UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK

ADRIANA AVILES, Individually and as Parent and Natural Guardian of N.A., N.A. and A.A., **STEPHANIE DENARO**, Individually and as Parent and Natural Guardian of D.D. and H.D., **CHRISTINE KALIKAZAROS,** Individually and as Parent and Natural Guardian of Y.K., **GAETANO LA MAZZA**, Individually and as Parent and Natural Guardian of R.L., **CRYSTAL LIA**, Individually and as Parent and Natural Guardian of F.L., and **CHILDREN'S HEALTH DEFENSE**, Plaintiffs, Against **BILL de BLASIO**, in his Official Capacity as Mayor of the City of New York, **DR. DAVID CHOKSHI**, in his Official Capacity of Health Commissioner of the City of New York, **NEW YORK CITY DEPARTMENT OF EDUCATION, RICHARD A. CARRANZA**, in his Official Capacity as Chancellor of the New York City Department of Education and **THE CITY OF NEW YORK**, Defendants.	**DECLARATION OF KEVIN MCKERNAN** Civil No.: 1:20−cv−09829−PGG

I, Kevin McKernan declare as follows:

1. 2. A true and correct copy of my Bio (Exhibit 1), and Resume (Exhibit 2) are hereby included.

2. From 1996 to 2000, I was the Team Leader for Research and Development at the Whitehead Institute/MIT, Center for Genome Research. Our team designed and constructed the robotics and DNA amplification pipeline for the Human Genome Project efforts under the leadership of Eric Lander.

3. In 2000, I founded Agencourt Biosciences. This company sold viral and pathogen DNA purification kits, and was the largest commercial DNA sequencing service company in the U.S. (Beckman Coulter acquired this company in 2005). During this acquisition we jointly spun out a new entity (Agencourt Personal Genomics) to build a next generation sequencer known as the SOLiD Sequencer. The SOLiD sequencer was 100,000x faster than the sequencer used to sequence the human genome in 1999. This new start-up was quickly acquired by the leader in DNA sequencing, Applied Biosystems in 2006.

4. From 2006-2011, I managed the Next Generation sequencing R&D at Applied Biosystems and Life Technologies. This company was acquired by Thermo Fischer and is now the largest C19 testing reagent provider in the world. Thermo did $2B in C19 testing in the Q3-2020 and is expecting 40% increases in Q4.

5. I hold many patents and peer reviewed articles on DNA sequencing, DNA and RNA isolation and PCR and was the CSO of Courtagen Life Sciences for 5 years. Courtagen was a CLIA and CAP certified high complexity laboratory that performed genetic testing on Children with Epilepsy, Austism and Mitochondrial disease. As a result, I have an intimate understanding of the medical experimentation and informed consent process required to perform genetic testing on symptomatic children.

6. I recently co-authored, along with 22 international authors who are among the world's leading experts in RT-PCR testing and pathology, a scientific article (Exhibit 3) demanding the retraction of a report regarding RT-PCR testing for SARS-CoV-2 by authors Corman and Drosten published in Eurosurveillance in Jan 2020 because of 10 major scientific flaws at the molecular and methodological levels. (Exhibit 3: Borger et al., *External peer review of the RT-PCR test to detect SARS-CoV-2 reveals 10 major scientific flaws at the molecular and*

methodological level: consequences for false positive results (Nov. 2020), https://www.researchgate.net/publication/346483715.)

7. Global PCR testing since the publication of the Corman-Drosten paper in February 2020, has been based on theoretical sequences of SARS-CoV-2 because the actual isolated genomic RNA was unavailable to the authors in February.

8. The review paper I co-authored points to several major concerns with the seminal Corman-Drosten paper regarding the global standard PCR protocol for diagnosis of SARS-CoV-2, including:

a) Erroneous primer concentrations

b) Unspecified primer and probe sequences

c) The test cannot discriminate between the whole virus and viral fragments. The test cannot be used as a diagnostic for SARS-viruses.

d) PCR data evaluated as positive after a Ct value of 35 cycles are completely unreliable.

e) Scientific studies show that only non-infectious (dead) viruses are detected with Ct values of 35.

f) The PCR products have not been validated at the molecular level with DNA sequencing, a "striking error of the protocol," making the test "useless" as a specific diagnostic tool to identify the SARS-CoV-2 virus.

g) Acknowledgement by the Corman-Drosten paper that it "generates false positives."

9. The authors of the Corman-Drosten paper were also on the editorial board, constituting a clear conflict of interest. The paper is now being re-reviewed under a community retraction request.

10. The paper was rushed through peer-reviewed in 24 hours. The average review time for Eurosurveillance is 179 days. (Exhibit 3.)

11. I am familiar with New York City School testing program initiated December 7, 2020.

12. NYC Dept. of Educ. has not disclosed the specifics of the test to which they seek "consent." They have not disclosed IF this is a PCR test. They have asked for "consent to test your child for COVID-19 infection."

13. Nonetheless, upon information and belief, NYC Dept. Of Educ. has contracted with laboratories to provide PCR testing, which is more likely than not based on the Corman-Drosten paper, the World Health Organization's "first gold standard" for PCR testing since February 2020.

14. PCR can test for the presence of viral RNA. PCR testing cannot test for viral infectiousness or illness. DoE (Department of Education) has inaccurately represented that they will be testing for infectiousness (as positive results lead to isolation), yet they are only providing genetic screening. Further testing is required for positive test to see if they are truly positive for SARS-CoV-2 infection and the patient is in fact infectious. Patients can be qPCR positive for 77 days post infection. (Exhibit 4: Liotti FM, Menchinelli G, Marchetti S, et al., *Assessment of SARS-CoV-2 RNA Test Results Among Patients Who Recovered From COVID-19 With Prior Negative Results*. JAMA Intern Med. (Nov. 12, 2020), https://jamanetwork.com/journals/jamainternalmedicine/fullarticle/2773053.) Complete live

viruses are necessary for transmission, not the fragments identified by PCR. (Exhibit 5: T Jefferson, E A Spencer, J Brassey, C Heneghan, *Viral cultures for COVID-19 infectious potential assessment – a systematic review*, Clinical Infectious Diseases, ciaa1764, OXFORD UNIVERSITY PRESS (Dec. 3, 2020), https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1764/6018217.)

15. The infectious period of this virus is only 7-10days. Both asymptomatic and symptomatic spread of this age group is rare as most don't develop symptoms. This means the majority of positive students will be falsely quarantined by this test and they are not informed of this deficit of qPCR testing.

16. Further, DoE has said that children will receive a "free diagnostic test." Without further testing, it CANNOT provide a diagnosis or determine whether the individual is infectious or not.

17. I have extensive experience in human subject research and the requirements of informed consent. I fully understand the requirements necessary for human subjects, and for parents on behalf of children, to be able to give prior, free and informed consent to any medical procedure (not just experiments), the hallmark of ethical medicine.

18. I concur with the findings of Exhibit 4 shows long periods of PCR positivity exists weeks to months past infectiousness. These are poorly designed PCR tests will quarantine mostly non-infectious people. (Exhibit 4: Liotti FM, Menchinelli G, Marchetti S, et al., *Assessment of SARS-CoV-2 RNA Test Results Among Patients Who Recovered From COVID-19 With Prior Negative Results*. JAMA Intern Med. (Nov. 12, 2020), https://jamanetwork.com/journals/jamainternalmedicine/fullarticle/2773053.)

19. I concur with the findings of Borry et al. Genetic Testing in Asymptomatic minors shows:

> As presymptomatic or predictive genetic testing may have far-reaching consequences for test applicants, their family members and society,62 concerns have always been raised about the pre-test and post-test counselling process, the provision of adequate information, the private and confidential character of the test result, the psychosocial impact of a test63 and the responsibility towards blood relatives.64, 65, 66 An even more cautious approach has been envisaged when considering such testing in children and adolescents. This originates from the fear that testing in childhood or adolescence could create devastating social, emotional, psychosocial and educational consequences in the child or in the adolescent.67, 68, 69"

(Exhibit 6: Borry, P., Evers-Kiebooms, G., Cornel, M. et al., *Genetic testing in asymptomatic minors*, EUR J HUM GENET 17, 711–719 (2009), https://www.nature.com/articles/ejhg200925.)

This peer reviewed Nature article by Borry et al goes on to conclude:

> In respect of national legislation, minors should be able to decide personally regarding a genetic test when they are **well informed**, have an adequate **understanding of the test** and its potential consequences, have the capacity to make this decision, are **not exposed to external pressure and have had appropriate counselling**.

(Exhibit 6: Borry, P., Evers-Kiebooms, G., Cornel, M. et al., *Genetic testing in asymptomatic minors*, EUR J HUM GENET 17, 711–719 (2009), https://www.nature.com/articles/ejhg200925.)

I see none of these informed consent features in mass genetic testing of asymptomatic minors as an educational requirement.

20. It is my opinion, that by all appearances, DoE has misrepresented the very nature of the testing it is providing to parents of children, thus making true INFORMED consent impossible.

21. DoE has not disclosed the parameters of its lab testing – the number of cycles it is using; the primers. Without making this information transparent, it is impossible to fully assess what DoE is actually doing with the samples.

22. Because manipulation of the Ct or cycle threshold determines the number of positive tests, current DoE testing practices leave open the possibility for arbitrary and capricious state or private actions to effectively close certain schools with a high positivity rate or to keep certain schools open with low positivity rates.

23. DoE has written in published materials that it destroys the samples after sending results to parents. This makes it impossible for families to challenge the accuracy of testing, thus making the test "irrefutable," even though the likelihood of false positives for PCR testing is extremely high.

24. The consent form is exceptionally vague, asking parents to consent to testing in their absence on a random basis for nasal testing "and/or collecting saliva (spit)" over the course of the next TEN months.

25. This information is too vague and uncertain as to timing and the nature of the test to constitute informed consent.

26. This "consent" is not properly considered "consent" to the extent that DoE has made clear that refusal to submit to testing results in eviction from any in-person schooling at least through September 2021. By requiring "consent" to continue in in-school participation, DoE is coercing parents on threat of deprivation of education. "Remote learning" is not equivalent.

27. DoE has failed to provide documentation of its contracts with testing providers that would prove that they are not selling or cataloguing students' and teachers' genetic material.

Parents cannot give true informed consent without knowledge of how the genetic material is being used.

28. The scientific literature on SARS-CoV-2 makes it clear that children are the least likely group in society to become ill from COVID or to transmit disease. For children is lower than annual influenza risk. The Infection fatality rate for 0-19 year olds according to the CDC is 0.00003. (Exhibit 7: *COVID-19 Pandemic Planning Scenarios*, CDC.GOV, (Sept. 10, 2020), https://www.cdc.gov/coronavirus/2019-ncov/hcp/planning-scenarios.html.)

29. There is no science to suggest that testing asymptomatic children has ANY benefit to society.

30. On the contrary, there is significant evidence that such testing:

a) "clogs the system," making it less likely that symptomatic carriers are detected and isolated;

b) Burdens the schools and children, taking time away from curricular activities;

c) Harms children psychologically by depriving them of the comfort and security of their parents and family physicians during such testing;

d) is supported by no empirical basis to believe that such testing is protective of the whole school body since DoE is randomly testing of 20% of the school population each week.

DoE has instructed parents that they will receive test results in 48-72 hours. But, this time lag further prevents this testing regime from being a potential way to control infection. In 2-3 days, an infectious person could have infected several others.

31. As a scientist who runs a testing company, I am in no way opposed to testing. Intelligent, useful testing and infection control in this situation would include:

- A focus on symptomatic testing.
- Age stratified testing priority since the elderly have 1000 fold higher risk than children.
- Transparent use of qPCR protocols that have been properly calibrated to know Ct predictiveness of infectiousness as seen in Jaafar et al. This requires public Ct scores and EUA documentation of the limit of detection on the tests being utilized.
- Elimination of asymptomatic testing on people who have had no contact with C19.
- Medical testing to call a 'case' requires physician review with symptoms. A single test can never be utilized to call a medical case without proper medical review of the patient.
- Accelerated regulatory approval of at-home testing or Point of Care testing where medical privacy is respected and rapid turn-around times actually useful for infection control.

32. It is my opinion that voluntary testing of the adult teacher population on demand – as they are at higher risk of infection, would be a more appropriate solution. Thereby, permitting teachers to receive testing from clinics or private providers and to provide waivers or certification to DoE.

33. As with all medical testing, physicians should be consulted to interpret the results. A positive or negative qPCR test in absence of any clinical data was never considered a medical

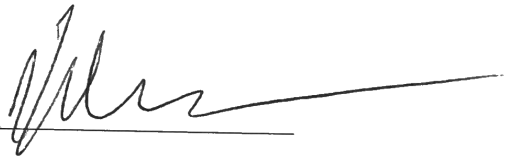
case in 2019. This is even more important when the tests are not Diagnostic grade. These are flawed Research Use Only (RUO) tests (with rapid EUA authorizations) being deployed on asymptomatic children used to falsely quarantine and isolate 5 times more non-infectious students than infectious students. This is causing physical and emotional harm to adolescents and is an embarrassment to medical testing ethics.

34. Temperature testing would be a better solution and less invasive solution.

35. Another solution would simply require symptomatic people stay home.

I declare under Penalty of Perjury under the Laws of the United States of America that the foregoing is true and correct.

Executed this 15th day of December, 2020 in Marblehead, MA_____(State).



__Kevin J McKernan
Kevin McKernan

EXHIBIT 1

Kevin McKernan
Medicinal Genomics

CSO & Founder

Kevin is the Chief Scientific Officer and Founder of Medicinal Genomics Corporation and has pioneered the genomics of cannabis and hemp to build a stronger scientific environment (Kannapedia.net) for the study of cannabis based therapeutics and blockchain technologies for tracking and verifying cannabis genetics.

Kevin has spent his career researching and developing various DNA sequencing technologies in both the research and clinical industries and has had a parallel interest in driving the tools used for personalized medicine into the world of cannabis medicine. Kevin believes the intersection of personalized medicine, genomics, blockchains and cannabis is one of the most exciting growth opportunities in our lifetime.

Medicinal Genomics made world-wide news in 2011 when it publically released the first genome sequence for *Cannabis Sativa L.* As a result of this work, Medicinal Genomics (MGC) launched a suite of qPCR tools for the detection of microbial contamination on *Cannabis*. In 2015, MGC was the largest provider of microbiological testing equipment in the cannabis space and has been selected to present on its genome sequencing, cannabis sex determination and microbiome work at ICRS 2014 and 2015.

Previously, Kevin was the CSO of Courtagen Life Sciences, Inc., and held the position of Vice President and Director of R&D of Life Technologies where he managed the development of Life Technologies next generation SOLiD sequencing technology. Integral to the SOLiD R&D process, Kevin oversaw over 100 research collaborations exploring the new biological frontiers with next generation sequencing and saw particular excitement and traction in human

Exhibit 1

tumor sequencing. Kevin initiated an R&D project to investigate chemFET semiconductor based DNA sequencing and spearheaded a process to acquire the DNA sequencing company Ion Torrent for $350M. These collaborations resulted in hundreds of publications and 7 Journal covers from Science Translational Medicine to Nature.

Kevin was the President and CSO of Agencourt Personal Genomics, a startup company he co-founded in 2005 to invent revolutionary sequencing technologies that dropped the cost of sequencing a human genome from $300M to $3,000; a 100,000-fold improvement in sequencing speed and cost in a few years. Kevin oversaw the growth and research of APG until it was sold it to Applied BioSystems. In 2000, Kevin Co-Founded Agencourt Biosciences Corporation and acted as the CSO until 2005 where it was acquired by Beckman Coulter. From 1996 to 2000 Kevin managed the Research and Development for the Human Genome Project at Whitehead Institute/MIT resulting in several patents for nucleic acid purification.

Kevin holds a B.S. in Biology from Emory University with a focus on cloning and expressing Norepinephrine Transporters.

EXHIBIT 2

Kevin McKernan

CSO and Founder
Medicinal Genomics

Kevin is the CSO and Founder of Medicinal Genomics and has pioneered the genomics of cannabis and hemp to build a stronger scientific environment (Kannapedia.net) for the study of cannabis based therapeutics and blockchain technologies for tracking and verifying cannabis genetics. Previously, Kevin was the CSO of Courtagen Life Sciences, Inc., and was Vice President and Director of R&D of Life Technologies where he managed the development of Life Technologies next generation SOLiD sequencing technology. Integral to the SOLiD R&D process, Kevin oversaw over 100 research collaborations exploring the new biological frontiers with next generation sequencing and saw particular excitement and traction in human tumor sequencing. Kevin initiated an R&D project to investigate chemFET semiconductor based DNA sequencing and spearheaded a process to acquire the DNA sequencing company Ion Torrent for $350M. These collaborations resulted in hundreds of publications and 7 Journal covers from Science Translational Medicine to Nature.

Kevin was the President and CSO of Agencourt Personal Genomics, a startup company he co-founded in 2005 to invent revolutionary sequencing technologies that dropped the cost of sequencing a human genome from $300M to $3,000; a 100,000-fold improvement in sequencing speed and cost in a few years. In 2000, Kevin Co-Founded Agencourt Biosciences Corporation and acted as the CSO until it was acquired by Beckman Coulter. Kevin also managed the R&D for the Human Genome Project at Whitehead Institute/MIT resulting in several patents for nucleic acid purification. Kevin holds a B.S. in Biology from Emory University with a focus on cloning and expressing Norepinephrine Transporters. When not decoding DNA and unraveling the mysteries of cannabis medicine, Kevin enjoy boating, skiing, and gardening.

Exhibit 2

EXHIBIT 3

External peer review of the RTPCR test to detect SARS-CoV-2 reveals 10 major scientific flaws at the molecular and methodological level: consequences for false positive results

Preprint · November 2020

DOI: 10.5281/zenodo.4298004

CITATIONS
0

READS
4,320

22 authors, including:

Klaus Steger
Justus-Liebig-Universität Gießen
247 PUBLICATIONS **7,814** CITATIONS
SEE PROFILE

Rainer J Klement
Leopoldina Hospital, Schweinfurt
130 PUBLICATIONS **1,663** CITATIONS
SEE PROFILE

Exhibit 3

External peer review of the RTPCR test to detect SARS-CoV-2 reveals 10 major scientific flaws at the molecular and methodological level: consequences for false positive results.

Pieter Borger [1*], Rajesh K. Malhotra [2], Michael Yeadon [3], Clare Craig [4], Kevin McKernan [5] Klaus Steger [6], Paul McSheehy [7], Lidiya Angelova [8], Fabio Franchi [9], Thomas Binder [10] Henrik Ullrich [11], Makoto Ohashi [12], Stefano Scoglio [13], Marjolein Doesburg-van Kleffens [14] Dorothea Gilbert [15], Rainer J. Klement [16], Ruth Schruefer [17], Berber W. Pieksma [18], Jan Bonte [19], Bruno H. Dalle Carbonara[20], Kevin P. Corbett [21], Ulrike Kämmerer [22].

* Corresponding author

ABSTRACT

In the publication entitled “Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR” (Eurosurveillance 25(8) 2020) the authors present a diagnostic workflow and RT-qPCR protocol for detection and diagnostics of 2019-nCoV (now known as SARS-CoV-2), which they claim to be validated, as well as being a *robust diagnostic methodology for use in public-health laboratory settings*.

In light of all the consequences resulting from this very publication for societies worldwide, a group of independent researchers performed a point-by-point review of the aforesaid publication in which 1) all components of the presented test design were cross checked, 2) the RT-qPCR protocol-recommendations were assesses w.r.t. good laboratory practice, and 3) parameters examined against relevant scientific literature covering the field.

The published RT-qPCR protocol for detection and diagnostics of 2019-nCoV and the manuscript suffer from numerous technical and scientific errors, including insufficient primer design, a problematic and insufficient RT-qPCR protocol, and the absence of an accurate test validation. Neither the presented test nor the manuscript itself fulfils the requirements for an acceptable scientific publication. Further, serious conflicts of interest of the authors are not mentioned. Finally, the very short timescale between submission and acceptance of the publication (24 hours) signifies that a systematic peer review process was either not performed here, or of problematic poor quality.

We provide compelling evidence of several scientific inadequacies, errors and flaws. Considering the scientific and methodological blemishes presented here, we are confident that the editorial board of Eurosurveillance has no other choice but to retract the publication.

Exhibit 3

CONCISE REVIEW REPORT

This paper will show numerous serious flaws in the Corman-Drosten paper, the significance of which has led to worldwide misdiagnosis of infections attributed to SARS-CoV-2 and associated with the disease COVID-19. We are confronted with stringent lockdowns which have destroyed many people's lives and livelihoods, limited access to education and these imposed restrictions by governments around the world are a direct attack on people's basic rights and their personal freedoms, resulting in collateral damage for entire economies on a global scale.

There are ten fatal problems with the Corman-Drosten paper which we will outline and explain in greater detail in the following sections.

The first and major issue is that the *novel* Coronavirus SARS-CoV-2 (in the publication named 2019-nCoV and in February 2020 named SARS-CoV-2 by an international consortium of virus experts) is based on *in silico* (theoretical) sequences, supplied by a laboratory in China [1], because at the time neither control material of infectious ("live") or inactivated SARS-CoV-2 nor isolated genomic RNA of the virus was available to the authors. To date no validation has been performed by the authorship based on isolated SARS-CoV-2 viruses or full length RNA thereof.

According to Corman et al.: *"We aimed to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available."* [1]

The focus here should be placed upon the two stated aims: a) *development* and b) *deployment* of a *diagnostic test for use in public health laboratory settings*. These aims are not achievable without having any actual virus material available (e.g. for determining the infectious viral load). In any case, only a protocol with maximal accuracy can be the mandatory and primary goal in any scenario-outcome of this magnitude. Critical viral load determination is mandatory information, and it is in Christian Drosten's group responsibility to perform these experiments and provide the crucial data.

Exhibit 3

Nevertheless these *in silico* sequences were used to develop a RT-PCR test methodology to identify the aforesaid virus. This model was based on the assumption that the *novel* virus is very similar to SARS-CoV from 2003 (Hereafter named SARS-CoV-1) as both are beta-coronaviruses.

The PCR test was therefore designed using the genomic sequence of SARS-CoV-1 as a control material for the Sarbeco component; we know this from our personal email-communication with [2] one of the co-authors of the Corman-Drosten paper. This method to model SARS-CoV-2 was described in the Corman-Drosten paper as follows:
"the establishment and validation of a diagnostic workflow for 2019-nCoV screening and specific confirmation, designed in absence of available virus isolates or original patient specimens. Design and validation were enabled by the close genetic relatedness to the 2003 SARS-CoV, and aided by the use of synthetic nucleic acid technology."

In short, a design relying merely on close genetic relatives does not fulfill the aim for a "robust diagnostic test" as cross reactivity and therefore false-positive results will inevitably occur.
Validation was only done in regards to *in silico* (theoretical) sequences and within the laboratory-setting, and not as required for in-vitro diagnostics with isolated genomic viral RNA. This very fact hasn't changed even after 10 months of introduction of the test into routine diagnostics.
There are numerous other severe scientific errors regarding the biomolecular design of the primers, the PCR method, as well as the molecular validation of the PCR products and methods described in the Corman-Drosten paper which are examined in detail in the following chapters. The paper itself already signifies that a large number of false positive results are generated by this test, even under controlled laboratory conditions, making it completely unsuitable as a reliable virus screening method for entire populations in an ongoing pandemic. Given the far-reaching implications, including quarantine measures, lockdowns, curfews and impacts on education etc., this paper must be immediately retracted.

DESIGN AND ERRORS in RT-PCR

The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is an important biomolecular technology to rapidly detect rare RNA fragments, which are known in advance. In the first step, RNA molecules present in the sample are reverse transcribed to yield cDNA. The cDNA is then amplified in the polymerase chain reaction using a specific primer pair and a thermostable DNA polymerase enzyme. The technology is highly sensitive and its detection limit is theoretically 1 molecule of cDNA. The specificity of the PCR is highly influenced by biomolecular design errors.

What is important when designing an RT-PCR Test and the quantitative RT-qPCR test described in the Corman-Drosten publication?

1. The primers and probes:

   a) the concentration of primers and probes must be of optimal range (100-200 nM)
   b) must be specific to the target-gene you want to amplify
   c) must have an optimal percentage of GC content relative to the total nitrogenous bases (minimum 40%, maximum 60%)
   d) for virus diagnostics at least 3 primer pairs must detect 3 viral genes (preferably as far apart as possible in the viral genome)

2. The temperature at which all reactions take place:

   a) DNA melting temperature (>92°)
   b) DNA amplification temperature (TaqPol specific)
   c) Tm; the annealing temperature (the temperature at which the primers and probes reach the target binding/detachment, not to exceed 2˚C per primer pair).
   Tm heavily depends on GC content of the primers

3. The number of amplification cycles (less than 35; preferably 25-30 cycles); In case of virus detection, >35 cycles only detects signals which do not correlate with infectious virus as determined by isolation in cell culture [reviewed in 2]; if someone is tested by PCR as positive when a threshold of 35 cycles or higher is used (as is the case in most laboratories in Europe & the US), the probability that said person is actually infected is less than 3%, the probability that said result is a false positive is 97%

[reviewed in 3]

4. Molecular biological validations; amplified PCR products must be validated either by running the products in a gel with a DNA ruler, or by direct DNA sequencing

5. Positive and negative controls should be specified to confirm/refute specific virus detection

6. There should be a Standard Operational Procedure (SOP) available, which unequivocally specifies the above parameters, so that all laboratories are able to set up the exact same test conditions. To have a validated universal SOP is essential, because it enables the comparison of data within and between countries.

MINOR CONCERNS WITH THE CORMAN-DROSTEN PAPER

1. In Table 1 of the Corman-Drosten paper, different abbreviations are stated - “nM” is specified, “nm” isn’t. Further in regards to correct nomenclature, nm means “nanometer” therefore nm should read nM here.

