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DOCUMENT	**EXPERT REPORT**
ADDRESS FOR SERVICE AND CONTACT INFORMATION OF PARTY FILING THIS DOCUMENT	Alberta Justice, Constitutional and Aboriginal Law 10th Floor, 102A Tower 10025 -102A Avenue Edmonton, Alberta T5J 2Z2 Attention: Nicholas Parker and Nicholas Trofimuk Tel: (780) 643-0853 Fax: (780) 643-0852

EXPERT REPORT OF DR NATHAN ZELYAS

1. My name is Dr Nathan Zelyas. I am a medical microbiologist with Alberta Precision Laboratories – Public Health Laboratory, where I am the Program Leader for Respiratory Viruses and Transplant Virology.

2. I have been asked by the Respondents to provide my opinion in response to the expert report of Dr Jay Bhattacharya regarding an analysis of polymerase chain reaction (PCR) diagnostic tests for COVID-19, including their accuracy/inaccuracy, their use to determine cases of COVID-19, and whether people who test positive from a PCR test are infected/contagious with COVID-19.

3. The substance of my opinion, including the information and assumptions upon which my opinion is based, is contained in **Schedule A**.

4. My qualifications and background are set out in my Curriculum Vitae, attached hereto as **Schedule B**, and the sources used in my report are attached hereto as **Schedule C**.

9 July 2021

Dr Nathan Zelyas

Schedule A

Molecular SARS-CoV-2 testing in Alberta

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen that causes coronavirus disease (COVID-19) and is responsible for the current ongoing pandemic. It is a virus belonging to the Coronaviridae family of viruses, and has a ribonucleic acid (RNA) genome which serves as its genetic material that is required for the production of different proteins, its replication, and overall survival and pathogenesis. While its RNA genome is responsible for its success as an infectious agent, it is also the target of tests used to detect its presence in clinical infections.

How samples are collected and processed for SARS-CoV-2 testing

In Alberta, the primary samples used to diagnose COVID-19 are nasopharyngeal (NP) swabs and throat swabs. An NP swab is a small flexible swab that must be inserted deep into a patient's nose to reach the NP region, the main area that SARS-CoV-2 (and most other respiratory viruses) replicates when causing an upper respiratory tract infection. Throat swabs, which are more rigid swabs that are used to sample the tonsillar pillars and posterior throat area, have also been determined to be acceptable specimen types compared to NP swabs (1). The swabs collect mucous, human cells, cellular debris, and the virus and its components. After a healthcare professional collects an NP or throat swab, it is typically placed in a tube containing a stabilizing solution (transport medium) that preserves the virus and inhibits the growth of bacteria and fungi (such as viral or universal transport medium). Once the swab is inserted into the transport medium, the human material and virus collected on the swab disperse into the transport medium. The swab contained in the tube of transport medium is transported to the laboratory for processing. Once it arrives in the laboratory, it is data entered into the laboratory information system and checked to ensure that the paperwork accompanying the sample matches the patient information on the tube. The transport medium, containing the human material and virus, is then subjected to nucleic acid extraction to break open the cells and virus to release and purify the nucleic acid encoding the SARS-CoV-2 genome. This process frees the nucleic acid of SARS-CoV-2 so that it is available for detection using advanced laboratory techniques.

How SARS-CoV-2 is detected in patient samples

Soon after the genome of SARS-CoV-2 was sequenced (very early after its initial discovery), laboratories and scientists were able to design specific tests to detect the virus. This is through the use of a powerful laboratory method called polymerase chain reaction (PCR). Soon after PCR was described, it was applied to the detection of microbes that cause infectious diseases (2). PCR takes advantage of the ability of deoxyribonucleic acid (DNA) to be replicated numerous times in an exponential fashion based on a specific DNA sequence. In a typical PCR reaction, the components include an enzyme called a polymerase, two short DNA sequences called primers, a buffered solution containing the ideal environment and cofactors needed for the polymerase to carry out the reaction, nucleotide molecules that form the building blocks of DNA strands, and a long strand of DNA that is to be used as the template for the reaction. The two primers bind to the template DNA based on their sequences being complementary to the template and this allows the polymerase to copy the template DNA between the primers. After the first cycle of the reaction is completed, this copied DNA strand can now also act as a template in future PCR cycles, allowing the generation of multiple new copies of the template DNA strand in an exponential fashion. A successful PCR (ie, one in which the primers bound to the target sequence and the polymerase produced copies of the template DNA) is detected by observing the amplified DNA product, which can be achieved in a number of different ways.

For COVID-19 testing, specific PCR assays have been developed targeting different areas of the genome of SARS-CoV-2. Because SARS-CoV-2 has an RNA genome and PCR normally requires a DNA template, an additional enzyme called reverse transcriptase is added to the reaction, which replicates the targeted region of the SARS-CoV-2 genome into the DNA template needed for PCR; this alternative form of PCR is referred to as reverse transcriptase-PCR (RT-PCR).

RT-PCR has become the method of choice for detecting SARS-CoV-2 for clinical diagnostic purposes, most commonly in a format known as real-time RT-PCR (rRT-PCR). rRT-PCR is an advanced version of RT-PCR whereby the amplification of target DNA can be visualized on a computer by the accumulation of fluorescence detected over time as the reaction progresses and more DNA product is generated. Once a pre-specified threshold of fluorescence is reached, a sample is considered positive for SARS-CoV-2 RNA. During rRT-PCR, the DNA target (if present) approximately doubles in amount with each cycle. The number of cycles that is required to reach the fluorescence threshold that is used to define whether a sample is positive or negative is referred to as the "cycle threshold," or CT, value. The higher the CT value, the lower the amount of starting of nucleic acid in a sample; accordingly, the lower the CT value, the higher the amount of starting nucleic acid in a sample. This principle is used to determine the viral load in quantitative real-time PCR tests (examples include human immunodeficiency virus, hepatitis C virus, and cytomegalovirus viral loads, among many others). There are currently no Health Canada-approved quantitative real-time PCR tests for COVID-19 – all approved tests are considered qualitative and therefore provide only a binary positive/negative result. It is important to note that though the CT value is an indication of the amount of viral RNA in a sample, a positive result, even at a high CT value, indicates that some viral RNA is present and that the patient was infected by the virus at some point. Likewise, a negative result indicates the absence of detectable viral RNA and therefore the result has no associated CT value as the fluorescence threshold was never reached despite the use of many amplification cycles in the test.

One of the challenges in using a highly sensitive technique such as rRT-PCR is that patients can remain positive months after their initial infection despite no longer harbouring infectious virus (3). Dead virus particles or RNA molecules targeted by an rRT-PCR test can be collected when a patient is swabbed and produce a positive result. While the patient may have been infected at some point, a positive rRT-PCR result alone does not indicate whether a patient was recently or remotely infected – nor does it provide insight into whether a patient is likely to be infectious.

Viral culture as a diagnostic test for SARS-CoV-2

To determine whether or not a patient harbours infectious SARS-CoV-2 virus, it is tempting to consider viral culture as a potential diagnostic modality. Viral culture consists of incubating swab material (or other sample type) collected from a patient in conditions that favour virus growth on a susceptible cell line culture and then observing the culture for the effects of replicating virus (cytopathic effect). This methodology would detect only viable virus capable of replicating and not merely non-transmissible fragments of a virus that are still detectable using rRT-PCR. While SARS-CoV-2 culture might appear to be a suitable laboratory technique to infer the infectious state of a patient, it has severe limitations that make it untenable for use in a diagnostic laboratory.

Culture is slow. While most nucleic acid testing takes between one and six hours to perform (depending on the specific platform), culture of SARS-CoV-2 can take up to three days or even longer to observe cytopathic effect (4,5). Waiting three days to determine whether a patient has COVID-19 or is infectious is too long to have be effective for clinical and public health management.

Additionally, culture is non-specific. The cytopathic effects of a different respiratory virus could be mistaken for those caused by SARS-CoV-2. As a result, a confirmatory method would have to be applied to potentially positive virus culture material – rRT-PCR would likely be the most specific and rapid method available to perform such a confirmation step, adding even more time to ascertain the diagnosis.

Viral culture requires specialized technical expertise that is not widely available anymore. While viral culture used to be a primary diagnostic method decades ago, it has fallen out of favor due to its much lower sensitivity than PCR and long turnaround time, making it less practiced and generally only available in reference or research laboratories. As well, SARS-CoV-2 culture requires containment level 3 laboratory facilities, which are not readily available in the vast majority of diagnostic microbiology laboratories in North America.

Finally, viral culture may not be an adequate proxy of infectiousness. In actual human infections, SARS-CoV-2 grows in nasopharyngeal, throat, and lung tissue, which are different from the cells used in viral culture. Because of this difference, a negative viral culture is not a perfect demonstration that a patient is not infectious.

The performance of SARS-CoV-2 rRT-PCR tests

The diagnostic foil to culture is rRT-PCR. As described above, rRT-PCR is a fast and highly sensitive, specific, and scalable method to detect a pathogen's nucleic acid. Even so, it is not a perfect test and has been estimated in a systematic review to have a sensitivity of 80% after three days from symptom onset (6). A local study found the rRT-PCR testing in Alberta early in the pandemic to have a sensitivity of 90.7% in patients with sufficiently high suspicion of disease to warrant repeat sample collection (7); this is similar to the findings of another Canadian study which found a sensitivity of 89% using NP swabs (8). The specificity of PCR for COVID-19 is estimated to be quite high. A review of a laboratory proficiency testing program showed that 98.3% of laboratories correctly identified a negative sample as negative (with the discrepant results likely attributable to clerical errors in filling out the proficiency testing paperwork) and another study using machine learning derived models estimated a false-positivity rate of 0.08% for all samples (9,10).

The use of CT values for determining transmissibility of SARS-CoV-2

There is some discussion in the literature regarding whether CT values generated by SARS-CoV-2 rRT-PCR tests can be used to infer whether someone is infectious. While CT values correlate with the amount of virus in a clinical sample and the amount of virus infecting someone correlates with virus cultivability (which in turn indicates the presence of transmissible virus), there are significant challenges with this idea. CT values are raw laboratory data that are not validated as quantitative measures. For a test to show that it can reliably identify the amount of virus in a sample, it needs to be compared to a reference (gold standard) method for a large number of samples with a variety of viral loads to demonstrate its accuracy and precision for determining the amount of virus in samples. None of the PCR tests used in COVID-19 clinical testing in Alberta have been validated in such a way. As well, reporting measures of viral loads in samples requires that a standard curve of samples with known virus concentrations be run on routine batches of PCR testing – this is not routinely done in the diagnostic laboratories in Alberta. Therefore, from an analytic perspective, the PCR tests used in Alberta are not suitable for reporting virus quantities in patient samples.

Studies examining the use of CT values to infer infectiousness have often led to broad sweeping generalizations to be made about their utility. For example, there have been claims that above a specific CT value (sometimes stated as 30, 35, 40 or some other number of cycles), a positive result is a false-positive regardless of the PCR test being used. However, this is a common fallacy as it is well known that CT values are heavily dependent on the specific test being used. This is demonstrated by a study comparing the CT values across different SARS-CoV-2 tests used by laboratories across North America in a proficiency testing survey; this study found that among the different tests used, there was a variation in as much as 14 CT values for results on the same sample (11). This lack of consistency indicates that CT values are not generalizable between different tests and statements that specific CT value cut-offs to define a sample as a false-positive demonstrates a lack of understanding of PCR testing in general.

Local data also indicates that CT values are not adequate predictors of infectiousness. An analysis of 5,756 positive cases with available CT values of the laboratory-developed COVID-19 rRT-PCR used initially in Alberta during the pandemic showed that while CT values generally correlated the time since symptom onset (which is a reliable predictor of infectiousness), 25% of individuals with symptom onset less than seven days prior to testing had CT values >29.1 and 10% of individuals with symptom onset less than seven days prior to testing had CT values >32.8 (12). This indicates that using a CT value cut-off to define infectiousness would likely run the risk of misclassifying a large number of people as non-infectious and therefore contribute to the spread of COVID-19.

Recently published guidance from the Public Health Agency of Canada (PHAC) explicitly states that "it is not possible to directly translate a CT value into degree or duration of infectiousness" (13). This is based on the inherent variability of CT values based on numerous factors: stage of infection, type of sample collected (eg, NP swab versus throat swab), quality of the sample, the PCR test used, the long duration of PCR positivity following an infection, and the potential impact of emerging SARS-CoV-2 variants (13). This guidance is consistent with that provided by the US Centers for Disease Control and the Association of Public Health Laboratories (14,15).

The importance of SARS-CoV-2 rRT-PCR for public health and infection prevention and control

The role of diagnostic SARS-CoV-2 PCR testing is to aid public health to identify cases of COVID-19 so that patients can isolate to avoid further transmission and to perform contact tracing to identify people at risk of being infected prior to them transmitting to others. As well, infection prevention and control (IPC) units that oversee acute care centres (such as hospitals) and long-term care facilities require the diagnostic SARS-CoV-2 results to manage the isolation of patients and limit spread in healthcare facilities where the most vulnerable populations can be found. These practices require a highly sensitive and rapid methodology such as rRT-PCR to accurately identify cases quickly so that immediate controls can be put into place to limit viral spread.

As discussed above, the CT values of positive results are not by themselves reliable in determining the transmissibility of an individual patient. However, in discussion with a trained virologist or microbiologist, a public health physician (a Medical Officer of Health, or MOH) or an IPC practitioner may request CT values to contribute information for a specific patient and their situation to help determine whether a positive SARS-CoV-2 PCR result is due to a new infection (and, hence, more likely to be infectious) or an old infection (where transmission is less likely). In these scenarios, the entire clinical and laboratory testing pictures are taken into account to come to an informed decision – they do not depend on specific CT value cut-offs alone to define a patient as infectious or non-infectious.

It is important to note that although CT values generally show an increasing trend as an individual progresses through their acute infection and eventually become non-infectious, this phenomenon is observed on a population level and cannot be safely applied on an individual level. Even if a patient is non-infectious at the time they are diagnosed as a case of COVID-19, it is still important that their contacts be identified to limit spread of disease in the community. Additionally, the CT value for a positive SARS-CoV-2 rRT-PCR test only represents the amount of viral RNA in a sample at a specific moment in time when the sample was collected; the trajectory of the viral replication (and, accordingly, the stage of infection) cannot be reliably predicted by the CT value at that point in time by itself. However, the utility of the positive/negative binary result yielded by rRT-PCR is that it identifies both those individuals who are or will become infectious and those who were previously infectious and may have recently transmitted the virus to others. Identifying these individuals for isolating and contact tracing is the role of this testing and supports public health and IPC efforts at controlling the spread of COVID-19.

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Schedule B

Nathan Zelyas, MD, MSc, BSc, FRCPC, D(ABMM)
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Current appointments

Medical Microbiologist – Alberta Precision Laboratories – Public Health Laboratory Program Leader for Respiratory Viruses and Transplant Virology	Jul 2016-present
Assistant Clinical Professor – Department of Laboratory Medicine and Pathology, Faculty of Medicine and Dentistry, University of Alberta	Jul 2020-present
Medical Microbiology Residency Program Director – University of Alberta	May 2019-present

Education

Post-graduate residency training in Medical Microbiology, University of Alberta	Jul 2011-Jun 2016
MD, University of Alberta	Sep 2007-Jun 2011
M.Sc., Biological Sciences with concentration in Microbiology and Biotechnology, University of Alberta	Sep 2004-Aug 2007

Examinations

Medical Council of Canada Qualifying Examination Part I – passed	Jun 2011
Medical Council of Canada Qualifying Examination Part II – passed	Dec 2012
Royal College of Physicians and Surgeons of Canada Examination in Medical Microbiology – passed	Jun 2016
American Board of Medical Microbiology Examination – passed	Aug 2016

Certifications

Licentiate of the Medical Council of Canada	Dec 2012
Fellow of the Royal College of Physicians of Canada	Jun 2016
Diplomate of the American Board of Medical Microbiology	Aug 2016

Awards and recognitions

Abbott Abstract Award for University of Alberta Medical Microbiology and Infectious Diseases trainees	2013
Canadian College of Microbiologists Poster Award at the Association of Medical Microbiology and Infectious Disease Canada Annual Conference	2013
First place student presentation on Provincial Laboratory for Public Health of Alberta Research Day	2008
Alberta Heritage Foundation for Medical Research (AHFMR) Summer Studentship Award	2008
First place poster in Microbiology and Biotechnology – Graduate Student Research Days, Department of Biological Sciences, Faculty of Science, University of Alberta	2007
Natural Sciences and Engineering Research Council (NSERC) PGS M; awarded for two years	Sep 2004 – Aug 2006
Walter H. Johns Graduate Fellowship; awarded twice	2004, 2005
Faculty of Science Graduate Scholarship	2004
NSERC Undergraduate Student Research Award; awarded twice	2003, 2004
Bill Paranchych Memorial Scholarship	2003
Jason Lang Scholarship; awarded three times	2001, 2002, 2003

Publications

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Professional association memberships

College of Physicians and Surgeons of Alberta	Jul 2011 – present
Canadian Medical Association	Sep 2007 – present
Alberta Medical Association	Sep 2007 – present
Association of Medical Microbiology and Infectious Disease Canada	May 2012 – present

Schedule C

Sensitivity of Nasopharyngeal, Nasal and Throat Swab for the Detection of SARS-CoV-2

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Running Title: Sensitivity of different swab sources for SARS-CoV-2

Key Words: Specimen handling, SARS-CoV-2, COVID-19 diagnostic testing, molecular diagnostic techniques, nasopharyngeal swab, throat swab, oropharyngeal swab, nasal swab

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Abstract

Nasopharyngeal (NP), nasal and throat swabs are the most practical specimen sources to test for upper respiratory pathogens. We compared the sensitivity of NP, nasal and throat swabs to detect SARS-CoV-2 in community patients. Using detection at any site as the standard, the sensitivities were 90%, 80% and 87% for NP, nasal and throat respectively (n=30 positive at any site). Throat swabs are likely a suitable alternative to NP swabs for the detection of COVID-19 infections.

Introduction

The specimen source of choice for the screening of coronavirus disease-2019 (COVID-19) varies globally. Generally, the ideal specimen type for viruses causing respiratory tract infections is a nasopharyngeal (NP) swab (1). Lower respiratory tract specimens may be of benefit in severe cases of COVID-19, but most cases have mild upper respiratory tract disease (2–4). However, due to worldwide shortages of swabs and collection media, it has become necessary to identify alternate methods to NP swabs for sample collection for COVID-19 testing. We enrolled COVID-19 positive community patients on home isolation to determine the sensitivity of NP, nasal and throat swabs.

Methods

Alberta Health Services (AHS) Public Health provided a list of people who had tested positive for COVID-19. Oral consent by phone was obtained for collection of NP, nasal, and throat swabs in the participant's home. NP swabs were collected using the Flexible Mini Tip Flocked Swab (Copan S.P.A, Italy) in Universal Transport Media (UTM, Copan), nasal swabs using APTIMA Unisex Collection Kit (Hologic Inc., Marlborough, Mass), and throat swabs using the APTIMA Multitest Collection Kit. Collectors were given instructions on how to perform swabs. For NP swabs the AHS collection guide was used (5). For nasal collection, both nares were swabbed to a depth of at least 3 cm (or until resistance felt) and rotated three times. Throat swabs were collected from both sides of the oropharynx and the posterior pharyngeal wall under the uvula. The University of Calgary Research Ethics board approved this study (REB20-444).

Testing for SARS-CoV-2 was performed by a multiplex reverse transcriptase real time-polymerase chain reaction (RT-PCR). The RT-PCR was developed, validated and performed at the Alberta Public Health Laboratory (ProvLab) targeting the envelope region (modified from (6)) and the RNA-dependent RNA polymerase encoding regions (E and RdRp genes, respectively). The test was validated against

proficiency panels provided by the Public Health Agency of Canada National Microbiology Laboratory (Winnipeg, MB). Spiking APTIMA media with a positive NP swab UTM specimen showed minimal difference in the Ct values for our SARS-CoV-2 PCR and a 2 log dilution series in APTIMA, stored at 4°C and room temperature for 48 h, showed minimal difference from time zero (≤0.6 change in Ct value). Graph Pad Prism v8.4.1 (Graphpad Prism Softwar L.L.C, San Diego, CA) was used for statistical analysis.

Results

Of 82 COVID-19 positive individuals contacted, 36 consented (41% female, mean age 44.6 (range 18-61)). Initial diagnosis was made by NP (n=15) or nasal swab (n=21). Participants were swabbed for the study a mean of 4.1 days (range 1 to 6) after the initial diagnostic test and 10 days (range 4 to 23) after symptom onset. Thirty of the thirty-six participants tested positive again at one or more of the three sites swabbed. The mean time from symptom onset and study swabs was 12.6 days (range 5-18) for those testing negative at all three sites and compared to 10.0 days (range 4-23) for those with a positive result at any site. Using a reference standard of a positive result at any site, NP swab had a sensitivity of 90% (95%CI 74.4-96.5), throat swab 87% (70.3-94.7) and nasal swab 80% (62.7-90.5) (Wilson/Brown Method, Table 1). In only two cases was only one specimen positive (both nasal). Seven participants were positive from only two sources (n=2 NP and nasal, n=5 NP and throat).

Comparing the samples where all targets were positive and Ct values were available (n=19 for E gene and n=18 RdRp), the Ct values for NP swabs was lower than throat swabs for the E gene ($p=0.028$, $p>0.22$ for other site comparisons) (Friedman Test). The median Ct values for the E gene were 25.5 (10^{th} to -90^{th} percentile: 20.5-29.5) for NP, 27.6 (24.7-32.4) for nasal and 28.7 (23.5-34.2) for throat; median Ct values for the RdRp gene were 27.9 (23.5-32.4), 30.5 (27.5-35.0), and 31.3 (26.5-35.5), for the same sites, respectively ($p>0.09$).

Discussion

Our study demonstrates that the sensitivity of nasal swabs is somewhat inferior to NP or throat swabs whereas throat and NP swabs have comparable sensitivity. This finding was despite the Ct value being higher in throat swabs compared to NP swabs. Consequently, when NP swabs are not available, throat swabs are a preferable alternative to nasal swabs for COVID-19 testing.

Péré *et al* also found nasal swabs to be less sensitive than NP swabs (8). They reported a sensitivity of 89.2% (4/37 NP positives were false negative) with NP swabs as the reference standard. Using NP swabs as the reference standard in our study, the sensitivity of nasal swabs was 82.5% (5/27 NP positives were false negative). It is important to note that in our study NP swabs missed 3 positives that other sources detected (n=2 detected by nasal and n=1 throat). Combining results from Péré *et al* and our study gives a sensitivity for nasal swabs of 85.9%. Differences between studies included a different patient population (patients seen in hospital vs. community), PCR assay used and collection media. Although our study represents the general population with COVID-19, the results may differ in inpatients as they may have higher viral loads (9).

Contrary to our findings, Wang *et al* (10) reported 73% of patients with a positive NP swab result tested negative by throat/oropharyngeal swab. A potential explanation is that Wang *et al* did not instruct collectors to swab under the epiglottis. Therefore, viral shedding from the nasopharynx may have not been optimally sampled in the Wang *et al* cohort.

One limitation of our study is the small number of samples validated. We chose thirty to have a high likelihood to detect 90% agreement (11). A hindrance of performing studies of this nature with a large sample size is that sampling puts collectors at risk of infection even with appropriate personal protective equipment. Our study was also performed a mean of 10 days after symptom onset, so the site of optimal sampling may have differed if participants were swabbed closer to symptom onset. In early

117 disease, throat swabs may be falsely negative compared to CT scan findings, though it is not clear
118 whether NP and nasal swabs also lack sensitivity early in the disease progress (12).

119 The sensitivity of the sample type is dependent on proper sampling procedure. In our jurisdiction, nasal
120 swabs were initially implemented due to reports of lower Ct values than those seen in throat swabs (13).
121 Despite education and routine observation of the technique used at COVID-19 community assessment
122 centres, multiple accounts of sampling the anterior nares instead of the posterior nares/lower
123 turbinates were reported to the laboratory by patients. Sampling errors may also occur with throat and
124 NP swab and may have contributed to some of the false-negative NP swab results despite our collectors
125 being trained health care professionals. Additionally, other studies have since reported that throat
126 swabs have equivalent or higher SARS-CoV-2 viral loads compared to NP or nasal swabs, respectively
127 (14,15). Based on our results and familiarity of health care providers with throat swabs (as opposed to
128 nasal swabs), we currently recommend in our jurisdiction the collection of throat swabs if NP swabs are
129 not available.

