, the full text was reviewed using the same screening procedure delineated above. However, additional filters were added to serial interval studies. Serial interval studies that refit data as well as small studies without supporting statistics, were excluded in this review. Studies excluded during full text analysis are marked 'initially included then excluded' in the supplement Table 1.

Article Types

There were initially 72 results. 2 additional records were added because studies in the review referenced or analyzed their data. After screening, 34 articles met all review criteria (see Fig. 1). These 34 articles fell into the broad categories of (1) case reports, (2) viral dynamic studies, or (3) analysis of serial interval between linked generations of cases. Each of these broad categories will be discussed separately in a subsequent section.

Data Extraction

Case reports and viral dynamic studies were analyzed qualitatively. In case reports, information about the location of the patients, number of patients observed, and notable conclusions were extracted. In viral dynamics studies, number of patients, location, disease severity, testing schedule and sample collection, and notable conclusion were extracted.

For studies that measured serial interval, the mean and/or median serial interval was extracted and compiled along with 95% confidence intervals although other comprehensive methods of expressing data distribution was allowed and noted. Information about the distribution, truncation, and standard deviation was also extracted when available. If study reported serial interval statistics for both high confidence and low or moderate confidence paired transmission statistics, both sets of data were extracted, but the high confidence data was preferentially reported.

Data extracted from serial interval studies (see Supplement Table 2):

- Date Range
- Location
- Number of patient pairs used in determination of serial interval
- Categorization of subsets of data (i.e. high/low confidence)
- Patient relationship (i.e. familial transmission)
- Any other notable features
- Type of distribution (i.e. normal, Weibull, gamma)
- Presence or absence of truncation (i.e. right truncation, not allowing negative values)
- Mean and/or median
- SD of mean/median
- CI on SD
- Type of error reported on mean/median (i.e. 95% CI, quartile, etc)
- Lower bound of error interval
- Upper bound of error interval
- Summary for inclusion in text

Risk of bias and quality of the studies were assessed through author consensus and discussed in the text of the review. Due to the circumstances of the global pandemic, there may be substantial systematic errors in the data being published. The general objective of this paper is to be descriptive, not to draw conclusions about the validity of individual estimates or determine predictive accuracy from the included studies.

Results

Evidence of Asymptomatic and Presymptomatic Transmission in SARS-CoV-2

The flow diagram documenting the results of the literature search are shown in Fig 1.

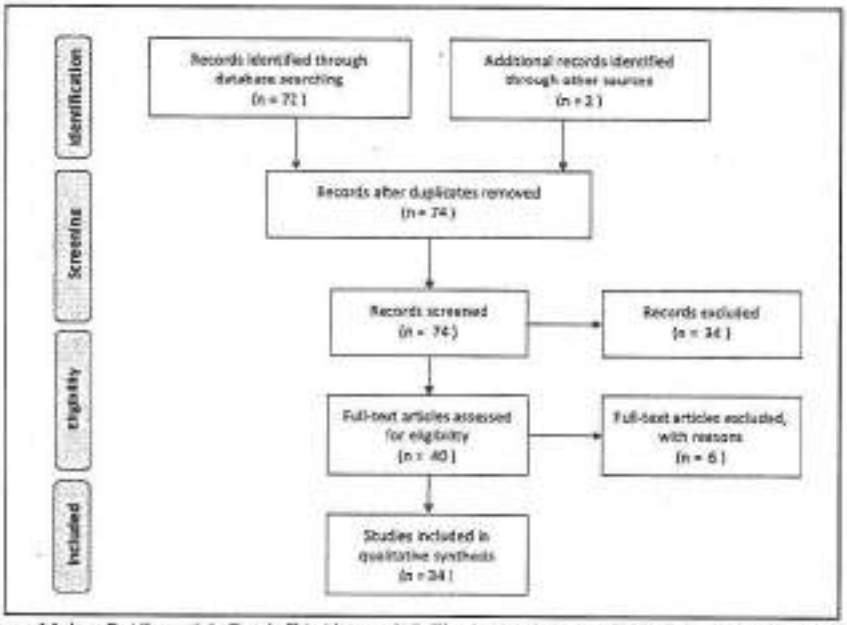

Figure 1. From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097. A complete list of studies retrieved from the search can be found in the appendix.

Case Studies Suggesting Asymptomatic or Presymptomatic Transmission

Case reports providing insight into asymptomatic and presymptomatic transmission are shown in Table 1.

Articles	Type	Description
Chen et al.[17]	Case Report	Reported on a family cluster in Hubei province where it appears a parent transmitted SARS-CoV-2 infection to their children while asymptomatic. Mother had traveled to Wuhan before returning home to Xiangyang.
Hu et al.[18]	Case Report	Contact tracing identified 24 asymptomatic COVID-19 infections in Nanjing, Jiangsu Province. One of these asymptomatic cases appears to be a possible source of infection in three relatives, one of which went on to develop severe pneumonia.
Huang et al.[19]	Case Report & Serial Interval Report	One 22-year-old from Wuhan appears to have infected his cousin and six classmates while presymptomatic.
Li et al.[20]	Case Report	Father appears to have infected his daughters, son-in-law, his son-in-law's workmate, and his workmate's family while asymptomatic. 2 family clusters of 6 patients stemming from one possible asymptomatic transmitter.
Lytras et al.[21]	Case Report	Noted the relatively high rates of asymptomatic SARS-CoV-2 infection in repatriation flights in flights from Spain, Turkey, and UK. Postulates about the possibility of presymptomatic transmission.

Ochiai et al.[31]	Case Report	62 obstetric patients were tested before hospital appointments at Keio University Hospital in Tokyo, Japan. 4% were found to be asymptomatic. No cases of asymptomatic transmission documented.
Qiu et al.[26]	Case Report	In 104 cases from Hunan province hospitals, 5 were identified as asymptomatic. Contact tracing suggests two of these cases infected family members.
Tong et al.[34]	Case Report	Reported on two people who were infected with SARS-CoV-2 and their infections appear to have stemmed from contact with a potentially asymptomatic/presymptomatic colleague. These individuals went on to infect other members of their household. However other sources of SARS-CoV-2 infection were not ruled out.
Wei et al.[28]	Case Report	Investigation into 157 locally acquired cases of SARS-CoV-2 infections in Singapore revealed ten cases in 7 family clusters where presymptomatic transmission appears to have occurred.
Wong et al.[36]	Case Report	Identifies two asymptomatic infected individuals from a cluster of cases in the Seri Petaling Mosque in Kuala Lumpur, Malaysia, who appear to have transmitted infection to others.
Ye et al.[37]	Case Report	Family cluster of five patients. One of the patients was believed to be the source of infection, and infected others during a family reunion. Asymptomatic transmission offered as possible explanation.

Table 1. A summary of case reports from the literature search that yielded insight into the question of asymptomatic and presymptomatic transmission.

The early literature of SARS-CoV-2 asymptomatic transmission was dominated by case reports of apparent asymptomatic transmission, and 9 studies that document cases of apparent asymptomatic or presymptomatic transmission were identified in this systematic review. A majority of these cases were individuals exposed during travel to Wuhan or other cities in Hubei Province, who later transmitted the infection to members of their household or other close contacts.[17, 18, 20, 23, 24, 27] Huang and colleagues reported a cluster of asymptomatic transmission among children, who had rapid onset of illness and various nonspecific or atypical manifestations of illness.[19] While many of these case reports took steps to ensure that those infected by asymptomatic or presymptomatic individuals did not have other plausible sources of infection, they were unable to definitively rule out other sources or community transmission. Other case reports center around regions that were believed to not have community transmission, where exposure to other sources of infection are less likely. One example is the case of a Chinese businesswoman who appeared to have asymptomatically infected some of her colleagues during a work trip in Germany.[28] However, after publication, the supplementary material was modified because the original patient recalled that she was experiencing symptoms during her meetings with colleagues. While this paper did not appear in the keyword search, and is not included in this review, it was frequently cited in other papers analyzed in this review. The subsequent update to the NEJM article is emblematic of the systematic biases in case reports documenting asymptomatic and presymptomatic transmission. Patients or practitioners may make errors when recalling or reporting symptom onset date. Another case report from the keyword search that focuses on areas without broad community transmission reports on seven clusters in Singapore where presymptomatic transmission appeared to be the most likely explanation.[25]

This study identified 10 cases where presymptomatic transmission appeared to occur 1-3 days before symptom onset in the initial patient. While compelling, the retrospective nature of these studies makes it difficult to rule out mild symptoms being present during transmission, or other sources of infection.

All case reports of asymptomatic and presymptomatic transmission are confounded by the highly subjective nature of reporting symptom onset and exposure date. Factors like age, cultural norms, and public communication about the pandemic may influence when people report their symptoms beginning. For example, an older person with chronic illness may attribute muscle and joint pain to age, whereas a younger person may call that a symptom. Additionally, as the pandemic has progressed, our categorization of what is considered a symptom has expanded. In February, the WHO said symptoms of COVID-19 included fever, dry cough, fatigue, sputum production, shortness of breath, sore throat, headache, myalgia or arthralgia, chills, nausea or vomiting, nasal congestion, diarrhea, hemoptysis, and conjunctival congestion.[29] In late February, Mao and colleagues first reported that anosmia, or loss of sense of smell, were symptoms of COVID-19, and this finding was supported in additional research.[30] On April 17th, the WHO added loss of smell or taste as well as rash and skin discolorations of fingers and toes as additional symptoms of COVID-19.[31] Knowledge of these changing definitions, differing levels of chronic illness, and varying levels of symptom awareness will alter when individuals first report experiencing symptoms.

Two additional reports included in this keyword search inferred the possibility of asymptomatic transmission from positive RT-PCR tests in asymptomatic and presymptomatic individuals. Lytras and colleagues noted a high prevalence of SARS-CoV-2 infection in asymptomatic cases in repatriation flights to Greece.[21] While this study supports the well-documented phenomenon of asymptomatic cases, the possibility of asymptomatic transmission is a hypothetical, as a positive RT-PCR test does not confirm that an individual is contagious. This study failed to provide insight into the feasibility of actual transmission during presymptomatic or asymptomatic infection because the authors failed to report Ct values of RT-PCR positive individuals, did not culture virus, and did not identify possible transmission chains. The study by Ochiai and colleagues had similar findings and limitations.

Viral Dynamics

Results from the literature search that documented viral dynamics are shown in Table 2.

Paper	Included/Excluded in Review	Category	Description
Ding et al.[32]	Included	Viral Dynamics	64 patients from Ruian People's Hospital in Zhejiang, China were retroactively enrolled in this study. Patients received lopinavir/ritonavir, interferon-α regimen, and some also received arbidol. Samples were taken at baseline and then every 3-5 days until discharge. Viral loads peak at start of observation.
He et al.[33]	Included	Viral Dynamics & Serial Interval	94 patients admitted to Guangzhou Eighth People's Hospital were studied. 414 throat swabs were collected between symptom onset up to day 32. At the time of this review, no information was found about frequency or duration of swab collection. Patients received treatment that is standard of care, including combinations of antivirals, antibiotics, corticosteroids, immunomodulatory agents and Chinese medicine preparations. Viral loads peak at start of observation.
Kim et al.[34]	Included	Viral Dynamics	10 asymptomatic and 3 presymptomatic individuals were studied. Viral load peaks at the beginning of observation. Patients were observed at Affiliated Hospitals of Chonnam National University between February 4 and April 7, 2020. At the time of this review, no information was found about the frequency or duration of swab collection. Viral loads peak at start of observation.
Liu et al.[35]	Included	Viral Dynamics	76 patients admitted to the First Affiliated Hospital of Nanchang University (Nanchang, China) from Jan 21 to Feb 4, 2020. 46 cases were mild and 30 were severe. At the time of this review, no information was found about frequency or duration of sample collection or treatment patients were receiving. Found that patients with severe COVID-19 tend to have a high viral load and a long virus-shedding period. Cite previous work showing viral loads peak during first week of disease onset.
Lui et al.[36]	Included	Viral Dynamics	A study of the first 11 laboratory-confirmed COVID-19 patients hospitalized in 2 hospitals in Hong Kong in February 2020. 6 had moderate/mild disease, 5 had severe/critical disease. Authors conclude that viral load appears to peak in the first week in mild cases, and potentially peak later in severe cases. All patients were taking antivirals including lopinavir/ritonavir, ribavirin, beta-interferon, and one patient was taking corticosteroid therapy.
To et al.[37]	Included, externally sourced	Viral Dynamics	23 patients from 2 hospitals in Hong Kong with laboratory confirmed COVID were entered in this cohort study. Patients were screened between Jan 22-Feb 11, 2020. Ten patients had severe COVID-19, 13 had mild disease. The median interval between symptom onset and hospitalization was 4 days. Five were admitted to ICU and 2 died. All patients produced an early morning saliva sample from the posterior oropharynx. Saliva viral load was also measured. In Fig 2, viral loads appear to peak a few days after symptom onset, but authors concluded viral loads peak around symptom onset.
Wölfel et al.[38]	Included	Viral Dynamics	Viral dynamics determined from 9 individuals from a single cluster in a single hospital in Munich, Germany. All patients were admitted after symptom onset. For most of the patients, viral loads appear to peak around the time observation began. At the time of this review, no information was found on patient treatments.
Yoon et al.[39]	Included	Viral Dynamics	Viral dynamics in diverse body fluids of 2 patients were studied. Patients were sampled every 1 days on hospital days 1-9. Patient 1 received lopinavir/ritonavir 400/100mg twice a day along with hydroxychloroquine 400 mg once daily. Patient 2 received lopinavir/ritonavir 400/100mg twice a day.
Young et al.[40]	Included, externally sourced	Viral Dynamics	Studied first 18 patients diagnosed with SARS-CoV-2 infection in Singapore between January 23 and February 3, 2020. 5 received lopinavir-ritonavir. For half of patients presented at hospital more than 3 days after symptom onset. Viral loads in nasopharyngeal samples from patients with COVID-19 peaked within the first few days of observation before declining.
Zhou et al.[41]	Included	Viral Dynamics	This study included 91 adults with confirmed SARS-CoV-2 infection who were asymptomatic on admission. No information about patient treatment. 22 of the patients went on to develop symptoms while 9 remained asymptomatic. When comparing the viral dynamics of asymptomatic and presymptomatic individuals, Zhou et al. found asymptomatic individuals had lower Ct values, and had peak viral loads in the second week of hospitalization.

Table 2. Results of literature search that yielded insight into the question of presymptomatic transmission that pertained to the study of viral dynamics. Two of the included studies did not appear in the PubMed literature search and were added later because they appeared as references in other reviewed studies (marked as externally sourced).

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Studying temporal viral dynamics allows for the prediction of peak infectiousness. In this review there were ten studies that measured viral temporal dynamics and kinetics of SARS-CoV-2. Eight of these studies measured viral dynamics by quantifying successive nasopharyngeal swabs in hospitalized patients. The two remaining papers focused exclusively on asymptomatic and presymptomatic individuals. From the eight studies of viral dynamics in hospitalized patients, all patients except one in the Zou *et al.* paper were symptomatic. The one asymptomatic individual in Zou *et al.* remained asymptomatic throughout the course of the study.

The eight studies reported viral loads were at their highest levels around the time observation began. Therefore, the authors of these studies concluded viral loads peak close to when symptoms emerge. However, this discovery must be prefaced by the limitation that all patients in the studies were enrolled after symptom onset, and therefore presymptomatic viral loads were not measured. This shortcoming is further propagated by the fact that patients often will not see a clinician immediately after symptom onset, in these cases we cannot rule out the possibility that viral load peaks after symptom onset. While studying COVID-19 in China, Zhang and colleagues found that an average of 2.5 days elapsed between symptom onset and first healthcare consultation.[56] Although this decreased from 3.0 to 1.6 days as the pandemic progressed. If individuals are only infectious for 8 days, as Bullard and colleagues report, this delay in seeking care greatly confounds our ability to measure comprehensive viral dynamics.[8,38] Additionally, the studies do not disclose how soon the first swab was taken after symptoms were reported; a margin of error of a day might dramatically change the viral load in patients. While the finding that viral load appears to peak soon after symptoms are detected in patients suggests that presymptomatic transmission is plausible, there is not enough information about the distribution of SARS-CoV-2 viral kinetics in presymptomatic stage to conclude when infectiousness begins.

When modeling viral dynamics, basic assumptions about the distribution will have dramatic effects on our prediction of when infectivity begins, and the specific time between symptom onset and viral load tests can dramatically change our understanding of transmissibility and infectiousness. Examples of hypothetical distributions of SARS-CoV-2 viral load and their effect of predicting transmissibility are shown in Fig 2. Knowledge of the shape of the distribution will impact our responses to curtail the pandemic.

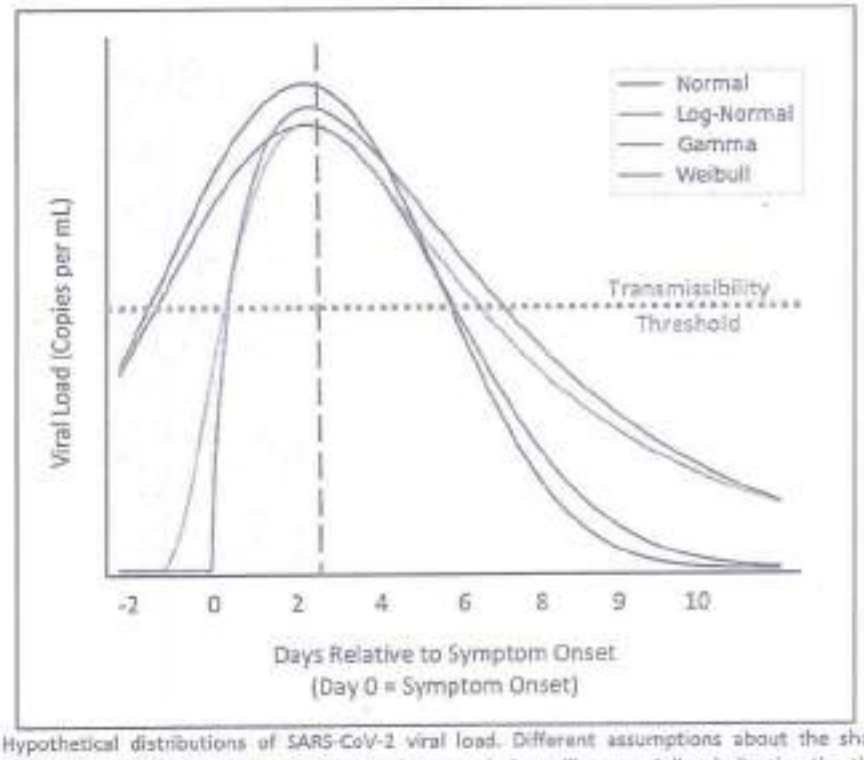

Figure 2. Hypothetical distributions of SARS-CoV-2 viral load. Different assumptions about the shape of the distributions will impact when and if presymptomatic transmission will occur. A line indicating the threshold of transmissibility is shown in purple, which is currently believed to be 10^6 copies per mL. The intersection of the purple line with the various curves would show when an individual becomes contagious. In these hypothetical distributions, a normal and Weibull distribution suggest significant presymptomatic transmission, while a gamma and lognormal distribution seem to suggest limited presymptomatic transmission. These conclusions can change with different transmission thresholds and distribution perameters. A vertical dashed line in grey shows when an individual might seek medical consultation, which Zhang and colleagues report as being 2.5 days after symptom onset in China during the COVID-19 pandemic.[53] Although this number decreased from 3.0 to 1.6 days as the pandemic progressed. Assuming patients don't seek medical care for 2.5 days, the light-yellow shaded region refers to the area where data is lacking. While many studies concluded viral load peaks when observation begins, for almost all of the studies, a significant portion of time elapsed between when symptoms first appeared and observation began.

Wolfel and colleagues attempted to relate RT-PCR quantification of viral load with infectivity. The authors combined RT-PCR measurement with viral culturing and found that the success of virus isolation in culture was a function of viral load: only samples that contained greater than 10^6 copies per mL yielded an isolate (although Ct value was not reported in this study, He *et al.* reports this corresponds to a Ct value of 24).[42] Interestingly no isolates were obtained after day 8, despite continuing high viral loads. This finding suggests persistent RNA detection represents non-viable virus that is not infectious. This finding demonstrates that while viral load can be predictive of transmissibility, it is not a perfect correlation. The

viral studies of Wolfel *et al.*, Lui *et al.*, To *et al.*, Young *et. al.* and Yoon *et al.* were limited by small sample size. However, He *et al.*, Liu *et al.*, and *Ding et al.* have similar findings with larger sample sizes.

Despite the attempt to comprehensively profile SARS-CoV-2 kinetics, all eight of these studies were limited in their scope because they were not able to swab patients before symptom onset. An additional limitation of these studies is that many failed to specify the exact schedule of when patient swabs were collected. Only one study, To *et al.* mentioned a precise collection schedule that applied to all patients. It is also worth noting that nasopharyngeal swabs are an imperfect proxy for viral production. Studies on influenza have shown variability in viral load when sampling left and right nostrils and this finding will likely be similar for SARS-CoV-2.[43] Perhaps the most important limitation of these studies is that the studies either did not specify or did not exclude individuals who were undergoing treatment. Undergoing antiviral, interferon, or steroid therapy may disrupt the natural progression of viral load. While the study by Ding and colleagues had the purpose of examining the viral kinetics during antiviral treatment, data focusing on viral load after therapeutic interventions cannot provide insight into the viral dynamics of the natural history of infection. Antiviral and interferon treatments should diminish viral replication and artificially cause viral load to peak at the start of treatment, while steroid treatment may dampen the immune response and potentially cause viral replication to increase. If the viral load data is a basis for clinical decision making, this will even further confound results because an increasing viral load would be the basis for more extensive interventions and therapeutic treatment.

There is an urgent need to study the viral kinetics in presymptomatic individuals. Kim *et al.* analyzed the Ct values of three presymptomatic patients and found the highest levels of virus were one to two days before symptom onset. However, this dataset is extremely small (n=3), and one of the patients was on the threshold of detection. It is hard to reliably extract general trends from this limited sample. Zhou *et al.* studied the viral dynamics of 31 patients who were asymptomatic upon hospital admission for laboratory confirmed SARS-CoV-2 infection. Twenty-two of the patients went on to develop symptoms while nine remained asymptomatic by the case definition used in the study. When comparing the viral dynamics of asymptomatic and presymptomatic individuals, Zhou *et al.* found asymptomatic individuals had lower Ct values, and had peak viral loads in the second week of hospitalization. This data cannot be extrapolated to inform our understanding of presymptomatic viral dynamics because symptom onset date was not disclosed, and therefore the viral load data cannot be ascertained in relation to symptom onset.

While there currently appears to be consensus that viral load appears to peak with the beginning of observation, these studies are preliminary and that there is a dearth of data regarding infectiousness during the presymptomatic interval. In evaluating viral dynamics, knowledge of the shape of the distribution would be valuable to our understanding of transmissibility of SARS-CoV-2.

Serial Interval Between Generations of Cases

Another approach to uncovering the prevalence of presymptomatic transmission has relied on calculations of serial interval. Serial interval is defined as the time between symptom onset in the first-generation case and the second-generation case. This method requires identification of serial cases where one individual (first-generation case) infected another individual (second-generation case). If the observed mean serial interval is shorter than the incubation period, this would support the conclusion that a significant portion of transmission may have occurred presymptomatically.

Fourteen papers in this review calculated serial interval by looking at paired cases with probable point transmission linkage (see Table 3).

Most of these fourteen reports in Table 3 calculated serial interval by compiling data from publicly available sources or from municipal datasets. It is difficult to control for quality and bias from these publicly available reports. These datasets are compiled from human-to-human transmission reports from different countries, jurisdictions, and points in time. These factors may impact standards of reporting cases or symptom onset. In addition to bias or error in the publicly sourced data, all of the serial interval studies are confounded by their reliance on self-reported symptom start date. As stated earlier in this paper, what is considered a symptom varies by region, culture, age, and time, and the definition of symptoms has become more expansive as time has progressed. For example, patients who notice loss of smell may have an earlier symptom start date than a patient who only reports fever and dry cough. The date reported as the onset of symptoms is also subject to error due to inherent inaccuracy of memory. Furthermore, in the datasets, the authors report the date of symptom onset rounded to the nearest day. This is especially problematic because the difference in serial interval and incubation period calculated in these studies often differed by less than a day. It is therefore not possible to ascertain if the difference between calculated serial interval and incubation period are true differences, or an artefact of rounding error.

Paper	Included/Excluded in Review	Category	Description
Aghaali et al.[44]	Included	Serial Interval	Study calculated serial interval for 37 linked cases in Qom, Iran, who were identified through contact tracing. Due to limited availability of RT-PCR tests, second generation cases were confirmed with chest CT. Authors assumed a gamma distribution of serial intervals.
Bi et al.[45]	Included	Serial Interval	Serial interval calculated from 48 pairs with clear relationship between index case and secondary case. Data released by Shenzhen CDC. A gamma distribution of serial interval times was used.
Böhmer et al.[46]	Included	Serial Interval	Data sourced from one outbreak cluster and their contacts in Germany. Altogether 16 paired transmission events were reported. At the time of this review, no distribution was reported.
Du et al. MedRxiv[47]	Included	Serial Interval	339 confirmed cases of COVID-19 identified from 264 cities in mainland China prior to February 19, 2020. Sourced public data. Authors looked at multiple distributions for serial interval, but ultimately chose a normal distribution. The authors found household transmission led to shorter serial interval than non-household transmission inside the household (4.57 days [95% CI 3.76–5.38]) versus outside the household (5.85 days [95% CI 5.06–6.64]).
Du et al.[48]	Included	Serial Interval	Identified 468 paired cases from provinces outside of Hubei Province in China. Similar to analysis listed above, which appears to be an earlier version of this study. Authors assumed a normal distribution of serial intervals. (The authors ruled out gamma or Weibull distribution).
Ganyani et al.[49]	Included	Serial Interval	Studied 54 cases in Singapore and 114 paired cases in Tianjin, China that were part of outbreak clusters. Authors included cases in clusters with likely but not definitive transmission links. Authors determined density function of serial intervals by using a Monte Carlo estimation. They then used bootstrap sampling to determine confidence intervals.
He et al.[50]	Included	Serial Interval and Viral Dynamics	77 transmission pairs were sourced from publicly available information from multiple countries. Data was fitted to a gamma distribution of serial intervals.
Kwok et al.[51]	Included	Serial Interval	Serial intervals were estimated from 26 (probable: 9; certain: 17) paired data from Hong Kong Centre for Health Protection (CHP) before February 13, 2020. Authors used a lognormal distribution of serial intervals, but gamma and Weibull distributions were also examined.
Nishiura et al.[52]	Included	Serial Interval	Identified 28 paired cases, 18 of which were considered high quality. The data was fit to many different distributions, but authors ultimately chose Weibull distribution of serial intervals as best fit for high quality data. Data sourced from articles and government documents.
Wang K. et al.[53]	Included	Serial Interval	27 cases with transmission chains were identified and studied in Shenzhen, China. Transmission events sourced from publicly released information and identified 27 transmission chains, including 23 infectees matched with only one infector. Authors used a Weibull distribution of serial intervals (but also looked at other distributions).
Wang X. et al.[54]	Included	Serial Interval	Enrolled 37 cases and found 9 transmission chains. From these 9 paired cases, the authors calculated serial interval and assumed gamma distribution of serial intervals. Patients were seen at Wuhan Union Hospital between January 5 to February 12, 2020.
Wu et al.[55]	Included	Serial Interval	Studied 48 secondary cases stemming from household transmission. Fit to lognormal distribution. Zhuhai, China. Enrolled index cases and studied their household members.
You et al.[56]	Included	Serial Interval	Data sourced from 198 linked transmission cases outside Hubei Province as of March 31, 2020. No information was found on the type of distribution used, and statistics were reported as interquartile range.
Zhang et al.[57]	Included	Serial Interval	Serial interval calculated from 35 secondary cases stemming from 28 primary cases. Serial interval was fit to a gamma distribution of serial intervals (although other distributions were analyzed as well). Data taken from provinces outside Hubei.
Huang et al.[58]	Initially included, then excluded from Serial Interval Data	Serial Interval and Case Report	Data about serial interval excluded because general population was not studied. Study focused exclusively on young individuals.
Li et al.[59]	Initially included then excluded	Serial Interval	This study used prior assumptions from SARS-CoV data in their calculation of serial interval, therefore study was excluded.
Pung et al.[60]	Initially included then excluded	Serial Interval	Study was of the first three clusters in Singapore, which identified 3 paired transmission cases. Study was excluded because no statistics or data were provided, and primary data could not be located.
Son et al.[61]	Initially included but then excluded	Serial Interval	Study of patients in Busan. Authors report mean serial interval as 5.54 days [95% CI 4.08-7.01 days]. Excluded because full article was not available in English.

Table 3. Results of literature search that yielded insight into the question of presymptomatic transmission that pertained to serial interval. Studies that were excluded after full text analysis were also included.

Many studies of serial interval are biased towards household transmission because it is more straightforward to isolate transmission chains and rule out other sources of infection in a household setting. In household transmission cases, newly infected individuals will likely be exposed to a much higher dose of viral particulates than would occur in a more casual transmission case. Exposure to higher inoculum may result in a decreased incubation period for household transmission. Given that the papers compared serial intervals to estimates of incubation period, the difference in inoculum between household transmission and community transmission may account for the difference between the calculated serial interval and incubation period. The interpretation of this data is further complicated by the fact that estimates of incubation period vary between studies.

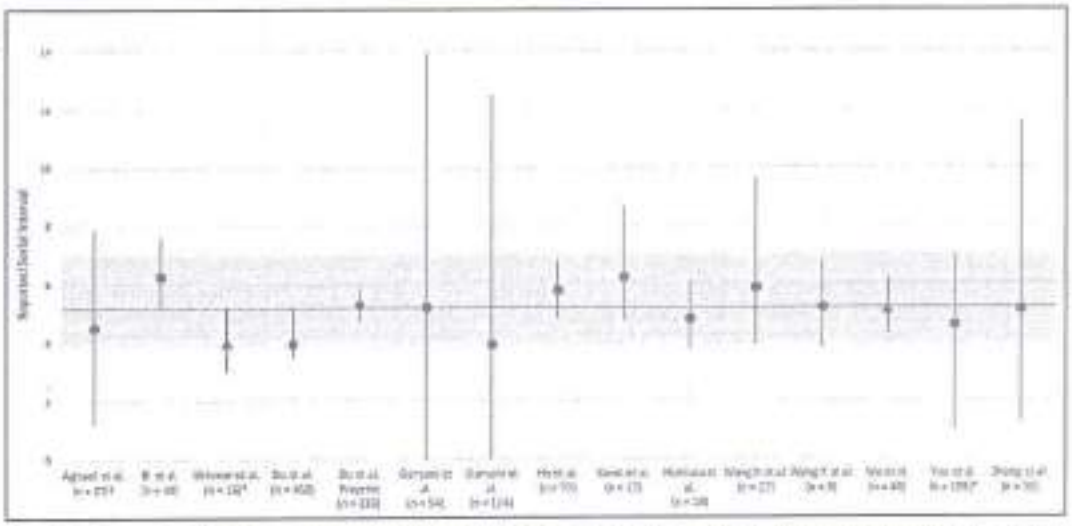

Figure 3. Green dotted line shows the reported mean incubation period of 5.2 days. Green shaded area shows 95% CI of incubation period as reported by Li *et al.* We preferentially reported the mean serial interval (red circle). If mean was not reported, median was used (red triangle). However, it should be noted that in skewed distributions such as gamma and lognormal, median is often less than mean. In the case of Wu *et al.* the mean was noted as 6.3, but no error terms were reported, therefore median was used in the figure. Error bars default to show 95% CI on serial interval on statistic, however if 95% CI was not reported, 1st and 3rd quartiles were used (denoted by *) or +/- 1 standard deviation (denoted by †). Error bars that extended below zero were not shown but are reported in supplemental Table 2. The two studies from Du *et al.* may use overlapping data, and if so, these serial intervals cannot be considered independently.

Mean serial interval was preferentially reported for the studies in this review and is denoted as a red circle on the graph of Figure 3. When mean was not reported, median was used (denoted with a red triangle). It is important to note that in skewed distributions such as gamma and lognormal, median is often less than mean. Despite the various possible sources of error and bias, it is notable that almost all of the studies have calculated serial intervals that fall within the 95% CI of the estimated incubation period as reported by Li and colleagues.[38] This finding is compatible with the hypothesis that infectiousness appears to

emerge at symptom onset. This interpretation is qualitative and should be revisited through meta-analysis and further study.

Discussion

This review focused on primary publications that reported asymptomatic and presymptomatic transmission through case reports, viral kinetics studies, and serial interval calculations. These different approaches have methodological shortcomings, which are summarized in Table 4.

Case Studies

While case studies, in aggregate, can offer compelling insight into the existence of asymptomatic and presymptomatic transmission, these reports have many limitations. Even if broad community transmission is not observed, it is still extremely difficult to rule out other sources of infection. Future studies should use viral sequence to more precisely determine sources of infection and transmission chains. Additionally, the temporal variation in what is classified as a symptom of COVID-19, combined with bias and reporting errors, make anecdotal reports of symptom start date unreliable. These factors confound the case reports that highlight asymptomatic or presymptomatic transmission and make it difficult to draw reliable conclusions.

Viral Dynamics

The preliminary SARS-CoV-2 viral dynamics studies demonstrate that viral titer peaks at patient presentation. Without more knowledge of the temporal distribution of viral load, presymptomatic transmission cannot be conclusively shown. In interpreting viral dynamic studies, a sharp rise in viral load, as would be observed if viral load followed a lognormal or gamma distribution, may link infectiousness with the start of symptom onset. On the other hand, a normal or Weibull distribution in viral load supports the possibility of presymptomatic transmission. It is important that the viral dynamics data be validated with culture data on infectivity. As Wolfel and colleagues demonstrated, while viral load is a proxy for infectivity and transmissibility, it is not perfectly correlated.

Nasopharyngeal swabs are an imperfect proxy for viral production, and any study on viral dynamics must account for high levels of variability in swab samples. Future research efforts should focus on other methods of virus harvesting including throat, blood, fecal, or urine samples, and must prioritize quantifying viral load from individuals in the presymptomatic stage.

Serial Interval

The shape of the distribution has the most direct impact on studies attempting to measure serial interval between successive generation of cases. Differences in the assumption about the distribution of the viral load curves can alter the calculation of how much presymptomatic transmission is occurring. Articles measuring serial interval in this review assumed Weibull, gamma, lognormal, and normal distributions. Furthermore, serial interval calculations in the reported literature rely heavily on cases of household transmission. It is not possible to differentiate an observation of shortened serial interval due to presymptomatic transmission from a decreased incubation period due to higher inoculum in household transmission.

This systematic review attempted to comprehensively document and analyze literature on asymptomatic and presymptomatic transmission of SARS-CoV-2. It is worth noting, especially because of the rapidly evolving nature of the COVID-19 pandemic, there is likely a risk of systemic bias in manuscripts published on this topic. Although all efforts were made to include a comprehensive review of the literature, the rapid progression and influx of new publications, as well as prevalence of preprint manuscripts on this topic, mean this literature review was likely affected by incomplete retrieval of identified research.

Shortcoming	Description	Type of Study Most Effected
Studying non representative populations	Age, chronic illness, and other factors can impact perception and reporting of symptoms. For example, it is difficult to recognize early signs and symptoms of respiratory viral infections in elderly populations, due to impaired immune responses associated with aging and the high prevalence of preexisting and underlying conditions, such as chronic cough and cognitive impairments. Furthermore, elderly and infirm patients have blunted physiological responses that may allow them to remain asymptomatic during infection. On the other hand, younger individuals may be more likely to remain asymptomatic. Studies can only represent the demographic they study.	All studies
Small sample size	Small sample sizes are more subject to bias and skewed results.	All studies
Errors when recalling or reporting symptom onset date	Many studies of transmission and serial interval recall on patients self-reported symptom onset date. Recall bias and other errors can alter when an individual reports symptom onset date.	Case reports and serial interval
Errors in determining sources of infection	In case report and serial interval studies it is impossible to rule out other sources of infection. This confounds determining if presymptomatic transmission occurred. Future studies can use viral genome sequence to better determine source of infection.	Case reports and serial interval
Varying definition of symptoms	What is considered a symptom varies by region, culture, age, and time. In February, symptoms of COVID-19 included fever, dry cough, fatigue, sputum production, shortness of breath, sore throat, headache, myalgia or arthralgia, chills, nausea or vomiting, nasal congestion, diarrhea, hemoptysis, and conjunctival congestion. In April, the WHO added loss of smell or taste as well as rash and skin discolorations of fingers and toes as additional symptoms of COVID-19.	Case reports, and serial interval
Household transmission altering incubation period	In household transmission cases, newly infected individuals will likely be exposed to a much higher dose of viral particulates than would occur in a more casual transmission case. Exposure to higher inoculum may result in a decreased incubation period for household transmission.	Serial interval
Effect of treatment on viral kinetics	Undergoing antiviral, interferon, or steroid therapy may disrupt the natural progression of viral load. Antiviral and interferon treatments should diminish viral replication and artificially cause viral load to peak at the start of treatment, while steroid treatment may dampen the immune response and potentially cause viral replication to increase. If the viral load data is a basis for clinical decision making, this will even further confound results because an increasing viral load would be the basis for more extensive interventions and therapeutic treatment.	Viral Dynamics
Using RT-PCR test as a proxy for infectiousness	RT-PCR testing informs clinicians whether there is detectable virus present, but it cannot determine whether an individual is contagious. Infectivity in cell culture is the standard for determining whether a patient is infectious, but even this is a proxy for transmissibility. Currently, it is believed a Ct value below 24 is the threshold for being infectious.	All studies
Inferences about viral load distribution before samples collected.	The finding that viral load is highest around the time symptoms are detected in patients suggests presymptomatic transmission is plausible. However, there is not enough information about the distribution of SARS-CoV-2 viral kinetics in presymptomatic stage to infer when infectiousness begins. Basic assumptions about the distribution will have dramatic effects on our prediction of when infectivity begins, and the specific time between symptom onset and viral load tests can dramatically change our understanding of transmissibility and infectiousness.	Viral dynamics, and serial interval
Not measuring viral load in presymptomatic stage	Viral loads generally appeared at their highest levels when observation in the clinical setting began. Therefore, authors have concluded viral loads peak when symptoms emerge. However, Zhang *et al.* has shown that multiple days elapse between symptom onset and seeking clinical care. This makes it even more difficult to extrapolate viral peak and presymptomatic viral dynamics. Without measuring viral load in the presymptomatic phase, the dynamics during the presymptomatic period can only be hypothesized.	Viral Dynamics
Rounding errors during calculation of incubation period and serial intervals	The datasets from the papers in this review that measured serial interval rounded the date of symptom onset to the nearest day. This is problematic because the difference in serial interval and incubation period calculated in these studies often differed by less than a day. It is therefore difficult to know if the difference between calculated serial interval and incubation period are true differences, or an artefact of rounding error.	Viral dynamics, and serial interval
Sampling errors in nasopharyngeal swabs	Nasopharyngeal swabs are an imperfect proxy for viral production. Studies on influenza have shown variability in viral load when sampling left and right nostrils and similar findings will be found in SARS-CoV-2. Any study on viral dynamics must account for high levels of variability in swab samples.	Viral Dynamics

Table 4. Summary of the major sources of error observed in the literature that was reviewed in this analysis.

Proposed study to characterize presymptomatic transmission

In order to ascertain the temporal viral dynamics and transmissibility of SARS-CoV-2, it is important to study a representative healthy population before, during, and after SARS-CoV-2 infection. It is essential to combine RT-PCR data with viral culturing data to ascertain transmissibility. In particular, such a study would clarify when viral load and transmissibility commence relative to the time of infection, and peak relative to the onset of symptoms. As well as provide insight into the relationship between viral load or Ct and the severity of symptoms. Additionally, this type of study would be instrumental in determining the most appropriate distribution curve to characterize the rise and decline of viral infectivity.

This study needs to involve a sufficient number of volunteers tested at frequent intervals to obtain a clear answer. Samples need to be collected in a consistent manner using the most reliable available tests. Because of the logistics of such a study, it would be valuable to collect additional information regarding subject demographic features as well as biochemical, immunological, and genetic markers that may be predictive of viral dynamics and transmissibility. Of particular interest will be the impact of age on viral load and infectivity. Among infected individuals, the additional determination of viral genomic sequences would allow for molecular epidemiological analysis of transmission between specific individuals as well as determine any differences in viral load profiles due to mutations in the virus.

One way to accelerate the determination of viral kinetics is to focus on a population with high risk of infection and low risk of complications, such as workers in factory at the start of an outbreak, or individuals identified through contact tracing. This population would be ideal to study because these individuals would likely not seek treatment for SARS-CoV-2 infection, therefore the viral dynamics data would not be confounded by therapeutic interventions like antiviral therapy.

While many of the research studies highlighted in this review have supported asymptomatic and presymptomatic transmission, these studies have been inadequate to ascertain the contribution of asymptomatic and presymptomatic transmission in the spread of SARS-CoV-2 infection. Understanding the temporal dynamics of SARS-CoV-2 transmission from asymptomatic and presymptomatic individuals is critical to our efforts to formulate effective and efficient policies to curtail the pandemic and to minimize the risks associated with phased reopening.

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EXHIBIT "O"

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Transmission of SARS-CoV-2: implications for infection prevention precautions

Source: World Health Organization

Link: https://www.who.int/news-room/commentaries/detail/transmission-of-sars-cov-2-implications-for-infection-prevention-precautions

Published on: 9 July 2020

Scientific Brief

This document is an update to the scientific brief published on 29 March 2020 entitled "Modes of transmission of virus causing COVID-19: implications for infection prevention and control (IPC) precaution recommendations" and includes new scientific evidence available on transmission of SARS-CoV-2, the virus that causes COVID-19.

Overview

This scientific brief provides an overview of the modes of transmission of SARS-CoV-2, what is known about when infected people transmit the virus, and the implications for infection prevention and control precautions within and outside health facilities. This scientific brief is not a systematic review. Rather, it reflects the consolidation of rapid reviews of publications in peer-reviewed journals and of non-peer-reviewed manuscripts on pre-print servers, undertaken by WHO and partners. Preprint findings should be interpreted with caution in the absence of peer review. This brief is also informed by several discussions via teleconferences with the WHO Health Emergencies Programme ad hoc

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Experts Advisory Panel for IPC Preparedness, Readiness and Response to COVID-19, the WHO ad hoc COVID-19 IPC Guidance Development Group (COVID-19 IPC GDG), and by review of external experts with relevant technical backgrounds.

The overarching aim of the global Strategic Preparedness and Response Plan for COVID-19(1) is to control COVID-19 by suppressing transmission of the virus and preventing associated illness and death. Current evidence suggests that SARS-CoV-2, the virus that causes COVID-19, is predominantly spread from person-to-person. Understanding how, when and in what types of settings SARS-CoV-2 spreads is critical to develop effective public health and infection prevention and control measures to break chains of transmission.

Modes of transmission

This section briefly describes possible modes of transmission for SARS-CoV-2, including contact, droplet, airborne, fomite, fecal-oral, bloodborne, mother-to-child, and animal-to-human transmission. Infection with SARS-CoV-2 primarily causes respiratory illness ranging from mild disease to severe disease and death, and some people infected with the virus never develop symptoms.

Contact and droplet transmission

Transmission of SARS-CoV-2 can occur through direct, indirect, or close contact with infected people through infected secretions such as saliva and respiratory secretions or their respiratory droplets, which are expelled when an infected person coughs, sneezes, talks or sings.(2-10) Respiratory droplets are >5-10 μm in diameter whereas droplets ≤5μm in diameter are referred to as droplet nuclei or aerosols.(11) Respiratory droplet transmission can occur when a person is in close contact (within 1 metre) with an infected person who has respiratory symptoms (e.g. coughing or sneezing) or who is talking or singing;

in these circumstances, respiratory droplets that include virus can reach the mouth, nose or eyes of a susceptible person and can result in infection. Indirect contact transmission involving contact of a susceptible host with a contaminated object or surface (fomite transmission) may also be possible (see below).

Airborne transmission

Airborne transmission is defined as the spread of an infectious agent caused by the dissemination of droplet nuclei (aerosols) that remain infectious when suspended in air over long distances and time.(11) Airborne transmission of SARS-CoV-2 can occur during medical procedures that generate aerosols ("aerosol generating procedures").(12) WHO, together with the scientific community, has been actively discussing and evaluating whether SARS-CoV-2 may also spread through aerosols in the absence of aerosol generating procedures, particularly in indoor settings with poor ventilation.

The physics of exhaled air and flow physics have generated hypotheses about possible mechanisms of SARS-CoV-2 transmission through aerosols.(13-16) These theories suggest that 1) a number of respiratory droplets generate microscopic aerosols (<5 μm) by evaporating, and 2) normal breathing and talking results in exhaled aerosols. Thus, a susceptible person could inhale aerosols, and could become infected if the aerosols contain the virus in sufficient quantity to cause infection within the recipient. However, the proportion of exhaled droplet nuclei or of respiratory droplets that evaporate to generate aerosols, and the infectious dose of viable SARS-CoV-2 required to cause infection in another person are not known, but it has been studied for other respiratory viruses.(17)

One experimental study quantified the amount of droplets of various sizes that remain airborne during normal speech. However, the authors acknowledge that

this relies on the independent action hypothesis, which has not been validated for humans and SARS-CoV-2.(18) Another recent experimental model found that healthy individuals can produce aerosols through coughing and talking (19), and another model suggested high variability between individuals in terms of particle emission rates during speech, with increased rates correlated with increased amplitude of vocalization.(20) To date, transmission of SARS-CoV-2 by this type of aerosol route has not been demonstrated; much more research is needed given the possible implications of such route of transmission.

Experimental studies have generated aerosols of infectious samples using high-powered jet nebulizers under controlled laboratory conditions. These studies found SARS-CoV-2 virus RNA in air samples within aerosols for up to 3 hours in one study (21) and 16 hours in another, which also found viable replication-competent virus.(22) These findings were from experimentally induced aerosols that do not reflect normal human cough conditions.

Some studies conducted in health care settings where symptomatic COVID-19 patients were cared for, but where aerosol generating procedures were not performed, reported the presence of SARS-CoV-2 RNA in air samples (23-28), while other similar investigations in both health care and non-health care settings found no presence of SARS-CoV-2 RNA; no studies have found viable virus in air samples.(29-36) Within samples where SARS-CoV-2 RNA was found, the quantity of RNA detected was in extremely low numbers in large volumes of air and one study that found SARS-CoV-2 RNA in air samples reported inability to identify viable virus. (25) The detection of RNA using reverse transcription polymerase chain reaction (RT-PCR)-based assays is not necessarily indicative of replication- and infection-competent (viable) virus that could be transmissible and capable of causing infection.(37)

Recent clinical reports of health workers exposed to COVID-19 index cases, not in the presence of aerosol-generating procedures, found no nosocomial transmission when contact and droplet precautions were appropriately used, including the wearing of medical masks as a component of the personal protective equipment (PPE). *(38, 39)* These observations suggest that aerosol transmission did not occur in this context. Further studies are needed to determine whether it is possible to detect viable SARS-CoV-2 in air samples from settings where no procedures that generate aerosols are performed and what role aerosols might play in transmission.

Outside of medical facilities, some outbreak reports related to indoor crowded spaces *(40)* have suggested the possibility of aerosol transmission, combined with droplet transmission, for example, during choir practice *(7)*, in restaurants *(41)* or in fitness classes.*(42)* In these events, short-range aerosol transmission, particularly in specific indoor locations, such as crowded and inadequately ventilated spaces over a prolonged period of time with infected persons cannot be ruled out. However, the detailed investigations of these clusters suggest that droplet and fomite transmission could also explain human-to-human transmission within these clusters. Further, the close contact environments of these clusters may have facilitated transmission from a small number of cases to many other people (e.g., superspreading event), especially if hand hygiene was not performed and masks were not used when physical distancing was not maintained.*(43)*

Fomite transmission

Respiratory secretions or droplets expelled by infected individuals can contaminate surfaces and objects, creating fomites (contaminated surfaces). Viable SARS-CoV-2 virus and/or RNA detected by RT-PCR can be found on those surfaces for periods ranging from hours to days, depending on the ambient

environment (including temperature and humidity) and the type of surface, in particular at high concentration in health care facilities where COVID-19 patients were being treated.(21, 23, 24, 26, 28, 31-33, 36, 44, 45) Therefore, transmission may also occur indirectly through touching surfaces in the immediate environment or objects contaminated with virus from an infected person (e.g. stethoscope or thermometer), followed by touching the mouth, nose, or eyes.

Despite consistent evidence as to SARS-CoV-2 contamination of surfaces and the survival of the virus on certain surfaces, there are no specific reports which have directly demonstrated fomite transmission. People who come into contact with potentially infectious surfaces often also have close contact with the infectious person, making the distinction between respiratory droplet and fomite transmission difficult to discern. However, fomite transmission is considered a likely mode of transmission for SARS-CoV-2, given consistent findings about environmental contamination in the vicinity of infected cases and the fact that other coronaviruses and respiratory viruses can transmit this way.

Other modes of transmission

SARS-CoV-2 RNA has also been detected in other biological samples, including the urine and feces of some patients.(46-50)One study found viable SARS-CoV-2 in the urine of one patient.(51)Three studies have cultured SARS-CoV-2 from stool specimens. (48, 52, 53) To date, however, there have been no published reports of transmission of SARS-CoV-2 through feces or urine.

Some studies have reported detection of SARS-CoV-2 RNA, in either plasma or serum, and the virus can replicate in blood cells. However, the role of bloodborne transmission remains uncertain; and low viral titers in plasma and serum suggest that the risk of transmission through this route may be

low.(48, 54) Currently, there is no evidence for intrauterine transmission of SARS-CoV-2 from infected pregnant women to their fetuses, although data remain limited. WHO has recently published a scientific brief on breastfeeding and COVID-19.(55) This brief explains that viral RNA fragments have been found by RT-PCR testing in a few breast milk samples of mothers infected with SARS-CoV-2, but studies investigating whether the virus could be isolated, have found no viable virus. Transmission of SARS-CoV-2 from mother to child would necessitate replicative and infectious virus in breast milk being able to reach target sites in the infant and also to overcome infant defense systems. WHO recommends that mothers with suspected or confirmed COVID-19 should be encouraged to initiate or continue to breastfeed.(55)

Evidence to date shows that SARS-CoV-2 is most closely related to known betacoronaviruses in bats; the role of an intermediate host in facilitating transmission in the earliest known human cases remains unclear.(56, 57) In addition to investigations on the possible intermediate host(s) of SARS-CoV-2, there are also a number of studies underway to better understand susceptibility of SARS-CoV-2 in different animal species. Current evidence suggests that humans infected with SARS-CoV-2 can infect other mammals, including dogs(58), cats(59), and farmed mink.(60) However, it remains unclear if these infected mammals pose a significant risk for transmission to humans.

When do people infected with SARS-CoV-2 infect others?

Knowing when an infected person can spread SARS-CoV-2 is just as important as how the virus spreads (described above). WHO has recently published a scientific brief outlining what is known about when a person may be able to spread, based on the severity of their illness.(61)

In brief, evidence suggests that SARS-CoV-2 RNA can be detected in people 1-3 days before their symptom onset, with the highest viral loads, as measured by RT-PCR, observed around the day of symptom onset, followed by a gradual decline over time.*(47, 62-65)* The duration of RT-PCR positivity generally appears to be 1-2 weeks for asymptomatic persons, and up to 3 weeks or more for patients with mild to moderate disease.*(62, 65-68)* In patients with severe COVID-19 disease, it can be much longer.*(47)*

Detection of viral RNA does not necessarily mean that a person is infectious and able to transmit the virus to another person. Studies using viral culture of patient samples to assess the presence of infectious SARS-CoV-2 are currently limited. *(61)* Briefly, viable virus has been isolated from an asymptomatic case,*(69)* from patients with mild to moderate disease up to 8-9 days after symptom onset, and for longer from severely ill patients.*(61)* Full details about the duration of viral shedding can be found in the WHO guidance document on "Criteria for releasing COVID-19 patients from isolation". *(61)* Additional studies are needed to determine the duration of viable virus shedding among infected patients.

SARS-CoV-2 infected persons who have symptoms can infect others primarily through droplets and close contact

SARS-CoV-2 transmission appears to mainly be spread via droplets and close contact with infected symptomatic cases. In an analysis of 75,465 COVID-19 cases in China, 78-85% of clusters occurred within household settings, suggesting that transmission occurs during close and prolonged contact.*(6)* A study of the first patients in the Republic of Korea showed that 9 of 13 secondary cases occurred among household contacts.*(70)* Outside of the household setting, those who had close physical contact, shared meals, or were in enclosed spaces for approximately one hour or more with symptomatic cases,

such as in places of worship, gyms, or the workplace, were also at increased risk of infection.*(7, 42, 71, 72)* Other reports have supported this with similar findings of secondary transmission within families in other countries.*(73, 74)*

SARS-CoV-2 infected persons without symptoms can also infect others

Early data from China suggested that people without symptoms could infect others.*(6)* To better understand the role of transmission from infected people without symptoms, it is important to distinguish between transmission from people who are infected who never develop symptoms*(75)* (asymptomatic transmission) and transmission from people who are infected but have not developed symptoms yet (pre-symptomatic transmission). This distinction is important when developing public health strategies to control transmission.

The extent of truly asymptomatic infection in the community remains unknown. The proportion of people whose infection is asymptomatic likely varies with age due to the increasing prevalence of underlying conditions in older age groups (and thus increasing risk of developing severe disease with increasing age), and studies that show that children are less likely to show clinical symptoms compared to adults.*(76)* Early studies from the United States *(77)* and China *(78)* reported that many cases were asymptomatic, based on the lack of symptoms at the time of testing; however, 75-100% of these people later developed symptoms. A recent systematic review estimated that the proportion of truly asymptomatic cases ranges from 6% to 41%, with a pooled estimate of 16% (12%–20%).*(79)* However, all studies included in this systematic review have important limitations.*(79)* For example, some studies did not clearly describe how they followed up with persons who were asymptomatic at the time of testing to ascertain if they ever developed symptoms, and others defined "asymptomatic" very narrowly as persons who never developed fever or respiratory symptoms, rather than as those who did not develop any symptoms.

at all.(76, 80) A recent study from China that clearly and appropriately defined asymptomatic infections suggests that the proportion of infected people who never developed symptoms was 23%.(81)

Multiple studies have shown that people infect others before they themselves became ill, (10, 42, 69, 82, 83) which is supported by available viral shedding data (see above). One study of transmission in Singapore reported that 6.4% of secondary cases resulted from pre-symptomatic transmission.(73) One modelling study, that inferred the date of transmission based on the estimated serial interval and incubation period, estimated that up to 44% (25-69%) of transmission may have occurred just before symptoms appeared.(62) It remains unclear why the magnitude of estimates from modelling studies differs from available empirical data.

Transmission from infected people without symptoms is difficult to study. However, information can be gathered from detailed contact tracing efforts, as well as epidemiologic investigations among cases and contacts. Information from contact tracing efforts reported to WHO by Member States, available transmission studies and a recent pre-print systematic reviews suggests that individuals without symptoms are less likely to transmit the virus than those who develop symptoms.(10, 81, 84, 85) Four individual studies from Brunei, Guangzhou China, Taiwan China and the Republic of Korea found that between 0% and 2.2% of people with asymptomatic infection infected anyone else, compared to 0.8%-15.4% of people with symptoms.(10, 72, 86, 87)

Remaining questions related to transmission

Many unanswered questions about transmission of SARS-CoV-2 remain, and research seeking to answer those questions is ongoing and is encouraged. Current evidence suggests that SARS-CoV-2 is primarily transmitted between

people via respiratory droplets and contact routes – although aerosolization in medical settings where aerosol generating procedures are used is also another possible mode of transmission - and that transmission of COVID-19 is occurring from people who are pre-symptomatic or symptomatic to others in close contact (direct physical or face-to-face contact with a probable or confirmed case within one meter and for prolonged periods of time), when not wearing appropriate PPE. Transmission can also occur from people who are infected and remain asymptomatic, but the extent to which this occurs is not fully understood and requires further research as an urgent priority. The role and extent of airborne transmission outside of health care facilities, and in particular in close settings with poor ventilation, also requires further study.

As research continues, we expect to gain a better understanding about the relative importance of different transmission routes, including through droplets, physical contact and fomites; the role of airborne transmission in the absence of aerosol generating procedures; the dose of virus required for transmission to occur, the characteristics of people and situations that facilitate superspreading events such as those observed in various closed settings, the proportion of infected people who remain asymptomatic throughout the course of their infection; the proportion of truly asymptomatic persons who transmit the virus to others; the specific factors that drive asymptomatic and pre-symptomatic transmission; and the proportion of all infections that are transmitted from asymptomatic and pre-symptomatic individuals.

Implications for preventing transmission

Understanding how, when and in which settings infected people transmit the virus is important for developing and implementing control measures to break chains of transmission. While there is a great deal of scientific studies becoming available, all studies that investigate transmission should be interpreted bearing

in mind the context and settings in which they took place, including the infection prevention interventions in place, the rigor of the methods used in the investigation and the limitations and biases of the study designs.

It is clear from available evidence and experience, that limiting close contact between infected people and others is central to breaking chains of transmission of the virus causing COVID-19. The prevention of transmission is best achieved by identifying suspect cases as quickly as possible, testing, and isolating infectious cases. *(88, 89)* In addition, it is critical to identify all close contacts of infected people *(88)* so that they can be quarantined *(90)* to limit onward spread and break chains of transmission. By quarantining close contacts, potential secondary cases will already be separated from others before they develop symptoms or they start shedding virus if they are infected, thus preventing the opportunity for further onward spread. The incubation period of COVID-19, which is the time between exposure to the virus and symptom onset, is on average 5-6 days, but can be as long as 14 days. *(82, 91)* Thus, quarantine should be in place for 14 days from the last exposure to a confirmed case. If it is not possible for a contact to quarantine in a separate living space, self-quarantine for 14 days at home is required; those in self-quarantine may require support during the use of physical distancing measures to prevent the spread of the virus.

Given that infected people without symptoms can transmit the virus, it is also prudent to encourage the use of fabric face masks in public places where there is community transmission[1] and where other prevention measures, such as physical distancing, are not possible.*(12)* Fabric masks, if made and worn properly, can serve as a barrier to droplets expelled from the wearer into the air and environment.*(12)* However, masks must be used as part of a comprehensive package of preventive measures, which includes frequent hand hygiene, physical distancing when possible, respiratory etiquette, environmental cleaning

and disinfection. Recommended precautions also include avoiding indoor crowded gatherings as much as possible, in particular when physical distancing is not feasible, and ensuring good environmental ventilation in any closed setting. *(92, 93)*

Within health care facilities, including long term care facilities, based on the evidence and the advice by the COVID-19 IPC GDG, WHO continues to recommend droplet and contact precautions when caring for COVID-19 patients and airborne precautions when and where aerosol generating procedures are performed. WHO also recommends standard or transmission-based precautions for other patients using an approach guided by risk assessment.*(94)* These recommendations are consistent with other national and international guidelines, including those developed by the European Society of Intensive Care Medicine and Society of Critical Care Medicine *(95)* and by the Infectious Diseases Society of America. *(96)*

Furthermore, in areas with COVID-19 community transmission, WHO advises that health workers and caregivers working in clinical areas should continuously wear a medical mask during all routine activities throughout the entire shift.*(12)* In settings where aerosol-generating procedures are performed, they should wear an N95, FFP2 or FFP3 respirator. Other countries and organizations, including the United States Centers for Diseases Control and Prevention *(97)* and the European Centre for Disease Prevention and Control *(98)* recommend airborne precautions for any situation involving the care of COVID-19 patients. However, they also consider the use of medical masks as an acceptable option in case of shortages of respirators.

WHO guidance also emphasizes the importance of administrative and engineering controls in health care settings, as well as rational and appropriate use of all PPE *(99)* and training for staff on these recommendations (IPC for

Novel Coronavirus [COVID-19] Course. Geneva; World Health Organization 2020, available at (https://openwho.org/courses/COVID-19-IPC-EN). WHO has also provided guidance on safe workplaces. *(92)*

Key points of the brief

Main findings

- Understanding how, when and in what types of settings SARS-CoV-2 spreads between people is critical to develop effective public health and infection prevention measures to break chains of transmission.
- Current evidence suggests that transmission of SARS-CoV-2 occurs primarily between people through direct, indirect, or close contact with infected people through infected secretions such as saliva and respiratory secretions, or through their respiratory droplets, which are expelled when an infected person coughs, sneezes, talks or sings.
- Airborne transmission of the virus can occur in health care settings where specific medical procedures, called aerosol generating procedures, generate very small droplets called aerosols. Some outbreak reports related to indoor crowded spaces have suggested the possibility of aerosol transmission, combined with droplet transmission, for example, during choir practice, in restaurants or in fitness classes.
- Respiratory droplets from infected individuals can also land on objects, creating fomites (contaminated surfaces). As environmental contamination has been documented by many reports, it is likely that people can also be infected by touching these surfaces and touching their eyes, nose or mouth before cleaning their hands.
- Based on what we currently know, transmission of COVID-19 is primarily occurring from people when they have symptoms, and can also occur just before they develop symptoms, when they are in close proximity to others

for prolonged periods of time. While someone who never develops symptoms can also pass the virus to others, it is still not clear to what extent this occurs and more research is needed in this area.

- Urgent high-quality research is needed to elucidate the relative importance of different transmission routes; the role of airborne transmission in the absence of aerosol generating procedures; the dose of virus required for transmission to occur; the settings and risk factors for superspreading events; and the extent of asymptomatic and pre-symptomatic transmission.

How to prevent transmission

The overarching aim of the Strategic Preparedness and Response Plan for COVID-19*(1)* is to control COVID-19 by suppressing transmission of the virus and preventing associated illness and death. To the best of our understanding, the virus is primarily spread through contact and respiratory droplets. Under some circumstances airborne transmission may occur (such as when aerosol generating procedures are conducted in health care settings or potentially, in indoor crowded poorly ventilated settings elsewhere). More studies are urgently needed to investigate such instances and assess their actual significance for transmission of COVID-19.

To prevent transmission, WHO recommends a comprehensive set of measures including:

- Identify suspect cases as quickly as possible, test, and isolate all cases (infected people) in appropriate facilities;
- Identify and quarantine all close contacts of infected people and test those who develop symptoms so that they can be isolated if they are infected and require care;

- Use fabric masks in specific situations, for example, in public places where there is community transmission and where other prevention measures, such as physical distancing, are not possible;
- Use of contact and droplet precautions by health workers caring for suspected and confirmed COVID-19 patients, and use of airborne precautions when aerosol generating procedures are performed;
- Continuous use of a medical mask by health workers and caregivers working in all clinical areas, during all routine activities throughout the entire shift;
- At all times, practice frequent hand hygiene, physical distancing from others when possible, and respiratory etiquette; avoid crowded places, close-contact settings and confined and enclosed spaces with poor ventilation; wear fabric masks when in closed, overcrowded spaces to protect others; and ensure good environmental ventilation in all closed settings and appropriate environmental cleaning and disinfection.

WHO carefully monitors the emerging evidence about this critical topic and will update this scientific brief as more information becomes available.

[1]Defined by WHO as "experiencing larger outbreaks of local transmission defined through an assessment of factors including, but not limited to: large numbers of cases not linkable to transmission chains; large numbers of cases from sentinel surveillance; and/or multiple unrelated clusters in several areas of the country/territory/area" (https://www.who.int/publications-detail/global-surveillance-for-covid-19-caused-by-human-infection-with-covid-19-virus-interim-guidance)

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M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
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EXHIBIT "P"

PLOS | PATHOGENS

RESEARCH ARTICLE

The blood DNA virome in 8,000 humans

Ahmed Moustafa[1], Chao Xie[2], Ewen Kirkness[1], William Biggs[1], Emily Wong[1], Yaron Turpaz[2], Kenneth Bloom[3], Eric Delwart[3], Karen E. Nelson[4], J. Craig Venter[1,4]*, Amalio Telenti[1,4]*

1 Human Longevity Inc., San Diego, California, United States of America, 2 Human Longevity Singapore Pte. Ltd., Singapore, 3 Blood Systems Research Institute, Department of Laboratory Medicine, University of California San Francisco, San Francisco, California, United States of America, 4 J. Craig Venter Institute, La Jolla, California, United States of America

* jcventer@jcvi.org (JCV); atelenti@humanlongevity.com (AT)

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Abstract

The characterization of the blood virome is important for the safety of blood-derived transfusion products, and for the identification of emerging pathogens. We explored non-human sequence data from whole-genome sequencing of blood from 8,240 individuals, none of whom were ascertained for any infectious disease. Viral sequences were extracted from the pool of sequence reads that did not map to the human reference genome. Analyses sifted through close to 1 Petabyte of sequence data and performed 0.5 trillion similarity searches. With a lower bound for identification of 2 viral genomes/100,000 cells, we mapped sequences to 94 different viruses, including sequences from 19 human DNA viruses, proviruses and RNA viruses (herpesviruses, anelloviruses, papillomaviruses, three polyomaviruses, adenovirus, HIV, HTLV, hepatitis B, hepatitis C, parvovirus B19, and influenza virus) in 42% of the study participants. Of possible relevance to transfusion medicine, we identified Merkel cell polyomavirus in 49 individuals, papillomavirus in blood of 13 individuals, parvovirus B19 in 6 individuals, and the presence of herpesvirus 8 in 3 individuals. The presence of DNA sequences from two RNA viruses was unexpected: Hepatitis C virus is revealing of an integration event, while the influenza virus sequence resulted from immunization with a DNA vaccine. Age, sex and ancestry contributed significantly to the prevalence of infection. The remaining 75 viruses mostly reflect extensive contamination of commercial reagents and from the environment. These technical problems represent a major challenge for the identification of novel human pathogens. Increasing availability of human whole-genome sequences will contribute substantial amounts of data on the composition of the normal and pathogenic human blood virome. Distinguishing contaminants from real human viruses is challenging.

Author summary

Novel sequencing technologies offer insight into the virome in human samples. Here, we identify the viral DNA sequences in blood of over 8,000 individuals undergoing whole genome sequencing. This approach serves to identify 94 viruses; however, many are shown to reflect widespread DNA contamination of commercial reagents or of environmental

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origin. While this represents a significant limitation to reliably identify novel viruses infecting humans, we could confidently detect sequences and quantify abundance of 19 human viruses in 42% of individuals. Ancestry, sex, and age were important determinants of viral prevalence. This large study calls attention on the challenge of interpreting next generation sequencing data for the identification of novel viruses. However, it serves to categorize the abundance of human DNA viruses using an unbiased technique.

Introduction

Research on the human microbiome has been primarily directed to the prokaryotic composition of the human microflora. Because most of the analyses use 16S rRNA gene-based amplification, the viral content has been rarely captured in large-scale microbiome studies. In contrast, analysis of the whole human genome by next-generation sequencing is an exercise in metagenomics: after mapping sequencing reads to the human reference genome, there is a significant proportion (generally 5% of all sequence data) that is left uncharacterized [1]. Bacterial but also archaea, non-human eukaryotic and viral sequences are thus a by-product of the sequencing of the human genome.

Previous studies of the human virome have addressed the viral component of the gut flora [2–4] and skin [5–7], with particular attention to the very abundant bacteriophages [7, 8]. A thorough review has been published recently [9]. Many viruses are present in peripheral blood —in particular, members of the *Herpesviridae* and *Anelloviridae* families are identified in the absence of disease. Metagenomic studies on blood have identified great genetic diversity of anelloviruses [10–12]. Metagenomic studies also lead to the identification of novel RNA viruses—for example the identification of two rhabdoviruses [13]. Other viral sequences in the blood of healthy individuals are related to members of the Picornaviridae, Poxviridae, Flaviviridae, and Phycodnaviridae families (reviewed in [9]). Finally, a number of viruses, prominently retroviruses, are integrated in the human genome as provirus, while others may integrate occasionally or accidentally [14].

The study of the human virome is particularly relevant in the context of current discussions of next-generation sequencing for surveillance of viruses in blood and for transfusion safety [11, 15, 16]. Only viruses that are both pathogenic and transfusion-transmissible are routinely tested for and excluded from blood-derived products. Rejecting all virus-infected donations irrespective of pathogenicity would not be sustainable as most donors are anellovirus positive. The time required to develop and implement specific virus nucleic acid tests to emerging viral pathogens in the blood supply has greatly improved as seen with the response to recent Zika virus outbreak [17]. Exclusionary steps for viruses can also vary depending on the recipients in whom sequelae may vary in severity such as the use of parvovirus B19-reduced plasma pool to derive products for pregnant B19 seronegative women and immunocompromised patients. Seasonal variation in virus prevalence can also affect when testing is implemented such during mosquitos season for West Nile virus RNA. As the rate of human genome and associated DNA viruses sequenced from blood continues to grow data a baseline will be available to compare rates of infections with various DNA viruses, as described in this study, to that in future populations.

There are many open questions on what could be considered a "normal" human blood virome. Recently, the National Heart, Lung, and Blood Institute of the National Institutes of Health convened a working group on the microbiome that identified studies of the human virome a key priority [18]. The present study aims at establishing the DNA virome in over 8,000 individuals participating in a large-scale sequencing effort of the whole human genome [1].

A careful definition is key to diagnosing infections, to understanding the role of the virome in chronic disease, and for settling claims for the identification of new viral species in humans.

Results

Viral sequences in the unmapped reads

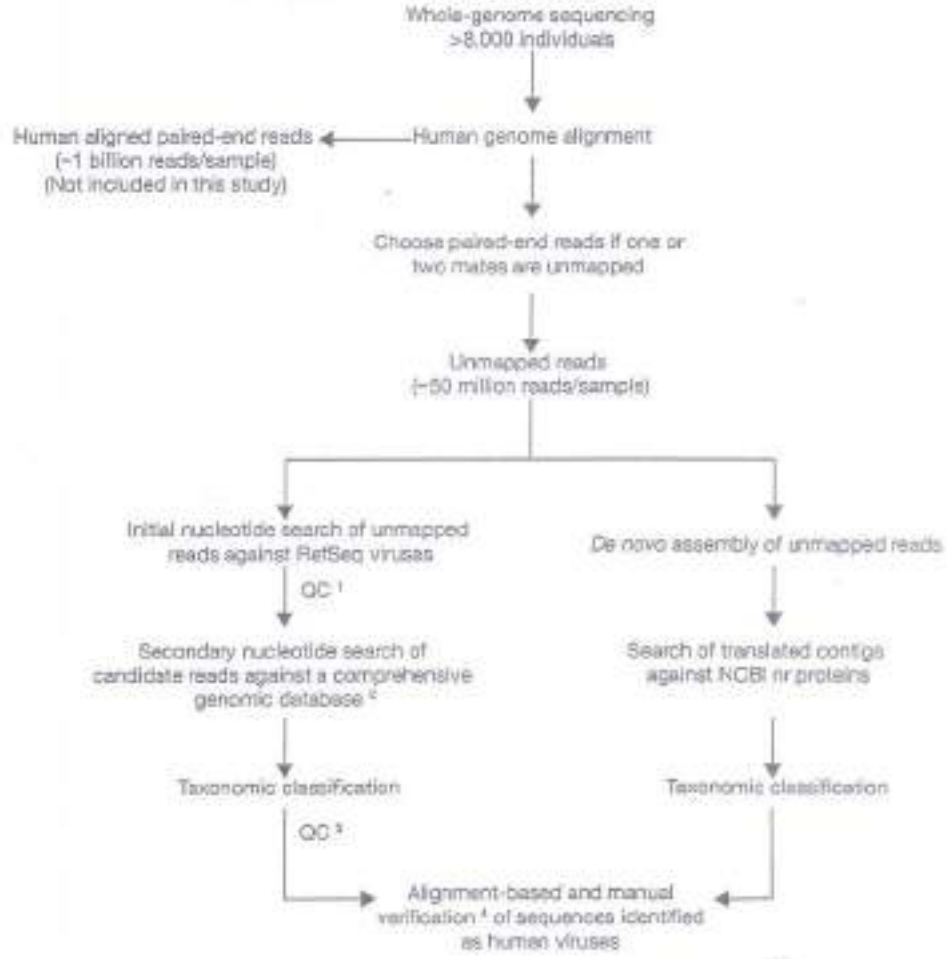
We sequenced the genomes of 8,240 individuals. On average, each sequencing reaction generated 1 billion reads. The total input approached 1 PB. The majority (95%) of reads were successfully mapped (S1 Fig) to the human reference genome GRCh38 (hg38). Among the remaining reads, similarity search assigned 9% to non-reference human sequences, 1% to other primate sequences, 0.2% to bacteria, and 0.01% to viruses. The bulk of unmapped reads mainly represents reads with multiple mappings to the human reference, but also microbial genomes absent in the database, and low quality reads.

We launched 0.5 trillion similarity searches against the NCBI viral genomes (Fig 1). This step mapped sequences to 94 viruses (S1 Table). Samples carried a median of about 400,000 viral reads. However, the majority corresponded to phiX174, used as spike-in control in the sequencing process, or to human endogenous retroviruses (HERV) that are discarded during alignment (Fig 2). Samples that carried phiX174 were also enriched in reads from multiple phages, which we interpret as contamination of the commercial preparation of phiX174. Epstein-Barr virus (EBV, HHV4) reads were abundant in sequences of the human reference genome NA12878 (www.nist.gov/programs-projects/genome-bottle) and in a subset (n = 148) of participant samples where the input DNA material was, in retrospect, from cell lines that use EBV in the process of cell immortalization. Furthermore, we observed cross-contamination from the EBV content in the human genome immortalized cell line NA12878 to other samples on the same flow cell (S2 Fig). The human reference genome NA12878 is used as standard reagent in sequencing laboratories.

In a second step, viral candidate reads were searched against a comprehensive database of viruses, vectors, bacteria, archaea, human, and other eukaryotes to reduce false-positive matches from the initial search. We identified 11% reads that would result from plasmid sequences engineered with sequences such as viral promoters. Therefore, we removed from downstream analysis reads of phiX174 and associated contaminant phages, HERVs, reads from samples containing EBV used in cell immortalization, and EBV reads from samples that were potentially contaminated and plasmids and vectors. Flow cells with high-titer samples of human papilloma virus (HPV) and parvovirus B12 contained other positive samples that were potential false positives (S3 Fig). Single indexing, where the barcodes are embedded in one of the sequencing library adapters, comes with a risk of misidentification of sequences sharing flow cells [19]. The quality control steps are depicted in Fig 1.

We compared the sensitivity of detection of viruses using nucleotide-based search with individual reads versus using protein-based search after *de novo* assembly of reads into contigs and translation (Fig 1 and S4 Fig). The mapping of single reads identified 19 human viruses. In contrast, contigs could only be assembled for 8 viruses because it required the presence of 1 to 4 orders of magnitude more viral reads in the sample (S4 Fig). Overall, viruses were detected by both read mapping or contigs in 137 samples, and only by read mapping in 3,342 samples. Because of the low sensitivity of the approach using contigs, the study proceeded using individual reads.

While it would have been ideal to perform a complete search of translated read-to-translated NCBI nt database using tools such as TBLASTX, this approach would be prohibiting in terms of computational demands. of translated read-to-translated NCBI nt database using tools such as TBLASTX, this approach would be prohibiting in terms of computational demands.



[1]Removal of reads of potential bar-code bleeding from EBV from immortalized cells, reads of the spiking-in control PhiX, and reads matching HERV-K113

[2]Database is composed of RefSeq viruses + representative bacteria and archaea + Genbank vectors and plasmids + UCSC genomes of human and other eukaryotes

[3]Removal of false-positive reads (e.g., vectors and plasmids)

[4]Removal of reads of potential cross-contamination from samples with B19 and HPV16

Fig 1. Study design. The flowchart summarizes the steps followed to identify viral content in the human blood DNA from whole-genome sequencing reads.

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Human DNA virome

Among the 94 different viruses identified in the study materials, we identified viral reads for 19 human viruses (Fig 3 and Table 1). Among the herpesvirus (HHV), HHV7 was found in 20%, and EBV was identified in 14% of the individuals. Analysis of sequence diversity

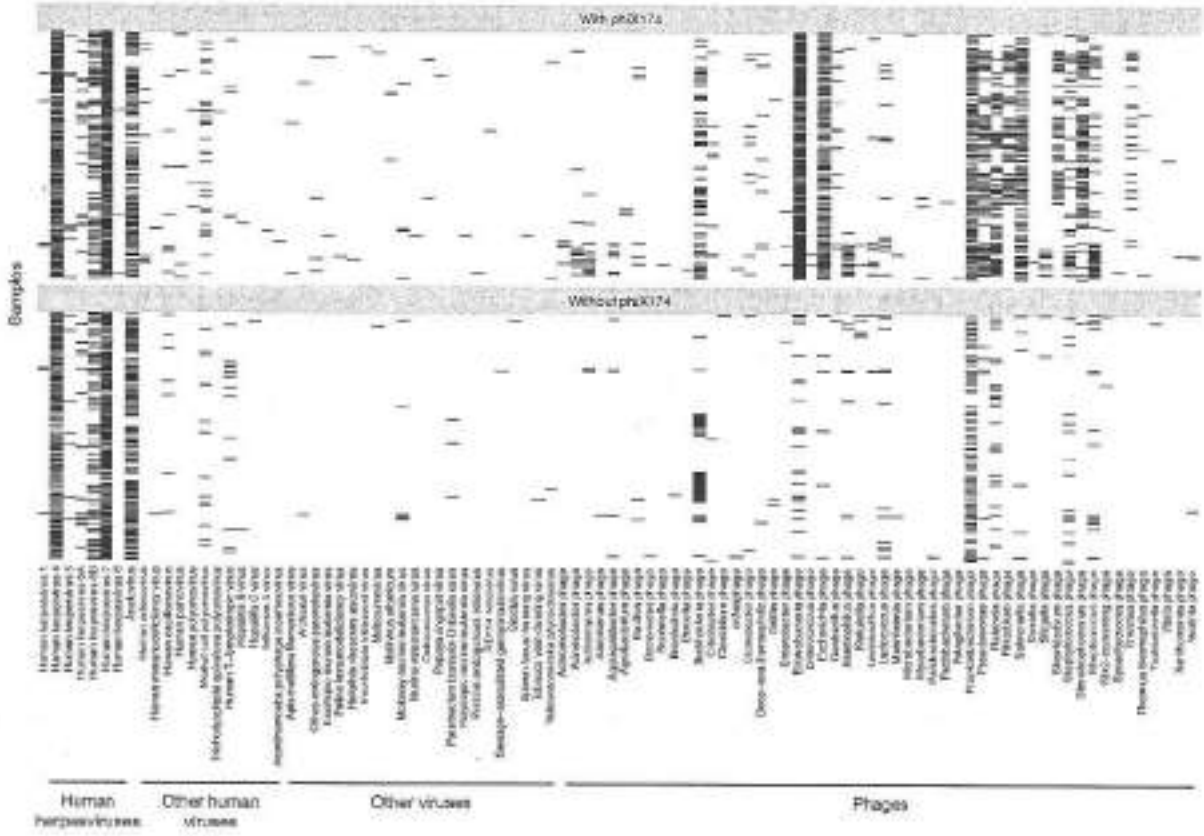

Fig 2. Viral content. The heatmap shows the presence of reads of viral nature in sequencing reactions of blood from 8,240 individuals. Extensive phage and other viral DNA is found in sequencing reactions, but it is almost universally associated to including phiX174 phage spike-in in the reaction (used in 80% of samples). For reference, we include the ubiquitous identification of human endogenous retrovirus (HERVs) sequences in the pool of unmapped reads.

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identified the presence of both EBV subtypes 1 and 2. The estimated proportion was 80% for subtype 1 and 20% for subtype 2, consistent with previous knowledge [28]. HHV6A and HHV6B were identified in 1.5% and 5% of individuals, respectively. We identified fewer individuals carrying sequences of other human herpesviruses: Herpes simplex 1 (HSV1), Cytomegalovirus (CMV, HHV5), and HHV8.

We identified a significant presence of anelloviruses (Torque teno virus [TTV] and TTV-like mini virus [TLMV]) in 9% of the individuals. Other viruses were identified in less that 1% of the study population (Fig 3 and Table 1). We took interest in the presence of sequence reads for papillomavirus (7 different types: 2, 10, 16, 92, 137, 163, and 179) in 17 individuals. Upon validation, we identified a cluster of individuals with the oncogenic type 16 in the same flow cell. We identified the wrong inclusion of a tumor sample in the analysis. This sample corresponded to a head and neck tumor containing large presence of papillomavirus 16 that led to contamination of samples sharing the same flow cell. Parvovirus B19 was identified in 10 individuals; however, four positive samples shared the flow cell with the sample with the highest

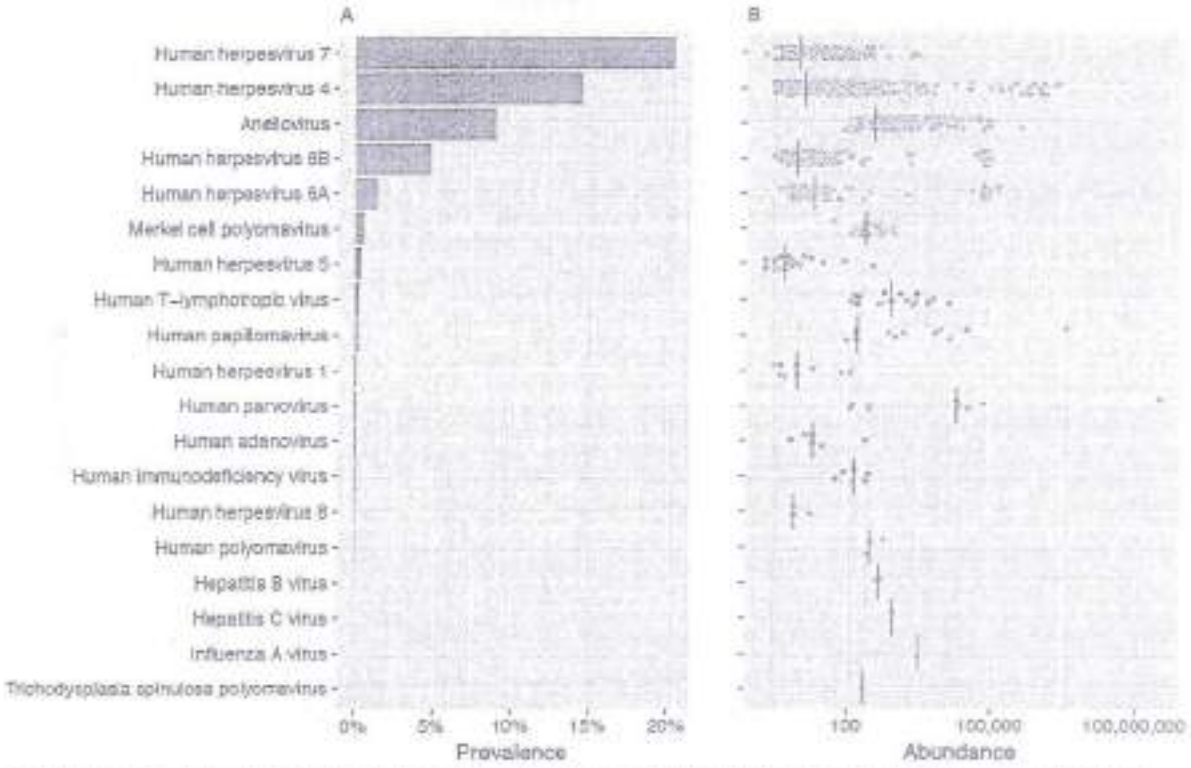

Fig 3. Prevalence and abundance of human DNA viruses and retroviruses in 8,240 individuals. A. Frequency of 19 human viruses in the study population ranked according to their prevalence. B. The viral load of human viruses represented on the x-axis as genome copies per 100,000 human cells; the bar represents the median.

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load of viral copies (> 300 million viral copies/100,000 cells) and where thus classified as contaminants.

We aimed at reconstructing viruses across many samples (Fig 4). The purpose of this step is to provide proof that the viral presence is confirmed by demonstrating broad and average coverage of each viral genome, and not the result of skewed accumulation of local reads—for example at CMV promoters in plasmids. It also offers a detailed view on viral polymorphism and subtypes. This was done for viruses with enough reads or present in numerous individuals, where we could reconstruct the viral genomes with significant coverage (Fig 4). For viruses where only a few reads could be identified, we checked them manually for unambiguous mapping.

Viral integration

HHV6 can integrate in the human genome in telomeric regions and can be inherited through the germline [21]. We identified integrated HHV6A/B in 0.5% of the individuals. Fig 5 depicts the expected binomial distribution where samples with integrated copies have 100, 000 viral copies per 100,000 human cells (one integration event in every cell). The precision of this number attests to the highly quantitative nature of the sequencing protocol. Actual proof of

Table 1. Detected human viruses in blood DNA of 8,240 individuals.

Virus	Number[1] of individuals	Percentage of individuals	Number of sequencing reads per individual		Abundance[2] of viral genomes per individual		Coverage of viral genome	
			Median	Maximum	Median	Maximum	Minimum	Maximum
Human herpesvirus 7 (HHV-7)	1,678	20.37%	2	702	10	2,860	0.001	0.688
Human herpesvirus 4 (HHV-4, EBV)	1,190	14.45%	4	732,061	12	2,404,531	0.001	637.336
Anellovirus (TTV & TLMV)	734	8.91%	2	2,416	359	392,179	0.049	110.236
Human herpesvirus 6B (HHV-6B)	396	4.80%	2	26,738	9	97,274	0.001	24.74
Human herpesvirus 6A (HHV-6A)	121	1.47%	5	38,254	20	134,595	0.001	36.016
Merkel cell polyomavirus (MCPyV)	49	0.59%	2	8	238	935	0.028	0.223
Human herpesvirus 5 (HHV-5, CMV)	29	0.35%	2	106	5	338	0.001	0.067
Human T-lymphotropic virus (HTLV-1/2)	22	0.27%	13	131	820	13,143	0.034	2.251
Human papillomavirus (HPV)	17[3]	0.19%	2	105,590	182	3,521,083	0.02	2,179.46
Human herpesvirus 1 (HHV-1, HSV-1)	10	0.12%	2	34	9	123	0.001	0.034
Human parvovirus B19	10[4]	0.12%	167	2,641,285	19,298	302,149,810	0.028	78,459.64
Human adenovirus	9	0.11%	1	11	19	235	0.004	0.046
Human immunodeficiency virus (HIV-1/2)	5	0.06%	2	3	142	275	0.015	0.046
Human herpesvirus 8 (HHV-8, KSHV)	3	0.04%	2	4	8	17	0.002	0.004
Human polyomavirus	3	0.04%	2	4	297	588	0.061	0.122
Hepatitis B virus (HBV)	2	0.02%	3	4	460	521	0.093	0.186
Trichodysplasia spinulosa polyomavirus	1	0.01%	2	2	219	219	0.057	0.057
Hepatitis C virus (HCV)	1	0.01%	18	18	912	912	0.286	0.286
Influenza A virus	1	0.01%	4	4	3,212	3,128	0.564	0.582

[1] Some individuals may carry more than one virus.

[2] Abundance is estimated per 100,000 human cells.

[3] Four samples possibly due to cross-contamination.

[4] Four samples possibly due to cross-contamination.

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integration was also obtained for most of those samples though the identification of chimeric reads or virus-host paired reads (Fig 5). In contrast, samples without integration have 3 to 4 orders of magnitude lower abundance.

Other than the integration events of HHV6 –and the presumed events (insufficient reads to identify the integration site) for human immunodeficiency virus (HIV) and human T lymphotropic virus (HTLV1/2)–we did not have direct proof for other integrated viruses. However, we identified two individuals carrying DNA sequence reads of RNA viruses, influenza and hepatitis C virus (HCV). In the first individual, we observed 4 reads of influenza virus. The reads were mapped to different regions of the viral matrix genes (M1 and M2) (S5 Fig), as well as in the terminal read, a short plasmid tail representing the cloning site of common vector backbones. A possible explanation is that this individual received a DNA-based vaccine. In the second individual, we identified 18 HCV reads. The resulting sequence is similar to HCV clone from Pakistan, which coincides with the demographic information on the presumed carrier (S5 Fig).

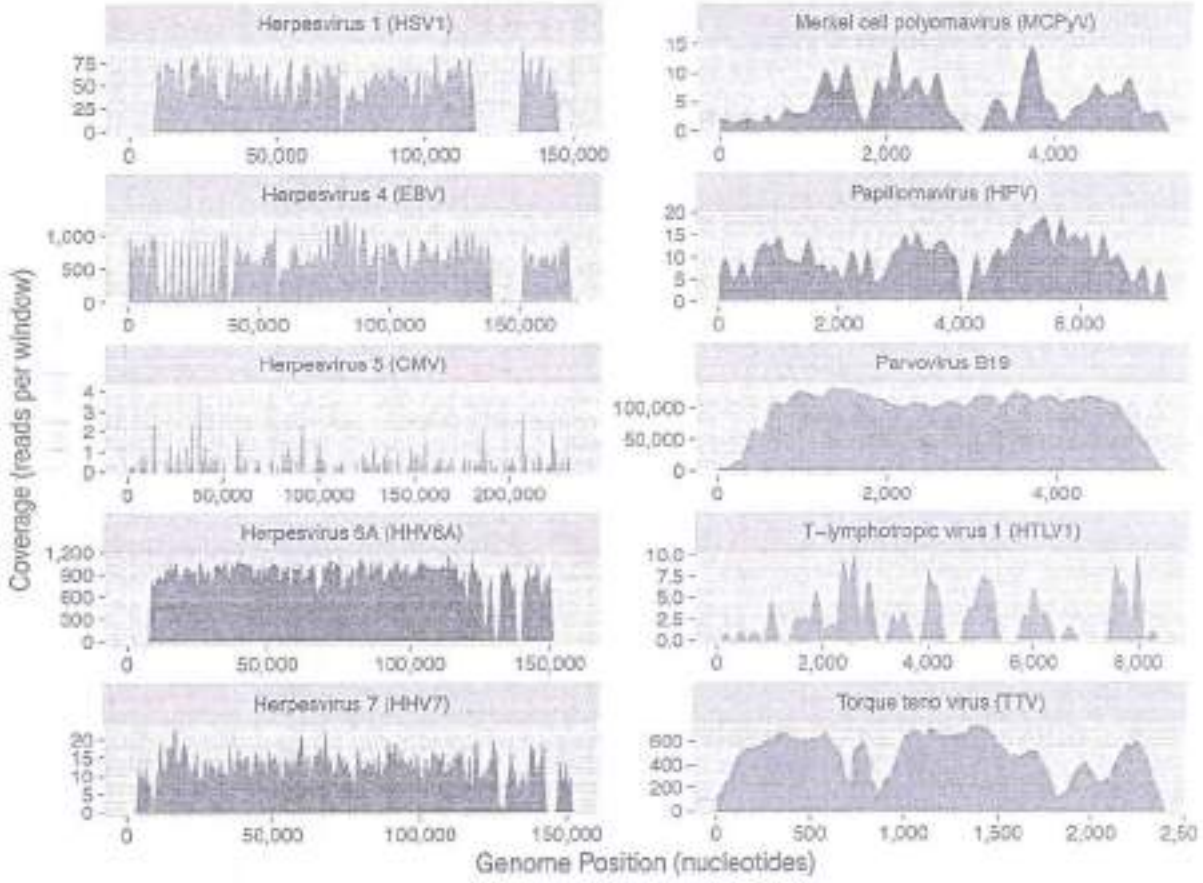

Fig 4. Genome coverage of selected human viruses. Shown are the alignment of reads contributed by all individuals carrying the corresponding virus. The depth of coverage (y-axis) changes in scale as a reflection of the viral abundance and prevalence. Gaps in coverage (e.g., in EBV) generally reflect repetitive regions that are masked during data processing.

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An additional sample contained many paired-end chimeras between CMV and human chromosome 11 and 15. Closer inspection revealed a lack of coverage of the CMV genome, with a large number of reads uniquely mapping to CMV regulatory elements used in expression vectors [22]. A similar situation was found in a sample that contained many reads of SV40 of plasmid origin.

Giant viruses and other viruses of interest

We identified a few viral sequences of Mollivirus in 8 individuals with a median of 2 reads per sample, Paramecium bursaria Chlorella virus in 3 individuals with a median of 2 sequence reads per sample, Apis mellifera filamentous virus in 2 individuals with a median of 2 sequence reads per sample, Melbournevirus in 2 individuals with a median of 3 sequence reads per sample, and Acanthamoeba polyphaga moumouvirus in 1 individual with 2 sequence reads.

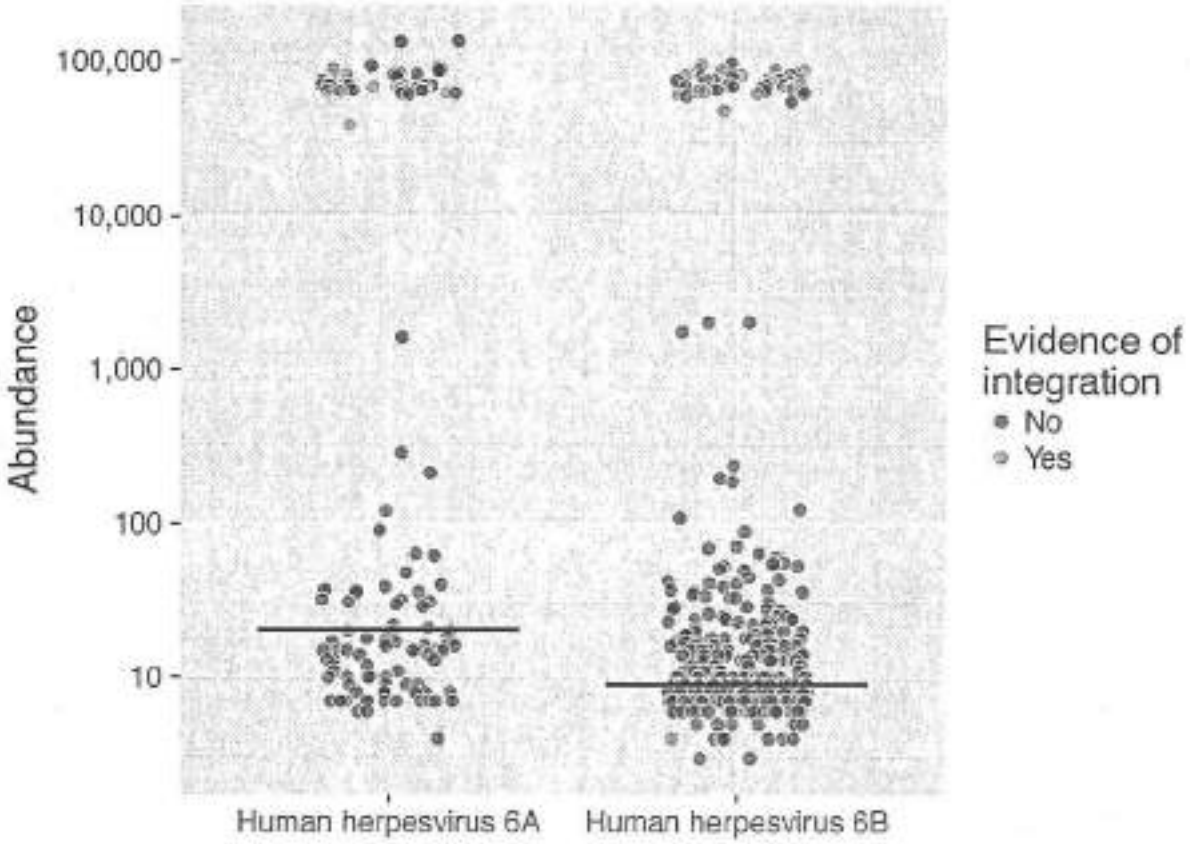

Fig 5. Integration of human herpesvirus 6. The two populations of HHV6A and HHV6B are present in a bimodal distribution. The frequency of integrated viruses, at approximately 0.5 per cell corresponds to the haploid nature of the integration in the case of inherited, vertical transmission—from one of the parents. The identification of chimeric reads, or paired human-virus reads is shown for a substantial proportion of integrated HHV6 (green dots). The bar represents the median.

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We observed the presence of occasional reads with correct match to animal retroviruses (Fig 1): Feline immunodeficiency virus and RD114 feline retrovirus, Ecotropic, Polytropic and Moloney murine leukemia virus, and Porcine endogenous retrovirus. The source of these viruses is likely to be through contamination of cell lines or the environment [23, 24].

We identified in a single individual the presence of 8 reads (abundance = 2,432 particles) of a virus corresponding to the sewage-associated gemycircularvirus. This virus was also identified in transfusion plasma pools and clinical samples [16], thus raising awareness for the possibility of gemycircularviruses infect humans or alternatively, reflecting contamination occurring during phlebotomy or plasma pool processing.

We identified a few viral sequences of archaeal viruses (Archaeal BJ1 virus and Haloviras) in 4 individuals with a median of 9 reads per sample. There is debate in the literature whether these viruses should be referred to as phages [25], and there is no sufficient information on whether archaea, and thus their viruses, may represent actual flora of humans [26].

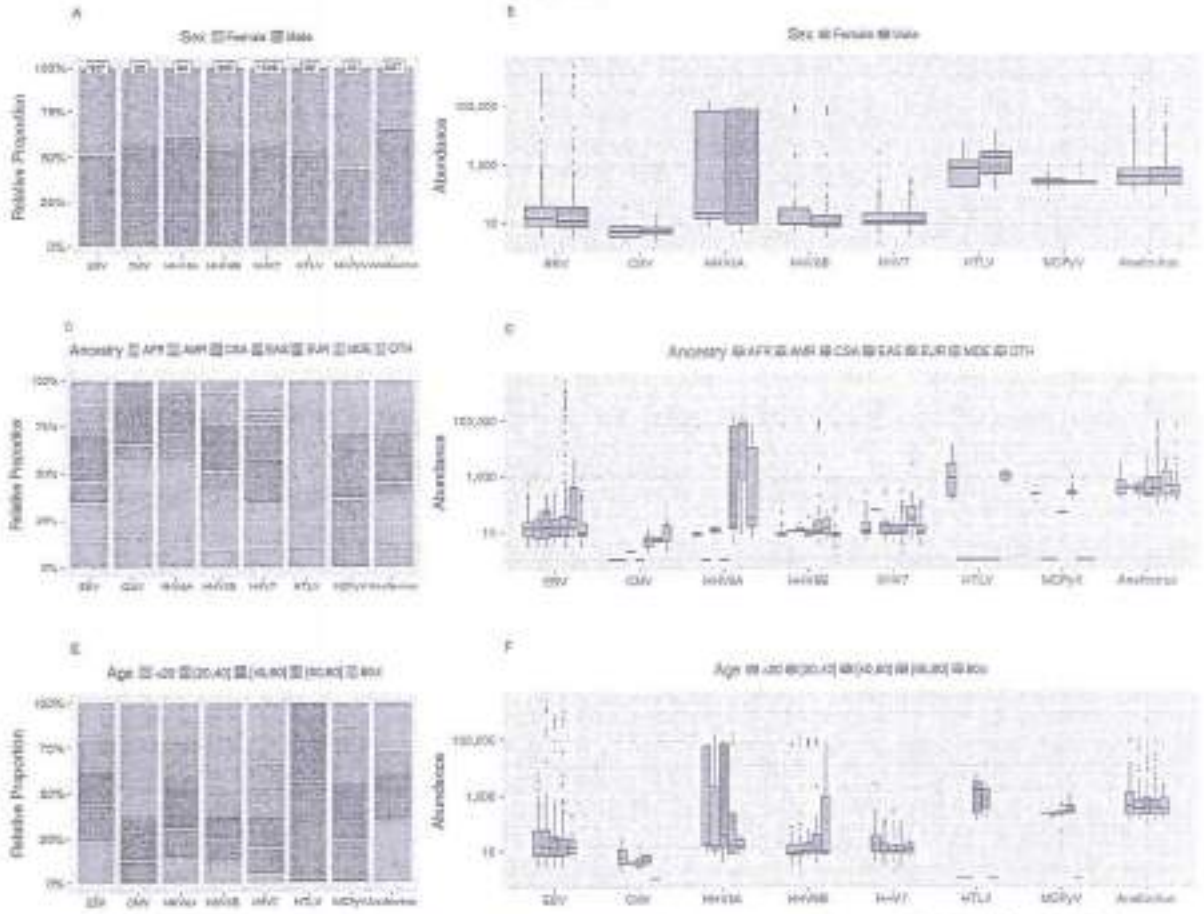

Fig 6. Relative proportion and viral load in the context of age, sex and ancestry. The relative proportion, normalized to 100% for visualization purposes (A, C and E) and distribution of observed viral loads (B, D and F) are depicted for the 8 viruses that have the largest prevalence in the study. Among the 4,506 with demographic information, the ancestries were: EUR, European = 3,049; AFR, African = 665; MDE, Middle Eastern = 94; EAS, East Asian = 91; CSA, Central South Asian = 54; AMR, Admixed American = 8; Multi-Racial and Others = 545.

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Associations with sex, ancestry and age

Complete demographic information was available for 4,505 individuals. We observed a greater prevalence of circulating viruses in men than in women (Fig 6 and S6 Fig). We also observed difference in viral prevalence in relation to age and ancestry (S6 Fig). Deltaretroviruses were predominantly identified in individuals of African ancestry from different geographical locations. Twenty out of 22 human T-lymphotropic virus (HTLV) infections (90%) were HTLV-2. CMV, HHV6A and B and HHV7 were more prevalent in the younger groups, with higher loads of HHV7 identified in them (Fig 6). Statistical significant differences for demographic characteristics and viral prevalence or viral load are summarized in S2 Table. Overall, viral presence associated with age (p-value = 5.6e-25) after adjustment for ancestry (p-value = 1.3e-20) and sex (p-value = 1.4e-9); (S6 Fig).

Discussion

The current work defines the human DNA blood virome in more than 8,000 individuals that we consider as representing a general population. The study leverages sequencing of the human genome that generates approximately 5% of reads (the sequence of a fragment of the genome) that do not map to the human reference genome. This large pool of reads primarily includes unmapped and repetitive human reads, bacterial reads, but also lesser numbers of sequences from archaea, eukaryotes, and viruses [1]. We identified 94 different viruses, including human DNA viruses, however, the pools of non-human reads are known to contain contaminant DNA from reagents [27, 28]. The routine process of sequencing human DNA does not capture RNA viruses except through the identification of proviruses and other possible viral integration events.

Among sequences that mapped to 94 viruses, we identified 19 human viruses in 42% of the study participants. In addition to a wide representation of human herpesviruses and anelloviruses, the study identified 7 different papillomavirus types, including the oncogenic type 16, HIV, HBV, 3 different polyomavirus types and parvovirus B19. These viruses generally correspond to those known to be highly seroprevalent in the human population [29]. Viral sequences in the study represent a concentration of two to millions of genome copies per 100,000 cells.

We identified sequences of most members of the herpesvirus with the notable exception of Varicella-Zoster virus. This virus is easily identified in blood from immunosuppressed hosts and in immunocompetent subjects with active herpes zoster disease [30, 31]. It is however reported absent in blood in the immunocompetent host [32]. We also observed papillomavirus reads in 0.2% of the study participants. Papillomavirus DNA was previously identified via PCR amplification in 8.3% (15/180) of healthy male blood donors [33]. The Merkel cell polyomavirus (MCPyV), found in 0.55% of the study participants, is highly seroprevalent in the population [34]. MCPyV was reported in 22% of blood samples from healthy donors using PCR [35]. We also identified Trichodysplasia spinulosa polyomavirus (TSPyV) [36], which is also seroprevalent in humans [37]. TSPyV viremia has been described, via PCR amplification, in immunosuppressed individuals but not in healthy controls [38].

The presence of viruses in blood products can be relevant for transfusion medicine. Currently, laboratory testing of donated blood prior to transfusion includes screening of HIV-1 and HIV-2, HTLV-1 and 2, HCV, HBV, West Nile virus, and Zika virus. The clinical impact, if any, of transmission of the highly prevalent GBV-C (aka pegivirus A) and of anelloviruses, is to be deciphered [39, 40]. Parvovirus B19 [41] and other parvoviruses [42] are of concern to transfusion safety because these viruses are not routinely screened for and they lack a lipid envelope, rendering pathogen inactivation procedures less effective. The observation of other human DNA viruses in the study population—for example HPV, MCPyV, HHV8 and adenovirus—adds to the list of viruses that could be potentially transmitted via blood products [43].

The coverage (30X) required for sequencing of the human genome [1] limits the ability to map integration events. This would rely on abundance of sequencing paired reads that encompass viral and human sequences. However, integration into the human genome was observed for HHV6A and B, known to occur in about 0.5% to 1% of humans [44, 45]. Integration by RNA viruses (other than retroviruses) has been described occasionally [14], and we were intrigued to identify one individual carrying few sequence reads of influenza virus that we attributed to the possible use of a DNA-based influenza vaccine (because of the presence of a small plasmid fragment in the sequence). The second surprising event was the identification of multiple sequence reads of HCV matching to viral clones from Pakistan, in an individual from the same geographical origin. There has been discussion on the role of reverse transcriptase

activity determining the accidental integration of viral RNA in the genome [46], and specific to HCV, the occasional claim of integration [47].

Younger study participants were more likely to have human viruses identified in blood—which is consistent with the impact of seroconversion window at younger age. Differences in viral prevalence and type of virus varied also by ancestry: geography and local epidemiology may be the driving epidemiological factor. We observed an unexpected bias towards greater prevalence of circulating viruses in men than in women that remained significant after adjusting for the other demographic factors. There have been many descriptions on differences in prevalence, susceptibility to infection and disease severity across sex. The current thinking is that females tend to mount higher innate, cell-mediated, and humoral immune responses than males [48].

Next-generation sequencing is used for the discovery of new human pathogens—particularly in the setting of acute infection. Although we identified 94 different viruses, we found that large numbers of viral sequences represented contamination. Specifically, we observed a very significant presence of phage DNA associated with use of phage phiX174 used to allow real-time quality metrics during sequencing. Although there is a possibility that some phage DNA could translocate from the gut [49], the presence of other phages and viruses each time that phiX174 was used is revealing of intrinsic contamination of the commercial phiX174 materials. Phage DNA can also derive from bacteria contaminating the reagents [22, 50]. Beyond phages, there are reports of false-positive results and claims of viral pathogen discovery traced back to specific steps in the process of sequencing; for example, the identification of parvovirus-like sequences in nucleic acid extraction columns [51, 52] or Moloney MuLV genome in cancer cell lines [53]. Therefore, the presence of a novel DNA virus in blood would require the use of numerous control experiments to exclude contamination. More generally, we identified animal retroviral sequences that likely reflect the contamination of cellular reagents or from environmental sources—a critical consideration given the past history of claims such as with Xenotropic murine leukemia virus-related retrovirus (XMRV) that was reported to be associated with prostate cancer and chronic fatigue syndrome. A massive effort was required to reverse those claims [54]. Finally, many reads were falsely attributed to viruses due to contamination with plasmid sequences that use viral regulatory cassettes.

We evaluated the presence of the recently discovered giant viruses [55]. Our finding of a small number of reads in only 0.2% of the study population suggests that giant virus DNA is not a frequent finding in blood or that its detection also reflects reagent or laboratory contamination [56]. In addition, the presence of samples with high viral-titers leads to misidentification of samples, due to sharing of barcodes in single-index sequencing libraries [19]. This problem has also been described as "sample bleeding" that refers to the incorrect assignment of reads to multiplexed samples that are being sequenced in the same sequencing lane [57]. Dual-indexing will be needed for more accurate studies of the human virome. Many of the observed viruses might be truly present in human blood—however, it is difficult to distinguish them from prevalent contaminant viral sequences. Study design, epidemiological setting and downstream validation by independent techniques are needed to propose novel viruses. Overall, the analysis aims at defining the normal DNA virome background in blood in a presumably healthy population against which novel discoveries can be proposed.

This study has the following limits. It analyzes a convenience population that does not contribute specific data on infectious diseases. However, this can be seen as an advantage in terms of better representing a general population. The nature of the sequencing protocol implies limited amplification of the viral genetic material, and a significant competition from the larger human genome. Therefore, this approach may not identify lower concentration viruses that could be revealed by using viral particles enrichment [58, 59] or viral genome capture [60, 61].

The latter methods rest on the ability to capture closely related sequences by hybridization to short conserved probes. Other recent approaches include methods that enable human viral epitope-wide exploration of immune responses in large numbers of individuals. This latter approach is effective for determining past viral exposure [62]. The study was not conceived for the discovery of highly divergent, novel human viruses, as this requires the use of less stringent similarity criteria for detecting divergent (relative to those already known) viral sequences. Lastly, the study did not address the RNA virome in human blood. Thus, the highly prevalent blood-borne RNA pegivirus A (GBV-C) in the *Flaviviridae* family was not detected here.

The interest of the study derives from the size of the investigation that serves to define the human DNA blood virome. The second, and equally important part of the study is the description of the contamination profile during genome sequencing that may confound the discovery of novel human viruses. Increasing numbers of humans undergoing whole genome and transcriptome sequencing will support the precise description of the human blood DNA and RNA virome.

Materials and methods

Study characteristics

Participants were representative of the spectrum of age (between 2 months and 102 years with a median of 56), and of major human populations and ancestries. Specifically, the study included EUR, European = 5,384; AFR, African = 1,049; MDE, Middle Eastern = 213; EAS, East Asian = 159; AMR, CSA, Central South Asian = 94; Admixed American = 16; and Multi-Racial and Others = 1,325. The study population was not ascertained for a specific infectious disease status. Other aspects of the study and the performance of genome sequence are detailed in Telenti et al. [1].

Ethics statement

New (Western Institutional Review Board, www.wirb.com) and existing IRB-approved consent forms for participation in research and collection of biological specimens and other data used in this publication were reviewed and confirmed to be appropriate for use. All adult subjects provided informed consent, and a parent or guardian of any child participant provided written informed consent on their behalf.

Sequencing

Library preparation was carried out using the TruSeq Nano DNA HT kit (Illumina Inc.). Libraries were combined into 6-sample pools and clustered. Flow cells were sequenced on the Illumina HiSeqX sequencer utilizing a 150 base paired-end single index read format. Despite of the use of TruSeq technology, several ssDNA viruses were identified. It is possible that this is a reflect of extensive secondary structure of the naked viral DNA [63] and of replicative intermediate forms that are dsDNA [64].

Identification of unmapped sequences

For each BAM file, we extracted read pairs with either one or both of the reads not mapping to hg38 using sambama [65] with filtering for "unmapped" or "mate_is_unmapped". Read pairs with average base quality below 30 were removed. Read pairs with low complexity identified using String Graph Assembler [66] with the following parameters dust-threshold = 2.5 and quality-filter = 50 then they were removed. Samples with more than 10% unmapped reads were excluded from further analysis.

Identification of viral sequences

Unmapped reads were in a first step searched for putative viral matches by blastn [67] against the NCBI RefSeq [68] viral reference genomes (> 8,000 viruses and phages) [69] using an e-value ≤ 1e-10. In a second step, candidate reads with viral hits were searched against a more comprehensive database comprised of NCBI RefSeq genomes of viruses, representative bacteria (1,636 species and strains), archaea (389 species and strains), and fungi (two species), and UCSC genomes of human, chimp, mouse, chicken, and fruit fly, and NCBI nt vectors (274,565 sequences) and plasmids (778 sequences) using blastn with e-value ≤ 1e-20. Viral hits were filtered for bit-score ≥ 190. Reads with hits other than viruses with bit scores greater than or equal to the viral hits were discarded. Finally, randomly selected reads with viral hits of the human viruses were manually and visually verified by searching (blastn) against NCBI nt (online) and by aligning the reads to the corresponding viral genomes.

Estimating viral abundances

The normalized abundance of a virus in a sample was estimated in genome copies per human cell (viral genomes per human diploid genome) with the following equation:

$$virus\ abundance = \frac{2 \times \frac{number\ of\ reads\ mapped\ to\ viral\ genome}{virus\ genome\ size}}{\frac{number\ of\ reads\ mapped\ to\ human\ genome}{human\ genome\ size}}$$

For ease of interpretation, values are referred to a "viral copies per 100,000 human cells". The fraction of viral reads has been shown to generally correspond to its viral load as determined by real time PCR [3, 58, 70].

Assembly of unmapped reads

The unmapped reads were also assembled in contigs using SOAPdenovo [71, 72] with *k*-mer size 91 for each sample. Contigs that were mapped to the human reference with > 90% identity on > 30% length were removed. The remaining contigs were then mapped to the hg38 regions that were masked as repeat in UCSC goldenPath using blastn [67] without low complexity filtering to remove contigs that contain > 20% repeat sequences. Contigs passing the above filtering steps were clustered into non-redundant set using CD-Hit [73, 74] with 90% global identity threshold. Non-redundant clusters were searched for matches to viral proteins using DIAMOND [75] against NBCI non-redundant proteins (nr).

Prediction of integration sites

To detect potential cases of integration between the viral genome and the human genome, identified viral reads were aligned to a database comprised of the viral genomes and the human reference genome hg38 to detect potential cases of integration, which were predicted via the identification of chimeric reads and chimeric mates using BWA [76] with the maximal exact matches algorithm "bwa mem". An integration event was predicted when either one mate of a paired-end read aligned to a virus genome and the other mate aligned to the human genome or a single mate chimerically split into two alignments where one part mapped to a virus genome and the other part mapped to the human genome.

Association with demographic characteristics

We conducted a logistic regression analysis under a generalized linear model (GLM) with binomial distribution for the presence of human viruses in response to the individuals' sex,

ancestry, and age along with the cohort information as the covariate using the 'glm' method in R, followed by the 'step'method for identifying the optimal model. The significance of the interactions was determined by chi-squared tests for the deviance table of the GLM. Statistical significances of the differences in prevalence and abundance across the demographic characteristics for each virus were estimated using chi-square test and Kruskal-Wallis test, respectively, followed by multiple test correction for the generated p-values.

Data access

Virome reads are available for downloading at www.HLI-OpenData.com/Virome/. In addition, see the Data Access Statement (www.humanlongevity.com/wp-content/uploads/HLIDataAccessAgreement020416.docx.) for information on extended access.

Supporting information

S1 Fig. Read mapping statistics. Unmapped reads in deep sequencing of the human genome using Illumina HiseqX10 technology. The average percentage of unmapped reads per sample is around 5.23%, and median is 4.91%.
(TIF)

S2 Fig. Abundance of EBV in association with use of human reference genome NA12878. The distribution of the abundance of EBV is shown for the EBV B95-8 strain-immortalized the cell line of NA12878, for samples sequenced sharing the same flow cell with human genome NA12878 and for samples sequenced in the absence of human genome NA12878 in the sequencing flow cell. We used the conservative approach of eliminating all the positive samples from flow cells containing NA12879 because the high counts indicated that most samples were contaminated. Only a minority of samples had low counts, and we did not attempt alignment to the EBV B95-8 genome because of the few available reads. The bars represent the median.
(TIF)

S3 Fig. Distribution of samples with viruses across the sequencing flow cells. The number of viral reads per samples are shown on the y-axis in relation to the number of samples per flow cell that are positive for the corresponding virus. The presence of multiple positive samples in flow cells that contain one high viral-titer sample is suggestive of contamination by misidentification by sharing of barcodes in single-index sequencing libraries. The bars represent the median.
(TIF)

S4 Fig. Assembly of contigs of human viruses. The sensitivity of identification of human viruses differs when using contigs from de novo assembly of reads, versus using individual reads. The upper panel is based on raw counts of the virus reads and the lower panels show the normalized viral abundances. The identification of viruses is improved by several orders when using read mapping. However, excessive number of reads (depth) may lead to failure of the assembly process Overall, viruses were detected by both read mapping or contigs in 137 samples, and only by read mapping in 3,342 samples. It came as a surprise that in 13 samples the identification of viral sequences (anellovirus, CMV, and HIV) was achieved using only contigs. After manual inspection, the CMV and HIV contigs represented plasmids sequences. Eleven samples with anelloviruses, represented by four clusters, were detected by contigs only because the individual reads had low identity (less than 70%) with the corresponding virus reference genome indicating the presence of divergent anelloviruses. Specifically, two contigs had the closest match as TTV-like mini LY1, one contig had the closest match as Torque teno mini

virus 3, and one contig had the closest match as unclassified Anelloviridae isolate TPK01. The bars represent the median.
(TIF)

S5 Fig. Sequence reads from RNA viruses. Panel A depicts the alignment of 4 reads from one individual to the influenza H1N1 reference sequence M1 and M2, segment seven. Closest match: serotype = H1N1, strain = A/Puerto Rico/8/1934. Panel B depicts the alignment of 18 reads from one individual to a HCV subtype 3 sequence. Closest match. HCV clone FG1-NS3-4a from Pakistan (https://www.ncbi.nlm.nih.gov/nuccore/KC825339). The number of reads represents and abundance is 912 HCV particles per 100,000 human cells. The viral reads are restricted to ~2Kb of the ~9Kb of HCV.
(TIF)

S6 Fig. Association of viral presence with demographic characteristics. Panel A-C depict the individual association of viral presence with sex, age and genetic ancestry. Panel D plots the results of the analysis of deviance (variance) for the presence of any human virus in response to the individuals' gender, ethnicity, age. AFR, African; AMR, Admixed American; EAS, East Asian; EUR, European; CSA, Central South Asian; MDE, Middle East.
(TIF)

S1 Table. Complete listing of viruses putatively identified or contaminating blood DNA of 8,240 individuals.
(PDF)

S2 Table. Statistical significant differences for demographic characteristics and viral prevalence or viral load.
(PDF)

Author Contributions

Conceptualization: AT JCV.

Data curation: AM CX EK EW.

Formal analysis: AM CX EK EW.

Funding acquisition: JCV.

Investigation: AT AM WB.

Methodology: AM CX.

Project administration: AT.

Resources: CX.

Software: AM CX.

Supervision: YT KB KEN JCV AT.

Validation: ED AT.

Visualization: AM.

Writing – original draft: AT ED AM.

Writing – review & editing: AT ED AM.

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EXHIBIT Q

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Metagenomic analysis of double-stranded DNA viruses in healthy adults

Kristine M Wylie[1,2*], Kathie A Mihindukulasuriya[2], Yanjiao Zhou[2], Erica Sodergren[2,3], Gregory A Storch[1] and George M Weinstock[2,3]

Abstract

Background: The Human Microbiome Project (HMP) was undertaken with the goal of defining microbial communities in and on the bodies of healthy individuals using high-throughput metagenomic sequencing analysis. The viruses present in these microbial communities, the 'human virome,' are an important aspect of the human microbiome that is particularly understudied in the absence of overt disease. We analyzed eukaryotic double-stranded DNA (dsDNA) viruses, together with dsDNA replicative intermediates of single-stranded DNA viruses, in metagenomic sequence data generated by the HMP. We studied 706 samples from 102 subjects were studied, with each subject sampled at up to five major body habitats: nose, skin, mouth, vagina, and stool. Fifty-one individuals had samples taken at two or three time points 30 to 359 days apart from at least one of the body habitats.

Results: We detected an average of 5.5 viral genera in each individual. At least one virus was detected in 92% of the individuals sampled. These viruses included herpesviruses, papillomaviruses, polyomaviruses, adenoviruses, anelloviruses, parvoviruses, and circoviruses. Each individual had a distinct viral profile, demonstrating the high interpersonal diversity of the virome. Some components of the virome were stable over time.

Conclusions: This study is the first to use high-throughput DNA sequencing to describe the diversity of eukaryotic dsDNA viruses in a large cohort of normal individuals who were sampled at multiple body sites. Our results show that the human virome is a complex component of the microbial flora. Some viruses establish long-term infections that may be associated with increased risk or possibly with protection from disease. A better understanding of the composition and dynamics of the virome may hold important keys to human health.

Keywords: Metagenomics, Microbiome, Virome

Background

The Human Microbiome Project (HMP) was undertaken to define microbial communities found in and on the bodies of healthy individuals [1]. Understanding the range of normal microbial flora will inform the design and interpretation of future studies aimed at associating microbial community states with disease. The HMP has generated the largest, most complex sequence data set from human microbial communities, with greater than seven terabases (about 70 billion sequences) of whole-genome shotgun data generated [2]. Recently the first large-scale analyses of the HMP data were published, focusing exclusively on bacteria [2,3]. Here we present a comprehensive analysis of eukaryotic DNA viruses in the HMP data set.

The viral component of the human microbiome, the human virome, is an important aspect of the HMP. While viruses that cause acute symptomatic infections clearly impact human health, viruses that establish acute or long-term apparently asymptomatic infections are part of the microbial flora and may have unappreciated effects on human health [4]. Describing the characteristics and dynamics of the human virome is a first step in understanding the role of the virome in human health. We report the first large-scale molecular analysis of the viral flora in a cohort of 102 healthy subjects sampled at as many as five major body habitats: nose, skin, mouth, vagina, and stool. Importantly, the analysis was based on the use of high-throughput deep sequencing, allowing the detection of a broad range of DNA viruses, both cultivable and non-cultivable.

* Correspondence: kwylie@genome.wustl.edu
[1]The Department of Pediatrics, Washington University School of Medicine, Campus Box 8116, 660 S. Euclid Avenue, St. Louis, MO 63110, USA
[2]The Genome Institute, Washington University School of Medicine, Campus Box 8501, 4444 Forest Park Avenue, St. Louis, MO 63108, USA
Full list of author information is available at the end of the article

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Results

The human virome in five body habitats in healthy, asymptomatic adults

We detected one to 15 viral genera (average 5.5) in 92% of the 102 individuals sampled (Figure 1A). Figure 1B illustrates the viromes of the 102 individuals defined by sampling up to five major body habitats, showing that a broad range of viruses was detected in healthy people. The 102 individuals carried seven distinct families of human DNA viruses (Figure 2A). The double-stranded DNA (dsDNA) viruses included members of the virus families *Herpesviridae*, *Papillomaviridae*, *Polyomaviridae*, and *Adenoviridae*. In addition to the dsDNA viruses, several genera of single-stranded DNA (ssDNA) viruses were detected, including members of the families *Anelloviridae*, *Parvoviridae*, and *Circoviridae*. These ssDNA viruses generate dsDNA intermediates during replication, which allowed them to be detected with the DNA preparation protocol employed that would not otherwise detect ssDNA viruses. The *Herpesviridae* genera included roseoloviruses (predominantly human herpesvirus (HHV)-7), herpes simplexvirus (HSV-1), lymphocryptovirus (Epstein Barr Virus), and human cytomegalovirus (HHV-5). The papillomaviruses detected were predominantly the most common human papillomaviruses (HPVs): alpha-, beta-, and gamma-papillomaviruses. These included high-risk papillomavirus types, such as HPV-16, HPV-18, and HPV-45. We also detected sequences, predominantly in the nose and skin, with more remote similarity to 17 papillomavirus genera that contain non-human viruses (Figure 2B). This suggests that numerous undescribed HPVs may exist, which is not surprising given that more than 150 HPV genera have been identified [5,6], and new HPVs continue to be discovered [7,8]. Polyomaviruses and adenoviruses were also relatively common. We detected polyomaviruses MWPyV [9], Merkel Cell [10,11], human polyomavirus (HPyV)-6, HPyV7 [12], and JC [13]. The anelloviruses were found in all of the body sites except stool. These viruses establish persistent infections in the blood in the majority of the population early in life [14]. The parvovirus detected was adeno-associated virus, which was detected in conjunction with adenovirus. The gyrovirus and circovirus sequences were found in the oral and gastrointestinal tracts.

Some viruses were found in a particular body site of greater than 30% of the subjects. Roseoloviruses, predominantly HHV-7 and to a lesser extent HHV-6, were present among 98% of the individuals who provided mouth samples, while skin samples collected from the retroauricular crease commonly contained a variety of alpha-, beta- and gamma-papillomaviruses (41%, 76%, and 76% of subjects, respectively). We detected beta- and gamma-papillomaviruses in the anterior nares of 48% of individuals, and roseoloviruses in 33%. The vagina was dominated by papillomaviruses, with 38% of those sampled carrying one or more alpha-papillomaviruses.

The same viruses were prevalent in multiple body habitats within individuals, and these shared components of the individual's virome were consistent with anatomic or functional links between these body sites. For instance, the beta- and gamma-papillomaviruses were the viruses most commonly found in the skin and the nose (anterior nares; Figure 2A,B), which may reflect proximity and similarities in microenvironments that support infection with these viruses. Roseoloviruses were common in mouth samples, and they were also common in skin and nose habitats (Figure 2A), which could result from transfer of material carrying these viruses. While viruses were detected in few stool samples, most components of the stool virome were shared with the mouth (Figure 2A), for example roseoloviruses and circoviruses, possibly reflecting the passage of oral viruses into the gut.

Viral profiles of individuals

Analysis on an individual level revealed distinct viral profiles and also supported the observation that some viruses are prevalent in multiple body habitats (Figure 1B). We describe a few individual cases here. Figure 3A shows an example in which numerous viruses were shared in the nose and skin samples from one individual, including human cytomegalovirus, several papillomaviruses including HPV-19, and MWPyV. Human adenoviruses were relatively common in oral and skin samples (17% of samples, including the subject in Figure 3B), with nearly all sequences mapping to human adenovirus C reference genomes. Figure 3C shows that roseolovirus HHV-7 was detected in samples from the nose, skin, and mouth of another individual. Some of the virome variation between individuals was found in sequences that lacked nucleotide sequence similarity but shared amino acid sequence similarity with a variety of papillomaviruses, which suggests the presence of novel human papillomavirus types or strains (Figure 3A-D, 'Other Papillomaviruses').

The stability of the human virome

Fifty-one individuals were sampled on two visits, which allowed us to assess the stability of an individual's virome (Figure 4 and Additional file 1: Tables S4 and S5). The roseolovirus HHV-7 was commonly detected in samples taken from the same individual on both visits (Figure 4A,B). In fact, if HHV-7 was detected in mouth samples taken during the first evaluation, it was also detected in samples from the second visit in greater than 90% of the subjects. For the individuals in whom alpha-, beta-, and gamma-papillomaviruses were detected in nasal or vaginal samples collected at one evaluation, the virus was also detected in samples from the other visit

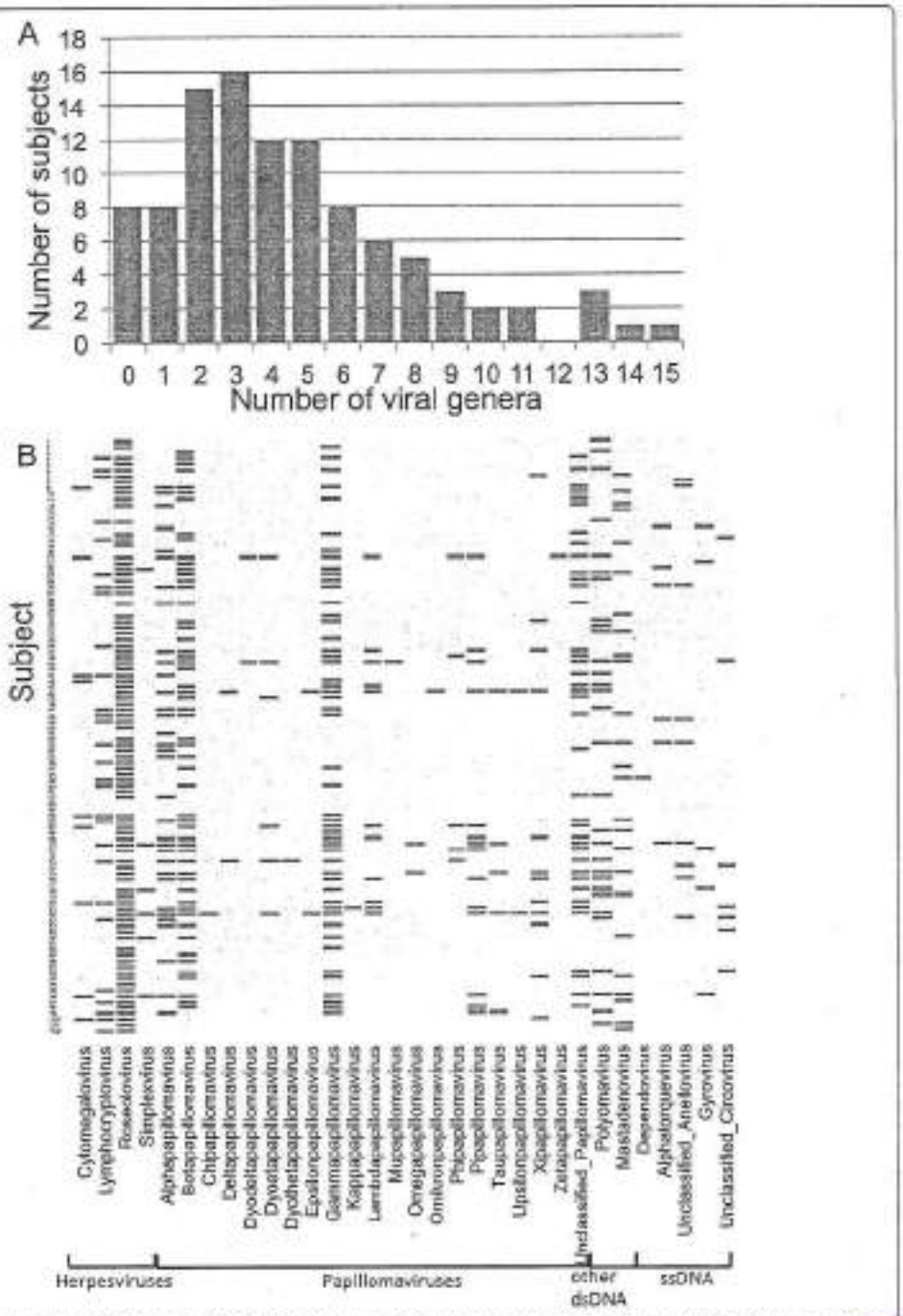

Figure 1 The human virome in healthy, asymptomatic adults. (A) The histogram shows the number of individuals (y-axis) who were positive for a given number of different viral genera (y-axis). **(B)** The viral genera (x-axis) detected in each subject (y-axis) are represented by black bars. The virome of each individual is viewed by looking at the black bars in a given row.

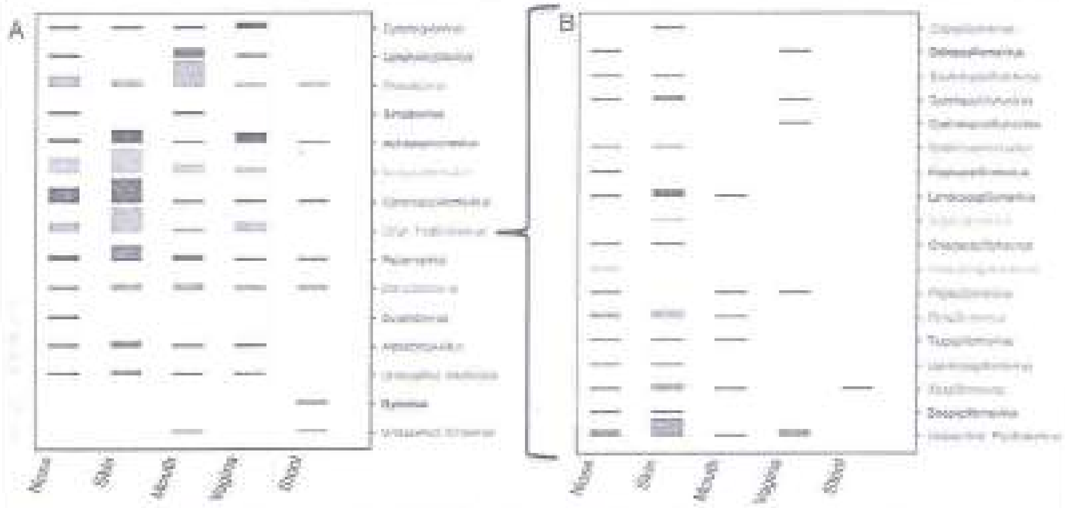

Figure 2 The human virome in five body habitats. **(A)** All of the viruses detected in the five body habitats are shown. Each virus is represented by a colored bar and labeled on the y-axis on the right side. The relative height of the bar reflects the percentage of subjects sampled at each body site in whom the virus was detected. In this panel, the bar representing roseoloviruses in the oral samples reflects the maximum bar height, because 98% of the individuals who were sampled in the mouth harbored roseoloviruses. **(B)** This panel shows papillomaviruses included in the category 'Other papillomaviruses'. The largest bar height shown represents the unclassified papillomaviruses found in skin samples from 85% of subjects. The percentages represented in this figure are shown in Additional file 1: Table S3.

in 30% to 50% of the subjects (Figure 4A). In samples from the mouth, papillomaviruses were detected less frequently over multiple visits.

Relationships between bacterial microbiome and virome in the vagina

Viruses and bacteria may have dynamic correlative and anti-correlative relationships within the microbiome. Therefore, we analyzed the relationship between the virome of individuals and the composition of their vaginal bacterial microbiome. Alpha-papillomaviruses were detected in 37.5% of subjects who had vaginal samples sequenced. Consistent with previous studies, we detected several types of vaginal bacterial communities [15,16]. Viruses were found in all of these microbial communities, but alpha-papillomaviruses were more common in samples from individuals with more bacterial diversity than those whose vaginal microbiomes were made up almost entirely of *Lactobacillus* (Figure 5). Looking only at the posterior fornix samples from the first visit of each individual (34 samples), subjects with communities containing less than 85% lactobacillus were more likely to have alpha-papillomaviruses in their samples compared with other subjects ($P = 0.0010$, Fisher's exact test). Subjects carried as many as four distinct alpha-papillomaviruses, including HPV-16, HPV-18, and others that are considered high-risk for tumorigenesis. For example, the vaginal sample from one subject contained HPV-45 (high risk), HPV-53 (probably high risk), and HPV-43 (low risk) [17]. We did not observe other statistically significant correlations of virus sequences with bacteria in other anatomic sites. Nor did we observe statistically significant correlations in other anatomic sites of virus sequences with patient data, such as body mass index, age, gender, or enrollment city (St. Louis, MO or Houston, TX).

Discussion

Here we report the most comprehensive metagenomic sequence-based analysis of eukaryotic viruses in the human virome to date. This description of the eukaryotic DNA virome is relatively unbiased in that it did not rely on virus culture or virus-specific assays to define the normal viral flora. Previous studies of the human virome have focused primarily on bacteriophages [18-21], which are closely tied to their bacterial hosts and have only recently been shown to have more direct effects on the human habitats they occupy [22]. We demonstrate that healthy humans harbor a surprising variety of DNA viruses that directly infect eukaryotic cells. Some of these viruses appear to be stable components of an individual's virome. Other components of the virome were detected at a single time point, suggesting these viruses were present transiently or that levels of some viruses in some samples may have fallen beneath the sensitivity of our assay. Future studies aimed at associating DNA viruses with disease in these body habitats will be able to use

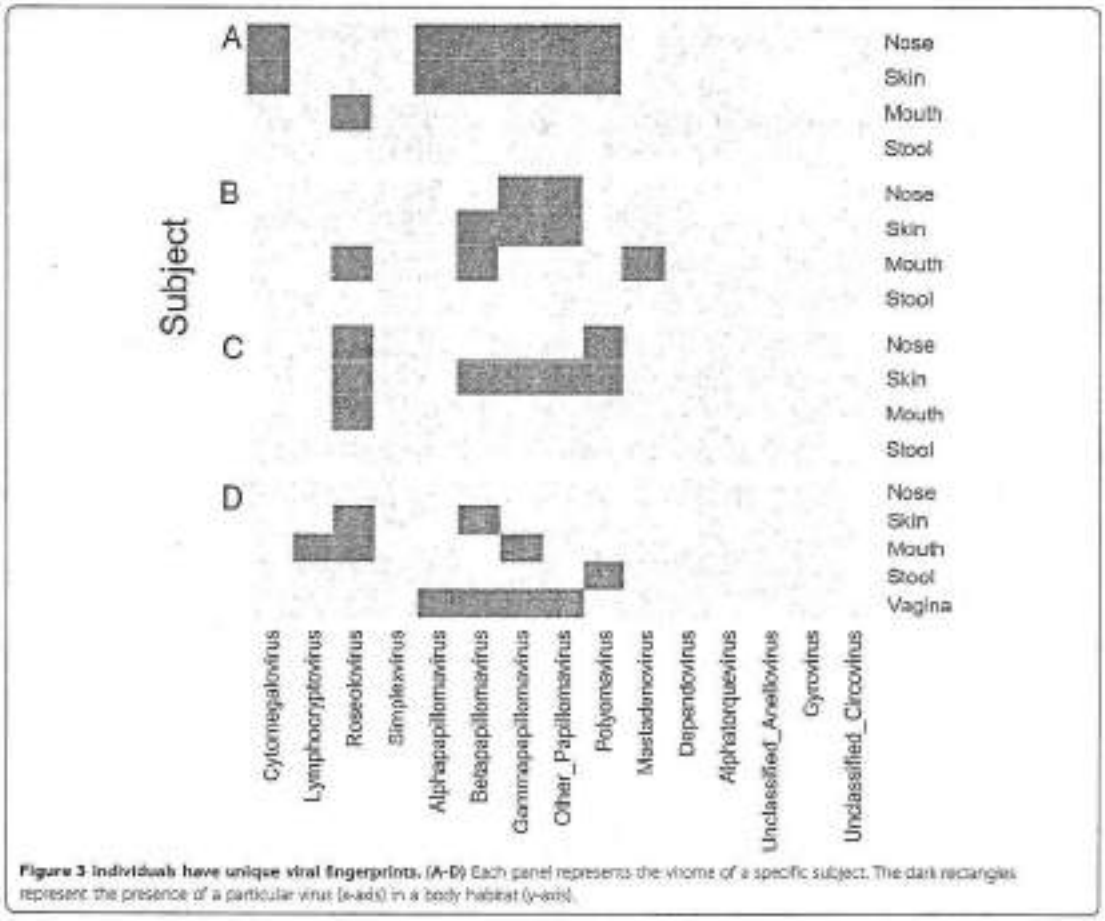

Figure 3 Individuals have unique viral fingerprints. (A-D) Each panel represents the virome of a specific subject. The dark rectangles represent the presence of a particular virus (x-axis) in a body habitat (y-axis).

the data from these healthy individuals to aid interpretation of their results.