2. It is the general consensus to write genetic sequences always in the 5’-3’ direction, including the reverse primers. It is highly unusual to do alignment with reverse complementary writing of the primer sequence as the authors did in figure 2 of the Corman-Drosten paper. Here, in addition, a wobble base is marked as “y” without description of the bases the Y stands for.

3. Two misleading pitfalls in the Corman-Drosten paper are that their Table 1 does not include Tm-values (annealing-temperature values), neither does it show GC-values (number of G and C in the sequences as %-value of total bases).

MAJOR CONCERNS WITH THE CORMAN-DROSTEN PAPER

A) BACKGROUND

The authors introduce the background for their scientific work as: "*The ongoing outbreak of the recently emerged novel coronavirus (2019-nCoV) poses a challenge for public health laboratories as virus isolates are unavailable while there is growing evidence that the outbreak is more widespread than initially thought, and international spread through travelers does already occur".*

According to BBC News [4] and Google Statistics [5] there were 6 deaths world-wide on January 21st 2020 - the day when the manuscript was submitted. Why did the authors assume a challenge for public health laboratories while there was no substantial evidence at that time to indicate that the outbreak was more widespread than initially thought?

As an aim the authors declared to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available. Further, they acknowledge that "The present study demonstrates the enormous response capacity achieved through coordination of academic and public laboratories in national and European research networks."

B) Methods and Results

1. Primer & Probe Design

1a) Erroneous primer concentrations

Reliable and accurate PCR-test protocols are normally designed using between 100 nM and 200 nM per primer [7]. In the Corman-Drosten paper, we observe unusually high and varying primer concentrations for several primers (table 1). For the RdRp_SARSr-F and RdRp_SARSr-R primer pairs, 600 nM and 800 nM are described, respectively. Similarly, for the N_Sarbeco_F and N_Sarbeco_R primer set, they advise 600 nM and 800 nM, respectively [1]. It should be clear that these concentrations are far too high to be optimal for specific amplifications of target genes. *There exists no specified reason to use these extremely high*

concentrations of primers in this protocol. Rather, these concentrations lead to increased unspecific binding and PCR product amplification.

Table1: Primers and probes (adapted from Corman-Drosten paper; erroneous primer concentrations are highlighted)

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

1b) Unspecified ("Wobbly") primer and probe sequences

To obtain reproducible and comparable results, it is essential to distinctively define the primer pairs. In the Corman-Drosten paper we observed six unspecified positions, indicated by the letters R, W, M and S (Table 2). The letter W means that at this position there can be either an A or a T; R signifies there can be either a G or an A; M indicates that the position may either be an A or a C; the letter S indicates there can be either a G or a C on this position.

This high number of variants not only is unusual, but it also is highly confusing for laboratories. These six unspecified positions could easily result in the design of several different alternative primer sequences which do not relate to SARS-CoV-2 (2 distinct RdRp_SARSr_F primers + 8 distinct RdRp_SARS_P1 probes + 4 distinct RdRp_SARSr_R). *The design variations will inevitably lead to results that are not even SARS-CoV-2 related. Therefore, the confusing unspecific description in the Corman-Drosten paper is not suitable as a Standard Operational Protocol. These unspecified positions should have been designed unequivocally.*

Exhibit 3

These wobbly sequences have already created a source of concern in the field and resulted in a Letter to the Editor authored by Pillonel *et al.* [8] regarding blatant errors in the described sequences. These errors are self-evident in the Corman *et al.* supplement as well.

Table 2: Primers and probes (adapted from Corman-Drosten paper; unspecified ("Wobbly") nucleotides in the primers are highlighted)

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

W is A/T; R is G/A; M is A/C; S is G/C; FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

The WHO-protocol (Figure 1), which directly derives from the Corman-Drosten paper, concludes that in order to confirm the presence of SARS-CoV-2, two control genes (the E- and the RdRp-genes) must be identified in the assay. It should be noted, that the RdPd-gene has one uncertain position ("wobbly") in the forward-primer (R=G/A), two uncertain positions in the reverse-primer (R=G/A; S=G/C) and it has three uncertain positions in the RdRp-probe (W=A/T; R=G/A; M=A/C). So, two different forward primers, four different reverse primers, and eight distinct probes can be synthesized for the RdPd-gene. Together, there are 64 possible combinations of primers and probes!

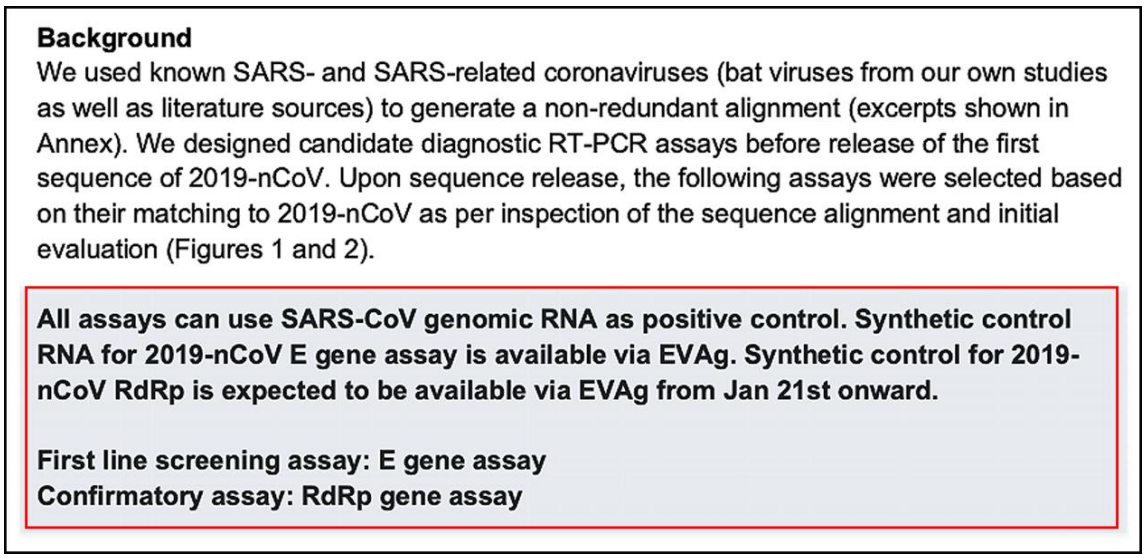
The Corman-Drosten paper further identifies a third gene which, according to the WHO protocol, was not further validated and deemed unnecessary: *"Of note, the N gene assay also performed well but was not subjected to intensive further validation because it was slightly less sensitive."*

This was an unfortunate omission as it would be best to use all three gene PCRs as

confirmatory assays, and this would have resulted in an almost sufficient virus RNA detection diagnostic tool protocol. Three confirmatory assay-steps would at least minimize-out errors & uncertainties at every fold-step in regards to “Wobbly”-spots. (Nonetheless, the protocol would still fall short of any “good laboratory practice”, when factoring in all the other design-errors).

As it stands, the N gene assay is regrettably neither proposed in the WHO-recommendation (Figure 1) as a mandatory and crucial third confirmatory step, nor is it emphasized in the Corman-Drosten paper as important optional reassurance “for a routine workflow” (Table 2).

Consequently, in nearly all test procedures worldwide, merely 2 primer-matches were used instead of all three. This oversight renders the entire test-protocol useless with regards to delivering accurate test-results of real significance in an ongoing pandemic.



Background

We used known SARS- and SARS-related coronaviruses (bat viruses from our own studies as well as literature sources) to generate a non-redundant alignment (excerpts shown in Annex). We designed candidate diagnostic RT-PCR assays before release of the first sequence of 2019-nCoV. Upon sequence release, the following assays were selected based on their matching to 2019-nCoV as per inspection of the sequence alignment and initial evaluation (Figures 1 and 2).

All assays can use SARS-CoV genomic RNA as positive control. Synthetic control RNA for 2019-nCoV E gene assay is available via EVAg. Synthetic control for 2019-nCoV RdRp is expected to be available via EVAg from Jan 21st onward.

First line screening assay: E gene assay
Confirmatory assay: RdRp gene assay

Figure 1: The N-Gene confirmatory-assay is neither emphasized as necessary third step in the official WHO Drosten-Corman protocol-recommendation [8] nor is it required as a crucial step for higher test-accuracy in the Eurosurveillance publication.

1c) Erroneous GC-content (discussed in 2c, together with annealing temperature (Tm))

1d) Detection of viral genes

RT-PCR is not recommended for primary diagnostics of infection. This is why the RT-PCR Test

used in clinical routine for detection of COVID-19 is not indicated for COVID-19 diagnosis on a regulatory basis.

"Clinicians need to recognize the enhanced accuracy and speed of the molecular diagnostic techniques for the diagnosis of infections, but also to understand their limitations. Laboratory results should always be interpreted in the context of the clinical presentation of the patient, and appropriate site, quality, and timing of specimen collection are required for reliable test results". [9]

However, it may be used to help the physician's differential diagnosis when he or she has to discriminate between different infections of the lung (Flu, Covid-19 and SARS have very similar symptoms). For a confirmative diagnosis of a specific virus, at least 3 specific primer pairs must be applied to detect 3 virus-specific genes. Preferably, these target genes should be located with the greatest distance possible in the viral genome (opposite ends included). Although the Corman-Drosten paper describes 3 primers, these primers only cover roughly half of the virus' genome. This is another factor that decreases specificity for detection of intact COVID-19 virus RNA and increases the quote of false positive test results.

Therefore, even if we obtain three positive signals (i.e. the three primer pairs give 3 different amplification products) in a sample, this does not prove the presence of a virus. A better primer design would have terminal primers on both ends of the viral genome. This is because the whole viral genome would be covered and three positive signals can better discriminate between a complete (and thus potentially infectious) virus and fragmented viral genomes (without infectious potency). In order to infer anything of significance about the infectivity of the virus, the Orf1 gene, which encodes the essential replicase enzyme of SARS-CoV-1 and SARS-CoV-2 viruses, should have been included as a target (Figure 2). The positioning of the targets in the region of the viral genome that is most heavily and variably transcribed is another weakness of the protocol.

Kim *et al.* demonstrate a highly variable 3' expression of subgenomic RNA in Sars-CoV-2 [23]. These RNAs are actively monitored as signatures for asymptomatic and non-infectious patients [10]. It is highly questionable to screen a population of asymptomatic people with qPCR primers that have 6 base pairs primer-dimer on the 3 prime end of a primer (Figure 3). Apparently the WHO recommends these primers. We tested all the wobble derivatives from

the Corman-Drosten paper with Thermofisher's primer dimer web tool [11]. The RdRp forward primer has 6bp 3prime homology with Sarbeco E Reverse. At high primer concentrations this is enough to create inaccuracies.

Of note: There is a perfect match of one of the N primers to a clinical pathogen (*Pantoea*), found in immuno-compromised patients. The reverse primer hits *Pantoea* as well but not in the same region (Figure 3).

<u>These are severe design errors, since the test cannot discriminate between the whole virus and viral fragments. The test cannot be used as a diagnostic for SARS-CoV-2 viruses.</u>

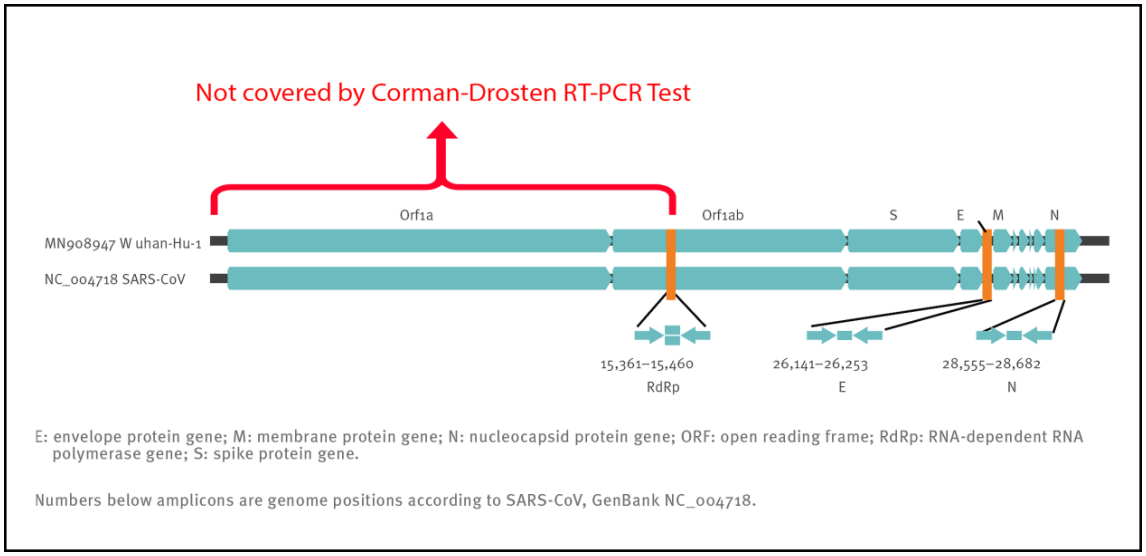


Figure 2: Relative positions of amplicon targets on the SARS-CoV-1 coronavirus and the 2019 novel coronavirus genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV-1, NC_004718 [1];

Exhibit 3

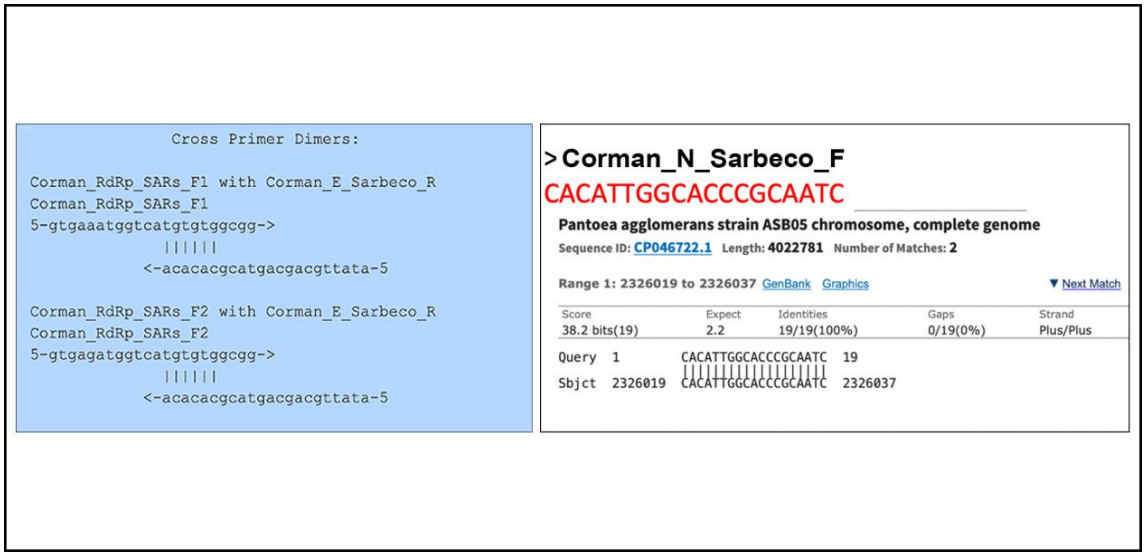


Figure 3: A test with Thermofischer's primer dimer web tool reveals that the RdRp forward primer has a 6bp 3`prime homology with Sarbeco E Reverse (left box). Another test reveals that there is a perfect match for one of the N-primers to a clinical pathogen (*Pantoea*) found in immuno-compromised patients (right box).

2. Reaction temperatures

2a) DNA melting temperature (>92°).

Adequately addressed in the Corman-Drosten paper.

2b) DNA amplification temperature.

Adequately addressed in the Corman-Drosten paper.

2c) Erroneous GC-contents and Tm

The annealing-temperature determines at which temperature the primer attaches/detaches from the target sequence. For an efficient and specific amplification, GC content of primers should meet a minimum of 40% and a maximum of 60% amplification. As indicated in table 3, three of the primers described in the Corman-Drosten paper are not within the normal range for GC-content. Two primers (RdRp_SARSr_F and RdRp_SARSr_R) have unusual and very low GC-values of 28%-31% for all possible variants of wobble bases, whereas primer E_Sarbeco_F has a GC-value of 34.6% (Table 3 and second panel of Table 3).

It should be noted that the GC-content largely determines the binding to its specific target due to its three hydrogen bonds in base pairing. Thus, the lower the GC-content of the primer, the lower its binding-capability to its specific target gene sequence (i.e. the gene to

be detected). This means for a target-sequence to be recognized we have to choose a temperature which is as close as possible to the actual annealing-temperature (best practise-value) for the primer not to detach again, while at the same time specifically selecting the target sequence.

If the Tm-value is very low, as observed for all wobbly-variants of the RdRp reverse primers, the primers can bind non-specifically to several targets, decreasing specificity and increasing potential false positive results.

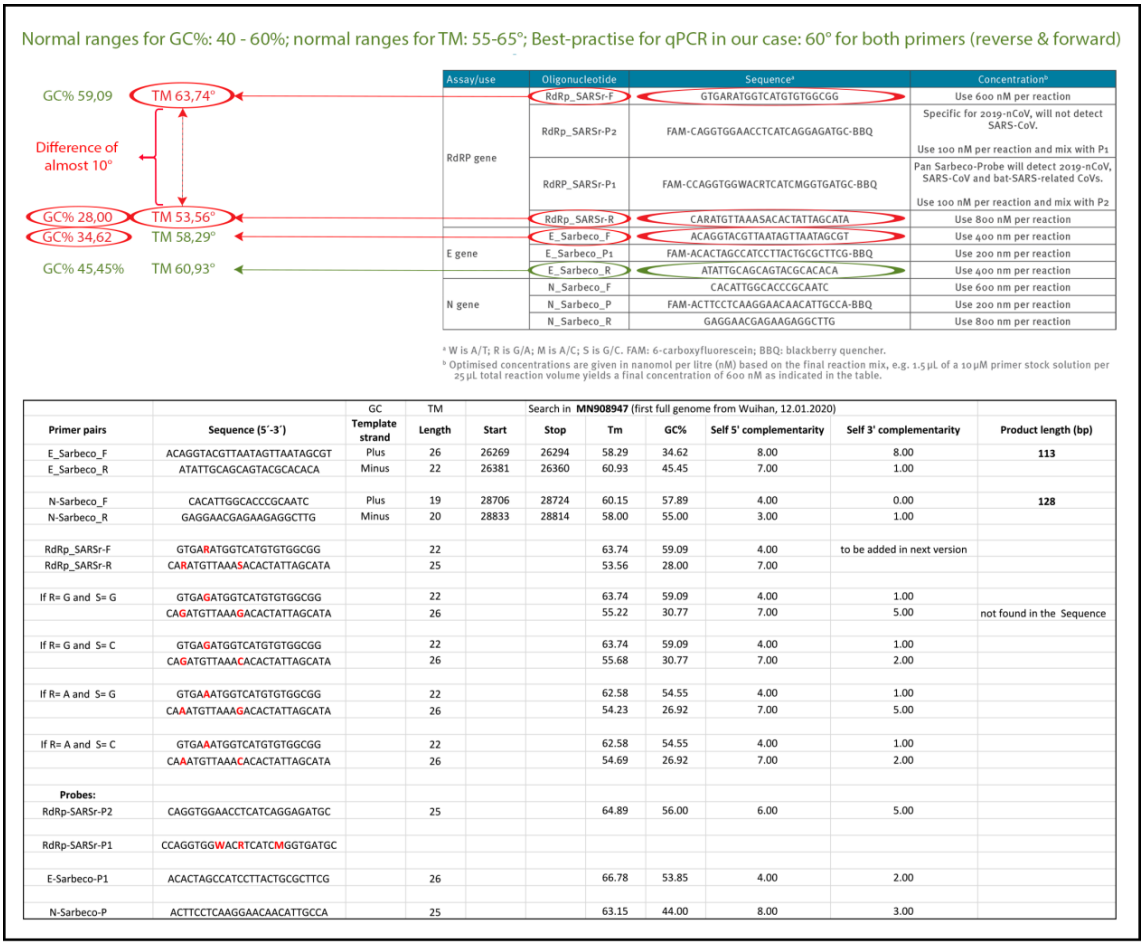
The annealing temperature (Tm) is a crucial factor for the determination of the specificity /accuracy of the qPCR procedure and essential for evaluating the accuracy of qPCR-protocols. Best-practice recommendation: Both primers (forward and reverse) should have an almost similar value, preferably the identical value.

We used the freely available primer design software Primer-BLAST [12, 25] to evaluable the best-practise values for all primers used in the Corman-Drosten paper (Table 3). We attempted to find a Tm-value of 60° C, while similarly seeking the highest possible GC%-value for all primers. A maximal Tm difference of 2° C within primer pairs was considered acceptable. Testing the primer pairs specified in the Corman-Drosten paper, we observed a difference of 10° C with respect to the annealing temperature Tm for primer pair1 (RdRp_SARSr_F and RdRp_SARSr_R). *This is a very serious error and makes the protocol useless as a specific diagnostic tool.*

Additional testing demonstrated that only the primer pair designed to amplify the N-gene (N_Sarbeco_F and N_Sarbeco_R) reached the adequate standard to operate in a diagnostic test, since it has a sufficient GC-content and the Tm difference between the primers (N_Sarbeco_F and N_Sarbeco_R) is 1.85° C (below the crucial maximum of 2° C difference). Importantly, this is the gene which was neither tested in the virus samples (Table 2) nor emphasized as a confirmatory test. In addition to highly variable melting temperatures and degenerate sequences in these primers, there is another factor impacting specificity of the procedure: the dNTPs (0.4uM) are 2x higher than recommended for a highly specific amplification. There is additional magnesium sulphate added to the reaction as well. This procedure combined with a low annealing temperature can create non-specific amplifications. When additional magnesium is required for qPCR, specificity of the assay should be further scrutinized.

The design errors described here are so severe that it is highly unlikely that specific amplification of SARS-CoV-2 genetic material will occur using the protocol of the Corman-Drosten paper.

Table 3: GC-content of the primers and probes (adapted from Corman-Drosten paper; aberrations from optimized GC-contents are highlighted. Second Panel shows a table-listing of all Primer-BLAST best practices values for all primers and probes used in the Corman-Drosten paper by Prof. Dr. Ulrike Kämmerer & her team



Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.
[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

		GC	TM		Search in **MN908947** (first full genome from Wuihan, 12.01.2020)					
Primer pairs	**Sequence (5'-3')**	**Template strand**	**Length**	**Start**	**Stop**	**Tm**	**GC%**	**Self 5' complementarity**	**Self 3' complementarity**	**Product length (bp)**
E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Plus	26	26269	26294	58.29	34.62	8.00	8.00	**113**
E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Minus	22	26381	26360	60.93	45.45	7.00	1.00	
N-Sarbeco_F	CACATTGGCACCCGCAATC	Plus	19	28706	28724	60.15	57.89	4.00	0.00	**128**
N-Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Minus	20	28833	28814	58.00	55.00	3.00	1.00	
RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG		22			63.74	59.09	4.00	to be added in next version	
RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA		25			53.56	28.00	7.00		
If R= G and S= G	GTGAGATGGTCATGTGTGGCGG		22			63.74	59.09	4.00	1.00	
	CAGATGTTAAAGACACTATTAGCATA		26			55.22	30.77	7.00	5.00	not found in the Sequence
If R= G and S= C	GTGAGATGGTCATGTGTGGCGG		22			63.74	59.09	4.00	1.00	
	CAGATGTTAAACACACTATTAGCATA		26			55.68	30.77	7.00	2.00	
If R= A and S= G	GTGAAATGGTCATGTGTGGCGG		22			62.58	54.55	4.00	1.00	
	CAAATGTTAAAGACACTATTAGCATA		26			54.23	26.92	7.00	5.00	
If R= A and S= C	GTGAAATGGTCATGTGTGGCGG		22			62.58	54.55	4.00	1.00	
	CAAATGTTAAACACACTATTAGCATA		26			54.69	26.92	7.00	2.00	
Probes:										
RdRp-SARSr-P2	CAGGTGGAACCTCATCAGGAGATGC		25			64.89	56.00	6.00	5.00	
RdRp-SARSr-P1	CCAGGTGGWACRTCATCMGGTGATGC									
E-Sarbeco-P1	ACACTAGCCATCCTTACTGCGCTTCG		26			66.78	53.85	4.00	2.00	
N-Sarbeco-P	ACTTCCTCAAGGAACAACATTGCCA		25			63.15	44.00	8.00	3.00	

3. The number of amplification cycles

It should be noted that there is no mention anywhere in the Corman-Drosten paper of a test being positive or negative, or indeed what defines a positive or negative result. These types of virological diagnostic tests must be based on a SOP, including a validated and fixed number of PCR cycles (Ct value) after which a sample is deemed positive or negative. The maximum reasonably reliable Ct value is 30 cycles. Above a Ct of 35 cycles, rapidly increasing numbers of false positives must be expected .