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138

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Table 1a. COVID-19 PCR Results for NP and Nasal swabs

		Nasal	
		Pos	Neg
NP	Pos	22	5
	Neg	2	7

Table 1b. COVID-19 PCR Results for Nasal and Throat Swabs

		Throat	
		Pos	Neg
Nasal	Pos	20	4
	Neg	6	6

Table 1c. COVID-19 PCR Results for NP and Throat Swabs

		Throat	
		Pos	Neg
NP	Pos	25	2
	Neg	1	8

The role of DNA amplification technology in the diagnosis of infectious diseases

Marie Louie,*† Lisa Louie,* Andrew E. Simor*†

Abstract

NUCLEIC ACID AMPLIFICATION AND DETECTION METHODS developed in the past decade are useful for the diagnosis and management of a variety of infectious diseases. The most widely used of these methods is the polymerase chain reaction (PCR). PCR assays can detect rapidly and accurately the presence of fastidious and slow-growing microorganisms, such as *Chlamydia*, mycoplasmas, mycobacteria, herpesviruses and enteroviruses, directly from clinical specimens. Commercial PCR assays for the diagnosis of tuberculosis and genital *C. trachomatis* infection are now routinely used in many diagnostic laboratories. Assays have also been developed that can detect antimicrobial resistance and are used to identify the cause of infection by organisms that cannot be cultivated. The value of viral load measurement by nucleic acid amplification in the management of patients with HIV infection or hepatitis C has also been well established. However, evaluations of this technology for rapid microbial diagnosis have generally been limited by small samples, and the cost of these assays may be as high as Can$125 per test. As nucleic acid amplification methods continue to evolve, their role in the diagnosis and management of patients with infectious diseases and their impact on clinical outcomes will become better defined.

Cases

A 58-year-old woman is being assessed for a 4-week history of low-grade fever and cough. A chest radiograph indicates the presence of disease in the left upper lobe airspace. Microscopic examination of a sputum specimen reveals a moderate number of acid-fast bacilli. Does this represent tuberculosis or the presence of nontuberculous mycobacteria?

A 19-year-old student is admitted to hospital with meningitis. Before her admission she had received 3 courses of oral cefaclor therapy. In consequence her blood and cerebrospinal fluid cultures are negative. She is responding to empiric antimicrobial therapy. Should her family or her roommates receive chemoprophylaxis for possible exposure to *Neisseria meningitidis*?

A 60-year-old man is admitted to hospital with the onset of encephalitis. Should he receive high-dose intravenous acyclovir therapy for presumed infection with herpes simplex virus?

Each of these clinical scenarios presents the medical practitioner with a problem that involves establishing a diagnosis of infection in a setting where routine laboratory investigations are likely to be nondiagnostic or will not provide results in a timely manner. In the past decade molecular techniques have been developed that allow the amplification and detection of minute amounts of nucleic acid sequences from tissues or body fluids. These nucleic acid amplification methods can create millions of identical copies of a DNA or RNA "target" sequence in a matter of hours. The ability to determine whether specific DNA or RNA sequences are present in clinical samples using molecular technology has dramatically changed our approach to the laboratory diagnosis of many diseases. For example, these methods have been useful in the diagnosis of genetic disorders such as sickle cell anemia, β-thalassemia and cystic fibrosis.[1] Recently the development of nucleic acid amplification technology has also had a significant impact on the diagnosis and management of many infectious diseases, including those represented by the 3 hypothetical cases described here.[2]

Review

Synthèse

From *the Department of Microbiology, SD Laboratory Services and the Division of Infectious Diseases, Sunnybrook & Women's College Health Sciences Centre, and †the Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ont.

This article has been peer reviewed.

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Several strategies for the amplification of nucleic acids have been described, including amplification of the nucleic acid target (e.g., polymerase chain reaction [PCR], strand-displacement amplification, self-sustaining sequence replication), amplification of a nucleic acid probe (e.g., ligase chain reaction, Qβ replicase) and signal amplification (e.g., branched-probe DNA assay). PCR is now the most widely used amplification method.

As these molecular methods are further refined and become more widely available in the next few years, physicians will need to understand their clinical applications and be aware of their potential advantages, limitations and clinical utility. In this paper we describe the principles behind PCR-based diagnosis and its applications for the diagnosis of infectious diseases. We review PCR tests that are currently available commercially and discuss assays that are under development. However, it is beyond the scope of this article to describe other nucleic acid amplification methods or to include a complete list of all PCR assays that have been developed; other recent reviews offer additional details.[2-4]

Polymerase chain reaction

PCR can amplify minute amounts of target DNA within a few hours.[1–3] Applications in microbiology and infectious diseases have included the diagnosis of infection due to slow-growing or fastidious microorganisms, detection of infectious agents that cannot be cultured and rapid identification of antimicrobial resistance.

The essential materials, reagents and equipment required for nucleic acid amplification and detection by PCR are summarized in Table 1. Nucleic acid amplification is performed in a thermocycler, which is an instrument that can hold the assay's reagents and allows the reactions to occur at the various temperatures required. In the initial step of the procedure, nucleic acid (e.g., DNA) is extracted from the microorganism or clinical specimen of interest. Heat (90°C–95°C) is used to separate the extracted double-stranded DNA into single strands (denaturation). Cooling to 55°C then allows primers specifically designed to flank the target nucleic acid sequence to adhere to the target DNA (annealing). Following this, the enzyme *Taq* polymerase and nucleotides are added to create new DNA fragments complementary to the target DNA (extension). This completes one cycle of PCR. This process of denaturation, annealing and extension is repeated numerous times in the thermocycler. At the end of each cycle each newly synthesized DNA sequence acts as a new target for the next cycle, so that after 30 cycles millions of copies of the original target DNA are created (Fig. 1). The result is the accumulation of a specific PCR product with sequences located between the 2 flanking primers.

Table 1: Reagents and equipment required for the polymerase chain reaction (PCR)

PCR target or "template"	The segment of nucleic acid (DNA or RNA) that is to be amplified
Nucleotides	Building blocks from which nucleic acids are constructed: adenine, guanine, cytosine, thymine and uracil
Primer	A short sequence of nucleotides complementary to, and binding (annealing) to, known sequences of the target nucleic acid; essential for "priming" the amplification reaction
Taq DNA polymerase	A heat-stable enzyme that makes a new complementary copy of the target nucleic acid by adding nucleotides to the annealed primer
Reverse transcriptase	An enzyme that converts RNA into a complementary DNA sequence (used in reverse transcription PCR)
Thermocycler	The equipment in which PCR reactions occur; it is able to change rapidly to the different temperatures required for repeated PCR cycles

Detection of the amplified products can be done by visualization with agarose gel electrophoresis, by an enzyme immunoassay format using probe-based colorimetric detection or by fluorescence emission technology. In multiplex PCR the assay is modified to include several primer pairs specific to different DNA targets to allow amplification and detection of several pathogens at the same time.

Reverse transcription PCR is a modification of this method used when the initial template is RNA rather than DNA. In this case the enzyme reverse transcriptase first converts the RNA target into a complementary DNA copy (cDNA). This cDNA can then be amplified by standard PCR methods as described earlier. Reverse transcription PCR can be used to amplify the much higher numbers of copies of messenger or ribosomal RNA than the number of DNA copies present in bacteria or fungi, and it may detect specific expression of certain genes during the course of infection. The detection of cDNA using reverse transcription PCR of messenger RNA encoded by a pathogen could be evidence of active infection,[5] in contrast to the detection of DNA from nonviable organisms using standard PCR.

Diagnosis of infectious diseases

Examples of infectious agents that have been detected by nucleic acid amplification assays are summarized in Table 2. Assays that are currently available commercially for use in diagnostic laboratories include tests for the detection of *Chlamydia trachomatis*,[9–12] *C. pneumoniae*,[8] *Mycobacterium tuberculosis*,[14,15] *Mycoplasma pneumoniae*,[17] *Neisseria gonorrhoeae*,[19] herpes simplex virus[30] and cytomegalovirus.[24] In addition there are PCR assays available for monitoring the viral load of HIV,[31–33] hepatitis C virus[29] and hepatitis B virus.[28] Unfortunately only a few of these commercially available assays have been extensively evaluated to determine their sensitivity, specificity or clinical utility. Two tests that have

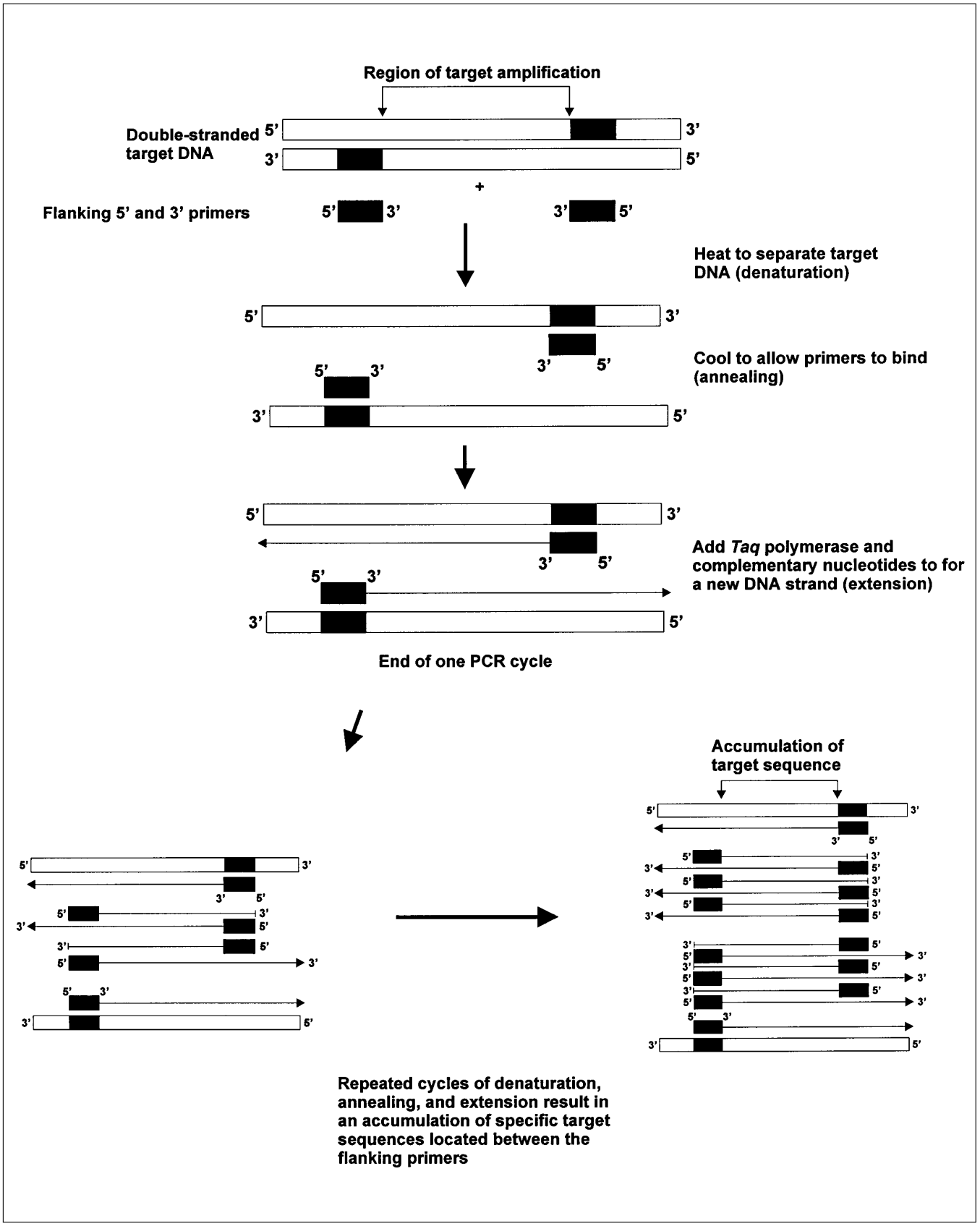

Fig. 1: Schematic representation of the polymerase chain reaction (PCR).

undergone such evaluations, and are currently among the most widely used PCR assays in diagnostic microbiology laboratories, are nucleic acid amplification assays for the detection of *C. trachomatis* and *M. tuberculosis* from clinical specimens.

One of the earliest commercial tests to become available was a PCR assay for the diagnosis of *C. trachomatis* genital tract infection. *C. trachomatis* is a fastidious microorganism, requiring specialized tissue culture facilities for laboratory isolation. Direct antigen detection of the organism by enzyme immunoassay or direct immunofluorescence is technically easier than culture but may lack sensitivity and specificity.[12,39] PCR assays have been found to be significantly more accurate, with sensitivities of 90%–100% and specificities greater than 97% for the detection of *C. trachomatis* from cervical or urethral specimens.[9-11] The positive predictive values reported in these studies ranged from 89% to 100%. A major advantage of these tests is the ability to detect *Chlamydia* in urine specimens. PCR testing of freshly voided urine was found to be the most sensitive (91%) and specific (100%) method for detecting asymptomatic *C. trachomatis* infection in men.[12] In addition, these assays have been automated, allowing for the processing of large numbers of specimens. They may be used for diagnosis or STD screening. A coamplification PCR assay for the direct detection of both *N. gonorrhoeae* and *C. trachomatis* from patients with STD has also been developed.[19] The sensitivity and specificity of PCR detection of *N. gonorrhoeae* from cervical and urethral specimens were found to be greater than 90% and 96% respectively.[19]

Table 2: Selected clinical applications of DNA amplification technology in infectious diseases and microbiology

Pathogens	Assay commercially available?	References
Bacteria		
Bordetella pertussis	No	Müller et al[6]
Borrelia burgdorferi	Yes	Brettschneider et al[7]
Chlamydia pneumoniae	Yes	Dalhoff et al[8]
Chlamydia trachomatis	Yes	Vincelette et al[9] Pasternack et al[10] Puolakkainen et al[11] Toye et al[12]
Escherichia coli O157:H7	No	Louie et al[13]
Mycobacterium tuberculosis	Yes	Piersimoni et al[14] D'Amato et al[15]
Mycobacterium avium complex	Yes	MacGregor et al[16]
Mycoplasma spp.	Yes	De Barbeyrac et al[17] Luki et al[18]
Neisseria gonorrhoeae	Yes	Crotchfelt et al[19]
Streptococcus pneumoniae	No	Matsumura et al[20] Chierian et al[21] Kearns et al[22]
Streptococcus pyogenes	No	Louie et al[23]
Viruses		
Cytomegalovirus	Yes	Long et al[24] Pellegrin et al[25]
Enterovirus	Yes	Van Vliet et al[26] Hadziyannis et al[27]
Hepatitis B virus	Yes	Pawlotsky et al[28]
Hepatitis C virus	Yes	Albadalejo et al[29]
Herpes simplex virus	Yes	Lakeman et al[30]
HIV	Yes	Nolte et al[31] Pachl et al[32] Segondy et al[33]
Fungi and parasites		
Cryptococcus neoformans	No	Vilgalys et al[34]
Plasmodium falciparum	No	Zhong et al[35]
Pneumocystis carinii	No	Helweg-Larsen et al[36]
Toxoplasma gondii	Yes	Burg et al[37]
Trichomonas vaginalis	No	Madico et al[38]

Direct amplification tests have also had a great impact on the rapid diagnosis of tuberculosis. Conventional culture methods for the isolation of mycobacteria generally take several weeks. Commercial amplification assays have been developed to provide accurate same-day results directly from clinical specimens.[14,15,40] These methods have been found to have sensitivities of about 90%–98%, as compared with culture of specimens that are smear-positive for acid-fast bacilli.[14,15] However, the performance of these amplification assays has been suboptimal for specimens without acid-fast bacilli seen on direct microscopic examination, with reported sensitivities as low as 46%.[15,41,42] The specificity of PCR-based assays for *M. tuberculosis* is excellent (> 98%).[14,15,42] Although these assays cannot replace mycobacterial cultures, their ability to determine rapidly the presence of *M. tuberculosis* directly from respiratory tract specimens has enabled more rapid institution of effective therapy and implementation of important infection control and public health interventions.

Nucleic acid amplification assays for the detection of viruses, such as herpes simplex virus, cytomegalovirus, enteroviruses and HIV, have proved to be useful for screening and for diagnosis and management. The Canadian Blood Services has recently adopted nucleic acid amplification methods to screen donated blood for hepatitis C and HIV because of the enhanced sensitivities of these assays. PCR detection of herpes simplex virus in cerebrospinal fluid has become the method of choice for the diagnosis of herpes encephalitis, with sensitivity and specificity of 95% and 94% respectively,[30] obviating the need for a brain biopsy.[2,30,43] Enteroviruses are among the most common causes of aseptic meningitis. PCR for the diagnosis of enteroviral meningitis using cerebrospinal fluid samples has been found to be significantly more sensitive than conventional viral isolation (14% of specimens positive v. 10% positive respectively).[26,27] Moreover, the PCR assay can be completed within 1 day, whereas cultures for enteroviruses typically require up to 5

days for isolation of the virus. A PCR assay for cytomegalovirus is available for detection of the virus in plasma or cerebrospinal fluid specimens and has been useful in monitoring HIV and bone marrow transplant patients with cytomegalovirus infection. The performance of this test has been comparable to that of antigen assays, with reported sensitivities and specificities of 95%–98% and 98%–100% respectively.[24,25] In contrast, the sensitivity of culture detection of cytomegalovirus was only 42%.[24]

In addition to these diagnostic applications, nucleic acid amplification procedures have also been modified to allow for the quantitative measurement of viral load in order to monitor response to therapy for patients with HIV, cytomegalovirus or hepatitis C virus infection.[25,29,31–33,44,45] For example, measuring HIV viral load in serum has had a major impact on the management of HIV-infected people. Viral load measurement is of prognostic importance, predicting progression of the disease, and is used to assist in making treatment decisions.[44,45]

A number of PCR assays that are not available commercially have potentially useful applications for the diagnosis of a variety of infectious diseases (Table 2).[6,13,20–23,34–36,38] Many of these tests are likely to become available in the near future. Multiplex PCR-based assays have been developed and have the advantage of detecting multiple pathogens in a single PCR reaction. These have been used to detect common bacterial and viral causes of respiratory tract infections,[8,46–49] bacteremia[50,51] and meningitis.[20–22,52,53]

PCR technology has also been used to identify infection owing to organisms that cannot be cultured. In order to accomplish this, investigators took advantage of the observation that portions of bacterial 16S ribosomal RNA sequences are highly conserved, whereas other regions are less well conserved and are species-specific. PCR amplification of 16S rRNA sequences of bacteria that cannot be cultured from tissues of patients with diseases such as Whipple's disease and bacillary angiomatosis allowed the discovery and identification of the etiologic agents.[54,55] Furthermore, using nucleic acid amplification methods, diseases previously thought to be noninfectious have been linked to infectious agents.[56]

Detection of antimicrobial resistance

As many of the genetic mechanisms of antimicrobial resistance have become better understood, nucleic acid amplification methods have proved to be useful for the confirmation of antimicrobial resistance in laboratory isolates and for the direct detection of such resistance in clinical specimens.[57] Conventional culture and susceptibility test procedures for most pathogenic bacteria generally take 48–72 hours. The performance of these tests may be erratic because factors such as inoculum size or variability in culture conditions may affect phenotypic expression of resistance. Amplification of genetic determinants may therefore be used to confirm antimicrobial resistance based on the organism's genotype rather than relying on the variability of phenotypic expression of the resistance (Table 3). More-

Table 3: PCR-based nucleic acid amplification for detection of antimicrobial resistance

Organism	Antimicrobial resistance	Gene targets for nucleic acid amplification (references)
Methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococci	Methicillin and all other β-lactam antibiotics	*mecA* (Vannuffel et al;[58] Murakami et al[59])
Vancomycin-resistant *Enterococcus* spp.	Vancomycin	*vanA, vanB, vanC1, vanC2, vanC3* (Satake et al;[60] Dutka-Malen et al;[61] Patel et al[62])
Streptococcus pneumoniae	Penicillin	*pbp1A* (du Plessis et al[63])
Enterobacteriaceae-producing extended-spectrum β-lactamase	Extended-spectrum penicillins and cephalosporins	SHV and TEM β-lactamase gene sequences (Arlet et al[64])
Mycobacterium tuberculosis	Isoniazid	*katG, inhA, ahpC* (Nachamkin et al[65])
	Rifampin	*rpoB* (Nachamkin et al;[65] Telenti et al[66])
Herpes simplex virus	Acyclovir	Thymidine kinase gene sequences (Sasadeusz et al[67])
Cytomegalovirus	Ganciclovir	Viral phosphotransferase gene (UL97), DNA polymerase gene (UL54) (Smith et al[68])
HIV	Reverse transcriptase inhibitors	Reverse transcriptase gene (Stuyver et al[69])
	Protease inhibitors	Protease gene (Vasudevachari et al[70])

over, these tests can be done within hours, providing clinically relevant information days before conventional susceptibility test results become available. Molecular assays to detect antimicrobial resistance directly from clinical samples have also been described.[58,60]

PCR-based methods for the detection of antimicrobial resistance have been applied to bacteria including methicillin-resistant *Staphylococcus aureus*,[58,59] vancomycin-resistant enterococci[60–62] and multidrug-resistant *M. tuberculosis*.[65,66] Detection of resistance to antiviral agents by molecular methods has also been described for acyclovir-resistant herpesviruses[67] and HIV resistant to reverse transcriptase inhibitors[69] and to protease inhibitors.[70] Currently none of these assays are available commercially, but they have been used in a number of reference and research laboratories.

The identification of methicillin resistance in *S. aureus* represents an ideal application of nucleic acid amplification methods. Methicillin-resistant *S. aureus* is an important hospital-acquired pathogen capable of causing life-threatening infections and nosocomial outbreaks. The incidence of infections from this pathogen in Canadian hospitals has increased dramatically in the past few years. Thus, the rapid and accurate identification of the pathogen is critical for patient management and for infection control programs in hospitals. However, the reliable detection of methicillin-resistant *S. aureus* using culture and susceptibility tests may be problematic because expression of resistance is usually heterogeneous and is influenced by culture conditions, especially in strains with low-level resistance.[71] All strains of methicillin-resistant *S. aureus* produce a unique penicillin-binding protein (PBP2′) that is encoded by a chromosomal gene, *mecA*. The *mecA* gene is not present in susceptible strains. PCR has been used successfully to amplify and detect *mecA* gene sequences from clinical isolates within a few hours.[59,72,73] These methods have also been used to detect methicillin-resistant *S. aureus* directly from clinical specimens such as blood cultures[74] and endotracheal aspirates.[58]

Vancomycin-resistant enterococci have also emerged as important nosocomial pathogens in North American hospitals. Identification using culture and susceptibility tests is even more problematic than that of methicillin-resistant *S. aureus*, primarily because of difficulties in detecting low levels of resistance[75] and because accurate identification using conventional laboratory procedures may take as long as 4–6 days. Vancomycin resistance in enterococci is mediated by one of several genes: *vanA*, *vanB*, *vanB2*, *vanC1*, *vanC2*, *vanC3* or *vanD*. PCR assays have been developed to recognize the *vanA*, *vanB* and *vanC* genotypes and have demonstrated value in characterizing enterococci in the laboratory when conventional laboratory test results have been inconclusive.[62,76] Another potential use of the assay is to assist in epidemiologic studies in the setting of an outbreak.[77] Finally, the ability to detect rapidly and accurately vancomycin-resistant enterococci directly from rectal swab specimens has also been reported.[60]

Incorporation of DNA amplification technology into the diagnostic microbiology laboratory

Newer DNA amplification methods have the potential to significantly influence the diagnosis and management of a variety of infectious diseases. Conventional laboratory diagnostic methods require a minimum of 24 hours, and in many cases significantly longer. Moreover, cultures may yield no bacterial growth if there has been a delay in transporting the specimen to the laboratory, if the number of viable infecting organisms is low, or if the patient was taking antibiotics by the time the culture specimen was obtained. Certain pathogenic organisms, such as *Mycoplasma* species, *Chlamydia* species, rickettsia and viruses, are not easily detected by routine culture methods and require specialized procedures. Rapid nonculture diagnostic tests relying on antigen detection by immunofluorescence or enzyme immunoassay, or using DNA probes, may have variable diagnostic sensitivities or specificities as compared with culture. Molecular methods with amplification and detection of target nucleic acids have generally been found to have superior sensitivity and specificity and have the potential to provide results within hours of collecting the specimen. As described here, currently available commercial tests using PCR for the diagnosis of infections include those able to detect *C. trachomatis*, *M. tuberculosis*, HIV, herpes simplex virus, cytomegalovirus, enterovirus, hepatitis C virus and other infectious agents. Many of these assays are now routinely being used in clinical microbiology laboratories. Diagnostic test kits for many other infectious agents are under development. Pilot studies have indicated the feasibility of designing broad-range multiplex PCR assays with the capability of detecting a panel of microorganisms from clinical specimens.[48,51,78,79] PCR-based methods have also been found to identify accurately antimicrobial resistance in clinical isolates and directly from patient specimens.[57,58,60,74]

Despite the obvious advantages to these newer procedures, there may be potential limitations to DNA amplification technology in the diagnostic microbiology laboratory (Table 4). The accuracy and reproducibility of PCR assays depend on the technical expertise and experience of the operator. Specificity of the test may be affected by contamination of the specimen during laboratory processing, if nonspecific primers are selected for the assay or if PCR conditions are not optimal, allowing nonspecific products to amplify. The most common sources of contamination are from other samples or from previous amplification procedures. Contamination or amplification product carry-over of even minute amounts of nucleic acid may result in the generation of billions of DNA copies that may lead to a false-positive test result. For this reason laboratories should have separate rooms for different steps of the PCR procedure and must follow stringent quality control measures to prevent contamination or carry-over. False-negative test

results may occur because of the presence of substances in the specimen that inhibit nucleic acid extraction or amplification. Certain specimen types (e.g., blood) are more likely to contain such inhibitors. The assays may also lack sensitivity if there is a low inoculum of the microorganism present in the clinical specimen. This may be exacerbated if an inadequate sample or very small specimen volume (i.e., < 20 μL) is available for testing.