While we observed viral nucleic acid sequences in the samples, we have not formally demonstrated that these viruses are replicating. However, we observed some of these viruses consistently in the same subject over multiple visits; we observed viruses in body sites associated with replication and shedding of virus particles rather than latency (HHV-7 in the saliva); and we detected viruses with ssDNA genomes whose dsDNA replication intermediate form could be detected in our sequencing assays (the dependovirus adeno-associated virus). Each of these suggests virus replication. For these reasons, we will use the term 'infection' in the discussion of these data, with the understanding that some of the viral sequences detected may represent virus latency or transient presence of viral nucleic acid rather than active infection.

The human body appears to dynamically maintain and control persistent infections, such as HHV-7 and many papillomaviruses. While symptomatic viral infections are currently most well known to medical science, future research is needed to explore the effects of long-term and asymptomatic infections on human health and disease. The virome may confer benefits on the host, the way the bacterial contingent of the microbiome does. For instance, murine herpesvirus infections have been shown to provide protection from infection with other pathogens in mice by up-regulating components of the immune system [23]. Members of the virome may play similar roles in maintaining human health, in a mutualistic relationship with the human host. Evolutionary considerations suggest that the universal presence of a specific microbe may reflect a beneficial interaction with the host. By contrast, some persistent DNA viral infections are clearly linked with disease, as exemplified by the increased risk for cervical cancer in women infected with high-risk HPV strains. Perhaps this is analogous to the persistence of bacterial pathogens in the microbiome

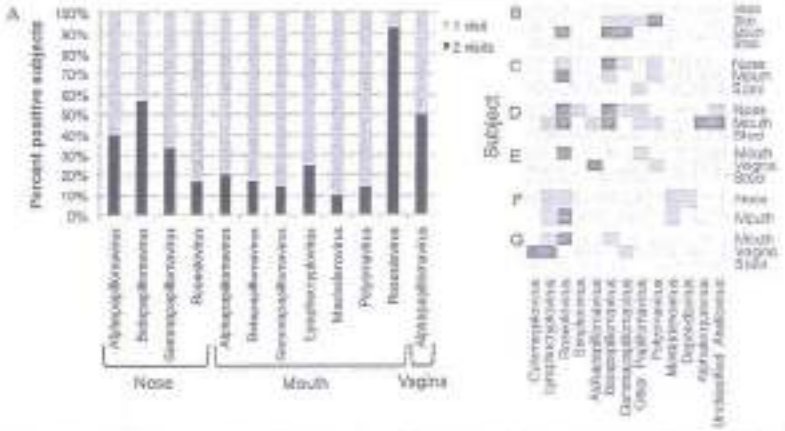

Figure 4 Stability of the human virome across two evaluations. The whole genome shotgun sequence data included a subset of samples from 51 individuals which were collected at two time points. For viruses detected in five or more individuals, Panel **A** shows the percentage of subjects in whom the virus was detected at only one (gray bar) or two consecutive (black bar) visits. The complete census of viruses that were detected in the longitudinal data set is presented in Additional file 1: Table S4. Panels **B–G** display the viruses detected in samples from a subject at one or both evaluations. Gray rectangles signify that the indicated virus was detected in a sample collected in one of two evaluations; black rectangles signify that the indicated virus was detected in the samples collected at both evaluations. Data from six individuals are shown in this figure for the purpose of illustration. Additional file 1: Table S5 shows the data for all subjects with multiple visits.

that present a potential for disease that is not always realized [24]. The idea that persistent viral infections can protect or predispose the host to other infections is a hypothesis we are pursuing.

Our expanded analysis of eukaryotic DNA viruses of 102 individuals at five major body habitats shows extensive interpersonal variation in virome composition. In addition, we show that although an individual's virome can change over time, viral presence can be remarkably stable in an individual. Inasmuch as humans are constantly exposed to viruses in the environment, the source of members of the variable virome is not difficult to envision. However, the mechanisms that favor viral-host coexistence likely consist of known protective host defenses

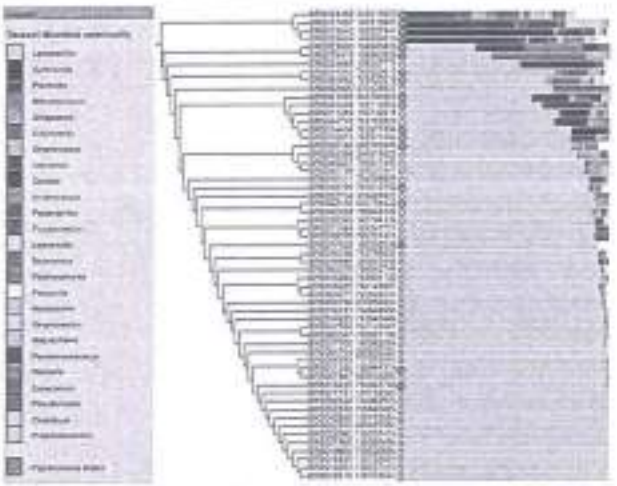

Figure 5 Variation in the vaginal microbiome. All posterior fornix samples were clustered based on bacterial community structure. The 20 most abundant components of the bacterial communities are shown in the stacked bar charts. The presence of alpha-papillomaviruses is indicated with a red circle at the base of each stacked bar.

and unappreciated processes. Viruses encode proteins to regulate the cell cycle [25], regulate host gene expression, and suppress or subvert host immune responses [26-30]. Viruses also encode micro RNAs that regulate cellular processes [31]. Thus, latent and persistently infecting viruses are continually interacting with the host in many ways. Many virologists are characterizing the complex effects of interactions that occur between virus and host during infection [25-30]. We know that viruses commonly target specific cellular pathways to regulate the cell and promote viral replication, but each virus can have its own mechanism for achieving the same control over a cellular pathway [31,32]. Future studies of the human virome will further address functional interactions between viruses and host cells.

Another important dimension of virome studies is possible interactions between the viral and bacterial communities harbored by complex organisms. To begin to probe this, we studied relationships between eukaryotic viruses and the composition of vaginal bacterial communities. Previous studies defined subtypes of vaginal bacterial microbiomes [15]. Here, using unbiased metagenomic sequencing we observed that alpha-papillomaviruses, which include the known oncogenic papillomavirus types, were more common in women with a bacterial community characterized by a decrease in *Lactobacillus* and an increase in anaerobic bacteria associated with bacterial vaginosis, such as *Gardnerella* [16]. This is consistent with other studies that show that persistent papillomavirus infection is associated with an increase in anaerobic bacteria [33,34]. Whether either member of this viral-bacterial correlate is causative, or whether each is simply present as a result of simultaneous exposure remains to be determined. The extension of this kind of analysis to disease etiologies and complex traits that are poorly understood may reveal new associations with viral infection and microbial community dynamics.

Virome analysis of the HMP and other data shows that more work is required to achieve an accurate understanding of the role of viruses in producing disease. The presence of certain viruses does not mean that there will invariably be clinical consequences. For example, we observed human adenovirus C in 17% of the nasal samples, yet these individuals did not have fever or acute illness. Likewise, in another study we found that 21% of afebrile children with minimal or no respiratory symptoms had rhinovirus present in nasopharyngeal secretions based on sequencing assays [35]. Taken together these data indicate that care is required in attributing disease manifestations to a virus that is found to be present, especially using sensitive molecular methods. We also detected adenovirus C in skin (retroauricular crease) samples from some healthy adults, which was unexpected because adenoviruses are typically associated with the respiratory or gastrointestinal tracts. Further work would need to be done to follow up this observation in a prospective study to determine whether the adenovirus sequences in skin samples represent infection or spread of virus-containing material from the respiratory tract. These early descriptions of the human virome suggest the need for additional studies to determine factors that distinguish asymptomatic infections from those that may explain a patient's symptoms and require interventions including treatment with antiviral drugs.

The data presented here show that an extensive, unbiased analysis - such as that achieved with high-throughput deep metagenomic shotgun sequencing of a large number of human-derived samples from different body habitats - is a sound approach to identifying the scope of viruses that comprise the human virome, including novel viruses. There are a number of ways this analysis can be extended in the future. Including RNA viruses in the sequencing and analysis and expanding the body sites sampled would lead to a more comprehensive view of the diversity in the human virome. Also, the methods used for DNA preparation and sequencing were limited to detecting ssDNA viruses with dsDNA intermediates, so future studies should include methods to assess ssDNA viral genomes. Deeper sequencing would allow the more robust detection of relatively rare viral sequences, improving the sensitivity of the sequencing assay. Deeper sequencing or the addition of quantitative follow-up assays would also allow us to assess viral dynamics by determining if the same virus strain is present in multiple body sites from the same subject or at multiple time points. Increasing the number of subjects would be beneficial because, while this is a large data set, eukaryotic virus communities are very simple and varied. Thus, some of the results from this set are anecdotal and bring attention to questions that can be better examined in future targeted studies. Sampling the same subjects at many time points would lead to an even more comprehensive understanding of the dynamics of the human virome, which is of particular interest for chronic infections. This study does not distinguish active and latent infections, and future studies could concentrate on active infections by assessing cell-free virus particles. This analysis relies on identifying sequence similarity to known viruses, and sequences from unknown novel viruses with no sequence similarity to known viruses may be present but not analyzed. New computational methods will thus be required to address this shortcoming. High-throughput sequencing technologies have the potential to play important roles in diagnostic virology, and expanding our knowledge of the human virome is a necessary first step to interpreting results from diagnostic tests and applying that information for effective diagnosis and treatment.

Conclusions

This study is the first to use high-throughput DNA sequencing to describe the diversity of eukaryotic DNA viruses in a large cohort of individuals who were sampled at a multiple body sites. This analysis demonstrates that there is a 'normal viral flora' in generally healthy, asymptomatic individuals. The normal flora includes viruses from seven families: *Herpesviridae*, *Polyomaviridae*, *Papillomaviridae*, *Adenoviridae*, *Anelloviridae*, *Parvoviridae*, and *Circoviridae*. Some viruses establish long-term infections that may be associated with increased risk or possibly with protection from disease. A better understanding of the composition and dynamics of the virome may hold important keys to human health.

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Authors' contributions

KMW contributed to figure production, data analysis, data interpretation, and writing. KAM, YZ, and ES contributed to data analysis. GAS and GMW contributed to data analysis, data interpretation, and writing. All authors read and approved the final manuscript.

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[1]The Department of Pediatrics, Washington University School of Medicine, Campus Box 8116, 660 S. Euclid Avenue, St Louis, MO 63110, USA. [2]The Genome Institute, Washington University School of Medicine, Campus Box 8501, 4444 Forest Park Avenue, St Louis, MO 63108, USA. [3]Current address: The Jackson Laboratory for Genomic Medicine, 10 Discovery Drive, Farmington, CT 06032, USA.

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EXHIBIT "R"

Healthy humans carry viruses too!

Source: The Economic Times – Panache

Link: https://economictimes.indiatimes.com/magazines/panache/healthy-humans-carry-viruses-too/articleshow/42716248.cms

Published on: Sep 17, 2014

Synopsis

The same viruses that make us sick can take up residence in and on the human body without provoking a sneeze, cough or other troublesome symptoms.

WASHINGTON: The same viruses that make us sick can take up residence in and on the human body without provoking a sneeze, cough or other troublesome symptoms, according to a new study.

In fact, healthy individuals carry about five types of viruses on their bodies, on average, researchers have found.

While everyone is familiar with the idea that a normal bacterial flora exists in the body, scientists have wondered whether there is a viral counterpart.

In 102 healthy young adults ages 18 to 40, the researchers in the new study sampled up to five body habitats: nose, skin, mouth, stool and vagina. The study's subjects were nearly evenly split by gender.

At least one virus was detected in 92 per cent of the people sampled, and some individuals harboured 10 to 15 viruses.

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NOTARY

"We were impressed by the number of viruses we found," said lead author Kristine M Wylie, an instructor of pediatrics at Washington University School of Medicine in St Louis.

"We only sampled up to five body sites in each person and would expect to see many more viruses if we had sampled the entire body," Wylie said.

Scientists led by George Weinstock, at Washington University's Genome Institute, sequenced the DNA of the viruses recovered from the body, finding that each individual had a distinct viral fingerprint.

About half of people were sampled at two or three points in time, and the researchers noted that some of the viruses established stable, low-level infections.

The researchers don't know yet whether the viruses have a positive or negative effect on overall health but speculate that in some cases, they may keep the immune system primed to respond to dangerous pathogens while in others, lingering viruses increase the risk of disease.

Study volunteers were screened carefully to confirm they were healthy and did not have symptoms of acute infection.

Analysing the samples, the scientists found seven families of viruses, including strains of herpes viruses that are not sexually transmitted.

For example, herpesvirus 6 or herpesvirus 7 was found in 98 per cent of individuals sampled from the mouth. Certain strains of papillomaviruses were found in about 75 per cent of skin samples and 50 per cent of samples from the nose. Novel strains of the virus were found in both sites.

Not surprisingly, the vagina was dominated by papillomaviruses, with 38 per cent of female subjects carrying such strains. Some of the women harboured certain high-risk strains that increase the risk of cervical cancer.

These strains were more common in women with communities of vaginal bacteria that had lower levels of Lactobacillus and an increase in bacteria such as Gardnerella, which is associated with bacterial vaginosis.

Adenoviruses, the viruses that cause the common cold and pneumonia, also were common at many sites in the body.

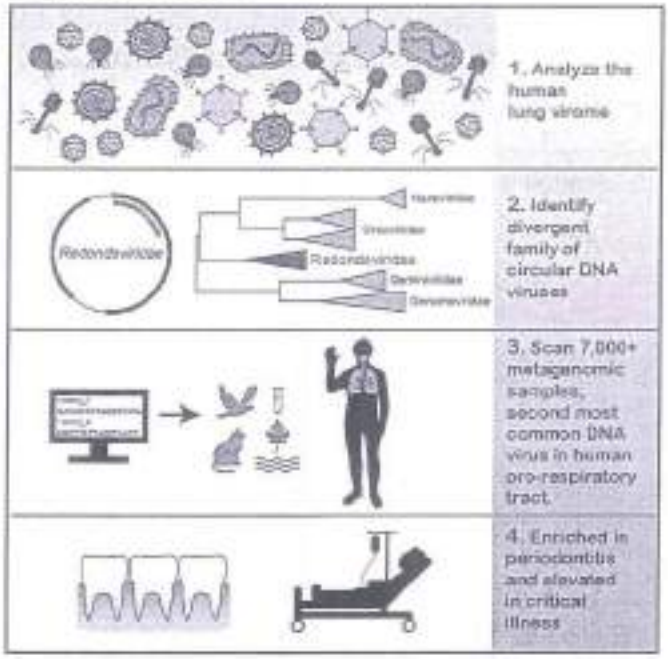
The study is published in the journal BioMed Central Biology.

EXHIBIT "S"

Cell Host & Microbe

Redondoviridae, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness

Graphical Abstract



Authors

Arwa A. Abbas, Louis J. Taylor, Marisol I. Dothard, ..., Layla A. Khatib, Ronald G. Collman, Frederic D. Bushman

Correspondence

collmanr@pennmedicine.upenn.edu (R.G.C.), bushman@pennmedicine.upenn.edu (F.D.B.)

In Brief

Abbas and Taylor et al. report the discovery and characterization of a family of circular DNA viruses subsequently named *Redondoviridae*. Redondoviruses are primarily found in the human oro-respiratory tract and reach high levels in subjects with periodontitis and critical illness.

Highlights

- A family of human DNA viruses was identified and named *Redondoviridae*
- Redondoviruses were the second most common virus in human respiratory virome samples
- Some subjects with periodontitis and critical illness had higher redondovirus levels

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CellPress

Redondoviridae, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness

Arwa A. Abbas,[1,3] Louis J. Taylor,[1,3] Marisol I. Dothard,[2] Jacob S. Leiby,[1] Ayannah S. Fitzgarald,[2] Layla A. Khatib,[2] Ronald G. Collman,[1,2,*] and Frederic D. Bushman[1,4,*]
[1]Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA
[2]Pulmonary, Allergy and Critical Care Division, Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA
[3]These authors contributed equally
[4]Lead Contact
*Correspondence: collman@pennmedicine.upenn.edu (R.G.C.), bushman@pennmedicine.upenn.edu (F.D.B.)
https://doi.org/10.1016/j.chom.2019.04.001

SUMMARY

The global virome is largely uncharacterized but is now being unveiled by metagenomic DNA sequencing. Exploring the human respiratory virome, in particular, can provide insights into oro-respiratory diseases. Here, we use metagenomics to identify a family of small circular DNA viruses—named *Redondoviridae*—associated with human diseases. We first identified two redondovirus genomes from bronchoalveolar lavage samples from human lung donors. We then queried thousands of metagenomic samples and recovered 17 additional complete redondovirus genomes. Detections were exclusively in human samples and mostly from respiratory tract and oro-pharyngeal sites, where *Redondoviridae* was the second most prevalent eukaryotic DNA virus family. Redondovirus sequences were associated with periodontal disease, and abundances decreased with treatment. Some critically ill patients in a medical intensive care unit were found to harbor high levels of redondoviruses in respiratory samples. These results suggest that redondoviruses colonize human oro-respiratory sites and can bloom in several human disorders.

INTRODUCTION

Viruses are the most abundant biological entities on Earth, but global viral populations (the "virome") are still mostly uncharacterized. Identifying novel viruses can be difficult if they have limited sequence homology to viral genomes in reference databases. Recent advances in sample preparation and sequencing techniques have uncovered a world of new viruses (Paez-Espino et al., 2016; Simmonds et al., 2017; Rosario and Breitbart, 2011; Minot et al., 2013; Minot et al., 2011). However, the majority of reads in most studies remain unclassified (Aggarwala et al., 2017; Krishnamurthy and Wang, 2017), leaving our understanding of the virome incomplete. Here, we describe the identification of a previously unstudied viral family, its localization in human oro-respiratory sites, and its association with disease states.

Methods for analyzing the virome are particularly efficient at recovering small circular DNA viruses. Metagenomic sample preparation commonly involves multiple displacement amplification (MDA) with a highly processive, strand-displacing DNA polymerase, which enriches for small, circular, single-stranded DNAs (ssDNA) (Rosario et al., 2012; Labonté and Suttle, 2013; Krupovic et al., 2016). Many ssDNA viruses encode a replication initiation protein (Rep); thus, this group is collectively known as circular Rep-encoding single-stranded DNA (CRESS) viruses (Rosario et al., 2012). Some aspects of genome architecture and functional domains of viral Rep and Capsid proteins are detectably conserved among CRESS viruses, though pairwise nucleotide identities are often low. A well-studied group of animal CRESS viruses is the *Circovirus* genus within the *Circoviridae* family, which includes pathogenic viruses of swine and birds (Ellis, 2014; Todd, 2000). The Circoviridae family also contains the genus *Cyclovirus*, which consists of viruses identified by metagenomic sequencing in samples from several mammalian species (Breitbart et al., 2017; Li et al., 2010), including some sporadically identified in human disease states (Phan et al., 2014; Smits et al., 2014). The recently identified *Smacoviridae* family has been detected in mammalian feces, though the definitive hosts are unknown (Varsani and Krupovic, 2018). Other CRESS families include viruses that infect plants, *Geminiviridae* and *Nanoviridae* (Harrison et al., 1977; Fauquet et al., 2005), fungi, *Genomoviridae* (Krupovic et al., 2016; Varsani and Krupovic, 2017), and additional apparent viruses detected as divergent sequences for which hosts are unknown (Simmonds et al., 2017).

We and others have previously investigated the human respiratory tract virome in health and disease. Typically, anelloviruses, herpesviruses, and bacteriophages dominate human respiratory tract samples (Willner et al., 2009; Abeles et al., 2015; Wylie et al., 2012; Pérez-Brocal and Moya, 2016; Young et al., 2015; Abbas et al., 2017; Abbas et al., 2019; Clarke et al., 2016). Recently, we identified short sequence reads with limited homology to a swine-associated CRESS virus (Cheung et al., 2014) in bronchoalveolar lavage (BAL) from human organ donors (Abbas et al., 2019; Abbas et al., 2017), raising the possibility that we had detected an undescribed human virus.

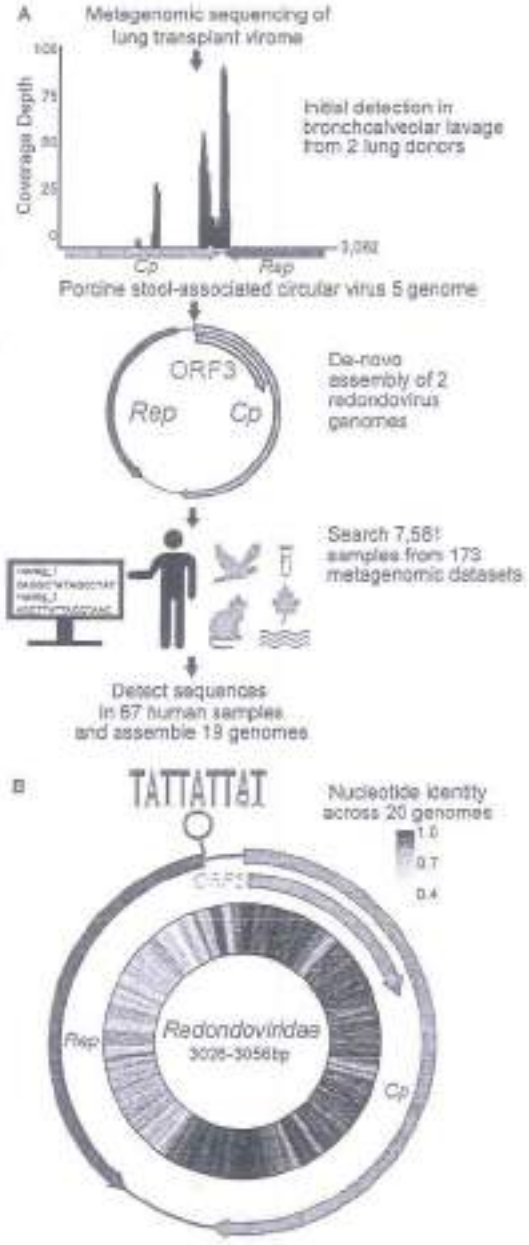

Figure 1. Discovery of Redondovirus Genomes in Metagenomic Samples

(A) Several hundred shotgun metagenomic reads from two organ donor BAL virome samples were identified as having limited homology to Porcine stool-associated circular virus 5 (PoSCV-5). Reads from these samples were assembled into two contigs, which were then cloned from multiple displacement amplified sample DNA using target specific primers and Sanger sequenced. The complete circular genomes were used to query additional internal and public microbial metagenomic datasets. Target-specific amplification, sequencing, and genome assembly were repeated for additional samples with sequences homologous to these novel genomes if the original DNA was available. In cases where original samples were not available, metagenomic contigs were checked for circularity and completeness. A total of 19 complete genomes were recovered from 67 human samples (bottom). See also Figure S1.

(B) The genomic architecture of redondoviruses shows antisense open-reading frames (ORFs) encoding a conserved capsid, replication associated protein (Rep), and unknown protein (ORF3). The average nucleotide identity of 20 *Redondoviridae* members (19 genomes discovered here and one genome previously reported [Cui et al., 2017]) is shown on the inside of the genome map as a heatmap. A putative origin of replication stem-loop structure with a conserved nonanucleotide motif is predicted to form in the 5′ end of the Rep coding region. The height of the letter in the motif represents its frequency.

Following up on this lead, we now report the identification of a group of CRESS viruses, highly divergent from other viral families, present in human respiratory and oral samples. These CRESS genomes are sufficiently different from previously described taxa that we propose establishing a viral family, which we name *Redondoviridae* (redondo—Spanish for "round"), containing the genera Vientovirus and Brisavirus (from the Spanish words for "wind" and "breeze," alluding to their discovery in the respiratory tract). A recently described genome identified in upper respiratory secretions of a febrile individual (Cui et al., 2017) also classified as a redondovirus, and we propose it as the type species for the Brisavirus genus. Analysis of the distribution of redondoviruses showed that they were the second most prevalent viruses in human respiratory samples, after anelloviruses, in samples from viral metagenomic studies. Analysis of redondovirus representation in numerous environmental and host-associated samples disclosed association of *Redondoviridae* with periodontal disease and acute critical illness.

RESULTS

Initial Discovery of Redondoviruses in Human BAL Fluid

In two previous studies of lung transplant recipients (Abbas et al., 2017; Abbas et al., 2019), samples of BAL were enriched for viral particles, and then RNA and DNA were purified and subjected to metagenomic sequence analysis. DNA fractions were amplified using MDA. Alignment of reads from two organ donor BAL samples to the NCBI viral genome database showed modest (14%) coverage of Porcine-stool-associated circular virus 5 (PoSCV-5) isolate CP3 (GenBank: NC_023878) (Figure 1). PoSCV-5 is currently an unclassified and unstudied member of the *Circoviridae* family.

After assembling reads from these samples into contigs, we found that sequences matching PoSCV-5 were present in circular contigs of approximately 3,000 base pairs (bp). Thus, whole viral genomes were present in the initial BAL samples, but only a small region of these genomes resembled PoSCV-5. Several sets of nested primers (Table S1; Figure S1) were used to amplify overlapping fragments from the original BAL samples. These fragments were sequenced using the Sanger method and assembled to construct two circular genomes of 3,026 bp (human lung-associated brisavirus RC; GenBank: MK059757) and 3,056 bp (human lung-associated vientovirus FB; GenBank: MK059763) (Figure S1).

Table 1. Comparison of Genomic Features between *Redondoviridae* and Other CRESS DNA Viruses

Feature	*Redondoviridae*	*Circoviridae*	*Nanoviridae*	*Geminiviridae*	*Genomoviridae*	*Smacoviridae*
Size (kb)	3.0–3.1	1.7–2.0	1.0 × 6 segments	2.5–3.0	2.1–2.2	2.6–2.9
ORFs	Cp, Rep, and ORF3	Cp, Rep, and ORF3/4	Cp, Rep, and others	Cp, Rep, and others	Cp and Rep	Cp and Rep
ORF orientation	ambisense	ambisense	segmented	ambisense (or segmented)	ambisense	ambisense
Origin sequence	TATTATTAT	TAGTATTAC	TATTATTAC	TAATATTAC	TAATATTAT	NAGTATTAC
Origin location	noncoding (upstream) and/or in Rep	noncoding (upstream) and/or in Rep	noncoding (upstream)	noncoding (upstream)	noncoding (upstream)	noncoding (downstream)

Contigs assembled from shotgun metagenomic reads of other BAL samples processed by our group were then queried for DNA sequence similarity to the two genomes described above. In total, seven complete *Redondoviridae* genomes were discovered and cloned from independent BAL samples from organ donors and patients with sarcoidosis (Figure S1). These genomes were then used as alignment targets to interrogate publicly available datasets. Twelve more samples had sufficient coverage of redondovirus sequences to allow assembly, yielding 19 complete genomes (Figure 1A; Table S2).

A danger is that small circular viruses may be derived from environmental contamination in clinical or laboratory reagents (Naccache et al., 2013; Salter et al., 2014). We queried 144 contamination controls from seven studies analyzed by shotgun metagenomics, six of which were from our laboratory (most viral metagenomic datasets in databases do not include sequenced contamination controls). None of these negative controls had any reads aligning to redondoviruses. This included 24 bronchoscope prewashes, which consist of a sterile saline solution passed through bronchoscopes before insertion into a patient. These were processed at our site in parallel with virome preps of multiple positive BAL samples; control samples were subjected to MDA, library preparation, and shotgun sequencing all in parallel (Clarke et al., 2018). In further tests, we used a qPCR assay targeting redondovirus genomes to check the 24 bronchoscope prewashes and two additional DNA extraction controls subjected to MDA. All were negative by qPCR analysis. As positive controls, we detected robust qPCR signals in MDA-amplified DNA extracted from the original acellular BAL samples from which these genomes were cloned (Figure S1C).

To strengthen the notion that *Redondoviridae* are of eukaryotic origin, we investigated whether they showed sequence signatures of bacteriophages. The presence of prokaryotic ribosomal binding sites (RBS) upstream of viral open reading frames (ORFs) can provide evidence for a prokaryotic host (Krishnamurthy and Wang, 2018). We implemented the algorithm described in Krishnamurthy and Wang (2018) and identified no prokaryotic RBS proximal to any redondovirus protein coding sequence. These data support the idea that redondovirus sequences were not derived from environmental contamination and are not bacteriophages.

Redondovirus Genomes Contain Conserved Features of CRESS Viruses and a Third Distinct ORF

Redondoviruses share some genomic features with other CRESS DNA viruses but display several unique characteristics (Table 1). Redondovirus genomes contain ambisense ORFs (Figure 1B) encoding a 334–363 amino acid Rep and a 449–631 amino acid capsid (Cp), which are only 10%–15% identical to those of porcine circovirus-1 and porcine circovirus-2, and 40%–55% for PoSCV-5, which provided the initial database "hit." All redondovirus genomes also contain a third ORF (ORF3) overlapping the capsid gene, which is not found in either porcine circoviruses or in PoSCV-5. ORF3 has no homology to any described protein family. Thus, while PoSCV-5 is the most closely related known virus to the redondoviruses, PoSCV-5 is markedly divergent in genome architecture and protein identity and does not meet criteria for a member of the *Redondoviridae* family.

Redondoviruses display considerable sequence divergence when comparing their Cp and Rep proteins. The range of pairwise amino acid identities of capsid is 67.5%–99.6% (median 82.3%) while the range of Rep amino acid identity is 36.6%–99.7% (median 54%) (Figure 1B). Surprisingly, Cp is more conserved than Rep. One might have expected that the capsid protein, which is presumably recognized by host antibodies, would be under stronger diversifying (positive) selection. Part of Cp overlaps ORF3 and so could be constrained in sequence drift for that reason, but even in the non-overlapping carboxy-terminal coding region (Figure 1B), the variability is still lower than in Rep.

To clarify the phylogenetic relationships between viral proteins within the *Redondoviridae* and other CRESS virus families, we built maximum-likelihood phylogenetic trees of Rep and Cp protein sequences. Redondoviruses are more similar to each other than to other CRESS families by protein identity and genome organization (Figure 2; Table 1). The capsid and Rep protein phylogenies show different relationships between the isolates, suggesting that recombination is common in redondoviruses, as in other circular ssDNA viruses (Ma et al., 2007; Lefeuvre et al., 2009; Rosenberger et al., 2017; Leppik et al., 2007). Based on previous definitions (Varsani and Krupovic, 2018) and analysis of the diversity of viral Rep proteins, redondovirus genomes can be grouped in two genera, demarcated by 50% Rep protein identity, which we propose to call Vientovirus and Brisavirus (Table S2).

The redondovirus Rep protein (Figure 3B) contains two domains found in many small DNA and RNA viruses: one involved in rolling-circle replication (Pfam: PF00799) and a second helicase domain within the P-loop NTPase superfamily (Pfam: PF00910) (Ilyina and Koonin, 1992; Gorbalenya et al., 1990).

Redondovirus capsids, like those of other ssDNA viruses, contain a basic amino terminus. Protein modeling by PHYRE2

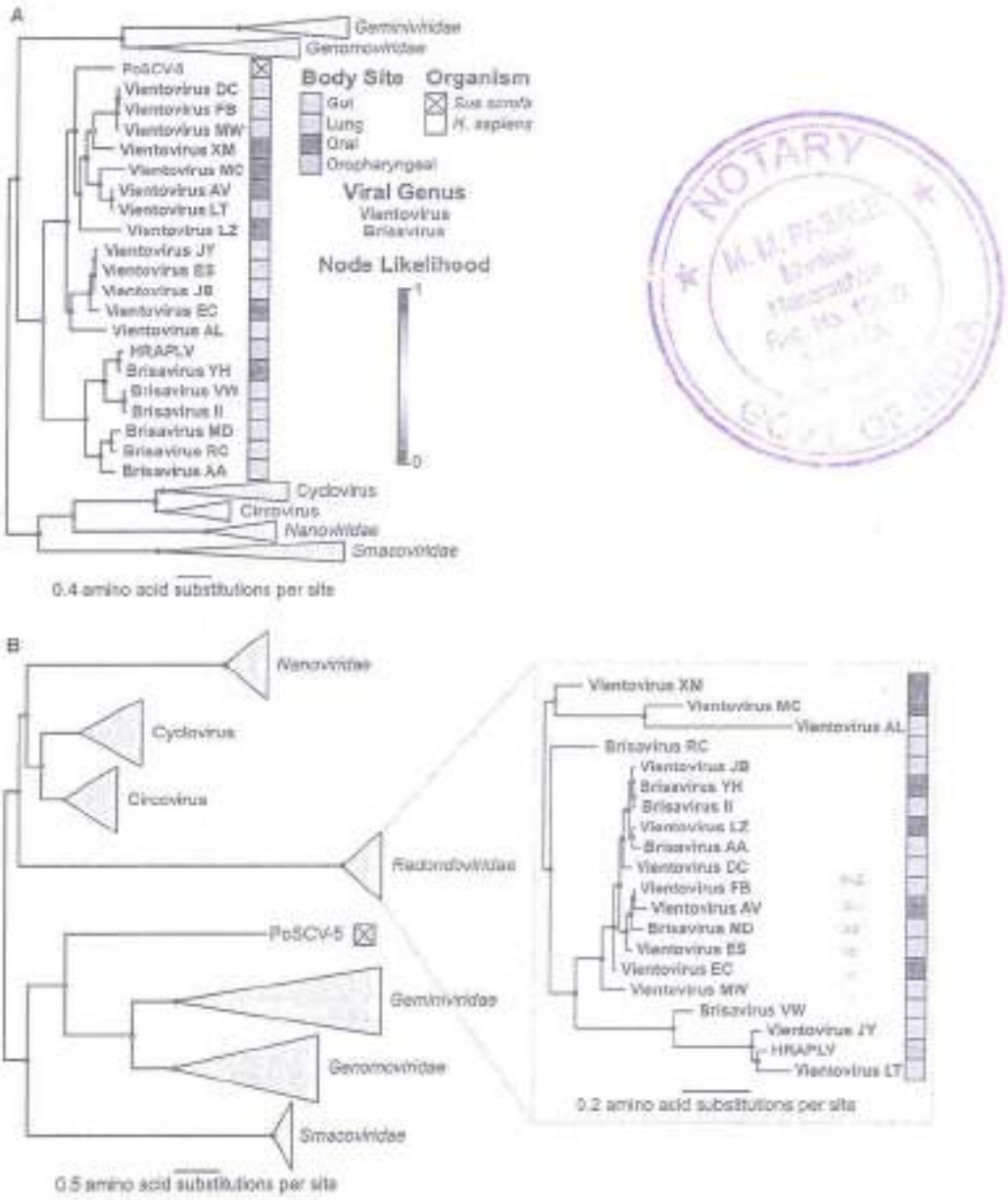

Figure 3. *Redondoviridae* Is a Distinct Virus Family Based on Capsid and Rep Identities Phylogenetic Trees of Redondovirus Rep (A) and capsid (B) proteins from CRESS DNA viruses. Collapsed viral genera or families are indicated by gray triangles. Branch likelihood, determined by approximate likelihood ratio test, is shown by colored circles at each node and the scale shows amino acid substitutions per site. The sample type of origin for each redondovirus is shown as colored boxes next to each virus' name, which is colored to reflect genus designation. See also Table S3.

(Kelley et al., 2015) weakly predicted folds similar to coat proteins of ssRNA viruses that infect plants (Figure 3C; 58% confidence over 7% of sequence).

Circovirus genomes typically contain a conserved motif ("*NANTATTAC*") within a stem-loop structure followed by short direct repeats, located in the intergenic region at the 5' end of

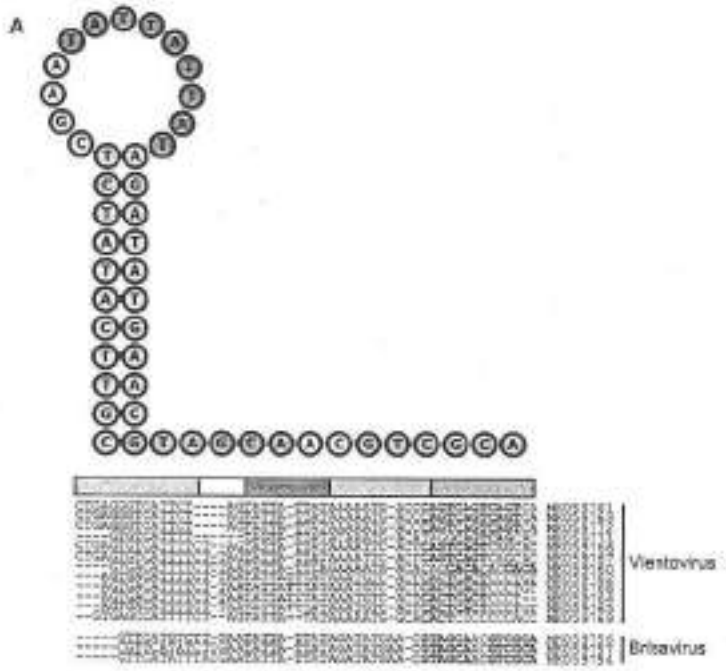

Figure 3. Redondovirus Genomes Contain Conserved Motifs Implicated in Rolling-Circle Replication

(A) The sequence and predicted structure of the putative replication origin of Human lung-associated brisavirus AA is shown in the top left. The inverted repeat forming the stem is shown in orange, the nonanucleotide motif within the loop is shown in green, and an imperfect 6 bp direct repeat sequence is shown in purple. Individual predicted stem loop sequences (threshold for stability: $\Delta G^{\circ} < -5$ kcal/mol) are shown to the right of the folded sequence. The calculated ΔG° of melting for the predicted stem loops ranges from −5.0 to −9.45 kcal/mol.

(B) Conserved rolling circle replication and superfamily 3 (SF3) helicase motifs were found in redondoviruses. The positions for the motifs are given using the Human lung-associated brisavirus AA genome sequence (GenBank: MK069754). The height of each letter represents its frequency. Amino acid positions identified as possibly under positive selection pressure are marked by a red star.

(C) The putative redondovirus capsid protein contains a basic amino-terminus and a predicted virus coat protein-like fold. The positions for the motifs correspond to Human lung-associated brisavirus AA, as above. Amino acid positions identified as possibly under positive selection pressure are marked by a red star.

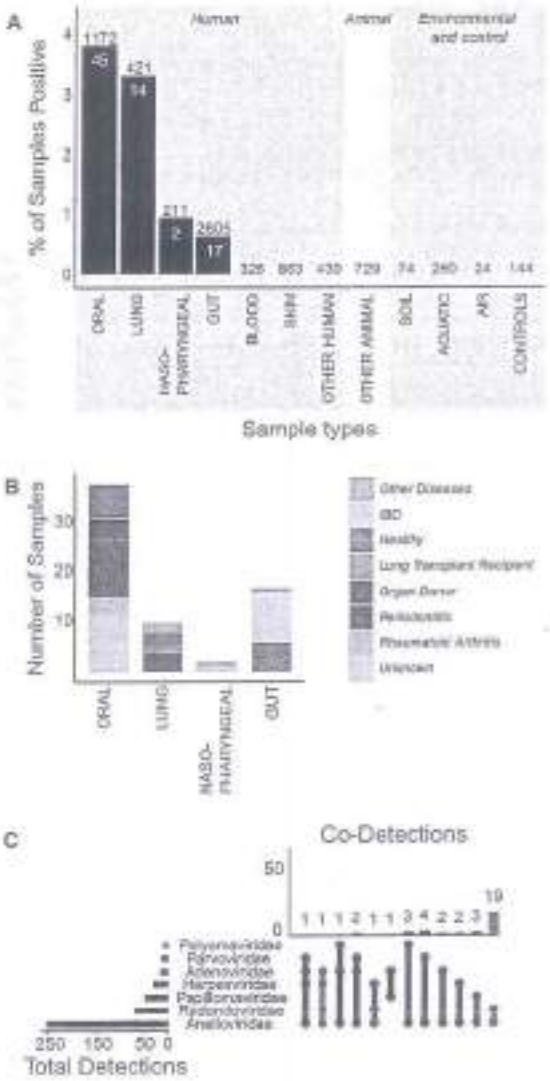

Figure 4. Frequency of Redondovirus Detection and Co-occurrence with Human DNA Viruses

(A) Reads from 173 metagenomic datasets encompassing different human and nonhuman sample types were aligned to redondovirus genomes. A positive hit was determined based on 25% coverage of any redondovirus genome by short-read alignment. The percentage of samples that were positive is plotted on the y axis and human body sites and other sample types are shown on the x axis. The total number of samples analyzed in each category is annotated above and the total number of positive samples is indicated within the bar. See also Table S3.

(B) The clinical status breakdown, if available, of redondovirus-positive samples is shown.

(C) Reads from a subset of 20 datasets across 9 body sites (n = 2,675) were analyzed for homology to 23 redondovirus genomes and 133 animal-cell DNA viruses from six viral families. The height of each column represents the total number of samples that had detections of multiple viral families (rows). The viral families included in the co-detections are depicted as filled dots connected with lines below. The length of the bars on the left represents the total number of samples in which that viral family was detected. Cases where redondoviruses were detected are indicated in blue. See also Figures S2 and S3. IBD, inflammatory bowel disease.

Cp and Rep. Such sequences are candidates for the origin of replication (Mankertz et al., 1997) where the viral-encoded Rep binds and cleaves, mediating replication by host polymerases. Such stem loops are found in other CRESS virus families. In the one previous report of a single redondovirus genome (Cui et al., 2017), the authors suggested a hairpin in the large intergenic region as the origin of replication. However, analysis of all 20 genomes showed that a conserved, stable stem loop structure is predicted to form in the second smaller intergenic region, partially overlapping Rep. Although the length of the stem, size of the loops, and presence of downstream direct repeats vary, most redondovirus genomes contain a nonanucleotide motif ("TATTATTAT") (Figure 1B) similar but not identical to that of other CRESS viruses. This structure is highly conserved among redondoviruses (Figure 3A), while the sequence of the alternative intergenic hairpin is not, suggesting that this is a more likely candidate for the replication origin.

Redondovirus Genomes Identified in Shotgun Metagenomic Data

To investigate redondovirus distribution in the biosphere, we surveyed metagenomic sequence datasets for homology to redondoviruses (Table S3 presents the datasets queried). Studies were favored for analysis if they (1) biochemically enriched for viral nucleic acids; (2) used MDA, which enriches for small circular viral genomes (Kim and Bae, 2011; Kim et al., 2008); (3) reported detection of Circovirus-like sequences; and/or (4) included a diverse range of sample types. In total, we queried 7,561 samples from 173 datasets covering 51 organisms or environments. Within human metagenomes, 18 body sites or fluids were examined. A positive hit was defined as 25% coverage of any redondovirus genome.

Redondoviruses were detected in metagenomic sequences from human oral cavity (3.8% of samples), lung (3.3%), nasopharynx (0.95%), and gut (0.58%). The most frequent sites of detection were the mouth and respiratory tract (Figure 4A). Redondovirus sequences were rare in human gut (17 detections total). Redondoviruses were not found in other animals, freshwater, marine, or soil samples (1,087 non-human biological samples) nor in laboratory reagents (144 contamination control samples). Importantly, 24 of the contamination controls analyzed were prepared side-by-side with redondovirus-positive samples and consist of saline bronchoscope pre-washes performed immediately prior to BAL sampling, which reflects the entire pipeline of specimen acquisition and nucleic acid processing. We thus conclude that redondoviruses are authentically present in the human oro-respiratory tract. Whether infrequent detection in gut samples reflects an authentic site of replication or transient passage after swallowing is uncertain. We cannot rule out that redondoviruses colonize other animal species, although thus far we have only identified hits in human samples.

Redondovirus Co-occurrence with Human DNA Viruses

Adeno-associated virus (AAV), an ssDNA virus of the *Parvoviridae* family, also encodes capsid and Rep proteins and is known to require co-infection with a helper virus such as adenovirus to replicate. We thus asked whether redondoviruses co-occurred with any other eukaryotic viral family suggestive of a helper virus. We analyzed a subset (20) of the 173 datasets we previously screened for redondoviruses for the presence of common human DNA virus families (*Adenoviridae*, *Anelloviridae*, *Herpesviridae*, *Papillomaviridae*, *Parvoviridae*, and *Polyomaviridae*). *Redondoviridae* was the second most frequent human DNA virus family detected, exceeded only by *Anelloviridae*, which are known to be ubiquitous in humans (Spandole et al., 2015)—this high frequency is likely affected by use of MDA for virome preps, which enriches for small circular DNAs. Figure 4C shows the representation of additional human DNA viruses that co-occurred with redondoviruses in metagenomic datasets. Only anelloviruses were found to co-occur significantly with redondoviruses (Figure 4C, $p = 5.7 \times 10^{-7}$, Fisher's exact test with Bonferroni correction). Anelloviruses are small ssDNA viruses that seem unlikely to contribute helper functions to redondovirus replication. We speculate that the inflammatory milieu known to favor anellovirus replication (Maggi et al., 2001; Mariscal et al., 2002) may be similarly favorable for redondoviruses. Alternatively, given the ubiquitous nature of anelloviruses in humans, this association may reflect the fact that MDA enriches for both anelloviruses and redondoviruses, resulting in their co-detection. Rarely, other human viruses were found in redondovirus positive samples; these included Human mastadenovirus C and Epstein-Barr virus.

Redondoviruses in the Respiratory Tract Are Elevated in Abundance in Critical Illness

Several sample sets were further queried using metagenomic analysis and qPCR to assess redondovirus abundance in the respiratory tract. We investigated 916 selected oro-respiratory samples using metagenomic analysis of datasets described above, reflecting a mixture of health and disease states, and found that redondoviruses were still the second most frequent DNA virus detected, after anelloviruses (Figure S3).

To investigate the presence of redondovirus in healthy subjects further, we tested DNA isolated from oropharyngeal swabs from 60 adults using qPCR (Charlson et al., 2010). DNA was subjected to selective whole-genome amplification (SWGA) (Clarke et al., 2017) to enrich for redondovirus sequences over the human genome background, followed by redondovirus qPCR. Nine of 60 healthy subjects were positive (15%), although quantities even following SWGA amplification showed generally modest levels (Figure 5A).

We then tested samples from 69 critically ill individuals (44 males, 25 females) using SWGA and qPCR (Figures 5A and 5B). Six (9%) had oropharyngeal samples positive for redondovirus (3 males and 3 females), indicating relevance to both genders (male 7% versus female 12%; $p = 0.66$, Fisher's exact test). Post-SWGA quantities were, on average, 10^4-fold greater than in healthy subjects, although the use of SWGA complicates quantitative comparisons between groups. Four of these six critically ill subjects also had lung secretions (endotracheal aspirates) available for testing; three were positive for redondovirus. In subjects with serial samples, redondovirus was generally detectable over a period of 2–3 weeks, suggesting persistent colonization or infection. We conclude that redondoviruses are found in both healthy and critically ill individuals, but their levels are elevated in illness. Furthermore, the upper and lower respiratory tracts appear to represent common niches with stable redondovirus detection over time.

Redondovirus Sequence Reads Are Associated with Periodontitis

The set of 97 metagenomic studies assessed for redondovirus sequences (Table S3) contained samples from several disease states, allowing us to assess possible associations of redondoviruses with human disorders. In addition to our initial detections in BAL from organ donors and lung transplant recipients (Abbas et al., 2017; Abbas et al., 2019), redondoviruses were found in (1) BAL from subjects with sarcoidosis and healthy adults (Clarke et al., 2018), (2) gingival samples from subjects with periodontitis (Wang et al., 2013; Shi et al., 2015; Califf et al., 2017), (3) oropharyngeal and nasopharyngeal samples from febrile subjects (Mokili et al., 2013; Wang et al., 2016), (4) oral samples from subjects with rheumatoid arthritis (Zhang et al., 2015), (5) stool samples from healthy individuals, (6) stool samples from subjects with inflammatory bowel disease (Norman et al., 2015), and (7) stool samples from subjects with HIV-associated immunodeficiency (Monaco et al., 2016) (Figure 4B).

A considerable proportion of redondovirus-positive samples were from studies of periodontal disease (Figure 4B), so we analyzed these further. Three studies queried gingival or oral samples from subjects suffering or recovered from periodontitis (detailed metadata in Table S4). One study queried samples before and after corrective treatment by scaling and root planing together with improved oral hygiene (Shi et al., 2015). Redondovirus representation was high prior to treatment and then fell substantially after treatment, as measured by the number of reads aligning to the most broadly covered redondovirus genome in each sample (Figure 5C). We averaged redondovirus reads across all individual tooth sites sampled for each subject and found lower redondovirus prevalence after recovery (Figure 5C, $p = 0.014$, Wilcoxon signed-rank test). The second study compared disease severity in two groups with chronic periodontitis; one group received treatment with 0.25% sodium hypochlorite rinse, while the other received a water rinse (Califf et al., 2017). We compared redondovirus representation in sub- and supra-gingival sites from subjects whose periodontitis did or did not improve and found that subjects that did not show improvement had greater numbers of reads mapping to redondovirus genomes (Figure 5D, $p = 0.028$, Wilcoxon rank-sum test). A third study analyzed two patients with severe periodontal disease before and after treatment; both subjects were positive for redondovirus prior to treatment, but no detections were found in samples taken after successful treatment (Kumar et al., 2018). Thus, we conclude that redondoviruses are associated with periodontitis in multiple studies and that levels are reduced with effective treatment.

DISCUSSION

Here, we introduce *Redondoviridae*, a family of small, circular DNA viruses discovered in metagenomic sequence data, which is

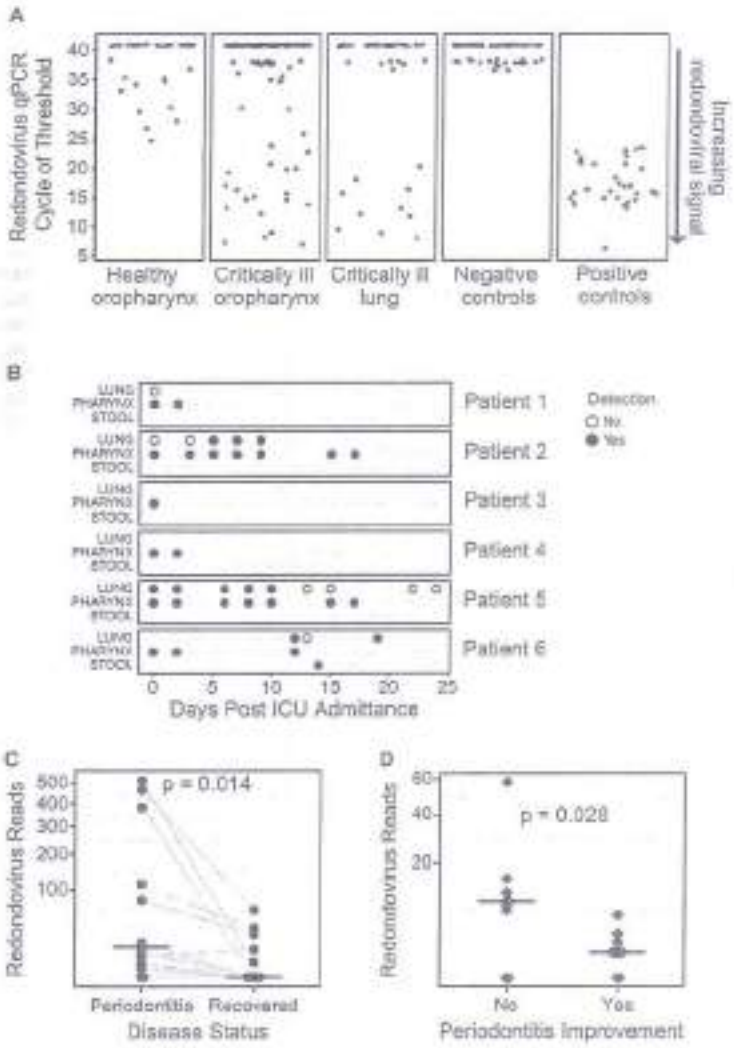

Figure 5. Redondoviruses in the Oro-Respiratory Tract in Humans with Critical Illness and Periodontitis

(A) Quantification of redondovirus genome sequences in post-SWGA DNA from oropharyngeal swabs (oropharynx) from 60 healthy volunteers and oropharyngeal swabs and endotracheal aspirates (lung aspirations) from 69 critically ill subjects. The average cycle of threshold (Ct) value of technical replicates is plotted on the y axis. Samples with undetermined (i.e., no amplification) value in all 3 replicates are assigned an arbitrary value above the Ct value of the limit of resolution of the assay (37) that corresponds to 11 target copies per reaction. Samples below this value are counted as authentic detections. Negative controls included extraction blanks, reagent blanks, and no template controls. Positive controls represent replicates of 10^4 copies of human lung-associated brisavirus RC spiked into DNA extracted from a redondovirus-negative lung sample, subjected to SWGA, and assayed by qPCR.

(B) qPCR was used for redondovirus detection in respiratory and/or stool samples from 69 subjects in the medical intensive care unit (ICU). Six total subjects, 3 males and 3 females, were positive for redondoviruses. The time point and type of sample surveyed for these six subjects is shown on the x and y axes, respectively. Postive samples are indicated by a filled-circle and negative samples by an open circle.

(C) Number of reads mapping to a redondovirus in periodontitis samples from (Shi et al., 2015). Each point represents the average of all samples from a particular individual either before treatment (red) or after disease resolution (blue). Points from the same subject are connected by gray lines. The horizontal black line indicates the median. The Wilcoxon signed-rank test was used to test for paired differences between groups.

(D) Each point represents the number of reads mapping to a redondovirus in samples from (Liem et al., 2017) from subjects with periodontitis whose disease either did (blue) or did not (red) improve during the study. The horizontal black line indicates the median. The Wilcoxon-rank-sum test was used to test for differences between groups. See also Table S4.

found selectively in human lung and oro-pharyngeal samples. We first identified redondovirus genomes by aligning metagenomic sequences from lungs of two organ donors to a viral genome database, resulting in weak hits to PcSCV-5. Assembly of shotgun metagenomic reads yielded complete circular genomes, which were then used to interrogate our collection of lung virome samples, allowing us to identify seven genomes. We then used these genomes to interrogate 7,691 metagenomic samples from diverse environmental sites, hosts, body sites, and disease states, detecting redondoviruses in 67 human samples and building 12 additional genomes. Independently, another group reported a single genome (Cui et al., 2017) in a sample from the throat of a febrile patient that we find is most closely related to Human oral-associated brisavirus YH (GenBank MK059758). Of the DNA viruses we surveyed in 20 human virome datasets, redondoviruses were the second most abundant, exceeded only by anelloviruses. The prevalence of redondoviruses was similar in cohorts of healthy subjects and critically ill subjects, although higher genome quantities suggested higher absolute levels in the ill subjects. Analysis of metagenomic samples revealed an association of redondoviruses with periodontal disease.

It is possible that redondovirus infection and replication may help maintain the inflammatory state associated with periodontitis and contribute to disease progression. A role in disease initiation seems less likely given the established roles of bacteria and oral hygiene (Edlund et al., 2013; Costalonga and Herzberg, 2014). Previous studies have tentatively implicated viruses in periodontitis based on alterations of subgingival bacteriophage

communities (Ly et al., 2014) and increased representation of some eukaryotic viruses including HIV, Human cytomegalovirus, and herpes simplex virus 1 (HSV-1) (Cappuyns et al., 2006; Li et al., 2017). The role of redondoviruses in periodontitis warrants further study. Similarly, the role redondoviruses play in diseases of the respiratory tract can now be investigated.

Do redondoviruses require helper viruses to replicate? The Dependoparvoviruses, which include AAV, are small, linear ssDNA viruses that require co-infection with larger DNA viruses to condition cells for efficient replication. Samples containing redondoviruses were scanned for other DNA viruses, but no large double stranded DNA viruses were consistently identified. Anelloviruses, small, circular ssDNA viruses, did co-occur. While we do not rule out that anelloviruses support redondovirus replication, it seems more likely that the inflammatory states known to promote anellovirus replication may do the same for redondoviruses or, alternatively, that the methods for virome sampling preferentially recover both redondoviruses and anelloviruses.

The high level of sequence variation in redondovirus Rep proteins is intriguing. Viruses encoding Reps are ubiquitous in both prokaryotes and eukaryotes. There are even transposon families that mobilize via ssDNA intermediates using Rep-like enzymes (Grabundzija et al., 2016). Cells have likely been opposing parasitism by Rep-encoding elements since the origins of cellular life. We conjecture that Rep amino acid variation reflects an ongoing Red Queen's Race between host intrinsic immunity and Rep enzymes. If so, there should be active host cell mechanisms targeting and inhibiting Rep proteins. The redondovirus Rep enzymes reported here provide an entry point to investigating this possibility.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chom.2019.04.001.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.A.A., L.J.T., R.G.C., and F.D.B.; Experimental Methodology, A.A.A., L.J.T., M.I.D., and J.S.L.; Specimen Collection, L.A.K. and A.S.F.; Formal Analysis, A.A.A., L.J.T., M.I.D., R.G.C., and F.D.B.; Writing – Original Draft, A.A.A. and L.J.T.; Writing – Review & Editing, A.A.A., L.J.T., R.G.C., and F.D.B., Supervision – R.G.C. and F.D.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
NEB 5-alpha Competent E. coli	New England BioLabs	Cat#C2987I
Biological Samples		
Bronchoalveolar lavage (BAL), BAL prewash, and reagent control samples	Abbas et al., 2017; Clarke et al., 2018; Abbas et al., 2019; Charlson et al., 2011; Charlson et al., 2010	N/A
Oral swabs, endotracheal aspirates, stool, and reagent control samples	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
AccuPrime™ Taq	ThermoFisher	Cat#12339016
Phi29 polymerase	New England BioLabs	Cat#M0269
Illustra GenomiPhi V2 DNA	GE Healthcare	Cat#25660031
Critical Commercial Assays		
DNeasy PowerSoil Kit	Qiagen	Cat#12888-100
Gibson Assembly Master Mix	New England BioLabs	Cat#E2611
TaqMan Fast Universal PCR Master Mix	Applied BioSystems	Cat#4364103
Deposited Data		
Genome accession numbers	GenBank	Accession #s: MK059754-MK059772
Oligonucleotides		
See Table S1	Integrated DNA Technologies	N/A
Recombinant DNA		
pUC57	BioBasic	Addgene 4508
pUC19	Norrander et al., 1983	Addgene 50005
Genome synthesis	BioBasic	https://www.biobasic.com/gene-splash
Software and Algorithms		
sra-tools	Leinonen et al., 2011	https://github.com/ncbi/sra-tools
SAMtools	Li et al., 2009	http://samtools.sourceforge.net/
BEDtools	Quinlan and Hall, 2010	https://bedtools.readthedocs.io/en/latest/
R	Ihaka and Gentleman, 1996	https://www.r-project.org/
Sunbeam	Clarke et al., 2018	https://github.com/sunbeam-labs/sunbeam
MEGAHit	Li et al., 2015	https://github.com/voutcn/megahit
BLASTn	Altschul et al., 1990	https://www.ncbi.nlm.nih.gov/books/NBK279690/
Cap3	Huang and Madan, 1999	http://doua.prabi.fr/software/cap3
CloneManager 9	Scientific & Educational Software	http://www.scied.com/pr_cmpro.htm
EMBOSS	Rice et al., 2000	http://emboss.sourceforge.net/
Mfold	Zuker, 2003	http://unafold.rna.albany.edu/?q=mfold
Forna	Kerpedjiev et al., 2015	http://rna.tbi.univie.ac.at/forna/
MUSCLE	Edgar, 2004	https://www.drive5.com/muscle/
PhyML	Guindon et al., 2010	http://www.atgc-montpellier.fr/phyml/
FigTree	N/A	http://tree.bio.ed.ac.uk/software/figtree/
haas pipeline	This study	https://github.com/louiejtaylor/hass
Ribosomal binding site analysis script	This study	https://github.com/louiejtaylor/find-prok-rbs
PRISNAL	Suyama et al., 2006	http://www.bork.embl.de/pal2nal/

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HyPhy	Pond et al., 2005	http://hyphy.org/
FUBAR	Murrell et al., 2013	N/A
pyvikO	Taylor and Strebel, 2017	https://github.com/louisjtaylor/pyViKO
WebLogo	Crooks et al., 2004	https://weblogo.berkeley.edu/logo.cgi
awga	Clarke et al., 2017	https://github.com/eclarke/awga
Snakemake	Köster and Rahmann, 2012	https://snakemake.readthedocs.io/en/stable/
Other		
QuantStudio 5 Real Time PCR System	Applied BioSystems	N/A
ABI 3730XL Sanger Sequencer	Applied BioSystems	NA

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Frederic Bushman (bushman@pennmedicine.upenn.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Studies

Samples analyzed here obtained in the context of studies previously reported were obtained with informed consent and under protocols approved by the Institutional Review Board at their respective institutes as detailed in (Abbas et al., 2017; Clarke et al., 2018; Abbas et al., 2019; Charlson et al., 2011; Charlson et al., 2010). Human subjects >18 years of age with varying critical illnesses were enrolled within 24 hours of admission at the Hospital of the University of Pennsylvania medical Intensive Care Unit (ICU). Individuals with an anticipated ICU length of stay <48 hours were excluded. Informed consent was obtained under IRB protocol 823392. Subjects were not involved in any other experimental procedures. Oropharyngeal swabs (n = 198), endotracheal aspirates (n = 87), and stool (n = 16) from 69 (44 males and 25 females) subjects were available to be queried by qPCR. Redondovirus positivity was similar in males and females, suggesting no obvious influence of gender. DNA from oropharyngeal swabs was extracted in single-tube DNeasy PowerSoil Kit (Qiagen; Hilden, Germany) and followed manufacturer's protocol except for two 50 μL elutions with buffer C6. Endotracheal aspirate was extracted with a 96-well format of the same kit. Due to sample availability limitations, a single biological replicate was used for each sample. No estimation for optimal sample size to detect statistical significance was performed for this initial survey. Additional metagenomic sequence data (n = 7,581 human, animal, and environmental samples) were derived from publically available data repositories or unpublished studies performed in our lab (n = 173 individual studies or datasets) (Table S3).

METHOD DETAILS

Discovery and Detection in Clinical Samples

Acellular bronchoalveolar lavage (BAL) samples were obtained from prior studies of organ donors and lung transplant recipients (Abbas et al., 2017; Abbas et al., 2019), subjects with sarcoidosis (Clarke et al., 2018) and healthy adults (Charlson et al., 2011). Virus-like particle purification, preparation of shotgun DNA libraries, metagenomic sequencing, within-sample contig assembly and annotation based on alignment to the NCBI viral database has been previously described (Abbas et al., 2017; Clarke et al., 2018; Abbas et al., 2019). Contigs found to have homology to redondovirus genomes were amplified and cloned from 7 samples.

Primers (Table S1) were designed to amplify redondovirus genome sequences from DNA extracted from BAL that underwent whole genome amplification with Illustra GenomiPhi V2 DNA (GE Healthcare; Little Chalfont, UK). PCR was performed with AccuPrime™ Taq DNA Polymerase System (ThermoFisher; Waltham, MA, USA) using 1 μL of whole-genome-amplified product, 20 μM of forward and reverse primers and 0.2 μL Taq polymerase in a total volume of 50 μL. Products were visualized on 1-1.5% ethidium bromide agarose gels (Figure S1). Amplicons were cloned and validated by using the Sanger sequence method on an ABI 3730XL (Applied BioSystems; Waltham, MA, USA) instrument. Full redondovirus genomes were either de-novo synthesized (BioBasic; Markham, ON, CA) or cloned by Gibson assembly (NEB; Ipswitch, MA, USA) and also verified by Sanger sequencing.

To detect redondovirus sequences in BAL samples, a TaqMan-based qPCR assay (Table S1) was designed targeting the genomic region encoding the capsid gene. For each sample, triplicate 20 μL reactions containing 4 μL of template DNA (depending on sample availability), 0.33 μL forward and reverse primers (18μM), 0.33 μL probe (5μM), 10 μL TaqMan Fast Universal PCR Master Mix (Applied Biosystems; Waltham, MA, USA) and 5 μL water were analyzed on a QuantStudio 5 Real Time PCR System (Applied Biosystems; Waltham, MA, USA) with the following cycling profile: 20 sec at 95°C for 1 cycle, and 40 cycles of 95°C for 3 sec and 60°C for

30 sec (signal collection). A linearized plasmid containing the complete Human lung-associated brisavirus RC genome in a pUC57 vector was used as a 7 point standard curve ranging from 75 to 30,000,000 copies/reaction. Amplification signal was required in 2 out of 3 wells to be scored as positive.

Endotracheal aspirates (lung secretions), oropharyngeal swabs and stool were collected from critically ill subjects and oropharyngeal swabs were collected from healthy volunteers, following written informed consent, under protocols approved by the University of Pennsylvania Institutional Review Board (protocols 823392 and 810987, respectively).

Extracted DNA was first subjected to SWGA using primers designed with the software described in Clarke et al., (2017). Each reaction contained 2 μL Phi29 10x Buffer (NEB; Ipswich, MA), 1 μL Phi29 polymerase, 0.2 μL bovine serum albumin (10 mg/mL), 100 μM total of 20 primers (final concentration of each primer was 2μM), 2 μL of 10mM dNTPs, and 1 μL of template DNA in a total volume of 20 μL. Reactions underwent a step-down amplification process by incubating at 35°C for 5 min, 34°C for 10 min, 33°C for 15 min, 32°C for 20 min, 31°C for 30 min and then 30°C for 16 hours, followed by a heat inactivation step (65°C for 15 min) as previously described (Clarke et al., 2017). After SWGA, 4 μL of product was queried in duplicate using a more sensitive TaqMan-based qPCR assay (Table S1) that was designed targeting a conserved region of the *Cp* gene. A linearized plasmid containing the complete genome of human lung-associated brisavirus RC was used a 9-point standard curve ranging from 10 to 10^8 copies per reaction. Negative and positive controls were included in each run to evaluate inter-assay variability. The positive control was 10^4 copies of the standard curve plasmid containing the viral genome spiked into DNA extracted from a redondovirus-negative endotracheal aspirate sample and also subjected to SWGA.

Querying Viral Metagenomic Datasets

Reads from viral and other shotgun metagenomic projects available in the Sequence Read Archive (SRA) (n = 146) or MG-RAST (n = 23) or the Human Oral Microbiome Database (n = 1) and from 3 unpublished datasets from the University of Pennsylvania (Table S4) were processed in the following steps: 1) adaptor-trimmed single or paired-end reads were downloaded using fastq-dump (Leinonen et al., 2011), 2) a sensitive local alignment of either single reads or read pairs to redondovirus genomes was performed using Bowtie2 (Langmead and Salzberg, 2012); 3) alignments were processed and genome coverage was calculated with SAMtools (Li et al., 2009) and BEDtools (Quinlan and Hall, 2010) and 4) alignments were visualized with a custom R (version 3.2.3) script (Ihaka and Gentleman, 1996) (R packages used: magrittr, ggplot2, reshape2). Based on the sigmoidal relationship between aligning reads and genome coverage (Figure S2), a minimum threshold of 25% genome coverage was used to determine detection of a redondovirus.

Genome Assembly

Samples in which 25% of any redondovirus genome was covered were further analyzed using the Sunbeam pipeline (Clarke et al., 2019) to process reads and to build and annotate contigs using MEGAhit (Li et al., 2015) and BLASTn (Altschul et al., 1990). Contigs were further refined by overlap consensus assembly using Cap3 (Huang and Madan, 1999) and manually checked for circularity and presence of key genomic features with CloneManager 9 (Scientific & Educational Software; Denver, CO).

DNA and Amino Acid Sequence Analysis

The EMBOSS einverted utility (Rice et al., 2000), Mfold (Zuker, 2003) or CloneManager Professional 9 (Scientific & Educational Software; Denver, CO) was used to predict and visualize energetically favorable DNA structural features potentially important for replication. Forna was used to plot stem loop structures (Kerpedjiev et al., 2015). Nucleotide and protein alignments were performed using MUSCLE (version 3.8.31) (Edgar, 2004). Phylogenetic trees were built using PhyML (Guindon et al., 2010) using the LG amino acid substitution model (Le and Gascuel, 2008) with sequences from 2-3 representative species of established viral families and all full-length protein sequences of novel redondoviruses. Branch support was quantified by the approximate likelihood-ratio test (Anisimova and Gascuel, 2006) and visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Consensus motif logos were generated using WebLogo (Crooks et al., 2004). Conserved domains within the Rep protein were detected using NCBI's CD-search against the Pfam database (v30.0, E-value < 10^{-2}). Protein folding predictions were done using PHYRE2 (Kelley et al., 2015) using default parameters.

To predict prokaryotic ribosomal binding sites (RBS), we implemented the algorithm described in Krishnamurthy and Wang (2018) in Python (version 3.6). Briefly, we extracted 18 nucleotides in the untranslated region immediately upstream of start codons and searched for prokaryotic ribosomal binding sites (full: AGGAGG; partial: AGGAG, GGAGG, AGGA, GGAG, GAGG).

We performed an exploratory analysis of synonymous and nonsynonymous substitution rates, as dN/dS as a marker of selective pressure is untested in CRESS viruses and may be confounded by overlap of unidentified coding sequence and/or functionally important DNA secondary structure elements (Zanini and Neher, 2013; Muhire et al., 2014). First, we aligned protein sequences using MUSCLE (Edgar, 2004), and built phylogenetic trees with PhyML (Guindon et al., 2010). We then generated codon alignments using PAL2NAL (Suyama et al., 2006) and used HyPhy (Pond et al., 2005) to perform dN/dS analysis. We used FUBAR (Murrell et al., 2013) to predict sites under positive selection. As dN/dS analysis in overlapping genes is overwhelmed by pressure to maintain the amino acid sequence of two genes, we only analyzed the portion of the Cp coding sequence that did not overlap with the ORF3 protein—overlapping coding regions were identified and excluded using pyviko (Taylor and Strebel, 2017).

QUANTIFICATION AND STATISTICAL ANALYSIS

Co-occurrence of Redondoviridae and Animal-Cell Viruses

Twenty datasets in which redondovirus genomes were found or were comprehensive studies of the human DNA virome were chosen for a targeted analysis of human viruses. Specifically, reads from these datasets (n = 2,675 samples) were aligned to 133 vertebrate viruses from the *Adenoviridae*, *Anelloviridae*, *Herpesviridae*, *Papillomaviridae*, *Parvoviridae* and *Polyomaviridae* families (downloaded from NCBI RefSeq on 20 August 2018). Alignments were done using the hisss pipeline as described above and analyzed in R (R packages used: tidyverse, reshape2, Biostrings, taxonomizr, UpSetR). Samples were considered positive for small (<10 kb) DNA viruses if greater than 25% of the target genome was covered. Samples were considered positive for large DNA viruses (>10 kb genomes), if greater than 10% of the target genome was covered (see Figure S3). The distribution of the frequency of *Redondoviridae* and other viral family detection was tested using the Fisher's exact test with Bonferroni correction for multiple testing.

Association with Human Clinical Disease States

In studies of periodontitis, the difference in number of redondovirus reads in disease versus nondisease states were tested using nonparametric Wilcoxon signed-rank or rank-sum tests, depending on whether samples were paired or not.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the viruses sequenced and reported in this paper are GenBank: MK059754-MK059772. Full details of each step of the Snakemake pipeline used in this report are available at https://github.com/louiejtaylor/hisss. The script used for RBS analysis is available at https://github.com/louiejtaylor/find-prok-rbs.

EXHIBIT" I

PCR (Polymerase Chain Reaction)

Source: Medicine Net

Link:

https://www.medicinenet.com/pcr_polymerase_chain_reaction/article.htm#what_is_pcr_polymerase_chain_reaction

Medical Author: Melissa Conrad Stöppler, MD

Medical Editor: William C. Shiel Jr., MD, FACP, FACR

What is PCR (polymerase chain reaction)?

PCR (polymerase chain reaction) is a method to analyze a short sequence of DNA (or RNA) even in samples containing only minute quantities of DNA or RNA. PCR is used to reproduce (amplify) selected sections of DNA or RNA. Previously, amplification of DNA involved cloning the segments of interest into vectors for expression in bacteria, and took weeks. But now, with PCR done in test tubes, it takes only a few hours. PCR is highly efficient in that untold numbers of copies can be made of the DNA. Moreover, PCR uses the same molecules that nature uses for copying DNA:

- Two "primers", short single-stranded DNA sequences that are synthesized to correspond to the beginning and ending of the DNA stretch to be copied;
- An enzyme called polymerase that moves along the segment of DNA, reading its code and assembling a copy; and
- A pile of DNA building blocks that the polymerase needs to make that copy.

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How is PCR (polymerase chain reaction) done?

As illustrated in the animated picture of PCR, three major steps are involved in a PCR. These three steps are repeated for 30 or 40 cycles. The cycles are done on an automated cycler, a device which rapidly heats and cools the test tubes containing the reaction mixture. Each step -- denataturation (alteration of structure), annealing (joining), and extension -- takes place at a different temperature:

1. **Denaturation:** At 94 C (201.2 F), the double-stranded DNA melts and opens into two pieces of single-stranded DNA.
2. **Annealing:** At medium temperatures, around 54 C (129.2 F), the primers pair up (anneal) with the single-stranded "template" (The template is the sequence of DNA to be copied.) On the small length of double-stranded DNA (the joined primer and template), the polymerase attaches and starts copying the template.
3. **Extension:** At 72 C (161.6 F), the polymerase works best, and DNA building blocks complementary to the template are coupled to the primer, making a double stranded DNA molecule.

With one cycle, a single segment of double-stranded DNA template is amplified into two separate pieces of double-stranded DNA. These two pieces are then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template is increased exponentially.

What is the purpose of doing a PCR (polymerase chain reaction)?

To do PCR, the original DNA that one wishes to copy need not be pure or abundant. It can be pure but it also can be a minute part of a mixture of materials.

So, PCR has found widespread and innumerable uses -- to diagnose genetic diseases, do DNA fingerprinting, find bacteria and viruses, study human evolution, clone the DNA of an Egyptian mummy, establish paternity or biological relationships, etc.. Accordingly, PCR has become an essential tool for biologists, DNA forensics labs, and many other laboratories that study genetic material.

How was PCR (polymerase chain reaction) discovered?

PCR was invented by Kary Mullis. At the time he thought up PCR in 1983, Mullis was working in Emeryville, California for Cetus, one of the first biotechnology companies. There, he was charged with making short chains of DNA for other scientists. Mullis has written that he conceived of PCR while cruising along the Pacific Coast Highway 128 one night on his motorcycle. He was playing in his mind with a new way of analyzing changes (mutations) in DNA when he realized that he had instead invented a method of amplifying any DNA region. Mullis has said that before his motorcycle trip was over, he was already savoring the prospects of a Nobel Prize. He shared the Nobel Prize in chemistry with Michael Smith in 1993.

As Mullis has written in the Scientific American: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat."

What is RT PCR?

RT-PCR (Reverse transcriptase-polymerase chain reaction) is a highly sensitive technique for the detection and quantitation of mRNA (messenger RNA). The technique consists of two parts:

- The synthesis of cDNA (complementary DNA) from RNA by reverse transcription (RT) and
- The amplification of a specific cDNA by the polymerase chain reaction (PCR).

RT-PCR has been used to measure viral load with HIV and may also be used with other RNA viruses such as measles and mumps.

Previous contributing author and editor:
Medical Author: Frederick Hecht, MD, FAAP, FACMG
Medical Editor: Leslie J. Schoenfield, M.D., Ph.D.

EXHIBIT "U"

Issues with the RT-PCR Coronavirus Test

Source: The Infectious Myth

Link: https://theinfectiousmyth.com/coronavirus/RT-PCR_Test_Issues.php

Author: David Crowe

Published on: April 23, 2020 Version 3

This is an analysis of the so-called coronavirus test, based on RT-PCR technology. It is based significantly on my recent reading of a 2017 article on potential problems with RT-PCR by Professor Stephen Bustin, a world expert, a podcast that I recently conducted with him and the MIQE guidelines for operating and reporting on RT-PCR data. This article does not question whether the RNA used in the test is viral or endogenous. If the RNA is not viral, then clearly the RT-PCR coronavirus test is of no value. This web page does not contain references, for those you should consult the fully referenced Coronavirus Panic Critique.

The PCR Cycle Number

The PCR algorithm is cyclical. At each cycle it generates approximately double the amount of DNA (which, in RT-PCR, corresponding to the RNA that the process started with). When used as a test you don't know the amount of starting material, but the amount of DNA at the end of each cycle will be shown indirectly by fluorescent molecules that are attached to the probes. The amount of light produced after every step will then approximately double, and when it reaches a certain intensity the process is halted and the sample is declared positive (implying infected). If, after a certain number of cycles, there is still not

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sufficient DNA, then the sample is declared negative (implying not infected). This cycle number (Ct) used to separate positive from negative is arbitrary, and is not the same for every organization doing testing. For example, there is a paper published that reported using 36 as the cutoff for positive, 37-39 as indeterminate, requiring more testing, and above 39 as negative. Another paper used 37 as the cutoff, with no intermediate zone. In a list of test kits approved by the US FDA one manufacturer each recommended 30 cycles, 31, 35, 36, 37, 38 and 39. 40 cycles was most popular, chosen by 12 manufacturers, and one each recommended 43 and 45.

Meaning of the Ct

Implicit in using a Ct number is the assumption that approximately the same amount of original RNA (within a multiple of two) will produce the same Ct number. However, there are many possibilities for error in RT-PCR. There are inefficiencies in extracting the RNA, even larger inefficiencies in converting the RNA to complementary DNA (Bustin noted that efficiency is rarely over 50% and can easily vary by a factor of 10), and inefficiencies in the PCR process itself. Bustin, in the podcast, described reliance on an arbitrary Ct number as "absolute nonsense, it makes no sense whatsoever". It certainly cannot be assumed that the same Ct number on tests done at different laboratories indicates the same original quantity of RNA.

Limits on Cycles

Professor Bustin stated that cycling more than 35 times was unwise, but it appears that nobody is limiting cycles to 35 or less (the MIQE guidelines recommend less than 40). Cycling too much could result in false positives as background fluorescence builds up in the PCR reaction.

Ct and Number of Positive Tests

The Ct cycle number will significantly influence the number of positive tests. If the Ct was changed from 37 to 35 there would be fewer positive tests, and if changed to 39 there would more positive tests. Even if the Ct number was standardized, it would still have different meaning depending on the specific machines, chemicals and procedures used by different labs, and even within the same lab changes could still be found between different runs of samples. Without simultaneously amplifying a known quantity of 'spiked' RNA, it cannot be assumed that with consistent Ct numbers can be used to consistently provide a boundary between positive and negative.

Is the Amount Meaningful?

If the process is efficient, a large number of cycles could detect as little as three molecules of RNA. If there are people who had such a small amount of virus in their body, causing no health problems, they would still test positive.

Is the Virus Functional?

If there are only parts of viruses present, or defective virus particles, that are not infectious, they would still produce positive tests. The tests do not prove that pathogenic, replicable virus is present.

Can RT-PCR Distinguish Infected from Uninfected

No.

How RT-PCR Works in More Detail

The following steps are used to test for particular RNA:

1. RNA must be extracted from a sample. This must be done carefully to ensure that DNA is eliminated, and that chemicals that might inhibit

further steps are not included. It is impossible to ensure absolute purity of the RNA.

2. RNA must be converted to complementary DNA (cDNA). This uses the enzyme Reverse Transcriptase and is never terribly efficient (50%). The amount of DNA produced can vary significantly, depending on numerous factors, perhaps by a factor of 10 (it used to be a factor of 100).
3. In the PCR part of the process, cDNA is present with primers and a probe (and possibly some stray DNA from the sample). The primers delimit the beginning and the ending of the cDNA that is intended to be duplicated. The probe helps ensure that RNA is only duplicated if it matches the primers (which are quite short) and the probe. At each cycle of this process (PCR proper) the amount of DNA is approximately doubled. Fluorescent markers are attached to the probe so that, at each step, the amount of light can be used to estimate how much DNA has been generated.
4. Optionally, the resulting DNA can be sequenced to determine exactly what the bases ('string of four different DNA beads') are.

Errors and inefficiencies can occur at every step. It is not possible to actually estimate quantities unless the reaction is 'spiked' with a known amount of a different RNA, which is also duplicated. Then the PCR cycle number can be roughly correlated with the original quantity of material.

Is There Proof There Are Problems, Or Is This Just a Hypothesis?

There are now several papers that illustrate essentially impossible testing results. A paper from China reported on consecutive testing results, defined as either Negative (N), Positive (P) or Dubious (D, presumably intermediate). Results for 29 people with inexplicable results out of about 600 patients were: 1 DDPDD 2 NNPN 3 NNNPN 4 DNPN 5 NNDP 6 NDP 7 DNP 8 NDDPN 9

NNNDPN 10 NNPD 11 DNP 12 NNNP 13 PPNDPN 14 PNPPP 15 DPNPNN 16 PNNP 17 NPNPN 18 PNP 19 NPNP 20 PNPN 21 PNP 22 PNP 23 PNP 24 PNDDP 25 PNPNN 26 PNPP 27 PNP 28 PNPN 29 PNP. A study from Singapore did tests almost daily on 18 patients and the majority went from Positive to Negative back to Positive at least once, and up to four times in one patient. In China they have found that 5-14% of patients who have been cleared, with two consecutive negative tests, have later tested positive again, usually without new symptoms. In South Korea they recently reported 91 such patients. A 68 year old Chinese man went to hospital with symptoms, and tested positive. After his symptoms resolved and he tested negative twice he was released. But he tested positive again, and was readmitted, was released again, tested positive again, was readmitted, and then was released for a third time.

Conclusions

RT-PCR testing for the Coronavirus seems to be designed to produce as many positive tests as possible. The fear of missing a true positive is so great that those designing the specific testing methodology based on RT-PCR completely ignore the risk of false positives. False positives make the epidemic appear larger, and justify the complete shutdown of the economy, locking people in their own homes, and destroying just about everything in the lives of people that brings them joy, such as playing ball in the park, going for coffee with a friend, going to the theater or a sports event, going swimming, going to the

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EXHIBIT "V"

CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel

For Emergency Use Only

Instructions for Use

NOTARY M. M. PABALL Mumbai Maharashtra Reg. No. 15

Catalog # 2019-nCoVEUA-01
1000 reactions

For *In-vitro* Diagnostic (IVD) Use

Rx Only

Centers for Disease Control and Prevention
Division of Viral Diseases
1600 Clifton Rd NE
Atlanta GA 30329

CDC
Centers for Disease Control and Prevention

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Intended Use

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of COVID-19 by their healthcare provider[1].

This test is also for the qualitative detection of nucleic acid from the SARS-CoV-2 in pooled samples containing up to four of the individual upper respiratory swab specimens (nasopharyngeal (NP), oropharyngeal (OP), NP/OP combined, or nasal swabs) that were collected using individual vials containing transport media from individuals suspected of COVID-19 by their healthcare provider. Negative results from pooled testing should not be treated as definitive. If a patient's clinical signs and symptoms are inconsistent with a negative result or results are necessary for patient management, then the patient should be considered for individual testing. Specimens included in pools with a positive, inconclusive, or invalid result must be tested individually prior to reporting a result. Specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet the requirements to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with SARS-CoV-2 but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

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[1] For this EUA, a healthcare provider includes, but is not limited to, physicians, nurses, pharmacists, technologists, laboratory directors, epidemiologists, or any other practitioners or allied health professionals.

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Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (2019-nCoV, also referred to as SARS-CoV-2), which has resulted in millions of confirmed human infections globally. Cases of asymptomatic infection, mild illness, severe illness, and deaths have been reported.

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is a molecular *in vitro* diagnostic test that aids in the detection and diagnosis of SARS-CoV-2 infection and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material used in rRT-PCR for the *in vitro* qualitative detection of 2019-nCoV RNA in respiratory specimens.

The term "qualified laboratories" refers to laboratories in which all users, analysts, and any person reporting results from use of this device should be trained to perform and interpret the results from this procedure by a competent instructor prior to use.

Principles of the Procedure

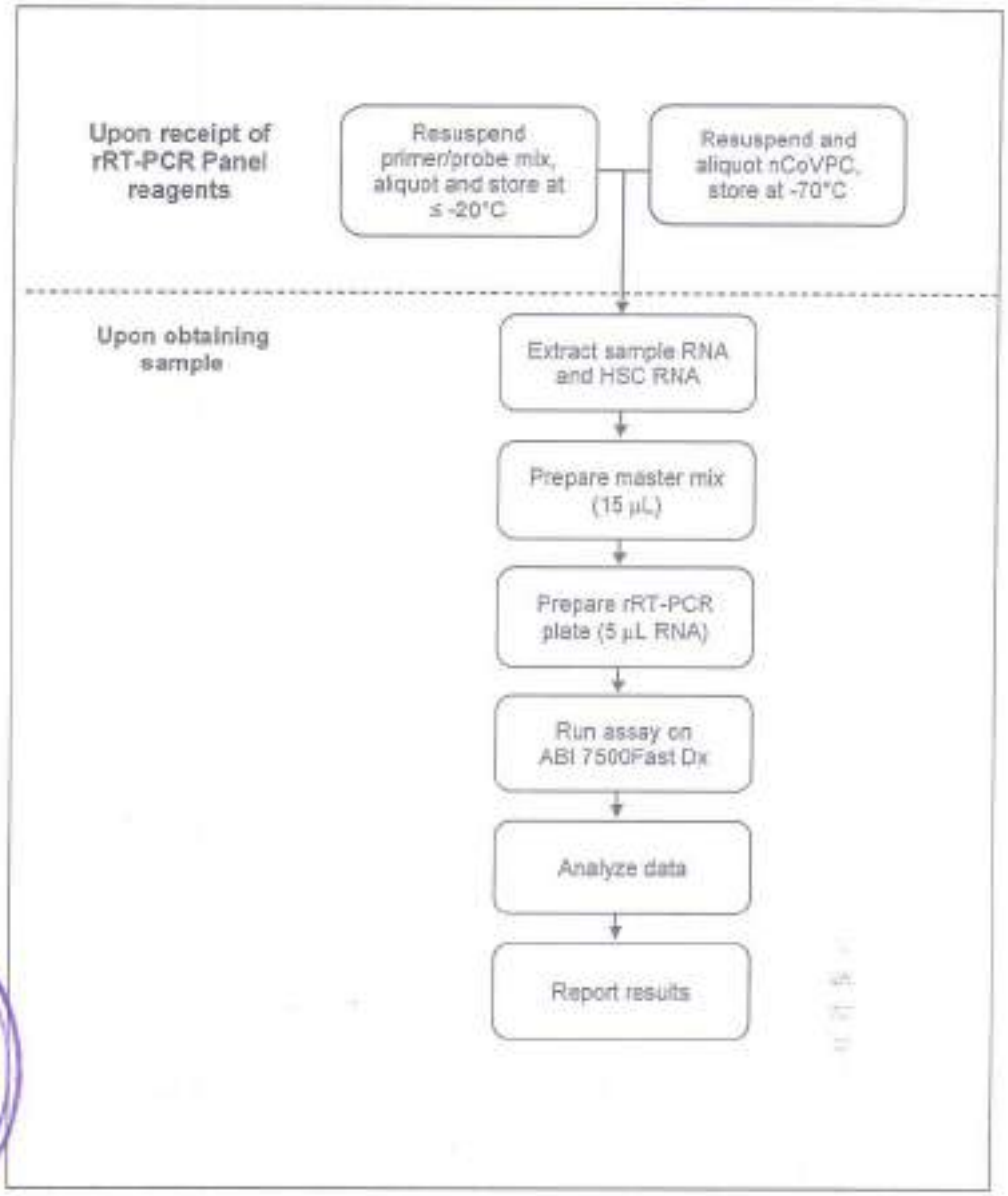
The oligonucleotide primers and probes for detection of 2019-nCoV were selected from regions of the virus nucleocapsid (N) gene. The panel is designed for specific detection of SARS-CoV-2 (two primer/probe sets). An additional primer/probe set to detect the human RNase P gene (RP) in control samples and clinical specimens is also included in the panel.

RNA isolated and purified from upper and lower respiratory specimens is reverse transcribed to cDNA and subsequently amplified in the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4 software. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by Applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4 software.

Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

NOTARY M. M. PA... Mumbai Maharashtra Reg. No. 15... GOVT...

Summary of Preparation and Testing Process



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Materials Required (Provided)

Note: CDC will maintain on its website a list of commercially available lots of primer and probe sets and/or positive control materials that are acceptable alternatives to the CDC primer and probe set and/or positive control included in the Diagnostic Panel. Only material distributed through the CDC International Reagent Resource and specific lots of material posted to the CDC website are acceptable for use with this assay under CDC's Emergency Use Authorization.

This list of acceptable alternative lots of primer and probe materials and/or positive control materials will be available at:
https://www.cdc.gov/coronavirus/2019-nCoV/lab/virus-requests.html

Primers and Probes:

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #1:

Reagent Label	*Part #*	*Description*	*Quantity / Tube*	*Reactions / Tube*
2019-nCoV_N1	RV202001 RV202015	2019-nCoV_N1 Combined Primer/Probe Mix	22.5 nmol	1000
2019-nCoV_N2	RV202002 RV202016	2019-nCoV_N2 Combined Primer/Probe Mix	22.5 nmol	1000
RP	RV202004 RV202018	Human RNase P Combined Primer/Probe Mix	22.5 nmol	1000

Positive Control (either of the following products are acceptable):

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #2:

Reagent Label	*Part #*	*Description*	*Quantity*	*Notes*
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Catalog #VTC-04 CDC 2019-nCoV Positive Control (nCoVPC)

Reagent Label	Part #	Description	Quantity	Notes
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Materials Required (But Not Provided)

Human Specimen Control (HSC)

Description	Quantity	CDC Catalog No.
Manufactured by CDC. For use as a nucleic acid extraction procedural control to demonstrate successful recovery of nucleic acid as well as extraction reagent integrity. The HSC consists of noninfectious (beta-Propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2-7.4.	10 vials x 500 µL	KT0189

Acceptable alternatives to HSC:

- Negative human specimen material: Laboratories may prepare a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.
- Contrived human specimen material: Laboratories may prepare contrived human specimen materials by suspending any human cell line (e.g., A549, Hela, or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.

CDC will maintain on its website a list of commercially alternative extraction controls, if applicable, that are acceptable for use with this assay under CDC's Emergency Use Authorization, at: https://www.cdc.gov/coronavirus/2019-nCoV/lab/virus-requests.html

rRT-PCR Enzyme Mastermix Options

Reagent	Quantity	Catalog No.
Quantabio qScript XLT One-Step RT-qPCR ToughMix	100 x 20 µL rxns (1 x 1 mL)	95132-100
	2000 x 20 µL rxns (1 x 20 mL)	95132-02K
	500 x 20 µL rxns (5 x 1 mL)	95132-500
Quantabio UltraPlex 1-Step ToughMix (4X)	100 x 20 µL rxns (500 µL)	95166-100
	500 x 20 µL rxns (5 x 500 µL)	95166-500
	1000 x 20 µL rxns (1 x 5 mL)	95166-01K
Promega GoTaq® Probe 1- Step RT-qPCR System	200 x 20 µL rxns (2 mL)	A6120
	1250 x 20 µL rxns (12.5 mL)	A6121
Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG	1000 reactions	A15299
	2000 reactions	A15300

RNA Extraction Options

For each of the kits listed below, CDC has confirmed that the external lysis buffer is effective for inactivation of SARS-CoV-2.

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[2]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
QIAGEN EZ1 Advanced XL	[2]EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073 or 19089) EZ1 Advanced XL DSP Virus Card (9018703)
	[2]EZ1 Virus Mini Kit v2.0	48 extractions (955134) Buffer AVL (19073 or 19089) EZ1 Advanced XL Virus Card v2.0 (9018708)
Roche MagNA Pure 24	[2]MagNA Pure 24 Total NA Isolation Kit	96 extractions (07 658 036 001) External Lysis Buffer (06 374 913 001, 12 239 469 103, 03 246 779 001 or 03 246 752 001)
Roche MagNA Pure 96	[2]DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001) External Lysis Buffer (06 374 913 001, 12 239 469 103, 03 246 779 001 or 03 246 752 001)
[1]Roche MagNA Pure LC	[2]Total Nucleic Acid Kit	192 extractions (03 038 505 001)

Instrument/Manufacturer	Extraction Kit	Catalog No.
[1]Roche MagNA Pure Compact	[2]Nucleic Acid Isolation Kit I	32 extractions (03 730 964 001)
Promega Maxwell® RSC 48 and Maxwell® CSC 48	[3]Maxwell® RSC Viral Total Nucleic Acid Purification Kit	48 extractions (AS1330) 144 extractions (AS81330)
[1]QIAGEN QIAcube	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[3]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
[1, 3]bioMérieux NucliSENS® easyMAG® and [2, 3]bioMérieux EMAG® (Automated magnetic extraction reagents sold separately. Both instruments use the same reagents and disposebles, with the exception of tips.)		EasyMAG® Magnetic Silica (280133) EasyMAG® Lysis Buffer (280134) EasyMAG® Lysis Buffer, 2 mL (200292) EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132) EasyMAG® Disposables (280135) Biohit Pipette Tips (easyMAG® only) (280146) EMAG®1000µL Tips (418922)

[1]Equivalence and performance of these extraction platforms for extraction of viral RNA were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302). Performance characteristics of these extraction platforms with 2019-nCoV (SARS CoV-2) have not been demonstrated.

[2] CDC has confirmed that the external lysis buffer used with this extraction method is effective for inactivation of SARS-CoV-2.

[3] CDC has compared the concentration of inactivating agent in the lysis buffer used with this extraction method and has determined the concentration to be within the range of concentrations found effective in inactivation of SARS-CoV-2.

Alternative to Extraction:

If a laboratory cannot access adequate extraction reagents to support testing demand due to the global shortage of reagents, CDC has evaluated a heat treatment procedure for upper respiratory specimens using the Quantabio UltraPlex 1-Step ToughMix (4X), CG. Though performance was comparable, this method has been evaluated with a limited number of clinical specimens and a potential reduction in sensitivity due to carryover of inhibitory substances or RNA degradation cannot be ruled out. It should only be used when a jurisdiction determines that the testing need is great enough to justify the risk of a potential loss of sensitivity. Heat-treated specimens generating inconclusive or invalid results should be extracted with an authorized extraction method prior to retesting. Details and procedure for the heat treatment alternative to extraction may be found in Appendix A.

Equipment and Consumables Required (But Not Provided)

- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)

- Multichannel micropipettes (5-50 µL)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks
- 7500 Fast Dx Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL, QIAGEN QIAcube, Roche MagNA Pure 24, Roche MagNA Pure 96, Promega Maxwell® RSC 48, Roche MagNA Pure LC, Roche MagNA Pure Compact, bioMérieux easyMAG, and bioMérieux EMAG
- Molecular grade water, nuclease-free
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNA*Zap*™ (Ambion, cat. #AM9890) or equivalent
- RNase AWAY™ (Fisher Scientific; cat. #21-236-21) or equivalent
- Disposable powder-free gloves and surgical gowns
- Aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)

Qualifying Alternative Components:

If a laboratory modifies this test by using unauthorized, alternative components (e.g., extraction methods or PCR instruments), the modified test is not authorized under this EUA. FDA's Policy for Diagnostic Tests for Coronavirus Disease-2019 during the Public Health Emergency, updated May 11, 2020, does not change this. As part of this policy, FDA does not intend to object when a laboratory modifies an EUA-authorized test, which could include using unauthorized components, without obtaining an EUA or EUA amendment, where the modified test is validated using a bridging study to the EUA-authorized test.

Warnings and Precautions

- For *in vitro* diagnostic use (IVD).
  - This test has not been FDA cleared or approved; this test has been authorized by FDA under an EUA for use by laboratories certified under CLIA, 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.
  - This test has been authorized only for the detection of nucleic acid from SARS CoV-2, not for any other viruses or pathogens.
  - This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.

- Handle all specimens as if infectious using safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with 2019-nCoV https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with 2019-nCoV is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Performance characteristics have been determined with human upper respiratory specimens and lower respiratory tract specimens from human patients with signs and symptoms of respiratory infection.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves, eye protection, and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
    - Maintain separate areas for assay setup and handling of nucleic acids.
    - Always check the expiration date prior to use. Do not use expired reagents. Do not substitute or mix reagents from different kit lots or from other manufacturers.
    - Change aerosol barrier pipette tips between all manual liquid transfers.
    - During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
    - Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
    - Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
    - Change gloves between samples and whenever contamination is suspected.
    - Keep reagent and reaction tubes capped or covered as much as possible.
    - Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on a cold block at all times during preparation and use.
    - Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, DNAZap™ and RNase AWAY™ to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- RNA should be maintained on a cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

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Reagent Storage, Handling, and Stability

- Store all dried primers and probes and the positive control, nCoVPC, at 2-8°C until re-hydrated for use. Store liquid HSC control materials at ≤ -20°C.
  Note: Storage information is for CDC primer and probe materials obtained through the International Reagent Resource. If using commercial primers and probes, please refer to the manufacturer's instructions for storage and handling.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.
- Do not refreeze probes.
- Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Specimen Collection, Handling, and Storage

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13-A may be referenced as an appropriate resource.

➢ Collecting the Specimen
- Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens for COVID-19 https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
- Follow specimen collection device manufacturer instructions for proper collection methods.
- Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 1-3 mL of appropriate transport media, such as viral transport media (VTM).

➢ Transporting Specimens
- Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. If a specimen is frozen at -70°C or lower, ship overnight to CDC on dry ice.

➢ Storing Specimens
- Specimens can be stored at 2-8°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimens at -70°C or lower.
- Extracted nucleic acid should be stored at -70°C or lower.

Specimen Referral to CDC

For state and local public health laboratories:

- Ship all specimens overnight to CDC.
- Ship frozen specimens on dry ice and non-frozen specimens on cold packs.
- Refer to the International Air Transport Association (IATA - www.iata.org) for requirements for shipment of human or potentially infectious biological specimens. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens.
- Prior to shipping, notify CDC Division of Viral Diseases (see contact information below) that you are sending specimens.
- Send all samples to the following recipient:

Centers for Disease Control and Prevention
c/o STATT
Attention: Unit 66
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-3931

The emergency contact number for CDC Emergency Operations Center (EOC) is 770-488-7100.

Reagent and Controls Preparation

NOTE: Storage information is for materials obtained through the CDC International Reagent Resource. If using commercial products for testing, please refer to the manufacturer's instructions for storage, handling, and preparation instructions.

Primer and Probe Preparation:

1) Upon receipt, store dried primers and probes at 2-8°C.
2) Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
3) Using aseptic technique, suspend dried reagents in 1.5 mL of nuclease-free water and allow to rehydrate for 15 min at room temperature in the dark.
4) Mix gently and aliquot primers/probe in 300 µL volumes into 5 pre-labeled tubes. Store a single, working aliquot of primers/probes at 2-8°C in the dark. Store remaining aliquots at ≤ -20°C in a non-frost-free freezer. Do not refreeze thawed aliquots (stable for up to 4 months at 2-8°C).

2019-nCoV Positive Control (nCoVPC) Preparation:

1) Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
2) Resuspend dried reagent in each tube in 1 mL of nuclease-free water to achieve the proper concentration. Make single use aliquots (approximately 30 µL) and store at ≤ -70°C.
3) Thaw a single aliquot of diluted positive control for each experiment and hold on ice until adding to plate. Discard any unused portion of the aliquot.

Human Specimen Control (HSC) (not provided):

1) Human Specimen Control (HSC) or one of the listed acceptable alternative extraction controls must be extracted and processed with each specimen extraction run.
2) Refer to the Human Specimen Control (HSC) package insert for instructions for use.

No Template Control (NTC) (not provided):

1) Sterile, nuclease-free water
2) Aliquot in small volumes
3) Used to check for contamination during specimen extraction and/or plate set-up

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 10% bleach, DNAzap™ and RNase AWAY™ to minimize the risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.

Nucleic Acid Extraction

NOTE: The extraction instructions below are for use when testing individual specimens ONLY. When pooling specimens, refer to Appendix B for modified extraction instructions.

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the panel:

Qiagen QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit

Recommendation(s): Utilize 100 µL of sample and elute with 100 µL of buffer or utilize 140 µL of sample and elute with 140 µL of buffer.

Qiagen EZ1 Advanced XL
Kit: Qiagen EZ1 DSP Virus Kit and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL DSP Virus Card
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Kit: Qiagen EZ1 Virus Mini Kit v2.0 and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL Virus Card v2.0
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Roche MagNA Pure 96
Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
Protocol: Viral NA Plasma Ext LysExt Lys SV 4.0 Protocol or Viral NA Plasma Ext Lys SV Protocol
Recommendation(s): Add 100 µL of sample to 350 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 450 µL). Proceed with the extraction on the MagNA Pure 96. (**Internal Control = None**). Elution volume is 100 µL.

Roche MagNA Pure 24
Kit: Roche MagNA Pure 24 Total NA Isolation Kit
Protocol: Pathogen 1000 2.0 Protocol
Recommendation(s): Add 100 µL of sample to 400 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 500 µL). Proceed with the extraction on the MagNA Pure 24. (**Internal Control = None**). Elution volume is 100 µL.

Promega Maxwell® RSC 48 and Maxwell® CSC 48
Kit: Promega Maxwell® Viral Total Nucleic Acid Purification Kit
Protocol: Viral Total Nucleic Acid
Recommendation(s): Add 120 µL of sample to 330 µL of pre-aliquoted External Lysis Buffer (300 µL Lysis Buffer plus 30 µL Proteinase K; supplied within the kit) (total input volume is 450 µL). Proceed with the extraction on the Maxwell® RSC 48. Elution volume is 75 µL.

Equivalence and performance of the following extraction platforms were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302) and based on those data are acceptable for use with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

QIAGEN QIAcube
Kit: QIAGEN QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit
Recommendations: Utilize 140 µL of sample and elute with 100 µL of buffer.

Roche MagNA Pure LC
Kit: Roche MagNA Pure Total Nucleic Acid Kit
Protocol: Total NA External_lysis
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

Roche MagNA Pure Compact
Kit: Roche MagNA Pure Nucleic Acid Isolation Kit I
Protocol: Total_NA_Plasma100_400
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

bioMérieux NucliSENS® easyMAG® Instrument
Protocol: General protocol (not for blood) using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of sample to 1000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 1100 µL). Incubate for 10 minutes at room temperature.
Elution volume is 100 µL.

bioMérieux EMAG® Instrument
Protocol: Custom protocol: **CDC Flu V1** using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of samples to 2000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 2100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL. The custom protocol, **CDC Flu V1**, is programmed on the bioMérieux EMAG® instrument with the assistance of a bioMérieux service representative. Installation verification is documented at the time of installation. Laboratories are recommended to retain a record of the step-by-step verification of the bioMérieux custom protocol installation procedure.

Manufacturer's recommended procedures (except as noted in recommendations above) are to be followed for sample extraction. HSC must be included in each extraction batch.

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

Assay Set Up

Reaction Master Mix and Plate Set Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTCs and nCoVPCs must be included in each run.

1) In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.

2) Mix buffer, enzyme, and primer/probes by inversion 5 times.
3) Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
4) Label one 1.5 mL microcentrifuge tube for each primer/probe set.
5) Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the rRT-PCR run), and RP reactions and for pipetting error. Use the following guide to determine N:
   - If number of samples (n) including controls equals 1 through 14, then N = n + 1
   - If number of samples (n) including controls is 15 or greater, then N = n + 2
6) For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	N x 5.0 µL
	Total Volume	N x 15.0 µL

Promega GoTaq® Probe 1- Step RT-qPCR System

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 3.1 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	GoTaq Probe qPCR Master Mix with dUTP	N x 10.0 µL
4	Go Script RT Mix for 1-Step RT-qPCR	N x 0.4 µL
	Total Volume	N x 15.0 µL

Quantabio qScript XLT One-Step RT-qPCR ToughMix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 3.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	qScript XLT One-Step RT-qPCR ToughMix(2X)	N x 10.0 µL
	Total Volume	N x 15.0 µL

Quantabio UltraPlex 1-Step ToughMix (4X)

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	UltraPlex 1-Step ToughMix (4X)	N x 5.0 µL
	Total Volume	**N x 15.0 µL**

7) Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. ***Do not vortex.***
8) Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
9) Set up reaction strip tubes or plates in a 96-well cooler rack.
10) Dispense 15 µL of each master mix into the appropriate wells going across the row as shown below (**Figure 1**):

Figure 1: Example of Reaction Master Mix Plate Set-Up

	1	2	3	4	5	6	7	8	9	10	11	12
A	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1
B	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2
C	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
D												
E												
F												
G												
H												

11) Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.
12) Pipette 5 µL of nuclease-free water into the NTC sample wells (**Figure 2**, column **1**). Securely cap NTC wells before proceeding.
13) Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

<u>**Nucleic Acid Template Addition**</u>

1) Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2) Centrifuge for 5 seconds to collect contents at the bottom of the tube.

3) After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
4) Samples should be added to columns 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 2**. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). *Keep other sample wells covered during addition. Change tips after each addition.*
5) Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
6) Change gloves often and when necessary to avoid contamination.
7) Repeat steps #4 and #5 for the remaining samples.
8) If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 2**, column 11). Securely cap wells after addition. NOTE: Per CLIA regulations, HSC must be tested at least once per day.
9) Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1) Pipette 5 µL of nCoVPC RNA to the sample wells of column 12 (**Figure 2**). Securely cap wells after addition of the control RNA.
   NOTE: *If using 8-tube strips, label the TAB of each strip to indicate sample position.* ***DO NOT LABEL THE TOPS OF THE REACTION TUBES!***
2) Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack.
   NOTE: *If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.*

Figure 2. 2019-nCoV rRT-PCR Diagnostic Panel: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11*	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoVPC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoVPC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoVPC
D												
E												
F												
G												
H												

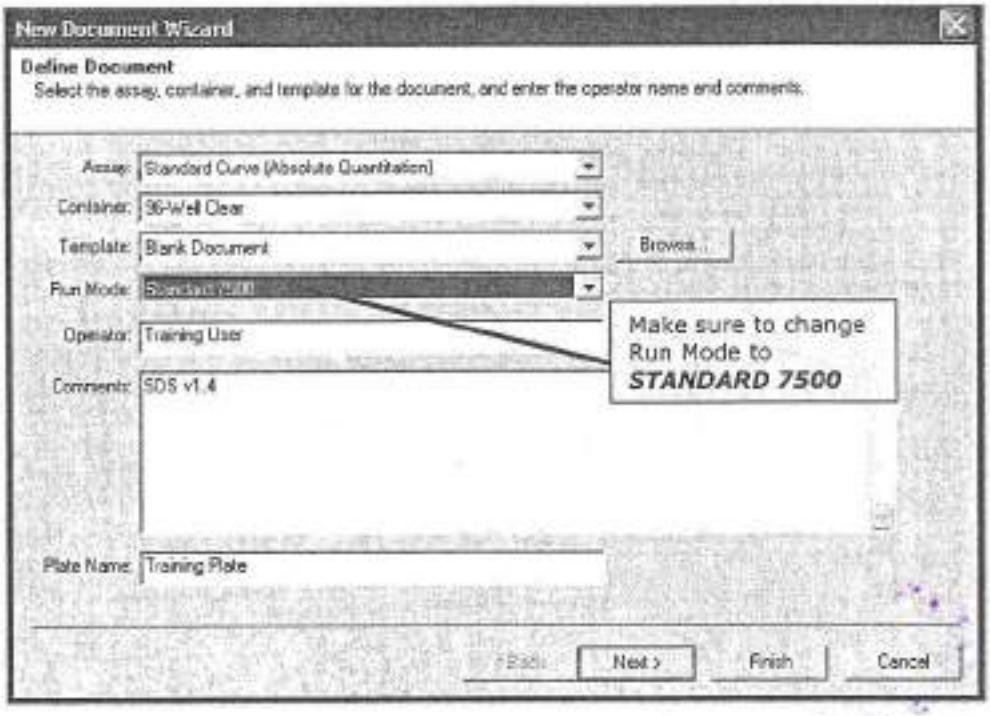
*Replace the sample in this column with extracted HSC if necessary

Create a Run Template on the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument (Required if no template exists)

If the template already exists on your instrument, please proceed to the **RUNNING A TEST** section.

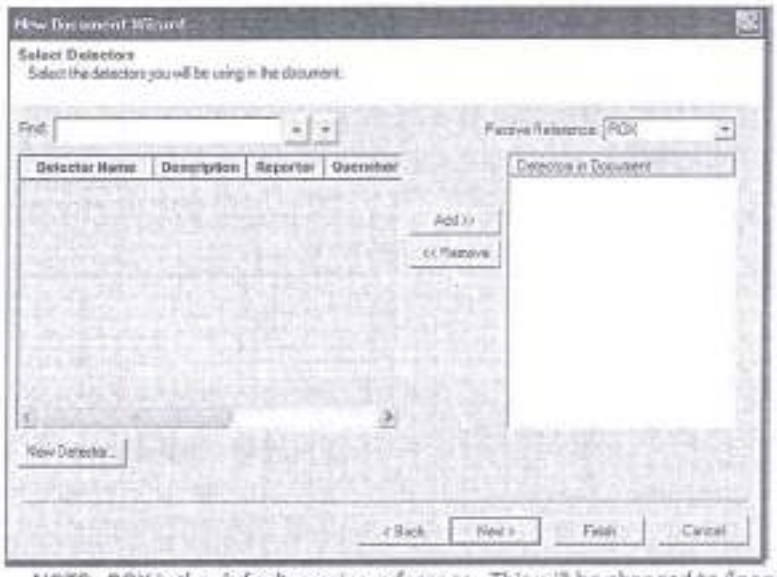
1) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument by double clicking on the Applied Biosystems 7500 Fast Dx System icon on the desktop.
2) A new window should appear, select **Create New Document** from the menu.

Figure 3. New Document Wizard Window



3) The **New Document Wizard** screen in **Figure 3** will appear. Select:
   a. Assay: **Standard Curve (Absolute Quantitation)**
   b. Container: **96-Well Clear**
   c. Template: **Blank Document**
   d. Run Mode: **Standard 7500**
   e. Operator: ***Your Name***
   f. Comments: **SDS v1.4**
   g. Plate Name: ***Your Choice***
4) After making selections click **Next** at the bottom of the window.

Figure 4. Creating New Detectors

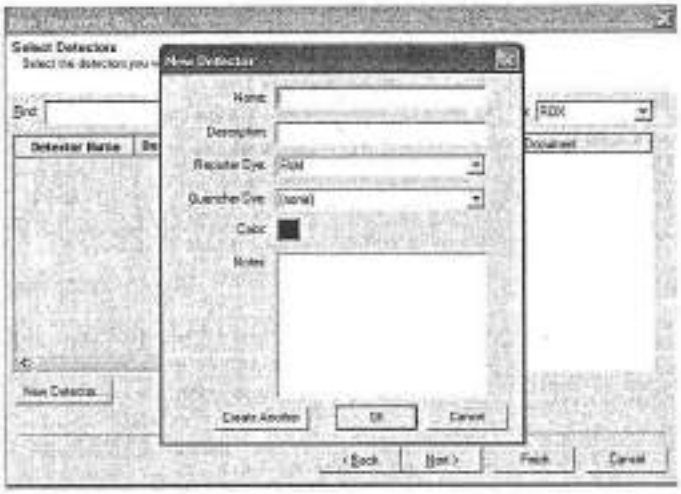


NOTE: ROX is the default passive reference. This will be changed to "none" in step 12.

5) After selecting next, the *Select Detectors* screen (**Figure 4**) will appear.
6) Click the **New Detector** button (see **Figure 4**).
7) The **New Detector** window will appear (**Figure 5**). A new detector will need to be defined for each primer and probe set. Creating these detectors will enable you to analyze each primer and probe set individually at the end of the reaction.

430

Figure 5. New Detector Window



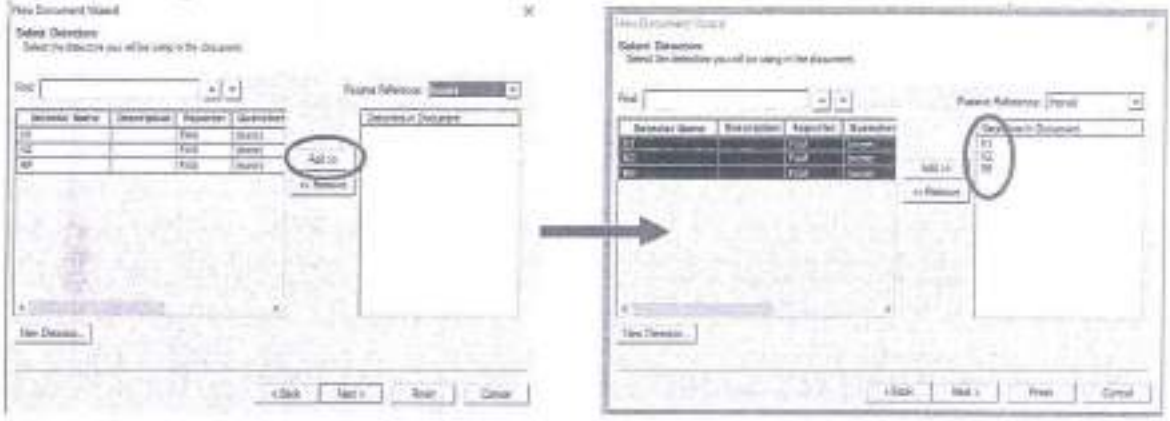
8) Start by creating the N1 Detector. Include the following:
   a. Name: **N1**
   b. Description: *leave blank*
   c. Reporter Dye: **FAM**
   d. Quencher Dye: **(none)**
   e. Color: *to change the color of the detector indicator do the following:*
      ⇒ Click on the color square to reveal the color chart
      ⇒ Select a color by clicking on one of the squares
      ⇒ After selecting a color click **OK** to return to the New Detector screen
   f. Click the **OK** button of the New Detector screen to return to the screen shown in **Figure 4.**
9) Repeat step 6-8 for each target in the panel.

Name	Reporter Dye	Quencher Dye
N1	FAM	(none)
N2	FAM	(none)
RP	FAM	(none)

10) After each Detector is added, the **Detector Name**, **Description**, **Reporter** and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 6**).

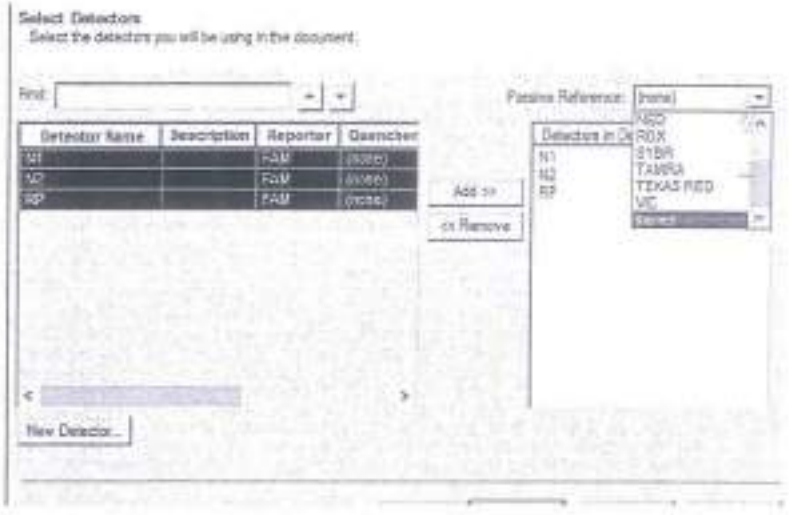
11) Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (see **Figure 6**). Detector names will appear on the right-hand side of the **Select Detectors** window (**Figure 6**).

Figure 6. Adding New Detectors to Document



12) Once all detectors have been added, select **(none)** for **Passive Reference** at the top right-hand drop-down menu (**Figure 7**).

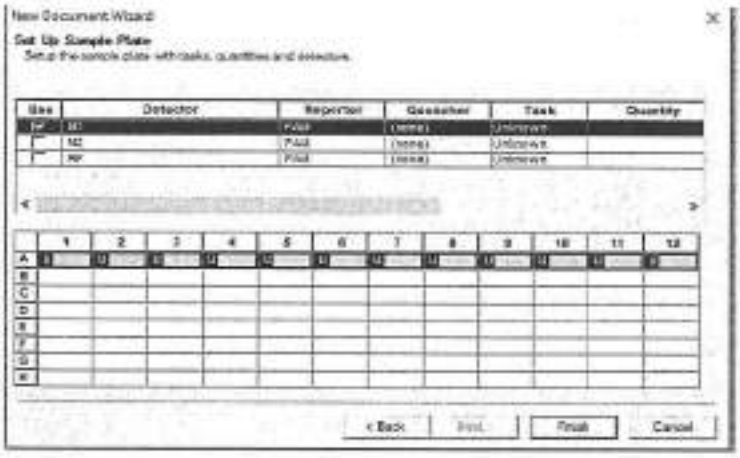
Figure 7. Select Passive Reference



Passive reference should be set to "(none)" as described above.

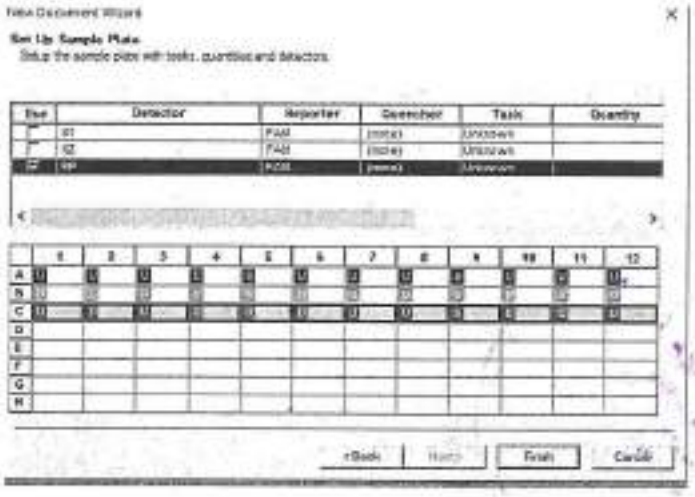
13) Click **Next** at the bottom of the **Select Detectors** window to proceed to the **Set Up Sample Plate** window (**Figure 8**).
14) In the **Set Up Sample Plate** window (**Figure 8**), use your mouse to select row A from the lower portion of the window, in the spreadsheet (see **Figure 8**).
15) In the top portion of the window, select detector **N1.** A check will appear next to the detector you have selected (**Figure 8**). You will also notice the row in the spreadsheet will be populated with a colored "U" icon to indicate which detector you've selected.
16) Repeat step 14-15 for each detector that will be used in the assay.

Figure 8. Sample Plate Set-up



17) Select **Finish** after detectors have been assigned to their respective rows. (**Figure 9**).

Figure 9. Finished Plate Set-up



18) After clicking "Finish", there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
19) After initialization, the **Plate** tab of the Setup (**Figure 10**) will appear.
20) Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager.**

Figure 10. Plate Set-up Window

21) The Detector Manager window will appear (**Figure 11**).

Figure 11. Detector Manager Window

22) Confirm all detectors are included and that each target has a **Reporter** set to **FAM** and the **Quencher** is set to **(none)**.
23) If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Defining the Instrument Settings

1) After detectors have been created and assigned, proceed to instrument set up.
2) Select the **Instrument** tab to define thermal cycling conditions.
3) Modify the thermal cycling conditions as follows (**Figure 12**):

Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix, CG

a. In Stage 1, Set to 2 min at **25°C**; **1 Rep.**
b. In Stage 2, Set to 15 min at **50°C**; **1 Rep.**
c. In Stage 3, Set to 2 min at **95°C**, **1 Rep.**
d. In Stage 4, Step 1 set to **3** sec at **95°C**.
e. In Stage 4, Step 2 set to **30** sec at **55.0°C**.
f. In Stage 4, Reps should be set to **45**.
g. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
h. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
i. Step 2 of Stage 4 should be highlighted in yellow to indicate data collection (see **Figure 12**).

OR

Quantabio qScript™ XLT One-Step RT-qPCR ToughMix or UltraPlex 1-Step ToughMix (4X)

a. In Stage 1, Set to 10 min at **50°C**; **1 Rep.**
b. In Stage 2, Set to 3 min at **95°C**, **1 Rep.**
c. In Stage 3, Step 1 set to **3** sec at **95°C**.
d. In Stage 3, Step 2 set to **30** sec at **55.0°C**.
e. In Stage 3, Reps should be set to **45**.
f. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
g. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 12**).

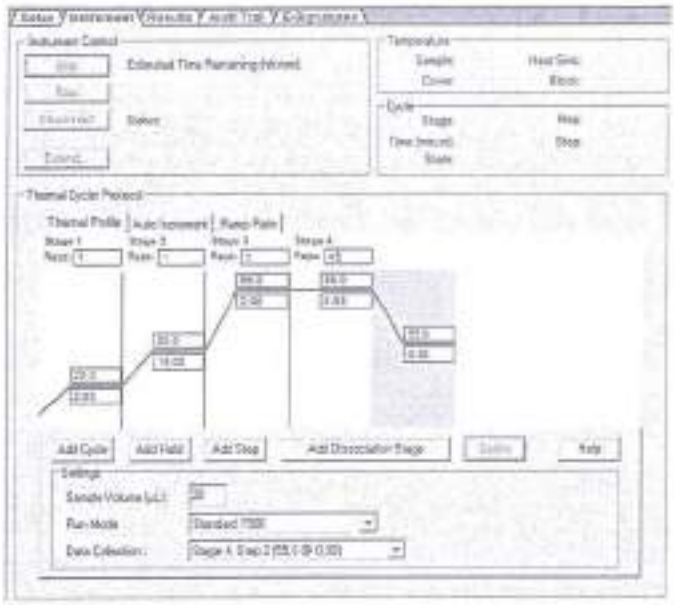
OR

Promega GoTaq® Probe 1-Step RT-qPCR System

a. In Stage 1, Set to 15 min at **45°C**; **1 Rep.**
b. In Stage 2, Set to 2 min at **95°C**, **1 Rep.**

c. In Stage 3, Step 1 set to **3 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
e. In Stage 3, Reps should be set to **45**.
f. Under **Settings (Figure 12)**, bottom left-hand box, change volume to 20 µL.
g. Under **Settings, Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 12**).

Figure 12. Instrument Window



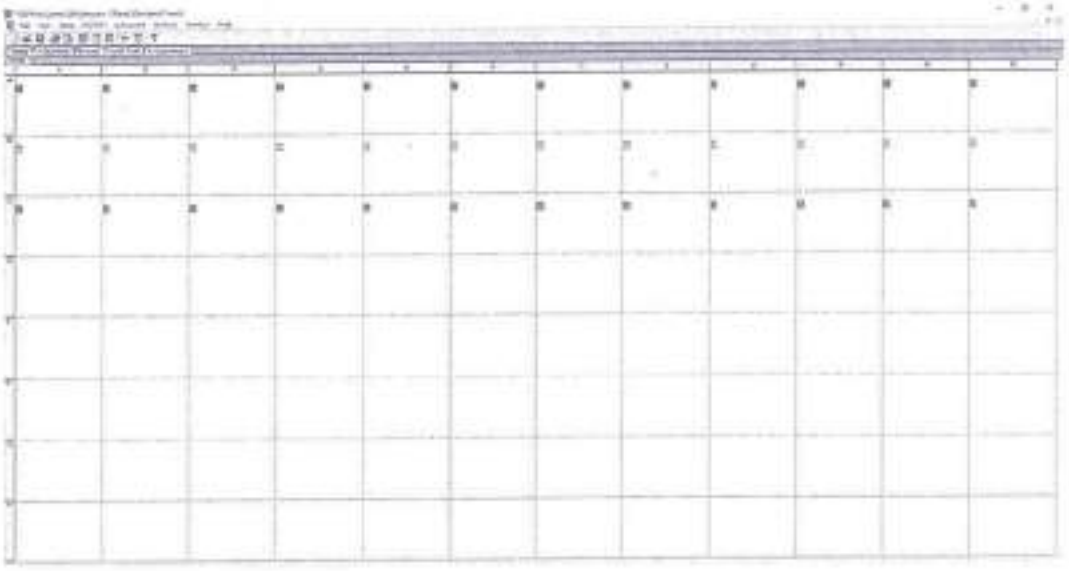
4) After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**. Since the enzyme options have different instrument settings, it is recommended that the template be saved with a name indicating the enzyme option.
5) Save the template as **2019-nCoV Dx Panel TaqPath** or **2019-nCoV Dx Panel Quanta** or **2019-nCoV Dx Panel Promega** as appropriate in the desktop folder labeled *"ABI Run Templates"* (you must *create this folder*). Save as type should be SDS Templates (*.sdt) (**Figure 13**).

Figure 13. Saving Template

Running a Test

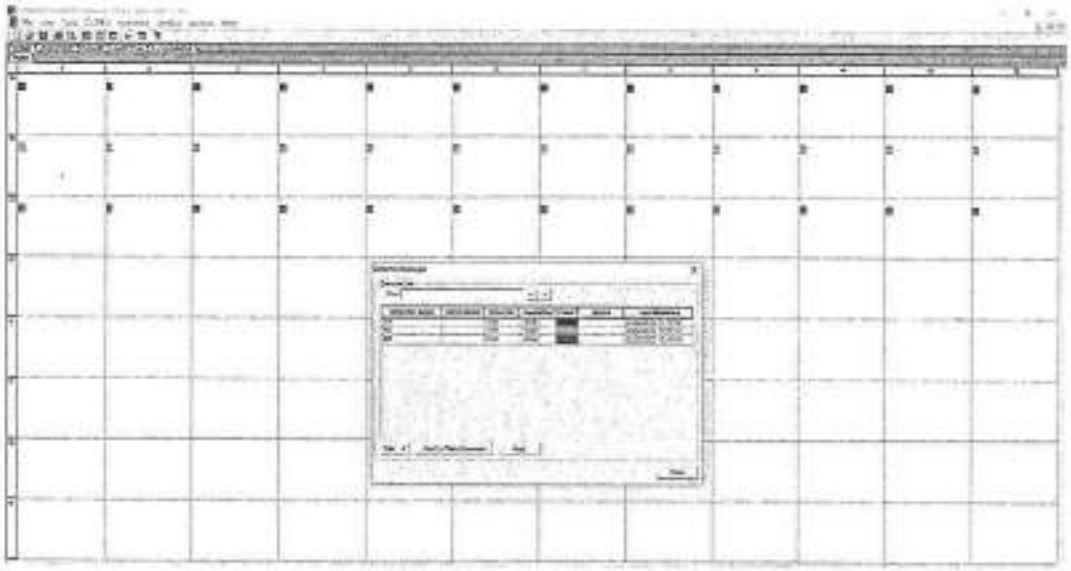
1) Turn on the ABI 7500 Fast Dx Real-Time PCR Instrument.
2) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3) A new window should appear, select **Open Existing Document** from the menu.
4) Navigate to select your ABI Run Template folder from the desktop.
5) Double click on the appropriate template file (**2019-nCoV Dx Panel TaqPath** or **2019-nCoV Dx Panel Quanta** or **2019-nCoV Dx Panel Promega)**
6) There will be a brief pause allowing the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***

Figure 14. Plate Set-up Window



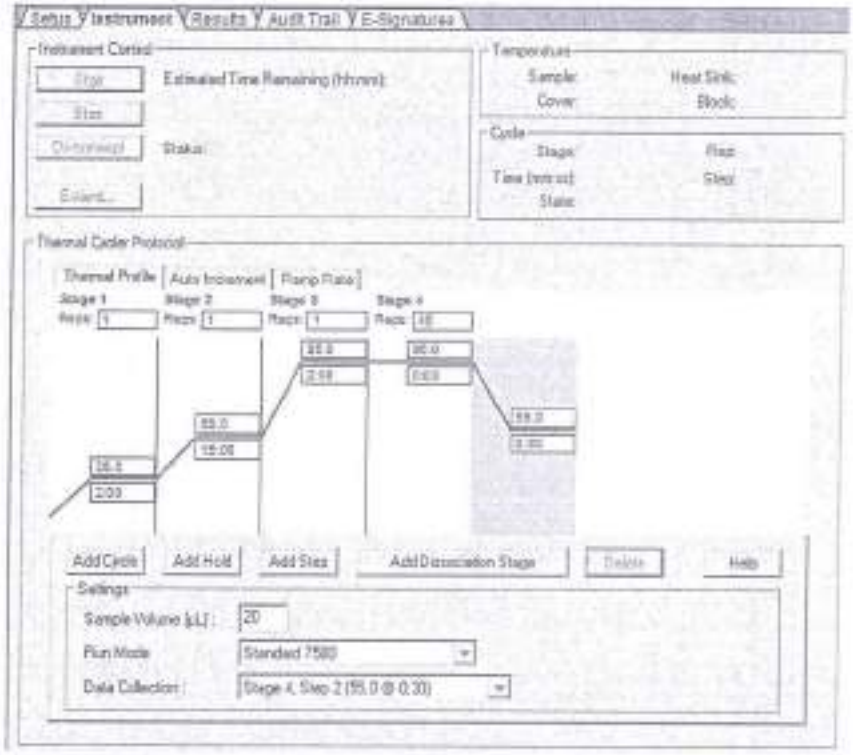
7) After the instrument initializes, a plate map will appear (**Figure 14**). The detectors and controls should already be labeled as they were assigned in the original template.
8) Click the **Well Inspector** icon from the top menu.
9) Highlight specimen wells of interest on the plate map.
10) Type sample identifiers to **Sample Name** box in the **Well Inspector** window (**Figure 15**).

Figure 15. Labeling Wells



11) Repeat steps 9-10 until all sample identifiers are added to the plate setup.
12) Once all specimen and control identifiers are added click the **Close** button on the **Well Inspector** window to return to the **Plate** set up tab.
13) Click the **Instrument** tab at the upper left corner.
14) The reaction conditions, volumes, and type of 7500 reaction should already be loaded (**Figure 16**).

Figure 16. Instrument Settings

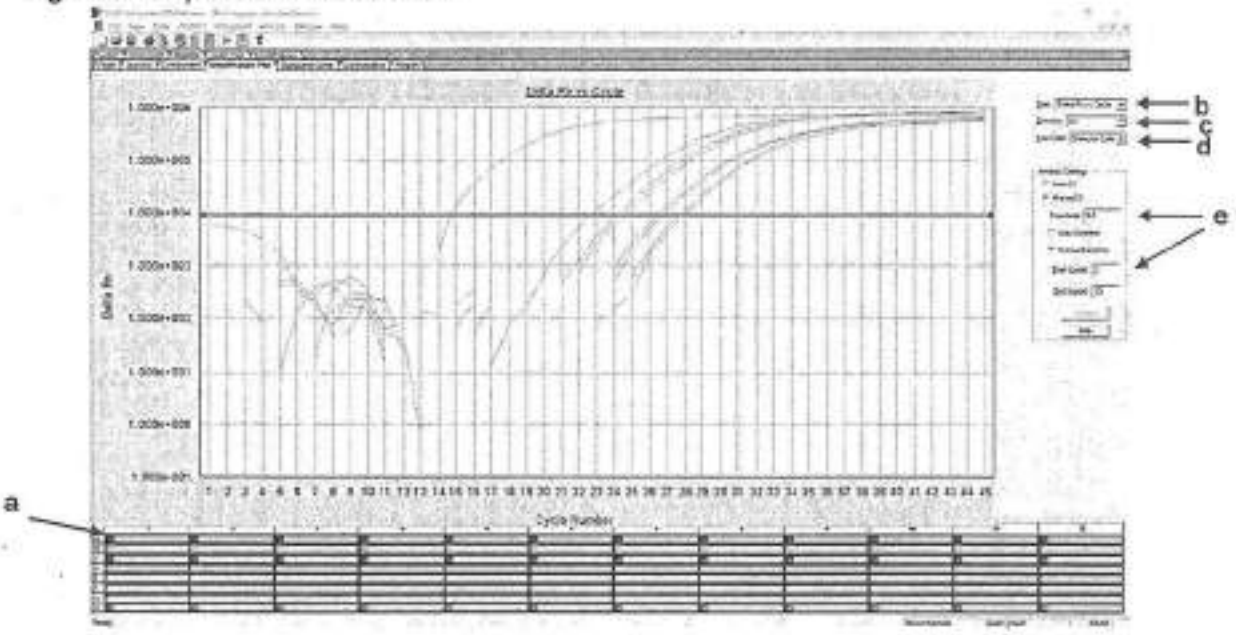


15) Ensure settings are correct (refer to the *Defining Instrument Settings*).
16) Before proceeding, the run file must be saved; from the main menu, select **File**, then **Save As**. Save in appropriate run folder designation.
17) Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
18) Once the run file is saved, click the **Start** button. *Note: The run should take approximately 1 hour and 20 minutes to complete.*

Data Analysis

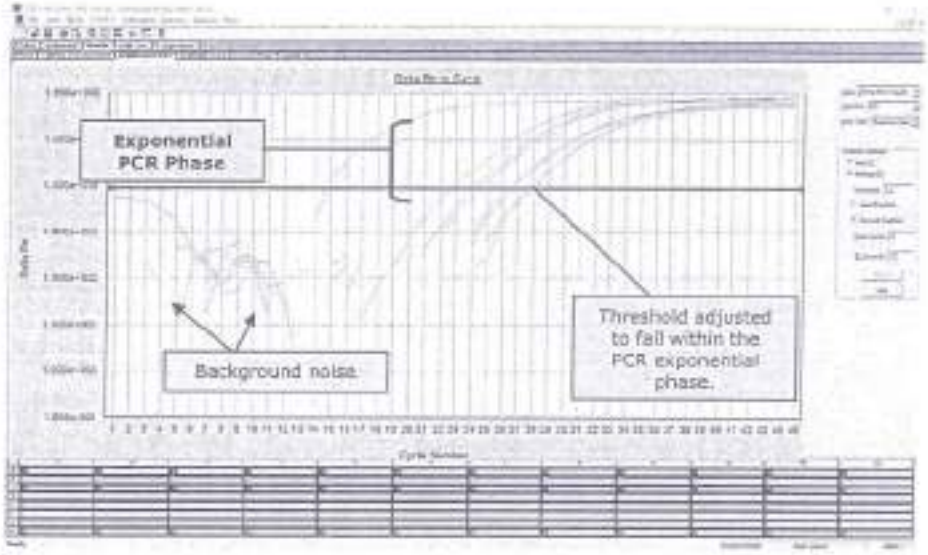
1) After the run has completed, select the **Results** tab at the upper left corner of the software.
2) Select the **Amplification Plot** tab to view the raw data (**Figure 17**).

Figure 17. Amplification Plot Window



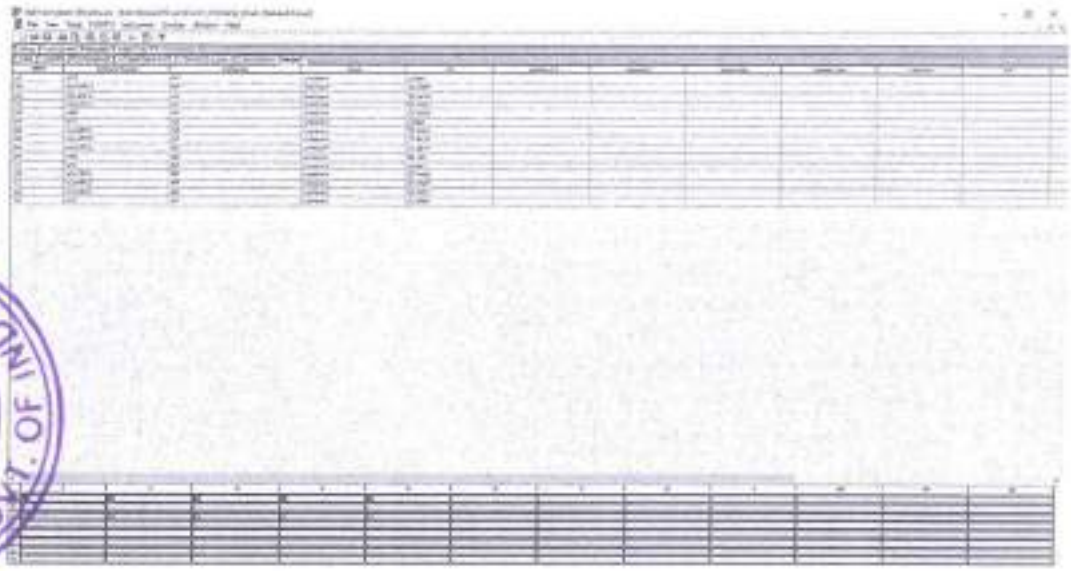
3) Start by highlighting all the samples from the run; to do this, click on the upper left-hand box (a) of the sample wells (**Figure 17**). All the growth curves should appear on the graph.
4) On the right-hand side of the window (b), the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
5) Select **N1** from (c), the **Detector** drop down menu, using the downward arrow.
    a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
6) In the **Line Color** drop down (d), **Detector Color** should be selected.
7) Under **Analysis Settings** select **Manual Ct** (e).
    b. Do not change the **Manual Baseline** default numbers.
8) Using the mouse, click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (**Figure 18**).