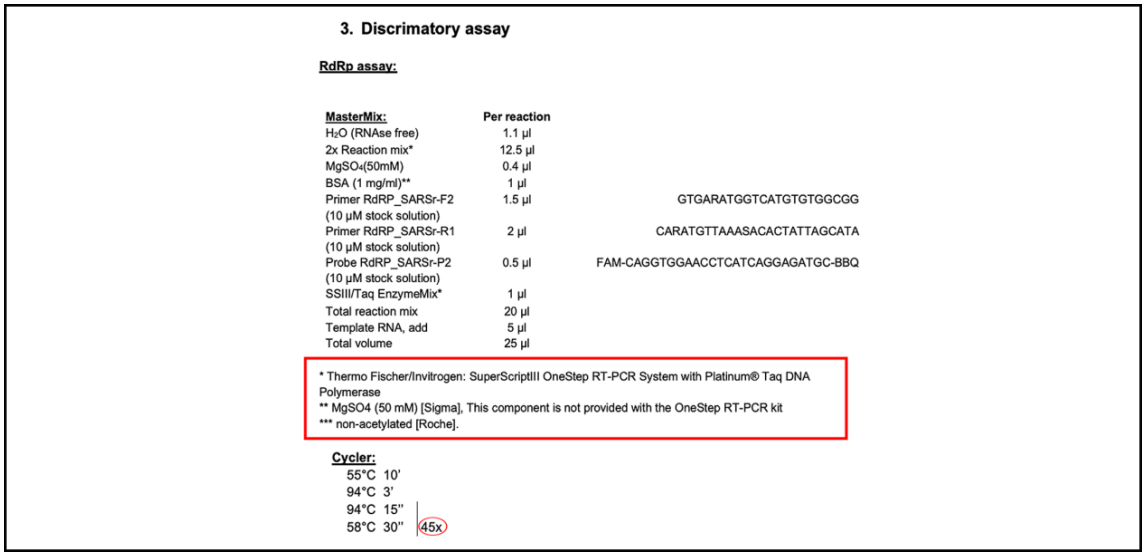
Exhibit 3

PCR data evaluated as positive after a Ct value of 35 cycles are completely unreliable.

Citing Jaafar *et al.* 2020 [3]: *"At Ct = 35, the value we used to report a positive result for PCR, <3% of cultures are positive."* In other words, there was no successful virus isolation of SARS-CoV-2 at those high Ct values.

Further, scientific studies show that only non-infectious (dead) viruses are detected with Ct values of 35 [22].

Between 30 and 35 there is a grey area, where a positive test cannot be established with certainty. This area should be excluded. Of course, one could perform 45 PCR cycles, as recommended in the Corman-Drosten WHO-protocol (Figure 4), but then you also have to define a reasonable Ct-value (which should not exceed 30). But an analytical result with a Ct value of 45 is scientifically and diagnostically absolutely meaningless (a reasonable Ct-value should not exceed 30). All this should be communicated very clearly. *It is a significant mistake that the Corman-Drosten paper does not mention the maximum Ct value at which a sample can be unambiguously considered as a positive or a negative test-result. This important cycle threshold limit is also not specified in any follow-up submissions to date.*



3. Discrimatory assay

RdRp assay:

MasterMix:	Per reaction	
H_2O (RNAse free)	1.1 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer RdRP_SARSr-F2 (10 µM stock solution)	1.5 µl	GTGARATGGTCATGTGTGGCGG
Primer RdRP_SARSr-R1 (10 µM stock solution)	2 µl	CARATGTTAAASACACTATTAGCATA
Probe RdRP_SARSr-P2 (10 µM stock solution)	0.5 µl	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:
55°C 10'
94°C 3'
94°C 15"
58°C 30" 45x

Figure 4: RT-PCR Kit recommendation in the official Corman-Drosten WHO-protocol [8]. Only a "Cycler"-value (cycles) is to be found without corresponding and scientifically reasonable Ct (Cutoff-value). This or any other cycles-value is nowhere to be found in the actual Corman-Drosten paper.

Exhibit 3

4. Biomolecular validations

To determine whether the amplified products are indeed SARS-CoV-2 genes, biomolecular validation of amplified PCR products is essential. For a diagnostic test, this validation is an absolute must.

Validation of PCR products should be performed by either running the PCR product in a 1% agarose-EtBr gel together with a size indicator (DNA ruler or DNA ladder) so that the size of the product can be estimated. The size must correspond to the calculated size of the amplification product. But it is even better to sequence the amplification product. The latter will give 100% certainty about the identity of the amplification product. Without molecular validation one can not be sure about the identity of the amplified PCR products. Considering the severe design errors described earlier, the amplified PCR products can be anything.

Also not mentioned in the Corman-Drosten paper is the case of small fragments of qPCR (around 100bp): It could be either 1,5% agarose gel or even an acrylamide gel.
The fact that these PCR products have not been validated at molecular level is another striking error of the protocol, making any test based upon it useless as a specific diagnostic tool to identify the SARS-CoV-2 virus.

5. Positive and negative controls to confirm/refute specific virus detection.

The unconfirmed assumption described in the Corman-Drosten paper is that SARS-CoV-2 is the only virus from the SARS-like beta-coronavirus group that currently causes infections in humans. The sequences on which their PCR method is based are *in silico* sequences, supplied by a laboratory in China [23], because at the time of development of the PCR test no control material of infectious ("live") or inactivated SARS-CoV-2 was available to the authors. The PCR test was therefore designed using the sequence of the known SARS-CoV-1 as a control material for the Sarbeco component (Dr. Meijer, co-author Corman-Drosten paper in an email exchange with Dr. Peter Borger) [2].

All individuals testing positive with the RT-PCR test, as described in the Corman-Drosten paper, are assumed to be positive for SARS-CoV-2 infections. There are three severe flaws in their assumption. First, a positive test for the RNA molecules described in the Corman-Drosten paper cannot be equated to "infection with a virus". A positive RT-PCR test merely indicates the presence of viral RNA molecules. As demonstrated under point 1d (above), the Corman-Drosten test was not designed to detect the full-length virus, but only a fragment of the virus. We already concluded that this classifies the test as unsuitable as a diagnostic test for SARS-virus infections.

Secondly and of major relevance, the functionality of the published RT-PCR Test was not demonstrated with the use of a positive control (isolated SARS-CoV-2 RNA) which is an essential scientific gold standard.

Third, the Corman-Drosten paper states:

"To show that the assays can detect other bat-associated SARS-related viruses, we used the E gene assay to test six bat-derived faecal samples available from Drexler et al. [...] und Muth et al. […]. These virus-positive samples stemmed from European rhinolophid bats. Detection of these phylogenetic outliers within the SARS-related CoV clade suggests that all Asian viruses are likely to be detected. This would, theoretically, ensure broad sensitivity even in case of multiple independent acquisitions of variant viruses from an animal reservoir."

This statement demonstrates that the E gene used in RT-PCR test, as described in the Corman-Drosten paper, is not specific to SARS-CoV-2. The E gene primers also detect a broad spectrum of other SARS viruses.

The genome of the coronavirus is the largest of all RNA viruses that infect humans and they all have a very similar molecular structure. Still, SARS-CoV-1 and SARS-CoV-2 have two highly specific genetic fingerprints, which set them apart from the other coronaviruses. First, a unique fingerprint-sequence (KTFPPTEPKKDKKKK) is present in the N-protein of SARS-CoV-1 and SARS-CoV-2 [13,14,15]. Second, both SARS-CoV-1 and SARS-CoV-2 do not contain the HE protein, whereas all other coronaviruses possess this gene [13, 14]. So, in order to specifically detect a SARS-CoV-1 and SARS-CoV-2 PCR product the above region in the N gene should have been chosen as the amplification target. A reliable diagnostic test should focus on this specific region in the N gene as a confirmatory test. The PCR for this N gene was not further validated nor recommended as a test gene by the Drosten-Corman paper, because of

being “not so sensitive” with the SARS-CoV original probe [1].

Furthermore, the absence of the HE gene in both SARS-CoV-1 and SARS-CoV-2 makes this gene the ideal negative control to exclude other coronaviruses. The Corman-Drosten paper does not contain this negative control, nor does it contain any other negative controls. The PCR test in the Corman-Drosten paper therefore contains neither a unique positive control nor a negative control to exclude the presence of other coronaviruses. This is another major design flaw which classifies the test as unsuitable for diagnosis.

6. Standard Operational Procedure (SOP) is not available

There should be a Standard Operational Procedure (SOP) available, which unequivocally specifies the above parameters, so that all laboratories are able to set up the identical same test conditions. To have a validated universal SOP is essential, because it facilitates data comparison within and between countries. It is very important to specify all primer parameters unequivocally. We note that this has not been done. Further, the Ct value to indicate when a sample should be considered positive or negative is not specified. It is also not specified when a sample is considered infected with SARS-CoV viruses. As shown above, the test cannot discern between virus and virus fragments, so the Ct value indicating positivity is crucially important. This Ct value should have been specified in the Standard Operational Procedure (SOP) and put on-line so that all laboratories carrying out this test have exactly the same boundary conditions. It points to flawed science that such an SOP does not exist. The laboratories are thus free to conduct the test as they consider appropriate, resulting in an enormous amount of variation. Laboratories all over Europe are left with a multitude of questions; which primers to order? which nucleotides to fill in the undefined places? which Tm value to choose? How many PCR cycles to run? At what Ct value is the sample positive? And when is it negative? And how many genes to test? Should all genes be tested, or just the E and RpRd gene as shown in Table 2 of the Corman-Drosten paper? Should the N gene be tested as well? And what is their negative control? What is their positive control? The protocol as described is unfortunately very vague and erroneous in its design that one can go in dozens of different directions. There does not appear to be any standardization nor an SOP, so it is not clear how this test can be implemented.

7. Consequences of the errors described under 1-5: false positive results.

The RT-PCR test described in the Corman-Drosten paper contains so many molecular biological design errors (see 1-5) that it is not possible to obtain unambiguous results. It is inevitable that this test will generate a tremendous number of so-called “false positives”. The definition of false positives is a negative sample, which initially scores positive, but which is negative after retesting with the same test. False positives are erroneous positive test-results, i.e. negative samples that test positive. And this is indeed what is found in the Corman-Drosten paper. On page 6 of the manuscript PDF the authors demonstrate, that even under well-controlled laboratory conditions, a considerable percentage of false positives is generated with this test:

“In four individual test reactions, weak initial reactivity was seen however they were negative upon retesting with the same assay. These signals were not associated with any particular virus, and for each virus with which initial positive reactivity occurred, there were other samples that contained the same virus at a higher concentration but did not test positive. Given the results from the extensive technical qualification described above, it was concluded that this initial reactivity was not due to chemical instability of real-time PCR probes and most probably to handling issues caused by the rapid introduction of new diagnostic tests and controls during this evaluation study.” [1]

The first sentence of this excerpt is clear evidence that the PCR test described in the Corman-Drosten paper generates false positives. Even under the well-controlled conditions of the state-of-the-art Charité-laboratory, 4 out of 310 primary-tests are false positives per definition. Four negative samples initially tested positive, then were negative upon retesting. This is the classical example of a false positive. In this case the authors do not identify them as false positives, which is intellectually dishonest.

Another telltale observation in the excerpt above is that the authors explain the false positives away as "handling issues caused by the rapid introduction of new diagnostic tests". Imagine the laboratories that have to introduce the test without all the necessary information normally described in an SOP.

8. The Corman-Drosten paper was not peer-reviewed

Before formal publication in a scholarly journal, scientific and medical articles are traditionally certified by "peer review." In this process, the journal's editors take advice from various experts ("referees") who have assessed the paper and may identify weaknesses in its assumptions, methods, and conclusions. Typically a journal will only publish an article once the editors are satisfied that the authors have addressed referees' concerns and that the data presented supports the conclusions drawn in the paper." This process is as well described for Eurosurveillance [16].

The Corman-Drosten paper was submitted to Eurosurveillance on January 21st 2020 and accepted for publication on January 22nd 2020. On January 23rd 2020 the paper was online. On January 13th 2020 version 1-0 of the protocol was published at the official WHO website [17], updated on January 17th 2020 as document version 2-1 [18], even before the Corman-Drosten paper was published on January 23rd at Eurosurveillance.

Normally, peer review is a time-consuming process since at least two experts from the field have to critically read and comment on the submitted paper. In our opinion, this paper was not peer-reviewed. Twenty-four hours are simply not enough to carry out a thorough peer review. Our conclusion is supported by the fact that a tremendous number of very serious design flaws were found by us, which make the PCR test completely unsuitable as a diagnostic tool to identify the SARS-CoV-2 virus. Any molecular biologist familiar with RT-PCR design would have easily observed the grave errors present in the Corman-Drosten paper before the actual review process. We asked Eurosurveillance on October 26th 2020 to send us a copy of the peer review report. To date, we have not received this report and in a letter dated November 18th 2020, the ECDC as host for Eurosurveillance declined to provide access without providing substantial scientific reasons for their decision. On the contrary, they write that "disclosure would undermine the purpose of scientific investigations." [24].

9. Authors as the editors

A final point is one of major concern. It turns out that two authors of the Corman-Drosten paper, Christian Drosten and Chantal Reusken, are also members of the editorial board of this journal [19]. Hence there is a severe conflict of interest which strengthens suspicions

that the paper was not peer-reviewed. It has the appearance that the rapid publication was possible simply because the authors were also part of the editorial board at Eurosurveillance. This practice is categorized as compromising scientific integrity .

SUMMARY CATALOGUE OF ERRORS FOUND IN THE PAPER

The Corman-Drosten paper contains the following specific errors:

1. There exists no specified reason to use these extremely high concentrations of primers in this protocol. The described concentrations lead to increased nonspecific bindings and PCR product amplifications, making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

2. Six unspecified wobbly positions will introduce an enormous variability in the real world laboratory implementations of this test; the confusing nonspecific description in the Corman-Drosten paper is not suitable as a Standard Operational Protocol making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

3. The test cannot discriminate between the whole virus and viral fragments. Therefore, the test cannot be used as a diagnostic for intact (infectious) viruses, making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus and make inferences about the presence of an infection.

4. A difference of 10° C with respect to the annealing temperature Tm for primer pair1 (RdRp_SARSr_F and RdRp_SARSr_R) also makes the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

5. A severe error is the omission of a Ct value at which a sample is considered positive and negative. This Ct value is also not found in follow-up submissions making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

6. The PCR products have not been validated at the molecular level. This fact makes the protocol useless as a specific diagnostic tool to identify the SARS-CoV-2 virus.

7. The PCR test contains neither a unique positive control to evaluate its specificity for SARS-CoV-2 nor a negative control to exclude the presence of other coronaviruses, making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

8. The test design in the Corman-Drosten paper is so vague and flawed that one can go in dozens of different directions; nothing is standardized and there is no SOP. This highly questions the scientific validity of the test and makes it unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

9. Most likely, the Corman-Drosten paper was not peer-reviewed making the test unsuitable as a specific diagnostic tool to identify the SARS-CoV-2 virus.

10. We find severe conflicts of interest for at least four authors, in addition to the fact that two of the authors of the Corman-Drosten paper (Christian Drosten and Chantal Reusken) are members of the editorial board of Eurosurveillance. A conflict of interest was added on July 29 2020 (Olfert Landt is CEO of TIB-Molbiol; Marco Kaiser is senior researcher at GenExpress and serves as scientific advisor for TIB-Molbiol), that was not declared in the original version (and still is missing in the PubMed version); TIB-Molbiol is the company which was “the first” to produce PCR kits (Light Mix) based on the protocol published in the Corman-Drosten manuscript, and according to their own words, they distributed these PCR-test kits before the publication was even submitted [20]; further, Victor Corman & Christian Drosten failed to mention their second affiliation: the commercial test laboratory “Labor Berlin”. Both are responsible for the virus diagnostics there [21] and the company operates in the realm of real time PCR-testing.

CONCLUSION

In light of our re-examination of the test protocol to identify SARS-CoV-2 described in the Corman-Drosten paper we have identified concerning errors and inherent fallacies which render the SARS-CoV-2 PCR test useless.

The decision as to which test protocols are published and made widely available lies squarely in the hands of Eurosurveillance. A decision to recognise the errors apparent in the Corman-Drosten paper has the benefit to greatly minimise human cost and suffering going forward. Is it not in the best interest of Eurosurveillance to retract this paper? Our conclusion is clear. In the face of all the tremendous PCR-protocol design flaws and errors described here, we conclude: There is not much of a choice left in the framework of scientific integrity and responsibility.

References

[1] Corman Victor M, Landt Olfert, Kaiser Marco, Molenkamp Richard, Meijer Adam, Chu Daniel KW, Bleicker Tobias, Brünink Sebastian, Schneider Julia, Schmidt Marie Luisa, Mulders Daphne GJC, Haagmans Bart L, van der Veer Bas, van den Brink Sharon, Wijsman Lisa, Goderski Gabriel, Romette Jean-Louis, Ellis Joanna, Zambon Maria, Peiris Malik, Goossens Herman, Reusken Chantal, Koopmans Marion PG, Drosten Christian. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):pii=2000045. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

[2] Email communication between Dr. Peter Borger & Dr. Adam Meijer: Supplementary Material

[3] Jafaar *et al.*, Correlation Between 3790 Quantitative Polymerase Chain Reaction–Positives Samples and Positive Cell Cultures, Including 1941 Severe Acute Respiratory Syndrome Coronavirus 2 Isolates https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1491/5912603

[4] BBC, January 21st 2020: https://www.bbc.com/news/world-asia-china-51185836; archive: https://archive.is/0qRmZ

[5] Google Analytics - COVID19-deaths worldwide: https://bit.ly/3fndemJ; archive: https://archive.is/PpqEE

[6] Laboratory testing for COVID-19 Emergency Response Technical Centre, NIVD under China CDC March 15th, 2020: http://www.chinacdc.cn/en/COVID19/202003/P020200323390321297894.pdf

[7] Real-Time PCR Handbook Life Technologies (https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf)

Nolan T, Huggett J, Sanchez E.Good practice guide for the application of quantitative PCR (qPCR) First Edition 2013

[8] Trestan Pillonel *et al*, Letter to the editor: SARS-CoV-2 detection by real-time RT-PCR: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7268274/

Exhibit 3

[9] Kurkela, Satu, and David WG Brown. "Molecular-diagnostic techniques." Medicine 38.10 (2009): 535-540.

[10] Wolfel *et al.,* Virological assessment of hospitalized patients with COVID-2019 https://www.nature.com/articles/s41586-020-2196-x

[11] Thermofischer Primer Dimer Web Tool: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html

[12] Primer-BLAST, NCBI - National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

[13] Marra MA, Steven JMJ, Caroline RA, Robert AH, Angela BW et al. (2003) Science. The Genome sequence of the SARS-associated coronavirus. Science 300(5624): 1399-1404.

[14] Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome: https://www.ncbi.nlm.nih.gov/nuccore/MN908947

[15] Borger P. A SARS-like Coronavirus was expected but nothing was done to be prepared. Am J Biomed Sci Res 2020. https://biomedgrid.com/pdf/AJBSR.MS.ID.001312.pdf https://www.researchgate.net/publication/341120750_A_SARS-like_Coronavirus_was_Expected_but_nothing_was_done_to_be_Prepared; archive: https://archive.is/i76Hu

[16] Eurosurveillance paper evaluation / review process: https://www.eurosurveillance.org/evaluation

[17] Official recommendation of the Corman-Drosten protocol & manuscript by the WHO, published on January 13th 2020 as version 1.0 of the document: https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf; archive: https://bit.ly/3m3jXVH

[18] Official WHO-recommendation for the Corman / Drosten RT-qPCR-protocol, which directly derives from the Eurosurveillance-publication, document-version 2-1, published on

17th January 2020: https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2

[19] Eurosurveillance Editorial Board, 2020: https://www.eurosurveillance.org/upload/site-assets/imgs/2020-09-Editorial%20Board%20PDF.pdf; archive: https://bit.ly/2TqXBjX

[20] Instructions For Use LightMix SarbecoV E-gene plus EAV Control, TIB-Molbiol & Roche Molecular Solutions, January 11th 2020:
https://www.roche-as.es/lm_pdf/MDx_40-0776_96_Sarbeco-E-gene_V200204_09164154001 (1).pdf
Archive, timestamp - January 11th 2020: https://archive.is/Vulo5; archive:
https://bit.ly/3fm9bXH

[21] Christian Drosten & Victor Corman, responsible for viral diagnostics at Labor Berlin: https://www.laborberlin.com/fachbereiche/virologie/; archive: archive.is/CDEUG

[22] Tom Jefferson, Elizabeth Spencer, Jon Brassey, Carl Heneghan Viral cultures for COVID-19 infectivity assessment. Systematic review. Systematic review doi:
https://doi.org/10.1101/2020.08.04.20167932;
https://www.medrxiv.org/content/10.1101/2020.08.04.20167932v4

[23] Kim *et al.,*The Architecture of SARS-CoV-2 Transcriptome:
https://www.sciencedirect.com/science/article/pii/S0092867420304062

[24] ECDC reply to Dr. Peter Borger, 18th November 2020: Supplementary Material

[25] Prof. Dr. Ulrike Kämmerer & team, survey & Primer-BLAST table: Supplementary Material

Additional literature:

Description RT-PCR RKI Germany, on page 10 of this link:
https://www.rki.de/DE/Content/Gesundheitsmonitoring/Gesundheitsberichterstattung/GBEDownloadsJ/JoHM_S5_2020_Studienprotokoll_CORONA_MONITORING_lokal.pdf?__blob=publicationFile

Exhibit 3

Author's Affiliations:

1) **Dr. Pieter Borger** (MSc, PhD), Molecular Genetics, W+W Research Associate, Lörrach, Germany,

2) Rajesh Kumar Malhotra (Artist Alias: **Bobby Rajesh Malhotra**), Former 3D Artist / Scientific Visualizations at CeMM - Center for Molecular Medicine of the Austrian Academy of Sciences (2019-2020), University for Applied Arts - Department for Digital Arts Vienna, Austria

3) **Dr. Michael Yeadon** BSs(Hons) Biochem Tox U Surrey, PhD Pharmacology U Surrey. Managing Director, Yeadon Consulting Ltd, former Pfizer Chief Scientist, United Kingdom,

4) **Dr. Clare Craig** MA, (Cantab) BM, BCh (Oxon), FRCPath, United Kingdom

5) **Kevin McKernan**, BS Emory University, Chief Scientific Officer, founder Medical Genomics, engineered the sequencing pipeline at WIBR/MIT for the Human Genome Project, Invented and developed the SOLiD sequencer, awarded patents related to PCR, DNA Isolation and Sequencing, USA

6) **Prof. Dr. Klaus Steger**, Department of Urology, Pediatric Urology and Andrology, Molecular Andrology, Biomedical Research Center of the Justus Liebig University, Giessen, Germany

7) **Dr. Paul McSheehy** (BSc, PhD), Biochemist & Industry Pharmacologist, Loerrach, Germany

8) **Dr. Lidiya Angelova**, MSc in Biology, PhD in Microbiology, Former researcher at the National Institute of Allergy and Infectious Diseases (NIAID), Maryland, USA

9) **Dr. Fabio Franchi**, Former Dirigente Medico (M.D) in an Infectious Disease Ward, specialized in "Infectious Diseases" and "Hygiene and Preventive Medicine", Società Scientifica per il Principio di Precauzione (SSPP), Italy

10) **Dr. med. Thomas Binder**, Internist and Cardiologist (FMH), Switzerland

11) **Prof. Dr. med. Henrik Ullrich**, specialist Diagnostic Radiology, Chief Medical Doctor at the Center for Radiology of Collm Oschatz-Hospital, Germany

12) **Prof. Dr. Makoto Ohashi**, Professor emeritus, PhD in Microbiology and Immunology, Tokushima University, Japan

13) **Dr. Stefano Scoglio**, B.Sc. Ph.D., Microbiologist, Nutritionist, Italy

14) **Dr. Marjolein Doesburg-van Kleffens** (MSc, PhD), specialist in Laboratory Medicine (clinical chemistry), Maasziekenhuis Pantein, Beugen, the Netherlands

15) **Dr. Dorothea Gilbert** (MSc, PhD), PhD Environmental Chemistry and Toxicology. DGI Consulting Services, Oslo, Norway

Exhibit 3

16) **Dr. Rainer J. Klement**, PhD. Department of Radiation Oncology, Leopoldina Hospital Schweinfurt, Germany

17) **Dr. Ruth Schruefer**, PhD, human genetics/ immunology, Munich, Germany,

18) **Dra. Berber W. Pieksma**, General Practitioner, The Netherlands

19) **Dr. med. Jan Bonte** (GJ), Consultant Neurologist, the Netherlands

20) Dr. Bruno H. Dalle Carbonare (Molecular biologist), IP specialist, BDC Basel, Switzerland

21) **Dr. Kevin P. Corbett**, MSc Nursing (Kings College London) PhD (London South Bank) Social Sciences (Science & Technology Studies) London, England, UK

22) **Prof. Dr. Ulrike Kämmerer**, specialist in Virology / Immunology / Human Biology / Cell Biology, University Hospital Würzburg, Germany

Author's Contributions:

PB: Planned and conducted the analyses and research, conceptualising the manuscript.

RKM: Planned and conducted the research, conceptualising the figures and manuscript.

MY: Conducted the analyses and research.

KMcK: Conducted the analyses and research, conceptualized the manuscript.

KS: Conducted the analyses and research.

PMcS: Proofreading the analyses and research.

LA: Proofreading the analyses and research.

FF: Proofreading the analyses and research.

TB: Proofreading the analyses and research.

HU: Proofreading the analyses and research.

MO: Proofreading the analyses and research.

SS: Proofreading the analyses and research.

MDvK: Proofreading the analyses and research.

DG: Proofreading the analyses and research.

RJK: Proofreading the analyses and research.

RS: Proofreading the analyses and research, and the manuscript.

BWK: Proofreading the analyses and research.

RvV: Proofreading the analyses and research.

JB: Proofreading the analyses and research.

KC: Proofreading the analyses and research.

UK: Planned and conducted the analyses and research, conceptualising the manuscript.

Acknowledgement:

We are grateful to Saji N Hameed (Environmental Informatics, University of Aizu, Tsuruga, Ikki-machi, Aizuwakamatsu-shi, Fukushima, Japan) and Howard R. Steen (MA Chem. Eng. Cantab (1969-'73), Former Research Manager, UK) for proofreading our manuscript.