Interpretation of nucleic acid amplification test results is not always clear-cut. For example, assays may detect the residual DNA of a pathogenic microorganism even after successful treatment,[80] and it is not clear whether this represents the presence of a small number of viable organisms or amplified DNA from nonviable organisms. Therefore, PCR tests should not be used to monitor the effectiveness of a course of therapy,[39] and physicians must be aware of the laboratory testing procedures. In addition, the meaning of a positive PCR test result has not been validated for all infections. For example, it is uncertain whether a positive PCR test result for cytomegalovirus from a patient's serum represents active disease or latent infection. Similarly, detection of pneumococcal DNA in blood samples has been reported in asymptomatic children colonized with *S. pneumoniae*[81] and therefore may not always indicate an invasive infection. These observations suggest that there is a need for interpretive guidelines based on a correlation of nucleic acid amplification test results with clinical outcome.

Finally, it must be acknowledged that performance of a PCR assay is generally more expensive than conventional diagnostic laboratory methods. The requirement of separate rooms for pre-PCR and post-PCR steps in order to reduce the risk of cross-contamination means that molecular laboratories use a disproportionate amount of laboratory space. There are capital costs associated with the initial equipment purchase (about Can$15 000), reagent costs for each clinical and control sample processed (Can$8–$40) and labour expenses. Therefore, the cost of these assays has been reported to be as high as Can$125 per test.[2]

Molecular technology involving nucleic acid amplification and detection is a promising tool for the rapid and accurate diagnosis of a variety of infectious diseases, and for the confirmation or detection, or both, of antimicrobial resistance (Table 4). Some of these tests are now widely used for the diagnosis of tuberculosis and *C. trachomatis* infection, and other assays have become important in the management of HIV infection and hepatitis C. A large number of PCR assays are still under development with the potential to provide accurate and rapid results when conventional methods are either not available, insensitive or too slow. To date, evaluations of this technology have generally been limited by small samples and have not considered how these assays should fit into routine laboratory procedures, particularly in smaller, nonreference laboratories. As this technology continues to evolve, it will be important to assess the cost-effectiveness of these procedures and their real impact on patient management and outcomes.

Table 4: Potential advantages and limitations of PCR in the diagnosis of infectious diseases

Advantages	Limitations
High sensitivity	Potential for false-positive test results (e.g., by amplification of "contaminating" DNA)
High specificity	
Good reproducibility	Potential for false-negative test results (e.g., because of presence of PCR inhibitors interfering with nucleic acid amplification)
Ability to detect the presence of infecting microorganisms that may not be identified by conventional methods	
Rapidity, able to provide same-day results	Interpretation of positive PCR test results not yet validated for all infectious diseases (e.g., latent v. active infection)
	Technically complex procedures
	Expensive equipment and reagents

Competing interests: None declared.

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KCDC
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FINDINGS FROM INVESTIGATION AND ANALYSIS OF RE-POSITIVE CASES

19 May 2020

SUMMARY

○ Epidemiological investigation and contact investigation have been completed for 285 (63.8%) of the total 447 re-positive cases (as of 15 May).

○ 59.6% were tested as a screening measure, and 37.5% were tested because of symptom onset. Of the 284 cases for which symptoms were investigated, 126 (44.7%) were symptomatic.

○ From the 285 re-positive cases, a total of 790 contacts were identified (351=family; 439=others). From the monitoring of contacts, as of now, no case has been found that was newly confirmed from exposure during re-positive period alone.

NOTE

○ In response to reports of multiple cases testing positive for SARS-CoV-2 after being discharged from isolation, on 14 April, KCDC began managing such cases with measures similar to those for confirmed cases, while further investigation, research and analysis continued. On 18 May, KCDC announced the findings and the conclusions of the advisory committee. The protocols for the management of such cases will be revised accordingly.

- Based on the findings, starting 0:00 of 19 May, KCDC has stopped applying the previous protocols for the management of confirmed cases after discharge from isolation and for the management of re-positive cases. Under the new protocols, no additional tests are required for cases that have been discharged from isolation.
- Reporting and investigation of re-positive cases and investigation of contacts of re-positive cases will be continued as before for the purposes of research and investigation. However, based on experts' recommendations, the terminology for referring to such cases will be changed from "re-positive" to "PCR re-detected after discharge from isolation".

○ In this document, "discharge" refers to discharge or release from isolation of confirmed cases after recovery and meeting all discharge criteria (in accordance with KCDC guidelines).

○ "Re-positive cases" are cases that test positive for SARS-CoV-2 after being discharged from isolation.

PROPORTION OF RE-POSITIVE CASES

○ Depending on the group, 25.9-48.9% of cases tested positive again after discharge.

Region	Group	Tested (n)		Re-positive (n)	(%)
Sejong City	All confirmed cases	27		7	25.9%
Daegu City	Confirmed cases related to schools (school staff, students)	Total	195	53	27.2%
		School staff	47	6	12.8%
		Students	148	47	31.8%
Gyeongbuk Province	Confirmed cases of Pureun Nursing Home	47		23	48.9%

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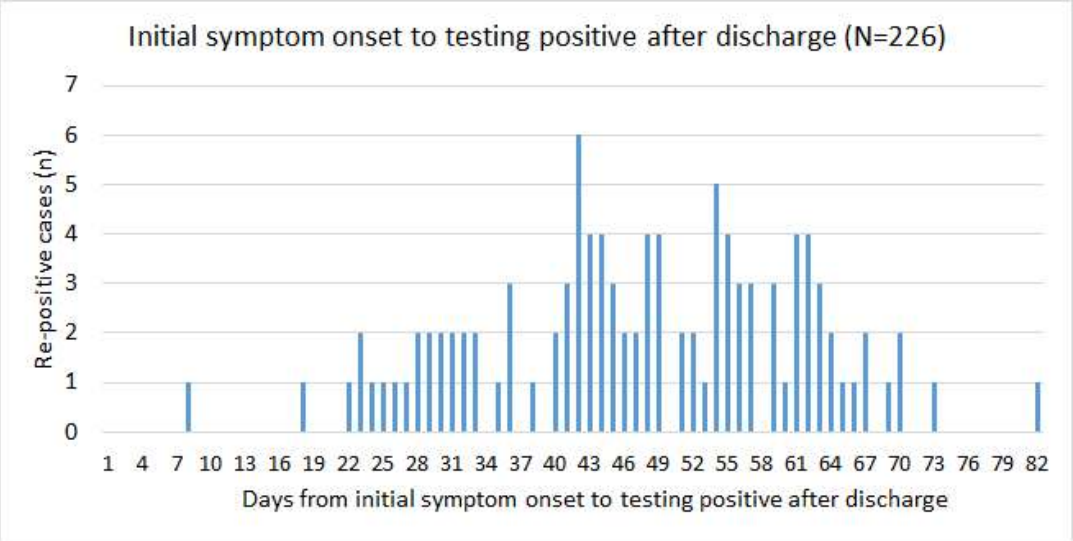
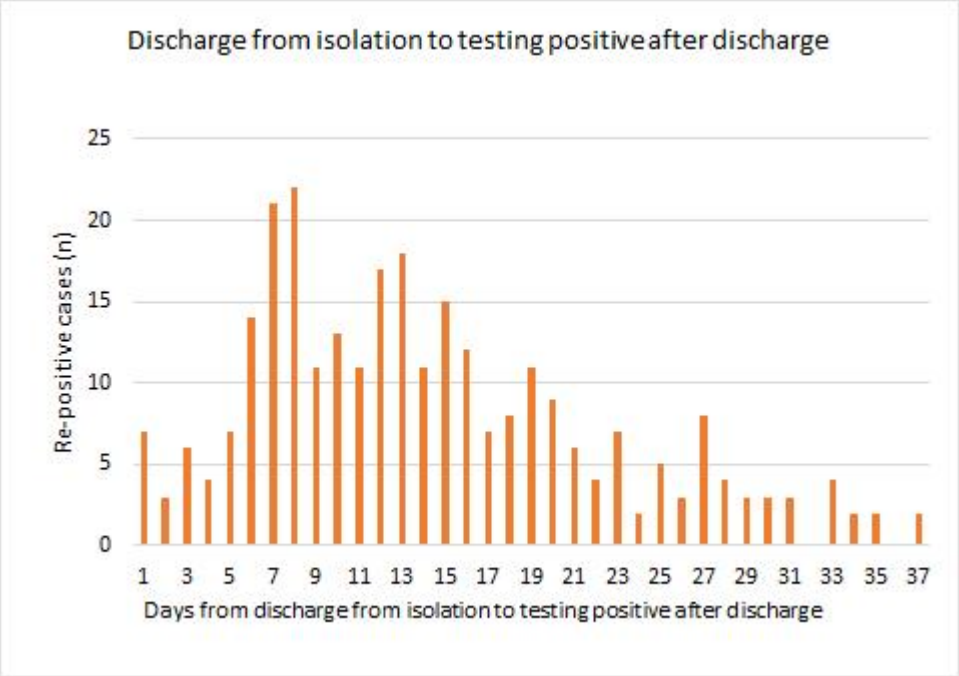
19 May 2020

TIMING OF TESTING RE-POSITIVE

○ On average, it took 44.9 days (range: 8-82 days) from initial symptom onset date to testing positive after discharge. (Based on 226 cases symptomatic at the time of initial confirmation)



○ On average, it took 14.3 days (range: 1-37 days) from discharge to testing positive. (Based on 285 cases)



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SYMPTOMS AND TESTING OF RE-POSITIVE CASES

○ 59.6% of the re-positive cases were tested for screening, regardless of symptoms.

○ 44.7% of re-positive cases had symptoms such as coughs, sore throat, etc.

(n, %)

		Re-positive cases	
Total		285	
Reason for testing	Symptoms present	107	(37.5)
	Investigation	170	(59.6)
	Requested (by self or guardian)	8	(2.8)
Symptoms *284 cases for which symptoms were checked	Symptoms present	126	(44.7)
	Symptoms absent	158	(56.6)

FINDINGS FROM MONITORING OF CONTACTS OF RE-POSITIVE CASES

○ For the 285 re-positive cases investigated, 790 contacts were found in total. Minimum 14-day monitoring found 27 of the contacts to be positive, 24 of which were cases that were previously confirmed.

○ There were 3 newly confirmed cases from the 790 contacts of re-positive cases.

- Other than their exposure to the re-positive cases during their respective re-positive period, all of the 3 newly confirmed cases had history of contact with Shincheonji religious group or a confirmed case in their family.
- Virus isolation cell culture result was negative for 2 of the newly confirmed cases. (Viral cell culture test was not possible for 1 case as the PCR result was indeterminate.)
- In all re-positive cases and newly confirmed cases, neutralizing antibody production was found from the first serum.

		Re-positive cases	Contacts	Confirmed cases among contacts
Total		285	790	27* (3.4)
Presence of symptoms in re-positive cases * 284 cases for which symptoms were checked	Yes	126 (44.2)	431 (54.6)	18 (4.2)
	No	158 (55.4)	359 (45.4)	9 (2.5)
Type of contact	Family	-	351 (44.4)	26 (7.4)
	Other	-	439 (55.6)	1 (0.2)

*24 of the 27 are previously confirmed and re-positive cases (included in the re-positive cases)

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VIRUS ISOLATION IN CELL CULTURE OF RE-POSITIVE CASES

○ Viral cell culture testing of 108 re-positive cases all had negative results. Basic analysis of 93 of the cases found the following results:

- From testing for 8 respiratory viruses, another respiratory virus was detected in 3 of the cases.
- The Ct values in real-time RT-PCR during re-positive period is found to be above 30 at 89.5%.

		N	(%)
Total		93	
Region	Seoul	2	(2.2)
	Daegu	47	(50.5)
	Incheon	7	(7.5)
	Sejong	2	(2.2)
	Gyeonggi	6	(6.5)
	Gangwon	4	(4.3)
	Gyeongbuk	22	(23.7)
	Gyeongnam	3	(3.2)
Sex	Male	31	(33.3)
	Female	62	(66.7)
Age	0-9	1	(1.1)
	10-19	4	(4.3)
	20-29	19	(20.4)
	30-39	10	(10.8)
	40-49	10	(10.8)
	50-59	18	(19.4)
	60-69	12	(12.9)
	70 or above	19	(20.4)
Symptoms	Symptoms present	45	(48.4)
	Symptoms absent	48	(51.6)
8 respiratory viruses*	Negative	90	(96.8)
	Adenovirus	2	(2.2)
	Bocavirus	1	(1.1)
Ct value in real-time RT-PCR** (RdRp gene)	25-30	8	(10.5)
	Above 30	68	(89.5)

* Influenza, parainfluenza, rhinovirus, metapneumovirus, human coronavirus, adenovirus, bocavirus, respiratory syncytial virus

** result upon testing re-positive (N=76)

RESULTS OF NEUTRALIZING ANTIBODY TESTING ON RE-POSITIVE CASES

○ Of the 23 re-positive cases from whom the first and the second serum samples were obtained, 96% were positive for neutralizing antibodies.

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FINDINGS FROM INVESTIGATION AND ANALYSIS OF RE-POSITIVE CASES

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[ATTACHMENT: EXPERT ADVISORY COMMITTEE RESULT ON RE-POSITIVE CASES]

FINDINGS FROM INVESTIGATION AND ANALYSIS OF RE-POSITIVE CASES

○ Based on active monitoring, epidemiological investigation, and laboratory testing of re-positive cases and their contacts, no evidence was found that indicated infectivity of re-positive cases.

- Of the 447 re-positive cases as of 15 May, epidemiological investigation was conducted on 285 cases and laboratory analysis on 108 cases. (*473 as of 18 May)
- From monitoring of 790 contacts of the 285 re-positive cases, no case was found that was newly infected solely from contact with re-positive cases during re-positive period.
- Virus isolation in cell culture of respiratory samples of 108 re-positive cases, all result was negative (i.e. virus not isolated).
- Of the 23 re-positive cases from which the first and the second serum samples were obtained, 96% were positive for neutralizing antibodies.

PROTOCOLS FOR MANAGEMENT OF CONFIRMED AND RE-POSITIVE CASES

○ Management of confirmed cases after discharge from isolation and management of re-positive cases will no longer be conducted. (Effective 0:00 of 19 May)

	Before	**After**
Management of confirmed cases after discharge from isolation	14 day self-isolation recommended after discharge from isolation	Not needed
	PCR test required if symptoms appear within 14 days of discharge from isolation	Not needed
Management of cases that test positive after discharge from isolation	Re-positive cases managed similar to management of confirmed cases (isolation)	Not needed
	Contacts managed similar to management of contacts of confirmed cases (quarantine)	Not needed
Investigation of re-positive cases	Reporting of re-positive cases and investigation	Same as before
	Investigation of contacts of re-positive cases	Same as before

* The new protocols will also be retroactively applied to the cases currently under management after discharge, re-positive cases currently under isolation, and contacts currently within monitoring period.

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Growth, detection, quantification, and inactivation of SARS-CoV-2

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ABSTRACT

Severe acute respiratory syndrome coronavirus (SARS-CoV)-2 is the agent responsible for the coronavirus disease 2019 (COVID-19) global pandemic. SARS-CoV-2 is closely related to SARS-CoV, which caused the 2003 SARS outbreak. Although numerous reagents were developed to study SARS-CoV infections, few have been applicable to evaluating SARS-CoV-2 infection and immunity. Current limitations in studying SARS-CoV-2 include few validated assays with fully replication-competent wild-type virus. We have developed protocols to propagate, quantify, and work with infectious SARS-CoV-2. Here, we describe: (1) virus stock generation, (2) RT-qPCR quantification of SARS-CoV-2 RNA; (3) detection of SARS-CoV-2 antigen by flow cytometry, (4) quantification of infectious SARS-CoV-2 by focus-forming and plaque assays; and (5) validated protocols for virus inactivation. Collectively, these methods can be adapted to a variety of experimental designs, which should accelerate our understanding of SARS-CoV-2 biology and the development of effective countermeasures against COVID-19.

1. Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV)-2 is an enveloped virus with a single-stranded positive-sense RNA genome. Zoonotic transmission of SARS-CoV-2 from an as yet unidentified animal reservoir occurred in late 2019. Subsequent human-to-human transmission by respiratory droplets has resulted in the ongoing Coronavirus disease 2019 (COVID-19) pandemic that has infected millions of people worldwide (Wu et al., 2020b; Zhou et al., 2020; Zhu et al., 2020). The rapid spread and relatively high case fatality rate of COVID-19 has led to an urgent need to develop diagnostics, therapeutics, and vaccines.

The SARS-CoV-2 genome is comprised of approximately 30,000 nucleotides. The first two-thirds of the genome encodes for non-structural proteins in open reading frames 1a and 1b that principally facilitate genome replication and viral RNA synthesis. The remaining one-third is comprised of genes encoding structural proteins such as spike (S), envelope (E), membrane (M), and nucleocapsid (N), which form the virion, and accessory proteins that regulate host cellular responses. Whole-genome phylogenetic analysis identified the SARS-like bat CoV (GenBank MG772933) as the closest known relative of SARS-CoV-2. Bats also are the reservoir host for SARS-CoV (Wu et al., 2020a). Alignment of SARS-CoV-2 to the consensus sequence of SARS-like CoV revealed 380 amino acid differences including 27 amino acid differences in the S protein and six substitutions in the receptor binding domain (RBD) (Wu et al., 2020a).

SARS-CoV entry is mediated by initial engagement of the RBD of the S protein with the human ACE2 receptor (Li et al., 2003, 2005), and recent studies have established that SARS-CoV-2 utilizes the same receptor for entry (Letko et al., 2020). The S protein also is a key target for neutralizing antibodies and vaccine strategies (Rockx et al., 2008; Sui et al., 2005; Zhu et al., 2007). Although the S protein of SARS-CoV and SARS-CoV-2 are structurally similar (Li et al., 2005; Walls et al., 2020; Wrapp et al., 2020), genetically similar (Walls et al., 2020), and use the same receptor (Lei et al., 2020; Li et al., 2003), neutralizing anti-SARS-CoV RBD antibodies (Abs) generally lack cross-reactivity to SARS-CoV-2 (Wrapp et al., 2020). However, polyclonal sera from mice immunized with recombinant SARS-CoV RBD protein inhibits SARS-CoV-2 infection (Walls et al., 2020). Recent studies have identified cross-reactive, non-neutralizing monoclonal Abs (mAbs) against SARS-CoV and SARS-CoV-2, which were isolated previously using phage display or hybridoma fusion screens (Joyce et al., 2020; ter Meulen

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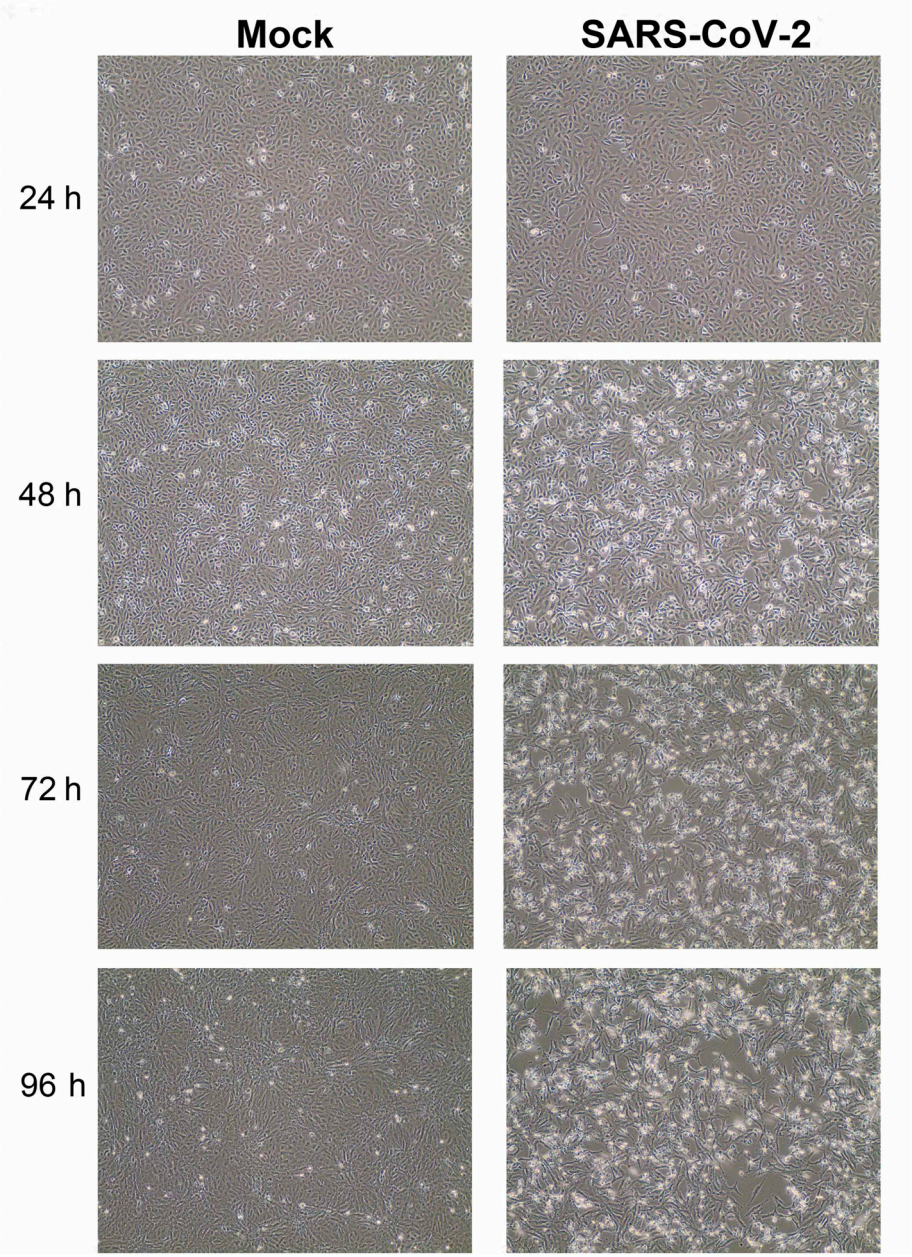

Fig. 1. SARS-CoV-2 causes cytopathic effect on Vero E6 cell monolayers. Vero E6 cells were inoculated with SARS-CoV-2 at an multiplicity of infection (MOI) of 0.01 plaque forming unit (PFU)/cell and monitored for cytopathic effect at the indicated timepoints. Images were collected using an EVOS XL Core Imaging System. Magnification is 10X for all images.

et al., 2006; Tian et al., 2020; Tripp et al., 2005; Yuan et al., 2020). Competition binding studies show that two of these mAbs, CR3022 and 240CD, both recognize the SARS-CoV-2 RBD. A co-crystal structure revealed that CR3022 binds an epitope on the RBD distal to the binding site of ACE2 and SARS-CoV neutralizing antibodies (Yuan et al., 2020).