Figure 18. Amplification Plot



9) Click the **Analyze** button in the lower right corner of the window. The red threshold line will turn to green, indicating the data has been analyzed.
10) Repeat steps 5-9 to analyze results generated for each set of markers (N1, N2, RP).
11) Save analysis file by selecting **File** then **Save As** from the main menu.
12) After completing analysis for each of the markers, select the **Report** tab above the graph to display the Ct values (**Figure 19**). To filter report by sample name in ascending or descending order, simply click on **Sample Name** in the table.

Figure 19. Report



Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

The NTC consists of using nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

2019-nCoV Positive Control (nCoVPC)

The nCoVPC consists of in vitro transcribed RNA. The nCoVPC will yield a positive result with the following primer and probe sets: N1, N2, and RP.

Human Specimen Control (HSC) (Extraction Control)

When HSC is run with the CDC 2019-nCoV rRT-PCR Diagnostic Panel (see previous section on Assay Set Up), the HSC is used as a nucleic acid extraction procedural control to demonstrate successful recovery of nucleic acid as well as extraction reagent integrity. The HSC control consists of noninfectious cultured human cell (A549) material. Purified nucleic acid from the HSC should yield a positive result with the RP primer and probe set and negative results with all 2019-nCoV markers.

Expected Performance of Controls Included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Control Type	External Control Name	Used to Monitor	2019 nCoV_N1	2019 nCoV_N2	RP	Expected Ct Values
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	< 40.00 Ct
Negative	NTC	Reagent and/or environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 40.00 Ct

If any of the above controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the 2019-nCoV N1 and N2 are positive even in the absence of a positive RP, the result should be considered valid. It is possible, that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RP signal does not preclude the presence of 2019-nCoV virus RNA in a clinical specimen.
  - If all 2019-nCoV markers AND RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

2019-nCoV Markers (N1 and N2)

- When all controls exhibit the expected performance, a specimen is considered negative if all 2019-nCoV marker (N1, N2) cycle threshold growth curves DO NOT cross the threshold line within 40.00 cycles (< 40.00 Ct) AND the RNase P growth curve DOES cross the threshold line within 40.00 cycles (< 40.00 Ct).
- When all controls exhibit the expected performance, a specimen is considered positive for 2019-nCoV if all 2019-nCoV marker (N1, N2) cycle threshold growth curves cross the threshold line within 40.00 cycles (< 40.00 Ct). The RNase P may or may not be positive as described above, but the 2019-nCoV result is still valid.
- When all controls exhibit the expected performance and the growth curves for the 2019-nCoV markers (N1, N2) AND the RNase P marker DO NOT cross the cycle threshold growth curve within 40.00 cycles (< 40.00 Ct), the result is invalid. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.
- When all controls exhibit the expected performance and the cycle threshold growth curve for any one marker (N1 or N2, but not both markers) crosses the threshold line within 40.00 cycles (< 40.00 Ct) the result is inconclusive. The extracted RNA should be retested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained,

report the inconclusive result. Consult with your state public health laboratory or CDC, as appropriate, to request guidance and/or to coordinate transfer of the specimen for additional analysis.

- If HSC is positive for N1 or N2, then contamination may have occurred during extraction or sample processing. Invalidate all results for specimens extracted alongside the HSC. Re-extract specimens and HSC and re-test.

2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide

The table below lists the expected results for the 2019-nCoV rRT-PCR Diagnostic Panel. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact CDC for consultation and possible specimen referral. See pages 13 and 53 for referral and contact information.

2019 nCoV_N1	2019 nCoV_N2	RP	Result Interpretation*	Report	Actions
+	+	±	2019-nCoV detected	Positive 2019-nCoV	Report results to CDC and sender.
If only one of the two targets is positive		±	Inconclusive Result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat rRT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
-	-	+	2019-nCoV not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses.[b]
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

[a]Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

[b]Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1256.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- Although HSC is not included with the 2019-nCov rRT-PCR Diagnostic Panel, the HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative template control (NTC) and the appropriate positive control (nCoVPC) in each amplification and detection run. All clinical samples should be tested for human RNase P gene to control for specimen quality and extraction.

Limitations

- All users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently.
- Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has only been established in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate).
- Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.
- A false-negative result may occur if a specimen is improperly collected, transported or handled. False-negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Positive and negative predictive values are highly dependent on prevalence. False-negative test results are more likely when prevalence of disease is high. False-positive test results are more likely when prevalence is moderate to low.
- Do not use any reagent past the expiration date.
- If the virus mutates in the rRT-PCR target region, 2019-nCoV may not be detected or may be detected less predictably. The clinical performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARSCoV-2 and their prevalence, which change over time.

- Inhibitors or other types of interference may produce a false-negative result. An interference study evaluating the effect of common cold medications was not performed.
- Test performance can be affected because the epidemiology and clinical spectrum of infection caused by 2019-nCoV is not fully known. For example, clinicians and laboratories may not know the optimum types of specimens to collect, and, during the course of infection, when these specimens are most likely to contain levels of viral RNA that can be readily detected.
- Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms.
- The performance of this test has not been established for monitoring treatment of 2019-nCoV infection.
- The performance of this test has not been established for screening of blood or blood products for the presence of 2019-nCoV.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Conditions of Authorization for the Laboratory

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas.

However, to assist clinical laboratories using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel ("your product" in the conditions below), the relevant Conditions of Authorization are listed below:

- Authorized laboratories using your product will include with test result reports, all authorized Fact Sheets available on the CDC website. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories using your product will use your product as outlined in the authorized labeling available on the CDC website. Deviations from the authorized procedures, including the authorized RT-PCR instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use your product are not permitted under this authorization.
- Authorized laboratories that receive the commercially manufactured and distributed primer and probe sets identified as acceptable on the CDC website for use with your product, and are not able to obtain the authorized Human Specimen Control and authorized Positive Control for 2019-nCoV (NCoVPC) materials described in your product's authorized labeling, may use appropriate materials identified as acceptable materials on the CDC website for use with your product. Authorized laboratories that receive your product will notify the relevant public health authorities of their intent to run your product prior to initiating testing.
- Authorized laboratories using your product will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

- Authorized laboratories will collect information on the performance of your product and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and CDC (respvirus@cdc.gov) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the test of which they become aware.
- Authorized laboratories using specimen pooling strategies when testing patient specimens with your product will include with negative test result reports for specific patients whose specimen(s) were the subject of pooling, a notice that pooling was used during testing and that "Patient specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing."
- Authorized laboratories implementing pooling strategies for testing patient specimens must use the "Implementation and Monitoring of Pooled Specimen Testing" available in the authorized labeling to evaluate the appropriateness of continuing to use such strategies based on the recommendations in the protocol.
- Authorized laboratories will keep records of specimen pooling strategies implemented including type of strategy, date implemented, and quantities tested, and test result data generated as part of the Protocol for Monitoring of Specimen Pooling Testing Strategies. For the first 12 months from the date of their creation, such records will be made available to FDA within 48 business hours (2 business days) for inspection upon request, and will be made available within a reasonable time after 12 months from the date of their creation.
- Authorized laboratories will report adverse events, including problems with your products performance or results, to MedWatch by submitting the online FDA Form 3500 (https://www.accessdata.fda.gov/scripts/medwatch/index.cfm?action=reporting.home) or by calling 1-800-FDA-1088.
- All laboratory personnel using the test must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit and use the test in accordance with the authorized labeling.
- CDC, IRR, manufacturers and distributors of commercial materials identified as acceptable on the CDC website, and authorized laboratories using your product will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

Performance Characteristics

Analytical Performance:

Limit of Detection (LoD):

LoD studies determine the lowest detectable concentration of 2019-nCoV at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized samples.

The analytical sensitivity of the rRT-PCR assays contained in the CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel were determined in Limit of Detection studies. Since no quantified virus isolates of the 2019-nCoV were available for CDC use at the time the test was developed and this study conducted, assays designed for detection of the 2019-nCoV RNA were tested with characterized stocks of in vitro transcribed full length RNA (N gene; GenBank accession: MN908947.2) of known titer (RNA copies/µL) spiked into a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM) to mimic clinical specimen. Samples were extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit (Cat# 62724) and manually with the QIAGEN DSP Viral RNA Mini Kit (Cat# 61904). Real-Time RT-PCR assays were performed using the Thermo Fisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG (Cat# A15299) on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use.

A preliminary LoD for each assay was determined testing triplicate samples of RNA purified using each extraction method. The approximate LoD was identified by extracting and testing 10-fold serial dilutions of characterized stocks of in vitro transcribed full-length RNA. A confirmation of the LoD was determined using 3-fold serial dilution RNA samples with 20 extracted replicates. The LoD was determined as the lowest concentration where ≥ 95% (19/20) of the replicates were positive.

Table 4. Limit of Detection Confirmation of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN EZ1 DSP

Targets	2019-nCoV_N1			2019-nCoV_N2		
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$
Positives/Total	20/20	19/20	13/20	20/20	17/20	9/20
Mean Ct[2]	32.5	35.4	NA	35.8	NA	NA
Standard Deviation (Ct)	0.5	0.8	NA	1.3	NA	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 5. Limit of Detection Confirmation CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN QIAmp DSP Viral RNA Mini Kit

Targets	2019-nCoV_N1			2019-nCoV_N2			
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$
Positives/Total	20/20	20/20	6/20	20/20	20/20	20/20	8/20
Mean Ct[2]	32.0	32.8	NA	33.0	35.4	36.2	NA
Standard Deviation (Ct)	0.7	0.8	NA	1.4	0.9	1.9	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 6. Limit of Detection of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	Material	Limit of Detection (RNA copies/µL)	
		QIAGEN EZ1 Advanced XL	QIAGEN DSP Viral RNA Mini Kit
2019 Novel Coronavirus	N Gene RNA Transcript	$10^{0.5}$	10^{0}

FDA Sensitivity Evaluation: The analytical sensitivity of the test will be further assessed by evaluating an FDA-recommended reference material using an FDA developed protocol if applicable and/or when available.

FDA SARS-CoV-2 Reference Panel Testing

The evaluation of sensitivity and MERS-CoV cross-reactivity was performed using reference material (T1), blinded samples and a standard protocol provided by the FDA. The study included a range finding study and a confirmatory study for LoD. Blinded sample testing was used to establish specificity and to confirm the LoD. Samples were extracted using the QIAGEN EZ1 Advanced XL with the QIAGEN EZ1 DSP Virus Kit. Extracted samples were then tested using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel on the Applied BioSystems 7500 Fast Dx Real-Time PCR Instrument using the ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The results are summarized in Table 7.

Table 7: Summary of LoD Confirmation Result using the FDA SARS-CoV-2 Reference Panel

Reference Materials Provided by FDA	Specimen Type	Product LoD	Cross-Reactivity
SARS-CoV-2	NP swab	1.8×10^4 NDU/mL	N/A
MERS-CoV		N/A	ND

NDU/mL = RNA NAAT detectable units/mL

N/A: Not applicable

ND: Not detected

In Silico Analysis of Primer and Probe Sequences:

The oligonucleotide primer and probe sequences of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel were evaluated against 831,910 recent high-quality genome sequences available in Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org) as of June 6, 2021 to demonstrate the predicted inclusivity of the 2019-nCoV real-time RT-PCR Diagnostic panel. In silico analysis monitoring for changes to the primer and probe binding regions are regularly conducted using a sliding window of the most recent 3 months of sequence data. The sliding window is employed to ensure that mismatches in newly reported sequences are easy to identify and their frequency among currently circulating strains and variants is emphasized. The analysis is conducted twice: once using the global GISAID database and again with U.S. sequences only.

Nucleotide mismatches in the primer/probe regions with frequency of >0.1% among currently circulating strains in the global and/or US strain analysis are shown in the Table 8 below. One thousand twenty-nine sequences out of 831,910 had two nucleotide mismatches within the same primer/probe sequence accounting for <1% of all global sequences deposited in GISAID in the past 3 months.

Two independent mismatches identified in an in-silico analysis performed on April 28, 2021 led CDC to further evaluate the impact on performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The first mismatch of interest was the single nucleotide change from C to T at position 3 (5' > 3') of the N1 probe. The mismatch, associated with the B.1.526.2 variant, was found to be present in 2.84% of recent U.S sequences. As of June 6, 2021, the prevalence of this mutation is 3.69% of recent U.S. sequences. Though the likelihood of significant impact from this single mismatch was low, the increasing frequency of the mismatch coupled with its position close to the FAM fluorophore toward the 5' end of the probe led CDC to check for impact on performance. 5 RNA samples from 5 separate clinical specimens containing the B.1.526.2 SARS-CoV-2 variant were tested with the 2019-nCoV Real-Time RT-PCR Diagnostic Panel using both the BioSearch and the IDT builds of the CDC Diagnostic Panel primer and probe sets. The specimens selected had been sequenced and confirmed to contain the mismatch. All 5 specimens generated positive results with the assay (both builds). However, with both builds, the Ct values generated by the N1 primer and probe set were observed to be slightly higher than the Ct values observed for the N2 primer and probe set. The difference ranged from 0.55 cycles to 2.07 cycles, with an average difference of 1.35 cycles. Testing of a 5-fold dilution series prepared from one of the B.1.526.2 RNA samples demonstrated a possible 0 to 0.5 log difference in sensitivity of the N1 primer and probe set compared to the N2 primer and probe set with this mutation. The impact of this mutation on the performance of the N1 primer and probe set is minimal. Overall diagnostic performance of the CDC 2019-nCoV Real-Time Rt-PCR should not be significantly impacted. Current presence of this mutation can be found in Table 8, from the in-silico analysis performed on June 6, 2021.

The second mismatch of interest identified in the in-silico analysis performed on April 28, 2021 was the single nucleotide change from C to T at position 10 (5' > 3') of the N2 probe. The mismatch, associated with the B.1.1.519 variant, was found to be present in 4.20% of recent U.S sequences. As of June 6, 2021, the prevalence of this mutation is 3.04% of recent U.S. sequences. Though the likelihood of significant impact from this single mismatch was low, the increasing frequency of the mismatch coupled with its position adjacent to the internal quencher on the IDT version of the N2 probe led CDC to check for impact on performance. 5 RNA samples from 5 separate clinical specimens containing the B.1.1.519 SARS-CoV-2 variant were tested with the 2019-nCoV Real-Time RT-PCR Diagnostic Panel using both the BioSearch and the IDT builds of the CDC Diagnostic Panel primer and probe sets. The specimens had been sequenced and confirmed to contain the mismatch. All 5 specimens generated positive results with the assay using both the IDT build (with internal quencher) and with the BioSearch build (no internal quencher). The Ct values for the N1 and N2 primer and probe sets across both builds of the primer and probe set were very

similar. Thus, no performance impact was observed with this mismatch. Current presence of this mutation can be found in Table 8. from the in-silico analysis performed on June 6, 2021.

In summary, the assessment of homology between available sequences of SARS CoV-2 as of June 6, 2021 and the CDC panel assay primers and probes shows that the risk of significant loss in reactivity and false negative results is very low due to the absence of significant numbers of mismatches. The design of the primers and probes, with melting temperatures of >60°C and an annealing temperature of 55°C, can tolerate up to two mismatches depending on location without significant loss in assay sensitivity.

Table 8. In Silico Inclusivity Analysis of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Among 831,910 Global Genome Sequences and 295,775 U.S Sequences Submitted to GISAID within three months of June 6, 2021.

Primer/probe	N2 forward			N2 probe			N2 reverse	
Location (5'>3')	4	8	16	7	10	13	4	13
Mismatch Nucleotide	C>T	C>T	G>T	T>C	C>T	C>T	C>A	G>A
Mismatch No. (global sequences)	1010	1,723	2,362	1051	13,621	1583	2,004	959
Mismatch Frequency (%, global sequences)	0.12	0.21	0.28	0.13	1.64	0.19	0.24	0.12
Mismatch No. (U.S. sequences)	285	873	231	1,018	8,997	569	1,273	377
Mismatch Frequency (%, U.S. sequences)	0.10	0.30	0.08	0.34	3.04	0.19	0.43	0.13

Primer/probe	N1 forward				N1 reverse	N1 probe							
Location (5'>3')	4	5	9	14	15	2	3	4	5	13	18	22	24
Mismatch Nucleotide	C>T	C>T	A>G	G>T	G>T	C>T	C>T	C>T	C>T	G>T	G>T	A>G	C>T
Mismatch No. (global sequences)	909	1,175	3,247	2,092	440	2,073	12,899	1958	2,387	699	859	753	397
Mismatch Frequency (%, global sequences)	0.11	0.14	0.39	0.70	0.05	0.03	1.55	0.24	0.29	0.12	0.10	.091	0.05
Mismatch No. (U.S. sequences)	131	526	1,024	520	425	193	10,913	1,163	1,107	147	373	716	310
Mismatch Frequency (%, U.S. sequences)	0.04	0.18	0.35	0.18	0.14	0.07	3.69	0.39	0.37	0.08	0.13	0.24	0.11

Specificity/Exclusivity Testing: In Silico Analysis

BLASTn analysis queries of the 2019-nCoV rRT-PCR assays primers and probes were performed against public domain nucleotide sequences. The database search parameters were as follows: 1) The nucleotide collection consists of GenBank+EMBL+DDBJ+PDB+RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 10/03/2019; 4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000; 5) The match and mismatch scores are 1 and -3, respectively; 6) The penalty to create and extend a gap in an alignment is 5 and 2 respectively.

2019-nCoV_N1 Assay:
Probe sequence of 2019-nCoV rRT-PCR assay N1 showed high sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. However, forward and reverse primers showed no sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. Combining primers and probe, there is no significant homologies with human genome, other coronaviruses or human microflora that would predict potential false positive rRT-PCR results.

2019-nCoV_N2 Assay:
The forward primer sequence of 2019-nCoV rRT-PCR assay N2 showed high sequence homology to Bat SARS-like coronaviruses. The reverse primer and probe sequences showed no significant homology with human genome, other coronaviruses or human microflora. Combining primers and probe, there is no prediction of potential false positive rRT-PCR results.

In summary, the 2019-nCoV rRT-PCR assay N1 and N2, designed for the specific detection of 2019-nCoV, showed no significant combined homologies with human genome, other coronaviruses, or human microflora that would predict potential false positive rRT-PCR results.

In addition to the *in silico* analysis, several organisms were extracted and tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel to demonstrate analytical specificity and exclusivity. Studies were performed with nucleic acids extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit. Nucleic acids were extracted from high titer preparations (typically ≥ 10^5 PFU/mL or ≥ 10^5 CFU/mL). Testing was performed using the Thermo Fisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument. The data demonstrate that the expected results are obtained for each organism when tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Table 9. Specificity/Exclusivity of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	Strain	Source	2019-nCoV_N1	2019-nCoV_N2	Final Result
Human coronavirus	229E	isolate	0/3	0/3	Neg.
Human coronavirus	OC43	isolate	0/3	0/3	Neg.
Human coronavirus	NL63	clinical specimen	0/3	0/3	Neg.
Human coronavirus	HKU1	clinical specimen	0/3	0/3	Neg.
MERS-coronavirus		isolate	0/3	0/3	Neg.
SARS-coronavirus		isolate	0/3	0/3	Neg.
bocavirus	-	clinical specimen	0/3	0/3	Neg.
Mycoplasma pneumoniae		isolate	0/3	0/3	Neg.
Streptococcus		isolate	0/3	0/3	Neg.
Influenza A(H1N1)		isolate	0/3	0/3	Neg.
Influenza A(H3N2)		isolate	0/3	0/3	Neg.
Influenza B		isolate	0/3	0/3	Neg.
Human adenovirus, type 1	Ad71	isolate	0/3	0/3	Neg.
Human metapneumovirus	-	isolate	0/3	0/3	Neg.
respiratory syncytial virus	Long A	isolate	0/3	0/3	Neg.
rhinovirus		isolate	0/3	0/3	Neg.
parainfluenza 1	C35	isolate	0/3	0/3	Neg.
parainfluenza 2	Greer	isolate	0/3	0/3	Neg.
parainfluenza 3	C-43	isolate	0/3	0/3	Neg.
parainfluenza 4	M-25	isolate	0/3	0/3	Neg.

Endogenous Interference Substances Studies:

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel uses conventional well-established nucleic acid extraction methods and based on our experience with CDC's other EUA assays, including the CDC Novel Coronavirus 2012 Real-time RT-PCR Assay for the presumptive detection of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel-Influenza A/H7 (Eurasian Lineage) Assay for the presumptive detection of novel influenza A (H7N9) virus that are both intended for use with a number of respiratory specimens, we do not anticipate interference from common endogenous substances.

Specimen Stability and Fresh-frozen Testing:

To increase the likelihood of detecting infection, CDC recommends collection of lower respiratory and upper respiratory specimens for testing. If possible, additional specimen types (e.g., stool, urine) should be collected and should be stored initially until decision is made by CDC whether additional specimen sources should be tested. Specimens should be collected as soon as possible once a PUI is identified regardless of symptom onset. Maintain proper infection control when collecting specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. Label each specimen container with the patient's ID number (e.g., medical record number), unique specimen ID (e.g., laboratory requisition number), specimen type (e.g., nasal swabs) and the date the sample was collected. Complete a CDC Form 50.34 for each specimen submitted.

Clinical Performance:

As of February 22, 2020, CDC has tested 2071 respiratory specimens from persons under investigation (PUI) in the U.S. using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Specimen types include bronchial fluid/wash, buccal swab, nasal wash/aspirate, nasopharyngeal swab, nasopharyngeal/throat swab, oral swab, sputum, oropharyngeal (throat) swab, swab (unspecified), and throat swab.

Table 10: Summary of CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Data Generated by Testing Human Respiratory Specimens Collected from PUI Subjects in the U.S.

Specimen Type	2019 nCoV Negative	2019 nCoV Positive	Inconclusive	Invalid	Total
Bronchial fluid/wash	2	0	0	0	2
Buccal swab	5	1	0	0	6
Nasal wash/aspirate	6	0	0	0	6
Nasopharyngeal swab	927	23	0	0	950
Nasopharyngeal swab/throat swab	4	0	0	0	4
Oral swab	476	9	0	0	485
Pharyngeal (throat) swab	363	10	0	1	374
Sputum	165	5	0	0	170
Swab (unspecified)[1]	71	1	0	0	72
Tissue (lung)	2	0	0	0	2
Total	2021	49	0	1	2071

[1]Actual swab type information was missing from these upper respiratory tract specimens.

Two thousand twenty-one (2021) respiratory specimens of the 2071 respiratory specimens tested negative by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Forty-nine (49) of the 2071 respiratory specimens tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Only one specimen (oropharyngeal (throat) swab) was invalid. Of the 49 respiratory specimens that tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, seventeen (17) were confirmed by genetic sequencing and/or virus culture (positive percent agreement = 17/17, 95% CI: 81.6%-100%)

During the early phase of the testing, a total of 117 respiratory specimens collected from 46 PUI subjects were also tested with two analytically validated real-time RT-PCR assays that target separate and independent regions of the nucleocapsid protein gene of the 2019-nCoV, N4 and N5 assays. The nucleocapsid protein gene targets for the N4 and N5 assays are different and independent from the nucleocapsid protein gene targets for the two RT-PCR assays included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, N1 and N2. Any positive result from the N4 and/or the N5 assay was further investigated by genetic sequencing.

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel testing these 117 respiratory specimens was estimated against a composite comparator. A specimen was considered comparator negative if both the N4 and the N5 assays were negative. A specimen was considered comparator positive when the N4 and/or the N5 assay generated a positive result, and the comparator positive result(s) were further investigated and confirmed to be 2019-nCoV RNA positive by genetic sequencing.

Table 11: Percent Agreement of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with the Composite Comparator

CDC 2019-nCoV Panel Result	Composite Comparator Result	
	Positive	Negative
Positive	13[1]	0
Inconclusive	0	0
Negative	0	104

[1]Composite comparator results were available for 13 of 49 CDC 2019-nCoV Panel positive specimens only.

Positive percent agreement = 13/13 = 100% (95% CI: 77.2% - 100%)
Negative percent agreement = 104/104 = 100% (95% CI: 96.4% - 100%)

Enzyme Master Mix Evaluation:

The limit of detection equivalence between the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix and the following enzyme master mixes was evaluated: Quantabio qScript XLT One-Step RT-qPCR ToughMix, Quantabio UltraPlex 1-Step ToughMix (4X), and Promega GoTaq® Probe 1- Step RT-qPCR System. Serial dilutions of 2019 novel coronavirus (SARS CoV-2) transcript were tested in triplicate with the CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel using all four enzyme master mixes. Both manufactured versions of oligonucleotide probe, BHQ and ZEN, were used in the comparison. The lowest detectable concentration of transcript at which all replicates tested positive using the Quantabio qScript XLT One-Step RT-qPCR ToughMix and Quantabio UltraPlex 1-Step ToughMix (4X) was similar to that observed for the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix. The lowest detectable concentration of transcript when using the Promega GoTaq® Probe 1- Step RT-qPCR System was one dilution above that observed for the other candidates when evaluated with the BHQ version of the CDC assays. The candidate master mixes all performed equivalently or at one dilution below the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix when evaluated with the ZEN version of the CDC assays.

Table 12: Limit of Detection Comparison for Enzyme Master Mixes – BHQ Probe Summary Results

Copy Number	Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix		Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1- Step RT-qPCR System	
	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2
10^2 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^1 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^0 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
10^{-1} copies µL	2/3	0/3	1/3	1/3	1/3	1/3	0/3	0/3

Table 13: Limit of Detection Comparison for Enzyme Master Mixes – ZEN Probe Summary Results

Copy Number	Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix		Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1- Step RT-qPCR System	
	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2
10^2 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^1 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^0 copies/µL	3/3	2/3	3/3	3/3	3/3	2/3	3/3	3/3
10^{-1} copies µL	1/3	1/3	0/3	0/3	0/3	1/3	1/3	1/3

Retrospective positive (18) and negative (17) clinical respiratory specimens were extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit and were tested with the CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel using the Quantabio qScript XLT One-Step RT-qPCR ToughMix, Quantabio UltraPlex 1-Step ToughMix (4X), and Promega GoTaq® Probe 1- Step RT-qPCR System master mixes. All three enzyme master mixes performed equivalently, demonstrating 100% positive and 100% negative agreement with expected results and a 95% confidence interval of 82.4%-100% and 81.6%-100%, respectively.

Table 14: Clinical Comparison – Retrospective Study Summary Results

CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel Result	Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1-Step RT-qPCR System	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	18	0	18	0	18	0
Negative	0	17	0	17	0	17

Roche MagNA Pure 24 and MagNA Pure 96 Extraction Platform Evaluation:

Performance of the 2019-CoV Real-time RT-PCR Diagnostic Panel using the Roche MagNA Pure 24 and MagNA Pure 96 extraction platforms was compared to performance with an authorized extraction method. Serial dilutions of quantified inactivated SARS-CoV-2 virus (USA-WA1/2020; 100 RNA copies/µL) in lysis buffer were added to pooled negative upper respiratory tract specimen matrix. Five samples of each dilution were extracted in parallel with the QIAGEN EZ1 Advanced XL (EZ1 DSP Virus Kit Cat# 62724) and the Roche MagNA Pure 24 (MagNA Pure 24 Total NA Isolation Kit Cat# 07658036001) and Roche MagNA Pure 96 (MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit Cat# 06543588001) extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The observed LoD was defined as the lowest concentration at which 100% (5 out of 5 total) of all replicates tested positive for both primer/probe sets (N1 and N2) in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The acceptance criteria for equivalence were defined as demonstrating an observed LoD either at the same endpoint or within a 3-fold dilution. The results showed that both the MagNA Pure 24 and MagNA Pure 96 extraction platforms performed equivalently or within one 3-fold dilution of the LoD observed when using the QIAGEN EZ1 Advanced XL extraction platform.

Table 15. Limit of Detection Comparison between the QIAGEN EZ1 Advanced XL, Roche MagNA Pure 96, and Roche MagNA Pure 24 Extraction Platforms using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Platform	Parameter	2019-nCoV_N1 Assay			2019-nCoV_N2 Assay			Observed LoD[1]
QIAGEN EZ1 Advanced XL	RNA copies/µL	$10^{1.3}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{0.5}$
	# pos./total	5/5	5/5	5/5	5/5	5/5	3/5	
	Mean Ct[2]	34.0	35.0	36.3	33.9	36.6	NA	
	Std. Deviation	0.2	0.8	0.2	0.4	0.9	NA	
Roche MagNA Pure 96	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{0.5}$
	# pos./total	5/5	5/5	5/5	5/5	5/5	2/5	
	Mean Ct[2]	33.3	34.6	36.1	33.2	35.7	NA	
	Std. Deviation	0.5	0.5	0.3	0.3	0.4	NA	
Roche MagNA Pure 24	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$
	# pos./total	5/5	3/5	3/5	5/5	5/5	5/5	
	Mean Ct[2]	34.4	NA	NA	35.2	36.9	36.2	
	Std. Deviation	0.6	NA	NA	0.5	1.0	0.8	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.
[2]Mean Ct reported for dilutions that show 100% positivity. Calculations only include positive results.
NA = not applicable

Previously characterized clinical remainder specimens (14 positive and 15 negative) were extracted using both the Roche MagNA Pure 96 and MagNA Pure 24 extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix. Acceptance criteria for clinical equivalence was defined as demonstrating 100% concurrence with qualitative results shown with the authorized comparator method (QIAGEN EZ1 Advanced XL). Results from this study showed 100% concurrence with the comparator method for both the Roche MagNA Pure

96 and Roche MagNA Pure 24 extraction platforms when used with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic panel.

Table 16. Clinical Comparison Results – Retrospective Study Results

Test Platform	Test Platform Result	QIAGEN EZ1 Advanced XL Result		Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
		Positive	Negative		
Roche MagNA Pure 96	Positive	14	0	100.0 (78.5 – 100.0)	100.0 (79.6 – 100.0)
	Negative	0	15		
Roche MagNA Pure 24	Positive	14	0	100.0 (78.5 – 100.0)	100.0 (79.6 – 100.0)
	Negative	0	15		

[1] CI = 95% confidence interval

Promega Maxwell® RSC 48 Extraction Platform Evaluation:

Performance of the 2019-CoV Real-time RT-PCR Diagnostic Panel using the Promega Maxwell® RSC 48 extraction platform was compared to performance with an authorized extraction method. Serial dilutions of quantified inactivated SARS-CoV-2 virus (USA-WA1/2020; 100 RNA copies/µL) in VTM were added to pooled negative upper respiratory tract specimen matrix. Five samples of each dilution were extracted in parallel with the QIAGEN EZ1® Advanced XL (EZ1 DSP Virus Kit Cat# 62724) and the Promega Maxwell® RSC 48 (Promega Maxwell® Viral Total Nucleic Acid Purification Kit Cat# AS1330) extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The observed LoD was defined as the lowest concentration at which 100% (5 out of 5 total) of all replicates tested positive for both primer/probe sets (N1 and N2) in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The acceptance criteria for equivalence were defined as demonstrating an observed LoD either at the same endpoint or within a 3-fold dilution. The results showed that the performance of the Maxwell® RSC 48 extraction platform performed equivalently or within one 3-fold dilution of the LoD observed when using the QIAGEN EZ1® Advanced XL extraction platform.

Table 17. Limit of Detection Comparison Between the QIAGEN EZ1® Advanced XL and Promega Maxwell® RSC 48 Extraction Platforms Using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Platform	Parameter	2019-nCoV_N1 Assay			2019-nCoV_N2 Assay			Observed LoD[1]
QIAGEN EZ1® Advanced XL	RNA copies/µL	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.0}$
	# pos./total	5/5	5/5	0/5	5/5	5/5	3/5	
	Mean Ct[2]	32.27	33.80	NA	35.13	36.41	NA	
	Std. Deviation	0.81	0.40	NA	0.81	0.40	NA	
Promega Maxwell® RSC 48	RNA copies/µL	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.0}$
	# pos./total	5/5	5/5	3/5	5/5	5/5	5/5	
	Mean Ct[2]	31.11	32.97	NA	31.89	33.93	35.17	
	Std. Deviation	0.24	0.34	NA	0.24	0.35	0.65	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.

[2]Mean cycle threshold (Ct) reported for dilutions that show 100% positivity. Calculations only include positive results.
NA = not applicable

Previously characterized clinical remainder specimens (15 positive and 15 negative) were extracted using the Promega Maxwell® RSC 48 extraction platform alongside the currently authorized QIAGEN EZ1® Advanced XL extraction platform and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix. Results from the Maxwell® RSC 48 were compared with the QIAGEN EZ1® Advanced XL extraction performed in parallel showing 100% (15/15) qualitative concurrence on positive samples and 93.3% (14/15) qualitative concurrence on negative samples. This evaluation showed that two originally negative (QIAGEN QIAamp® DSP Viral RNA Mini Kit) specimens (Specimens 16 and 24) yielded an inconclusive result after extraction using the QIAGEN EZ1® Advanced XL. Repeat of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel resolved one of the two specimens (Specimen 24, negative result). The second specimen (Specimen 16) remained inconclusive. Both these specimens yielded a negative result on the Maxwell® RSC 48.

Table 18. Clinical Comparison Results – Retrospective Study Results

Test Platform		Promega Maxwell® RSC 48			Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
	Result	Positive	Negative	Inconclusive		
QIAGEN EZ1® Advanced XL	Positive	15	0	0	100.0 (79.6-100.0)	93.3 (70.2-98.9)
	Negative	0	14	0		
	Inconclusive	0	1	0		

[1] CI = 95% confidence interval

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

References

1. Ballew, H. C., *et al.* "Basic Laboratory Methods in Virology," DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

2. Clinical Laboratory Standards Institute (CLSI), "Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Proposed Guideline," MM13-A

3. Lieber, M., *et al.* "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.

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Revision History

Revision #	Effective Date	Summary of Revisions
1	February 4, 2020	Original Instructions for Use
2	March 15, 2020	• Intended use update • Removal of N3 primer and probe set from Diagnostic Panel • Performance data update • Addition of alternative nucleic acid extraction platforms • Addition of acceptable alternatives to HSC and addition of QIAGEN RUO extraction reagents • Positive results no longer presumptive. No confirmation of positive results required
3	March 30, 2020	• Addition of alternative enzyme master mix options
4	June 12, 2020	• Addition of MagNA Pure 24 extraction method • Addition of performance data for the MagNA Pure 96 extraction method with SARS-CoV-2 • Addition of heat treatment alternative to specimen extraction • Addition of Roche and QIAGEN external lysis buffer alternatives • Acknowledgment of FDA policy permitting end users to qualify alternative components without seeking an EUA or EUA amendment
5	July 13, 2020	• Addition of Promega Maxwell® RSC 48 extraction method • Update to *in silico* inclusivity analyses
6	December 1, 2020	• Addition of specimen pooling instructions and monitoring procedure (Appendices B and C) • Addition of Promega Maxwell CSC 48 extraction instrument • Addition of data for CDC testing of FDA reference panel
7	July 21, 2021	• Update in-silico analysis of primer/probe sequences

Contact Information, Ordering, and Product Support

For technical and product support, contact the CDC Division of Viral Diseases directly.

Send email to: respvirus@cdc.gov

Note: If your laboratory is using reagents sourced from someone other than the CDC International Reagent Resource, please refer to the manufacturer's instructions provided with the commercial materials.

Appendix A: Heat Treatment Alternative to Extraction
UltraPlex 1-Step ToughMix (4X)

This procedure is only for use by public health laboratories.

Purpose:

In response to a global shortage of nucleic acid extraction reagents causing significant delays in testing, the CDC has investigated the use of a heat treatment method requiring minimal reagents as a specimen processing alternative to nucleic acid extraction for use with the 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Where possible, laboratories should use qualified RNA or total nucleic acid extraction methods for processing of specimens for subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Extraction removes inhibitory substances from specimens that could negatively impact PCR performance.

This procedure for use of heat treatment for specimen processing is only recommended when a shortage of qualified extraction reagents is a limiting factor in a laboratory's ability to meet urgent COVID-19 testing demand.

Precautions/Warnings/Limitations:

- CDC has evaluated this heat treatment process and has determined that this process is effective for inactivation of SARS-CoV-2 in patient specimens.
- Performance was evaluated with only upper respiratory specimens. Heat treatment of lower respiratory specimens for subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has not been evaluated.
- This procedure for heat treatment of specimens is only for use with the Quantabio UltraPlex 1-Step ToughMix (4X).
- Heat treatment should only be conducted when a lab is ready to test the specimens by PCR. Testing of heat-treated specimens must be conducted the same day.

Acceptable Specimens:

- Upper respiratory specimens
  Note: Do not use heat treatment to process specimens that appear bloody or that contain particulate matter. Such specimens should be extracted using a qualified RNA or TNA extraction method prior to testing.

Materials Required (not provided):

- 70% ethanol
- 10% bleach, freshly prepared
- 96-well PCR reaction plates (Applied Biosystems catalog # 4346906, 4366932, 4346907, or equivalent)
- Optical strip caps (Applied Biosystems 4323032, or equivalent)

- 1.5 mL Sarstedt tubes or equivalent
- Aerosol resistant micropipette tips
- Micropipettes
- 96-well cold block
- Cold blocks for 1.5 mL - 2.0 mL tubes
- Vortex mixer
- 96-well plate centrifuge or equivalent
- Thermal cycler or equivalent
- Class II Biological Safety Cabinet (BSC)

Procedure:

Sample Preparation

1) Decontaminate BSC with 10% bleach followed by 70% ethanol.
2) If samples are frozen, thaw on ice or at 4°C. Wipe the outside of the sample tube with 70% ethanol. Place thawed sample on cold rack or ice in BSC.
3) Pulse vortex each sample and briefly spin down in a centrifuge to collect the liquid at the bottom of the tube.

Heat Treatment

1) Place a thermal cycler in the BSC, turn on, and program for 95°C for 1 min followed by 4°C hold.
2) Place a 96-well PCR plate onto a cold rack or ice in the BSC.
3) Transfer 100 µL of each sample to the 96-well PCR plate and securely cap each well using optical strip caps.
   NOTE: Ensure that an HSC extraction control is included in each batch run as required under CLIA.
4) Place this 96-well PCR plate on the pre-heated thermal cycler and start run. Leave plate on thermal cycler at 4°C, or place on ice or a cold block.
5) Remove plate and centrifuge for 1 minute at 500 x *g* to pellet cellular debris.
6) Place plate on a cold rack or ice and proceed to testing the supernatant by rRT-PCR.
7) Testing of heat-treated specimens must be conducted the same day heat treatment is performed. For long term storage, keep the original specimen at ≤-70°C.

Special Testing Considerations for Heat Treated Specimens:

Enzyme Master Mix

- Testing of specimens that have been processed with heat treatment should be conducted with the **Quantabio UltraPlex 1-Step ToughMix (4X)**, which demonstrated the best performance with heat treated specimens. PCR testing of heat-treated specimens should follow the instructions in the main body of this Instructions for Use document.

Resolution of Inconclusive and Invalid Results

- Retesting of heat-treated specimens that generated an inconclusive or invalid result must include extraction of the original specimen with a qualified RNA or total nucleic acid (TNA) extraction method, if available. Do not re-test the heat-treated specimen material to resolve inconclusive or invalid test results.

Verification:

CDC recommends performance of verification studies for the heat treatment method prior to diagnostic use that includes side-by-side preparation of a panel of positive and negative clinical specimens using a qualified extraction method and this heat treatment method with subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Performance Characteristics:

Quantabio UltraPlex 1-Step ToughMix (4X)

Limit of Detection Comparison

Serial dilutions of inactivated SARS-CoV-2 [SARS-CoV-2 USA-WA1/2020] were prepared in simulated specimen material (human A549 cells suspended in viral transport medium). Each concentration was prepared side-by-side five times by both EZ1 extraction and by heat treatment. Each extracted or heat-treated sample was subsequently tested by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel using the Quantabio UltraPlex 1-Step ToughMix (4X) on the Applied Biosystems 7500 Fast Dx instrument. Observed detection was similar between the two specimen preparation methods.

Table 1: UltraPlex Limit of Detection Comparison between QIAGEN EZ1 Advanced XL extraction and heat treatment (95°C for 1 min) method – Summary Results

Enzyme	Platform	Parameter	2019-nCoV_N1 Assay					2019-nCoV_N2 Assay					Observed LoD[1]
Quantabio UltraPlex 1-Step ToughMix (4X) 5 µL Template Addition	QIAGEN EZ1 Advanced XL	RNA copies/µL	$10^{1.0}$	$10^{0.3}$	$10^{0.0}$	$10^{-0.3}$	$10^{-1.0}$	$10^{0.8}$	$10^{0.3}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$	$10^{0.3}$
		# pos./total	5/5	5/5	4/5	4/5	3/5	5/5	5/5	5/5	2/5	2/5	
		Mean Ct[2]	34.11	34.59	NA	NA	NA	31.97	33.76	34.70	NA	NA	
		Std. Deviation	0.75	0.99	NA	NA	NA	0.33	0.72	0.98	NA	NA	
	Heat Treatment 95°C for 1 min	RNA copies/µL	$10^{1.3}$	$10^{0.3}$	$10^{0.0}$	$10^{-0.3}$	$10^{-1.0}$	$10^{1.0}$	$10^{0.3}$	$10^{0.0}$	$10^{-0.3}$	$10^{-1.0}$	$10^{0.3}$
		# pos./total	5/5	5/5	4/5	5/5	1/5	5/5	5/5	4/5	2/5	1/5	
		Mean Ct[2]	33.41	34.32	NA	36.73	NA	33.45	35.25	NA	NA	NA	
		Std. Deviation	0.82	0.40	NA	0.82	NA	0.40	0.80	NA	NA	NA	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.

[2]Mean Ct reported for dilutions that show 100% positivity. Calculations only include positive results.

NA = not applicable

Clinical Comparison

A panel of 39 upper respiratory specimens were tested side-by-side using extraction with the Qiagen EZ1 extraction instrument and heat treatment. Extracted and heat-treated specimens were subsequently tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel using the Quantabio UltraPlex 1-Step ToughMix (4X). Qualitative results were compared to demonstrate agreement.

Table 2: Clinical Comparison Results Summary – Heat Treatment versus QIAGEN EZ1 Advanced XL

	Test Result	Heat Treatment			Total	Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
		Positive	Inconclusive	Negative			
QIAGEN EZ1 Advanced XL	Positive	18	1	0	19	94.7 (75.4-99.1)	100 (83.9-100)
	Inconclusive	0	0	0	0		
	Negative	0	0	20	20		
	Total	18	1	20	39		

[1] CI = 95% confidence interval

Questions and Comments:

If you have questions or comments about this procedure, please send by email to: respvirus@cdc.gov

Appendix B: Pooled Specimen Preparation and Processing

Purpose:

In response to strong demand for higher throughput testing approaches as well as a global shortage of nucleic acid extraction reagents causing significant delays in testing, the CDC has evaluated specimen pooling and determined that pooling of up to 4 specimens is suitable for use with the 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Specimen pooling may cause a slight reduction in test sensitivity and therefore may be most appropriate for screening or diagnostic testing when laboratory staff, equipment or reagents are insufficient to accommodate testing demand. Specimen pooling only presents a throughput advantage when the disease prevalence is low. Therefore, laboratories should monitor specimen positivity rates over time to determine if pooling of specimens continues to provide a test throughput advantage over individual specimen testing. Please see Appendix C for more detail on implementation considerations.

While this procedure describes the process to prepare, process and test a pool size of up to 4 specimens, specimen pool sizes from 2-4 are authorized for use with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. When using a pool size of less than 4 specimens, please use the following instructions as a model. Pooled specimen input volume and the pooled specimen to lysis buffer volume ratios must remain as prescribed below (not a lower proportion of lysis buffer) to ensure inactivation of SARS-CoV-2 in patient specimens. An N-pool specimen approach should include equal volumes of each of the N specimens pooled together to create the total pooled specimen input volume required under the below pooled specimen extraction instructions.

Precautions/Warnings/Limitations:

- Pooling of specimens has the potential to decrease sensitivity. The specimens in a pooled procedure are diluted, which could result in a low concentration of viral genetic material below the limit of detection of a given test.
- When pooling specimens, the laboratory cannot ensure the diagnostic integrity of an individual specimen because it is combined with other specimens before testing. Specimen integrity can be affected by the quality of swab specimen collection, which could result in some swabs having limited amounts of viral genetic material for detection. Inadequate individual specimens, including those with limited amounts of viral genetic material, might not be eliminated from the pooled specimen before testing and may be reported as negative through this process.
- Performance of this specimen pooling process was evaluated with nasopharyngeal (NP) swabs. Pooling of other types of specimens for subsequent testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has not been evaluated, however evidence suggests the Ct value distribution for other upper respiratory swab specimens is similar to that observed for NP swab specimens.
- Pooling should only be implemented in laboratories with a minimum of 10 days experience using the CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel for diagnostic testing in the population of specimens being considered for pooling.

- This procedure for pooling of specimens has only been evaluated for use with the QIAGEN EZ1 Advanced XL, Roche MagNA Pure 96, and the Promega Maxwell RSC 48 instruments. Pooling using other extraction methods is not authorized under this EUA.
- Sufficient volume of specimens must be available to allow subsequent extraction and testing of individual specimens should the specimen pool test positive, inconclusive or invalid.
- Appropriate interpretation, reporting and next steps may be different for surveillance testing and are not limited by the diagnostic interpretation instructions below.
- Laboratories are encouraged to revisit the question of whether pooling continues to make sense when testing demand no longer exceeds laboratory capacity and/or when testing reagents are no longer in short supply.

Acceptable Specimen Types:

- Upper respiratory swabs (e.g., nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, NP/OP swabs combined, nasal swabs)
  Note: Specimens must have adequate volume to support pooled specimen testing and any subsequent individual re-testing.

NOTARY
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Mumbai
Maharashtra
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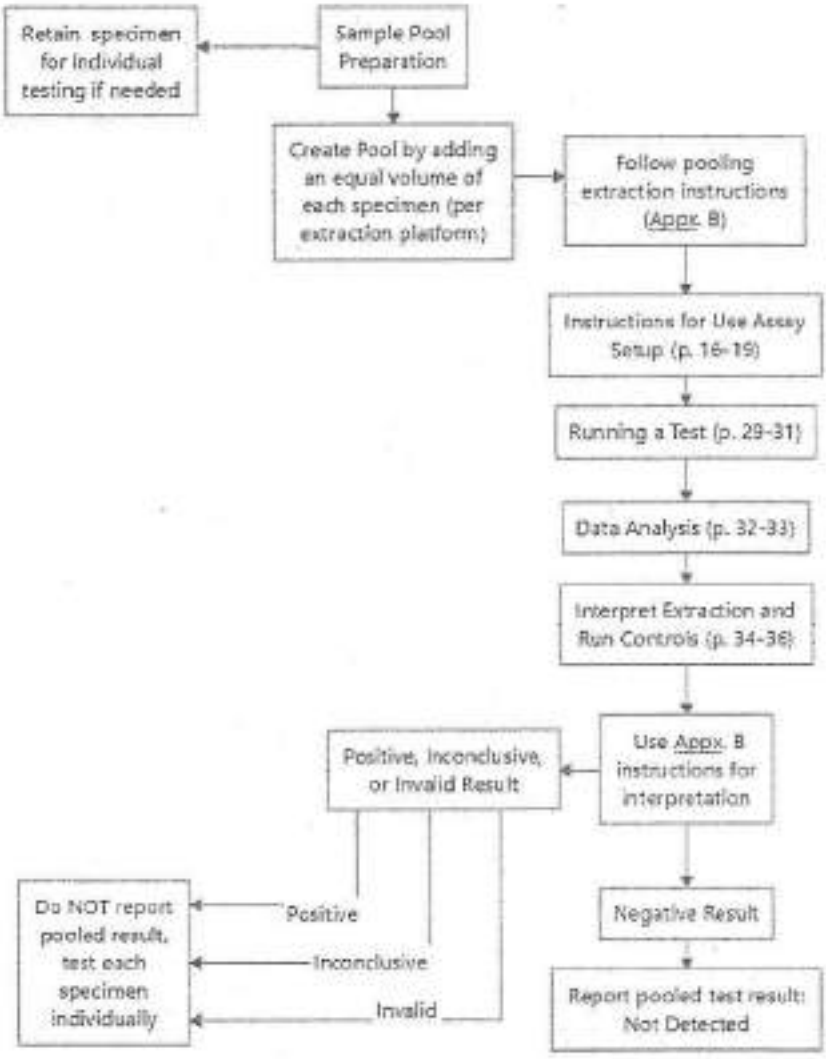
Overview of Pooled Specimen Testing:

This flow chart provides an overview of the steps involved in pooled specimen testing and location of the instructions for each step within the document. For those steps that are identical for both individual specimen testing and for pooled specimen testing, instructions are found in the main body of the instructions for use. For steps in which pooled specimen testing differs from individual specimen testing, instructions specific to pooled specimen testing are presented in this Appendix.



Procedure:

Specimen Pool Preparation

1) Decontaminate BSC with 10% bleach followed by 70% ethanol.
2) If specimens are frozen, thaw on ice or at 4°C. Wipe the outside of the specimen tube with 70% ethanol. Place thawed specimen on cold rack or ice in BSC.
3) Pulse vortex each specimen and briefly spin down in a centrifuge to collect the liquid at the bottom of the tube.
4) For each specimen pool, add an equal volume of each specimen (depending on total volume needed for extraction platform) to be included into the pool, into a sterile, nuclease-free tube.
5) Pulse vortex each specimen pool and briefly centrifuge to collect the liquid at the bottom of the tube.
6) Proceed to extraction using the modified extraction parameters below.

Extraction Instructions

NOTE: These pooled specimen extraction instructions have been modified to optimize recovery of nucleic acid when processing pooled specimens. When testing individual specimens, please follow the individual specimen extraction instructions (p. 14-16).

QIAGEN EZ1 Advanced XL

- Kit: Qiagen EZ1 DSP Virus Kit and Buffer AVL (supplied separately) for offboard lysis
- Card: EZ1 Advanced XL DSP Virus Card
- Instructions: Add 200 µL of pooled specimen to 200 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 60 µL.

Roche MagNA Pure 96

- Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
- Protocol: Viral NA Plasma Ext LysExt Lys SV 4.0 Protocol or Viral NA Plasma Ext Lys SV Protocol
- Instructions: Add 225 µL of pooled specimen to 225 µL of pre-aliquoted MagNA Pure 96 External Lysis Buffer (supplied separately) for total input sample volume of 450 µL. Proceed with the extraction on the MagNA Pure 96. (Internal Control = none).
- Elution volume is 50 µL.

Promega Maxwell RSC 48 or Maxwell CSC 48

- Kit: Promega Maxwell® Viral Total Nucleic Acid Purification Kit
- Protocol: Viral Total Nucleic Acid
- Instructions: Add 240 µL of pooled specimen to 660 µL of pre-aliquoted External Lysis Buffer (600 µL Lysis Buffer plus 60 µL Proteinase K; supplied within the kit) (total input volume is 900 µL). Proceed with the extraction on the Maxwell® RSC 48 or Maxwell CSC 48.
- Elution volume is 50 µL.

Testing, Interpretation, and Reporting Instructions

1) After extraction, continue PCR testing using the steps outlined in the following sections of the Instructions for Use: Assay Set Up (p. 16-20), Running a Test (p. 29-32) and Data Analysis (p. 33-34).
2) Interpret controls and PCR test results as outlined in the following sections of the Instructions for Use: Interpretation of Results and Reporting (p. 35-37), 2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide (p. 38). All extraction and PCR run controls must perform as expected.
   A. If a specimen pool generates **negative** results, report "Not Detected" for each specimen included in the pool. The test result report should include a statement that the specimen was tested in a pooled format. No further testing is required.
   B. If a specimen pool generates a **positive** result, do not report the result. Test each specimen included in the pool individually, according to the Instructions for Use. Report the individual result for each specimen.
   C. If a specimen pool generates an **inconclusive** result, do not report the result. Test each specimen included in the pool individually, according to the Instructions for Use. Report the individual result for each specimen.
   D. If a specimen pool generates an **invalid** result, do not report the result. Test each specimen within the pool individually, according to the Instructions for Use. Report the individual result for each specimen.

Verification:

Verification should be conducted by all laboratories prior to implementing pooling for diagnostic testing. In addition to method verification studies, verification must include a validation of the accuracy and function of the laboratory's process of specimen and pool labeling, tracking and reporting through the laboratory information management system(s).

Performance Characteristics:

Pooling Validation

A panel of 20 positive, previously characterized nasopharyngeal (NP) specimens, with 25% (5) representing the weak positive range (Ct 36.00-39.99), were used for evaluation of specimen pooling with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel; 80 negative NP specimens were also included. These specimens were used to create twenty positive 4-specimen pools, by combining one positive specimen with three negative specimens. Twenty negative pools were also created by combining equal volumes of four negative specimens. These pools were tested and qualitative results from pooled-testing were compared with the results from individual-testing to determine the positive and negative percent agreement of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for testing using 4-specimen pools. The performance of 4-specimen pooling was evaluated using the QIAGEN EZ1® Advanced XL, the Promega Maxwell RSC 48, or the Roche MagNA Pure 96 platforms. Modifications were made to the input and output volume for each extraction platform, for pooling patient specimen, to compensate for the dilution effect due to pooling.

QIAGEN EZ1® Advanced XL

Individual positive panel members and positive and negative pools were extracted using the QIAGEN EZ1® Advanced XL platform for subsequent testing on the Applied Biosystems 7500 Fast Dx with the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix, CG. Results obtained for twenty, 4-specimen pools were compared with the results from individual testing. Pools containing a positive specimen were considered to be in agreement with results from individual testing if the pool generated positive or inconclusive results (i.e., at least one of the SARS-CoV-2-specific primer and probe sets generated a Ct value less than 40). Negative specimen pools were considered to be in agreement with expected results if the pooled specimen result was negative (i.e., neither SARS-CoV-2-specific primer and probe set generated positive results).

A comparison of qualitative test results from individual testing of panel members with pooled specimen testing is summarized below in Table 1, along with testing agreement with results from individual testing for positive and negative specimen pools. Of the five positive specimen panel members in the weak positive category (determined by original diagnostic results): one returned an inconclusive result when tested individually, but returned a positive result when tested in a 4-specimen pool; one returned a negative result when tested individually, but returned a positive result when tested in a 4-specimen pool; and three were in agreement when tested individually and in 4-specimen pools.

Table 1 -Summary of Pooled vs. Individual-testing - QIAGEN EZ1® Advanced XL

Test Platform		Individual testing result			Positive Pools Percent Agreement (CI)[2]	Negative Pools Percent Agreement (CI)[2]
	Result	Positive	Inconclusive[1]	Negative		
4-specimen Pooling Result	Positive	17	1	1	100% (82.4-100)	95.2% (77.3-99.2)
	Inconclusive[4]	1	0	0		
	Negative	0	0	20		

[2]CI = 95% confidence interval
[1]Inconclusive individually-tested results are not included in the final performance calculations.
[4]Inconclusive pooled results are considered in agreement with the positive individually-tested results for the final performance calculations.

Promega Maxwell RSC 48

Individual positive panel members and positive and negative pools were extracted using the Promega Maxwell RSC 48 platform for subsequent testing on the Applied Biosystems 7500 Fast Dx with the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix, CG. Results obtained for twenty, 4-specimen pools were compared with the results from individual testing. Pools containing a positive specimen were considered to be in agreement with the results from individual testing if the pooled specimen generated positive or inconclusive results (i.e., at least one of the SARS-CoV-2-specific primer and probe sets generated a Ct value less than 40). Twenty negative specimen pools (made from 4 known negative specimens) were considered to be in agreement with expected results if the pooled specimen result was negative (i.e., neither SARS-CoV-2-specific primer and probe set generated positive results).

A comparison of qualitative test results from individual testing of panel members with pooled specimen testing is summarized below in Table 2, along with testing agreement with results from individual testing for positive and negative specimen pools. Of the five positive specimen panel members in the weak positive category (determined by original diagnostic results): one returned a positive result when tested

individually, but returned a negative result when tested in a 4-specimen pool; and four were in agreement when tested individually and in 4-specimen pools. Additionally, one moderate to high positive panel member returned an inconclusive result when tested individually but returned a positive result when tested in a 4-specimen pool.

Table 2 -Summary of Pooled vs. Individual-testing Results – Promega Maxwell RSC 48

Test Platform		Individual-testing result			Positive Pools Percent Agreement (CI)[2]	Negative Pools Percent Agreement (CI)[2]
	Result	Positive	Inconclusive[3]	Negative		
4-specimen Pooling Result	Positive	16	1	0	94.7% (75.4-99.1)	100% (83.9-100)
	Inconclusive[4]	2	0	0		
	Negative	1	0	20		

[2]CI = 95% confidence interval
[3]Inconclusive individually-tested results are not included in the final performance calculations.
[4]Inconclusive pooled results are considered in agreement with the positive individually-tested results for the final performance calculations.

MagNA Pure 96

Individual positive panel members and positive and negative pools were extracted on the MagNA Pure 96 platform for subsequent testing on the Applied Biosystems 7500 Fast Dx using the Thermo Fisher TaqPath™ 1-Step RT-qPCR Master Mix, CG. Results obtained for twenty, 4-specimen pools were compared with the results from individual testing. Pools containing a positive specimen were considered to be in agreement with the results from individual testing if the pooled specimen generated positive or inconclusive results (i.e., at least one of the SARS-CoV-2-specific primer and probe sets generated a Ct value less than 40). Twenty negative specimen pools (made from 4 known negative specimens) were considered to be in agreement with expected results if the pooled specimen result was negative (i.e., neither SARS-CoV-2-specific primer and probe set generated positive results).

A comparison of qualitative test results from individual testing of panel members with pooled specimen testing is summarized below in Table 3, along with testing agreement with results from individual testing for positive and negative specimen pools. Of the five positive specimen panel members in the weak positive category (determined by original diagnostic results): one returned an inconclusive result when tested individually, but returned a positive result when tested in a 4-specimen pool; and four were in agreement when tested individually and in 4-specimen pools.

Table 3 -Summary of Pooled vs. Individual-testing Results – Roche MagNA Pure 96

Test Platform		Individual-testing result			Positive Pools Percent Agreement (CI)[1]	Negative Pools Percent Agreement (CI)[1]
	Result	Positive	Inconclusive[2]	Negative		
4-specimen Pooling Result	Positive	18	1	0	100% (83.2-100)	100% (83.9-100)
	Inconclusive[3]	1	0	0		
	Negative	0	0	20		

[1]CI = 95% confidence interval
[2]Inconclusive individually-tested results are not included in the final performance calculations.
[3]Inconclusive pooled results are considered in agreement with the positive individually-tested results for the final performance calculations.

In Silico *Sensitivity*

An *in silico* analysis was conducted to evaluate the effect of 4-sample pooling on the clinical sensitivity of the CDC 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel using the QIAGEN EZ1® Advanced XL extraction platform. This analysis was conducted by performing a Passing-Bablok regression using the "Pooling Validation" data to calculate the Ct shifts resulting from the dilution effect of 4-specimen pools (1 positive specimen combined with 3 negative specimens) for each target. In the regression analysis, the X-axis displayed individual Ct values for positive specimens and the Y-axis displayed Ct values for the corresponding pools with one positive specimen and 3 negative specimens. The regression analysis was used to calculate an interval of Ct values [X*, 40] where individual specimens with Ct values within this interval would have negative results in 4-specimen pools (1 positive and 3 negative).

The results from individually-tested NP swab specimens processed at the CDC using the QIAGEN EZ1® Advanced XL extraction platform (n= 381), were analyzed to determine an *in silico* PPA for 4-specimen pooling. The number of individual specimens with Ct values ranging from [X*, 40] was determined. The X* value for the N1 target was 37.2 and the X* value for the N2 target was 37.5. The results, summarized Table 4, show that 97.1% (370/381 95% CI 94.9-98.3%) of the specimens would not have negative results when combined into 4-specimen pools.

Table 4. *in silico* Sensitivity for Pooled Specimens Extracted using the QIAGEN EZ1® Advanced XL

n	N1 Interval [X*, 40]	Number of samples with N1 Ct values in the interval	N2 Interval [X*, 40]	Number of samples with N2 Ct values in the interval	Number of Samples with both target Ct values in intervals	Neg	Inc	Pos	% Positive Percent Agreement*
381	[37.2, 40]	16	[37.5, 40]	63	11	11	57	313	97.1

*Since any pool that is not negative is re-tested as individual samples, the Positive Percent Agreement includes all pools that were not negative.

The specimens submitted to CDC for testing included many that had generated inconclusive results in the hands of public health laboratories and were forwarded to CDC for further testing. Thus, specimens received by CDC for testing overrepresent the weak positive category. The CDC also obtained the results of individually-tested NP swab specimens from three additional geographic locations: state lab 1 (n= 2217), state lab 2 (n= 6559), and state lab 3 (n= 2315). These data sets were analyzed with regard to the

percent of weak positives (Ct 36.00-39.99) collected at each site. The results, summarized in Table 5, suggest that the percent of weak positive NP specimens received at State Health Department laboratories is smaller than that obtained at CDC. Therefore, the *in silico* analysis, summarized in Table 4, presents a PPA in a scenario where 4-specimen pooling is applied to testing populations with a greater than average number of weak positive specimens.

Table 5. Percent of Weak Positive Specimens at CDC Compared with Three Geographic Locations

		Ct < 36.0		Ct 36.0 - 39.99	
	n	N1	N2	N1	N2
CDC	381	314 (82.4 %)	254 (66.7 %)	67 (17.6 %)	127 (33.3 %)
State 1	2217	2121 (95.7 %)	2021 (91.2 %)	150 (6.8 %)	250 (11.3%)
State 2	6559	6343 (96.7 %)	5883 (89.7 %)	216 (3.3%)	676 (10.3%)
State 3	2315	2073 (89.5 %)	1884 (81.4 %)	242 (10.5 %)	431 (18.6 %)

References:

1. Abdalhamid, B., *et al.* "Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing Resources." *Am J Clin Path* 2020, 153, 715-718.

Questions and Comments:

If you have questions or comments about this procedure, please send by email to: respvirus@cdc.gov

Appendix C: Implementation and Monitoring of Pooled Specimen Testing

Purpose:

These instructions are intended to assist laboratories and jurisdictions to implement and monitor the efficiency and effectiveness of pooled specimen testing over time. A laboratory should have a minimum of 10 days experience performing the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for diagnostic testing within the intended population prior to implementation of a pooling strategy.

Laboratories should weigh the potential risks associated with pooling as described in Appendix B against the benefits of pooling for their laboratory and patient population. When pooling has been implemented to address overwhelming testing demands and/or scarcity of testing materials, laboratories should consider a return to individual specimen testing when laboratory capacity and resources permit.

Prior to Implementation of a Specimen Pooling Strategy:

- Determine the individual specimen positivity rate ($P_{individual}$) for your testing population.
  Before implementation of specimen pooling, evaluate test data from the testing population for the previous 7-10 days to estimate the positivity rate ($P_{individual}$) which is the number of positive results divided by the total number of specimens tested during these 7-10 days. $P_{individual}$ will be used to determine if pooling should be considered and will be used to monitor the performance of your pooling strategy.

  Note: To calculate the efficiency of 4-sample pooling, using $P_{individual}$, apply the formula $F=1/(1+1/n-(1-P_{individual})^n)$, where F is the efficiency and n is the pool size. For example, when $P_{individual}$ is 1%, the efficiency, F, is 3.46 for n = 4. This means that 1,000 tests can cover testing of 3,460 patients on average, which translates to a reduction in test volume of 71%. Using this formula, a pool size of n = 3 produces a greater efficiency than n = 4 at $P_{individual} \geq 13\%$.

- Identify your positivity thresholds.

Table 1: Association Between Individual Specimen Positivity Rates and Reduction in Test Volume with Pooling

Specimen Positivity Rate (P)	No. tests with 4 specimen pooling for 300 patients	Reduction in testing volume with 4 specimen pooling	No. tests with 3 specimen pooling for 300 patients	Reduction in testing volume with 3 specimen pooling	No. tests with 2 specimen pooling for 300 patients	Reduction in testing volume with 2 specimen pooling
1%	87	71%	109	64%	156	48%
2%	98	67%	118	61%	162	46%
3%	109	64%	126	58%	168	44%
4%	120	60%	135	55%	174	42%
5%	131	56%	143	52%	179	40%
6%	141	53%	151	50%	185	38%
8%	160	47%	166	45%	191	36%
10%	178	41%	181	40%	207	31%
11%	187	38%	189	37%	212	29%
12%	195	35%	196	35%	218	27%
13%	203	32%	202	33%	223	26%
14%	211	30%	209	30%	228	24%

Specimen Positivity Rate (P)	No. tests with 4 specimen pooling for 300 patients	Reduction in testing volume with 4 specimen pooling	No. tests with 3 specimen pooling for 300 patients	Reduction in testing volume with 3 specimen pooling	No. tests with 2 specimen pooling for 300 patients	Reduction in testing volume with 2 specimen pooling
15%	218	27%	216	28%	233	22%
16%	226	25%	222	26%	238	21%
17%	233	22%	228	24%	243	19%
18%	239	20%	235	22%	248	17%
19%	246	18%	241	20%	253	16%
20%	252	16%	246	18%	258	14%

Note: Table 1 does not account for running the positive, negative, and extraction controls with each plate.
Note: Unshaded cells indicate the optimal reduction in testing volume compared to other the pool sizes.

Table 1 presents the estimated increase in efficiency achieved through pooling at different rates of specimen positivity. Laboratories are encouraged to use this table and factor in additional testing necessary to monitor the effectiveness of pooling to determine the specimen positivity thresholds ($P_{Threshold}$) at which (1) four-specimen pooling is appropriate, (2) smaller specimen pools are appropriate, and (3) the threshold at which specimen pooling offers no testing capacity/reagent consumption advantages. These thresholds should be documented for your laboratory's pooling monitoring process.

Example: a laboratory might consider a 30% reduction in test volume as the minimum required reduction in test volume to be considered beneficial (this is subjective). Thus, the laboratory would set a positivity threshold ($P_{Threshold}$) at 12%[2] for 4 specimen pooling, 14% for 3 specimen pooling, and 10% or less for 2 specimen pooling.

NOTE: It is recommended that $P_{Threshold}$ should not be greater than 20% for any pool size. Moreover, laboratories may choose a threshold positivity rate lower than 20% for when to consider a pooling strategy.

- Define and document a specimen pooling strategy/process for your laboratory.
- Verify laboratory performance of pooled specimen testing (CLIA requirement).
- Establish and implement a pooling monitoring process appropriate to assure the quality of results generated through the laboratory's pooling strategy. The pooling monitoring process should document the laboratory's $P_{Threshold}$ for each pool size, $P_{Individual}$ and the laboratory's process to address the monitoring requirements below.
- Please note that information from the monitoring process described in this Appendix may not be the only information useful to monitor for pooling performance issues or to aid in decision-making for pooling strategy. Thus, laboratories are encouraged to monitor other sources of COVID-19 prevalence or test positivity rate information for their target population, comparing those trends to

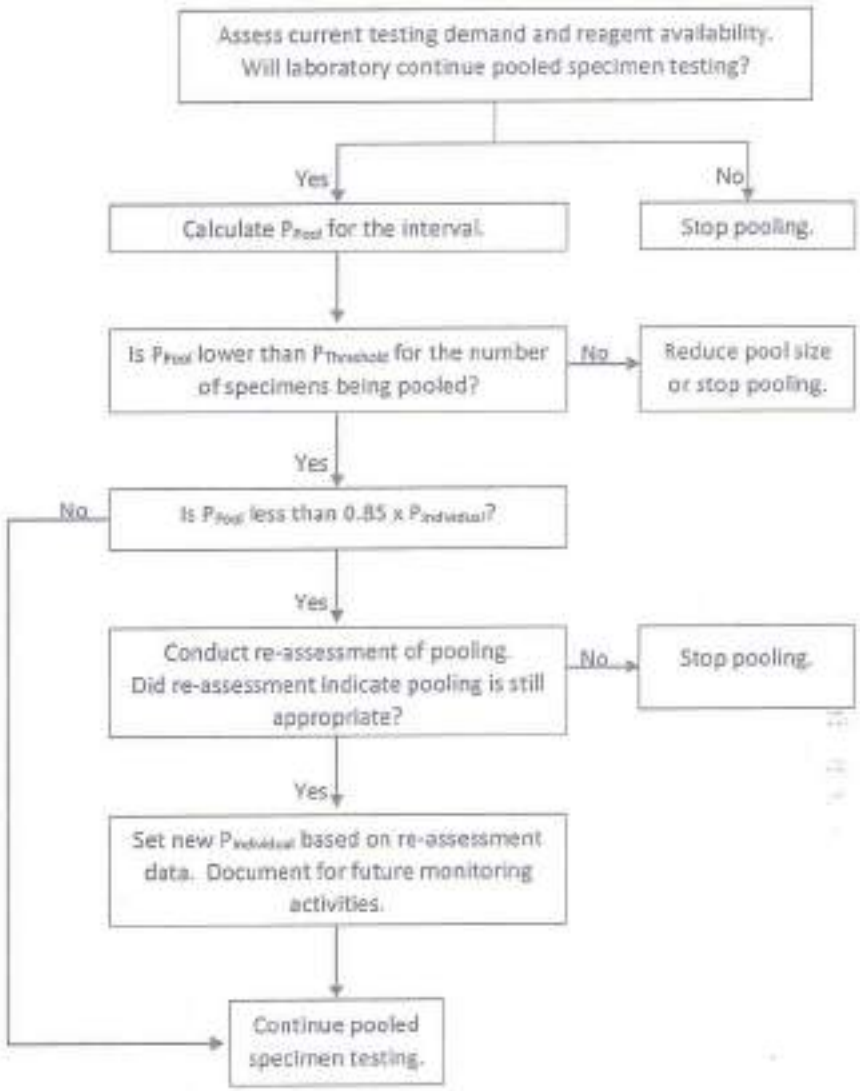
[2] At $P_{Individual} \geq 13\%$, n=3 offers a greater efficiency than n=4, therefore it is recommended that $P_{Threshold}$ should not be greater than 12% for n=4.

their pooling testing trends, as part of the laboratory's pooling monitoring process in addition to the approach below.

Monitoring of the Pooling Strategy

At regular intervals (weekly at a minimum, continual analysis with a moving 7-10 day average preferred), laboratories must monitor the positivity rate of specimen pools as described below.

Figure 1: Overview of Pooling Monitoring Process to be Conducted at Defined Intervals



Data Monitoring Steps to Be Conducted at Each Interval

1. Evaluate need for pooled specimen testing going forward.

   Evaluate test demand and testing resource availability. When testing capacity and resource availability are sufficient to meet testing demand without the use of specimen pooling, consider whether the risks of reduced test sensitivity with pooling and extra effort of pooling outweigh the benefits of pooled specimen testing.

2. Calculate Pooled Percent Positive (P_{Pool}) for the interval.

$$P_{Pool} = \frac{\text{Specimens tested under pooling strategy generating positive results}}{\text{Total specimens tested under pooling strategy}}$$

3. Check Interval P_{Pool} Against Positivity Threshold ($P_{Threshold}$) for the pool size in use.

   Compare the P_{Pool} for the current interval to the specimen positivity threshold established during pooling implementation.

   - If P_{Pool} is greater than $P_{Threshold}$ for the pool size in use, consider a reduction in pool size or discontinuation of specimen pooling until specimen positivity rates decrease in your testing population.
   - If P_{Pool} is less than $P_{Threshold}$, proceed to step 4.

4. Check Interval P_{Pool} Against Re-assessment Criteria

   Compare P_{Pool} to $P_{Individual}$.
   - If P_{Pool} is equal to or greater than 85% of $P_{Individual}$. ($P_{Pools} \geq 0.85 \times P_{Individual}$), then monitoring checks are complete for this interval. Proceed with the current pooling strategy.
   - If P_{Pool} is less than 85% of $P_{Individual}$. ($P_{Pools} < 0.85 \times P_{Individual}$), re-assessment is necessary to determine if pooled-specimen testing process is still acceptable. Please proceed to the Re-assessment of Pooling section below.

5. Update $P_{Individual}$ if Indicated by Re-assessment.

   If re-assessment is conducted and indicates n-specimen pooling is still acceptable, re-establish $P_{Individual}$ in your laboratory using the data generated during re-assessment.

   If 10 positive specimens were tested during re-assessment:

$$P_{Individual} = \frac{\text{10 positive specimens used for re-assessment}}{\text{The total number of specimens tested to reach 10 positive specimens}} \times \frac{10}{11}$$

   If 20 positive specimens were tested during re-assessment:

$$P_{Individual} = \frac{\text{20 positive specimens used for re-assessment}}{\text{The total number of specimens tested to reach 20 positive specimens}} \times \frac{20}{21}$$

   This updated new positivity rate should be used as $P_{Individual}$ in the future monitoring.

Explanation: This calculation attempts to estimate the individual testing positivity rate in the population from which the 10 (or 20) individual positive samples were collected for re-assessment. Since the total number of samples (N*) that needed to be tested to obtain 10 (or 20) consecutive positive samples for re-assessment is stopped at the 10th (or 20th) positive sample, then the positivity rate of 10/N* (or 20/N*) may be overestimated. $P_{Individual}$ is corrected in the above calculations by including an appropriate multiplier: estimated positivity rate for 10 positive sample re-assessment data is (10/N*)×(10/11) and for 20 samples is (20/N*)×(20/21).

Re-assessment of Pooling:

Re-assessment of pooling should be conducted as indicated by monitoring activities. Two possible approaches are presented below.

Option 1

Incoming patient specimens should be tested individually until the collection contains 10 positive specimens.

- Using these specimens, 10 *n*-specimen pools should be created and tested, each with 1 positive and (*n*-1) negative specimens.
- Calculate the PPA between individual testing results and pooled testing results.

  Note: For the calculation of the PPA, all pools that generate positive or inconclusive result are considered in agreement with the individually-tested positive result.

Option 2

Incoming patient specimens can continue to be tested in pools.

- Re-assessment study should start from time T0 and should consist of individual sample testing in parallel with the pooled testing. However, since all non-negative sample pools require individual testing of all individual samples included in the pool as a part of the n-sample pooling and deconvoluting workflow, the re-assessment study essentially consists of testing individual samples from the negative n-sample pools.
- Re-assessment study may pause at time T1 when a minimum of 10 consecutive positive individual results are obtained, including both positive individual results generated from individual testing of samples from the non-negative sample pools following the n-sample pooling and deconvoluting workflow, and positive individual results obtained from individual testing of samples from the negative sample pools for the time period from T0 to T1 [T0, T1].
- Considering that number of positive individual sample results among negative pools is K, PPA between testing n-sample pools and assaying single specimens using the candidate test should be calculated as PPA (EUA Test pool vs. EUA Test individual) = 100% x (10-K)/10. It is critical that all consecutive positive samples from time period [T0, T1] are included in the PPA calculations. With regard to calculating the PPA, all non-negative results testing pooled samples should be counted as in agreement with positive individually tested results.

Re-assessment Acceptance Criteria for Option 1 and Option 2

- If the PPA between pooled-testing results and individual-testing results is ≥ 90% (9 out of 10 or 10 out of 10), then continuation of testing using *n*-specimen pooling is acceptable.
- If the PPA between pooled-testing results and individual-testing results is less than 85%:
    - If PPA ≤ 70% (7 out of 10), reduce the pool size (consider n = 3) and repeat the re-assessment testing with the new pool size until PPA of pooled compared to individual testing is ≥ 90% OR consider cessation of pooling patient specimens.
    - If PPA is 80% (8 out of 10), collect an additional 10 consecutive individually positive samples. Then, calculate the PPA from the combined data of 20 samples, between pooled testing results and individual testing results. If the PPA is ≥ 85%, then implementation of testing using *n*-sample pooling is acceptable. Or, to compensate for lost sensitivity, reduce the pool size (consider n = 3) and repeat the re-assessment testing with the new pool size until PPA of pooled compared to individual testing is ≥ 85%.
- If PPA of at least 85% cannot be reached for any pool size, cease pooling patient specimens.
- If *n*-sample pooling is acceptable, return to step 5 in "Data Monitoring" to re-establish $P_{individual}$.

Additional Resources:

1. Abdalhamid, et al., Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing resources, Amer J Clin Pathol., Vol 153, June 2020, Pages 715–718. https://doi.org/10.1093/ajcp/aqaa064.
2. Advisory on feasibility of using pooled samples for molecular testing of COVID-19. https://www.icmr.gov.in/pdf/covid/strategy/Advisory_on_feasibility_of_sample_pooling.pdf
3. CDC, Interim Guidance for Use of Pooling Procedures in SARS-CoV-2 Diagnostic, Screening and Surveillance Testing. https://www.cdc.gov/coronavirus/2019-ncov/lab/pooling-procedures.html

Questions and Comments:

If you have questions or comments about this procedure, please send by email to: respvirus@cdc.gov.

*** DO NOT DISCARD: Important product-specific information ***

CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel – Verification Requirements

Please consult the following guidance from the Centers for Medicare & Medicaid Services (CMS) regarding diagnostic tests under Emergency Use Authorization (EUA): https://www.cms.gov/Medicare/Provider-Enrollment-and-Certification/SurveyCertificationGenInfo/Policy-and-Memos-to-States-and-Regions-Items/QSO18-19-CLIA

INTENDED USE

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of COVID-19 by their healthcare provider[1].

This test is also for the qualitative detection of nucleic acid from the SARS-CoV-2 in pooled samples containing up to four of the individual upper respiratory swab specimens (nasopharyngeal (NP), oropharyngeal (OP), NP/OP combined, or nasal swabs) that were collected using individual vials containing transport media from individuals suspected of COVID-19 by their healthcare provider. Negative results from pooled testing should not be treated as definitive. If a patient's clinical signs and symptoms are inconsistent with a negative result or results are necessary for patient management, then the patient should be considered for individual testing. Specimens included in pools with a positive, inconclusive, or invalid result must be tested individually prior to reporting a result. Specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet the requirements to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with SARS-CoV-2 but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

REQUIRED MATERIALS

The 2019 novel coronavirus positive control (nCoVPC) is provided with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel and should be prepared according to the Instructions for

[1] For this EUA, a healthcare provider includes, but is not limited to, physicians, nurses, pharmacists, technologists, laboratory directors, epidemiologists, or any other practitioners or allied health professionals.

*** DO NOT DISCARD: Important product-specific information ***

Use. The nCoVPC consists of an RNA transcript of the 2019-nCoV N gene as well as human RNase P gene segment. nCoVPC will yield a positive result with the following primer and probe sets: 2019-nCoV_N1, 2019-nCoV_N2, and RP.

Approximately 2 mL of an upper respiratory specimen (e.g. nasopharyngeal swab (NPS) in transport media) are needed for testing. Specimens may be pooled if less than 2 mL of one specimen is available.

Refer to CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel package insert (manufacturer instructions) for additional reagents, materials, and instructions.

PRECAUTIONS

This reagent should be handled in an approved biosafety level 2 (BSL-2) handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. This product is an RNA transcript and is non-infectious. However, the nCoVPC should be handled in accordance with Good Laboratory Practices.

Store reagent at appropriate temperatures (see Instructions for Use) and hold on ice when thawed.

Please use standard precautions when handling respiratory specimens.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAamp® DSP VIRAL RNA MINI KIT OR THE QIAamp® VIRAL RNA MINI KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 560 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 140 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 14 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 14 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 14 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAGEN EZ1® ADVANCED XL

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 280 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 120 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.

*** DO NOT DISCARD: Important product-specific information ***

- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE TOTAL NUCLEIC ACID KIT OR THE ROCHE MagNA PURE NUCLEIC ACID ISOLATION KIT I

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 300 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE 24 AND TOTAL NUCLEIC ACID ISOLATION KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 400 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE 96 DNA AND VIRAL NA SMALL VOLUME KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 350 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.

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- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE PROMEGA MAXWELL® RSC 48

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 330 µL of lysis buffer (300 µL of lysis buffer + 30 µL Proteinase K, included in the kit) into each of nine tubes labeled 1-9.
- Add 120 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE BIOMÉRIEUX NucliSENS easyMAG OR THE BIOMÉRIEUX EMAG

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 1000 µL or 2000 µL of pre-aliquoted easyMAG lysis buffer into each of nine tubes labeled 1-9 for the easyMAG or eMAG, respectively.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

PROCEDURE

Follow the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for testing the nine extracted samples at least once.

EXPECTED RESULTS

Moderate nCoVPC samples should be positive for 2019-nCoV.
Low nCoVPC samples should be positive for 2019-nCoV.
Negative upper respiratory samples should be negative for 2019-nCoV.

≥90% of test results should be in agreement with the expected results. If test results are <90% in agreement with expected results, contact CDC at respvirus@cdc.gov.

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*** DO NOT DISCARD: Important product-specific information ***

LIMITATIONS

This test has not been FDA cleared or approved.

This test has been authorized by FDA under an EUA for use by authorized laboratories.

This test has been authorized only for the detection of nucleic acid from 2019-nCoV, not for any other viruses or pathogens.

This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of SARS-CoV-2 under Section 564(b)(1) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.

QUESTIONS

Please send questions or comments by email to respvirus@cdc.gov.

DISTRIBUTION

Distributed to qualified laboratories by Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA, 30329 USA

	Division of Viral Diseases/Respiratory Viruses Branch
2019-nCoVEUA-01	**CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Product Information Sheet**

****DO NOT DISCARD: Important product-specific information****

CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel
For use under EMERGENCY USE AUTHORIZATION (EUA) only.
Rx only

CATALOG: 2019-nCoVEUA-01
KIT LOT:
EXPIRATION DATE:

INTENDED USE
The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of COVID-19 by their healthcare provider[1].

This test is also for the qualitative detection of nucleic acid from the SARS-CoV-2 in pooled samples containing up to four of the individual upper respiratory swab specimens (nasopharyngeal (NP), oropharyngeal (OP), NP/OP combined, or nasal swabs) that were collected using individual vials containing transport media from individuals suspected of COVID-19 by their healthcare provider. Negative results from pooled testing should not be treated as definitive. If a patient's clinical signs and symptoms are inconsistent with a negative result or results are necessary for patient management, then the patient should be considered for individual testing. Specimens included in pools with a positive, inconclusive, or invalid result must be tested individually prior to reporting a result. Specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet the requirements to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with SARS-CoV-2 but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

PACKAGE CONTENTS

PACKAGING	COMPONENT	PART NUMBER	COMPONENT LOT NUMBER	VIALS PER KIT	QUANTITY /VIAL	STATE
Oligonucleotide Box	2019-nCoV_N1 Combined Primer/Probe Mix			1	22.5 nmol	Dried
	2019-nCoV_N2 Combined Primer/Probe Mix			1	22.5 nmol	Dried
	RP Combined Primer/Probe Mix			1	22.5 nmol	Dried
Control Box	nCoVPC 2019-nCoV Positive Control (non-infectious)			4	1×10^4 copies/µL	Dried

IVD

[1] For this EUA, a healthcare provider includes, but is not limited to, physicians, nurses, pharmacists, technologists, laboratory directors, epidemiologists, or any other practitioners or allied health professionals.

	Division of Viral Diseases/Respiratory Viruses Branch
2019-nCoVEUA-01	**CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Product Information Sheet**

STORAGE INSTRUCTIONS

Upon receipt, store at 2-8°C. Refer to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use before opening and preparing reagents for use.

PROCEDURE/INTERPRETATION/LIMITATIONS

Users should refer to the **CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use** posted on the FDA website for all IVD products used under Emergency Use Authorization, http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm.

This test has not been FDA cleared or approved.

This test has been authorized by FDA under an EUA for use by authorized laboratories.

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PRECAUTIONS

This reagent should be handled in an approved BSL-2 handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. This product is non-infectious. However, this product should be handled in accordance with Good Laboratory Practices.

REAGENT COMPLAINTS/QUESTIONS

If you have a question/comment about this product, please contact the CDC Division of Viral Diseases/Respiratory Viruses Branch by email at respvirus@cdc.gov.

DISTRIBUTED BY

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IVD

EXHIBIT "W"

Review Article

COVID Diagnostics: Do We Have Sufficient Armamentarium for the Present and the Unforeseen?

Bineeta Kashyap, Nisha Goyal[1], Anupam Prakash[2]

Department of Microbiology, University College of Medical Sciences and Guru Teg Bahadur Hospital, [1]Department of Microbiology, G B Pant Institute of Postgraduate Medical Education and Research, [2]Department of Medicine, Lady Hardinge Medical College and SSK Hospital, New Delhi, India

Abstract

The COVID-19 pandemic has taken the world by storm, and nations world over are battling this unprecedented health crisis. Diagnostics play the most important part in the "test, track, and treat" strategy being used in most of the nations to combat COVID-19. Although viral culture is the gold standard, it is not pursued because of the associated biohazard risks. Short of that, nucleic acid amplification tests (NAATs) are the present gold standard and are being used in several ways. Real-time reverse transcriptase-polymerase chain reaction is being widely used, although cartridge-based NAAT and TrueNat™ testing are also in vogue. Serological testing is also being used as an adjunct specially for screening (rapid antigen testing kits), while antibody (specially IgG) testing is being used as a serosurveillance strategy. Radiological investigations, especially computed tomography scan of the thorax, give peculiar peripheral ground-glass opacities which are quite characteristic in the present COVID pandemic and need to be ascertained together with other clinical features and diagnostic tools. Although the present tools have been able to support the diagnosis of COVID to quite an extent, there are limitations, and as the whole spectrum of COVID disease unfolds, the diagnostic armamentarium will also continue to expand, and we will need to use the diagnostic strategies further to be able to contain this pandemic at the earliest.

Keywords: Coronavirus, COVID-19, polymerase chain reaction, serology, viral pneumonia

We will not be going back to the "old normal." The pandemic has already changed the way we live our lives. Part of adjusting to the "new normal" is finding ways to live our lives safely.

Dr. Tedros, Director-General, World Health Organization in his media briefing on July 23, 2020.[1]

Testing is The Way Out...

Since its origin in the end of December 2019, SARS-CoV-2 has affected 15,296,926 individuals and is responsible for 628,903 deaths globally. India alone has reported 1,287,945 confirmed cases and 30,601 deaths due to COVID-19 till date.[1] There is a wide consensus over the perception of "test, track, and treat strategy" to be the only possible way to limit the COVID-19 spread and save precious human lives. The majority of COVID-19 cases develop mild illness and eventually recover uneventfully. However, some develop severe disease with progression to pneumonia, hypoxemia, systemic inflammatory illness, and hypercoagulability.[2] It is crucial to scale up the testing for SARS-CoV-2 and make it available even in distant outreach areas. This review intends to explore the selection of the most appropriate specimens and also discuss the availability, utility, or accuracy of the most reliable diagnostic methods for COVID-19 so as to make the clinicians better aware and also timely manage the patients.

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SARS-CoV-2 the Virus!

Coronaviruses (CoVs), enveloped viruses with nonsegmented positive sense RNA, belong to the family Coronaviridae and order Nidovirales. Four genera of CoVs (α, β, γ, and δ) have been identified; the human CoVs (HCoVs)

Address for correspondence: Dr. Bineeta Kashyap, Flat No. C-402, Vimal CGHS LTD., Plot-3, Sector-12, Dwarka, New Delhi - 110 078, India.
E-mail: dr_bineetakashyap@yahoo.co.in

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are included in the α-coronavirus (HCoV-229E and NL63) and β-coronavirus (Middle-East respiratory syndrome coronavirus [MERS-CoV], SARS-CoV, HCoV-OC43, and HCoV-HKU1) genera. SARS-CoV-2, classified under the subgenus Sarbecovirus, subfamily Orthocoronavirinae, is a group 2B coronavirus. The immune-dominant spike (S) protein, envelope (E) protein, nucleocapsid (N) protein, and membrane (M) protein constitute the four structural proteins of CoVs. The transmembrane spike glycoprotein, homotrimers projecting from the viral surface of coronavirus, mediates its entry into the host cells. S consists of two functional subunits: S_1 subunit is involved in binding to the host cell receptor, whereas the S_2 subunit is responsible for the fusion of viral and host cell membranes. In several CoVs, S cleaves at the border connecting the S_1 and S_2 subunits which have noncovalent bonding in prefusion conformation.[3] The distal S_1 subunit besides containing the receptor-binding domain (s), also provides the stabilization to membrane-anchored S_2 subunit in prefusion state.[4] Host proteases further cleave the S at S_2 cleavage site that leads to exhaustive irreversible conformational modifications responsible for the activation of protein associated with membrane fusion.[4,5] Thus, the entry of coronavirus into susceptible host cell is mediated through a complex process involving the binding to receptor and proteolytic processing of S protein. Coronavirus S glycoprotein, being surface exposed, is the primary target for the neutralizing antibodies and also the central point for therapeutic and vaccine research.[6] Distinct domains inside S_1 subunit are utilized by various CoVs for the identification of different attachment and entry receptors. S domain A (S^A) attaches to 5-N-acetyl-9-O-acetyl-sialosides of host cell surface to gain entry in endemic human CoVs OC43 and HKU1.[6] In MERS-CoV, S^A is utilized for the identification of nonacetylated sialoside attachment receptors that may further promote the binding of S^B to dipeptidyl peptidase 4 entry receptor.[7] S^B connects directly with angiotensin-converting enzyme 2 (ACE2) for entry in target cells in SARS-CoV and several SARS-related CoVs. ACE2 is also the functional receptor for SARS-CoV-2 that mediates its entry into host cell via SARS-CoV-2 S. The analogous affinity of SARS-CoV-2 S^B and SARS-CoV S^B for human ACE2, partly explains the similar efficient transmission in humans of SARS-CoV-2 as that of SARS-CoV.[8]

Diagnostic Strategies for SARS-CoV-2: A Review of Available Approaches

Nucleic acid amplification tests

Table 1 depicts the various diagnostic techniques available for COVID-19 and Table 2 shows the interpretation of various microbiological test results in COVID-19. The primary challenges for nucleic acid amplification tests (NAATs) are (i) to minimize the false negatives by efficiently detecting the small number of viral RNA copies in the sample, (ii) to reduce the false positives by effectively differentiating the positive signals emitted by other pathogens, and (iii) to have the ability to test a large number of samples accurately in limited time. SARS-CoV-2 is an enveloped virus containing positive-sense RNA as its genetic material. The genome of SARS-CoV-2 consists of approximately 30,000 nucleotides and 15 genes. Many of these genes, such as spike (S), nucleocapside (N), envelop (E), RNA-dependent RNA polymerase (RdRp), helicase (Hel), nonstructural protein 10 (nsp10), and nonstructural protein 14 (nsp14), have been utilized as probes or primer targets in the diagnostic NAATs. Initial researches demonstrated that by targeting the S gene, SARS-CoV-2 could be differentiated from SARS-CoV-1 with good specificity but low sensitivity.[2] In order to improve the sensitivity, additional viral-specific genes, such as RdRp/Hel, were integrated.[9] E and RdRp primers were considered to be most sensitive and were extensively used all over Europe, though RdRp primers were found to be cross reactive to SARS-CoV RNA.[2,9] The WHO recommends the usage of E, N, S, and RdRp genes in various combinations for optimum diagnosis.[10] Similar to other RNA viruses, SARS-CoV-2 also has a tendency to mutate. However, the proofreading property of Nsp14 restricts the nucleotide misincorporation rate.[11] Variation in nucleotide sequence may lead to diminished recognition by distinct primer-probe set. The use of minimum two molecular targets, ideally at least one targeting the conserved/specific region, would help to mitigate the effects of a potential SARS-CoV-2 genetic drift or its cross-reaction with any other circulating coronavirus.

Real-time reverse transcription-polymerase chain reaction

Real-time reverse transcription-polymerase chain reaction (rRT-PCR) remains the validated assay for prompt diagnosis in suspected SARS-CoV-2 infection. Amplification and analysis being carried out together in a closed system, the chances of false positives in these assays are minimized. The approximate turnaround time (TAT) is 4–5 h. The high diagnostic accuracy of rRT-PCR along with the capacity to test up to ninety specimens in a single run makes it the frontline test for diagnosis of COVID-19. The need of trained workforce, specialized laboratory equipment, and specific biosafety requirements, limits its use at peripheral centers in developing countries like India.

Loop-mediated isothermal amplification is another alternative to RT-PCR which uses amplification under isothermal condition with more rapidity and specificity.

With the surge in cases of COVID-19 in India and over 150 countries across the globe, a rapid point-of-care assay is considered to be a major tool toward the containment of cases. To reduce the TAT of NAATs, many rapid platforms of microarray or sequencing solutions co multi-RT-PCR panels with automation are being developed. Though initially intended to be used for the diagnosis of tuberculosis and other infectious diseases, TrueNat™ and CBNAAT-based nucleic acid amplification test (CBNAAT) are now widely deployed for the detection of COVID-19 cases. The real-time micro-PCR system is achieved through a combination of

Table 1: Diagnostic techniques for COVID-19

Technique	Time needed	Working principle	Advantage	Limitation
RT-PCR	3–4 h	Specific primer-probe based detection	High sensitivity (only small amount of RNA needed) Well-established methodology	High costs Detection is also complex and time-consuming Large number of samples tested in each run
TrueNat and CBNAAT	1 h	Specific primer-probe based detection	Fast results Closed system with lesser chances of contamination Minimal biosafety concerns	High costs Limited number of samples tested in each run
LAMP	1 h	More than two sets of specific primers Pair-based detection	Highly accurate and repeatable Single working temperature	Too sensitive, highly prone to false positives due to cross-contamination or carry-over
NGS	1–2 days	Whole-genome sequencing	High sensitivity and specificity Ability to identify novel strains Provides detailed information	Need of highly trained experts Highly sophisticated equipment required High cost
Serology (traditional)	4–6 h	IgG/IgM	Identify exposure	Limited sensitivity at an early stage
Rapid serological	15-30 min	IgG/IgM	Point-of-care test	Limited sensitivity at an early stage
Rapid antigen test	30 min	Antigen detection	Point-of-care test High specificity	Limited sensitivity
CT scan	1 h	Chest images	Increased sensitivity if findings combined with RT-PCR results	Not able to distinguish from other viral pneumonia
Virus isolation	5–15 days	*In vitro* live virus isolation and propagation	Highly (100%) specific Gold standard	Low sensitivity as isolation is not 100% Biosafety concerns

NGS: Next-generation sequencing, CT: Computed tomography, LAMP: Loop-mediated isothermal amplification, CBNAAT: Cartridge-based nucleic acid amplification test, RT-PCR: Real-time reverse transcriptase-polymerase chain reaction, IgM: Immunoglobulin M, IgG: Immunoglobulin G

Table 2: Interpretation of various microbiological test results in COVID-19

	Acute infection	Recent infection	Late-onset infection	Old infection	Absence of infection
Clinical symptoms	Variable	Positive	Positive	Negative	Negative
NAATs	Positive	Negative	Negative	Negative	Negative
IgM	Variable	Positive, may be negative	Positive	Negative	Negative
IgG	Variable	Positive	Positive	Variable	Negative

NAATs: Nucleic acid amplification test, IgM: Immunoglobulin M, IgG: Immunoglobulin G

the cartridge-based RNA extraction system and real-time micro-PCR analyzer. These are cartridge-based closed NAAT systems that can be performed with minimal hands-on training. CBNAAT (GeneXpert® SARS-CoV-2 test Cepheid®) is a simple, highly performing test with a short TAT. TrueNat is a chip-based RT-PCR test for the semi-quantitative detection of beta-coronavirus and SARS coronavirus RNA. The target sequence is the E gene of Sarbecovirus for beta-coronavirus and Rdrp gene for SARS coronavirus. As the viral lysis buffer used in the processing inactivates the virus, these techniques impose a minimal biosafety hazard. The TAT for these platforms is approximately 1 h. However, only 1–4 samples can be processed in a single run, limiting the samples that can be tested in a single day to 24–48. These systems have the capability of being utilized at grass-root levels.

Cycle threshold value

Real time-PCR assay measures the viral RNA in terms of cycle threshold (Ct), which is the number of cycles the fluorescent signal requires to become detectable and is inversely proportional to the viral load. The results are interpreted based on the Ct values and value <40 are generally considered positive. In RT-PCR, false-negative result may occur due to sampling error or incorrect timing of sampling.

The overall limit of detection for NAATs ranges from 100 to 1000 copies. Thus, these tests have much desired high analytical sensitivity along with very high specificity.[11]

Choosing the Right Specimens

Selection of an appropriate specimen is very crucial for the correct diagnosis of infected COVID-19 patients. COVID-19 patients typically shed high load of culturable virus starting from about 5–6 days of becoming symptomatic though viral RNA remains detectable in the respiratory samples for longer. Severely ill patients can continue to shed the virus for weeks to months. Fecal shedding contributing to the spread of infection remains a concern too. Nasopharyngeal (NP) swab

collected by trained health-care workers (HCWs) constitutes a standard sample for RT-PCR as per the recommendation by the Centers for Disease Control and Prevention. The process of obtaining an NP swab is uncomfortable for the patient and may elicit coughing or sneezing. Hence, the use of adequate personal protective equipment and adherence to standard protocol for infection prevention and control is warranted by the HCW for this procedure. Operational difficulty in the collection of NP swabs has led to the assessment of other comparatively easily available alternative samples such as saliva, oropharyngeal swabs, nasal swabs, NP wash/aspirate, and mid-turbinate swabs. Oropharyngeal swabs are comparatively less sensitive than NP and nasal swabs.[15] Testing of simultaneously collected nasal and oropharyngeal swabs, either independently or together in a single aliquot, is an attractive alternative option to increase the chances of positivity. The collection and processing of saliva, though an appealing sample, possesses its own challenges. Nasal swabs have comparable sensitivity to NP swabs. Many studies have reported a comparable sensitivity of self-collected samples by patients with those collected by health-care personnel.[11,16] Any upper respiratory specimen, however, may miss early infection; when a repeat testing must be performed preferably on a lower respiratory specimen, as the main site of replication by then might possibly be the lower respiratory tract. Moreover, sputum and bronchoalveolar lavage (BAL) have shown higher sensitivity in comparison to upper respiratory samples, probably due to the presence of greater viral loads in these specimens.[12] Collecting different specimen types in highly clinically suspected cases will improve detection rate by reducing false negativity. However, due to these being invasive procedures with the enhanced associated risk of aerosol generation, collection of these samples is done in selective instances.[2] Shipping of all these specimens to the reference laboratory must be done following triple packaging system ensuring appropriate labeling and sealing of the samples as per the standard protocol.

A false-negative nucleic acid amplification test: Significance?

It is of paramount importance to understand that a negative RT-PCR test result does not rule out COVID-19. There are several factors influencing the positivity of a RT-PCR test result, some of which include: low viral load in case of an incubation period or convalescent stage; or primary replication of the virus at other sites in the body (lower respiratory tract). There have been negative RT-PCR test reports with upper respiratory tract specimens in cases with suggestive pulmonary computed tomography (CT) scan findings.[16] This viral tropism for the lower respiratory tract is probably due to the inconsistent distribution of ACE2 viral receptors throughout the respiratory tract.[2,17] A suboptimal sampling technique may also affect the RT-PCR test results. In the instances of high clinical suspicion, it is prudent to repeat testing, as the sensitivity of NP swab is below ideal. Furthermore, in a high prevalence milieu, researchers have shown a significant increase in the positivity of these tests.

A positive nucleic acid amplification test previously declared negative

The criterion most often applied for discontinuation of isolation is two negative RT-PCR test results at least 24 h apart. Nevertheless, some of such cases report positive again despite having two negative test results.[18] This may be due to the alterations in the shedding of viral RNA during convalescence. However, the prognosis of such cases seems to be good in the absence of an actual clinical or virological relapse.[2]

Does nucleic acid amplification test positivity measure infectiousness?

As the viral RNA can be demonstrated from the samples of patients during recovery, nucleic acid amplification tests are not very useful in monitoring the infectivity of COVID-19 cases.[19] Although the ability of virus present in the sample to grow in culture constitutes a better measure of infectivity, it is rarely practiced due to biosafety concerns.[20]

Quantitative nucleic acid amplification test: Any prognostic value?

Instances show the presence of high viral loads even among asymptomatic cases as determined by the real-time PCR Ct values. Therefore, the prognostic utility of viral load in isolation is limited.[21] Though some correlations have been revealed between the severity of the disease and viral load, the viral load determined by these assays in terms of Ct value should not be used for prognosis or monitoring treatment response.[22] In all probabilities, irrespective of the course of the disease, viral loads typically regress with time.[23,24] Lower Ct values indicate high viral load and hence can be suggestive of transmissibility.

Adjuncts to Molecular Diagnosis

Serological evidence of SARS-CoV-2 diagnosis

Immunological tests can either measure the antibodies produced during the host immune response to infection or the antigenic viral particles in the respiratory specimens. The techniques commonly used for the demonstration of SARS-CoV-2-specific antibodies are immunochromatographic tests, enzyme-linked immunosorbent assays (ELISA), neutralization assays, and chemiluminescent immunoassays. Serologic tests are less dependable than the NAATs for the detection of SARS-CoV-2. The prevalence of infection also plays a crucial role in determining the positive or negative predictive values of a given test. In a low-prevalence setting, a positive serologic test with limited specificity is more likely to be a false-positive test result. Concerns were raised regarding the cross-reactivity of antibodies against SARS-CoV-2 and various related or distant viral families. Other human CoVs (such as HKU1, OC43, 229E, and NL63) causing mild-to-moderate seasonal respiratory symptoms are antigenically closely related to SARS-CoV-2.[25] The chances of cross-reactions are furthermore plausible with SARS-CoV or MERS-CoV. However, majority of the commercially available

serologic assays demonstrate a specificity above 98%. The target antigen used in a serological assay also influences the sensitivity and specificity of that assay. S protein, produced at a much advanced stage of COVID-19, has lower sensitivity but higher specificity (particularly with S1 subunit) in comparison to N protein targets.[26]

Serological evidence: The relevance?

Serologic tests are mostly used to determine the exposure to SARS-CoV-2 in the past. These tests may also prove useful in establishing the diagnosis of COVID-19 in cases with negative NAATs with high clinical suspicion. Though IgM and IgG antibodies have been demonstrated as early as 3–6 days following the onset of symptoms, the seroconversion has been reported to occur by 3 weeks in majority of cases.[27] IgM is generally the first class of antibody to be produced in any infection followed by IgG immunity. IgM can be detected from the 2nd week, with the titers touching the peak in the 3rd week from the onset of symptoms and then slowly declining over time. IgG is reported to stabilize around 4 weeks.[28] However, in COVID-19, it is believed that IgM may be short lasting and IgG response may occur earlier than usual, and how long this IgG lasts is yet not known. IgM and IgG against SARS-CoV-2 can be determined qualitatively by immunochromatography assays and quantitatively by ELISA. Detection rates improve with the progression of illness.[29] A recent study has reported a higher accuracy of IgM and IgG ELISA in comparison to lateral flow assays.[30]

Though the antibody responses are demonstrable in majority of the COVID-19 cases, seroconversion may not be observed in immunocompromised patients or in a few with asymptomatic/very mild infections. The presence of specific antibodies against SARS-CoV-2 is most probably linked with some level of protection, though cutoff values of these antibodies are yet not established.[31] Neutralizing antibodies are generally considered more directly connected with the protective immunity. Furthermore, the production of neutralizing antibodies is complemented with T-cell responses. Low titers of antibodies are not considered protective and high titers are often encountered in severe COVID-19.[3] The recovery of mild cases even with low antibody levels and persistence of the disease in the presence of high antibody titers in severe cases raise queries about the role of neutralizing antibodies in providing immunity. Therefore probably, the therapeutic benefit of convalescent plasma has been attributed to other components by some researchers.[32]

Several lateral flow assays have been developed to detect the antigens of COVID-19 as point-of-care platform. The widely used antigen detection tests have a moderate sensitivity (ranging from 50.6% to 84%) with a high specificity (99.3% to 100%). The rapid chromatographic immunoassay may aid in the qualitative determination of SARS-CoV-2-specific antigens. The positive test results by antigen detection test can be regarded as true positives. However, the negative test results in symptomatic cases need further confirmation by real-time PCR test.[33] Although these assays have the theoretical advantage of being rapid and low cost, the viral load of the patient and the variability in specimen collection could result into lower sensitivities of these tests, early in infection.

Due to the kinetics of antibody formation or variabilities in the sensitivities of these assays, clinical decision-making should not be solely relied upon these tests unless strong evidence exists. Though impractical in early stage, antibody detection tests may be used for retrospective evaluation and epidemiological surveillance in terms of the burden of infection, significance of asymptomatic infections, basic reproduction number of the virus, or the overall mortality.

Computed tomography scan

CT scan is often regarded as an important auxiliary investigation for COVID-19. The researchers from Wuhan have reported a considerably higher sensitivity of CT scan in comparison to PCR tests in COVID-19 cases.[34] CT scan plays a pivotal role in the early diagnosis and timely management of COVID-19 cases. The characteristic features of COVID-19 infection comprise bilateral multi-lobar ground-glass opacities with differential distribution, subpleural ascendance, thickened lobular septa with inconsistent alveolar filling, and amalgation.[34,35] However, these findings of CT scan are suggestive and not confirmatory for COVID-19 diagnosis.

Viral culture

Though viral culture is the gold standard for the isolation and characterization of the virus, it is not used for the diagnosis of COVID-19 due to the process being labor intensive and also due to the requirement of biosafety level 3 facility with skilled workforce. Vero, Huh, and human airway epithelial cells lines have been used by several researchers to observe the cytopathic effects, which are confirmed by RT-PCR.[36-38]

Biomarkers

Several biomarkers are routinely used in clinical practice for their possible predictive role in the assessment of disease progression. They are crucial in identifying the cases at higher risk of developing complications. In addition, these also help in deciding the treatment protocols in COVID-19 cases. Figure 1 shows the commonly used biomarkers in COVID-19 patients.

Figure 1: Biomarkers with predictive role in COVID-19 progression

Diagnosis of SARS-CoV-2: Challenges and Limitations!

Despite an upstanding accomplishment of validated NAATs, there are some inherent challenges. NAATs carry the risk of false-negative results due to several pre-analytical factors that can influence the end results such as the inappropriate timing of collection of specimen (too early or too late in the course of illness); poor-quality specimen; type of sample (lower respiratory tract specimens, such as BAL and induced sputum, have better sensitivity than upper respiratory tract specimens such as NP and oropharyngeal swabs); and lapse in sample transportation (unsuitable container, inappropriate viral transport medium, or inadequate maintenance of cold chain, etc.).[31] Serological assays have a low sensitivity in the early course of COVID-19. The clinical utility of serodiagnosis is confined to the convalescent patients with negative molecular test results. In addition, researchers have documented the ongoing evolution of SARS-CoV-2 genome through active mutations and genetic recombination.[40,41] Being RNA virus, SARS-CoV-2 is also deficient in effective proofreading machinery needed to secure the RNA replication fidelity.[40] Mutations may change the sequence of primers hybridizing regions, thus yielding false-negative results.[41,42] However, this issue can be addressed by targeting more than one (two or three) sequence in viral genome.

What Do We Need to Remember?

- The complex scenario of the ongoing and rapidly evolving COVID-19 pandemic warrants the concerted use of various available modalities and their interpretation in relation to the clinical milieu of individual case
- The diagnosis not only needs to be timely and accurate, but should also contribute toward providing relevant epidemiological information so as to assess the actual burden and spread of the disease. In this context, the serological assays can complement molecular diagnosis, especially among those who are still asymptomatic and not hospitalized or may be used as screening assays as adjunct to diagnosis, though NAAT continues to remain the reference standard for COVID-19 diagnosis
- Monitoring the viral load along with being observant of the technique and timing of sample collection will help better interpret different stages of the disease. The type of sample to be collected, whether upper or lower respiratory, will further reduce the false negativity
- Clinical corroboration completed by serological evidence will help to reach a prompt management decision.
- The patients' microbiota and the immune system will finally contribute to the varied clinical manifestations present and the prognosis. Hence, continuous addition to the existing knowledge needs to be made as further studies get published.

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None.

Conflicts of interest

There are no conflicts of interest.

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EXHIBIT "X"

Are you infectious if you have a positive PCR test result for Covid-19?

Source: CEBM – University of Oxford

Link: https://www.cebm.net/covid-19/infectious-positive-pcr-test-result-covid-19/

Author: Tom Jefferson, Carl Heneghan, Elizabeth Spencer, Jon Brassey

Published on: August 5, 2020

PCR detection of viruses is helpful so long as its accuracy can be understood: it offers the capacity to detect RNA in minute quantities, but whether that RNA represents infectious virus may not be clear.

During our Open Evidence Review of oral-fecal transmission of Covid-19, we noticed how few studies had attempted or reported culturing live SARS-CoV-2 virus from human samples.

This surprised us, as viral culture is regarded as a gold standard or reference test against which any diagnostic index test for viruses must be measured and calibrated, to understand the predictive properties of that test. In viral culture, viruses are injected in the laboratory cell lines to see if they cause cell damage and death, thus releasing a whole set of new viruses that can go on to infect other cells.

We, therefore, reviewed the evidence from studies reporting data on viral culture or isolation as well as reverse transcriptase-polymerase chain reaction (RT-PCR), to understand more about how the PCR results reflect infectivity.

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What did we find?

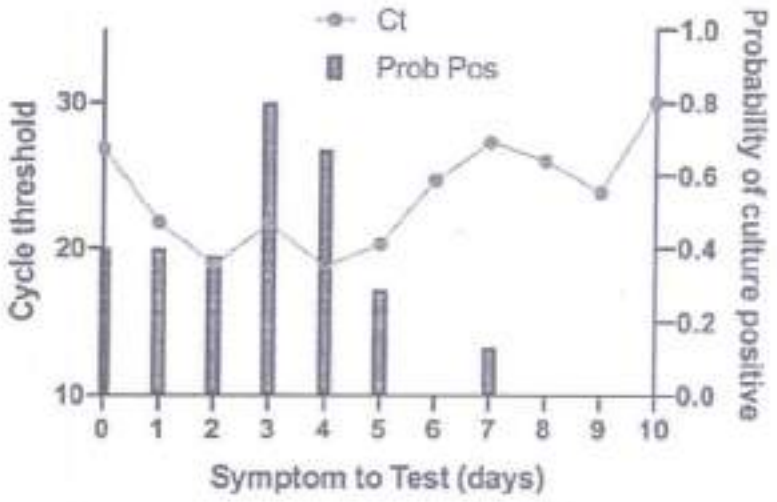
We searched for studies that reported culture or isolation of SARS CoV-2 using samples from Covid-19 patients.

We identified fourteen studies that succeeded in culturing or observing tissue invasion by SARS-CoV from various samples from patients diagnosed with Covid-19. The quality of these studies was moderate with a lack of protocols, standardised methods and reporting.

Data are sparse on how the PCR results relate to viral culture results. There is some evidence of a relationship between the time from collection of a specimen to test, symptom severity and the chances that someone is infectious.

One of the studies we found (Bullard et al) investigated viral culture in samples from a group of patients and compared the results with PCR testing data and time of their symptom onset.

The figure below reported in Bullard shows how the probability of SARS CoV-2 infectious virus is greater (the red bars) when the cycle threshold is lower (the blue line) and when symptoms to test time is shorter - beyond 8 days, no live virus was detected.



Shedding of infectious virus in hospitalized patients with coronavirus disease 2019 (COVID 19): duration and key determinants medRxiv 2020.06.08.20125310.

Kampen and colleagues studied the shedding of infectious virus in 129 hospitalized patients with COVID-19. The duration of infectious virus shedding ranged from 0 to 20 days post-onset of symptoms, and the probability of detecting infectious virus dropped below 5% after 15 days post-onset of symptoms. They also report that the amount of virus is associated with the detection of infectious SARS-CoV-2, and once neutralizing antibodies are detected in the serum the virus becomes non-infectious.

When the samples were taken seemed important for viral culture. In a case report, SARS-CoV-2 RT-PCR continued to detect the virus until the 63rd day after symptom onset whereas the virus could only be isolated from respiratory specimens collected within the first 18 days. In a cohort of 59 patients, fecal

discharge was longer after respiratory shedding stopped. Gupta et al. [w15] reported the duration for fecal shedding of viral RNA after clearance of respiratory samples ranged from 1 to 33 days and in one patient was up to 47 days from symptom onset.

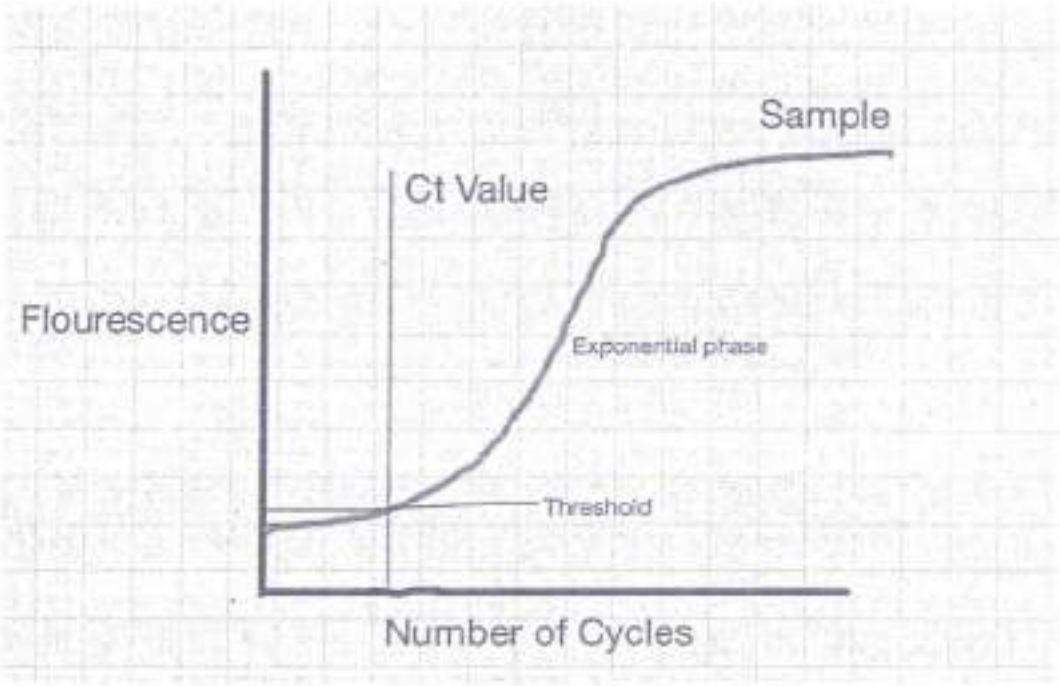
It was not possible to make a precise quantitative assessment of the association between RT-PCR results and the success rate of viral culture within these studies. These studies were not adequately sized nor performed in a sufficiently standardised manner and may be subject to reporting bias.

Furthermore, context matters. The cycle threshold level for detecting live virus will vary by setting (hospital vs. community); depending on the symptom severity and the duration of symptoms, as well as the quality of the testing. Cycle thresholds are the times that the amplifying test has to be repeated to get a positive result. The higher the viral concentration the lower amplification cycles are necessary.

Why does the cycle threshold cut-off matter?

RT-PCR uses an enzyme called reverse transcriptase to change a specific piece of RNA into a matching piece of DNA. The PCR then amplifies the DNA exponentially, by doubling the number of molecules time and again. A fluorescent signal can be attached to the copies of the DNA, and a test is considered positive when the fluorescent signal is amplified sufficiently to be detectable.

The cycle threshold (referred to as the Ct value) is the number of amplification cycles required for the fluorescent signal to cross a certain threshold. This allows very small samples of RNA to be amplified and detected.

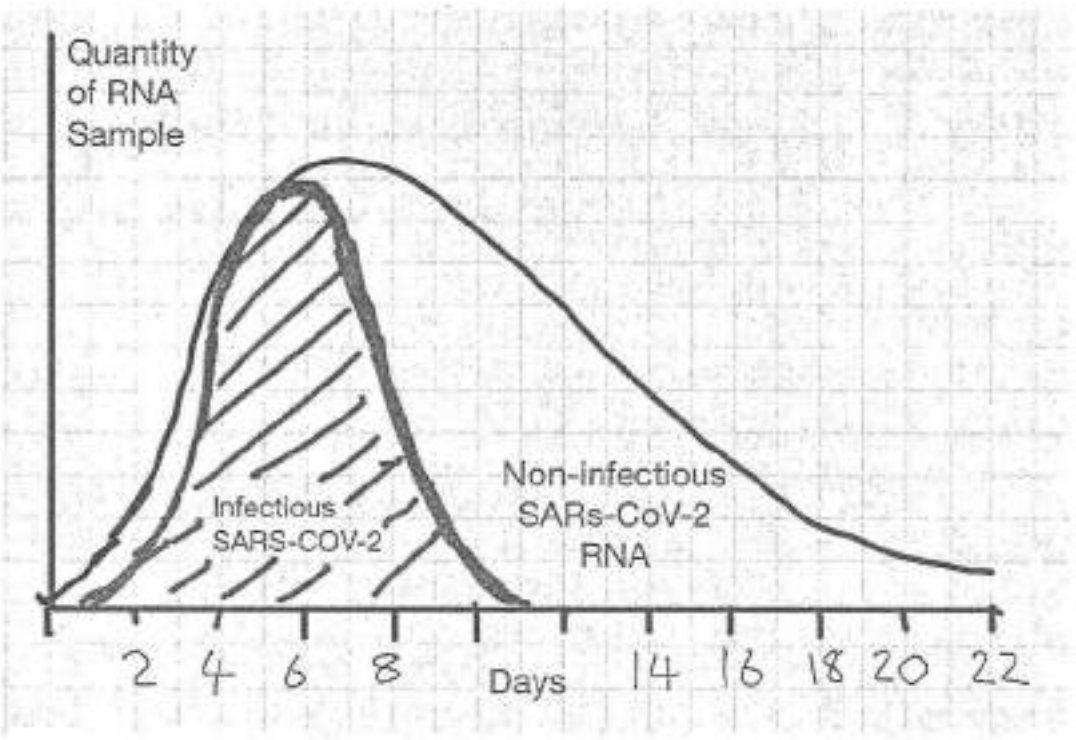


The lower the cycle threshold level the greater the amount of RNA (genetic material) there is in the sample. The higher the cycle number, the less RNA there is in the sample.

What does this mean?

This detection problem is ubiquitous for RNA viruses detection. SARS-CoV, MERS, Influenza Ebola and Zika viral RNA can be detected long after the disappearance of the infectious virus.

The immune system works to neutralise the virus and prevent further infection. Whilst an infectious stage may last a week or so, because inactivated RNA degrades slowly over time it may still be detected many weeks after infectiousness has dissipated.



PCR detection of viruses is helpful so long as its limitations are understood; while it detects RNA in minute quantities, caution needs to be applied to the results as it often does not detect infectious virus.

What can we conclude?

These studies provided limited data of variable quality that PCR results per se are unlikely to predict viral culture from human samples. Insufficient attention may have been paid how PCR results relate to disease. The relation with infectiousness is unclear and more data are needed on this.

If this is not understood, PCR results may lead to restrictions for large groups of people who do not present an infection risk.

The results indicate that viral RNA load cut-offs should be used: to understand who is infectious, the extent of any outbreak and for controlling transmission.

What next?

Our review is an Open Evidence Review. We will update the findings as additional evidence become available. WE submitted the manuscript to the preprint server MedRxiv. (See here) We will continue to search for and find further studies (such as Kampen et al) that will be included in updates.

Meanwhile, if you have comments, if you have other studies to be included, and especially if you have been diagnosed as infected or infectious please send them to tom.jefferson@conted.ox.ac.uk.

We will read all comments but we cannot promise to respond.

EXHIBIT "Y"

Review Article

COVID Diagnostics: Do We Have Sufficient Armamentarium for the Present and the Unforeseen?

Bineeta Kashyap, Nisha Goyal[1], Anupam Prakash[2]

Department of Microbiology, University College of Medical Sciences and Guru Teg Bahadur Hospital, [1]Department of Microbiology, G B Pant Institute of Postgraduate Medical Education and Research, [2]Department of Medicine, Lady Hardinge Medical College and SSK Hospital, New Delhi, India

Abstract

The COVID-19 pandemic has taken the world by storm, and nations world over are battling this unprecedented health crisis. Diagnostics play the most important part in the "test, track, and treat" strategy being used in most of the nations to combat COVID-19. Although viral culture is the gold standard, it is not pursued because of the associated biohazard risks. Short of that, nucleic acid amplification tests (NAATs) are the present gold standard and are being used in several ways. Real-time reverse transcriptase-polymerase chain reaction is being widely used, although cartridge-based NAAT and TrueNat™ testing are also in vogue. Serological testing is also being used as an adjunct specially for screening (rapid antigen testing kits), while antibody (specially IgG) testing is being used as a serosurveillance strategy. Radiological investigations, especially computed tomography scan of the thorax, give peculiar peripheral ground-glass opacities which are quite characteristic in the present COVID pandemic and need to be ascertained together with other clinical features and diagnostic tools. Although the present tools have been able to support the diagnosis of COVID to quite an extent, there are limitations, and as the whole spectrum of COVID disease unfolds, the diagnostic armamentarium will also continue to expand, and we will need to use the diagnostic strategies further to be able to contain this pandemic at the earliest.

Keywords: Coronavirus, COVID-19, polymerase chain reaction, serology, viral pneumonia

We will not be going back to the "old normal." The pandemic has already changed the way we live our lives. Part of adjusting to the "new normal" is finding ways to live our lives safely.

Dr. Tedros, Director-General, World Health Organization in his media briefing on July 23, 2020.[1]

Testing is The Way Out...

Since its origin in the end of December 2019, SARS-CoV-2 has affected 15,296,926 individuals and is responsible for 628,903 deaths globally. India alone has reported 1,287,945 confirmed cases and 30,601 deaths due to COVID-19 till date.[1] There is a wide consensus over the perception of "test, track, and treat strategy" to be the only possible way to limit the COVID-19 spread and save precious human lives. The majority of COVID-19 cases develop mild illness and eventually recover uneventfully. However, some develop severe disease with progression to pneumonia, hypoxemia, systemic inflammatory illness, and hypercoagulability.[2] It is crucial to scale up the testing for SARS-CoV-2 and make it available even in distant outreach areas. This review intends to explore the selection of the most appropriate specimens and also discuss the availability, utility, or accuracy of the most reliable diagnostic methods for COVID-19 so as to make the clinicians better aware and also timely manage the patients.

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SARS-CoV-2 the Virus!

Coronaviruses (CoVs), enveloped viruses with nonsegmented positive sense RNA, belong to the family Coronaviridae and order Nidovirales. Four genera of CoVs (α, β, γ, and δ) have been identified; the human CoVs (HCoVs)

Address for correspondence: Dr. Bineeta Kashyap, Flat No. C-402, Vimal CGHS LTD., Plot-3, Sector-12, Dwarka, New Delhi - 110 078, India.
E-mail: dr_bineetakashyap@yahoo.co.in

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are included in the α-coronavirus (HCoV-229E and NL63) and β-coronavirus (Middle-East respiratory syndrome coronavirus [MERS-CoV], SARS-CoV, HCoV-OC43, and HCoV-HKU1) genera. SARS-CoV-2, classified under the subgenus Sarbecovirus, subfamily Orthocoronavirinae, is a group 2B coronavirus. The immune-dominant spike (S) protein, envelope (E) protein, nucleocapsid (N) protein, and membrane (M) protein constitute the four structural proteins of CoVs. The transmembrane spike glycoprotein, homotrimers projecting from the viral surface of coronavirus, mediates its entry into the host cells. S consists of two functional subunits: S_1 subunit is involved in binding to the host cell receptor, whereas the S_2 subunit is responsible for the fusion of viral and host cell membranes. In several CoVs, S cleaves at the border connecting the S_1 and S_2 subunits which have noncovalent bonding in prefusion conformation.[3] The distal S_1 subunit besides containing the receptor-binding domain (s), also provides the stabilization to membrane-anchored S_2 subunit in prefusion state.[4] Host proteases further cleave the S at S_1 cleavage site that leads to exhaustive irreversible conformational modifications responsible for the activation of protein associated with membrane fusion.[3,5] Thus, the entry of coronavirus into susceptible host cell is mediated through a complex process involving the binding to receptor and proteolytic processing of S protein. Coronavirus S glycoprotein, being surface exposed, is the primary target for the neutralizing antibodies and also the central point for therapeutic and vaccine research.[6] Distinct domains inside S_1 subunit are utilized by various CoVs for the identification of different attachment and entry receptors. S domain A (S^A) attaches to 5-N-acetyl-9-O-acetyl-sialosides of host cell surface to gain entry in endemic human CoVs OC43 and HKU1.[6] In MERS-CoV, S^A is utilized for the identification of nonacetylated sialoside attachment receptors that may further promote the binding of S^B to dipeptidyl peptidase 4 entry receptor.[7] S^B connects directly with angiotensin-converting enzyme 2 (ACE2) for entry in target cells in SARS-CoV and several SARS-related CoVs. ACE2 is also the functional receptor for SARS-CoV-2 that mediates its entry into host cell via SARS-CoV-2 S. The analogous affinity of SARS-CoV-2 S^B and SARS-CoV S^B for human ACE2, partly explains the similar efficient transmission in humans of SARS-CoV-2 as that of SARS-CoV.[3]

Diagnostic Strategies for SARS-CoV-2: A Review of Available Approaches

Nucleic acid amplification tests

Table 1 depicts the various diagnostic techniques available for COVID-19 and Table 2 shows the interpretation of various microbiological test results in COVID-19. The primary challenges for nucleic acid amplification tests (NAATs) are (i) to minimize the false negatives by efficiently detecting the small number of viral RNA copies in the sample, (ii) to reduce the false positives by effectively differentiating the positive signals emitted by other pathogens, and (iii) to have the ability to test a large number of samples accurately in limited time.

SARS-CoV-2 is an enveloped virus containing positive-sense RNA as its genetic material. The genome of SARS-CoV-2 consists of approximately 30,000 nucleotides and 15 genes. Many of these genes, such as spike (S), nucleocapside (N), envelop (E), RNA-dependent RNA polymerase (RdRp), helicase (Hel), nonstructural protein 10 (nsp10), and nonstructural protein 14 (nsp14), have been utilized as probes or primer targets in the diagnostic NAATs. Initial researches demonstrated that by targeting the S gene, SARS-CoV-2 could be differentiated from SARS-CoV-1 with good specificity but low sensitivity.[2] In order to improve the sensitivity, additional viral-specific genes, such as RdRp/Hel, were integrated.[3] E and RdRp primers were considered to be most sensitive and were extensively used all over Europe, though RdRp primers were found to be cross reactive to SARS-CoV RNA.[2,3] The WHO recommends the usage of E, N, S, and RdRp genes in various combinations for optimum diagnosis.[1,8] Similar to other RNA viruses, SARS-CoV-2 also has a tendency to mutate. However, the proofreading property of Nsp14 restricts the nucleotide misincorporation rate.[2] Variation in nucleotide sequence may lead to diminished recognition by distinct primer-probe set. The use of minimum two molecular targets, ideally at least one targeting the conserved/specific region, would help to mitigate the effects of a potential SARS-CoV-2 genetic drift or its cross-reaction with any other circulating coronavirus.

Real-time reverse transcription-polymerase chain reaction

Real-time reverse transcription-polymerase chain reaction (rRT-PCR) remains the validated assay for prompt diagnosis in suspected SARS-CoV-2 infection. Amplification and analysis being carried out together in a closed system, the chances of false positives in these assays are minimized. The approximate turnaround time (TAT) is 4–5 h. The high diagnostic accuracy of rRT-PCR along with the capacity to test up to ninety specimens in a single run makes it the frontline test for diagnosis of COVID-19. The need of trained workforce, specialized laboratory equipment, and specific biosafety requirements, limits its use at peripheral centers in developing countries like India.

Loop-mediated isothermal amplification is another alternative to RT-PCR which uses amplification under isothermal condition with more rapidity and specificity.

With the surge in cases of COVID-19 in India and over 150 countries across the globe, a rapid point-of-care assay is considered to be a major tool toward the containment of cases. To reduce the TAT of NAATs, many rapid platforms of microarray or sequencing solutions on multi-RT-PCR panels with automation are being developed. Though initially intended to be used for the diagnosis of tuberculosis and other infectious diseases, TrueNat™ and cartridge-based nucleic acid amplification test (CBNAAT) are now widely deployed for the detection of COVID-19 cases. The real-time micro-PCR system is achieved through a combination of

Table 1: Diagnostic techniques for COVID-19

Technique	Time needed	Working principle	Advantage	Limitation
RT-PCR	3-4 h	Specific primer-probe based detection	High sensitivity (only small amount of RNA needed) Well-established methodology	High costs Detection is also complex and time-consuming Large number of samples tested in each run
TrueNat and CBNAAT	1 h	Specific primer-probe based detection	Fast results Closed system with lesser chances of contamination Minimal biosafety concerns	High costs Limited number of samples tested in each run
LAMP	1 h	More than two sets of specific primers Pair-based detection	Highly accurate and repeatable Single working temperature	Too sensitive, highly prone to false positives due to cross-contamination or carry-over
NGS	1-2 days	Whole-genome sequencing	High sensitivity and specificity Ability to identify novel strains Provides detailed information	Need of highly trained experts Highly sophisticated equipment required High cost
Serology (traditional)	4-6 h	IgG/IgM	Identify exposure	Limited sensitivity at an early stage
Rapid serological	15-30 min	IgG/IgM	Point-of-care test	Limited sensitivity at an early stage
Rapid antigen test	30 min	Antigen detection	Point-of-care test High specificity	Limited sensitivity
CT scan	1 h	Chest images	Increased sensitivity if findings combined with RT-PCR results	Not able to distinguish from other viral pneumonia
Virus isolation	5–15 days	*In vitro* live virus isolation and propagation	Highly (100%) specific Gold standard	Low sensitivity as isolation is not 100% Biosafety concerns

NGS: Next-generation sequencing, CT: Computed tomography, LAMP: Loop-mediated isothermal amplification, CBNAAT: Cartridge-based nucleic acid amplification test, RT-PCR: Real-time reverse transcriptase-polymerase chain reaction, IgM: Immunoglobulin M, IgG: Immunoglobulin G

Table 2: Interpretation of various microbiological test results in COVID-19

	Acute infection	Recent infection	Late-onset infection	Old infection	Absence of infection
Clinical symptoms	Variable	Positive	Positive	Negative	Negative
NAATs	Positive	Negative	Negative	Negative	Negative
IgM	Variable	Positive, may be negative	Positive	Negative	Negative
IgG	Variable	Positive	Positive	Variable	Negative

NAATs: Nucleic acid amplification test, IgM: Immunoglobulin M, IgG: Immunoglobulin G

the cartridge-based RNA extraction system and real-time micro-PCR analyzer. These are cartridge-based closed NAAT systems that can be performed with minimal hands-on training. CBNAAT (GeneXpert® SARS-CoV-2 test Cepheid®) is a simple, highly performing test with a short TAT. TrueNat is a chip-based RT-PCR test for the semi-quantitative detection of beta-coronavirus and SARS coronavirus RNA. The target sequence is the E gene of Sarbecovirus for beta-coronavirus and Rdrp gene for SARS coronavirus. As the viral lysis buffer used in the processing inactivates the virus, these techniques impose a minimal biosafety hazard. The TAT for these platforms is approximately 1 h. However, only 1–4 samples can be processed in a single run, limiting the samples that can be tested in a single day to 24–48. These systems have the capability of being utilized at grass-root levels.

Cycle threshold value

Real time-PCR assay measures the viral RNA in terms of cycle threshold (Ct), which is the number of cycles the fluorescent signal requires to become detectable and is inversely proportional to the viral load. The results are interpreted based on the Ct values and value <40 are generally considered positive. In RT-PCR, false-negative result may occur due to sampling error or incorrect timing of sampling.

The overall limit of detection for NAATs ranges from 100 to 1000 copies. Thus, these tests have much desired high analytical sensitivity along with very high specificity.[11]

Choosing the Right Specimens

Selection of an appropriate specimen is very crucial for the correct diagnosis of infected COVID-19 patients. COVID-19 patients typically shed high load of culturable virus starting from about 5–6 days of becoming symptomatic though viral RNA remains detectable in the respiratory samples for longer. Severely ill patients can continue to shed the virus for weeks to months. Fecal shedding contributing to the spread of infection remains a concern too. Nasopharyngeal (NP) swab

collected by trained health-care workers (HCWs) constitutes a standard sample for RT-PCR as per the recommendation by the Centers for Disease Control and Prevention. The process of obtaining an NP swab is uncomfortable for the patient and may elicit coughing or sneezing. Hence, the use of adequate personal protective equipment and adherence to standard protocol for infection prevention and control is warranted by the HCW for this procedure. Operational difficulty in the collection of NP swabs has led to the assessment of other comparatively easily available alternative samples such as saliva, oropharyngeal swabs, nasal swabs, NP wash/aspirate, and mid-turbinate swabs. Oropharyngeal swabs are comparatively less sensitive than NP and nasal swabs.[10] Testing of simultaneously collected nasal and oropharyngeal swabs, either independently or together in a single aliquot, is an attractive alternative option to increase the chances of positivity. The collection and processing of saliva, though an appealing sample, possesses its own challenges. Nasal swabs have comparable sensitivity to NP swabs. Many studies have reported a comparable sensitivity of self-collected samples by patients with those collected by health-care personnel.[11,14] Any upper respiratory specimen, however, may miss early infection; when a repeat testing must be performed preferably on a lower respiratory specimen, as the main site of replication by then might possibly be the lower respiratory tract. Moreover, sputum and bronchoalveolar lavage (BAL) have shown higher sensitivity in comparison to upper respiratory samples, probably due to the presence of greater viral loads in these specimens.[15] Collecting different specimen types in highly clinically suspected cases will improve detection rate by reducing false negativity. However, due to these being invasive procedures with the enhanced associated risk of aerosol generation, collection of these samples is done in selective instances.[2] Shipping of all these specimens to the reference laboratory must be done following triple packaging system ensuring appropriate labeling and sealing of the samples as per the standard protocol.

A false-negative nucleic acid amplification test: Significance?

It is of paramount importance to understand that a negative RT-PCR test result does not rule out COVID-19. There are several factors influencing the positivity of a RT-PCR test result, some of which include: low viral load in case of an incubation period or convalescent stage; or primary replication of the virus at other sites in the body (lower respiratory tract). There have been negative RT-PCR test reports with upper respiratory tract specimens in cases with suggestive pulmonary computed tomography (CT) scan findings.[16] This viral tropism for the lower respiratory tract is probably due to the inconsistent distribution of ACE2 viral receptors throughout the respiratory tract.[2,17] A suboptimal sampling technique may also affect the RT-PCR test results. In the instances of high clinical suspicion, it is prudent to repeat testing, as the sensitivity of NP swab is below ideal. Furthermore, in a high prevalence milieu, researchers have shown a significant increase in the positivity of these tests.

A positive nucleic acid amplification test previously declared negative

The criterion most often applied for discontinuation of isolation is two negative RT-PCR test results at least 24 h apart. Nevertheless, some of such cases report positive again despite having two negative test results.[18] This may be due to the alterations in the shedding of viral RNA during convalescence. However, the prognosis of such cases seems to be good in the absence of an actual clinical or virological relapse.[2]

Does nucleic acid amplification test positivity measure infectiousness?

As the viral RNA can be demonstrated from the samples of patients during recovery, nucleic acid amplification tests are not very useful in monitoring the infectivity of COVID-19 cases.[19] Although the ability of virus present in the sample to grow in culture constitutes a better measure of infectivity, it is rarely practiced due to biosafety concerns.[20]

Quantitative nucleic acid amplification test: Any prognostic value?

Instances show the presence of high viral loads even among asymptomatic cases as determined by the real-time PCR Ct values. Therefore, the prognostic utility of viral load in isolation is limited.[21] Though some correlations have been revealed between the severity of the disease and viral load, the viral load determined by these assays in terms of Ct value should not be used for prognosis or monitoring treatment response.[22] In all probabilities, irrespective of the course of the disease, viral loads typically regress with time.[23,24] Lower Ct values indicate high viral load and hence can be suggestive of transmissibility.

Adjuncts to Molecular Diagnosis

Serological evidence of SARS-CoV-2 diagnosis

Immunological tests can either measure the antibodies produced during the host immune response to infection or the antigenic viral particles in the respiratory specimens. The techniques commonly used for the demonstration of SARS-CoV-2-specific antibodies are immunochromatographic tests, enzyme-linked immunosorbent assays (ELISA), neutralization assays, and chemiluminescent immunoassays. Serologic tests are less dependable than the NAATs for the detection of SARS-CoV-2. The prevalence of infection also plays a crucial role in determining the positive or negative predictive values of a given test. In a low-prevalence setting, a positive serologic test with limited specificity is more likely to be a false-positive test result. Concerns were raised regarding the cross-reactivity of antibodies against SARS-CoV-2 and various related or distant viral families. Other human CoVs (such as HKU1, OC43, 229E, and NL63) causing mild-to-moderate seasonal respiratory symptoms are antigenically closely related to SARS-CoV-2.[25] The chances of cross-reactions are furthermore plausible with SARS-CoV-1 or MERS-CoV. However, majority of the commercially available

serologic assays demonstrate a specificity above 98%. The target antigen used in a serological assay also influences the sensitivity and specificity of that assay. S protein, produced at a much advanced stage of COVID-19, has lower sensitivity but higher specificity (particularly with S1 subunit) in comparison to N protein targets.[26]

Serological evidence: The relevance?