Exhibit 3

EXHIBIT 4

Letters

RESEARCH LETTER

Assessment of SARS-CoV-2 RNA Test Results Among Patients Who Recovered From COVID-19 With Prior Negative Results

Some patients who have recovered from coronavirus disease 2019 (COVID-19) with documented negative real-time polymerase chain reaction (RT-PCR) results at the time of recovery have had subsequent positive RT-PCR test results for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1,2] in the absence of any symptoms suggestive of new infection.[3] It is unknown whether such patients are infectious and whether they should be quarantined. Real-time PCR is not a viral culture and does not allow determination of whether the virus is viable and transmissible. We investigated RT-PCR retested positive nasal/oropharyngeal swab (NOS) samples from recovered patients with COVID-19 with prior negative results for the presence of replicative SARS-CoV-2 RNA.[4]

Editor's Note

Supplemental content

Table. Testing Results for NOS Samples Obtained at COVID-19 Diagnosis or After COVID-19 Recovery in 32 Study Patients[a]

Sample	COVID-19 samples tested											Days of recovery sampling since diagnosis
	Diagnosis				Recovery							
	Genomic RNA (C_T value)			Subgenomic RNA (C_T value)	Genomic RNA (C_T value)			Subgenomic (C_T value)	RNA load, copies/mL	Serology (positive or negative result)		
Sample No.	*E* gene	*RdRP* gene	*N* gene	*E* gene	*E* gene	*RdRP* gene	*N* gene	*E* gene	*N* gene	IgG	IgA	
1	31.6	31.3	31.2	34.5	29.3	30.7	31.2	39.1	1.2×10^4	Positive	Positive	39
2	27.0	26.9	30.0	36.0	30.0	30.5	31.2		8.9×10^3	Positive	Positive	31
3	19.3	20.8	22.1	35.2	31.5	34.7	32.8		3.3×10^3	Positive	Negative	44
4	21.6	22.0	22.9	36.4	31.8	31.4	32.3		5.5×10^3	Positive	Positive	34
5	30.0	32.8	38.1	30.2	31.8	34.3	34.5		3.2×10^3	Positive	Positive	62
6	20.8	20.9	22.3	37.3	32.2	32.8	34.1		5.3×10^3	Positive	Positive	37
7	27.3	29.9	31.3	36.9	32.3	30.9	32.7		6.4×10^3	Positive	Positive	39
8	26.9	27.0	31.2	38.1	35.0	34.4	36.1		4.0×10^2	Positive	Positive	71
9	22.5	23.7	24.9	31.0	38.8	33.6	33.9		2.6×10^3	Negative	Negative	42
10	21.3	21.4	28.9	38.9		32.2	33.4		1.2×10^4	Positive	Positive	56
11	26.6	26.9	28.1	33.0		32.8	33.2		1.3×10^4	Positive	Positive	54
12	22.8	24.2	25.3	31.0		34.2	33.7		6.9×10^3	Positive	Positive	55
13	25.8	25.8	26.1	39.8	NA	34.8	39.1		3.0×10^2	Positive	Positive	36
14	20.8	20.4	21.1	32.0		35.0	35.1		1.9×10^3	Positive	Positive	56
15	29.4	30.1	32.2	37.0		36.5	39.2		3.2×10^3	Positive	Positive	36
16	27.9	29.1	31.1	32.0		38.1	39.3		1.6×10^1	Positive	Positive	77
17	30.6	29.9	31.8	32.1			35.7	NA	5.4×10^3	Positive	Positive	53
18	28.5	29.1	30.8	36.8			36.8		2.9×10^3	Positive	Positive	43
19	26.9	22.2	26.1	30.1			37.5		1.1×10^3	Positive	Positive	36
20	25.7	25.2	28.9	38.0			37.9		2.6×10^3	Positive	Positive	48
21	27.0	29.0	30.2	32.3			38.1		1.9×10^3	Positive	Positive	41
22	28.5	29.4	30.0	32.3			38.4		4.9×10^1	Positive	Negative	76
23	27.1	28.6	29.3	36.1			38.9		4.5×10^2	Positive	Positive	29
24	25.4	22.9	24.1	34.8			39.0		5.6×10^1	Positive	Positive	70
25	28.7	29.5	31.4	37.3	NA		39.1		5.4×10^3	Negative	Positive	46
26	27.1	27.7	29.2	37.1			39.1		1.9×10^3	Positive	Positive	34
27	26.7	27.7	29.6	39.2			39.2		2.0×10^3	Positive	Positive	45
28	17.1	19.1	19.9	33.0			39.2		8.5×10^2	Positive	Positive	40
29	27.0	28.9	30.0	32.1			39.3		5.0×10^1	Positive	Positive	56
30	22.9	23.8	25.8	37.1			39.4		1.6×10^2	Positive	Positive	55
31	28.6	30.4	30.9	33.0			39.6		5.3×10^2	Positive	Positive	61
32	29.1	28.0	30.9	36.2			39.8		3.4×10^2	Positive	Positive	53

Abbreviations: COVID-19, coronavirus disease 2019; C_T, cycle threshold; *E* gene, envelope gene; NA, not applicable; *N* gene, nucleocapsid gene; *RdRP*, RNA-dependent RNA polymerase; RT-PCR, real-time polymerase chain reaction.

[a] For RT-PCR testing, the Seegene Allplex 2019-nCoV and Clonit Quanty COVID-19 assays were used for total RNA detection and quantification, respectively, whereas replicative (*E* gene) RNA was detected by an in-house RT-PCR assay.[4] Results were expressed as C_T values (<40 for positive detection) or quantified as RNA (*N* gene) copies per mL. NA indicates the absence of positive detection for the indicated gene. For serological testing, SARS-CoV-2 IgG/IgA Euroimmun enzyme-linked immunoassays were used, and positive and negative results were assessed using the 1.1 or greater or less than 1.1 times the manufacturer's cutoffs as reference IgG/IgA values, respectively.

Exhibit 4

Methods | We studied 176 recovered patients with COVID-19 who were admitted to the postacute outpatient service of our institution (Rome, Italy) from April 21 to June 18, 2020, for COVID-19 follow-up.[5,6] Before that, patients had discontinued isolation according to current criteria,[5] which require no fever for 3 consecutive days, improvement in other symptoms, and 2 negative RT-PCR results for SARS-CoV-2 RNA 24 hours apart.

Nasal/oropharyngeal swab samples from patients at follow-up were analyzed for total (genomic) and replicative (subgenomic) SARS-CoV-2 RNA using RT-PCR assays (eMethods in the Supplement). For patients with positive results for total RNA, samples previously obtained at the time of COVID-19 diagnosis and kept at −112 °F until testing were also tested for replicative RNA. Serological testing was performed for SARS-CoV-2 IgG/IgA detection (eMethods in the Supplement). The ethics committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS (Rome, Italy) approved the study, and written informed consent was obtained from each patient.

Results | As shown in the **Table**,[4] 32 of 176 NOS samples (18.2%) tested positive for total SARS-CoV-2 RNA, with viral loads ranging from 1.6×10^1 to 1.3×10^4 SARS-CoV-2 RNA copies per mL. One of the 32 samples (3.1%) had replicative SARS-CoV-2 RNA. Samples from the 32 patients at the time of COVID-19 diagnosis were also tested and, expectedly, had replicative SARS-CoV-2 RNA. All but 1 of 32 patients had a positive serology result against SARS-CoV-2 (Table), as well as 139 of remaining 144 patients (data not shown), at COVID-19 follow-up. The patient who tested serologically negative was not the one with a positive test result for replicative SARS-CoV-2 RNA. The mean (SD) time from COVID-19 diagnosis to follow-up was 48.6 (13.1) days in 32 patients (Table) and 57.7 (16.9) days in 144 patients (data not shown).

Discussion | Similar to that reported elsewhere,[2] 18% of patients with COVID-19 in our institution became RT-PCR positive for SARS-CoV-2 RNA after clinical recovery and previous negative results.[5] As positivity in the patients was suggestive, but not necessarily a reflection, of viral carriage, we used replicative SARS-CoV-2 RNA detection as a proxy for virus replication in culture.[4]

Only 1 of 32 patients retesting positive had replicating virus in the NOS sample, suggesting either recurrent infection or reinfection, which is impossible to separate because no whole-genome sequencing and phylogenetic analyses were performed.[3] The patient retested positive 16 days after COVID-19 recovery (ie, 39 days from COVID-19 diagnosis) and was symptomatic. The patient was an older adult with hypertension, diabetes, and cardiovascular disease but no evidence of close contacts with people with SARS-CoV-2 infection or persons who became RT-PCR positive. In the 31 remaining patients (who were asymptomatic), their positive result likely represented either recurrent or resolving infection, but in either case, they were unlikely to be infectious. The limitations of our study are the lack of data from viral cultures or whole-genome sequencing analysis and the small sample size.

Conclusions | This study highlights that many patients who recovered from COVID-19 may be still positive (albeit at lower levels) for SARS-CoV-2 RNA, but only a minority of the patients may carry a replicating SARS-CoV-2 in the respiratory tract. Further studies are needed to verify whether such patients can transmit the virus.

Flora Marzia Liotti, PhD
Giulia Menchinelli, PhD
Simona Marchetti, BSc
Brunella Posteraro, PhD
Francesco Landi, MD
Maurizio Sanguinetti, MD
Paola Cattani, MD

Author Affiliations: Dipartimento di Scienze Biotecnologiche di Base, Cliniche Intensivologiche e Perioperatorie, Università Cattolica del Sacro Cuore, Rome, Italy (Liotti, Menchinelli, Posteraro, Sanguinetti, Cattani); Dipartimento di Scienze di Laboratorio e Infettivologiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy (Liotti, Menchinelli, Sanguinetti, Cattani); Dipartimento di Scienze Mediche e Chirurgiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy (Posteraro); Dipartimento di Scienze dell'Invecchiamento, Neurologiche, Ortopediche e della Testa-Collo, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy (Landi).

Accepted for Publication: October 25, 2020.

Published Online: November 12, 2020. doi:10.1001/jamainternmed.2020.7570

Corresponding Author: Brunella Posteraro, PhD, Dipartimento di Scienze Mediche e Chirurgiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Largo A. Gemelli 8, 00168 Rome, Italy (brunella.posteraro@unicatt.it).

Author Contributions: Drs Sanguinetti and Cattani had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Drs Liotti and Menchinelli contributed equally to the study. Drs Sanguinetti and Cattani contributed equally as senior authors.
Concept and design: Liotti, Posteraro, Landi, Sanguinetti, Cattani.
Acquisition, analysis, or interpretation of data: Liotti, Menchinelli, Marchetti, Posteraro, Sanguinetti, Cattani.
Drafting of the manuscript: Liotti, Menchinelli, Posteraro, Sanguinetti, Cattani.
Critical revision of the manuscript for important intellectual content: Liotti, Marchetti, Landi, Sanguinetti, Cattani.
Statistical analysis: Menchinelli.
Obtained funding: Sanguinetti.
Supervision: Posteraro, Landi.
Other: Liotti.

Conflict of Interest Disclosures: None reported.

Funding/Support: This work was funded by donations from Reale Group and Fondazione Valentino Garavani & Giancarlo Giammetti to support the COVID-19 research in our institution.

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Additional Contributions: We thank Franziska Lohmeyer, PhD (Scientific Direction, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy) for English revision of the manuscript. She was not compensated for her contributions.

1. Lan L, Xu D, Ye G, et al. Positive RT-PCR test results in patients recovered from COVID-19. *JAMA*. 2020;323(15):1502-1503. doi:10.1001/jama.2020.2783

2. Kang H, Wang Y, Tong Z, Liu X. Retest positive for SARS-CoV-2 RNA of "recovered" patients with COVID-19: Persistence, sampling issues, or re-infection? *J Med Virol*. 2020;1-3. doi:10.1002/jmv.26114

3. Alvarez-Moreno CA, Rodríguez-Morales AJ. Testing dilemmas: post negative, positive SARS-CoV-2 RT-PCR—is it a reinfection? *Travel Med Infect Dis*. 2020;35:101743. doi:10.1016/j.tmaid.2020.101743

4. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature*. 2020;581(7809):465-469. doi:10.1038/s41586-020-2196-x

5. Carfì A, Bernabei R, Landi F; for the Gemelli Against COVID-19 Post-Acute Care Study Group. Persistent symptoms in patients after acute COVID-19. *JAMA*. 2020;324(6):603-605. doi:10.1001/jama.2020.12603

6. Gemelli Against COVID-19 Post-Acute Care Study Group. Post-COVID-19 global health strategies: the need for an interdisciplinary approach. *Aging Clin Exp Res*. 2020;32(8):1613-1620.

Exhibit 4

EXHIBIT 5

Viral cultures for COVID-19 infectious potential assessment – a systematic review

Jefferson T[1]; Spencer EA[1]; Brassey J[2]; Heneghan C[1].

1. **Nuffield Department of Primary Care Health Sciences, University of Oxford, Radcliffe Observatory Quarter, Oxford, OX2 6GG**
2. **Trip Database Ltd**

Corresponding author:

Jefferson (tom-jefferson@conted.ox.ac.uk)

Alternate corresponding author

Carl Heneghan

Professor of EBM & Director CEBM,

Nuffield Dept. of Primary Care Health Sciences,

University of Oxford

E: carl.heneghan@phc.ox.ac.uk

Summary

The reliability of RT-qPCR for assessing infectious potential of Covid-19 positives is defined by testing reference and culture specimens and their relation to patient characteristics (date and severity of symptoms, medical history) and test factors (cycle threshold).

Exhibit 5

ABSTRACT:

Objective to review the evidence from studies relating SARS-CoV-2 culture with the results of reverse transcriptase polymerase chain reaction (RT-PCR) and other variables which may influence the interpretation of the test, such as time from symptom onset

Methods We searched LitCovid, medRxiv, Google Scholar and the WHO Covid-19 database for Covid-19 to 10 September 2020. We included studies attempting to culture or observe SARS-CoV-2 in specimens with RT-PCR positivity. Studies were dual extracted and the data summarised narratively by specimen type. Where necessary we contacted corresponding authors of included papers for additional information. We assessed quality using a modified QUADAS 2 risk of bias tool.

Results We included 29 studies reporting attempts at culturing, or observing tissue infection by, SARS-CoV-2 in sputum, nasopharyngeal or oropharyngeal, urine, stool, blood and environmental specimens. The quality of the studies was moderate with lack of standardised reporting. The data suggest a relationship between the time from onset of symptom to the timing of the specimen test, cycle threshold (Ct) and symptom severity. Twelve studies reported that Ct values were significantly lower and log copies higher in specimens producing live virus culture. Two studies reported the odds of live virus culture reduced by approximately 33% for every one unit increase in Ct. Six of eight studies reported detectable RNA for longer than 14 days but infectious potential declined after day 8 even among cases with ongoing high viral loads. Four studies reported viral culture from stool specimens.

Conclusion

Complete live viruses are necessary for transmission, not the fragments identified by PCR. Prospective routine testing of reference and culture specimens and their relationship to symptoms, signs and patient co-factors should be used to define the reliability of PCR for assessing infectious potential. Those with high cycle threshold are unlikely to have infectious potential.

Keywords: Covid-19; mode of transmission, viral culture; symptom onset to test date; polymerase chain reaction; SARS-CoV-2; infectious potential.

Introduction

Effective prevention and management of SARS-CoV-2 infections relies on our capacity to identify those who are infected or potentially infectious. In the absence of predictive clinical signs or symptoms, the major means of detection is testing using Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) [1, 2, 3]

The test amplifies genomic sequences identified in specimens, and is highly sensitive, being capable of generating observable signals from specimens containing minute amounts of matching genomic sequence. Amplification of genomic sequence is measured in cycle thresholds (Ct), each cycle being a cut off for positive detection. There may be a correlation between Ct values from respiratory specimens, symptom onset to test (STT) date and positive viral culture. Evidence suggests the lower the Ct value and the shorter the STT, the higher the infectious potential. [4] If this is so, we should be able to identify those with the highest infectious potential.

Identification 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. [5] RT-qPCR cannot distinguish between the shedding of live virus or of viral fragments with no infectious potential, and it cannot measure the quantity of live virus present in a person's excreta. Although viral culture is difficult, time consuming and requires specialised facilities it potentially represents the best indicator of infection and infectious potential. We, therefore, set out to review those studies attempting viral culture, regardless of specimen type tested. We investigated the probability of successful culture with time from symptom onset to test and cycle threshold. We also examined the relationship between specimen cycle threshold and infectious potential.

Methods

We searched four databases: LitCovid, medRxiv, Google Scholar and the WHO Covid-19 database, using the terms 'viral culture' or 'viral replication' and associated synonyms on 10 September 2020. For relevant articles, citation matching was undertaken and relevant results identified.

We included studies reporting attempts to culture SARS-CoV-2 and those which also estimated the potential infectivity of the isolates or observed tissue infection by SARS CoV-2 and related them to other clinical variables such as date of symptom onset to test and patient characteristics.

Isothermal methods of detection are not included in our review, as they do not provide a Ct value

One reviewer extracted data for each study and a second reviewer checked the extraction. Heterogeneity and lack of detail of some of the reported data in the included studies prevented pooling. We tabulated data and summarised it descriptively by specimen: fecal, respiratory,

environment or mixed. Where possible, we also reported the duration of detectable RNA and the relationship of PCR cycle threshold and log 10 copies to positive viral culture.

Where necessary we contacted corresponding authors of the cited papers for additional information. We assessed quality using the QUADAS 2 risk of bias tool, simplified because the included studies were not designed as primary diagnostic accuracy studies. [6] Our methods are more fully described in our protocol (published on the 4th of July and updated on 5th of October 2020). [7]

Results

We identified 145 possible articles for inclusion and after screening, 29 full texts were read and included (see PRISMA [8] flow chart - Figure 1)**.** One unpublished study was not included as no permission was given by the authors. The included studies were published in 30 articles (see web appendix references w1-w29), four of which were in pre-print servers. The characteristics of each study are shown in Table 1. All included studies were case series of **moderate quality** (Table 2. Quality of included studies). We could not identify a protocol for any of the studies. All had been made public in 2020. We received five author responses regarding clarifying information (see Acknowledgments).

Studies using fecal specimens

Nine studies assessed viral viability from fecal specimens positive for SARS-CoV-2 based on RT-PCR result [W10, W11, W13, W17, W22, W23, W25-W27] One study reported infecting ferrets with stool supernatant; [w10] two reported visual growth in tissue [w19, w22[and four reported achieving viral replication [w13, w23, w24, w26]. In one further study, methods were unclear.[W28]

Studies using respiratory specimens

Seventeen studies reported attempting viral isolation and culture from respiratory specimens [W3, W4, W6-10, W13-16, W18, W21-23, W26, W27] One study successfully cultured 26/90 nasopharyngeal specimens: positive cultures were observed only up to day eight post-symptom onset; [w7] another study obtained cultures from 31/46 nasopharyngeal and oropharyngeal specimens. [w3] The largest study came from the La Scola group publications [w15] with positive cultures of 1,941 from 3,790 specimens. Another study of UK health care workers during a period of low viral circulation isolated SARS Cov-2 from 1/19 specimens. [w5]

Two more studies reported a clear correlation between symptoms onset, date of sampling, Ct and likelihood of viral culture. [w18, w21]

One study [w14] of nasopharyngeal specimens from 638 patients aged <16 years reported achieving culture from 12 (52%) of the 23 who tested positive for SARS CoV-2 with a Ct of around 28. Gniazdowski [w8] assessed RNA and infectious virus detection in 161 nasopharyngeal specimens from hospitalised Covid-19 patients. Positive culture was associated with Ct values of 18.8 ± 3.4 (median 18.7); negative culture was associated with mean Ct values 27.1 ± 5.7 (median 27.5). Over 90% of the virus isolates were obtained from specimens with a Ct value below 23

Basile [w4] reported 24% culture positivity, with specimens significantly more likely to be positive from ICU. A report by the Korean Centres for Disease Control failed to grow live viruses from 108 respiratory specimens from "re-positives" i.e. people who had tested positive after previously testing negative.[w12]

Ladhani [w16] and colleagues reported a successful culture rate of 87/158 RT-PCR positive naso-pharyngeal specimens from six nursing homes in London.

Studies using environmental specimens

Two possible (the text is unclear) positive cultures were obtained from 95 environmental specimens in one study that assessed aerosol and surface transmission potential of SARS-CoV-2 [w20]. No viruses could be grown from specimens from seven areas of a large London hospital from specimens with a cut-off RT-PCR Ct > 30. [w29]

Ahn and colleagues [w1] failed to grow live virus from an unspecified number of air specimens from isolation rooms of patients with severe Covid-19, but were able to grow virus from swabs of handrails, and the external surfaces of intubation cannulae.

Mixed sources

Some studies labelled as mixed source specimens are also reported by indvidual specimen in this text.

Eight studies reported viral culture from mixed sources: 12 oropharyngeal, nine nasopharyngeal and two sputum specimens [w9], one stool specimen and an unreported number of other specimens[w10], from saliva, nasal swabs, urine, blood and stool collected from nine Covid-19 and a possible

specimen stool culture [[w23], nine nasopharyngeal, oropharyngeal, stool, serum and urine specimens [w13], seven sputum specimens, three stool specimens and one nasopharyngeal specimen of 11 patients. [w26] . In this study all specimens had been taken within 5 days of symptom onset and there was a relationship between copy thresholds and cytopathic effect observed in infected culture cells.

Kim and colleagues reported no viral growth from an unclear number of serum, urine and stool specimens, despite these specimens being collected soon after admission [w11] . Lu and colleagues also reported no viral growth, however their specimens were from 87 cases tested "re-positive". [w17]

One study [w27] reported 21 positive cultures from from naso-pharyngeal specimens of 19 hospitalised patients in Singapore but no growth from specimens with a Ct value >30, or collected >14 days after symptoms onset. No culture was achieved from the urine or stool specimens.

Blood cultures

In one study by Andersson [w2] et al 20 RT-PCR positive serum specimens from 12 individual patients were selected at random from a Covid-19 specimen bank at 3 to 20 days following onset of symptoms. None of the 20 serum specimens produced a viral culture.

Post mortem study

One study on alveolar specimens from 68 elderly deceased reported postmortem studies on lung tissues from six cases were available for viral isolation. The evaluation showed viable SARS-CoV-2 in all six cases - in one case on day 26 from symptom onset. [w6]

Duration of RNA viral detection

Table 3 shows that nine studies report on the duration of viral RNA detection as assessed by PCR for SARS-CoV-2 RNA. [w7, w8, w10, w12, w13, w21, w24, w25, w27] All nine studies reported RNA detection for longer than 7 days. Young et al [w27] reported that SARS-CoV-2 was detectable from nasopharyngeal swabs by PCR up to 48 days after symptom onset.

Live viral culture window

The live viral culture time window was much shorter than for viral RNA identification, ranging from less than 8 days from symptom onset to test [w23] and Ct < 24 [w7]. Median duration of viral RNA identification in culture was 4 days (InterQuartile Range: 1 to 8) [w21].

The relationship between RT-PCR results and viral culture of SARS-CoV-2

Table 4 shows that ten studies analysed the relationship between Ct values and the possibility of culturing live virus [w4, w5, w7, w8, w9 w15, w16, w21, w23, w27] and three quantified the mean log copies of detected virus and live culture [w9, w14, w18]. All reported that Ct were significantly lower and log copies were significantly higher in those with live virus culture. Five studies reported no growth in specimens based on a Ct cut-off value [w5, w7, w9, w16, w27] ranging from CT > 24 [w7] to 35 [w15].

The estimated probability of recovery of virus from specimens with Ct > 35 was 8.3% (95% CI: 2.8% to 18.4%)[w21]. All donors above the Ct threshold of 35 (n=5) producing live culture were symptomatic.

In six London nursing homes there was no correlation between Ct values and symptoms in either residents or staff, [w16] although nearly 50% of both categories were asymptomatic.

One study [w9] reported different cut-off thresholds depending on the gene fragment analysed[34]. No growth was found for the NSP 12 fragment at Ct > 31.5, whereas the value was higher for the N gene fragment (>35.2).

The odds for culturing live virus decreased by 0.64 for every one unit increase in Ct (95%CI 0.49 to 0.84, p<0.001) [w7]; another study[w21] reported similar results in line with empirical evidence of an increased Ct of 0.58 per day since symptoms started. [9]

Discussion

The studies in this review attempted, and some successfully achieved, culture of SARS-CoV-2 in the laboratory, using a range of different specimens. There is evidence of a positive relationship between lower cycle count threshold, likelihood of positive viral culture and date of symptom onset. [10] This is seen clearly in the two studies assessing the infectious potential of "re-positives", i.e. COVID-19 cases who had been discharged from hospital after testing negative repeatedly and who then tested positive again after discharge: Lu 2020 [w17], Korean CDC [w12].

Lu and colleagues considered four hypotheses for the origin of "re-positives" [w17]. On the basis of their evidence they discarded re-infection and latency as explanations, and concluded that the most plausible explanations were either contamination of the specimen by extraneous material or

identification in the specimen of minute and irrelevant particles of dead SARS-CoV-2 representing virus long neutralised by the immune system.

Rapid expansion in testing capability requires training protocols and precautions to avoid poor laboratory practice which may not be possible in the time pressure of a pandemic. The evidence in this review shows that those with high cycle threshold are unlikely to have infectious potential.

Interpreting the results of RT-PCR requires consideration of patient characteristics such as symptoms and their severity, contacts history, presence of pre-existing morbidities and drug history, the cycle threshold value, the number of days from symptom onset to test and the specimen donor's age.[11 12]

Several of our included studies assessed the relationship of these variables and there appears to be a time window during which RNA detection 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.[11] We propose that further work should be done on this with the aim of constructing an algorithm for integrating the results of PCR with other variables, to increase the effectiveness of detecting infectious patients.

PCR should be continuously calibrated against a reference culture in Vero E6 cells in which cytopathic effect has been observed [w6]. Confirmation of visual identification using methods, such as an immunofluorescence assay may also be needed to aid diagnosis. [13] Henderson and colleagues have called for a multicentre 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. [14]

Ascertainment of infectious potential is all the more important as there is good evidence of viral RNA persistence across a whole range of different viral diseases with little or no infectious potential in the post infectious phase of MERS,[15] measles,[16] other coronoviridae, HCV and a variety of animal RNA viruses.[17]

In one COVID-19 (former) case, viral RNA was detectable until day 78 from symptoms onset with a very high Ct [18] but no culture growth, implying a lack of infectious potential.