SARS-CoV-2 research must be performed in a biosafety level 3 laboratory by personnel equipped with a powered air-purifying respirator (PAPR). This limitation has compelled the development of many *in vitro* assays that utilize heterologous pseudotyped viruses expressing the SARS-CoV-2 S protein (Lei et al., 2020; Letko et al., 2020). However, this approach only can be used to study cellular and antibody interactions involving the S protein that principally affect attachment and entry. Here, we developed or adapted multiple methodologies to quantify SARS-CoV-2 infection *in vitro* using a patient isolate of SARS-CoV-2: 1) RT-qPCR quantification of viral RNA; 2) detection of viral antigen by flow cytometry; 3) focus-forming assay through immunostaining of the S protein and 4) plaque assay. We also have identified and validated chemical and heat treatment methods to inactivate replication-competent virions, which are compatible with downstream quantification assays. Together, the methodologies can be used to examine SARS-CoV-2 pathogenesis and antibody responses, and to screen for potential inhibitors of infection.

2. Results and discussion

Propagation of SARS-CoV-2 *in vitro*. Isolates of SARS-CoV-2 from patients or animals often need to be propagated to generate high-titer virus stocks. We have tested several cell types and found African Green Monkey cell lines and derivatives thereof to be most permissive to SARS-CoV-2 infection. These include Vero-CCL81 (ATCC-CCL81), Vero-furin (Mukherjee et al., 2016), Vero E6 (ATCC-CRL1586), Vero-TMPRSS2 (Matsuyama et al., 2020), and MA104 (ATCC-CRL-2378.1)

cells. Each cell type is sufficient to propagate SARS-CoV-2 using the protocol detailed below. All procedures should be completed only after appropriate safety training is obtained and using aseptic technique within a certified biosafety cabinet under BSL-3 containment.

2.1. Materials needed

Chosen cell type (Vero-CCL81, Vero-furin, Vero E6, Vero-TMPRSS2, and MA104 cells)
Standard media for chosen cell type (see Recipes)
Infection media (see Recipes)
SARS-CoV-2 seed stock
150 cm^2 (T150) tissue culture flasks
15 mL disposable polystyrene conical tubes with screw caps (*e.g.*, Falcon)
50 mL disposable polystyrene conical tubes with screw caps (*e.g.*, Falcon)
1.5 mL or 0.5-mL O-ring tubes

1.) In a standard BSL2 laboratory, plate cells for infection one day prior into two T150 flasks in standard media for the chosen cell type. One flask serves as a mock-infected control and the other for infection. Plate cells so they will be ~80–90% confluent the following day. **For instance, plate 1 x 10^7 Vero CCL81 cells* per *T150 flask.* Place flasks in a humidified 37 °C incubator with 5% CO_2 overnight.
2.) Transfer flasks into BSL3 facility the following day. Rapidly thaw a SARS-CoV-2 stock at 37 °C. Calculate the volume of virus needed to infect at the desired multiplicity of infection (MOI) using the following formula:

$$\frac{(\#\text{ of cells in a confluent T150}) \times (\%\text{ confluency at present}) \times (\text{desired MOI})}{\text{Virus titer in PFU/mL}} = \text{Volume of virus needed (mL)}$$

3.) Add the volume of virus calculated above to 20 mL of infection medium.
4.) Remove medium from T150 flasks. Replenish with 20 mL of fresh infection medium for mock-infected flask. Add 20 mL of infection medium containing virus from step #3 to flask for infection.
5.) Incubate for 48–72 h at 37 °C monitoring daily for evidence of cytopathic effect (CPE) (Fig. 1). Use the mock-infected flask as a control for subtle CPE.

**Harvesting at 48–72 h post-inoculation has yielded the best titers in our hands; although, titers remain roughly the same when incubated for longer periods (4–5 days). CPE should be apparent by day 3 in Vero or MA104 cells.*

6.) To harvest virus, collect the cell culture supernatant by pipetting the media into two 15 mL conical tubes. Centrifuge at 450 × *g* for 5 min at 4 °C to clarify supernatants and pellet cell debris. Combine the supernatant from all tubes into a single vessel and gently mix using a serological pipette to ensure homogeneity across aliquots of the stock. Pipette the supernatant into small aliquots (200–500 µL) in O-ring tubes. Store at −80 °C.

Real-time PCR assay for SARS-CoV-2 detection. Detection of viral RNA by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) using a TaqMan probe is a highly-sensitive and specific method for measuring viral burden in a variety of specimens. Because CoVs generate subgenomic RNAs as a template for translation, the abundance of viral RNA varies for each gene and depends upon the gene position within the genome. Genes located closer to the 3′ end of the (+) sense genome will have a greater abundance of transcripts than those located at the 5′ end of the (+) sense genome. This should be considered when designing primer/probe combinations, as "N gene" transcripts will be more abundant than genomic RNA copies, which can be quantified by targeting sequences within the ORF1a gene. Many primer/probe combinations have been designed and validated, several of which are used in clinical diagnosis (CDC, 2020; Corman et al., 2020). In the clinical setting, precise copy-number quantitation of viral RNA is not necessary and instead sensitivity is paramount. However, quantitative assays are desirable for research applications, and may have utility in longitudinal studies of infected human subjects. RT-qPCR cycle threshold (Ct) values can be converted to transcript or genome copy number equivalents by generating an RNA standard curve, the design and production of which is described below.

2.2. Design of the primer/probe combination

The CoV replication strategy should be considered when designing a RT-qPCR assay. Primer/probe combinations targeting the N gene are most sensitive; those targeting the spike gene can also be used to titer spike-containing pseudoviruses; those targeting the ORF1a gene provide genome equivalents; and those targeting the leader sequence can give an estimation of the total number of viral transcripts (Table 1). For a given viral gene target, a template (~500–1000 bp) for *in vitro* transcription can be generated by RT-PCR using primers that flank the intended target, with the forward (F) primer also including a 5′ T7 promoter sequence (Vogels et al., 2020). If multiple targets are desired, a single dsDNA fragment can be synthesized to include concatenated gene fragments, each of which spans the entirety of the target amplicons. This strategy also can be used to quantify host genes of interest (*e.g.*, ACE2).

2.3. Construction of the RNA standard

1. (Day 1) The DNA fragment/amplicon containing the primer/probe targets to be used in the RT-qPCR assay should be introduced into a vector containing a T7 (or other DNA-dependent RNA-polymerase) promoter sequence using Gibson Assembly, restriction enzyme cloning, blunt-end ligation, or gene synthesis. These vectors should be transformed into competent *E. coli* (*e.g.*, DH5α) for antibiotic selection.
2. (Day 2) Pick clones and amplify to miniprep scale. *We normally pick*

Table 1
Primer/probe combinations for detection of SARS-CoV-2 RNA.

Assay name	Target	F primer sequence	R primer sequence	Probe Sequence	Designer
5′UTR	5′UTR	ACTGTCGTTGACAGGACACG	AACACGGACGAAACCGTAAG	CGTCTATCTTCTGCAGGCTG	ALB
ORF1a	ORF1a	TTCAGTTGACTTCGCAGTGG	GGACGGGTTTGAGTTTTTCA	AACTAACATCTTTGGCACTGTTT	ALB
nCoV_ALB	N gene	ATGCTGCAATCGTGCTACAA	GACTGCCGCCTCTGCTC	TCAAGGAACAACATTGCCAA	ALB
N1	N gene	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	ACCCCGCATTACGTTTGGTGGACC	CDC
N2	N gene	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	ACAATTTGCCCCCAGCGCTTCAG	CDC
N3	N gene	GGGAGCCTTGAATACACCAAAA	TGTAGCACGATTGCAGCATTG	AYCACATTGGCACCCGCAATCCTG	CDC

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CDC = Centers for Disease Control and Prevention.

6 to 12 clones to ensure proper cloning.

3. (Day 3) Purify plasmid from clones, and identify a clone with the proper insert using restriction enzyme digestion and/or Sanger sequencing.
4. (Day 4) Linearize ~2–4 μg of the DNA in preparation for *in vitro* transcription by performing an overnight restriction digest using a high-fidelity restriction enzyme that cuts each plasmid only once in a position 3′ to the insert. The distance between the T7 transcriptional start-site and the 3′ end restriction site should be ~500–1500 nucleotides.
5. (Day 5) Run the linearized product on a 1% agarose gel. A shift in fragment size should be apparent relative to the non-linearized plasmid. Extract and cleanup the linearized product with a commercially-available gel-extraction (*e.g.,* Qiagen) kit.
6. Perform *in vitro* transcription using a commercially available kit (*e.g.*, MEGAscript T7). Note: to prevent contamination of PCR workstations with transcribed RNA, all steps hereafter should be performed in a contained hood/workspace that is separate from the area where PCR reaction setup is performed.
7. Digest DNA with DNase, then perform RNA cleanup using a commercially available kit (*e.g.*, MEGApure).
8. Quantify the RNA using a spectrophotometer (*e.g.,* Nanodrop or Qubit) by diluting the RNA with RNase-free water until the concentration is within the analytical measurement range of the spectrophotometer.
9. Calculate the copies of RNA transcript within each μL:

$$\frac{\text{concentration of RNA } (\frac{\text{ng}}{\mu\text{L}}) \times \text{Avogadro's number}}{\text{molecular weight of transcript } (\frac{\text{g}}{\text{mol}}) \times 1{,}000{,}000{,}000} = \textit{RNA copies}/\mu L$$

Note: the molecular weight can be calculated online (e.g., OligoCalc website).

10. Dilute the transcript with RNase-free water containing 1% of added ribonuclease inhibitor (*e.g.*, RNaseOUT) to obtain 1–2 mL of standard at a 1×10^{10} copies/μL. Mix by pipette.
11. Aliquot the diluted RNA transcript into PCR strip tubes (with individual caps) in aliquots of 6–12 μL/aliquot.
12. Freeze at −80 °C. The remaining concentrated RNA can be frozen and re-quantified later as needed.

2.4. Validation and use of the RNA standard

The RNA standard is concentrated and poses a risk for contamination of reagents and specimens. Follow best-practices for PCR preparation (Standards Unit, 2010) and only handle RNA standards after all reagents and specimens have been stored. Appropriate no-template controls must be used to eliminate and track possible contamination. Wipe down work areas and pipettes with 10% bleach followed by 70% ethanol. Bleach pipette tips.

1. Create a 20x stock of primer/probe mix by diluting primers to a concentration of 10 μM and probe to a concentration of 2 μM.
2. For "n" number of reactions, create a master-mix for n + 1 by combining one-step RT-qPCR reaction buffer, primer/probe mix, and reverse-transcriptase enzyme at the appropriate concentration/volumes. Aliquot master-mix into wells of a RT-qPCR-compatible plate.
3. Separate a single tube containing the RNA standard from the stock. Work quickly to avoid thawing other aliquots in the adjacent strip tubes.
4. Thaw the aliquot and briefly centrifuge to collect contents at the bottom of the tube.
5. Dilute the standard into a volume of RNase-free water to obtain 1.0×10^9 RNA copies per reaction. Mix gently but thoroughly by pipette. Change gloves.
6. Make 10-fold serial dilutions in a PCR strip-tube by transferring 10 μL into 90 μL of RNase-free water. Mix each dilution thoroughly with a p100 pipette set to 70 μL. Discard tips between each dilution.

Note: When testing a new RNA standard, perform serial dilutions several-fold below 1 copy per reaction. Reactions containing less that 1–10 copies/well should fail to amplify.

7. Transfer the appropriate volume of RNA standard from each dilution into the reaction plate using a multichannel pipette.
8. Perform real-time PCR using the following thermocycling parameters:
   1. 48 °C for 15 min
   2. 95 °C for 10 min
   3. 95 °C for 15 s
   4. 60 °C for 1 min – Acquire Signal
   5. Go to "step 3″ 49x (*i.e.,* 50 cycles)

Note: these parameters may vary depending on the specific RT-qPCR kit used; our parameters have been tested using the TaqMan RNA-to-CT 1-step kit (Applied Biosystems) on the QuantStudio 6 flex Real-time PCR system (Applied Biosystems).

9. Upon completion of the run, examine your standard curve. Approximately 3.3 Ct should separate each dilution, which corresponds to a change of one $\log_{10}$ copies for a reaction that is > 90% efficient.

Quantification of SARS-CoV-2 by plaque assay. The plaque assay is the gold standard test for quantifying infectious virus in a sample. The plaque assay measures "plaques," which describe the zone of cellular death that occurs after one infectious unit has entered a cell and spread to adjacent cells over the time period of incubation (Fig. 2). The assay does not rely on the use of any virus-specific reagents, which is beneficial when reagents are unavailable. As this cell-based assay typically is performed in 6-well plates, it is relatively low-throughput, labor-intensive, and may not be reliable when the samples themselves are cytotoxic (*e.g.,* homogenate from certain tissues) or when the virus is poorly cytopathic in a given cell type. Thus, it is important to choose a highly permissive cell type (*e.g.*, Vero E6 cells) for which SARS-CoV-2 causes substantive cell death.

2.5. Materials needed

Vero E6 or Vero-furin cells
Vero cell culture medium (see Recipes)
Infection media (see Recipes)
Virus to be titered
96-well U-bottom plates
6-well or 12-well tissue culture plates
2X MEM + 4% FBS (see Recipes)
2% methylcellulose (see Recipes)
4% paraformaldehyde solution (in PBS)
Crystal violet staining solution (see Recipes)

1. Plate approximately 7.5×10^5 Vero E6 or Vero-furin cells/well into 6-well plates. Plate enough wells to test each dilution in duplicate (starting from 10^{-1} to 10^{-6}; 10-fold dilutions). Incubate cells overnight (12–18 h) at 37 °C.

**12-well tissue culture plates also will work. Plate approximately 2.5×10^5 cells/well.*

2. Dilute samples to be titered in infection media in 96-well U-bottom plates. Make a 10-fold dilution series, providing enough volume to add 200 μL per 6-well plate.

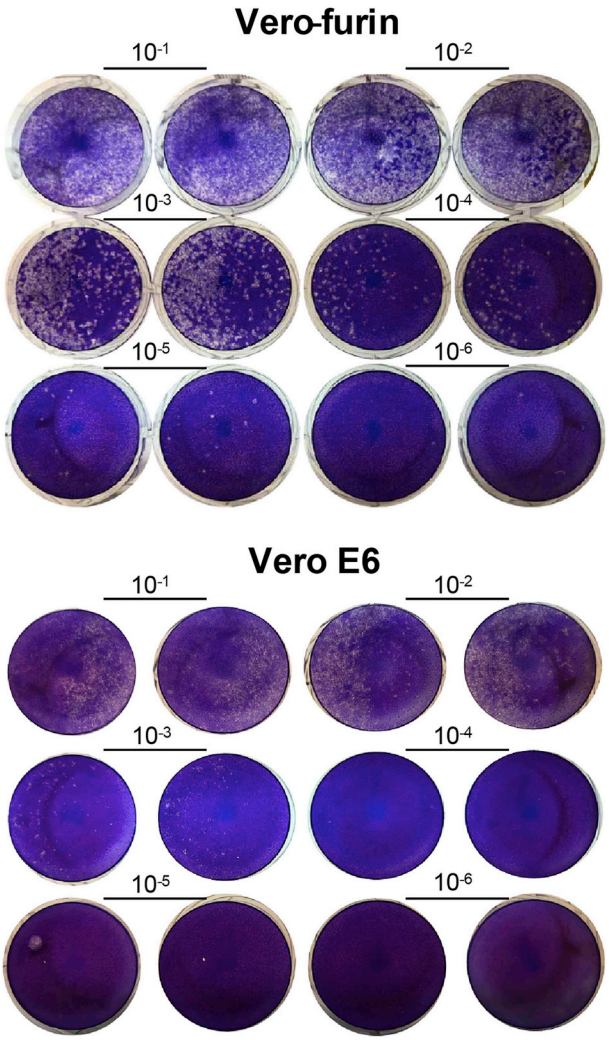

Fig. 2. Crystal violet stained plaque assay plates. Vero-furin or Vero E6 cells were inoculated with 10-fold serial dilutions of a SARS-CoV-2 stock. Plates were fixed three days post-infection and stained with crystal violet. Wells with individual plaques were used to determine the virus titer (Vero-furin 10^{-4}, Vero E6 10^{-3}). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Remove existing cell culture media from 6-well plates. Add 200 µL of each dilution to one well of a 6-well plate (200uL to 12-well plate) starting with most diluted so the same pipette tip can be used up the dilution series.
4. Incubate 6-well plates at 37 ˚C in 5% CO_2 for 1 h, rocking plates every 15 min to prevent cells from drying out.
5. Meanwhile, mix 2X MEM + 4% FBS with 2% methylcellulose in a 1:1 ratio. Place in 37 ˚C incubator while plates are incubating to decrease viscosity of the solution.
6. After 1 h incubation, add 2 mL of MEM:methylcellulose mixture to each well of the 6-well plates (1 mL–12-well plate).
7. Incubate plates at 37 ˚C in 5% CO_2 for 3 days.

**After 3 days, plaques should be visible by eye when carefully held up to the light. If plaques are too small to discern, plates can be incubated for an additional day.*

8. On day 3, gently remove methylcellulose overlays with a pipette and fix cells by adding 3 mL of 4% paraformaldehyde (PFA) in PBS to each well. Incubate at room temperature for 20 min.
9. Remove 4% PFA into an appropriate hazardous waste container. If this concentration of PFA has been approved as a method of SARS-CoV-2 inactivation by the Institutional Biosafety Committee, plates can be removed from the BSL3.
10. Add 1 mL of 0.05% (w/v) crystal violet in 20% methanol to each well. Incubate for 20–30 min. Remove crystal violet with a pipette and wash twice with dH_2O or until excess crystal violet is removed, and plaques are easily visualized.
11. Count the plaques at the dilution in which there are 10–100 plaques. Calculate titer in PFU/mL using the following formula: Titer (PFU/mL) = number of plaques counted × $10^{\text{dilution counted}}$ × 5 (to get to mL because we added 200 µL of diluted sample)

$$\text{Titer}\left(\frac{\text{PFU}}{\text{mL}}\right) = \frac{\text{Number of plaques counted}}{0.2\ \text{mL}} \text{x}\ 10^{\text{Dilution counted}}$$

Quantification of SARS-CoV-2 by focus-forming assay. A focus-forming assay is similar to a plaque assay in that it detects infectious virus in a sample. A "foci" describes the zone of cells that have become infected from a single infectious unit. These foci of cells express high amounts of viral antigen, which can be detected using a virus-specific antibody that is directly conjugated to a colorimetric readout (*e.g.* peroxidase) or through use of secondary antibodies (Fig. 3). This approach adds specificity to the assay, but also increases the number of processing steps post-infection. However, because the focus-forming assay captures infected foci before the cells die and develop into plaques, this assay typically requires shorter incubation times than the plaque assay. It also can be performed in 96-well plate format, which can increase throughput.

2.6. Materials needed

Vero E6 or Vero-furin cells
Vero cell culture medium (see Recipes)
Infection media (see Recipes)
Viral sample to be titered
96-well U-bottom plates
96-well flat-bottom plates
2X MEM + 4% FBS (see Recipes)
2% methylcellulose (see Recipes)
4% PFA
Permeabilization (Perm) wash buffer (see Recipes)
Anti-SARS-CoV-2 antibody (and secondary antibody if primary is not directly conjugated)
Automated cell counter (*e.g.* CTL BioSpot)

1. Plate Vero E6 or Vero-furin cells in 96-well flat bottom plates at a cell density of 2.5 × 10^4 cells/well in a total volume of 100 µL/well.
2. Incubate cells overnight (12–18 h) in humidified incubator at 37 ˚C in 5% CO_2.
3. The next day, make 10-fold serial dilutions of viral sample to be titered in infection media in 96-well U-bottom plates. Change tips between dilutions and mix each row well before transferring to the next.
4. Remove existing cell culture media from cells. Transfer 100 µL of the dilutions generated in step #3 to corresponding wells on cell plate starting with the most diluted so the same pipette tip can be used up the dilution series.
5. Incubate at 37 ˚C for 1 h.
6. Meanwhile, mix 2X MEM + 4% FBS with 2% methylcellulose in a 1:1 ratio. Warm in 37 ˚C incubator while plates are incubating to decrease viscosity of the solution.
7. Add 100 µL/well MEM:methylcelluose overlay to each well.
8. Incubate at 37 ˚C for 30 h.
9. Remove methylcellulose:MEM overlays from each well.
10. Add 300 µL of 4% PFA in PBS (see Recipes) to each well.
11. Incubate at room temperature for 20 min.

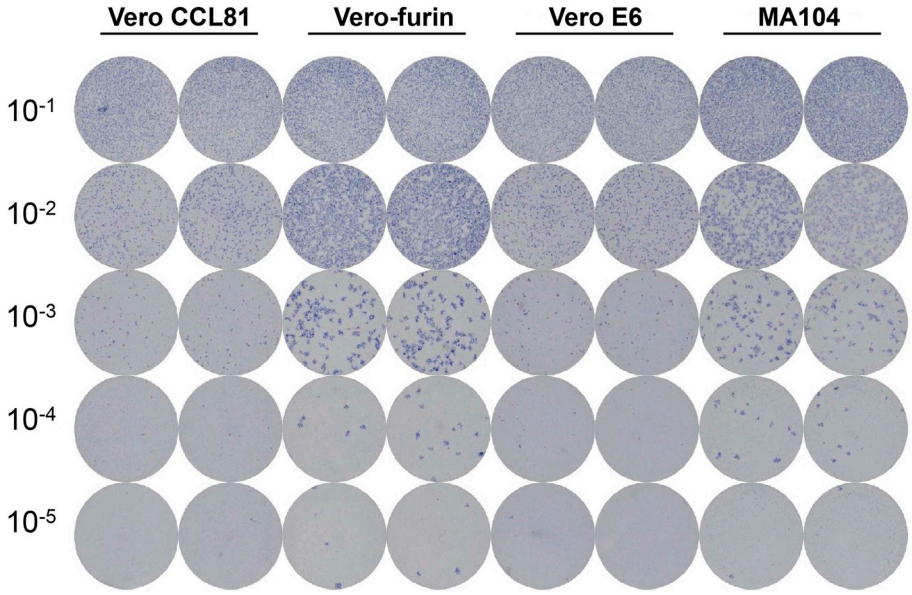

Fig. 3. SARS-CoV-2 focus-forming assay. CCL81, Vero-furin, Vero E6, and MA104 cells were inoculated with 10-fold serial dilutions of a SARS-CoV-2 stock. Plates were fixed 30 h post-infection and stained with CR3022 anti-SARS-CoV-2 antibody (1 μg/mL) overnight followed by anti-human IgG-HRP (1:500) for 2 h. Foci were visualized using TrueBlue substrate and wells with discrete foci were used to determine virus titer (10^{-3} - 10^{-4}).

12. Remove 4% PFA into appropriate waste container.
13. Wash cells with 300 μL of Perm wash (see Recipes) six times to remove any remaining overlay and 4% PFA.

**Plates can now be removed safely from the BSL3 after obtaining Institutional Biosafety Committee approval.*

14. Add 50 μL primary antibody/well in Perm wash. Incubate at 4 °C overnight with no rocking or 2 h at room temperature with rocking.