Serologic tests are mostly used to determine the exposure to SARS-CoV-2 in the past. These tests may also prove useful in establishing the diagnosis of COVID-19 in cases with negative NAATs with high clinical suspicion. Though IgM and IgG antibodies have been demonstrated as early as 3–6 days following the onset of symptoms, the seroconversion has been reported to occur by 3 weeks in majority of cases.[27] IgM is generally the first class of antibody to be produced in any infection followed by IgG immunity. IgM can be detected from the 2nd week, with the titers touching the peak in the 3rd week from the onset of symptoms and then slowly declining over time. IgG is reported to stabilize around 4 weeks.[28] However, in COVID-19, it is believed that IgM may be short lasting and IgG response may occur earlier than usual, and how long this IgG lasts is yet not known. IgM and IgG against SARS-CoV-2 can be determined qualitatively by immunochromatography assays and quantitatively by ELISA. Detection rates improve with the progression of illness.[29] A recent study has reported a higher accuracy of IgM and IgG ELISA in comparison to lateral flow assays.[30]

Though the antibody responses are demonstrable in majority of the COVID-19 cases, seroconversion may not be observed in immunocompromised patients or in a few with asymptomatic/very mild infections. The presence of specific antibodies against SARS-CoV-2 is most probably linked with some level of protection, though cutoff values of these antibodies are yet not established.[31] Neutralizing antibodies are generally considered more directly connected with the protective immunity. Furthermore, the production of neutralizing antibodies is complemented with T-cell responses. Low titers of antibodies are not considered protective and high titers are often encountered in severe COVID-19.[3] The recovery of mild cases even with low antibody levels and persistence of the disease in the presence of high antibody titers in severe cases raise queries about the role of neutralizing antibodies in providing immunity. Therefore probably, the therapeutic benefit of convalescent plasma has been attributed to other components by some researchers.[32]

Several lateral flow assays have been developed to detect the antigens of COVID-19 as point-of-care platform. The widely used antigen detection tests have a moderate sensitivity (ranging from 50.6% to 84%) with a high specificity (99.3% to 100%). The rapid chromatographic immunoassay may aid in the qualitative determination of SARS-CoV-2-specific antigens. The positive test results by antigen detection test can be regarded as true positives. However, the negative test results in symptomatic cases need further confirmation by real-time PCR test.[33] Although these assays have the theoretical advantage of being rapid and low cost, the viral load of the patient and the variability in specimen collection could result into lower sensitivities of these tests, early in infection.

Due to the kinetics of antibody formation or variabilities in the sensitivities of these assays, clinical decision-making should not be solely relied upon these tests unless strong evidence exists. Though impractical in early stage, antibody detection tests may be used for retrospective evaluation and epidemiological surveillance in terms of the burden of infection, significance of asymptomatic infections, basic reproduction number of the virus, or the overall mortality.

Computed tomography scan

CT scan is often regarded as an important auxiliary investigation for COVID-19. The researchers from Wuhan have reported a considerably higher sensitivity of CT scan in comparison to PCR tests in COVID-19 cases.[34] CT scan plays a pivotal role in the early diagnosis and timely management of COVID-19 cases. The characteristic features of COVID-19 infection comprise bilateral multi-lobar ground-glass opacities with differential distribution, subpleural ascendance, thickened lobular septa with inconsistent alveolar filling, and amalgation.[34,35] However, these findings of CT scan are suggestive and not confirmatory for COVID-19 diagnosis.

Viral culture

Though viral culture is the gold standard for the isolation and characterization of the virus, it is not used for the diagnosis of COVID-19 due to the process being labor intensive and also due to the requirement of biosafety level 3 facility with skilled workforce. Vero, Huh, and human airway epithelial cells lines have been used by several researchers to observe the cytopathic effects, which are confirmed by RT-PCR.[36-38]

Biomarkers

Several biomarkers are routinely used in clinical practice for their possible predictive role in the assessment of disease progression. They are crucial in identifying the cases at higher risk of developing complications. In addition, these also help in deciding the treatment protocols in COVID-19 cases. Figure 1 shows the commonly used biomarkers in COVID-19 patients.

Figure 1: Biomarkers with predictive role in COVID-19 progression

Diagnosis of SARS-CoV-2: Challenges and Limitations!

Despite an upstanding accomplishment of validated NAATs, there are some inherent challenges. NAATs carry the risk of false-negative results due to several pre-analytical factors that can influence the end results such as the inappropriate timing of collection of specimen (too early or too late in the course of illness); poor-quality specimen; type of sample (lower respiratory tract specimens, such as BAL and induced sputum, have better sensitivity than upper respiratory tract specimens such as NP and oropharyngeal swabs); and lapse in sample transportation (unsuitable container, inappropriate viral transport medium, or inadequate maintenance of cold chain, etc.).[31] Serological assays have a low sensitivity in the early course of COVID-19. The clinical utility of serodiagnosis is confined to the convalescent patients with negative molecular test results. In addition, researchers have documented the ongoing evolution of SARS-CoV-2 genome through active mutations and genetic recombination.[40,41] Being RNA virus, SARS-CoV-2 is also deficient in effective proofreading machinery needed to secure the RNA replication fidelity.[42] Mutations may change the sequence of primers hybridizing regions, thus yielding false-negative results.[41,42] However, this issue can be addressed by targeting more than one (two or three) sequence in viral genome.

What Do We Need to Remember?

- The complex scenario of the ongoing and rapidly evolving COVID-19 pandemic warrants the concerted use of various available modalities and their interpretation in relation to the clinical milieu of individual case
- The diagnosis not only needs to be timely and accurate, but should also contribute toward providing relevant epidemiological information so as to assess the actual burden and spread of the disease. In this context, the serological assays can complement molecular diagnosis, especially among those who are still asymptomatic and not hospitalized or may be used as screening assays as adjunct to diagnosis, though NAAT continues to remain the reference standard for COVID-19 diagnosis
- Monitoring the viral load along with being observant of the technique and timing of sample collection will help better interpret different stages of the disease. The type of sample to be collected, whether upper or lower respiratory, will further reduce the false negativity
- Clinical corroboration completed by serological evidence will help to reach a prompt management decision.
- The patients' microbiota and the immune system will finally contribute to the varied clinical manifestations present and the prognosis. Hence, continuous addition to the existing knowledge needs to be made as further studies get published.

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Conflicts of interest

There are no conflicts of interest.

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Correlation between 3790 qPCR positives samples and positive cell cultures including 1941 SARS-CoV-2 isolates

Rita Jaafar[a,b], Sarah Aherfi[a,b], Nathalie Wurtz[a,b], Clio Grimaldier[a,b], Van Thuan Hoang[a,c,d], Philippe Colson[a,b], Didier Raoult[a,b], Bernard La Scola[a,b,*]

[a]IHU-Méditerranée Infection, Marseille, France.

[b]Aix Marseille Univ, IRD, APHM, MEPHI, Marseille, France.

[c]Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France.

[d]Thai Binh University of Medicine and Pharmacy, Thai Binh, Viet Nam

*Corresponding author:

Bernard La Scola bernard.la-scola@univ-amu.fr

Dear Editor -

Since the beginning of the outbreak of the emerging epidemic (Covid-19) due to SARS-CoV-2, declared as a pandemic on March 12th 2020 by the WHO [1], a major issue has been to correlate viral RNA load obtained after RT-PCR and expressed in cycle threshold (Ct) with contagiousness and therefore duration of eviction from contacts and discharge from specialized infectious disease wards. Several works published recently and based on more than 100 studies attempt to propose such cut off for Ct value and duration of eviction with a consensus at approximately Ct > 30 and at least 10 days, respectively [2–5]. However, in an article published in this journal, a group reported that patients could be not be contagious above 25 Ct as the virus was not detected in culture above this Ct [6]. This limit was then evoked in the French media during the interview with the member of the French Scientific Council Covid-19 as a possible value above which patients are no longer contagious [7]. At the beginning of the outbreak, we correlated the Ct values obtained by our PCR technique based on the amplification of the E gene and the results of the culture [8]. Since the beginning of the epidemic, we have performed in our institute 250,566 SARS-CoV-2 RT-PCR for 179,151 patients, of which 13,161 (7.3%) tested positive. Up to the end of May, 3 790 of these samples reported positives on naso-pharyngeal samples were inoculated and managed for culture as previously described [8]. Of these 3 790 inoculated samples, 1941 SARS-Cov-2 isolates could be obtained after the first inoculation or up to 2 blind subcultures. The correlation between the scanner values and the positivity of the culture allows us to observe that the image obtained with ten times more isolates than our preliminary work (1941 versus 129) does not change significantly (Figure 1). It can be observed that at Ct=25, up to 70% of patients remain positive in culture and that at Ct=30 this value drops to 20%. At Ct=35, the value we used to report positive result for PCR, less than 3% of culture are negative. Our Ct value of 35 initially based on the results obtained by RT-PCR on control negative samples in

our laboratory and initial results of cultures [8] is validated by the present work and is in correlation with what was proposed i.e. in Korea [9] or Taiwan [10]. We could observe that subcultures, especially the first one, allow increasing percentage of viral isolation on high Ct samples, confirming that these high Ct are mostly correlated with low viral loads. From our cohort, we now need to try to understand and define the duration and frequency of live virus shedding in patients on a case-by-case basis, in the rare cases where the PCR is positive beyond 10 days, often at a Ct above 30. In any cases, these rare cases should not impact public health decisions.

Acknowledgments.

Ethical approval : The protocol was approved by the ethical committee of the University Hospital Institute Méditerranée Infection (N°: 2020-01). All subjects provided informed consent in accordance with the Declaration of Helsinki.

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Conflict of Interest: D.R. reports grants from Hitachi High-Tech Corporation, outside the submitted work. The others authors declare no conflict of interest.

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Figure 1. Percentage of positive viral culture of SARS-CoV-2 PCR-positive nasopharyngeal samples from Covid-19 patients, according to Ct value (plain line). The dashed curve indicates the polynomial regression curve.

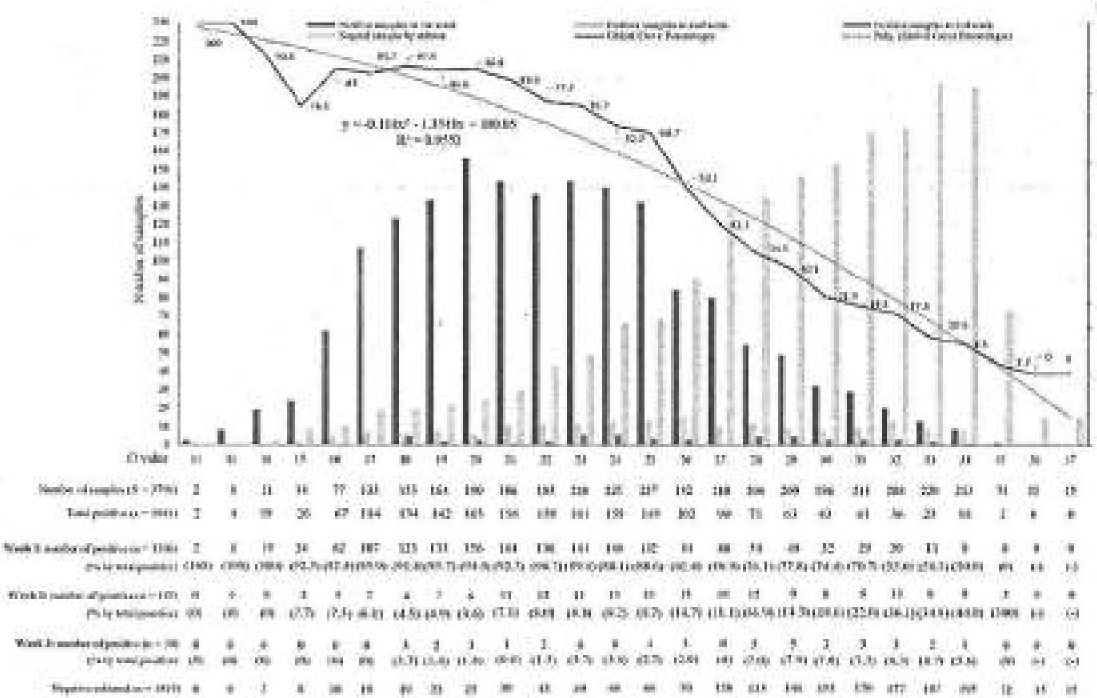

Figure 1

Predicting Infectious SARS-CoV-2 from diagnostic samples

Jared Bullard MD[1,2,3], Kerry Dust PhD[1], Duane Funk MD[4,5], James E. Strong MD,PhD[1,2,3,4], David Alexander PhD[1,3], Lauren Garnett BSc[3,4], Carl Boodman MD[1], Alexander Bello PhD[3,4], Adam Hedley BSc[1], Zachary Schiffman BSc[3,4], Kaylie Doan BSc[4], , Nathalie Bastien PhD[3,4], Yan Li PhD[3,4], Paul G. Van Caeseele MD [1,2,3] and Guillaume Poliquin MD, PhD[1,3,4]

1. Cadham Provincial Laboratory, Manitoba Health, Winnipeg, Manitoba, Canada

2. Department of Pediatrics & Child Health, University of Manitoba, Winnipeg, Manitoba, Canada

3. Department of Medical Microbiology & Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada

4. National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada

5. Departments of Anaesthesiology and Medicine, Section of Critical Care, University of Manitoba, Winnipeg, Manitoba, Canada

Corresponding Author:

Jared Bullard

750 William Avenue

Winnipeg, Manitoba, Canada

R3C 3Y1

Email: jared.bullard@gov.mb.ca

Phone: (204)945-1306 Fax: (204)786-4770

Summary: Respiratory samples from COVID-19 patients with ≥ 8 days of symptoms and a SARS-CoV-2 E gene RT-PCR Ct value ≥ 24 may predict lack of infectivity of those patients in a clinical and community context.

Abstract

Background: RT-PCR has become the primary method to diagnose viral diseases, including SARS-CoV-2. RT-PCR detects RNA, not infectious virus, thus its ability to determine duration of infectivity of patients is limited. Infectivity is a critical determinant in informing public health guidelines/interventions. Our goal was to determine the relationship between E gene SARS-CoV-2 RT-PCR cycle threshold (Ct) values from respiratory samples, symptom onset to test (STT) and infectivity in cell culture.

Methods: In this retrospective cross-sectional study, we took SARS-CoV-2 RT-PCR confirmed positive samples and determined their ability to infect Vero cell lines.

Results: Ninety RT-PCR SARS-CoV-2 positive samples were incubated on Vero cells. Twenty-six samples (28.9%) demonstrated viral growth. Median TCID50/ml was 1780 (282-8511). There was no growth in samples with a Ct > 24 or STT > 8 days. Multivariate logistic regression using positive viral culture as a binary predictor variable, STT and Ct demonstrated an odds ratio for positive viral culture of 0.64 (95% CI 0.49-0.84, p<0.001) for every one unit increase in Ct. Area under the receiver operating characteristic curve for Ct vs. positive culture was OR 0.91 (95% CI 0.85-0.97, p<0.001), with 97% specificity obtained at a Ct of >24.

Conclusions: SARS-CoV-2 Vero cell infectivity was only observed for RT-PCR Ct < 24 and STT < 8 days. Infectivity of patients with Ct >24 and duration of symptoms >8 days may be low. This information can inform public health policy and guide clinical, infection control and occupational health decisions. Further studies of larger size are needed.

Keywords: SARS-COV-2, COVID-19, RT-PCR, infectivity, public health

Introduction

The emergence of SARS-CoV-2, the causative agent of COVID-19, represents a public health emergency of historic proportion. The global containment efforts have had broad societal and economic impacts. Policy decisions to relax public health measures will require a better understanding of duration of infectivity. This information will also impact infection control practices and occupational health.

To date, the diagnosis of COVID-19 has relied on the detection of SARS-CoV-2 through molecular detection. While this method is both rapid and highly sensitive, there are important limitations. Several studies describe the persistence of SARS-CoV-2 RNA within different body sites (1,2). It is known from other viruses that viral RNA can persist beyond infectivity (3,4). As a result, demonstration of *in vitro* infectiousness on cell lines is a more informative surrogate of viral transmission. The ability of viral culture to inform infectivity is an important aspect of diagnostics but its use is hampered by its difficult and labour-intensive nature. This is further complicated by the need for containment level 3 facilities in the case of SARS-CoV-2. In a recent cohort study of nine patients, no virus could be recovered beyond 7 days post symptom onset (1). This important study is limited by the small number of patients examined and the fact that all nine cases are linked, therefore the data may represent a unique viral subpopulation. Here we add to the existing body of literature by presenting viral culture results on a larger cross-sectional group of patients, compared to PCR data and time of symptom onset.

Methods

SARS-CoV-2 RT PCR cycle threshold values and symptom onset to test

All samples in this study were obtained to support routine care and surveillance of the public health response in the province of Manitoba, Canada. All suspect COVID-19 cases had SARS-CoV-2 RT-PCR performed on nasopharyngeal (NP) or endotracheal (ETT) samples at Cadham Provincial Laboratory (CPL), the public health laboratory.

NP swabs and ETT specimens in viral transport media were stored at 4°C for 24-72 hours until they were tested for the presence of SARS-CoV-2 RNA using real-time RT-PCR targeting a 122nt portion of the Sarbecovirus envelope gene (E gene) (5). Fifty-five microliters of RNA were extracted from 200 µL of a respiratory specimen using the Ambion AM1836 RNA kit (Thermofisher) paired with the Kingfisher Flex instrument (Thermofisher). The 20 µL reactions, comprised of Taqman Fast Virus One-step master mix and 5 µL of RNA, were cycled for 5 min@ 50°C, 20 sec@ 95°C followed by 40 cycles of 5 sec@ 95°C and 30 sec @ 58°C on a Biorad CFX96 thermal cycler. RT-PCR results were analyzed with the CFX manager software (version 3.1).

Through public health and epidemiology/surveillance and laboratory records, date of symptom onset was determined. Time from symptom onset to RT-PCR, or symptoms to test (STT), was calculated based on laboratory records. For all positive samples, the cycle threshold (Ct) was obtained. The study was performed in accordance with protocol H523906 (H2020:211), approved by the University of Manitoba Research Ethics Board.

Tissue Culture Infectious Dose 50% (TCID50) Assay

Samples were stored at -80°C for between 2 to 4 weeks before being processed for culture. Viral titers of patient samples were determined through TCID50 assays inside a biocontainment level 4 laboratory (BSL4). Briefly, Vero cells (ATCC: CCL-81), maintained in Modified Eagles Medium (MEM) supplemented with 5% Fetal Bovine Serum (FBS), 1% penicillin/ streptomycin (P/S), 0.5 µg/mL Amphotericin B and 1% L-glutamine, were seeded into 96 well plates (Thermo Scientific, 167008) at 70% confluency. Using dilution blocks, patient samples were serially diluted 10-fold from 10^{-1} to 10^{-8} in MEM supplemented with 2% FBS, 1% Penicillin/Streptomycin, 0.5 µg/mL Amphotericin B and 1% L-glutamine. Dilutions were placed onto the Vero cells in triplicate and incubated at 37°C with 5% CO2 for 96 hours. Following incubation of 4 days, cytopathic effect (CPE) was evaluated under a microscope and recorded. TCID50 and TCID50/mL were calculated using the Reed and Muench method previously described (6)

Statistical Methods

Data are presented as mean ± standard deviation for normally distributed data and as median [interquartile range] for non-normally distributed data. P values are reported as two tailed. All statistical analysis was performed with Stata V14.2 (College Station, Texas, USA). Between group comparisons were performed using a Students t test or Mann-Whitney test. Normality was assessed using the Kolmorgorov-Smirnov test, and logistic regression was performed with robust standard errors.

Results

A total of 90 samples were analyzed. Median age of the patients sampled was 45 (30-59). Forty nine percent of our samples were from males. SARS-CoV-2 was successfully cultivated from 26 (28.9%) of the samples. The samples included in this study included those positive for SARS-CoV-2 by RT-PCR from day of symptom onset (Day 0) up to 21 days post symptom onset. Within this range of samples, positive cultures were only observed up to day 8 post symptom onset (Figure 1). Median Ct count of all samples was 23 (IQR 17-32). The median TCID50/ml was 1780 (282-8511). Positive culture samples had a significantly lower Ct when compared to culture negative samples (17 [16-18] vs 27 [22-33], p<0.001, Figure 2). Symptom to test time was also significantly lower in culture positive vs. culture negative samples (3 [2-4] vs. 7 [4-11], p<0.001, Figure 2).

Multivariate logistic regression using positive culture as a predictor variable (binary result) and STT, age and gender as independent variables showed Ct as being significant (OR 0.64 95% CI 0.49-0.84, p<0.001). This implies that for every one unit increase in Ct, the odds of a positive culture decreased by 32%. Increasing symptom to test time was also significantly associated with a negative culture (OR 0.63, 95% CI 0.42-0.94, p=0.025). For every one day increase in STT, the odds ratio of being culture positive was decreased by 37%. Receiver operating characteristic curves constructed using Ct vs. positive culture showed an area of 0.91 (95% CI 0.85-0.97, p<0.001) with 97% specificity obtained at a Ct of greater than 24. Similarly, STT vs. positive culture showed an area of 0.81 (95% CI 0.0.73-0.90, p<.001), with 96% specificity at >8 days. The probability of successfully cultivating SARS-CoV-2 on Vero cell culture compared to STT is demonstrated in Figure 3. The probability of obtaining a positive viral culture peaked on day 3 and decreased from that point.

Discussion

PCR and other nucleic amplification (NA) strategies have surpassed viral culture as the gold standard viral diagnostic, because of their wider application, higher sensitivity, rapid performance, and ability for field deployment. A major drawback to PCR and other diagnostic approaches (including other NA, serology, antigen detection) is that they all fail to determine virus infectivity: PCR sensitivity is excellent but specificity for detecting replicative virus is poor (13). Our study utilized a cross-sectional approach to correlate COVID-19 symptom onset to specimen collection with SARS-CoV-2 E gene RT-PCR and virus viability as determined by cell culture.

These results demonstrate that infectivity (as defined by growth in cell culture), is significantly reduced when RT-PCR Ct values are greater than 24. For every 1 unit increase in Ct, the odds ratio for infectivity decreased by 32%. The high specificity of Ct and STT suggests that Ct values greater than 24, along with duration of symptoms greater than 8 days may be used in combination to determine duration of infectivity in patients. Positive cell culture results in our study were most likely between days one and five. This finding is consistent with existing literature (1,2).

This study is the first to report a large enough data set that demonstrates a link between *in vitro* viral growth, Ct value and STT.

These results have implications for clinical care, infection prevention and control and public health. These data can be used to efficiently target case finding efforts by better defining the period of maximal transmission risk. This will be of particular importance in the maintenance phase of the response, where case finding efforts to rapidly interrupt chains of transmission will be essential. Isolation of COVID-19 cases in the community is typically recommended for at least ten days after symptom onset. Our data supports this approach. Jurisdictions across Canada and the US are recommending a variety of strategies to discontinue isolation of hospitalized COVID-19 cases (7-12). Clinical criteria including 14 days from symptom onset or 72 hours symptom free (whichever is

longer) are being used in some while other jurisdictions are using two negative NP RT-PCR results 48 hours apart after 14 days of symptoms. Our data supports the former approach since RT-PCR positivity persists significantly beyond infectivity; the alternative approach may lead to unnecessary isolation, and use of PPE and testing resources. The qualitative reporting of results of SARS-CoV-2 RT-PCR as positive or negative is sufficient for diagnosis but may be supplemented by Ct, a semi-quantitative value, as well as time of symptom onset to guide infection control, public health and occupational health decisions.

Our study has important limitations. First, our study utilized a single SARS-CoV-2 gene target (E gene). Though other gene targets may offer greater specificity, SARS-CoV-2 E-gene is more consistently used in both laboratory-developed tests (LDT) and commercial assays. The testing criteria in Manitoba had sufficient pre-test probability to make the likelihood of a false positive remote. In addition, the first 71 of 90 samples were confirmed using the described protocol with CDC N1-gene target (14). Second target confirmation was discontinued at that time based on being satisfied with testing criteria and assay sensitivity to accurately identify true COVID-19 cases. Reagent supply also played a role. Second, the recall bias of symptom onset is possible, but this likely would have been equally distributed between those who were culture positive and negative. Third, the infectivity of certain individual cases and the accuracy of our culture assay may have unique variations. Though some individuals in our cross-sectional study would be considered immunocompromised, patients with these conditions could have prolonged shedding of infective SARS-CoV-2 and may not be fully represented here. Few children have been diagnosed with COVID-19 in our province (Median age of positive PCR = 45 [30-59]). With other respiratory viruses, children may have prolonged shedding. Finally, our patient numbers remain small and larger studies are needed to establish Ct criteria that reliably correlates with loss of infectivity and that utilize additional SARS-CoV-2 gene targets.

In conclusion, the SARS-CoV-2/COVID-19 pandemic represents a dynamic situation where decisions and policy must be guided by evidence. Our study showed no positive viral cultures with a Ct greater than 24 or STT greater than 8 days. The odds of a positive culture were decreased by 32% for each unit increase in Ct. This data, if confirmed, may help guide isolation, contact tracing, and testing guidelines.

Acknowledgments

This work was supported by the collaborative efforts in the public health response to the SARS-CoV-2/COVID-19 pandemic by Manitoba Health and Cadham Provincial Laboratory (CPL) and the Public Health Agency of Canada and the National Microbiology Laboratory. A special acknowledgement to the Medical Laboratory Technologists in the Virus Detection Section of CPL. We would be blind without you.

Potential conflicts of interest: The authors have no conflicts to report.

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Figure Legends:

Figure 1: SARS-CoV-2 viral dynamics as expressed by E gene RT-PCR Cycle threshold (Ct) value and cell culture TCID50/mL, over time (days). Squares represent Ct values while triangles reflect TCID50.

Figure 2: SARS-CoV-2 E gene RT-PCR Cycle Threshold (Ct) values and symptom to test time (STT) in samples that were culture positive (Ct +, STT +), or negative (Ct -, STT -). Positive SARS-CoV-2 culture samples had a significantly lower Ct when compared to culture negative samples (17 [16-18] vs 27 [22-33], p<0.001). Symptom to test time was also significantly lower in culture positive vs. culture negative samples (3 [2-4] vs. 7 [4-11], p<0.001).

Figure 3: Comparison of symptom onset to test (days) to the probability of successful cultivation on Vero cells and SARS-CoV-2 E gene RT-PCR Cycle threshold (Ct) value. Ct values are represented by the line graph with circles. Probability of SARS-CoV-2 culture is shown by the bar graph.

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Figure 1

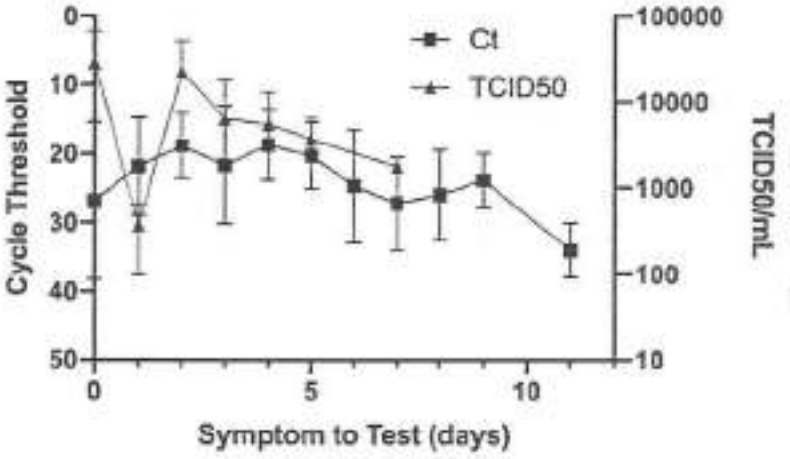
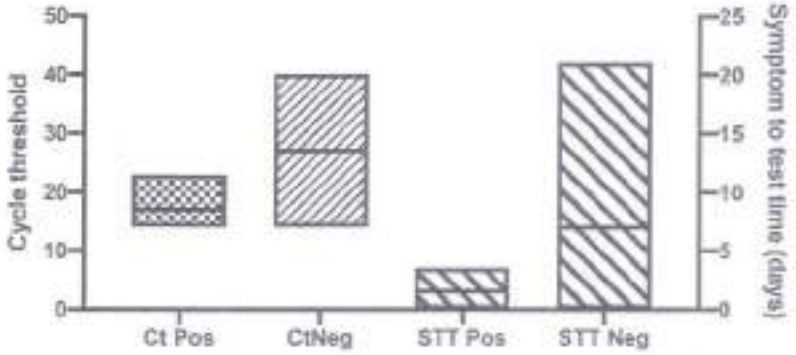


Figure 2



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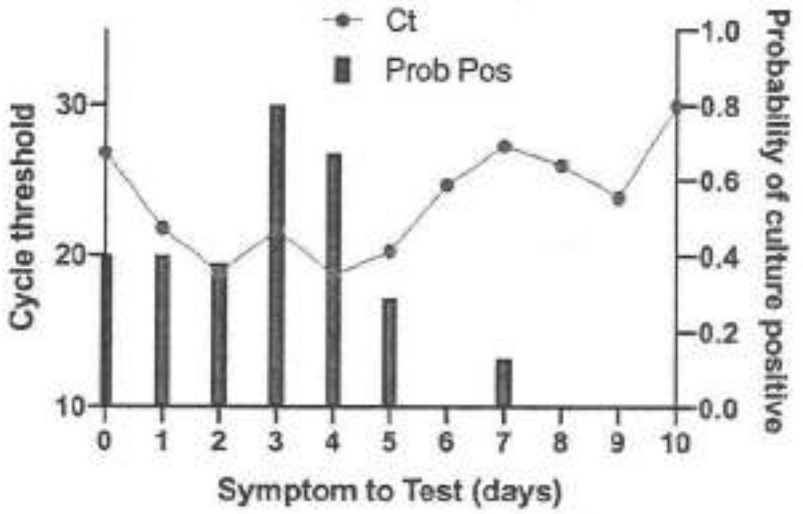

EXHIBIT "BB"

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Viral cultures for COVID-19 infectivity assessment – a systematic review (Update 4)

Jefferson T[1]; Spencer EA[1]; Brassey J[2]; Heneghan C[1].

Affiliations

1. **Nuffield Department of Primary Care Health Sciences, University of Oxford, Radcliffe Observatory Quarter, Oxford, OX2 6GG**
2. **Trip Database Ltd**

Keywords: Covid-19; mode of transmission, viral culture; symptom onset to test data; polymerase chain reaction; SARS-CoV-2; infectivity.

Joint corresponding authors:
Jefferson (tom-jefferson@contod.ox.ac.uk)
Heneghan (Carl.heneghan@phc.ox.ac.uk)

Summary

Objective to review the evidence from studies comparing SARS-CoV-2 culture, the best indicator of current infection and infectiousness with the results of reverse transcriptase polymerase chain reaction (RT-PCR).

Methods We searched LitCovid, medRxiv, Google Scholar and the WHO Covid-19 database for Covid-19 using the terms 'viral culture' or 'viral replication' and associated synonyms up to 18 September 2020. We carried out citation matching and included studies reporting attempts to culture or observe SARS-CoV-2 matching with cutoffs for RT-PCR positivity. One reviewer extracted data for each study and a second reviewer checked and edited the extraction and summarised the narratively by sample: fecal, respiratory, environment or mixed.
Where necessary we wrote to corresponding authors of the included or background papers for additional information. We assessed quality using a modified QUADAS 2 risk of bias tool.
This review is part of an Open Evidence Review on Transmission Dynamics of COVID-19. Summaries of the included studies and the protocol (v1) are available at: https://www.cebm.net/evidence-synthesis/transmission-dynamics-of-covid-19/ . Searches are updated every 2 weeks. This is the fourth version of this review that was first published on the 4th of August and updated on the 21t of August

Results We included 29 studies reporting culturing or observing tissue invasion by SARS-CoV in sputum, naso or oropharyngeal, urine, stool, blood and environmental samples from patients diagnosed with Covid-19. The data are suggestive of a relation between the time from collection of a specimen to test, cycle threshold and symptom severity. The quality of the studies was moderate with lack of standardised reporting.

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Twelve studies reported that Ct values were significantly lower and log copies higher in samples producing live virus culture. Five studies reported no growth in samples based on a Ct cut-off value. These values ranged from CT > 24 for no growth to Ct ≥ 34. Two studies report a strong relationship between Ct value and ability to recover infectious virus and that the odds of live virus culture reduced by 33% for every one unit increase in Ct. A cut-off RT-PCR Ct > 30 was associated with non-infectious samples. One study that analysed the NSP, N and E gene fragments of the PCR result reported different cut-off thresholds depending on the gene fragment analysed. The duration of RNA shedding detected by PCR was far longer compared to detection of live culture. Six out of eight studies reported RNA shedding for longer than 14 days. Yet, infectivity declines after day 8 even among cases with ongoing high viral loads. A very small proportion of people re-testing positive after hospital discharge or with high Ct are likely to be infectious.

Conclusion

Prospective routine testing of reference and culture specimens are necessary for each country involved in the pandemic to establish the usefulness and reliability of PCR for Covid-19 and its relation to patients' factors. Infectivity is related to the date of onset of symptoms and cycle threshold level.
A binary Yes/No approach to the interpretation RT-PCR unvalidated against viral culture will result in false positives with possible segregation of large numbers of people who are no longer infectious and hence not a threat to public health.

Introduction

The ability to make decisions on the prevention and management of COVID-19 infections rests on our capacity to identify those who are infected and infectious. In the absence of predictive clinical signs or symptoms[1], the most widely used means of detection is molecular testing using Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR)[2,3].

The test amplifies genomic sequences identified in samples. As it is capable of generating observable signals from small samples, it is very sensitive. Amplification of genomic sequence is measured in cycle thresholds (Ct). There appears to be a correlation between Ct values from respiratory samples, symptom onset to test (STT) date and positive viral culture. The lower the Ct value and the shorter the STT, the higher the infectivity potential[4].

Whether probing for sequences or whole genomes[5], in the diagnosis of Covid-19 a positive RT-qPCR cannot tell you whether the person is infectious or when the infection began, nor the provenance of the genetic material. Very early in the COVID-19 outbreak it was recognised that cycle threshold values may be a proxy for quantitative measure of viral load, but correlation with clinical progress and transmissibility was not yet known[4]. A positive result indicates that a person has come into contact with the genomic sequence or some other viral antigen at some time in the past. However, presence of viral genome on its own is not sufficient proof of infectivity and caution is needed when evaluating the infectivity of specimens simply based on the detection of viral nucleic acids[6]. In addition, viral genomic material can be still be present weeks after infectious viral clearance.[7] Like all tests, RT-qPCR requires validation against a gold standard. In this case isolation of a whole virion (as opposed to fragments) and proof that the isolate is capable of replicating its progeny in culture cells is the closest we are likely to get to a gold standard.[8] The inability of PCR to distinguish between the shedding of live virus or of viral debris, means that it cannot measure a person's viral load (or quantity of virus present in a person's excreta).

Our Open Evidence Review of transmission modalities of SARS CoV-2 identified a low number of studies which have attempted viral culture. There are objective difficulties in doing such cultures such as the requirement for a level III laboratory, avoidance of contamination, time and the quality of the specimens as well as financial availability of reagents and culture media to rule out the presence of other pathogens.

As viral culture represents the best indicator of infection and infectiousness, we set out to review the evidence on viral culture compared to PCR, and report the results of those studies attempting viral culture regardless of source (specimen type) of the sample tested.

Methods

We searched four main databases: LitCovid, medRxiv, Google Scholar and the WHO Covid-19 database for Covid-19 using the terms 'viral culture' or 'viral replication' and associated synonyms. Searches were last updated on 10 September 2020. Searches are conducted on a per calendar month basis and for databases which do not support such date granularity, the date of publication is approximated. For articles that looked particularly relevant, citation matching was undertaken and relevant results were identified.

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101 We included studies reporting attempts to culture SARS-CoV-2 and those which also estimated the
102 infectiousness of the isolates or observed tissue invasion by SARS CoV-2. One reviewer extracted data for
103 each study and a second review checked and edited the extraction. We tabulated the data and summarised
104 data narratively by mode of sample: fecal, respiratory, environment or mixed.

105 Where necessary we wrote to corresponding authors of the included or background papers for additional
106 information. We assessed quality using a modified QUADAS 2 risk of bias tool. We simplified the tool as the
107 included studies were not designed as primary diagnostic accuracy studies.[9]
108 This review is part of an Open Evidence Review on Transmission Dynamics of COVID-19. Summaries of the
109 included studies and the protocol (v1) are available at: https://www.cebm.net/evidence-
110 synthesis/transmission-dynamics-of-covid-19/ . Searches are updated every 2 weeks.
111
112 This is the fourth update of this review with the addition of four studies identified in the two weeks since the
113 last update.
114
115 **Results**
116 We identified 145 articles of possible interest and after screening full texts included 29 (see PRISMA[10] flow
117 chart - Figure 1). We identified one unpublished study which was not included as no permission to do so was
118 given by the authors. The salient characteristics of each included study are shown in Table 1.
119 All included studies were case series of **moderate quality** (Table 2. Quality of included studies). We could
120 not identify a protocol for any of the studies. All the included studies had been either published or were
121 available as preprints. All had been made public in 2020. We received five responses from authors regarding
122 clarifying information (see Acknowledgments).
123
124 **Studies using fecal samples**
125 Nine studies assessed viral viability from fecal samples which were positive for SARS-CoV-2 based on RT-
126 PCR result [11-13, 16-19]. One study reported infecting ferrets with stool supernatant[11], two reported visual growth
127 in tissue[12, 20] and five reported achieving viral replication[13-16]. One laboratory study[27] found that SARS-CoV-2
128 infected human small intestinal organoids.
129
130 **Studies using respiratory samples**
131 Sixteen studies on respiratory samples reported achieving viral isolation[4, 22, 11, 23, 24, 14, 15, 16, 25, 26, 15, 20, 21] . One
132 study assessed 90 nasopharyngeal samples and cultured 26 of the samples, and positive cultures were only
133 observed up to day eight post symptom onset; [4] another study obtained 31 cultures from 46 nasopharyngeal
134 and oropharyngeal samples; [22] while 183 nasopharyngeal and sputum samples produced 124 cases in
135 which a cytopathic effect was observed although the denominator of samples taken was unclear [22]. Another
136 study in health care workers in UK hospitals isolated one SARS Cov-2 from nineteen specimens in a
137 situation of low viral circulation.[27]
138

Two more studies reported a clear correlation between symptoms onset, date of sampling, Ct and likelihood of viral culture. [29,30]

L'Huillier and colleagues[29] sampled nasopharyngeal swabs in 638 patients aged less than 16 years in a Geneva Hospital: 23 (3.6%) tested positive for SARS CoV-2 - median age of 12 years and 12 (52% were culture positive). The Ct was around 28 for the children whose samples grew viable viruses. Gniazdowski[29] probably assessed 161 nasopharyngeal specimens. A positive culture was associated with Ct values of 18.8 ± 3.4. Infectious viral shedding occurred in specimens (a Ct ≥ 23 yielded 8.5% of virus isolates).

Basile and colleagues [30] found a culture positivity rate of 24%, which was significantly more likely to be positive in ICU patients compared with other inpatients or outpatients.
A report by the Korean Centres for Disease Control failed to grow live viruses from 108 respiratory samples from "re-positives" i.e. people who had tested positive after previously testing negative[33]

Ladhani [31] and colleagues reported a successful culture rate of out 31 of 86 RT-PCR positive naso-pharyngeal samples from six nursing home in London.
The largest number of positive culture came from the La Scola group publications[37] with 1941 positive cultures from 3790 samples.

<u>Studies using environmental samples</u>

Two possible positive cultures were obtained from 95 environmental samples in one study that assessed the aerosol and surface transmission potential of SARS-CoV-2 [34]. Zhou and colleagues reported on samples taken from seven areas of a large London hospital. Despite apparent extensive air and surface contamination of the hospital environment, no infectious samples were grown[35]. For air samples, 2/31 (6.4%) were positive and 12/31 (39%) suspect for SARS-CoV-2 RNA but no virus was cultured. Similarly, 91 of 218 surface samples were suspect (42%) or 23 positive (11%) for SARS-CoV-2 RNA but no virus was cultured. The authors noted that a cut-off RT-PCR Ct > 30 was associated with non-infectious specimens.

Ahn and colleagues[36] failed to grow live virus from an unspecified number of air samples in isolation rooms of patients with severe Covid-19 but were able to grow virus from swabs of hand rails, and the external surfaces of intubation cannulae.

<u>Mixed sources</u>

Some of the studies labelled as mixed source samples are also reported in individual provenance breakdown in this text because of lack of clarity of the text.

Eight studies reported viral culture from mixed sources. Using 60 samples from 50 cases of Covid-19, viral culture was achieved from 12 oropharyngeal, nine nasopharyngeal and two sputum samples[1]. Jeong et al [11]

who reported isolation live virus from a stool sample also reported that from of an unreported number of nasopharyngeal, oropharyngeal, saliva, sputum and stool samples, one viral culture was achieved: ferrets inoculated with these samples became infected; SARS-CoV-2 was isolated from the nasal washes of the two urine-treated ferrets and one stool-treated ferret[11]. An unreported number of samples from saliva, nasal swabs, urine, blood and stool collected from nine Covid-19 patients produced positive cultures and a possible specimen stool culture[14]. One study showed that from nine nasopharyngeal, oropharyngeal, stool, serum and urine samples, all nine were culturable, including two from non-hospitalised Covid-19 patients[15].

Yao and colleagues cultured viable viral isolates from seven sputum samples, three stool samples and one nasopharyngeal sample of 11 patient aged 4 months to 71 years, indicating that the SARS-CoV-2 is capable of replicating in stool samples as well as sputum and the nasopharynx.[16] All samples had been taken within 5 days of symptom onset. The authors also report a relationship between viral load (copy thresholds) and cytopathic effect observed in infected culture cells.[17]

Kim and colleagues reported no viral growth from and unclear number of serum, usirne and stool samples despite collection very soon after admission[17]. Lu and colleagues also reported no viral growth, however their specimens were from 87 cases tested "re-positive".[18]

Young and colleagues [19] from Singapore had 21 positive cultures from 19 hospitalised patients in Singapore. No virus was isolated from samples with a Ct value >30, or when the sample was collected >14 days after symptoms onset. All positive cultures came from naso-pharyngeal samples, none of the 24 urine or 35 stool samples exhibited viral growth

Blood cultures

In one study by Andersson[20] et al 20 RT-PCR positive serum samples were selected at random from a Covid-19 sample bank, representing samples from 12 individual patients (four individuals were represented at two timepoints), collected at 3 to 20 days following onset of symptoms. None of the 20 serum samples produced a viral culture

Post mortem study

One study on alveolar samples from 88 elderly deceased gre iable virus from 6 out 6 different samples, in one case on day 26 from symptom onset. [39]

Duration of viral shedding

Nine studies report on the duration of viral shedding as assessed by PCR for SARS-CoV-2 RNA[4 11 28 13 14 15 19 28 40]. The minimum duration of RNA shedding detected by PCR was seven days reported in Bullard, the maximum duration of shedding was 35 days after symptom onset in Qian. Seven out of eight studies reported RNA shedding for longer than 14 days (see Table 3).

Young et al[19] reported that 91% of patients had ceased viral shedding by day 20 from symptom onset.

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220 Duration of live viral culture detection

221 The duration of live viral culture detection was much shorter than viral shedding. Wölfel et al [14] reported that
222 virus could not be isolated from samples taken after day 8 even among cases with ongoing high viral loads
223 of approximately 105 RNA copies/mL.

224

225 Bullard et al similarly reported that SARS-CoV-2 Vero cell infectivity of respiratory samples from SARS-CoV-
226 2 positive individuals was only observed for RT-PCR Ct < 24 and symptom onset to test of < 8 days[4].

227

228 Singanayagam and colleagues [25] reported the median duration of virus shedding as measured by viral
229 culture was 4 days (Inter Quartile Range: 1 to 8)[25].

230

231 The relationship between RT-PCR results and viral culture of SARS-CoV-2

232 Fifteen studies attempted to quantify the relationship between cycle threshold (Ct) and likelihood of culturing
233 live virus[4 5 12 32 13 15 14 16 25 26 27 29-31]. Table 4 shows that nine studies analysed the relationship between Ct
234 values and live viral culture[4 5 32 26 27 29 30 31 15] and three quantified the mean log copies of detected virus and
235 live culture[5 30 28]. All reported that Ct were significantly lower and log copies were significantly higher in those
236 with live virus culture. Five studies reported no growth in samples based on a Ct cut-off value[4 5 27, 15 31]. These
237 values for no growth ranged from CT > 24[4] to Ct ≥ 35[31].

238

239 Singanayagam et al [32] reported the estimated probability of recovery of virus from samples with Ct ≥ 35
240 was 8.3% (95% CI: 2.8%–18.4%). All donors above the Ct threshold of 35 (n=5) with live culture were
241 symptomatic.

242

243 The study in London nursing homes by Ladhani and colleagues found no correlation between Ct values with
244 presence or absence of symptoms in either residents or staff[31], although nearly 50%of both categories were
245 asymptomatic.

246

247 Huang and colleagues[5] analysed the NSP, N and E gene fragments of the PCR result, which reported
248 different cut-off thresholds depending on the gene fragment analysed[5]. No growth was found for the NSP 12
249 fragment at Ct > 31.47, whereas the value was higher for the N gene fragment at >35.2.

250

251 Bullard et al [4] reported a reduction in the odds ratio for culturing live virus of 0.64 for every one unit increase
252 in Ct (95%CI 0.49 to 0.84, p<0.001). Similar to Bullard and colleagues, Singanayagam [32] reported a strong
253 relationship between Ct value and ability to recover infectious virus: estimated OR of recovering infectious
254 virus decreased by 0.67 for each unit increase in Ct value (95% CI: 0.58–0.77). This value is very close to
255 that of other empirical studies (an increased Ct of 0.58 per day since symptoms started)[31].

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Young et al[19] reported no viral isolation from samples where the Ct value was >30, or when the sample was collected >14 days after symptoms onset.

Discussion

Society is attempting to interrupt transmission of SARS-CoV-2 by identifying and isolating those who are sick and those who are infectious. As there are no Covid-19-specific mass treatments or preventive measures, such a strategy relies on our capability to identify infected and infectious persons with a reasonable amount of certainty to avoid isolation of those who pose little threat to the public health. An increasing body of evidence shows that such identification cannot be accurately achieved through the simplistic division of those who test positive and who do not, on the basis of the results of RT-PCR. The sensitivity and specificity of RT-PCR needs comparing to the gold standard of infectiousness: the capacity to grow live virus from a specimen.

Some of the authors of the studies in our review have attempted and successfully achieved culture of SARS-CoV-2 in the laboratory, using a range of respiratory, fecal or environmentally collected samples. However the simplistic dichotomous division into positive/negative is insufficient to accurately identify infectiousness as detection of viral RNA cannot support an inference of contagiousness[40]. The evidence shows that there is a positive relationship between lower cycle count threshold, likelihood of positive viral culture[40] and date of symptom onset. Nowhere can this be seen as clearly as in the two studies assessing the infectiousness of "re-positives", i.e. those COVID-19 cases who had been discharged from hospital after testing negative repeatedly and then testing positive after discharge: Lu 2020[18], Korean CDC[39].

In a very tightly designed and argued study Lu and colleagues tested four hypotheses for the origin of "re-positives"[18]. After discarding the first two (re-infection and latency) on the basis of their evidence, they reached the conclusions that the most plausible explanations were either contamination of the sample by extraneous material or identification in the sample of minute and irrelevant particles of SARS-CoV-2 debris representing virus long neutralised by the immune system.

Both explanations fit the facts, the others do not. It is very likely that a huge expansion in testing capability requires training protocols and precautions to avoid poor laboratory practice which are simply not possible in the restricted times of a pandemic. We equally know that weak positives (those with high Ct) are unlikely to be infectious, as a whole live virus is the prime requirement for transmission, not the fragments identified by PCR.

The purpose of viral testing is to assess the relation of the micro-organism and hazard to humans, i.e. its clinical impact on the individual providing the sample for primary care and the risk of transmission to others for public health. PCR on its own is unable to provide such answers. When interpreting the results of RT-PCR it is important to take into consideration the clinical picture, the cycle threshold value, the number of days from symptom onset to test (STT) and the specimen donor's age [44,42,43]. Several of our included studies assessed the relationship of these variables and there appears to be a time window during which shedding is at its highest with low cycle threshold and higher possibility of culturing a live virus, with viral load and probability of growing live virus of SARS-CoV2 peaking much sooner than that of SARS CoV-1 or MERS-CoV[42]. We propose that further work should be done on this with the aim of constructing a calibrating

algorithm for PCR which are likely to detect infectious patients. PCR should be continuously calibrated against a reference culture in Vero cells in which cytopathic effect has been observed[4]. Confirmation of visual identification using methods, such as an immunofluorescence assay may also be relevant for some virus types[5]. Henderson and colleagues have called for a multicenter study of all currently manufactured SARS-CoV-2 nucleic acid amplification tests to correlate the cycle threshold values on each platform for patients who have positive and negative viral cultures. Calibration of assays could then be done to estimate virus viability from the cycle threshold with some certainty.[42]

Ascertainment of infectiousness is all the more important as there is good evidence of viral RNA persistence across a whole range of different viral RNA disease with little or no infectivity in the post infectious phase on MERS[46], measles [47], other coronoviridae[48], HCV and a variety of animal RNA viruses[49]. In one COVID-19 (former) case this persisted until day 78 from symptoms onset with a very high Ct [47] but no culture growth, showing its lack of infectiousness.

We are unsure whether SARS CoV-2 methods of cell culture have been standardised. Systems can vary depending upon the selection of the cell lines; the collection, transport, and handling of and the maintenance of viable and healthy inoculated cells[49]. We therefore recommend that standard methods for culture should be urgently developed and external quality assessment schemes be extended to to all laboratories offering testing for SARS CoV2.[50]. If identification of viral infectivity relies on visual inspection of cytopathogenic effect, then a reference culture of cells must also be developed to test recognition against infected cells. Viral culture may not be appropriate for routine daily results, but specialized laboratories should rely on their own ability to use viruses as controls, perform complete investigations when needed, and store representative clinical strains whenever possible[49]. In the absence of culture, ferret inoculation of specimen washings and antibody titres could also be used. It may be impossible to produce a universal Cycle threshold value as this may change with circumstances (e.g. hospital, community, cluster and symptom level), laboratory methods[51] and the current evidence base is thin.

We suggest the WHO produce a protocol to standardise the use and interpretation of PCR and routine use of culture or animal model to continuously calibrate PCR testing, coordinated by designated Biosafety Level III laboratory facilities with inward directional airflow[52]. Further studies with standardised methods[51] and reporting are needed to establish the magnitude and reliability of this association.

The results of our review are similar to those of the scoping review by Byrne and colleagues on infectivity periods[53] and those of the living review by Cevick and colleagues[12]. Although the inclusion criteria are narrower than ours, the authors reviewed 79 studies on the dynamics, load and shedding for SARS CoV-1, MERS and SARS CoV-2 from symptoms onset. They conclude that although SARS-CoV-2 RNA shedding in respiratory (up to 83 days) and stool (35 days) can be prolonged, duration of viable virus is relatively short-lived (up to a maximum of 8 days from symptoms onset). Results that are consistent with Bullard et al who found no growth in samples with a cycle threshold greater than 24 or when symptom onset was greater than

8 days, and Wölfel et al [14] who reported that virus could not be isolated from samples taken after day 8 even among cases with ongoing high viral loads.

The review by Rhee and colleagues also reaches conclusion similar to ours.[43]

The evidence is increasingly pointing to the probability of culturing live virus being related to the amount of viral RNA in the sample and, therefore, inversely related to the cycle threshold. Thus, blanket detection of viral RNA cannot be used to infer infectiousness. Length of excretion is also linked to age, male gender and possibly use of steroids and severity of illness.

The limits of our review are the low number of studies of relatively poor quality with lack of standardised reporting and lack of gold testing for each country involved in the pandemic. We plan to keep updating this review with emerging evidence.

Conclusion

The current data are suggestive of a relation between the time from collection of a specimen to test, copy threshold, and symptom severity, but the quality of the studies limits drawing firm conclusions. We recommend that a uniform international standard for reporting of comparative SARS-CoV-2 culture with index test studies be produced. Particular attention should be paid to the relationship between the results of testing, clinical conditions and the characteristics of the source patients, description of flow of specimens and testing methods. Extensive training of operators and avoidance of contamination should take place on the basis of fixed and internationally recognised protocols. Defining cut off levels predictive of infectivity should be feasible and necessary for diagnosing viral respiratory infections using molecular tests[54].

We will contact the corresponding authors of the 11 studies correlating Ct with likelihood of culture to assess whether it is possible to aggregate data and determine a firm correlation to aid decision making.

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377 **Data Availability**

378 All data included in the review are from publications or preprints. All extractions sheets with direct links to the
379 source paper are available from https://www.cebm.net/evidence-synthesis/transmission-dynamics-of-covid-
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382 **Authors:**

383 Tom Jefferson is a Senior Associate Tutor and Honorary Research Fellow, Centre for Evidence-Based
384 Medicine, University of Oxford. Disclosure statement is here

385

386 Elizabeth Spencer is Epidemiology and Evidence Synthesis Researcher at the Centre for Evidence-Based
387 Medicine. (Bio and disclosure statement here)

388

389 Jon Brassey is the Director of Trip Database Ltd, Lead for Knowledge Mobilisation at Public Health Wales
390 (NHS) and an Associate Editor at the BMJ Evidence-Based Medicine.

391 Carl Heneghan is Professor of Evidence-Based Medicine, Director of the Centre for Evidence-Based
392 Medicine and Director of Studies for the Evidence-Based Health Care Programme. (Full bio and disclosure
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540 **Figure 1 - PRISMA 2009 Flow Diagram**

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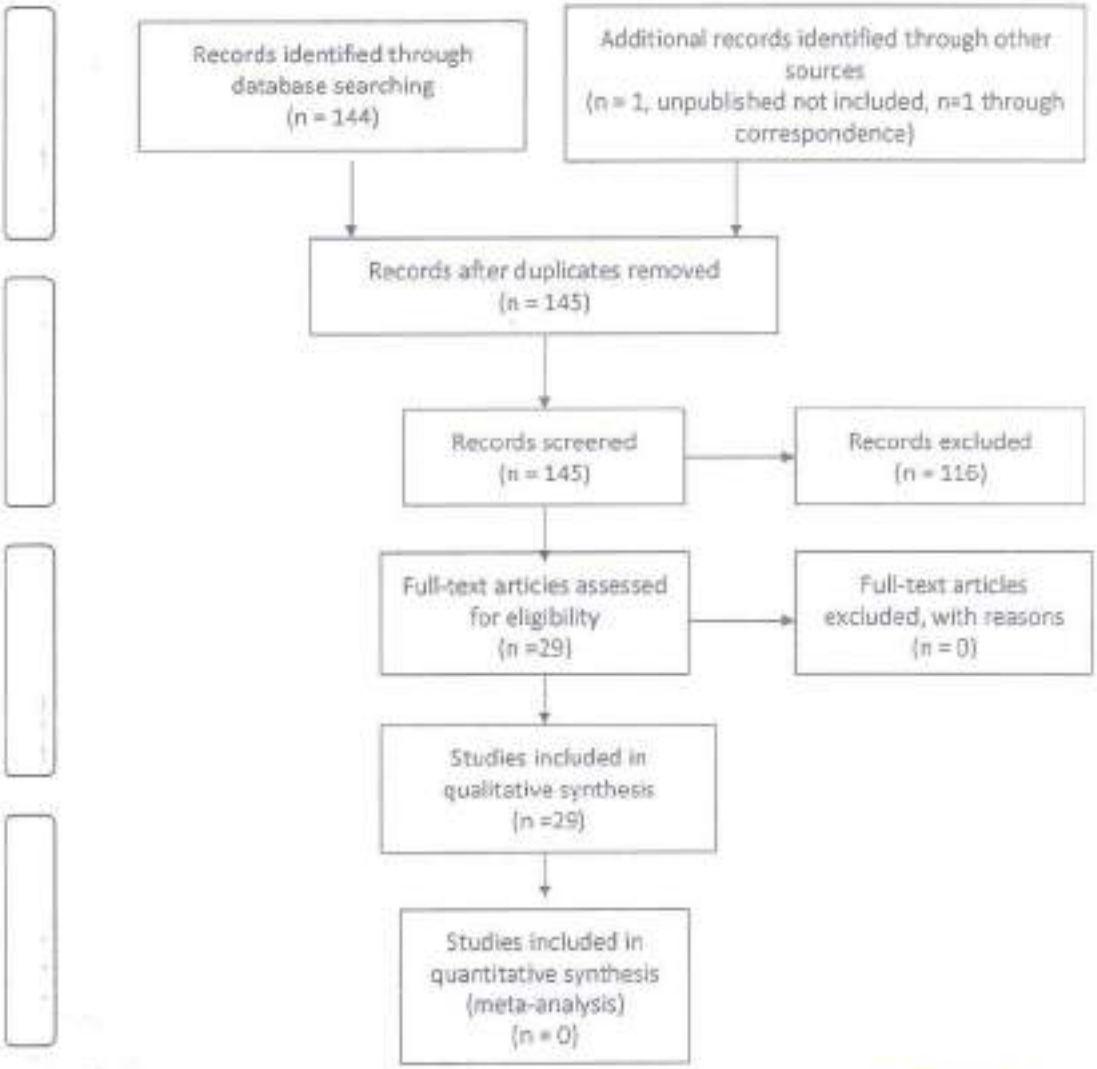

Serial	Study	Samples (source)	Samples (n) [SST]	Culture methods	Culture Positive	Additional notes
1.	Rulland[4]	Nasopharyngeal (NP) or endotracheal (ETT) from COVID-19 patients (mean age 45 years)	90 [0 to 7 days]	NP swabs and ETT specimens in viral transport media were stored at 4°C for 24-72 hours until they were tested for the presence of SARS-CoV-2 RNA using real-time RT-PCR targeting a 122nt portion of the Sarbecovirus envelope gene (E gene). Dilutions were placed onto the Vero cells in triplicate and incubated at 37°C with 5% CO2 for 96 hours. Following incubation of 4 days, cytopathic effect was evaluated under a microscope and recorded.	26	The range of symptoms onset to negative PCT was 21 days. Within this period, positive cultures were only observed up to day 8 post symptom onset
2.	Huang[3]	Oropharyngeal (OP) or nasopharyngeal (NP) swabs, or sputum (SP)	60 specimens from 50 cases [3,4 days mean but see table 1 for freeze thaw cycles delays]	SARS-CoV-2 cDNA was prepared using RNA extracted from the specimens of the first patient with confirmed COVID-19. RT was performed using the MMLV Reverse transcription kit. All procedures for viral culture were conducted in a biosafety level-3 facility. Vero-E6 and MK-2 (ATCC) cells were maintained in a virus culture medium and the cells were maintained in a 37°C incubator with daily observations of the cytopathic effect.	12 OP, 9 NP and two from SP specimens were culturable	Specimens with high copy numbers of the viral genome, indicative of higher viral load, were more likely to be culturable.
3.	Jeong[11]	Nasoloropharyngeal swabs, saliva, urine, and stool	5 patients	Specimens positive by qPCR were subjected to virus isolation in Vero cells. Urine and stool samples were inoculated intranasally in ferrets and they evaluated the virus titers in nasal washes on 2, 4, 6, and 8 days post-infection (dpi). Immunofluorescence antibody assays were also done.	Naso/ oropharyngeal saliva, urine and stool Samples were collected between days 8 to 30 of the clinical course. Viable SARS-CoV-2 was isolated from 1 naso / oropharyngeal swab. Ferrets inoculated	Viral loads in urine, saliva, and stool samples were almost equal to or higher than those in naso / oropharyngeal swabs. After symptom resolution, patients shed viable virus in their saliva and urine up to day 15 of illness.

					with patient urine or stool were infected. SARS-CoV-2 was isolated from the nasal washes of the 2 urine-treated ferrets and one stool-treated ferret	
4.	Qian[20]	Rectal tissue obtained from a surgical procedure was available.	1 [1 to 3 days post op]	Ultrathin sections of tissue fixed in epoxy resin on formvar-coated copper grids were observed under electron microscope under 200kV. Immunohistochemical staining was used to establish expression and distribution of SARS-CoV-2 antigen.	1	No culture performed. Visualisation of virions in rectal tissue and detection of SARS-CoV-2 antigen in the rectal tissue.
5.	Wang[17]	Bronchoalveolar fluid, sputum, feces, blood, and urine specimens from hospital in-patients with COVID-19	4 fecal samples with sufficiently high copy numbers from 1,070 specimens collected from 205 patients with COVID-19 (mean age of 44 years and 68% male [1 to 3 days from hospital admission)	rRT-PCR targeting the open reading frame 1ab gene of SARS-CoV-2; cycle threshold values of rRT-PCR were used as indicators of the copy number of SARS-CoV-2 RNA in specimens with lower cycle threshold values corresponding to higher viral copy numbers. A cycle threshold value less than 40 was interpreted as positive for SARS-CoV-2 RNA. Four SARS-CoV-2 positive fecal specimens with high copy numbers were cultured, and then electron microscopy was performed to detect live virus.	4 viewed by electron microscope	The details of how the 4 samples were cultured were not reported. The patients did not have diarrhoea.
6.	Xiao F, Sun J [18]	Serial feces samples collected from 26 hospitalised COVID-19 patients: 3 samples from 3 RNA-positive patients were tested for possible viral	3, one patient admitted day 7 post onset	Inoculation of Vero 6 cells. Cycle threshold values for the fecal sample were 23.34 for the open reading frame 1lab gene and 20.52 for the nucleoprotein gene. A cytopathic effect was visible in Vero E cells 2 days after a second-round passage. The researchers negatively stained culture supernatant and visualized	2/3 (infectious virus was present in faeces from two cases)	Selection of samples is not entirely clear.

		culture.		by transmission electron microscopy. Viral particles that were visible were spherical and had distinct surface spike protein projections, consistent with a previously published SARS-CoV2 image.		
7.	Arons [23]	nasopharyngeal and oropharyngeal swabs	46 rRT-PCR–positive specimens [For asymptomtic median 4 days, Ct 23.1]	All rRT-PCR positive samples shipped to USA CDC for viral culture using Vero-CCL-81 cells. Cells showing cytopathic effects were used for SARS-CoV-2 rRT-PCR to confirm isolation and viral growth in culture.	31 [no relation to symptoms presence. Culturable virus isolated from 6 days before to 9 days after symptom onset]	
8.	La Scola [32]	Naso pharyngeal swabs or sputum samples Only Naso pharyngeal samples from the subsequent Jaafar et al letter.	183 [4384 samples from 3466 patients) [not reported]	From 1,049 samples, 611 SARS-CoV-2 isolates were cultured. 183 samples testing positive by RT-PCR (9 sputum samples and 174 nasopharyngeal swabs) from 155 patients, were inoculated in cell cultures. SARS-CoV-2. RNA rtPCR targeted the E gene. Nasopharyngeal swab fluid or sputum sample were filtered and then inoculated in Vero E6 Cells. All samples were inoculated between 4 and 10 h after sampling and kept at + 4 °C before processing. After centrifugation they were incubated at 37 °C. They were observed daily for evidence of cytopathogenic effect. Two subcultures were performed weekly and scanned by electron microscope and then confirmed by specific RT-PCR targeting E gene.	Of the 183 samples inoculated in the studied period of time, 129 led to virus isolation. Of these 124 samples had detectable cytopathic effect between 24 and 96 h The letter by Jaafar et al adds that 1941 SARS-Cov-2 30 isolate cultures were positive out 3 790 inoculated samples. These could be seen after the first inoculation or up to 2 blind subcultures. At at Ct of ≥ 34 2.6% of samples yielded a	There was a significant relationship between Ct value and culture positivity rate: samples with Ct values of 13–17 all had positive culture. Culture positivity rate decreased progressively according to Ct values to 12% at 33 Ct. No culture was obtained from samples with Ct > 34. The 5 additional isolates obtained after blind subcultures had Ct between 27 and 34, thus consistent with low viable virus load.

					positive culture.	
9.	Santarpia[21]	Windowsill and air, mean 7.3 samples per room. The percentage of PCR positive samples from each room was 40% -100%	13 patients [days 5 to 9 and day 18 of isolation in a quarantine unit]	Vero E6 cells were used to culture virus from environmental samples. The cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with heat inactivated fetal bovine serum (10%), Penicillin/Streptomycin (10,000 IU/mL &10,000 µg/mL) and Amphotericin B (25 µg/mL).	Possibly 2 with weak cytopathic effect	Isolates were from days 5 and 6 of occupancy of hospital/isolation rooms
10.	Wölfel[24]	Saliva, nasal swabs, urine, blood and stool	9 patients [2 to 4 days]	The average virus RNA load was 6.76 × 105 copies per the whole swab until day 5, and the maximum load was 7.11 × 108 copies per swab. The last swab sample that tested positive was taken on day 28 after the onset of symptoms.	Yes in respiratory samples, and indicative in stool	
11.	Kuiawski [19] (for The COVID-19 Investigation Team)	Nasopharyngeal (NP), oropharyngeal (OP), stool, serum and urine specimens	9 from 9 patients	SARS-CoV-2 real-time PCR with reverse transcription (rRT–PCR) cycle threshold (Ct) values of virus isolated from the first tissue culture passage were 12.3 to 35.7 and for one patient, virus isolated from tissue culture passage 3 had a titer of 7.75 × 10^6 median tissue culture infectious dose per ml; these data were likely more reflective of growth in tissue culture than patient viral load.	9 (including two non- hospitalised)	Viable SARS-CoV-2 was cultured at day 9 of illness (patient 10), but was not attempted on later specimens. SARS-CoV-2 rRT–PCR Ct values of virus isolated from the first tissue culture passage were 12.3 to 35.7. Mean Ct values in positive specimens were 17.0 to 38.0 for NP, 22.3 to 39.7 for OP and 24.1 to 39.4 for stool. All blood and urine isolates were negative. Ct values of upper respiratory tract specimens were lower in the first week of illness than the second in most patients, low Ct values continued into the second and third week of

						illness.
12.	Zhang[36]	Stool	Unknown [not reported]	Vero cells were used for viral isolation from stool samples of Covid-19 patients. A 2019-nCoV strain was isolated from a stool specimen of a laboratory-confirmed COVID-19 severe pneumonia case, who experienced onset on January 16, 2020 and was sampled on February 1, 2020. The interval between sampling and onset was 15 days. The full-length genome sequence indicated that the virus had high-nucleotide similarity (99.98%) to that of the first isolated novel coronavirus isolated from Wuhan, China. In the Vero cells, viral particles with typical morphology of a coronavirus could be observed under the electron microscope.	1	We do not know what influenced successful virus culture e.g. methods optimal, or concentration of virus optimal. More information needed.
13.	Xiao F, Tang M[35]	Esophageal, gastric, duodenal, and rectal tissues were obtained from 1 COVID-19 patients by endoscopy.	1 plus an unknown additional number of fecal samples from RNA-positive patients. [not reported]	Histological staining (H&E) as well as viral receptor ACE2 and viral nucleocapsid staining were performed.	1/1 RNA-positive patient. Positive staining of viral nucleocapsid protein was visualized in the cytoplasm of gastric, duodenal, and rectum glandular epithelial cell, but not in esophageal epithelium of the 1 patient providing these tissues. Additionally, positive staining of ACE2 and SARS-CoV-2 was also observed in gastrointestinal epithelium from other patients who tested positive for SARS-CoV-2 RNA	Total sample numbers are not reported.

					in feces, results not shown.	
14.	Yao [16]	Sputum (n=7), stool (n=3) and one nasopharyngeal sample	11 patients admitted to hospital: 9 classified as serious or critical, 1 moderate, 1 mild symptoms [0 to 16 days]	The samples of the 11 patients involved in this study were collected during the early phase of the Covid-19 break out in China, dates ranging from 2nd of January to the 2nd of April 2020. All except one of the patients had moderate or worse symptoms. Three patients had co-morbidities and one patient needed ICU treatment. Seven patients had sputum samples, one nasopharyngeal and three had stool samples The samples were pre-processed by mixing with appropriate volume of MEM medium with 2% FBS, Amphotericin B, Penicillin G, Streptomycin and TPCK-trypsin. The supernatant was collected after centrifugation at 3000 rpm at room 434 temperature. Before infecting Vero-E6 cells, all collected supernatant was filtered using a 435 0.45 μm filter to remove cell debris etc. Vero-E6 cells were infected with 11 viral isolates and quantitatively assessed their viral load at 1, 2, 4, 8, 24, and 48 hours post-infection (PI) and their viral cytopathic effects (CPE) at 48 and 72 hours PI and examined whether the viral isolates could successfully bind to Vero-E6 243 cells as expected. Super-deep sequencing of the 11 viral isolates on the Novaseq 6000 platform was performed.	11 samples taken up to 16 days from admission to hospital.	Cultured viruses were inoculated in Vero cells. At 8 hours post-infection there was a significant decrease in Ct value (increases in viral load) for five isolates. At 24 hours significant decreases in the Ct values for all of the viral isolates were observed. Mutations of the viruses are also reported
15.	Singanayagam[20]	324 samples: nose,	293 positive	Vero E6 cells were inoculated with	133 (41%) samples	RT-PCR cycle threshold

		throat, combined nose-and throat and nasopharyngeal swabs and aspirates	case [-10 to 60 days]	clinical specimens and incubated at 37 °C, 5% CO_2. Cells were inspected for cytopathic effect daily up to 14 days. Presence of SARS-CoV-2 was confirmed by SARSCoV-2 nucleoprotein staining by enzyme immunoassay on infected cells.	(from 111 cases)	values correlate strongly with cultivable virus i.e. likelihood of infectiousness. Median Ct of all 324 samples was 31.15. Probability of culturing virus declines to 8% in samples with Ct > 35 and to 6% 10 days after onset and was similar in asymptomatic and symptomatic persons. Asymptomatic persons represent a source of transmissible virus but there is no difference in Ct values and culturability by age group.
16.	Perera [26]	68 specimens: nasopharyngeal aspirates combined with throat swab (n=49), nasopharyngeal aspirate (n=2), nasopharyngeal swab combined with throat swab (n=3), nasopharyngeal swab (n=2), sputum (n=11) and saliva (n=1).	35 patients, 32 with mild disease [1 to 67 days]	Specimens were tested for sgRNA with ≥5 log10 N gene copies per mL. The complementary DNA obtained was subjected to PCR (40 cycles). Vero E6 cells were seeded and incubated for 24 hours in a CO_2 incubator. The culture medium was removed and 125 µL of the clinical specimen in virus transport medium diluted and was inoculated into 2 wells. After 2 hours incubation in a CO_2 incubator at 37°C, the plates were incubated at 37°C in a CO_2 incubator. A sample (100 µL) of supernatant was sampled for a quantitative real-time RT-PCR at 0 and 72 hours post inoculation. At 72 hours, cells were scraped into the supernatant and transferred onto fresh cells in 24-well plates and monitored for an additional 72 hours. A final quota of cells was collected for quantitative real-time RT-PCR. Cells were observed for cytopathic effect daily and harvested for testing if 25%–50% of cells showed a	16/35 at a median 26 Ct	Culturable SARS CoV-2 and sub-genomic RNA (good indicator of replication) was rarely detectable beyond 8 days after onset of illness although virus RNA by RT-PCR remained for up to 70 days.

				cytopathic effect.		
17.	Brown[27]	Combined viral throat and nose swab from each participant n=1,152	Health care workers in six UK hospitals	Specimens were sent on the same day for detection of SARS-CoV-2 RNA by RT-PCR to the PHE national reference laboratory (five hospitals) or one hospital laboratory. The PHE laboratory used an Applied Biosystems 7500 FAST system targeting a conserved region of the SARS-CoV-2 open reading frame (ORF1ab) gene. The hospital laboratory used a different CE-IVD kit, targeting 3 SARS-CoV-2 genes (RdRp, E, and N). Both PCRs had internal controls. Viral culture of PHE laboratory positives was attempted in Vero E6 cells with virus detection confirmed by cytopathic effect up to 14 days post- inoculation.	SARS-CoV-2 virus was isolated from only one (5%) of nineteen cultured samples. It had a Ct value of 28.2.	Symptoms in the past month were associated with threefold increased odds of testing positive (aOR 3.46, 95%CI 1.38 to 8.67; p =< 0.008). 23 of 1,152 participants tested positive (2.0%) with a median Ct of 35.70 (IQR 32.42 to 37.57).
18.	L'Huillier[28]	Nasopharyngeal swabs in 636 patients aged less than 18 years in Geneva Hospital	23 (3.6%) tested positive for SARS CoV-2 - median age of 12 years (range 7 days to 14.9 years) [1-4]	Observation of cytopathic effect on days 2,4, and 6 of inoculum in Vero cells in two passages.	12 (52% of PCR positive)	Ct was around 28 for the children whose samples grew viable viruses
19.	Gniazdowski [29]	161 probably nasopharyngeal specimens	161 cases with positive PCR (not reported)	Ct values were calculated of only one gene target per assay: the Spike (S) gene for the RealStar® SARS-CoV-2 and the nonstructural protein 101 (Nsp) 2 gene for the NeuMoDx™ SARS-CoV-2 assays. Genome sequencing was carried out. Incubation of the inoculum in VeroE6 cells cultured at 37°C was observed for 4 days for cytopathic effect and immunofluorescence used to identify viral presence	Unclear possibly 47 isolates	Positive culture was associated with Ct values of 18.8 ± 3.4. Infectious viral shedding occurred in specimens collected up to 20 days after the first positive result in symptomatics. Mean and 184 median Ct values associated with recoverable virus were 18.8 ± 3.4 and 18.17 respectively, which was significantly lower than

						the mean and median Ct values that did not correlate with infectious virus recovery: 27.1 ± 5.7 and 27.5 respectively. PCR results should be interpreted alongside symptoms
20.	Basile [30]	234 samples, 228 (97%) from the upper respiratory tract (sputum, naso pharyngeal swabs, bronchial lavage from 195 individuals with Covid-19.	Samples from routine laboratory tests or from patients admitted to ICU or from a physician request [mean 4.5 days, 0-18, only one day to day 18]	Probes targets for PCR included E, RdRp, N, M, and ORF1ab for samples from ICU patients and 1 to 4 E, RdRp, N and Orf1ab for all other samples. After stabilization at 4 degrees centigrade samples were inoculated into Vero E6 cells and incubated at 370C in 5% CO2 for 5 days (day 0 to 4). Cultures were observed daily for cytopathic effect (CPE). CPE when it occurred took place between days 2 and 4. Day 4 was chosen for terminal sampling.	Culture positivity rate was 56 (24%) and significantly more likely positive in ICU patients compared with other inpatients or outpatients and significantly more likely positive in samples from inpatients	The highest Ct value with a successful culture was 32 (N gene target). A Ct cut-off of ≥37 was not indicative of viable virus
21.	Zhou 2020[35]	218 surface samples 31 air samples	7 areas of large London hospital	RT-PCR with primers and probes for the envelope (E) gene. Duplicate PCR was carried out and samples were considered positive if both duplicates had Ct< 40.4, or suspect if one of the two have Ct<40.4 (equivalent to one genome copy. For culture Vero E6 and Caco2 cells were used from air and environmental samples using a method adapted from one previously used to culture influenza virus. On day 0 and after 5-7 days, cell supernatants were collected, and RT-qPCR to detect SARS-CoV-2 performed as described above. Samples with at least one log increase in copy numbers for the E gene (reduced Ct values relative to the original samples) after 5-7 days propagation in cells compared with the	No cultures were positive	The pre-defined cycle threshold cut off was too high

				starting value were considered positive by viral culture.		
22.	Kim 2020[37]	Unclear. Possibly 323 serum 247 urine and 129 stool samples	74 COVID-19 hospital patients	RT-PCR was performed on the target genes were E and RdRp. Cell culture was performed in a Level III facility by inoculum into CaCo-2 cell line after stabilisation at 4C and harvested after 5 days and the supernatant after centrifugation was re-inoculated for another 5 days and assessed with RT-PCR.	No viral growth was detected in any specimen despite a positive RT-PCR very soon after admission	
23.	Lu 2020[38]	87 cases testing "re-positive" at RT-PCR 137 swabs (51 nasopharyngeal, 18 throat and 68 anal)	619 hospital discharges of which tested positive after discharge	137 swabs and 59 serum samples from 70 "repositive" cases to assess the immunological and virologic characteristics of the SARS-CoV-2 "repositive" cases. From 23 January, hospital dischargees followed a strict isolation protocol living (for example) in single dedicated hotel rooms and went home only when nucleic acid tests were negative on both respiratory tract and digestive tract samples. Samples (nasopharyngeal, throat and anal swabs), were collected for RT-PCR diagnosis at 7 and 14 days after discharge. Culture was carried out by inoculating Vero E6 cells with patient sample. CPE were observed daily at 7 days with a second round of passage. RT-PCR diagnosis was carried out on RNA using three RT-PCR kits to conduct nucleic acid testing, in an attempt to avoid false negatives. Ct varied from 29 to 39 depending on gene and kit	No cultures were positive	"Re-positive" cases are unlikely to be infectious as no intact RNA single helix was detected or viral isolated grew. Prolonged detection of viral RNA is a challenge for public health interventions targeted at isolating infectious cases. "Re-positive" discharged cases are caused by intermittent shedding of cells containing remnant RNA.

24.	Andersson[58]	20 RT-PCR positive serum samples, selected at random from a Covid-19 sample bank, representing samples from 12 individual patients (four individuals were represented at two timepoints), collected at 3 to 20 days following onset of symptoms.	20 serum samples from 12 hospitalised Covid-19 patients	Samples VC01-20 were provided blinded for viral culture experiments. 50 µL aliquots of samples VC1-VC20 were separately added to 2.4 x 105 Vero E6 cells in 24-well plates. Cells were propagated in DMEM supplemented with 10% FBS. Virus growth assays were done in DMEM supplemented with 1% FBS, glutamine and penicillin/streptomycin, according to published methods. In parallel, wells of the same number of cells were cultured in triplicate without virus challenge but with 50 µL control serum (VC21), or in duplicate with a stock of Victoria/01/2020 SARS-CoV-2 passage 4 (Oxford) at calculated ten-fold serial dilutions per well of 78, 7.8, 0.78 and 0.078 plaque forming units (pfu) in 50 µL of control serum (VC21). Wells were observed daily for cytopathic effects (CPE), and 50 L samples were taken for vRNA extraction on day 3 post-challenge. On day 4, 50 L aliquots of supernatants from cells challenged with VC01-20 were "blind passaged" to fresh cells, and the remaining supernatants were harvested and stored separately at -80C for future analysis. After a further 3 days, CPE was recorded, if any, for second passage cultures.	0 / 20 these serum samples produced positive viral culture	Serum samples.
25.	Korean CDC[59]	Respiratory swab samples for individuals testing	108 samples	Methods not reported	0 / 108 respiratory samples	This report does not report the laboratory

		positive after having previously tested positive, then negative.				methods used.
26.	Alin [30]	Air and surfaces of isolation room of 3 patients with severe Covid 19	48 [not reported]	Only positive samples (Ct value ≤35 for the RdRp and E genes) were cultured in Vero E6 cells 10-fold dilutions of the SARS-CoV-2 supernatants from the environmental samples was used. The inoculated cultures were grown in a humidified 37°C incubator with 5% CO2. After 72 hours, areas of cell clearance with crystal violet staining were used to demonstrate the cytopathic effect. In the presence of cytopathic effect was observed, detection of nucleic acid of SARS-CoV-2 by rRT-PCR in the supernatant was performed to confirm a successful culture.	External surfaces of intubation cannulae and surfaces in the room of patient not intubated	No air samples grew virus Ct values of samples who grew virus were uniformly low below 30 except in one case.
27.	Young [79]	Naso pharyngeal swabs, stool, fresh urine	152 of 74 patients	Material from nasopharyngeal swabs was inoculated in Vero-E6 cells in a Level 3 laboratory. Urine and stool samples were collected and transported fresh for virus culture but stools were filtered before inoculation. Cells were cultured at 37C for seven days or less if cytopathic effect (CPE) was observed by day 4 and confirmed by PCR.	21 naso pharyngeal specimens from 19 (14%) patients	No virus was isolated when the PCR cycle threshold (Ct) value was >30 or >14 days from symptom onset. Urine and stool samples at admission did not grow virus
28.	Ladhani [31]	Naso pharyngeal swabs	87 [Residents post, pre and	All SARS-CoV-2 positive samples with a Ct value of <35 were incubated on Vero E6 mammalian cells and	87	Ct values ≤ 35 Higher Ct values (lower

			symptomatic, _5 (_6 to _3) 4 (2 to 11) _7 (_10 to _4). Staff post, pre and symptomatic _7 (_9 to _4) 3 (2_5) _5 (_9 to _3)]	virus detection was confirmed by cytopathic effect (CPE) up to 14 days post-inoculation. Whole genome sequencing (WGS) was carried out on all RT-PCR positive samples		virus load) samples were associated with decreasing ability to recover infectious virus from 100% (2/2) with Ct <20.00 to 17.0% (9/53) with Ct 30.00_34.99 (x2 for trend, P<0.001)
29.	Borczuk [39]	Post mortem lung tissue from 68 elderly deaths (median age 73)	Six	When a cytopathic effect was seen, the Vero cell culture supernatant was passed to a fresh Vero cell culture tube to ensure reproducibility. SARS-CoV-2 in the supernatant was further confirmed by RT-PCR	6	No ct reported. In one case virus grew on day 26 from symptoms kick off

Table 1. Characteristics of included studies. Key: STT = symptom onset to test date.

Study	Description of methods and sufficient detail to replicate	Sample sources clear	Analysis & reporting appropriate	Is bias dealt with	Applicability
Bullard 2020[8]	Yes	Yes	yes	unclear	unclear
Santarpia 2020[11]	Yes	Yes	yes	unclear	unclear
Wölfel 2020[14]	Yes	Yes	yes	unclear	unclear
Huang 2020[9]	yes	Yes	yes	unclear	unclear
Wang W [12] 2020	No	Yes	yes	no	unclear
Zhang Y 2020[30]	Partly	Yes	yes	no	unclear
Xiao 2020b[29]	No	Yes	yes	no	unclear
Qian Q 2020[22]	Yes	Yes	yes	unclear	unclear
Arons 2020[13]	Yes	Yes	yes	yes	unclear
Xiao F 2020[13]	Yes	Yes	yes	no	unclear
Kujawski 2020[15]	Yes	Yes	yes	unclear	unclear
Jeong 2020[11]	Yes	Yes	yes	no	unclear
La Scola 2020[23]	Yes	Yes	yes	unclear	unclear
Yoa H 2020[16]	Yes	Yes	yes	unclear	unclear
Singanayagam[25]	Yes	No	Yes	unclear	unclear
Perera [20]	Yes	Yes	Yes	unclear	unclear
Brown[27]	Yes	Yes	Yes	Unclear	unclear
Gniazdowski[28]	Yes	Yes	Yes	Unclear	unclear
Basile[30]	Yes	Yes	Yes	Unclear	unclear
L'Huillier[18]	Yes	Yes	Yes	Unclear	unclear
Zhou 2020[35]	Yes	Yes	Yes	Unclear	Unclear
Kim[37]	No	No	No	Unclear	Unclear

Lu[19]	Yes	Yes	Yes	Partly	Yes
Andersson[28]	Yes	Yes	Yes	Partly	Yes
Korean CDC[22]	No	Partly	Partly	No	Unclear
Ahn[29]	Yes	Yes	Yes	Partly	Unclear
Young[17]	Yes	Yes	Yes	Yes	Yes
Ladhani[31]	Yes	Yes	Yes	Yes	Likely
Borczuk[30]	Yes	Yes	Yes	Yes	Unclear

Table 2. Quality of included studies

Table 3. Duration of viral shedding in the included studies.

Study	Duration of viral shedding as assessed by PCR for SARS-CoV-2 RNA	Range of duration	Median of duration	Notes on clinical course
Rutiert[4]	Day 0 to day 7 at least.	NR	NR	SARS-CoV-2 Vero cell infectivity of respiratory samples from SARS-CoV-2 positive individuals was only observed for RT-PCR CI < 24 and symptom onset to test of < 8 days.
Jeong[11]	At least 8 days to at least 30 days	NR	NR	5 positive-PCR patients, day 8 to day 30 after symptom onset. At the time of sampling, patients 1, 2, 3, and 5 were on days 8, 13, 11, and 30 of illness, respectively, and their clinical symptoms had resolved completely. Patient 4 was on day 15 of illness with a ventilator and extracorporeal membrane oxygenation support. All clinical specimens collected from the five patients were positive for the SARS-CoV-2 spike gene by qPCR, even though four of the patients no longer displayed clinical symptoms.
Qian[30]	SARS-CoV-2 RNA detected day 10 to between day 18 and day 35 after symptom onset.			Covid-19 symptoms began on day 3 after surgery on day 0. SARS-CoV-2 PCR test done on day 7 after surgery. PCR on day 14 and day 18 post-surgery were positive. PCR on day 37 and day 38 after surgery were negative. Patient was discharged on day 41 after surgery following the 2 sequential negative PCR tests plus absence of clinical symptoms and radiological abnormalities. Fecal samples day 35 after discharge were negative.
Xing F. Sun [...]	Day 7 after symptom onset to at least day 28.			1 patient. SARS-CoV-2 RNA PCR positive at day 7 after symptom onset.

				Patient died two weeks after final sample.
Wölfel[14]	Up to day 28 after onset of symptoms.	NR	NR	9 cases. All swabs taken between day 1 and day 5 were positive by PCR. Virus could not be isolated from samples taken after day 8 even among cases with ongoing high viral loads of approximately 105 RNA copies/mL
Kujawski [15] (for The COVID-19 Investigation Team)	Duration of SARS-CoV-2 detection by RT-PCR was 7 to 22 days	7 to 22 days		First 12 identified patients in the US. Respiratory specimens collected between illness days 1 to 9 (median, day 4) All patients had SARS-CoV-2 RNA detected in respiratory specimens, typically for 2 to 3 weeks after illness onset. Mean duration of fever was 9 days. Two patients received a short course of corticosteroids.
Xiao[99], Tang M	1 to 12 days (stool samples) Duration of detection of SARS-CoV-2 respiratory samples not reported.	1 to 12 days	NR	Positive stool results duration ranged from 1 to 12 days. 17 (23%) patients continued to have positive results in stool after showing negative results in respiratory samples.
Singanayagam[25]	At least day 20 post symptom onset, upper respiratory tract swabs PCR	NR	NR	Median duration of virus shedding as measured by viral culture was 4 days (IQR: 1 to 8; range: −13 to 12, with symptom onset dates based on symptom recall)
Perera [26]	>30 days in 10 patients	NR	NR	
Brown[27]	NR	NR	NR	
Gniazdowski [28]	Up to 22 days in subset of 29 patients	1-22 days	NR	Ct values reported in aggregate and for subset of 20 patients but retrospective nature of specimens precluded details description

Lu[18]	Not reported in paper or suppl material (no linking of patient number with type of sample but may be available from the authors			
Andersson[20]	Not included in this paper			
Korean CDC[25]	Time to retesting positive via PCR is reported, among this specific group of individuals who retested positive by PCR	On average, it took 44.9 days (range: 8 to 82 days) from initial symptom onset date to testing positive after discharge. (Based on 226 cases symptomatic at the time of initial confirmation)		This may indicate an overall duration of viral shedding, indicating that shedding of RNA may detected over a long period of time and inconsistently. These data may not be comparable with information from studies specifically observing duration of viral shedding as an outcome.
Young [19]	16.7 days	(95% CI 15.2 to 18.3)		Cessation of viral shedding by PCR occurred in 4% by day 7, 30% by day 14, 78% by day 21 and 91% by day 28. There were no differences by disease severity
Ladhani [21]				
Borczuk [22]	Culture positive around 2 weeks of duration except for one case up to 25 days	NR	NR	Post mortem study

Table 4: Relationship of PCR Cycle threshold and Log 10 copies to Positive Viral Culture

	Sample				Cycle Threshold			Log 10 copies			
Study	RT-PCR SARS-CoV-2 positive samples (n)	Viral Culture growth (n)	No growth (n)	Gene fragment sampled on PCR Test	Positive culture Ct value	Negative culture Ct Value	No growth in samples based on Ct	Log 10 copies positive culture (unless otherwise stated)	Log 10 copies negative culture	No growth based on log copies	ORs for Viral Culture
Bullard J 2020 [4]	90	26	64	E gene	17 [16-18]	27 [22-33]	Ct > 24				OR 0.64 (95%CI 0.49 to 0.84, p<0.001) for every one unit increase in Ct.
	60	23	34	Nsp 12	Mean 23.9 ± SEM 0.78	Mean 29.26 ± SEM 0.78	Ct >31.47	mean 7.37 ± SEM 0.20	Mean 5.96 ± SEM 0.18		
Huang 2020 [5]		23	37	E	Mean 22.39 ± SEM 0.75	Mean 28.92 ± SEM 0.65	Ct >31.46	mean 8.21 ± SEM 0.18	Mean 6.62 ± SEM 0.16		
		21	31	N	Mean 27.29 ± SEM 0.77	Mean 31.49 ± SEM 0.59	Ct >35.2	mean 7.87 ± SEM 0.21	Mean 6.70 ± SEM 0.17		
La Scola 2020 [19] (Jaafar 2020)	611 (3790)	129(1941)	482 (1849)	E			Ct ≥ 34 (2.6% positives)				
Bcroon CS [17]	23	1	22	RdRp, E, and N	28.16	35.16 ± SEM 0.63	Ct >26.2				
Perera [21]	68	16	52	N				7.5 [2]	3.8	<5.0	
Singanayagam 2020 [23]	324	133	191	Unclear			Ct > 35 probability of no growth was 8.3% (95% CI: 2.8%–18.4%) [1]				OR 0.67 for each unit increase in Ct value (95% CI: 0.58–0.77)
Wölfel 2020 [26]	45	9	36	E, Subgenomic mRNA							
L'Huillier 2020 [15]	23 [4]	12	11					Mean 7.9×10^7 IQR 4.7×10^6- 1.0×10^8	Mean 5.4×10^7 IQR 4.2×10^6– 1.8×10^8		
Gniazdowski R 2020 [14]	132	47	85	S, Nsp 2	Mean 12.8 ± 3.4 Median 18.17	Mean 27.1 ± 6.7 Median 27.5	Ct ≥ 23 yielded 8.8% of virus isolates				
Basile K 2020 [10]	234	56	178	E, RdRp, N, M, and	26.01	27.75	Ct >32 with the N gene target [3]				

				ORF1ab for ICU patients		
Ladhani [11] 2020	87	31	56	ORF1ab	100% cultures (2/2) with Ct <20.00 to 17.0% (9/53) with Ct 30.00-34.99	Cutoff <35
Young [18] 2020	100	21	79	N, S, and ORF1ab	28.2 (24.3 to 33.3	<30

1 All above CT (n=5) 35 were symptomatic

2. Of the 16 culture positive specimens, 15 (94%) had viral RNA load >6 log10 copies/mL (p<0.01). All of them were collected within the first 8 days of illness

3. no CPE visualised but a decrease in CT values between the Ct of the original clinical sample-PCR (Ct_{sample}) and the terminal culture (day four) supernatant PCR ($Ct_{culture}$) of ≥3 (equivalent to a 1 log increase in virus quantity) i.e. $Ct_{sample} - Ct_{culture} \geq 3$ = culture positive. The authors hypothesized that a Ct_{sample} minus $Ct_{culture}$ <3 was due to residual inoculated clinical sample and not replicating virus

4.23 SARS-CoV-2-infected children

JAMA | Original Investigation

Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore

Barnaby Edward Young, MB, BChir; Sean Wei Xiang Ong, MBBS; Shirin Kalimuddin, MPH; Jenny G. Low, MPH; Seow Yen Tan, MBBS; Jiashen Loh, MBBS; Oon Tek Ng, MPH; Kalisvar Marimuthu, MBBS; Li Wei Ang, Msc; Tze Minn Mak, PhD; Sok Kiang Lau, PhD; Danielle E. Anderson, PhD; Kian Sing Chan, MBBS; Thean Yen Tan, MBBCh; Tong Yong Ng, MBBS; Lin Cui, PhD; Zubaidah Said, MSc; Lalitha Kurupatham, MPH; Mark I-Cheng Chan, PhD; Monica Chan, BMBS; Shawn Vasoo, MBBS; Lin-Fa Wang, PhD; Boon Huan Tan, PhD; Raymond Tzer Pin Lin, MBBS; Vernon Jian Ming Lee, PhD; Yee-Sin Leo, MPH; David Chien Lye, MBBS; for the Singapore 2019 Novel Coronavirus Outbreak Research Team

IMPORTANCE Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, in December 2019 and has spread globally with sustained human-to-human transmission outside China.

OBJECTIVE To report the initial experience in Singapore with the epidemiologic investigation of this outbreak, clinical features, and management.

DESIGN, SETTING, AND PARTICIPANTS Descriptive case series of the first 18 patients diagnosed with polymerase chain reaction (PCR)–confirmed SARS-CoV-2 infection at 4 hospitals in Singapore from January 23 to February 3, 2020; final follow-up date was February 25, 2020.

EXPOSURES Confirmed SARS-CoV-2 infection.

MAIN OUTCOMES AND MEASURES Clinical, laboratory, and radiologic data were collected, including PCR cycle threshold values from nasopharyngeal swabs and viral shedding in blood, urine, and stool. Clinical course was summarized, including requirement for supplemental oxygen and intensive care and use of empirical treatment with lopinavir-ritonavir.

RESULTS Among the 18 hospitalized patients with PCR-confirmed SARS-CoV-2 infection (median age, 47 years; 9 [50%] women), clinical presentation was an upper respiratory tract infection in 12 (67%), and viral shedding from the nasopharynx was prolonged for 7 days or longer among 15 (83%). Six individuals (33%) required supplemental oxygen; of these, 2 required intensive care. There were no deaths. Virus was detectable in the stool (4/8 [50%]) and blood (1/12 [8%]) by PCR but not in urine. Five individuals requiring supplemental oxygen were treated with lopinavir-ritonavir. For 3 of the 5 patients, fever resolved and supplemental oxygen requirement was reduced within 3 days, whereas 2 deteriorated with progressive respiratory failure. Four of the 5 patients treated with lopinavir-ritonavir developed nausea, vomiting, and/or diarrhea, and 3 developed abnormal liver function test results.

CONCLUSIONS AND RELEVANCE Among the first 18 patients diagnosed with SARS-CoV-2 infection in Singapore, clinical presentation was frequently a mild respiratory tract infection. Some patients required supplemental oxygen and had variable clinical outcomes following treatment with an antiretroviral agent.

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Video and Supplemental content

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Author Affiliations: Author affiliations are listed at the end of this article.

Group Information: Members of the Singapore 2019 Novel Coronavirus Outbreak Research Team are listed in the Supplement.

Corresponding Authors: Barnaby Edward Young, MB, BChir (barnaby_young@nccid.sg) and Yee-Sin Leo, MPH (Yee_Sin_Leo@nccid.sg), National Centre for Infectious Diseases, 16 Jln Tan Tock Seng, Singapore 308442.

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The third novel coronavirus in 17 years emerged in Wuhan, China, in December 2019.[1] Phylogenetics has indicated that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is closely related to bat-derived SARS-like coronaviruses.[2] Early reports from Wuhan described the associated coronavirus disease 2019 (COVID-19) as a SARS-like atypical pneumonia in which 26% to 33% of patients required intensive care and 4% to 15% died.[1,3,4] A large case series of 72 314 infected individuals has since refined these initial estimates in China to severe disease in 14% and a case-fatality rate of 2.3%.[5]

On January 23, 2020, the first imported SARS-CoV-2 infection in Singapore was detected in a visitor from Wuhan. Subsequently, COVID-19 has been diagnosed among other visitors and returning travelers, and from limited local transmission.[6]

While a proven effective antiviral treatment in COVID-19 is not available, the antiretroviral drug lopinavir-ritonavir has been proposed, as its potential effectiveness in the treatment of SARS was suggested in 2 case series.[7,8] In Middle East respiratory syndrome, lopinavir-ritonavir also showed potential activity in marmosets,[9] but in a mouse model it did not reduce virus titer or lung pathology, although it improved lung function.[10]

This case series describes the epidemiologic features, clinical presentation, treatment, and outcomes of the first 18 patients in Singapore with confirmed COVID-19.

Key Points

Question What was the initial experience in Singapore with the outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)?

Findings In this descriptive case series of the first 18 patients diagnosed with SARS-CoV-2 infection in Singapore between January 23 and February 3, 2020, clinical presentation was a respiratory tract infection with prolonged viral shedding from the nasopharynx of 7 days or longer in 15 patients (83%). Supplemental oxygen was required in 6 patients (33%), 5 of whom were treated with lopinavir-ritonavir with variable clinical outcomes following treatment.

Meaning These findings provide clinical features and course among patients diagnosed with SARS-CoV-2 infection in Singapore.

Methods

Outbreak Response

On January 2, 2020, after first reports of an outbreak of atypical pneumonia in Wuhan, China, the Singapore Ministry of Health issued a health alert that patients with pneumonia and recent travel to Hubei Province should be screened for SARS-CoV-2 infection. All individuals with suspected SARS-CoV-2 infection were isolated with airborne and contact precautions, and attending staff wore personal protective equipment in accordance with the US Centers for Disease Control and Prevention guidelines.[11] Extensive contact tracing followed by quarantine of asymptomatic contacts and hospital isolation and screening of symptomatic contacts was strictly enforced.

Data and Specimen Collection

Individuals confirmed to have COVID-19 by SARS-CoV-2 real-time reverse transcriptase–polymerase chain reaction (RT-PCR) were eligible for inclusion in this study (eMethods in the Supplement). Data were collected at the 4 hospitals that provided care for these patients.

Waiver of informed consent for collection of clinical data from infected individuals was granted by the Ministry of Health, Singapore, under the Infectious Diseases Act as part of the COVID-19 outbreak investigation. Written informed consent was obtained from study participants for collection of biological samples after review and approval of the study protocol by the institutional ethics committee.

Data from electronic health records were summarized using a standardized data collection form. Two researchers independently reviewed the data collection forms for accuracy.

Specimens (blood, stool, and urine samples; nasopharyngeal swabs) were collected at multiple time points in the first 2 weeks following study enrollment and tested by RT-PCR for the presence of SARS-CoV-2. RT-PCR cycle threshold values were collected. The cycle threshold value correlates with the number of copies of the virus in a biological sample, in an inversely proportional and exponential manner. Sequencing of PCR products of the RNA-dependent RNA polymerase (*RdRp*) gene were used to construct phylogenetic trees (eMethods in the Supplement).

Clinical Management

As part of standard of care, complete blood cell count, tests of kidney and liver function, and measurement of C-reactive protein and lactate dehydrogenase levels were performed. Respiratory samples were tested for influenza and other respiratory viruses with a multiplex PCR assay.

All patients received supportive therapy, including supplemental oxygen when saturations as measured by pulse oximeter dropped below 92%. Patients clinically suspected of having community-acquired pneumonia were administered empirical broad-spectrum antibiotics and oral oseltamivir. Coformulated lopinavir-ritonavir (200 mg/100 mg twice daily orally for up to 14 days) was prescribed to selected patients at the treating physicians' discretion after shared decision-making and provision of oral informed consent. Corticosteroids were avoided, reflecting increased mortality with their use in severe influenza.[12]

Respiratory samples were sent daily for SARS-CoV-2 PCR on clinical recovery. Deisolation was contingent on at least 2 consecutive negative PCR assay results more than 24 hours apart.

Results

Epidemiologic Features

Between January 23 and February 3, 2020, 18 patients infected with SARS-CoV-2 were diagnosed in Singapore, with symptom onset from January 14 to January 30, 2020. All patients reported travel to Wuhan, China, in the 14 days prior to

Table. Clinical Features of Patients Infected With SARS-CoV-2

	All patients (N = 18)	Did not require supplemental O_2 (n = 12)	Required supplemental O_2 (n = 6)
Demographics			
Age, median (range), y	47 (31-73)	37 (31-56)	56 (47-73)
Male sex, No. (%)	9 (50)	7 (58)	2 (33)
Any comorbidity, No. (%)[a]	5 (28)	1 (8)	4 (67)
Signs and symptoms on presentation, No. (%)			
Fever	13 (72)	7 (58)	6 (100)
Cough	15 (83)	10 (83)	5 (83)
Shortness of breath	2 (11)	1 (8)	1 (17)
Rhinorrhea	1 (6)	1 (8)	0
Sore throat	11 (61)	8 (67)	3 (50)
Diarrhea	3 (17)	3 (25)	0
Vital signs at presentation, median (range)			
Temperature, °C	37.7 (36.1-39.6)	38.3 (36.6-39.6)	37.7 (36.1-38.1)
Respiratory rate, breaths/min	18 (16-21)	18 (17-19)	20 (16-21)
Pulse oximeter O_2 saturation, %	98 (95-100)	98 (95-100)	97 (95-98)
Systolic blood pressure, mm Hg	131 (103-167)	131 (104-167)	136 (108-141)
Heart rate, /min	97 (75-118)	99 (75-118)	91 (78-102)
Baseline investigations, median (range)			
WBCs, $\times 10^9$/L	4.6 (1.7-6.3)	4.6 (1.7-6.3)	3.4 (2.6-5.8)
Hemoglobin, g/dL	13.5 (11.7-17.2)	13.9 (11.7-17.2)	13.2 (11.7-14)
Platelets, $\times 10^9$/L	159 (116-217)	159 (128-213)	156 (116-217)
Neutrophils, $\times 10^9$/L	2.7 (0.7-4.5)	2.6 (0.7-4.5)	1.8 (1.2-3.7)
Lymphocytes, $\times 10^9$/L	1.2 (0.8-1.7)	1.2 (0.8-1.6)	1.1 (0.8-1.7)
C-reactive protein, mg/L (n = 16)	16.3 (0.9-97.5)	11.1 (0.9-19.1)	65.6 (47.5-97.5)
LDH, U/l (n = 13)	512 (285-796)	424 (285-748)	550 (512-796)
Abnormal chest radiograph, No. (%)	6 (33)	3 (25)	3 (50)
Duration of symptoms, median (range)			
Fever, d	4 (0-15)	1 (0-7)	5 (4-15)
Any symptoms, d	13 (5-24)	12 (5-24)	16 (10-30)

Abbreviations: LDH, lactate dehydrogenase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WBC, white blood cell.

SI conversion factor: To convert LDH values to µkat/L, multiply by 0.0167.

[a] Group not requiring supplemental oxygen: hypertension, diabetes. Group requiring supplemental oxygen: hypertension (n = 4), type 2 diabetes (n = 1), hyperlipidemia (n = 1).

Illness onset (eTable 1 in the Supplement). Four patients (22%) were identified through contact tracing, while 3 (17%) were identified through border screening. Of the 18 patients, 16 (89%) were Chinese nationals, while 2 (11%) were Singapore residents. There were 5 clusters comprising family, traveling companions, or other close contacts (eFigure 1 in the Supplement). Contact tracing of the 18 patients identified a total of 264 close contacts in Singapore (eFigure 2 in the Supplement). As of February 25, 2020, no infections had been detected among health care workers involved in the care of patients with COVID-19.

Clinical Features

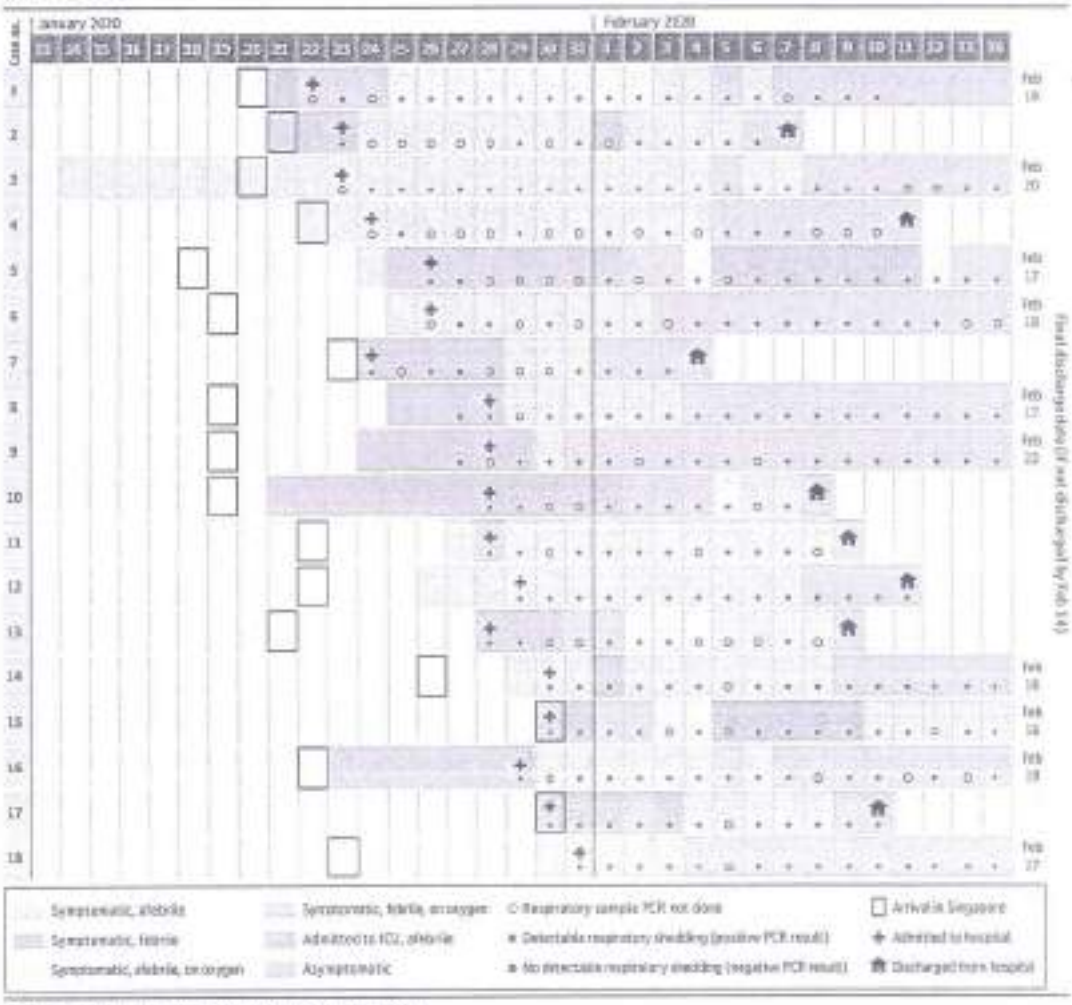
Clinical features are summarized in the **Table**. Fever (13 [72%]), cough (15 [83%]), and sore throat (11 [61%]) were common symptoms. Rhinorrhea was infrequent (1 [6%]), while 6 patients (33%) had an abnormal chest radiograph finding or lung crepitations. No patients presented with a severe acute respiratory distress syndrome, and only 1 required immediate supplemental oxygen. Lymphopenia (<1.1 $\times 10^9$/L) was present in 7 of 16 patients (39%) and an elevated C-reactive protein level (>20 mg/L) in 6 of 16 (38%), while kidney function remained normal.

The clinical course was uncomplicated for 12 patients (67%), but 6 patients (33%) desaturated and required supplemental oxygen (**Figure 1**). Chest radiographs showed no pulmonary opacities at presentation in 12 patients (67%) and remained clear throughout the acute illness in 9 (50%). Three patients with initially normal chest radiograph findings developed bilateral diffuse airspace opacities; of these, 2 had been persistently febrile for more than 1 week. Two individuals (11%) required admission to the intensive care unit because of increasing supplemental oxygen requirements, and 1 (6%) required mechanical ventilation. No concomitant bacterial or viral infections were detected, and there were no deaths as of February 25, 2020.

Clinical Outcomes

Of the 6 patients who required supplemental oxygen, 5 received lopinavir-ritonavir (**Figure 2**). For 3 of 5 patients (60%),

Figure 1. Time Course of Symptoms, Supplemental Oxygen Requirements, Hospital Admission, and Discharge of Patients Infected With SARS-CoV-2



ICU indicates intensive care unit; PCR, polymerase chain reaction.

initiation of lopinavir-ritonavir was followed by a reduction in supplemental oxygen requirements within 3 days, and viral shedding in nasopharyngeal swabs cleared within 2 days of treatment for 2 of 5 (40%).

Two patients, however, deteriorated and experienced progressive respiratory failure while receiving lopinavir-ritonavir, with 1 requiring invasive mechanical ventilation. Virus continued to be detected by nasopharyngeal swab or endotracheal tube aspirate for these 2 patients for the duration of their admission to intensive care.

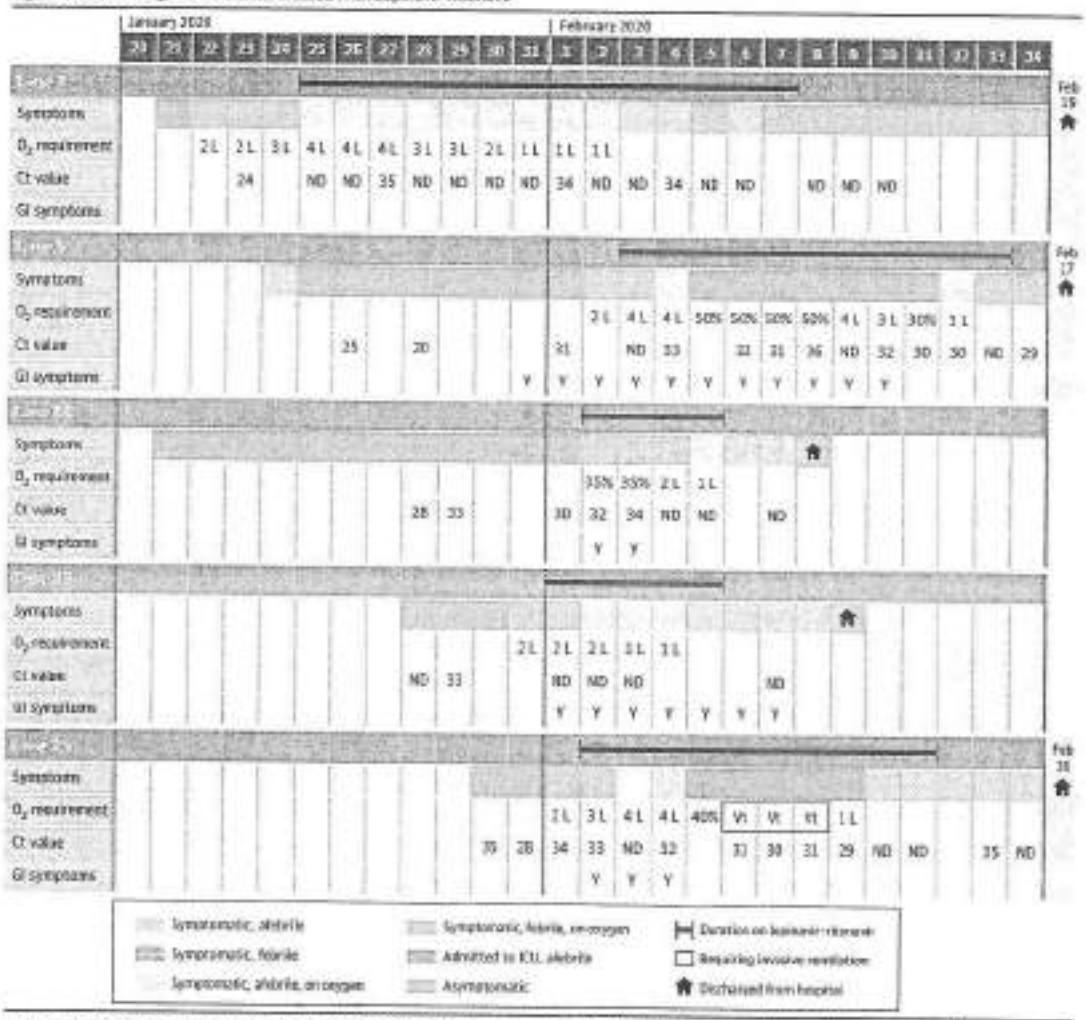
Four of the 5 patients treated with lopinavir-ritonavir developed nausea, vomiting, and/or diarrhea, and 3 developed abnormal liver function test results. Only 1 individual completed the planned 14-day treatment course as a result of these adverse events.

Virologic Features

Median duration of viral shedding from first to last positive nasopharyngeal swab collected as part of clinical care was 12 days (range, 1-24), and 15 patients (83%) had viral shedding from the nasopharynx detected for 7 days or longer. Daily serial RT-PCR cycle threshold values for all 18 patients are shown in eFigure 3 in the Supplement. The time course of serial cycle threshold values by day of illness for 12 patients not receiving supplemental oxygen and 6 patients receiving supplemental oxygen (of whom 5 were treated with lopinavir-ritonavir) appeared similar (eFigure 4 in the Supplement).

Virus was detected by PCR in stool (4/8 patients [50%]) and in whole blood (1/12 [8%]); virus was not detected in urine (0/10 samples) (eTable 2 in the Supplement). Viral

Figure 2. Clinical Progress in Patients Treated With Lopinavir-Ritonavir



Cycle threshold (Ct) value corresponds to the number of copies of the virus in a biological sample, in an inversely proportional and exponential manner. ICU indicates intensive care unit; GI, gastrointestinal; ND, not detected; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Y, yes; Vt, mechanical ventilation.

culture results were not available at the time of writing to determine the viability of virus detected outside the respiratory tract.

Sequences for phylogenetic analysis were available for 6 viruses (eFigure 5 in the Supplement). All clustered together with other SARS-CoV-2 sequences reported from China and other countries and are available in public databases.

Discussion

This descriptive case series reports the clinical features of the first 18 patients with laboratory-confirmed SARS-CoV-2 infection in Singapore, reporting epidemiologic features and clinical course in detail.

Perhaps reflecting the intensive efforts at contact tracing, 28% of patients were without fever vs 1.4% to 17% in 3 previously reported studies from Wuhan, China.[1,4,13] In the current study, 6 of 18 patients (33%) experienced oxygen desaturation to 92% or less, in contrast to the 76% to 90% supplemental oxygen use from the other 3 studies.[1,4,13]

In 4 of 8 patients, virus was detected in stool, regardless of diarrhea, over 1 to 7 days. Viremia was detected in 6 patients[1] and 1 of 5 patients[14] in China but only in 1 of 12 in this study. In the family cluster in Shenzhen, the virus was not detected in urine or stool.[14] In SARS, viremia was observed

in the first week, with peak respiratory viral shedding in the second week and persistent stool viral shedding beyond the second week.[15] In Middle East respiratory syndrome, viral shedding was greater in lower respiratory tract samples than in blood and stool.[16] Viral load in nasopharyngeal samples and viremia was associated with disease severity in SARS.[17]

In this study, viral load in nasopharyngeal samples from patients with COVID-19 peaked within the first few days after symptom onset before declining. The duration of viral shedding from nasopharyngeal aspirates was prolonged up to at least 24 days after symptom onset. This was longer than reported from a comparable series from China.[18] Toward the end of this period virus was only intermittently detected from nasopharyngeal swabs. It is unclear whether this is attributable to biological differences in the intensity of shedding or to sampling variability when low amounts of virus are present. Determining whether the virus remains transmissible throughout the period of detectability is critical to control efforts.

Five patients were treated with lopinavir-ritonavir within 1 to 3 days of desaturation, but evidence of clinical benefit was equivocal. While defervescence occurred within 1 to 3 days of lopinavir-ritonavir initiation, it was unable to prevent progressive disease in 2 patients. Decline in viral load as indicated by the cycle threshold value from nasopharyngeal swabs also appeared similar between those treated and not treated with lopinavir-ritonavir. The effectiveness of lopinavir-ritonavir treatment in COVID-19 needs to be examined in an outbreak randomized trial, given a lack of clear signal in this small case series.

Limitations

This study has several limitations. First, it was a case series of 18 patients who acquired their infection following travel to Wuhan, China. Findings from this study are valuable early data from a high-resource setting but may change as this outbreak continues to evolve and local transmission clusters emerge. Second, 9 individuals (50%) presented to the hospital more than 2 days after symptom onset. As a result, sample collection early during the course of illness was limited. Third, biological samples were collected systematically when possible, but not all patients consented to sample collection. Baseline laboratory data were also not available for all patients. Fourth, cycle threshold values are a quantitative measure of viral load, but correlation with clinical progress and transmissibility is not yet known.

Conclusions

Among the first 18 patients diagnosed with SARS-CoV-2 infection in Singapore, clinical presentation was frequently a mild respiratory tract infection. Some patients required supplemental oxygen and had variable clinical outcomes following treatment with an antiretroviral agent.

ARTICLE INFORMATION

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Author Affiliations: National Centre for Infectious Diseases, Singapore (Young, Ong, O.-T. Ng, Marimuthu, Ang, Mak, Cui, Chen, M. Chan, Vasoo, Lin, Leo, Lye); Tan Tock Seng Hospital, Singapore (Young, Ong, O.-T. Ng, Marimuthu, M. Chan, Vasoo, Leo, Lye); Lee Kong Chian School of Medicine, Singapore (Young, O.-T. Ng, B. H. Tan, Lee, Lye); Singapore General Hospital, Singapore (Kalimuddin, Low, K. S. Chan); Duke-NUS Medical School, Singapore (Kalimuddin, Low, Anderson, T. Y. Tan, Wang); Changi General Hospital, Singapore (S. Y. Tan, T. Y. Tan); Sengkang General Hospital, Singapore (Loh, T. Y. Ng); Yong Loo Lin School of Medicine, Singapore (Marimuthu, Leo, Lye); DSO National Laboratories, Singapore (Lau, B. H. Tan); Ministry of Health, Singapore (Said, Kurupatham, Lee); Saw Swee Hock School of Public Health, Singapore (Chen, Lee, Leo).

Author Contributions: Dr Young had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Concept and design: Young, Ong, O.-T. Ng, Marimuthu, Ang, Anderson, M. Chan, Vasoo, B. Tan, Leo, Lye.

Acquisition, analysis, or interpretation of data: Young, Ong, Kalimuddin, Low, S. Tan, Loh, O.-T. Ng, Marimuthu, Ang, Mak, Lau, Anderson, K. Chan, T. Tan, T. Ng, Cui, Said, Kurupatham, Chen, Wang, B. Tan, Lin, Lee, Leo, Lye.

Drafting of the manuscript: Young, Ong, Low, O.-T. Ng, Ang, Mak, Anderson, Said, Lee, Lye.

Critical revision of the manuscript for important intellectual content: Young, Ong, Kalimuddin, Low, S. Tan, Loh, O.-T. Ng, Marimuthu, Lau, Anderson, K. Chan, T. Tan, T. Ng, Cui, Said, Kurupatham, Chen, M. Chan, Vasoo, Wang, B. Tan, Lin, Lee, Leo, Lye.

Statistical analysis: Young, Ang, Anderson, Said.

Obtained funding: Young, Marimuthu, Anderson, B. Tan.

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Supervision: Young, Kalimuddin, O.-T. Ng, Marimuthu, Anderson, T. Ng, M. Chan, Vasoo, Lin, Wang, B. Tan, Lee, Leo, Lye.

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EXHIBIT "DD"

Your Coronavirus Test Is Positive. Maybe It Shouldn't Be.

Source: The New York Times
Link: https://www.nytimes.com/2020/08/29/health/coronavirus-testing.html
Author: Apoorva Mandavilli
Published on: Aug. 29, 2020

The usual diagnostic tests may simply be too sensitive and too slow to contain the spread of the virus.

Some of the nation's leading public health experts are raising a new concern in the endless debate over coronavirus testing in the United States: The standard tests are diagnosing huge numbers of people who may be carrying relatively insignificant amounts of the virus.

Most of these people are not likely to be contagious, and identifying them may contribute to bottlenecks that prevent those who are contagious from being found in time. But researchers say the solution is not to test less, or to skip testing people without symptoms, as recently suggested by the Centers for Disease Control and Prevention.

Instead, new data underscore the need for more widespread use of rapid tests, even if they are less sensitive.

"The decision not to test asymptomatic people is just really backward," said Dr. Michael Mina, an epidemiologist at the Harvard T.H. Chan School of Public Health, referring to the C.D.C. recommendation.

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"In fact, we should be ramping up testing of all different people," he said, "but we have to do it through whole different mechanisms."

In what may be a step in this direction, the Trump administration announced on Thursday that it would purchase 150 million rapid tests.

The most widely used diagnostic test for the new coronavirus, called a PCR test, provides a simple yes-no answer to the question of whether a patient is infected.

But similar PCR tests for other viruses do offer some sense of how contagious an infected patient may be: The results may include a rough estimate of the amount of virus in the patient's body.

"We've been using one type of data for everything, and that is just plus or minus — that's all," Dr. Mina said. "We're using that for clinical diagnostics, for public health, for policy decision-making."

But yes-no isn't good enough, he added. It's the amount of virus that should dictate the infected patient's next steps. "It's really irresponsible, I think, to forgo the recognition that this is a quantitative issue," Dr. Mina said.

The PCR test amplifies genetic matter from the virus in cycles; the fewer cycles required, the greater the amount of virus, or viral load, in the sample. The greater the viral load, the more likely the patient is to be contagious.

This number of amplification cycles needed to find the virus, called the cycle threshold, is never included in the results sent to doctors and coronavirus patients, although it could tell them how infectious the patients are.

In three sets of testing data that include cycle thresholds, compiled by officials in Massachusetts, New York and Nevada, up to 90 percent of people testing positive carried barely any virus, a review by The Times found.

On Thursday, the United States recorded 45,604 new coronavirus cases, according to a database maintained by The Times. If the rates of contagiousness in Massachusetts and New York were to apply nationwide, then perhaps only 4,500 of those people may actually need to isolate and submit to contact tracing.

One solution would be to adjust the cycle threshold used now to decide that a patient is infected. Most tests set the limit at 40, a few at 37. This means that you are positive for the coronavirus if the test process required up to 40 cycles, or 37, to detect the virus.

Tests with thresholds so high may detect not just live virus but also genetic fragments, leftovers from infection that pose no particular risk — akin to finding a hair in a room long after a person has left, Dr. Mina said.

Any test with a cycle threshold above 35 is too sensitive, agreed Juliet Morrison, a virologist at the University of California, Riverside. "I'm shocked that people would think that 40 could represent a positive," she said.

A more reasonable cutoff would be 30 to 35, she added. Dr. Mina said he would set the figure at 30, or even less. Those changes would mean the amount of genetic material in a patient's sample would have to be 100-fold to 1,000-fold that of the current standard for the test to return a positive result — at least, one worth acting on.

The Food and Drug Administration said in an emailed statement that it does not specify the cycle threshold ranges used to determine who is positive, and that "commercial manufacturers and laboratories set their own."

The Centers for Disease Control and Prevention said it is examining the use of cycle threshold measures "for policy decisions." The agency said it would need to collaborate with the F.D.A. and with device manufacturers to ensure the

measures "can be used properly and with assurance that we know what they mean."

The C.D.C.'s own calculations suggest that it is extremely difficult to detect any live virus in a sample above a threshold of 33 cycles. Officials at some state labs said the C.D.C. had not asked them to note threshold values or to share them with contact-tracing organizations.

For example, North Carolina's state lab uses the Thermo Fisher coronavirus test, which automatically classifies results based on a cutoff of 37 cycles. A spokeswoman for the lab said testers did not have access to the precise numbers.

This amounts to an enormous missed opportunity to learn more about the disease, some experts said.

"It's just kind of mind-blowing to me that people are not recording the C.T. values from all these tests — that they're just returning a positive or a negative," said Angela Rasmussen, a virologist at Columbia University in New York.

"It would be useful information to know if somebody's positive, whether they have a high viral load or a low viral load," she added.

Officials at the Wadsworth Center, New York's state lab, have access to C.T. values from tests they have processed, and analyzed their numbers at The Times's request. In July, the lab identified 872 positive tests, based on a threshold of 40 cycles.

With a cutoff of 35, about 43 percent of those tests would no longer qualify as positive. About 63 percent would no longer be judged positive if the cycles were limited to 30.

In Massachusetts, from 85 to 90 percent of people who tested positive in July with a cycle threshold of 40 would have been deemed negative if the threshold were 30 cycles, Dr. Mina said. "I would say that none of those people should be contact-traced, not one," he said.

Other experts informed of these numbers were stunned.

"I'm really shocked that it could be that high — the proportion of people with high C.T. value results," said Dr. Ashish Jha, director of the Harvard Global Health Institute. "Boy, does it really change the way we need to be thinking about testing."

Dr. Jha said he had thought of the PCR test as a problem because it cannot scale to the volume, frequency or speed of tests needed. "But what I am realizing is that a really substantial part of the problem is that we're not even testing the people who we need to be testing," he said.

The number of people with positive results who aren't infectious is particularly concerning, said Scott Becker, executive director of the Association of Public Health Laboratories. "That worries me a lot, just because it's so high," he said, adding that the organization intended to meet with Dr. Mina to discuss the issue.

The F.D.A. noted that people may have a low viral load when they are newly infected. A test with less sensitivity would miss these infections.

But that problem is easily solved, Dr. Mina said: "Test them again, six hours later or 15 hours later or whatever," he said. A rapid test would find these patients quickly, even if it were less sensitive, because their viral loads would quickly rise.

PCR tests still have a role, he and other experts said. For example, their sensitivity is an asset when identifying newly infected people to enroll in clinical trials of drugs.

But with 20 percent or more of people testing positive for the virus in some parts of the country, Dr. Mina and other researchers are questioning the use of PCR tests as a frontline diagnostic tool.

People infected with the virus are most infectious from a day or two before symptoms appear till about five days after. But at the current testing rates, "you're not going to be doing it frequently enough to have any chance of really capturing somebody in that window," Dr. Mina added.

Highly sensitive PCR tests seemed like the best option for tracking the coronavirus at the start of the pandemic. But for the outbreaks raging now, he said, what's needed are coronavirus tests that are fast, cheap and abundant enough to frequently test everyone who needs it — even if the tests are less sensitive.

"It might not catch every last one of the transmitting people, but it sure will catch the most transmissible people, including the superspreaders," Dr. Mina said. "That alone would drive epidemics practically to zero."

Correction: *Sept. 8, 2020*

An earlier version of this article, using information provided by a laboratory spokesman, misstated the number of positive coronavirus tests in July processed by Wadsworth Center, New York's state lab. It was 872 tests, not 794. Based on that error, the article also misstated the number of tests that would no longer qualify as positive with a C.T. value of 35 cycles. It is about 43 percent of the tests, not about half of them. Similarly, the article misstated the number of tests that would no longer qualify as positive if cycles were limited to 30. It is about 63 percent of the tests, not about 70 percent.

EXHIBIT "EE"

PCR positives: What do they mean?

Source: CEBM – University of Oxford

Link: https://www.cebm.net/covid-19/pcr-positives-what-do-they-mean/

Published on: 17 September, 2020

PCR Positives: What do they mean?

Download Pdf:
https://www.cebm.net/wp-content/uploads/2020/09/PCRtestREFERENCE_Infectivity2020Nov5.pdf

Watch video:
https://www.youtube.com/watch?v=muGTrS6DfPM&feature=youtu.be

Contents

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Is the PCR test sensitive enough?

The PCR is very sensitive and will detect the presence of viral RNA (with PCR the virus is detected by targeting one or more gene fragments). The gene fragment might be detected and the virus "positively found". But is this viral RNA active? That is, does the detected viral RNA have the capacity to reproduce or infect the person (virulence) or get transmitted to other people (infectivity)?

The Centre for Evidence-Based Medicine (CEBM) says [1, 2]:

"PCR detection of viruses is helpful so long as its accuracy can be understood: it offers the capacity to detect RNA in minute quantities, but whether that RNA represents infectious virus may not be clear."

Culturing a virus as reference test

What are a reference test and a baseline? Many experiments in science are relative in the sense that they do not give absolute values or need to account for context dependent data. In this sense, it is typical of scientific instrumentation and measurements to require calibration or a baseline. The baseline and calibration allow the scientist to interpret the results. In this respect, the CEBM writes:

"Viral culture [acts] as reference test against which any diagnostic index test for viruses must be measured and calibrated, to understand the predictive properties of that test."

Does a PCR "TRUE POSITIVE" mean INFECTIVITY OR VIRULENCE?
What does viral culture tell about PCR positives?

A PCR test might find the virus it was looking for. This results in a PCR positive, but a crucial question remains: is this virus active, i.e. infectious, or virulent? The PCR alone cannot answer this question. The CEBM explains why culturing the virus is needed to answer this question:

"In viral culture, viruses are injected in the laboratory cell lines to see if they cause cell damage and death, thus releasing a whole set of new viruses that can go on to infect other cells."

That is, if the PCR detects the virus in the human sample, this detection might correspond to a virus that is now incapable of infecting cells and reproducing. Biologists can tell if the virus is infectious by injecting it into cells (culture cells). If these cells are not affected by the virus and the virus does not reproduce in them, then the PCR test found a virus that is no longer active.

The meaning is that the PCR positive is a **non-infectious positive.**

PCR true positives versus infectivity and virulence
Does a PCR positive mean TRUE POSITIVE if the gene fragments targeted in the PCR are unique to the virus and the PCR is VERY ROBUST?

There is speculation as to whether the PCR can indeed find the virus from a person's sample or maybe the PCR is not specific enough and might give positive when other viruses are present. Some PCR manufacturers tell us there

is "cross contamination" and "non-specific" interference with a list of viruses and other in their instructions manuals [3, 4].

POSSIBILITY ONE: the PCR test is positive, but this was due to cross contamination or non-specific interactions. Then the test would be a FALSE POSITIVE because the SARS Cov2 virus is not present in the sample. This means the PCR positive is a FALSE POSITIVE rather than a TRUE POSITIVE. But this is not the only possibility. We want to focus on the CEBM argument that depends on viral culture.

POSSIBILITY TWO: Even if the PCR test only detects TRUE POSITIVES in the sense that the SARS Cov2 virus, or better, the target gene fragment, is present in the sample, it remains to be seen whether the person can infect others or even if the virus is still infecting the very person carrying the virus.

What did Tom Jefferson et al. find in their investigation regarding viral culture of SARS Cov2 in order to assess infectivity (horizontal transmission or capacity for a virus to spreads among hosts) and virulence (a pathogen's ability to infect or damage a host):

"We, therefore, reviewed the evidence from studies reporting data on viral culture or isolation as well as reverse transcriptase-polymerase chain reaction (RT-PCR), to understand more about how the PCR results reflect infectivity."

What does this mean? The authors wanted to find out if **1) PCR TRUE POSITIVE meant that the virus found in the person could be transmitted to other people or was virulent or 2) the virus was no longer infective or virulent.**

Explanation of the experiment that shows whether a virus is still infective

Imagine that a virus enters your body. In a few months it might not do anything to you anymore. It might not do anything to your cells (virulence), and it might also lack the capacity to move into another person (infectivity) when you speak or sneeze. It is also possible that this virus simply never did anything to you and lacked infectivity from the very beginning. But traces of the virus might still be present in the person. In this case, the virus is present but inactive.

So how do you know if the virus is active? You do the PCR. If the virus is found in the person (PCR TRUE POSITIVE), that virus is injected into a culture cell. If by injecting that virus into culture cells, the virus is not able to reproduce in the cells, that virus cannot infect anybody any longer. This means that even if you are a PCR positive, you are no longer contagious, that is, the virus in you is no longer active.

Conclusion: A TRUE POSITIVE in PCR does not always mean that the person presents any danger to society. The virus cannot be transmitted when cell culture shows that the virus is not infective. Unfortunately relating PCR POSITIVE to infectivity is not easy if we consider the whole population. This would need 1) a model (correlation) that maps PCR POSITIVES and/or symptoms to infectivity as tested by viral culture or 2) viral culture for every individual case. See next.

Is there evidence that someone is infectious after PCR results?

Tom Jefferson et al. claim that after searching for the PCR to viral culture correlation no conclusion was found since time from collection and symptoms severity are needed for the correlation amongst other to find an appropriate model. We recall that currently they (governments) hardly look for symptoms in people. Positives are called PCR Positive asymptomatic if they present no symptoms. In the article the authors say:

"Data are sparse on how the PCR results relate to viral culture results. There is some evidence of a relationship between the time from collection of a specimen to test, symptom severity and the chances that someone is infectious.

One of the studies we found (Bullard et al) investigated viral culture in samples from a group of patients and compared the results with PCR testing data and time of their symptom onset." Figure 1.

Conclusion:

"It was not possible to make a precise quantitative assessment of the association between RT-PCR results and the success rate of viral culture within these studies.

This means that PCR Positives might or might not lead to concluding that a subject testing positive by PCR is infectious. Why? Because PCR positives have not been carried to the growth of the virus in culture. They continue to explain why this correlation is not possible:

"These studies were not adequately sized nor performed in a sufficiently standardised manner and may be subject to reporting bias."

Can successive tests on the same person give contradictory results?
That a PCR test gives positive or negative depends on how the experiment is conducted. Furthermore, since it is not known whether and how PCR positives correlates to infectivity and how it is that this correlation must be interpreted, the interpretation of a PCR POSITIVE is inconclusive. The authors claim:

"Cycle thresholds are the times that the amplifying test has to be repeated to get a positive result. The higher the viral concentration the lower amplification cycles are necessary."

Some people might give positive after running the PCR test with a high threshold and others with a low threshold. The threshold alone might or might not tell whether someone carries infective viral RNA.

How long can an inactive virus remain in a body?

This is inconclusive since PCR positives to viral culture studies are lacking and cycle thresholds should also be considered. See above. The way in which the experiment is carried out however, matters. This is because one might be PCR Positive long after the virus is no longer active. The authors briefly explain why:

"This detection problem is ubiquitous for RNA virus's detection. SARS-CoV, MERS, Influenza Ebola and Zika viral RNA can be detected long after the disappearance of the infectious virus. ...because inactivated RNA degrades slowly over time it may still be detected many weeks after infectiousness has dissipated."

The authors show a figure (figure 2) where it is noted that the presence and detection of viral RNA by PCR does not imply that the virus is infectious or virulent any longer.

PCR kits for SARS Cov2 (manufacturers and asymptomatic)

PCR positives on asymptomatic people should be treated with care since it is possible that the asymptomatic people are not infectious. This is even when the PCR tests or the antibody tests are positive. This is because viral culture is required to establish if the viral RNA is capable of infecting cells and "reproduce".

PCR manufacturers typically remind the users that "the detection result of this product is only for clinical reference, and it should not be used as the only evidence for clinical diagnosis and treatment [3]" and "designed for the specific

identification and differentiation of the new coronavirus (SARS-CoV-2) in clinical samples from patients with signs and symptoms of Covid19".

Conclusion in relation to PCR positives and an advancing pandemic

Conclusion: symptoms and signs of Covid19 are necessary to support the claim that the subject is or can be infectious. But calling PCR positives "cases" does not specify whether the persons have carried the virus for long or whether it is "active". This could lead to the finding of many "cases" as a function of the number of PCR tests conducted. For example, if 20% of a population are PCR positive, the number of PCR positives will depend on the size of the sample. This means that the more PCR test are carried out the larger the fraction of the population that is confirmed but this might not speak of changes in the population. That is, it is possible that the population was infected already long before deciding to test and PCR positives would therefore not speak of "an advancing pandemic"

Are PCR tests helpful?

It is typical now to call PCR positives that present no symptoms asymptomatic (see above). It is highly likely that these tests are detecting viral RNA in patients where the virus is no longer capable of infecting. A statistical test where biological equipment would not be required could involve correlating deaths to PCR positives (we discuss this next) The CEBM authors claim:

"PCR detection of viruses is helpful so long as its limitations are understood; while it detects RNA in minute quantities, caution needs to be applied to the results as it often does not detect infectious virus."

Statistical analysis: PCR positives and deaths (excess deaths

We start by claiming that **if PCR positives have any predictive power on the number of deaths expected, there should be some correlation,** i.e. the more PCR positives (SARS Cov2) today the more deaths by Covid19 in the future (at least a few days later but presumably 2-4 weeks later at least if the PCR is taken just after infection). Figure 3 illustrates this.

However, in figure 4 we show PCR positives versus Covid 19 deaths as labeled by the Spanish ministry of health.
https://www.mscbs.gob.es/profesionales/saludPublica/ccaves/alertasActual/nCov/documentos/Actualizacion_207_COVID-19.pdf

Covid19 labelled death versus TRUE death by Covid19

We differentiate between labelled Covid19 and death by Covid19 as the true cause of death. Since we cannot know the true cause of death (this is done by medical examiners but the results are or can be relatively subjective) we will also discuss excess deaths later. A ratio between infections and deaths is the typical way in which mortality is considered [5]. A simple function between PCR positives to Covid19 could be a linear function (Eq. 1). We can add a time delay indicating that it takes time for people to die after being infected (Figures 3 and 4). This function should have some predictive power to be useful. If so, there should be correlation. Here D(t) is the number of deaths at time t (or a given day) and P(t*) is the number of PCR positives at an earlier time $t^*=t-t_0$, where t_0 is the time between the number of deaths D recorded and the number of PCR Positives recorded (typically days to weeks as shown in Figure 5). Here a is the effective mortality rate, i.e. for a number of PCR Positives P, D deaths should be expected after a t_0 ($a = D/P$).

$$D(t) = aP(t^*) \qquad \text{Eq. 1}$$

Figure 5 shows schematically that t_0 is expected to be between 20 and 30 days roughly (4 weeks) and on average. Ideally and accordingly, if the PCR tests were performed during the very first days of infection, Eq. 1 would give us some predictive power over the number of deaths by Covid19 expected in t_0 days (time).