SARS CoV-2 methods of cell culture vary and to our knowledge have not been standardised. Methods vary depending upon the selection of the cell lines; the collection, transport, and handling of and the maintenance of viable and healthy inoculated cells. [19] We therefore urgently recommend the development of standard culture methods and external quality assessment schemes for laboratories offering testing for SARS CoV2. [20 21] If identification of viral infectious potential 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 use viruses as controls, perform complete investigations when needed, and store representative clinical strains whenever possible. [22] Current evidence is too limited to establish the feasibility of generating a universal cycle threshold value as this may change with circumstances (e.g. hospital, community, cluster and symptom level), laboratory methods, so more information is urgently needed [23].

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.[24] Further studies with standardised methods [25] and reporting are needed to establish the magnitude and reliability of this association.

The results of our review agree with the scoping review by Byrne and colleagues on infectious potential periods [26] and those of the living review by Cevick and colleagues[11]. The authors reviewed 79 studies on the dynamics, load and RNA detection for SARS CoV-1, MERS and SARS CoV-2 from symptoms onset. They concluded that although SARS-CoV-2 RNA identification 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). Those results are consistent with Bullard et al who found no growth in specimens with a cycle threshold greater than 24 [w7] or when symptom onset was greater than 8 days, and Wölfel et al [w23] who reported that virus could not be isolated from specimens taken after day 8 even among cases with ongoing high viral loads. The review by Rhee and colleagues reaches conclusion similar to ours.[10]

The importance of symptom onset and reported PCR threshold is shown in a study that collected test data during a prospective household transmission study. The authors found that Ct values were lowest soon after symptom onset and correlated with time elapsed since symptom onset (within 7 days after symptom onset, the median Ct value was 26.5 compared with a median of 35.0 21 days after onset). Ct values were significantly higher among those participants reporting no symptoms, and lower in those reporting upper respiratory symptoms at the time of specimen collection.[28]

The evidence is increasingly pointing to the probability of culturing live virus being related to the amount of viral RNA in the specimen and, therefore, inversely related to the cycle threshold. Thus, detection of viral RNA *per se* cannot be used to infer infectiousness. Duration of excretion may also be linked to age, male gender and possibly use of steroids and severity of illness.

Our review is limited by the lack of standardised reporting and lack of standard testing methods amongst the included studies[20]. Ct threshold reporting was inconsistent, preventing pooling or further in-depth analysis of the data, and insufficient clinical details were reported to define the possible role of asymptomatics or pre-symptomatics in transmission. The included studies were case reports or case series with a mixture of laboratory and clinical data, and variable in reporting the relation between donor characteristics and PCR results.

We may have missed some studies or new studies as they are published and we aim to update this review with emerging evidence.

Conclusion

The evidence gathered in this review points to a relationship between the time from collection of a specimen to test, cycle threshold, and symptom severity. 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. Defining cut off levels predictive of infectious potential [27]should be feasible and is necessary for diagnosing viral respiratory infections using molecular tests.

Acknowledgments

Drs Susan Amirian, Siyuan Ding, Long Rong, Sravanthi Parasato and Bernard La Scola provided additional information for this review. Dr Maryanne DeMasi helped with reference identification. All data included in the review are from publications or preprints.

Funding

The review was partly funded by NIHR Evidence Synthesis Working Group project 380.

Disclaimer: The views expressed in this commentary represent the views of the authors and not necessarily those of the host institution, the UK NHS, the NIHR, or the Department of Health and Social Care. The views are not a substitute for professional medical advice. It will be regularly updated see the evidence explorer at https://www.cebm.net/evidence-synthesis/transmission-dynamics-of-covid-19/ for regular updates to the evidence summaries and briefs.

Potential conflicts:

Tom Jefferson is a Senior Associate Tutor and Honorary Research Fellow, Centre for Evidence-Based Medicine, University of Oxford. Disclosure statement is here

TJ was in receipt of a Cochrane Methods Innovations Fund grant to develop guidance on the use of regulatory data in Cochrane reviews (2015-018). In 2014–2016, TJ was a member of three advisory boards for Boehringer Ingelheim. TJ was a member of an independent data monitoring committee for a Sanofi Pasteur clinical trial on an influenza vaccine. TJ is occasionally interviewed by market research companies about phase I or II pharmaceutical products for which he receives fees (current). TJ was a member of three advisory boards for Boehringer Ingelheim (2014-16). TJ was a member of an independent data monitoring committee for a Sanofi Pasteur clinical trial on an influenza vaccine (2015-2017). TJ is a relator in a False Claims Act lawsuit on behalf of the United States that involves sales of Tamiflu for pandemic stockpiling. If resolved in the United States' favor, he would be entitled to a percentage of the recovery. TJ is co-holder of a Laura and John Arnold Foundation grant for development of a RIAT support centre (2017-2020) and Jean Monnet Network Grant, 2017-2020 for The Jean Monnet Health Law and Policy Network. TJ is an unpaid collaborator to the project Beyond Transparency in Pharmaceutical Research and Regulation led by Dalhousie University and funded by the Canadian Institutes of Health Research (2018-2022). TJ consulted for Illumina LLC on next generation gene sequencing (2019-2020). TJ was the consultant scientific coordinator for the HTA Medical Technology programme of the Agenzia per i Serivizi Sanitari Nazionali (AGENAS) of the

Italian MoH (2007-2019). TJ is Director Medical Affairs for BC Solutions, a market access company for medical devices in Europe. TJ is funded by NIHR UK and the World Health Organization (WHO) to update Cochrane review A122, "Physical Interventions to interrupt the spread of respiratory viruses". TJ is funded by Oxford University to carry out a living review on the transmission epidemiology of COVID-19. Since 2020, TJ receives fees for articles published by The Spectator and other media outlets.

Elizabeth Spencer is Epidemiology and Evidence Synthesis Researcher at the Centre for Evidence-Based Medicine. Disclosure statement is here.

Jon Brassey is the Director of Trip Database Ltd, Lead for Knowledge Mobilisation at Public Health Wales (NHS) and an Associate Editor at the BMJ Evidence-Based Medicine. Jon is a shareholder in Trip Database Ltd.

Carl Heneghan is Professor of Evidence-Based Medicine, Director of the Centre for Evidence-Based Medicine and Director of Studies for the Evidence-Based Health Care Programme. Disclosure statement is here.

References

1. Wynants L, Van Calster B, Collins GS, et al. Prediction models for diagnosis and prognosis of covid-19: systematic review and critical appraisal. BMJ 2020;369:m1328. doi: 10.1136/bmj.m1328

2. Transmission of SARS-CoV-2: implications for infection prevention precautions. Scientific brief.

3. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19) 16-24 February 2020. 2020

4. Bullard J, Dust K, Funk D, et al. Predicting infectious SARS-CoV-2 from diagnostic s. LID - 10.1093/cid/ciaa638 [doi] LID - ciaa638. (1537-6591 (Electronic))

5. Hematian A, Sadeghifard N, Mohebi R, et al. Traditional and Modern Cell Culture in Virus Diagnosis. Osong public health and research perspectives 2016;7(2):77-82. doi: 10.1016/j.phrp.2015.11.011 [published Online First: 2016/01/08]

6. Whiting PF, Rutjes Aw Fau - Westwood ME, Westwood Me Fau - Mallett S, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. (1539-3704 (Electronic))

7. Analysis of the evidence of transmission dynamics of COVID-19 Protocol for a scoping evidence review. Jefferson T, Plüddemann A; Spencer EA; Roberts N; Heneghan C [https://www.cebm.net/evidence-synthesis/transmission-dynamics-of-covid-19/

8. Moher D, Shamseer L, Clarke M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Syst Rev 2015;4(1):1-1. doi: 10.1186/2046-4053-4-1

9. Lesho E, Reno L, Newhart D, et al. Temporal, Spatial, and Epidemiologic Relationships of SARS-CoV-2 Gene Cycle Thresholds: A Pragmatic Ambi-directional Observation. Clinical Infectious Diseases 2020 doi: 10.1093/cid/ciaa1248

10. Rhee C, Kanjilal S, Baker M, et al. Duration of SARS-CoV-2 Infectivity: When is it Safe to Discontinue Isolation? Clinical Infectious Diseases 2020 doi: 10.1093/cid/ciaa1249

11. Cevik M, Tate M, Lloyd O, et al. SARS-CoV-2, SARS-CoV-1 and MERS-CoV viral load dynamics, duration of viral shedding and infectiousness: a living systematic review and meta-analysis. medRxiv 2020:2020.07.25.20162107. doi: 10.1101/2020.07.25.20162107

12. Tom MR, Mina MJ. To Interpret the SARS-CoV-2 Test, Consider the Cycle Threshold Value. Clinical Infectious Diseases 2020 doi: 10.1093/cid/ciaa619

13. Hematian A, Sadeghifard N, Mohebi R, et al. Traditional and Modern Cell Culture in Virus Diagnosis. Osong public health and research perspectives 2016;7(2):77-82. doi: 10.1016/j.phrp.2015.11.011 [published Online First: 2016/01/08]

14. Henderson DK, Weber DJ, Babcock H, et al. The perplexing problem of persistently PCR-positive personnel. Infection Control & Hospital Epidemiology 2020:1-2. doi: 10.1017/ice.2020.343 [published Online First: 2020/07/20]

15. Bin SY, Heo JY, Song M-S, et al. Environmental Contamination and Viral Shedding in MERS Patients During MERS-CoV Outbreak in South Korea. Clinical Infectious Diseases 2015;62(6):755-60. doi: 10.1093/cid/civ1020

16. Lin W-HW, Kouyos RD, Adams RJ, et al. Prolonged persistence of measles virus RNA is characteristic of primary infection dynamics. Proceedings of the National Academy of Sciences 2012;109(37):14989-94. doi: 10.1073/pnas.1211138109

17. Owusu M, Annan A, Corman VM, et al. Human Coronaviruses Associated with Upper Respiratory Tract Infections in Three Rural Areas of Ghana. PLOS ONE 2014;9(7):e99782. doi: 10.1371/journal.pone.0099782

18. Lesho E, Reno L, Newhart D, et al. Temporal, Spatial, and Epidemiologic Relationships of SARS-CoV-2 Gene Cycle Thresholds: A Pragmatic Ambi-directional Observation. Clinical Infectious Diseases 2020 doi: 10.1093/cid/ciaa1248

19. Hodinka RL. Point: is the era of viral culture over in the clinical microbiology laboratory? J Clin Microbiol 2013;51(1):2-4. doi: 10.1128/JCM.02593-12 [published Online First: 2012/10/10]

20. Rhoads D, Peaper DR, She RC, Nolte FS, Wojewoda CM, Anderson NW, et al. College of American Pathologists (CAP) Microbiology Committee Perspective: Caution must be used in interpreting the Cycle Threshold (Ct) value. LID - ciaa1199 [pii] LID - 10.1093/cid/ciaa1199 [doi]

21. Matheeussen V, Corman VM, Donoso Mantke O, et al. International external quality assessment for SARS-CoV-2 molecular detection and survey on clinical laboratory preparedness during the COVID-19 pandemic, April/May 2020. Eurosurveillance 2020;25(27):2001223. doi: doi:https://doi.org/10.2807/1560-7917.ES.2020.25.27.2001223

22. Hodinka RL. Point: is the era of viral culture over in the clinical microbiology laboratory? J Clin Microbiol 2013;51(1):2-4. doi: 10.1128/JCM.02593-12 [published Online First: 2012/10/10]

23. Binnicker MA-O. Challenges and Controversies Related to Testing for COVID-19. LID - JCM.01695-20 [pii] LID - 10.1128/JCM.01695-20 [doi]. (1098-660X (Electronic))

24. Laboratory support for COVID-19 in the EU/EEA. Testing for SARS-CoV-2 virus European Centre for Disease Prevention and Control. 2020 https://www.ecdc.europa.eu/en/novel-coronavirus/laboratory-support

25. Binnicker MA-O. Challenges and Controversies Related to Testing for COVID-19. LID - JCM.01695-20 [pii] LID - 10.1128/JCM.01695-20 [doi]. (1098-660X (Electronic))

26. Byrne AW, McEvoy D, Collins AB, et al. Inferred duration of infectious period of SARS-CoV-2: rapid scoping review and analysis of available evidence for asymptomatic and symptomatic COVID-19 cases. BMJ Open 2020;10(8):e039856. doi: 10.1136/bmjopen-2020-039856

27. Jansen RR, Wieringa J, Koekkoek SM, et al. Frequent Detection of Respiratory Viruses without Symptoms: Toward Defining Clinically Relevant Cutoff Values. J Clin Microbiol 2011;49(7):2631-36. doi: 10.1128/jcm.02094-10

28. Salvatore PP, Dawson P, Wadhwa A, Rabold EM, Buono S, Dietrich EA, et al. Epidemiological Correlates of PCR Cycle Threshold Values in the Detection of SARS-CoV-2. Clinical Infectious Diseases. 2020.

Tables:

Table 1. Characteristics of included studies

Table 2. Quality of included studies

Table 3. Duration of viral detection

Table 4. Relationship of PCR Cycle threshold and Log 10 copies to Positive Viral Culture

Figures: Figure 1 PRISMA flow chart.

Table 1 Characteristic of Included Studies

Study [ref id]	Specimens (source)	Specimens (n) [SST]	Culture methods	Culture Positive	Additional notes
Ahn 2020 [W1]	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 specimens 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 specimens grew virus Ct values of specimens who grew virus were uniformly low below 30 except in one case.
Andersson 2020 [W2]	20 RT-PCR positive serum specimens, selected at random from a Covid-19	20 serum specimens from 12 hospitalised	Specimens VC01-20 were provided blinded for viral culture experiments. 50 µL aliquots of specimens VC1-VC20 were separately added to 2.4 x 105 Vero E6 cells in 24-well plates.	0 / 20 these serum specimens produced positive viral culture	Serum specimens.

	specimen bank, representing specimens from 12 individual patients (four individuals were represented at two timepoints), collected at 3 to 20 days following onset of symptoms.	Covid-19 patients	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 specimens 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.			
Arons 2020 [W3]	nasopharyngeal and oropharyngeal swabs	48 rRT-PCR–positive specimens [For asymptomatic median 4 days, Ct 23.1]	All rRT-PCR positive specimens 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]		

Basile 2020 [W4]	234 specimens, 228 (97%) from the upper respiratory tract (sputum, naso pharyngeal swabs, bronchial lavage from 195 individuals with Covid-19.	Specimens 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 specimens from ICU patients and 1 to 4 E, RdRp, N and Orf1ab for all other specimens. After stabilization at 4 degrees centigrade specimens 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 specimens 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
Borczuk 2020 [W5]	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
Brown 2020 [W6]	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	SARS-CoV-2 virus was isolated from only one (5%) of nineteen cultured specimens. It had a Ct value of 26.2.	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

			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.			tested positive (2.0%) with a median Ct of 35.70 (IQR:32.42 to 37.57).
Bullard 2020 [W7]	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 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
Gniazdowski 2020 [W8]	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
Huang 2020 [W9]	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.	Obtained 23 isolates from different specimen types (12 from OP, nine from NP, and two from SP).	Specimens with high copy numbers of the viral genome, indicative of higher viral load, were more likely to be culturable.
Jeong 2020 [W10]	Naso/oropharyngeal swabs, saliva, urine, and stool	5 patients	Specimens positive by qPCR were subjected to virus isolation in Vero cells. Urine and stool specimens 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 Specimens were collected between days 8 to 30 of the clinical course. Viable SARS-CoV-2 was isolated from 1 naso / oropharyngeal swab. Ferrets inoculated with patient urine or stool were	Viral loads in urine, saliva, and stool specimens 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.

				infected. SARS-CoV-2 was isolated from the nasal washes of the 2 urine-treated ferrets and one stool-treated ferret	
Kim 2020 [W11]	Unclear. Possibly 323 serum 247 urine and 129 stool specimens	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	
Korean CDC 2020 [W12]	Respiratory swab specimens for individuals testing positive after having previously tested positive, then negative.	108 specimens	Methods not reported	0 / 108 respiratory specimens	This report does not report the laboratory methods used.
Kujawski 2020 (for The COVID-19 Investigation Team) [W13]	Nasopharyngeal (NP), oropharyngeal (OP), stool, serum and urine specimens	12 patients had initial respiratory specimens collecte	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 × 106 median tissue culture infectious dose per ml; these data were likely more reflective of growth in tissue culture than patient viral load.	Viral culture was attempted on initial respiratory specimens from 9 patients and was successful in all 9, including 2 patients who not hospitalized	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.

Exhibit 5

	L'Huillier 2020 [W14]	Nasopharyngeal swabs in 638 patients aged less than 16 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 specimens grew viable viruses
	La Scola 2020 [W15]	Naso pharyngeal swabs or sputum specimens Only Naso pharyngeal specimens from the	183 (4384 specimens from 3466 patients) [not reported]	From 1,049 specimens, 611 SARS-CoV-2 isolates were cultured. 183 specimens testing positive by RT-PCR (9 sputum specimens 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 specimen were filtered and then inoculated in Vero E6 Cells. All specimens were inoculated	Of the 183 specimens inoculated in the studied period of time, 129 led to virus isolation. Of these 124 specimens had detectable cytopathic effect	There was a significant relationship between Ct value and culture positivity rate: specimens with Ct values of 13–17 all had positive culture. Culture positivity rate decreased progressively according to Ct values to 12% at 33 Ct.

Exhibit 5

	subsequent Jaafar et al letter.		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.	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 specimens. These could be seen after the first inoculation or up to 2 blind subcultures. At at Ct of > 34 2.6% of specimens yielded a positive culture.	No culture was obtained from specimens with Ct > 34. The 5 additional isolates obtained after blind subcultures had Ct between 27 and 34, thus consistent with low viable virus load.
Ladhani 2020 [W16]	Naso pharyngeal swabs	87 [Residents post, pre and 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)]	All SARS-CoV-2 positive specimens with a Ct value of <35 were incubated on Vero E6 mammalian cells and 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 specimens	87	Ct values < 35 Higher Ct values (lower virus load) specimens 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)
Lu 2020 [W17]	87 cases testing “re-positive” at RT-PCR	619 hospital discharges of which tested positive after	137 swabs and 59 serum specimens from 70 “re-positive” cases to assess the immunological and virologic characteristics of the SARS-CoV-2 “re-positive” cases. From 23 January, hospital	No cultures were positive	“Re-positive” cases are unlikely to be infectious as no intact RNA single helix was detected or viral

	137 swabs (51 nasopharyngeal, 18 throat and 68 anal)	discharge	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 specimens. Specimens (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 specimen. 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		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.
Perera 2020 [W18]	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	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 CO2 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 CO2 incubator at 37°C, the plates were incubated at 37°C in a CO2 incubator. A specimen (100 µL) of supernatant was specimend for a quantitative real-time RT-PCR at 0 and 72 hours post inoculation. At 72 hours, cells were scraped into	Virus was isolated from 16 specimens for 16 patients out of a total of 35 specimens	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.

	(n=1).		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 cytopathic effect.		
Qian 2020 [W19]	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.
Santarpia 2020 ([W20]	Windowsill and air, mean 7.3 specimens per room. The percentage of PCR positive specimens 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 specimens. The cells were cultured in Dulbeccos'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 cyotopathic effect	Isolates were from days 5 and 8 of occupancy of hospital/isolation rooms
Singanayagam 2020 [W21]	324 specimens: nose, throat, combined nose-and throat and nasopharyngeal swabs and aspirates	253 positive case [-10 to 60 days]	Vero E6 cells were inoculated with clinical specimens and incubated at 37 °C, 5% CO2. 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	133 (41%) specimens (from 111 cases)	RT-PCR cycle threshold values correlate strongly with cultivable virus i.e. likelihood of infectiousness. Median Ct of all 324 specimens was 31.15. Probability of culturing virus

			cells.		declines to 8% in specimens 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.
Wang 2020 [W22]	Bronchoalveolar fluid, sputum, feces, blood, and urine specimens from hospital in-patients with COVID-19	1,070 specimens collected from 205 patients with COVID-19	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.	Live SARS-CoV-2 was observed in the stool specimen from 2 patients who did not have diarrhea.	The details of how the specimens were cultured were not reported.
Wölfel 2020 [W23]	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 specimen that tested positive was taken on day 28 after the onset of symptoms.	Yes in respiratory specimens, and indicative in stool	

Xiao F SJ 2020 [W24]	Esophageal, gastric, duodenal, and rectal tissues were obtained from 1 COVID-19 patients by endoscopy.	1 plus an unknown additional number of fecal specimens from RNA-positive patients.	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 in feces, results not shown.	Total specimen numbers are not reported.
Xiao F 2020 [W25]	Serial feces specimens collected from 28 hospitalised COVID-19 patients: 3 specimens from 3 RNA-positive patients were tested for possible viral culture.	3, one patient admitted day 7 post onset	Inoculation of Vero 6 cells. Cycle threshold values for the fecal specimen were 23.34 for the open reading frame 1lab gene and 20.82 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 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.	Infectious virus was present in faeces from two cases)	Selection of specimens is not entirely clear.

Yao 2020 [W26]	Sputum (n=7), stool (n=3) and one nasopharyngeal specimen	11 patients admitted to hospital: 9 classified as serious or critical, 1 moderate, 1 mild symptoms [0 to 16 days]	The specimens 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 specimens, one nasopharyngeal and three had stool specimens The specimens 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 specimens 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

Young 2020 [W27]	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 specimens 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 specimens at admission did not grow virus
Zhang 2020 [W28]	Stool	Unknown [not reported]	Vero cells were used for viral isolation from stool specimens 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 specimend 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.
Zhou 2020 [W29]	218 surface specimens 31 air specimens	7 areas of large London hospital	RT-PCR with primers and probes for the envelope (E) gene. Duplicate PCR was carried out and specimens 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	No cultures were positive	The pre-defined cycle threshold cut off was too high

			specimens 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. Specimens with at least one log increase in copy numbers for the E gene (reduced Ct values relative to the original specimens) after 5-7 days propagation in cells compared with the starting value were considered positive by viral culture.	

Key: STT = symptom onset to test date.

Exhibit 5

Table 2. Quality of included studies

Study	Description of methods and sufficient detail to replicate	Sample sources clear	Analysis & reporting appropriate	Is bias dealt with	Applicability
Ahn 2020 [W1]	Yes	Yes	Yes	Partly	Unclear
Andersson 2020 [W2]	Yes	Yes	Yes	Partly	Yes
Arons 2020 [W3]	Yes	Yes	yes	Yes	Unclear
Basile 2020 [W4]	Yes	Yes	Yes	Unclear	unclear
Borczuk 2020 [W5]	Yes	Yes	Yes	Yes	Unclear
Brown 2020 [W6]	Yes	Yes	Yes	Unclear	Unclear
Bullard 2020 [W7]	Yes	Yes	yes	unclear	Unclear
Gniazdowski [W8]	Yes	Yes	Yes	Unclear	Unclear
Huang 2020 [W9]	yes	Yes	Yes	Unclear	Unclear
Jeong 2020 [W10]	Yes	Yes	Yes	No	Unclear
Kim 2020 [W11]	No	No	No	Unclear	Unclear
Korean CDC [W12]	No	Partly	Partly	No	Unclear
Kujawski 2020 [W13]	Yes	Yes	Yes	Unclear	Unclear

L'Huillier 2020 [W14]	Yes	Yes	Yes	Unclear	Unclear
La Scola 2020 [W15]	Yes	Yes	Yes	Unclear	Unclear
Ladhani 2020 [W16]	Yes	Yes	Yes	Yes	Likely
Lu 2020 [W17]	Yes	Yes	Yes	Partly	Yes
Perera 2020 [W18]	Yes	Yes	Yes	Unclear	Unclear
Qian Q 2020 [W19]	Yes	Yes	Yes	Unclear	Unclear
Santarpia 2020 [W20]	Yes	Yes	Yes	Unclear	Unclear
Singanayagam [W21]	Yes	No	Yes	Unclear	Unclear
Wang W 2020 [W22]	No	Yes	Yes	No	Unclear
Wölfel 2020 [W23]	Yes	Yes	Yes	Unclear	Unclear
Xiao FSJ 2020 [W24]	No	Yes	Yes	No	Unclear
Xiao F 2020 [W25]	Yes	Yes	Yes	No	Unclear
Yoa H 2020[W26]	Yes	Yes	Yes	Unclear	Unclear
Young 2020 [W27]	Yes	Yes	Yes	Yes	Yes
Zhang 2020 [W28]	Partly	Yes	yes	No	Unclear
Zhou 2020 [W29]	Yes	Yes	Yes	Unclear	Unclear

Table 3. Duration of **detectable SARS-CoV-2 RNA** in the included studies.

Study	**Duration of detectable SARS-CoV-2 RNA as assessed by PCR**	**Comments on the clinical course**
Bullard [w7]	Specimens 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.	SARS-CoV-2 Vero cell infectivity of respiratory specimens from SARS-CoV-2 positive individuals was only observed for RT-PCR Ct < 24 and symptom onset to test of < 8 days.
Gniazdowski [w8]	Patients that received repeated testing with longitudinal positive results were tested within a time frame that ranged from less than one day to more than 45 days	Four patients had infectious virus recovered from specimens collected in up to 22 days after the first positive result. Many patients who tested negative for SARS-COV-2 showed a subsequent positive result
Jeong [w10]	Five positive-PCR patients, day 8 to day 30 after symptom onset.	Viable SARS-CoV-2 was demonstrated in saliva, urine and stool specimens from COVID-19 patients up to days 11-15 of the clinical course.