**These conditions are optimized for using CR3022 (*Yuan et al., 2020*) at* 1 μg/mL.

15. Wash three times with 300 μL/well of PBS + Tween wash (see Recipes).
16. Add 50 μL of secondary antibody/well in Perm wash.

**We use goat anti-human IgG-HRP at 1:500 (Sigma).*

17. Incubate plates for 2 h at room temperature with rocking or gentle agitation.
18. Wash 3x with 300 μL/well of PBS + Tween wash.
19. Add 50 μL of KPL TrueBlue substrate (from Seracare Life Sciences Inc).
20. Incubate plates at room temperature with rocking until foci are fully developed and visible by eye (~15–30 min).
21. Wash 3x with 300 μL/well of dH_2O.
22. Tap plate dry on a paper towel and image with CTL Immunospot plate reader.

Detection of SARS-CoV-2 antigen by flow cytometry. Detection of viral antigen in infected cells using flow-cytometry has a range of applications. In particular, multiplexing of viral staining with live/dead dyes and additional antigen stains can be used to screen infected cells for antibody binding or interrogate a range of biological variables.

2.7. Materials needed

Infected cells
Cell culture medium
96-well V-bottom plates
Dissociation reagent (*e.g.* Trypsin-EDTA or TrypLE)
FACS wash (see Recipes)
Anti-SARS-CoV-2 primary antibody
Secondary antibody (if primary is not directly conjugated)

1. If cells are adherent, dissociate SARS-CoV-2-infected cells into single-cell suspension using trypsin or EDTA-based dissociation agent.
2. Add cellular growth media and centrifuge in a swinging bucket rotor for 5 min at 500 × *g*.

Note: this must be performed in an aerosol-tight bucket with gasketed lid.

3. Open the gasketed centrifuge lid in the biosafety cabinet, remove samples, and pipette off supernatant. Resuspend cells in 4% PFA (final concentration) diluted in PBS and incubate for 10 min at room temperature.
4. Centrifuge at 600 × *g* for 3 min at 4 °C, remove supernatant, and resuspend cells in FACS wash.

**Cells can now be removed safely from the BSL3 after obtaining Institutional Biosafety Committee approval.*

5. Count cells and add 3 × 10^4–1 × 10^6 cells/well in a 96-well U-bottom plate for each sample to be tested.

**If desired, cells can be permeabilized using Perm wash (see Recipe below) and stained for intracellular antigen.*

6. Centrifuge at 600 × *g* for 3 min at 4 °C, remove supernatant, and resuspend cells with 50μL/well FACS wash containing primary antibody (*e.g.* containing 2 μg/mL of CR3022). Incubate at 4 °C for 45 min–1 h.
7. Wash twice with FACS wash by repeated centrifugation at 600 × *g* for 3 min at 4 °C, removal of supernatant, and resuspension of pellet in FACS wash.
8. Upon completing the second spin, resuspend cells with FACS wash containing fluorophore-conjugated secondary antibody that recognizes the primary antibody (*e.g.*, goat anti-human IgG Alexa 647 at 1:1000 dilution) for 1 h at 4 °C.
9. Wash twice with FACS wash by repeated centrifugation at 600 × *g* for 3 min at 4 °C, removal of supernatant, and resuspension of pellet in FACS wash. Resuspend after final spin in an appropriate volume for the flow cytometer you will use.
10. Analyze cells on a flow cytometer (Fig. 4).

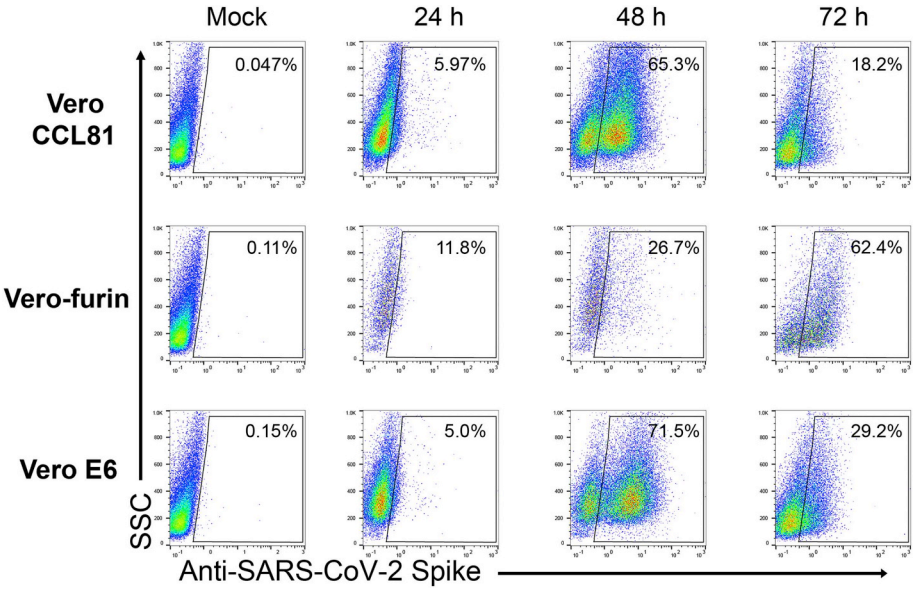

Fig. 4. SARS-CoV-2 infected cell flow cytometry plots. Indicated cell types were inoculated with SARS-CoV-2 at an MOI of 0.01 PFU/cell. At each indicated timepoint post-infection, cells were collected and prepared for flow cytometry using CR3022 anti-S as the primary antibody followed by goat-anti-human IgG Alexa 647 as the secondary antibody.

Table 2
Methods for inactivation of infectious SARS-CoV-2.

Method	Specimen type(s)	Specific reagent(s)/method(s) used	Incubation time
Trizol*	Tissue homogenate Cells Biological fluids	Per manufacturer instructions	
Paraformaldehyde*	Cells Biological fluids	4% final concentration	10 min
Formalin*	Tissues	10% at a ratio of 1:10 (tissue:formalin)	7 days
Triton-X-100	Serum Tissue culture media** Cell homogenate***	1% triton-X (final conc.) (Millipore-Sigma cat #11332481001)	20 min
Triton-X-100	Lung homogenate		60 min
MagMAX	Serum Tissue culture media**	MagMAX viral RNA kit (ABI cat # AM1939)	5 min
MagMAX	Tissue homogenate Cell homogenate***	MagMAX mirVana kit (ABI cat # A27828)	5 min
Heat	Urine Tissue culture media	50 °C for 5 min followed by 95 °C for 5 min	10 min
Paraformaldehyde	Lung homogenate	1% (final concentration)	60 min

*Gold standard methods.
**Note: tissue culture media is less complex than serum and in some cases is inferred from data showing 100% inactivation in serum.
**Note: cells from tissue culture are less complex than tissues from an infected animal, and this data is inferred from data showing 100% inactivation in homogenized lung tissue.

SARS-CoV-2 outgrowth assay for validation of inactivation methods. To evaluate many aspects of COVID-19 biology, methods for inactivating SARS-CoV-2 infectivity are needed so that samples can be worked with safely outside of the BSL3. To test whether a specific method or treatment completely inactivates SARS-CoV-2, a virus outgrowth assay should be used. This type of assay is highly sensitive in that it allows for the outgrowth of as little as a single infectious unit. However, it is not quantitative and must be adapted to the application in question. Alternate agents and methods are benchmarked against "gold-standard" methods of inactivation of SARS-CoV-2 (Table 2). We describe methods that we have tested and validated, although before use, individual Institutional Biosafety Committees likely will need to review data before providing clearance.

2.8. Materials needed

Inactivation agent/method of choice
Vero E6 cells
Vero cell culture medium

2.9. Selecting an inactivation agent/method

Selecting the appropriate inactivation agent/method requires a detailed understanding of the project in question: specifically, the properties of the measurand (*e.g.*, DNA, RNA, protein, and cells); the effect of the agent/method on the integrity of the measurand (*e.g.*, fragmentation of DNA by formaldehyde or lysis of RNA by boiling); and the types of specimens that will be treated for inactivation (*e.g.*, whole blood, plasma, cell culture media, cells, and tissues). No one reagent or method works for all applications, and ideally, the activity of each inactivation agent should be tested against each specimen type that will be used in the project. Validation of reagents/methods with a diverse array of applications may result in fewer hours spent performing validation of inactivation reagents.

2.10. Determination of the inactivation agent's cytotoxicity

Many commonly used chemical inactivation agents (*e.g.*, chaotropic salts, detergents, and formaldehyde-based solutions) are toxic to cells (Fig. 5A). Because viruses require infection of a cell to replicate, this

A

% PFA	1	2	3	4	5	6	7	8	9	10	11	12
A	1	3.33E-01	1.11E-01	3.70E-02	1.23E-02	4.12E-03	1.37E-03	4.57E-04	1.52E-04	5.08E-05	1.69E-05	Un
B	1.00E-01	3.33E-02	1.11E-02	3.70E-03	1.23E-03	4.12E-04	1.37E-04	4.57E-05	1.52E-05	5.08E-06	1.69E-06	Un
C	1.00E-02	3.33E-03	1.11E-03	3.70E-04	1.23E-04	4.12E-05	1.37E-05	4.57E-06	1.52E-06	5.08E-07	1.69E-07	Un
D	1.00E-03	3.33E-04	1.11E-04	3.70E-05	1.23E-05	4.12E-06	1.37E-06	4.57E-07	1.52E-07	5.08E-08	1.69E-08	Un
E	1.00E-04	3.33E-05	1.11E-05	3.70E-06	1.23E-06	4.12E-07	1.37E-07	4.57E-08	1.52E-08	5.08E-09	1.69E-09	Un
F	1.00E-05	3.33E-06	1.11E-06	3.70E-07	1.23E-07	4.12E-08	1.37E-08	4.57E-09	1.52E-09	5.08E-10	1.69E-10	Un
G	1.00E-06	3.33E-07	1.11E-07	3.70E-08	1.23E-08	4.12E-09	1.37E-09	4.57E-10	1.52E-10	5.08E-11	1.69E-11	Un
H	1.00E-07	3.33E-08	1.11E-08	3.70E-09	1.23E-09	4.12E-10	1.37E-10	4.57E-11	1.52E-11	5.08E-12	1.69E-12	Un

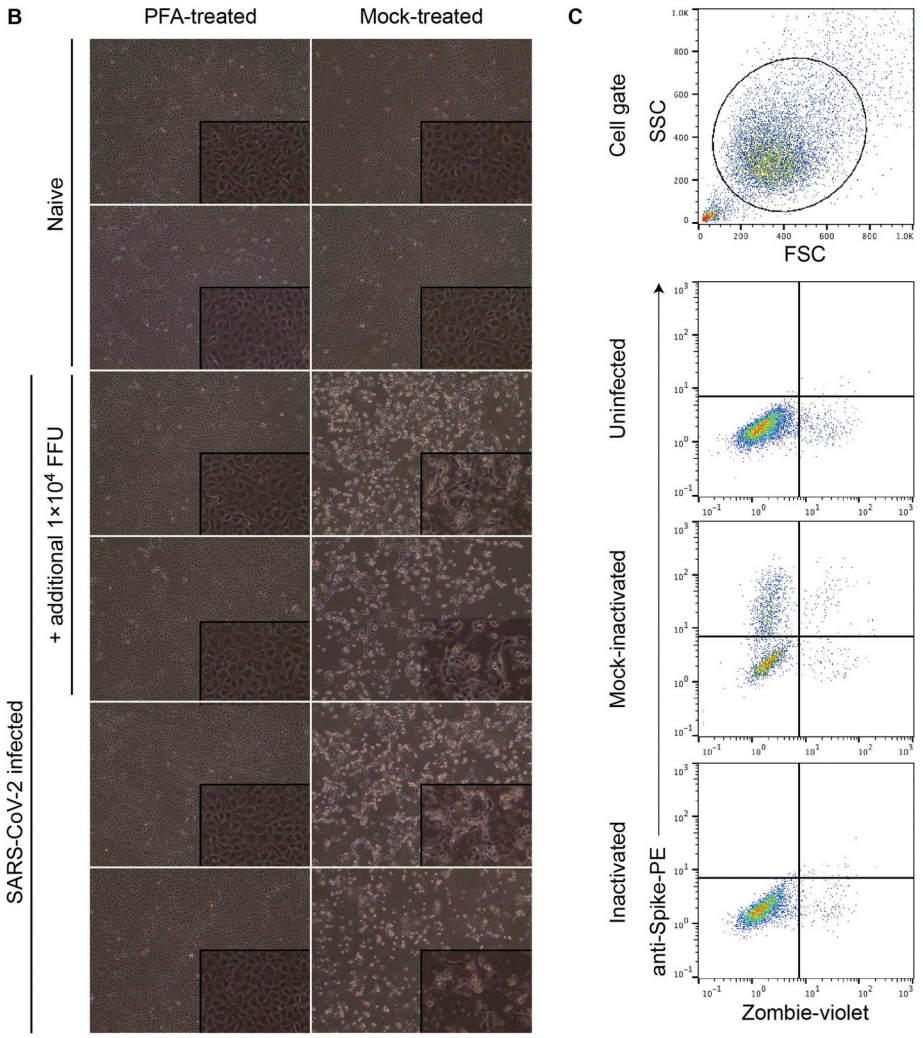

Fig. 5. Virus outgrowth assay. (A) Titration of paraformaldehyde toxicity on Vero E6 cells plated in 96-well format as described in the protocol. Yellow shading indicates wells in which cytopathic effect was observed. (B) Cytopathic effect observed in Vero E6 cells following inoculation with SARS-CoV-2 infected lung homogenate, treated with or without 1% PFA for 60 min and diluted 1:15,000. Photographs show cells under phase-contrast at 20X (and 40X, inset) magnification. (C) Flow cytometric analysis of Vero E6 cells inoculated with SARS-CoV-2 following treatment with an inactivation agent or PBS (mock). Cells were dissociated to single-cell suspension once the mock-treated culture displayed CPE consistent with SARS-CoV-2 infection. Viability staining with Zombie violet was performed prior to fixation. Antibody staining was performed on 4% paraformaldehyde-fixed and permeabilized cells using the CR3022 anti-SARS-CoV-2 spike antibody followed by anti-human IgG-BV421 labelled secondary antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

toxic effect of the inactivation agent must be diluted sufficiently after the sample has been treated to enable virus outgrowth.

1. (Day −1) Add 2 × 10^4 cells per well in a flat-bottom tissue-culture 96 well plate and incubate at 37 °C overnight (12–18 h).
2. (Day 0) In a separate U-bottom plate, add 200 µL of the fully-reconstituted inactivation agent to well A1. Make serial 10-fold dilutions down the first column by transferring 18 µL into 162 µL. Make serial 3-fold dilutions across the plate using a multichannel pipette by transferring 60 µL into 120 µL; add only media to column 12.

This setup will create a dilution scheme in which the dilution that renders the inactivation agent non-toxic can be assessed in multiple rows.

3. Remove the supernatant from the plate containing Vero cells, and add 100 µL from each corresponding well in the plate containing diluted inactivation agent. Incubate cells at 37 °C overnight.
4. (Day 1) Observe the cells under the microscope, noting wells with obvious cell death. Longer incubation times may be necessary. Mark wells in which toxicity is no longer obvious, and test the cells in these wells for viability and total cell number using trypan blue staining (or a variety of other live/dead counting methods). Use the media-only wells in column 12 for comparison. Wells that have the same viability and number of cells (± 10%) should be used to calculate the required dilution factor.

2.11. Sample inactivation and virus outgrowth assay

To determine the ability of the agent/method to fully inactivate infectious virus, several high-titer specimens should be identified. If

these are not available, then these specimens can be created by spiking specimens with SARS-CoV-2 virus stock (1:10) or infected cells. Ideally, specimens with the range of characteristics that will be encountered during the project (*e.g.*, icteric, hemolyzed, and lipemic serum specimens) should be tested.

1. Prepare Vero E6 cells in a volume that enables the dilution of inactivation agent to a non-toxic level when > 1 μL of treated sample is added. The number of cells also should be such that they will reach approximately 50% confluency upon adhering.

For example, for diluting an agent 1:1000, plate 3-4 × 10^4 Vero E6 cells in 4 mL and add 4 μL of the inactivated sample.

2. For the specimen(s) to be tested, split into two equal aliquots. Subject one to inactivation and the other to mock-inactivation (*e.g.*, with addition of saline or medium instead of inactivation reagent). This should be performed at the temperature and for the duration of time that will be used for inactivation of experimental specimens.
3. Add the appropriate volume of inactivated (and mock-inactivated) sample to the Vero cells.
4. Incubate at 37 ˚C and observe daily (Fig. 5B). Once obvious signs of CPE are observed, examine the cells and/or supernatant for infection using the flow cytometry (Figure.5C) and/or focus forming assay described above to confirm viral infection.

Conclusions. The emergence of SARS-CoV-2 and the resulting COVID-19 pandemic has strained biomedical resources throughout the world. Necessarily, pressure has been placed on the scientific community to deliver countermeasures for this continually evolving threat. Although new technologies are being applied to address this problem, classical virological methods, such as those presented here, remain important. Within a remarkably short period of time, the scientific community has built an infrastructure for studying SARS-CoV-2, especially given the biosafety concerns surrounding SARS-CoV-2 research. However, given the complex nature of COVID-19 pathophysiology, a critical need remains for developing new modalities for studying and combating this novel disease. Further optimization of assays will be required, and these will include a need to amplify high-titer virus stocks from low-passage patient isolates and develop new culture models to evaluate infectivity, host responses, and outcomes. New methods for SARS-CoV-2 inactivation will be developed, and these will require rigorous validation before wide-scale implementation. Issues regarding SARS-CoV-2 biosafety and biocontainment will continue to evolve as the pandemic progresses, and methods for safely working with and titrating SARS-CoV-2 will require further evaluation.

3. Recipes

3.1. Vero cell culture medium

For 1 L:
Dulbecco's Modified Eagle Medium (high glucose) supplemented to contain:
10% heat-inactivated fetal bovine serum
1% Glutamax
10 mM HEPES
100 U/mL penicillin/100 U/mL streptomycin
Sterile filter and store at 4 ˚C.
**Vero-furin media is as stated above with the addition of 5 μg/mL blasticidin.*

3.2. MA104 culture medium

For 1 L:
M199 medium with Earle's salts supplemented to contain:
5% fetal bovine serum
10 mM HEPES
100 U/mL penicillin/100 U/mL streptomycin
2.5 μg/mL amphotericin B

3.3. Infection medium

For 1 L:
Dulbecco's Modified Eagle Medium (high glucose) supplemented to contain:
2% heat-inactivated fetal bovine serum
10 mM HEPES
100U/mL penicillin/100U/mL streptomycin

3.4. 2X minimal essential medium + 4% FBS

For 1 L:
200 mL 10X MEM (Sigma #M0275)
20 mL of 1 M L-glutamine
20 mL 1 M HEPES
40 mL heat-inactivated fetal bovine serum
4.2 g sodium bicarbonate
20 mL of 10,000 IU/ml penicillin +10,000 μg/ml streptomycin
To volume with deionized distilled (Milli-Q) water
Sterile filter and store at 4 ˚C.

3.5. 2% methylcellulose

Autoclave a 250 mL glass bottle containing 2 g carboxymethylcellulose powder (Sigma #M0512) and a stir bar.
Autoclave 100 mL deionized distilled (Milli-Q) water.
When water is cool enough to handle, add to methylcellulose containing bottle.
Stir mixture overnight at 4 ˚C and then store at 4 ˚C until ready for use.

3.6. Crystal violet staining solution

For 100 mL:
50 mg of crystal violet powder (Fisher #C581-100)
20 mL of 100% methanol
80 mL of deionized distilled (Milli-Q) water
Store at room temperature and mix well before each use.

3.7. Perm wash

For 1L:
1 g of saponin (Sigma, Cat. No: S7900)
1 g of bovine serum albumin (Fraction V)
To volume with 1 L phosphate buffered saline (without Ca or Mg)
Filter sterilize and store at 4 ˚C until ready for use.

3.8. FACS wash

For 1 L:
100 mL 10X phosphate-buffered saline
5 mL 1 M EDTA solution
50 mL 5% heat-inactivated fetal bovine serum
10 mL 5% sodium azide solution
835 mL deionized distilled (Milli-Q) water
Filter sterilize and store at 4 ˚C until ready for use.

3.9. PBS + Tween wash

1 L 10X PBS
9 L ddH_2O
50 mL 10% Tween-20

Author contributions

J.B.C. developed the protocols and performed the infection studies with SARS-CoV-2. A.L.B. designed and tested the SARS-CoV-2 primer/probes and developed the inactivation protocols. J.B.C. and A.S.K. performed flow cytometry assays. R.E.C. provided experimental assistance with focus-forming and plaque assays. A.L.B., J.B.C., A.S.K., and M.S.D. wrote the initial manuscript draft.

Declaration of competing interest

M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM Biopharmaceuticals, and on the Scientific Advisory Board of Moderna.

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Original Article

Identification of Coronavirus Isolated from a Patient in Korea with COVID-19

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ABSTRACT

Objectives: Following reports of patients with unexplained pneumonia at the end of December 2019 in Wuhan, China, the causative agent was identified as coronavirus (SARS-CoV-2), and the 2019 novel coronavirus disease was named COVID-19 by the World Health Organization. Putative patients with COVID-19 have been identified in South Korea, and attempts have been made to isolate the pathogen from these patients.
Methods: Upper and lower respiratory tract secretion samples from putative patients with COVID-19 were inoculated onto cells to isolate the virus. Full genome sequencing and electron microscopy were used to identify the virus.
Results: The virus replicated in Vero cells and cytopathic effects were observed. Full genome sequencing showed that the virus genome exhibited sequence homology of more than 99.9% with SARS-CoV-2 which was isolated from patients from other countries, for instance China. Sequence homology of SARS-CoV-2 with SARS-CoV, and MERS-CoV was 77.5% and 50%, respectively. Coronavirus-specific morphology was observed by electron microscopy in virus-infected Vero cells.
Conclusion: SARS-CoV-2 was isolated from putative patients with unexplained pneumonia and intermittent coughing and fever. The isolated virus was named BetaCoV/Korea/KCDC03/2020.

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Introduction

Coronavirus is an RNA virus consisting of positive-sense single-stranded RNA of approximately 27-32 kb. Coronavirus belong to the family *Coronaviridae*, which comprises of alpha, beta, delta, and gamma coronaviruses [1,2]. As the name indicates, the spherical external spike protein displays a characteristic crown shape when observed under an electron microscope [3,4]. The virus is known to infect a wide range of hosts including humans, other mammals, and birds. Infected hosts exhibit different clinical courses, ranging from asymptomatic to severe symptoms in their respiratory, digestive, and genital organs [1,2]. There are 6 known coronaviruses that typically cause infection in humans. Among these, coronavirus 229E, OC43, NL63, and HKU1 generally cause mild cold-like symptoms, whereas severe acute respiratory syndrome-coronavirus (SARS-CoV) in 2003, and Middle East respiratory syndrome-coronavirus (MERS-CoV) in 2012, caused severe respiratory diseases such as pneumonia and death [5,6].

Following the first outbreaks of unexplained pneumonia in

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Wuhan, China, in late 2019, a new coronavirus was identified as the causative agent in January 2020 [7]. As of February 11th, 2020, a total of 45,000 cases of pneumonia, have been reported from 26 countries, including China. Approximately 96.8% of all cases have been reported in China, and patients in the Hubei province account for 75% of all cases [8-10]. In Korea, the first case was reported on January 20, 2020, when SARS-CoV-2 was detected in a traveler entering Korea from Wuhan, China [11].

This study reports the full genome sequencing of SARS-CoV-2 isolated from putative the 2019 novel coronavirus disease (COVID-19) patients in Korea, by cell culture. The isolated SARS-CoV-2 was named BetaCoV/Korea/KCDC03/2020.

Materials and Methods

1. Clinical specimens and RNA extraction

Nasopharyngeal and oropharyngeal swab and sputum samples were collected from symptomatic patients to detect SARS-CoV-2 by real-time reverse transcriptase (RT)-PCR. RNA was extracted from clinical samples with a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. All specimens were handled under a biosafety cabinet according to laboratory biosafety guidelines of Korea Centers for Disease Control and Prevention for COVID-19.