For the Spanish data (Figures 4, 6 and 7) the key points are:

- Figure 4 shows that the same order of magnitude of positives was recorded in March-April 2020 as in July-August-September 2020 but the number of deaths was much lower in August to September (data from the Spanish Ministry of Health). This means that 1) either we do not have the true infection fatality ratio (IFR) but a (CFR), 2) the cases in March April correspond to different phenomena to those in July-September, or 3) the virus has mutated so rapidly that the true IFR has changed already and dramatically. We believe that the second point here is key and the explanation is that the cases in March-April were cases of truly infected people whereas in July-September the cases correspond to people that have mostly passed the infection already, i.e. will not die. The confirmation of this hypothesis would be given by viral culture experiments as discussed by Jefferson et al. above.
- Figure 6 shows that the peak in PCR positives in March-April does not lead to a peak in deaths at the end of April. We ran a correlation test and got numbers in the 0.4-0.2 range. If a delay of 10-20 days is allowed, implying that we want to predict deaths in the future from PCR positives today, the correlation coefficient gave us numbers below 0.2 (not shown). The same happens with the more decent data in July August (not shown).

What if we take into account excess deaths instead?

The data for total deaths in 2020 in Spain, mean number of deaths for the year 2010 to 2019 and confidence interval for those years is provided by the Spainsh Ministerio de Ciencia e Innovacion at https://www.isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/MoMo/Paginas/Informes-MoMo-2020.aspx

We might argue that labelled deaths are not in agreement with the true number of deaths by Covid19. If we take excess deaths instead, this being the number of deaths in 2020 compared to previous years (2010 – 2019) we can plot the normalised excess deaths (blue) against normalised PCR positives (black) in Figure 7. There is no time delay between PCR tests and excess deaths as shown in Figure 7 and it could be argued that this could explain the lack of correlation. We applied a time delay and checked the coefficient of determination for delays ranging from 0 to 45 days (Figure 8).

The highest value for the coefficient of determination R^2 was found by applying no delay as seen in Figure 8. The implication is that the number of positive PCR cases is proportional to the excess deaths reported that day, i.e. with no time delay. If that was the case the PCR testing would be ultimately redundant since knowing the excess deaths tells you at once excess deaths that day which is the variable targeted in the study. We will find no meaningful correlation (correlation coefficients still much below 0.5. figure 8) by applying delays as shown in Figure 8. Data from May to the end of August is shown in a scatter diagram, i.e. PCR positives versus excess deaths, in Figure 9.

CONCLUSIONS

As shown in Figure 8, the more delay we give to the PCR positives recorded on a given day in relation to the excess deaths recorded, the lower R^2. A delay of at least a few days to weeks would be meaningful, i.e. would imply PCR positives

predict the number of deaths in the future since governments could "expect" what is to come in the future on the basis of the number of PCR positive cases recorded on a given day. The R2 number however, and Figures 4, 7, 8 and 9, show that PCR positives do not correlate to excess deaths in the future. The implication is that PCR positives lack predictive power in terms of telling whether people will die in the future.

A possible explanation could be that the PCR positives simply measure the number of PCR tests taken on a given day, i.e. they might be somewhat proportional to the number of PCR taken on a given day, and positives might or might not be "infectious" positives. Ultimately, this means PCR positives cannot be used to tell if the pandemic is advancing if for that we understand that deaths are to increase or decrease. This agrees with the interpretation of CEBM above.

Finally, we want to point out that the same can be said for all countries we have examined, i.e. other than Spain. For example, in the months of July to September positive cases in Europe are said to have risen, but we find no evidence of excess deaths in the countries in Europe reported by euromomo.eu (Figure 10). We believe the rise in deaths toward August and September corresponds to the heat wave. It seems like this year the heat wave has been displaced toward August and September, rather than July and August as in previous years, in some European countries. In this work we have dedicated most attention to the Spanish data but more curves providing Positive PCR cases versus deaths (not excess but Covid19 as reported by each country) can be found at worldometers.info (https://www.worldometers.info/coronavirus/), John Hopkins, and other sources. Such data can be submitted to either visual inspection or PCR positive to excess death correlation as shown here. Our impression is that most data for all countries is in agreement with our interpretation, namely, PCR positives do not correlate to deaths in the future and

are therefore meaningless, on their own, to interpret the spread of the virus in terms of potential deaths.

We suggest that the hypothesis of CEBM, i.e. that viral culture is required as a reference to test for infectivity, and other similar ones such as that by Jared Bullard et al[6]., i.e. search for relations between cycle threshold (Ct), symptom onset and infectivity in cell culture, should be explored in order to increase the predictive power of tests. Such predictive power is central provided the possible advance of the pandemic is to be understood and provided we understand that an advancing pandemic must be related to excess deaths in the future. Finally, regarding deaths, we must consider carefully Covid19 labelled deaths versus excess deaths. Covid19 labelled deaths depend on subjective parameters whether excess deaths have the advantage of being a standard relative to a reference, namely, the number of deaths in previous years. If we find many Covid19 deaths during a period but excess deaths are low or negative, it is likely that we are inflating Covid19 numbers. Furthermore, excess deaths typically depend on high/low temperature, i.e. cold winters or heat waves (Figure 10). Therefore, any light increase/decrease in deaths should be contrasted to be temperature. For example, heat waves might come in June, July, August or even in September (2020 –Spain[7]) in Europe and direct comparison between years should consider this.

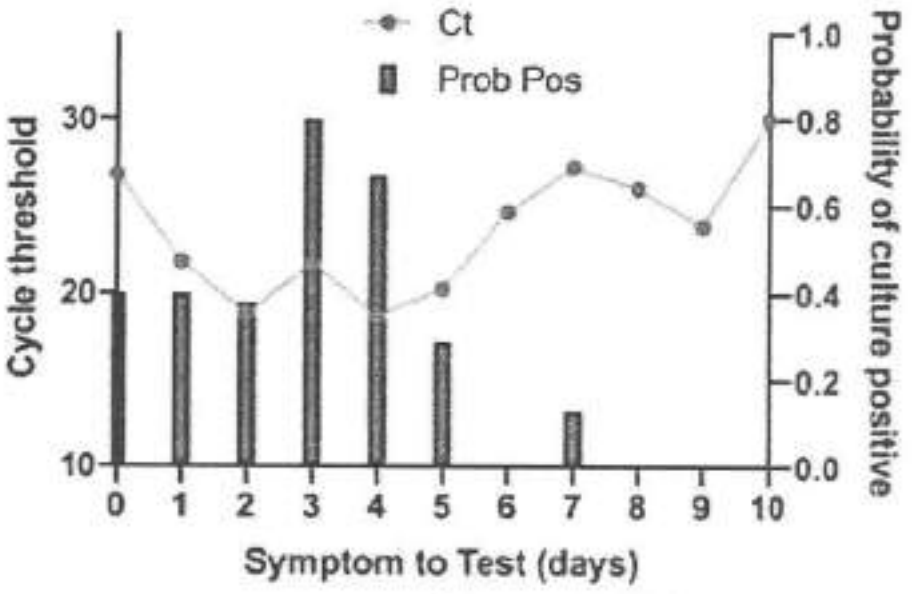


Figure 1. Time from symptom onset to RT-PCR, or symptoms to test (STT), was calculated based on laboratory records. The probability of successfully cultivating SARCoV-2 on Vero cell culture compared to STT is demonstrated in Figure 3. The probability of obtaining a positive viral culture peaked on day 3 and decreased from that point. [6]

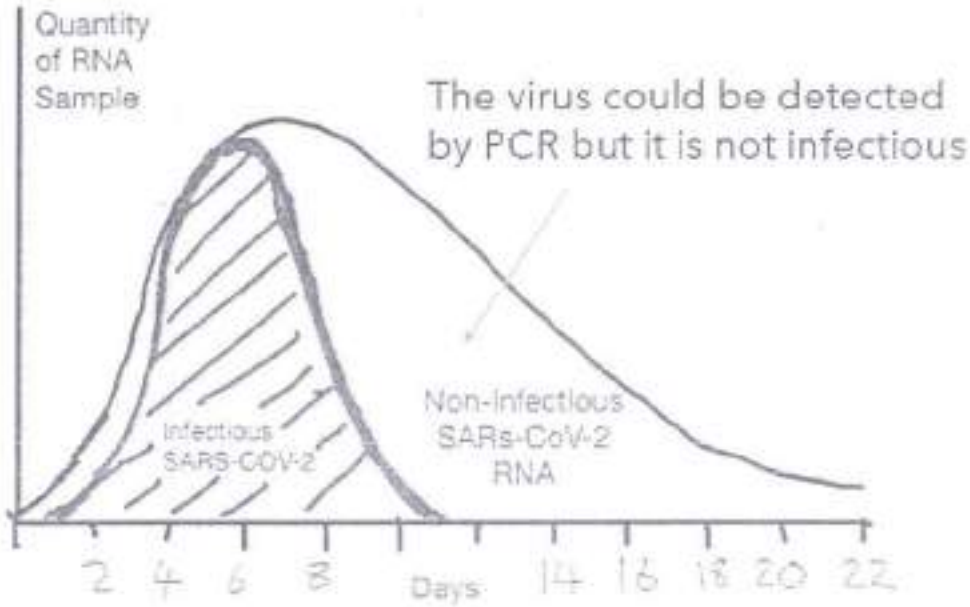


Figure 2. The shaded area shows that up to X days, i.e. 10 days approximately after infection, the virus is infectious. But then the virus is still present many days after. This could result in PCR positive but it does not mean that the virous is virulent or infectious, rather it means that residues and "non active" viral RNA is still detectable by PCR.

Figure 3. True infections today (PCR positives that are taken from a sample where the virus is still infectious or virulent) should lead to deaths in the future.

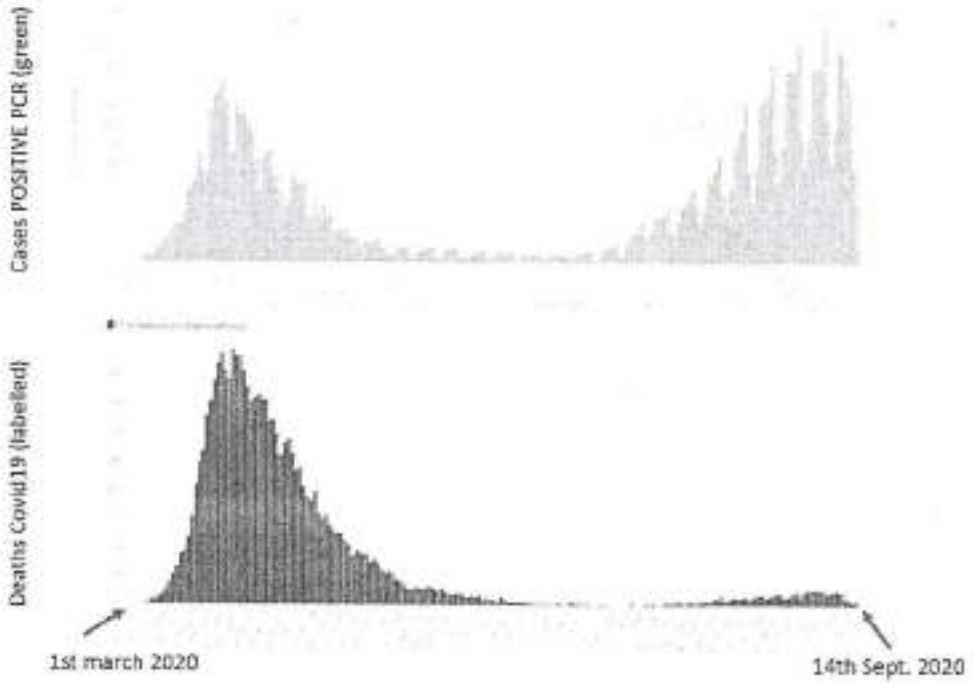
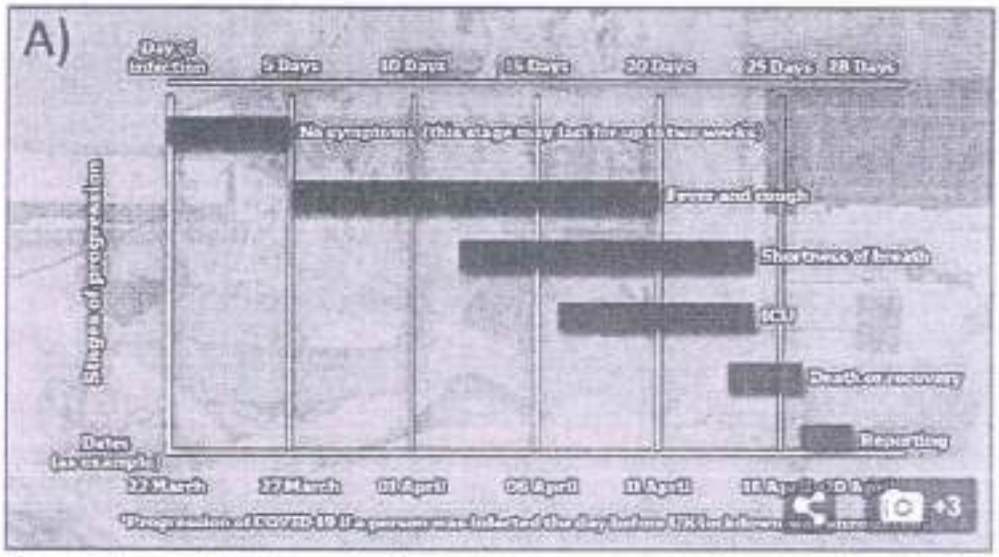


Figure 4. PCR positives in Spain (Top in green) versus deaths labeled as Covid 19 deaths (Bottom brown) from March to the 14th of September in Spain according to the Ministry of health.
https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/documentos/Actualizacion_207_COVID-19.pdf

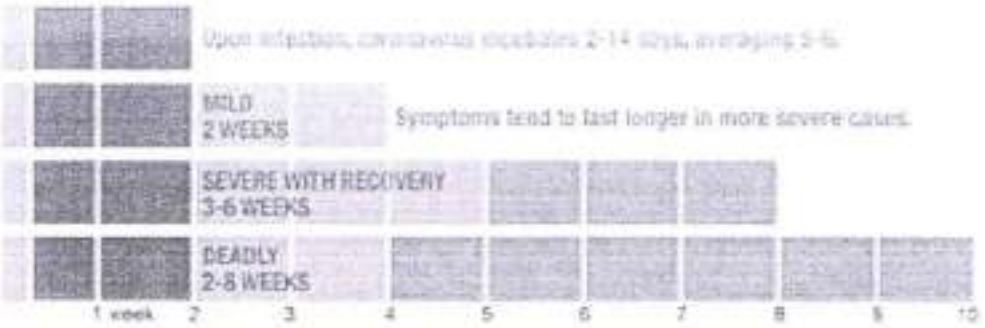


There can be a time lag of more than three weeks between someone becoming infected with coronavirus and dying. Symptoms take days - if not weeks - to become life-threatening. The death has to be recorded and reported, and the family notified, in a process that takes days

B)

COVID-19 Timeline: Infection to Recovery or Death

The course of the disease can run from around 16 days after a short incubation period and mild case to 10 weeks given a long incubation period and worst-case outcome. The following scenarios are estimates based on research to date and input from multiple epidemiologists and medical professionals.



A person can be infectious before symptoms start until symptoms are gone, with a peak about 5 days after symptoms onset. But this timeframe is not well understood, and some people with COVID-19 have no symptoms yet can infect others.

Figure 5. Time sequence from infection to recovery or death from different sources as in a) 4 weeks approx. [8]. and b) 2 to 8 weeks approx.. [9]

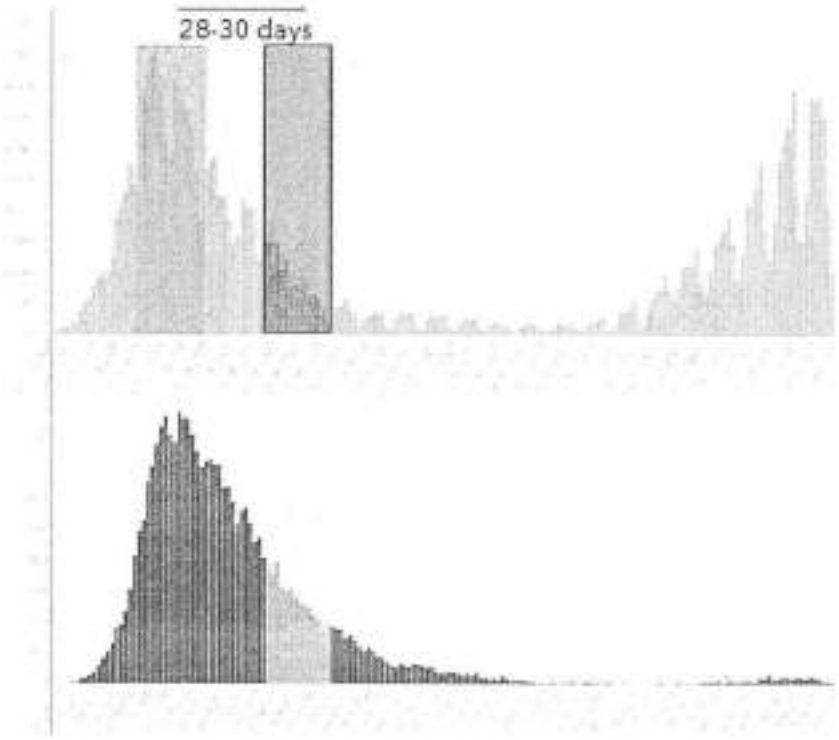


Figure 6. The peak in PCR positives in March-April in Spain (top green) does not lead to a peak in deaths 20-40 days latter (bottom brown).

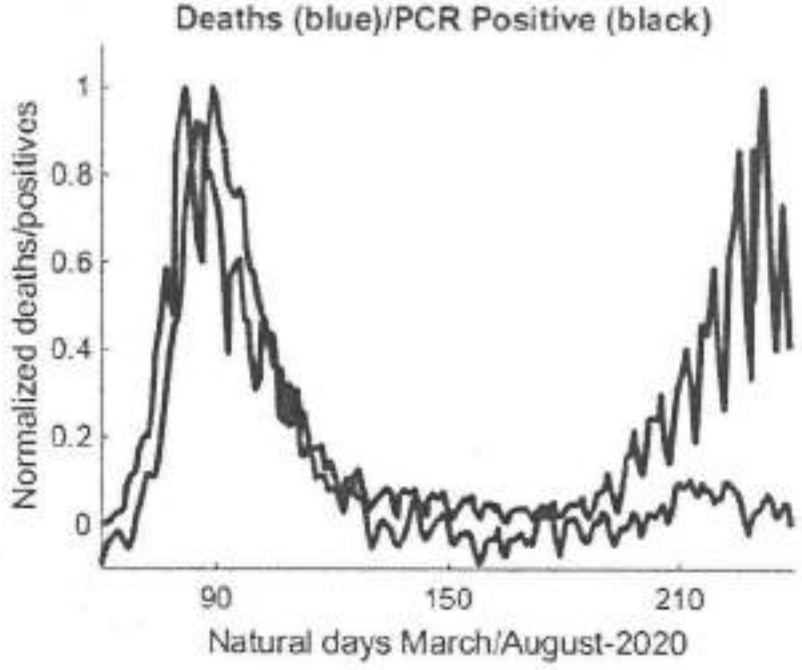


Figure 7. Normalized excess deaths in Spain (blue) against PCR positives (black).

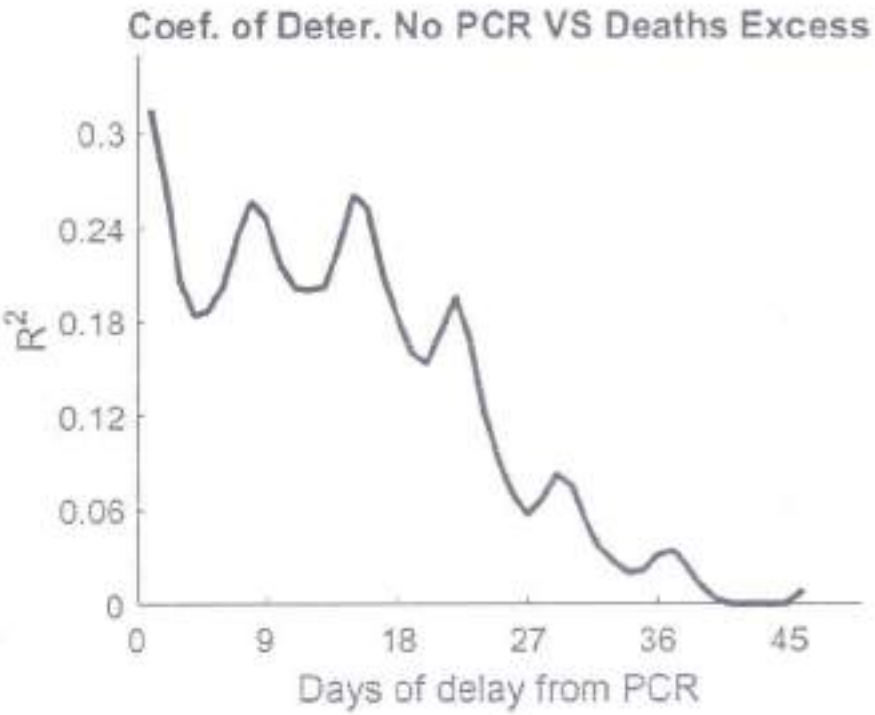


Figure 8. The x axis stands for the days of delay from the number of PCR positive recorded to the number of excess deaths. For example, if the X PCR positives were recorded today, 27 days of delay would mean that X is mapped to the excess deaths 27 days after the recording of the PCR positives. The y axis gives the coefficient of determination R2 as a function of days of delay. The highest values correspond to the proportionality between excess deaths "today" and "PCR positives today" implying that PCR tests lack any predictive power by being redundant at most.

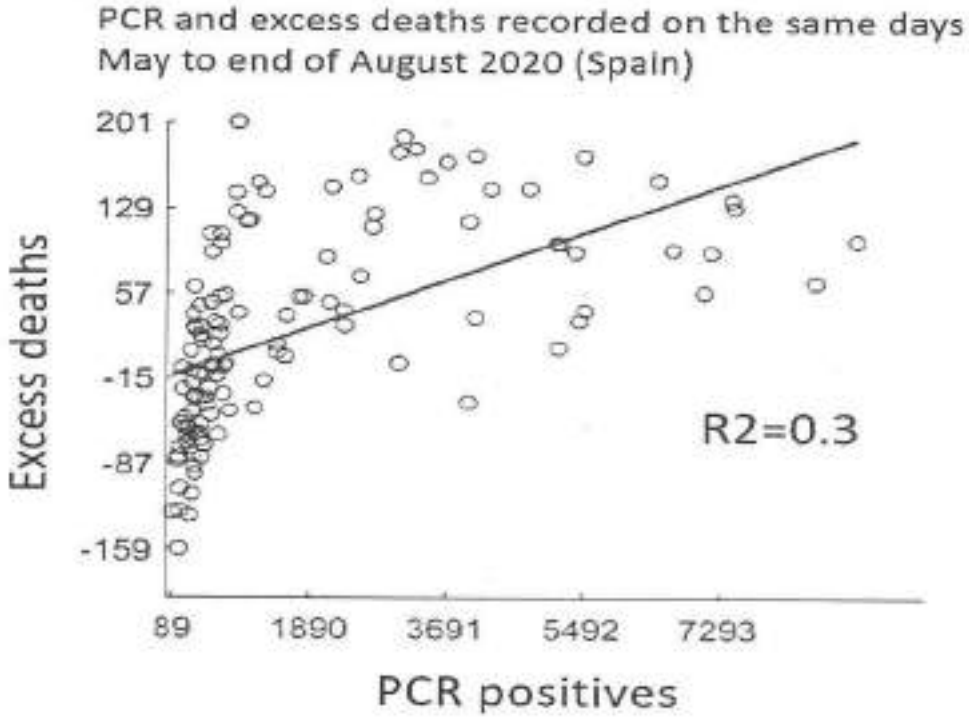


Figure 9. Scatter plot showing PCR positives versus excess deaths from may to the end of August. The coefficient of determination R^2 is 0.3 and is highest when plotting the PCR positives recorded on the same day that excess deaths are recorded. The implication is that PCR positives have no "predictive power" since in this way they cannot predict if excess deaths will follow from PCR positives. As shown in Figure 8, the more delay we give to PCR in relation to excess deaths, the lower R^2. A delay of at least a few days to weeks would be meaningful since governments could "expect" what is to come in the future on the basis of the number of PCR positive cases recorded. As shown the PCR positives do not correlate to excess deaths in the future and therefore lack predictive power.

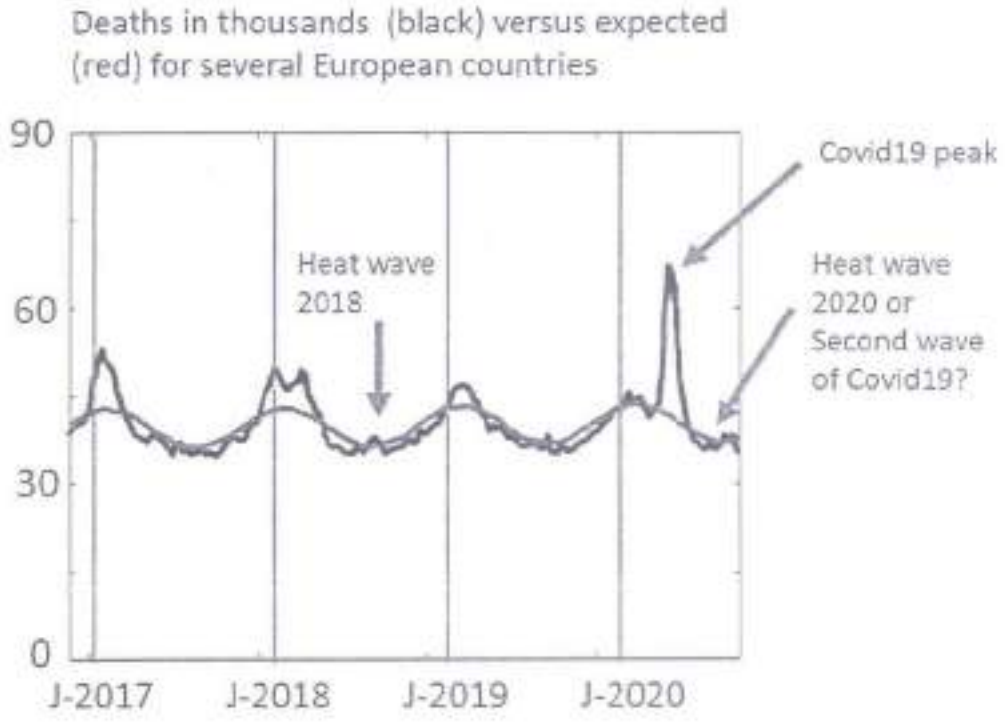


Figure 10. Deaths from 2017 to September of 2020 for several countries in Europe as recorded by euromomo.eu (https://www.euromomo.eu/graphs-and-maps)

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EXHIBIT "FF"

Revised Discharge Policy for COVID-19

The revised discharge policy is aligned with the guidelines on the 3 tier COVID facilities and the categorization of the patients based on clinical severity (Available at: https://www.mohfw.gov.in/pdf/FinalGuidanceonMangaementofCovidcasesversion2.pdf)

1. Mild/very mild/pre-symptomatic cases

Mild/very mild/pre-symptomatic cases admitted to a COVID Care Facility will undergo regular temperature and pulse oximetry monitoring. The patient can be discharged after 10 days of symptom onset and no fever for 3 days. There will be no need for testing prior to discharge.

At the time of discharge, the patient will be advised to isolate himself at home and self-monitor their health for further 7 days.

At any point of time, prior to discharge from CCC, if the oxygen saturation dips below 95%, patient is moved to Dedicated COVID Health Centre (DCHC).

After discharge from the facility, if he/she again develops symptoms of fever, cough or breathing difficulty he will contact the COVID Care Centre or State helpline or 1075. His/her health will again be followed up through tele-conference on 14th day.

2. Moderate cases admitted to Dedicated COVID Health Centre (Oxygen beds)

2.1. Patients whose symptoms resolve within 3 days and maintains saturation above 95% for the next 4 days

Cases clinically classified as "moderate cases" will undergo monitoring of body temperature and oxygen saturation. If the fever resolve within 3 days and the patient maintains saturation above 95% for the next 4 days (without oxygen support), such patient will be discharged after 10 days of symptom onset in case of:

- Absence of fever without antipyretics
- Resolution of breathlessness
- No oxygen requirement

There will be no need for testing prior to discharge.

At the time of discharge, the patient will be advised to isolate himself at home and self-monitor their health for further 7 days.

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2.2. Patient on Oxygenation whose fever does not resolve within 3 days and demand of oxygen therapy continues

Such patients will be discharged only after

- resolution of clinical symptoms
- ability to maintain oxygen saturation for 3 consecutive days

3. Severe Cases including immunocompromised (HIV patients, transplant recipients, malignancy)

Discharge criteria for severe cases will be based on

- Clinical recovery
- Patient tested negative once by RT-PCR (after resolution of symptoms)

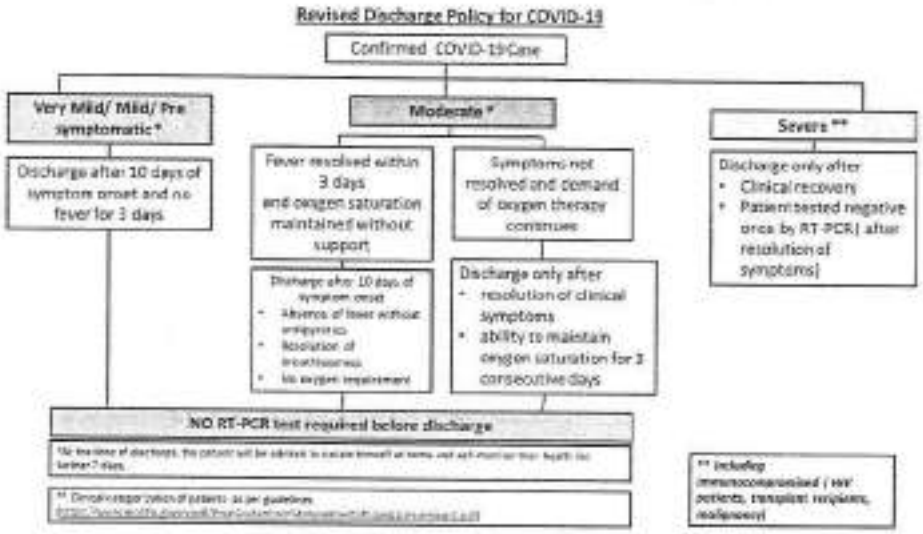

EXHIBIT "G G"

भारतीय आयुर्विज्ञान अनुसंधान परिषद
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार
कल्याण मंत्रालय, भारत सरकार

Indian Council of Medical Research
Department of Health Research, Ministry of Health
and Family Welfare, Government of India

Date: 04/05/2021

Advisory for COVID-19 testing during the second wave of the pandemic

Context

An unprecedented upsurge of COVID-19 cases and deaths is currently being witnessed across India. The overall nationwide test positivity rate is above 20%. Testing-tracking-tracing, isolation and home-based treatment of positive patients is the key measure to curb transmission of SARS-CoV-2, the causative agent of COVID-19. As on today, India has a total of 2506 molecular testing laboratories including RTPCR, TrueNat, CBNAAT and other platforms. The total daily National testing capacity is close to 15 lakh tests considering a three- shift operationalization of the existing laboratory network. At present, the laboratories are facing challenges to meet the expected testing target due to extraordinary case load and staff getting infected with COVID-19. In view of this situation, it is imperative to optimize the RTPCR testing and simultaneously increase the access and availability of testing to all citizens of the country.

Recommended measures to optimize RTPCR testing

i. RTPCR test must not be repeated in any individual who has tested positive once either by RAT or RTPCR.
ii. No testing is required for COVID-19 recovered individuals at the time of hospital discharge in accordance with the discharge policy of MoH&FW (*https://www.mohfw.gov.in/pdf/ReviseddischargePolicyforCOVID19.pdf*).
iii. The need for RTPCR test in healthy individuals undertaking inter-state domestic travel may be completely removed to reduce the load on laboratories.
iv. Non-essential travel and interstate travel of symptomatic individuals (COVID-19 or flu like symptoms) should be essentially avoided to reduce the risk of infection.
v. All asymptomatic individuals undertaking essential travel must follow COVID appropriate behavior.
vi. Mobile testing laboratories are now available on GeM portal. States are encouraged to augment RTPCR testing through mobile systems.

Measures to improve access and availability of testing:

Rapid antigen tests (RATs) were recommended in India for COVID-19 testing in June 2020. However, the use of these tests is currently limited to containment zones and health care settings. RAT has a short turn-around time of 15-30 minutes and thus offers a huge advantage of quick detection of cases and opportunity to isolate and treat them early for curbing transmission. So far, ICMR has approved 36 RATs of which 10 are on GeM portal. To meet the overwhelming testing demand, it will be prudent to upscale testing using RATs.

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Measures to ramp up testing through RAT

i. RAT may be allowed at all available Government and private healthcare facilities.

ii. Set up dedicated RAT booths in cities, towns and villages to offer testing to people.

iii. Testing booths may be set up at multiple locations including healthcare facilities, RWA, offices, schools, colleges, community centers and other available vacant spaces.

iv. These booths should be operational on a 24X7 basis to improve access and availability of testing.

v. Drive-through RAT testing facilities may be created at convenient locations as identified by local administration.

vi. Stringent measures must be instituted to avoid overcrowding at RAT testing facilities.

Additional advice

- All states are advised to ensure full utilization of the available RTPCR testing capacity, both in public and private laboratories.
- RAT testing must be conducted in compliance with the ICMR advisory available at:
  https://www.icmr.gov.in/pdf/covid/strategy/Testing_Strategy_v6_04092020.pdf
- Symptomatic individuals identified positive by RAT should not be re-tested and advised to go through home-based care as per ICMR guidelines available at: *https://www.icmr.gov.in/pdf/covid/techdoc/COVID_HOME_CARE.pdf.*
- Symptomatic individuals identified negative by RAT should be linked with RTPCR test facility and in the meantime be urged to follow home isolation and treatment.
- All RTPCR and RAT test results should be uploaded on ICMR portal at: *https://cvstatus.icmr.gov.in.*
- During the current upsurge of COVID-19 cases, any individual presenting with fever with / without cough, headache, sore throat, breathlessness, bodyache, recent loss of taste or smell, fatigue and diarrhea should be considered as suspect case of COVID-19 unless proven otherwise by confirmation of another etiology.
- The vaccination status of all individuals tested for COVID-19 must be entered into the Sample Referral Form (SRF) in the RTPCR app both for individuals tested by RTPCR and RAT. This information is of critical importance.
- Payment modalities for upscaled RAT testing may be decided by the state health authorities.

EXHIBIT HH

609

World Health Organization

WHO Information Notice for Users 2020/05

Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2

20 January 2021 | Medical product alert | Geneva | Reading time: 1 min (370 words)

Français | Español

Product type: Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2

Date: 13 January 2021

WHO-identifier: 2020/5, version 2

Target audience: laboratory professionals and users of IVDs.

Purpose of this notice: clarify information previously provided by WHO. This notice supersedes WHO Information Notice for In Vitro Diagnostic Medical Device (IVD) Users 2020/05 version 1, issued 14 December 2020.

Description of the problem: WHO requests users to follow the instructions for use (IFU) when interpreting results for specimens tested using PCR methodology.

Users of IVDs must read and follow the IFU carefully to determine if manual adjustment of the PCR positivity threshold is recommended by the manufacturer.

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WHO guidance Diagnostic testing for SARS-CoV-2 states that careful interpretation of weak positive results is needed (*1*). The cycle threshold (Ct) needed to detect virus is inversely proportional to the patient's viral load. Where test results do not correspond with the clinical presentation, a new specimen should be taken and retested using the same or different NAT technology.

WHO reminds IVD users that disease prevalence alters the predictive value of test results; as disease prevalence decreases, the risk of false positive increases (*2*). This means that the probability that a person who has a positive result (SARS-CoV-2 detected) is truly infected with SARS-CoV-2 decreases as prevalence decreases, irrespective of the claimed specificity.

Most PCR assays are indicated as an aid for diagnosis, therefore, health care providers must consider any result in combination with timing of sampling, specimen type, assay specifics, clinical observations, patient history, confirmed status of any contacts, and epidemiological information.

Actions to be taken by IVD users:

1. Please read carefully the IFU in its entirety.
2. Contact your local representative if there is any aspect of the IFU that is unclear to you.
3. Check the IFU for each incoming consignment to detect any changes to the IFU.
4. Provide the Ct value in the report to the requesting health care provider.

Contact person for further information:

Anita SANDS, Regulation and Prequalification, World Health Organization, e-mail: rapidalert@who.int

References:

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EXHIBIT "II"

Faith in Quick Test Leads to Epidemic That Wasn't

Dr. Brooke Herndon of Dartmouth-Hitchcock Medical Center, shown at left this month, was told last spring that she appeared to have whooping cough.
Jon Gilbert Fox for The New York Times

By Gina Kolata
Jan. 22, 2007

Dr. Brooke Herndon, an internist at Dartmouth-Hitchcock Medical Center, could not stop coughing. For two weeks starting in mid-April last year, she coughed, seemingly nonstop, followed by another week when she coughed sporadically, annoying, she said, everyone who worked with her.

Before long, Dr. Kathryn Kirkland, an infectious disease specialist at Dartmouth, had a chilling thought: Could she be seeing the start of a whooping cough epidemic? By late April, other health care workers at the hospital were coughing, and severe, intractable coughing is a whooping cough hallmark. And if it was whooping cough, the epidemic had to be contained immediately because the disease could be deadly to babies in the hospital and could lead to pneumonia in the frail and vulnerable adult patients there.

It was the start of a bizarre episode at the medical center: the story of the epidemic that wasn't.

For months, nearly everyone involved thought the medical center had had a huge whooping cough outbreak, with extensive ramifications. Nearly 1,000 health care workers at the hospital in Lebanon, N.H., were given a preliminary test and furloughed from work until their results were in; 142 people, including Dr. Herndon, were told they appeared to have the disease; and thousands were given antibiotics and a vaccine for protection. Hospital beds were taken out of commission, including some in intensive care.

Then, about eight months later, health care workers were dumbfounded to receive an e-mail message from the hospital administration informing them that the whole thing was a false alarm.

Not a single case of whooping cough was confirmed with the definitive test, growing the bacterium, Bordetella pertussis, in the laboratory. Instead, it appears the health care workers probably were afflicted with ordinary respiratory diseases like the common cold.

Now, as they look back on the episode, epidemiologists and infectious disease specialists say the problem was that they placed too much faith in a quick and highly sensitive molecular test that led them astray.

Infectious disease experts say such tests are coming into increasing use and may be the only way to get a quick answer in diagnosing diseases like whooping cough, Legionnaire's, bird flu, tuberculosis and SARS, and deciding whether an epidemic is under way.

There are no national data on pseudo-epidemics caused by an overreliance on such molecular tests, said Dr. Trish M. Perl, an epidemiologist at Johns Hopkins and past president of the Society of Health Care Epidemiologists of America. But, she said, pseudo-epidemics happen all the time. The Dartmouth case may have been one the largest, but it was by no means an exception, she said.

There was a similar whooping cough scare at Children's Hospital in Boston last fall that involved 38 adults and 2 children. Definitive tests, though, did not find pertussis.

"It's a problem; we know it's a problem," Dr. Perl said. "My guess is that what happened at Dartmouth is going to become more common."

Many of the new molecular tests are quick but technically demanding, and each laboratory may do them in its own way. These tests, called "home brews," are not commercially available, and there are no good estimates of their error rates. But their very sensitivity makes false positives likely, and when hundreds or thousands of people are tested, as occurred at Dartmouth, false positives can make it seem like there is an epidemic.

"You're in a little bit of no man's land," with the new molecular tests, said Dr. Mark Perkins, an infectious disease specialist and chief scientific officer at the Foundation for Innovative New Diagnostics, a nonprofit foundation supported by the Bill and Melinda Gates Foundation. "All bets are off on exact performance."

Of course, that leads to the question of why rely on them at all. "At face value, obviously they shouldn't be doing it," Dr. Perl said. But, she said, often when answers are needed and an organism like the pertussis bacterium is finicky and hard to grow in a laboratory, "you don't have great options."

Waiting to see if the bacteria grow can take weeks, but the quick molecular test can be wrong. "It's almost like you're trying to pick the least of two evils," Dr. Perl said.

At Dartmouth the decision was to use a test, P.C.R., for polymerase chain reaction. It is a molecular test that, until recently, was confined to molecular biology laboratories.

"That's kind of what's happening," said Dr. Kathryn Edwards, an infectious disease specialist and professor of pediatrics at Vanderbilt University. "That's the reality out there. We are trying to figure out how to use methods that have been the purview of bench scientists."

The Dartmouth whooping cough story shows what can ensue.

To say the episode was disruptive was an understatement, said Dr. Elizabeth Talbot, deputy state epidemiologist for the New Hampshire Department of Health and Human Services.

"You cannot imagine," Dr. Talbot said. "I had a feeling at the time that this gave us a shadow of a hint of what it might be like during a pandemic flu epidemic."

Yet, epidemiologists say, one of the most troubling aspects of the pseudo-epidemic is that all the decisions seemed so sensible at the time.

Dr. Katrina Kretsinger, a medical epidemiologist at the federal Centers for Disease Control and Prevention, who worked on the case along with her colleague Dr. Manisha Patel, does not fault the Dartmouth doctors.

"The issue was not that they overreacted or did anything inappropriate at all," Dr. Kretsinger said. Instead, it is that there is often is no way to decide early on whether an epidemic is under way.

Before the 1940s when a pertussis vaccine for children was introduced, whooping cough was a leading cause of death in young children. The vaccine led to an 80 percent drop in the disease's incidence, but did not completely eliminate it. That is because the vaccine's effectiveness wanes after about a decade, and although there is now a new vaccine for adolescents and adults, it is only starting to come into use. Whooping cough, Dr. Kretsinger said, is still a concern.

The disease got its name from its most salient feature: Patients may cough and cough and cough until they have to gasp for breath, making a sound like a whoop. The coughing can last so long that one of the common names for whooping cough was the 100-day cough, Dr. Talbot said.

But neither coughing long and hard nor even whooping is unique to pertussis infections, and many people with whooping cough have symptoms that like those of common cold: a runny nose or an ordinary cough.

"Almost everything about the clinical presentation of pertussis, especially early pertussis, is not very specific," Dr. Kirkland said.

That was the first problem in deciding whether there was an epidemic at Dartmouth.

The second was with P.C.R., the quick test to diagnose the disease, Dr. Kretsinger said.

With pertussis, she said, "there are probably 100 different P.C.R. protocols and methods being used throughout the country," and it is unclear how often any of them are accurate. "We have had a number of outbreaks where we believe that despite the presence of P.C.R.-positive results, the disease was not pertussis," Dr. Kretsinger added.

At Dartmouth, when the first suspect pertussis cases emerged and the P.C.R. test showed pertussis, doctors believed it. The results seem completely consistent with the patients' symptoms.

"That's how the whole thing got started," Dr. Kirkland said. Then the doctors decided to test people who did not have severe coughing.

"Because we had cases we thought were pertussis and because we had vulnerable patients at the hospital, we lowered our threshold," she said. Anyone who had a cough got a P.C.R. test, and so did anyone with a runny nose who worked with high-risk patients like infants.

"That's how we ended up with 134 suspect cases," Dr. Kirkland said. And that, she added, was why 1,445 health care workers ended up taking antibiotics and 4,524 health care workers at the hospital, or 72 percent of all the health care workers there, were immunized against whooping cough in a matter of days.

"If we had stopped there, I think we all would have agreed that we had had an outbreak of pertussis and that we had controlled it," Dr. Kirkland said.

But epidemiologists at the hospital and working for the States of New Hampshire and Vermont decided to take extra steps to confirm that what they were seeing really was pertussis.

The Dartmouth doctors sent samples from 27 patients they thought had pertussis to the state health departments and the Centers for Disease Control. There, scientists tried to grow the bacteria, a process that can take weeks. Finally, they had their answer: There was no pertussis in any of the samples.

"We thought, Well, that's odd," Dr. Kirkland said. "Maybe it's the timing of the culturing, maybe it's a transport problem. Why don't we try serological testing? Certainly, after a pertussis infection, a person should develop antibodies to the bacteria."

They could only get suitable blood samples from 39 patients — the others had gotten the vaccine which itself elicits pertussis antibodies. But when the Centers for Disease Control tested those 39 samples, its scientists reported that only one showed increases in antibody levels indicative of pertussis.

The disease center did additional tests too, including molecular tests to look for features of the pertussis bacteria. Its scientists also did additional P.C.R. tests on samples from 116 of the 134 people who were thought to have whooping cough. Only one P.C.R. was positive, but other tests did not show that that person was infected with pertussis bacteria. The disease center also interviewed patients in depth to see what their symptoms were and how they evolved.

"It was going on for months," Dr. Kirkland said. But in the end, the conclusion was clear: There was no pertussis epidemic.

"We were all somewhat surprised," Dr. Kirkland said, "and we were left in a very frustrating situation about what to do when the next outbreak comes."

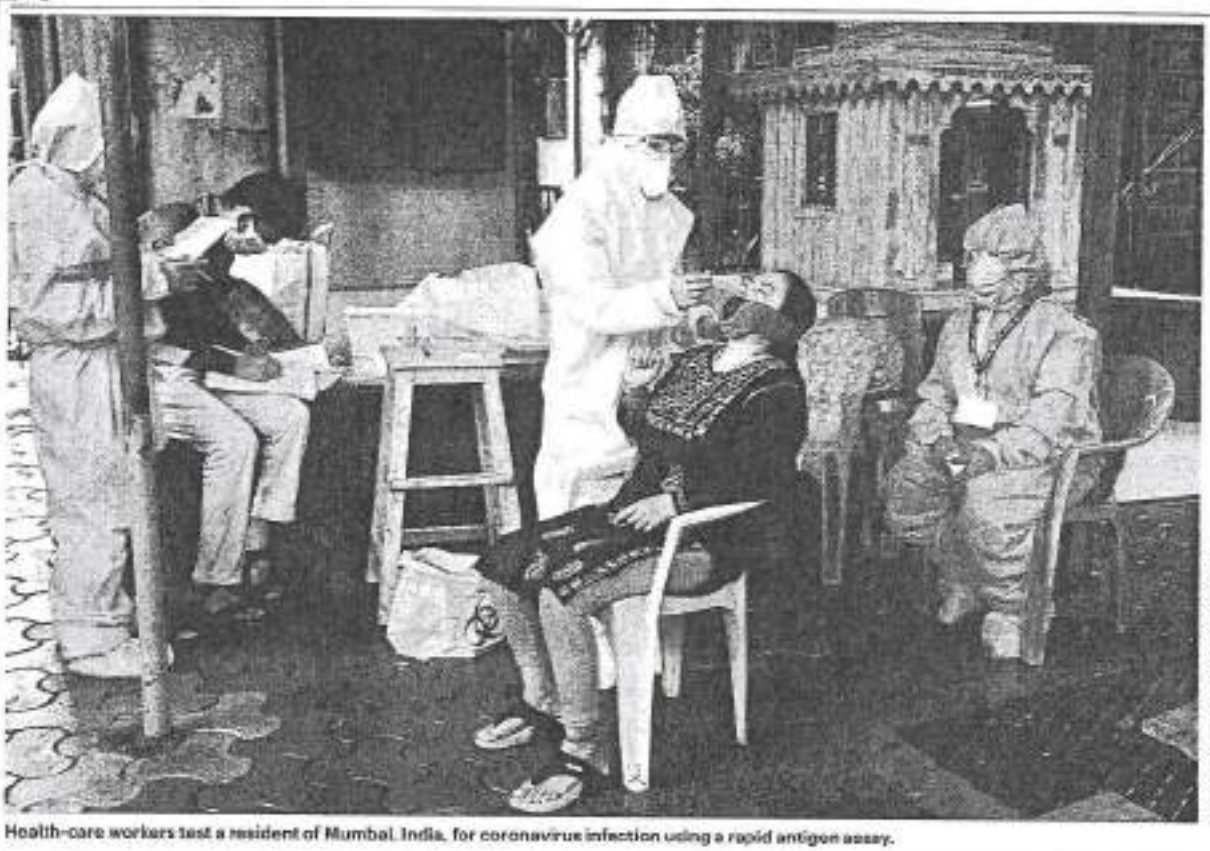
Dr. Cathy A. Petti, an infectious disease specialist at the University of Utah, said the story had one clear lesson.

"The big message is that every lab is vulnerable to having false positives," Dr. Petti said. "No single test result is absolute and that is even more important with a test result based on P.C.R."

As for Dr. Herndon, though, she now knows she is off the hook.

"I thought I might have caused the epidemic," she said.

Correction: Jan. 29, 2007
The credit for pictures last Monday with the continuation of a front-page article about a whooping cough scare at Dartmouth-Hitchcock Medical Center omitted the photographer's surname. He is Jon Gilbert Fox.



Health-care workers test a resident of Mumbai, India, for coronavirus infection using a rapid antigen assay.

FAST CORONAVIRUS TESTS ARE COMING

Rapid antigen tests are designed to tell in a few minutes whether someone is infectious. Will they be game changers?

By Giorgia Guglielmi

The United States leads the world in COVID-19 deaths but lags behind many countries – both large and small – in testing capacity. That could soon change.

At the end of August, the US Food and Drug Administration (FDA) granted emergency-use approval to a new credit-card-sized testing device for the coronavirus that costs US$5, gives results in 15 minutes and doesn't require a laboratory or a machine for processing. The United States is spending $760 million on 150 million of these tests from health-care company Abbott Laboratories, headquartered in Abbott Park, Illinois, which plans to ramp up production to 50 million per month in October.

The tests detect specific proteins – known as antigens – on the surface of the virus, and can identify people who are at the peak of infection, when virus levels in the body are likely to be high. Proponents argue that this could be a game changer. Antigen tests could help to keep the pandemic at bay, because they can be rolled out in vast numbers and can spot those who are at greatest risk of spreading the disease. These tests are also a key element in the testing strategies of other countries, such as India and Italy.

Antigen assays are much faster and cheaper than the gold-standard tests that detect viral RNA using a technique called the polymerase chain reaction (PCR). But antigen tests aren't as sensitive as the PCR versions, which can pick up minuscule amounts of the SARS-CoV-2 virus that causes COVID-19.

This difference raises some concerns among specialists, who worry that antigen tests will miss infectious people and result in outbreaks in countries that have largely controlled coronavirus transmission. Others view the lower sensitivity as an attribute, because some people who receive positive PCR test results are infected, but are no longer able to spread the virus to others. So antigen tests could shift the focus to identifying the most infectious people.

At present, antigen tests are administered by trained professionals, but some companies are developing versions that are simple enough to be used at home – similar to pregnancy tests.

"Making the tests faster, cheaper, easier is definitely the goal – and I think the antigen test is the way to get there," says Martin Burke, a chemist at the University of Illinois at Urbana-Champaign, who is co-developing rapid tests, including antigen-based assays.

"This is by no means the perfect solution, it's just the fastest thing we could get going now," he says.

What tests are there and how do they work?

Tests for COVID-19 fall into two categories: diagnostic tests such as PCR and antigen assays, which detect parts of the SARS-CoV-2 virus, and antibody tests that sense molecules that people produce when they have been infected by the virus. Antibodies can take several days to develop after an infection and often stay in the blood for weeks after recovery, so antibody tests have limited use in diagnosis (see 'Catching COVID-19').

The high-sensitivity PCR tests are almost 100% accurate in spotting infected people, when they are administered properly. But such tests generally require trained personnel, specific reagents and expensive machines that take hours to provide results.

Countries such as South Korea and New Zealand have succeeded in boosting PCR-based testing, but scaling up these tests has proved difficult elsewhere. The United States, for example, has seen a slow and poorly coordinated response to outbreaks, faulty tests from the Centers for Disease Control and Prevention (CDC) and problems with the supply chain. All of this has hindered efforts to collect and process samples for PCR, pushing waiting times to days or even weeks. These delays, along with a lack of tests, have contributed to the rampant spread of COVID-19 across the country, which by 18 September had seen almost 200,000 deaths from the disease.

A typical antigen test starts with a healthcare professional swabbing the back of a person's nose or throat — although companies are developing kits that use saliva samples, which are easier and safer to collect than a swab. The sample is then mixed with a solution that breaks the virus open and frees specific viral proteins. The mix is added to a paper strip that contains an antibody tailored to bind to these proteins. If they're present in the solution, a positive test result can be detected either as a fluorescent glow or as a dark band on the paper strip.

Antigen tests give results in less than 30 minutes, don't have to be processed in a lab and are cheap to produce. Yet that speed comes with a cost in sensitivity. Whereas a typical PCR test can detect a single molecule of RNA in a microlitre of solution, antigen tests need a sample to contain thousands — probably tens of thousands — of virus particles per microlitre to produce a positive result[2]. So, if a person has low amounts of virus in their body, the test might give a false-negative result.

When used on people who were positive for SARS-CoV-2 in a standard PCR test, Abbott's antigen assay correctly spotted the virus in 95–100% of cases if the samples were collected within a week of the onset of symptoms. But that proportion dropped to 75% if samples were taken more than a week after people first showed symptoms. The sensitivity — or the rate of detecting infections correctly — of the other antigen tests used in the United States is between 84% and 98% if a person is tested in the week after showing symptoms.

Companies and academic research labs are also rolling out other tests that are faster, cheaper and more user-friendly than standard PCR assays, although they are not being produced on the same scale as antigen tests. Some of these other tests use the gene-editing tool CRISPR to zero in on genetic snippets of the coronavirus. Others are quicker variants of the PCR test that use different reagents, meaning they're not limited by the same supply-chain problems. Saliva-based PCR tests, for example, are being used as screening tools in universities and for professional basketball teams.

Which tests tell whether someone is infectious?

Although the PCR method can test whether someone is infectious, it also detects people who have the virus but are not likely to spread it.

Antigen-based testing, by contrast, could help to rapidly identify people who have high levels of virus — those who are most likely to be infectious to others — and isolate them from the community, says Marion Koopmans, a virologist at the Erasmus University Medical Centre in Rotterdam, the Netherlands. "The question is, what is the safe limit? Because the moment you get that wrong, the whole idea implodes," she says. It's still unclear what viral load is the threshold below which a person is no longer contagious, says Koopmans, who is working with the World Health Organization (WHO) to determine a standard to validate rapid tests. "It would be very worrying if everyone does that on their own, using different criteria," she says.

Viral load peaks early in SARS-CoV-2 infections and then gradually declines, with tiny amounts of virus RNA staying in someone's nose or throat for weeks or possibly months[3]. And although there are not enough data to equate different viral levels with how infectious people are, there is evidence that individuals are unlikely to spread the virus about eight to ten days after showing symptoms[3].

"If you're at risk of transmitting the virus to somebody else, you're going to have plenty of viral particles — those would certainly show up in antigen tests," says Michael Mina, an infectious-disease immunologist at the Harvard T. H. Chan School of Public Health in Boston, Massachusetts, who has been a vocal proponent of antigen tests.

There are challenges at the start of the infection, when people have low levels of the virus. The answer, says Mina, is frequent testing — done multiple times per week. This could quickly identify infected people, even if the assays are less sensitive than a PCR-based test, because the amount of virus in their noses and throats rises within hours, he says.

Mina and his colleagues have used statistical models to assess this strategy. In a preprint updated on 8 September, they suggest that testing people twice a week with a relatively insensitive test could be more effective at curbing the spread of SARS-CoV-2 than are more accurate tests done once every two weeks[3]. Another study that modelled different scenarios for safely reopening university campuses reported similar findings[4].

To slow outbreaks, the focus should be on identifying those who are at risk of spreading SARS-CoV-2 to other people, rather than on spotting anyone who is infected with it, some experts say.

When used as a screening tool to frequently assess as many people as possible, rapid antigen tests could be "a game changer", says Rebecca Lee Smith, an epidemiologist at the University of Illinois.

How do countries plan to use antigen tests?

At the beginning of April, as coronavirus outbreaks raged across the world, India had tested only about 150,000 people — one of the lowest testing rates per capita worldwide. On 21 August, the country conducted more than one million coronavirus tests in a single day. It reached that milestone after Indian authorities began using antigen assays to boost testing capacity.

Delhi was the first Indian state to begin using rapid antigen tests in June. By mid-July, the

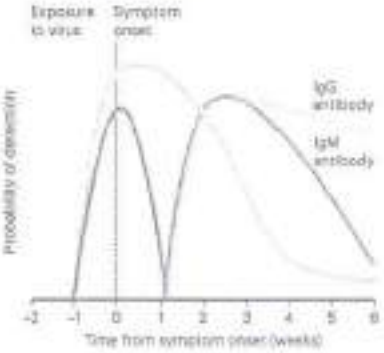
CATCHING COVID-19

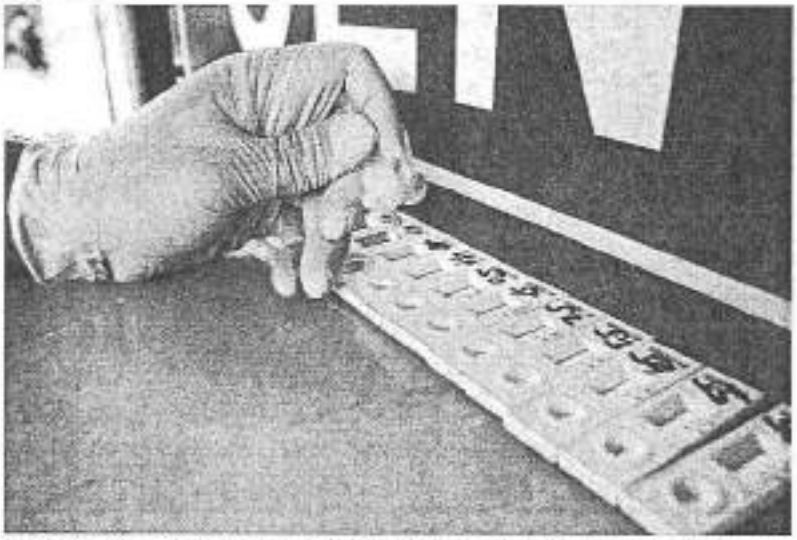
Different types of COVID-19 test can detect the presence of the SARS-CoV-2 virus or the body's response to infection. The probability of a positive result varies with each test before and after symptoms appear.

— **PCR-based tests** detect small amounts of viral genetic material, so a test can be positive long after a person stops being infectious.

— **Rapid antigen tests** detect the presence of viral proteins and can return positive results when a person is most infectious.

— **Antibody tests** detect the body's immune response to the virus and are not effective at the earliest phase of infection.





A technician in a mobile unit conducts rapid antigen tests for COVID-19 in New Delhi.

number of cases there had decreased and the daily death counts had plateaued, suggesting that the tests might have played some part in controlling the spread of the virus. Epidemiologist K. Srinath Reddy, president of the Public Health Foundation of India, a non-profit organization in New Delhi, says that the Delhi example is interesting, but not clear-cut: he notes that the government started to lift lockdown restrictions in August, which led to a surge in infections. "Rapid antigen tests have picked up the increased number of cases, but whether they have been successful in limiting the spread of COVID, we'll only know in the next couple of months," Reddy says.

So far, India has approved the use of three antigen tests for screening large numbers of people, whether or not they have symptoms. One of the kits was evaluated by the Indian Council of Medical Research (ICMR) and the All India Institute of Medical Sciences, which found that the test detected infections between 51% and 84% of the time. Guidance from the ICMR says that people who have a negative result from an antigen test should also get a PCR test if they show symptoms, to rule out the possibility that the rapid test missed an infection.

The WHO and the US CDC have also advised getting a PCR test if people showing symptoms test negative with a rapid antigen test. The US FDA has so far granted emergency use authorization for four antigen tests, each of which has a higher sensitivity than those used in India. The 150 million tests bought from Abbott will be used in schools and "other special needs populations", according to the Department of Health and Human Services. The FDA, however, has authorized antigen-based tests only for people who have had symptoms for 12 days or fewer. Tests must be prescribed by a physician and administered by a health-care professional.

Other countries are also considering the use of rapid antigen tests to meet targets. In July, the Philippine Society for Microbiology and Infectious Diseases issued temporary guidelines for clinicians and health-care workers, saying that antigen tests could be used as an alternative to PCR for diagnosing a coronavirus infection during the first week in people with symptoms. But it also recommend15 ss that all negative results should be confirmed with a PCR-based assay, says Edsel Salvaña, an infectious-diseases expert at the University of the Philippines Manila, who is advising Philippine officials on rapid testing.

Antigen-based tests are being used in some of Italy's major airports to screen people who arrive from four Mediterranean countries considered to have a high risk of infection. Negative results do not have to be confirmed with a PCR test. The Italian health minister, Roberto Speranza, has announced plans to use antigen tests to screen passengers at all of the country's airports, and a group of experts has urged the Italian government to use the rapid tests in schools and universities.

> "Testing should become a part of life: in the morning you take your cereals, your vitamins, and you quickly check your status."

But others don't think rapid antigen tests are a good idea. When trying to contain small outbreaks, such as those happening in Italy, public-health authorities should use assays that are highly accurate, because missing even just one positive individual could lead to a steep increase in the total number of cases, says Andrea Crisanti, a microbiologist at the University of Padua.

Some researchers worry that there won't be enough antigen tests available to greatly expand their use. "Rapid tests right now are for the happy few," Koopmans says. "If we want to take these assays responsibly forward, we should talk about whether they can be produced to levels that would make them globally available."

Could antigen assays be used at home like pregnancy tests?

Several experts have promoted the idea of developing an antigen test that is cheap and simple enough to use at home, without a health-care worker administering it.

Burke says what's needed is something as easy as a pregnancy test. "You just spit into a tube, put a piece of paper in it and you get the result within minutes," Burke says. "Testing should become a part of life: in the morning you take your cereals, your vitamins, and you quickly check your status," he says.

A few companies are developing simple paper-strip antigen tests. But drug regulators have not yet approved them for emergency use. "We don't have a lot of real-life experience with these tests, and a lot of the validations have only been done in the laboratory," Salvaña says.

Beyond concerns about costs and availability, researchers worry that, with an over-the-counter test, people who get positive results might not follow up with public-health authorities, so their contacts won't be traced. Another risk would be people "gaming the system", Smith says — for example, getting someone else to take their test — so they can be sure of a negative result and avoid quarantine. Without incentives such as freely available tests and a living salary for those who have to isolate, testing and self-isolation could become a luxury reserved for wealthier people, others have argued.

Another concern is that people will get a false sense of security from tests that have only limited accuracy. "There's a big risk that the moment these tests become widely available, people will just use them and say, 'It's negative, so I'm clear'," Koopmans says.

Even when testing negative, people should continue to wash their hands, wear masks and avoid gathering in big groups, she says. Testing, she adds, "cannot replace the basic control measures that need to be in place to keep this virus controlled".

Giorgia Guglielmi is a science journalist in Basel, Switzerland.

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EXHIBIT "KK"

50% Covid Tests In Mumbai Are Less Reliable Antigen Tests, Data Shows.

Source: NDTV

Link: https://www.ndtv.com/india-news/50-covid-tests-in-mumbai-are-less-reliable-antigen-tests-data-shows-2402588

Written By: Purva Chitnis

Published on: March 30, 2021.

"We have exhausted our RT-PCR capacity, and we would love to enhance this further but now we can either stop at that or increase our chances with RAT, so that we can at least have the chance of finding more positives," BMC Commissioner Iqbal Chahal told NDTV.

As India's financial capital braces for 10,000 fresh Covid cases a day, random tests are being conducted to arrest the spread of the infection. On Tuesday, random tests were conducted at the sessions court in Mumbai.

With the fear of Covid spreading, the Brihanmumbai Corporation has decided to ramp up testing. The Centre wants states to do 70 per cent of the more reliable RT-PCR tests.

"RTPCR is preferred. And of number of tests, 70 per cent tests should be RTPCR," the Union health secretary said in Delhi this evening.

NOTARY M. M. PABALE Mumbai Maharashtra Reg. No. 15220 Expiry Dt. 20-10-2024 GOVT. OF INDIA

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NDTV analysed the data for a week till March 28, which showed that about half the Covid tests are the less-reliable antigen tests, rather than the gold-standard RT-PCR.

The RT-PCR has a much higher positivity rate, so much so that if all tests conducted last week were RT-PCR, there would be roughly 50 per cent more cases. This means that instead of an average of a slightly below 4,000 cases daily, there would be almost 6,000 cases.

Mumbai's municipal chief said they do not have the capacity to conduct more RT-PCR tests.

"We have exhausted our RT-PCR capacity, and we would love to enhance this further but now we can either stop at that or increase our chances with RAT, so that we can at least have the chance of finding more positives," BMC Commissioner Iqbal Chahal told NDTV.

"Our positivity is 14 per cent currently. We are going to increase our testing to 60,000 by Thursday. So the figure can go up to 10,000, he said.

There is nothing to worry as "most of the patients are asymptomatic," he added. In the current situation, that might be the silver lining, as authorities are scrambling for more hospital beds.

But the danger of false negatives is that people can roam around freely spreading the virus and to arrest the spread, as the union health ministry is advising, it is important for the BMC to increase the capacity for RT-PCR tests.

The second wave of coronavirus in Maharashtra started on February 10 and till March 20, Mumbai logged 85,000 cases, said Mr Chahal has said.

EXHIBIT "LC"

Need to stop second COVID-19 peak; 70% RT-PCR test must for states: PM Modi.

Source: MINT

Link: https://www.livemint.com/news/india/need-to-stop-second-covid-19-peak-70-rt-pcr-test-must-for-states-pm-modi-11615972632349.html

Published on: March 17, 2021

To prevent the spread of coronavirus infection in the country, Prime Minister Narendra Modi on Wednesday said RT-PCR test should make up for over 70% of overall coronavirus testing. States likes Chhattisgarh, Uttar Pradesh, Kerala and Odisha have been mostly dependent on antigen tests, he further noted."Test, track and treat should be taken as seriously now as it has been for over a year since the outbreak," Modi said in a meeting with state chief ministers.

India's daily coronavirus infections jumped by 28,903 on Wednesday, for the highest increase since 13 December. According to the data released by ministry of health and family welfare, five states — Maharashtra, Punjab, Karnataka, Gujarat and Tamil Nadu — accounted for 71.10% of the total infections reported in the last 24 hours.

Taking the stock of the COVID-19 situation in the country, Modi said, "I think it's a time of test for good governance. Our confidence should not turn into overconfidence. Our success should not turn into negligence."

"It has now become necessary for us to become pro-active. The option of making micro-containment zones wherever necessary, we should not bring leniency," Prime Minister added.

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Maharashtra has been severely affected by coronavirus pandemic. The western state alone recorded 61.8% of the daily new cases. At least 17,864 people tested positive for novel coronavirus in the last 24 hours in Maharashtra.

Kerala witnessed second highest daily COVID-19 cases on Wednesday with 1,970 fresh cases. However, the southern state has been reporting a consistently declining trend over the last month. At least 1,463 tested positive for novel coronavirus in Punjab on Wednesday.

"Maharashtra, Kerala and Punjab account for 76.4% of India's total active cases, with Maharashtra alone contributing nearly 60 per cent," the ministry highlighted.

Modi said, "70 districts in 16 states have seen an increase in the positivity rate by over 150% in last 15 days." "If we do not stop it here, then a condition for a nationwide outbreak may emerge," he said and called for quick and decisive steps to stop it.

EXHIBIT "MM"

Antigen-based testing but not real-time PCR correlates with SARS-CoV-2 virus culture

Andrew Pekosz, PhD,[1,2] Charles K. Cooper, MD,[3] Valentin Parvu, PhD,[2] Maggie Li, MS,[1] Jeffrey C. Andrews, MD,[3] Yukari C. Manabe, MD,[2,4] Salma Kodsi, MS,[2] Jeffry Leitch, PhD,[3] Devin S. Gary, PhD,[2] Celine Roger-Dalbert, MS[3]

[1]W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, [2]Department of Emergency Medicine
[3]Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions, 7 Loveton Circle, Sparks, MD, USA,
[4]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

#To whom correspondence should be addressed:
Andrew Pekosz, PhD
615 N. Wolfe Street
Room W2116
Baltimore, Maryland 21205
Phone: 410-502-9306
Email: apekosz1@jhu.edu

Charles K. Cooper, MD
Vice President of Medical Affairs
Becton, Dickinson and Company, BD Life Sciences – Integrated Diagnostic Solutions
7 Loveton Circle, Sparks MD 21152, USA
Phone: 410-316-4984
E-mail: Charles_K_Cooper@bd.com

WORD COUNT
1,668 (Introduction, Results, and Discussion); 942 (Methods)

KEY WORDS: SARS-CoV-2; Antigen testing; SARS-CoV-2 contagious; viral load; live culture; RT-PCR testing

RUNNING TITLE: SARS-CoV-2 antigen testing and infectiousness

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SUMMARY

Individuals can test positive for SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR) after no longer being infectious.[1-8] Positive SARS-CoV-2 antigen-based testing exhibits a temporal pattern that corresponds with active, replicating virus and could therefore be a more accurate predictor of an individual's potential to transmit SARS-CoV-2.[2,3,9] Using the BD Veritor System for Rapid Detection of SARS-CoV-2 later flow antigen detection test, we demonstrate a higher concordance of antigen-positive test results with the presence of cultured, infectious virus when compared to RT-PCR. When compared to infectious virus isolation, the sensitivity of antigen-based testing is similar to RT-PCR. The correlation between SARS-CoV-2 antigen and SARS-CoV-2 culture represents a significant advancement in determining the risk for potential transmissibility beyond that which can be achieved by detection of SARS-CoV-2 genomic RNA. Coupled with a rapid time-to-result, low cost, and scalability, antigen-based testing should facilitate effective implementation of testing and public health interventions that will better contain COVID-19.

INTRODUCTION

The SARS-CoV-2 causes COVID-19 and is spread from human-to-human primarily through airborne transmission.[10] The mean incubation time, or presymptomatic period, for SARS-CoV-2 is approximately 5.8 days (95% CI 5.0-6.7),[11,12] and the period of transmission (the total time during which a patient is contagious) begins around one to three days prior to symptom onset, with a subsequent reduction in contagiousness occurring 7-10 days following symptom onset.[8,13,14] Recent work in a golden hamster SARS-CoV-2 model demonstrated that although the presence of genomic RNA in nasal washes extends to 14 days post-inoculation, the detection of infectious virus and the communicable period both end well before 14 days.[15] In addition, four previous studies, utilizing culture-based virus detection from human specimens, demonstrated an absence of infectious isolates from most specimens taken eight days after symptom onset, despite measurable viral RNA loads using RT-PCR.[1,3,5,7]

Several SARS-CoV-2 antigen-based tests, which work via a lateral flow immunoassay mechanism, have recently received Emergency Use Authorization (EUA) from the Food and Drug Administration.[16-19] Several lines of indirect evidence suggest that antigen-based testing may align better with culture-based test results compared to RT-PCR. For example, higher RT-PCR Ct values from specimens are observed when individuals are negative by antigen testing or culture-based testing compared to those from individual that are antigen test [9] or culture–based test positive.[6] In addition, current EUA SARS-CoV-2 antigen tests have optimal performance profiles[16-19] at time points that overlap with the temporal expression profile of SARS-CoV-2 sub-genomic RNA (a marker for active, replicating virus).[3] Despite the recognition that point-of-care or other testing modalities might be more effective at discerning contagious from non-contagious

individuals,[2] no study has directly compared antigen-based testing with RT-PCR in the same study using a reference method for infectiousness.

The objective of this study was to determine whether SARS-CoV-2 antigen testing differentiates SARS-CoV-2-contagious individuals (e.g., those still shedding infectious virus) from non-contagious individuals compared to RT-PCR methodology. To address this, we utilized Quidel Lyra® SARS-CoV-2 Assay ("RT-PCR assay") positive and negative specimens obtained from a diverse set of collection sites across the USA. The RT-PCR assay and the BD Veritor™ System for Rapid Detection of SARS-CoV-2 ("antigen test") were compared to SARS-CoV-2 TMPRSS2 culture (a sensitive virus culture test utilizing the VeroE6TMPRSS2 cell line), which served as the reference method for determining infectiousness.

49 **RESULTS**

50 The 38 RT-PCR positive specimens were tested for the presence of SARS-CoV-2 using infection
51 of VeroE6TMPRSS2 cell cultures (SARS-CoV-2 TMPRSS2 culture). Overall, 28 RT-PCR-
52 positive specimens were also positive by SARS-CoV-2 TMPRSS2 culture and 10 of 38 RT-
53 PCR-positive specimens were negative by SARS-CoV-2 TMPRSS2 culture (Figure 1A). SARS-
54 CoV-2 TMPRSS2 culture-positive specimens had a mean log10 viral RNA copy number of 7.16
55 compared to 4.01 from specimens that were SARS-CoV-2 TMPRSS2 culture-negative (p-value
56 <0.001; two-sample t-test, 2-tailed analysis). Further stratification by results from the antigen test
57 showed that 27 of 28 RT-PCR-positive/SARS-CoV-2 TMPRSS2 culture-positive specimens
58 were also positive in the antigen test; only two of the ten RT-PCR-positive/ SARS-CoV-2
59 TMPRSS2 culture-negative specimens were positive by the antigen test.
60
61 Of the 38 RT-PCR-positive results utilized for these analyses, nine were antigen test negative.
62 These nine negative results showed a trend towards longer time from symptom onset compared
63 to the 29 RT-PCR assay-positive/antigen test-positive specimens (4.4 days on average versus
64 2.9, p-value = 0.108).[9] Of the nine samples that were RT-PCR-positive/antigen test-negative, the
65 viral RNA copy number was significantly lower than that observed for the 29 RT-PCR-
66 positive/antigen test-positive specimens (mean 4.3 log10 cp/mL versus 7.0 log10 cp/mL, p-
67 value<0.001, Figure S1). Symptom day was not a significant factor in multivariate models, while
68 viral RNA load continued to be significant (p-value = 0.002).
69
70 Probit models for percent positivity by viral RNA load corresponding to the RT-PCR assay,
71 antigen test, SARS-CoV-2 TMPRSS2 culture, and SARS-CoV-2 culture with VeroE6 cells

72 ("SARS-CoV-2 VeroE6 culture;" data integrated into the probit model using previous data; see
73 Methods)[4] are provided in Figure 1B. The SARS-CoV-2 VeroE6 culture yielded a positive result
74 at a rate of 5% for a viral load of 5.75 log10 cp/ml, whereas the SARS-CoV-2 TMPRSS2 culture
75 corresponded to a positive result with a rate of 5% at a viral load of 4.5 log10 cp/mL. At a viral
76 load of 2.6 log10 cp/mL, the antigen test yielded a positive result at a rate of 5%. In a
77 multivariate generalized linear model with viral RNA load and test type, the SARS-CoV-2
78 TMPRSS2 culture was not significantly different from the antigen test (p-value = 0.953). Both
79 the SARS-CoV-2 TMPRSS2 culture and antigen test were significantly more likely to yield
80 positive results than SARS-CoV-2 VeroE6 culture (p-value<0.001 for both). Unlike the antigen
81 test, the RT-PCR assay showed very little overlap with SARS-CoV-2 TMPRSS2 culture,
82 yielding positive results at much lower viral loads.
83
84 As shown in Table 1, the antigen test demonstrated a sensitivity and specificity of 96.4% (95%
85 CI: 82.3, 99.4) and 98.7% (96.1, 99.7), respectively. The RT-PCR assay demonstrated a
86 sensitivity and specificity of 100% (87.7, 100) and 95.5% (91.1, 97.8), respectively. Based on the
87 study prevalence of 11.2%, as determined by SARS-CoV-2 TMPRSS2 culture positivity and a
88 total specimen number of 251 (based on the total, evaluable specimen set utilized for the Veritor
89 EUA study), the positive predictive value (PPV) for the antigen test was 90.0% (76.3, 97.6),
90 while the PPV for the RT-PCR assay was only 73.7% (60.8, 85.3).

91 **DISCUSSION**

92 The results here show similar sensitivity between the SARS-CoV-2 antigen test and the SARS-
93 CoV-2 RT-PCR assay (96.4% and 100%, respectively) over a time range of <8 days post
94 symptom onset. However, the SARS-CoV-2 antigen test had a PPV of 90.0%, whereas the RT-
95 PCR assay showed a PPV of only 73.7%. In addition, the probit model for percent positivity
96 employed in this study showed considerable overlap between the antigen test and the SARS-
97 CoV-2 TMPRSS2 culture, with little overlap between the SARS-CoV-2 TMPRSS2 culture and
98 RT-PCR.
99
100 Ct values are inversely correlated with the viral load thresholds corresponding to infectious virus
101 isolation. Because limits of detection vary between RT-PCR assays, however, Ct values reported
102 by specific RT-PCR assays correspond to different viral RNA loads.[6,7,20-25] Here we utilized the
103 Lyra assay to establish a probit model of percent positivity by viral load, which facilitates a
104 better comparison of these results with previous work. Recent studies involving upper respiratory
105 swab specimens reported no cases of COVID-19 with SARS-CoV-2 viral RNA loads below 4
106 log10 cp/mL.[1,3,5,6,8,11,26,27] Other work has shown that specimens with viral RNA loads ≤6 log10
107 cp/ml have minimal or no culturable SARS-CoV-2 virus.[3,5,28-30] Here, a low percent positivity (5-
108 10%) was observed for the SARS-CoV-2 VeroE6- culture test below 6 Log10 cp/mL. The
109 SARS-CoV-2 TMPRSS2 culture test, however, showed 90% positivity at 5.6 log10 cp/mL.
110 Although antigen test had a larger distribution of positivity, it overlapped considerably with the
111 SARS-CoV-2 TMPRSS2 culture test and approached 90% positivity at a viral RNA load of
112 6.4Log10. This is consistent with the WHO target product profile for priority diagnostics, which

113 supports viral RNA load based methodologies and includes an acceptable limit of detection for
114 point-of-care tests of 6Log10 cp/mL.[31]
115
116 As with other viruses, RT-PCR-based methodologies may be detecting SARS-CoV-2 RNA even
117 after infectious virus is no longer present;[32-37] especially at time periods beyond 7 days from
118 symptom onset.[1,3] For most patients with COVID-19, efforts to isolate live virus from upper
119 respiratory tract specimens have been unsuccessful ≥10 days from symptom onset; it is unlikely
120 that these individuals pose a transmission risk to others.[25] In addition, there is no evidence to date
121 that persistent or recurrent detection of viral RNA, following recovery from COVID-19, poses a
122 risk of SARS-CoV-2 transmission.[25]
123
124 This work highlights a key potential value of decentralized POC antigen-based testing and
125 furthers our understanding of the interpretation of antigen test results. Antigen testing facilitates
126 accurate and rapid detection of infectious individuals who may not require direct medical
127 management (due to mild/non-severe disease), but for whom infection control measures have the
128 potential to interrupt community transmission. While RT-PCR is highly sensitive when
129 compared to SARS-CoV-2 TMPRSS2culture, antigen testing also showed excellent sensitivity
130 (96.4%) coupled with better PPV relative to RT-PCR (90.0 versus 73.7) and rapid time to results.
131
132 This study had limitations. It only included specimens from patients within seven days of
133 symptom onset. Several studies have demonstrated an inability to culture SARS-CoV-2 beyond
134 day eight, despite ongoing RT-PCR positivity.[1,3,5] Serial sampling of COVID-19 patients is
135 needed to determine if there is a propensity to have viral antigen test positive results after a

negative result, as can sometimes be seen with RT-PCR tests. Results from this study likely underestimate the difference in specificity between RT-PCR and antigen testing that would be expected in a set that included specimens collected at later times post symptom onset. In this study, while three subjects were antigen test false positives versus SARS-CoV-2 TMPRSS2 culture, as many as ten subjects were RT-PCR false positives versus culture (viral RNA loads ranging from 2.6 to 5.4 log10 copies/mL). Although the sample size was adequate in this study, the confidence intervals in the probit model were too wide to establish a definitive viral load cut-off. To improve the precision associated with the point estimates, either a larger study or a meta-analysis, involving multiple studies, would be required. Also, there are limitations associated with the use of culture positivity or viral RNA load as a surrogate for infectiousness or transmissibility that require further investigation. Finally, it is unclear how well the results here will extrapolate to the other antigen tests due to variability in limit of detection or other test characteristics.

Conclusion

Point-of-care SARS-CoV-2 antigen tests have the potential to significantly change the public health interventions needed to minimize the spread of COVID-19 by providing a better test to identify individuals that are likely to be shedding infectious virus and therefore transmit SARS-CoV-2. This will allow for rapid identification of asymptomatic COVID-19 cases and inform shorter periods of self-isolation for COVID-19 infected individuals. In addition, the low cost and scalability in low and middle-income countries associated with antigen-based testing will be an important tool in the diagnostic armamentarium to contain and suppress COVID-19 community transmission.

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167

168 **AUTHOR CONTRIBUTIONS**

169 All authors contributed to the interpretation of the data, critically revised the manuscript for
170 important intellectual content, approved the final version to be published, and agree to be
171 accountable for all aspects of the work.

172

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177

178 **POTENTIAL CONFLICTS OF INTEREST**

179 CKC, VP, JCA, SK, JL, DSG, and CR-D are employees of Becton, Dickinson and Company
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181 ML—None
182 YM—None

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300 **METHODS**

301 *Study design and specimen collection*

302 Prospective specimen collection, specimen use, and participant demographics for the parent
303 study were described previously.[9] This study involved the use of residual respiratory swab
304 specimens from the previous antigen test Food and Drug Administration-Emergency Use
305 Authorization (EUA) study, which occurred across 21 geographically diverse study sites, from
306 June 5-11, 2020. Briefly, eligible participants were ≥18 years of age and had one or more self-
307 reported COVID-19 symptoms between 0-7 days from symptom onset.[38,39] Nasal swab
308 specimens for use with antigen testing were collected only after the standard of care (SOC) swab.
309 Nasopharyngeal (NP) swab specimens were collected after the nasal swab specimen for use with
310 the RT-PCR assay (the laboratory reference standard in the EUA study); if an NP was collected
311 as part of the SOC procedure at a collection site, the participant was given the choice of having
312 an oropharyngeal (OP) swab specimen collected in lieu of a second NP swab for use with the
313 RT-PCR assay. Overall, 76 specimen sets (consisting of one nasal and either one NP or one OP
314 swab) were utilized from the original 251 evaluable specimen sets in the EUA study. The 76
315 specimens consisted of all 38 RT-PCR assay positive specimens, and 38, randomly selected RT-
316 PCR assay negative specimens from the parent study. Specimens for the RT-PCR assay
317 consisted of 71 NP swabs (37 and 34 positive and negative, respectively) and five OP swabs (1
318 and 4 positive and negative swabs, respectively). For the EUA study, reference testing was
319 performed at TriCore Reference Laboratories while the antigen testing was performed internally
320 at BD (San Diego, CA, USA). No study-related procedures were performed without an informed
321 consent process or signature of a consent form. This research was performed in alignment with

322 principles set forth by Good Clinical Practice guidelines and the Declaration of Helsinki. This
323 article was prepared according to STARD guidelines for diagnostic accuracy studies reporting.[40]
324
325 *Test/assay procedures*
326 Antigen test and RT-PCR assay
327 The antigen test (Becton, Dickinson and Company, BD Life Sciences—Integrated Diagnostic
328 Solutions, San Diego, CA) and RT-PCR assay (Quidel Corporation. Athens, OH) were
329 performed according to the manufacturers' IFU.[19,41] The only exception was that nasal swabs
330 were shipped on dry ice (-70°) to the testing site prior to preparation for the antigen test. The RT-
331 PCR assay reports cycle number in a manner that omits the first 10 cycles; here cycle numbers
332 for the RT-PCR assay are reported with the addition of first 10 cycles.
333
334 SARS-CoV-2 virus culture
335 VeroE6TMPRSS2 was adapted from the VeroE6 cell line (ATCC CRL-1586) to express the
336 TMPRSS2 protease at levels approximately 10-fold higher than that found in the human lung.[42]
337 The cells were cultured in complete medium (CM) consisting of Dulbecco's modified Eagle
338 Medium, supplemented with 10% fetal bovine serum (Thermo Fisher Scientific-Gibco,
339 Waltham, MA), 1mM glutamine (Thermo Fisher Scientific-Invitrogen, Waltham, MA), 1mM
340 sodium pyruvate (Thermo Fisher Scientific-Invitrogen, Waltham, MA), 100µg/mL penicillin
341 (Thermo Fisher Scientific-Invitrogen, Waltham, MA) and 100 µg/mL streptomycin (Thermo
342 Fisher Scientific-Invitrogen, Waltham, MA), at 37°C in a humidified chamber with 5% carbon
343 dioxide. Cells were grown to 75% confluence in a 24 well plate format and the CM was removed
344 and replaced with 150 µL of infection media (IM) which is identical to CM but with the final

345 bovine serum reduced to 2.5%. One hundred microliters (100 µL) of the clinical specimen was
346 added to each assay well and the cells were incubated at 37°C for two hours. The inoculum was
347 then aspirated and replaced with 0.5 ml IM; the cells were then maintained at 37°C for four days.
348 When a cytopathic effect was visible in most of the cells in a given well, the IM was harvested
349 and stored at -70°C. The presence of SARS-CoV-2 was confirmed through quantitative RT-PCR
350 as described previously,[7,45] by extracting RNA from the cell culture supernatant using the Qiagen
351 viral RNA isolation kit and performing RT-PCR using the N1 and N2 SARS-CoV-2 specific
352 primers and probes in addition to primers and probes for human RNaseP gene using synthetic
353 RNA target sequences to establish a standard curve.
354
355 *Probit models for probability of positive SARS-CoV-2 result*
356 The RT-PCR assay was performed on serially diluted samples containing SARS-CoV-2 related
357 genomic RNA prepared in universal transport media (containing human lung epithelial cells at
358 130,000 cells per mL) at concentrations ranging from 1.27 log10 copies/mL (cp/ml) to 4.27
359 log10 cp/ml (Table S1). The RT-PCR assay probability of positive result was fit using a probit
360 model linking the Lyra results to viral RNA load. Linear regression was performed linking log10
361 cp/ml viral RNA load to Lyra Ct score using all samples with at least 3 log10 cp/ml (for which
362 observed Lyra positivity was 100%).
363
364 Antigen test positivity and SARS-CoV-2 TMPRSS2 culture positivity (a surrogate for
365 contagiousness), with RT-PCR confirmation, were fit with a probit model as a function of viral
366 load, using results from the Veritor EUA study;[9] RT-PCR assay Ct scores were used to estimate
367 viral RNA loads, as described above. SARS-CoV-2 VeroE6 culture positivity linkage to viral

load was inserted into the probit model for probability of a positive result using data from Huang et al (2020).[6] Virus isolation in Huang et al was attempted for a total of 60 specimens, positive by RT-PCR for SARS-CoV-2. Of those, 23 were positive by culture. Ct scores of the SARS-CoV-2 envelope, nucleocapsid, and non-structural protein-12 RT-PCR targets were linked to the viral load (log10 cp/ml) through quadratic regressions. The empirical equation for envelope target Ct score was then used to calculate viral load for the 23 culture positive and 37 culture negative specimens (Figure S2). All analyses were performed using the R software system and the ggplot2 R package.[44,45]

Table 1. Performance of the antigen test and the RT-PCR assay for detection of SARS-CoV-2 infectivity based on virus culture positive results within 0-7 days from symptom onset

Prevalence: 11.2%		
Performance values	**Antigen test performance**	**RT-PCR assay performance**
Sensitivity	96.4 [82.3, 99.4]	100 [87.7, 100]
Specificity	98.7 [96.1 99.7]	95.5 [91.1, 97.8]
PPV	90.0 [76.3, 97.6]	73.7 [60.8, 85.3]
NPV	99.5 [97.7, 100]	100 [98.4, 100]
Accuracy	99.4 [98.0, 99.4]	96.0 [92.8, 97.8]
TP	27	28
FP	3	10
FN	1	0
TN[a]	220	213

Abbreviations: RT-PCR, real-time polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; TP, true positive; FP, false positive; FN, false negative; TN, true negative

[a]Includes 176 specimen sets that were RT-PCR and antigen negative, with unavailable culture results.

FIGURES
FIGURE 1

Figure 1A.

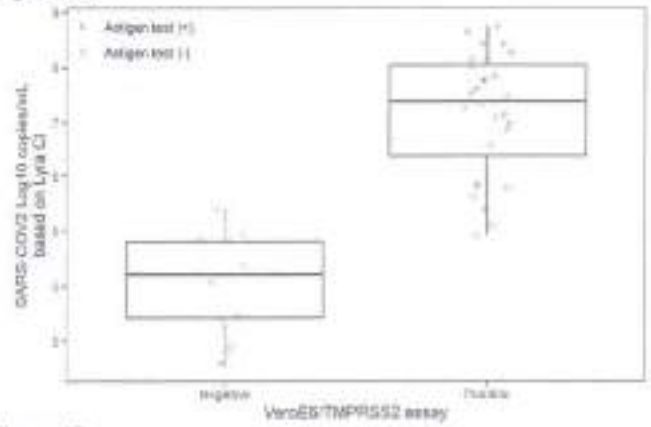

Figure 1B.

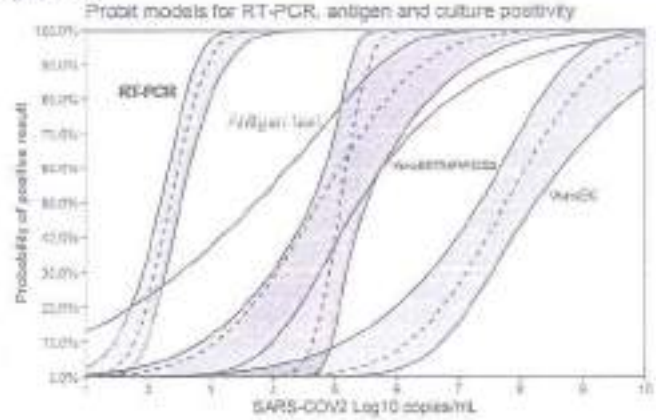

Figure 1. (A) The 38 RT-PCR assay positive specimens are plotted by Log10 copies/mL (y-axis) and are stratified by the SARS-CoV-2 live culture results (negative, n=10; positive, n=28). The median and inter-quartile range values, respectively, for the RT-PCR-positive/SARS-CoV-2 TMPRSS2 culture-negative were 4.21 and 1.37; the median and inter-quartile range values, respectively, for the RT-PCR-positive/SARS-CoV-2 TMPRSS2 culture-negative were 7.39 and 1.66. The mean values for the SARS-CoV-2 TMPRSS2 culture-negative and SARS-CoV-2 TMPRSS2 assay-positive specimen groups were significantly different (4.01 versus 7.16, respectively; p-value <0.001 based on two-sample t-test [2-tailed]). Antigen test positive results are indicated as red data points (n=29) and the antigen test negative results (n=9) are indicated by the green data points. (B) Probit models linking viral load to the probability of positive result of RT-PCR (Lyra), antigen test (Veritor), SARS-CoV-2 TMPRSS2 culture and SARS-CoV-2 VeroE6 culture (refer to Huang et al 2020). Viral load levels at which there is a 5% chance of positive result: 1.6, 2.6, 4.5, and 5.75 log10 cp/ml for RT-PCR, antigen, SARS-CoV-2 TMPRSS2 culture, and SARS-CoV-2 VEroE6 culture, respectively.

458 SUPPLEMENTAL MATERIAL
459 TABLE S1
460
461 **Table S1.** Corresponding values for Ct score and viral RNA load during limit of
462 detection analysis involving Lyra SARS-CoV-2 RT-PCR assay-positive specimens
463

Viral Load (copies/mL)	Log10 Viral Load	#Positive/ #Tested	PCR assay Ct score; Mean (SD)
0	-	0/5	- (-)
19	1.27	0/9	- (-)
40	1.60	1/10	38.03 (-)
86	1.94	1/9	38.83 (-)
186	2.27	4/10	37.33 (1.20)
400	2.60	8/10	35.89 (1.69)
862	2.94	8/10	34.51 (1.62)
1857	3.27	10/10	32.66 (1.07)
4000	3.60	10/10	31.08 (0.75)
8618	3.94	9/9	30.09 (1.15)
18566	4.27	9/9	29.51 (1.45)

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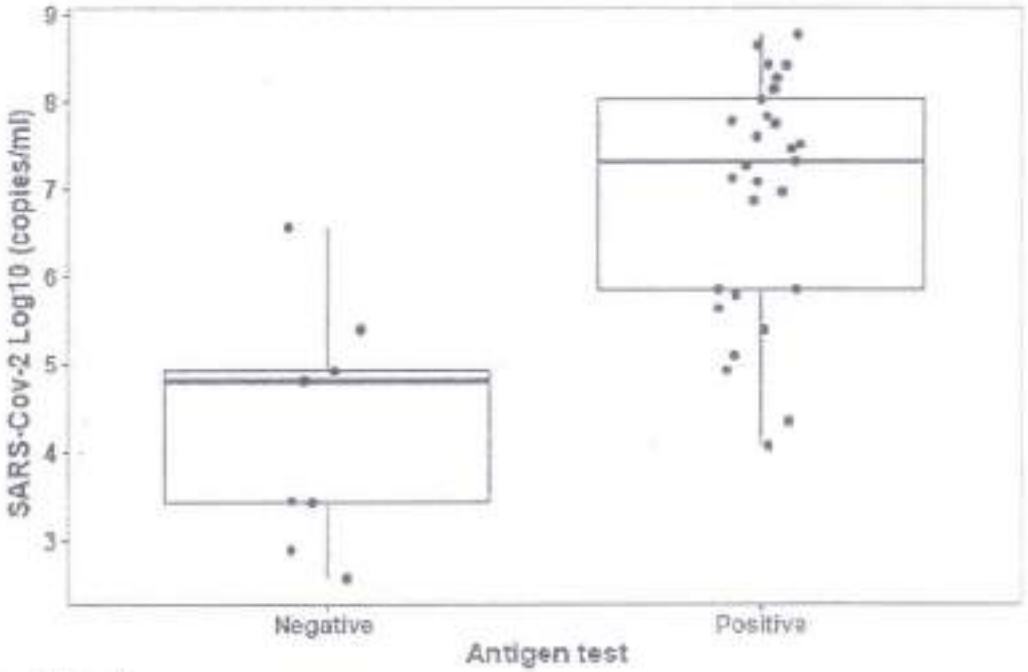
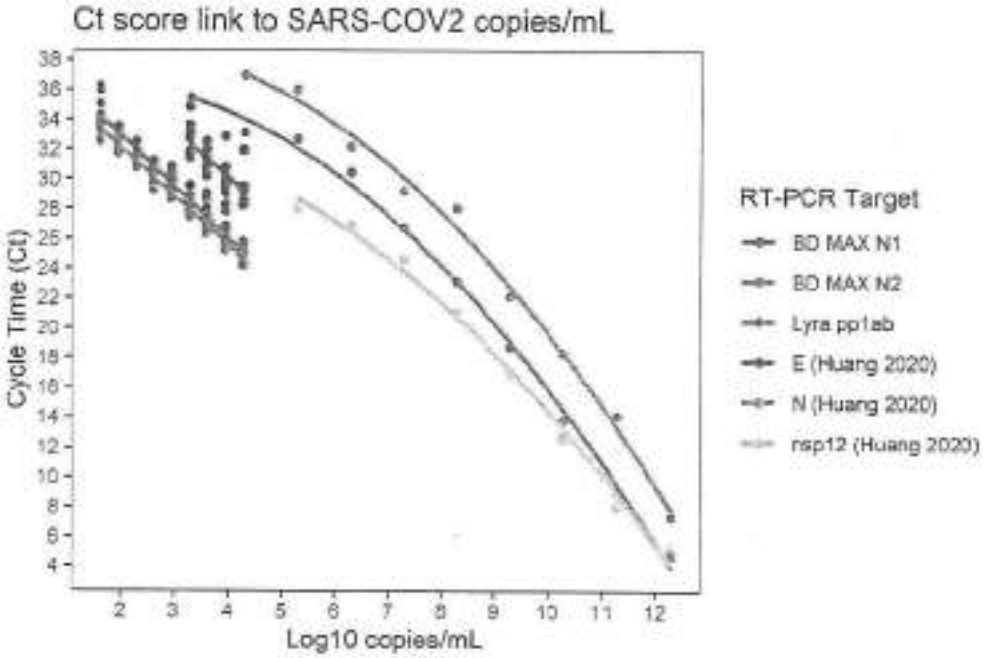


477 FIGURE S1

478 **Figure S1.** Box plots showing the median values for SARS-CoV-2 viral RNA loads from
479 antigen-positive and -negative results within the 38 RT-PCR-positive results from the
480 Veritor test EUA study. A two-sample t-test (2-tailed) analysis indicated a significantly
481 higher mean (7.0 log10 cp/ml) for RT-PCR-positive/antigen-positive results compared to
482 that (4.3 log10 cp/mL) for RT-PCR-positive/antigen negative results (p-value<0.001).



483 FIGURE S2

484

485 **Figure S2.** Relation of the RT-PCR Ct scores for the BD MAX assay, the RT-PCR

486 assay, and the RT-PCR method used in Huang et al (2020)[6] to viral load. Empirical

487 equation for the RT-PCR assay Ct = 42.69 - 3.14 Log10 copies/mL. Empirical equation

488 for the E target in Huang et al: Log10 copies/mL = 12.377 – 0.052 Ct – 0.005 Ct^2

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EXHIBIT "NN"

Evaluation of Abbott BinaxNOW Rapid Antigen Test for SARS-CoV-2 Infection at Two Community-Based Testing Sites — Pima County, Arizona, November 3–17, 2020

Jessica L. Prince-Guerra, PhD[1]; Olivia Almendares, MSPH[1]; Leisha D. Nolen, MD, PhD[1]; Jaylem K. L. Gunn, PhD[1]; Arielle P. Dale, PhD[1,2,3]; Sean A. Buono, PhD[1]; Molly Deutsch-Feldman, PhD[1,2]; Suganthi Suppiah, PhD[1]; Lijuan Hao, MD[1]; Yan Zeng, MS[1]; Valerie A. Stevens[1]; Kirsten Knipe, MS[1]; Justine Pompey, PhD[1]; Christine Atherstone, PhD[1,2]; David P. Bui, PhD[1,2]; Tracy Powell, PhD[1]; Anaibi Tamin, PhD[1]; Jennifer L. Harcourt, PhD[1]; Patricia L. Shewmaker, PhD[1]; Magdalena Medrzycki, PhD[1]; Phili Wong, MS[1]; Shilpi Jain, PhD[1]; Alexandra Tejada-Strop, MS[1]; Shannon Rogers, MS[1]; Brian Emery[1]; Houping Wang, PhD[1]; Maria Perway, MPH[1]; Caitlin Bohannon, PhD[1]; Jennifer M. Folster, PhD[1]; Adam MacNeil, PhD[1]; Reynolds Salerno, PhD[1]; Wendi Kuhnert-Tallman, PhD[1]; Jacqueline E. Tate, PhD[1]; Natalie J. Thornburg, PhD[1]; Hannah L. Kirking, MD[1]; Khalilallah Shriban, MD[4]; Julie Kudrna, MPA[4]; Theresa Cullen, MD[4]; Kenneth K. Komatsu, MPH[2]; Julie M. Villanueva, PhD[1]; Dale A. Rose, PhD[1]; John C. Neatherlin, MPH[1]; Mark Anderson, MD[1]; Paul A. Rota, PhD[1]; Margaret A. Honein, PhD[1]; William A. Bower, MD[1]

On January 19, 2021, this report was posted as an MMWR *Early Release on the* MMWR *website (https://www.cdc.gov/mmwr).*

Rapid antigen tests, such as the Abbott BinaxNOW COVID-19 Ag Card (BinaxNOW), offer results more rapidly (approximately 15–30 minutes) and at a lower cost than do highly sensitive nucleic acid amplification tests (NAATs) (*1*). Rapid antigen tests have received Food and Drug Administration (FDA) Emergency Use Authorization (EUA) for use in symptomatic persons (*2*), but data are lacking on test performance in asymptomatic persons to inform expanded screening testing to rapidly identify and isolate infected persons (*3*). To evaluate the performance of the BinaxNOW rapid antigen test, it was used along with real-time reverse transcription–polymerase chain reaction (RT-PCR) testing to analyze 3,419 paired specimens collected from persons aged ≥10 years at two community testing sites in Pima County, Arizona, during November 3–17, 2020. Viral culture was performed on 274 of 303 residual real-time RT-PCR specimens with positive results by either test (29 were not available for culture). Compared with real-time RT-PCR testing, the BinaxNOW antigen test had a sensitivity of 64.2% for specimens from symptomatic persons and 35.8% for specimens from asymptomatic persons, with near 100% specificity in specimens from both groups. Virus was cultured from 96 of 274 (35.0%) specimens, including 85 (57.8%) of 147 with concordant antigen and real-time RT-PCR positive results, 11 (8.9%) of 124 with false-negative antigen test results, and none of three with false-positive antigen test results. Among specimens positive for viral culture, sensitivity was 92.6% for symptomatic and 78.6% for asymptomatic individuals. When the pretest probability for receiving positive test results for SARS-CoV-2 is elevated (e.g., in symptomatic persons or in persons with a known COVID-19 exposure), a negative antigen test result should be confirmed by NAAT (*1*). Despite a lower sensitivity to detect infection, rapid antigen tests can be an important tool for screening because of their quick turnaround time, lower costs and resource needs, high specificity, and high positive predictive value (PPV) in settings of high pretest probability. The faster turnaround time of the antigen test can help limit transmission by more rapidly identifying infectious persons for isolation, particularly when used as a component of serial testing strategies.

Paired upper respiratory swabs were collected at the same timepoint from persons aged ≥10 years receiving testing for SARS-CoV-2, the virus that causes coronavirus disease 2019 (COVID-19), at two Pima County Health Department community testing sites during November 3–17 (site A) and November 8–16 (site B). The sites offered SARS-CoV-2 testing to anyone in the community who wanted testing. A questionnaire capturing demographic information and current and past–14-day symptoms was administered to all participants. At both sites, a health care professional first collected a bilateral anterior nasal swab, using a swab provided in the BinaxNOW kit, immediately followed by a bilateral nasopharyngeal (NP) swab for real-time RT-PCR testing. Anterior nasal swabs were immediately tested on-site using the BinaxNOW antigen test according to the manufacturer's instructions (*4*). NP swabs were stored in phosphate buffered saline at 39°F (4°C) and analyzed within 24–48 hours by real-time RT-PCR using either the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for detection of SARS-CoV-2 (*5*) (2,582 swabs) or the Fosun COVID-19 RT-PCR Detection Kit (*6*) (837 swabs). Viral culture*,† was attempted on 274 of 303 residual real-time RT-PCR specimens if either the real-time RT-PCR or BinaxNOW antigen test result was positive (the remaining 29 were not available for viral culture). Results from real-time RT-PCR and the BinaxNOW antigen test were compared to evaluate sensitivity, specificity, negative predictive value (NPV), and PPV. Statistical analyses were performed using SAS (version 9.4; SAS Institute). Cycle threshold (Ct) values from real-time RT-PCR were compared using a Mann-Whitney U Test; 95% confidence intervals (CIs)

* Specimens were used to perform a limiting-dilution inoculation of Vero CCL-81 cells, and cultures showing evidence of cytopathic effect were tested by real-time RT-PCR for the presence of SARS-CoV-2 RNA. Viral recovery was defined as any culture in which the first passage had an NT Ct value at least two Ct values lower than the corresponding clinical specimen.

† https://www.biorxiv.org/content/10.1101/2020.[illegible]v1.

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were calculated using the exact binomial method. The investigation protocol was reviewed by CDC and determined to be nonresearch and was conducted consistent with applicable federal law and CDC policy.[§]

Paired upper respiratory swabs were collected from 3,419 persons, including 1,458 (42.6%) from site A and 1,961 (57.4%) from site B (Table 1). Participants ranged in age from 10 to 95 years (median = 41 years) with 236 (6.9%) aged 10–17 years, 1,885 (55.1%) aged 18–49 years, 743 (21.7%) aged 50–64 years, and 555 (16.2%) aged ≥65 years. Approximately one third (31.4%) of participants identified as Hispanic or Latino, and three quarters (75.1%) identified as White.

At the time of testing, 827 (24.2%) participants reported at least one COVID-19–compatible sign or symptom,[¶] and 2,592 (75.8%) were asymptomatic. Among symptomatic participants, 113 (13.7%) received a positive BinaxNOW antigen test result, and 176 (21.3%) received a positive real-time RT-PCR test result. Among asymptomatic participants, 48 (1.9%) received a positive BinaxNOW antigen test result, and 123 (4.7%) received a positive real-time RT-PCR test result.

Testing among symptomatic participants indicated the following for the BinaxNOW antigen test (with real-time RT-PCR as the standard): sensitivity, 64.2%; specificity, 100%; PPV, 100%; and NPV, 91.2% (Table 2); among asymptomatic persons, sensitivity was 35.8%; specificity, 99.8%; PPV, 91.7%; and NPV, 96.9%. For participants who were within 7 days of symptom onset, the BinaxNOW antigen test sensitivity was 71.1% (95% CI = 63.0%–78.4%), specificity was 100% (95% CI = 99.3%–100%), PPV was 100% (95% CI = 96.4%–100%), and NPV was 92.7% (95% CI = 90.2%–94.7%). Using real-time RT-PCR as the standard, four false-positive BinaxNOW antigen test results occurred, all among specimens from asymptomatic participants. Among 299 real-time RT-PCR positive results, 142 (47.5%) were false-negative BinaxNOW antigen test results (63 in specimens from symptomatic persons and 79 in specimens from asymptomatic persons).

Virus was recovered from 96 (35.0%) of 274 analyzed specimens that were positive by either test, including 85 (57.8%) of 147 with concordant positive results and 11 (8.9%) of 124 with false-negative BinaxNOW antigen test results. Virus was not recovered from any of the three available specimens with false-positive BinaxNOW antigen test results. Among the 224 specimens undergoing viral culture that were analyzed with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for detection of SARS-CoV-2, median Ct values[**] were significantly higher for specimens with false-negative BinaxNOW antigen test results, indicating lower viral RNA levels than in those with concordant positive results (33.9 versus 22.0 in specimens from symptomatic persons [p<0.001] and 33.9 versus 22.5 in specimens from asymptomatic persons [p<0.001]) (Figure). Median Ct values for SARS-CoV-2 culture-positive specimens (22.1) were significantly lower than were those for culture-negative specimens (32.8) (p<0.001), indicating higher levels of viral RNA in culture-positive specimens. Among specimens with positive viral culture, the sensitivity of the BinaxNOW antigen test compared with real-time RT-PCR in specimens from symptomatic participants was 92.6% (95% CI = 83.7%–97.6%) and in those from asymptomatic participants was 78.6% (95% CI = 59.1%–91.7%).

Discussion

In this evaluation, using real-time RT-PCR as the standard, the sensitivity of the BinaxNOW antigen test was lower among specimens from asymptomatic persons (35.8%) than among specimens from symptomatic persons (64.2%). Specificity (99.8%–100%) was high in specimens from both asymptomatic and symptomatic groups. The prevalence of having SARS-CoV-2 real-time RT-PCR positive test results in this population was moderate (8.7% overall; 4.7% for asymptomatic participants); administering the test in a lower prevalence setting will likely result in a lower PPV.[††] Among 11 participants with antigen-negative, real-time RT-PCR–positive specimens with positive viral culture, five were symptomatic and six asymptomatic. Some antigen-negative, real-time RT-PCR–positive specimens possibly could represent noninfectious viral particles, but some might also represent infectious virus not detected by the antigen test. In a clinical context, real-time RT-PCR provides the most sensitive assay to detect infection. Viral culture, although more biologically relevant than real-time RT-PCR, is still an artificial system and is subject to limitations. Numerous biological (e.g., individual antibody status and specific sequence of the virus) and environmental (e.g., storage conditions and number of freeze-thaw cycles) variables can affect the sensitivity and

[§] 45 C.F.R. part 46.102(l)(2), 21 C.F.R. part 56; 42 U.S.C. Sect. 241(d); 5 U.S.C. Sect. 552a; 44 U.S.C. Sect. 3501 et seq.

[¶] Participants were asked whether they had each sign or symptom from a list based on Council for State and Territorial Epidemiologists clinical criteria for COVID-19 that included fever, cough, shortness of breath, fatigue, sore throat, headache, muscle aches, chills, nasal congestion, difficulty breathing, diarrhea, nausea, vomiting, abdominal pain, rigors, loss of taste, and loss of smell. https://odn.ymaws.com/www.cste.org/resource/resmgr/ps/positionstatement2020/Interim-20-ID-02_COVID-19.pdf.

[**] Ct values from the N1 viral nucleocapsid protein gene region from real-time RT-PCR were compared only for specimens that were analyzed with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for detection of SARS-CoV-2. Lower Ct values represent higher levels of viral RNA in the specimen and higher Ct values represent lower levels of viral RNA.

[††] https://www.cdc.gov/coronavirus/2019-ncov/lab/faqs.html#Interpreting-Results-of-Diagnostic-Tests.

TABLE 1. Characteristics of persons providing paired upper respiratory swabs (N = 3,419)* for the Abbott BinaxNOW COVID-19 Ag Card Point of Care Diagnostic Test and real-time reverse transcription–polymerase chain reaction (RT-PCR) testing† for SARS-CoV-2 at two community-based testing sites, by test results — Pima County, Arizona, November 2020

Characteristic	Total no. of persons (column %)	No. of persons (row %)§			
		Antigen-positive	Real-time RT-PCR–positive	Real-time RT-PCR–positive, antigen-negative	Real-time RT-PCR–negative, antigen-positive
Total	3,419 (100)	161 (4.7)	299 (8.7)	142 (4.2)	4 (0.1)
Testing site					
A	1,458 (42.6)	72 (4.9)	145 (9.9)	74 (5.1)	1 (0.1)
B	1,961 (57.4)	89 (4.5)	154 (7.9)	68 (3.5)	3 (0.2)
Sex					
Male	1,290 (37.7)	74 (5.7)	138 (10.7)	63 (5.0)	1 (0.1)
Female	1,681 (49.2)	76 (4.5)	127 (7.6)	54 (3.2)	3 (0.2)
Undisclosed	448 (13.1)	11 (2.5)	34 (7.6)	23 (5.1)	0 (—)
Ethnicity					
Hispanic/Latino	1,075 (31.4)	86 (8.0)	150 (14.0)	65 (6.0)	1 (0.1)
Not Hispanic or Latino	1,930 (56.4)	63 (3.3)	118 (6.1)	58 (3.0)	3 (0.2)
Undisclosed	414 (12.1)	12 (2.9)	31 (7.5)	19 (4.6)	0 (—)
Race					
White	2,567 (75.1)	110 (4.3)	204 (7.9)	98 (3.8)	4 (0.2)
Black/African American	83 (2.4)	3 (3.6)	8 (9.6)	5 (6.0)	0 (—)
American Indian/Alaska Native	69 (2.0)	1 (1.4)	2 (2.9)	1 (1.4)	0 (—)
Asian	84 (2.5)	4 (4.8)	10 (11.9)	6 (7.1)	0 (—)
Native Hawaiian/Pacific Islander	24 (0.7)	1 (4.2)	1 (4.2)	0 (—)	0 (—)
Undisclosed	592 (17.3)	42 (7.1)	74 (12.5)	32 (5.4)	0 (—)
Age group, yrs					
10–17	336 (6.9)	10 (4.2)	22 (9.3)	13 (5.5)	1 (0.4)
18–49	1,885 (55.1)	91 (4.8)	178 (9.4)	89 (4.7)	2 (0.1)
50–64	743 (21.7)	41 (5.5)	69 (9.3)	29 (3.9)	1 (0.1)
≥65	555 (16.2)	19 (3.4)	30 (5.4)	11 (2.0)	0 (—)
Median age (range)	41 (10–85)	40 (13–84)	38 (11–84)	35 (11–83)	27 (16–63)
Current symptoms¶					
≥1	827 (24.2)	113 (13.7)	176 (21.3)	63 (7.6)	0 (—)
None	2,592 (75.8)	48 (1.9)	123 (4.7)	79 (3.0)	4 (0.2)
Days from symptom onset**					
Median (range)	4 (0–210)	3 (0–14)	4 (0–65)	4 (0–65)	2 (0–12)
0–3	382 (11.2)	59 (15.4)	84 (22.0)	25 (6.5)	0 (—)
4–7	280 (8.2)	42 (15.0)	58 (20.7)	16 (5.7)	0 (—)
8–10	43 (1.3)	6 (14.0)	12 (27.9)	6 (14.0)	0 (—)
11–14	63 (1.8)	6 (9.5)	16 (25.4)	10 (15.9)	0 (—)
>14	55 (1.6)	0 (—)	6 (10.9)	6 (10.9)	0 (—)
≥7	662 (19.4)	101 (15.3)	142 (21.5)	41 (6.2)	0 (—)
Exposure to a diagnosed COVID-19 case††					
Yes	1,138 (33.3)	93 (8.2)	162 (14.2)	71 (6.2)	2 (0.2)
No/Unknown	2,281 (66.7)	68 (3.0)	137 (6.0)	71 (3.1)	2 (0.1)
Days since last exposure, median (range)	5 (0–14)	4 (0–14)	3 (0–14)	1 (0–14)	9 (4–14)
Positive test results in past 90 days§§					
Yes	179 (5.2)	22 (12.3)	83 (46.4)	62 (34.6)	1 (14.3)
No/Unknown	3,239 (94.7)	139 (4.3)	216 (6.7)	80 (2.5)	3 (42.9)

Abbreviation: COVID-19 = coronavirus disease 2019.

* Includes 113 persons who received testing multiple times and were included more than once in the analysis.

† Testing with real-time RT-PCR was performed using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for detection of SARS-CoV-2 (2,582 participants) or Fosun assay (837 participants).

§ Only selected categories shown; therefore, row numbers and percentages do not sum to total or 100%.

¶ Participants were asked whether they had each individual sign or symptom from a list based on the Council of State and Territorial Epidemiologists' clinical criteria for COVID-19 interim case definition, which include fever, cough, shortness of breath, fatigue, sore throat, headache, muscle aches, chills, nasal congestion, difficulty breathing, diarrhea, nausea, vomiting, abdominal pain, rigors, loss of taste, and loss of smell (https://cdn.ymaws.com/www.cste.org/resource/resmgr/ps/positionstatement2020/Interim-20-ID-02_COVID-19.pdf).

** Based on one or more symptoms.

†† Exposure was defined as close contact (within 6 ft for ≥15 min) in the 14 days before the day of testing with a person with diagnosed COVID-19.

§§ Received positive real-time RT-PCR or antigen test result.

TABLE 2. Test results and performance characteristics of the Abbott BinaxNOW COVID-19 Ag Card Point of Care Diagnostic Test (BinaxNOW antigen test) compared with real-time reverse transcription–polymerase chain reaction (RT-PCR) for testing received among asymptomatic and symptomatic persons at two community-based testing sites — Pima County, Arizona, November 2020

Results and Performance	Real-time RT-PCR, no. of tests		
	Positive	Negative	Total
BinaxNOW antigen test result			
All participants (N = 3,419)			
Positive	157	4	161
Negative	142	3,116	3,258
Total	299	3,120	3,419
Symptomatic (≥1 symptom) (n = 827)			
Positive	113	0	113
Negative	63	651	714
Total	176	651	827
Asymptomatic (n = 2,592)			
Positive	44	4	48
Negative	79	2,465	2,544
Total	123	2,469	2,592
BinaxNOW antigen test performance, % (95% CI)			
All participants (N = 3,149)			
Sensitivity	52.5 (46.7–58.3)		
Specificity	99.9 (99.7–100.0)		
PPV	97.5 (93.8–99.3)		
NPV	95.6 (94.9–96.3)		
Symptomatic (n = 827)			
Sensitivity	64.2 (56.7–71.3)		
Specificity	100.0 (99.4–100.0)		
PPV	100.0 (96.8–100.0)		
NPV	91.2 (88.6–93.1)		
Asymptomatic (n = 2,592)			
Sensitivity	35.8 (27.3–44.9)		
Specificity	99.8 (99.6–100.0)		
PPV	91.7 (80–97.7)		
NPV	96.9 (96.1–97.5)		

Abbreviations: CI = confidence interval; COVID-19 = coronavirus disease 2019; NPV = negative predictive value; PPV = positive predictive value.

outcome of viral culture. Despite the limitations of interpreting culture-negative specimens, a positive viral culture is strong evidence for the presence of infectious virus. The performance of the BinaxNOW antigen test compared with real-time RT-PCR was better for those specimens with positive viral culture than for all specimens, with a sensitivity of 92.6% for specimens from symptomatic persons and 78.6% for those from asymptomatic persons. The results of the current evaluation differ from those of an evaluation of the BinaxNOW antigen test in a community screening setting in San Francisco (*7*), which found a BinaxNOW antigen test overall sensitivity of 89.0% among specimens from all 3,302 participants, regardless of the Ct value of the real-time RT-PCR–positive specimens.

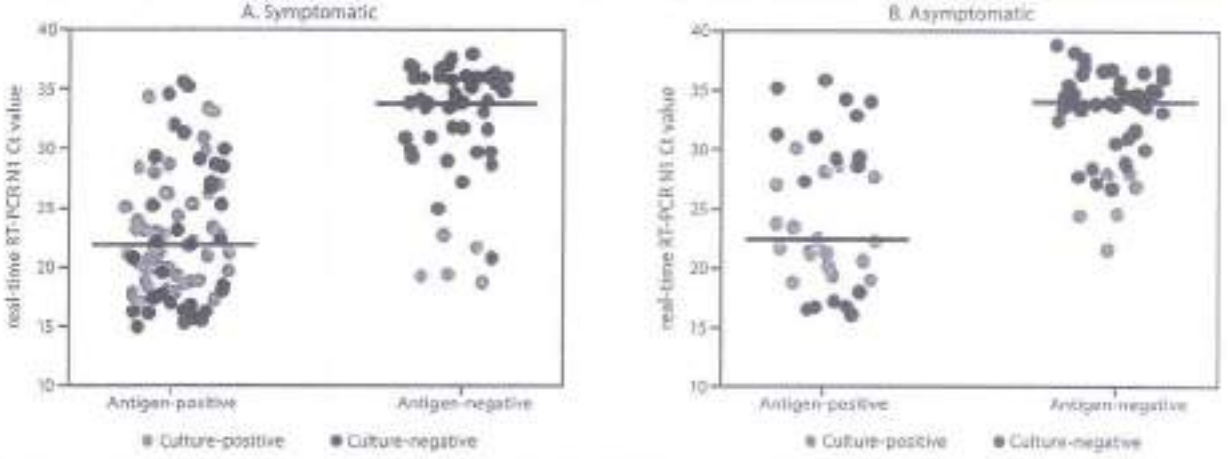
The findings in this investigation are subject to at least five limitations. First, anterior nasal swabs were used for BinaxNOW antigen testing, but NP swabs were used for real-time RT-PCR testing, which might have contributed to increased detection for the real-time RT-PCR assay (*8*). Second, participants might have inadvertently reported common nonspecific symptoms as COVID-19–compatible symptoms. Third, this investigation evaluated the BinaxNOW antigen test, and results presented here cannot be generalized to other FDA-authorized SARS-CoV-2 antigen tests. Fourth, the BinaxNOW antigen test characteristics might be different depending on whether an individual had previously tested positive. Finally, many factors might limit the ability to culture virus from a specimen, and the inability to detect culturable virus should not be interpreted to mean that a person is not infectious.

Public health departments are implementing various strategies to reduce or prevent SARS-CoV-2 transmission, including expanded screening testing for asymptomatic persons (*3*). Because estimates suggest that over 50% of transmission occurs from persons who are presymptomatic or asymptomatic (*9*), expanded screening testing, potentially in serial fashion for reducing transmission in specific venues (e.g., institutions of higher education, schools, and congregate housing settings), is essential to interrupting transmission (*3*).

Rapid antigen tests can be an important tool for screening because of their quick turnaround time, lower requirement for resources, high specificity, and high PPV in settings of high pretest probability (e.g., providing testing to symptomatic persons, to persons with a known COVID-19 exposure, or where community transmission is high). Importantly, the faster time from testing to results reporting can speed isolation of infectious persons and will be particularly important in communities with high levels of transmission.

Although the sensitivity of the BinaxNOW antigen test to detect infection was lower compared with real-time RT-PCR, it was relatively high among specimens with positive viral culture, which might reflect better performance for detecting infection in a person with infectious virus present. Community testing strategies focused on preventing transmission using antigen testing should consider serial testing (e.g., in kindergarten through grade 12 schools, institutions of higher education, or congregate housing settings), which might improve test sensitivity in detecting infection (*10*). When the pretest probability for receiving positive SARS-CoV-2 test results is elevated (e.g. for symptomatic persons or for persons with a known COVID-19 exposure) a negative antigen test result should be confirmed by NAAT. Asymptomatic persons who receive a positive BinaxNOW antigen test result in a setting with a high risk for adverse consequences resulting from false-positive results (e.g. in long-term care facilities) should also receive confirmatory testing by NAAT (*1*).

FIGURE. Abbott BinaxNOW COVID-19 Ag Card Point of Care Diagnostic Test (antigen test) results, N1 cycle threshold (Ct) values,* and viral culture results† among A) symptomatic (N = 136)§ and B) asymptomatic (N = 88)¶ participants receiving positive SARS-CoV-2 real-time reverse transcription–polymerase chain reaction (RT-PCR) test results at two community-based testing sites — Pima County, Arizona, November 2020



* Only those specimens that were analyzed using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel for detection of SARS-CoV-2 and that were analyzed using viral culture are included in the graph.

† Twenty specimens with Ct values <18 had positive antigen and real-time RT-PCR results but were culture negative. The culture showed evidence of cytopathic effects and had presence of SARS-CoV-2 RNA as detected by real-time RT-PCR in the first passage culture, but viral recovery was not two Ct values lower than the corresponding clinical specimen Ct.

§ Antigen test results: 88 positive and 48 negative; median Ct values indicated with black line: 22.0 for antigen-positive specimens and 33.9 for antigen-negative specimens.

¶ Antigen test results: 37 positive and 51 negative; median Ct values indicated with black line: 22.5 for antigen-positive specimens and 33.9 for antigen-negative specimens.

Summary

What is already known about this topic?

The BinaxNOW rapid antigen test received Emergency Use Authorization by the Food and Drug Administration for testing specimens from symptomatic persons; performance among asymptomatic persons is not well characterized.

What is added by this report?

Sensitivity of the BinaxNOW antigen test, compared with polymerase chain reaction testing, was lower when used to test specimens from asymptomatic (35.8%) than from symptomatic (64.2%) persons, but specificity was high. Sensitivity was higher for culture-positive specimens (92.6% and 78.6% for those from symptomatic and asymptomatic persons, respectively); however, some antigen test-negative specimens had culturable virus.

What are the implications for public health practice?

The high specificity and rapid BinaxNOW antigen test turnaround time facilitate earlier isolation of infectious persons. Antigen tests can be an important tool in an overall community testing strategy to reduce transmission.

Despite their reduced sensitivity to detect infection compared with real-time RT-PCR, antigen tests might be particularly useful when real-time RT-PCR tests are not readily available or have prolonged turnaround times. Persons who know their positive test result within 15–30 minutes can isolate sooner, and contact tracing can be initiated sooner and be more effective than if a test result is returned days later. Serial antigen testing can improve detection, but consideration should be given to the logistical and personnel resources needed. All persons receiving negative test results (NAAT or antigen) should be counseled that wearing a mask, avoiding close contact with persons outside their household, and washing hands frequently remain critical to preventing the spread of COVID-19.[55]

[55] https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/prevention.html

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Corresponding author: Jessica L. Prinzi-Guerra, yov1@cdc.gov.

[1]CDC COVID-19 Response Team; [2]Arizona Department of Health Services; [3]Epidemic Intelligence Service, CDC; [4]Pima County Health Department, Tucson, Arizona.

All authors have completed and submitted the International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest. No potential conflicts of interest were disclosed.

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EXHIBIT "OO"

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Evaluation of a SARS-CoV-2 rapid antigen test: Potential to help reduce community spread?

Tuna Toptan [a,*], Lisa Eckermann [a], Annika E. Pfeiffer [a], Sebastian Hoehl [a], Sandra Ciesek [a], Christian Drosten [b,c], Victor M. Corman [b,c]

[a] Institute for Medical Virology, University Hospital Frankfurt, Goethe University Frankfurt am Main, Germany
[b] Institute of Virology, Charité-Universitätsmedizin Berlin, Humboldt-Universität zu Berlin, Berlin, Germany
[c] German Centre for Infection Research (DZIF), Berlin, Germany

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ABSTRACT

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can spread from symptomatic persons with COVID-19, but also from asymptomatic individuals. Therefore, robust surveillance and timely interventions are essential for the control of virus spread within the community. In this regard the frequency of testing and speed of reporting, but not the test sensitivity alone, play a crucial role.

Objectives: In order to reduce the costs and meet the expanding demands in real-time RT-PCR (rRT-PCR) testing for SARS-CoV-2, complementary assays, such as rapid antigen tests, have been developed. Rigorous analysis under varying conditions is required to assess the clinical performance of these tests and to ensure reproducible results.

Results: We evaluated the sensitivity and specificity of a recently licensed rapid antigen test using 117 clinical samples in two institutions. Test sensitivity was between 88.2–89.6 % when applied to samples with viral loads typically seen in infectious patients. Of 32 rRT-PCR positive samples, 19 demonstrated infectivity in cell culture, and 84 % of these samples were reactive with the antigen test. Seven full-genome sequenced SARS-CoV-2 isolates and SARS-CoV-1 were detected with this antigen test, with no cross-reactivity against other common respiratory viruses.

Conclusions: Numerous antigen tests are available for SARS-CoV-2 testing and their performance to detect infectious individuals may vary. Head-to-head comparison along with cell culture testing for infectivity may prove useful to identify better performing antigen tests. The antigen test analyzed in this study is easy-to-use, inexpensive, and available. It can be helpful in monitoring infection trends and thus has potential to reduce transmission.

1. Introduction

Since the beginning of COVID-19 outbreak in December 2020, the global demand for the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing has been steadily increasing. Already back in March 2020, hospitals and laboratories around the world announced their concerns about reagent, consumable material shortages, and limited personal protective equipment. Yet, timely detection and isolation of SARS-CoV-2 infected cases and identification of their contacts are pivotal to slowing down the pandemic.

The main public health strategy during a pandemic relies on robust and easy to perform diagnostic tools that can be used to test large number of samples in a short time. To date the gold standard diagnostic method for SARS-CoV-2 detection [1] is based on real time reverse transcription-PCR (rRT-PCR) technology which has been promptly implemented by the World Health Organization (WHO) [2], Center for Disease Control and Prevention (CDC) [3] protocols, and a number of commercial assays [4]. The SARS-CoV-2 rRT-PCR has high specificity and sensitivity [5,6]. However, the type and quality of the patient specimen [7,8], stage of the disease, and the degree of viral replication and/or clearance have an impact on the test outcome [9]. These factors are critical not only for PCR-based but also for other diagnostic test systems aiming to detect the presence of the virus. Hence interpreting a test result for SARS-CoV-2 depends on the accuracy of the test, but the

* Corresponding author at: Institute for Medical Virology, University Hospital Frankfurt, Paul-Ehrlich-Str. 40, 60590, Frankfurt am Main, Germany.
E-mail address: tuna.toptan@kgu.de (T. Toptan).

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prevalence and the estimated risk of disease before testing should also be taken into consideration.

In many countries SARS-CoV-2 testing is extended to asymptomatic population, e.g. in schools, airports, nursing-homes, and workplaces. This leads to a growing gap between the large number of demand and the laboratory capacities to preform rRT-PCR tests, especially in developing countries. Despite high specificity and sensitivity, rRT-PCR has a disadvantage in point of care testing, because it usually requires professional expertise, expensive reagents and specialized equipment. Therefore, alternative assays, such as rapid antigen detection tests, which can also detect the presence of the virus directly in respiratory samples, have been developed [4] and tested by different groups [10–14]. However, it is vital to determine the sensitivity, specificity of such tests relative to standard rRT-PCR in order to identify the ideal circumstances that their application would be beneficial.

This study was performed to evaluate a novel antigen test produced by R-Biopharm for the detection of SARS-CoV-2 in different specimens and to identify its limitations and potential usage. Different types of materials and verification analysis were used by two institutions independently to assure the reproducibility of the testing and to analyze the potential caveats.

2. Materials and methods

2.1. Specimen collection

At the Institute of Virology, Charité Berlin stored specimens taken after routine diagnostic were used with no extra procedures required for the study. Cell culture supernatants of respiratory viruses other than SARS-CoV-2 were available at the Institute of Virology, Charité through a EVD-LabNet EQA (https://www.evd-labnet.eu/) [25].

At the Institute of Virology, Frankfurt, the clinical samples were collected from subjects as part of registered protocols. Combined oropharyngeal/nasal swabs were collected, stored in 2 ml PBS at 4 °C and processed for further analysis within 24 h.

2.2. Cell culture and virus stocks

Caco-2 (human colon carcinoma) were cultured in Minimum Essential Medium (MEM) supplemented with 10 % final calf serum (FCS), 100 IU/mL of penicillin and 100 g/mL of streptomycin. All culture reagents were purchased from Sigma (St. Louis, MO, USA). The Caco-2 cells were originally obtained from DSMZ (Braunschweig, Germany, no.: ACC 169), differentiated by serial passaging and selected for high permissiveness to virus infection. Caco-2 cells were infected with different viral isolates (FFM1-FFM7) [15] at an MOI 0.1. Cell culture supernatant was harvested 48 h after infection, precleared at 2000 × g for 10 min at room temperature. Aliquots of virus particle containing supernatant were kept at −80 °C.

2.3. Detection of infectious virus in cell culture

Of the swab-dilution, 500 µL were mixed with 1.5 ml of MEM containing 1% FCS (Sigma-Aldrich; St. Louis, Missouri, USA), 7.5 µg/mL Amphotericin B, and 0.1 mg/mL Primocin, (InvivoGen; San Diego, California, USA). Swab-inoculums were transferred to Caco-2 cells seeded in 5.5 cm^2 culture tubes. Cytopathogenic effect (CPE) was assessed daily for up to seven days or until cell lysis occurred.

2.4. Rapid antigen test

Rapid antigen test was provided by R-Biopharm. Test was performed according to the manufacturer's recommendations and evaluated visually by four or six-eye principle. Briefly, samples were vortexed for 20 s. 50 µl from Solution A (blue) and B (yellow) were dispensed in clean 1.5 ml reaction tubes which leads to green coloring. Immediately 50 µl of the test samples were added to the reaction mixture, mixed briefly and incubated for 10 min at room temperature. Test strips were placed into the mixture vertically to allow absorption. Test results were evaluated after 10 min. Intensities of the test bands were compared to control bands and categorized as follows: +++ (test band intensity stronger than the control), ++ (test and control band intensity are similar), + (test band intensity is weaker than the control). Antigen testing for viable SARS-CoV-2 and SARS-CoV-1 cell culture supernatants was performed in a BSL-3 laboratory.

2.5. RNA extraction and rRT-PCR analysis

At the Institute of Virology, Charité Berlin, stored samples (swab resuspended in 1.5 mL of phosphate-buffered saline) were anonymized before testing. After thawing at room temperature all samples were analyzed by antigen test and rRT-PCR in parallel. RNA extraction for rRT-PCR was done by using the MagNA Pure 96 system, using 100 µl of sample, eluted in 100 µl. The rRT-PCR was performed as published previously [1].

At the Institute of Virology in Frankfurt the SARS-CoV-2 rRT-PCR analysis (Cobas, Roche, Basel, Switzerland) was performed on the automated Cobas 6800 system. Of the swab-dilution, 1000 µl aliquots were mixed with lysis buffer (1:1 ratio) and 500 µL aliquots were transfered to barcoded secondary tubes, loaded on the Cobas 6800 system, tested with Cobas SARS-CoV-2 master mix containing an internal RNA control, and primer-probe sets towards ORF1 and E-gene according to the manufacturer's instructions.

Within seven days of virus inoculation using clinical sample material, culture supernatant was collected to perform rRT-PCR in order to confirm productive virus replication. RNA was isolated from 100 µL cell culture supernatant using the QIAcube HT instrument and QIAamp 96 Virus QIAcube HT Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. SARS-CoV-2 RNA was analyzed by rRT- PCR using the Luna Universal One-Step RT-qPCR Kit (New England Biolabs; Ipswich, Massachusetts, USA) and primers targeting RNA-dependent RNA polymerase (RdRp) as described [16]. RdRP_SARSr-F2: GTGARATGGTCATGTGTGGGGG, RdRP_SARSr-R1: CARATGTTAAASACACTATTAGCATA.

2.6. Statistical analysis

The number of positive samples were compared using two by two contingency table. The agreement between the antigen test and rRT-PCR techniques was evaluated using the Cohen's weighted kappa index (K value) [17]. K value interpretations were categorized as follows: <0.20 is poor, 0.21–0.40 is fair, 0.41–0.60 is moderate, 0.61–0.80 is substantial, and 0.81–1.00 is almost perfect agreement [17].

2.7. Ethical statement

The use of stored clinical samples for validation of diagnostic methods without person related data is covered by Section 2.5 of the Berlin hospital law and does not require ethical or legal clearance.

The use of anonymized clinical samples for validation of diagnostic methods does not require ethical clearance by the Goethe University, Frankfurt.

3. Results

Rapid antigen test sensitivity and specificity were evaluated by two independent institutions using various number of clinical samples. rRT-PCR was used as a reference test system. We deemed individuals to be uninfected with SARS-CoV-2 when a negative result was obtained by rRT-PCR.

In the Institute of Virology, Charité, Berlin, a total of 67 stored patient samples were available for the study. Of these, 38 were rRT-PCR positive with cycle threshold (Ct) range between 18.77–30

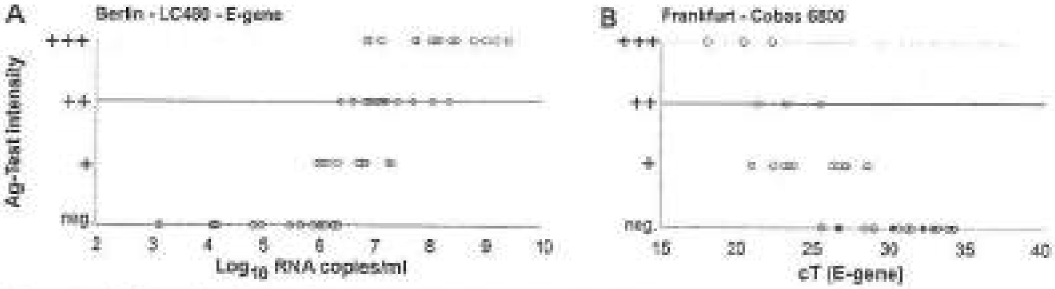

Fig. 1. Antigen test analysis performed in Berlin (A) and Frankfurt (B). A. Log_{10} RNA copies/mL and corresponding antigen (Ag) detection test results (red circles positive n: 45, blue circles negative n: 13) for each rRT-PCR positive sample (n: 58). **B.** Cycle threshold (cT) value and corresponding antigen (Ag) detection test results (red circles positive n: 16, blue circles negative n: 16) for each rRT-PCR positive sample (n: 32). 32 rRT-PCR positive samples were tested in cell culture for infectivity. All Ag-test positive (n:16, red circles) and three Ag-test negative (red-filled blue circles) samples displayed CPEs after inoculating in Caco-2 cells (Table S2). Intensities of the test bands were compared to control band and designated as follows: +++ (test band intensity stronger than the control), ++ (test and control band intensity are similar), + (test band intensity is weaker than the control).

Table 1
Sensitivity and specificity of the antigen detection test in comparison to rRT-PCR.

		Antigen test				
		Negative	Positive	Marginal row	Sensitivity (%)	Specificity (%)
rRT-PCR	Negative	9	0	9		100
	Positive	13	45	58	77.6	
genomic copies/mL	$\geq 1.09 \times 10^{6}$	5	43	48	89.6	
	1.38×10^{3}–9.76×10^{5}	8	2	10	20	

Table 2
Cohen's weighted kappa coefficient between rapid antigen test and rRT-PCR.