Korean CDC [w12]	On average, it took 45 days (range: 8 to 82 days) from the initial symptom onset date to testing re positive after discharge. (Based on 226 cases symptomatic at the time of initial confirmation)	This may indicate duration of viral RNA detection over a long period of time and inconsistently. These data may not be comparable with information from studies specifically observing the duration of viral RNA detection as an outcome. Time to retesting positive via PCR is reported, among this specific group of individuals who retested positive by PCR.
Kujawski (for The COVID-19 Investigation Team) [w13]	Duration of SARS-CoV-2 detection by RT-PCR was 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.
Singanayagam [w21]	SARS-CoV-2 viral load identified that the level of SARS-CoV-2 RNA in the URT was greatest around symptom onset, steadily decreased during the first 10 days after illness onset and then plateaued up to day 21	Probability of culturing virus declined to 8% in specimens with Ct > 35 and to 6% 10 days after onset;
Xiao F SJ [w24]	The viral load was higher in feces than in respiratory specimens collected at multiple time points (17–28 days after symptom onset)	Isolation of virus from feces specimens collected at later time points was not successful, although results for virus RNA remained positive, indicating only RNA fragments, not infectious virus, in feces of this patient collected at later time points of disease onset.
Xiao F w25	The duration time of positive stool results ranged from 1 to 12 days.	17 (23%) patients continued to have positive results in stool after showing negative results in respiratory specimens.

	Young w27	SARS-CoV-2 RNA was detectable from nasopharyngeal swabs by PCR up to 48 days after symptom onset.	Mean duration of viral RNA detection by PCR was 16.7 days (95% CI 15.2-18.3). C Cessation of viral RNA detection 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 No virus was isolated when the PCR cycle threshold (Ct) value was >30 or >14 days after symptom onset.

Table 4: Relationship of PCR Cycle threshold and Log ^{10} copies to Positive Viral Culture

	Specimen				Cycle Threshold			Log ^{10} copies			
Study	RT-PCR SARS-CoV-2 positive specimens (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 specimens 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
Basile 2020 [w4]	234	56	178	E, RdRp, N, M, and ORF1ab for ICU patients;	25.01	27.75	Ct >32 with the N gene target [3]				
Brown 2020 [w5]	23	1	22	RdRp, E, and N	26.16	35.16 ± SEM 0.63	Ct >26.2				
Bullard 2020 [w7]	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.
Gniazdowski 2020 [w8]	132	47	85	S, Nsp 2	mean 12.8 ± 3.4 median 18.17	mean 27.1 ± 5.7 median 27.5	Ct ε 23 yielded 8.5% of virus isolates				
Huang 2020	60	23	34	Nsp 12	mean 23.9 ±	mean 29.26 ±	Ct >31.47	mean 7.37 ±	mean 5.98 ±		

[w9]							SEM 0.78	SEM 0.78		SEM 0.20	SEM 0.18		
							mean 22.39 ± SEM 0.75	mean 28.92 ± SEM 0.65		mean 8.21 ± SEM 0.18	mean 6.62 ± SEM 0.16		
					23	37	E		Ct >31.46				
					21	31	N	mean 31.49 ± SEM 0.59	Ct >35.2	mean 7.87 ± SEM 0.21	mean 6.70 ± SEM 0.17		
L'Huillier 2020 [w14]		23[4]								mean 7.9×10[8] IQR 4.7·10[6] - 1.0·10[9]	mean 5.4×10[7] IQR 4.2×10[3]– 1.8×10[6]		
			12	11			mean 27.29 ± SEM 0.77						
La Scola 2020 (Jaafar 2020) [w15]		611 (3790)							Ct ε 34 (2,6% positives)				
			129(1941)	482 (1849)	E								
Ladhani 2020 [w16]		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					
Perera 2020 [w18]		68	16	52	N					7.5[2]	3.8	<5.0	
Singanayagam 2020 [w21]		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 [w23]	45	9	36	E, Subgenomic mRNA.						
Young 2020 [w27]	100	21	79	N, S, and ORF1ab	28.2 (24.3 to 33.3	>30				

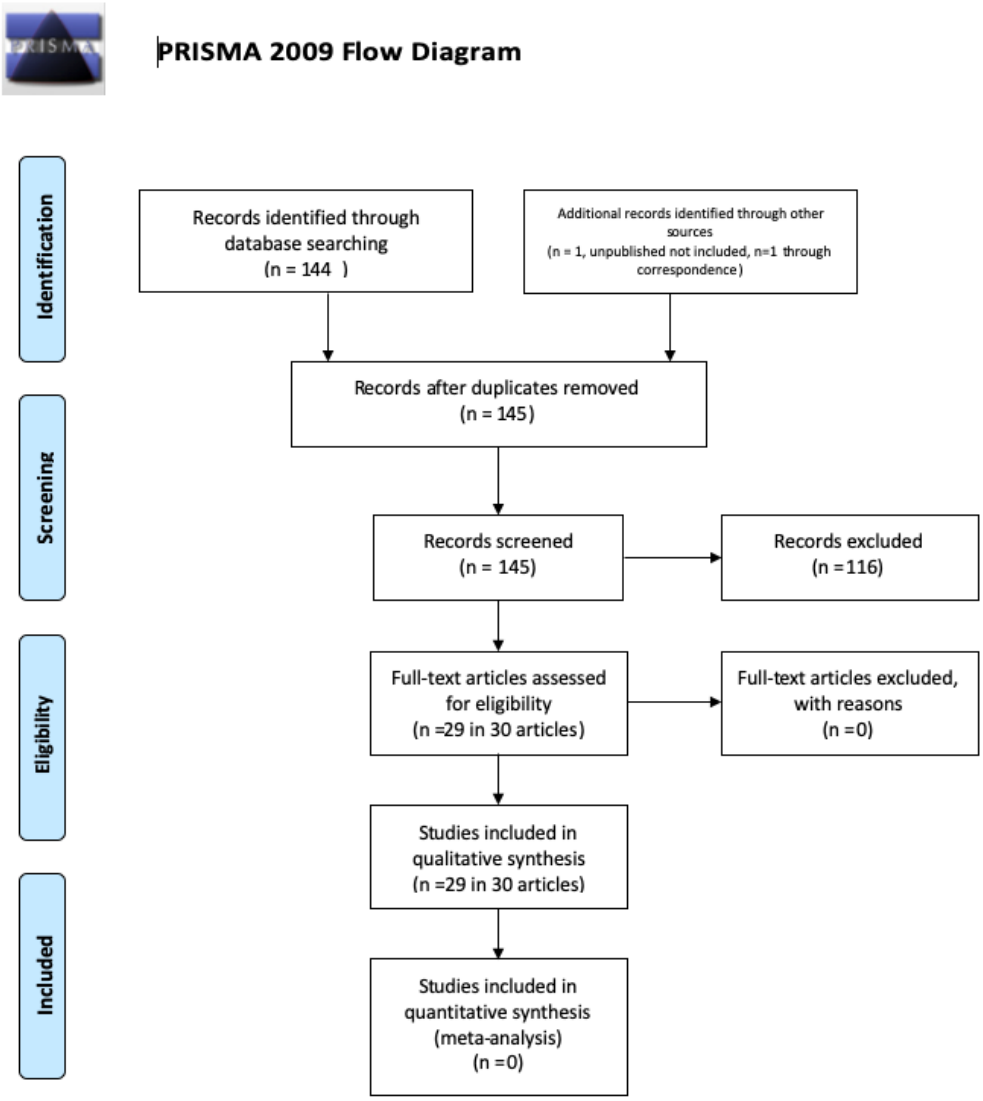
1 All above CT (n=5) 35 were symptomatic

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 specimen PCR ($Ct_{specimen}$) and the terminal culture (day four) supernatant PCR ($Ct_{culture}$) of ≥3 (equivalent to a 1 log increase in virus quantity) i.e. $Ct_{specimen} - Ct_{culture} \geq 3$ = culture positive. The authors hypothesized that a $Ct_{specimen}$ minus $Ct_{culture}$ <3 was due to residual inoculated clinical specimen and not replicating virus.

4.23 SARS-CoV-2–infected children

Figure 1



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Exhibit 5

EXHIBIT 6

European Journal of Human Genetics (2009) 17, 711 – 719

www.nature.com/ejhg

POLICY

Genetic testing in asymptomatic minors

Background considerations towards ESHG Recommendations

Pascal Borry*[,1], Gerry Evers-Kiebooms[2], Martina C Cornel[3], Angus Clarke[4] and Kris Dierickx[1] on behalf of the Public and Professional Policy Committee (PPPC) of the European Society of Human Genetics (ESHG)

[1]*Research Fund Flanders, Centre for Biomedical Ethics and Law, Faculty of Medicine, Katholieke Universiteit Leuven, Belgium;* [2]*Psychosocial Genetics Unit University Hospitals, Center for Human Genetics, Katholieke Universiteit Leuven, Belgium;* [3]*Community Genetics, Department of Clinical Genetics/EMGO Institute, VU University Medical Center, Amsterdam, The Netherlands;* [4]*Institute of Medical Genetics, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, Wales, UK*

Although various guidelines and position papers have discussed, in the past, the ethical aspects of genetic testing in asymptomatic minors, the European Society of Human Genetics had not earlier endorsed any set of guidelines exclusively focused on this issue. This paper has served as a background document in preparation of the development of the policy recommendations of the Public and Professional Committee of the European Society of Human Genetics. This background paper first discusses some general considerations with regard to the provision of genetic tests to minors. It discusses the concept of best interests, participation of minors in health-care decisions, parents' responsibilities to share genetic information, the role of clinical genetics and the health-care system in communication within the family. Second, it discusses, respectively, the presymptomatic and predictive genetic testing for adult-onset disorders, childhood-onset disorders and carrier testing.
European Journal of Human Genetics (2009) **17,** 711–719; doi:10.1038/ejhg.2009.25; published online 11 March 2009

Although various guidelines and position papers have discussed, in the past, the ethical aspects of genetic testing in asymptomatic minors,[1,2] the European Society of Human Genetics had not earlier endorsed any set of guidelines exclusively focused on this issue. This background paper was preceded by an in-depth research on the topic by Eurogentest.[3] Eurogentest (http://www.eurogentest.org) aims to develop the necessary infrastructure, tools, resources, guidelines and procedures that will structure, harmonize and improve the overall quality of all the EU genetic services at the molecular, cytogenetic, biochemical and clinical level.[4] Attention has also been paid to the provision of appropriate counselling related to genetic testing, the education of patients and professionals, as well as to the ethical, legal and social issues surrounding testing. The focus of the ethics unit of Eurogentest was oriented towards the study of the ethical issues related to genetic testing in minors. This work was the starting point for this background paper, which has been prepared and supported by different types of evidence. First, research has been performed on the existing recommendations regarding predictive genetic testing in minors[1] and carrier testing,[2] with the intention of identifying areas of agreement and disagreement. Second, the literature on medico–ethical and medico–legal aspects of predictive genetic testing in minors,[5] carrier testing,[6,7] the position of minors[8] and patient rights[9] was studied. Third, a systematic literature review was performed to gather information regarding the attitudes of the different stakeholders (minors, health-care professionals, parents and relatives of the affected individuals) towards genetic testing in asymptomatic minors.[10,11] Fourth, the attitudes of European clinical geneticists regarding genetic testing in asymptomatic minors were gathered.[12–14]

*Correspondence: Dr P Borry, Research Fund Flanders, Centre for Biomedical Ethics and Law, Faculty of Medicine, Katholieke Universiteit Leuven, Kapucijnenvoer 35/3, 3000 Leuven, Belgium.
E-mail: Pascal.Borry@med.kuleuven.be

In 2007, contacts were made with the Public and Professional Policy Committee of the European Society of Human Genetics with the aim of developing policy recommendations on the issue. On the basis of a decision of the PPPC meeting during the ESHG conference in Nice (June 2007), an *ad hoc* committee, consisting of Pascal Borry (Eurogentest), Kris Dierickx (Eurogentest), Angus Clarke, Gerry Evers-Kiebooms (PPPC) and Martina Cornel (PPPC), was created. This *ad hoc* committee met on 15 November 2007 to discuss a first draft of a background paper and recommendations that were prepared by Pascal Borry under the supervision of Kris Dierickx. A revised version was discussed during a PPPC meeting in Amsterdam (April 2008) and Barcelona (June 2008). In order not to repeat issues that have been discussed elsewhere, reference will often be made to the above-referenced publications.

General considerations

The concept of 'best interests'

It is a well-known rule of biomedical ethics that treatment may only be carried out after a patient has been informed of the purpose, nature, risks and consequences of the intervention, and has freely consented to it.[15] When talking about health-care decisions involving children, the concept of 'best interests' takes a more central position than the concept of informed consent. Parents are responsible for their children and have the authority to make decisions on their behalf. This is not different from the context in which parents make decisions regarding genetic testing of asymptomatic minors. This responsibility includes the moral and legal right to make decisions regarding the health of their children. In most cases, as parents are those who care the most about their children and know them best, they are expected to make the best decisions for their children, guided by the standard of 'the best interest of the child' ('acting to promote the good of the individual to the maximum extent').[16] This concept is frequently used in the ethical literature[17–21] and in international documents,[22,23] and it has been argued that it should be a standard in health-care decisions involving children, even if the more general character of the concept often creates difficulties in interpretation when applied to concrete cases.[24,25] (eg, the distinction between benefit in terms of physical gains or in terms of social, psychological and emotional gains is often a reason for conflict). Article 3.1 of the Convention on the Rights of the Child stipulated that 'in all actions concerning children, whether undertaken by public or private social welfare institutions, courts of law, administrative authorities or legislative bodies, the best interests of the child shall be a primary consideration.'[26]

Participation of minors in health-care decisions

Although the 'best interest standard' is important in cases in which children cannot participate in the decision-making process, this standard is becoming less adequate, as children acquire more intellectual and psychosocial capacities and can take part in decisions regarding their health. Most medical–ethical literature[27] on the subject emphasizes that as soon as children are able to communicate and participate in decisions that affect them, they should be encouraged to participate in all aspects of the decision-making process. They should be properly informed about the medical issues that affect them, should be able to express their views, ask questions or communicate their worries.[28] It is clear that during their development, children acquire cognitive, social and emotional skills. However, intellectual capacity and emotional understanding do not necessarily develop in the same way. There is a huge individual and societal variation regarding the moment when particular levels of competence are achieved. As a consequence from an ethical perspective, a rule about competence that is solely based on age cannot be satisfactory. When assessing competence, it is important not to assess general competence, but to assess a patient's level of understanding in relation to a specific choice that has to be made. 'The nature and complexity of the decision or task, the person's ability to understand, at the time the decision is made, the nature of the decision required and its implications, are all relevant. Thus the graver the impact of the decision, the commensurately greater the competence needed to make it.'[29] Moreover, in the context of genetic testing, the opinion of minors should be taken into consideration, as an increasingly determining factor in proportion to his or her age and degree of maturity. All children do not develop in the same way. Children of the same age may have a different level of development or maturity. Therefore, the competence of children should be assessed on a case-by-case basis in order to take this reality into consideration. Decision-making should include, to the greatest extent possible, the assent or consent of the minor who is involved. For 'assent', we understand that health-care professionals should help 'the patient achieve a developmentally appropriate awareness of the nature of his or her condition'; 'tell the patient what he or she can expect with tests and treatment(s)'; make a 'clinical assessment of the patients understanding of the situation and the factors influencing how he or she is responding (including whether there is inappropriate pressure to accept testing or therapy)'; and solicit 'an expression of the patient's willingness to accept the proposed care.'[30] As children develop, they should gradually become the primary guardians of personal health and the primary partners in medical decision-making. Thus, they should be able to provide consent themselves. This should include from the health-care professionals, a 'provision of information: patients should have explanations, in understandable

language, of the nature of the ailment or condition; the nature of proposed diagnostic steps and/or treatment(s) and the probability of their success; the existence and nature of the risks involved; and the existence, potential benefits, and risks of recommended alternative treatments (including the choice of no treatment)'; the 'assessment of the patient's understanding of the above information'; the 'assessment, if only tacit, of the capacity of the patient or surrogate to make the necessary decision(s)'; and the 'assurance, insofar as is possible, that the patient has the freedom to choose among the medical alternatives without coercion or manipulation.'[30]

The European legislation with regard to the legal position of minors related to interventions in the health field is different in the various European nations.[8] However, at the European level, the European Convention on Human Rights and Biomedicine of the Council of Europe[31] contains a specific provision – Article 6 – related to the protection of persons who are unable to consent. Paragraph 2 of this article, furthermore, stipulates: 'Where, according to law, a minor does not have the capacity to consent to an intervention, the intervention may only be carried out with the authorisation of his or her representative or an authority or a person or body provided for by law. The opinion of the minor shall be taken into consideration as an increasingly determining factor in proportion to his or her age and degree of maturity.' In view of the preservation of the autonomy of persons with regard to interventions affecting their health, the Explanatory Report, furthermore, states that 'in certain situations which take account of the nature and seriousness of the intervention as well as the minor's age and ability to understand, the minor's opinion should increasingly carry more weight in the final decision'. According to the Explanatory Report in some cases, it could therefore even be concluded that, the consent of a minor should be necessary, or at least sufficient for some interventions. In this respect, a reference is made to Article 12 of the United Nations Convention on the Rights of the Child, which stipulates that 'States Parties shall assure the child, who is capable of forming his or her own views the right to express those views freely in all matters affecting the child, the views of the child being given due weight in accordance with the age and maturity of the child'. Therefore, in the context of genetic tests that can easily be postponed until the minor can participate in the decision-making process, this should be carried out as much as possible in order to enable the minor to realize his decision-making capacities.

Parents' responsibility to share genetic information: prerequisites and difficulties

The communication of genetic information is often a difficult issue. It has been reported that the desire not to cause anxiety or alarm, geographical distances, family conflicts, relational ruptures, adoption, generational gaps or complex family relations have been reported as issues that might make it more difficult to convey information to relatives or children.[32] Moreover, the decision not to provide relevant genetic information to relatives might be based on, the one hand, a the deliberate choice of a person not to disclose results[33] or, on the other hand, the inability to communicate genetic risk information. In addition, several empirical studies have shown that adults may encounter difficulties in understanding and assessing genetic risk,[34,35] as well as in understanding the recessive patterns of inheritance.[36,37] Several studies[38–40] have reported that parents may experience difficulties in the retention of test results, and other studies[39,41–44] have observed a low recall of residual risk after a negative test in the long term in spite of post-test counselling. Although a majority of the studies understand the concept and relevance of carrier status, almost all studies report that some individuals experience difficulties in understanding carrier status. Furthermore, different studies[45] observed that some parents continue to have difficulties regarding information about the carrier status of their children.[36,46] Mischler *et al*[47] reported that a few families did not understand the meaning of being a carrier, and seemed to believe that their carrier children might develop cystic fibrosis. Another study found that 1 year after the carrier detection, through neonatal screening, 15% of the families were not sure whether carrier status implied health difficulties.[48] It is more than likely that these parents will not be able to transmit accurate information to their offspring regarding their genetic risk. Children in these families might make the same erroneous assumptions and believe that they are or will become sick. Parents might initiate a socialization of the child into a sick role.[36] Some evidence suggests that although parents are the best placed to inform their children of their genetic risk, some of them decline to tell their children or family members,[49–53] defer disclosure of genetic risk,[49] encounter difficulties in telling their children or family members[54,55] or share the information in a way that results in many family members not being fully aware of their risk of being a carrier.[37] Therefore, parents have an important obligation to make a reasonable effort to understand the nature and implications of genetic information, to provide appropriate information to their children and to share their concerns and needs.[56] They may assist their children in contacting genetic services later for further information and genetic counselling. In general, clinical genetic services and the health-care system may have an important role in this communication process. Although genetic services are not currently set up to recontact individuals regarding genetic test results at a later age,[57] they have a responsibility towards supporting parents in the communication of genetic risk information to their children.

The role of clinical genetics and the health-care system in the communication within the family

The role of genetic counselling in genetic services is mainly to support, insofar as possible, decisions regarding genetic testing. It has been emphasized that the goal of genetic counselling is to provide accurate, full and unbiased information to individuals and families. Non-directive counselling does not mean just presenting information and letting people make their own decisions without any help or support. The counselling sessions should be oriented to empower individuals and families to make their own decisions. It should guide and help people to work towards their own decisions, *a priori* with regard to reproductive decisions, and if adequate preventative interventions or therapies are not immediately available. It is linked to the original intent of genetic counselling to respect the profoundly personal nature of decision-making.[58] It is clear that the counsellor is not completely unbiased, but he should be aware of his personal values and should not attempt to impose them on individuals or families.[59,60] However, genetic counsellors and clinical geneticists cannot be obliged to perform actions that are opposed to good clinical practice. On the one hand, they can refuse actions that are not in the best interests of a child (eg, childhood genetic testing for adult-onset disorders, see below). On the other hand, if parents refuse genetic testing and eventually therapeutic actions or preventive measures aimed at therapeutic interventions that might be life saving for a child, health professionals have the responsibility to use all means for promoting the benefit of the child. Preliminary results of a recent research[61] showed that parents and children often felt that minors were not engaged sufficiently in the decision-making process by suitably trained professionals. Parents and children often felt their needs as a family were not considered, not only in relation to genetic testing but also in dealing and coping with the outcomes. Parents were often present during consultation about a minor, and parents and children were aware that parental anxieties and concerns were more focused on by the health-care professional than the child's. This suggests that there is probably a need for developing skills and expertise of specialist health-care professionals in working specifically with young people about making these decisions.

Presymptomatic and predictive genetic testing

Presymptomatic and predictive genetic testing makes it possible to provide information regarding future health risks in asymptomatic persons. As presymptomatic or predictive genetic testing may have far-reaching consequences for test applicants, their family members and society,[62] concerns have always been raised about the pre-test and post-test counselling process, the provision of adequate information, the private and confidential character of the test result, the psychosocial impact of a test[63] and the responsibility towards blood relatives.[64–66] An even more cautious approach has been envisaged when considering such testing in children and adolescents. This originates from the fear that testing in childhood or adolescence could create devastating social, emotional, psychosocial and educational consequences in the child or in the adolescent.[67–69]

Presymptomatic and predictive genetic testing for adult-onset disorders

In the past, presymptomatic and predictive genetic testing in minors has been the subject of up to 27 guidelines and position papers.[1] Despite the extensive number of guidelines published and the variety of guideline developers, a great unanimity has been observed with regard to the issue of predictive genetic testing for adult-onset disorders. They all clearly suggested that, when talking specifically about predictive and presymptomatic tests for late-onset disorders, such testing is only recommended when 'established, effective, and important medical treatment'[2,70] can be offered or when testing 'provides scope for treatment which to any essential degree prevents, defers or alleviates the onset of disease or the consequences of the development of the disease.'[71] In a similar way, they emphasize that presymptomatic and predictive genetic testing should be delayed until adulthood, except for disorders for which preventive actions (preventive surgery or early detection strategies aimed at therapeutic interventions) could be initiated before that time. A similar attitude was reported in a recent survey of European clinical geneticists.[14]

Considering that minors do not have any prospect of effective treatment to benefit from in this case, it has been questioned whether non-medical benefits might provide convincing arguments in favour of predictive genetic testing in minors with adult-onset disorders.[72] First, it has been argued[73] that persons who undergo genetic testing and receive 'good news' may learn definitively, or with a high probability, that they will not develop the disease and that those individuals who have inherited the mutation are able to anticipate the future and plan their lives. However, various concerns have been raised. It has been reported that receiving good news may also lead to psychological and social distress and troubling family relations.[74] A study also showed that receiving such DNA results did not always reassure the parents about the health situation of their children.[75] Even receiving favourable news and reassurance might affect people's self-image and the family dynamics.[76] Second, it has been argued[77] that by testing early in life, this 'information becomes part of personal identity. When a child learns personal genetic information early in life, it can be absorbed and accommodated into their identity. When the information is disclosed later in life, it can conflict with their self-image and be very hard to internalize and accept.'[78] Studies have indeed shown that

'it is easier for a young person to deal with the news of risk than it would be at an older age'[79] However, it has been emphasized that there is a difference between being told to be at risk for a disease that exists within a family on the one hand, and performing a genetic test for an adult-onset disorder on the other.[72] Third, various studies[80–82] have shown that parents might believe that detection in childhood might help prepare their children and themselves psychologically for the future. Various parents who have been tested for a specific disease and who know that their children are at risk, might argue that the uncertainty of not knowing is more burdensome than receiving a negative or positive test result. Therefore, some parents consider that they should be able to consent to genetic testing in their children for diseases that only have their onset in adulthood. However, the risk to relatives, the absence of an effective cure, the potential loss of health insurance, the financial costs of testing and the inability to 'undo' the knowledge have been identified as reasons why adults decide not to undergo predictive genetic tests for adult-onset disorders.[76] Considering that minors, far more than their parents, will be living with the repercussions of the test results, there are good reasons that they should be able to decide about the participation in such a genetic test.[83] The presence of severe anxieties and uncertainties in parents about a potential genetic mutation might be an indication for further psychological support in order to address these emotions rather than a clear indication for testing.

Presymptomatic and predictive genetic testing for preventable or treatable childhood-onset disorders

In an earlier study,[1] it has been reported that professional guidelines and position papers recommended that the presence of medical benefit should be the primary justification of genetic testing in children and adolescents. Therefore, from an ethical point of view, in the case of preventable or treatable childhood-onset disorders, the most crucial question is not whether the test should be done, but when it should be done. In this context, various other guidelines have referred to the fact that testing should be recommended when the results are of 'immediate' relevance[84,85] for their health or may offer 'timely'[86–89] medical benefit.

Presymptomatic and predictive genetic testing for unpreventable or untreatable childhood-onset disorders

Various positions have been advanced regarding the issue of predictive genetic testing for unpreventable or untreatable childhood-onset disorders.[1] On the basis of a medical benefit argumentation, some[66,70] have argued that the absence of measures to prevent the disease or its complications or to treat the disease is a reason not to perform this test. Although still acknowledging the importance of medical benefit as a justification for predictive genetic testing, it has been recognized that there are circumstances in which not testing might create more harm than testing.[89–93] Therefore, it has been advanced that 'parents should have discretion to decide about genetic testing for childhood diseases that are unpreventable and untreatable.'[89] 'Since, with unpreventable and untreatable genetic diseases, there are both benefits and risks to genetic testing, and neither the benefits or risks clearly outweigh the other, parents generally should be allowed to decide about testing for their children.'[89] For these guidelines, testing is considered appropriate on condition that 'testing would be in the child's best interests'.[89] As best interests cannot be understood in this context as a medical benefit, it should be understood here as a psychological or social benefit.