2. Real-time RT-PCR

The optimal concentration of primers and probes, which were synthesized using a published sequence [12], was determined with the RNA transcripts of SARS-CoV. The primer and probe sequences used for RNA-dependent RNA polymerase gene detection were: 5′- GTGARATGGTCATGTGTGGCGG-3' (Forward), 5'- CARATGTTAAASACACTATTAGCATA-'3 (Reverse) and 5'-CAGGTGGAACCTCATCAGGAGATGC-3' (Probe in 5-FAM/3'-BHQ format) and the primer and probe sequences used for E gene detection were: 5'- ACAGGTACGTTAATAGTTAATAGCGT-3' (Forward), 5'- ATATTGCAGCAGTACGCACACA-3' (Reverse) and 5'- ACACTAGCCATCCTTACTGCGCTTCG-3' (Probe in 5-FAM/3'-BHQ format). A 25-μL reaction was setup that contained 5 μL of RNA, 12.5 μL of 2 × reaction buffer provided with the Agpath IDTM 1 step RT-PCR system (Thermo Fisher Scientific, Waltham, USA), 1 μL of 25 × enzyme mixture, 1 μL of forward and reverse primers at 10 pM, and 0.5 μL of each probe at 10 pM. Reverse transcription was performed at 50°C for 30 minutes, followed by inactivation of the reverse transcriptase at 95°C for 10 minutes. PCR amplification was performed with 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using an ABI 7500 Fast instrument (Thermo Fisher Scientific).

3. Virus isolation

The virus was isolated from nasopharyngeal and oropharyngeal samples from putative COVID-19 patients. Oropharyngeal samples were diluted with viral transfer medium containing nasopharyngeal swabs and antibiotics (Nystadin, penicillin-streptomycin 1:1 dilution) at 1:4 ratio and incubated for 1 hour at 4°C, before being inoculated onto Vero cells. Inoculated Vero cells were cultured at 37°C, 5% CO_2 in 1× Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum and penicillin-streptomycin. Virus replication and isolation were confirmed through cytopathic effects, gene detection, and electron microscopy. Viral culture of SARS-CoV-2 was conducted in a biosafety Level-3 facility according to laboratory biosafety guidelines of Korea Centers for Disease Control and Prevention.

4. Next generation sequencing of viral full-length genome

Using reverse transcriptase, cDNA was synthesized from RNA extracted from the cultured cell medium in which the virus was replicated. A next generation sequencing (NGS) library was constructed after amplifying the full-length genes of the isolates using the synthesized cDNA and primers designed based on published SARS-CoV-2 DNA sequence. The prepared library was purified and analyzed with Miseq 150 PE. De novo assembly was performed on the sequenced product using Megahit to secure a full-length genome.

5. Sequencing analysis

Gene sequencing was performed using CLC Main Workbench 7.9.1. Alignment was conducted using human and animal coronavirus sequences registered in Global Initiative on Sharing All Influenza Data (GISAID) and NCBI GenBank. The phylogenetic tree was analyzed using MEGA6 with the neighbor-joining method, maximum composite likelihood-parameter distance matrix, and bootstrap values of 1,000 replicates.

6. Transmission electron microscopy

For transmission electron microscopy, the inoculated cells were prefixed by incubating in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) to prevent the autolysis of the cells infected with virus. To minimize the chemical reaction between pre- and post-fixation, the slides were washed 3 times using the same buffer as in the fixative solution and post-fixed with 1% osmium tetroxide. After washing 3 times with deionized water, en bloc staining was performed using 0.5% uranyl acetate. Thereafter, 30%, 50%, 70%, 80%, 90%, and 100% ethanol were used sequentially in ascending concentration for dehydration, which was

substituted with propylene oxide. The slides were then embedded in Epon812 plastic resin, and polymerized at 70°C for 48 hours. The prepared plastic block was cut to 70-nm thick sections using an ultramicrotome and mounted on a 100-mesh nickel grid, and electrostained with 5% uranyl acetate. The sections were observed with a transmission electron microscope (Libra120, Carl Zeiss, Germany) at an acceleration voltage of 120 kV [13-15].

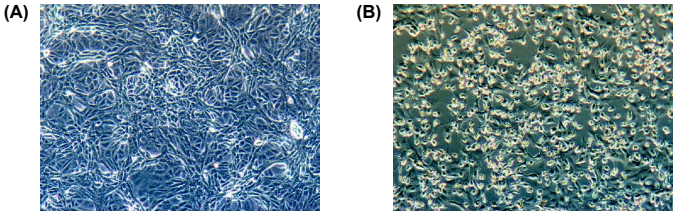

Figure 1. Cytopathic effect of SARS-CoV-2 on Vero cells. (A) Mock inoculated cells (B) SARS-CoV-2 inoculated cells.

Results

1. Virus isolation from Vero cells

Following inoculation of Vero cells with the nasopharyngeal and oropharyngeal samples, they were observed at 24-hour intervals, and the cytopathic effects were observed from 3 days after inoculation (Figure 1). The inoculated cells were harvested on the 4th day when more than 80% of the cells exhibited cytopathic effects. Virus replication was confirmed using real-time RT-PCR with RNA extracted from the cell culture medium. The Ct values were 14.40 and 18.26 for the nasopharyngeal and oropharyngeal samples, respectively, which were lower than the cycle threshold (Ct) values of 20.85 and 21.85 in the pre-inoculated samples. The number of virus copies in the samples before inoculation was 7.6×10^8 and 3.9×10^8 copy/mL, respectively, and increased by 10-70-fold to 5.4×10^{10} and 4.2×10^9, respectively, in the cell culture supernatants.

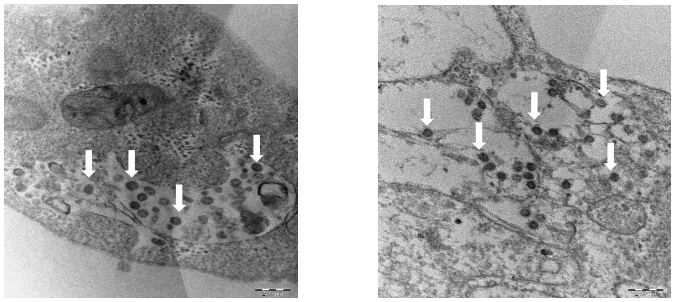

Figure 2. Thin section electron micrographs of Vero cells infected with SARS-CoV-2. Electron micrographs show representative thin sections of Vero cells infected with SARS-CoV-2; cells were collected at 48 hours after infection for examination by electron microscopy. White arrows point to aggregates of assembled intracellular virions.

2. Analysis of the structure of the virus by electron micrographs

The structure of the virus in the cytoplasm of 3-day post-inoculation cells was examined by electron microscopy (Figure 2). Coronavirus-specific morphology was observed. Virus particle size ranged from 70-90 nm and the virus was observed in a wide range of intracellular organelles, especially in vesicles.

3. Full-length genome and phylogenetic analysis

After inoculating cells with the nasopharyngeal and oropharyngeal samples, RNA was extracted from the virus-replicated cell culture medium. The RNA was amplified with primers for full-length gene analysis, and NGS was performed using Miseq. De novo assembly of the NGS sequence secured 28,818 bp of the full-length gene. The acquired gene was compared with 57 human and animal coronaviruses, including Wuhan/IVDC-HB-01/2019(GISAID accession ID: EPI_ISL_402119~121), which was first reported in Wuhan, 54 reported full-length SARS-CoV-2 genes, Bat-SARS-like CoV, and human SARS-CoV (Figure 3). The analysis showed that the sequence was included in the same cluster as the previously reported SARS-CoV-2 sequence and showed high homology of > 99.5% with other isolated SARS-CoV-2 sequences. The virus was named BetaCoV/Korea/KCDC03/2020, and its full-length gene sequence was registered in WHO GISAID (GISAID accession ID: EPI_ISL_407193).

Discussion

As of February 12th, 2020, 28 cases of COVID-19 have been reported in Korea, with the first case observed in a traveler residing in Wuhan, China. The SARS-CoV-2 was isolated from a Korean patient who had self-administered antipyretics for initial symptoms such as chills and fever. The patient had experienced intermittent coughing with sputum 3 days after the administration of antipyretics. The SARS-CoV-2 could replicate in other cells (Vero E6 and Caco-II cells), in addition to Vero cells (data not shown). The first SARS-CoV-2 was successfully isolated by inoculating human airway epithelial cells with bronchoalveolar-lavage fluid samples from a patient with pneumonia [16]. Since human airway epithelial cells (because of their resemblance to pseudostratified mucociliary

Figure 3. Phylogenetic tree analysis of SARS-CoV-2 based on full genome nucleotide sequences using the neighbor-joining tree. Values on branches are shown as percentages based on 1,000 bootstrap replicates. (A) Gene analysis of SARS-CoV-2 and other coronaviruses, (B) gene analysis of SARS-CoV-2 and BetaCoV/Korea/KCDC03/2020.

epithelium) require 4-6 weeks to differentiate in vivo, isolation of SARS-CoV-2 using Vero cells or Caco-II cells is more convenient. Further studies are needed to select more sensitive cell lines suitable for virus isolation from low viral load samples.

The sequence of the suspected novel coronavirus (KCDC03) was analyzed using the sequences of 54 SARS-CoV-2 including 6 human coronaviruses, Bat-CoVs, and 51 SARS-CoV-2 that have been registered in GISAID by several countries including China, and a phylogenetic tree was produced. Results show that the isolate from Korea was clustered with the new coronavirus, SARS-CoV-2 and classified as betacoronavirus. High homology (99.94%-99.99%) was confirmed with the viral sequences reported from other countries, such as those from Wuhan/IVDC-HB-01/2019(GISAID accession ID: EPI_ISL_402119). Homology with bat CoV (bat-SL-CoVZC45), SARS-CoV (AY278741) and MERS-CoV (JX869059) was 89.1%, 77.5%, and 50%, respectively. However, the lowest homology of 99.5% was observed with BetaCoV/Wuwhan/IVDCHB-04/2020, which could be because of the inaccurate sequence of IVDCHB-04, Hence, it has been excluded from the comparative analysis in other studies.

Prior to identification of SARS-CoV-2 as the causative agent of the unknown pneumonia in Wuhan, China, pan-CoV RT-PCR was being used to detect SARS-CoV-2 in Korea. The Pan-CoV RT-PCR detects all human coronaviruses and animal-derived coronaviruses (personal communication). Since the release of the SARS-CoV-2 sequence, a real-time RT-PCR method has been established in the diagnosis of COVID-19 patients. Currently, the diagnosis of COVID-19 is based on gene detection via real-time RT-PCR. With the isolation of the causative agent, development of serological tests and rapid diagnostic tests in addition to virus detection will be required.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Annals of Internal Medicine ORIGINAL RESEARCH

Variation in False-Negative Rate of Reverse Transcriptase Polymerase Chain Reaction–Based SARS-CoV-2 Tests by Time Since Exposure

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Background: Tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) based on reverse transcriptase polymerase chain reaction (RT-PCR) are being used to "rule out" infection among high-risk persons, such as exposed inpatients and health care workers. It is critical to understand how the predictive value of the test varies with time from exposure and symptom onset to avoid being falsely reassured by negative test results.

Objective: To estimate the false-negative rate by day since infection.

Design: Literature review and pooled analysis.

Setting: 7 previously published studies providing data on RT-PCR performance by time since symptom onset or SARS-CoV-2 exposure using samples from the upper respiratory tract (n = 1330).

Patients: A mix of inpatients and outpatients with SARS-CoV-2 infection.

Measurements: A Bayesian hierarchical model was fitted to estimate the false-negative rate by day since exposure and symptom onset.

Results: Over the 4 days of infection before the typical time of symptom onset (day 5), the probability of a false-negative result in an infected person decreases from 100% (95% CI, 100% to 100%) on day 1 to 67% (CI, 27% to 94%) on day 4. On the day of symptom onset, the median false-negative rate was 38% (CI, 18% to 65%). This decreased to 20% (CI, 12% to 30%) on day 8 (3 days after symptom onset) then began to increase again, from 21% (CI, 13% to 31%) on day 9 to 66% (CI, 54% to 77%) on day 21.

Limitation: Imprecise estimates due to heterogeneity in the design of studies on which results were based.

Conclusion: Care must be taken in interpreting RT-PCR tests for SARS-CoV-2 infection—particularly early in the course of infection—when using these results as a basis for removing precautions intended to prevent onward transmission. If clinical suspicion is high, infection should not be ruled out on the basis of RT-PCR alone, and the clinical and epidemiologic situation should be carefully considered.

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Tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) based on reverse transcriptase polymerase chain reaction (RT-PCR) are often used to "rule out" infection among high-risk persons, such as exposed inpatients and health care workers. Hence, it is critical to understand how the predictive value changes in relation to time since exposure or symptoms, especially when using the results of these tests to make decisions about whether to stop using personal protective equipment or allow exposed health care workers to return to work. The sensitivity and specificity of PCR-based tests for SARS-CoV-2 are poorly characterized, and the "window period" after acquisition in which testing is most likely to produce false-negative results is not well known.

Accurate testing for SARS-CoV-2, followed by appropriate preventive measures, is paramount in the health care setting to prevent both nosocomial and community transmission. However, most hospitals are facing critical shortages of SARS-CoV-2 testing capacity, personal protective equipment, and health care personnel (1). As the epidemic progresses, hospitals increasingly have to decide how to respond when a patient or health care worker has a known exposure to SARS-CoV-2. Although 14 days of airborne precautions or quarantine would be a conservative approach to minimizing transmission per guidelines from the Centers for Disease Control and Prevention (2), this is not feasible for many hospitals given starkly limited resources.

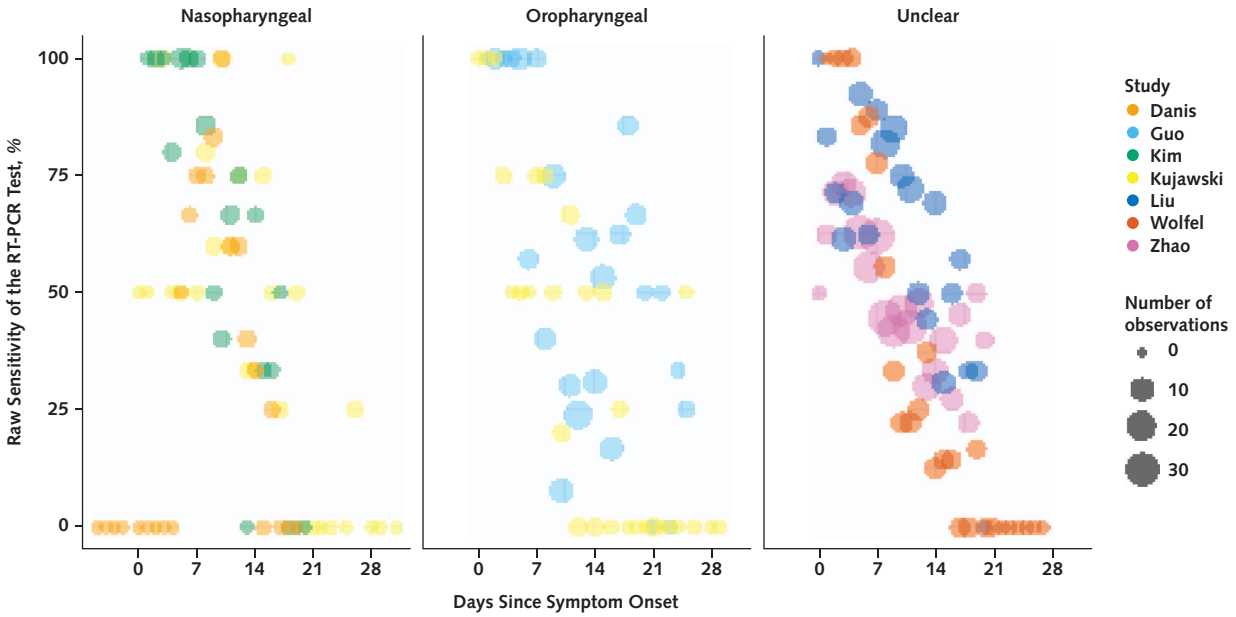
As RT-PCR–based tests for SARS-CoV-2 are becoming more available, they are increasingly being used to "rule out" infection to conserve scarce personal protective equipment and preserve the workforce. When exposed health care workers test negative, they may be cleared to return to work; similarly, when exposed patients test negative, airborne or droplet precautions may be removed. If negative results from tests done during the window period are treated as strong evidence that an exposed person is SARS-CoV-2–negative, preventable transmission could occur.

It is critical to understand how the predictive value of the test varies with time from exposure and symptom onset to avoid being falsely reassured by negative results from tests done early in the course of infection. The goal of our study was to estimate the false-negative rate by day since infection.

See also:

Web-Only
Supplement

Figure 1. Sensitivity of RT-PCR tests, by study and days since symptom onset, for nasopharyngeal samples (*left*), oropharyngeal samples (*middle*), and unspecified upper respiratory tract (*right*).



RT-PCR = reverse transcriptase polymerase chain reaction.

METHODS

Source Data

As part of a broader effort to provide critical evaluation of emerging evidence, the Novel Coronavirus Research Compendium at the Johns Hopkins School of Public Health did a literature review to identify preprint and peer-reviewed articles on SARS-CoV-2 diagnostics (3). Investigators searched PubMed, bioRxiv, and medRxiv using a strategy detailed in Supplement Table 1 (available at Annals.org). The search was last updated on 15 April 2020. From the broader search, we identified articles that provided data on RT-PCR performance by time since symptom onset or exposure using samples derived from nasal or throat swabs among patients tested for SARS-CoV-2. Inclusion criteria were use of an RT-PCR–based test, sample collection from the upper respiratory tract, and reporting of time since symptom onset or exposure. We excluded articles that did not clearly define time between testing and symptom onset or exposure. We identified 7 studies (2 preprints and 5 peer-reviewed articles) (4–10) with a total of 1330 respiratory samples analyzed by RT-PCR. Figure 1 summarizes the source data. One study by Kujawski and colleagues (10) provided both nasal and throat samples for each patient; we used only the nasal samples in our analysis.

How Cases Were Defined

Most studies (Danis and colleagues [6], Wölfel and colleagues [4], Kim and colleagues [7], Kujawski and colleagues [10], and Zhao and colleagues [8]) did serial testing and required at least 1 positive RT-PCR result to consider a case confirmed. Our pooled analysis included only confirmed cases from those studies. The studies by Liu and colleagues (9) and Guo and colleagues (5) included both confirmed cases (≥1 positive RT-PCR result, similar to other studies; n = 153 for Liu and n = 82 for Guo) and probable cases as determined by a set of clinical criteria (n = 85 for Liu and n = 58 for Guo). In both studies, most probable case patients were positive for IgM or IgG SARS-CoV-2 antibodies (67 of 85 probable cases for Liu were IgM- or IgG-positive, and 54 of 58 for Guo were IgM-positive). Thus, 22 participants were considered case patients on the basis of clinical criteria alone because we could not separate them out using the information provided. Supplement Table 2 (available at Annals.org) provides additional details on the source data used in our calculations. As a sensitivity analysis to assess the effect of individual studies on our inferences, we excluded each study in turn from calculations of the posttest probability of infection after a negative RT-PCR result (Supplement Figure 3, available at Annals.org).

Statistical Analysis

Model for Estimating False-Negative Rate and False Omission Rate by Time Since Exposure

Using an approach similar to that of Leisenring and colleagues (11) and Azman and colleagues (12), we fitted a Bayesian hierarchical logistic regression model for test sensitivity pj,t with a random effect for study j and a cubic polynomial spline for log-time t since exposure:

$$x_{j,t} \sim \text{Binomial}(n_{j,t}, p_{j,t})$$
$$logit(p_{j,t}) = \beta_j + \beta_1 log(t) + \beta_2 log(t)^2 + \beta_3 (t)^3$$
$$\beta_j \sim \text{Normal}(\beta_0, \sigma^2)$$

where $x_{j,t}$ is the number of patients who tested positive on RT-PCR out of $n_{j,t}$ total tests t days after exposure in study j. The exposure was assumed to have occurred 5 days before symptom onset based on the median incubation period previously estimated in a large study of transmission in household contacts (13) and among publicly confirmed cases (14). From the sensitivity, we calculated the expected false-negative rate on each day. We also calculated the posttest probability of infection, assuming a pretest probability based on the attack rate in close household contacts of SARS-CoV-2 case patients in Shenzhen, China (77 of 686 [11.2%]) (14). We assumed a specificity of 100% for RT-PCR, as reported in the U.S. Food and Drug Administration package insert for the Quest RT-PCR assay for SARS-CoV-2, which based its estimate on testing in 72 presumed negative samples from the upper respiratory tract and 30 from the lower respiratory tract (15). This specificity is further supported by a European study that showed no cross-reactivity with other coronaviruses in 297 clinical samples (16).

Sensitivity Analyses

Although the Food and Drug Administration reported that specificity for SARS-CoV-2 RT-PCR is 100%, many of the supporting studies were done outside the United States, and we cannot exclude variability in test performance. Thus, we repeated our analysis assuming 90% specificity to assess the sensitivity of our results to this assumption. A second assumption of our model, the 5-day incubation period, was based on a large study of household contacts in Shenzhen (13) and on publicly confirmed cases (14). We did additional analyses varying the incubation period to 3 and 7 days to assess the sensitivity of our results to this assumption. We also repeated analyses excluding 1 study each time to assess the effect on our inferences.

Code and Data Availability

The data and code used to run this analysis are publicly available at https://github.com/HopkinsIDD/covidRTPCR (17).

Role of the Funding Source

The funders had no influence on the study's design, conduct, or reporting.

RESULTS

Probability of a False-Negative Result Among SARS-CoV-2–Positive Patients, by Day Since Exposure

Over the 4 days of infection before the typical time of symptom onset (day 5), the probability of a false-negative result in an infected person decreases from 100% (95% CI, 100% to 100%) on day 1 to 67% (CI, 27% to 94%) on day 4, although there is considerable uncertainty in these numbers. On the day of symptom onset, the median false-negative rate was 38% (CI, 18% to 65%) (Figure 2, *top*). This decreased to 20% (CI, 12% to 30%) on day 8 (3 days after symptom onset) then began to increase again, from 21% (CI, 13% to 31%) on day 9 to 66% (CI, 54% to 77%) on day 21.

Posttest Probability of Infection if RT-PCR Result is Negative (1 Minus Negative Predictive Value)

Translating these results into a posttest probability of infection, a negative result on day 3 would reduce our estimate of the relative probability that a case patient was infected by only 3% (CI, 0% to 47%) (for example, from 11.2%, the rate seen in a large study of household contacts, to 10.9%) (Figure 2, *bottom*). Tests done on the first day of symptom onset are more informative, reducing the inferred probability that a case patient was infected by 60% (CI, 33% to 80%).

Variation in Posttest Probability of Infection if RT-PCR Result is Negative, by Pretest Probability

The posttest probability of infection in a patient with a negative RT-PCR result varies with the pretest probability of infection—that is, how likely infection is on the basis of the magnitude of exposure or clinical presentation. When we assumed a high pretest probability of infection (4 times the attack rate observed in a large cohort study), the posttest probability of infection was at minimum 14% (CI, 9% to 20%) 8 days after exposure (Figure 3). When we assumed a lower pretest probability of 5.5% (half the observed attack rate), the negative posttest probability of infection was still minimized 8 days after exposure (1.2% [CI, 0.7% to 2.0%]).