	Antigen test		
rRT-PCR	Negative	Positive	Row marginal
Negative	9	0	9 (13.4%)
Positive	13	45	58 (86.6%)
Column marginal	22 (32.8%)	45 (67.2%)	67
Weighted Kappa	0.482		
Standard error	0.110		
95% CI	0.266 to 0.698		

corresponding to 2.5×10^{8} -1380 RNA copies/mL (Table S1), representing 86.6 % (58/67) of the clinical samples analyzed (Fig. 1A). When the rRT-PCR results were used as a reference, the antigen test diagnosed SARS-CoV-2 infection status with a sensitivity of 77.6 % (45/58) and a specificity of 100 % (9/9) (Table 1). After re-evaluating the data based on the acceptable analytic sensitivity and limit of detection suggested by WHO [18], we identified 48 samples with $\geq 10^{6}$ RNA copies/mL. Rapid antigen test performed with 89.6 % sensitivity for this sample set (Table 1). Of these, 40 samples had $\sim 2.23 \times 10^{6}$ or more RNA copies/mL and reacted positive with the antigen test(Table S1). In contrast samples with less than 7.63×10^{5} RNA copies/mL were negative (Fig. 1A, Table S1). Cohen's weighted kappa value of 0.482 indicated moderate agreement between the rRT-PCR and the rapid antigen test (Table 2). The overall concordance between the rRT-PCR and the antigen test was 80.6 % (54/67).

Certain rapid tests may be used at the point-of-care and thus offer benefits for the detection and management of infectious diseases. In order to assess the potential of the rapid antigen test in this context, 70 nasopharyngeal samples freshly collected from individuals living in a shared housing were analyzed head to head by rRT-PCR using Cobas 6800 system, rapid antigen test, and cell culture using Caco-2 cells to determine the infectivity (Institute of Medical Virology, Goethe University, Frankfurt). 45.7 % (32/70) of the clinical samples were diagnosed positive for SARS-CoV-2 by rRT-PCR with cT values ranging between 18.01–35.98 (Fig. 1B, Table S2). The antigen test diagnosed the infection status with a sensitivity of 50 % (16/32) and a specificity of 100 % (Table 3). Re-evaluating the data based on the limit of detection, sensitivity was determined to be 88.2 % for samples with cT values <28, and it was reduced in the group of samples with cT values ≥28 (6.7 %) (Table 3). Cohen's weighted kappa value of 0.521 indicated moderate agreement between rRT-PCR and the rapid antigen test (Table 4). The overall concordance between the rRT-PCR and the antigen test was 77.1 % (54/70) (Table 4).

rRT-PCR is a highly sensitive method to detect viral RNA molecules from clinical samples. However, viral RNA can persist in different body parts and can be detected in specimens for much longer than the presence of viable virus [19]. Thus demonstration of infectivity on permissive cell lines in vitro is a more reliable surrogate for infectivity and virus transmission. Therefore, we attempted virus isolation by inoculating

Table 3
Comparison of the clinical diagnostic performance of rapid antigen test with rRT-PCR.

		Antigen test				
		Negative	Positive	Marginal row	Sensitivity (%)	Specificity (%)
rRT-PCR	Negative	38	0	38		100
	Positive	16	16	32	50	
cT<28	Positive	2	15	17	88.2	
cT≥28	Positive	14	1	15	6.7	

Table 4
Cohen's weighted kappa coefficient between rapid antigen test and rRT-PCR.

	Antigen test		
rRT-PCR	Negative	Positive	Row marginal
Negative	38	0	38 (54.3%)
Positive	16	16	32 (45.7%)
Column marginal	54 (77.1%)	16 (22.9%)	70
Weighted Kappa	0.521		
Standard error	0.092		
95% CI	0.339 to 0.702		

Table 5
Rapid Antigen Test results using different respiratory virus cell culture supernatant stocks.

Cell culture supernatant with virus	Antigen-Test
SARS-CoV-1	+
SARS-CoV-2	+
HCoV-229E	-
HCoV-NL63	-
MERS-CoV	-
Enterovirus	-
Rhinovirus	-
Parainfluenzavirus 1	-
Parainfluenzavirus 2	-
Parainfluenzavirus 3	-
Parainfluenzavirus 4	-
hMPV A	-
hMPV B	-
RSV	-
Influenzavirus A H1N1	-
Influenzavirus A H3N2	-
Influenza B	-

rRT-PCR positive samples in Caco-2 cells. Cytopathic changes were monitored daily by microscopy for a week and subsequently aliquots of culture supernatant were tested to verify viral RNA copies (Table S2). For samples that are positive for both antigen test and rRT-PCR (16/32, cT 18.01–28.45), we observed cytopathic effects (CPE) in cell culture 1–3 days after inoculation (Fig. 1B, Table S2). Three samples that had a negative result in the antigen test, but were positive by rRT-PCR (cT values 26.68, 30.12, and 32.13) displayed CPE as well. Other 13 antigen-test negative samples with higher cT values (indicating lower viral load) between 28.34–34.12 were not infectious in cell culture. Interestingly, one sample with a relatively low cT value 25.53, did not show any CPE in cell culture and was also negative for the antigen test (Table S2).

In order to investigate potential cross reactivity among common coronaviruses and other respiratory viruses, infectious and heat inactivated (4 h at 60 °C) cell culture supernatants were tested (Table 5). SARS-CoV-1 and SARS-CoV-2 tested positive with the antigen test, as expected. The antigen test did not display any cross-reactivity with the other respiratory and endemic corona viruses listed in Table 5.

We further evaluated the detection sensitivity among different SARS-CoV-2 isolates. Here we used cell culture supernatant collected from Caco-2 cells infected with seven different isolates [15] and SARS-CoV-1 (Fig. 2). The virus stocks were thawed at room temperature and a total of six 10-fold dilutions were prepared in PBS. The antigen test was performed and evaluated immediately (Fig. 2A). In parallel, aliquots of the dilutions were mixed with lysis buffer used for RNA extraction to inactivate the virus. rRT-PCR was performed for two different gene targets ORF1 and E-gene that resulted in similar cT values (Fig. 2B, Table S3). 10-fold serial dilutions led to ~3 cT difference in rRT-PCR for each set as anticipated. According to our results the limit of detection was between 100–560 RNA copies/mL which is in line with the manufacturer's findings. We previously identified RG203KR mutations in FFM3, FFM4 and FFM6 and S→L mutation in FFM1 within the nucleocapsid protein coding region [15]. According to GISAID classification the GR clade, carrying the combination of Spike D614G and nucleocapsid RG203KR mutations, is currently the most common representative of the SARS-CoV-2 population worldwide [20]. Our results suggest that the presence of the RG203KR mutation did not interfere with the antigen test performance.

4. Discussion

In this study we validated the assay performance of a recently approved rapid antigen test in two independent institutions using a total of 137 clinical samples. Although the test specificity was 100 % for this particular sample set, overall sensitivity was low (50–77.6 %), yet re-analyzing samples with higher viral loads, typically observed during early stages of the infection, showed good correlation (88.2–89.6 %). Previous studies reported that lower cT values are associated with higher viral culture positivity [21,22]. There is currently no direct evidence whether cell culture positivity or higher viral load correlates with contagiousness of an individual, however, it is commonly recognized as the surrogate of infectivity [23]. Since an important aspect of using point-of-care testing is to able to identify infected individuals who are infectious and can potentially transmit the virus, we performed correlation analysis within a group of clinical samples tested. 19 out of 32 SARS-CoV-2 infected individuals were positive in cell culture. The antigen test detected 16 out of 19 these (84 %). In contrast 43.7 % (14/32) of the samples were not infectious in cell culture, yet positive by rRT-PCR, probably due to persisting genomic and subgenomic viral RNA within the collected sample. We detected an excess amount of viral RNA in cell culture supernatants due to high replication capacity of the virus in permissive cells, despite a negative antigen test result. This might explain the cT discrepancy between the cell culture supernatant and clinical samples. Small sample size is the major limitation of this study. Future efforts should aim to monitor frequent sampling of larger groups and to compare different rapid antigen tests, different sampling sites along with infectivity correlation in cell culture.

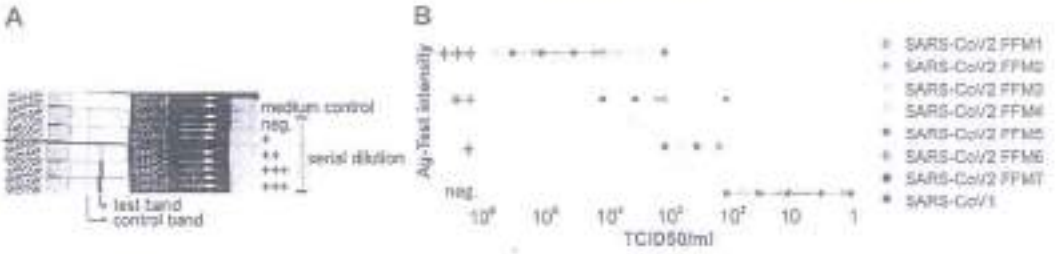

Fig. 2. Rapid Antigen Test Results for SARS-CoV-1 and SARS-CoV-2 isolates. A. Representative lateral flow assay using serially diluted virus stocks. Intensities of the test bands were compared to control band and designated as follows: +++ (test band intensity stronger than the control), ++ (test and control band intensity are similar), + (test band intensity is weaker than the control). B. TCID50/mL values and corresponding antigen (Ag) detection test intensity for serially diluted SARS-CoV-2 isolates FFM1-7 and SARS-CoV-1 are shown. Representative result of two experiments.

Our results suggest that the rapid antigen test can detect SARS-CoV-2 infected individuals with high viral loads and has potential in determining highly contagious individuals. Despite low analytic sensitivity, rapid antigen tests are inexpensive and therefore can be used frequently for detecting infected individuals who are asymptomatic, pre-symptomatic and without known or suspected exposure to SARS-CoV-2 [24]. They can be beneficial in congregate settings, such as a long-term care facility or a correctional facility, workplace, or a school testing its students, faculty, and staff. Rapid antigen tests likely perform best during the early stages of SARS-CoV-2, when the viral loads are higher.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2020.104713.

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EXHIBIT "PP"

Potential for False Positive Results with Antigen Tests for Rapid Detection of SARS-CoV-2 - Letter to Clinical Laboratory Staff and Health Care Providers

The U.S. Food and Drug Administration (FDA) is alerting clinical laboratory staff and health care providers that false positive results can occur with antigen tests, including when users do not follow the instructions for use of antigen tests for the rapid detection of SARS-CoV-2. Generally, antigen tests are indicated for the qualitative detection of SARS-CoV-2 antigens in authorized specimen types collected from individuals who are suspected of COVID-19 by their healthcare provider within a certain number of days of symptom onset. The FDA is aware of reports of false positive results associated with antigen tests used in nursing homes and other settings and continues to monitor and evaluate these reports and other available information about device safety and performance.

The FDA reminds clinical laboratory staff and health care providers about the risk of false positive results with all laboratory tests. Laboratories should expect some false positive results to occur even when very accurate tests are used for screening large populations with a low prevalence of infection. Health care providers and clinical laboratory staff can help ensure accurate reporting of test results by following the authorized instructions for use of a test and key steps in the testing process as recommended by the Centers for Disease Control and Prevention (CDC), including routine follow-up testing (reflex testing) with a molecular assay when appropriate, and by considering the expected occurrence of false positive results when interpreting test results in their patient populations.

Recommendations

The FDA recommends clinical laboratory staff and health care providers who use antigen tests for the rapid detection of SARS-CoV-2:

- Be aware that the Conditions of Authorization in the antigen Emergency Use Authorizations specify that authorized laboratories are to follow the manufacturer's instructions for use, typically found in the package insert, when performing the test and reading test results. If you no longer have the package insert for the test you are using, you can contact the manufacturer. The authorized instructions for use for each test can also be found on the FDA's COVID-19 IVD EUA webpage f/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas).
    - For example, the package insert for tests include instructions for handling of the test cartridge/card, such as ensuring it is not stored open prior to use. If the test components are not stored properly, this can affect the performance of the test.

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- The package insert for tests also includes instructions about reading the test results, including the appropriate time to read the results. **Reading the test *before or after* the specified time could result in false positive or false negative results.**

- Be aware that processing multiple specimens in batch mode may make it more challenging to ensure the correct incubation time for each specimen. Refer to the package insert and ensure proper timing for each specimen when processing the specimen in the test device and reading the results.
- Be careful to minimize the risks of cross-contamination when testing patient specimens, which can cause false positive results. Insufficient cleaning of the workspace, insufficient disinfection of the instrument, or inappropriate use of protective equipment (for example, failing to change gloves between patients) can increase the risk of cross-contamination between specimens with subsequent false positive results. Consider the CDC guidance (https://www.cdc.gov/coronavirus/2019-ncov/lab/point-of-care-testing.html) for changing gloves and cleaning work area between specimen handling and processing.
- Consider the CDC's recommendations (https://www.cdc.gov/coronavirus/2019-ncov/hcp/nursing-homes-antigen-testing.html) when using antigen testing in nursing homes and other settings. For positive results, especially in low incidence counties, consider performing confirmatory RT-PCR test within 48 hours.
- Remember that positive predictive value (PPV) varies with disease prevalence when interpreting results from diagnostic tests. PPV is the percent of positive test results that are true positives. As disease prevalence decreases, the percent of test results that are false positives increase.
    - For example, a test with 98% specificity would have a PPV of just over 80% in a population with 10% prevalence, meaning 20 out of 100 positive results would be false positives.
    - The same test would only have a PPV of approximately 30% in a population with 1% prevalence, meaning 70 out of 100 positive results would be false positives. This means that, in a population with 1% prevalence, only 30% of individuals with positive test results actually have the disease.
    - At 0.1% prevalence, the PPV would only be 4%, meaning that 96 out of 100 positive results would be false positives.
    - Health care providers should take the local prevalence into consideration when interpreting diagnostic test results.
- Consider positive results in combination with clinical observations, patient history, and epidemiological information.
- Be aware that the Conditions of Authorization in the antigen EUAs specify that Authorized Laboratories are to collect information on the performance of antigen tests and report any suspected occurrence of false positive or false negative results and

significant deviations from the established performance characteristics of which they become aware to both the FDA and the test manufacturer.

Background

The FDA issued the first Emergency Use Authorization (EUA) for a COVID-19 antigen test in May 2020. These diagnostic tests quickly detect fragments of proteins found on or within the virus by testing samples collected from the nasal cavity using swabs. A list of the FDA-authorized antigen tests are available on the FDA's In Vitro Diagnostics EUA page (https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas#individual-antigen).

Antigen tests are an important tool in the overall response against COVID-19 and benefit public health. One of the main advantages of an antigen test is the speed of the test, which can provide results in minutes. The availability of these types of tests may provide the ability to test millions of Americans rapidly.

In general, antigen tests are not as sensitive as molecular tests. Due to the potential for decreased sensitivity compared to molecular assays, negative results from an antigen test may need to be confirmed with a molecular test prior to making treatment decisions. Negative results from an antigen test should be considered in the context of clinical observations, patient history and epidemiological information.

Like molecular tests, antigen tests are typically highly specific for the SARS-CoV-2 virus. However, all diagnostic tests may be subject to false positive results, especially in low prevalence settings. Health care providers should always carefully consider diagnostic test results in the context of all available clinical, diagnostic and epidemiological information. Test interference from patient-specific factors, such as the presence of human antibodies (for example, Rheumatoid Factor, or other non-specific antibodies) or highly viscous specimens could also lead to false positive results.

FDA Actions

The FDA continues to work with other agencies, such as the Centers for Disease Control and Prevention (CDC) and the Centers for Medicare and Medicaid Services (CMS) to safeguard COVID test use in nursing homes and other settings.

The FDA is also working with test manufacturers to ensure that their instructions for use are as clear as possible to minimize the occurrence of false results.

The FDA will continue to keep clinical laboratory staff, health care providers, manufacturers, and the public informed of new or additional information.

Additional Resources

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- CDC's Considerations for Use of SARS-CoV-2 Antigen Testing in Nursing Homes (https://www.cdc.gov/coronavirus/2019-ncov/hcp/nursing-homes-antigen-testing.html)
- FAQs on Testing for SARS-CoV-2 (/medical-devices/coronavirus-covid-19-and-medical-devices/faqs-testing-sars-cov-2)
- In Vitro Diagnostics EUAs (/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas)

Reporting Problems to the FDA

The FDA encourages stakeholders to report any adverse events or suspected adverse events experienced with antigen tests for rapid detection of SARS-CoV-2.

- Voluntary reports can be submitted through MedWatch, the FDA Safety Information and Adverse Event Reporting program (/safety/medwatch-fda-safety-information-and-adverse-event-reporting-program/reporting-serious-problems-fda).
- Generally, as specified in a test's EUA, device manufacturers must comply with applicable Medical Device Reporting (MDR) regulations (/medical-devices/postmarket-requirements-devices/mandatory-reporting-requirements-manufacturers-importers-and-device-user-facilities).
- Health care personnel and clinical laboratory staff employed by facilities that are performing COVID-19 testing should follow the reporting requirements for authorized laboratories as specified in the test's EUA.

Prompt reporting of adverse events can help the FDA identify and better understand the risks associated with medical devices.

Contact Information

If you have questions about this letter, contact COVID19DX@fda.hhs.gov (mailto:COVID19DX@fda.hhs.gov).

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EXHIBIT "99"

Journal of Clinical Microbiology®

Challenges and Controversies to Testing for COVID-19

Matthew J. Binnicker[a,b]

[a]Division of Clinical Microbiology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA
[b]Director of Clinical Virology, Mayo Clinic, Rochester, Minnesota, USA

ABSTRACT The coronavirus disease (COVID-19) pandemic has placed the clinical laboratory and testing for SARS-CoV-2 front and center in the worldwide discussion of how to end the outbreak. Clinical laboratories have responded by developing, validating, and implementing a variety of molecular and serologic assays to test for SARS-CoV-2 infection. This has played an essential role in identifying cases, informing isolation decisions, and helping to curb the spread of disease. However, as the demand for COVID-19 testing has increased, laboratory professionals have faced a growing list of challenges, uncertainties, and, in some situations, controversy, as they have attempted to balance the need for increasing test capacity with maintaining a high-quality laboratory operation. The emergence of this new viral pathogen has raised unique diagnostic questions for which there have not always been straightforward answers. In this commentary, the author addresses several areas of current debate, including (i) the role of molecular assays in defining the duration of isolation/quarantine, (ii) whether the PCR cycle threshold value should be included on patient reports, (iii) if specimen pooling and testing by research staff represent acceptable solutions to expand screening, and (iv) whether testing a large percentage of the population is feasible and represents a viable strategy to end the pandemic.

The coronavirus disease (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2), has been confirmed in nearly 20 million cases and caused over 730,000 deaths worldwide (https://coronavirus.jhu.edu). As the virus has spread across the globe, laboratory testing has played an important role in diagnosing those with disease, as well as identifying individuals who are asymptomatic yet have the potential to serve as a source of viral transmission. Molecular tests, such as real-time PCR, have been the most common laboratory tool used to detect cases of COVID-19. In fact, SARS-CoV-2 molecular assays have become an integral component in a multipronged strategy aimed at reducing transmission of the virus. This strategy has consisted of (i) case identification (i.e., through testing), (ii) quarantine or isolation of exposed/infected individuals, and (iii) contact tracing. Despite the broad application of this strategy, cases of COVID-19 have continued to surge, especially in the United States, where the number of confirmed infections has surpassed 5 million (~25% of the global case count).

The COVID-19 pandemic is an unprecedented health care crisis that has required clinical and laboratory professionals to rapidly adapt to new information, innovate, and, in some situations, implement practices that would not be considered under normal circumstances. The emergence of SARS-CoV-2 has forced clinical laboratories and test manufacturers to develop novel diagnostic assays in a time frame that previously would not have been considered feasible. As testing options have become available, health care professionals and diagnostics experts have had to learn how to best apply these tools to diagnose and manage patients with COVID-19 and slow the spread of disease. Some of these lessons have shown routine testing approaches to be effective, while

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Address correspondence to binnicker.matthew@mayo.edu.
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others have taught us that a new pathogen, such as SARS-CoV-2, may require a new diagnostic playbook.

As SARS-CoV-2 has spread globally, it has required laboratory professionals to venture into uncharted territory. Not only is testing being used to diagnose patients with symptomatic disease, it is being incorporated into local, state, and federal strategies to reopen the economy. Businesses, schools, and even athletic teams are considering how laboratory testing can be used to demonstrate that asymptomatic employees, customers, students, and athletes are safe to return to normal activities. Some hospitals and clinics are utilizing SARS-CoV-2 testing prior to certain procedures and surgeries to reduce nosocomial transmission and prevent poor outcomes in high-risk patients. This trend toward large-scale screening of asymptomatic individuals has placed an incredible burden on the global testing infrastructure and has created challenges with regard to how testing should be utilized and results interpreted. Therefore, it is not surprising that a number of diagnostic uncertainties and controversies have arisen. In this commentary, I will address several topics that have been the focus of continued discussion and debate, including (i) the clinical sensitivity of molecular assays and their role in defining the duration of quarantine/isolation, (ii) whether the PCR cycle threshold value should be included on patient reports, (iii) the potential for specimen pooling and testing by research staff to expand testing capacity, and (iv) whether testing a large percentage of the population represents a viable strategy to end the pandemic.

CLINICAL SENSITIVITY OF PCR AND ITS USE AS A TEST OF CURE

Molecular tests, such as real-time PCR, have become a cornerstone in the diagnosis of infectious diseases and have been the most common laboratory method utilized during the COVID-19 pandemic. The inherent sensitivity of real-time PCR allows for detection of minute amounts (e.g., <100 copies/ml) of target nucleic acid in clinical samples. Despite this, one of the earliest challenges arising during the COVID-19 pandemic was the concern that cases were going undetected by real-time PCR (1–3). Several studies reported the clinical sensitivity of SARS-CoV-2 real-time PCR assays performed on upper respiratory swab samples to be in the range of 60 to 70% (4, 5). However, as experience was gained with testing and additional data published, it was recognized that the likelihood of detecting SARS-CoV-2 RNA is highly dependent on the timing of sample collection, the type of specimen that is obtained, and the quality of the sample. SARS-CoV-2 is present at the largest amounts in the upper respiratory tract (URT) (i.e., the nasopharynx) during the first several days following symptom onset (typically 5 to 7 days following exposure) and subsequently declines in the URT over the course of the following week (6). During the later stages of disease (e.g., >7 days postonset of symptoms), lower respiratory tract (LRT) samples, such as sputum, bronchoalveolar lavage (BAL) fluid, or tracheal secretions, may yield higher rates of detection (4). Due to these nuances, it has been challenging for laboratory professionals to truly define the clinical sensitivity of SARS-CoV-2 real-time PCR and has required that negative results be interpreted in the context of the timing of sample collection (early postonset versus late postonset), the type of specimen tested (e.g., nasopharyngeal [NP] swab versus throat swab), and the performance characteristics of the assay.

Despite the observation that some COVID-19 patients initially test negative by real-time PCR (1, 2), a dichotomous observation has been the persistent detection of SARS-CoV-2 RNA in other patients (7). A number of published reports have demonstrated that SARS-CoV-2 real-time PCR assays may be positive for weeks, even following the resolution of clinical symptoms (7, 8). The author is aware of a case where a patient with an underlying health condition tested positive by PCR for over 100 days (unpublished data). Many health care institutions have followed a test-based strategy—initially recommended by the U.S. Centers for Disease Control and Prevention (CDC)—in which two negative PCR results (obtained on serial samples collected at least 24 h apart) were required prior to releasing a patient from isolation. This approach, whereby qualitative molecular assays have been used to assess whether an individual is infectious, has led

to prolonged isolation, loss of work, extended use of personal protective equipment for hospitalized patients, and psychological distress for patients and their family members.

Laboratory professionals have recognized for years that qualitative molecular assays for infectious diseases can remain positive following the resolution of disease, and therefore, these methods are not typically recommended as "tests of cure" (9, 10). Due to this limitation, the U.S. CDC no longer recommends the use of a SARS-CoV-2 test-based strategy to determine when to discontinue transmission-based precautions, instead relying on a symptom-based strategy in the majority of situations (https://www.cdc.gov/coronavirus/2019-ncov/hcp/duration-isolation.html).

Author's opinion. Molecular methods, including real-time PCR, should not be used following an initial diagnosis of COVID-19 to determine whether an individual continues to shed infectious SARS-CoV-2. Repeat molecular testing may be indicated in patients who recover and subsequently develop new COVID-19-related symptoms.

PROVIDING THE PCR CYCLE THRESHOLD VALUE IN THE PATIENT REPORT

Due to the possibility that SARS-CoV-2 molecular assays can remain persistently reactive in patients who have recovered from COVID-19 or remain asymptomatic, there has been interest in whether semiquantitative or quantitative data can assist in result interpretation. For many commercial and laboratory-developed real-time PCR assays, the cycle threshold (C_T) value associated with a PCR result is available to the laboratory staff. The C_T value is inversely proportional to the amount of target nucleic acid and can be used as a relative indicator of the concentration of a pathogen in a clinical specimen. For example, a positive PCR result with an associated C_T value of 15 would indicate a very high concentration of the target nucleic acid in a sample, whereas a C_T of 35 may suggest that the target is present but near the assay's limit of detection. Importantly, the C_T value is nonnormalized and, therefore, cannot be considered a quantitative result. This is because the C_T value is dependent on a number of variables, including the assay's gene target, the extraction platform, PCR amplification chemistry, and even the quality of specimen collection.

Despite these limitations, several studies have assessed whether a correlation can be made between the PCR C_T value and the presence of replication-competent virus by using viral culture as a surrogate for a patient's infectious status. Bullard et al. (11) compared viral culture and C_T values for 90 respiratory samples that were positive by PCR and demonstrated that a C_T >24 showed a strong correlation with reduced recovery of SARS-CoV-2 in cell culture. Similarly, La Scola et al. inoculated 183 PCR positive respiratory samples in a Vero E6 cell line (12). This group demonstrated a similar reduction in culture positivity as the C_T value increased; however, in this study, a C_T >34 was proposed as a threshold to estimate that an individual is no longer shedding infectious virus. Unfortunately, PCR C_T values may vary significantly between assays, even those using the same gene target (13, 14). During the COVID-19 pandemic, it has become common for clinical laboratories to perform multiple real-time PCR assays to detect SARS-CoV-2. Therefore, including the C_T value on all positive results may be confusing and misleading to ordering providers. Since the C_T value is not normalized against a human gene internal control, it is possible that a high C_T value (e.g., >30) could be due to an inadequate sample collection rather than a low level of target nucleic acid or "noninfectious" virus. Additional data are needed before a definitive viral load threshold correlating with infectivity can be established. Finally, we should be cautious to equate viral culture negativity with an individual's inability to serve as a source of viral transmission. Due to inferior sensitivity, viral culture has been replaced by molecular assays for the diagnosis of a number of respiratory viral infections.

Author's opinion. The PCR C_T value for qualitative SARS-CoV-2 real-time PCR assays should not be routinely reported. On a case-by-case basis, the C_T value may be provided (i.e., verbally) to the ordering physician upon request. This approach allows for clarification of the assay used for testing and a discussion of the limitations associated with using the C_T value while interpreting the result.

SPECIMEN POOLING AS AN APPROACH TO INCREASE TESTING CAPACITY

One of the most challenging aspects of the COVID-19 pandemic for clinical laboratories has been ongoing supply chain shortages and disruptions. Laboratories have not only struggled with maintaining an adequate supply of test reagents, but also other essential supplies, including consumables (e.g., pipette tips, 96-well plates), swabs, and viral transport media. As the demand for testing has increased alongside a global shortage of necessary supplies, laboratory professionals have been forced to identify ways to "do more with less." One potential solution receiving a significant amount of attention is specimen pooling, whereby aliquots from a predefined number of individual samples (e.g., 3, 5, or 10) are combined and the mixture is subsequently tested. If the mixture, or pool, tests negative, then all of the individual samples making up the pool are considered negative. However, if the pool is positive, then each of the samples making up that particular mixture must be tested individually. The concept of specimen pooling has been applied in the past for large-scale screening of other infectious diseases (e.g., HIV, hepatitis B virus) (15, 16) and has been shown to increase testing capacity and reduce reagent use and expenses. A number of recent studies have assessed the potential of specimen pooling for SARS-CoV-2 PCR testing and have demonstrated similar findings. Abdalhamid et al. evaluated the performance of PCR using experimental specimen pools consisting of aliquots from 3 to 10 individual samples (17). This group demonstrated that the qualitative detection of SARS-CoV-2 RNA was not impacted in any of the 25 specimen pools (i.e., all expected positive pools tested positive) with a maximum pool size of 5 individual samples. However, the C_T value of the pooled specimens increased by as much as 5.03 compared to the individual sample result. Similarly, Wacharapluesadee et al. (18) showed that pooling did not impact the sensitivity of detecting SARS-CoV-2 RNA when the PCR C_T value of an individual sample was <35. However, 2 (13.3%) of 15 pools consisting of positive samples with a C_T >35 tested falsely negative. Both of these studies estimated significant improvements in testing efficiency and reductions in cost when pooling is applied in a low-prevalence setting (e.g., <10%) and when pools consist of less than 10 samples.

Despite these potential advantages, a number of important factors should be considered prior to implementing a specimen pooling strategy for SARS-CoV-2 testing. First, as published studies have confirmed, specimen pooling increases the likelihood of low-level positive samples (i.e., those with high C_T values) going undetected. As underscored above, it may be premature to conclude that a positive sample with a C_T >35 is insignificant. Second, many respiratory samples testing positive for SARS-CoV-2 contain large amounts of viral RNA (e.g., as evidenced by associated C_T values <20) and, therefore, pose a significant risk to the laboratory for specimen and/or amplicon contamination. Although pooling can increase testing capacity, it requires samples to be manipulated (e.g., uncapped or pipetted) on a number of occasions, thereby increasing the potential of a contamination event. Third, any manipulation of clinical samples may increase the incidence of sample labeling and/or reporting errors. For example, if a laboratory performing 2,500 individual tests/day implements a protocol allowing for pooling of 4 specimens, that laboratory could theoretically increase testing capacity to nearly 10,000 per day. Even at low disease prevalence, "decoupling" of tens (or hundreds) of positive pools may result in reporting errors. Finally, it remains unclear how billing should be handled in situations where specimen pooling is applied. It is unlikely that laboratories will be reimbursed for multiple tests on a single sample (i.e., a positive pool and a subsequent test on the individual samples), and therefore, this will need to be carefully considered.

Author's opinion. Specimen pooling represents an option to improve efficiency and reduce costs; however, it should only be considered when the testing demand for an individual laboratory far outstrips the resources available to perform testing on individual samples. If specimen pooling is pursued, pool sizes should be kept as small as possible (e.g., ≤5) and automated solutions for sample pipetting and specimen

identification used, whenever possible, to reduce the risk of contamination and reporting errors.

RESEARCH STAFF PERFORMING COVID-19 CLINICAL TESTING

An additional strategy that has been discussed to address the increasing demand for testing has been to redeploy personnel in research laboratories to COVID-19 testing. Obviously, there are thousands of highly trained research scientists worldwide who have extensive expertise in performing and troubleshooting molecular and serologic tests. Therefore, given the shortage of certified medical laboratory scientists, it is plausible to propose that those working in research laboratories could be utilized for clinically related COVID-19 testing. However, there are a number of important factors to consider prior to pursuing this option. First, there are a number of regulations requiring that a laboratory, and the staff working within it, be certified to perform testing on human specimens when results are used for clinical diagnosis and management (19, 20). Specifically, the Clinical Laboratory Improvement Amendments of 1988 (CLIA) state that laboratories performing nonwaived testing are "subject to inspection, and must meet the CLIA quality system standards, such as those for proficiency testing, quality control and assessment, and personnel requirements" (19). In addition, the College of American Pathologists General Checklist specifies that all personnel performing moderate- or high-complexity clinical testing must meet minimum requirements, including completion of a certified clinical laboratory sciences training program or at least 3 months of documented laboratory training in the specialty where the individual will be working. Furthermore, the individual must demonstrate and maintain competency for all testing they will perform within the clinical laboratory (20).

These regulations are in place to ensure that a clinical laboratory is performing testing at a required standard and, most importantly, reporting accurate results for patient diagnosis and management. This is an essential component of the clinical laboratory profession, and ensuring high-quality test results is as important—if not more important—during a pandemic as it is during normal times. That being said, the COVID-19 pandemic has highlighted a key vulnerability within the diagnostic community, that being a shortage in the number of trained, certified personnel to perform clinical testing. We must rapidly respond to address this gap, potentially by establishing a clinical laboratory "national guard" as proposed by Bertuzzi and Patel (https://www.nytimes.com/2020/04/27/opinion/biomedical-national-guard-covid.html). Doing so would help ensure there is an infrastructure in place to provide clinical laboratories with the necessary staff that are trained and deemed competent to provide essential testing services during a public health emergency such as COVID-19. Until this is available, there are opportunities to utilize the assistance of research staff and other volunteers aside from clinical testing, including the preparation of sterile aliquots of transport media, routine decontamination of the clinical laboratory, and providing technical guidance and troubleshooting recommendations when testing issues arise (https://www.pnas.org/content/pnas/117/18/9656.full.pdf).

Author's opinion. Personnel from research laboratories should not be utilized for clinical testing unless they have met the required criteria to perform moderate- to high-complexity testing as outlined by CLIA and other regulatory agencies. Although research scientists are highly trained with extraordinary expertise, enlisting their service in a clinical laboratory prior to meeting these requirements sets a dangerous precedent for the clinical laboratory profession.

SHOULD EVERYONE WHO WANTS A TEST GET A TEST?

In recent years, clinical microbiologists have made a concerted effort to promote the judicious use of laboratory tests, emphasizing the importance of stewardship and data-driven decision-making. Many diagnostics experts have been actively involved in the creation of testing algorithms and clinical decision support tools, which guide providers to order the most appropriate tests, assist with result interpretation, and inform follow-up testing recommendations (21, 22). These efforts have been necessary

in order to quell the rising costs of health care and ensure that limited resources are used wisely. Despite these efforts, testing for SARS-CoV-2 has been applied in the asymptomatic population, and unnecessary repeat molecular testing is common. This has led to a significant detrimental impact on the global supply chain, delays in result turnaround time, and, most importantly, a shortage of tests for those who need testing most.

Recently, there has been discussion of the potential merits of testing a large percentage of the population with rapid and inexpensive at-home assays (23). The concept proposes testing asymptomatic individuals with high frequency (e.g., every few days), which may counterbalance lower sensitivity. While interesting in theory, this approach is unlikely to be feasible and may be problematic. Consider, for example, the strategy of testing students in the United States in order for them to attend school. According to the National Center for Education Statistics (https://nces.ed.gov/fastfacts/display.asp?id=372), there are ~56 million students attending elementary, middle, and high schools in the United States. Even if 25% of those students were tested regularly (e.g., three times each week), that would equate to 42 million tests per week (i.e., 756 million tests between 1 September and 31 December 2020). Assuming an overall disease prevalence of 5% and a screening assay with 98% specificity, nearly 800,000 false-positive results would occur weekly (i.e., >14 million false-positive results by year's end). False-positive results for SARS-CoV-2 are not inconsequential, as they may lead to loss of work, separation from family members, and unnecessary psychological distress. Furthermore, there is no reason to assume that rapid at-home tests will be immune to the same supply chain challenges that have plagued clinical laboratories during the COVID-19 pandemic.

Author's opinion. At this stage of the pandemic, a "test everyone" strategy is unlikely to be feasible and will prevent access of limited resources to those who need testing most. Testing should be prioritized for those situations where the result will inform patient management, personal protective equipment use, and isolation decisions.

SUMMARY

The COVID-19 pandemic has presented a significant challenge to the diagnostic community; however, the valiant efforts of clinical laboratory professionals and public health and industry partners have made a tremendous impact on improving the diagnosis and management of infected individuals and reducing the spread of disease. Given that the virus is now well entrenched in the United States, a successful end to the outbreak will require the thoughtful application of testing, consistent/universal masking policies, and continued physical distancing measures. As a profession, clinical microbiologists should continue to promote diagnostic stewardship and its importance during a pandemic. In addition, we should highlight the COVID-19 pandemic as an example of why the rapid development and implementation of diagnostic assays are necessary to prevent the spread of future novel infectious diseases.

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EXHIBIT" RR

TRL JUDGMENTS

JUDGMENT OF THE LISBON COURT OF APPEAL

Process: 1783/20.7T8PDL.L1-3

Reporter: MARGARIDA RAMOS DE ALMEIDA

Descriptors: HABEAS CORPUS

INTEREST IN ACT: SARS-COV-2, RT-PCR TESTS

DEPRIVATION OF FREEDOM ILLEGAL DETENTION

Agreement Date: 11/11/2020

Vote: UNANIMOUSNESS

Procedural Means: CRIMINAL APPEAL

Decision: PROVISION DENIED

Summary: I. The ARS cannot appeal a decision that ordered the immediate release of four people, for illegal detention, in the scope of a habeas corpus process (art. 220 als. c) and d) of the CPPenal), asking that the confinement be validated mandatory for applicants, for being carriers of the SARS-CoV-2 virus (A....) and for being under active surveillance, for high-risk exposure, decreed by the health authorities (B..., C.... and D.....) for not having legitimacy, nor interest in acting.

II. The request made would also be manifestly unfounded because:

A. Prescription and diagnosis are medical acts, the exclusive responsibility of a physician, registered with the Portuguese Medical Association (Regulation No. 698/2019, of 5.9).

Thus, the prescription of auxiliary diagnostic methods (such as viral infection detection tests), as well as the diagnosis of the existence of a disease, for any and all persons, is a matter that cannot be carried out by law. , Resolution, Decree, Regulation or any other way rules , as they are acts of our legal system reserves the exclusive competence of a doctor, given that this, the advice of your sick, you should always try to get their informed consent (nº 1 of article 6 of the Universal Declaration on Bioethics and Human Rights).

B. In the case we are dealing with, there is no indication or proof that such a diagnosis was actually carried out by a qualified professional under the terms of the Law and who had acted in accordance with good medical practices. In fact, what follows from the facts given as established is that none of the applicants was even seen by a doctor, which is frankly inexplicable, given the alleged seriousness of the infection.

TRUE COPY

ADVOCATE FOR

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C. The only element that appears in the proven facts, in this regard, is the performance of RT-PCR tests, one of which presented a positive result in relation to one of the applicants.

D. Given the current scientific evidence, this test alone is unable to determine, without a reasonable margin of doubt, that such positivity in fact corresponds to the infection of a person by the SARS-CoV-2 virus, for various reasons, , of which we highlight two (in addition to the issue of the gold standard which, due to its specificity, we will not even address):

Because this reliability depends on the number of cycles that make up the test;

Because this reliability depends on the amount of viral load present.

III. Any diagnosis or any act of health surveillance (such as the determination of the existence of viral infection and high risk of exposure, which are covered by these concepts)carried out without prior medical observation of patients and without the intervention of a physician registered with the OM (who carried out the assessment of their signs and symptoms, as well as the exams they deemed appropriate to their condition), violates Regulation No. 698/2019, of 5.9. , as well as the provisions of article 97 of the Statute of the Medical Association, being liable to configure the crime of usurpation of functions, p. and p. by article 358 al b), of the Criminal Code.

IV. Any person or entity that issues an order, the content of which leads to the deprivation of physical freedom, ambulatory, of others (whatever the nomenclature this order takes: confinement, isolation, quarantine, prophylactic protection, health surveillance, etc.), that if it does not fit the legal provisions, namely in the provisions of art. 27 of the CRP , it will be carrying out an illegal detention , because it was ordered by an incompetent entity and because it was motivated by a fact for which the law does not allow it.

(Summary prepared by the reporter)

Partial Text Decision:

Full Text Decision:They agree in a conference at the 3rd Criminal section of the Lisbon Court of Appeal

I – report

1. By decision of 26-08-2020, the request for habeas corpus was granted, as its detention was illegal , and it was determined that immediate return to freedom of Claimants SH__SWH___, AH___ and NK___. 2. Then came the REGIONAL HEALTH AUTHORITY, represented by the Regional Directorate of Health of th

Autonomous Region of the Azores, to file an appeal against this decision, requesting that it be validated.

mandatory confinement of applicants, for being carriers of the SARS-CoV-2 virus (AH___) and for being under active surveillance, for high-risk exposure, decreed by health authorities (SH__, SWH__ and NK___).

4. The appeal has been admitted.

5. The M° P°, in his answer, defends that the present appeal should be considered unfounded.

6. In this court, the former PGA applied for a visa.

II – previous point.

Since the appeal filed by the appellant must be rejected, the court will limit itself, pursuant to paragraphs 1, a) and 2 of article 420 of the Code of Criminal Procedure, to summarily specifying the grounds of the decision.

III – reasoning.

1. The decision rendered by the "a quo" court has the following content:

Proven facts:

1. On 01/08/2020 the applicants arrived on the island of São Miguel, coming by plane from the Federal Republic of Germany, where, at 72 (seventy and two) hours prior to arrival, they had performed a COVID test19, with a negative result, copies of which were presented and delivered to the Regional Health Authority, upon arrival at the airport in Ponta Delgada.

2. On 08/07/2020 and already during their stay on the island of São Miguel, applicants AH___ and NK___ performed a second test to COVID19.

3. On 08/10/2020 and also during their stay on the island of São Miguel, applicants SH___ and SWH___ performed a second test to COVID19.

4. On 08/08/2020, applicant AH___ was informed by telephone that her test carried out the day before had accused her of being "detected".

5. As of that day 08/08/2020, applicant AH___ no longer cohabits with the other three applicants, having always maintained a distance of no less than 2 (two) meters from them.

6. On 08/10/2020 applicants SH___, SWH___ and NK___ were informed by telephone that their tests had been "negative".

7. On 08/10/2020, the document was sent to all applicants by e-mail, attached to pages 25, 25verse, 26 and 26 verse, signed by the Health Delegate of the municipality of Lagoa, in office, Dr. Magno José Viveiros Silva, called Notification

of Prophylactic Isolation – Coronavirus SARS- CoV-2/COVID Disease – 19, and two attachments (only one of them in English) and which reads (equal content except for the identification of each of the Applicants herein):

"Isolation (...)

Notification of

Prophylactic Isolation

Coronavirus SARS- CoV-2/COVID Disease – 19

Mário Viveiros Silva Lagoa Health Authority

Pursuant to Normative Circulars No. DRSCINF/2020/22 of 2020/03/25 and DRS CNORM2020/39B of 2020/08/04 of the REGIONAL HEALTH AUTHORITY (attached) and of Standard No. 015/2020, of 24/ 07/2020 of the Directorate General of Health (attached) I determine the

PROPHYLACTIC ISOLATION

OF

(...)

Holder of the Citizen Card/PASSPORT No. (...), valid ... until ... with the number of social security identification for the period from 08/08/2020 to 22/08/2020 due to the danger of contagion and as a containment measure of COVID 19 (SARS-Cov-2)

Date 2020/08/10 (...)

8. The Claimants requested that they send the said results, and the test report was sent to Claimants AH___ and NK___ by email on 08/13/2020 and to Claimants SH___ and SWH___ yesterday, 24/ 08/2020, by e-mail, reports written in Portuguese.

9. Between August 1st and 14th, applicants were accommodated at the Marina Mar II accommodation, in Vila Franca do Campo.

10. From August 14th onwards, applicants are accommodated at "THE LINCE AZORES GREAT HOTEL, CONFERENCE & SPA", in Ponta Delgada (where they are currently located), by order of the Health Delegate under the terms described in 7 as follows:

- In room 502 are the applicants SH___ and SWH___.

- In room 501 is the applicant AH___.

- In room 506 is the applicant NK___.

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Maharashtra
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11. The applicants have tried at least 3 times to contact the telephone helpline they know (296 249 220) to be clarified in their language or at least in English, but they have never had any success, as they only answer and respond in Portuguese, which the applicants do not understand.

12. At the hotel, meals are delivered to your room by hotel services at pre-determined times and according to a choice made by a third party, except during the first 3 days at the Hotel Lynce in which breakfast was served and the remaining meals through room service.

13. On August 15, while complying with the prophylactic isolation determined by the Health Delegate, the applicant AH___ began to suffer from an inflammation in her mouth, apparently resulting from the dental appliance she was wearing.

14. Having, by telephone, to the number 296 249 220, I shared this situation with the Regional Health Authority, who requested the necessary medical support.

15. This request was ignored by the aforementioned helpline, which did not provide the respondent AH___ with the necessary support.

16. Not seeing any support, two days later, on August 17th, duly protected by a mask and gloves, the applicant SWH___ left her room, went to the nearest pharmacy to the hotel, where she purchased an ointment to temporarily overcome the situation, and immediately returned to the hotel and to her room.

17. On 19/08/2020, the Health Delegate, Dr. JMS___, sent an e-mail to the Claimants, which specifically reads:

"(...) AH___ is only considered cured after having a negative test and a 2nd negative cure test, when that happens the health delegation will contact you (...) (sic).

18. On 8/21/2020 the following message was sent to the four applicants by the Health Delegate Dr. JMS___, via email: "In other words, when they finish the quarantine they must take a test and if it is negative they can leaving home" (sic).

19. On that same August 21st, applicant SH___ questioned the aforementioned physician and Health Delegate, Dr. JMS___, by email that he sent, the following (translated into Portuguese language free of charge):

"Dear Dr. JMS___ ,

We have already done two COVID/person tests, all were negative (SH___, SWH___, NK___). ..and after that we spent 2 weeks in isolation, and none of us report any symptoms!!

We have Dr. MMS___'s documents, confirm.

Nobody told us anything about the new tests after the isolation time?!

We have already rescheduled our flights and are planning to leave the island.

Explain the reason for your statement.

Why wasn't the COVID test of AH___ done yesterday?

Greetings,

SH___"

20. The applicants did not receive any response to this email, with the exception of the Claimant AH___, who was informed that a new screening test was scheduled to be carried out, specifically, for the next day of 08/29/2020.

21. On 08/20/2020, applicant AH___ performed a third test to COVID19, and on the following day (08/21/2020), only by telephone, she was informed that the result had been "detected".

22. Applicant AH___ requested that written evidence of this positive result be sent to her, which was sent to her by email yesterday, 08/24/2020.

23. The Applicants questioned the reception staff of the hotel where they are located, having been told that none of the four applicants, without exception, may be absent from the rooms.

24. Applicants do not have, and have never had, any symptoms of the disease (fever, cough, muscle pain, sneezing, lack of smell or palate).

25. The applicants were not explained the content of the two documents sent to them with the writings listed in point 7.

26. The applicants have their habitual residence in the Federal Republic of Germany, identified in these records.

Rationale:

The question raised here is that the Applicants are deprived of their liberty (from the 10th of August to the present date, as follows from the proven facts) and, consequently, can avail themselves of the present institute of habeas corpus - as we will go on to explain -, it leads to knowing whether or not there is a legal basis for this deprivation of liberty.

In fact, without even questioning the organic constitutionality of Regional Government Council Resolution 207/2020, of 31 July 2020, currently in force within the scope of the procedures approved by the Government of the Azores to contain the spread of the SARS-COV- virus 2 in this Autonomous Region, in the situation in question, the detention/confinement of the Applicants since the past

NOTARY
M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15229
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10th of August is materialized by a communication carried out by e-mail, in Portuguese, in the terms given as proven under point 7.

As it is clear from the aforementioned point 7 of the proven facts, the regional health authority, through the respective Health Delegate of the territorial area where the Applicants were staying, determined the prophylactic isolation of these under the Normative Circulars no DRSCINF/2020/ 22 of 2020/03/2025 and DRS CNORM2020/39B of 2020/08/04 of the REGIONAL HEALTH AUTHORITY and of Standard No. 015/2020, of 24/07/2020 of the General Directorate of Health. And, it was through a communication with the aforementioned support, it should be noted, in normative circulars and a norm of the General Directorate of Health, that the Regional Health Authority deprived the Applicants of their freedom, as the proven facts derive from the satiety that these, in the rigor of the concepts ,were detained from the 10th to the 14th of August 2020 in a hotel development in Vila Franca do Campo and from the 14th of August 2020 to the present date confined, and therefore detained, in a hotel room in this city of Ponta Delgada. We cannot forget, even because it stands out from the list of proven facts, that the power of movement and the right of mobility of the Applicants - or of any other individual in the same situation - are so limited that the first exit from the rooms where they are found was to go to this court and make statements (with the exception of the trip to the Applicant SWH__'s pharmacy in clear desperation to attend to her daughter's pains in the proven terms).We cannot forget, even because it stands out from the list of proven facts, that the power of movement and the right of mobility of the Applicants - or of any other individual in the same situation - are so limited that the first exit from the rooms where they are found was to go to this court and make statements (with the exception of the trip to the Applicant SWH__'s pharmacy in clear desperation to attend to her daughter's pains in the proven terms).We cannot forget, even because it stands out from the list of proven facts, that the power of movement and the right of mobility of the Applicants - or of any other individual in the same situation - are so limited that the first exit from the rooms where they are found was to go to this court and make statements (with the exception of the trip to the Applicant SWH__'s pharmacy in clear desperation to attend to her daughter's pains in the proven terms).that the power of movement and right of mobility of the Applicants - or of any other individual in the same situation - are so limited that the first exit from the rooms where they are found was to go to this court and make statements (with except for the trip to the Applicant's pharmacy SWH__ in clear desperation to attend to her daughter's pains in the proven terms).that the power of movement and right of mobility of the Applicants - or of any other individual in the same situation - are so limited that the first exit from the rooms where they are found was to go to this court and make

statements (with except for the trip to the Applicant's pharmacy SWH___ in clear desperation to attend to her daughter's pains in the proven terms).

In short, after analyzing the ascertained factuality, it is inexorable to conclude that we are facing a real deprivation of personal and physical freedom of the applicants, not consented by them, which prevents them not only from moving, but also from being with their family, living for about 16 days separated (applicants SH___ and SWH___ and her daughter, here Applicant, AH___) and, in the case of Applicant NK___ all alone, without any physical contact with anyone. To say that there is no deprivation of liberty because they can leave their rooms at any time, in which they are, is a fallacy, just paying attention to the communications made to them after August 10th, none of them in German,and the conditions in which they have been living (not forgetting that they are foreign citizens with the inherent language barrier) or requesting their return to their place of origin is a fallacy, and for such a conclusion it is enough to pay attention to the latest communications made in Portuguese, it should be underlined from which the given as proven under point 8 stands out, specifically "In other words, when they finish the quarantine they have to take a test and if it is negative they can leave the house such as the hotel where they are confined in 3 bedrooms.when they finish the quarantine they have to take a test and if this is negative they can leave the house as the hotel where they are confined in 3 rooms.when they finish the quarantine they have to take a test and if this is negative they can leave the house as the hotel where they are confined in 3 rooms.

Therefore, since the Applicants are deprived of their freedom, given the proven circumstances, it is necessary to trace the path on which we are moving, starting the journey through the guiding light of the Portuguese legislative system: the Constitution of the Portuguese Republic.

Thus, in terms of the hierarchy of norms, it is important to remember that, as provided for in article 1 of the CRP, "Portugal is a sovereign Republic, based on the dignity of the human person and on the popular will and committed to building a free, just society and solidary.". Hence, it is clear that the unity of meaning in which our system of fundamental rights is based is based on human dignity – the principle of the dignity of the human person is the axial reference of the entire system of fundamental rights.

One of them, the most relevant given its structuring nature of the democratic state itself, is the principle of equality, provided for in article 13 of the CRP, which states, in paragraph 1, that "All citizens have the same social dignity and are equal before the law.", adding paragraph 2, that "No one may be privileged, benefited, harmed, deprived of any right or exempt from any duty on account of

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M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
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ancestry, sex, race, language, territory of origin, religion, political or ideological convictions, education, economic status, social status or sexual orientation.".

And, in what is particularly important here, under the heading "right to freedom and security" article 27, paragraph 1, of the CRP provides, "Everyone has the right to freedom and security", referring to José Lobo Moutinho, in an annotation to such article, that "Freedom is an absolutely decisive and essential moment - not to say, the very and constitutive way of being - of the human person (Ac. No. 607/03: "ontic demand"), which lends him that dignity in which finds its granitic foundation in the Portuguese legal (and, above all, legal-constitutional) order (Article 1 of the Constitution). In this sense, it can be said the cornerstone of the social edifice" (Ac. n° 1166/96)" (aut.cit., in op. cit., p. 637).

Since human freedom is not one-dimensional, it can take on multiple dimensions, such as articles 37 and 41 of the CRP, the freedom in question in article 27 is physical freedom, understood as freedom of bodily movement, of coming and going, freedom of movement or mobility, providing in paragraph 2 of this last article that " No one may be totally or partially deprived of liberty, except as a result of a judgment condemning the practice of an act punishable by law with the penalty of imprisonment or judicial application of security measure." – our underscore.

The exceptions to this principle are typified in paragraph 3, which provides that:

"Deprivation of liberty is excluded from this principle, for the time and under the conditions that the law determines, in the following cases:

a) Detention in flagrante delicto;

b) Detention or preventive detention for strong evidence of the commission of a felony which corresponds to a prison sentence whose maximum limit is greater than three years;

c) Arrest, detention or other coercive measure subject to judicial control, of a person who has entered or remains illegally in the national territory or against whom an extradition or expulsion process is in progress;

d) Disciplinary imprisonment imposed on military personnel, with a guarantee of appeal to the competent court;

e) Subjection of a minor to measures of protection, assistance or education in an adequate establishment, decreed by the competent judicial court;

f)Detention by court order due to disobedience to a decision taken by a court or to ensure appearance before the competent judicial authority;

g) Detention of suspects, for identification purposes, in cases and for the time strictly necessary;

h) Internment of a patient with a mental anomaly in an appropriate therapeutic establishment, decreed or confirmed by a competent judicial authority."

Finally, it should be recalled that, in the event of deprivation of liberty against the provisions of the Constitution and the Law, the State is constituted with the duty to compensate the injured party under the terms established by the law, as follows from paragraph 5 of article 27, noting that , in accordance with article 3 of the CRP:

(...) 2. The State is subordinate to the Constitution and is based on democratic legality.

3. The validity of laws and other acts of the State, autonomous regions, local authorities and any other public entities depends on their compliance with the Constitution.

Having arrived here, having traced the legal territory, let's take a closer look at the situation in which the Regional Health Authority moved in the situation under analysis.

Claimants SH_SWH_ and NK_ performed a screening test for the SARS-CoV-2 virus, the result of which was negative for all of them, with the same positive test being obtained for Claimant AH___, which led to the aforementioned order of prophylactic isolation and consequent permanence of these under the terms set out and tasted.

Therefore, given the content of the notification made to the Applicants, this court cannot fail to express, ab initio, its perplexity at the determination of prophylactic isolation to the four Applicants.

As follows from the definition given by the Directorate General of Health, "Quarantine and isolation are essential measures of social distancing in public health. They are especially used in response to an epidemic and are intended to protect the population from person-to-person transmission. The difference between quarantine and isolation stems from the state of illness of the person who wants to be socially withdrawn. In other words:

"quarantine is used on people who are supposed to be healthy, but may have been in contact with an infected patient;

isolation is the measure used in sick people, so that through social distancing they do not infect other citizens." (at https://www.sns24.gov.pt/tema/doencas-infecciosas/covid-19/isolamento/?fbclid=IwAR34hD77oLCpxUVYJ9OI4ttgwo4tsTOvPflq[illegible]yoH0E JEbCs3jEihkaEPAY#sec-0).

Returning to the present case, the Regional Health Authority decided to erase essential concepts, as they delimit the differentiated treatment (because it is different, pass the pleonasm), of the situations of infected people and of those who were in contact with them, before the order of prophylactic isolation to all applicants, despite only one of them having positive results in the aforementioned screening test. Furthermore, it decided to make a dead letter of the Government Council Resolution No. 207/2020 of 31 July, interfering with the mandatory submission to judicial validation by the competent court decreed that it be mandatory quarantine, when it comes to the satiety of the facts proven that Claimants SH__SWH__ and NK___, at best, are subject to mandatory quarantine.

It did not do so within the 24 hours provided for in point 6 of the aforementioned Resolution, not even within a longer period - as in the 48 hours provided for in article 254, paragraph 1, subparagraph a) of the Code of Criminal Procedure, or in article 26, no. 2, of the LSM – continuing to make any communication and, in this way, the evident restriction of the freedom of the Claimants SH__SWH__ and NK_ will always be illegal.

In this step, the aforementioned Resolution of the Government Council No. 207/2020, of July 31, 2020, provides in its point 4 that in cases where the result of the virus test for SARS-CoV-2 is positive, the authority of within its competences, will determine the procedures to be followed. The Applicant AH___ positive in the screening test for the virus in question was notified, reiterate in the same terms as the other Applicants, of the order of prophylactic isolation between 08/10/2020 and 08/22/2020.

At this point, it should be made clear that the notification made as proven under point 7, is brought from what is contained in the Standard of DGS015/2020, a rule to which it alludes in addition to the normative circulars (available for consultation athttps://www.dgs.pt/directrizes-da-dgs/normas-e-circulares-normativas/norma-n-0152020-de-24072020-pdf.aspx), and tells us, in what matters here: (. ..) Contacts with High Risk Exposure

15. A contact classified as having high risk exposure, in accordance with Annex 1, is subject to:

a.Active surveillance for 14 days from the date of the last exposure;

b.Determination of prophylactic isolation, at home or in another place defined at the local level, by the Health Authority, until the end of the period of active surveillance, in accordance with the model of Dispatches No. 2836-A/2020 and/or n. 3103-A/20202 (model accessible at http://www.scg-

social.pt/documents/10152/16819997/GIT_70.docx/e6940795-8bd0-4fad-b850-ce9e05d80283)

Following this standard of the General Directorate of Health, one can read, among others, in normative circular No. DRSCNORM/2020/39B, of 2020-08-04 (available for consultation at http://www.azores.gov.en/NR/rdonlyres/25F80DC1-51E6-4447-8A38-19529975760/1125135/CN39B_signed1.pdf),

(...)

a.High-risk

close contacts High-risk close contacts are treated as suspected cases until the laboratory result of the suspected case. These close contacts should screen for SARS-CoV-2. The following are considered high risk contacts: i. Cohabitation with a confirmed case of COVID-19; (...)

ii. Surveillance and Control of Close Contacts

3. Close contacts of high risk, considering that, currently, it is estimated that the period of incubation of the disease (time elapsed from exposure to the virus until the onset of symptoms) is between 1 and 14 days, they must comply with 14 days of prophylactic isolation, even if they present negative screening tests during this period, and the test should be carried out on the 14th day. If the test result on the 14th day is negative, they are discharged. If close high-risk contacts coexist with the positive case, they should only be discharged when the positive case is cured, and, in this way, the respective prophylactic isolation should be extended.

(...)

13. Compliance with prophylactic isolation

All persons identified as suspected cases, until the negative results are known, undergo prophylactic isolation;

All persons who tested positive for Covid-19 and who are discharged after a cure test (inpatient or at home) do not need to undergo another 14-day isolation period or repeat a new test on the 14th day.

All passengers arriving at the Region's airports from airports located in areas considered to be areas of active community transmission or with active transmission chains of the SARS-CoV-2 virus must comply with the procedures in force in the Region at the time.

Having arrived here, let us analyze the legal value of norms/guidance of the General Directorate of Health and normative circular 39B, of 08/04/2020, duly

NOTARY
M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
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Regional Health Directorate,leaving no doubt that we have entered the sphere of administrative guidelines.

In this regard, with the specificity of reporting to the Tax Authority - which has the same administrative legal position as the National Health Authority in the State's ius imperium -, CASALTA NABAIS (Tax Law, 6th ed., Almedina, p. 197), "the so-called administrative guidelines, traditionally presented in the most diverse forms as instructions, circulars, circular letters, circular letters, normative orders, regulations, opinions, etc.", which are very frequent in tax law constitute "internal regulations that, as they are only addressed to the tax administration, only this one owes them obedience, being, therefore, mandatory only for the bodies located hierarchically below the authorizing body.

Therefore, they are not binding on individuals or on the courts. And this is either organizational regulations, which define rules applicable to the internal functioning of the tax administration, creating working methods or modes of action, or interpretative regulations, which interpret legal (or regulatory) precepts.

It is true that they densify, explain or develop the legal precepts, previously defining the content of the acts to be performed by the administration when they are applied. But that does not make them the standard of validity of the acts they support. In fact, the assessment of the legality of the acts of the tax administration must be carried out through direct confrontation with the corresponding legal norm and not with the internal regulation, which intervened between the norm and the act".

However, the issue of the normative relevance of the Administration Circulars (Tax) has already been raised and considered in the Constitutional Court Judgments No. 583/2009 and 42/14, of 11.18.2009 and of 12.09.2014, respectively, with that Court having decided, with which we agree, that the prescriptions contained in the Tax Administration Circulars, regardless of their persuasive effect on the practice of citizens, do not constitute norms for the purposes of the constitutionality control system entrusted to the Constitutional Court.

As that edge underlined (Rule 583/2009) "(...) These acts, in which the "circulars" loom large, emanate from the power of self-organization and the hierarchical power of the Administration. They contain generic service orders and it is for this reason and only in the respective subjective scope (of the hierarchical relationship) that compliance is assured. They incorporate guidelines for future action, transmitted in writing to all subordinates of the administrative authority that issued them. They are standardized decision-making modes, assumed to rationalize and simplify the operation of services.

This is worth saying that, although they may indirectly protect legal certainty and ensure equal treatment through uniform application of the law, they do not regulate the matter they deal with in confrontation with individuals, nor do they constitute a decision rule for the courts."

Consequently, lacking heteronomous binding force for individuals and not imposing themselves on the judge except for the doctrinal value they may have, the provisions contained in the "circulars" do not constitute norms for the purposes of the constitutionality review system within the competence of the Constitutional Court.

What has been said, allows us to conclude that the administrative guidelines conveyed in the form of a normative circular, as in the present case, do not constitute provisions of legislative value that can be the object of a formal declaration of unconstitutionality - see Judgment of the Supreme Administrative Court, of 21/06/2017, available for consultation in www.dgsi.pt .

And, to make it clear that the regulations invoked by the Regional Health Authority that upheld the deprivation of liberty imposed on the Claimants through notification of prophylactic isolation are non-binding administrative guidelines for the Claimants. By the way, just pay attention to who they are addressed respectively to:

Normative Circular No. DRSCNORM/2020/39B:"For: Health Units of the Regional Health Service, Municipal Health Delegates (C/c Regional Civil Protection Service and Azores Fire Service, Line de Saúde Azores) Subject: Screening of SRAS-CoV-2 and approach of suspected or confirmed cases of infection by SARS-CoV-2 Source: Regional Directorate of Health (...)

Standard 015/2020, of 07/24/2020: "TOPIC: COVID-19: Contact Tracing KEYWORDS: Coronavirus, SARS-CoV-2, COVID-19, Contact Tracing, Epidemiological Investigation

FOR: Health system (...).

In this sequence, and, in summary, this court cannot fail to emphasize that the present case, we allow ourselves to say aberrant, of deprivation of liberty of persons, absolutely lacks any legal basis, and do not come back with the argument that the defense of public health is at stake because the court always acts in the same way, that is, in accordance with the law, in fact, hence the need for judicial confirmation enshrined in the Mental Health Law in the case of compulsory internment, as from the facts found and from the above results:

- The Applicants have been confined to the space of one room for about 10 days, based on a notification of "prophylactic isolation" until 08/22/2020, a period that has already been exceeded and the notification operated, which in any case

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it is illegal as a means of detaining people for the reasons already explained (just paying attention to the constitutional norms set out above), has expired;

- The Applicants were never transmitted any information, communication, notification, as appropriate, in their mother tongue, nor were they provided with an interpreter, immediately in flagrant violation of the European Convention on Human Rights (articles 5, no. 2 and 6, no. 3, subsection a) and of the criminal procedural rules (cf. art. 92 of the Criminal Procedure Code), that is, in our legal system a foreign person detained and without control of the Portuguese language is immediately appointed interpreter, and, in the case of the Applicants who limited themselves to travel to this island and enjoy its beauty, they were never granted such a possibility;

- Applicants after 08/22/2020 are confined to the space of a room based on the following communications:

- On 8/19/2020 the Health Delegate, Dr. JMS___, was sent to the Claimants by e-mail, which specifically reads:

"(...) AH___ is only considered cured after having a negative test and a 2nd negative cure test, when this happens the health delegation will contact you (...) (sic).

- On 8/21/2020, the following message was sent to the four applicants by the Health Delegate Dr. JMS___, via email: "In other words, when they finish the quarantine they have to take a test and if it is negative they can leave from home" (sic);

- The deprivation of liberty of the Applicants was not subject to any judicial review.

As we said initially, we could also consider the organic constitutionality of Government Council Resolution no. 1207/2020, of 31 June, however, we believe it to be a negligible issue for the object of the decision to be rendered, which is intended to be swift, because even the In light of such a resolution, the decision cannot be different, based on the decision of the Constitutional Court, of 07/31/2020, in the scope of case No. 424/2020, and, because the position of the Regional Health Authority in the present circumstances leads to to the application of normative circulars, with the value explained above.

Finally, and because this court has been pronouncing successively and recently within the scope of this institute of "habeas corpus" in light of the orders issued by the Regional Health Authority, we allow ourselves to subscribe and underline the following excerpt from the first decision of this Criminal Investigation Court

"The issue of compulsory confinement in case of contagious diseases, and the terms under which it should occur, is a pressing issue, and one that is not supported by article 27, paragraph 3, of the CRP, namely in its subparagraph h), where only provision is made for the hospitalization of a patient with a mental anomaly in an appropriate therapeutic establishment, decreed or confirmed by a competent judicial authority. It is urgent to legislate on this matter, clearly establishing the fundamental principles to which it must comply, leaving the detailed aspects to secondary law - and only these.

For, as Professor Gian Luigi Gatta, who we quote here in a free translation, says,"Right now, the country's energies are focused on emergency. But the need to protect fundamental rights, also and above all in an emergency, requiring the Courts to do their part. Because, in addition to medicine and science, also law - and human rights law in the first place - must be at the forefront: not to prohibit and sanction - as is being stressed too much these days - but to guarantee and protect everyone we. Today the emergency is called a coronavirus. We don't know tomorrow. And what we do or don't do today, to maintain compliance with the system's fundamental principles, can condition our future." (in "I diritti fondamentali all the proof of the coronavirus. Perché it is necessary a legge sulla quarantine",)".

It will not be difficult to admit and accept that the legislative turmoil generated around the containment of the spread of COVID-19 had - and will continue to have - in its raison d'être the protection of public health, but this turmoil can never harm the right to death, freedom and security and, ultimately, the absolute right to human dignity.

It remains to decide accordingly.

[...]

Therefore, in light of the above, as illegal the detention of Claimants SH__SWH___, AH___ and NK___, I decide to uphold the present request for habeas corpus and, consequently, I order their immediate return to freedom.

2. The appellant made the following conclusions, which he drew from his motivation:

1. The object of the present appeal is the decision rendered by the learned Court a quo considered "illegal the detention of the Claimants SH__SWH___, AH___ and NK___" and decided to "uphold the present request for habeas corpus and, consequently, I order the immediate restitution from them to freedom.";

2.Only for reasons of procedural economy, that is, because it is of little relevance to the assessment of the merits of the case, the factuality given as proven is not

appealed, however, it should be noted that it was based solely on the statements of the applicants themselves.

3. The contested decision, claiming that the appellant did not comply with point 6 of the Resolution of the Council of the Regional Government of the Azores no. 207/2020, of 31 July 2020, violated the scope of application of the same Resolution, defined in point 1 of the same Resolution;

4. The judicial validation of mandatory quarantine, provided for in point 6 of said resolution, only applies to mandatory quarantine decreed to passengers who do not accept, alternatively, any of the procedures, provided for in point 1 of the aforementioned Resolution;

5. The applicants complied with the procedure provided for in subparagraph a) of point 1 of Resolution 207/2020, of July 31, 2020, so they could never be subject to mandatory quarantine, under that Resolution and, consequently, there is no place for judicial validation, provided for in point 6 of Resolution No. 207/2020, of 31 July 2020.

6. Contrary to what is defended in the contested decision, the Portuguese legal system allows the adoption of exceptional measures, including separation of persons, consequent decree of mandatory confinement of infected persons with a high probability of being infected, through the mechanism provided for in article 17 of Law No. 81/2009, of 21 August;

7. The Council of Ministers legitimately made use of the exceptional regulatory power, provided for in article 17 of Law No. 81/2009, through Resolutions of the Council of Ministers No. 55-A/2020, of July 31, 2020 and No. 63-A/2020, of August 14th;

8.No. 2 of the Resolution of the Council of Ministers No. 55-A/2020, of 31 July 2020, ordered exceptional measures to be applied throughout the national territory, necessary to combat COVID -19, namely those provided for in the regime attached to that resolution;

9. Article 2 of the Annex decreed that:

"Article 2

Mandatory confinement

1 - They are in mandatory confinement, in a health establishment, in their home or in another place defined by the health authorities:

a) Patients with COVID -19 and those infected with SARS -CoV-2;

b) Citizens for whom the health authority or other health professionals have determined active surveillance.

2 – (...)"

10. The applicant AH___ being infected with the SARS-CoV-2 virus, in compliance with article 2, paragraph 1, subparagraph a) of Annex I of the Resolution of the Council of Ministers 55-A/2020, had to be in mandatory confinement;

11. The lower court, by decreeing the habeas corpus of AH___ and allowing its free circulation, violated article 17 of Law no. 81/2009, of 21 August, by reference to article 2, no. 1, paragraph a) of Annex I of the Resolution of the Council of Ministers No. 55-A/2020;

12. Applicants SH__SWH__ and NK_ in accordance with the rules stipulated by the National Health Authority, contained in Norm 015/2020, of 07/24/2020, are contacts with High Risk Exposure, and shall be subject to:

a. Active surveillance for 14 days from the date of the last exhibition;

b. Determination of prophylactic isolation, at home or in another place defined at the local level, by the Health Authority, until the end of the period of active surveillance, in accordance with the model of Dispatches No. 2836-A/2020 and/or n. º 3103-A/20202"

13. The applicants SH__SWH__ and NK_ are subject to active surveillance, in compliance with article 2, paragraph 1, subparagraph b) of Annex I of the Resolution of the Council of Ministers no. 55-A/2020, had to be in mandatory confinement;

14. The lower court, by decreeing the habeas corpus of SH__SWH__ and NK_ and allowing their free circulation, violated article 17 of Law no. 81/2009, of August 21, by reference to article 2, no. 1, subparagraph b) of Annex I of the Resolution of the Council of Ministers No. 55-A/2020.

15. The contested decision must be revoked and replaced by another that validates the mandatory confinement of the applicants, as they are carriers of the SARS -CoV-2 (AH___) virus and because they are under active surveillance, due to high risk exposure, decreed by the health authorities (SH__SWH__ and NK___).

3. In his response, the M°P° drew the following conclusions:

1— The judgment of the Constitutional Court of 07/31/2020 (Proc. 403/2020; 1st Section; Cons. José António Teles Pereira), after concluding that mandatory confinement, either through quarantine or through prophylactic isolation, constitutes a true deprivation of liberty not provided for in art. 27, no. 2, of the CRP, and that all deprivations of liberty require the prior authorization of the Assembly of the Republic, which was not the case with the Resolutions of the

Regional Government of the Azores that imposed a mandatory quarantine, considered the organic unconstitutionality of the aforementioned norms.

2 — These norms, declared unconstitutional by the Constitutional Court, are in all materially identical to those contained in Resolutions of the Council of Ministers nos. 55-A/2020, of 07-31, 63-A/2020, of 08-14 , and 70-A/2020, of 9/11, and No. 88-A/2020, of 10/14, insofar as they provide for deprivations of liberty not provided for in an appropriate legal diploma issued by the competent entity, as well as are not found in the exceptions provided for in art. 27, paragraph 3, of the CRP, so they must also be unapplied for violation of art. 27(1) of the CRP.

3 — Foreseeing art. 5, no. 1, al. e) of the European Convention on Human Rights (Convention for the Protection of Human Rights and Fundamental Freedoms — Rome, 11-04-1950), on the right to liberty and security, which states that "Everyone has the right to freedom and security" and that "No one may be deprived of their liberty, except in the following cases and in accordance with the legal procedure: (...) "If it is the legal detention of a person liable to spread a contagious disease, a mentally insane person, an alcoholic, a drug addict or a vagabond', we can conclude that the deprivation of liberty of a person likely to spread a contagious disease is a form of detention and that, according to the Convention, States provide in their domestic legislation for the detention of these persons.

4 — Taking into account the constitutional principle of the typicality of measures depriving liberty, and not providing for art. 27 of the CRP, in none of its paragraph 3, the deprivation of liberty of a person "likely to spread a contagious disease", 5 - And having paragraph h) - which provides for the hospitalization of a patient with a mental disorder

in an adequate therapeutic establishment —has been added by art. 11.0, no. 6, of Constitutional Law no. 1/97, of 20 September (4th constitutional revision), at a time when the European Convention on Human Rights already expressly provided for the detention of a person liable to propagate contagious disease,

6 — E que o legislador constitucional, nem na referida revisão constitucional nem noutra posterior, acrescentou outra alínea ao n.º 3 do art. 27.º a prever esta possibilidade, como fez relativamente ao internamento de portador de anomalia psíquica, podemos concluir que estamos perante uma decisão consciente do legislador constitucional em não permitir que se proceda à privação da liberdade de pessoa susceptível de propagar doença contagiosa, apenas por esse facto.

7 — From the analysis of the constitutional regime of the right to liberty and security provided for in art. 27, no. 1, of the CRP, we can thus conclude that it

is not possible for the legislator, even through the Assembly of the Republic or the Government authorized by it, to create deprivations of liberty that are not provided for in no. 3 of the aforementioned constitutional regulation, namely with regard to people with infectious and contagious diseases, whether these deprivations of freedom are confinement, quarantines or prophylactic isolation, without incurring any rules created for this purpose in material unconstitutionality for violation of said constitutional regulation.

8 — Turning now to the legal regime for the internment of patients with contagious diseases, Law No. 2036 of 09-08-1949 provided for the possibility of promoting the isolation or internment of people with infectious diseases, but only in this last case, in situations where there was a serious danger of contagion, with an appeal to an authority for the decision of isolation or internment.

9 — In turn, art. 17 of Law No. 81/2009, of 21-08, which repealed Law No. 2036 of 09/08-1949, allows the member of the Government responsible for the health area a special regulatory power, in accordance with the stipulated by base XX of Law No. 48/90, of 24-08 (Basic Health Law), namely, "take essential exceptional measures in case of public health emergency, including restriction, suspension or closure of activities or separation of people who are not sick, means of transport or goods that have been exposed, in order to avoid the possible spread of infection or contamination".

10— From this, it follows, from the outset, that the possibility of promoting the isolation or hospitalization of people with infectious-contagious diseases is not provided for in this law, as was provided for in Law No. 2036 of 09-08-1949. On the other hand, since the measures taken by the health authorities must respect the Constitution and the law and the Constitutional Law does not provide for the deprivation of liberty of people with infectious diseases, the interpretation to be given to the expression «separation of people who are not patients, means of transport or goods that have been exposed", in order to comply with the Constitution of the Portuguese Republic, it cannot reach the core of the right to liberty, that is, it must not constitute a total deprivation of liberty.

11 — On the other hand, the current Basic Health Law — Law No. 95/2019, of 04-09 — provides in Base 34, on the defense of public health, that the public health authority may «b) Unleash , in accordance with the Constitution and the law, the internment or the compulsory provision of health care to persons who otherwise constitute a danger to public health».

12 – Law No. 82/2009, of 02-04, which regulates the legal regime for the designation, competence and operation of entities that exercise the power of health authorities, provides in its art. 5th the competences of the health authority, namely, «c) To trigger, in accordance with the Constitution and the

law, the internment or the compulsory provision of health care to individuals in a situation of harming public health».

13 — It follows that, since the measures taken by the health authorities must respect the Constitution and the law, and the Constitutional Law does not provide for the deprivation of liberty of people with infectious and contagious diseases, if the interpretation to be given to the expression "hospitalization or the compulsory provision of health care to individuals in a situation of harming public health' either in the sense that health authorities may order the internment, or other measure restricting the freedom of movement, or the compulsory provision of health care from people with infectious and contagious diseases, such interpretation of the law is materially unconstitutional for violation of art. 27(1) of the CRP.

14 — Defining Law No. 27/2006, of 03-07 (Basic Civil Protection Law) "Serious accident" as an unusual event with relatively limited effects in time and space, capable of affecting people and other beings living, the goods or the environment, but establishing in art. 5, no. 1, al. a), the principle of priority of public interest relating to civil protection in relation to the interests of national defense, internal security and public health, we can conclude that serious situations of public health, such as the current pandemic, are not included in the public interest relating to civil protection, therefore, are not included in the concepts of "serious accident" and "catastrophe" referred to in art. 3 of the Civil Protection Law.

15 — From this it can also be concluded that the Resolutions of the Council of Ministers — and the Resolutions of the Council of the Regional Government — which were based on the Basic Civil Protection Law to declare "the contingency and alert situation, within the scope of the disease pandemic COVID-19", namely the Resolutions of the Council of Ministers No. 55-A/2020, of 07/31/2020, of 08/14, 68-A/2020, of 08/28, and 70-A/2020, of 11-09 — revoked by Resolution of the Council of Ministers no. 88-A/2020, of 14-10, currently in force —, which provide in point 2 the "mandatory confinement, in establishment of health, in the respective household or in another place defined by the health authorities: (...) «a) Patients with COVID-19 and those infected with SARS-CoV-2; (...) "b) Citizens for whom the health authority or other health professionals have determined active surveillance", have no legal basis, asthe Civil Protection Law does not apply to situations of danger to public health.

16 - We can thus conclude that the Resolutions of the Council of Ministers No. 55-A/2020, of 07-31, 63-A/2020, of 08-14, 68-A/2020, of 08-28 , 81/2020, of 29-09 - the latter revoked by Resolution of the Council of Ministers No. 88-A/2020, of 14-10, currently in force -, and respective Annex, which were issued by the Government, in use administrative powers, created a regime that restricts

the freedom of citizens with infectious and contagious diseases (quarantines, prophylactic isolation, etc.) and, to reinforce the application of a deprivation of liberty not allowed by the Constitution or provided for in the law enabling situations of people with a contagious disease or danger to public health, established the commission of a crime of disobedience for such violations and the aggravation of the penalty provided for such crime,directly violate art. 27, no. 1, of the CRP, therefore, as unconstitutional, they should be disapplied in the present case, contrary to the request made by the applicant,

17 — The sub judice decision being maintained.

4. The applicant is the regional health authority, represented by the Regional Health Directorate of the Autonomous Region of the Azores.

Decree-Law No. 11/93, of 1993-01-15, in its current version (Statute of the National Health Service) determines that (our underlining):

Article 1.

The National Health Service , hereinafter referred to as NHS, is an ordered and hierarchical set of institutions and official services providing health care, operating under the supervision or supervision of the Minister of Health .

Article 3

1 - The NHS is organized in health regions.

2 - The health regions are divided into health sub-regions, integrated by health areas.

Article 6

1 - In each health region there is a regional health administration, hereinafter referred to as the ARS .

2-the ARS have legal personality, administrative and financial autonomy and own assets.

3-the ARS have functions of planning, distribution of resources, guidance and coordination of activities, human resources management, technical and administrative support and also evaluating the functioning of institutions and services providing health care.

4 – (...).

In turn, Decree-Law No. 22/2012 stipulates

Article 1

1 - The Regional Health Administrations, IP, for short referred to as ARS , IP., are public institutes integrated in the indirect administration of the State , endowed with administrative, financial autonomy and their own assets.

2-the ARS, IP, carry out their duties, under the supervision and supervision of the member of the Government responsible for the health area.

3-the ARS, IP, are governed by the rules contained in this decree-law, by the provisions of the framework law of public institutes and the Statute of the National Health Service and other rules that are applicable to it .

Article 3

1 - The ARS, IP, have the mission of guaranteeing the population of the respective geographic area of intervention access to the provision of health care, adapting the available resources to the needs and complying with and enforcing health policies and programs in their area of intervention.

2 - The responsibilities of each ARS, IP, within the scope of their respective territorial constituencies:

a) To carry out the national health policy, in accordance with global and sectoral policies, aiming at its rational ordering and the optimization of resources ;

b) Participate in the definition of intersectoral planning coordination measures, with the aim of improving the provision of health care;

c) Collaborate in the preparation of the National Health Plan and monitor its execution at the regional level;

d) Develop and promote activities within the scope of public health, in order to ensure the protection and promotion of the health of populations;

e) Ensuring the execution of local intervention programs with a view to reducing the consumption of psychoactive substances, preventing addictive behaviors and reducing dependence;

f) Develop, consolidate and participate in the management of the National Network for Continued Integrated Care in accordance with the defined guidelines;

g) Ensure the regional planning of human, financial and material resources, including the execution of the necessary investment projects, of institutions and services providing health care, supervising their allocation;

h) Draw up, in line with the guidelines defined at national level, the map of facilities and equipment;

i) Allocate, in accordance with the guidelines defined by the Central Administration of the Health System, IP, financial resources to institutions and health care providers integrated or financed by the National Health Service and private entities with or without profit , who provide health care or work within the areas referred to in subparagraphs e) and f);

j) Signing, monitoring and reviewing contracts within the scope of public-private partnerships, in accordance with the guidelines defined by the Central Administration of the Health System, IP, and allocating the respective financial resources;

l) Negotiate, celebrate and monitor, in accordance with the guidelines defined at national level, the contracts, protocols and conventions of regional scope, as well as carry out the respective evaluation and review, in the context of the provision of health care as well as in the aforementioned areas in sub-paragraphs e) and f);

m) To guide, provide technical support and assess the performance of institutions and services providing health care, in accordance with the defined policies and with the guidelines and regulations issued by the competent central services and bodies in the various fields of intervention;

n) Ensuring adequate articulation between health care services in order to guarantee compliance with the referral network;

o) Allocate financial resources, through the signing, monitoring and review of contracts in the context of integrated continuing care;

p) Develop functional programs for health establishments;

q) Licensing private health care units and units in the area of dependencies and additive behavior in the social and private sector;

r) Issue opinions on master plans for health units, as well as on the creation, modification and merger of services;

s) Issue opinions on the acquisition and expropriation of land and buildings for the installation of health services, as well as on projects for the installations of health care providers.

3 - In order to carry out their duties, the ARS, IP may collaborate with each other and with other entities in the public or private sector, with or without profit, under the terms of the legislation in force.

5. The requested habeas corpus measure falls within the provisions of article 220 of the CPPenal, which reads as follows:

Habeas corpus due to illegal detention

1 - Those detained to the order of any authority may request the investigating judge of the area where they are located to order their immediate judicial presentation, on any of the following grounds:

a) The deadline for delivery to the judiciary has been exceeded;

b) Keeping detention outside legally permitted places;

c) The arrest was made or ordered by an incompetent entity;

d) Be the detention motivated by a fact for which the law does not allow it.

2 - The application can be signed by the detainee or by any citizen in the enjoyment of their political rights.

3 — Any authority that raises an illegitimate obstacle to the submission of the application referred to in the preceding paragraphs or to its referral to the competent judge is punishable with the penalty provided for in article 382 of the Penal Code.

6. Appreciating.

Article 401 of the Criminal CP stipulates the following:

1 - The following are entitled to appeal:

a) The Public Prosecutor's Office, of any decisions, even if in the exclusive interest of the accused;

b) The defendant and the assistant, of decisions rendered against them;

c) The civil parts, from the part of the decisions against each one rendered;

d) Those who have been sentenced to pay any sums, under the terms of this Code, or have to defend a right affected by the decision.

2 - Anyone who has no interest in acting cannot appeal.

7. The first question that arises here is that of the appellant's legitimacy, in the context of an appeal in criminal proceedings.

I. We are within the scope of a criminal jurisdiction, whose purpose is to ensure the effective exercise of the State 's jus puniendi , that is, which is dedicated to investigating and deciding on behavior that constitutes a crime or administrative offence.

It is in this context and with such purpose in mind that the Law determines who has the legitimacy to be able to discuss the goodness of a decision rendered by a criminal court.

ii.In this case, we find that the appellant is not a defendant, is not an assistant and has not made any civil claim that, in view of the principle of adhesion, would determine her status as plaintiff or defendant.

iii. Thus, under the Law and given the list of interveners that the legislator understood may have legitimacy to intervene in a process in this type of jurisdiction, on appeal, we must immediately conclude that the appellant lacks legitimacy to be able to discuss the content of a court decision in this context.

iv.In fact, the practice of any crime, nor any illicit offense of an administrative nature is not discussed here, given that the issue of possible consequences at the criminal level, the recognition of the existence of an illegal detention, is a matter that will have to be discussed in its own seat - that is, in an inquiry that may be opened for this purpose, being completely foreign to the decision of the present case.

v. We conclude, therefore, that the appellant lacks legitimacy to file an appeal against the decision rendered by the "a quo" court.

8. Regardless of the question of legitimacy, it appears that, similarly, the appellant lacks an interest in bringing proceedings.

i.As follows from peaceful jurisprudence and doctrine in this regard, the interest in acting means the need for someone to use the appeal mechanism as a way of reacting against a decision that entails a disadvantage for the interests that it defends or that has frustrated one of its legitimate expectation or benefit.

ii. Now, in the present case, the question is – did the decision rendered entail any disadvantage for the interests that ARS defends? Or a legitimate expectation or benefit of yours?

The answer is manifestly negative.

If not, let's see.

iii. The ARS continues its attributions, under the supervision and supervision of the member of the Government responsible for the health area .

Thus, and from the outset, either in view of the functions entrusted to it, or in view of their manifest hierarchy, before the tutelage, it will have to be concluded that no ARS pursues its own autonomous interest, which it is responsible for defending. Eventually, it will be the respective Minister or the Government in which it operates, as the "interests" of the ARS will not be its own, but will be included in the health policy of the ministry that oversees such entity.

It should be noted, moreover, that in the definition of its attributions [1] it is not assigned any specific defense function, autonomously and in its own name,

in court, of any interests that fall within its functions which, in regarding criminal or administrative offence activities, they are none...

iv.In turn, the interest that the appellant itself intends to defend and which appears in the application, at the end of this appeal - the validation of the mandatory confinement of the applicants, for being carriers of the SARS -CoV-2 (AH___) virus and for being in active surveillance, for high risk exposure, decreed by the health authorities (SH__SWH__ and NK___) - is something in itself contradictory and goes beyond the purpose and scope of powers of a criminal court.

Contradictory because the appellant does not admit that confinement corresponds to deprivation of liberty. If so, it is not clear in which seat the appellant founds the competence of a criminal court to validate "confinements". And outside the scope of action of a criminal court, because it is not responsible for making declarative decisions to validate infections or diseases...

v . Finally, one does not see that a legitimate expectation or benefit has an entity under the tutelage of a Government agency, seen frustrated by the decision now being criticized. saw. It follows from this that the appellant has no interest in acting, which is why, under the provisions of paragraph 2 of article 401 of the Criminal Code, it cannot appeal against the decision rendered. 9. The decision rendered by the "a quo" court to receive this appeal is not binding on this court (article 414 of the CPPenal), so there is nothing to prevent its rejection being determined. 10.

Nevertheless, and for the peace and tranquility of consciences, the following will be added:

Even if it were not understood that way, the appeal presented would be manifestly unfounded, for the following succinct reasons :

i. First, for the exhaustive and correct reasoning set out in the decision, by the "a quo" court, whose content is fully endorsed.

Actually,in view of the Constitution and the Law, the health authorities do not have the power or legitimacy to deprive any person of their freedom - even under the label of "confinement", which effectively corresponds to a detention - since such a decision can only be determined or validated by a judicial authority, that is, the exclusive competence, in view of the Law that still governs us, to order or validate such deprivation of liberty, is assigned exclusively to an autonomous power, to the Judiciary.

It follows that any person or entity that issues an order, the content of which leads to the deprivation of physical freedom, ambulatory, of others (whatever the nomenclature that this order takes: confinement, isolation, quarantine,

prophylactic protection, etc.), which does not comply with the legal provisions, namely the provisions of art. 27 of the CRP and without having been granted such decision-making power, by virtue of Law - from the AR, within the strict scope of the declaration of a state of emergency or siege , subject to the principle of proportionality - which mandates and specifies the terms and conditions of such deprivation , will be carrying out an illegal detention, because ordered by an incompetent entity and because motivated by a fact that the law does not allow (it should be said, in fact, that this issue has been debated, over time, in relation to other public health phenomena, namely with regard to HIV infection and tuberculosis, for example. And, as far as is known, no one has ever been deprived of their freedom, for suspicion or certainty of suffering from such diseases, precisely because the law does not allow it).

It is in this context that, without any shadow of doubt, the situation under consideration in this case is included, given that the appropriate means of defense against illegal detention is subject to the appeal for habeas corpus , provided for in art. 220, als. c) and d) of the CPPenal.

And, correctly, the "a quo" court ordered the immediate release of four people who were illegally deprived of liberty.

ii.Second, because the request made in the appeal proves to be impossible .

If not, let's see:

11. In fact, it is requested that "the mandatory confinement of the applicants be validated, as they are carriers of the SARS-CoV-2 virus (AH___) and because they are under active surveillance, for high risk exposure , decreed by the authorities of health (SH__SWH__ and NK_)."

12. It is with great astonishment that this court is faced with such a request, especially considering that the appellant is active in the health sector.

Since when is it the responsibility of a court to make clinical diagnoses, on its own initiative and based on the eventual results of a test? Or to ARS? Since when is the diagnosis of a disease made by decree or by law?

13. As the appellant has more than an obligation to know, a diagnosis is a medical act, the sole responsibility of a doctor .

This is what results unequivocally and peremptorily from Regulation No. 698/2019, of 5.9 (regulation that defines the proper acts of physicians), published in DR.

There it is determined, in an imperative way (which imposes its compliance by all, including the applicant) that (our emphasis):

Article 1

Object

This regulation defines the professional acts of physicians, their responsibility, autonomy and limits, within the scope of their performance.

Article 3

Qualification

1 — The doctor is the professional legally qualified to practice medicine , qualified for the diagnosis , treatment, prevention or recovery of diseases and other health problems , and able to provide care and intervene on individuals, groups of individuals or population groups, sick or healthy, with a view to protecting, improving or maintaining their state and level of health.

two -Physicians who are currently registered with the Portuguese Medical Association are the only professionals who can practice the actions of physicians , under the terms of the Statute of the Medical Association, approved by Decree-Law No. 282/77, of 5 July, with the amendments introduced by Law No. 117/2015, of 31 August and this regulation.

Article 6

Medical act in general

1 — The medical act consists of the diagnostic , prognostic , surveillance , investigation, medico-legal expertise, clinical coding, clinical audit, prescription and execution of pharmacological and non-pharmacological therapeutic measures, pharmacological, medical techniques , surgical and rehabilitation, health promotion and disease prevention in all its dimensions, namely physical, mental and social of people, population groups or communities, respecting the deontological values of the medical profession.

Article 7

Diagnosis

The identification of a disorder, disease or the state of a disease by studying its symptoms and signs and analyzing the examinations carried out constitutes a basic health procedure that must be carried out by a doctor and in each specific area , by a specialist physician and aims to establish the best preventive, surgical, pharmacological, non-pharmacological or rehabilitation therapy. 14.

Even under the Mental Health Law, Law No. 36/98, of 24 July, the diagnosis of the pathology that can lead to compulsory hospitalization is obligatorily carried out by specialist physicians and their technical-scientific judgment - inherent to the assessment clinical-psychiatric - is excluded from the judge's discretion (see articles 13 n°3, 16 and 17 of the said Law).

15. Thus, any diagnosis or any act of health surveillance (such as the determination of the existence of viral infection and high risk of exposure, which are covered by these concepts) made without prior medical observation to the applicants, without the intervention of a doctor registered with the OM (which carried out the assessment of its signs and symptoms, as well as the exams it deemed appropriate to its condition), violates such Regulation, as well as the provisions of article 97 of the Statute of the Medical Association, being liable to configure the crime P. and p. by article 358 al.b) (Usurpation of functions) of the Criminal Code, if dictated by someone who does not have such quality, that is, who is not a physician registered with the Medical Association.

viola also paragraph 1 of article 6 of the Universal Declaration on Bioethics and Human Rights, which Portugal has subscribed to and is internally and externally obliged to respect, since there is no document in the file proving that the informed consent that this Declaration imposes has been given. .

It is thus clear that the prescription of auxiliary diagnostic methods (such as tests to detect viral infection), as well as the diagnosis of the existence of a disease, in relation to anyone and everyone, is a matter that cannot be carried out by Law, Resolution, Decree, Regulation or any other normative means, as they are acts that our legal system reserves the exclusive competence of a doctor, given that the doctor, in advising his patient, should always try to obtain the your informed consent.

16. In the case we are dealing with, there is no indication or proof that such a diagnosis was actually carried out by a qualified professional under the terms of the Law and who had acted in accordance with good medical practices.

In fact, what follows from the facts given as established is that none of the applicants was even seen by a doctor, which is frankly inexplicable, given the alleged seriousness of the infection.

17. In fact, the only element that appears in the proven facts, in this regard, is the performance of RT-PCR tests, one of which had a positive result in relation to one of the applicants.

i. Now, given the current scientific evidence, this test is, by itself, unable to determine, without a reasonable margin of doubt, that such positivity corresponds, in fact, to the infection of a person by the SARS-CoV-2 virus, by several reasons, of which we highlight two (in addition to the issue of the gold standard which, due to its specificity, we will not even address):

Because this reliability depends on the number of cycles that make up the test;

Because this reliability depends on the amount of viral load present.

ii. In fact, the RT-PCR (Polymerase Chain Reaction) tests, molecular biology tests that detect the RNA of the virus, commonly used in Portugal to test and enumerate the number of infected (after nasopharyngeal collection), are performed by amplification of samples , through repetitive cycles.

From the number of cycles of such amplification, the greater or lesser reliability of such tests results.

iii. And the problem is that this reliability proves, in terms of scientific evidence (and in this field, the judge will have to rely on the knowledge of experts in the field) more than debatable.

This is what results, among others, from the very recent and comprehensive study Correlation between 3790 qPCR positive samples and positive cell cultures including 1941 SARS-CoV-2 isolates , by Rita Jaafar, Sarah Aherfi, Nathalie Wurtz, Clio Grimaldier, Van Thuan Hoang, Philippe Colson, Didier Raoult, Bernard La Scola, Clinical Infectious Diseases, ciaa1491, https://doi.org/10.1093/cid/ciaa1491, at https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1491/5912603 , published at the end of September this year, by Oxford Academic , carried out by a group that brings together some of the greatest European and world experts in the field.

This study concludes [2] , in free translation:

"At a cycle threshold [ct] of 25, about 70% of the samples remain positive in cell culture (ie were infected): at a ct of 30, 20% of the samples remain positive; in a ct of 35, 3% of the samples remained positive; and in a ct above 35, no sample remained positive (infectious) in cell culture (see diagram).

This means that if a person has a positive PCR test at a cycle threshold of 35 or higher (as is the case in most laboratories in the US and Europe), the chances of a person being infected are less than 3%. The probability of the person receiving a false positive is 97% or higher".

iv. What follows from these studies is simple -the eventual reliability of the PCR tests carried out depends, from the outset, on the threshold of amplification cycles they support, such that, up to the limit of 25 cycles, the test reliability will be around 70%; if 30 cycles are performed, the degree of reliability drops to 20%; if 35 cycles are reached, the degree of reliability will be 3%.

v. In the present case, the number of amplification cycles with which PCR tests are carried out in Portugal, including the Azores and Madeira, is unknown, since we were unable to find any recommendation or limit in this regard.

saw. In turn, in a very recent study by Elena Surkova, Vladyslav Nikolayevskyy and Francis Drobniewski, accessible inhttps://www.thelancet.com/journals/lanres/article/PIIS2213-2600(20)30453-7/fulltext , published in the equally prestigious The Lancet, Respiratory Medicine , refers (in addition to the multiple issues that the Lancet itself). The accuracy of the test raises, regarding the specific detection of the sars-cov 2 virus, strong doubts regarding compliance with the so-called gold standard | that (free translation):

"Any diagnostic test must be interpreted in the context of the actual possibility of the disease, existing before its realisation . For Covid-19, this decision to perform the test depends on the prior assessment of the existence of symptoms, previous medical history of Covid 19 or the presence of antibodies, any potential exposure to this disease and no likelihood of another possible diagnosis. " [3] "One of the potential reasons for presenting positive results could be the prolonged shedding of viral RNA, which is known to extend for weeks after recovery in those who were previously exposed to SARS-CoV-2. However, and most importantly, there is no scientific data to suggest that low levels of viral RNA by RT-PCR equate to infection, unless the presence of infectious viral particles has been confirmed by laboratory culture methods .

In summary, Covid-19 tests that accuse false positives are increasingly likely in the current epidemiological climate scenario in the UK, with substantial consequences at the personal, health system and societal level ." [4]

18. Thus, with so many scientific doubts, expressed by experts in the field, which are the ones that matter here, as to the reliability of such tests, ignoring the parameters of their performance and with no diagnosis made by a doctor, in the sense of the existence of infection and risk, it would never have been possible for this court to determine that AH___ was a carrier of the SARS-CoV-2 virus, nor that SH__SWH__ and NK_ had been exposed to high risk.

19.In a final summary, it will be said that, since the appeal filed is inadmissible, due to lack of legitimacy and lack of interest in acting on the part of the appellant, as well as manifestly unfounded, it will have to be rejected, under the provisions in articles 401 nº 1 al. a), 417 nº6 al. b) and article 420 nº 1 paragraphs. a) and b), all from the Criminal CP. iv – decision. In view of the above, and pursuant to the provisions of articles 417, no. 6, al. b) and 420 nº 1 als. a) and b), both of the Code of Criminal Procedure, the appeal filed by the REGIONAL HEALTH AUTHORITY, represented by the Regional Directorate of Health of the Autonomous Region of the Azores , is rejected .

Pursuant to paragraph 3 of article 420 of the CPPenal, the appellant is sentenced to a procedural sanction of 4 UC, as well as in the TJ of 4 UC and in the costs.

Immediately inform the "a quo" court of the content of this judgment. Lisbon, November 11th, 2020 Margarida Ramos de Almeida Ana Paramés

EXHIBIT "SS"

Portuguese Court Rules PCR Tests "Unreliable" & Quarantines "Unlawful"

Source: Off-Guardian

Link: https://off-guardian.org/2020/11/20/portuguese-court-rules-pcr-tests-unreliable-quarantines-unlawful/

Published On: November 20, 2020.

Important legal decision faces total media blackout in Western world

An appeals court in Portugal has ruled that the PCR process is not a reliable test for Sars-Cov-2, and therefore any *enforced quarantine based on those test results is unlawful.*

Further, the ruling suggested that any forced quarantine applied to healthy people could be a violation of their fundamental right to liberty.

Most importantly, the judges ruled that *a single positive PCR test cannot be used as an effective diagnosis of infection.*

The specifics of the case concern four tourists entering the country from Germany – all of whom are anonymous in the transcript of the case – who were quarantined by the regional health authority. Of the four, only one had tested positive for the virus, whilst the other three were deemed simply of "high infection risk" based on proximity to the positive individual. All four had, in the previous 72 hours, tested negative for the virus before departing from Germany.

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In their ruling, judges Margarida Ramos de Almeida and Ana Paramés referred to several scientific studies. Most notably **this study by Jaafar et al.**, which found that – when running PCR tests with 35 cycles or more – the accuracy dropped to 3%, meaning up to 97% of positive results could be false positives.

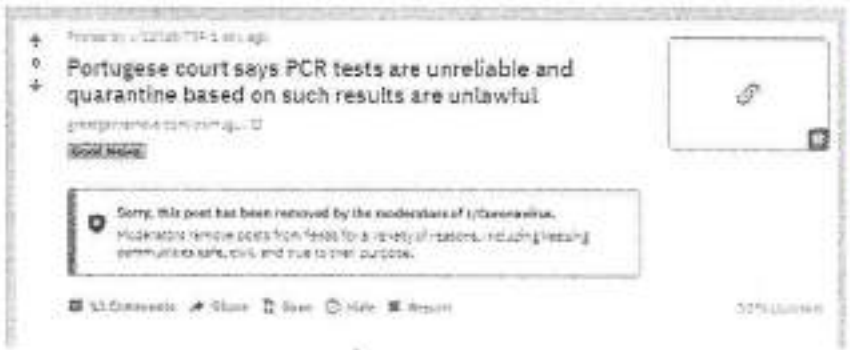
The ruling goes on to conclude that, based on the science they read, any PCR test using over 25 cycles is totally unreliable. Governments and private labs have been very tight-lipped about the exact number of cycles they run when PCR testing, but it is known to sometimes be **as high as 45**. Even fearmonger-in-chief Anthony Fauci has publicly stated anything over 35 is **totally unusable**.

You can read the complete ruling in the original Portuguese **here**, and translated into English **here**. There's also a good write up on it on **Great Game India**, plus a Portuguese professor sent a long email about the case to **Lockdown Sceptics**.

The media reaction to this case has been entirely predictable – they have not mentioned it. At all. Anywhere. Ever.

The ruling was published on November 11th, and has been referenced by many alt-news sites since...but the mainstream outlets are maintaining a complete blackout on it.

The reddit Covid19 board actually **removed the post**, because it was "not a reliable source", despite relying on the official court documents:



Lookout for a forced and disingenuous "fact-check" on this issue from HealthFeedback or some other "non-partisan" outlet in the near future. But until they find some poor shlub to lend their name to it, the media blackout will continue.

Whatever they say, this is a victory for common sense over authoritarianism and hysteria.

EXHIBIT "TT"

NEGATIVE TEST MANDATORY FOR ENTRY TO NL

Source: DutchNews.nl

Link: https://www.thehagueonline.com/news/2021/01/04/negative-test-mandatory-for-entry-to-nl

Published on: Jan 4, 2021

The government is sticking to its requirement that everyone travelling to the Netherlands, including Dutch nationals, should have a negative PCR coronavirus test no older than 72 hours if they come from a risky area, the cabinet confirmed on Sunday evening.

The requirement is now being incorporated into public health law via the emergency coronavirus legislation from January 4, and thus remains mandatory for all travellers, the ministry said in a statement.

On December 31, anti-coronavirus activists won a court case against the Dutch state to ensure a family can return from holiday in Tanzania without having to produce negative coronavirus tests.

The court in The Hague ruled that the family can return from the high risk country on January 3 without a negative test and ordered the state to pay the legal costs.

The judge said the family have the right to protest about being forced to undergo a PCR test against their will. 'Introducing such a requirement for citizens of the

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Netherlands who want to return home requires legal grounding, and this is not covered by article 53 or 54 of the public health act,' the judge is quoted as saying.

Legal basis

The fact that further spreading of the virus needs to be tackled urgently is not up for discussion, the judge said. 'But such a far reaching obligation as this, which concerns physical integrity, requires a concrete legal basis.'

The government said immediately that it would appeal against the court ruling and has now confirmed it will amend the public health act to incorporate the requirement. 'The cabinet is doing all this to limit the spread of the virus as much as possible,' the statement said.

'The government continues to emphasise that people should only travel if strictly necessary. Holidays and family visits are non essential trips.'

EXHIBIT UU

Austrian Court Rules PCR Unsuited For COVID, Lockdowns Unlawful

Source: Principia Scientific International

Link: https://principia-scientific.com/austrian-court-rules-pcr-unsuited-for-covid-lockdowns-unlawful/

Written by: greatgameindia.com

Published on: April 15, 2021

Following the Portuguese, German and Dutch rulings, now the Austrian court has ruled that PCR tests are not suitable for COVID-19 diagnosis and that lockdowns has no legal or scientific basis.

The Vienna Administrative Court granted a complaint by the FPÖ against the prohibition of its meeting registered for January 31 in Vienna.

"*The prohibition was wrong*," the court said says in the ruling (read judgement below).

The court stated on the basis of scientific studies that the grounds for the prohibition put forward by the Vienna State Police Department are completely unfounded.

The court agrees with the statements in the complaint "*on all points*" and even goes far beyond the arguments put forward by the FPÖ itself.

In particular, the criteria and definitions used to determine the number of corona infections are being massively questioned.

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It is expressly pointed out that, even according to the World Health Organization (which took a u-turn only after Biden took office), *"a PCR test is not suitable for diagnosis and therefore does not in itself say anything about the disease or infection of a person".*

"However, the Minister of Health uses a completely different, much broader case definition for Covid-19 diagnosis, which cannot be used to justify the prohibition of a meeting."

The court came to the conclusion that the "information" from the Vienna City Health Service, on which the prohibition by the Vienna State Police Department was based, *"did not contain any valid and evidence-based statements or findings on the pandemic".*

Based on the definitions of the Minister of Health, "Case definition Covid-19" from December 23, 2020, a "confirmed case"

1. *is any person with evidence of SARS-CoV-2-specific nucleic acid (PCR test), regardless of clinical manifestation or*
2. *any person with evidence of SARS-CoV-specific antigen that meets the clinical criteria or*
3. *any person with evidence of SARS-CoV-specific antigen that meets the epidemiological criteria.*

Thus none of the three "confirmed cases" defined by the Minister of Health meets the requirements of the WHO concept of "sick / infected person". The WHO refuses to rely solely on the PCR test (confirmed case 1).

Below is the Austrian court judgement translated into English.

ADMINISTRATIVE COURT
WIEN

IN THE NAME OF THE REPUBLIC

Worldwide Action On Scamdemic

In a similar ruling, a Portuguese appeals court has ruled that *PCR tests are unreliable and that it is unlawful to quarantine people based solely on a PCR test.*

A German court in a landmark ruling has declared that COVID-19 lockdowns imposed by the government *are unconstitutional.*

Thuringia's spring lockdown was a "*catastrophically wrong political decision with dramatic consequences for almost all areas of people's lives,*" the court said, justifying its decision.

It was then revealed that the *German Interior Ministry hired scientists to develop fake coronavirus model in order to justify strict lockdown*, according to extensive email exchanges obtained by a group of lawyers in a legal dispute.

Earlier, an American federal judge ruled *coronavirus restrictions in Pennsylvania as unconstitutional.*

Even the Hague Court has ruled that the COVID-19 lockdown which was imposed by the Dutch Govt had no legal basis *and that it was illegal.*

However, the Dutch appeals court overturned the ruling within hours which ordered the government to lift the "*illegitimate*" measure immediately.

The appeals judges argued that they wanted to avoid what they called a "*yo-yo effect,*" referring to public confusion around whether the curfew was still in force.

Last year, an Italian politician had demanded arrest of Bill Gates in the Italian Parliament.

Sara Cunial, MP for Rome denounced Bill Gates as "*Vaccine Criminal*" and urged Italian President to hand him over to the International Criminal Court for crimes against humanity.

Then an Austrian parliamentary member exposed the defectiveness of the government's COVID-19 tests by demonstrating in the parliament how a glass of Coca Cola tested positive for COVID-19.

Earlier, the Tanzanian President John Magufuli growing suspicious of the World Health Organization (WHO), decided to investigate the claims of the tests himself. He sent the WHO samples of *a goat, a papaya and a quail for testing, all of which came COVID-19 positive.*

NOTARY
M. M. PARAB
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

Magufuli was known as "The Bulldozer" for his tough stance against corruption and for his hardline policies, which helped him to victory in October 2015 as Tanzania's fifth president.

The European Union gave 27 million euros to Tanzania to impose COVID-19 measures prescribed by the WHO like strict lockdowns, masks and mass-scale vaccination.

Tanzania took the money, then declared the country coronavirus free. This led to the Europeans venting their anger in the parliament.

His sudden death has raised many questions including *whether Tanzania's President was assassinated for exposing the COVID-19 plandemic* by taking controversial action against Big Pharma and the WHO and their global push for vaccines.

Because of such massive COVID-19 false positive cases scandal, the Australian Government has scrapped a billion dollar coronavirus vaccine agreement with Australian biotech company CSL Limited to supply 51 million doses of a Covid-19 vaccine being developed by the University of Queensland after *several trial participants returned false positive HIV test results.*

Even the Belgian health experts have demanded an investigation into the WHO for faking a pandemic.

In 2010, the WHO was caught faking a pandemic and was forced to admit that its methodology of measuring the virality or the spread of the disease, instead of its severity was incorrect.

It was also revealed in an intercepted human intelligence report that *Bill Gates offered $10 million bribe for a forced Coronavirus vaccination program in Nigeria*

As reported by *GreatGameIndia* last year, the *President of Belarus had exposed that the World Bank coronavirus aid comes with conditions for imposing extreme lockdown measures*, to model their coronavirus response on that of Italy and even changes in the economic policies which he refused as being "*unacceptable*".

A major lawsuit has also been filed against the PM of Denmark for strict COVID-19 restrictions and for killing almost 17 million minks.

A three judge court in Peru has ruled that the COVID-19 pandemic was started by the billionaires Bill Gates, George Soros and Rockefeller.

Despite the ruling (read the resolution in full at above link), *"fact-checkers" concluded that the Peruvian court had no evidence to make such a judgement* citing declaration from the WHO and CDC who have lost all its credibility.

EXHIBIT "VV"

80% Covid patients in India are asymptomatic, health ministry analysis finds.

Source: ThePrint

Link: https://theprint.in/health/80-covid-patients-in-india-are-asymptomatic-health-ministry-analysis-finds/487761/

Written By: Abantika Ghosh

Published on: 24 August, 2020

Analysis of cases across India until 23 August shows about 25.93% of the symptomatic patients reported with fever and 17.18% with cough.

New Delhi: About 80 per cent of Covid-19 patients in India are asymptomatic, an internal analysis by the Integrated Disease Surveillance Programme (IDSP) has concluded after extensive discussions with states, ThePrint has learnt.

The last documented estimate on this was 28 per cent, published in a study in the Indian Journal of Medical Research (IJMR) in May that looked at cases until 30 April.

Of the symptomatic patients, analysis of all cases until 23 August shows, about 25.93 per cent reported with fever, 17.18 per cent with cough, 7.83 per cent with sore throat and 5.54 per cent with breathlessness, sources said.

However, the largest proportion is of people who reported with other symptoms such as gastrointestinal problems, loss of smell and taste and body ache, added the sources.

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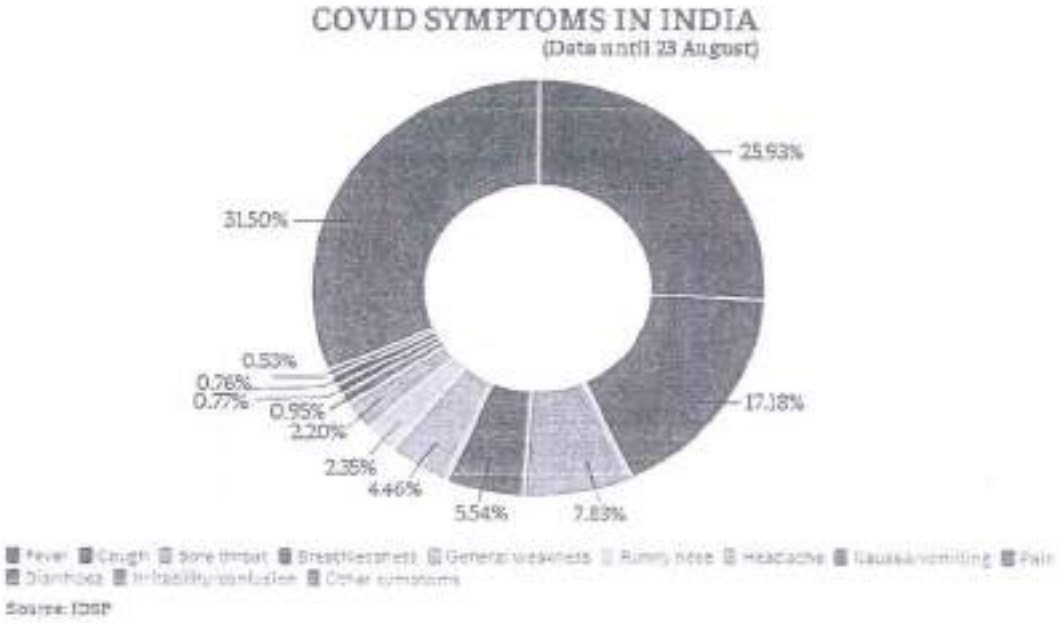

Graphic: Ramandeep Kaur/The Print

"This is an analysis we have done of all the symptoms that patients have reported with. However, it is important to understand that after many meetings with states, it is now clear that about 80% patients in India are asymptomatic. This is a large number," said a senior IDSP official, who did not wish to be named.

"We earlier thought that these are presymptomatic people who have been traced and tested during the contact tracing exercise and are essentially presymptomatic, that is they go on to develop symptoms later. However, states have now emphatically told us that these are asymptomatic people not presymptomatic. This is the largest chunk of patients," added the official.

As of Sunday, India recorded 30.44 lakh Covid cases and 56,706 deaths.

Asymptomatic patients driving the epidemic

A large number of asymptomatic patients are widely recognised as a pool for further spread of the disease.

Younger people who are in their 20s, 30s and 40s often tend to be asymptomatic and are, hence, the drivers of the disease, the World Health Organization (WHO) said this week. Analysis of data from the top five high burden states in India also bears this trend.

Initially, asymptomatic patients were estimated to be in the 69-80 per cent range in India. Dr Raman Gangakhedkar, former head of epidemiology, Indian Council of Medical Research (ICMR), had given the 80 per cent figure in one of the Covid briefings in April, only to revise it to 69 per cent a few days later. There was a sharp downward revision in this after the IJMR study of over 40,000 patients in May.

However, India's testing strategy banks heavily on whether a person shows symptoms. According to experts, this is so because a person is less likely to test positive unless symptoms are showing. This is provided a person is presymptomatic, which is a transient phase, and not asymptomatic — a condition that can last the duration of the infection.

With such a high incidence of asymptomatic cases, this may need to be revised at some point.

Analysis of signs

The IDSP also analysed the signs as reported by doctors in their forms.

"Symptoms are the complaints that patients come to us for; signs are what doctors discover during their examination," explained the IDSP official quoted above.

This shows that 8.46 per cent doctors reported an abnormal lung X-ray or CT scan, 2.78 per cent reported tachypnea or rapid breathing, 1.89 per cent reported abnormal lung sounds, 0.75 per cent reported redness of eyes, 0.66 per cent wrote coma, 0.51 per cent reported seizures and 0.60 per cent reported abnormal sounds.

The IDSP official explained that while the reporting form is periodically updated, a lot of the newer signs and symptoms have not been included and are in the process of being added. These include dehydration, alteration of acid base balance of the body, abnormal heartbeats etc. These have all been categorised as "other signs" that constitute 83.54 per cent of the reported signs.

EXHIBIT "WW"

71% active Covid cases in Mumbai asymptomatic

Source: Hindustan Times.

Link: https://www.hindustantimes.com/mumbai-news/71-active-covid-cases-in-mumbai-asymptomatic/story-r5x39rSpDHRBpYjazpUnDK.html

Written by: Rupa Chakraborty

Published on - December 07, 2020.

Doctors say good sign as chances of spread from asymptomatic patients are lower, want more RT-PCR tests than rapid antigen tests

Of the 12,926 active Covid-19 patients in Mumbai, 9,155 (71%) are asymptomatic, displaying no symptoms before undergoing tests for the presence of Sars-CoV-2, the pathogen that causes the disease, data from the Brihanmumbai Municipal Corporation (BMC) shows. A total of 835 patients are in critical condition.

While doctors call it a good sign, as chances of transmission and spread of infection from asymptomatic patients are lower, they stress the need to conduct more reverse transcription polymerase chain reaction (RT-PCR) tests – considered the gold standard in detection of the novel coronavirus – and warn against overdependence on the quick-result rapid antigen tests (RAT).

Every day, the civic body conducts around 15,000 Covid-19 tests, of which a majority – almost 50-55% – are antigen tests. Similar to RT-PCR, rapid antigen kits detect the presence of molecular pathogens of the novel coronavirus. But RT-PCR test results takes almost eight hours as compared to within 30 minutes via RAT.

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Asymptomatic transmission refers to transmission of the virus from a person who does not develop Covid-19 symptoms. As per the World Health Organization (WHO), there are few reports of laboratory-confirmed cases, who are truly asymptomatic, and there is no documented asymptomatic transmission till date.

"The infectious period of the virus that causes Covid-19 in symptomatic individuals may begin within two days. It persists for more than 10 days after symptoms show," said Dr Pradeep Awate, state surveillance officer. "In asymptomatic patients, however, the chances of transmitting the virus are lower, as these individuals don't sneeze or cough. Hence, having more asymptomatic patients helps break the chain of infection."

Most asymptomatic patients are identified through contact-tracing. In Mumbai, more than 40 lakh close contacts of infected patients have been traced by the civic body since the outbreak of the virus in March.

"Asymptomatic patients don't know they are infected because they do not have symptoms. Contact-tracing becomes important to identify this set," said Dr Mangala Gomare, executive health officer, BMC. "Many asymptomatic patients get detected when they are admitted to hospitals for other ailments."

However, a section of medical experts call asymptomatic patients "silent spreaders", suggesting that more Covid-19 tests via RT-PCR be undertaken across the city that will help in early diagnosis.

Since the outbreak of the infection in March till December 5, the civic body has conducted 19,71,736 Covid-19 tests, of which 5,25,143 tests were done through rapid antigen kits. "We get less than 2% false positive reports using rapid antigen tests. Also, people get results within 30 minutes, which helps reduce the burden on labs," said Suresh Kakani, additional commissioner, BMC.

However, doctors have highlighted a loophole in the system. As per rules of the Indian Council of Medical Research (ICMR), every positive report through RAT is treated as true positive, while those with negative report have to be mandatorily re-tested through RT-PCR only if they display symptoms. "Asymptomatic patients don't show symptoms and as per ICMR, the sensitivity of rapid antigen kits is only 50%. So, thousands of asymptomatic patients are slipping through the loophole in diagnosis, as the civic body is relying more on rapid antigen tests," said a senior epidemiologist. "While there is no scientific evidence that asymptomatic patients can't infect others and the spread of infection rate may be less, they could still transmit," added the doctor

"Post Diwali, many migrants who had gone back to their hometowns are returning to the city, and many will be asymptomatic. So, it is essential to run tests to find silent carriers. Considering they live in densely populated slums, they can contribute further to the spread of the virus," said Dr Om Srivastava, epidemiologist and member of the state's Covid-19 taskforce.

EXHIBIT "XX"

85,000 Covid Cases In Second Wave, Most Asymptomatic: Mumbai Civic Body.

Source: NDTV

Link: https://www.ndtv.com/india-news/85-000-covid-cases-in-second-wave-most-asymptomatic-mumbai-civic-body-2402202

Edited by: Anindita Sanyal

Published on: 30th March 2021

Brihanmumbai Commissioner said they are conducting more than 25,000 RT-PCR tests. "We would love to go further, but we have exhausted our capacity," he added.

Mumbai: The second wave of coronavirus in Maharashtra started on February 10 and till March 20, Mumbai logged 85,000 cases, said Iqbal Chahal, the Commissioner of Brihanmumbai corporation, the civic body of Mumbai. Of the total number of cases, 69,500 are asymptomatic, he added. The remaining 8,000 patients reached hospitals with mild symptoms. In the current cycle, the city might see 6000 to 7000 cases a day. The current positivity rate is 14 per cent.

Mumbai recorded 5,890 cases yesterday but because reporting and testing is less on Sunday, the numbers dropped.

"We are going to increase our testing to 60,000 by Thursday. So figure can go to 10,000. But nothing to worry... most are asymptomatic," he said.

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The BMC chief admitted that there are certain limitations on the RT-PCR tests. "Right now, we are conducting more than 25,000 RT-PCR tests. We would love to go further, but we have exhausted our capacity," he added.

At the current rate, the city will need 1,400 to 1,500 beds daily.

Assuring there is sufficient number of beds, he said, "We have acquired additional number of beds and also in private hospitals. So people requiring private hospitals will also now have beds".

By next Monday, 7,000 beds will be available in private hospitals, he said. There is also sufficient number of ventilators and ICU beds available.

Mr. Chahal, who got his first dose of vaccination today, said till yesterday, 10 lakh people have been vaccinated. The target is to give vaccine to 1 lakh people per day, the current number is around 45,000.

Most of the vaccination is happening in hospitals run by the Brihanmumbai Corporation and the government. The Maharashtra government, he added, is requesting the Centre to administer vaccine door-to-door.

EXHIBIT "YY"

Majority of Bengaluru's Covid-19 patients are asymptomatic

Source: Deccan Herald

Link – https://www.deccanherald.com/city/top-bengaluru-stories/majority-of-bengalurus-covid-19-patients-are-asymptomatic-976071.html

Published on: APRIL 19 2021

The highest number of symptomatic cases in the state (28.02 per cent) have been reported from the Uttara Kannada district

India reported 2.73 lakh fresh Covid-19 cases and 1,619 deaths on Monday, with Karnataka among the states accounting for the highest caseload in the country. However, reports show that 95.9 per cent of the state's cases are 'asymptomatic'.

The percentage of asymptomatic cases in the state capital of Bengaluru is even higher – 99.4 per cent, according to a report by Bangalore Mirror quoting data from the state's Covid-19 war room.

The highest number of symptomatic cases in the state (28.02 per cent) have been reported from the Uttara Kannada district.

Most infections in the pandemic's second wave have been asymptomatic or mildly symptomatic, according to Lancet's Covid-19 Commission India Task Force. However, they have stated that the infection is spreading more rapidly in the new wave.

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A Bengaluru doctor told the publication that he has mostly been seeing patients who do not possess any symptoms and if some exhibit mild symptoms those have been going away in a couple of days since being infected.

Another doctor pointed out the problem of mainly asymptomatic cases being reported – the difficulty of containing the spread of the virus. Despite not showing symptoms, people infected with the virus can spread it as easily as their symptomatic counterparts.

A Covid-19 analysis by Jeevan Rekha has held both the government and people's negligence accountable for the recent rise in cases.

"In a pandemic until a natural herd immunity is developed as well as critical mass is vaccinated in the region, cases surge and recede as a function of testing, both qualitative and quantitative. Bengaluru cannot afford to repeat the mistake; it is the question of life and death of 1.2 million people.

Due to low and slow testing, Bengaluru witnessed a sharp surge in covid cases in the June-October period," Sanjeev Mysore, convenor of Jeevan Rekha, told Bangalore Mirror the publication.

Karnataka has a total of 1,141,998 infections as on April 19, 2021.

EXHIBIT "ZZ"

Need To Focus On Asymptomatic COVID Patients: ICMR

Source: Punekar News

Link: https://www.punekarnews.in/need-to-focus-on-asymptomatic-covid-patients-icmr/

Published on: 4 April 2021

Pune, 4 April 2021: The Indian Medical Research Council (ICMR) warned about asymptomatic patients that they can be hidden super-spreaders of the Coronavirus in the country. There is a need to give more attention to those patients said the experts.

In India, 69 percent of the total Covid-19 positive people do not show any symptoms of the virus infection. Such patients can't be identified until they have tested. In various cases, the symptoms can not be seen for seven days even though the person is infected. In such a case, these asymptomatic people can spread the virus widely, if not isolated. People who came in contact can get infected, the condition may be worsened in the case of a senior citizen. Therefore the ICMR suggested paying more attention to such asymptomatic patients.

Pune Divisional Commissioner Saurabh Rao also expressed the need to pay more attention to asymptomatic people, in a recent press conference.

Necessary precautions:

– If a person came in to contact with an infected person, he/she should immediately test himself/ herself

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– an asymptomatic patient can stay in home quarantine according to the doctor's advice.

– Maintain a 6 ft distance from the family members. Use 3 layered masks.

– Use a mask and maintain a safe distance at public places.

– People with co-morbidity must avoid going to crowded places.

EXHIBIT" AAA

Online RTI Request Form Details

RTI Request Details :-

RTI Request Registration number	INCMR/R/E/21/00909
Public Authority	Indian Council of Medical Research

Personal Details of RTI Applicant:-

Name	[redacted]
Gender	[redacted]
Address	[redacted]
Pincode	[redacted]
Country	India
State	[redacted]
Status	Rural
Educational Status	Literate
Phone Number	Details not provided
Mobile Number	[redacted]
Email-ID	[redacted]

Request Details :-

Citizenship	Indian
Is the Requester Below Poverty Line ?	No

Description of information sought (upto 500 characters)

Description of Information Sought

1. Request count of people all over India who tested positive for Covid-19 after 1st and 2nd dose of vaccination. Request split of this data between the different kinds of vaccines including Covishield and Covaxin and also the split between the different kinds of tests. This data is requested in tabular form as specified in table 1 of attachment.

2. Request count of Covid-19 tests done all over India. Request split of this data between the different kinds of tests and between the symptomatic status. This data is requested in tabular form as specified in table 2 of attachment.

4. Request information on the protocol of Covid-19 testing with respect to testing data collection, test result data storage, and data segregation on the basis of vaccination status , symptomatic status and hospitalization/quarantine status.

5. Request information on whether a Covid-19 positive person admitted to hospital is retested for Covid-19 prior to allowing the person to leave hospital. Request documents that justify the above.

6. Request information whether there are adverse effects or side effects of the RT-PCR tests and if ICMR has conducted studies on the same.

7. Kindly inform if the RT-PCR test kits currently in use in India are fully approved or approved under emergency use authorization or if there is some other kind of approval. If not fully approved, then request the steps taken for its full approval.

8. Kindly inform if an unvaccinated person can refuse being Covid-19 tested in order to go to work, in order to travel within India or has been in contact with a COVID-19 positive person.

Concerned CPIO	Dr Nivedita Gupta
Supporting document *(only pdf upto 1 MB)*	

Print Close

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Select Language: English

Public Authorities Available

RTI Online

Version 3.0
An Initiative of Department of Personnel & Training, Government of India

Home Submit Request Submit First Appeal View Status View History HELP User Manual FAQ

Online RTI Status Form

Note:Fields marked with * are Mandatory

Enter Registration Number	ICMR/R/I/21/00689
Name	[redacted]
Date of filing	14/07/2021
Public Authority	Indian Council of Medical Research
Status	REQUEST DISPOSED OF
Date of action	11/08/2021
Reply :- Please find the relevant information as available with ICMR. Attached Pdf file.	
View Document	
CPIO Details :-	Dr Nivedita Gupta Phone: 011-26588980 ngupta[at]icmr[dot]org[dot]in
First Appellate Authority Details :-	Dr Samiran Panda1 Phone: 011-26588773 samiranpanda[dot]ha[at]icmr[dot]gov[dot]in
Nodal Officer Details :-	
Telephone Number	011-26588980
Email id	wahedwhand[dot]hq[at]icmr[dot]gov[dot]in

Print RTI Application Print Status Go Back

Point wise information is as follows:-

1. ICMR- MoH&FW are developing a COVID-19 Na onel vaccina on tracker. This informa on will be available soon in the upcoming vaccines tracker.

2.

Symptomatic status count of people who tested Covid-19 positive -Data		
	Count of symptoma c people who tested posi ve	Count of asymptoma c people who tested posi ve
RT-PCR	8047959	232861528
Rapid An gen	4255956	206382651
Other Tests	454275	11361871

4. The SRF (Specimen Referral Form) is itself a protocol with respect to tes ng data collec on, test result data storage and data segrega on for di erent parameters involved. The SRF form is mandatory for the collec on centres or laboratories doing COVID-19 diagnosis to enter details of the samples. The format of SRF form can be found at
h ps://www.icmr.gov.in/pdf/covid/labs/Revised_SRF_Form_26052021_1.pdf

5. This informa on is not available with ICMR.

6. Repeated tes ng at very frequent intervals can lead to injury in nasal cavity and throat.

7. This does not pertain to ICMR. The informa on can be seeked from DCGI.

8. This informa on is not available with ICMR.

EXHIBIT "BBB"

Source: WorldOmeter

Link: https://www.worldometers.info/coronavirus/country/india/

Updated on: January 06, 2022

WORLD / COUNTRIES / INDIA

Last updated: January 07, 2022, 09:25 GMT

India

Coronavirus Cases:

35,226,386

Deaths:

483,178

Recovered:

34,371,845

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CLOSED CASES

34,855,023

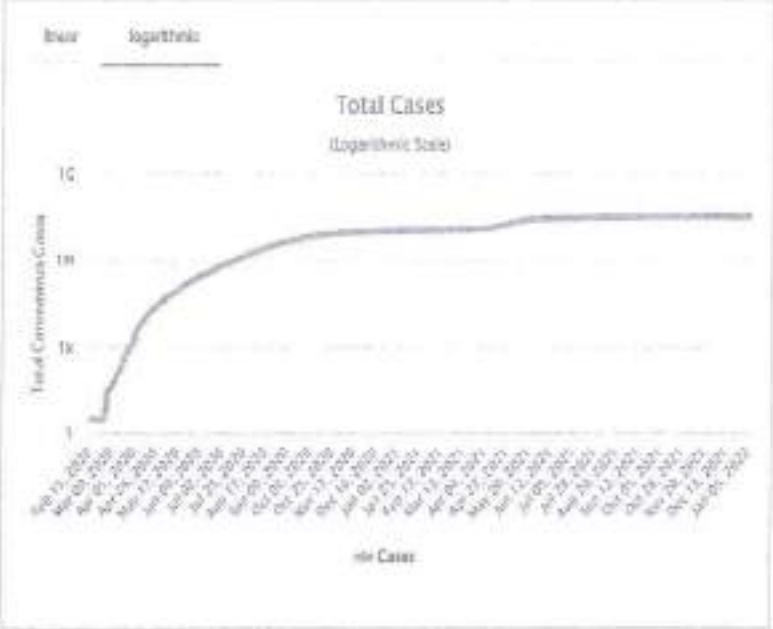
Cases which had an outcome:

34,371,845 (99%) Recovered / Discharged

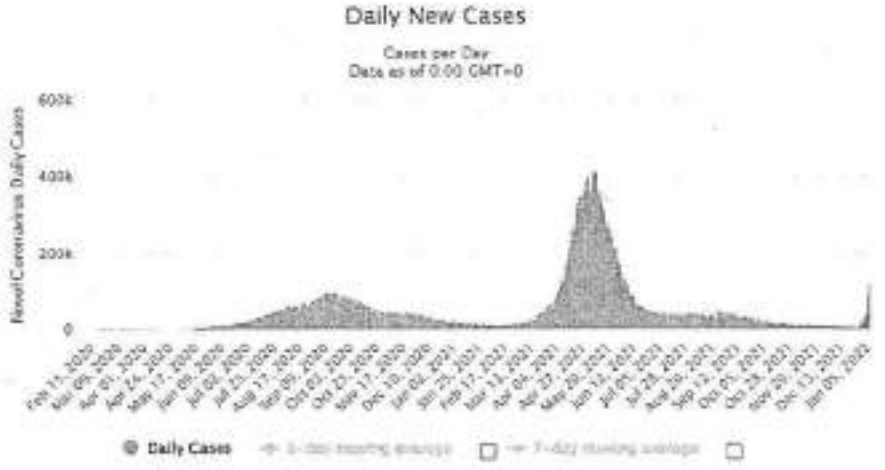
483,178 (1%) Deaths

Show Graph

Total Coronavirus Cases in India



Daily New Cases in India

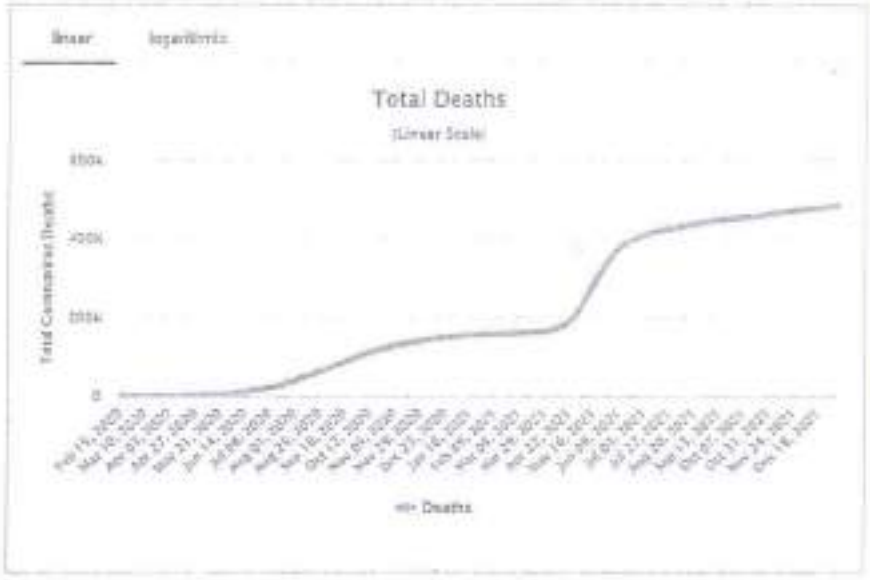


See also: Daily Deaths Graph

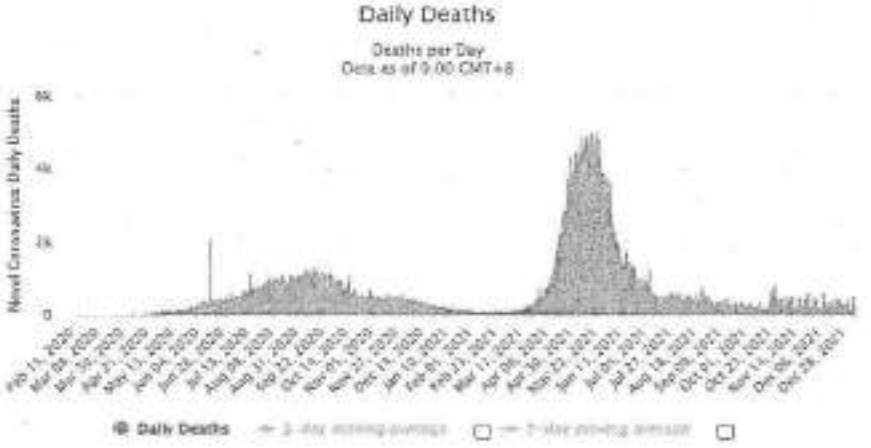
See also: Daily Deaths Graph

Active Cases in India

Total Coronavirus Deaths in India

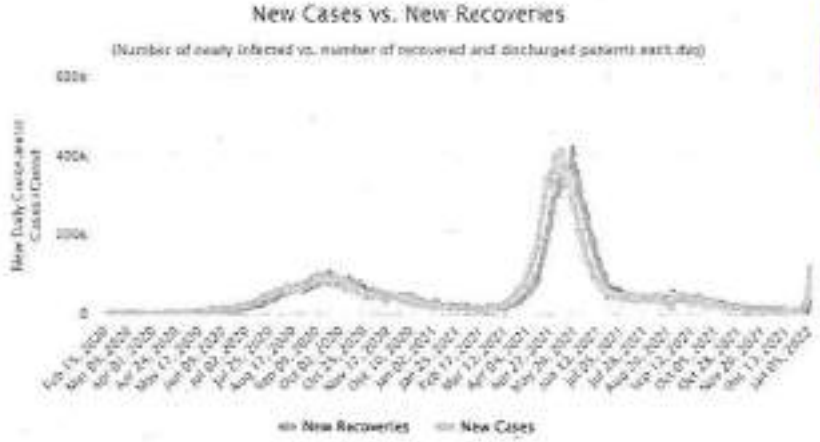


Daily New Deaths in India



See also: Daily Cases Graph

Newly Infected vs. Newly Recovered in India



Outcome of Cases (Recovery or Death) in India

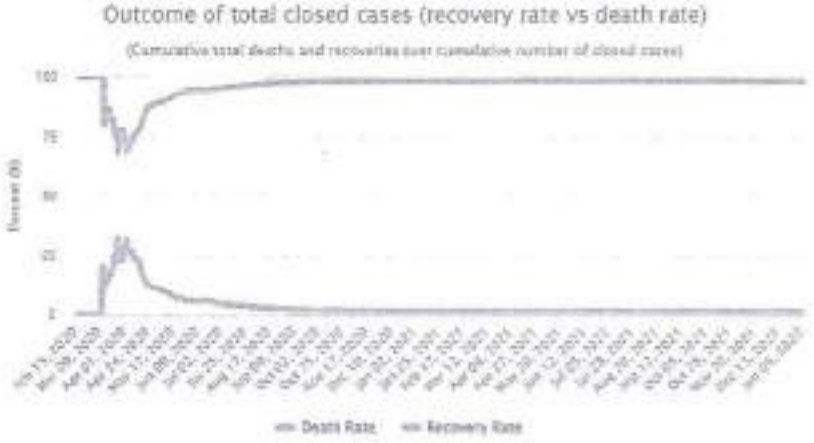


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EXHIBIT CCC

One year of Covid-19: How India fought the virus

Source: Hindustan Times

Link: https://www.hindustantimes.com/india-news/one-year-of-covid-19-how-india-fought-the-virus-101614553310402.html

Written By: Binayak Dasgupta, Anonna Dutt

Published on: Mar 01, 2021

In March of 2020, when the world first caught a glimpse of the devastation that the coronavirus would go on to unleash, experts feared India would suffer heavily as well.

There are several ways to look at India's Covid-19 year. On the face of it, it's the story of a country battered by the world's second-highest number of infections. The country's 1.3 billion-plus people spent more fatigued weeks in a hard lockdown than most others and, in turn, faced one of the sharpest economic contractions seen anywhere in the world.

At least 11 million have contracted the virus, and close to 160,000 died due to it.

But, it is also a story of intrigue and, often, surprise. It has one of the lowest deaths rates seen in any large country and, leaving out trends that have become visible only in the latest fortnight, gone through only a single, gigantic wave of infections that appeared to be well-controlled by the country's precarious health infrastructure.