Carrier testing

Borry *et al*[2] reported that professional recommendations were in agreement that carrier testing of minors in families affected by autosomal recessive or X-linked disorders or by balanced chromosomal rearrangements, ideally should be deferred. As carrier testing has the potential of affecting the future reproductive prospects of a child, the studied guidelines emphasized that the decision to test should be made by the child when he reaches the age of maturity. This view is based on the basic ethical principle of informed consent, by which an individual can freely and voluntarily give, without external pressure, his consent to be tested after being informed of the benefits, risks, procedures and other pertinent information relating to the carrier test. As carrier testing performed during childhood only affects the future of that child, not that of his parents or guardians, the guidelines stated that it is wiser to defer testing until the child himself is able to give proper informed consent, than to acquiesce to the wishes of his parents or guardians to go forward with testing. The child's personal consent takes precedence over the wishes of third parties, including parents, either to carry out or to refuse genetic testing. Knowledge of carrier status critically impacts future decisions regarding reproduction (eg, carrier testing of partner, prenatal diagnosis, artificial insemination, pre-implantation genetic diagnosis, adoption, not to have children). Some guidelines suggested that carrier testing performed during childhood also denies the child of confidentiality, a right he would expect if tested as an adult. The majority of European clinical geneticists also supports this position.[12] This stance holds for autosomal recessive disorders, in which the risk for offspring would generally be $<1\%$, and also for the X-linked disorders and balanced chromosomal rearrangements carrier, in which risks for the offspring can be much higher (25% for X-linked disorders, for instance).

However, various studies[11] have shown that an important group of parents are in favour of carrier testing in their children before the age of legal majority and some parents are even in favour of testing in early childhood.[94] Arguments in favour of carrier testing on parental request concentrate on the issues that learning one's carrier status while young may help their children adapt to the carrier status, reduce the uncertainty about the carrier status, avoid resentment from children later in life and may be in accordance with the conviction that parents have the right and the ability to make decisions regarding their children's health.[95] An important parental concern is that their children become aware of their genetic risk before becoming sexually active and that their child is able to chose a partner, informed of his carrier status. Some parents consider that a good parent should know as much as possible about their children, and that it is to the emotional benefit of the child to grow up knowing his or her carrier status before becoming sexually active.[96]

A recent development that challenges the governing professional recommendation is that DNA testing becomes more and more integrated in newborn screening programmes. This recent development offers ethical challenges that were not present in the context of the traditionally used biochemical testing methods for detecting inherited disorders. The use of DNA mutation analysis might, in addition to identifying affected infants, also inadvertently identify mutation carriers who will be unaffected, but at risk of having children with the disorder for which they underwent the screening. In the past, various newborn screening programmes often did not report the identification of the detected carriers.[97] Moreover, professional guidelines from the American Medical Association and the German Society of Human Genetics recommended that this information should not be disclosed to parents or to third parties. Rather, they recommended that this information should be discussed with the child when he or she reaches reproductive age. The guidelines from the American Medical Association provided instructions for maintaining the confidentiality of this genetic information, stating that this privileged information should be kept in a separate portion of a patient's medical record to prevent accidental disclosure. However, no clear instructions are offered as to at what age and by whom this information should be given. However, new screening programmes[98,99] seem to orient practice more and more into the direction that parents should be told about this possibility before the test, and that results should be given to the parents together with adequate counselling by a health-care professional. This is in line with recommendations offered by the British Medical Association and by the American Academy of Pediatrics, who had earlier already defended the concept that carrier status results obtained incidentally (eg, after screening or prenatal diagnosis) should be conveyed to the parents. Therefore, it was reported that rigid and diametrically opposed recommendations regarding the disclosure of carrier status in two different settings (ie, clinical setting and screening context) is conflicting, and should be harmonized.[7] Incidental discovery of carrier status in a parent may occur when investigating whether a possible pathogenic finding in a child is a *de novo* occurrence, when using array-based genome investigations for CNVs. An accidental discovery that a woman has a deletion affecting, eg, DMD or BRCA1 might occur. At present, it may not possible to counsel all parents about this possibility beforehand. With the increasing use of high-throughput technology and the decreasing prices of genomic information, the problem of incidental findings needs to be discussed urgently. For practical purposes, and before consensus is reached on the reporting of incidental findings, it may be advisable to ignore data that are not relevant for the pathology in the patient.

Conclusions

Recent developments in genetics have created expanding possibilities for genetic testing. Similar to many other fields of human activity, larger choice means a larger responsibility. Genetic testing offers the possibility to know the individual risk for a genetic disorder. When adult relatives of an affected individual are at risk for a disorder, they can decide for themselves whether to undergo a genetic test. In this background paper, we described that those health-care decisions that affect minors should be considered with special caution. A great unanimity has been reported in situations, in which predictive genetic testing might lead to an established and effective medical treatment or provides the possibility of preventive actions that can be initiated before the onset of the disorder. As soon as minors, in proportion to their age and degree of maturity, are able to participate in the decision-making, their opinion should be taken increasingly into consideration. In respect of national legislation, minors should be able to decide personally regarding a genetic test when they are well informed, have an adequate understanding of the test and its potential consequences, have the capacity to make this decision, are not exposed to external pressure and have had appropriate counselling. This background paper concisely reviews the major discussions with regard to predictive genetic testing for adult-onset disorders, preventable or treatable childhood-onset disorders, unpreventable or untreatable childhood-onset disorders and carrier testing.

Acknowledgements

Part of this contribution was supported by the Eurogentest Network of Excellence of the EU, FP6-512148.

Members of the PPPC in 2007–2008 were:
Martina Cornel, chair, MD, Epidemiologist, Professor of Community Genetics and Public Health Genomics, Department of Clinical Genetics/EMGO Institute, VU University Medical Center, Amsterdam, The Netherlands; Gerry Evers-Kiebooms PhD, Psychologist, Psychosocial Genetics Unit University Hospitals, Katholieke Universiteit Leuven, Belgium; Ségolène Aymé MD, Medical Geneticist and Epidemiologist, Project Leader of ORPHANET, INSERM, Paris, France; Suzanne Braga MD, Medical Geneticist, Psychotherapist, Family therapist and counselor, SGMG/SSGM, Bern, Switzerland; Franca Dagna Bricarelli Professor, Human Genetics Laboratory, Galliera Hospital, Genoa, Italy; Shirley Hodgson Professor of Cancer Genetics, Honorary Consultant in Clinical Genetics, Department of Clinical Genetics, St George's Hospital, University of London, London, UK; Gyorgy Kosztolany Professor, Director of the Department of Medical Genetics, University of Pécs, Hungary; Jan Lubinski, MD, PhD, Professor, Department of Genetics and Pathology, International Hereditary Cancer Center, Pomeranian Medical University, Szczecin, Poland; Meral Özgüc, Professor, Department of Medical Biology, Faculty of Medicine, Hacettepe University Ankara, Turkey; Christine Patch PhD, RN, Consultant Genetic Counsellor and Manager, Guys and St Thomas' NHS Foundation Trust, London, UK; Jorge Sequeiros MD, Professor in Medical Genetics, Head of the Department of Human Genetics, University of Porto, Porto, Portugal; Lisbeth Tranebjaerg MD, PhD, Professor in Genetic Audiology, Department of Audiology, H:S Bispebjerg Hospital and Wilhelm Johannsen Centre of Functional Genomics, University of Copenhagen, Denmark; Veronica van Heyningen Dphil, FRS, FRSE, FMedSci, Honorary Professor of Edinburgh University, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK

References

1 Borry P, Stultiens L, Nys H, Cassiman JJ, Dierickx K: Presymptomatic and predictive genetic testing in minors: a systematic review of guidelines and position papers. *Clin Genet* 2006; **70**: 374–381.
2 Borry P, Schotsmans P, Fryns JP, Dierickx K: Carrier testing in minors: a systematic review of guidelines and positions statements. *Eur J Hum Genet* 2006; **14**: 133–138.
3 Hayhurst R, Cassiman JJ: EuroGentest standing up to scrutiny - first year demonstrates good progress harmonizing community approaches. *J Appl Genet* 2006; **47**: 5–7.
4 Cassiman JJ: EuroGentest - a European Network of Excellence aimed at harmonizing genetic testing services. *Eur J Hum Genet* 2005; **13**: 1103–1105.
5 Borry P, Fryns JP, Dierickx K: Predictive genetic testing in children. A clinical-ethical analysis; in de Bouvet A, Boitte P, Aiguier G (eds): *Questions éthique en médicine predictive*. Montrouge, Editions John Libbey Eurotext, 2006, pp 89–98.
6 Borry P, Dierickx K: Carrier testing for congenital adenal hyperplasia: ethical issues. *Cares* 2007; **6**: 16–18.
7 Borry P, Nys H, Dierickx K: Ethics watch – carrier testing in minors: conflicting views. *Nat Rev Genet* 2007; **8**: 828.
8 Stultiens L, Goffin T, Borry P, Dierickx K, Nys H: Minors and informed consent: a comparative approach. *Eur J Health Law* 2007; **14**: 21–46.
9 Nys H, Stultiens L, Borry P, Goffin T, Dierickx K: Patient rights in EU member states after the ratification of the convention on human rights and biomedicine. *Health Policy* 2007; **83**: 223–235.
10 Borry P, Stultiens L, Nys H, Dierickx K: Attitudes towards predictive genetic testing in minors for familial breast cancer: a systematic review. *Crit Rev Oncol Hematol* 2007; **64**: 173–181.
11 Borry P, Schotsmans P, Fryns JP, Dierickx K: Attitudes of minors, parents and health professionals towards carrier testing in minors: a systematic review. *Genet Couns* 2005; **16**: 341–352.
12 Borry P, Goffin T, Nys H, Dierickx K: Attitudes regarding carrier testing in incompetent children. A survey of European clinical geneticists. *Eur J Hum Genet* 2007; **15**: 1211–1217.
13 Borry P: Minors and informed consent in carrier testing. A survey of European clinical geneticists. *J Med Ethics* 2008; **34**: 370–374.
14 Borry P, Goffin T, Nys H, Dierickx K: Attitudes regarding predictive genetic testing in minors. A survey of European clinical geneticists. *Am J Med Genet C Semin Med Genet* 2008; **148C**: 78–83.
15 Andorno R: The Oviedo Convention: a European framework at the intersection of human rights and health law. *J Int Biotechnol Law* 2005; **2**: 133–143.
16 Buchanan AE, Brock DW: *Deciding for Others*. Cambridge: University Press, 1989.
17 Dawson A: The determination of the best interests in relation to childhood immunisation. *Bioethics* 2005; **19**: 72–89.
18 Downie RS, Randall F: Parenting and the best interests of minors. *J Med Philos* 1997; **22**: 219–231.
19 Ross LF: Health care decision making by children. Is it in their best interest? *Hastings Cent Rep* 1997; **27**: 41–45.
20 Spence K: The best interest principle as a standard for decision making in the care of neonates. *J Adv Nurs* 2000; **31**: 1286–1292.
21 Trau JM, McCartney JJ: In the best interest of the patient. Applying this standard to healthcare decision making must be done in a community context. *Health Prog* 1993; **74**: 50–56.
22 Charter of Fundamental Rights of the European Union, http://www.europarl.europa.eu/charter/pdf/text_en.pdf, 2000.
23 Universal Declaration on the Human Genome and Human Rights; Unesco, http://portal.unesco.org/shs/en/ev.php-URL_ID=1881&URL_DO=DO_TOPIC&URL_SECTION=201.html, 1997.
24 Kopelman LM: The best-interests standard as threshold, ideal, and standard of reasonableness. *J Med Philos* 1997; **22**: 271–289.
25 Kopelman LM: Children and bioethics: uses and abuses of the best-interests standard. *J Med Philos* 1997; **22**: 213–217.
26 United Nations. Convention on the Rights of the Child, http://www.unhchr.ch/html/menu3/b/k2crc.htm, 1989.
27 Alderson P, Montgomery J: *Health Care Choices: Making Decisions with Children*. London: Institute for Public Policy Research, 1996.
28 Royal College of Psychiatrists Report: *Appendix 2 – Children's Level of Understanding of Medical Decisions; Guidance for Researchers and for Ethics Committees on Psychiatric Research Involving Human Participants*. London: The Royal College of Psychiatrists, 2001.
29 British Medical Association: *Consent, Rights and Choices in Health Care for Children and Young People*. London: BMJ Books, 2001.
30 Informed consent, parental permission, and assent in pediatric practice: Committee on Bioethics, American Academy of Pediatrics. *Pediatrics* 1995; **95**: 314–317.
31 Council of Europe. Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the application of Biology and Medicine: Convention on Human Rights and Biomedicine. Oviedo, Council of Europe, 1997.
32 Clarke A, Richards M, Kerzin-Storrar L *et al*: Genetic professionals; reports of nondisclosure of genetic risk information within families. *Eur J Hum Genet* 2005; **13**: 556–562.
33 Loud JT, Weissman NE, Peters JA *et al*: Deliberate deceit of family members: a challenge to providers of clinical genetics services. *J Clin Oncol* 2006; **24**: 1643–1646.
34 Fanos JH: Developmental tasks of childhood and adolescence: implications for genetic testing. *Am J Med Genet* 1997; **71** (1): 22–28.
35 Denayer L, Welkenhuysen M, Evers-Kiebooms G, Cassiman JJ, Van den BH: Risk perception after CF carrier testing and impact of the test result on reproductive decision making. *Am J Med Genet* 1997; **69**: 422–428.
36 Fanos JH, Johnson JP: Perception of carrier status by cystic fibrosis siblings. *Am J Hum Genet* 1995; **57**: 431–438.

37 Denayer L, De Boeck K, Evers-Kiebooms G, Van den BH: The transfer of information about genetic transmission to brothers and sisters of parents with a CF-child. *Birth Defects Orig Artic Ser* 1992; **28**: 149–158.
38 Axworthy D, Brock DJ, Bobrow M, Marteau TM: Psychological impact of population-based carrier testing for cystic fibrosis: 3-year follow-up. UK Cystic Fibrosis Follow-Up Study Group. *Lancet* 1996; **347**: 1443–1446.
39 Bekker H, Modell M, Denniss G *et al*: Uptake of cystic fibrosis testing in primary care: supply push or demand pull? *BMJ* 1993; **306**: 1584–1586.
40 Bekker H, Denniss G, Modell M, Bobrow M, Marteau T: The impact of population based screening for carriers of cystic fibrosis. *J Med Genet* 1994; **31**: 364–368.
41 Watson EK, Mayall E, Chapple J *et al*: Screening for carriers of cystic fibrosis through primary health care services. *BMJ* 1991; **303**: 504–507.
42 Mennie ME, Gilfillan A, Compton M *et al*: Prenatal screening for cystic fibrosis. *Lancet* 1992; **340**: 214–216.
43 Callanan NP, Cheuvront BJ, Sorenson JR: CF carrier testing in a high risk population: anxiety, risk perceptions, and reproductive plans of carrier by 'non-carrier' couples. *Genet Med* 1999; **1**: 323–327.
44 Marteau TM, Michie S, Miedzybrodzka ZH, Allanson A: Incorrect recall of residual risk three years after carrier screening for cystic fibrosis: a comparison of two-step and couple screening. *Am J Obstet Gynecol* 1999; **181**: 165–169.
45 Fanos JH, Mackintosh MA: Never again joy without sorrow: the effect on parents of a child with ataxia-telangiectasia. *Am J Med Genet* 1999; **87**: 413–419.
46 Fanos JH, Nickerson BG: Long-term effects of sibling death during adolescence. *J Adolesc Res* 1991; **6**: 70–82.
47 Mischler EH, Wilfond BS, Fost N *et al*: Cystic fibrosis newborn screening: impact on reproductive behavior and implications for genetic counseling. *Pediatrics* 1998; **102**: 44–52.
48 Ciske DJ, Haavisto A, Laxova A, Rock LZ, Farrell PM: Genetic counseling and neonatal screening for cystic fibrosis: an assessment of the communication process. *Pediatrics* 2001; **107**: 699–705.
49 Forrest K, Simpson SA, Wilson BJ *et al*: To tell or not to tell: barriers and facilitators in family communication about genetic risk. *Clin Genet* 2003; **64**: 317–326.
50 Varekamp I, Suurmeijer TP, Brocker-Vriends AH *et al*: Carrier testing and prenatal diagnosis for hemophilia: experiences and attitudes of 549 potential and obligate carriers. *Am J Med Genet* 1990; **37**: 147–154.
51 Varekamp I, Suurmeijer T, Brocker-Vriends A, Rosendaal FR: Hemophilia and the use of genetic counseling and carrier testing within family networks. *Birth Defects Orig Artic Ser* 1992; **28**: 139–148.
52 Varekamp I, Suurmeijer TP, Rosendaal FR, Brocker-Vriends AH: The use of preventive health care services: carrier testing for the genetic disorder haemophilia. *Soc Sci Med* 1993; **37**: 639–648.
53 Sorenson JR, Jennings-Grant T, Newman J: Communication about carrier testing within hemophilia A families. *Am J Med Genet* 2003; **119C**: 3–10.
54 Fanos JH, Johnson JP: Barriers to carrier testing for adult cystic fibrosis sibs: the importance of not knowing. *Am J Med Genet* 1995; **59**: 85–91.
55 Duster T: The social consequences of genetic disclosure; in: Carson RA, Rothstein MA (eds): *Behavioral Genetics. The Clash of Culture and Biology*. London: John Hopkins University Press, 1999, pp 172–188.
56 Sharpe NF: The duty to recontact: benefit and harm. *Am J Hum Genet* 1999; **65**: 1201–1204.
57 Borry P, Dierickx K: What are the limits of the duty of care? The case of clinical genetics. *Per Med* 2008; **5**: 101–104.
58 Biesecker BB: Future directions in genetic counseling: practical and ethical considerations. *Kennedy Inst Ethics J* 1998; **8**: 145–160.
59 Elwyn G, Gray J, Clarke A: Shared decision making and non-directiveness in genetic counselling. *J Med Genet* 2000; **37**: 135–138.
60 Oduncu FS: The role of non-directiveness in genetic counseling. *Med Health Care Philos* 2002; **5**: 53–63.
61 Metcalfe A: Consultation on testing in minors recommendations, personal communication, 2008.
62 Harper P, Clarke A: *Genetics, Society and Clinical Practice*. Oxford: Bios, 1997.
63 Codori AM, Zawacki KL, Petersen GM *et al*: Genetic testing for hereditary colorectal cancer in children: long-term psychological effects. *Am J Med Genet A* 2003; **116A**: 117–128.
64 Allan D: Ethical boundaries in genetic testing. *CMAJ* 1996; **154**: 241–244.
65 Godard B, Kaariainen H, Kristofferson U *et al*: Provision of Genetic Services in Europe - current practices and issues. *Eur J Hum Genet* 2003; **11**(Suppl 2): S13–S48.
66 European Society of Human Genetics: Provision of Genetic services in Europe - current practices and issues. *Eur J Hum Genet* 2003; **11**(Suppl 2): S2–S4.
67 Clarke A: *The Genetic Testing of Children*. Oxford: Bios, 1998.
68 Clarke A, Flinter F: The genetic testing of children: a clinical perspective, in: Marteau T, Richards M (eds): *The Troubled Helix: Social and Psychological Implications of the New Human Genetics*. Cambridge: Cambridge University Press, 1996, pp 164–176.
69 Clarke A: The genetic testing of children. *J Med Genet* 1995; **32**: 492.
70 German Society of Human Genetics: Statement on genetic diagnosis in children and adolescents, http://www.gfhev.de/en/gfh/, 1995.
71 Danish Council of Ethics: Genetic Investigation of Healthy Subjects - Report on Presymptomatic Gene Diagnosis. http://www.etiskraad.dk/sw293.asp, 2001.
72 Borry P, Goffin T, Nys H, Dierickx K: Predictive genetic testing in minors for adult-onset genetic diseases. *Mt Sinai J Med* 2008; **75**: 287–296.
73 Rhodes R: Why test children for adult-onset genetic diseases? *Mt Sinai J Med* 2006; **73**: 609–616.
74 Hayes CV: Genetic testing for Huntington's disease–a family issue. *N Engl J Med* 1992; **327**: 1449–1451.
75 Grosfeld FJ, Beemer FA, Lips CJ, Hendriks KS, ten Kroode HF: Parents' responses to disclosure of genetic test results of their children. *Am J Med Genet* 2000; **94**: 316–323.
76 Quaid KA, Morris M: Reluctance to undergo predictive testing: the case of Huntington disease. *Am J Med Genet* 1993; **45**: 41–45.
77 Malpas PJ: Why tell asymptomatic children of the risk of an adult-onset disease in the family but not test them for it? *J Med Ethics* 2006; **32**: 639–642.
78 Rhodes R: Why test children for adult-onset genetic diseases? *Mt Sinai J Med* 2006; **73**: 609–616.
79 Skirton H: Telling the children; in: Clarke A (eds): *The Genetic Testing of Children*. Oxford: Bios, 1998, pp 103–111.
80 Campbell E, Ross LF: Parental attitudes and beliefs regarding the genetic testing of children. *Community Genet* 2005; **8**: 94–102.
81 Hamann HA, Croyle RT, Venne VL, Baty BJ, Smith KR, Botkin JR: Attitudes toward the genetic testing of children among adults in a Utah-based kindred tested for a BRCA1 mutation. *Am J Med Genet* 2000; **92**: 25–32.
82 Benkendorf JL, Reutenauer JE, Hughes CA *et al*: Patients' attitudes about autonomy and confidentiality in genetic testing for breast-ovarian cancer susceptibility. *Am J Med Genet* 1997; **73**: 296–303.
83 Evers-Kiebooms G: Predictief testen van minderjarigen voor aandoeningen die later in het leven tot uiting komen: psychologische aspecten en multidisciplinaire aanpak. *Tijdschrift voor geneeskunde* 2006; **62**: 463–471.
84 Swiss Academy of Medical Sciences: Genetic investigations in humans, http://www.samw.ch/, 1993.

85 Human Genetics Society of Australasia: DNA presymptomatic and predictive testing for genetic disorders, http://www.hgsa.com.au/policy/dnaptgd.html, 2002.
86 The American Society of Human Genetics Board of Directors and The American College of Medical Genetics Board of Directors: Points to consider: ethical, legal, and psychological impications of genetic testing in children and adolescents. *Am J Hum Genet* 1995; **57**: 1233–1241.
87 Task Force on Genetic Testing: Promoting safe and effective genetic testing in the United States, http://www.genome.gov/10001733, 1997.
88 Canadian College of Medical Geneticists: Genetic testing of children, http://ccmg.medical.org/, 2000.
89 Bioethics Committee Canadian Paediatric Society: Guidelines for genetic testing of healthy children. *Paediatr Child Health* 2003; **8**: 42–45.
90 Committee on Bioethics: Ethical issues with genetic testing in pediatrics. *Pediatrics* 2001; **107**: 1451.
91 Dalby S: GIG response to the UK Clinical Genetics Society report 'The genetic testing of children'. *J Med Genet* 1995; **32**: 490–491.
92 Clarke A: The genetic testing of children. Working Party of the Clinical Genetics Society (UK). *J Med Genet* 1994; **31**: 785–797.
93 American Medical Association: Testing children for genetic status, http://www.ama-assn.org/, 1995.
94 Barnes C: *Carrier testing in childhood: conflict or compromise*. http://www.medacad.org/eshg/abstracts/eshgplenary.htm, 2002.
95 McConkie-Rosell A, Spiridigliozzi GA, Rounds K *et al*: Parental attitudes regarding carrier testing in children at risk for fragile X syndrome. *Am J Med Genet* 1999; **82**: 206–211.
96 James CA, Holtzman NA, Hadley DW: Perceptions of reproductive risk and carrier testing among adolescent sisters of males with chronic granulomatous disease. *Am J Med Genet* 2003; **119C**: 60–69.
97 Green NS, Dolan SM, Murray TH: Newborn screening: complexities in universal genetic testing. *Am J Public Health* 2006; **96**: 1955–1959.
98 UK Newborn Screening Programme Centre: Newborn blood spot screening in the UK Policies and Standards. http://www.newbornbloodspot.screening.nhs.uk/download/policies_standards.pdf, 2005.
99 Health Resources and Services Administration: Newborn Screening: Toward a Uniform Screening Panel and System, ftp://ftp.hrsa.gov/mchb/genetics/screeningdraftforcomment.pdf, 2005.

EXHIBIT 7

Centers for Disease Control and Prevention

COVID-19 (Coronavirus Disease)

MENU >

COVID-19 Pandemic Planning Scenarios

Updated Sept. 10, 2020 Print

Summary of Recent Changes

Updates as of September 10

As of September 10, 2020

- The Infection Fatality Ratio parameter has been updated to include age-specific estimates
- The parameter for Number of Days from Symptom Onset to Seeking Outpatient Care—which was based on influenza care seeking data—has been replaced with the Median Number of Days from Symptom Onset to SARS-CoV-2 Test among SARS-CoV-2 Positive Patients
- A new parameter for the likelihood of an infection being reported has been added: The Ratio of Estimated Infections to Reported Case Counts

CDC and the Office of the Assistant Secretary for Preparedness and Response (ASPR) have developed five COVID-19 Pandemic Planning Scenarios that are designed to help inform decisions by public health officials who use mathematical modeling, and by mathematical modelers throughout the federal government. Models developed using the data provided in the planning scenario tables can help evaluate the potential effects of different community mitigation strategies (e.g., social distancing). The planning scenarios may also be useful to hospital administrators in assessing resource needs and can be used in conjunction with the COVID-19Surge Tool.