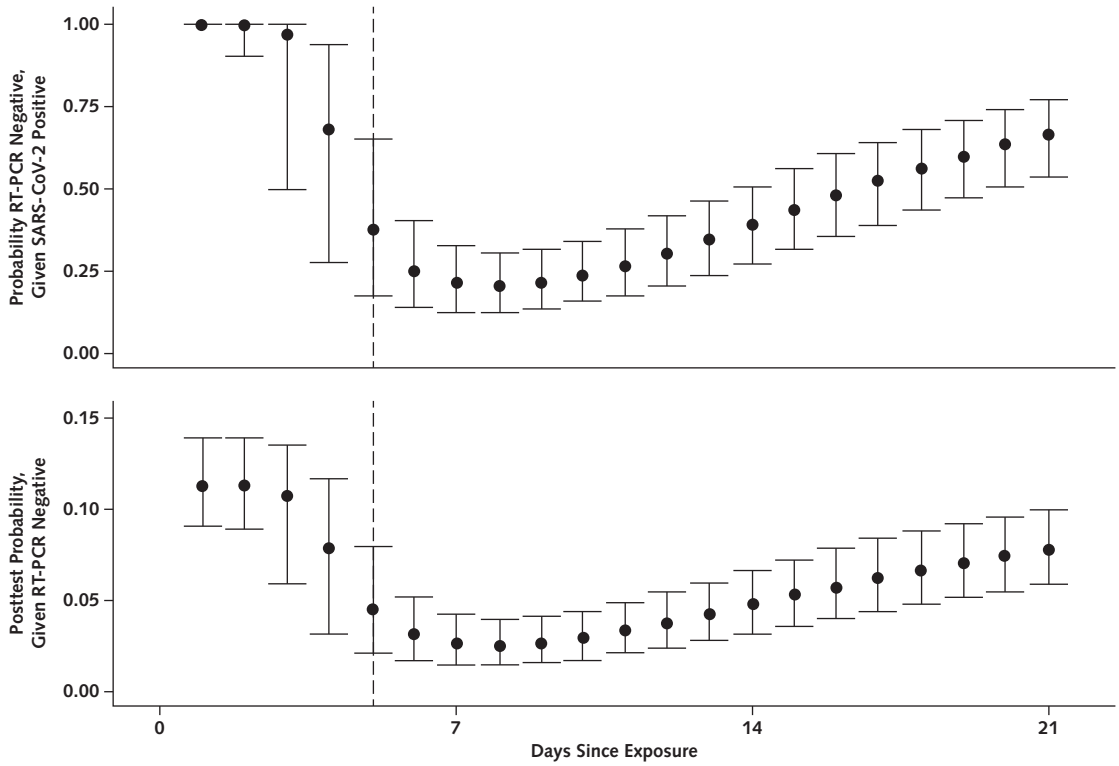
Sensitivity Analyses

When we repeated our analysis assuming a specificity of RT-PCR of 90% rather than 100%, results were very similar (Supplement Figure 1, available at Annals.org). We found a higher probability of infection in the setting of a negative RT-PCR result, with the greatest difference occurring on day 2 (12.4% vs. 11.3% [1.1 percentage point higher]). When we repeated our analyses varying the incubation period, we found that an earlier onset time of symptoms led to a quicker decrease in false omission rate and a later onset time led to a slower decrease; however, curves were similar overall, and our primary inferences remained the same relative to the date of onset (Supplement Figure 2, available at Annals.org). When we repeated our analysis of the posttest probability of infection excluding a different study each time, our inferences were unchanged (Supplement Figure 3).

DISCUSSION

Over the 4 days of infection before the typical time of symptom onset (day 5), the probability of a false-negative result in an infected person decreased from 100% on day 1 to 68% on day 4. On the day of symptom onset, the median false-negative rate was 38%. This decreased to 20% on day 8 (3 days after symptom onset) then began to increase again, from 21% on day 9 to 66% on day 21. The false-negative rate was minimized 8 days after exposure—that is, 3 days after the onset of symptoms on average. As such, this may be

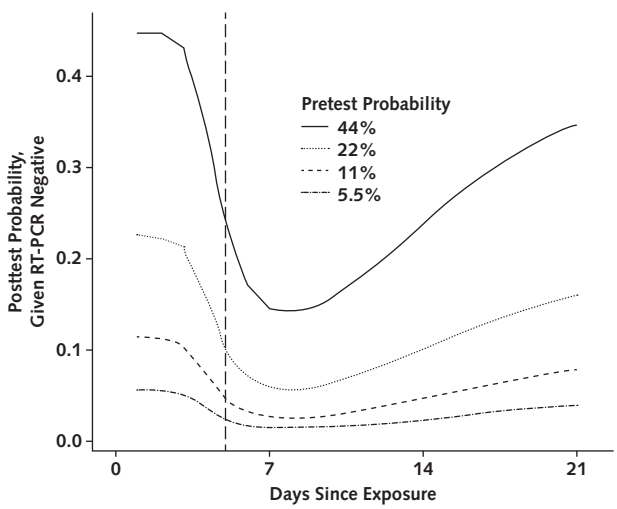
Figure 2. Probability of having a negative RT-PCR test result given SARS-CoV-2 infection (*top*) and of being infected with SARS-CoV-2 after a negative RT-PCR test result (*bottom*), by days since exposure.



RT-PCR = reverse transcriptase polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

the optimal time for testing if the goal is to minimize false-negative results. When the pretest probability of infection is high, the posttest probability remains high even with a negative result. Furthermore, if testing is done immediately after exposure, the pretest probability is equal to the negative posttest probability, meaning that the test provides no additional information about the likelihood of infection.

Figure 3. Posttest probability of SARS-CoV-2 infection after a negative RT-PCR result, by pretest probability of infection.



RT-PCR = reverse transcriptase polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

Since the outbreak began, concerns have been raised about the poor sensitivity of RT-PCR–based tests (18); 1 study has suggested that this might be as low as 59% (19). We have designed a publicly available model that provides a framework for estimating the performance of these tests by time since exposure and can be updated as additional data become available.

Tests for SARS-CoV-2 based on RT-PCR added little diagnostic value in the days immediately after exposure. This is consistent with a window period between acquisition of infection and detectability by RT-PCR seen in other viral infections, such as HIV and hepatitis C (20, 21). Our study suggests a window period of 3 to 5 days, and we would not recommend making decisions regarding removing contact precautions or ending quarantine on the basis of results obtained in this period in the absence of symptoms. Although the false-negative rate is minimized 1 week after exposure, it remains high at 21%. Possible mechanisms for the high false-negative rate include variability in individual amount of viral shedding and sample collection techniques.

One consideration is whether serial testing would offer any benefit in test performance compared with a single test. If we assume independence of the test re-

sults, serial testing would almost certainly reduce the false-negative rate; however, without more data on the underlying mechanism for the high false-negative rate, this assumption may not be warranted. For example, if the rate were due to individual variability in viral shedding, performance would likely not be improved by serial tests. Although we are aware of no large-scale studies, some preliminary reports suggest lack of independence; for example, in 1 case report of a person with infection confirmed on the basis of both radiologic findings and RT-PCR positivity from endotracheal aspirates, RT-PCR results from nasopharyngeal swabs were negative throughout the clinical course (6). Further studies to better characterize the underlying mechanism for poor diagnostic performance of SARS-CoV-2 RT-PCR are needed to inform testing strategies.

The relationship between a false-negative result and infectiousness is unclear, and patients who test negative on samples from nasopharyngeal swabs may be less likely to transmit the virus regardless of true case status. We found an increase in the false-negative rate starting 9 days after exposure; however, it is possible that some of the later results were not true false negatives but rather represented clearance of the infection. Thus, interpretation later in the clinical course depends on the purpose of testing: If the goal is to clear a patient from isolation, these negative results may be correct, although more data are needed given studies showing viral replication in other sites. However, if the goal of the test is to evaluate whether additional follow-up is needed or whether the patient should be treated as SARS-CoV-2–positive for the purpose of contact tracing, the test may not be providing the desired information and caution should be used in decision making. Because antibodies appear later in the course of infection, a combination of antibody testing and RT-PCR might be most useful for patients more remote from symptoms or exposure.

Our study has several limitations. There was significant heterogeneity in the design and conduct of the underlying studies from which the data used in our analyses were drawn. However, when we did a sensitivity analysis excluding each study in turn, we found that no 1 study was especially influential and inferences were largely unchanged. Sample collection techniques varied across studies (oropharyngeal vs. nasopharyngeal swabs), and several studies stated that samples were from the upper respiratory tract without providing further details. Thus, we could not fully account for differences in sample collection techniques. Most studies tested samples at time of symptom onset rather than time of exposure, leading to high variance in estimates in the first few days after exposure. Our model is applicable only in the setting of a known, one-time exposure, not in the setting of continuous exposure, such as in health care workers who may be exposed daily to SARS-CoV-2–positive patients. Finally, most studies defined true-positive cases as those with at least 1 positive RT-PCR result, meaning that patients who never tested positive would not be included; this could lead to underestimation of the true false-negative rate. Two studies included probable cases based on clinical and epidemiologic characteristics even if the patients had never had a positive RT-PCR result or serology. Because such criteria as fever, respiratory symptoms, and imaging findings are nonspecific, misclassification is likely, wherein some proportion of probable cases are actually true negatives rather than false negatives. We believe that this effect was small because excluding these studies from our analysis did not change our primary inferences.

In summary, care must be taken when interpreting RT-PCR tests for SARS-CoV-2 infection, particularly early in the course of infection and especially when using these results as a basis for removing precautions intended to prevent onward transmission. If clinical suspicion is high, infection should not be ruled out on the basis of RT-PCR alone, and the clinical and epidemiologic situation should be carefully considered. In many cases, time of exposure is unknown and testing is done on the basis of time of symptom onset. The false-negative rate is lowest 3 days after onset of symptoms, or approximately 8 days after exposure. Clinicians should consider waiting 1 to 3 days after symptom onset to minimize the probability of a false-negative result. Further studies to characterize test performance and research into higher-sensitivity approaches are critical.

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Reproducible Research Statement: *Study protocol:* Further details are available from Dr. Kucirka (e-mail, lauren@jhmi.edu). *Statistical code and data set:* Available at https://github.com/HopkinsIDD/covidRTPCR.

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Virology Journal

SHORT REPORT Open Access

False negative rate of COVID-19 PCR testing: a discordant testing analysis

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Abstract

Background: COVID-19 is diagnosed via detection of SARS-CoV-2 RNA using real time reverse-transcriptase polymerase chain reaction (rtRT-PCR). Performance of many SARS-CoV-2 rtRT-PCR assays is not entirely known due to the lack of a gold standard. We sought to evaluate the false negative rate (FNR) and sensitivity of our laboratory-developed SARS-CoV-2 rtRT-PCR targeting the envelope (E) and RNA-dependent RNA-polymerase (RdRp) genes.

Methods: SARS-CoV-2 rtRT-PCR results at the Public Health Laboratory (Alberta, Canada) from January 21 to April 18, 2020 were reviewed to identify patients with an initial negative rtRT-PCR followed by a positive result on repeat testing within 14 days (defined as discordant results). Negative samples from these discordant specimens were re-tested using three alternate rtRT-PCR assays (targeting the E gene and N1/N2 regions of the nucleocapsid genes) to assess for false negative (FN) results.

Results: During the time period specified, 95,919 patients (100,001 samples) were tested for SARS-CoV-2. Of these, 49 patients were found to have discordant results including 49 positive and 52 negative swabs. Repeat testing of 52 negative swabs found five FNs (from five separate patients). Assuming 100% specificity of the diagnostic assay, the FNR and sensitivity in this group of patients with discordant testing was 9.3% (95% CI 1.5–17.0%) and 90.7% (95% CI 82.6–98.9%) respectively.

Conclusions: Studies to understand the FNR of routinely used assays are important to confirm adequate clinical performance. In this study, most FN results were due to low amounts of SARS-CoV-2 virus concentrations in patients with multiple specimens collected during different stages of infection. Post-test clinical evaluation of each patient is advised to ensure that rtRT-PCR results are not the only factor in excluding COVID-19.

Keywords: SARS-CoV-2, COVID-19, Discordant testing, False negative rate

Background

Accurate case detection with rapid isolation and contact tracing form critical elements of the public health response to COVID-19. With most emerging infections, initially available nucleic acid tests (NATs) may lack data on the frequency of false negative results which can unnecessarily lead to repeated testing.

Studies of false-negative (FN) results from respiratory samples for SARS-CoV-2 are variable demonstrating FN rates (FNRs) ranging from 1 to 30% [1, 2]. FN results can occur for numerous reasons including suboptimal specimen collection, testing too early in the disease process, low analytic sensitivity, inappropriate specimen type, low viral load, or variability in viral shedding [3–9].

Implications of FN results can be significant, potentially leading to positive case clusters and negative outcomes [10]. Current guidance from the World Health Organization (WHO) and others calls for repeat testing (including sampling of the lower respiratory tract) in individuals

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who continue to display symptoms of COVID-19 with continued infection prevention measures [9, 11, 12]. The optimal interval of repeat testing is not clear with different studies suggesting a range from 1 to 6 days following the first negative test [13, 14].

The current study was designed to assess the FNR and sensitivity for the laboratory-developed test rtRT-PCR (LDT) used for frontline SARS-CoV-2 testing in Alberta, Canada, by determining the number of FN results in patients with repeat specimens submitted.

Methods

Setting, patients, and clinical samples

In the province of Alberta, Canada (population 4.4 million people), SARS-CoV-2 testing was conducted exclusively at the provincial Public Health Laboratory for symptomatic patients during the first four months of the pandemic [15–17]. The first case was confirmed on March 5, 2020 [18]. Test results and patient demographics were extracted from the laboratory information system to identify patients between January 21 and April 18, 2020, with an initial negative SARS-CoV-2 result followed by a positive result on repeat testing within 14 days (one incubation period) hereon defined as discordant test results [11].

Acceptable specimens for SARS-CoV-2 testing included nasopharyngeal (NP), oropharyngeal (OP), deep nasal turbinate swabs, endotracheal aspirates, and bronchoalveolar lavages (see Additional file 1: Table S1). All collection kits were internally validated prior to use.

SARS-CoV-2 RNA detection

Nucleic acid extraction was performed on one of several platforms (see Additional file 1: Table S1). A LDT rtRT-PCR targeting the envelope (E) and RNA-dependent RNA-polymerase (RdRp) genes was used to detect SARS-CoV-2 RNA [19]. Samples with cycle threshold (Ct) values > 35 cycles were repeated in duplicate and considered positive if ≥ 2 of three results had an amplification curve. Invalid was used to refer to samples with PCR run errors such as instrument or internal control failure. The assay parameters and comparison to other assays used across Canada has been published [19, 20].

The negative samples from sets of discordant specimens were re-tested by rtRT-PCR for SARS-CoV-2 to evaluate for FNs. This was carried out by extracting nucleic acid from the original sample followed by testing using assays targeting three different genes: the E gene (using only the E gene target from the LDT in a singleplex format) and the N1/N2 portions of the nucleocapsid gene (see Additional file 1: Table S1) [21]. Evaluation of the CDC N1/N2 assay compared to the LDT demonstrated 94% positive agreement (95% CI 87.7–100%) and 100% negative agreement (see Additional file 2: Table S2).

The discordant samples were retrieved from storage at − 70 °C and underwent one freeze–thaw cycle. Samples that had tested positive were assumed to be true positives (based on the validation study of the LDT assay demonstrating analytic specificity of 100%) [19]. A negative sample was considered to be a FN if repeat testing yielded a positive result for ≥ 2 of three gene targets (E gene, N1, and/or N2).

Evaluation of discordant swab quality

All swab sets identified as discordant were tested for the presence of human ribonuclease P (RNAse P) using an RT-PCR assay (see Additional file 1: Table S1) [21].

Statistical analysis

Statistical comparison of parametric variables was done using independent t-tests and non-parametric variables using the Wilcoxon matched-pairs signed rank test. Data analyses were conducted in Stata 14.2 software (Statacorp LP, 2015, College Station, USA).

Results

Between January 21 and April 18, 2020, 100,001 COVID-19 tests (95,919 patients) were completed with 1954 (2%) individual cases confirmed (see Additional file 3: Figure S1). Including repeat tests, the overall positivity rate was 2.2%.

Forty-nine (0.05%) were found to have discordant results (total 101 swabs including 46 patients with two swabs and 3 patients with three swabs). The median age of these patients was 72 years (range 25–97) with 69.4% being female and 26.5% requiring hospitalization (Table 1).

All 101 discordant swabs were available for further evaluation (herein identified as swab 1, swab 2, and swab 3) (Table 2). Original testing results of these 49 patients showed: swab 1 for all 49 patients was negative; swab 2 for 46/49 patients was positive, and swab 3 was positive for 3/3 patients. Repeat testing of swab 1 for each of the 49 patients using a combination of three alternate assays revealed five FN results (Table 2). Of these, 3/5 were NP swabs in UTM and 2/5 were Aptima® swabs used for deep nasal sampling. Ct values for repeat testing of swab 1 specimens among the three different assays ranged from 32.7 to 38.8 cycles (median 35.5). Five swab 1 specimens re-tested positive on the E gene assay and the CDC N2 assay; two swab 1 specimens re-tested positive by all three alternate assays. The mean times of collection (in days) between swab 1 and swab 2 for the FN and non-FN discrepant specimens were 6.1 ($p = 0.06$) and 3.3 ($p = 0.20$), respectively.

Table 1 Demographic characteristics of 49 patients with discordant swab results for COVID-19

Variable		%
Age (years)		
Median	72	
Range	25–97	
Sex		
Male	15	30.6
Female	34	69.4
Exposure to a known case		
Yes	38	77.6
Acquisition		
Health care	9	18.4
Community	38	77.6
Unknown	2	4.0
Healthcare worker	7	14.3
Hospitalization	13	26.5
Travel history	4	8.2

No significant differences in the Ct values for human RNAse P were noted between swabs 1, 2, and 3 (see Additional file 4: Figure S2; all p-values > 0.05).

From the five FN specimens, 4/5 had swab 1 collected on or the day after date of symptom onset (DSO) (Table 3). The maximum duration between DSO and swab 1 was 9 days and swab 2 was eleven days. Swab 2 for all five patients was collected post-DSO (4–11 days). All patients with FN results had community-acquired SARS-CoV-2 infection; three were healthcare workers and three had exposure to a confirmed COVID-19 case.

Based on the additional testing conducted, 5/101 negative swabs were considered FNs with 49/101 presumed to be true positives (TPs). Therefore, FNR (FN/[FN + TP]) in this subset of patients with discordant swabs is 9.3% (95% CI 1.5–17.0%). By extension, the sensitivity (1-FNR) of testing in this subset of discordant swabs is 90.7% (95% CI 82.6–98.9%).

Discussion

The major strength of this study lies in the large sample size (100,001 SARS-CoV-2 rtRT-PCR tests from 95,919 patients) from which discordant results were identified. Discordant results were found for 0.05% of all patients tested. Based on re-testing of 49 patients with discordant results, the FNR and sensitivity of our LDT in this subgroup of patients was approximately 9.3% and 90.7%, respectively.

The FNR calculated from our data analysis is comparable to other reports. Data from earlier in the pandemic reported FNRs of up to 30% [6] with a systematic review on the topic reporting ranges from 2 to 29% [2]. A large study from New York evaluating the clinical performance of SARS-CoV-2 molecular testing found that on average up to 17% of positives were missed by the first test [22], while another American study reported a FNR of 3.5% in patients with discordant swab results within a 7-day period [1]. Two other studies have estimated sensitivities ranging from 89 to 94.6% [22, 23].

In our study, specimen quality was not considered a contributing factor given human DNA content did not differ significantly across all the swabs. A similar approach using RNase P as a surrogate for quality of

Table 2 Evaluation of 49 patients (101 swabs) with discordant COVID-19 testing and confirmatory testing results

	Swab 1 (n = 49 swabs)	Swab 2 (n = 49 swabs)	Swab 3 (n = 3 swabs)[c]
NP swab in UTM (%)	29 (59.2)	32 (65.3)	2 (66.7)
Deep nasal turbinate swab (%)	20 (40.8)	9 (18.4)	1 (33.3)
Oropharyngeal swab (%)	0	8 (16.3)	0
Original swab test result[a] (E gene Ct median; range) (RdRp gene Ct median; range)	49 negative	46 positive 3 negative (19.5; 13.0–35.5) (22.4; 16.2–37.8)	3 positive 0 negative (13.9; 12.5–24.7) (16.5; 14.8–28.0)
Positive result on E gene assay[b] (Ct range)	5/49 (34.1–37.9)	0/3	ND
Positive result on CDC N1 assay[b] (Ct range)	2/49 (32.7–35.9)	0/3	ND
Positive result on CDC N2 assay[b] (Ct range)	5/49 (33.2–38.8)	0/3	ND

Ct cycle threshold (cycles), *E* envelope, *LDT* laboratory developed test, *ND* not done, *NP* nasopharyngeal, *RdRp* RNA dependent RNA polymerase, *UTM* universal transport media

[a] Testing done using E gene/RdRp gene LDT SARS-CoV-2 rtRT-PCR

[b] Repeat testing conducted only on negative swabs to evaluate for false-negative results

[c] Only 3 of 49 patients had a third swab done

Table 3 Timeline (in days) of swab collection and epidemiologic information of five false negative discordant specimens

Patient	−5	−4	−3	−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	Exposed to confirmed case?	Healthcare worker?	Community- or healthcare-acquired?
1						DSO	S1			S2								Yes	Yes	Community-acquired
2						DSO		S1				S2						No	Yes	Community-acquired
3						DSO									S1		S2	Yes	No	Community-acquired
4						DSO, S1						S2						Unknown	No	Community-acquired
5	S1					DSO						S2						Yes	Yes	Community-acquired

Negative numbers indicate days before DSO; day 0 indicates DSO; positive numbers indicate days post-DSO

DSO date of symptom onset, *S1* swab 1, *S2* swab 2

swab collection has been used in several other studies [3, 23, 24].

The five FNs were likely caused by changes in viral load and shedding over time. Based on Ct values, all FNs were found to have low levels of viral RNA. Four of five FN samples had early collections related to the DSO (from 5 days prior to symptom onset to 2 days post-symptom onset). The other FN sample was collected 9 days post-symptom onset with the swab found to be positive for this patient with routine testing having been collected 2 days later, which could be related to variable shedding after the acute phase of infection [8]. Variable shedding dynamics have also been noted by authors of a pooled analysis of 1330 samples with FNR estimated as 20% at three days post DSO, 38% on the DSO, and 67% on the day prior to DSO [4].

Three of five FN swabs were collected using an NP flocked swab in UTM and the other two were collected using the Aptima® swab and transport medium. While this may indicate that these swab types and media did not influence the FNR, more data is needed to support this. However, one study indicated that Aptima® products are as good or better than routine flocked NP/UTM swabs for detecting SARS-CoV-2, attributed in part to the preservatives in the Aptima® transport solution preventing RNA degradation [25].

The principal limitations of this study are its retrospective nature and that FN samples were biased towards patients undergoing repeat swab collection, likely due to high suspicion of COVID-19. Ideally, a cohort of negative patients would be tested using multiple NAT tests and re-tested prospectively, but this poses logistical challenges and would require a large number of patients to be screened. Another limitation is the assumption that all positives by the local LDT were true positives. However, the analytical specificity of the LDT is reported as 100% [19] and it demonstrated a high negative percent agreement with the CDC N1/N2 assay. Most other SARS-CoV-2 rtRT-PCR assays have shown high clinical specificities, making this a reasonable assumption [26, 27].

Conclusions

This work adds to the literature by demonstrating that the FNR of SARS-CoV-2 molecular assays is low [1, 27, 28] and subject to viral load dynamics over time. However, the interpretation of COVID-19 test results should be conducted in the overall context of each patient's clinical presentation [9, 29], with repeat testing advised should post-test probability upon follow-up clinical evaluation remain high.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-021-01489-0.

Additional file 1: Table S1. Commercial products utilised in the process of SARS-CoV-2 rtRT-PCR testing.

Additional file 2: Table S2. Comparison of LDT to CDC N1/N2 assay using 100 specimens selected randomly from those tested.

Additional file 3: Figure S1. Results of COVID-19 testing from the first 100,001 tests completed.

Additional file 4: Figure S2. RNAse P detection (based on Ct value) between swab sets **(a)** on 46 patients who had two swabs that were discordant; and **(b)** on 3 patients with three swabs.

Abbreviations

Ct: Cycle threshold; DSO: Date of symptom onset; FN: False-negative; FNR: False-negative rate; LDT: Laboratory developed test; NATs: Nucleic acid tests; NP: Nasopharyngeal; OP: Oropharyngeal; PCR: Polymerase chain reaction; RdRp: RNA-dependent RNA-polymerase; RNAse P: Human ribonuclease P; rtRT-PCR: Real-time reverse-transcriptase polymerase chain reaction; UTM: Universal transport media; WHO: World Health Organization.