In March of 2020, when the world first caught a glimpse of the devastation that the coronavirus would go on to unleash, experts feared India would suffer heavily as well. Tried-and-tested epidemiological models predicted 97,000-1.3 million infections by mid-May, to perhaps even 300 million infections by the end of the year.

But, largely, the worst of the fears did not pan out. During the highest of the infection peaks, hospitals appeared to be on top of the situation – unlike in New York or Milan. The season of festivals, which touches almost every region, religion and custom in the pre-winter months of October-November, led to spikes, but not of the nature seen in other parts of the world. "Reported cases mean very little in India. The estimated number of true infections from models as well as sero surveys is huge and point to about 20-25% of the adult population being infected. That is nearly 170-200 million infections," said Bhramar Mukherjee, the chair of biostatistics at the University of Michigan School of Public Health.

Even if one takes this into account and looks at fatalities to calculate what is known as the infection fatality rate (IFR), she added, India's story seems different. "Even if you believe not all deaths were reported, this points to a low IFR compared to the rest of the world," she added.

India's IFR is 0.1% and this number may be a better measure of an epidemic since deaths are more likely to be recorded than infections, particularly in the case of a disease like Covid-19, which infects many without symptoms. But, Mukherjee added, "41% of India's population is in the age group 0-18, in which the infection rate is really low -- so the age-adjusted mortality does not look as low as the overall one."

In the week beginning March 2, there were nearly 50 cases. The infected were not only those who had history of recent international travel, but, for the first time, were also people who picked it up locally. The month of March is crucial – not only because this is when the nationwide lockdown would come into force later – but because the most critical first step of a scientific response to an epidemic was missing: Testing.

It would not be until March 20 that authorities would allow a person without a travel history to take a Covid-19 test. But people would still be eligible only if they had symptoms, travel history or high-risk contacts – a condition that was relaxed in September.

The most effective intervention, then, was a crude one: the hard, 68-day lockdown that began on March 25. It was also unique since India was the only country that shut down while cases were still low, and actually surmounted the peak while reopening.

According to Mukherjee, the lockdown did not help stop transmission in the end but it "really helped us scale up health care". "Testing became accessible and affordable, and isolation beds, ICUs, Covid care centres were set up. That helped reduce mortality. After opening up when cases surged and hospital beds were filling up, we were much more prepared," she said, while adding that the hard lockdown also helped send a message of seriousness from authorities, "which was not the case all over the world."

SK Sarin, the director of Institute of Liver and Biliary Sciences, headed Delhi government's first committee on Covid-19 control and management. Having been on the frontlines in the fight in the Capital, he identified several things that helped eventually, but the first was to plan for infection surges: "Even in the

initial days, we planned for situations in which 1,000 or even 5,000 cases were reported in a day."

Then, he added, protocols were defined to manage the various ways in which the disease manifested. "We also worked on scaling up testing. ILBS had one of the biggest labs at that time with a capacity for conducting 1,000 RT-PCR tests a day. And Delhi was the first to set up the flu clinics in hospitals to check patients coming in for symptoms of Covid-19."

But this was the story of Delhi, the country's capital. An analysis of infection and fatality trends suggests India's rural regions reported similar fatality trends as more urbanised regions, where health infrastructure is arguably better.

Mukherjee, who was among the dozen-odd researchers from three American universities who collaborated for the Crisis of Virus in India (COV-IND) study group, identifies the trends in rural India as particularly surprising. "I spent quite a bit of time in my parents' farmhouse. In the villages they do not wear masks, and yet there were no severe cases that they could report. Our fear seemed like an urban myth or fiction to them. What happened there?"

The second question of intrigue is the dramatic drop in infections in India. The Indian Council of Medical Research (ICMR) in February released the latest nationwide sero survey that showed roughly four in five Indians was still vulnerable to an infection. Mukherjee described the end of the first wave as something that struck her, in addition to big festivals like Diwali and Dussehra not triggering major outbreaks. She notes that, as reflected in the sero surveys, which found higher exposure in cities compared to rural areas, the receding of the pandemic may perhaps be due to separate characteristics in these areas.

"I think the antibodies in metros/urban areas are still protecting us from massive surges, but we cannot wait too long. Rural India is a mystery to me. I believe it

is a confluence of cross-immunity, genetics, lifestyle, lower obesity and heart disease prevalence and also a more outdoor lifestyle with natural air ventilation that helped villages," she said, while adding: "I really want to understand why despite tourism and travel by migrant workers, the number of infections remained low in rural areas."

Lalit Kant, the former head of epidemiology and infectious diseases at the Indian Council of Medical Research saw much progress in how India's government and citizens have learned to manage the pandemic. "We have come a long way. This time in 2020, we did not know much about the virus or the treatment. There was an acute shortage of masks, PPE kits, sanitisers, and, when cases started rising, hospital beds. Many permutations and combinations of medicines were tried out and it took doctors some time to standardise the treatment, and this was happening across the globe."

But now, he added, "testing has since improved, including the quality of tests available and people's awareness about the disease and how it spreads has increased, even if precautions are not followed all the time."

As the country turns the page on its first year of Covid-19, it will begin to open up the vaccination drive to its general public.

Experts see this as the most crucial objective now. "It has been a year of challenge, but it has also showed the power of science. But, the virus is likely to persist. While there wouldn't be big outbreaks like we saw last year, summers in India is when there is indoor time (staying indoors increases the risk of transmission) and if we do not take precautions there could be an increase in the number of cases, said Anurag Agarwal, director, CSIR-Institute of Genomics and Integrative Biology. "Also, by the time the peak summers come, immunity

will decline from last years' outbreak leaving more people susceptible. Over the next six months we just have to vaccinate, vaccinate, vaccinate."

EXHIBIT "DDD"

Estimating the wave 1 and wave 2 infection fatality rates from SARS-CoV-2 in India.

Source:- BMC Research Notes

Link:- Estimating the wave 1 and wave 2 infection fatality rates from SARS-CoV-2 in India | BMC Research Notes | Full Text (biomedcentral.com)

Published on - 08 July 2021

Abstract

Objective

There has been much discussion and debate around the underreporting of COVID-19 infections and deaths in India. In this short report we first estimate the underreporting factor for infections from publicly available data released by the Indian Council of Medical Research on reported number of cases and national seroprevalence surveys. We then use a compartmental epidemiologic model to estimate the undetected number of infections and deaths, yielding estimates of the corresponding underreporting factors. We compare the serosurvey based ad hoc estimate of the infection fatality rate (IFR) with the model-based estimate. Since the first and second waves in India are intrinsically different in nature, we carry out this exercise in two periods: the first wave (April 1, 2020–January 31, 2021) and part of the second wave (February 1, 2021–May 15, 2021). The latest national seroprevalence estimate is from January 2021, and thus only relevant to our wave 1 calculations.

Results

Both wave 1 and wave 2 estimates qualitatively show that there is a large degree of "covert infections" in India, with model-based estimated underreporting factor for infections as 11.11 (95% credible interval (CrI) 10.71–11.47) and for

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deaths as 3.56 (95% CrI 3.48–3.64) for wave 1. For wave 2, underreporting factor for infections escalate to 26.77 (95% CrI 24.26–28.81) and to 5.77 (95% CrI 5.34–6.15) for deaths. If we rely on only reported deaths, the IFR estimate is 0.13% for wave 1 and 0.03% for part of wave 2. Taking underreporting of deaths into account, the IFR estimate is 0.46% for wave 1 and 0.18% for wave 2 (till May 15). Combining waves 1 and 2, as of May 15, while India reported a total of nearly 25 million cases and 270 thousand deaths, the estimated number of infections and deaths stand at 491 million (36% of the population) and 1.21 million respectively, yielding an estimated (combined) infection fatality rate of 0.25%. There is considerable variation in these estimates across Indian states. Up to date seroprevalence studies and mortality data are needed to validate these model-based estimates.

Introduction

Main text

In late August 2020, India was predicted to surpass the United States in terms of reported case counts from SARS-CoV-2 infections. To the surprise of many modelers the curve turned corner in late September with the highest number (97,894) of daily new cases reported on 16 September 2020 [1]. After a steady decline for nearly five months, the curve started rising again, growing into an astronomic second wave. The highest number (414,280) of daily new cases in wave 2 was reported on May 6, 2021. As of May 15, 2021, India has reported 24.7 million cases, the second highest in the world, and nearly 270 thousand deaths, the third highest in the world. In this brief report, we reconcile estimates of the infection fatality rate (IFR) inferred from seroprevalence studies with epidemiologic model-based estimates that account for underreporting of infections and deaths in India for wave 1. We then proceed to compute, compare and combine wave 1 with wave 2 IFR estimates.

Methods

Synthesizing evidence from seroprevalence studies

We review available seroprevalence results that vary across states and specifically across rural versus urban areas. Whereas in many major metros and slum areas the seroprevalences were reported to be more than 50%, in rural areas there is a wide variation (Table 1). The latest national serosurvey (from 17 December 2020 to 8 January 2021) reports 21.4% of all Indians above age 18 have antibodies present that indicate past SARS-CoV-2 infection [2]. Since approximately 59% [3] of India's 1.36 billion citizens are above age 18 and 10.45 million infections were reported as of 8 January 2021, this points to approximately 172.47 million infections, with an implied underreporting factor of 16.5 (172.47/10.45). In other words, only 6% of India's COVID-19 infections are reported, while 94% remained undetected or unreported. We use this estimated number of infections to calculate the IFR. Regional studies based on crematorium data and counting obituaries in India have suggested an underreporting factor in the range of 2 to 5 for COVID-deaths; this is at best ad hoc and anecdotal in nature and no rigorous quantification of missing death numbers is currently available [4].

Table 1 Summary of results from various serological surveys conducted in India during 2020–21

Full size table

Model-based estimates

Using a compartmental epidemiologic model (as explained in the *Supplementary Methods*) with a compartment for unascertained cases and deaths after accounting for the false negative rates of RT-PCR and rapid antigen

tests used in India [5] we estimate the national and state-level IFR in India by inferring underreporting factors for cases and deaths. We assume that the estimated total infections (deaths) are comprised of reported and unreported infections (deaths). The model divides the population into ten disjoint compartments: S (Susceptible), E (Exposed), T (Tested), U (Untested), P (Tested positive), F (Tested False Negative), RR (Reported Recovered), RU (Unreported Recovered), DR (Reported Deaths) and DU (Unreported Deaths), as described in Additional file 1: Figure S1. A set of nine differential equations govern the transmission dynamics, which are approximated by means of discrete recurrence relations. For any compartment XX, the instantaneous rate of change at time tt (given by dXdtdXdt) is approximated by the difference of counts in that specific compartment on the (t+1)(t+1) th day and the (t)(t) th day, i.e., say X(t+1)−X(t)X(t+1)−X(t). Parameters are estimated using Bayesian techniques by generating samples from the posterior distribution using a Metropolis–Hastings algorithm with Gaussian proposal density, with 95% credible intervals (CrI) to quantify uncertainty of the estimates. Additional file 1: Table T4 presents an overview of the parameter descriptions and settings for this model.

Comparing and combining waves 1 and 2

Due to the stress on the healthcare and reporting infrastructure, the fatality and underreporting processes were very different across the two waves. Thus, we consider two separate phases of the pandemic, with wave 1 from April 1, 2020–January 31, 2021 and wave 2 starting on February 1, 2021. This definition is artificial and is guided by the fact that the national effective reproduction number (R_{eff}) crossed unity for the first time in 2021 on February 14 and we allow a two-week incubation period before that date. Using daily time series of case, death and recovery counts we compare fatality rates and underreporting factors associated with the two time periods using the compartmental models.

Further, using observed data from the two waves and the model-based underreporting factor estimates, we compute cumulative case and death counts for the total duration of waves 1 and 2. We multiply the wave-specific cumulative counts with relevant underreporting factors and sum over both waves to get combined counts of cases and deaths. The estimated numbers of cumulative deaths and infections provide us with a combined IFR estimate for India as of May 15.

Results

IFR estimates for wave 1 using seroprevalence surveys

The observed case fatality rate (CFR) in India is low. With 154,428 deaths and 10.76 million cases reported as of January 31, 2021 the estimated CFR for wave 1 is 1.435% (95% confidence interval 1.428–1.442%) [1]. The estimated number of infections from the January seroprevalence survey imply an approximate infection fatality rate of 0.09% (i.e. 154,428/172.47 M). The anecdotal underreporting factor for deaths (in the range of 2–5) implies an ad hoc estimate of IFR in the range of 0.19–0.45%.

Estimates from epidemiological models

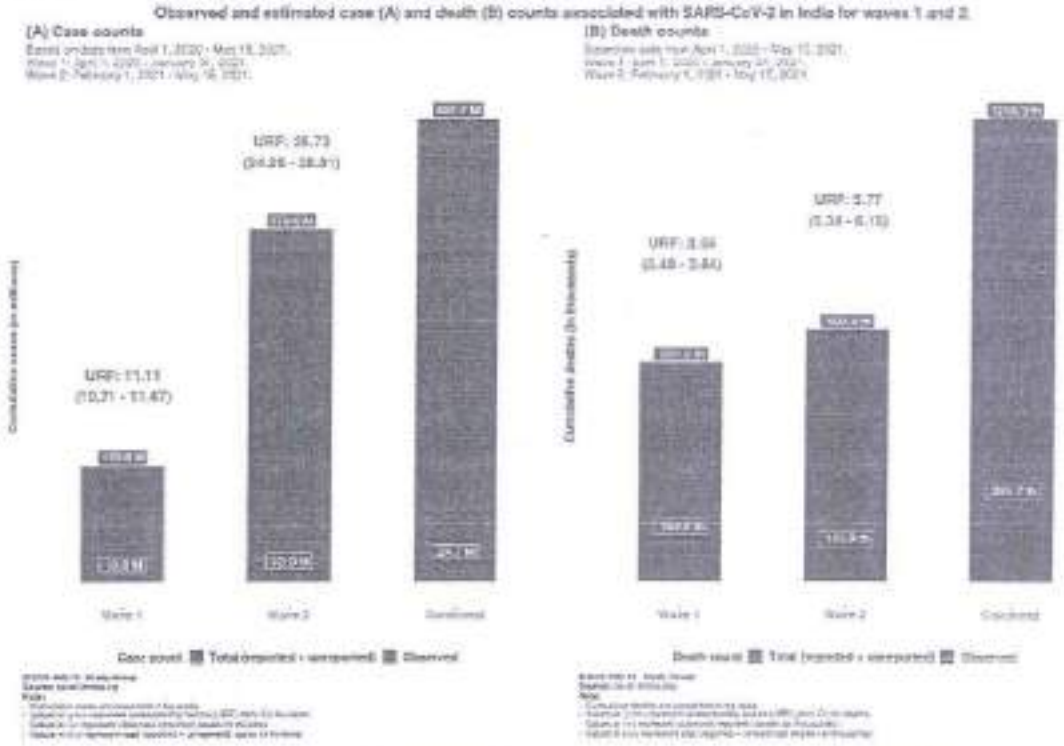
For wave 1 our estimate for the national IFR_1 (observed cumulative deaths/estimated cumulative total infections) is 0.129% (95% CrI 0.125–0.134%) and IFR_2 (estimated total cumulative deaths/estimated total cumulative infections) is 0.461% (95% CrI 0.455–0.468%) with an underreporting factor for cases estimated at 11.11 (95% CrI 10.71–11.47) and for deaths at 3.56 (95% CrI 3.48–3.64). These model-based estimates in wave 1 are largely consistent with the estimates from the latest and third nationwide seroprevalence study.

In wave 2, using the same model we see a stark contrast with wave 1, with case and death underreporting factor estimates escalate to 26.73 (95% CrI 24.26–

28.81) and 5.77 (95% CrI 5.34–6.15) respectively, leading to IFR_1 estimate of 0.032% (95% CrI 0.029–0.035%) and IFR_2 estimate of 0.183% (95% CrI 0.18–0.186%). This pattern is consistent with wave 2 CFR being estimated at 0.845% (95% CrI 0.840–0.849%), 59% of wave 1 estimate.

Figure 1 shows underreporting factors and estimated infections and deaths in waves 1 and 2 for India while Fig. 2 highlights state-level variations in IFR_1, IFR_2, CFR for waves 1 and 2 for 20 states in India with large case/death counts.

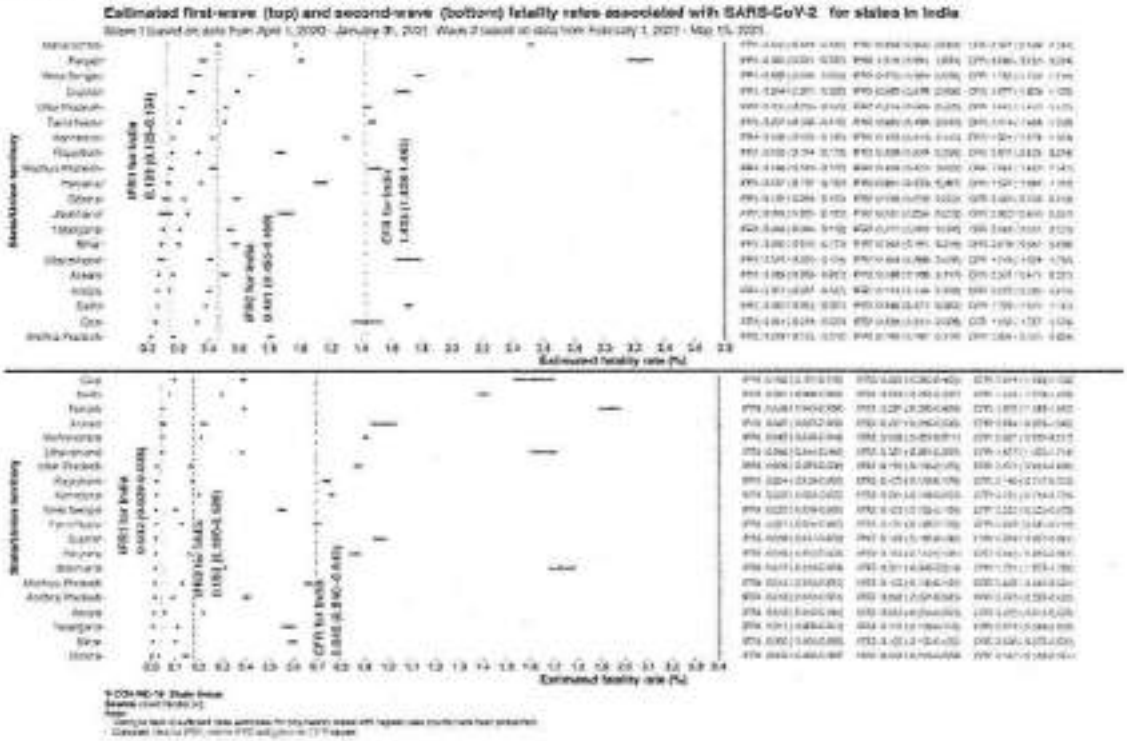
Fig. 1



Comparison of observed and estimated case and death counts and associated underreporting factors from waves 1, 2 and both waves combined

Full size image

Fig. 2



Forest plot of wave 1 and wave 2 infection fatality rates (IFR) and case fatality rates (CFR) associated with SARS-CoV-2 in various states in India. IFR1 is based on reported deaths whereas IFR2 estimates and includes the unreported deaths

Full size image

Combining waves 1 and 2

The composite CFR as of May 15 stands at 1.1%. The estimate for total (reported + unreported) cumulative case count for waves 1 and 2 combined is 491.73 (95% CrI 453.03–524.56) million, while the estimated number of total (reported + unreported) deaths is 1216.35 (95% CrI 1154.21–1272.70) thousand. This leads to a combined IFR_1 estimate of 0.06% and IFR_2 estimate of 0.24%.

Detailed numerical estimates of underreporting factors across states for waves 1 and 2 are presented in Additional file 1: Tables T1, T2 and T3 and Additional file 1: Figures S2 and S3.

Discussion

Despite accounting for underreported deaths, the large number of asymptomatic/undetected infections (more than 90% by any calculation) indicate a lower IFR in India in comparison with other Western countries. A meta-analysis across the world places the pooled mean of IFRs at 0.68% (95% CI: 0.53–0.82%) [6], while another meta-analysis places the median at 0.27% [7] (with a range of 0–1.63%). Seroprevalence surveys and epidemiologic models qualitatively agree on the estimated IFR for India for wave 1. Up to date serosurvey and excess death/mortality data are needed to validate wave 2 and combined estimates. The estimated number of total infections as of May 15 suggests roughly 36% of Indians have an active or past infection, a number that will need to be verified with synchronous sero-surveys.

The current reduction in fatality rates in wave 2 that we notice could be primarily due to two reasons, one is that we do not have the same length of follow-up period and complete data on the decay phase of wave 2 curve. The second could be the different age composition of the infected populations in the two waves; it has been reported that the younger population got infected in larger numbers in wave 2 and they have lower risk of COVID-19 mortality. A fraction of the older population (aged 65 + years) also got vaccinated during wave 2. However, this hypothesis about reduced fatality rates in wave 2 cannot be verified without more granular, age-sex stratified nationwide time-series data on case and death counts, which is currently unavailable.

Limitations

We do not have a rigorous way to validate the extent of underreporting of deaths. An excess death calculation based on historical mortality data is infeasible at this point due to absence of all-cause-mortality data in the last three years from India. India has a very young population with only 6.4% in age group 65 + (compared to the US where this proportion is 16.5%) so a comparison of overall IFR between India and say the US is not fair, and only age-specific IFRs should be calculated and compared when more data become available. We do recognize that wave 2 information is appreciably incomplete, and the estimates will change as we have more complete information on deaths. For example, while our wave 2 analysis period ended on May 15, the highest daily number of deaths (4529 daily new deaths) were reported shortly after on May 18. Thus, our analysis presents an updated but incomplete picture of wave 2.

Availability of data and materials

All data are publicly available at covid19india.org. We used reported daily case, death and recovery counts for India and its states and union territories from April 1, 2020 to May 15, 2021. The statistical package SEIR-Fansy developed by the authors is available at covind19.org.

Abbreviations

CFR:

Case fatality rate

CI:

Confidence interval

CrI:

Credible interval

IFR:

Infection fatality rate

RT-PCR:

Real time reverse transcript polymerase chain reaction

SEIR:

Susceptible-exposed-infected-recovered

URF:

Underreporting Factor

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Author information

Affiliations

1. **Department of Biostatistics, University of Michigan, Ann Arbor, MI, 48109, USA**
   Soumik Purkayastha, Daniel Barker, Michael Kleinsasser & Bhramar Mukherjee
2. **Indian Statistical Institute, Kolkata, West Bengal, 700108, India**
   Ritoban Kundu & Ritwik Bhaduri
3. **Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 21205, USA**
   Debashree Ray
4. **Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 21205, USA**
   Debashree Ray
5. **Center for Precision Health Data Science, University of Michigan, Ann Arbor, MI, 48109, USA**
   Bhramar Mukherjee
6. **Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, MI, 48109, USA**
   Bhramar Mukherjee

Contributions

SP created the initial draft, conducted the literature review and collaborated on the analysis. RK and RB created the R package SEIR-fansy and implemented the epidemiologic models. DB and MK collaborated on analysing data from the second wave. DR helped create and modify the final draft and address issues raised by the review panel. BM conceived the project, planned the analysis and wrote the draft of the paper. All authors read, reviewed, edited and approved the manuscript for submission.

Corresponding author

Correspondence to Bhramar Mukherjee.

Ethics declarations

Ethics approval and consent to participate

Not applicable. The analysis is based on publicly available completely de-identified aggregate counts. The study is exempt from IRB review as no patient participation or contact is involved.

Consent for publication

Not applicable.

Competing interests

There are no conflicts of interest perceived or declared by any of the authors.

Additional information

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Supplementary Information

Additional file 1 of Estimating the wave 1 and wave 2 infection fatality rates from SARS-CoV-2 in India

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Supplementary material: Estimating the
Wave 1 and Wave
2
infection fatality rate from SARS-CoV-2 in India

Soumik Purkayastha, M.Stat.
1
, Ritoban Kundu, B.Stat.
2
, Ritwik Bhaduri, B.Stat.
2
,
Daniel Barker B.S.
1
, Michael Kleinsasser, M.S.
1
, Debashree Ray, Ph.D
3,4
and Bhramar
Mukherjee, Ph.D
1,5,6 (*)
1
Department of Biostatistics, University of Michigan, Ann Arbor, MI 48109, USA.
2

Indian Statistical Institute, Kolkata, West Bengal 700108, INDIA.

3

Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health,

Baltimore, MD 21205, USA.

4

Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA.

5

Center for Precision Health Data Science, University of Michigan, Ann Arbor, MI

48109, USA.

6

Department of Epidemiology, University of Michigan School of Public Health, Ann

Arbor, MI 48109, USA.

(*)

Corresponding author (Email:

bhramar@umich.edu

).

Soumik Purkayastha (

soumikp@umich.edu

)

Ritoban Kundu (

ritoban.kundu@gmail.com

)

Ritwik Bhaduri (

ritwik.bhaduri@gmail.com

)

Daniel Barker (
danbarke@umich.edu
)
Michael Kleinsasser (mkleinsa@umich.edu)
Debashree Ray (dray@jhu.edu)

SEIR-fansy model

Introduction

Here we are using the SEIR-fansy model
1
and software package
2
which uses
a compartmental model accounting for false negative rates and preferential diagnostic testing for SARS-CoV-2 infections. The SEIR-fansy model can be represented by the compartmental model in Figure S1.

1.
Bhaduri R, Kundu R, Purkayastha S, Kleinsasser M, Beesley LJ, Mukherjee B.
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SEIRfansy: Extended Susceptible-Exposed-Infected-Recovery Model. R package version 1.1.0. https://CRAN.R-project.org/package=SEIRfansy

Figure S1: Schematic diagram for the SEIR-fansy model with imperfect testing and misclassification.

Mathematical framework

The following differential equations summarize the transmission dynamics being modeled.

$$\frac{\partial S}{\partial t} = -\beta S(t)N(\alpha P P(t) + \alpha$$

$$U$$
$$U$$
$$($$
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$$)$$
$$+$$
$$F$$
$$($$
$$t$$
$$)$$
$$)$$
$$+$$
$$\lambda N$$
$$-$$
$$\mu S$$
$$($$
$$t$$
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$$\partial$$
$$E$$
$$\partial$$
$$t$$
$$=$$
$$\beta$$
$$S$$
$$($$
$$t$$
$$)$$
$$N$$

$$(\alpha PPP(t)+\alpha UU(t)+F(t))-E(t)De^{-\mu E}$$

(
t
)

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Additional file 1

Additional file 1: Figure S1. Schematic diagram for the SEIR-fansy model with imperfect testing and misclassification. Figure S2. Estimated first wave underreporting factors for cases and deaths associated with SARS-CoV-2 for states in India. Figure S3. Estimated first wave underreporting factors for cases and deaths associated with SARS-CoV-2 for states in India. Table T1. Summary of the different metrics for the states and the nation for wave 1, on 31st January, 2021. Table T2. Summary of the different metrics for the states and the nation for wave 2, on 15th May, 2021. Table T3. Summary of the different metrics for the states and the nation for waves 1 and 2 combined, on 15th May, 2021. Table T4. Parameter values and descriptions for the SEIRfansy model.

Additional file 1

: **Figure S1**. Schematic diagram for the SEIR-fansy model with imperfect testing and misclassification. **Figure S2.** Estimated first wave underreporting factors for cases and deaths associated with SARS-CoV-2 for states in India. **Figure S3.** Estimated first wave underreporting factors for cases and deaths associated with SARS-CoV-2 for states in India. **Table T1.** Summary of the different metrics for the states and the nation for wave 1, on 31st January, 2021. **Table T2.** Summary of the different metrics for the states and the nation for wave 2, on 15th May, 2021. **Table T3.** Summary of the different metrics for

the states and the nation for waves 1 and 2 combined, on 15th May, 2021. **Table T4.** Parameter values and descriptions for the SEIRfansy model.

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Infection fatality rate of COVID-19 inferred from seroprevalence data

John P A Ioannidis[a]

Objective To estimate the infection fatality rate of coronavirus disease 2019 (COVID-19) from seroprevalence data.

Methods I searched PubMed and preprint servers for COVID-19 seroprevalence studies with a sample size ≥ 500 as of 9 September 2020. I also retrieved additional results of national studies from preliminary press releases and reports. I assessed the studies for design features and seroprevalence estimates. I estimated the infection fatality rate for each study by dividing the cumulative number of COVID-19 deaths by the number of people estimated to be infected in each region. I corrected for the number of immunoglobin (Ig) types tested (IgG, IgM, IgA).

Findings I included 61 studies (74 estimates) and eight preliminary national estimates. Seroprevalence estimates ranged from 0.02% to 53.40%. Infection fatality rates ranged from 0.00% to 1.63%, corrected values from 0.00% to 1.54%. Across 51 locations, the median COVID-19 infection fatality rate was 0.27% (corrected 0.23%); the rate was 0.09% in locations with COVID-19 population mortality rates less than the global average (< 118 deaths/million), 0.20% in locations with 118–500 COVID-19 deaths/million people and 0.57% in locations with > 500 COVID-19 deaths/million people. In people younger than 70 years, infection fatality rates ranged from 0.00% to 0.31% with crude and corrected medians of 0.05%.

Conclusion The infection fatality rate of COVID-19 can vary substantially across different locations and this may reflect differences in population age structure and case-mix of infected and deceased patients and other factors. The inferred infection fatality rates tended to be much lower than estimates made earlier in the pandemic.

Abstracts in عربي, 中文, Français, Русский and Español at the end of each article.

Introduction

The infection fatality rate, the probability of dying for a person who is infected, is one of the most important features of the coronavirus disease 2019 (COVID-19) pandemic. The expected total mortality burden of COVID-19 is directly related to the infection fatality rate. Moreover, justification for various non-pharmacological public health interventions depends on the infection fatality rate. Some stringent interventions that potentially also result in more noticeable collateral harms[1] may be considered appropriate, if the infection fatality rate is high. Conversely, the same measures may fall short of acceptable risk–benefit thresholds, if the infection fatality rate is low.

Early data from China suggested a 3.4% case fatality rate[2] and that asymptomatic infections were uncommon,[3] thus the case fatality rate and infection fatality rate would be about the same. Mathematical models have suggested that 40–81% of the world population could be infected,[4,5] and have lowered the infection fatality rate to 1.0% or 0.9%.[4,6] Since March 2020, many studies have estimated the spread of the virus causing COVID-19 – severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) – in various locations by evaluating seroprevalence. I used the prevalence data from these studies to infer estimates of the COVID-19 infection fatality rate.

Methods

Seroprevalence studies

The input data for calculations of infection fatality rate were studies on the seroprevalence of COVID-19 done in the general population, or in samples that might approximately represent the general population (e.g. with proper reweighting), that had been published in peer-reviewed journals or as preprints (irrespective of language) as of 9 September 2020. I considered only studies with at least 500 assessed samples because smaller data sets would result in large uncertainty for any calculations based on these data. I included studies that made seroprevalence assessments at different time intervals if at least one time interval assessment had a sample size of at least 500 participants. If there were different eligible time intervals, I selected the one with the highest seroprevalence, since seroprevalence may decrease over time as antibody titres decrease. I excluded studies with data collected for more than a month that could not be broken into at least one eligible time interval less than one month duration because it would not be possible to estimate a point seroprevalence reliably. Studies were eligible regardless of the exact age range of participants included, but I excluded studies with only children.

I also examined results from national studies from preliminary press releases and reports whenever a country had no other data presented in published papers or preprints. This inclusion allowed these countries to be represented, but information was less complete than information in published papers or preprints and thus requires caution.

I included studies on blood donors, although they may underestimate seroprevalence and overestimate infection fatality rate because of the healthy volunteer effect. I excluded studies on health-care workers, since this group is at a potentially high exposure risk, which may result in seroprevalence estimates much higher than the general population and thus an improbably low infection fatality rate. Similarly, I also excluded studies on communities (e.g. shelters or religious or other shared-living communities). Studies were eligible regardless of whether they aimed to evaluate seroprevalence in large or small regions, provided that the population of reference in the region was at least 5000 people.

I searched PubMed® (LitCOVID), and medRxiv, bioRxiv and Research Square using the terms "seroprevalence" OR "antibodies" with continuous updates. I made the first search in early May and did monthly updates, with the last update

[a] Meta-Research Innovation Center at Stanford (METRICS), Stanford University, 1265 Welch Road, Stanford, California 94305, United States of America.
Correspondence to John P A Ioannidis (email: jioannid@stanford.edu).
(Submitted: 13 May 2020 – Revised version received: 13 September 2020 – Accepted: 15 September 2020 – Published online: 14 October 2020)

on 9 September 2020. I contacted field experts to retrieve any important studies that may have been missed.

From each study, I extracted information on location, recruitment and sampling strategy, dates of sample collection, sample size, types of antibody measured (immunoglobulin G (IgG), IgM and IgA), the estimated crude seroprevalence (positive samples divided by all samples tested), adjusted seroprevalence and the factors that the authors considered for adjustment.

Inferred infection fatality rate

If a study did not cover an entire country, I collected information on the population of the relevant location from the paper or recent census data so as to approximate as much as possible the relevant catchment area (e.g. region(s) or county(ies)). Some studies targeted specific age groups (e.g. excluding elderly people and/or excluding children) and some estimated numbers of people infected in the population based on specific age groups. For consistency, I used the entire population (all ages) and, separately, the population 0–70 years to estimate numbers of infected people. I assumed that the seroprevalence would be similar in different age groups, but I also recorded any significant differences in seroprevalence across age strata so as to examine the validity of this assumption.

I calculated the number of infected people by multiplying the relevant population size and the adjusted estimate of seroprevalence. If a study did not give an adjusted seroprevalence estimate, I used the unadjusted seroprevalence instead. When seroprevalence estimates with different adjustments were available, I selected the analysis with largest adjustment. The factors adjusted for included COVID-19 test performance, sampling design, and other factors such as age, sex, clustering effects or socioeconomic factors. I did not adjust for specificity in test performance when positive antibody results were already validated by a different method.

For the number of COVID-19 deaths, I chose the number of deaths accumulated until the date 1 week after the midpoint of the study period (or the date closest to this that had available data) – unless the authors of the study had strong arguments to choose some other time point or approach. The 1-week lag accounts for different delays in developing antibodies versus dying from infection. The number of deaths is an approximation because it is not known when exactly each patient who died was infected. The 1-week cut-off after the study midpoint may underestimate deaths in places where patients are in hospital for a long time before death, and may overestimate deaths in places where patients die soon because of poor or even inappropriate care. Whether or not the health system became overloaded may also affect the number of deaths. Moreover, because of imperfect diagnostic documentation, COVID-19 deaths may have been both overcounted and undercounted in different locations and at different time points.

I calculated the inferred infection fatality rate by dividing the number of deaths by the number of infected people for the entire population, and separately for people younger than 70 years. I took the proportion of COVID-19 deaths that occurred in people younger than 70 years from situational reports for the respective locations that I retrieved at the time I identified the seroprevalence studies. I also calculated a corrected infection fatality rate to try and account for the fact that only one or two types of antibodies (among IgG, IgM, IgA) might have been used. I corrected seroprevalence upwards (and inferred infection fatality rate downwards) by one tenth of its value if a study did not measure IgM and similarly if IgA was not measured. This correction is reasonable based on some early evidence,[7] although there is uncertainty about the exact correction factor.

Data synthesis

The estimates of the infection fatality rate across all locations showed great heterogeneity with I^2 exceeding 99.9%; thus, a meta-analysis would be inappropriate to report across all locations. Quantitative synthesis with meta-analysis across all locations would also be misleading since locations with high COVID-19 seroprevalence would tend to carry more weight than locations with low seroprevalence. Furthermore, locations with more studies (typically those that have attracted more attention because of high death tolls and thus high infection fatality rates) would be represented multiple times in the calculations. In addition, poorly conducted studies with fewer adjustments would get more weight because of spuriously narrower confidence intervals than more rigorous studies with more careful adjustments which allow for more uncertainty. Finally, with a highly skewed distribution of the infection fatality rate and with large between-study heterogeneity, typical random effects models would produce an incorrectly high summary infection fatality rate that approximates the mean of the study-specific estimates (also strongly influenced by high-mortality locations where more studies have been done); for such a skewed distribution, the median is more appropriate.

Therefore, in a first step, I grouped estimates of the infection fatality rate from studies in the same country (or for the United States of America, the same state) together and calculated a single infection fatality rate for that location, weighting the study-specific infection fatality rates by the sample size of each study. This approach avoided inappropriately giving more weight to studies with higher seroprevalence estimates and those with seemingly narrower confidence intervals because of poor or no adjustments, while still giving more weight to larger studies. Then, I used the single summary estimate for each location to calculate the median of the distribution of location-specific infection fatality rate estimates. Finally, I explored whether the location-specific infection fatality rates were associated with the COVID-19 mortality rate in the population (COVID-19 deaths per million people) in each location as of 12 September 2020; this analysis allowed me to assess whether estimates of the infection fatality rate tended to be higher in locations with a higher burden of death from COVID-19.

Results

Seroprevalence studies

I retrieved 61 studies with 74 eligible estimates published either in the peer-reviewed literature or as preprints as of 9 September 2020.[8–68] Furthermore, I considered another eight preliminary national estimates.[69–76] This search yielded a total of 82 eligible estimates (Fig. 1).

The studies varied substantially in sampling and recruitment designs (Table 1; available at: http://www.who.int/bulletin/volumes/99/1/20-265892). Of the 61 studies, 24 studies[illegible]

explicitly aimed for random sampling from the general population. In principle, random sampling is a stronger design. However, even then, people who cannot be reached (e.g. by email or telephone or even by visiting them at a house location) will not be recruited, and these vulnerable populations are likely to be missed. Moreover, several such studies[8,18,19,37,42] focused on geographical locations with high numbers of deaths, higher than other locations in the same city or country, and this emphasis would tend to select eventually for a higher infection fatality rate on average.

Eleven studies assessed blood donors,[12,13,15,17,20,21,31,40,45,50,60] which might underestimate COVID-19 seroprevalence in the general population. For example, 200 blood donors in Oise, France showed 3.00% seroprevalence, while the seroprevalence was 25.87% (171/661) in pupils, siblings, parents, teachers and staff at a high school with a cluster of cases in the same area; the true population seroprevalence may be between these two values.[19]

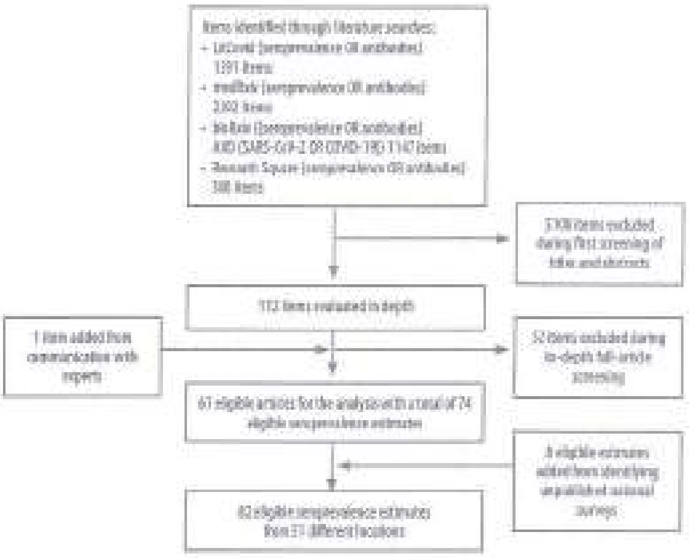
For other studies, healthy volunteer bias[38] may underestimate seroprevalence, attracting people with symptoms[39] may overestimate seroprevalence, and studies of employees,[14,21,28,33,48] grocery store clients[23] or patient cohorts[11,13,27,30,34,36,41,43,51,56,58,63,64,67] risk sampling bias in an unpredictable direction.

All the studies tested for IgG antibodies but only about half also assessed IgM and few assessed IgA. Only seven studies assessed all three types of antibodies and/or used pan-Ig antibodies. The ratio of people sampled versus the total population of the region was more than 1:1000 in 20 studies (Table 2; available at: http://www.who.int/bulletin/volumes/99/1/20-265892).

Seroprevalence estimates

Seroprevalence for the infection ranged from 0.02% to 53.40% (58.40% in the slum sub-population in Mumbai; Table 3). Studies varied considerably depending on whether or not they tried to adjust their estimates for test performance, sampling (to get closer to a more representative sample), clustering (e.g. when including household members) and other factors. The adjusted seroprevalence occasionally differed substantially from the unadjusted value. In studies that used samples from multiple locations, between-location heterogeneity was seen (e.g. 0.00–25.00% across 133 Brazilian cities).[25]

Fig. 1. **Flowchart for selection of seroprevalence studies on severe acute respiratory syndrome coronavirus 2, 2020**



COVID-19: coronavirus disease 2019; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Inferred infection fatality rate

Inferred infection fatality rate estimates varied from 0.00% to 1.63% (Table 4). Corrected values also varied considerably (0.00–1.54%).

For 15 locations, more than one estimate of the infection fatality rate was available and thus I could compare the infection fatality rate from different studies evaluating the same location. The estimates of infection fatality rate tended to be more homogeneous within each location, while they differed markedly across locations (Fig. 2). Within the same location, infection fatality rate estimates tend to have only small differences, even though it is possible that different areas within the same location may also have real differences in infection fatality rate. France is one exception where differences are large, but both estimates come from population studies of outbreaks from schools and thus may not provide good estimates of population seroprevalence and may lead to an underestimated infection fatality rate.

I used summary estimates weighted for sample size to generate a single estimate for each location. Data were available for 51 different locations (including the inferred infection fatality rates from the eight preliminary additional national estimates in Table 5).

The median infection fatality rate across all 51 locations was 0.27% (corrected 0.23%). Most data came from locations with high death tolls from COVID-19 and 32 of the locations had a population mortality rate (COVID-19 deaths per million population) higher than the global average (118 deaths from COVID-19 per million as of 12 September 2020;[79] Fig. 3). Uncorrected estimates of the infection fatality rate of COVID-19 ranged from 0.01% to 0.67% (median 0.10%) across the 19 locations with a population mortality rate for COVID-19 lower than the global average, from 0.07% to 0.73% (median 0.20%) across 17 locations with population mortality rate higher than the global average but lower than 500 COVID-19 deaths per million, and from 0.20% to 1.63% (median 0.71%) across 15 locations with more than 500 COVID-19 deaths per million. The corrected estimates of the median infection fatality rate were

Table 3. **Estimated prevalence of COVID-19 and estimated number of people infected, 2020**

Country (location)	Seroprevalence, %			Estimated no. of people infected
	Crude	Adjusted		
		Value	Adjustments	
Argentina (Barrio Padre Mugica)[47]	ND	53.4	Age, sex, household, nonresponse	26 691
Belgium[48]	5.7	6.0	Sampling, age, sex, province	695 377
Brazil (133 cities)[49]	1.09	1.62 overall (0 – 25.0 across the 133 cities)	Test design	1 209 405[a]
Brazil (Espirito Santo)[50]	2.1	ND	NA	84 391
Brazil (Maranhao)[51]	37	40.4	Clustering, stratification, non-response	2 877 454
Brazil (Rio de Janeiro), blood donors[52]	4	4.7	Age, sex, test	811 452
Brazil (Rio Grande do Sul)[53]	0.222	0.332[a]	Sampling	25 283
Brazil (Sao Paulo)[54]	5.2	4.7	Sampling design	14 017
Canada (British Columbia)[55]	0.45	0.55	Age	27 880
Chile (Vitacura)[56]	11.2	ND	NA	9 500
China, blood donors[57]				
Wuhan	3.87	ND	NA	433 827
Shenzhen	0.06	ND	NA	7 818
Shijiazhuang	0.02	ND	NA	2 206
China (Wuhan)[58]	10	ND	NA	1 108 000
China (Wuhan)[59]	8.36	ND	NA	926 288
Entire period	3.53	2.80	Age, sex, test	–
China (Guangzhou), blood donors[60]	0.09	ND	NA	104 783
China (several regions)[61]				
Hubei (not Wuhan)	3.6	ND	NA	1 718 110
Chongqing	3.8	ND	NA	11 956 109
Sichuan	0.6	ND	NA	487 847
Guangdong	2.2	ND	NA	2 522 010
Croatia[62]	1.27[a]	ND	NA	51 765
Denmark, blood donors[63]	2	1.9	Test	109 065
Denmark (Faroe Islands)[64]	0.6	0.7	Test	365
France (Crepy-en-Valois)[65]	10.4	ND	NA	620 105
France (Oise)[66]	25.9	ND	NA	1 548 000
Germany (Gangelt)[67]	15	23.0	Test, cluster, symptoms	2 919
Germany (Frankfurt)[68]	0.6	ND	NA	16 086
Greece[69]	0.42 (Apr 8)	0.49[a]	Age, sex, region	51 013
Hungary[70]	0.97	0.68	Design, age, sex, district	65 611
Iceland[71]	2.3 (quarantined), 0.3 (unknown exposure)	0.9	Including those positive by RT-PCR	3 177
India (Mumbai)[72]				334 750
Slum areas	54.1	58.4	Test, age, sex	–
Non-slum areas	16.1	17.3	Test, age, sex	–
India (Srinagar)[73]	3.8	3.6	Age, sex	54 000
Islamic Republic of Iran (Gulan)[74]	22	33.0	Test, sampling	770 000
Italy (Apulia), blood donors[75]	0.99	ND	NA	39 887
Japan (Kobe)[76]	3.3	2.7	Age, sex	48 999
Japan (Tokyo)[77]	3.83	ND	NA	532 450
Japan (Utsunomiya City)[78]	0.4	1.23	Age, sex, distance to clinic, district, cohabitants	6 378
Kenya, blood donors[79]	5.6	5.2	Age, sex, region, test	2 783 453
Luxembourg[80]	1.9	2.1	Age, sex, district	12 684
Netherlands, blood donors[81]	2.7	ND	NA	461 622
Netherlands (Rotterdam)[82]	3	ND	NA	512 910
Pakistan (Karachi)[83]	16.3	11.9	Age, sex	1 987 300
East	20.0	15.1	Age, sex	–
Malir	12.7	8.7	Age, sex	–
Pakistan (urban)[84]	17.5	ND	NA	13 825 000
Qatar[85]	30.4	ND	NA	851 200

(continues)

(. . . *continued*)

Country (location)	Seroprevalence, %			Estimated no. of people infected
	Crude	Adjusted		
		Value	Adjustments	
Entire period	24.0	ND	NA	–
Republic of Korea[13]	0.07	ND	NA	1867
Spain[14]	ND	5.0[a]	Sampling, age, sex, income	2 347 000
Spain (Barcelona)[15]	14.3	ND	NA	1 081 998
Switzerland (Geneva)[16]	10.6	10.9	Test, age, sex	54 500
Switzerland[17]				
Zurich[f]	Unclear	1.3	Multivariate Gaussian conditioning	19 773
Zurich and Lucerne[g]	Unclear	1.6	Multivariate Gaussian conditioning	30 888
United Kingdom (England)[18]	5.6	6.0	Test, sampling	3 360 000
United Kingdom (Scotland) blood donors[19]	1.2	ND	NA	64 800
USA (10 states)[20]				
Washington, Puget Sound	1.3	1.1	Age, sex, test	48 291
Utah	2.4	2.2	Age, sex, test	71 550
New York, New York City	5.7	6.9	Age, sex, test	641 778
Missouri	2.9	2.7	Age, sex, test	161 936
Florida, south	2.2	1.9	Age, sex, test	117 389
Connecticut	4.9	4.9	Age, sex, test	176 012
Louisiana	ND	5.8	Age, sex, test	267 033
California, San Francisco Bay	ND	1	Age, sex, test	94 626
Pennsylvania, Philadelphia	ND	3.3	Age, sex, test	136 633
Minnesota, Minneapolis	ND	2.4	Age, sex, test	90 651
USA (California, Bay Area) blood donors[21]	0.4	0.1	Test and confirmation	7 752
USA (California, Los Angeles)[22]	4.06	4.35	Test, sex, race and ethnicity, income	367 000
USA (California, San Francisco), in census tract 022.901[23]	4.3	6.1	Age, sex, race and ethnicity, test	316
USA (California, Santa Clara)[24]	1.5	2.5	Test, sampling, cluster	51 000
USA (Idaho, Boise)[25]	1.79	ND	NA	8620
USA (Georgia, DeKalb and Fulton counties)[26]	2.7	2.5	Age, sex, race and ethnicity	45 567
USA (Idaho, Blaine County)[27]	22.4	23.4	Test, age, sex, household	5 403
USA (Indiana)[28]	2.3 (IgG and RT-PCR)[h]	2.8	Age, race, Hispanic ethnicity	187 802
USA (Louisiana, Baton Rouge)[29]	6	6.6	Census, race, parish, including RT-PCR positives	46 147
USA (Louisiana, Orleans and Jefferson Parish)[30]	6.9 (IgG and RT-PCR)[h]	6.9 for IgG	Census weighting, demographics	96 578
USA (New York)[31]	12.5	14.0	Test, sex, age race and ethnicity, region	2 723 000
USA, New York[32]				
Columbia University Medical Center, New York City	5	ND	NA	463 044
CareMount central laboratory, five New York state counties	1.8	ND	NA	183 404
USA (New York, Brooklyn)[33]	4.7	ND	NA	1 203 154
USA (Rhode Island), blood donors[34]	3.9	ND	NA	41 384

COVID-19, coronavirus disease 2019; NA, not applicable; ND, no data available; RT-PCR, real-time polymerase chain reaction; test, test performance.

[a] The authors calculated 260 000 to be infected in the 90 cities that had 200–250 samples tested, but many of the other 43 cities with <200 samples may be equally or even better represented since they tended to be smaller than the 90 cities (mean population 356 213 versus 639 336).

[b] An estimate is also provided adjusting for test performance, but the assumed specificity of 99.0% seems inappropriately low, since as part of the validation process the authors found that several of the test-positive individuals had household members who were also infected, thus the estimated specificity was deemed by the authors to be at least 99.95%.

[c] 1.20% in workers in Spain without mobility restrictions, 5.37% in workers in Rioja without mobility restrictions, 1.37% for all workers without mobility restrictions. Spain and Rioja tended to have somewhat higher death rates than nationwide Croatia, but residence of workers is not given, so the entire population of the country is used in the calculations.

[d] An estimate is also provided adjusting for test performance resulting in adjusted seroprevalence of 0.23%, but this seems inappropriately low, since the authors report that all positive results were further validated by ELISA (enzyme-linked immunosorbent assay).

[e] 5.0% with point of care test, 4.8% with immunoassay, 3.7% with both tests positive, 6.2% with at least one test positive.

[f] Patients during 1–15 April.

[g] Blood donors in May.

[h] The study counts in prevalence also those who were currently/recently infected as determined by a positive RT-PCR.

Notes: Of the studies where seroprevalence was evaluated at multiple consecutive time points, the seroprevalence estimate was the highest in the most recent time interval with few exceptions, for example in the Switzerland (Geneva) study,[17] the highest value was seen 2 weeks before the last time interval; in the Switzerland (Zurich) study,[17] the highest value was seen in the period 1–15 April for patients at the university hospital and in May for blood donors; and in the China (Wuhan) study,[18] the highest value was seen about 3 weeks before the last time interval.

Table 4. **Deaths from COVID-19 and inferred infection fatality rates, overall and in people younger than 70 years, by location, 2020**

Location	No. of site-specific cumulative deaths from COVID-19 (to date)[a]	Inferred infection fatality rate, % (corrected)	% of site-specific cumulative deaths from COVID-19 in people < 70 years[a]	Infection fatality rate in people < 70 years, % (corrected)
Argentina (Barrio Padre Mugica)[47]	64 (1 July)	0.16 (0.13)	~70	0.11 (0.09)
Belgium[48]	7594 (30 April)	1.09 (0.87)	10	0.13 (0.10)
Brazil (133 cities)[49]	–[b]	Median 0.30 (0.27)	31 (<60 years)	0.10 (0.09)
Brazil (Espirito Santo)[50]	363 (21 May)	0.43 (0.39)	31 (Brazil, <60 years)	0.14 (0.13)
Brazil (Maranhao)[51]	4272 (8 August)	0.15 (0.14)	23	0.04 (0.03)
Brazil (Rio de Janeiro), blood donors[52]	1019 (3 May)	0.12 (0.11)	31 (Brazil, <60 years)	0.04 (0.04)
Brazil (Rio Grande do Sul)[53]	124 (14 May)	0.49 (0.39)	31 (Brazil, <60 years)	0.19 (0.15)
Brazil (Sao Paulo)[54]	NA[c] (15 May)	Unknown, but likely > 0.4	31 (Brazil, <60 years)	Unknown, but likely > 0.1
Canada (British Columbia)[55]	164 (28 May)	0.59 (0.59)	13	0.08 (0.08)
Chile (Vitacura)[56]	NA[c] (18 May)	Unknown, but likely < 0.2	38 (Chile)	Unknown, but likely < 0.1
China, blood donors[57]				
Wuhan	1935 (20 February)	0.45 (0.41)	50	0.24 (0.22)
Shenzhen	1 (5 March)	0.01 (0.01)	About 50 (if similar to Wuhan)	0.01 (0.01)
Shijiazhuang	1 (27 February)	0.05 (0.04)	About 50 (if similar to Wuhan)	0.03 (0.02)
China (Wuhan)[58]	3869 (2 May)	0.35 (0.31)	50	0.19 (0.15)
China (Wuhan)[59]	3869 (13 April)	0.42 (0.38)	50	0.23 (0.21)
China (Guangzhou), blood donors[60]	8 (5 April)	0.00 (0.00)	About 50 (if similar to Wuhan)	0.00 (0.00)
China (several regions)[45]				
Hubei (not Wuhan)	643 (12 April)	0.04 (0.03)	About 50 (if similar to Wuhan)	0.02 (0.02)
Chongqing	6 (12 April)	0.00 (0.00)	About 50 (if similar to Wuhan)	0.00 (0.00)
Guangdong	8 (12 April)	0.00 (0.00)	About 50 (if similar to Wuhan)	0.00 (0.00)
Sichuan	3 (12 April)	0.00 (0.00)	About 50 (if similar to Wuhan)	0.00 (0.00)
Croatia[61]	79 (3 May)	0.15 (0.14)	13	0.02 (0.02)
Denmark, blood donors[62]	370 (21 April)	0.34 (0.27)	12	0.05 (0.04)
Faroe Islands[63]	0 (5 May)	0.00 (0.00)	9	0.00 (0.00)
France (Crepy-en-Valois)[64]	2325 (5 May)[d]	0.37 (0.30)	7 (France, <65 years)	0.04 (0.03)
France (Oise)[65]	932 (7 April)[d]	0.06 (0.05)	7 (France, <65 years)	0.01 (0.01)
Germany (Gangelt)[66]	7 (15 April)	0.28 (0.25)	9	0.00 (0.00)
Germany (Frankfurt)[67]	42[e] (17 April)	0.26 (0.21)	14 (Germany)	0.04 (0.03)
Greece[68]	121 (22 April)	0.24 (0.19)	30	0.09 (0.07)
Hungary[69]	442 (15 May)	0.67 (0.54)	No data	No data
Iceland[70]	10 (1 June)	0.30 (0.30)	30	0.10 (0.10)
India (Mumbai)[71]	495 (13–20 July)	0.09 (0.07)	50 (<60 years, India)	0.04 (0.03)
India (Srinagar)[72]	35 (15 July)[f]	0.06 (0.05)	50 (<60 years, India)	0.03 (0.03)
Islamic Republic of Iran (Guilan)[73]	617 (23 April)	0.08 (0.07)	No data	No data
Italy (Apulia), blood donors[74]	530 (22 May)	1.33 (1.20)	15 (Italy)	0.24 (0.22)
Japan (Kobe)[75]	10 (mid-April)	0.02 (0.02)	21 (Japan)	0.01 (0.01)
Japan (Tokyo)[76]	189 (11 May)	0.04 (0.03)	21 (Japan)	0.01 (0.01)
Japan (Utsunomiya City)[77]	0 (14 June)	0.00 (0.00)	0	0.00 (0.00)
Kenya, blood donors[78]	64 (31 May)	0.00 (0.00)	58 (< 60 years)	0.00 (0.00)
Luxembourg[79]	92 (2 May)	0.73 (0.58)	9	0.07 (0.06)
Netherlands, blood donors[80]	3134 (15 April)	0.68 (0.68)	11	0.09 (0.09)
Netherlands (Rotterdam)[81]	3134 (15 April)	0.65 (0.52)	11	0.08 (0.06)
Pakistan (Karachi)[82]	~1500 (9 July)[g]	0.08 (0.07)	~70	0.06 (0.05)
Pakistan (urban)[83]	5266 (13 July)[h]	0.04 (0.04)	~70	0.03 (0.03)
Qatar[84]	93 (19 June)	0.01 (0.01)	74	0.01 (0.01)
Republic of Korea[85]	2 (3 June)[i]	0.10 (0.09)	0	0.00 (0.00)
Spain[86]	26 920 (11 May)	1.15 (0.92)	13	0.18 (0.14)
Spain (Barcelona)[87]	5137 (2 May)	0.48 (0.48)	13 (Spain)	0.07 (0.07)
Switzerland (Geneva)[88]	243 (30 April)	0.45 (0.36)	8	0.04 (0.03)

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Location	No. of site-specific cumulative deaths from COVID-19 (to date)[a]	Inferred infection fatality rate, % (corrected)	% of site-specific cumulative deaths from COVID-19 in people < 70 years[b]	Infection fatality rate in people < 70 years, % (corrected)
Switzerland (Zurich)[c,d]	107 (15 April, Zurich), 147 (22 May, Zurich and Lucerne)	0.31 (0.41)	8 (Switzerland)	0.05 (0.04)
England[e]	38 854 (5 July)	1.16 (0.93)	23	0.27 (0.22)
Scotland, blood donors[e]	47 (1 April)	0.07 (0.06)	9 (< 65 years)	0.01 (0.01)
USA (10 states)[f,g]				
Washington, Puget Sound	267 (4 April)	0.43 (0.43)	10 (state, < 60 years)	0.03 (0.03)
Utah	58 (4 May)	0.08 (0.08)	28 (< 65 years)	0.03 (0.03)
New York	4146 (4 April)	0.65 (0.65)	34 (state)	0.25 (0.25)
Missouri	329 (30 April)	0.20 (0.20)	23	0.05 (0.05)
Florida, south	295 (15 April)	0.25 (0.25)	28 (state)	0.08 (0.08)
Connecticut	2718 (6 May)	1.54 (1.54)	18	0.31 (0.31)
Louisiana	806 (11 April)	0.30 (0.30)	32	0.10 (0.10)
California, San Francisco Bay	321 (1 May)	0.50 (0.50)	25	0.14 (0.14)
Pennsylvania, Philadelphia	697 (26 April)	0.45 (0.45)	21 (state)	0.10 (0.10)
Minnesota, Minneapolis	436 (13 May)	0.48 (0.48)	20 (state)	0.10 (0.10)
USA (California, Bay Area)[h]	12 (22 March)	0.15 (0.12)	25	0.04 (0.03)
USA (California, Los Angeles)[i]	724 (19 April)	0.20 (0.18)	24 (< 65 years)	0.06 (0.05)
USA (California, San Francisco)[j]	0 (4 May)	0.00 (0.00)	0	0.00 (0.00)
USA (California, Santa Clara)[j]	84 (22 April)	0.18 (0.17)	35	0.07 (0.06)
USA (Idaho, Boise)[k]	14 (24 April)	0.16 (0.13)	14 (Idaho)	0.02 (0.02)
USA (Georgia)[l]	198 (7 May)	0.64 (0.44)	30	0.15 (0.15)
USA (Idaho, Blaine County)[m]	5 (19 May)	0.10 (0.08)	14 (Idaho)	0.02 (0.01)
USA (Indiana)[n]	1099 (30 April)	0.58 (0.46)	24	0.16 (0.13)
USA (Louisiana, Baton Rouge)[o]	420 (30 July)	0.91 (0.73)	32 (Louisiana)	0.32 (0.25)
USA (Louisiana, Orleans and Jefferson Parish)[p]	925 (16 May)	1.63 (1.31)	32	0.57 (0.46)
USA (New York)[q,r]	18 610 (30 April)	0.68 (0.54)	34	0.24 (0.23)
USA (New York Columbia University Medical Center, New York City and CareMount central laboratory, five New York state counties)[s,t]	965 (28 March, New York state)	0.15 (0.14)	34	0.08 (0.05)
USA (New York, Brooklyn)[u]	4894 (19 May)[v]	0.41 (0.33)[v]	34 (New York state)	0.15 (0.14)
USA (Rhode Island), blood donors[w]	430 (11 May)	1.04 (0.83)	17	0.23 (0.19)

COVID-19: coronavirus disease 2019; NA: not available.

[a] Whenever the number or proportion of COVID-19 deaths at age < 70 years was not provided in the paper, I retrieved the proportion of these deaths from situation reports of the relevant location. If I could not find this information for the specific location, I used a larger geographic area. For Brazil, the closest information that I found was from a news report.[77] For Croatia, I retrieved data on age for 45/105 deaths through Wikipedia.[78] Geographical location in parentheses specifies the population.

[b] Data are provided by the authors for deaths per 100 000 population in each city along with inferred infection fatality rate in each city, with wide differences across cities; the infection fatality rate shown here is the median across the 16 cities with 300–350 samples and at least one positive sample (the interquartile range for the uncorrected infection fatality rate is 0.26–0.67% and across all cities is 0–2.4%, but with very wide uncertainty in each city). A higher infection fatality rate is alluded to in the preprint, but the preprint also shows a scatter diagram for survey-based seroprevalence versus reported deaths per population with a regression slope that agrees with an infection fatality rate of 0.3%.

[c] Information on deaths was not available for the specific locations. In the Sao Paulo study, the authors selected six districts of Sao Paulo most affected by COVID-19; they do not name the districts and the number of deaths as of mid-May is not available, but using data for death rates across all Sao Paulo would give an infection fatality rate of > 0.4% overall. In the Veneza study, similarly one can infer from the wider Santiago metropolitan area that the infection fatality rate in the Vitacura area would probably be < 0.2% overall.

[d] For France, government situation reports provide the number of deaths per region only for in-hospital deaths; therefore, I multiplied the number of in-hospital deaths by a factor equal to: total number of deaths/in-hospital deaths for all of France.

[e] Estimated from number of deaths in Hesse province on 17 April x proportion of deaths in the nine districts with key enrolment (enrolment ratio > 1:10 000) in the study among all deaths in Hesse province.

[f] I calculated the approximate number of deaths assuming the same case fatality ratio in the Srinagar district as in the Jammu and Kashmir state where it is located.

[g] For Karachi, it is assumed that about 30% of COVID-19 deaths in Pakistan are in Karachi (since about 30% of the cases are there).

[h] The number of deaths across all Pakistan, I assumed that this number is a good approximation of deaths in urban areas (most deaths occur in urban areas and there is some potential underreporting).

[i] I calculated the approximate number of deaths from the number of cases in the study areas in south-western Seoul, assuming a similar case fatality as in the rest of the city.

[j] Confirmed COVID-19 deaths; inclusion of probable COVID-19 deaths would increase the infection fatality rate estimates by about a quarter.

Note: Cumulative deaths are sourced from the specific study or from situation report on the same location unless otherwise stated.

0.09%, 0.20% and 0.57%, respectively, for the three location groups.

For people younger than 70 years old, the infection fatality rate of COVID-19 across 40 locations with available data ranged from 0.00% to 0.31% (median 0.05%); the corrected values were similar.

Discussion

The infection fatality rate is not a fixed physical constant and it can vary substantially across locations, depending on the population structure, the case-mix of infected and deceased individuals and other, local factors. The studies analysed here represent 82 different estimates of the infection fatality rate of COVID-19, but they are not fully representative of all countries and locations around the world. Most of the studies are from locations with overall COVID-19 mortality rates that are higher than the global average. The inferred median infection fatality rate in locations with a COVID-19 mortality rate lower than the global average is low (0.09%). If one could sample equally from all locations globally, the median infection fatality rate might even be substantially lower than the 0.23% observed in my analysis.

COVID-19 has a very steep age gradient for risk of death.[10] Moreover, in European countries that have had large numbers of cases and deaths[11], and in the USA[12], many, and in some cases most, deaths occurred in nursing homes. Locations with many nursing home deaths may have high estimates of the infection fatality rate, but the infection fatality rate would still be low among non-elderly, non-debilitated people.

Within China, the much higher infection fatality rate estimates in Wuhan compared with other areas of the country may reflect widespread nosocomial infections,[13] as well as unfamiliarity with how to manage the infection as the first location that had to deal with COVID-19. The very many deaths in nursing homes, nosocomial infections and overwhelmed hospitals may also explain the high number of fatalities in specific locations in Italy[14] and New York and neighbouring states.[12,15,16,18] Poor decisions (e.g. sending COVID-19 patients to nursing homes), poor management (e.g. unnecessary mechanical ventilation and hydroxychloroquine) may also have contributed to worse outcomes.

Fig. 2. **Estimates of infection fatality rates for COVID-19 in locations that had two or more estimates, 2020**

COVID-19: coronavirus disease 2019.
Notes: Locations are defined at the level of countries, except for the United States of America where they are defined at the level of states and China is separated into Wuhan and non-Wuhan areas. Corrected infection fatality rate estimates are shown (correcting for what types of antibodies were assayed).

High levels of congestion (e.g. in busy public transport systems) may also have exposed many people to high infectious loads and, thus, perhaps more severe disease. A more aggressive viral clade has also been speculated.[15] The infection fatality rate may be very high among disadvantaged populations and in settings with a combination of factors predisposing to higher fatalities.[17]

Very low infection fatality rates seem common in Asian coun-

Table 5. **Infection fatality rates for COVID-19 inferred from preliminary nationwide seroprevalence data, 2020**

Country	Sample size	Date	Reported seroprevalence (%)	Population, no.	Deaths, no. (date)	Inferred infection fatality rate (corrected), %
Afghanistan[?]	9 500 (NR)	NR	31.5	39 021 453	1 300 (8 May)	0.01 (0.01)
Czechia[?]	26 549 (IgG)	23 April–1 May	0.4	10 710 000	252 (4 May)	0.59 (0.47)
Finland[a]	674 (IgG)	20–26 April[a]	2.52	5 541 000	211 (30 April)	0.15 (0.12)
Georgia[b]	1 068 (NR)	18–27 May	1	3 988 264	12 (30 May)	0.03 (0.03)[b]
Israel[?]	1 709 (NR)	May	2–3	9 198 000	299 (10 June)	0.13 (0.10)[c]
Russian Federation[?]	650 000 (NR)	NR	14	145 941 776	5 859 (7 June)	0.03 (0.03)
Slovenia[?]	1 368 (NR)	April	3.1	2 079 000	92 (1 May)	0.14 (0.11)
Sweden[?]	1 200 (IgG)	18–24 May	6.3	10 101 000	4 501 (28 May)	0.71 (0.57)

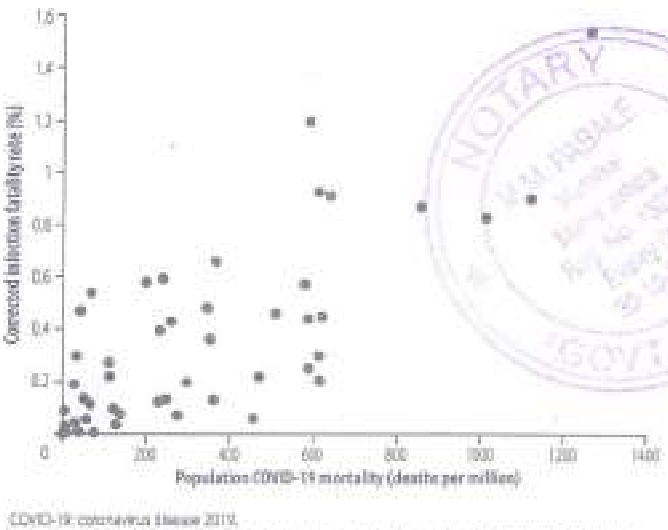
COVID-19: coronavirus disease 2019; Ig: immunoglobin; NR: not reported.
[a] The seroprevalence was slightly lower in subsequent weeks.
[b] The survey was done in Tbilisi, the capital city with a population 1.1 million. I could not retrieve the count of deaths in Tbilisi, but if more deaths happened in Tbilisi, then the infection fatality rate may be higher, but still < 0.1%.
[c] Assuming a seroprevalence of 2.5%.
Notes: These are countries for which no eligible studies were retrieved in the literature search. The results of these studies have been announced in the press and/or in preliminary reports, but are not yet peer reviewed and published.

tries.[illegible] A younger population in these countries (excluding Japan), previous immunity from exposure to other coronaviruses, genetic differences, hygiene etiquette, lower infectious load and other unknown factors may explain these low rates. The infection fatality rate is low also in low-income countries in both Asia and Africa,[illegible] perhaps reflecting the young age structure. However, comorbidities, poverty, frailty (e.g. malnutrition) and congested urban living circumstances may have an adverse effect on risk and thus increase infection fatality rate.

Antibody titres may decline with time[illegible] and this would give falsely low prevalence estimates. I considered the maximum seroprevalence estimate when multiple repeated measurements at different time points were available, but even then some of this decline cannot be fully accounted for. With four exceptions,[illegible] the maximum seroprevalence value was at the latest time point.

Positive controls for the antibody assays used were typically symptomatic patients with positive polymerase chain reaction tests. Symptomatic patients may be more likely to develop antibodies.[illegible] Since seroprevalence studies specifically try to reveal undiagnosed asymptomatic and mildly symptomatic infections, a lower sensitivity for these mild infections could lead to substantial underestimates of the number of

Fig. 3. **Corrected estimates of COVID-19 infection fatality rate in each location plotted against COVID-19 cumulative deaths per million as of September 12 2020 in that location**



COVID-19: coronavirus disease 2019.
Notes: Locations are defined at the level of countries, except for the United Kingdom of Great Britain and Northern Ireland where they are defined by jurisdiction, United States of America (USA) are defined at the level of states and China is separated into Wuhan and non-Wuhan areas. Included locations are: Afghanistan; Argentina; Belgium; Brazil; Canada; Chile; China (non-Wuhan and Wuhan); Croatia; Czechia; Denmark; Faroe Islands; Finland; France; Georgia; Germany; Greece; Hungary; Iceland; India; Iran (Islamic Republic of); Israel; Italy; Japan; Kenya; Luxembourg; Netherlands; Pakistan; Qatar; Republic of Korea; Russian Federation; Slovenia; Spain; Sweden; Switzerland; United Kingdom (England, Scotland); and USA (California, Connecticut, Florida, Georgia, Idaho, Indiana, Louisiana, Minnesota, Missouri, New York, Pennsylvania, Rhode Island, Utah, Washington). When several infection fatality rate estimates were available from multiple studies for a location, the sample size-weighted mean is used. One outlier location with very high deaths per million population (1 702 for New York) is not shown.

infected people and overestimates of the inferred infection fatality rate.

A main issue with seroprevalence studies is whether they offer a representative picture of the population in the assessed region. A generic problem is that vulnerable people at high risk of infection and/or death may be more difficult to recruit in survey-type studies. COVID-19 infection is particularly widespread and/or lethal in nursing homes, in homeless people, in prisons and in disadvantaged minorities.[34] Most of these populations are very difficult, or even impossible, to reach and sample and they are probably under-represented to various degrees (or even entirely missed) in surveys. This sampling obstacle would result in underestimating the seroprevalence and overestimating infection fatality rate.

In principle, adjusted seroprevalence values may be closer to the true estimate, but the adjustments show that each study alone may have unavoidable uncertainty and fluctuation, depending on the type of analysis chosen. Furthermore, my corrected infection fatality rate estimates try to account for undercounting of infected people when not all three antibodies (IgG, IgM and IgA) were assessed. However, the magnitude of the correction is uncertain and may vary in different circumstances. An unknown proportion of people may have responded to the virus using immune mechanisms (mucosal, innate, cellular) without generating any detectable serum antibodies.[35–37]

A limitation of this analysis is that several studies included have not yet been fully peer-reviewed and some are still ongoing. Moreover, despite efforts made by seroprevalence studies to generate estimates applicable to the general population, representativeness is difficult to ensure, even for the most rigorous studies and despite adjustments made. Estimating a single infection fatality rate value for a whole country or state can be misleading, when there is often huge variation in the population mixing patterns and pockets of high or low mortality. Furthermore, many studies have evaluated people within restricted age ranges, and the age groups that are not included may differ in seroprevalence. Statistically significant, modest differences in seroprevalence across some age groups have been observed in several studies.[illegible] Lower values have been seen in young children and higher values in adolescents and young adults, but these patterns are inconsistent and not strong enough to suggest that major differences are incurred by extrapolating across age groups.

Acknowledging these limitations, based on the currently available data, one may project that over half a billion people have been infected as of 12 September 2020, far more than the approximately 29 million documented laboratory-confirmed cases. Most locations probably have an infection fatality rate less than 0.20% and with appropriate, precise non-pharmacological measures that selectively try to protect high-risk vulnerable populations and settings, the infection fatality rate may be brought even lower. ■

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ملخص

معدل وفيات عدوى كوفيد 19 المستدل عليه من بيانات الانتشار المصلي

الغرض تقدير معدل الوفيات الناجمة عن الإصابة بمرض فيروس كورونا 2019 (كوفيد 19) من بيانات الانتشار المصلي.

الطريقة قمت بالبحث في عرادم PubMed وغرادم ما قبل الطباعة عن دراسات الانتشار المصلي لكوفيد 19، بحجم عينة أكبر من أو تساوي 500 بدءاً من 9 سبتمبر/أيلول 2020. كما أنني استرجعت النتائج الإضافية للدراسات الوطنية من البيانات الصحفية والتقارير الأولية. قمت بتقييم دراسات ميزات التصميم وتقديرات الانتشار المصلي. لقد قمت بتقدير معدل الوفيات الناجمة عن الإصابة لكل دراسة عن طريق قسمة العدد الإجمالي للوفيات الناجمة عن جائحة كوفيد 19، على عدد الأشخاص المقدر إصابتهم في كل منطقة. وقمت بتصحيح عدد أنواع الأجسام المضادة التي تم اختبارها (الغلوبين المناعي IgA، IgM، IgG).

النتائج قمت بتضمين 61 دراسة (74 تقديرًا) وثمانية تقديرات وطنية أولية. تراوحت تقديرات الانتشار المصلي من 0.02% إلى 53.40%. تراوحت معدلات وفيات العدوى من 0.00% إلى 1.63%، وتم تصحيح القيم من 0.00% إلى 1.54%. عبر 51 موقعًا، كان متوسط معدل وفيات عدوى كوفيد 19 هو 0.27% (تصحيح بنسبة 0.23%): كان المعدل 0.09% في المواقع التي تقل فيها معدلات وفيات السكان المصابين بكوفيد 19 عن المتوسط العالمي (أكثر من 118 حالة وفاة/مليون نسمة)، و0.20% في المواقع التي يوجد بها من 118 إلى 500 حالة وفاة/مليون نسمة مصابين بكوفيد 19، و0.57% في مواقع بها أكثر من 500 حالة وفاة/مليون نسمة بسبب كوفيد 19. في الأشخاص الذين تقل أعمارهم عن 70 عامًا، تراوحت معدلات وفيات الإصابة بالعدوى من 0.00% إلى 0.31% بمتوسطات مبدئية ومصححة قدرها 0.05%.

الاستنتاج يمكن أن يختلف معدل وفيات الإصابة بفيروس كوفيد 19 بشكل كبير عبر المواقع المختلفة، وقد يعكس هذا الاختلافات في التركيب العمري للسكان، ومزيج الحالات من المرضى المصابين والمتوفين، وعوامل أخرى. تميل معدلات الوفيات المستدل عنها من العدوى إلى أن تكون أقل بكثير من التقديرات التي تم إجراؤها في وقت سابق في الجائحة.

摘要

根据血清阳性率数据推断新型冠状病毒肺炎的感染死亡率

目的 根据血清阳性率数据估计 2019 年冠状病毒病（新型冠状病毒肺炎）的感染死亡率。

方法 在 PubMed 和预印本服务器上查找截至 2020 年 9 月 9 日新型冠状病毒肺炎相关的血清阳性率研究。样本量为 500 个。另外根据初步新闻稿和报告检索了其他全国性研究结果。并评估了与设计特征和血清阳性率估计值相关的研究。通过将新型冠状病毒肺炎累计死亡人数除以每个地区估计感染人数，估算出了每项研究的感染死亡率。然后校正了测试的抗体类型（免疫球蛋白。免疫球蛋白 G、免疫球蛋白 M、免疫球蛋白 A）的数量。

结果 我汇总了 61 项研究（74 个估计值）和 8 个全国性初步估计值。血清阳性率估计值介于 0.02% 至 53.40% 之间。感染死亡率介于 0.00% 至 1.63% 之间。校正值则介于 0.00% 至 1.54% 之间。在 51 个地区中，新型冠状病毒肺炎感染死亡率的中位数为 0.27%（校正值为 0.23%）；在新型冠状病毒肺炎导致的人口死亡率低于全球平均水平（每一百万人口中死亡病例介于 118 例）的地区中，该比率为 0.09%；在每一百万人口中新型冠状病毒肺炎死亡病例介于 118 – 500 例之间的地区，该比率为 0.20%；而在每一百万人口中新型冠状病毒肺炎死亡病例大于 500 例的地区，该比率则为 0.57%。70 岁以下人群的感染死亡率介于 0.00% 至 0.31% 之间，经粗略校正后该比率的中位数为 0.05%。

结论 不同地区的新型冠状病毒肺炎感染死亡率可能存在很大的差异，据此可反映出在人口年龄结构、感染和死亡病例组合以及其他因素方面存在差异。推断的感染死亡率往往比全球性流行病爆发初期的估计值要低得多。

Résumé

Ratio de létalité réel de la COVID-19 déduit à partir des données de séroprévalence

Objectif Estimer le ratio de létalité réel de la maladie à coronavirus 2019 (COVID-19) à partir des données de séroprévalence.

Méthodes J'ai effectué des recherches sur PubMed et sur les serveurs de prépublication afin de trouver des études consacrées à la séroprévalence de la COVID-19, avec des échantillons ≥ 500, au 9 septembre 2020. J'ai également prélevé des résultats supplémentaires dérivés d'études nationales qui figurent dans les versions préliminaires de divers rapports et communiqués de presse. J'ai analysé les études pour y déceler des caractéristiques de conception et des estimations de séroprévalence. Ensuite, j'ai calculé le ratio de létalité réel pour chaque étude en divisant le nombre cumulé de décès dus à la COVID-19 par le nombre d'individus qui auraient été infectés dans chaque région. Enfin, j'ai apporté des corrections en fonction des types d'anticorps testés (immunoglobulines, IgG, IgM, IgA).

Résultats J'ai pris 61 études en compte (74 estimations) et huit estimations nationales préliminaires. Les estimations en matière de séroprévalence étaient comprises entre 0,02% et 53,40%. Les ratios de létalité réels allaient de 0,00% à 1,63%, les valeurs corrigées de 0,00% à 1,54%. Dans les 51 lieux étudiés, la médiane du ratio de létalité réel pour la COVID-19 s'élevait à 0,27% (0,23% après correction). Le ratio était de 0,09% dans les endroits où le taux de mortalité dû à la COVID-19 était inférieur à la moyenne mondiale (< 118 décès/million d'habitants), de 0,20% dans les endroits dénombrant 118–500 décès COVID-19/million d'habitants, et de 0,57% là où la COVID-19 était responsable de > 500 décès/million d'habitants. Chez les personnes de moins de 70 ans, les ratios de létalité réels se situaient entre 0,00% et 0,31% avec des médianes brutes et corrigées de 0,05%.

Conclusion Le ratio de létalité réel de la COVID-19 peut considérablement varier d'un endroit à l'autre, ce qui pourrait correspondre aux différences de structure de pyramide des âges au sein de la population, au calendrier entre patients infectés et décédés, ainsi qu'à d'autres facteurs. Les ratios de létalité réels que j'ai pu déduire avaient tendance à être nettement inférieurs aux estimations formulées précédemment durant la pandémie.

Резюме

Показатель летальности при инфицировании COVID-19, определенный на основании данных о серораспространенности

Цель Оценить показатель летальности при инфицировании коронавирусным заболеванием 2019 г. (COVID-19) на основании данных о серораспространенности.

Методы Автор провел поиск на серверах PubMed и серверах предварительной публикации на предмет исследований серораспространенности COVID-19 с размером выборки ≥500 по состоянию на 9 сентября 2020 года. Были также получены дополнительные результаты национальных исследований из предварительных пресс-релизов и отчетов. Автор оценил исследования по элементам дизайна и оценкам серораспространенности. Автор оценил показатель летальности при инфицировании для каждого исследования, разделив общее количество смертей от COVID-19 на количество людей, предположительно инфицированных в каждом регионе. При этом была сделана поправка на количество протестированных типов антител (иммуноглобины, IgG, IgM, IgA).

Результаты В работу вошло 61 исследование (74 прогноза) и восемь предварительных национальных прогнозов. Прогнозы серораспространенности варьировались в диапазоне от 0,02 до 53,40%. Показатели летальности при инфицировании варьировались в диапазоне от 0,00 до 1,63%, скорректированные значения — от 0,00 до 1,54%. В 51 регионе средний показатель летальности при инфицировании COVID-19 составил 0,27% (скорректированный показатель 0,23%); этот показатель составил 0,09% в регионах с уровнем летальности населения от COVID-19 ниже, чем в среднем по миру (<118 смертей на миллион), 0,20% в регионах, в которых зарегистрировано 118–500 случаев смерти от COVID-19 на миллион человек, и 0,57% в регионах, где зарегистрировано более 500 случаев смерти от COVID-19 на миллион человек. У людей младше 70 лет показатель летальности при инфицировании колебался в пределах от 0,00 до

0,31 % с приблизительными и скорректированными медианными значениями 0,05%.

Вывод Показатель летальности при инфицировании COVID-19 может существенно различаться в разных регионах, и это может отражать различия в возрастной структуре населения, структуре случаев инфицирования и смерти пациентов, а также в других факторах. Предполагаемые показатели летальности при инфицировании, как правило, были намного ниже, чем прогнозы, сделанные ранее во время пандемии.

Resumen

Tasa de letalidad por la infección de la COVID-19 calculada a partir de los datos de seroprevalencia

Objetivo Estimar la tasa de letalidad por la infección de la enfermedad por coronavirus de 2019 (COVID-19) a partir de los datos de seroprevalencia.

Métodos Se buscaron los estudios de seroprevalencia de la COVID-19 con un tamaño de muestra mayor o igual a 500 a partir del 9 de septiembre de 2020 en PubMed y en los servidores de preimpresión. Además, se recuperaron los resultados adicionales de los estudios nacionales a partir de los comunicados de prensa y de los informes preliminares. Se evaluaron los estudios para determinar las características de diseño y las estimaciones de seroprevalencia. Para calcular la tasa de letalidad por la infección de cada estudio, se dividió la cantidad acumulada de muertes por la COVID-19 por la cantidad de personas que se estima que están infectadas en cada región. Asimismo, se corrigió la cantidad de tipos de anticuerpos probados (inmunoglobulinas, IgG, IgM, IgA).

Resultados Se incluyeron 61 estudios (74 estimaciones) y 8 estimaciones nacionales preliminares. Las estimaciones de seroprevalencia oscilaban entre el 0,02 % y el 53,40 %. Las tasas de letalidad por la infección oscilaron entre el 0,00 % y el 1,63 %. los valores corregidos entre el 0,00 % y el 1,54 %. En 51 lugares, la mediana de la tasa de letalidad por la infección de la COVID-19 fue del 0,27 % (corregida en un 0,23 %); la tasa fue del 0,09 % en lugares donde las tasas de letalidad de la población con la COVID-19 eran inferiores al promedio mundial (menos de 118 muertes/millón), del 0,20 % en lugares con 118-500 muertes a causa de la COVID-19/millón de personas y del 0,57 % en lugares con más de 500 muertes a causa de la COVID-19/millón de personas. En personas menores de 70 años, las tasas de letalidad por la infección oscilaron entre el 0,00 % y el 0,31 % con medianas brutas y corregidas del 0,05 %.

Conclusión La tasa de letalidad por infección de la COVID-19 puede variar de manera sustancial en diferentes lugares y esto puede reflejar diferencias en la estructura de edad de la población y en la variedad de casos de los pacientes infectados y fallecidos, así como en otros factores. Las tasas de letalidad por infección que se calculan tienden a ser mucho más bajas que las estimaciones realizadas a principios de la pandemia.

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Table 1. **Eligible seroprevalence studies on COVID-19 published or deposited as preprints as of 9 September 2020: dates, sampling and recruitment**

Author	Country (location)	Dates	Sampling and recruitment

(...continued)

Author	Country (location)	Dates	Sampling and recruitment
Kraehling et al.[47]	Germany (Frankfurt)	6–14 April	Employees of Infraserv Höchst, a large industrial site operator in Frankfurt am Main. No exclusion criteria
Bogogiannidou et al.[48]	Greece	March and April (April data used)	Leftover blood samples collected from a nationwide laboratory network, including both private and public hospital laboratories (27 laboratories in total)
Merkely et al.[49]	Hungary	1–16 May	Representative sample (n = 17 787) of the Hungarian population ≥ 14 years living in private households (8 283 810)
Gudbjartsson et al.[50]	Iceland	Several cohorts between April and June[a]	30 576 people in Iceland, including those documented to be infected, those quarantined and people not known to have been exposed
Malani et al.[51]	India (Mumbai)	29 June–19 July	Geographically-spaced community sampling of households, one individual per household was tested in slum and non-slum communities in three wards, one each from the three main zones of Mumbai
Khan et al.[52]	India (Srinagar)	1–15 July	Adults (> 18 years) who visited selected hospitals across the Srinagar District
Shakiba et al.[3]	Islamic Republic of Iran (Gulan)	April (until 21 April)	Population-based cluster random sampling design through telephone call invitation, household-based
Fiore et al.[53]	Italy (Apulia)	1–31 May	Blood donors 18–65 years old free of recent symptoms possibly related to COVID-19, no close contact with confirmed cases, symptom-free in the preceding 14 days, no contact with suspected cases
Doi et al.[54]	Japan (Kobe)	31 March–7 April	Randomly selected patients who visited outpatient clinics and received blood testing for any reason. Patients who visited the emergency department or the designated fever consultation service were excluded
Takita et al.[55]	Japan (Tokyo)	21 April–20 May	Two community clinics in the main railway stations in Tokyo (Navitas Clinic Shinjuku and Tachikawa)
Nawa et al.[56]	Japan (Utsunomiya City)	14 June–5 July	Invitations enclosed with a questionnaire were sent to 2280 people in 1 000 households randomly selected from Utsunomiya City's basic resident registry; 742 completed the study
Uyoga et al.[57]	Kenya	30 April–16 June (~90% of samples in last 30 days)	Residual blood donor serum samples from donors 18–65 years in four sites (Mombasa, Nairobi, Eldoret and Kisumu)
Snoeck et al.[58]	Luxembourg	16 April–5 May	Representative sample (no details how ensured); 1 807 of 2000 contacted provided data, were < 79 years and had serology results
Slot et al.[59]	Netherlands	1–15 April	Blood donors. Donors must be completely healthy, but they may have been ill in the past, provided that they recovered at least 2 weeks before
Westerhuis et al.[60]	Netherlands (Rotterdam)	Early March and early April	Left-over plasma samples from patients of nine age categories in Erasmus Medical Center in Rotterdam; 879 samples in early March and 729 in early April
Nisar et al.[61]	Pakistan (Karachi)	25 June–11 July (after baseline on 15–25 April)	Cross-sectional household surveys in a low- (District Malir) and high-transmission (district East) area of Karachi with households selected using simple random sampling (Malir) and systematic random sampling (East)
Javed et al.[62]	Pakistan (urban Karachi, Lahore, Multan, Peshawar and Quetta)	Up to 6 July	Adult, working population aged 18–65 years, recruited from dense, urban workplaces including factories, businesses, restaurants, media houses, schools, banks, hospitals (health-care providers), and from families of positive cases in cities in Pakistan
Abu Raddad et al.[63]	Qatar	12 May–12 July (highest seroprevalence on 12–31 May)	Convenience sample of residual blood specimens collected for routine clinical screening or clinical management from 33 970 outpatient and inpatient departments for a variety of health conditions (n = 957 in 12–31 May)
Noh et al.[64]	Republic of Korea	25–29 May	Outpatients who visited two hospitals in south-west Seoul which serve six administrative areas
Pollán et al.[65]	Spain	27 April–11 May	35 883 households selected from municipal rolls using two-stage random sampling stratified by province and municipality size, with all residents invited to participate (75.1% of all contacted individuals participated)

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Author	Country (location)	Dates	Sampling and recruitment
Crovetto et al.[20]	Spain (Barcelona)	14 April–5 May	Consecutive pregnant women for first trimester screening or delivery in two hospitals
Stringhini et al.[14]	Switzerland (Geneva)	6 April–9 May (5 consecutive weeks)	Randomly selected previous participants of the Bus Santé study with an email (or telephone contact, if email unavailable); participants were invited to bring all members of their household aged 5 years and older
Emmenegger et al.[21]	Switzerland (Zurich)	Prepandemic until June (patients) and May (blood donors)	Patients at the University Hospital of Zurich and blood donors in Zurich and Lucerne
Ward et al.[15]	United Kingdom (England)	20 June–13 July	Random population sample of 100 000 adults over 18 years
Thompson et al.[16]	United Kingdom (Scotland)	21–23 March	Blood donors. Donors should not have felt unwell in the past 14 days; some other deferrals also applied regarding travel and COVID-19 symptoms
Havers et al.[17]	USA (10 states)	23 March–1 April (Washington, Puget Sound and New York, New York City), 1–8 April (Louisiana), 5–10 April (Florida, south), 13–25 April (Pennsylvania, Philadelphia, metropolitan area), 20–26 April (Missouri), 23–27 April (California, San Francisco Bay Area), 20 April–3 May (Utah), 26 April–3 May (Connecticut), 30 April–12 May (Minnesota, Minneapolis)	Convenience samples using residual sera obtained for routine clinical testing (screening or management) by two commercial laboratory companies
Ng et al.[24]	USA (California, Bay Area)	March	1000 blood donors in diverse Bay Area locations (excluding those with self-reported symptoms or abnormal vital signs)
Sood[22]	USA (California, Los Angeles)	10–14 April	Proprietary database representative of the county. A random sample of these residents was invited, with quotas for enrolment for subgroups based on age, sex, race and ethnicity distribution
Charnle et al.[23]	USA (California, San Francisco)	25–28 April	United States census tract 022901 population-dense area (58% Latin American) in San Francisco Mission district, expanded to neighbouring blocks on 28 April
Bendavid et al.[18]	USA (California, Santa Clara)	2–3 April	Facebook advertisement with additional targeting by zip code
Biggs et al.[25]	USA (Georgia, DeKalb and Fulton)	28 April–3 May	Two-stage cluster sampling design used to randomly select 30 census blocks in DeKalb County and 30 census blocks in Fulton County, with a target of seven participating households per census block
McLaughlin et al.[26]	USA (Idaho, Blaine County)	4–19 May	Volunteers who registered via a secure web link, using poststratification weighting to the population distribution by age and sex within each zip code
Bryan et al.[27]	USA (Idaho, Boise)	Late April	People from the Boise, Idaho metropolitan area, part of the Crush the Curve initiative
Menachemi et al.[28]	USA (Indiana)	25–29 April	Stratified random sampling among all persons aged ≥ 12 years using Indiana's 10 public health preparedness districts as sampling strata
Feehan et al.[29]	USA (Louisiana, Baton Rouge)	15–31 July	Representative sample in a method developed by Public Democracy
Feehan et al.[29]	USA (Louisiana, Orleans and Jefferson Parish)	9–15 May	Pool of potential participants reflecting the demographics of the parishes was based on 50 characteristics, then a randomized subset of 150 000 people was selected, and 25 000 were approached with digital apps, and 2640 were recruited

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Author	Country (location)	Dates	Sampling and recruitment
Rosenberg et al.[33]	USA (New York)	19–28 April	Convenience sample of people ≥ 18 years living in New York State, recruited consecutively on entering 99 grocery stores and through an in-store flyer
Meyers et al.[34]	USA (New York)	2–30 March (Columbia University Medical Center, New York City); 13–28 March (CareMount central laboratory)	Discarded clinical samples in Columbia Medical Center, New York City (n = 814 in 24 February–30 March, 742 of those in the period 2–30 March) and samples from CareMount central laboratory (960 samples on 13/14 March, 505 samples on 20/21 March, and 376 samples on 27/28 March) from its network of clinics in five counties north of New York City
Reifer et al.[35]	USA (New York, Brooklyn)	Early May	Patients seen in an urgent care facility in Brooklyn
Nesbitt et al.[36]	USA (Rhode Island)	27 April–11 May	Consecutive blood donors

COVID-19: coronavirus disease 19; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

* Sample collection time for some sub-cohorts may have exceeded 1 month, but more than half of the cases were already documented by polymerase chain reaction testing before any antibody testing and the last death occurred on 28 April.

Note: Some studies included additional data sets that did not fulfil the eligibility criteria (e.g. had sample size < 500 or were health-care workers) and they are not presented here.

Table 2. **Sample size, types of antibodies assessed and population size in the studies included to assess COVID-19 infection fatality rate, 2020**

Country (location)	Sample size[a], no.	Antibody	Population,[b,c] no.	% of population <70 years[c]
Argentina (Barrio Padre Mugica)[40]	873	IgG	49 983	99
Belgium[41]	3 391 (20–26 April)	IgG	11 589 623	86
Brazil (133 cities)[42]	24 995	IgG and IgM	74 656 499	94 (Brazil)
Brazil (Espirito Santo)[43]	4 608	IgG and IgM	4 018 650	94 (Brazil)
Brazil (Maranhao)[43]	3 156	IgG and IgM	7 114 598	92
Brazil (Rio de Janeiro), blood donors[44]	669 (24–27 April)	IgG and IgM	17 264 943	94 (Brazil)
Brazil (Rio Grande do Sul)[45]	4 500	IgG	11 377 239	91
Brazil (Sao Paulo)[46]	517	IgG and IgM	298 240 (6 districts)	94 (Brazil)
Canada (British Columbia)[47]	885	IgG, IgM and IgA	5 071 000	94
Chile (Vitacura)[48]	1 244	IgG and IgM	85 000	92 (Chile)
China, blood donors[49]				
Wuhan	930 (3–23 February)	IgG and IgM	11 210 000	93 (China)
Shenzhen	3 507 (24 February–15 March)	IgG and IgM	13 030 000	93 (China)
Shijiazhuang	6 455 (10 February–1 March)	IgG and IgM	11 030 000	93 (China)
China (Wuhan)[50]	1 401	IgG and IgM	11 080 000	93 (China)
China (Wuhan)[51]	1 186 (4–8 April)	IgG and IgM	11 080 000	93 (China)
China (Guangzhou), blood donors[52]	2 199	IgG, IgM and IgA	115 210 000 (Guangdong)	93 (China)
China (several regions)[53]				
Hubei (not Wuhan)	979	IgG and IgM	48 058 000	93 (China)
Chongqing	993	IgG and IgM	31 243 200	93 (China)
Sichuan	9 442	IgG and IgM	83 750 000	93 (China)
Guangdong	1 005	IgG and IgM	115 210 000	93 (China)
Croatia[54]	1 494	IgG and IgM	4 076 000	86
Denmark blood donors[55]	20 640	IgG and IgM	5 771 876	86
Denmark (Faroe Islands)[56]	1 075	IgG and IgM	52 428	88
France (Crepy-en-Valois)[57]	1 340	IgG	5 978 000 (Hauts-de-France)	89
France (Oise)[58]	661	IgG	5 978 000 (Hauts-de-France)	89
Germany (Gangelt)[59]	919	IgG and IgA	12 597	86
Germany (Frankfurt)[60]	1 000	IgG	2 681 000[d]	84 (Germany)
Greece[61]	6 586 (4 511 in April)	IgG	10 412 967	84
Hungary[62]	10 504	IgG (also had RT-PCR)	9 657 451	88
Iceland[63]	30 576	Pan-Ig	366 854	90
India (Mumbai)[64]	6 904 (4 202 in slums, 2 702 not in slums)	IgG	1 414 917 (705 523 in slums, 709 394 in non-slums) in the 3 ward areas	98
India (Srinagar)[65]	2 906	IgG	1 500 000	97
Islamic Republic of Iran (Guilan)[66]	551	IgG and IgM	2 354 848	95
Italy (Apulia), blood donors[67]	909	IgG and IgM	4 029 000	84
Japan (Kobe)	1 000	IgG	1 518 870	75 (Japan)
Japan (Tokyo)[68]	1 071	IgG	13 902 077	75 (Japan)
Japan (Utsunomiya City)[69]	742	IgG	518 610	75 (Japan)
Kenya, blood donors[70]	3 098	IgG	47 564 296	99
Luxembourg[71]	1 807	IgG and IgA[e]	615 729	90
Netherlands blood donors[72]	7 361	IgG, IgM and IgA	17 097 123	86
Netherlands (Rotterdam)[73]	729 (early April)	IgG	17 097 123 (Netherlands)	86
Pakistan (Karachi)[74]	1 004	IgG and IgM	16 700 000	98 (Pakistan)
Pakistan (urban)[75]	24 210	IgG and IgM	79 000 000 (urban)	98
Qatar[76]	937	IgG	2 800 000	99
Republic of Korea[77]	1 500	IgG	2 667 341	90 (Republic of Korea)
Spain[78]	61 075	IgG	46 940 000	85
Spain (Barcelona)[79]	874	IgG, IgM and IgA	7 566 000 (Catalonia)	86
Switzerland (Geneva)[80]	577 (20–27 April)	IgG	500 000	88

[continues...]

(...continued)

Country (location)	Sample size[a], no.	Antibody	Population,[b,c] no.	% of population < 70 years[c]
Switzerland (Zurich)[20]	1 644 patients (1–15 April)	IgG	1 520 968 (Zurich canton)	88
Switzerland (Zurich and Lucerne)[21]	1 640 blood donors (May)	IgG	1 930 523 (Zurich and Lucerne)	88
United Kingdom (England)[22]	105 076	IgG	56 287 000	86
United Kingdom (Scotland), blood donors[23]	500	IgG	5 400 000	88
USA (10 states)[24]				
Washington, Puget Sound	3 264	Pan-Ig	4 273 548	90 (Washington)
Utah	1 132	Pan-Ig	3 282 120	92
New York, New York City	2 482	Pan-Ig	9 260 870	89
Missouri	1 882	Pan-Ig	6 110 800	88
Florida, south	1 742	Pan-Ig	6 343 345	86 (Florida)
Connecticut	1 431	Pan-Ig	3 562 989	88
Louisiana	1 184	Pan-Ig	4 644 049	92
California, San Francisco Bay	1 224	Pan-Ig	2 173 082	90
Pennsylvania, Philadelphia	824	Pan-Ig	4 910 139	90
Minnesota, Minneapolis	860	Pan-Ig	3 857 479	90
USA (California, Bay Area)[25]	1 000	IgG	7 753 000	90
USA (California, Los Angeles)[26]	863	IgG and IgM	7 892 000	92
USA (California, San Francisco)[27]	3 953	IgG and RT-PCR	5 174 (in census tract 022 901)	95
USA (California, Santa Clara)[28]	3 300	IgG and IgM	1 928 000	90
USA (Idaho, Boise)[29]	4 856	IgG	481 567 (Ada County)	93
USA (Georgia, DeKalb and Fulton Counties)[30]	696	Total Ig	1 806 672	88 (Georgia)
USA (Idaho, Blaine County)[31]	917	IgG	23 089	92
USA (Indiana)[32]	3 629	IgG and RT-PCR	6 730 000	89
USA (Louisiana, Baton Rouge)[33]	130	IgG	699 200 (East Baton Rouge, West Baton Rouge, Ascension, Livingston)	92 (Louisiana)
USA (Louisiana, Orleans and Jefferson Parish)[34]	2 640	IgG	825 057	92 (Louisiana)
USA (New York)[35]	15 101	IgG	19 450 000	90
USA, New York[36]				
Columbia University Medical Center, New York City	742 (2–30 March)	IgG and IgM	9 260 870	89
CareMount central laboratory, five New York state counties	1 841	IgG and IgM	10 189 130 (New York state excluding New York City)	89
USA (New York, Brooklyn)[37]	11 092	IgG	2 559 903	91
USA (Rhode Island), blood donors[38]	1 996	IgG and IgM	1 059 000	88

COVID-19: coronavirus disease 19; Ig: immunoglobin; RT-PCR: real-time polymerase chain reaction.

[a] Dates in brackets are the specific dates used when seroprevalence was evaluated at multiple consecutive time points or settings.

[b] Some studies focused on age-restricted populations of the specific location under study, for example: people 1–70 years in the Denmark blood donor study (n = 3 800 000); people 18–79 years in the Luxembourg study (n = 483 000); people < 70 years in the Netherlands blood donor study (n = 13 745 768); people ≥ 18 years in the New York state study (n = 15 280 000); people > 19 years in the Utah population of the 10-state United States of America study (n = 2 173 082); people ≥ 18 years in Blaine County, Idaho (n = 17 611); people 15–64 years in the Kenya blood donor study (n = 27 150 165); people > 14 years living in private premises in Hungary (n = 8 281 870); people > 18 years (n = 631 186) in Baton Rouge, Louisiana; people 18–65 years working in urban locations in Pakistan (n = 22 100 000); and people > 18 years in Srinagar District, India (n = 1 000 000). In this table and subsequent analyses, the entire population in the location is considered for consistency across studies.

[c] Information in parentheses specifies the population.

[d] When the population of the relevant location was not given in a specific study, information on recent estimates of the pertinent population was obtained by standard online sources such as: populationpyramid.net, worldpopulationreview.com, worldometers.info/coronavirus, and Wikipedia.

[e] Participants were recruited from a large number of districts, but most districts had very few participants; here I included the population of the nine districts with > 1:10 000 sampling rate (846/1 000 participants came from these nine districts).

[f] Considered positive if both IgG and IgA were positive; in the other studies, detection of any antibody was considered positive.

EXHIBIT "FFF"

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MINI REVIEW OPEN ACCESS

COVID-19 Case Fatality Rate: Misapprehended Calculations

Krishna Prasad Acharya[1], Ranjit Sah[2]*, Supram Hosuru Subramanya[3], Dinesh Neupane[4], Binay Kumar Panjiyar[2], Ali A. Rabaan[5], Kranti Suresh Vora[6,7], SenthilKumar Natesan[6], Kuldeep Dhama[8] and Alfonso J. Rodriguez-Morales[9,10,11]*

[1]Animal Quarantine Office-Kathmandu, Department of Livestock Services, Kathmandu, Nepal.
[2]Tribhuvan University Teaching Hospital, Institute of Medicine, Kathmandu, Nepal.
[3]Manipal College of Medical Sciences, Pokhara, Nepal.
[4]John Hopkins Bloomberg School of Public Health, Baltimore, USA.
[5]Molecular Diagnostic Laboratory, Johns Hopkins Aramco Healthcare, Dhahran, Saudi Arabia.
[6]Department of Infectious Diseases, Indian Institute of Public Health Gandhinagar, Lekawada, Gandhinagar, Gujarat, 382042, India.
[7]Institute of Health Research, University of Canberra, ACT 2617, Australia.
[8]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar 243 122, Bareilly, Uttar Pradesh, India.
[9]Public Health and Infection Research Group, Faculty of Health Sciences, Universidad Tecnologica de Pereira, Pereira, Risaralda, Colombia.
[10]Grupo de Investigacion Biomedicina, Faculty of Medicine, Fundacion Universitaria Autonoma de lasAmericas, Sede Pereira, Pereira, Risaralda, Colombia.
[11]School of Medicine, Universidad Privada Franz Tamayo (UNIFRANZ), Cochabamba, Bolivia.

*Correspondence: ranjitsah57@gmail.com; arodriguezm@utp.edu.co

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) pandemic, posed by saver acute respiratory syndrome coronavirus – 2 (SARS-CoV-2), is killing tens of thousands of people globally. It is disrupting societies and economies across the globe with widespread uncertainty. As of August 10, 2020, the total cases of COVID–19 have risen above 20 million and death tolls to nearly 0.74 million[1] with the case fatality rate (CFR) of 3.65[1]. The CFR indicates proportion of persons who die from a specified disease out of all individuals diagnosed positive over a specific period. Thus, the CFR is based on the number of deaths and confirmed cases. However, number of confirmed cases is not including asymptomatic cases or patients with mild symptoms, which in turn may not get tested and may not reflect the actual CFR. CFR is also proportionately variable depending on the level of testing done in a population or a country. When testing is done very extensively covering large population including symptomatic patients and non-symptomatic contacts, the denominator of the CFR calculation increases which will reduce the CFR compared to limited testing carried out only on symptomatic patients. Huge variation in the CFR was observed between different countries. The CFR in early stage of the epidemic was 0.15% (95% CI: 0.12-0.18%) in China excluding Hubei and 1.41% (95% CI: 1.38-1.45%) in Hubei province excluding Wuhan city, and it was 5.25% (95% CI: 4.98-5.51%) in Wuhan[1] named COVID-19, hit a major city of China, Wuhan in December 2019 and subsequently spread to other provinces/regions of China and overseas. Several studies have been done to estimate the basic reproduction number in the early phase of this outbreak, yet there are no reliable estimates of case fatality rate (CFR. The CFR rate of was found very high among countries such as France, Italy, Spain and USA viz., 13.7%, 13.5%, 10.2%, and 5.7%, respectively[4]. Fig. 1 shows CFR of world and selected countries as of 22nd July 2020. It highlights the differences among countries while noting changes in the protocols of testing (www.ourworlddata.org/coronvirus).

The CFR is also influenced by the demographic nature of the population including

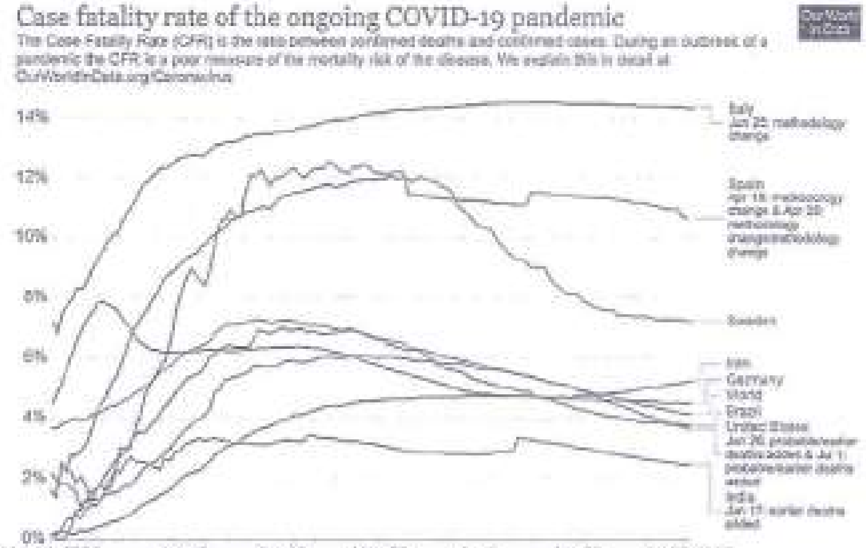

Fig. 1. COVID-19 Case Fatality Rates of World and selected countries as of 22nd July 2020. Assesed from https://ourworldindata.org. on 23rd July 2020.

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factors such as age and sex of population and underlying co-morbidities. Meta-analysis of the 10 published data showed a fatality rate of 5%[4]. Different approaches for estimating the CFR accurately has also been suggested[5-8].

Moreover, confirmed cases included in calculating CFR are derived from the cases which were reported to hospitals or laboratory testing at other places, which is not the accurate representation of total cases of COVID-19. This method is likely to miss the cases not reported to the hospitals because of many mild and asymptomatic cases. Therefore it becomes crucial to assess exact fatality rate by dividing total number of deaths to total number of confirmed cases including patients with mild symptoms, symptomatic as well as asymptomatic cases diagnosed through extensive surveillance using reliable accurate testing[9]. Currently, most countries test only the symptomatic patients with influenza like respiratory symptoms using the RT-PCR method of detecting the SARS COV-2 genome.

Onder et al.,[9] identified the fatality rates in COVID-19 patients varying from 1% to 7%; however, this information must be interpreted with caution because these calculations probably are misapprehended. The variation of CFR might differ from countries to countries, which bring discrepancies in determining overall fatality rates. For example, the massive screening of COVID-19 with very high tests per million of the population was performed in the entire community in South Korea, Singapore, Hongkong, and New Zealand, which had higher chances of detection of mild and asymptomatic cases with the inclusion of both mild and asymptomatic cases in the denominator. That has resulted in less than a 3% fatality rate[3,10,11]. Conversely, in countries like Italy, Spain, France, screening for COVID-19 was performed only to hospitals (having low tests per million population) attending symptomatic patients and high-risk group[12], which ultimately decreased the total count of actual cases; thus, more than 10% CFR was reported as of July 8, 2020[10,11].

Spychalski and colleagues suggest CFR overestimation owing to under-testing and time-lag bias[13]. It is, therefore, very crucial to understand the difference between the CFR and infection fatality rate (IFR) before drawing any such conclusions. CFR is the proportion of the number of deaths divided by all confirmed cases by molecular detection method. Infection fatality rate (IFR) is proportion of deaths divided by all infection (includes mild and even asymptomatic individuals). Considering the current selection bias testing strategies, that is, the polymerase chain reaction (PCR) based test is mainly utilized for the detection of individuals with coronavirus symptoms and high-risk patients. Understanding the true IFR has strategic significances for designing control measures for COVID-19. A realistic estimation of CFR is even more important for low- and middle-income countries because of their limited testing capacity, deaths of older people usually occur at home, and there is no proper system to assign the cause of mortality. The Centre for Evidence-Based Medicine (CEBM) at University of Oxford[10] estimates the CFR globally at 0.51% and IFR between 0.1% to 0.26%, with all caveats concerning that.

Though the significant causes of death include respiratory failure, shock, multiple organ failure, actual death process in COCID-19 patients is still poorly understood[14]. Moreover, the effect of the secondary bacterial infection triggering the death of patients is also not well-defined, highlighting that deaths of patients were not just confined to viral infections but also various other parameters. In marginalized and resource-driven countries, they lack medicines, hospital beds, intensive care, mechanical ventilation, extracorporeal membrane oxygenation, vasopressors, and renal replacement. Therefore, the actual death may not be attributed to COVID-19, but due to the lack of necessary equipment and facilities. Also, in high infection zones, patients with trauma or acute brain injury might be admitted to the ICU, who later acquired COVID-19 during the hospital stay and died after that. These deaths are still accountable to COVID-19[13]. An autopsy study on COVID-19 patients showed advanced diffuse alveolar and superimposed bacterial pneumonia along with changes in organs such as liver and heart[14,15].

Moreover; some patients are on the verge of death due to the elderly or some other underlying diseases such as cancer or terminal organ failure. In such cases, the COVID-19 infection plays a minimal role in the deaths. Therefore, it becomes difficult to affirm the CFR due to COVID-19 among patients with pre-

existing comorbidities / illnesses. A study from NewYork city showed that age-adjusted CFRs in cancer patients were significantly higher than non-cancer ones. It showed a CFR of 37% for hematologic malignancies while 25% for solid cancers. The increase in mortality among cancer patients due to COVID-19 was markedly related with older age, other comorbidities, elevated profiles of biomarkers such as D-dimer, lactate dehydrogenase, and lactate[16].

Mortality in mainland China has shown the highest death rates (10.5%) in patients with cardiovascular disease, then by diabetes (7.3%), chronic respiratory disease (6.3%), hypertension (6%), and cancer (5.6%)[17]. The study also found that the highest health risk and mortality (14.8%) were detected in older adults aged over 60 years, while no death was reported in the 0-9 years age group[18]. However, patients with no pre-existing conditions had fewer death rates (0.9%). Similarly in the USA, data collected from January 22 to May 30 , 2020, showed the highest mortality rate of 28.7% in the COVID-19 patients ≥ 80 years old, whereas 0.1% deaths in the patients ≤ 19 years old[19]. Likewise, a mortality rate of 19.5% (38,812/198,879) had been reported in patients with pre-existing illness whereas only 1.6% (1,431/88,441) of COVID-19 patients with no preexisting health illness have died[19]. Similar higher mortality rate of 42.5% (7558/36398) among the COVID-19 patients with pre-existing illness has also been reported by Khan and his colleagues in their systematic review on COVID-19 patients[20]. These results vividly illustrate that the patients are dying due to pre-existing conditions rather than COVID-19. The actual reporting is lacking since the data regarding pre-existing conditions of COVID-19 patients are confined to very few countries. Most of the reported COVID-19 cases are from the hospitals, which is likely to bias the cases that are not hospitalized. WHO–China Joint Mission on COVID-19 reported that 80% of 55,924 laboratory-confirmed COVID-19 patients in China to February 20, 2020, revealed mild disease, both pneumonia and non-pneumonia, whereas 13.8% reported a severe illness and 6.1% reached to critical stage demanding intensive care[18]. Considering the fact that many observed mild conditions, highest death rates were reported from older people, also those with the pre-existing condition, we can infer that death rates of COVID-19 patients are over-estimation than actual. Therefore, it seems like most of the patients are dying "with" COVID-19 rather than "of" COVID-19.

In conclusion, CFR of various countries is variable and it is difficult to judge the change in same. There are several caveats in estimating the CFR currently as there is no uniform approach for SARS-CoV-2 testing for diagnosis of COVID-19. Such lack of uniformity in testing is also contributing to the wide variation in CFR reported from different countries. The patients tested today positive may counted in the confirmed cases, however, their fatality may not be included in the time point when the CFR is estimated. The exclusion of mild or non-symptomatic patients from testing will also affect the CFR. The number of tests done for a specific number of people in a population should also be considered. All these factors can affect the CFR estimation. Underestimation of CFR may provide a false sense of security and overestimation may lead to panic among people. Therefore, there is a need to follow a standard approach for accurate estimation of CFR which is acceptable across different geographical regions. Based on evidence examined it is suggested to use the total confirmed cases (symptomatic as well as asymptomatic cases) to interpret the actual CFR through an intensive population-based surveillance system using the standard scientific approach. Moreover, it is imperative to investigate the reason and process of deaths of patients, including aspects such as lack of treatment facilities, beds, personnel, equipment, pre-existing conditions (patient's age, pre-existing diseases), and the prevalence of antimicrobial resistance in the region or other demographic information. CFR is an effective measure of diagnostic and treatment capacity of a health system along with surveillance ability. Accurate calculation of CFR is need of the hour to measure the progress made and future actions at the country and global levels.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All the listed author(s) have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

Not applicable.

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EXHIBIT "GGG"

Covid-19 CFR and IFR Confused

Source: Watts Up With That?

Link: https://wattsupwiththat.com/2020/09/08/covid-19-cfr-and-ifr-confused/

Written By: Charles Rotter

Published on: September 8, 2020

New article in journal published by Cambridge Univ Press says that testimony to House Oversight Committee in March 2020 mixed up case fatality rate (CFR) and infection fatality rate (IFR) for influenza, resulting in major error. (I report this w/o parsing)

Disaster Medicine and Public Health Preparedness

Article Metrics

Accepted manuscript August 2020 , pp. 1-24

Public health lessons learned from biases in coronavirus mortality overestimation

Ronald B. Brown

DOI: https://doi.org/10.1017/dmp.2020.268 Published online by Cambridge University Press: 12 August 2020

Abstract

In testimony before U.S. Congress on March 11, 2020, members of the House Oversight and Reform Committee were informed that estimated mortality for the novel coronavirus was ten-times higher than for seasonal influenza. Additional evidence, however, suggests the validity of this estimation could benefit from vetting for biases and miscalculations. The main objective of this article is to critically appraise the coronavirus mortality estimation presented to Congress. Informational leads from the World Health Organization and the Centers for Disease Control and Prevention are compared with coronavirus mortality calculations in Congressional testimony. Results of this critical appraisal reveal information bias and selection bias in coronavirus mortality overestimation, most likely caused by misclassifying an influenza infection fatality rate as a case fatality rate. Public health lessons learned for future infectious disease pandemics include: safeguarding against research biases that may underestimate or overestimate an associated risk of disease and mortality; reassessing the ethics of fear based public health campaigns; and providing full public disclosure of adverse effects from severe mitigation measures to contain viral transmission.

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2/ author Ronald Brown (of University of Waterloo in Ontario) said that House Committee was told that estimated mortality was 10 times higher than seasonal influenza. This was prime argument for lockdown.

involving a U.S. government agency: On March 11, 2020, the U.S. Congress House Oversight and Reform Committee received information from the National Institute of Allergy and Infectious Diseases (NIAID) concerning the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and coronavirus-disease 2019 (COVID-19).[3] Based on the data available at the time, Congress was informed that the estimated mortality rate for the coronavirus was ten-times higher than for seasonal influenza, which helped launch a campaign of social distancing, organizational and business lockdowns, and shelter-in-place orders.

3/ Brown observed that New England Journal article, just prior to House testimony, had (incorrectly) said that CFR for seasonal influenza was 0.1% whereas 0.1% is actually the value for IFR (WHO).

Almost as a parenthetical afterthought, the NEJM editorial inaccurately stated that 0.1% is the approximate case fatality rate of seasonal influenza. By contrast, the World Health Organization (WHO) reported that 0.1% or lower is the approximate influenza infection fatality rate,[5] not the

case fatality rate. To fully appreciate the significance of discrepancies in fatality rate usage by NIAID, the CDC, and the WHO, brief definitions of relevant epidemiological terms follow.

4/ Brown observed that CFR and IFR have different definitions in epidemiology and gives a lengthy exposition.

as COVID-19 according to standard criteria in a case definition.[7] A case fatality rate (CFR) is defined as the proportion of deaths among confirmed cases of the disease. CFRs indicate the disease severity, while an infection fatality rate (IFR) is defined as the proportion of deaths relative to the prevalence of infections within a population.[8] IFRs are estimated following an outbreak, often based on representative samples of blood tests of the immune system in individuals exposed to a virus. Estimation of the IFR in COVID-19 is urgently needed to assess the scale of the coronavirus pandemic.[9]

5/ Brown curiously didn't identify the person who, according to Brown, gave the wrong benchmark information on fatality rates for seasonal influenza to Congress, linking to CSPAN in his footnotes.

3. Fifer J. House Oversight and Reform Committee Hearing on Coronavirus Response, Day 1. C-SPAN Web site. https://www.c-span.org/video/?c4860450/user-clip-dr-anthony-fauci-addresses-covid-19-mortality-rate%20. Published March 11, 2020. Accessed April 30, 2020.

6/ the expert who, according to Brown, made the 10x error in testimony to Congress is, by now, well known to all of us. It was, needless to say, Anthony Fauci.

7/ I urge readers to read original article. I am not personally familiar with definitional distinctions emphasized by Brown and haven't verified his claims. Brown is at reputable university and journal is by reputable publisher (Cambridge Univ).

EXHIBIT HHH

Mumbai: BMC to conduct 47,000 random Covid tests everyday, refusing one can land you in trouble.

Source: India Today

Link: https://www.indiatoday.in/cities/mumbai/story/mumbai-random-covid-corona-testing-crowded-places-bmc-rules-cost-punishment-1781633-2021-03-20

Author: Pankaj Upadhyay

Published on: March 21, 2021 00:33 IST

The BMC has decided to carry out random Covid-19 tests in crowded places across Mumbai. Refusing these Rapid Antigen Tests will land one in trouble.

Mumbai's civic body, the BMC has decided to conduct Covid-19 tests at crowded places in light of the recent surge in cases. On Saturday, the BMC (Brihanmumbai Municipal Corporation) issued guidelines for the Rapid Antigen Testing (RAT) that will be carried out at crowded places.

The latest order by the BMC in this regard says Covid-19 tests will be conducted at malls, railway stations (for inbound trains), MSRTC Bus Depots, khau gullies, hawkers, market places, tourist spots, and various government offices.

Testing for Covid-19 will be done randomly, the order states.

The BMC has also clarified that the Covid-19 tests will be carried out without the consent of citizens who are present at these crowded places.

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"If the citizen refuses the test, it would amount to an offense under the Epidemic Act, 1897. Hence, the action shall be initiated under the Epidemic Act, 1897 against the offender," the BMC has said.

Cost of testing at malls to be borne by visitor

The civic body intends to test at least 400 people visiting malls each day. For the 27 malls in Mumbai, the BMC will conduct 10,800 random tests each day.

The cost incurred for carrying out the Rapid Antigen Test shall be borne by the person concerned visiting the mall, the BMC says.

If an individual entering a mall is asked to undergo the Covid-19 test and refuses to pay for the same, it would amount to an offence under the Epidemic Act. Action will then be initiated against the person.

Each ward given Covid-19 testing target

Apart from malls, the BMC will bear the cost of Covid-19 testing at other crowded places.

Similarly, at least 1,000 passengers of inbound trains will be tested for Covid-19 at each railway station in Mumbai. A similar pattern will be followed at bus depots operated by the Maharashtra State Road Transport Corporation (MSRTC).

In addition to crowded places, the BMC has also instructed each ward to carry out at least 1,000 Rapid Antigen Tests at restaurants, market places, government offices and beaches among other crowded places.

The BMC has set a target of 47,000 random Covid-19 tests in crowded places across Mumbai every day. Each of Mumbai's 24 wards has also been given a testing target.

EXHIBIT "III"

Why Mumbai is relying more on antigen tests than RT-PCR as it doubles Covid testing.

Source: The Print

Link: https://theprint.in/india/why-mumbai-is-relying-more-on-antigen-tests-than-rt-pcr-as-it-doubles-covid-testing/632919/

Author: Manasi Phadke

Published on: 02 April, 2021

BMC has instructed all its 24 administrative wards, 7 railway stations, bus depots, and 27 malls in the city to randomly conduct antigen tests on visitors to check the rise on cases.

Mumbai: Mumbai, which is once again struggling to contain its Covid caseload, has nearly doubled the number of tests in the last one month — from about 20,000 tests a day to over 40,000.

However, the percentage of RT-PCR tests, considered to be the gold standard in testing for Covid, has plummeted to a seven-day average of 48 per cent in the past week, from 69 per cent in the last week of February.

Civic officials in Mumbai, however, say the emphasis on more rapid antigen tests (RATs) is deliberate because it is the city's floating population that needs to be kept in check in the current wave.

Over the past seven days from 24 March to 30 March, the Brihanmumbai Municipal Corporation (BMC) tested 41,271 samples a day, compared to an average of 20,714 between 22 February and 28 February.

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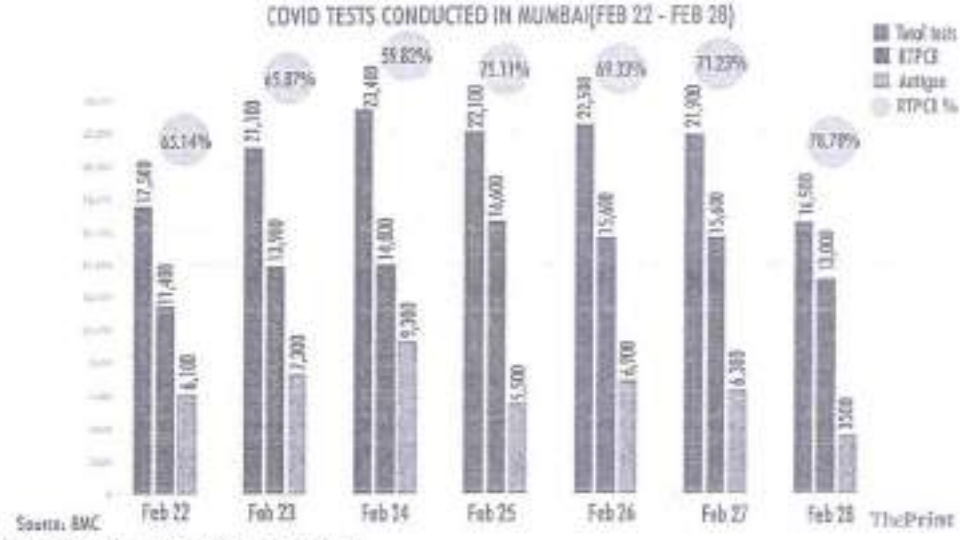
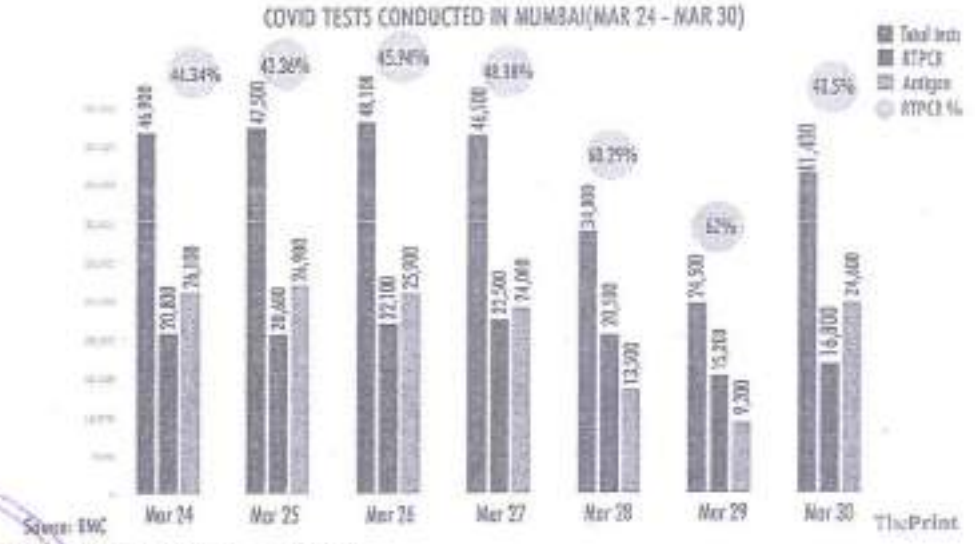
Most of this rise has come from aggressively increasing RATs, which give quick results (within 30 minutes) but are less reliable than RT-PCR, the results of which can take up to a day.

The Centre has time and again advised states to ensure that its cities and districts are conducting at least 70 per cent tests through RT-PCR.



Graphic by Ramandeep Kaur | ThePrint



Graphic by Ramandeep Kaur | ThePrint

Mumbai has so far recorded 4,23,360 Covid positive cases, of which 55,005 are active. The city has been recording new peaks in its battle against the virus almost every day. On 1 April, it recorded its highest ever daily caseload with 8,646 cases.

'Can't target floating population with RT-PCR'

The BMC has instructed its 24 administrative wards, seven railway stations and the Maharashtra State Road Transport Corporation's bus depots, as well as private malls in the city, to randomly test visitors for Covid.

According to officials, Mumbai has a very large floating population that comes for work and needs to be tested, but can't be targeted with RT-PCR tests.

"In Mumbai, the floating population is a very high in number. These are the people traveling by local trains, visiting malls and restaurants. They quickly vanish into the crowd and it is difficult to trace them," said Suresh Kakani, additional municipal commissioner at BMC.

"People are wary of giving out their addresses and contact numbers when they visit crowded public places, so an RT-PCR here doesn't help. With an antigen test, we get results in 20-30 minutes and take immediate action."

In the last week of February, the BMC was conducting about 14,300 RT-PCR tests a day. While this number has grown to 19,788 now, most of the increase in the daily testing figures has come from the less reliable RATs.

On an average, in the past week, the BMC has conducted 21,457 antigen tests a day as against 6,414 a day about a month ago between 22 February and 28 February.

Kakani said, "It is not that we haven't increased our RT-PCR testing, which has also shot up from about 15,000 a day last month to 20,000 now. We have the capacity and infrastructure to increase it further, but we are using RT-PCR only for persons with permanent establishments such as shop owners."

On 20 March, the BMC instructed all its 24 administrative wards to randomly conduct antigen tests on visitors. It also drew up a list of 27 malls in the city and gave each a target of conducting 400 RATs every day.

Similarly, seven railway stations were asked to randomly test 1,000 inbound passengers each day, while bus depots of the MSRTC were directed to test at least 1,000 passengers a day.

Each of the BMC's 24 administrative wards have been given a target of testing 1,000 persons a day, focusing on crowded places such as restaurants, hawkers, market places and tourist attractions.

EXHIBIT "JJJ"

Daily Covid-19 tests must be raised to 40,000 in Mumbai: Iqbal Singh Chahal.

Source: The Times of India.
Link: https://timesofindia.indiatimes.com/city/mumbai/daily-covid-19-tests-must-be-raised-to-40000-in-mumbai-iqbal-singh-chahal/articleshow/82372451.cms
Published On: May 3, 2021

MUMBAI: The number of daily Covid-19 tests in Mumbai needs to be increased to at least 40,000, municipal commissioner Iqbal Singh Chahal said on Monday.

He said the number of tests being conducted per day fell from over 50,000 earlier to 38,000 on May 1 and further to 28,000 on May 2.

Chahal said this number could fall further on weekends.

"We need to increase the testing to 40,000 per day at least, if not more," Chahal said in a message and appealed to the people to come forward for testing.

He said the BMC has been consistently conducting maximum tests to "flush out coronavirus from Mumbai's environment".

During the first wave of the pandemic, the highest single-day testing figure for Mumbai was 24,500 between March 1, 2020, and February 10, 2021, he said.

"During the second wave, testing in the city has more than doubled and touched 56,000 a day last month. Our average daily testing in April was nearly 44,000 per day," Chahal added.

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The municipal commissioner had last week said the Covid-19 test positivity rate in Mumbai had dropped below 10 per cent to 9.94 per cent on April 29, when 4,328 people tested positive for Covid-19 out of the 43,525 samples that were examined.

On Monday, he attributed the fall in the positivity rate to the aggressive testing policy being adopted by the Brihanmumbai Municipal Corporation (BMC).

"This (BMC's testing policy) might have reduced the natural demand for home collection of swabs from our citizens in the last few days," Chahal said.

The test positivity rate (TPR) is defined as the proportion of samples that return positive among the total tested.

As per the BMC's data, a total of 54,90,241 tests have been conducted so far in Mumbai.

Mumbai's overall infection tally stood at 6,56,204 as of May 2 and the death toll 13,330, as per the civic body.

There are 57,342 active patients in the Maharashtra capital as of Sunday.

EXHIBIT "KKK"

Covid-19: Big dip in Mumbai cases, the worst may be over.

Source: The Times of India.

Link: https://timesofindia.indiatimes.com/city/mumbai/covid-19-big-dip-in-mumbai-cases-the-worst-may-be-over/articleshow/82379993.cms

Author: Malathy Iyer & Bhavika Jain

Published on: May 04, 2021.

MUMBAI: Almost a month after Mumbai registered its worst single-day tally of 11,206 Covid-19 cases, the graph of the second wave dipped sharply on Monday, with 2,624 cases detected in a 24-hour period. While the 23,542 tests done to detect these cases were lower than the city's daily average of 44,000 (in April), officials said the worst appears to be over as far as the second wave is concerned.

However, the daily toll continued to be high with 78 deaths recorded on Monday, showing a daily case fatality rate (CFR) of 2.9%, almost twice the daily reported last week. BMC officials have maintained that the daily toll will plateau this week and start dropping thereafter. The city's tally so far is 6.58 lakh cases and 13,372 deaths.

Similarly, Maharashtra reported a 15% dip in daily detection and fatalities on Monday, the second consecutive day of a decline in cases. The state reported 48,621 cases and 567 deaths, taking total cases to 47.7 lakh while the toll touched 70,851.

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Officials said daily detections were down owing to fewer tests over the weekend and fatalities had dipped due to delay in updating data. The state conducted 2.1 lakh tests in the last 24 hours, a dip from the 2.5-2.7 lakh tests it conducts during the weekdays.

"Mumbai's daily positivity rate is low (11.3%) though we did fewer tests over the weekend," said additional municipal commissioner Suresh kakani. In contrast, positivity rate on April 4 was 30%.

BMC commissioner I S Chahal, in a statement on Monday, said "our aggressive testing policy has resulted in reduction of positivity rate."

However, he noted that there has been a reduction in demand for tests in the last few days. "It has been the consistent policy of MCGM to do maximum testing to flush out coronavirus from Mumbai's environment" he said.

EXHIBIT "LLL"

800

As cases drop, number of covid tests halved in Mumbai

Source: Hindustan Times

Link: https://www.hindustantimes.com/cities/mumbai-news/as-cases-drop-number-of-covid-tests-halved-in-mumbai-101621278073321.html

Published on: May 18, 2021.

With a constant decrease in the number of daily Covid-19 cases reported in Mumbai for the past two weeks, the tests conducted in the city have also almost halved

With a constant decrease in the number of daily Covid-19 cases reported in Mumbai for the past two weeks, the tests conducted in the city have also almost halved. While an average of 50,000 tests was conducted per day in Mumbai during mid-April, they have now come down to an average of 20,000-25,000 daily.

Officials have attributed the decrease in the number to fewer people coming forward for a test. Experts believe that Mumbai needs to continue testing more people. They also believe that it is too early to tell if the second wave is declining, but the city must not assume that the threat of Covid-19 has passed.

About 35%-40% of the tests conducted per day are rapid antigen tests, which on an average have a lower positivity rate than RT-PCR (reverse transcription polymerase chain reaction) tests.

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On Sunday, Mumbai conducted only 17,640 tests, the results of which were revealed on Monday. Of these, Mumbai reported 1,232 cases with a positivity rate of 6.9%. Mumbai also reported 48 deaths, with a case fatality rate of 3.8%.

So far, Mumbai has conducted a total of 5,916,245 tests. The total number of positive cases in Mumbai is 689,062, with a cumulative positivity rate of 11.9%.

The average positivity per day in Mumbai has now dropped to 6% to 8%, down from 20% and above seen in mid-April. Positivity rate is the number of positive Covid-19 cases reported as a percent of the total tests done.

By mid-March, Mumbai had started conducting at least 50,000 tests a day and on some occasions up to 55,000 tests. Experts and authorities believe the second wave started around March 11. On April 4, Mumbai reported the highest number of Covid-19 cases in a single day – 11,204 – of 51,319 tests conducted, revealed BMC data. By April 20, the daily tests dropped to below 40,000. For the past five days, Mumbai has been conducting lower than 30,000 tests per day. However, on all these days Mumbai reported less than 2,000 cases, with a positivity rate of 6% to 8%.

The steady decline in cases reported per day is in no way indicative of the larger picture that the second wave may soon pass, experts say.

Dr Om Shrivastav, who is a part of Maharashtra government's Covid task force, said, "We can't look at Mumbai in isolation. The cases have come down, but cases in the rest of the state or country have not. So what happens when restrictions are lifted? To say that the second wave is declining, we should wait till the end of September at least and then take a relook at the figures and decide."

Experts also believe that the city needs to keep up high testing numbers despite the lower positivity rate. Dr Rahul Pandit, another member of the task force,

EXHIBIT "MMM"

Maharashtra sees spike in single-daily Covid-19 cases as testing increases.

Source: Hindustan Times

Link: https://www.hindustantimes.com/cities/mumbai-news/maharashtra-sees-spike-in-single-daily-covid-19-cases-as-testing-increases-101624983236317.html

Published on: Jun 29, 2021

- *Tuesday's daily spike marks an increase over the 6,727 positive Covid-19 cases Maharashtra had recorded on Monday*

Maharashtra recorded 8,085 new cases of Covid-19 on Tuesday, taking the state-wide tally to 6,051,633. The death toll reached 121,804 after 231 more individuals succumbed to the infection. As many as 8,623 people made a full recovery in the previous 24 hours, taking the total recoveries in the state to 5,809,548, according to the state health bulletin.

The capital city of Mumbai reported 556 fresh cases of Covid-19 on Tuesday, the city has recorded 721,516 cases since the pandemic started. An addition of 12 fatalities pushed the death toll to 15,426. Breaking with Maharashtra's trend, Mumbai reported a decline in its daily tally. The city had reported 611 cases on Monday.

Tuesday's daily spike marks an increase over the 6,727 positive cases the state had recorded on Monday. The state also witnessed a drop in the number of daily

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recoveries, after recording 8,623 recoveries on Tuesday as opposed to the 10,812 recorded a day prior.

The state tested 190,140 samples for coronavirus marking an increase from the 166,163 samples tested a day before. Tuesday marks the sixth consecutive day of the state, which was once reeling under a vicious second wave, recording less than 10,000 cases a day.

The state has been trying to ramp up vaccination drive before a possible third wave hits. Minority Affairs Minister Nawab Malik said that "If Maharashtra gets adequate stock of vaccine doses, the state can vaccinate its entire population in two months," He also told reporters that of the 21 people infected with the Delta plus variant only one had been administered a single shot of the Covid-19 vaccine.

EXHIBIT "NNN"

With a drop in testing over the weekend, fewer Covid-19 detections in Maharashtra & Mumbai

Source: The Times of India
Link: https://timesofindia.indiatimes.com/city/mumbai/mumbai-with-a-drop-in-testing-over-the-weekend-fewer-covid-19-detections-in-state-city/articleshow/85193228.cms
Published on: Aug 10, 2021

MUMBAI: Maharashtra and Mumbai reported a significant dip in daily detections in Covid cases on Monday. Maharashtra reported 4,505 new cases, the lowest in nearly six months, since February 16 (3,663). Mumbai reported 208 cases on Monday, lowest since April 2020 (183).

Officials attributed the dip to a drop in testing over the weekend. Maharashtra conducted only 1.5 lakh tests on Sunday, as against over 2 lakh on weekdays.