Each scenario is based on a set of numerical values for biological and epidemiological characteristics of COVID-19 illness, which is caused by the SARS-CoV-2 virus. These values—called *parameter values*—can be used in models to estimate the possible effects of COVID-19 in U.S. states and localities. This document was first posted on May 20, 2020, with the understanding that the parameter values in each scenario would be updated and augmented over time, as we learn more about the epidemiology of COVID-19. The September 10 update is based on data received by CDC through August 8, 2020.

In this update, age-specific estimates of Infection Fatality Ratios have been updated, one parameter measuring healthcare usage has been replaced with the median number of days from symptom onset to positive SARS-CoV-2 test, and a new parameter has been included: Ratio of Estimated Infections to Reported Case Counts, which is based on recent serological data from a commercial laboratory survey in the U.S.[1]

New data on COVID-19 are available daily, yet information about the biological aspects of SARS-CoV-2 and epidemiological characteristics of COVID-19 remain limited, and uncertainty remains around nearly all parameter values. For example, current estimates of infection-fatality ratios do not account for time-varying changes in hospital capacity (e.g., in bed capacity, ventilator capacity, or workforce capacity) or for differences in case ascertainment in congregate and community settings or in rates of underlying health conditions that may contribute to a higher frequency of severe illness in those settings. A nursing home, for example, may have a high incidence of infection (due to close contacts among many individuals) and severe disease (due to a high rate of underlying conditions) that does not reflect the frequency or severity of disease in the broader population of older adults. In addition, the practices for testing nursing home residents for SARS-CoV-2 upon identification of

Exhibit 7

a positive resident may be different than testing practices for contacts of confirmed cases in the community. Observed parameter values may also change over time (e.g., the percentage of transmission occurring prior to symptom onset will be influenced by how quickly and effectively both symptomatic people and the contacts of known cases are quarantined).

The parameters in the scenarios:

- Are estimates intended to support public health preparedness and planning.
- Are not predictions of the expected effects of COVID-19.
- Do not reflect the impact of any behavioral changes, social distancing, or other interventions.

CDC and the Office of the Assistant Secretary for Preparedness and Response (ASPR) have developed five COVID-19 Pandemic Planning Scenarios that are designed to help inform decisions by public health officials who use mathematical modeling, and by mathematical modelers throughout the federal government. Models developed using the data provided in the planning scenario tables can help evaluate the potential effects of different community mitigation strategies (e.g., social distancing). The planning scenarios may also be useful to hospital administrators in assessing resource needs and can be used in conjunction with the COVID-19Surge Tool.

CDC and the Office of the Assistant Secretary for Preparedness and Response (ASPR) have developed five COVID-19 Pandemic Planning Scenarios that are designed to help inform decisions by public health officials who use mathematical modeling, and by mathematical modelers throughout the federal government. Models developed using the data provided in the planning scenario tables can help evaluate the potential effects of different community mitigation strategies (e.g., social distancing). The planning scenarios may also be useful to hospital administrators in assessing resource needs and can be used in conjunction with the COVID-19Surge Tool.

Each scenario is based on a set of numerical values for biological and epidemiological characteristics of COVID-19 illness, which is caused by the SARS-CoV-2 virus. These values—called *parameter values*—can be used in models to estimate the possible effects of COVID-19 in U.S. states and localities. This document was first posted on May 20, 2020, with the understanding that the parameter values in each scenario would be updated and augmented over time, as we learn more about the epidemiology of COVID-19. The September 10 update is based on data received by CDC through August 8, 2020.

In this update, age-specific estimates of Infection Fatality Ratios have been updated, one parameter measuring healthcare usage has been replaced with the median number of days from symptom onset to positive SARS-CoV-2 test, and a new parameter has been included: Ratio of Estimated Infections to Reported Case Counts, which is based on recent serological data from a commercial laboratory survey in the U.S.[1]

New data on COVID-19 are available daily, yet information about the biological aspects of SARS-CoV-2 and epidemiological characteristics of COVID-19 remain limited, and uncertainty remains around nearly all parameter values. For example, current estimates of infection-fatality ratios do not account for time-varying changes in hospital capacity (e.g., in bed capacity, ventilator capacity, or workforce capacity) or for differences in case ascertainment in congregate and community settings or in rates of underlying health conditions that may contribute to a higher frequency of severe illness in those settings. A nursing home, for example, may have a high incidence of infection (due to close contacts among many individuals) and severe disease (due to a high rate of underlying conditions) that does not reflect the frequency or severity of disease in the broader population of older adults. In addition, the practices for testing nursing home residents for SARS-CoV-2 upon identification of a positive resident may be different than testing practices for contacts of confirmed cases in the community. Observed parameter values may also change over time (e.g., the percentage of transmission occurring prior to symptom onset will be influenced by how quickly and effectively both symptomatic people and the contacts of known cases are quarantined).

The parameters in the scenarios:

- Are estimates intended to support public health preparedness and planning.
- Are not predictions of the expected effects of COVID-19.
- Do not reflect the impact of any behavioral changes, social distancing, or other interventions.

The five COVID-19 Pandemic Planning Scenarios (Page 1) [illegible] for COVID-19

Exhibit 7

The five COVID-19 Pandemic Planning Scenarios (Box 1) represent a range of possible parameters for COVID-19 in the United States. All parameter values are based on current COVID-19 surveillance data and scientific knowledge.

- Scenarios 1 through 4 are based on parameter values that represent the lower and upper bounds of disease severity and viral transmissibility (moderate to very high severity and transmissibility). The parameter values used in these scenarios are likely to change as we obtain additional data about the upper and lower bounds of disease severity and the transmissibility of SARS-CoV-2, the virus that causes COVID-19.
- Scenario 5 represents a current best estimate about viral transmission and disease severity in the United States, with the same caveat: the parameter values will change as more data become available.

Parameter values that vary among the Pandemic Planning Scenarios are listed in Table 1, while parameter values common to all five scenarios are listed in Table 2. Definitions of the parameters are provided below, and the source of each parameter value is indicated in the Tables.

Parameter values that vary across the five COVID-19 Pandemic Planning Scenarios (Table 1) include measures of viral transmissibility, disease severity, and pre-symptomatic and asymptomatic disease transmission. Age-stratified estimates are provided, where sufficient data are available.

Viral Transmissibility

- **Basic reproduction number (R_0):** The average number of people that one person with SARS-CoV-2 is likely to infect in a population without any immunity (from previous infection) or any interventions. R_0 is an estimate of how transmissible a pathogen is in a population. R_0 estimates vary across populations and are a function of the duration of contagiousness, the likelihood of infection per contact between a susceptible person and an infectious person, and the contact rate.[2]

Disease Severity

- **Infection Fatality Ratio (IFR):** The number of individuals who **die** of the disease among all infected individuals (symptomatic and asymptomatic). This parameter is not necessarily equivalent to the number of reported deaths per reported case because many cases and deaths are never confirmed to be COVID-19, and there is a lag in time between when people are infected and when they die. This parameter also reflects the existing standard of care, which may vary by location and may be affected by the introduction of new therapeutics.

Pre-symptomatic and Asymptomatic Contribution to Disease Transmission

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. Parameter values that measure the pre-symptomatic and asymptomatic contribution to disease transmission include:

- **Percentage of infections that are asymptomatic:** The percentage of persons who are infected with SARS-CoV-2 but never show symptoms of disease. Asymptomatic cases are challenging to identify because individuals do not know they are infected unless they are tested over the course of their infection, which is typically only done systematically as a part of a scientific study.
- **Infectiousness of asymptomatic individuals relative to symptomatic individuals:** The contribution to transmission of SARS-CoV-2 from asymptomatic individuals compared to the contribution to transmission of SARS-CoV-2 from symptomatic individuals. For example, a parameter value of 50% means that an asymptomatic individual is half as infectious as a symptomatic individual, whereas a parameter value of 100% means that an asymptomatic individual is just as likely to transmit infection as a symptomatic individual.
- **Percentage of transmission occurring prior to symptom onset:** Among symptomatic cases, the percentage of new cases of COVID-19 due to transmission from a person with COVID-19 who infects others before exhibiting symptoms (pre-symptomatic).

Parameter values that do not vary across the five Pandemic Planning Scenarios (Table 2) are:

- **Level of pre-existing immunity to COVID-19 in the community:** The percentage of the U.S. population that had existing immunity to COVID-19 prior to the start of the pandemic beginning in 2019.

Exhibit 7

- **Ratio of estimated infections to reported case counts:** The estimated number of infections divided by the number of reported cases. The level of case detection likely varies by the age distribution of cases, location, and over time.
- **Time from exposure to symptom onset:** The number of days from the time a person has contact with an infected person that results in COVID-19 infection and the first appearance of symptoms.
- **Time from symptom onset in an individual and symptom onset of a second person infected by that individual:** The number of days from the time a person becomes symptomatic and when the person who they infect becomes symptomatic.

Additional parameter values common to the five COVID-19 Pandemic Planning Scenarios are these ten measures of healthcare usage:

- Median number of days from symptom onset to SARS-CoV-2 test among SARS-CoV-2 positive patients
- Median number of days from symptom onset to hospitalization
- Median number of days of hospitalization among those not admitted to the ICU
- Median number of days of hospitalization among those admitted to the ICU
- Percentage of patients admitted to the ICU among those hospitalized
- Percentage of patients on mechanical ventilation among those hospitalized (includes both non-ICU and ICU admissions)
- Percentage of patients who die among those hospitalized (includes both non-ICU and ICU admissions)
- Median number of days on mechanical ventilation
- Median number of days from symptom onset to death
- Median number of days from death to reporting of that death

These healthcare-related parameters (Table 2) are included to assist in assessment of resource needs as the pandemic progresses.

Box 1 Description of the Five COVID-19 Pandemic Planning Scenarios

For each Pandemic Planning Scenario:

- Parameter value for **viral transmissibility** is the Basic Reproduction Number (R_0)
- Parameter value for **disease severity** is the Infection Fatality Ratio (IFR)
- Parameter values for the **pre-symptomatic and asymptomatic contribution** to disease transmission are:
  - Percentage of transmission occurring prior to symptom onset (from pre-symptomatic individuals)
  - Percentage of infections that are asymptomatic
  - Infectiousness of asymptomatic individuals relative to symptomatic individuals

For Pandemic Scenarios 1-4:

- These scenarios are based on parameter values that represent the lower and upper bounds of disease severity and viral transmissibility (moderate to very high severity and transmissibility). The parameter values used in these scenarios are likely to change as we obtain additional data about the upper and lower bounds of disease severity and viral transmissibility of COVID-19.

For Pandemic Scenario 5:

- This scenario represents a current best estimate about viral transmission and disease severity in the United States, with the same caveat: that the parameter values will change as more data become available.

Scenario 1:

- Lower-bound values for virus transmissibility and disease severity
- Lower percentage of transmission prior to onset of symptoms

Exhibit 7

- Lower percentage of infections that never have symptoms and lower contribution of those cases to transmission

Scenario 2:

- Lower-bound values for virus transmissibility and disease severity
- Higher percentage of transmission prior to onset of symptoms
- Higher percentage of infections that never have symptoms and higher contribution of those cases to transmission

Scenario 3:

- Upper-bound values for virus transmissibility and disease severity
- Lower percentage of transmission prior to onset of symptoms
- Lower percentage of infections that never have symptoms and lower contribution of those cases to transmission

Scenario 4:

- Upper-bound values for virus transmissibility and disease severity
- Higher percentage of transmission prior to onset of symptoms
- Higher percentage of infections that never have symptoms and higher contribution of those cases to transmission

Scenario 5:

- Parameter values for disease severity, viral transmissibility, and pre-symptomatic and asymptomatic disease transmission that represent the best estimate, based on the latest surveillance data and scientific knowledge. Parameter values are based on data received by CDC through August 8, 2020.

Table 1. Parameter Values that vary among the five COVID-19 Pandemic Planning Scenarios. The scenarios are intended to advance public health preparedness and planning. They are **not** predictions or estimates of the expected impact of COVID-19. The parameter values in each scenario will be updated and augmented over time, as we learn more about the epidemiology of COVID-19. Additional parameter values might be added in the future (e.g., population density, household transmission, and/or race and ethnicity).

Parameter	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5: Current Best Estimate
R_0*	2.0		4.0		2.5
Infection Fatality Ratio†	0-19 years: 0.00002 20-49 years: 0.00007 50-69 years: 0.0025 70+ years: 0.028		0-19 years: 0.0001 20-49 years: 0.0003 50-69 years: 0.010 70+ years: 0.093		0-19 years: 0.00003 20-49 years: 0.0002 50-69 years: 0.005 70+ years: 0.054
Percent of infections that are asymptomatic§	10%	70%	10%	70%	40%
Infectiousness of asymptomatic individuals relative to symptomatic¶	25%	100%	25%	100%	75%
Percentage of transmission occurring prior to symptom onset**	30%	70%	30%	70%	50%

Exhibit 7

*The best estimate representative of the point estimates of R_0 from the following sources:
Chinazzi M, Davis JT, Ajelli M, et al. The effect of travel restrictions on the spread of the 2019 novel coronavirus (COVID-19) outbreak. *Science*. 2020;368(6489):395-400; Imai N., Cori, A., Dorigatti, I., Baguelin, M., Donnelly, C. A., Riley, S., Ferguson, N.M. (2020). Report 3: Transmissibility of 2019-nCoV. *Online report*
Li Q, Guan X, Wu P, et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. *N Engl J Med*. 2020;382(13):1199-1207
Munayco CV, Tariq A, Rothenberg R, et al. Early transmission dynamics of COVID-19 in a southern hemisphere setting: Lima-Peru: February 29th-March 30th, 2020 [published online ahead of print, 2020 May 12]. *Infect Dis Model*. 2020; 5:338-345
Salje H, Tran Kiem C, Lefrancq N, et al. Estimating the burden of SARS-CoV-2 in France [published online ahead of print, 2020 May 13] [published correction appears in Science. 2020 Jun 26;368(6498):]. *Science*. 2020;eabc3517.
The range of estimates for Scenarios 1-4 represent the upper and lower bound of the widest confidence interval estimates reported in: Li Q, Guan X, Wu P, et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. *N Engl J Med*. 2020;382(13):1199-1207.
Substantial uncertainty remains around the R_0 estimate. Notably, Sanche S, Lin YT, Xu C, Romero-Severson E, Hengartner N, Ke R. High Contagiousness and Rapid Spread of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg Infect Dis*. 2020;26(7):1470-1477 (https://dx.doi.org/10.3201/eid2607.200282) estimated a median R_0 value of 5.7 in Wuhan, China. In an analysis of 8 Europe countries and the US, the same group estimated R_0 of between 4.0 and 7.1 in the pre-print manuscript: Ke R., Sanche S., Romero-Severson, & E., Hengartner, N. (2020). Fast spread of COVID-19 in Europe and the US suggests the necessity of early, strong and comprehensive interventions. *medRxiv.*

† These estimates are based on age-specific estimates of infection fatality ratios from Hauser, A., Counotte, M.J., Margossian, C.C., Konstantinoudis, G., Low, N., Althaus, C.L. and Riou, J., 2020. Estimation of SARS-CoV-2 mortality during the early stages of an epidemic: a modeling study in Hubei, China, and six regions in Europe. *PLoS medicine*, *17*(7), p.e1003189. Hauser et al. produced estimates of IFR for 10-year age bands from 0 to 80+ year old for 6 regions in Europe. Estimates exclude infection fatality ratios from Hubei, China, because we assumed infection and case ascertainment from the 6 European regions are more likely to reflect ascertainment in the U.S. To obtain the best estimate values, the point estimates of IFR by age were averaged to broader age groups for each of the 6 European regions using weights based on the age distribution of reported cases from COVID-19 Case Surveillance Public Use Data (https://data.cdc.gov/Case-Surveillance/COVID-19-Case-Surveillance-Public-Use-Data/vbim-akqf). The estimates for persons ≥70 years old presented here do not include persons ≥80 years old as IFR estimates from Hauser et al., assumed that 100% of infections among persons ≥80 years old were reported. The consolidated age estimates were then averaged across the 6 European regions. The lower bound estimate is the lowest, non-zero point estimate across the six regions, while the upper bound is the highest point estimate across the six regions.

§ The percent of cases that are asymptomatic, i.e. never experience symptoms, remains uncertain. Longitudinal testing of individuals is required to accurately detect the absence of symptoms for the full period of infectiousness. Current peer-reviewed and preprint studies vary widely in follow-up times for re-testing, or do not include re-testing of cases. Additionally, studies vary in the definition of a symptomatic case, which makes it difficult to make direct comparisons between estimates. Furthermore, the percent of cases that are asymptomatic may vary by age, and the age groups reported in studies vary. Given these limitations, the range of estimates for Scenarios 1-4 is wide. The lower bound estimate approximates the lower 95% confidence interval bound estimated from: Byambasuren, O., Cardona, M., Bell, K., Clark, J., McLaws, M. L., & Glasziou, P. (2020). Estimating the extent of true asymptomatic COVID-19 and its potential for community transmission: systematic review and meta-analysis. *Available at SSRN 3586675*. The upper bound estimate approximates the upper 95% confidence interval bound estimated from: Poletti, P., Tirani, M., Cereda, D., Trentini, F., Guzzetta, G., Sabatino, G., Marziano, V., Castrofino, A., Grosso, F., Del Castillo, G. and Piccarreta, R. (2020). Probability of symptoms and critical disease after SARS-CoV-2 infection. *arXiv preprint arXiv:2006.08471*. The best estimate is the midpoint of this range and aligns with estimates from: Oran DP, Topol EJ. Prevalence of Asymptomatic SARS-CoV-2 Infection: A Narrative Review [published online ahead of print, 2020 Jun 3]. *Ann Intern Med*. 2020; M20-3012.

¶ The current best estimate is based on multiple assumptions. The relative infectiousness of asymptomatic cases to symptomatic cases remains highly uncertain, as asymptomatic cases are difficult to identify, and transmission is difficult to observe and quantify. The estimates for relative infectiousness are assumptions based on studies of viral shedding dynamics. The upper bound of this estimate reflects studies that have shown similar durations and amounts of viral shedding between symptomatic and asymptomatic cases: Lee, S., Kim, T., Lee, E., Lee, C., Kim, H., Rhee, H., Park, S.Y., Son, H.J., Yu, S., Park, J.W. and Choo, E.J., Clinical Course and Molecular Viral Shedding Among Asymptomatic and Symptomatic Patients With SARS-CoV-2 Infection in a Community Treatment Center in the Republic of Korea. *JAMA Internal Medicine*; Zou L, Ruan F, Huang M, et al. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *N Engl J Med*. 2020;382(12):1177-1179; and Zhou R, Li F, Chen F, et al. Viral dynamics in asymptomatic patients with COVID-19. *Int J Infect Dis*. 2020; 96:288-290. The lower bound of this estimate reflects data indicating that viral loads are higher in severe cases relative to mild cases (Liu Y, Yan LM, Wan L, et al. Viral dynamics in mild and severe cases of COVID-19. *Lancet Infect Dis*. 2020;20(6):656-657) and data showing that viral loads and shedding durations are higher among symptomatic cases relative to asymptomatic cases (Noh JY, Yoon JG, Seong H, et al. Asymptomatic infection and atypical manifestations of COVID-19: Comparison of viral shedding duration [published online ahead of print, 2020 May 21]. *J Infect*. 2020; S0163-4453(20)30310-8).

Exhibit 7

** The lower bound of this parameter is approximated from the lower 95% confidence interval bound from: He, X., Lau, E.H., Wu, P., Deng, X., Wang, J., Hao, X., Lau, Y.C., Wong, J.Y., Guan, Y., Tan, X. and Mo, X. (2020). Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nature medicine*, *26*(5), pp.672-675. The upper bound of this parameter is approximated from the higher estimates of individual studies included in: Casey, M., Griffin, J., McAloon, C.G., Byrne, A.W., Madden, J.M., McEvoy, D., Collins, A.B., Hunt, K., Barber, A., Butler, F. and Lane, E.A. (2020). Estimating pre-symptomatic transmission of COVID-19: a secondary analysis using published data. *medRxiv.*The best estimate is the geometric mean of the point estimates from these two studies.

Table 2. Parameter Values Common to the Five COVID-19 Pandemic Planning Scenarios. The parameter values are likely to change as we obtain additional data about disease severity and viral transmissibility of COVID-19.

Parameter values are based on data received by CDC through August 8, 2020, including COVID-19 Case Surveillance Public Use Data (https://data.cdc.gov/Case-Surveillance/COVID-19-Case-Surveillance-Public-Use-Data/vbim-akqf); data from the Hospitalization Surveillance Network (COVID-NET) (through August 1); and data from Data Collation and Integration for Public Health Event Response (*DCIPHER*).

Pre-existing immunity Assumption, ASPR and CDC	No pre-existing immunity before the pandemic began in 2019. It is assumed that all members of the U.S. population were susceptible to infection prior to the pandemic.
Time from exposure to symptom onset*	~6 days (mean)
Time from symptom onset in an individual and symptom onset of a second person infected by that individual†	~6 days (mean)
Mean ratio of estimated infections to reported case counts, Overall (range)§	11 (6, 24)
Parameter Values Related to Healthcare Usage	
Median number of days from symptom onset to SARS-CoV-2 test among SARS-CoV-2 positive patients (interquartile range)¶	Overall: 3 (1, 6) days
Median number of days from symptom onset to hospitalization (interquartile range)**	18-49 years: 6 (3, 10) days 50-64 years: 6 (2, 10) days ≥65 years: 4 (1, 9) days
Median number of days of hospitalization among those not admitted to ICU (interquartile range)††	18-49 years: 3 (2, 5) days 50-64 years: 4 (2, 7) days ≥65 years: 6 (3, 10) days
Median number of days of hospitalization among those admitted to ICU (interquartile range)††,§§	18-49 years: 11 (6, 20) days 50-64 years: 14 (8, 25) days ≥65 years: 12 (6, 20) days

Exhibit 7

Percent admitted to ICU among those hospitalized††	18-49 years: 23.8% 50-64 years: 36.1% ≥65 years: 35.3%
Percent on mechanical ventilation among those hospitalized. Includes both non-ICU and ICU admissions††	18-49 years: 12.0% 50-64 years: 22.1% ≥65 years: 21.1%
Percent that die among those hospitalized. Includes both non-ICU and ICU admissions††	18-49 years: 2.4% 50-64 years: 10.0% ≥65 years: 26.6%
Median number of days of mechanical ventilation (interquartile range)**	Overall: 6 (2, 12) days
Median number of days from symptom onset to death (interquartile range)**	18-49 years: 15 (9, 25) days 50-64 years: 17 (10, 26) days ≥65 years: 13 (8, 21) days
Median number of days from death to reporting (interquartile range)¶¶	18-49 years: 19 (5, 45) days 50-64 years: 21 (6, 46) days ≥65 years: 19 (5, 44) days

* McAloon, C.G., Collins, A., Hunt, K., Barber, A., Byrne, A., Butler, F., Casey, M., Griffin, J.M., Lane, E., McEvoy, D. and Wall, P. (2020). The incubation period of COVID-19: A rapid systematic review and meta-analysis of observational research. *medRxiv*.

† He, X., Lau, E.H., Wu, P., Deng, X., Wang, J., Hao, X., Lau, Y.C., Wong, J.Y., Guan, Y., Tan, X. and Mo, X. (2020). Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nature medicine*, *26*(5), pp.672-675.

§ The point estimate is the geometric mean of the location specific point estimates of the ratio of estimated infections to reported cases, from Havers, F.P., Reed, C., Lim, T., Montgomery, J.M., Klena, J.D., Hall, A.J., Fry, A.M., Cannon, D.L., Chiang, C.F., Gibbons, A. and Krapiunaya, I., 2020. Seroprevalence of antibodies to SARS-CoV-2 in 10 sites in the United States, March 23-May 12, 2020. *JAMA Internal Medicine*. The lower and upper bounds for this parameter estimate are the lowest and highest point estimates of the ratio of estimated infections to reported cases, respectively, from Havers et al., 2020.

¶ Estimates only include symptom onset dates between March 1, 2020 – July 15, 2020. Estimates represent time to obtain SARS-CoV-2 tests among cases who tested positive for SARS-CoV-2. Estimates based on and data from Data Collation and Integration for Public Health Event Response (*DCIPHER*).

** Estimates only include symptom onset dates between March 1, 2020 – July 15, 2020 to ensure cases have had sufficient time to observe the outcome (hospital discharge or death). Data for 17 year olds and under are suppressed due to small sample sizes.

†† Based on data reported to COVID-NET by Aug 1, 2020. Data for 17 year olds and under are suppressed due to small sample sizes. https://gis.cdc.gov/grasp/COVIDNet/COVID19_5.html.

§§ Cumulative length of stay for persons admitted to the ICU, inclusive of both ICU and non-ICU days.

Exhibit 7

¶¶ Estimates only include death dates between March 1, 2020 – July 15, 2020 to ensure sufficient time for reporting. Data for 17 year olds and under are suppressed due to small sample sizes.

References

1. Havers, F.P., Reed, C., Lim, T., Montgomery, J.M., Klena, J.D., Hall, A.J., Fry, A.M., Cannon, D.L., Chiang, C.F., Gibbons, A. and Krapiunaya, I., 2020. Seroprevalence of antibodies to SARS-CoV-2 in 10 sites in the United States, March 23-May 12, 2020. *JAMA Internal Medicine*.
2. Dietz K. The estimation of the basic reproduction number for infectious diseases. Stat Methods Med Res. 1993;2:23–41.

Archive

COVID-19 Pandemic Planning Scenarios – May 20, 2020 [7 pages]

COVID-19 Pandemic Planning Scenarios – July 10, 2020 [9 pages]

Last Updated Sept. 10, 2020

Exhibit 7