The state and city also reported a dip in fatalities. Maharashtra reported 63 deaths, of which three were in Mumbai. Cases in the state rose to 63.5 lakh and toll touched 1,34,064. Mumbai on Monday reported 7.3 lakh cases and 15,954 deaths. As per civic data of 4,000-odd active cases, 2,700 are symptomatic, while 1,400 are asymptomatic. Critical cases dropped to 475. Since the state has allowed relaxations, civic hospitals have been told to stay prepared for an increase in admissions. SevenHills Hospital dean Dr Balkrishna Adsul said their current occupancy was little over 400, out of the 1,850 beds. "We are ready. Besides adults, we created an-80 bed pediatric isolation ward and 20-bed paediatric ICU," he said, adding that the hospital is admitting 40-50 patients daily.

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EXHIBIT "OOO"

806

As tests increase, Covid cases tick up in Mumbai, positivity still under 1%

Source: The Times of India

Link: https://timesofindia.indiatimes.com/city/mumbai/mumbai-as-tests-increase-cases-tick-up-in-city-ity-still-under-1/articleshow/85440355.cms

Published on: August 19, 2021

MUMBAI: Daily case detections and deaths showed an upward tick in the state as well as the city on Wednesday.

After reporting under-4,500 cases for the last two days, Maharashtra listed 5,132 cases and 158 deaths, including five in Mumbai. The city added 285 patients testing positive for Covid as against 196 on Tuesday. The authorities are keep a watch on the numbers since train travel and other relaxations were granted since Sunday.

In KEM Hospital in Parel, the Covid ward has only 10 patients and there is no admission on some days. "The positivity rate in KEM is below 1% and the general observation is that the severity of Covid is lesser now," said KEM Hospital dean Dr Hemant Deshmukh. The hospital, reserved for severe patients, used to have over 500 Covid patients at any given time during the second wave.

Across Mumbai, the daily positivity rate is 0.7%. After two days of conducting fewer than 35,000 tests daily, the BMC ran over 38,703 tests in a single day. The active cases in the city are below 3,000.

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The total caseload in the state rose to 64.1 lakh and deaths touched 1.4 lakh, of which 7.4 lakh cases and 15,930 deaths were in Mumbai.

At the state level, 15 districts have less than 100 active cases. Of them, six — Dhule, Washim, Yavatmal, Bhandara, Wardha, and Gondia — have less than 10. Officials said the positivity rate of Kolhapur and Ratnagiri have dropped below the state's average weekly positivity rate.

Eye on third wave, govt to spend Rs 22.5 crore on 25 lakh RAT test kits.

The public health department is spending Rs 22.5 crore to procure 25 lakh Rapid Antigen Test kits, reports Prafulla Marpakwar. "Rapid Antigen Test will be useful for treatment as well as prevention of ," said its order. The funds have been sanctioned by the relief and rehabilitation department.

Former medical education director T P Lahane did not rule out a third wave. "If we study the situation across the world, the possibility of a third wave is not ruled out. However, it will be difficult to predict its intensity," he said.

"In certain countries, the third wave hit areas where the number of Covid patients was less than 1%. We do not know if a similar situation will be seen in Maharashtra. Currently, we have 19 districts where the number of patients is less than 1%, while in thhe remaining districts, the percentage ranges between 2-2.5%," said Lahane.

EXHIBIT "PPP"

Maharashtra: Covid dip likely due to weekend effect on tests.

Source: The Times of India.

Link: https://timesofindia.indiatimes.com/city/mumbai/mumbai-covid-dip-likely-due-to-weekend-effect-on-tests/articleshow/85751885.cms?utm_source=contentofinterest&utm_medium=text&utm_campaign=cppst&peode=461

Published on: August 30, 2021.

MUMBAI: After two consecutive days of an increase in new Covid-19 cases, the state and city saw a marginal drop in new detections. The decline in cases is likely due to fewer tests performed during the weekend.

CORONAVIRUS UPDATE

As of August 29, Sunday

		Maha	Mumbai
New	Cases	4,666	345
	Deaths	**131**	**2**
Total	Cases	64,56,939	7,43,499
	Deaths	**1,37,157**	**15,974**
Discharged		62,63,416	7,22,039

MAHARASHTRA MUMBAI

Case fatality rate (in %) 2.12 2.14

52,844 Active cases 3,378

- Marginal drop in daily detections in Mumbai and Maharashtra
- Fatalities drop to two in Mumbai. Active Covid-19 cases report a slight rise in Maharashtra

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The state on Sunday reported 4,666 new cases, taking the overall tally to 64,56,939. has been reporting less than 5,000 cases for the past three days. Mumbai reported 345 cases. The city's case tally now stands at 7,43,499. Deaths have remained stagnant in the state, but dropped in the city. Maharashtra reported 131 deaths taking the toll to 1,37,157. Mumbai reported two Covid fatalities on Sunday. The city has been reporting Covid deaths in single digit for nearly a month now. Mumbai's cumulative Covid toll has reached 15,974. Both the deceased on Sunday were senior citizens and had comorbidities.

Cautioning that Maharashtra could see a spurt in cases post festivities like Kerala has done, public health Rajesh Tope said called on districts to exercise caution, especially after the Centre advised a night curfew ahead of the festive season such as Gauri-Ganpati, Dussehra and Diwali. "In light of the Covid surge seen in Kerala after Onam, the Centre has recommended night curfew for Maharashtra too. However, a final decision on the issue will be taken by chief minister Uddhav Thackeray soon," Tope said, while speaking to the media in Jalna, his hometown.

He also said that a special vaccination drive will be started for teachers and non-teaching staff by September 5 on Teachers' Day.

Active coronavirus cases increased in the state on Sunday, while Mumbai noted a fall. The state had 52,844 active cases, up from 51,821. Mumbai's active cases dropped to 3,378 from 3,406. Pune district has the highest number of active cases (13,503), followed by Satara (5,515), Ahmednagar (5,168) and Sangli (4,712). Pune circle had the highest fatalities— 61 —on Sunday, followed by 38 in Kolhapur circle and 14 in Nashik circle. Mumbai Metropolitan Region recorded 11 deaths.

EXHIBIT "QQQ"

Covid test must for visitors to Navi Mumbai malls.

Source: The Times of India.

Link: https://timesofindia.indiatimes.com/city/navi-mumbai/covid-test-must-for-visitors-to-navi-mumbai-malls/articleshow/83556738.cms

Published on: June 16, 2021.

NAVI MUMBAI: The Navi Mumbai Municipal Corporation (NMMC) has decided to make rapid antigen tests mandatory for all who visit shopping during the weekends — Friday, Saturday and Sunday.

Visitors will only be allowed, if their Covid tests are negative. NMMC area has been classified under level-2 of state's fivelevel unlock plan based on the positivity rate and oxygen bed occupancy. All essential and non-essential shops, including those in the malls, are to remain open till 10pm. "Malls are allowed to function till 10 pm with 50% outlets operational. We can't afford the spurt in cases as the third wave is predicted in August-September," said civic chief Abhijit Bangar.

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EXHIBIT "RRR"

BMC braces for Covid's third wave in Mumbai. Here's how.

Source: India Today

Link:- https://www.indiatoday.in/cities/mumbai/story/bmc-braces-for-covid-third-wave-in-mumbai-here-how-1839757-2021-08-11

Author: Pankaj Upadhyay

Published on: August 11, 2021

In preparation for a possible third wave of Covid-19 in Mumbai, the Brihanmumbai Municipal Corporation (BMC) will expand testing and contact tracing, as well as increase additional medical resources.

The Brihanmumbai Municipal Corporation (BMC) is bracing for a potential third wave of Covid-19 in Mumbai by expanding testing and contact tracing and boosting other medical resources.

Given the third wave of the pandemic in various countries, essential planning and preparations are being undertaken to deal with similar circumstances more effectively, BMC said on Wednesday.

These primarily involve expanding hospitals, smaller treatment centres, beds and tests, besides implementing a more effective containment strategy, BMC said.

Suresh Kakani, Additional Municipal Commissioner (Western Suburbs), said that BMC's 7 circles and 24 divisional offices have been given specific instructions. The most crucial are:

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Review of all facilities:

Authorities to visit all areas where Covid-19 treatment facilities were constructed between the first and second waves. They will update the list of treatment facilities and related details, as well as document staffing and service requirements.

More testing kits:

Authorities to provide more testing kits, both RT-PCR as well as Rapid Antigen. They will also ensure the supply of the requisite number of kits to testing facilities.

Expanding contact tracing:

Contact tracing is carried out on people who have come into close contact with patients to avoid the spread of the virus. Previously, contact tracing of 15 persons was done for each patient. This number will now be 20.

Follow-up of restricted areas and affected patients:

More effective and consistent follow-up has been mandated of patients and suspects in restricted areas.

Review and training of divisional control rooms:

Ward War Rooms are operational in all 24 divisions of BMC for effective Covid-19 management. Simultaneously, given the likelihood of children getting infected, control room personnel will receive requisite training.

Jumbo Covid hospitals and care centres:

BMC has ordered an assessment of amenities at Covid Hospitals and Covid Care Centres, including the supply of medically necessary material, cleaning, hygiene and catering services.

Absolute compliance with Covid prevention rules:

To make residents more compliant in following masking, spacing and hand hygiene, the awareness campaign is being made more effective, and disciplinary action is being taken more sternly. Disciplinary measures against people who spit in public will also be strengthened.

Latest statistics:

At July-end, 30,364 beds were available in the BMC area. There are 17,697 oxygen beds, 3,788 intensive care beds, 1,460 beds for children, 230 beds in Paediatric ICUs, and 53 beds in Infant ICUs.

EXHIBIT "SSS"

814

As tests increase, Covid cases tick up in Mumbai, positivity still under 1%

Source: The Times of India

Link: https://timesofindia.indiatimes.com/city/mumbai/mumbai-as-tests-increase-cases-tick-up-in-city-ity-still-under-1/articleshow/85440355.cms

Updated on: Aug 19, 2021

MUMBAI: Daily case detections and deaths showed an upward tick in the state as well as the city on Wednesday.

After reporting under-4,500 cases for the last two days, Maharashtra listed 5,132 cases and 158 deaths, including five in Mumbai. The city added 285 patients testing positive for Covid as against 196 on Tuesday. The authorities are keep a watch on the numbers since train travel and other relaxations were granted since Sunday.

In KEM Hospital in Parel, the Covid ward has only 10 patients and there is no admission on some days. "The positivity rate in KEM is below 1% and the general observation is that the severity of Covid is lesser now," said KEM Hospital dean Dr Hemant Deshmukh. The hospital, reserved for severe patients, used to have over 500 Covid patients at any given time during the second wave.

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At the state level, 15 districts have less than 100 active cases. Of them, six — Dhule, Washim, Yavatmal, Bhandara, Wardha, and Gondia — have less than 10. Officials said the positivity rate of Kolhapur and Ratnagiri have dropped below the state's average weekly positivity rate.

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"In certain countries, the third wave hit areas where the number of Covid patients was less than 1%. We do not know if a similar situation will be seen in Maharashtra. Currently, we have 19 districts where the number of patients is less than 1%, while in thhe remaining districts, the percentage ranges between 2-2.5%," said Lahane.

EXHIBIT "TTT"

Third wave: Mandatory testing of all close contacts of Covid patients in Mumbai.

Source: Hindustan Times

Link:- https://www.hindustantimes.com/cities/mumbai-news/third-wave-mandatory-testing-of-all-close-contacts-of-covid-patients-in-mumbai-101630079669711.html

Published on: Aug 27, 2021

On Thursday, Mumbai reported 397 new Covid-19 cases, the highest daily count since July 28, when the daily count was 404

With the gradual rise in the daily count of Covid-19 cases, the doubling rate has plunged by 232 days in the past 10 days — from 2,057 on August 18 to 1,825 on August 27 — indicating the start of a third wave. Against this backdrop, the Brihanmumbai Municipal Corporation (BMC) has changed its contact tracing policy. Ward officers have been instructed to mandatorily test all close contacts of Covid positive patients immediately to avoid transmission of the infection amid relaxations in Covid-19 restrictions.

As HT reported on August 11, municipal commissioner Iqbal Singh Chahal along with Kakani, in a review meeting of the health department, decided to increase their contact tracing from 10 to 20 per infected patient. However, this week, the target of contact tracing was increased to 25 per infected patient.

The city has been witnessing a steady rise in cases for the past few days. On August 17, Mumbai had reported 198 cases and 283 infections were registered on August 18 and 19. The next day, 322 cases were recorded. On August 21, 259 new patients were identified. The following day, 294 cases were recorded.

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which plunged to 226 the next day. On August 24, the daily count rose to 270, which surged to 343 cases on August 25. On Thursday, Mumbai reported 397 new Covid-19 cases, the highest daily count since July 28, when the daily count was 404.

As per BMC data, the number of daily tests dropped from over 50,000 to around 30,000 a few days last week. With the reopening of establishments like shops, hotels and malls, BMC has instructed the ward officers to test all the close contacts of the infected patients immediately, even if they don't show symptoms.

"So far, we used to test close contacts after 4-5 days if they develop any symptoms. Till then, they were being instructed to get home quarantined. But now, we have instructed ward officers to test them immediately, without any delay," said Suresh Kakani, additional municipal commissioner, BMC. "The movement of the people has increased with the relaxation. So, we don't want any asymptomatic patient to infect others in the crowd," he added

During the outbreak of the second wave, the civic body was blamed for inadequate contact tracing. Though the Union Health Ministry advised tracking 30 close contacts per patient, between January and February, the civic body traced only 4-5 close contacts for every patient.

"With the rise of Delta Plus variants, along with vaccination, BMC needs to focus on contact tracing. Or else, it can lead to an influx of another pandemic wave," said Dr Ravikant Singh, founder of the NGO — Doctors for You.

But civic officials often hit a roadblock in contact tracing due to improper information shared by the patients.

"Many times, infected patients don't reveal the names of their close contacts. In fact, some families don't even inform their buildings or societies about their infection. These problems are most common in non-slum areas," said Dr Bhagyashree Kapse, ward officer, R-Central that covers Borivli.

Also, instruction has been given to keep an eye on families and societies where more than one Covid-19 patient is recorded. "If any family or society is recording high transmission rate, we are sending their samples for genome sequencing to find out any possible infection from Delta or Delta plus variants," said Kakani.

EXHIBIT "UUU"

Navi Mumbai to increase Covid-19 testing ahead of festive season

Source: Hindustan Times

Link: https://www.hindustantimes.com/cities/mumbai-news/navi-mumbai-to-increase-covid-19-testing-ahead-of-festive-season-101630789285245.html

Written By: Raina Shine

Published on: Sep. 05, 2021

The artificial ponds which were 132 last year have been increased to 156 this year in order to make sure that there is not much crowding at one place. There were 132 applications for the pandals this year of which 120 were granted permission while there were 90 applications last year of which 84 were granted permission.

Ahead of Ganeshotsav, the Navi Mumbai Municipal Corpocation has increased random testing in Navi Mumbai especially at the railway stations. Currently, around 500 random antigen tests are being conducted at railway stations. The corporation plans to conduct similar random tests at busy markets, pandals and immersion sites as well.

Pramod Patil, medical health officer, NMMC, said, "A total of 7,000 Rapid Antigen Tests and around 2,000 RT-PCR tests are conducted daily. This year during the festival, we have to fight Malaria, Dengue as well as Covid-19. So along with making sure that the Covid-19 norms are followed, we also need to ensure preventive measures for dengue and malaria. Regular checking of stagnant water and fogging at the pandal areas also needs to be done."

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Depending on the availability of vaccines, the corporation also plans to have vaccination drive at the pandals. "If there is enough supply, we will have vaccination drives at whichever pandals possible. We will also be putting up hoardings and signages regarding Covid-19 at the pandals. We had a joint meeting with the police wherein we have informed them as well about the actions they should take against people not following the Covid-19 norms," Assistant Municipal Commissioner Sanjay Kakade said.

The Corporation also plans to have an arrangement wherein the residents not willing to step out for immersion can handover the idol to the designated persons visiting their area and they would further immerse it.

The artificial ponds which were 132 last year have been increased to 156 this year in order to make sure that there is not much crowding at one place. There have been 132 applications for the pandals this year of which 120 were granted permission while there were 90 applications last year of which 84 were granted permission.

"Every pandal should use only the one fourth of the road and there should be area left for the people to travel. The pandals should not be blocking any footpath or roadway. Such applications which can create blockage to the movement of public, have been rejected and in some cases, people have made double applications thinking that if one is rejected, another might be considered," an official from NMMC said.

Meanwhile, the cases of not wearing masks and not maintaining social distancing have also increased, ahead of the festival. In the month of August, around 1272 people were fined for not wearing masks and a total of ₹6.36 lakh was collected, 2075 were fined for not maintaining social distancing and fined ₹4.15 lakh.

EXHIBIT "YYY"

State COVID Task Force Cautions Towards New Symptoms Of COVID-19

Source: Mumbai Live

Link: https://www.mumbailive.com/en/civic/state-covid-task-force-cautions-towards-new-symptoms-of-covid-19-68158

Published:- August 2021.

Personnel of the state covid task force encouraged doctors to diagnose coronavirus even in those cases wherein the usual signs aren't present

Some of the newer symptoms in covid-19 patients include conjunctivitis, a dry mouth, reduction in hearing and a throbbing headache, stated a report by the Times of India. Personnel of the state covid task force encouraged doctors to diagnose coronavirus even in those cases wherein the usual signs such as sore throat, cough, shortness of breath isn't present.

Dr Rahul Pandit, a member of the task force, listed a few of the developing symptoms that comprise conjunctivitis, intense weakness, problems in hearing, dry mouth and less saliva secretion, persistent headache and rashes on skins. These can be indications of covid19 as well, stated the account.

On Sunday, Chief Minister Uddhav Thackeray chaired an online conference, wherein he elaborated that whilst the virus has been around for 17 months, new symptoms are constantly developing which needs to be watched.

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The chairman of the task force, Dr. Sanjay Oak stated that in the Delta variant driven, second wave many showcased gastrointestinal symptoms that comprised diarrhoea, vomiting and nausea. In different forms, fever can be persistent, some don't have a fever, some get it in snaps, others in intervals, while in others it goes away to return with intensity, he said, according to the account.

Members of the task force stressed the importance of early testing. Dr Oak stated that family doctors have an important role in encouraging a covid test ideally through an RT-PCR. He said that if tests are promptly done, the patient can be given antivirals or monoclonal antibodies which are efficient when given sooner.

In the conference, an endocrinologist and member of the task force, Dr Shashank Joshi, elaborated that those who tested positive should check their temperature, pulse, blood pressure, respiratory rate, oxygen saturation and blood sugar levels.

The specialists emphasized that complete recovery can take nearly three weeks. Dr Ajit Desai, a cardiologist stated that after covid-19, nearly 40% of patients may wrestle with anxiety, depression, sleep problems, post-traumatic stress disorder, while some 5% could have thromboembolic events, highlighted the story.

EXHIBIT "WWW"

Maharashtra Lockdown Update: Third Wave of Corona Hits Nagpur, Fresh Restrictions to be Announced Soon, Says Minister

Source: India.com

Link: https://www.india.com/maharashtra/maharashtra-lockdown-news-today-7-september-2021-third-wave-arrived-in-nagpur-fresh-restrictions-to-be-announced-soon-in-mumbai-nagpur-pune-uddhav-thackeray-big-announcement-awaited-4939878/

Published on: 7 September, 2021

Maharashtra lockdown Update: Enhanced restrictions could include restaurants being allowed to remain open till 8pm instead of 10pm, shops and other establishments till 4pm

Mumbai: 'Third wave of coronavirus has arrived in Nagpur, thus fresh restrictions will be reimposed in the city soon', said State Energy Minister Nitin Raut after holding a review meeting with senior administrative officers from various state departments. Speaking to reporters, Raut said that the third wave, which was predicted to hit the country anytime in September-October, has reached here (Nagpur) as the city has logged COVID cases in double digits.

Notably, the COVID-19 tally in Nagpur increased by 12 during the day to reach 4,93,072, while the death toll remained unchanged at 10,119, leaving the district with an active caseload of 56. Raut, speaking to reporters after the meeting at the divisional commissionerate said the daily addition was in single digits till a

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couple of days ago, but on Monday it was 12. He said 78 samples have been sent for genome sequencing.

On being asked what kind of curbs can be imposed in the city, Raut asserted enhanced restrictions could include restaurants being allowed to remain open till 8 pm instead of 10 pm, shops and other establishments till 4 pm and a complete shutdown on weekends, with only essential services being allowed to operate.

Earlier, in the wake of the rising number of Delta plus variant cases in Maharashtra, the Nagpur Municipal Corporation (NMC) had ordered mandatory institutional quarantine or hospitalisation for every patient testing positive for the deadly COVID-19 in the city.

Municipal commissioner Radhakrishnan B had issued an order regarding this directing all zone commissioners to put COVID patients under institutional quarantine or ensure their hospitalization, an NMC release said.

EXHIBIT "XXX"

"Covid Third Wave Here Already," Says Mumbai Mayor.

Source: NDTV

Link: https://www.ndtv.com/india-news/covid-third-wave-here-already-says-mumbai-mayor-2532865

Edited by: Anindita Sanyal

Published on: September 07, 2021

Mumbai has logged 28 per cent of cases recorded in August in just the first six days of September.

Mumbai: The third wave of Covid is already upon Mumbai, the city's Mayor has said, pointing to a sudden spike in cases. "The third-wave of Covid-19 is not coming, it is here," said Kishori Pednekar, adding that an announcement regarding the matter had already been made in Nagpur, ANI reported.

Speaking of the coming festive season in Maharashtra, the Mayor urged people to remain in their homes. The Mandals have been asked to allow only 10 people and have them work in shifts, she said.

Mumbai has logged 28 per cent of cases recorded in August in just the first six days of September.

On Monday, Mumbai reported 379 fresh cases of coronavirus and five fatalities, reported news agency Press Trust of India, quoting an official of the Brihanmumbai Municipal Corporation.

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The new additions pushed the total in the city to 7,46,725, the deaths to 15,998, and the number of recoveries to 7,24,494.

The surge comes ahead of the festival season, when there are concerns of massive spike. The first wave of Covid last year had started amid the festive season.

Chief Minister Uddhav Thackeray has urged cancellation of political rallies and religious events in view of a possible surge.

"Public health is very important. Festivals can be celebrated in future. The situation can worsen if the cases of Covid increase. One must give priority to the health of the people so we can avoid the third wave," Mr Thackeray was quoted as saying by news agency Press Trust of India.

"One must not bring about a situation where strict restrictions need to be imposed. We have an idea of how the second wave of Covid started," he added.

EXHIBIT "YYY"

Maharashtra Lockdown Big Update: Deputy CM Warns of Shutting Down Everything if Covid Third Wave Hits

Source: India.com

Link: https://www.india.com/news/india/maharashtra-lockdown-big-update-deputy-cm-ajit-pawar-warns-of-shutting-down-everything-if-covid-third-wave-hits-uddhav-thackeray-rajesh-tope-shopping-malls-temples-markets-coronavirus-4932761/

Published on: September 3, 2021.

Speaking to reporters, Pawar said the Centre has already cautioned all the states, citing that Kerala and Maharashtra have been reporting the highest number of cases.

Maharashtra Lockdown Update: Maharashtra Deputy chief minister Ajit Pawar on Friday asked citizens to follow all the covid protocols and guidelines set by the government to contain the spread of the virus in the state. Expressing concerns over people letting their guard down against Covid-19 in rural areas Pawar urged citizens not to put the state government in a position where it has to shut everything in the event of a third wave of the pandemic.

"Unfortunately, in rural areas, some people are becoming lax. They are not scared of coronavirus. They don't use masks, don't follow physical distancing, and they have assumed that everything (Covid-19 pandemic) is over. This has led to rise in infections," Pawar said.

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Speaking to reporters, Pawar said the Centre has already cautioned all the states, citing that Kerala and Maharashtra have been reporting the highest number of cases. The chief minister has appealed to people from time to time, but some people politicise the issue and resort to celebrating festivals, he said.

Here are some of the important statements made by Ajit Pawar:

"All this should stop somewhere. People should not put the state government and administrations in a position where they have to close everything if the third wave hits," he appealed.

When asked about reopening of schools, Pawar said discussions are being held with the concerned experts and a decision will be taken.

"There are two opinions. Some say schools should open after Diwali, while others say they should be reopened in places where the Covid-19 positivity rate is zero. The chief minister, however, will take the decision," he said.

On BJP and MNS Demand To Open Temples:

Speaking about the BJP and MNS' demand to open temples in the state, Pawar said as the civic elections are round the corner, every party was striving to make its presence felt, which is why this "emotional" issue was being raised.

Pawar, who is also the guardian minister of Pune, was speaking to the media after holding a review meeting of the Covid-19 situation in the district.

The divisional commissioner (Pune division) has informed that at least 5 lakh doses may be available for the district soon, and the authorities will try to speed up the vaccination in slums, he said.

Pawar further said that while Ganesh Utsav is drawing closer, people must refrain from celebrating the festival on a grand scale.

"Since all big mandals (organisers) have decided to celebrate the festival in a simple manner and as there will be no decorations, there is no scope for crowding. However, we will monitor the situation from day one, and if we find that gatherings are taking place, stricter measures will be taken from the second day," he said.

EXHIBIT "222"

Odisha issues guidelines for stricter implementation of COVID norms in schools amid surge in infection among children.

Source: Money Control

Link: https://www.moneycontrol.com/news/trends/current-affairs-trends/odisha-issues-guidelines-for-stricter-implementation-of-covid-norms-in-schools-amid-surge-in-infection-among-children-7445271.html

Published on: September 08, 2021

The fresh guideline was issued by the School and Mass Education Department on Tuesday after some children and teachers tested positive for the infection in Dhenkanal and Bargarh districts.

The Odisha government has issued a fresh guideline calling for stricter implementation of COVID norms in schools amid a surge in infection among children.

Of the 762 new COVID-19 patients in the state, 102 are in the 0-18 age group. However, the infection rate among the children slightly dipped to 13.38 per cent on Wednesday from 14.57 per cent on the previous day.

The fresh infections reported in 29 of the 30 districts pushed the state's caseload to 10,13,567. Eight fresh fatalities took the state's coronavirus death toll to 8,070, a health official said.

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The fresh guideline was issued by the School and Mass Education Department on Tuesday after some children and teachers tested positive for the infection in Dhenkanal and Bargarh districts.

It said that students, teachers and other staffers have to mandatorily wear masks to enter the schools where classes are being held for students of standards 9, 10 and 12.

A COVID monitor, preferably the PET of the school, will have to ensure strict adherence to social distancing norms. Anyone exhibiting even mild symptoms must be refrained from entering the schools. Thermal screening and availability of sanitiser at the entrance have to be ensured, the notification said.

While both offline and online classes will continue, no student must be forced to attend the physical classes, it said.

The second wave of the pandemic has claimed the lives of 39 children, with 13 deaths being reported between August 10 and 31.

Of the 762 new cases, 443 were reported in quarantine centres, while 319 infections were detected during contact tracing.

Khurda district, under which Bhubaneswar falls, registered the highest number of new cases at 314, followed by Cuttack (119). Boudh did not report any fresh infection.

Dhenkanal and Khurda districts registered two fresh fatalities each, while one death each was reported in Angul, Balasore, Keonjhar and Koraput. Fifty-three patients have died due to comorbidities so far.

Odisha now has 6,853 active cases. Khurda remains in the 'red zone' with 2,617 patients still infected with the disease.

As many as 9,98,591 people have recovered from the infection in the state so far, including 801 on Tuesday.

The state has thus far tested over 1.85 crore samples for COVID-19, including 64,602 on Tuesday. The daily test positivity rate stood at 1.17 per cent.

The administration has inoculated over 2.42 crore people, of whom 59.98 lakh have been fully vaccinated.

EXHIBIT "AAAA"

भारतीय आयुर्विज्ञान अनुसंधान परिषद
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार
कल्याण मंत्रालय, भारत सरकार

Indian Council of Medical Research
Department of Health Research, Ministry of Health
and Family Welfare, Government of India

Date: 04/05/2021

Advisory for COVID-19 testing during the second wave of the pandemic

Context

An unprecedented upsurge of COVID-19 cases and deaths is currently being witnessed across India. The overall nationwide test positivity rate is above 20%. Testing-tracking-tracing, isolation and home-based treatment of positive patients is the key measure to curb transmission of SARS-CoV-2, the causative agent of COVID-19. As on today, India has a total of 2506 molecular testing laboratories including RTPCR, TrueNat, CBNAAT and other platforms. The total daily National testing capacity is close to 15 lakh tests considering a three- shift operationalization of the existing laboratory network. At present, the laboratories are facing challenges to meet the expected testing target due to extraordinary case load and staff getting infected with COVID-19. In view of this situation, it is imperative to optimize the RTPCR testing and simultaneously increase the access and availability of testing to all citizens of the country.

Recommended measures to optimize RTPCR testing

i. RTPCR test must not be repeated in any individual who has tested positive once either by RAT or RTPCR.
ii. No testing is required for COVID-19 recovered individuals at the time of hospital discharge in accordance with the discharge policy of MoH&FW (*https://www.mohfw.gov.in/pdf/ReviseddischargePolicyforCOVID19.pdf*).
iii. The need for RTPCR test in healthy individuals undertaking inter-state domestic travel may be completely removed to reduce the load on laboratories.
iv. Non-essential travel and interstate travel of symptomatic individuals (COVID-19 or flu like symptoms) should be essentially avoided to reduce the risk of infection.
v. All asymptomatic individuals undertaking essential travel must follow COVID appropriate behavior.
vi. Mobile testing laboratories are now available on GeM portal. States are encouraged to augment RTPCR testing through mobile systems.

Measures to improve access and availability of testing:

Rapid antigen tests (RATs) were recommended in India for COVID-19 testing in June 2020. However, the use of these tests is currently limited to containment zones and health care settings. RAT has a short turn-around time of 15-30 minutes and thus offers a huge advantage of quick detection of cases and opportunity to isolate and treat them early for curbing transmission. So far, ICMR has approved 36 RATs of which 10 are on GeM portal. To meet the overwhelming testing demand, it will be prudent to upscale testing using RATs.

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icmr
INDIAN COUNCIL OF MEDICAL RESEARCH

Measures to ramp up testing through RAT

i. RAT may be allowed at all available Government and private healthcare facilities.

ii. Set up dedicated RAT booths in cities, towns and villages to offer testing to people.

iii. Testing booths may be set up at multiple locations including healthcare facilities, RWA, offices, schools, colleges, community centers and other available vacant spaces.

iv. These booths should be operational on a 24X7 basis to improve access and availability of testing.

v. Drive-through RAT testing facilities may be created at convenient locations as identified by local administration.

vi. Stringent measures must be instituted to avoid overcrowding at RAT testing facilities.

Additional advice

- All states are advised to ensure full utilization of the available RTPCR testing capacity, both in public and private laboratories.
- RAT testing must be conducted in compliance with the ICMR advisory available at:

  https://www.icmr.gov.in/pdf/covid/strategy/Testing_Strategy_v6_04092020.pdf
- Symptomatic individuals identified positive by RAT should not be re-tested and advised to go through home-based care as per ICMR guidelines available at: *https://www.icmr.gov.in/pdf/covid/techdoc/COVID_HOME_CARE.pdf.*
- Symptomatic individuals identified negative by RAT should be linked with RTPCR test facility and in the meantime be urged to follow home isolation and treatment.
- All RTPCR and RAT test results should be uploaded on ICMR portal at: *https://cvstatus.icmr.gov.in.*
- During the current upsurge of COVID-19 cases, any individual presenting with fever with / without cough, headache, sore throat, breathlessness, bodyache, recent loss of taste or smell, fatigue and diarrhea should be considered as suspect case of COVID-19 unless proven otherwise by confirmation of another etiology.
- The vaccination status of all individuals tested for COVID-19 must be entered into the Sample Referral Form (SRF) in the RTPCR app both for individuals tested by RTPCR and RAT. This information is of critical importance.
- Payment modalities for upscaled RAT testing may be decided by the state health authorities.

EXHIBIT BBBB

835

भारत सरकार
स्वास्थ्य एवं परिवार कल्याण विभाग
स्वास्थ्य एवं परिवार कल्याण मंत्रालय
Government of India
Department of Health and Family Welfare
Ministry of Health and Family Welfare

राजेश भूषण, आईएएस
सचिव

RAJESH BHUSHAN, IAS
SECRETARY

D.O. No.01/S(HFW)/Omicron/Maha/2021
01 December, 2021

Dear Dr. Vyas,

This is with reference to the Govt. Of Maharashtra Order No. DMU/2020/CR.92/DisM-1 dated 30th Nov. 2020, vide which the following restrictions have been imposed:

i. Mandatory RTPCR testing of all international travellers at the Mumbai airport, irrespective of country of origin
ii. Mandatory 14-day home quarantine for all international passengers, despite being tested RTPCR Negative upon arrival
iii. Mandatory RTPCR test for passengers planning to undertake connecting flights after disembarking at Mumbai and further travel subject to a negative RTPCR result
iv. Requirement of negative RTPCR test 48 hours prior to date of journey, for domestic passengers travelling from other States to Maharashtra

2. This is in divergence with the SoPs & Guidelines issued by Ministry of Health & Family Welfare, Govt. of India. I would, therefore, urge you to align the Orders issued by the State with the Guidelines issued by the Ministry of Health & Family Welfare, Govt. Of India, so that uniform implementation of the guidelines may be ensured across all States/UTs. I would also advise that such modified orders of the State Government are given wide publicity to obviate any inconvenience to travellers.

Warm Regards.

Yours sincerely

(Rajesh Bhushan)

Dr. Pradeep Kumar Vyas
Additional Chief Secretary
Department of Health & Family Welfare
Government of Maharashtra
Mumbai
Email : psec.pubhealth@maharashtra.gov.in

NOTARY
M. PARALE
Mumbai
Maharashtra
Regd. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

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Room No. 156, A-Wing, Nirman Bhawan, New Delhi-110 011
Tele : (O) 011-23061863, 23063221, Fax : 011-23061252, E-mail : secyhfw@nic.in

ADVOCATE FOR

EXHIBIT "CCCC"

836

DIRECTOR GENERAL
HEALTH
REPUBLIC OF SOUTH AFRICA

PRETORIA
Private Bag X828, PRETORIA, 0001, Dr AB Xuma Building, 1112 Voortrekker Rd, Pretoria Townlands 351-JR, Pretoria, 0187, Tel (012) 395 8000
CAPE TOWN
P.O. Box 1675, CAPE TOWN, 8000, 103 Parliament Towers, Room 618, Plein Street, CAPE TOWN, 8000 Tel (021) 461 2040 Fax (021) 461 6984

TO: HEADS OF PROVINCIAL HEALTH DEPARTMENTS

SUBJECT: REVISION TO CONTACT TRACING, QUARANTINE AND ISOLATION PROTOCOLS: 23.12.2021

1. The following revisions have been accepted based on the -COVID-19 MAC advisories of 16.12.2021

1.1 Proportion of people with some immunity from infection and/or vaccination is high
- past infection in 60-80% in several sero-surveys

1.2 Containment strategies are no longer appropriate – mitigation is the only viable strategy
- Especially true of the newer, more infectious/transmissible variants like OMICRON

1.3 New knowledge about the virus:
a) high proportion of asymptomatic disease,
b) high degree of asymptomatic and pre-symptomatic spread,
c) aerosol spread.
d) Only a small proportion of cases are diagnosed.

1.4 We never identify most high risk patients
a) Testing skewed towards symptomatic (minority)
b) Not all symptomatic people test
c) Not all negative tests are true negatives

1.5 "High risk" definition probably isn't meaningful anymore
a) Doesn't take into account aerosol spread
b) Doesn't take into account the newer variants (increased transmissibility)
c) Doesn't take into account pre-existing immunity

1.6 Quarantine has been costly to essential services and society as many people stay away from their work and thus lose their income and children miss on their schooling.

Thus, the following is applicable with immediate effect :

2. Contact Tracing

2.1 All contact tracing be stopped with immediate effect except in congregate settings and cluster outbreak situations or self-contained settings.

2.2 All contacts must continue with their normal duties with heightened monitoring (daily temperature testing, symptom screening) of any early signs. If they develop symptoms then they should be tested and be managed according to the severity of the symptoms

2.3 All contacts must not be tested unless if they develop symptoms

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3. **Quarantining for contacts of confirmed cases of Covid -19**

3.1 All quarantine is to be stopped with immediate effect

3.2 This applies to both vaccinated and unvaccinated contacts

3.3 No testing for Covid -19 is required irrespective of the risk exposure unless the contact becomes symptomatic

4. **Isolation**

- Isolation rules are applicable to both vaccinated and unvaccinated individuals
- Isolation rules are applicable to high and low risk individuals
- Return to work from Day 10 onwards must as always take into consideration the individual's clinical status. Only those patients well enough to work should do so.

4.1 Asymptomatic Individuals

a) No isolation period required
b) To do self-observation for 5-7 days for development of any symptoms with enhanced precautions including avoiding attending settings where many people gather, mask wearing and social distancing.

4.2 Mild disease

Mild diseases refers to persons who have symptoms and have tested positive but who do not require hospitalization, do not have shortness of breath, dyspnoea or abnormal chest imaging.

Mild disease symptoms and signs include but are not limited to the following: fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhoea, loss of taste and smell.

a) Isolation period is maintained at 8 days.
b) The person in this category must wear a mask at all times (even at home, work and all public spaces) for the duration of the 8 days period of isolation.
c) Where a health care worker returns to work after Day 8 such a worker must wear a N95 mask at all times and must at all times avoid contacts with extremely high risk patients (especially severely immune-compromised patients).
d) There is no need for Covid-19 test (either PCR or antigen test) be performed prior to returning to work after 8 days isolation period.
e) For mild cases, isolation beyond 8 days must be supported by the medical report

4.3 Severe Disease:

a) Severe disease refers to persons who test positive and have exacerbated symptoms i.e shortness of breath, dyspnoea, chest pain and abnormal chest imaging and who require hospitalisation to manage the clinical presentation.
b) Isolation period is maintained at 10 days after clinical stability is achieved
c) The person in this category must wear a mask at all times (even at home, work and all public spaces) for the duration of the 10 days period of isolation.
d) Where a health care worker returns to work after Day 10, such a worker must wear a N95 mask at all times and must at all times avoid contacts with extremely high risk patients (especially severely immune-compromised patients).

e) There is no need for Covid-19 test (either PCR or antigen test) be performed prior to returning to work after 10-day isolation period
f) For severe cases, isolation beyond 10 days must be supported by the medical report

5. Return to work

All people that have been infected and have been in isolation, must be ready to return to work after completing mandatory period of isolation as above and no further testing is required after either 8 or 10 days of isolation.

DR SSS BUTHELEZI
DIRECTOR-GENERAL: HEALTH
DATE: 23 December 2021

EXHIBIT "DDDD"

'महावसुली सरकार' चे घोटाळे

-डॉ. किरीट सोमैया

ठाकरे सरकार मधील परिवहन मंत्री श्री अनिल परब यांचा अनधिकृत रिसोर्ट

श्री मिलिंद नार्वेकर यांचा दापोली समुद्र किनार्यावरील सीआरझेड ३ क्षेत्रामध्ये अनधिकृत बेकायदेशीर बंगला पाडला

1. २४ महिन्यात १०० घोटाळे...
2. ६ नेता / मंत्रींना / अटक / जेल
3. २४ हून अधिक घोटाळ्याची ई डी, सी बी आय, आयकर, पोलीस .....द्वारा चौकशी सुरु
4. १८ प्रकरणात न्यायालय, लोकायुक्त, NGT ग्रीन प्राधिकरण, मानव अधिकार आयोग, पोलीस प्राधिकरण.....सुनावणी सुरु

भारतीय जनता पार्टी

NOTARY M.M. PARALE ... Reg. No. 15220 ... GOVT. OF INDIA

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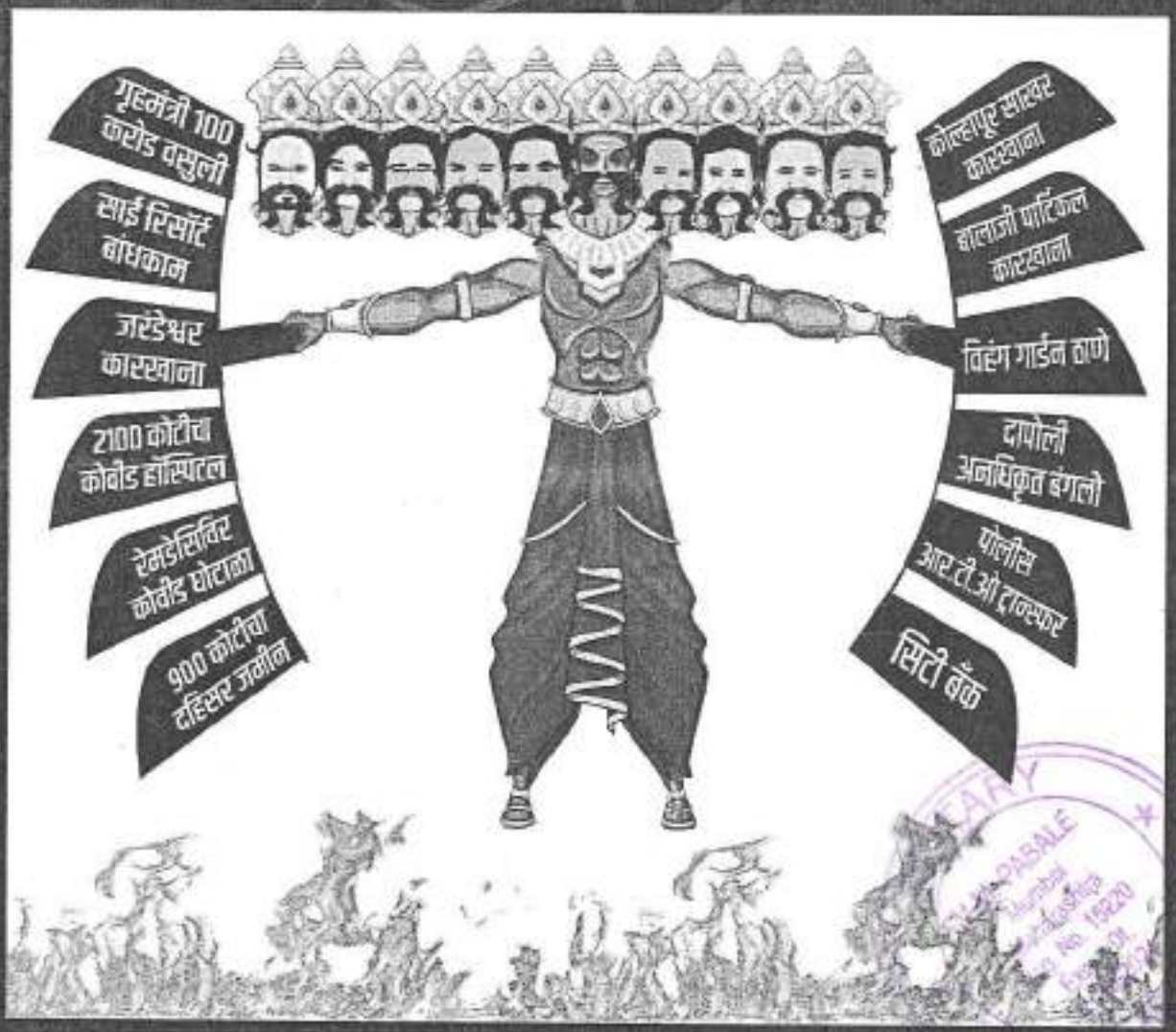
840

'महावसूली' सरकार



ठाकरे सरकारचे घोटाळे

गेल्या २७ महिन्यात ठाकरे सरकारचे २ डझनहून अधिक मंत्री / नेते / अधिकारी घोटाळ्यात सापडले आहेत. हे घोटाळे उघडकीस आले, पुराव्यांसह विभिन्न यंत्रणेमार्फत त्याचा पाठपुरावा ही सुरु आहे. इन्कमटॅक्स, प्रवर्तन निदेशालय (ED), न्यायालय, लोकायुक्त, विभिन्न प्राधिकरण, सहकार मंत्रालय, आर्थिक गुन्हे अन्वेषण विभाग (EOW), कंपनी मंत्रालय अशा विभिन्न तपास यंत्रणेद्वारा तपास सुरु आहे. अनेकांवर कारवाई सुद्धा झाली आहे.

१. मंत्री अनिल देशमुख वसुली व मनी लॉंडरिंग
२. संजय राऊत रु.५५ लाख घोटाळा
३. मुख्यमंत्र्यांचे सचिव मिलिंद नार्वेकर पाच अघिकृत बंगला
४. मंत्री हसन मुशरीफ रु.१५००० कोटी जरंडेश्वर कंपनी घोटाळा
५. शिवसेना नेते श्री. आनंद अडसूळ सिटी बँक घोटाळा
६. पोलीस अधिकारी/ शिवसेना प्रवक्ते सचिन वाझे– वसुली कांड
७. शिवसेनेचे नेते/ पोलीस अधिकारी श्री. प्रदीप शर्मा– वसुली कांड
८. पोलीस आयुक्त श्री. परमबीर सिंह– फरार
९. उपमुख्यमंत्री अजित पवार मित्र – परिवार रु.१०५० कोटी बेनामी व्यवहार
१०. शिवसेनेचे नेते आमदार प्रताप सरनाईक अनेक घोटाळे
११. मंत्री अनिल परब अनधिकृत रिसॉर्ट
१२. खासदार भावना गवळी रु. १०० कोटी घोटाळा
१३. ठाकरे सरकार/ बृहन्मुंबई महानगरपालिका रु २.५५ कोटींची दहिसर येथील जमिन रु. ९०० कोटींमध्ये घेण्याचा घोटाळा
१४. कोविड रेमडेसिवीर घोटाळा
१५. मुंबई महानगरपालिका १ कोटी कोविड वॅक्सिन (लस) जागतिक निविदा घोटाळा
१६. मुंबई महानगरपालिका/ पालक मंत्री कोविड ऑक्सिजन टैंक/ टाकी घोटाळा
१७. मुंबई महानगरपालिका/ मुख्यमंत्री कार्यालय " ५००० खाटांचे कोविड हॉस्पिटल रु. २२०० कोटींचा घोटाळा"
१८. शिवसेना नेते रविंद्र वायकर व भागिदार यांचा कोलंबई, अलिबाग जमिन/ बंगले घोटाळा
१९. मंत्री जितेंद्र आव्हाड व पोलिसांद्वारा ठाणे येथील व्यवसायिक अपहरण, मारहाण
२०. नांदेड धर्नाबाद येथील सहकारी पतसंस्था १२५० शेतकऱ्यांच्या नावे करोडो रुपयांचे बेनामी आर्थिक व्यवहार, रु २३५ कोटी कर्ज घोटाळा
२१. अर्जुन खोतकर मित्र – परिवार/ भागिदार बेनामी व्यवहार जालना सहकार कारखाना घोटाळा
२२. लातुर बालाघाट, प्रियदर्शनी, जागृती कारखाना अपारदर्शक आर्थिक व्यवहार घोटाळा
२३. जरंडेश्वर साखर कारखाना घोटाळा
२४. पुणे येथील वक्फ बोर्ड जमिन घोटाळा

घोटाळ्यांचा पाठपुरावा-

- केंद्र सरकार, राज्य सरकार, मंत्रालयात पाठपुरावा
- मुंबई उच्च न्यायालयात १ + १ याचिका
- लोकायुक्तांकडे ७ याचिका (सुनावणी सुरु)
- मानव अधिकार आयोग ३ याचिका
- पोलिस प्राधिकरण २ याचिका
- नॅशनल ग्रीन ट्रिब्युनल (NGT) १ याचिका
- TRAI मध्ये १ याचिका
- अनुसूचित जाती आयोगमध्ये १ याचिका
- महिला आयोगाकडे १ याचिका

६ नेते / अधिकारी यांना अटक/जेल

- गृहमंत्री अनिल देशमुख जेल मध्ये
- आनंद अडसूळ ईडीद्वारा अटक, हॉस्पिटल मध्ये भरती
- मंत्री जितेंद्र आव्हाड अटक व जामिनावर सुटका
- शिवसेना उमेदवार प्रदिप शर्मा (पोलिस अधिकारी) जेल मध्ये
- शिवसेना प्रवक्ते पोलिस API सचिन वाझे जेल मध्ये
- मुंबई पोलिस आयुक्त श्री. परमबीर सिंह फरार

इकडे तिकडे चोही कडे..... घोटाळेच घोटाळे
महिन्याची रु. १०० कोटीची वसूली ते मनसुख हिरेन हत्या ....

१. मंत्री **श्री. अनिल देशमुख** जेल मध्ये महिन्याची १०० कोटींची वसुली, १०० हून अधिक कोटींचे मनी लाँडरिंग, बेनामी संपत्ती.....

२. **श्री. संजय राऊत** यांना भ्रष्ट पद्धतीने आलेले रु. ५५ लाख ईडीला परत द्यावे लागले.

"चोरी का माल वापस करना पडा"

पीएमसी बँक – एचडीआयएल – प्रवीण राऊतकडून हे ५५ लाख रुपये श्री. संजय राऊत परिवाराच्या खात्यात आले होते.

३. मुख्यमंत्री उद्धव ठाकरे यांचे सचिव **श्री. मिलिंद नार्वेकर** दापोली येथे समुद्र किनाऱ्यावर अनधिकृत बेकायदेशीर बंगला बांधत होते. आम्ही आक्षेप घेतला. भारत सरकारच्या पर्यावरण मंत्रालयाच्या टीमने भेट दिली. श्री. मिलिंद नार्वेकर यांनी स्वत: तो बंगला पाडला.

४. राष्ट्रवादी काँग्रेसचे मंत्री **श्री. हसन मुश्रीफ** यांनी रु. १५,००० कोटींचे त्यांच्याच ग्रामविकास खात्याचे कंत्राट आपल्याच कुटुंबाच्या जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनीला दिले. आम्ही हा विषय मांडला अलेरीस ठाकरे सरकारलाहा कॉन्ट्रक्टरद्द करावा लागला.

५. शिवसेना नेते माजी खासदार **श्री. आनंद अडसूळ** सीटी को-ऑपरेटीव्ह बँकेच्या घोटाळ्यात गुंतले आहेत (रु. १२० कोटी). त्यांना ईडीने अटक केली पण लगेचच त्यांना रुग्णालयात दाखल करून घ्यावे लागले.

६. निलंबित पोलीस अधिकारी व शिवसेना प्रवक्ते **श्री. सचिन वाझे** यांना ठाकरे सरकारनी परत नौकरीत घेतले. वाझेला ५० कोटी रुपयांच्या वसुलीची धमकी व मनसुख हिरेनच्या हत्या बद्दल वाझेची अटक झाली, ते सध्या तुरुंगात आहेत.

७. शिवसेनेचे २०१९ विधानसभा निवडणुकीतील उमेदवार **श्री. प्रदिप शर्मा** यांना ही नोकरीत परत घेण्यात आले वसुली व सुपारी घेऊन हत्याप्रकरणात ते ही तुरुंगात आहेत.

८. ठाकरे सरकारने **श्री. परमबीरसिंह** यांची मुंबई पोलिस आयुक्त म्हणून नियुक्ती केली व आता त्याच **परमबीर सिंह** वर ठाकरे सरकारने डझनभर खंडणीचे गुन्हे दाखल केले आहेत, परमबीर सिंह सध्या फरार आहेत.

९. उपमुख्यमंत्री **श्री. अजित पवार** अर्थमंत्री असताना ते महाराष्ट्र राज्य सहकारी बँकेचे सर्वेसर्वा होते. त्यांनी जरंडेश्वर साखर कारखाना बेनामी पद्धतीने आपल्याच एका कंपनीच्या ताब्यात आणला, बळकावला. ईडीने जरंडेश्वर साखर कारखाना नुकताच जप्त केला आहे.

१०. ईडी व आयकर खात्याची **श्री. अजित पवार, मित्र, परिवार, कंपन्यांवर** १९ दिवस धाडी घातल्या. रु. १०५० कोटींची बेनामी संपत्ती बाहेर आली.

११. शिवसेनेचे नेते व प्रवक्ते **आ. प्रताप सरनाईक** यांनी एनएसईएल गैरव्यवहातील ३५ कोटी रुपये बळकावल्याचे आढळले. त्यांनी टिटवाळा येथे ७८ एकर जमीन खरेदी केली व ती ईडीने जप्त केली आहे.

१२. **श्री. प्रताप सरनाईक** यांनी त्यांच्या विहंग गार्डन बिल्डिंग प्रकल्पामध्ये बेकायदेशीरपणे पाच मजले बांधले आहेत. त्यांना २१ कोटी रुपये दंड भरण्यास सांगण्यात आले आहे. हा गैरव्यवहार आम्ही उघडकीस आणला आहे. लोकायुक्तांसमोर सुनावणी चालू आहे.

१३. शिवसेनेचे मंत्री **श्री. अनिल परब** / सहकारी यांचे वांद्रा पूर्व, मुंबई येथील म्हाडा इमारतीमधील कार्यालय बेकायदेशीर असल्याचे आढळले. लोकायुक्तांनी ते पाडण्याचे आदेश दिले.

१४. **श्री. अनिल परब** यांनी कोरोनाच्या कालावधीत दापोली येथे समुद्रकिनाऱ्यावर एक अनधिकृत पंचतारांकित रिसॉर्ट बांधले. आम्ही हा गैरव्यवहार उघड केला. अखेरीस महाराष्ट्र सरकारला अनिल परब यांचे रिसॉर्ट पाडण्याचा आदेश देणे भाग पडले आहे. सध्या लोकायुक्तांसमोर सुनावणी चालू आहे.

१५. शिवसेनेच्या **खा. भावना गवळी** या रु. १०० कोटींच्या गैरव्यवहारात गुंतल्याचे आढळले आहे. त्यांचे भागीदार आणि सहकारी **श्री. सईद खान** यांना एक महिन्यापूर्वी अटक केली असून ते तुरुंगात आहेत. भावना गवळी यांच्या विरोधात कठोर कारवाई अपेक्षित आहे.

१६. मुंबई महानगरपालिका **२१०० कोटी रुपयांच्या कोविड रुग्णालय गैरव्यवहारात** गुंतलेली आढळली आहे. ठाकरे सरकारने ही जमीन ६ ऑक्टोबर २०२० रोजी एका बिल्डरला ६२ कोटी रुपयांना मालकी हक्काने हस्तांतरित केली. तावडतोब काही तासात मुंबई महानगरपालिकेने हीच जमीन त्या बिल्डरकडून २१०० कोटी रुपयांना खरेदी करण्याची तयारी दर्शविली. लोकायुक्तांसमोर झालेल्या सुनावणीत ठाकरे सरकारने आणि मुंबई महानगरपालिकेने मान्य केले की अपारदर्शीपणे व्यवहार झाला आणि करार रद्द केले.

१७. **शिवसेना, मुंबई महानगरपालिका** यांनी जागतिक टेंडर काढून एक कोटी कोरोना व्हॅक्सिन खरेदी करण्याची घोषणा केली. या जागतिक टेंडरसाठी जे अकरा प्रस्ताव आले होते ते सर्व संशयास्पद प्रस्ताव होते. मुख्यमंत्री श्री. उद्धव ठाकरे यांना, मुंबई महानगरपालिकेला सर्व प्रस्ताव फेटाळणे भाग पडले आणि एक कोटी व्हॅक्सिन्ससाठीचे संशयास्पद जागतिक टेंडरही रद्द करावे लागले.

१८. **ठाकरे सरकारचा रेमडेसिव्हीर घोटाळा** हाफकीन इन्स्टिट्यूटने ५२,००० रेमडिसिव्हिर इंजेक्शनची खरेदी प्रत्येकी ६६८ रुपयांना केली. त्याच वेळी मुंबई महानगरपालिकेने प्रत्येकी १,६६८ रुपयांनी ७२,००० रेमडिसिव्हिर इंजेक्शन घेतले. एकाच कालावधीत हाफकीन इन्स्टिट्यूट या राज्य सरकारच्या संस्थेकडून रेमडिसिव्हिरची प्रत्येकी ६६८ रुपये दराने खरेदी आणि मुंबई महानगरपालिकेकडून प्रत्येकी १,६६८ रुपये दराने.

१९. शिवसेना पक्षप्रमुख श्री. उद्धव ठाकरे यांनी २८ नोव्हेंबर २०१९ रोजी मुख्यमंत्रिपदाची शपथ घेतली. **मुंबई महानगरपालिकेने** दुसऱ्याच दिवशी २९ नोव्हेंबर २०१९ रोजी **अल्पेश अजमेरा बिल्डर्सकडून** ९०० कोटी रुपयांना जमीन खरेदी केली आणि ३४१ कोटी रुपये दिले सुद्धा. बिल्डरने ही जमीन २ कोटी ५५ लाख रुपयांना खरेदी केली होती.

२०. **शिवसेना नेते श्री. रविंद्र वायकर आणि भागीदार** यांनी कै. अन्वय नाईक यांच्याकडून जमीन आणि त्याच जमिनीवरील संशयास्पद १९ बंगलेही खरेदी केले. आम्ही हा विषय उपस्थित केला, घोटाळा उघड केला. अखेरीस घोटाळा सिद्ध झाला.

२१. **श्री अनंत करमुसे** यांचे त्यांच्या घरातून राज्य साडेअकरा वाजता **मंत्री श्री. जितेंद्र आव्हाड व पोलिस सुरक्षा रक्षकांनी** अपहरण केले होते. गेले १५ महिने अनंत करमुसे उच्च न्यायालयात न्याय मिळण्यासाठी धडपडत होते. काही दिवसांपूर्वी आव्हाड यांना अटक करण्यात आली आणि नंतर त्यांची जामीनावर मुक्तता करण्यात आली.

२२. **मुंबईच्या महापौर श्रीमती किशोरी पेडणेकर** यांनी वरळी येथील गोमाता जनता एसआरएस सोसायटी मध्ये अर्धा डझनहून अधिक गरिबांचे / लाभार्थींचे गाळे अनधिकृतरित्या स्वत:च्या ताब्यात घेतले आहेत. फोर्जरी ही केली आहे बनावटी / खोट्या सह्या व कागदपत्राद्वारा हे गाळे ताब्यात घेतल्याचे पुरावे ही न्यायालयात सुपूर्त करण्यात आले आहेत व सुनावणी सुरु आहे.

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अनिल परब यांचा रिसॉर्ट अनधिकृत - महाराष्ट्र सरकार

ठाकरे सरकारचे मंत्री श्री अनिल परब यांनी मौजे मुरुड ता. दापोली येथे अनधिकृत साई रिसॉर्ट बांधला आहे त्या संबंधात भाजपा नेते डॉ. किरीट सोमैया यांनी महामहीम राज्यपाल यांच्याकडे तक्रार केली होती, डॉ. सोमैया समवेत खा. श्री गोपाळ शेट्टी, आमदार श्री मिहिर कोटेचा, आमदार श्री राम कदम, श्री राहुल नार्वेकर, श्री संजय उपाध्याय हे राज्यपालांना भेटले होते.

राज्यपालांनी याची चौकशी करण्याची सूचना लोकायुक्त यांना केली होती / आहे.

महाराष्ट्राचे लोकायुक्त जस्टिस विद्यासागर कानडे यांच्याकडे या याचिकेची सुनावणी सुरू झाली आहे. लोकायुक्त यांनी श्री किरीट सोमैया यांनाही या सुनावणीच्या वेळी बोलावले होते. 5 ऑक्टोबर रोजी लोकायुक्तांकडे सुनावणी झाली.

1. लोकायुक्त यांनी या याचिके संबंधी आंतरिम निर्देश सुनावणी दरम्यान दिले ज्यात, या रिसॉर्ट मध्ये झालेल्या फोर्जरी, फ्रॉड संबंधात चौकशी साठी महाराष्ट्राचे गृह सचिवांना 7 डिसेंबरला हजर राहाण्याचे निर्देश दिले.

2. महाराष्ट्र पर्यावरण मंत्रालय व महाराष्ट्र सागरी किनारा प्राधिकरण यांना सुद्धा पुढच्या सुनावणी दरम्यान हजर राहण्यास सांगितले आहे.

3. सुनावणीत महाराष्ट्र सरकारच्या वतीने हा रिसॉर्ट अनधिकृत असल्याचे मान्य करण्यात आले आहे.

4. महाराष्ट्र सरकारच्या वतीने महाराष्ट्राचे अतिरिक्त सचिव श्री नितीन करीर यांनी लोकायुक्तांकडे निवेदन करताना जी अकृषिक परवानगी घेतली गेली त्यात बनावट पद्धतीने फसवणूक केली गेल्याचे सांगितले.

5. पर्यावरण मंत्रालयाने सुद्धा हा रिसॉर्ट CRZ च्या प्रतिबंधीत क्षेत्रात (no development zone) बांधला आहे व तो तोडावाच लागणार असे म्हंटले.

6. अश्या प्रकारे फसवणूक, घोटाळा करून अकृषिक परवानगी देण्यात आली त्या अधिकाऱ्यांविरुद्ध चौकशी चे आदेश महाराष्ट्र सरकारने दिले आहेत.

श्री अनिल परबांचा रिसॉर्ट अनधिकृत आहे हे आता ठाकरे सरकारनेच मान्य केले आहे, हा रिसॉर्ट तावडतोब तोडावा व अनिल परबांची मंत्रिमंडळातून त्वरित हकालपट्टी करावी अशी मागणी भाजपा नेते डॉ. किरीट सोमैया यांनी केली आहे.

मंत्री अनिल परब दापोली रिसॉर्टच्या विरुद्ध नॅशनल ग्रीन ट्रिब्यूनल (NGT) मध्ये सुनावणी सुरू

ठाकरे सरकारचे मंत्री श्री अनिल परब यांनी मौजे मुरुड, ता. दापोली, जि. रत्नागिरी येथे अनधिकृत साई रिसॉर्ट बांधले आहे त्या विरोधात भाजपा नेते डॉ किरीट सोमैया यांनी नॅशनल ग्रीन ट्रिब्यूनल (NGT) येथे याचिका दाखल केली आहे.

नॅशनल ग्रीन ट्रिब्यूनलने २९ सप्टेंबर २०२१ च्या सुनावणी दरम्यान श्री अनिल परब, श्री सदानंद कदम, श्री विभास साठे यांच्या विरुद्ध नोटीस जारी करण्याचे निर्देश दिले आहेत. त्या प्रमाणे श्री अनिल परब, श्री सदानंद कदम आणि श्री विभास साठे यांना या संबंधीची नोटीस जारी करण्यात आली आहे.

या अनधिकृत रिसॉर्टची मालकी कोणाची?

याचिकाकर्ते डॉ. किरीट सोमैया यांनी हा रिसॉर्ट अनधिकृतरीत्या श्री अनिल परब यांनी बांधला. ही जागा श्री विभास साठे यांच्याकडून श्री अनिल परब यांनी विकत घेतली. श्री विभास साठे यांनी किंवा त्यांच्या नावाने खाडाखोड (फोर्जरी) करून अकृषिक परवाना घेण्यात आला, तसेच हा अनधिकृत रिसॉर्ट श्री सदानंद कदम यांनी विकत घेतला असे आपल्या याचिकेत म्हटले आहे.

श्री अनिल परब, श्री सदानंद कदम आणि श्री विभास साठे यांना या संबंधात नोटीस जारी करण्यात आल्या आहेत, पुढची सुनावणी २५ नोव्हेंबर २०२१ ला होणार.

BEFORE THE NATIONAL GREEN TRIBUNAL,
WESTERN ZONE BENCH, PUNE

Original Application No. 58/2021 (WZ)
I.A. No. 73/2021 (WZ)

Dr. Kirit Somaiya
V/s.
State Of Maharashtra & Ors.

To

Shri Anil Dattatray Parab — Proposed Respondent No. 6
Transport Minister, Govt. Of Maharashtra
Ravi Kiran 58/2025, Gandhi Nagar,
Near Bank Of Maharashtra, Bandra (E), Mumbai- 400051
(9820413605; anilparbatrav@gmail.com)

NOTICE

1. The above titled Original Application is posted for **admission** on **25.11.2021 at 10.30 AM through Video Conferencing** before The National Green Tribunal, Western Zone Bench, Pune.

2. Please note that you shall make yourself available, or represent through authorized legal representative, on the date and place indicated herein above, in default, the said Application will be heard and determined in your absence.

3. Given under my hand and the seal of this Tribunal, this the 05.10.2021.

Assistant Registrar, NGT

End: Completion I.A. if along with the order
(For Orders, Cause Lists & other information, please visit our website www.greentribunal.gov.in)

NOTARY
GOVT. OF INDIA

राष्ट्रवादी काँग्रेसचे मंत्री श्री. हसन मुश्रीफ यांनी रु. १५,००० कोटींचे त्यांच्याच ग्रामविकास खात्याचे कंत्राट आपल्याच कुटुंबाच्या जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनीला दिले. आम्ही हा विषय मांडला अखेरीस ठाकरे सरकारलाहा कॉन्ट्रक्ट रद्द करावा लागला.

नवभारत

भाजपा के आरोपों से डरी आघाड़ी सरकार

मुश्रीफ के दामाद का ठेका रद्द

■ **मुंबई,** नवभारत न्यूज नेटवर्क. भाजपा नेता एवं पूर्व सांसद किरीट सोमैया ने राज्य के ग्रामीण विकास मंत्री हसन मुश्रीफ पर एक बार फिर हमला बोला है. उन्होंने कहा कि कोरोना काल में जहां एक तरफ मुख्यमंत्री उद्धव ठाकरे ठाकरे सहित तमाम लोग घर में बैठे थे, वहीं दूसरी तरफ सरकार में बड़े पैमाने पर भ्रष्टाचार चल रहा था. सोमैया ने कहा कि शत-प्रतिशत मंत्रालय बंद होने के बावजूद शरद पवार के शिष्य 15,000 करोड़ रुपये लूट रहे थे. भाजपा कार्यालय में आयोजित पत्रकार परिषद में सोमैया ने कहा कि हसन मुश्रीफ के दामाद मतीन मंगोली की जयोस्तुते मैनजमेंट कंपनी को दिया गया 1,500 करोड़ रुपये का ठेका आखिरकार रद्द कर दिया गया है.

₹ 15,000
करोड़ के भ्रष्टाचार का आरोप

परब पर भी साधा निशाना

सोमैया ने मुश्रीफ के साथ ही शिवसेना नेता और परिवहन मंत्री अनिल परब पर भी निशाना साधा है. उन्होंने बताया कि अनिल परब को नेशनल ग्रीन ट्रिब्यूनल के सामने पेश होने का आदेश दिया गया है.

संजय राऊत को चुनौती

सोमैया ने पिंपरी-चिंचवड़ नगर निगम में कथित घोटाले के संबंध में संजय राऊत की तरफ से लिखे गए एक पत्र का भी जवाब दिया. उन्होंने कहा कि आपको जांच पड़ताल करने का अधिकार है. क्या मुख्यमंत्री पूछताछ नहीं कर सकते? जांच करें कि घोटाला कहां हुआ, कार्रवाई करें. आपको कौन रोक रहा है? अगर ईओडब्ल्यू की बात है तो पूछने दीजिए.

■ सोमैया ने बताया कि 27,000 ग्राम पंचायतों के टीडीएस रिटर्न का भुगतान करने के लिए 10 वर्षों के लिए अनुबंध दिया गया था. कंपनी को मतीन ने 8 महीने पहले खरीदा था.

Mumbai Edition
22-october-2021 Page No. 1
epaper.enavabharat.com

राज्यातील ग्रामपंचायती, पंचायत समित्या व जिल्हा परिषदांमध्ये तसेच ग्रामविकास विभागाच्या अधिपत्याखालील इतर कार्यालयांमध्ये GST TDS, Income Tax वरील TDS/TCS तसेच Labour Cess, रॉयल्टी आणि इन्श्युरन्स इत्यादी प्रकारच्या वजावटी यांचा भरणा/ रिटर्न्स भरण्यासाठी एजन्सीची निवड करणेबाबत.

महाराष्ट्र शासन
ग्राम विकास विभाग
शासन निर्णय क्रमांक : संकीर्ण-२४२०/प्र.क्र.१९ /आपले सरकार कक्ष
बांधकाम भवन,२५,मर्झबान पथ,
फोर्ट,मुंबई-४०००० १
दिनांक: १३ सप्टेंबर, २०२१

वाचा : १. ग्रामविकास विभाग शासन निर्णय क्रमांक संकिर्ण-२४२०/प्र.क्र.१९/आसक, दि.१० मार्च, २०२१

२.ग्रामविकास विभाग शासन शुध्दीपत्रक क्रमांक संकिर्ण-२४२०/प्र.क्र.१९/आसक, दि.२९ जुलै, २०२१

३.ग्रामविकास विभाग शासन निर्णय क्रमांक संकिर्ण-२४२०/प्र.क्र.१९/आसक, दि.६ सप्टेंबर, २०२१

प्रस्तावना :-

वित्त मंत्रालय, भारत सरकार यांची शासन अधिसुचना क्रमांक ५०/२०१८, दि. १३ सप्टेंबर, २०१८ तसेच त्या अनुषंगाने वित्त विभाग, महाराष्ट्र शासन यांनी दि.१८ सप्टेंबर, २०१८ रोजी काढलेली अधिसुचना आणि वित्त विभाग परिपत्रक क्रमांक: संकीर्ण-२०१८/प्र.क्र.१४४/२०१८/ कोषा-५, दि. २८ सप्टेंबर, २०१८ अन्वये सर्व शासकीय, निम शासकीय संस्था, स्थानिक स्वराज्य संस्था, सार्वजनिक उपक्रमातील आस्थापनांना वस्तू व सेवा कर अधिनियमाखाली GST करावर २% TDS (१% CGST आणि १% SGST किंवा २% IGST) भरणे अनिवार्य आहे. त्याचप्रमाणे आयकर कायद्यातील तरतुदी नुसार आयकरावरील TDS/TCS भरणे अनिवार्य आहे. सदर TDS/TCS ची रक्कम शासनाने निर्धारीत केलेल्या वेळेपुर्वी भरणे अनिवार्य आहे. अन्यथा, या

देखील अद्याप कोणत्याही ग्रामपंचायतीने सदर ससंस्थेकडून काम करून घेण्यासाठी प्रतिसाद दिलेला नसल्याची बाब निदर्शनास आली. वास्तविक GST वा तत्सम करांचे रिटर्न्स भरण्यासाठी शासनाने ग्रामपंचायतींना प्रशिक्षित मनुष्यबळ उपलब्ध करून दिले होते. परंतु ग्रामपंचायती सदर संस्थेकडून काम करून घेण्यास इच्छुक नसतील तर त्यांना सक्ती करणे योग्य नसल्याने यासंदर्भात शासनाने घेतलेल्या निर्णयाचा पुर्नविचार करण्याची बाब शासनाच्या विचाराधीन होती.

शासन निर्णय :

राज्यातील ग्रामपंचायती, पंचायत समित्या व जिल्हा परिषदांमध्ये तसेच या विभागाच्या अधिनस्त इतर शासकीय कार्यालयांमध्ये GST TDS, Income Tax वरील TDS/TCS तसेच Labour Cess, रॉयल्टी आणि इंन्श्युरन्स इत्यादी प्रकारच्या वजावटी यांचा भरणा/ रिटर्न्स भरणेकरिता मे जयोस्तुते मॅनेजमेंट प्रा.लि. या संस्थेची निविदा प्रक्रियेद्वारे केलेली निवड रद्द करण्यात येत आहे.

२. यासंदर्भात शासनाने निर्गमित केलेला ग्रामविकास विभाग शासन निर्णय क्रमांक संकिर्ण-२४२०/प्र.क्र.१९/आसक, दि.१० मार्च, २०२१ व त्याअनुषंगाने निर्गमित केलेले शासन शुध्दीपत्रक दि.२९ जुलै, २०२१ आणि शासन निर्णय दि.६ सप्टेंबर, २०२१ रद्द करण्यात येत आहेत.

३. यासंदर्भात शासनाने मे जयोस्तुते मॅनेजमेंट प्रा.लि. या संस्थेसोबत दि.३० मार्च, २०२१ रोजी केलेला करारनामा रद्द करण्यात येत आहे.

सदर शासन निर्णय महाराष्ट्र शासनाच्या www.maharashtra.gov.in या संकेतस्थळावर उपलब्ध करण्यात आले असून त्याचा संकेताक २०२११०१४०९४९३८३५२० असा आहे. हा आदेश डिजीटल स्वाक्षरीने साक्षांकित करुन काढण्यात येत आहे.

महाराष्ट्राचे राज्यपाल यांच्या आदेशानुसार व नावाने,

(प्रविण देविचंद जैन)
उप सचिव, महाराष्ट्र शासन

प्रत,

१. मा. राज्यपाल यांचे सचिव
२. मा.मुख्यमंत्री यांचे प्रधान सचिव
३. मा. उप मुख्यमंत्री यांचे प्रधान सचिव
४. मा. सभापती विधान परिषद यांचे खाजगी सचिव
५. मा. अध्यक्ष विधानसभा यांचे खाजगी सचिव, विधानभवन मुंबई
६. मा. विरोधी पक्ष नेते विधान परिषद/ विधानसभा यांचे खाजगी सचिव, विधानभवन मुंबई
७. सर्व विधानमंडळ सदस्य, विधानभवन मुंबई
८. शासनाचे मुख्य सचिव

Dr. Kirit Somaiya
Chartered Accountant,Ph.D
Former Member of Parliament

Bharatiya Janata Party

कृपया प्रकाशनार्थ

दि. २१ ऑक्टोबर, २०२१

श्री हसन मुश्रीफ यांच्या जावयाला दिलेला १५०० कोटींचा कॉन्ट्रॅक्ट रद्द- डॉ. किरीट सोमैया

ठाकरे सरकारचे ग्राम विकास मंत्री श्री हसन मुश्रीफ यांनी आपल्या जावयाच्या जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनीला १० वर्षांचा १५०० कोटींचा कॉन्ट्रॅक्ट दिला होता. महाराष्ट्रातील २७००० ग्राम पंचायतींचे TDS रिटर्न पुढच्या १० वर्षांपर्यंतजयोस्तुते मॅनेजमेंट प्रा. लि. कंपनी फाइल करणार आणि त्यासाठी प्रत्येक ग्रामपंचायत ने सुमारे ५०,००० रुपये ची वर्षी द्यावयाचा हा कॉन्ट्रॅक्टहोता. दर वर्षी जयोस्तुते मॅनेजमेंट कंपनीला यातून १५०० रुपये आवक होणार होती.

भाजपा नेते डॉ. किरीट सोमैया यांनी या घोटाळेबाज कंपनीला घोटाळेबाज मंत्री श्री हसन मुश्रीफ यांनी अपारदर्शकरित्या १० मार्च २०२१ रोजी १० वर्षांचाकॉन्ट्रॅक्ट दिला हा घोटा ळा सप्टेंबर महिन्यात उघडकिस आणला होता.जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनीची स्थापना जरी २०१२-१३ मध्ये झाली असेल, परंतु श्री हसन मुश्रीफ यांचे जावईश्री मतीन यांनी ही कंपनी ८ महिन्यापूर्वीच विकत घेतली. गेल्या ८ वर्षात या कंपनीची काहीही आवक नाही, व्यवसाय नाही.२०१९-२० चे उलाढाल (Turnover) शून्य.

कशा पद्धतीने हा घोटाळा करण्यात आला, कंपन्याची सेयर उभी करुन श्री हसन मुश्रीफ यांच्या जावयाची मालकी लपवण्याचा अट्टाहास श्री मुश्रीफ यांनी कैला होता, काल ग्रामविकास मंत्रालयात माहिती अधिकार अंतर्गत डॉ. किरीट सोमैया यांनी या कॉन्ट्रॅक्ट दिलेल्या फाईलचे इन्स्पेक्शन, अवलोकन केले त्यात धक्कादायक बाब उघडकीस आली आहे.

1. अशा पद्धतीने केंद्र सरकारने जोनिधी ग्रामपंचायतच्या खात्यात जमा केला आहे तोखुटण्याचा डाव हा मे २०२० मध्ये रचण्यात आला.
2. ५ मे २०२० ला ग्रामविकास मंत्रालय, ग्रामविकास मंत्री यांनी अशा पद्धतीने सगळ्या ग्रामपंचायतींचा कॉन्ट्रॅक्ट एका कंपनीला देण्याचा व त्याच्या टेंडर प्रक्रियेचा प्रारंभ करावा असा निर्णय घेतला.
3. डॉ. किरीट सोमैया यांनी काल फाईल नोटिंग पाहिले त्यात आश्चर्यजनक बाब समोर आली की ५ मे २०२० ला हा निर्णय घेण्यात आला त्याचे फाईल नोटिंगग्रामविकास मंत्रालयाच्या फाईलीत उपलब्ध आहेत.
4. ज्यावेळी संपूर्ण महाराष्ट्र १००% लॉकडाऊन मध्ये होता, मंत्रालय, सरकारी कार्यालय, न्यायालय, मुख्यमंत्री सगळेच आपापल्या घरी बंद होते त्यावेळेला हा निर्णय करण्यात आला.
5. १० मार्च २०२१ ला मुश्रीफ साहेबांनी आदेश देऊन जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनी ला हा कॉन्ट्रॅक्ट देण्यात आला.
6. भाजपा ने हा घोटाळा बाहेर काढला प्रदेशाध्यक्ष श्री चंद्रकांत पाटील यांनी या संबंधी आक्षेप घेतला. ग्रामविकास मंत्रालय, भारत सरकार यांनी या संबंधी महाराष्ट्र सरकारकडून स्पष्टता मागविली.
7. आता १४ ऑक्टोबर २०२१ रोजी श्री प्रविण देविचंद जैन या उपसचिवांच्या नावाने ग्रामविकास खात्याचे एक परिपत्रक हे सरकारी संकेतस्थळावर अपलोड करण्यात आली आहे त्यात १० मार्च २०२१ चा जयोस्तुते मॅनेजमेंट प्रा. लि. कंपनीला देण्यात आलेला कॉन्ट्रॅक्ट रद्द करण्यात येत आहे असे म्हटले आहे.

१५०० कोटींचा हा ठाकरे सरकारचे मंत्री श्री हसन मुश्रीफ यांचा घोटाळा आता सिद्ध झाला आहे त्यांची ताबडतोब मंत्रीमंडळातून हकालपट्टी करावी अशी डॉ. किरीट सोमैया यांनी मागणी केली आहे.

(कार्यालय सचिव)

kiritsomaiya@gmail.com www.kiritsomaiya.com KiritSomaiyaBJP @kiritsomaiya

Neelam Nagar, Mulund, Mumbai-400 081 • Tel.: 022-21634152 • Mob.: 9869220027

850 कार परिवाराचे जावई "श्री मोहन पाटील" करोडो चे आर्थिक व्यवहार

ठाकरे सरकारचे उपमुख्यमंत्री श्री. अजित पवार यांचे आर्थिक व्यवहार, उलाढाल आश्चर्य देणारीच आहे. पवारांचे मित्र, बिल्डरांकडून श्री. अजित पवार आणि त्यांच्या परिवाराच्या सदस्यांच्या खात्यात कोट्यावधी रुपयांची (शंभरहून अधिक) अपारदर्शक नामी व बेनामी आवक. पवार परिवारचे जावई आणि अन्य सदस्यांच्या खात्यात कोट्यावधी रुपयांची ट्रान्सफर.

1. श्री. अजित पवार (स्वत:)
2. सौ. सुनेत्रा अजित पवार (पत्नी)
3. श्री. पार्थ अजित पवार (मुलगा)
4. श्रीमती आशाताई अनंतराव पवार (आई)
5. सौ. विजया मोहन पाटील (बहीण)
6. श्री. मोहन पाटील (जावई)
7. सौ. नीता पाटील (बहीण)

गेली १९ दिवस इन्कम टॅक्स आता ईडी ची धाड, शोध (सर्च) सुरु आहेत. १,०५० कोटी रुपयांची बेनामी संपत्ती समोर आली आहे, त्याचबरोबर १८४ कोटी रुपयांची रोख रक्कम, दागिने (ज्वेलरी), आर्थिक व्यवहारांचे कागदपत्र इ. इन्कम टॅक्सच्या हाती लागले.

जरंडेश्वर साखर कारखाना, दौंड साखर कारखाना, श्री. अंबालिका शुगर प्रा. लि. व अन्य कंपन्यांचे आर्थिक व्यवहार, तसेच श्री. अजित पवार मित्र परिवाराच्या, समूहाच्या विभिन्न कंपन्यांच्या बरोबरची हेराफेरी समोर येत आहे.

डझनावारी कंपन्यांचा आर्थिक व्यवहार अंतर्गत ट्रान्सफर हे बेनामी संपत्ती व मनी लॉंड्रींगसाठी कंपनीची लेयर / शिडी तयार करणे, करोडो रुपयांची हेराफेरी.

श्री. अजित पवारांनी नामीबेनामी संपत्तीसाठी पवार परिवाराचे जावई श्री. मोहन पाटील यांचा ही उपयोग केलेला दिसत आहे. ईडी व इन्कम टॅक्सची चौकशी चालू आहे.

अजित पवार, मित्र परिवार घोटाळा

गुरू कमोडिटीजने स्पार्कलिंग सॉईल (अजित पवार), शिवालिक बिल्डर्स (विवेक जाधव), ओंकार रियल्टर्स (बाबुलाल वर्मा) यांच्या भागीदारीत जरंडेश्वर साखर कारखाना विकत घेतला.

गुरू कमोडिटीजने जरंडेश्वर साखर कारखाना जरंडेश्वर शुगर मिल्स लिमिटेडला ४५ वर्षांच्या दीर्घ मुदतीच्या भाडेतत्त्वावर दिला.

जरंडेश्वर साखर कारखाना हा स्तरित कंपन्यांच्या मालकीचा आहे, ज्यांचे मालक आहेत...

- मोहन पाटील (अजित पवार यांची बहीण सौ. विजया पाटील यांचे पती)
- नीता पाटील
- सुनेत्रा पवार

जरंडेश्वर साखर कारखाना

जरंडेश्वर साखर कारखाना भेट

852 ठाकरे सरकार - बीएमसी १०० कोटींचा दहिसर जमीन घोटाळा

उद्धव ठाकरे यांनी २८ नोव्हेंबर रोजी मुख्यमंत्रीपदाची शपथ घेतली. ठाकरे सरकारने सत्तेवर आल्या आल्या कोणता निर्णय घेतला तर दहिसर येथील एका बिल्डरला ९०० कोटींची जमीन बहाल करण्याचा.

गेल्या १० वर्षांत दहिसर येथील ७ एकर जमिनीच्या खरेदीचा प्रस्ताव मांडला गेला होता , मात्र तो स्वीकारला गेला नव्हता. निशल्प रिऍलिटी ने हा भूखंड खरेदी करण्याचा प्रस्ताव दिला होता.

या जागेवर १०० टक्के अतिक्रमणे आहेत. बृहन्मुंबई महानगरपालिकेने या जमिनीच्या खरेदीचा प्रस्ताव फेटाळून लावला होता. ही जमीन खरेदी करण्याचा प्रस्ताव म्हणजे बृहन्मुंबई महापालिकेची कोट्यवधींची उधळपट्टी करण्यासारखे आहे, असे स्पष्ट मत नोंदविले होते.

बृहन्मुंबई महानगरपालिकेचे त्यावेळचे आयुक्त प्रवीण परदेशी यांनी महापालिकेच्या सुधार समितीला पत्र पाठवून या जमिनीचा ताबा घेण्यास स्पष्ट विरोध दर्शविला होता.

२ नोव्हेंबर २०१९ रोजी महापालिकेच्या सुधार समितीने या भूखंडाच्या ताब्याचा प्रस्ताव मांडला होता.

मात्र परदेशी यांनी तो फेटाळला. निशल्प रिऍलिटी ने मांडलेला हा प्रस्ताव कसा अव्यवहार्य आहे आणि त्यामुळे तो फेटाळणे कसे आवश्यक आहे, याबाबतची सविस्तर टिप्पणी दिली होती.

२ नोव्हेंबर रोजी झालेल्या सुधार समितीच्या बैठकीत या जागेची प्रत्यक्ष पाहणी करण्याचा प्रस्ताव मांडला.

२८ नोव्हेंबर रोजी उद्धव ठाकरे यांनी मुख्यमंत्रीपदाची शपथ घेतली.

२९ नोव्हेंबर रोजी सुधार समितीची विविध विषयांवर चर्चा करण्यासाठी बैठक होणार होती. या बैठकीत दहिसर एकसर येथील भूखंडाच्या खरेदीचा विषय नव्हता. मात्र हा विषय तातडीचा विषय म्हणून हा भूखंड खरेदी विषय बैठकीच्या कार्यक्रमपत्रिकेत घुसडण्यात आला.

दहिसर येथील प्रस्तावीत जागा

ठाकरे सरकार - बीएमसी १०० कोटींचा दहिसर जमीन घोटाळा

दहिसर येथील जागेची भेट

प्रवीण परदेशी यांनी महापालिका आयुक्त असताना या भूखंडाची किमत ५४ कोटी च्या आसपास आहे, असे स्पष्ट मत नोंदविले होते.

१५ ऑक्टोबर २०१९ रोजी तत्कालीन आयुक्त प्रवीण परदेशी यांनी काढलेल्या सूचनापत्रकात, अशा कोणत्याही जागेचा ताबा कोणत्याही अडचणींविना मिळाला पाहिजे, असे मत नोंदविले होते.

या भूखंडाचे मूल्य निर्धारण ३५४ कोटी करण्यात आले त्यावेळीही परदेशी यांनी त्यास तीव्र आक्षेप घेतला होता.

असे असताना ठाकरे सरकारकडून महापालिकेच्या वित्त विभागाला या भूखंडाच्या खरेदीपोटी ३४९ कोटी, १४ लाख ११ हजार १३ रु. इतकी रक्कम तातडीने देण्यात यावी, अशी सूचना देण्यात आली. बिल्डरकडून ५४ कोटी रु. चा भरणा अनामत रक्कम म्हणून अगोदरच करण्यात आला होता. त्यामुळे १५ फेब्रुवारी २०२० रोजी उर्वरीत २९४ कोटी रु. चा मोबदला निशल्प रिऍलिटी ला देण्यात आला.

आता संबंधित बिल्डर या जागेचे ३४९ कोटी रु. हे मूल्यनिर्धारण चुकीचे आहे, असा दावा करीत आहे. या बिल्डरने नागपूर येथील महसूल न्यायाधिकरणाकडे अपील करून या भूखंडाची किमत ९०० कोटी रु . असल्याचा दावा केला आहे. या बिल्डरने मुंबई महापालिकेकडे उर्वरीत ५५० कोटी रु. देण्याची मागणी केली आहे.

सदर भूखंडाची मी प्रत्यक्ष पाहणी केली असून या भूखंडावरील अतिक्रमणे हटविणे शक्य नाही असे माझे स्पष्ट मत बनले आहे.

राज्य सरकार आणि बृहन्मुंबई महापालिका २८ नोव्हेम्बर २०१९ पर्यंत अतिक्रमण असलेला हा भूखंड एवढ्या चढ्या किमतीत घेण्यास विरोध करीत होते. मात्र उद्धव ठाकरे मुख्यमंत्री झाल्यावर एका रात्रीत राज्य सरकारची आणि महापालिकेची भूमिका बदलली आणि ९०० कोटींच्या भूखंडाची भेट एका बिल्डरला देण्यात आली.

मिलिंद नार्वेकरनी स्वतःचा बंगला पाडला : ठाकरे सरकार

रत्नागिरी जिल्हाधिकारी नी माझा तक्रारीचा उत्तरात मला कळविले आहे की " मौजे मुरुड ता. दापोली येथील गट नं. ४१० मधील बांधकाम श्री. मिलींद केशव नार्वेकर यांनी स्वतःच निष्कासीत केलेले असल्यामुळे सदयस्थितीमध्ये कोणतेही बांधकाम जागेवर अस्तित्वात नाही"

महाराष्ट्र शासन
महसूल व वनविभाग
जिल्हाधिकारी कार्यालय रत्नागिरी

[illegible] प्रशासकीय इमारत [illegible] महसूल शाखा [illegible] रत्नागिरी

दूरध्वनी क्रमांक : [illegible] फॅक्स क्रमांक : [illegible]
[illegible] Email ID:- revenuecollector@yahoo.in

क्र. महसूल/कार्या.ओ.के.-9/CRZ/SR-40/कावि.484/2021 दिनांक : २८ /०९/2021

प्रति,

(१५११२) ✓डॉ. किरीट सोमैया
माजी संसद सदस्य,
रा. 9बी, नीलम नगर, फेज-2,
मुलुंड (पुर्व), मुंबई-400 081

विषय : मौजे मुरुड ता. दापोली येथील श्री. मिलींद नार्वेकर यांच्या अनधिकृत बंगल्याचे बांधकामाबाबत .

संदर्भ : 1. आपला अर्ज क्र. KS/MUM/770/2021 दिनांक 26/08/2021

महोदय,

मौजे मुरुड ता. दापोली येथील स.नं. 410 मध्ये श्री. मिलिंद केशव नार्वेकर यांनी सीआरझेड क्षेत्रामध्ये विनापरवाना बांधकाम केले असलेबाबत संदर्भीय तक्रार अर्ज या कार्यालयाकडे प्राप्त झाले आहेत. सदर तक्रार अर्जाप्रकरणी उपविभागीय अधिकारी दापोली, उपविभाग दापोली यांचेमार्फत चौकशी करण्यात आली असून, उपविभागीय अधिकारी दापोली, उपविभाग दापोली यांचा चौकशी अहवाल दिनांक 22/09/2021 रोजीचे पत्रान्वये या कार्यालयाकडे प्राप्त झाला आहे.

उपविभागीय अधिकारी दापोली, उपविभाग दापोली यांच्या अहवालाचे अवलोकन केले असता मौजे मुरुड ता. दापोली येथील गट नं. 410 मधील बांधकाम श्री. मिलिंद केशव नार्वेकर यांनी स्वत:च निष्कासीत केलेले असलेमुळे सद्यस्थितीमध्ये कोणतेही बांधकाम जागेवर अस्तित्वात नाही. सबब जागेवरील बांधकाम पुर्णत: निष्कासीत झालेले आहे. सोबत उपविभागीय अधिकारी दापोली, उपविभाग दापोली यांच्या अहवालाची प्रत माहितीस्तव सादर करणेत येत आहे.

आपला विश्वासु,

(संजय शिंदे)
अपर जिल्हाधिकारी
जिल्हाधिकारी कार्यालय रत्नागिरी

मा. जिल्हाधिकारी रत्नागिरी यांच्या मान्यतेने

प्रत : कक्ष अधिकारी, महसूल व वनविभाग, मंत्रालय, मुख्य इमारत, पहिला मजला, मादाम कामा मार्ग, हुतात्मा राजगुरु चौक, मुंबई-400 032 यांजकडे माहितीसाठी सादर

NOTARY
GOVT. OF INDIA
Maharashtra
Reg No. 15220

Dr. Kirit Somaiya
Chartered Accountant,Ph.D
Former Member of Parliament

Bharatiya Janata Party

कृपया प्रसिद्धीसाठी

मुंबई, 16 डिसेंबर 2020

श्री. प्रताप सरनाईकचे "विहंग गार्डन बी" बिल्डिंग्स अनधिकृत -किरीट सोमैया

विहंग ग्रुप ऑफ़ बिल्डर्स ने बांधलेले विहंग गार्डन ठाणे, चे बी 1 आणि बी 2 अशा दोन इमारती, अनधिकृत असून कारवाई करण्याचे आदेश 2012 मध्ये देण्यात आले होते. ह्या दोन्ही बी 1 आणि बी 2 ला अजून पर्यंत वापर परवाना (Occupation Certificate OC) मिळाले नाही. ह्या इमारतीचे 9 ते 13 मजले अनधिकृत आहे आणि ताबडतोब तोडण्याचे आदेश ही, 2012 मध्ये देण्यात आले होते.

भाजप नेते डॉ. किरीट सोमैया यांनी माहिती अधिकारा खाली केलेल्या अर्जांच्या उत्तरात ठाणे महापालिकांनी त्यांना ही माहिती दिली आहे.

ज्याच्या अंतर्गत विहंग गार्डनच्या इमारत 'A' साठी वापर परवाना प्रदान करण्यात आलेला आहे. परंतु, B1 व B2 इमारतीसाठी कोणताही वापर परवाना (O.C.) देण्यात आले नाही.

यासंबंधात ठाणे महानगरपालिकेकडे श्री. प्रताप सरनाईक यांनी अपिल केल्यानंतर काही अटीवर अनधिकृत बांधकाम तोडण्याचे काम तात्पुरते थांबवण्यात आले. ठाणे महापालिकेचे तत्कालीन आयुक्तांनी एकमहिन्याची मुदत दिली होती त्याला आता ८ वर्षे झाली.

शिवसेना नेते श्री. प्रताप सरनाईक यांनी विहंग गार्डनच्या B1 व B2 तेरा मजली इमारतीचे अनधिकृत बांधकाम केले, यातील सदनिकेची विक्री करून प्रामाणिक मध्यमवर्गीय ग्राहकांची फसवणूक केली आहे, शिवसेनेच्या अखत्यारीतील ठाणे महापालिकेने श्री. प्रताप सरनाईकच्या या फसवणूकीला संरक्षण दिले आहे.

डॉ. किरीट सोमैया यांनी या फसवणूकीच्या विरोधात, अनधिकृत बांधकामाच्या विरोधात श्री. प्रताप सरनाईक, विहंग ग्रुप ऑफ बिल्डर्सच्या विरोधात ताबडतोब कारवाई करावीअशी मागणी केली आहे.

डॉ. सोमैया यांनी भाजपात ठाणे महापालिका गटाचे नेते श्री. संजय वाघुले सोबत काल ठाणे महापालिकेच्या संबंधित अधिकाऱ्यांशी प्रत्यक्ष भेट घेतली. या अनधिकृत बांधकामा विरोधात आतापर्यंत कारवाई का नाही? यासंबंधी तक्रारही केली.

श्री. सोमैया व श्री. वाघुले यांनी विहंग गार्डन या इमारतीची पण प्रत्यक्ष पाहणी केली.

या इमारतीत श्री. प्रताप सरनाईक यांचे कार्यालयही आहे, तसेच प्रताप सरनाईक यांचे घोटाळेबाज भागीदार श्री. अमित चंदोळे तेही या इमारतीत 12/13 व्या मजल्यावर राहत आहेत.

(सचित्र)

kiritsomaiya@gmail.com www.kiritsomaiya.com KiritSomaiyaBJP @kiritsomaiya
Neelam Nagar, Mulund, Mumbai-400 081 • Tel.: 022-21634152 • Mob.: 9869220027

NOTARY M. PABLE Mumbai Maharashtra Reg. No. Exp. Dt. GOVT. OF INDIA

शिवसेनेचे नेते व प्रवक्ते आ. प्रताप सरनाईक यांनी एनएसईएल गैरव्यवहारातील ३५ कोटी रुपये वळकावल्याचे आढळले. त्यांनी टिटवाळा येथे ७८ एकर जमीन खरेदी केली व ती ईडीने जप्त केली आहे. श्री. प्रताप सरनाईक यांनी त्यांच्या विहंग गार्डन बिल्डिंग प्रकल्पामध्ये बेकायदेशीरपणे पाच मजले बांधले आहेत. त्यांना २१ कोटी रुपये दंड भरण्यास सांगण्यात आले आहे. हा गैरव्यवहार आम्ही उघडकीस आणला आहे. लोकायुक्तांसमोर सुनावणी चालू आहे.

ठाणे महानगरपालिका, ठाणे

ठाणे महानगरपालिका, ठाणे

Thane Municipal Corporation File/Noting

Thane Municipal Corporation File/Noting

Dr. Kirit Somaiya
Chartered Accountant/Ph.D
Former Member of Parliament

Bharatiya Janata Party

कृपया प्रकाशनार्थ

दि. 10 जानेवारी, 2021

ED यांनी श्री.प्रताप सरनाईक यांच्या बिहेन आस्था हौसिंग कंपनीच्या गुरवली, टिटवाळा येथील 112 जमिनी/भिळकतींचा ताबा घेतला !! - किरीट सोमैया

शिवसेना नेते श्री. प्रताप सरनाईक आणि श्री. मोहित अग्रवाल यांनी NSEL घोटाळ्याचे ₹100 कोटी बिहेन आस्था हौसिंग कंपनी LLP मधे वळविले (diverted) होते. टिटवाळा येथील गुरवली गावात 112 जमिनी घेतल्या होत्या.

दि. 31 जानेवारी, 2014 रोजी प्रवर्तन निदेशालय (ED) यांनी या जमिनी संबंधी Attachment/जप्तीचे आदेश दिले होते. न्यायालयीन प्रक्रियेत ट्रिब्यूनल Tribunal ने त्याला मान्यता ही दिली होती/आहे.

आता या जमिनींचा प्रत्यक्ष ताबाही ED ने काल घेतला. श्री. प्रताप सरनाईक यांचा असा एकशे बारा सात बारा (112, 7/12) एकंदर 78.27 हक्टर जमिनीचा ताबा काल ED ने घेतला. ED ने या जागेवर आपले बोर्डही लावले आहेत.

"PMLA कायद्यानुसार या जमिनींचा कब्जा Directorate of Enforcement, Mumbai तर्फे घेण्यात आला आहे, Prevention of Money Laundering Act, 2002 च्या अंतर्गत या जमिनींचा ताबा अधिकृतरित्या घेण्यात आला आहे. या जमिनीवर अतिक्रमण (Trespassing Prohibited) करण्यास बंदी आहे. या जमिनी संबंधी कोणीही कोणत्याही प्रकारचा आर्थिक व्यवहार करण्यात येऊ नये बोर्ड ही ED अधिकारी यांच्या आदेशावरून लावण्यात आले आहे.

भाजप नेते श्री. किरीट सोमैया यांनी एक महिन्यापूर्वी या जागेची भेट घेतली होती. श्री. प्रताप सरनाईकच्या NESL घोटाळ्यातील या जमिनींवर ED ने जप्तीची नोटिस काढली होती. या जमिनींचे पाठच्या दरवाजे व्यवहार होत असल्याची तक्रार श्री. किरीट सोमैया यांनी ED कडे केली होती. याचा भाग म्हणून ED ने आता या तक्रारी संदर्भात कारवाई करताना ED नेच जागेचा ताबा घेतला आहे.

श्री. प्रताप सरनाईकचे भागीदार श्री. मोहित अग्रवाल यांनी NSEL चा 250 कोटी रुपयांचा घोटाळा केला होता, त्यातले ₹100 कोटी, ह्या बिहेन आस्था कंपनी मधे, अशा जमिनी/भिळकती घेण्यासाठी वापरले होते. त्यामुळे ही जप्तीची कारवाई झाली आहे.

सचिव

kiritsomaiya@gmail.com www.kiritsomaiya.com KiritSomaiyaBJP @kiritsomaiya
Neelam Nagar, Mulund, Mumbai-400 081 • Tel.: 022-21634102 • Mob.: 9892220627

श्री. अनंत करमुसे यांचे त्यांच्या घरातून राली साडेअकरा वाजता मंत्री श्री. जितेंद्र आव्हाड व पोलिस सुरक्षा रक्षकांनी अपहरण केले होते. गेले १५ महिने अनंत करमुसे उच्च न्यायालयात न्याय मिळण्यासाठी धडपडत होते. काही दिवसांपूर्वी आव्हाड यांना अटक करण्यात आली आणि नंतर त्यांची जामीनावर मुक्तता करण्यात आली.

आव्हाड यांना अटक, सुटका

करमुसे मारहाणप्रकरणी गृहनिर्माणमंत्र्यांवर अखेर कारवाई

म. टा. प्रतिनिधी, ठाणे

लावकर सुनावणीच्या विनंतीनंतर...

66 दीड वर्षांनंतर संबंधी न्यायालयाने न्याय दिला. गृहनिर्माणमंत्री जितेंद्र आव्हाड यांना अटक करण्याचे आदेश दिले. ठाणे पोलिसांनी त्यांना अटक केली. अनंत करमुसेला न्याय मिळाला. आता प्रश्न गृह मंत्र्यांचा मंत्रिमंडळात ठेवू नये. आम्ही राज्यपालांकडे जितेंद्र आव्हाड यांची हकालपट्टी करण्याची मागणी करणार आहोत.

– किरीट सोमय्या, भाजपचे नेते

सकाळ

दै. Sakal

'जरंडेश्वर'चा मालक कोण?

» पान II वरून

तुम्हाला सात तासांमध्ये ८५ कोटींचे कर्ज कसे मिळाले. लवकरच हा घोटाळा समोर येणार आहे. मी पाच

वक्तव्यासंदर्भात 'आता अजित पवार यांची नजर किसन वीर कारखान्यावर गेली आहे का?' असा प्रतिप्रश्न करत श्री. सोमय्या यांनी थेट बोलणे

'जरंडेश्वर'संबंधित कंपन्यांचा अजित पवार याच्याशा संबंध

(पान १ वरून) उपमुख्यमंत्री अजित पवार यांच्यासह खासदार सुप्रिया सुळे, मुख्यमंत्री उद्धव ठाकरे तसेच महाविकास आघाडी सरकारमधील मंत्र्यांवर शरसंधान साधले.

सोमय्या म्हणाले, उपप्रादेशिक परिवहन अधिकारी बजरंग खरमाटे यांच्या मालमत्तेची पाहणी करण्यासाठी वारामतीत आलो आहे. खरमाटे यांच्यासंबंधी बुधवारी लोकायुक्तांपुढे झालेल्या सुनावणीत राज्य सरकारने स्वतंत्र चौकशी करणार

...मग रश्मी ठाकरेंच्या बंगल्यांची माहिती कोणी दिली?

मंत्री अनिल परब यांच्यासंबंधी शिवसेनेतील काही नेत्यांकडून माहिती पुरवली जात असल्याच्या आरोपावर सोमय्या म्हणाले, मला यासंबंधी रामदास कदम यांच्याकडून माहिती दिली गेली असेल, तर मग रश्मी उद्धव ठाकरे यांच्या १९ बंगल्यांची माहिती कोणी दिली, हेही आरोप करणाऱ्यांनी सांगावे

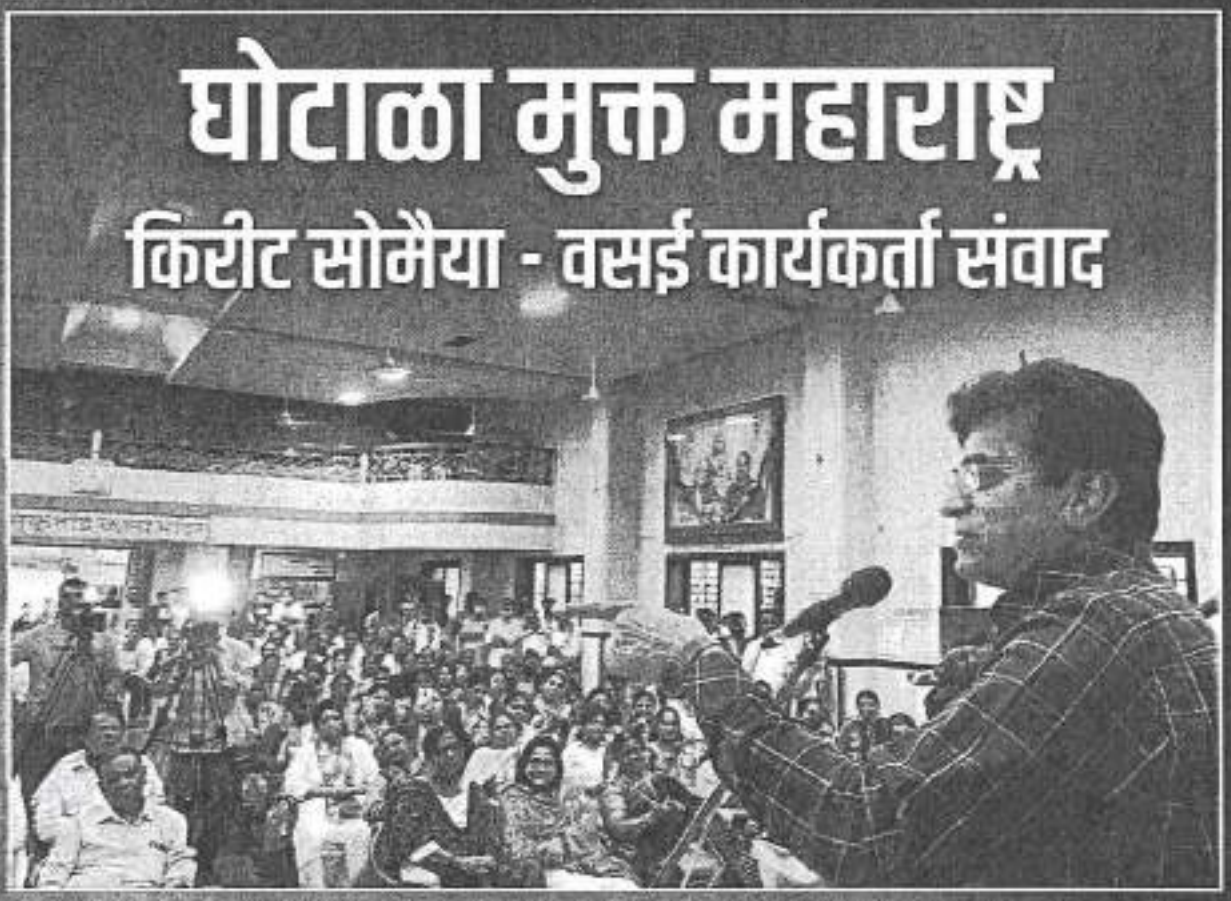
मेसर्स शिवालिक बिल्डर्स, जरंडेश्वर शुगर मिल्स प्रा. लि. या सगळ्यांचा एकमेकांशी, अजित पवार यांच्याशी संबंध व भूमिका आहे, असा आरोप करून त्यांची चौकशी व्हावी अशी

प्रश्नावर त्यांनी हा विषय राज्याच्या पर्यावरण खात्याचा आहे. मुख्यमंत्री उद्धव ठाकरे, पर्यावरणमंत्री आदित्य ठाकरे यांनी त्यात लक्ष घालावे, अशी मागणी त्यांनी केली.

जरंडेश्वर साखर कारखाना चे संस्थापक शेतकऱ्या सोबत ई डी कार्यालयात तक्रार दाखल केली

घोटाळा मुक्त महाराष्ट्र

किरीट सोमैया - वसई कार्यकर्ता संवाद



भारतीय जनता पार्टी

Neelam Nagar, Mulund, Mumbai-400 081• Tel.: 022-21634152

EXHIBIT "FFFF"

GOVERNMENT OF MAHARASHTRA
Department of Revenue and Forest, Disaster Management,
Relief and Rehabilitation, Mantralaya, Mumbai- 400 032
No: DMU/2020/CR. 92/DisM-1, Dated: 27th November, 2021

ORDER

Reference:

a. The Epidemic Diseases Act, 1897
b. The Disaster Management Act, 2005

The State Government is currently recording fewer number of COVID 19 positive cases consistently over the past few months. Also, there has been a steady as well as consistent decline in the trend curve of COVID cases in the country and nearby states. All these successes are due to discipline in adherence by various establishments to various necessary restrictions that have been imposed on various activities as well as discipline in Covid Appropriate Behaviour shown by majority of public at large. Vaccination drive in the state and the country also has seen reasonable uptake and has contributed immensely to reduction in pressure on health infrastructure, public as well as private. In the light of the said fact the State Government is now considering to open up economic, social, entertainment and cultural activities with fewer restrictions, especially for fully vaccinated persons.

Thus in exercise of the powers conferred under the Disaster Management Act, 2005, the undersigned in the capacity of the Chairperson of the State Executive Committee of the State Disaster Management Authority, in super cession of all earlier orders by the State Government with regard to imposition of restrictions for preventing the transmission of the COVID 19 virus, decrees with immediate effect that all economic, cultural, social, sports and entertainment activities will now be allowed as per the normal timings decided by various local or other competent authorities before the advent of the COVID 19 pandemic, subject to the following conditions:

1. **Adherence to CAB (Covid Appropriate Behaviour):** Strict adherence to CAB as laid down by the State and Central Government from time to time shall be observed by all, including the service providers, owners of premises, licensees, organizers etc. as well as all visitors, service takers, customers, guests etc. Detailed guidelines for CAB (Covid Appropriate Behaviour) as well as fines in case of breach shall be as per CAB Guidelines and Fines stated hereinafter.

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2. **Requirement of being Fully Vaccinated:**
   a. All persons connected with the organization of any program, event or show, ticketed or non-ticketed, as well as all service providers and participants (like players, actors etc.), visitors, guests, customers shall be fully vaccinated as per the definition of the same given hereinafter.
   b. Any shop, establishment, mall, event, gathering etc. where a member of public has a right to come and get services must be manned by fully vaccinated persons and all visitors, customers for such places shall be fully vaccinated.
   c. All public transport shall be used only by fully vaccinated persons.
   d. The Universal Pass created by the State Government (https://epassmsdma.mahait.org or telegram-MahaGovUniversalPass Bot) shall be a valid proof for status of full vaccination. Or else, Cowin Certificate with a valid ID proof carrying photo may be taken a valid proof for the same. For citizens bellow 18 years, other Government or school issued photo identity and for those who are unable to take the vaccine due to medical reasons, a certificate from a certified medical practitioner may serve as documentary evidence for entry.
   e. Though offices and other establishments where there is no visit by any person of general public as well as private transport does not have this requirement of being open to fully vaccinated persons, they are strongly advised to go for full vaccination.
3. **Travel into Maharashtra State:** All travellers into state from any international destination shall be governed by directions of Government of India in this respect. All domestic travellers into the state shall either be fully vaccinated as defined hereinafter or shall carry a RT-PCR test valid for 72 hours.
4. **Restriction on attendance in any program, event etc.:**
   a. In case of any program/ event/ activity happening in an enclosed/ closed space like a cinema hall, theatre, marriage hall, convention hall etc, people up to 50 percent of the capacity of the space will be allowed.
   b. In the case of open to sky spaces, for any events or gatherings, people up to 25 percent of space capacity will be allowed. Concerned DDMA shall have authority to decide the capacity in case of such locations of gatherings or events, if not already declared formally (like stadiums).

c. In the case the total number of people present for any gathering in accordance with the above rules exceeds 1 thousand, then the local disaster management authority will have to be informed of the same and the local disaster management authority may send their representative to supervise as observers any such gathering and to ensure that there is strict adherence to the above mentioned rules. The said representative of DDMA shall have authority to order closure of part or full activity if CAB is seen to be violated in large scale endangering spread of Covid 19.

5. **Other reasonable restrictions by DDMA:** Restrictions and conditions mentioned herein may be augmented, but not diluted, by any DDMA for their respective jurisdiction, if deemed fit at any moment of time, but not without giving 48 hours of information through public notice. Any restrictions that are in force on the date of this order, levied by DDMA shall cease to operate after 48 hours unless re-issued with a public notice for continuation.

6. **Definition of being Fully Vaccinated:**

A fully vaccinated person will mean-

- Any person who has received both doses of the vaccine and 14 days have lapsed since the administration of the second dose; or
- Any person having a medical condition that does not allow him or her to take the vaccine and has a certificate to that extent from a recognised doctor; or
- A person who is less than 18 years of age.

7. CAB rules and fines:

Definition: *CAB can be defined as the everyday common behaviour needed to be followed by individuals and organisations to curb the spread of the COVID 19 virus and thereby breaking the chain of transmission of the same. Aspects of behaviour that's characterised as CAB include those mentioned below and also all such rational aspects that may hinder spread of Covid 19 virus given its methodology of spread indicated herein.*

Following are some aspects of basic COVID appropriate behaviour that have to be followed by everyone at all times. All organisations are to ensure that all their employees, visitors to their campuses, customers or anyone engaged in any activity of the organisations, directly or indirectly, follow the same and shall be responsible for enforcing the same on their campus and/ or while transacting the transactions related to business or other activities related to concerned organization. Organisations are also responsible for

NOTARY M.M. PABALE Thane Maharashtra Regd. No. 15220 GOVT. OF INDIA

availability of hand sanitisers, soap & water, thermal scanners etc. required for following CAB by all such personnel at all such locations that are under its control or where it is transacting its business or other activities.

1. Wear a mask at all times in the right way. Mask should cover nose and mouth at all times. (An handkerchief will not be considered as mask & person using would be liable for fine.)
2. Maintain social distancing (6 feet distance) at all times wherever possible.
3. Wash hands frequently and thoroughly by soaps or sanitisers.
4. Avoid touching nose/ eyes/ mouth without washing your hands with soap or without use of sanitiser.
5. Maintain proper respiratory hygiene.
6. Regularly clean and disinfect frequently cleaned surfaces.
7. When coughing or sneezing, cover the mouth and nose using tissue and throw used tissues in the trash; if one doesn't have tissue, one should cough and sneeze into bent elbow and not one's hand.
8. Do not spit in public places.
9. Avoid crowds and maintain safe distance *(6 feet distance)* in public places.
10. Greet anyone without physical contact.
11. Any other rational behaviour required for avoiding spread of Covid 19 virus.

Penalties:

- Any individual not following CAB expected is these rules shall be fined Rs. 500/- for each instance of default.
- If the default by an individual is seen in any premises of organization or establishment that is supposed to impose CAB on their visitors, customers etc., in addition to imposing fine on the individual, these organizations or establishments shall also be fined Rs. 10,000/-. If any organization or establishment is seen to be a regular defaulter in ensuring discipline for CAB in its visitors, customers etc. such organization or establishment shall be closed till the notification of Covid 19 as a disaster remains in force.
- If an organization or establishment fails to follow CAB or SOP itself, it will be liable to be fine of Rs. 50000/- for each instance. Frequent

NOTARY GOVT. OF INDIA

defaults shall lead to closure of the organization or establishment till the notification of Covid 19 as a disaster remains in force.

- If a default is found inside any taxi or private transport four- wheeler or inside any bus, along with the individual defaulting CAB being fined Rs. 500/-, driver, helper or conductor who are providing service shall also be fined Rs. 500/-. Owner transport agency in cases of buses shall be fined Rs. 10000/- for each instance of default. Frequent defaults shall lead to withdrawal of license or closure of operations for the owner agency till the notification of Covid 19 as a disaster remains in force.
- The above mentioned rules regarding COVID appropriate behavior to be followed mandatorily and violation of the same will result in fines and penalties as stated above as well as any other fine or penalty may be levied on the violators by any disaster management authority in accordance with the Disaster Management Act, 2005. The rules/ polices for CAB shall be in accordance with the above and any other issue regarding CAB not specifically mentioned herein shall be in accordance with the current rules/ orders of the State Government that are in force.

BY ORDER OF AND IN THE NAME OF THE GOVERNOR OF MAHARASHTRA

27/11/21
(Sitaram Kunte)
Chief Secretary

NOTARY GOVT. OF INDIA

EXHIBIT "FFFF"

868

दूरध्वनी : २५३३१२११
: २५३३१५९०

ठाणे महानगरपालिका,ठाणे

महानगरपालिका भवन, सरसेनानी जनरल अरुणकुमार वैद्य मार्ग, चंदनवाडी,पांचपखाडी, ठाणे (प)- ४०० ६०२
THE MUNICIPAL CORPORATION OF THE CITY OF THANE

संदर्भ क्र : जा.क्र./ठामपा/मुख्य-१/आवि-३०/ 3894 दिनांक: ६/१/22

प्रति,
अध्यक्ष/सचिव/खजिनदार,
गृहसंकुलन (सर्व),
ठाणे.

विषय: कोव्हिड-१९ च्या सुरक्षीततेकरीता ईमारतींमध्ये उपाययोजना करणेबाबत सुचना.

उपरोक्त विषयान्वये आपणांस कळविण्यात येते की, ठाणे महानगरपालिका क्षेत्रात गेल्या आठवड्यापासून कोव्हिड-१९ चा वाढता प्रादुंभाव पाहता रुग्णाच्या संख्येमध्ये झपाटयाने वाढ होत असून सदर रुग्णापैकी जास्तीचे रुग्ण हे मोठ्या ईमारती मध्ये असल्याचे आढळून आलेले आहे.

शासन निर्देशानूसार जर एखाद्या मजल्यावर कोव्हिड-१९ सक्रीय रुग्ण आढळल्यास तो मजला पुढील १० दिवसाकरीता बंद करण्यात येईल. तसेच सदर ईमारतीमध्ये १० पेक्षा जास्त कोव्हिड-१९ चे सक्रीय रुग्ण आढळून आल्यास संपूर्ण ईमारत बंद करण्यात येईल. सदर बाबतीत ठाणे महानगरपालिकेमार्फत खालील प्रमाणे मार्गदर्शक सूचना देण्यात येत आहेत.

१) गृहसंकुलातील व्यवस्थापकीय समितीने कोव्हिड पॉझिटिव्ह आलेल्या रुग्णांची सेवा करणाऱ्या (Caregiver) व्यक्तीशी दररोज संपर्कात राहणे व तसेच सदर सेवा देणारी व्यक्ती पूर्णपणे कोव्हिड लसीकरण झाल्याबद्दलची खात्री करुन घेणे.

२) सदर कोव्हिड पॉझिटिव्ह रुग्णाला व त्यांच्या कुटूंबाना दैनंदिन मूलभूत सुविधा पुरविण्याची जबाबदारी सदर संकुलातील व्यवस्थापकीय समितीची असेल.

३) एखाद्या ईमारतीमध्ये (१० पेक्षा कमी) कोव्हिड रुग्ण आढळल्यास,

i) तो रुग्ण राहत असलेल्या संपूर्ण मजल्यावरच्या लोकांची RT-PCRचाचणी ५ ते ७ व्या दिवशी ठाणे महानगरपालिकेमार्फत करण्यात येईल.

ii) तसेच सदर रुग्णाच्या वरच्या व खालच्या मजल्यावरील लोकांची ॲन्टिजेन चाचणी ५ ते ७ व्या दिवशी करण्याची जबाबदारी संबंधीतांची राहील.

४) एखाद्या ईमारतीमध्ये (१० पेक्षा जास्त) कोव्हिड रुग्ण आढळल्यास,

NOTARY M M PARALE Mumbai Maharashtra Regd No. 15220 GOVT. OF INDIA

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ADVOCATE FOR

i) तो रुग्ण राहत असलेल्या संपूर्ण मजल्यावरच्या लोकांची RT-PCRचाचणी ५ ते ७ व्या दिवशी ठाणे महानगरपालिकेमार्फत करण्यात येईल.

ii) तसेच सदर संपूर्ण ईमारीमधील लोकांची ॲन्टिजेन चाचणी करण्याची जबाबदारी संबंधीतांची राहील.

५) कोव्हिड रुग्णांच्या घरातील जैव-वैद्यक कचरा (मास्क, ग्लॉज, इंजेक्शन, सिरिंजेस, रेझर ब्लेड इ.) या वर्गीकृत करुन मार्गदर्शक सूचनानूसार ठाणे महानगरपालिकेमार्फत पुरविण्यात येणाऱ्या पिवळ्या पिशवी मध्येच जमा करण्यात यावा.

कोव्हिड-१९ रुग्ण आढळलेल्या ईमारतीमधील त्या मजल्यातील रहिवाश्यांना ईमारती बाहेर येण्यास व जाण्यास सक्त मनाई असेल. रहिवाशीयांनी कोव्हिड-१९ टेस्ट करण्यास टाळाटाळ किंवा मनाई केल्यास बंद केलेली ईमारत जो पर्यंत सर्व रहिवाशी यांचे आरटीपीसीआर / ॲन्टिजेन टेस्ट होत नाही तोपर्यंत उघडण्यात येणार नाही. तसेच उपरोक्त नमूद सुचनांचे उल्लघन करणाऱ्यां विरुध साथरोग अधिनियमाअंतर्गत कायदेशीर कार्यवाही करण्यात येईल.

तरी सर्व रहिवाशी यांनी कोव्हिड-१९ चा प्रसार आटोक्यात आणण्यासाठी ठाणे महानगरपालिकेशी सहकार्य करावे.

मनिष जोशी
उपआयुक्त (आरोग्य)
ठाणे महानगरपालिका, ठाणे.

प्रत: माहितीस्तव सविनय सादर,
१) मा. महापौर, ठा.म.पा. ठाणे.
२) मा. आयुक्त सो. ठा.म.पा. ठाणे.
३) मा. अति. आयुक्त-१ सो. ठा.म.पा. ठाणे.
४) वैद्यकीय आरोग्य अधिकारी, ठा.म.पा. ठाणे.
५) सहा. आयुक्त (सर्व) ठा.म.पा. ठाणे.
६) वैद्यकीय अधिकारी (सर्व) ठा.म.पा. ठाणे.

5th January 2022

Government of India
Ministry of Health & Family Welfare

Revised guidelines for Home Isolation of mild /asymptomatic COVID-19 cases

1. Background

Over the past two years, it has been seen globally as well as in India that majority of cases of COVID-19 are either asymptomatic or have very mild symptoms. Such cases usually recover with minimal interventions and accordingly may be managed at home under proper medical guidance and monitoring.

Ministry of Health & FW has thus issued and updated guidelines for home isolation from time to time to clarify selection criteria, precautions that need to be followed by such patients and their families, signs that require monitoring and prompt reporting to health facilities.

The present guidelines are applicable to COVID-19 patients who have been clinically assessed and assigned as **mild /asymptomatic cases of COVID-19.**

2. Asymptomatic cases; mild cases of COVID-19

The asymptomatic cases are laboratory confirmed cases who are not experiencing any symptoms and have oxygen saturation at room air of more than 93%.

Clinically assigned mild cases are patients with upper respiratory tract symptoms with or without fever, without shortness of breath and having oxygen saturation at room air of more than 93%.

3. Patients eligible for home isolation

i. The patient should be clinically assigned as mild/ asymptomatic case by the treating Medical Officer. Further a designated control room contact number at the district /sub district level shall be provided to the family to get suitable guidance for undertaking testing, clinical management related guidance, assignment of a hospital bed, if warranted.
ii. Such cases should have the requisite facility at their residence for **self-isolation** and for **quarantining the family contacts.**
iii. A caregiver **(ideally someone who has completed his COVID-19 vaccination schedule)** should be available to provide care on 24 x7 basis. **A communication link between the**

caregiver and a Medical Officer is a prerequisite for the entire duration of home isolation.

iv. Elderly patients aged more than 60 years and those with co-morbid conditions such as Hypertension, Diabetes, Heart disease, Chronic lung/liver/ kidney disease, Cerebro-vascular disease etc **shall only be allowed home isolation after proper evaluation by the treating medical officer.**

v. Patients suffering from immune compromised status (HIV, Transplant recipients, Cancer therapy etc.) **are not recommended** for home isolation and **shall only be allowed home isolation after proper evaluation by the treating Medical Officer.**

vi. While a patient is allowed home isolation, all other members in the family including other contacts shall follow the **home quarantine guidelines** available at: https://www.mohfw.gov.in/pdf/GuidelinesforhomequarantIne.pdf.

4. **Instructions for the patient**

i. Patient must isolate himself from other household members, stay in the identified room and away from other people in home, especially elderly and those with co-morbid conditions like hypertension, cardiovascular disease, renal disease etc.

ii. The patient should stay in a well-ventilated room with cross ventilation and windows should be kept open to allow fresh air to come in.

iii. Patient should at all times use triple layer medical mask. They should discard mask after 8 hours of use or earlier if the mask becomes wet or is visibly soiled. In the event of Caregiver entering the room, both Caregiver and patient may preferably consider using N-95 mask.

iv. Mask should be discarded after cutting them to pieces and putting in a paper bag for a minimum of 72 hours.

v. Patient must take rest and drink lot of fluids to maintain adequate hydration.

vi. Follow respiratory etiquettes at all times.

vii. Undertake frequent hand washing with soap and water for at least 40 seconds or clean with alcohol-based sanitizer.

viii. The patients shall not share personal items including utensils with other people in the household.

ix. Need to ensure cleaning of frequently touched surfaces in the room (tabletops, doorknobs, handles, etc.) with soap/detergent & water. The cleaning can be undertaken either by the patient or the caregiver duly following required precautions such as use of masks and gloves.

x. Self-monitoring of blood oxygen saturation with a pulse oximeter for the patient is advised.

xi. The patient shall self-monitor his/her health with daily temperature monitoring (as given below) and report promptly if any deterioration of symptom is noticed. The status shall be shared with the treating Medical Officer as well as surveillance teams/Control room.

Patients Self -health monitoring Chart

Date and time	Temperature	Heart rate (from pulse oximeter)	SpO2 % (from pulse oximeter) *	Feeling: (better /same /worse)	Breathing: (better / same/ worse) **

*For self-monitoring blood oxygen saturation with a pulse oximeter, place the index finger (after cleaning hands and removing nail polish, if any) in the pulse oximeter probe and take the highest steady reading after a few seconds.

**The patient may self-monitor breathing rate/respiratory rate in sitting position, breathe normally and count the number of breaths taken in 1 full minute.

5. Instructions for Care Giver

i. **Mask:**
- The caregiver should wear a triple layer medical mask. N95 mask may be considered when in the same room with the ill person.
- Front portion of the mask should not be touched or handled during use.
- If the mask gets wet or dirty with secretions, it must be changed immediately.
- Mask should be discarded after cutting them to pieces and putting in a paper bag for a minimum of 72 hours.
- Perform hand hygiene after disposal of the mask.
- He/she should avoid touching own face, nose or mouth.

ii. **Hand hygiene**
- Hand hygiene must be ensured following contact with ill person or his immediate environment.
- Use soap and water for hand washing at least for 40 seconds. Alcohol-based hand rub can be used, if hands are not visibly soiled.
- After using soap and water, use of disposable paper towels to dry hands is desirable. If not available, use dedicated clean cloth towels and replace them when they become wet.
- Perform hand hygiene before and after removing gloves.

iii. **Exposure to patient/patient's environment**
- Avoid direct contact with body fluids (respiratory, oral secretions including saliva) of the patient. Use disposable gloves while handling the patient.

- Avoid exposure to potentially contaminated items in his immediate environment (e.g. avoid sharing eating utensils, dishes, drinks, used towels or bed linen).
- Food must be provided to the patient in his room. Utensils and dishes used by the patient should be cleaned with soap/detergent and water while wearing gloves. The utensils may be re-used after proper cleaning.
- Clean hands after taking off gloves or handling used items. Use triple layer medical mask and disposable gloves while cleaning or handling surfaces, clothing or linen used by the patient.
- Perform hand hygiene before and after removing gloves.

iv. **Biomedical Waste disposal**

Effective and safe disposal of general wastes such as disposable items, used food packets, fruit peel offs, used water bottles, left-over food, disposable food plates etc. should be ensured. They should be collected in bags securely tied for handing over to waste collectors.

Further, the used masks, gloves and tissues or swabs contaminated with blood / body fluids of COVID-19 patients, including used syringes, medicines, etc., should be treated as biomedical waste and disposed of accordingly by collecting the same in a yellow bag and handed over to waste collector separately so as to prevent further spread of infection within household and the community. Else they can be disposed of by putting them in appropriate deep burial pits which are deep enough to prevent access to rodents or dogs etc.

6. Treatment for patients with mild /asymptomatic disease in home isolation

i. Patients must be in communication with a treating Medical Officer and promptly report in case of any deterioration.
ii. The patient must continue the medications for other co-morbidities/ illness after consulting the treating Medical Officer.
iii. Patient may utilize the tele-consultation platform made available by the district/state administration including the e-Sanjeevani tele-consultation platform available at https://esanjeevaniopd.in/
iv. Patients to follow symptomatic management for fever, running nose and cough, as warranted.
v. Patients may perform warm water gargles or take steam inhalation thrice a day.
vi. If fever is not controlled with a maximum dose of Tab. Paracetamol 650 mg four times a day, consult the treating doctor.

vii. Information floating through social media mentioning non-authentic and non-evidence-based treatment protocols can harm patients. Misinformation leading to creation of panic and in-turn undertaking tests and treatment which are not required has to be avoided. Clinical management protocol for asymptomatic/mild patients as available on the website of Ministry of Health & FW (https://www.icmr.gov.in/pdf/covid/techdoc/COVID_Management_Algorithm_23092021.pdf) may be referred to by the treating Medical Officer to aid management of the case.

viii. Do not rush for self-medication, blood investigation or radiological imaging like chest X ray or chest CT scan without consultation of your treating Medical Officer.

ix. Steroids are not indicated in mild disease and shall not be self-administered. Overuse & inappropriate use of steroids may lead to additional complications.

x. Treatment for every patient needs to be monitored individually as per the specific condition of the patient concerned and hence generic sharing of prescriptions shall be avoided.

xi. In case of falling oxygen saturation or shortness of breath, the person may require hospital admission and shall seek immediate consultation of their treating Medical Officer/surveillance team /Control room.

7. When to seek medical attention

Patient / Care giver will keep monitoring their health. Immediate medical attention must be sought if serious signs or symptoms develop. These could include-

i. Unresolved High-grade fever (more than 100° F for more than 3 days)
ii. Difficulty in breathing,
iii. Dip in oxygen saturation (SpO2 ≤ 93% on room air at least 3 readings within 1 hour) or respiratory rate >24/ min
iv. Persistent pain/pressure in the chest,
v. Mental confusion or inability to arouse,
vi. Severe fatigue and myalgia

8. Monitoring of the Patient during Home Isolation

The concerned district administration under the overall supervision of State Health Authority shall be responsible for monitoring the patient under home isolation.

8.1. Responsibilities of grass root level Surveillance Teams

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M.M. PARALE
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i. The Surveillance Teams (ANM, Sanitary inspector, MPHW etc) shall be responsible for initial assessment of the patient and whether the requisite facilities are there for home isolation.

ii. The health worker should contact the patient daily preferably in-person or over telephone/ mobile and obtain the details of temperature, pulse, oxygen saturation, patients overall wellness and worsening of signs/ symptoms.

iii. The Surveillance Team may provide Home Isolation Kits to the patient/ caregiver as per the policy of the State Government. The Kit may contain masks, hand sanitizers, paracetamol along with a detailed leaflet to educate patients and family members in local language.

iv. If there is reported worsening of signs/ symptoms and/or fall in oxygen saturation, the Surveillance team shall re-assess the patient and inform the Control Room for shifting the patient to hospital.

v. The surveillance Team shall also undertake the patient education on the disease, its symptoms, warning signs, COVID appropriate behaviour and need for vaccination for all eligible members.

8.2. Responsibilities of the District/ Sub-District Control Room.

District and sub-district control rooms will be made operational and their telephone numbers should be well publicised in public so that people under home-isolation may contact the control rooms for seamless transfer of patients through ambulance from home to the dedicated hospital.

These Control Rooms shall also make outbound calls to the patients under home isolation to monitor their status.

8.4. Role of District Administration

The district administration should monitor all cases under home isolation on a daily basis.

9. When to discontinue home isolation

Patient under home isolation will stand discharged and end isolation after at least 7 days have passed from testing positive and no fever for 3 successive days and they shall continue wearing masks. **There is no need for re-testing after the home isolation period is over.**

Asymptomatic contacts of infected individuals need not undergo Covid test & monitor health in home quarantine.

Guidelines for Home Isolation (Dated 5th January 2022)

Patient Tested Positive

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Patients clinically assessed and assigned as mild /asymptomatic cases of COVID-19 or patients experiencing no symptoms and have oxygen saturation at room air of 93% or more.

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Management of cases under Home Isolation

Instructions for the patient	• Identify separate, well-ventilated room; • Use triple layer mask and discard in a paper bag after 72 hours, cutting into pieces; • Maintain adequate hydration; • Follow respiratory etiquettes; • Follow hand hygiene;	• Do not share personal items including utensils with others; • Clean frequently touched surfaces with soap/detergent and water; • Monitor blood oxygen saturation and temperature regularly; • Report promptly in case of any deterioration
Instructions for caregivers (caregiver must be fully vaccinated)	• Use triple layer mask and discard in a paper bag after 72 hours, cutting into pieces; • Replace mask immediately if wet or dirty with secretion; • Follow hand hygiene; • Avoid touching face, nose or mouth;	• Use gloves and perform hand hygiene before and after using gloves; • Avoid direct contact with body fluids of patient; • Avoid exposure to contaminated items in patient's immediate environment; • Ensure effective waste disposal;
Treatment for patients with mild /asymptomatic disease	• Patient must be in communication with a Medical Officer; • Medication for co-morbidities must be continued after consulting treating Medical Officer; • Leverage Tele-consultation platform; • Follow symptomatic management for fever, cough, etc.; • Avoid misinformation leading to panic;	• Do not rush for self-medication, blood investigation or radiological imaging without consultation of your treating Medical Officer. • Steroids are not indicated in mild disease and shall not be self-administered; • Only Medical Officer must decide about drugs, hoarding any drugs is not useful
Monitoring of the Patient during Home Isolation by District administration	• The concerned district administration under the overall supervision of State Health Authority responsible for monitoring the patient under home isolation • Initial assessment to be conducted by surveillance teams at ground level; • Adequately staffed and well-equipped control rooms to provide end-to-end support to the patient under home isolation;	• Contact numbers of Control Rooms should be well publicised for seamless transfer of patients through ambulance from home to the dedicated hospital • Necessary coordination with respect to infrastructure to be ensured by the district administration;

Patient / Caregiver to monitor health of patient. Immediate medical attention must be sought if serious signs or symptoms develop. These could include-

↓

- Unresolved high grade Fever; >100° F for more than 3 days
- Difficulty in breathing
- SpO2 < 93% on room air at least 3 reading within 1 hour or Respiratory rate >24/ min
- Persistent pain/ pressure in the chest
- Mental confusion or inability to arouse
- Severe fatigue and myalgia

Discontinue Home Isolation: Patient under home isolation will stand discharged and end isolation after at least 7 days have passed from testing positive and no fever for 3 successive days and they shall continue wearing masks. There is no need for re-testing after the home isolation period is over. Asymptomatic contacts of infected individuals need not undergo Covid test & monitor health in home quarantine.

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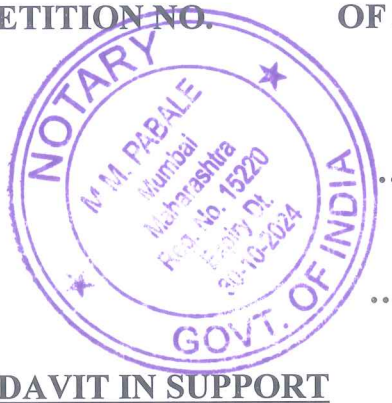
IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022



Amber H. Koiri **.... Petitioner**

Versus

State of Maharashtra & Ors. **...Respondents**

AFFIDAVIT IN SUPPORT

I, **Amber H. Koiri**, Aged about [redacted], the Petitioner do hereby solemnly state and affirm as under:

1. That, I am Petitioner of the instant Writ Petition and being conversant with the facts and circumstances of the case, am competent to swear this Affidavit.

2. That, I have read and understood the contents of the abovementioned writ petition and I state that the same are true and correct to my knowledge.

3. That, all the Exhibit annexed to the Writ Petition are true copies of their respective originals.

4. I have done whatsoever inquiry/investigation which was in my power to do, to collect all data/material which was available on public domin and which was relevant for this court to entertain the present petition.

5. That I have read and understood the content of Writ Petition. I have read and understood the contents of the accompanying synopsis at **Pages A to B**, Writ Petition at **Pages 1 to 119**, **Para 1 to 40**, and all accompanying Applications. I state that the facts therein are true and correct to the best of my knowledge and

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belief. I further state that the Exhibit annexed to the Writ Petition are true copies of their respective originals.

DEPONENT

VERIFICATION:

Verified at Mumbai on this 10 day of January, 2022 that the contents of my aforesaid affidavit are true and correct to my knowledge and belief. No part of it is false nor anything material has been concealed therefrom.

NOTARY
M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
30-10-2024
GOVT. OF INDIA

DEPONENT

BEFORE ME

MANISH M. PABALE
B.Sc.,LL.M.
ADVOCATE & NOTARY (GOVT. OF INDIA)
104, Natwar Chambers,
94 Nagindas Master Road,
Fort, Mumbai - 400 001.

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NOTARY
M. M. PABALE
Mumbai
Maharashtra
Reg. No. 15220
Expiry Dt.
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GOVT. OF INDIA

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NOTED & REGISTERED
Page No. 93/24 Sr. No. 637
Date 10 JAN 2022

ID / Aadhar / PAN / DL: 534203880642
Seen Org. / POA / Board Resol.:.....

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IN THE HIGH COURT OF JUDICATURE AT BOMBAY

ORDINARY ORIGINAL CIVIL JURISDICTION

WRIT PETITION NO. OF 2022

Amber H. Koiri .... Petitioner

Versus

State of Maharashtra & Ors. ...Respondents

ADVOCATE'S CERTIFICATE

To,

The Prothonotary & Senior Master,

High Court,

Bombay.

I, do certify and state that, the issue involved in the present Writ Petition is required to be entertained by Hon'ble Division Bench of this High Court as per amended **Rule 636 (1)(a)** of the Bombay High Court Original Side rules.

Therefore, the matter pertains to Division Bench of this Hon'ble High Court as per amended rule **636 (1)(a)** of the Bombay High Court Original Side rules.

Yours faithfully,

Advocate for the Petitioner

NOTARY M.M. PABALE Mumbai Maharashtra R.J. No. 15220 Exp. Dt. 30-10-2024 GOVT. OF INDIA