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Authors' contributions

JNK participated in the formal data analysis, writing of the original draft, as well as review and editing of the manuscript. NZ conceptualised the idea for the project and contributed to data curation, validation, project administration, methodology, investigation, as well as review and editing of the manuscript. CM contributed to the conceptualisation, methodology, formal analysis, data curation, writing of the original draft of the manuscript, as well as review and editing of manuscript. KP contributed to the methodology, investigation, provided laboratory resources, spent time with data curation, as well as review and editing of the manuscript. MNK contributed to data curation as well as review and editing of the draft. AP contributed to data curation as well as writing and review of the first draft of the manuscript. JH provided oversight of the project, administrative support, as well as review and editing of the manuscript. MD provided support with regards to manuscript review and editing along with initial critique of the project writeup. BMB provided support with regards to review and editing of the manuscript. GP provided support with regards to supervision, project administration, and review and editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The Human Research Ethics Board at the University of Alberta approved this study protocol (Reference Number Pro00100001).

Consent to publication

Not applicable.

Competing interests

All authors declare that there are no conflicts of interests or competing interests with regards to the material in this manuscript.

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Publisher's Note

Clinical Infectious Diseases

BRIEF REPORT

Sensitivity of Nasopharyngeal Swabs and Saliva for the Detection of Severe Acute Respiratory Syndrome Coronavirus 2

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We enrolled 91 consecutive inpatients with COVID-19 at 6 hospitals in Toronto, Canada, and tested 1 nasopharyngeal swab/saliva sample pair from each patient using real-time RT-PCR for severe acute respiratory syndrome coronavirus 2. Sensitivity was 89% for nasopharyngeal swabs and 72% for saliva ($P = .02$). Difference in sensitivity was greatest for sample pairs collected later in illness.

Keywords. COVID-19; SARS-CoV-2; nasopharyngeal swab; saliva.

Rapid and accurate detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in patient specimens is critical to controlling the coronavirus disease 2019 (COVID-19) pandemic. As yet, there are few data comparing sensitivity of different specimen types for SARS-CoV-2 detection.

In Canada, nasopharyngeal (NP) swabs are the preferred collection site for SARS-CoV-2 testing [1, 2], and preliminary data suggest that they may be more sensitive than oropharyngeal swabs for SARS-CoV-2 detection [3, 4]. However, collection of both NP and oropharyngeal swabs is uncomfortable for patients and may pose a risk to healthcare workers. Moreover, recent global supply-chain shortages have resulted in limited access to various swab types. Saliva, in contrast, can be easily self-collected by most adolescents and adults. Other groups have demonstrated successful detection of SARS-CoV-2 in saliva specimens and use of saliva for serial sampling [5–7]. We aimed to compare the sensitivity of NP swabs and saliva for SARS-CoV-2 detection in hospitalized patients.

METHODS

The Toronto Invasive Bacterial Disease Network (TIBDN) performs population-based surveillance for select infectious diseases in metropolitan Toronto and the regional municipality of Peel (population base, 4.2 million in 2016), Ontario, Canada. For COVID-19, clinical microbiology laboratories report specimens testing positive for SARS-CoV-2 to the central study office. Starting on 16 March 2020, study staff enrolled consecutive inpatients at 6 TIBDN hospitals. Patient demographic, exposure, and medical data were collected by interview and chart review. An NP swab and saliva specimen were collected on the day of enrollment, and then 3 subsequent pairs of samples were obtained at 72-hour intervals if the patient remained hospitalized. The NP swabs were collected as per standard procedures and placed into UTM viral transport medium (COPAN Diagnostics, Murrietta, CA) [8]. For saliva specimens, patients were asked to spit 1 teaspoon (5 mL) of saliva into a sterile specimen container and then 2.5 mL of phosphate-buffered saline was added.

Samples were transported to the research microbiology laboratory, where they were aliquoted and frozen at −80°C within 8 hours of collection. On 14 April, we selected each patient's most recent NP swab/saliva sample pair for SARS-CoV-2 real-time reverse transcriptase–polymerase chain reaction (RT-PCR) testing. On 1 June, we repeated the selection for new patients enrolled since 14 April. Laboratory testing was with the Allplex 2019-nCoV Assay (100T) (Seegene Inc, Seoul, Korea) to detect RNA-dependent RNA polymerase (RdRp), envelope (E), and nucleocapsid (N) genes at Sinai Health System (Toronto, Canada).

RESULTS

Ninety-one inpatients were included; all were confirmed to have COVID-19 with an NP, midturbinate, or nasal swab tested in a clinical laboratory in Toronto. The median age was 66 years (range, 23–106 years), 39 (43%) were female, 70 (77%) had at least 1 comorbidity, and 12 (13%) were immunocompromised. Eighteen (20%) had a household contact as the suspected source of exposure. On admission, 66 (73%) had fever and 68 (75%) had cough. The median time from illness onset to hospital admission was 6 days (interquartile range [IQR], 2–9 days) and 27 (30%) required intensive care. The median time from illness onset to collection of the tested specimens was 12 days (IQR, 9–15 days). As of 5 June, 3 (3%) patients remained hospitalized, 82 (90%) were discharged, and 6 (7%) had died.

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Table 1. Results of Testing of Nasopharyngeal Swab and Saliva for SARS-CoV-2 RNA in Hospitalized Patients With COVID-19, by Time From Illness Onset to Collection of Sample Pair

Time From Illness Onset to Specimen Collection	No. of Patients (%)			
	With NP Swabs and Saliva Both Positive	With NP Swab Only Positive	With Saliva Only Positive	With NP Swab and Saliva Both Negative
0–7 days (n = 18)	14 (78)	2 (11)	1 (6)	1 (6)
8–14 days (n = 43)	21 (49)	13 (30)	4 (9)	5 (12)
≥15 days (n = 30)	9 (30)	5 (17)	3 (10)	13 (43)
Any (n = 91)	44 (48)	20 (22)	8 (9)	19 (21)

N = 91.

Abbreviations: COVID-19, coronavirus disease 2019; NP, nasopharyngeal; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Of 91 patients with paired samples tested, 72 (79%) had at least 1 positive specimen. In 44 (61%) of these 72 patients, both NP swab and saliva were positive, in 20 (28%) only the NP swab was positive, and in 8 (11%) only saliva was positive ($P = .02$) (Table 1). Thus, using NP swabs only would have detected 64 of 72 (89%) patients with at least 1 positive specimen and using saliva only would have detected 52 of 72 (72%) patients with at least 1 positive specimen. Using NP swabs only would have detected 16 of 17 (94%), 34 of 38 (89%), and 14 of 17 (82%) patients in their first, second, and third/fourth week of illness, respectively (Table 1). Using saliva only would have detected 15 of 17 (88%), 25 of 38 (66%), and 12 of 17 (71%) patients in their first, second, and third/fourth week of illness, respectively (Table 1).

The median N gene cycle threshold (Ct) for NP swabs was 30 (IQR, 26–35) when the saliva specimen in the pair was positive (n = 44) versus 34 (IQR, 31–37) when the saliva specimen in the pair was negative (n = 20) ($P = .003$). N gene Ct values were higher if samples were collected later in illness (Spearman's $\rho = 0.3$, $P = .0003$). Results were similar when Ct values of the E and RdRp genes were used (data not shown).

DISCUSSION

In this sample of 91 inpatients, NP swabs were 17% more sensitive than saliva overall. Sensitivity of both types of specimens was highest in the first week of illness, when viral concentrations have been reported to be highest [4, 9]. The difference in sensitivity between NP swabs and saliva was 6% if collected in the first week of illness and 20% if collected in the second week of illness or later. Our data suggest that NP swabs are more sensitive than saliva for SARS-CoV-2 detection, especially if the patient is later in illness.

Our data also suggest that neither a single NP swab nor a single saliva specimen is 100% sensitive for the detection of COVID-19. This is consistent with prior literature [10], emphasizing that a single negative test does not rule out disease in patients with a high pretest probability of COVID-19. Repeated samples may improve yield. For example, among patients with a high pretest probability for COVID-19 and a negative NP swab, repeating the NP swab and also collecting a saliva sample may be considered, as saliva sampling is noninvasive and 11% of patients in this study with at least 1 positive specimen were only positive in their saliva.

There are several limitations to this analysis. As these patients were originally diagnosed using NP, midturbinate, or nasal swabs, it is possible that there is a bias towards subsequent NP swabs versus other specimens being positive. We used a single detection system (Seegene), and other platforms may have yielded different results. We simply asked patients to spit a teaspoon of saliva into a specimen container; many patients were unable to provide a full teaspoon of saliva, and this may in part explain the gap in sensitivity between NP swabs and saliva. It is also possible that other methods, such as throat washing with normal saline, would have improved yield. One small study found throat washing to be significantly more sensitive than NP swabs for SARS-CoV-2 detection, possibly enabling the acquisition of more epithelial cells [11]. Throat washing is easy to self-collect and should be further investigated as a noninvasive alternative to NP swabs and other invasive swabs such as oropharyngeal swabs.

In conclusion, NP swabs were more sensitive than saliva for SARS-CoV-2 detection, particularly among patients beyond the first week of illness. Notably, however, NP swabs were only 6% more sensitive than saliva among the 18 sample pairs collected in the first week of illness in this study. This raises the possibility that NP swabs and saliva are equivalent early in illness, but this requires study in a larger sample. More data are also needed to assess testing on different platforms and to assess the sensitivity of different specimen types in asymptomatic patients or those whose illness does not require hospitalization.

Notes

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Identification of SARS-CoV-2 in a Proficiency Testing Program

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ABSTRACT

Objectives: *At the onset of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic in the United States, testing was limited to the Centers for Disease Control and Prevention–developed reverse transcription polymerase chain reaction assay. The urgent and massive demand for testing prompted swift development of assays to detect SARS-CoV-2. The objective of this study was to assess the accuracy of these newly developed tests.*

Methods: *The American Proficiency Institute sent 2 test samples to 346 clinical laboratories in order to assess the accuracy of SARS-CoV-2 assays. The positive sample, containing 5,175 viral copies/mL, was fully extractable with SARS-CoV-2 viral capsid protein and RNA. The negative sample, with 3,951 viral copies/mL, contained recombinant virus particles with sequences for targeting human RNAase P gene sequences.*

Results: *Of the laboratories submitting results, 97.4% (302/310) correctly detected the virus when present and 98.3% (296/301) correctly indicated when the virus was not present. Among incorrect results reported in this proficiency challenge, 76.9% (10/13) were likely related to clerical error. This accounts for 1.6% (10/611) of all reported results.*

Conclusions: *Overall performance in this SARS-CoV-2 RNA detection challenge was excellent, providing confidence in the results of these new molecular tests and assurance for the clinical and public health decisions based on these test results.*

Key Points

- Overall performance in this SARS-CoV-2 RNA detection challenge was excellent, providing confidence in the results of these new molecular tests.
- Demand for quality SARS-CoV-2 tests is universal. Laboratories from 46 states and 4 countries participated in the first US assessment of test accuracy.
- Over 30 tests methods were reported by the more than 300 respondents in this challenge.

In December 2019 a cluster of respiratory disease cases were recognized in Wuhan, China.[1] By January 2020, the cause of the infections was identified as a novel coronavirus that was later designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[2] The first confirmed case of SARS-CoV-2 infection in the United States was identified in Washington in a traveler returning from Wuhan.[3] As of June 1, 2020, over 1.7 million cases and over 102,000 deaths have been reported in the United States.[4] The rapid and widespread transmission of the virus led to unprecedented social and economic disruptions as governments ordered schools and businesses closed.

At the onset of the US SARS-CoV-2 epidemic, testing was limited to the Centers for Disease Control and Prevention (CDC) and state public health laboratories using the CDC-developed reverse transcription (RT) polymerase chain reaction (PCR) assay. In early 2020, the urgent and massive demand for testing led to rapid development and validation of commercial and laboratory-developed assays to detect SARS-CoV-2.

In the United States, each clinical laboratory is required to verify performance of Food and Drug

Administration (FDA)-approved and/or validated laboratory diagnostic tests. However, there is no standard for the number of samples to be included in a verification or the acceptable performance level.[5] This means there is considerable variation among laboratory verification studies, leading to concerns about reliability of test results.

Globally, SARS-CoV-2 molecular tests are being performed in laboratories with a range of experience and technical capacity for nucleic acid amplification testing. The commercial and laboratory-developed tests, even if validated and approved for use by a regulatory body, have little performance history in wide deployment. Therefore, an objective measure of system-wide product and laboratory quality is needed.

Results of SARS-CoV-2 testing are used not just for patient management but also for infection control in health care settings and for surveillance data that drive decisions on community-wide sheltering orders. SARS-CoV-2 test results are the cornerstone of contact tracing activities to control ongoing disease transmission. This study was undertaken to evaluate the performance of laboratories enrolled in a SARS-CoV-2 RT-PCR proficiency testing (PT) program and to assess reliability of test kits and methods.

The American Proficiency Institute (API) is a PT provider approved by the federal Centers for Medicare and Medicaid Services under the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88).[5] API provides PT samples and performance analysis to over 20,000 laboratories with over 350 programs. Subscribing laboratories are shipped samples and instructed to test them in the same manner as patient specimens. Each laboratory reports its results to API. API then provides performance feedback to subscribers. Analysis of PT results has been useful in the past to reveal deficiencies in testing quality and has led to recommendations that improved testing accuracy.[6,7] The ability of laboratories to correctly detect the presence or absence of SARS-CoV-2 has not previously been studied by CLIA-approved PT organizations. Data from these studies are important because they provide a snapshot of current laboratory practices and accuracy.

In this report we present the results of the first US study of SARS-CoV-2 accuracy by API participant laboratories from the 2020 First Test Event.

Materials and Methods

Data were acquired from a single PT event, 2020 First Test Event, by API. As part of this PT event, 2 samples (both noninfectious and manufactured by SeraCare) were shipped overnight in May 2020 to 346 laboratories enrolled in the API program. These subscribers represented domestic and international commercial laboratories, public health laboratories, clinics, hospitals, and diagnostic kit manufacturers.

The positive sample (COV-01), containing 5,175 viral copies/mL, was fully extractable with SARS-CoV-2 RNA encoding viral capsid protein and RNase P. It was prepared to be compatible with assays targeting the following regions: ORF1a, RdRp, S (spike), E (envelope), and N (nucleocapsid). The negative sample (COV-02), with 3,951 viral copies/mL, contained recombinant virus particles with sequences for targeting sequences from human RNase P gene. The proficiency samples were formulated in viral transport media consisting of Tris-buffered saline, with added antimicrobial agents, glycerol, and human proteins.

Participating testing sites were located in 50 clinical point-of-care testing sites, 182 hospital-based laboratories, 37 independent laboratories, 3 diagnostic kit manufacturers, and 5 government care facilities; 17 testing sites did not indicate laboratory type. Participating laboratories were located in 46 US states and 4 international sites (Malaysia, Pakistan, Taiwan, and Vietnam.)

Laboratories were instructed to submit the interpreted results (detected or not detected) for COV-01 and COV-02, and to provide the instrument and test kit used for testing the samples. Since laboratories using traditional PCR methods could participate, cycle threshold (Ct) values were not requested on the report. Of the 346 laboratories that received proficiency materials, 310 submitted results by the reporting deadline. The results from these samples were processed with proprietary software developed at API.

Results

Correct positive results were reported by 302 laboratories (97.4%), with 8 laboratories incorrectly reporting negative results for COV-01 (2.6%). Negative results were reported by laboratories using Applied Biosystems/Quidel Lyra SARS-CoV-2 and Luminex ARIES SARS-CoV-2 and had correct negative results for COV-02. For COV-02, 306 laboratories correctly reported negative results (98.3%) **Table 1**. The 9 laboratories who reported a testing problem with sample COV-02 all used the BioGx SARS-CoV-2 reagent on the BD Max System. BioGx users indicated that their results for sample COV-02 were "unresolved" due to an internal control failure and were thus nonreportable. The BioGx SARS-CoV-2 reagent (along with several other testing methods) requires human RNase P to be present in a sample to serve as

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Table 1

Overall Performance by Test Method of 310 Testing Sites Participating in SARS-CoV-2 RNA Detection Proficiency Testing Challenge[a]

Method	COV-01, No. (% Correct)	COV-02, No. (% Correct)
Abbott m2000/Abbott RealTime SARS-CoV-2 assay	5 (100)	5 (100)
Agilent AriaMx/Light Power IVASARS-CoV-2	1 (100)	1 (100)
Applied Biosystems PCR/Altona RealStar SARS-CoV-2	1 (100)	1 (100)
Applied Biosystems PCR/CDC 2019-nCoV RT-PCR	13 (100)	13 (100)
Applied Biosystems PCR/Lab Corp COVID-19 RT-PCR	1 (0)	1 (0)
Applied Biosystems PCR/NY Wadsworth SARS-CoV-2 RT-PCR	1 (100)	1 (100)
Applied Biosystems PCR/Quest Diagnostics SARS-CoV-2	1 (100)	1 (100)
Applied Biosystems PCR/Quidel Lyra SARS-CoV-2	2 (0)	2 (100)
Applied Biosystems PCR/Thermo Fisher TaqPath COVID-19	9 (100)	9 (100)
BD Max /BD SARS-CoV-2	5 (100)	5 (100)
BD Max /BioGx SARS-CoV-2	25 (100)	16 (100)[b]
Bio-Rad CFX/ADT LyteStar 2019-nCOV	1 (100)	1 (100)
Bio-Rad CFX/CDC 2019-nCoV RT-PCR	1 (100)	1 (100)
Bio-Rad CFX/Curative-Korva SARS-CoV-2	1 (100)	1 (100)
Bio-Rad CFX/Logix Smart SARS-CoV-2	1 (100)	1 (100)
Bio-Rad CFX/Thermo Fisher TaqPath COVID-19	3 (100)	3 (100)
Cepheid Xpert Xpress SARS CoV-2	189 (99.5)	189 (99.5)
DiaSorin Simplexa COVID-19	4 (100)	4 (100)
GenMark ePlex SARS-CoV-2	2 (100)	2 (100)
Hologic Panther Fusion SARS-CoV-2	1 (100)	1 (100)
Luminex ARIES SARS-CoV-2	6 (83.3)	6 (100)
Luminex NxTag CoV	2 (50)	2 (50)
Mesa Biotech Accula SARS-CoV-2	1 (100)	1 (100)
NeuMoDx SARS-CoV-2	2 (100)	2 (100)
QIAstat-Dx Respiratory SARS-CoV-2 Panel	5 (100)	5 (100)
QuantStudio/CDC 2019-nCoV RT-PCR	1 (0)	1 (100)
QuantStudio/LabTurbo AIO COVID-19	1 (100)	1 (100)
QuantStudio/Thermo Fisher TaqPath COVID-19	2 (50)	2 (50)
Roche cobas 6800, 8800/cobas SARS-CoV-2	16 (100)	16 (100)
Roche cobas Z480/cobas SARS-CoV-2	1 (100)	1 (100)
Roche LightCycler/CDC 2019-nCoV RT-PCR	3 (100)	3 (100)
Sacace PCR/Sansure Biotech (2019-nCOV)	1 (100)	1 (100)
Sentosa SA201/ViroKey SARS-CoV-2	2 (100)	2 (100)
Total	302 (97.4)	296 (98.3)

COVID-19, coronavirus disease 2019; RT-PCR, reverse transcription polymerase chain reaction.
[a]Sample COV-01 contained 5,175 copies/mL of SARS-CoV-2 virus; sample COV-02 was negative for SARS-CoV-2.
[b]Nine laboratories reported a testing problem with sample COV-02 due to lack of internal amplification control.

an endogenous nucleic acid extraction control, which is present in all properly collected patient samples. Sample COV-02 did contain 3,951 copies/mL of RNase P; however, this was very near the limit of detection for the BioGx reagent, resulting in 9 of 25 laboratories using the reagent recovering levels of RNase P below the required threshold of detection.

Table 2 shows that all types of laboratories performed well, with consensuses higher than 90%. The manufacturer's category was excluded due to a low number of participants. False-negative results were reported by 4 independent laboratories, 4 hospital-based laboratories, and 1 diagnostic kit manufacturer. False-positive results were reported by 1 hospital-based laboratory and 4 independent laboratories. All 5 laboratories that reported false-negative results for COV-01 also reported a false-positive result for COV-02, indicating probable clerical errors during testing or reporting.

Among incorrect results reported in this proficiency challenge, 76.9% (10/13) were likely related to clerical error. This accounts for 1.6% (10/611) of all reported results.

Discussion

Before passage of CLIA '88, participation in PT was voluntary for many clinical laboratories. With the implementation of the CLIA '88 rules, PT evolved from an educational self-assessment tool to a measure that is fundamental for trend analysis, risk management, and laboratory accreditation. Performance on PT is a vital, objective indicator of the quality of clinical testing.

Monitoring and analyzing PT results from a large group of participating clinical laboratories helps to assess the accuracy of test methods applied in a variety of settings and individual laboratory performance. The significance of this

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Table 2

Overall Performance by Type of Laboratory of 310 Testing Sites Participating in SARS-CoV-2 RNA Detection Proficiency Challenge[a]

Method	COV-01, No. (% Correct)	COV-02, No. (% Correct)
Clinic/Physician office laboratory	50 (100)	50 (100)
Hospital laboratory ≤100 beds	57 (100)	57 (100)
Hospital laboratory 101-200 beds	47 (97.9)	47 (100)
Hospital laboratory 201-300 beds	44 (95.5)	44 (97.7)
Hospital laboratory 301-400 beds	23 (100)	23 (100)
Hospital laboratory >400 beds	19 (100)	19 (100)
Independent laboratory	41 (90.2)	41 (90.2)
Manufacturer	3 (66.7)	3 (100)
Point of care	1 (100)	1 (100)
VA hospital/clinic	6 (100)	6 (100)
Not indicated	17 (100)	17 (100)
Total	310	310

[a]Sample COV-01 contained 5,175 copies/mL of SARS-CoV-2 virus; sample COV-02 was negative for SARS-CoV-2.

measure is compounded when a test is new and widely practiced and results have several applications. SARS-CoV-2 RNA amplification test results are used for patient management, infection control in health care settings, contact tracing, and epidemiologic surveillance data. SARS-CoV-2 test results are the cornerstone of contact tracing activities, community-wide sheltering orders, and control of ongoing disease transmission during this historic pandemic.

With an overall consensus greater than 97%, this SARS-CoV-2 RNA detection challenge indicates excellent accuracy by test participants when evaluated by testing method and type of laboratory. The results of this study should provide confidence in clinical laboratory test results for patient management and public health decisions.

There are several limitations to this study. While PT materials are designed to mimic patient specimens and be stable, they are not exactly the same matrix and do not have the entire viral genome for safety considerations. This limitation is demonstrated by the challenges that laboratories using Applied BioSystems/Quidel Lyra reported. SARS-CoV-2 is not an analyte that CLIA requires PT for at this time. Therefore, the results of this voluntary program do not reflect performance across all laboratories performing SARS-CoV-2 amplification assays. Laboratories may have deployed multiple testing methods, but results are reported for only 1 method per laboratory. This study may not accurately represent the true scope of method deployment. Finally, Ct values were not collected. Ct value comparison for sites using the same quantitative PCR method may be valuable to assess interlaboratory variability.

Negative results for the COV-01 sample were reported by laboratories using Applied Biosystems/Quidel Lyra SARS-CoV-2 and Luminex ARIES SARS-CoV-2; these laboratories had correct negative results for the COV-02 sample. Upon further review, sample COV-01 lacked the target region (pp1ab) of the SARS-CoV-2 genome that is detected by the Quidel Lyra test system. Future PT challenge samples should include target gene sequences detected by all FDA-approved SARS-CoV-2 RNA assays. One of 6 laboratories using Luminex ARIES SARS-CoV-2 reported a negative result for COV-01 but reported expected results for COV-02. This result pattern is not typical of clerical errors, and laboratories using this method should closely monitor test performance through heightened ongoing verification activities.

Five testing sites reported both a false negative for COV-01 and a false positive for COV-02, suggesting clerical errors. Typical rate for clerical errors in reporting PT results is historically about 1%. While clerical errors do not reveal lack of sensitivity or specificity of the test method, they have an equal impact on treatment, infection control, and disease control efforts. Laboratories that do not meet expected performance on PT challenges due to clerical errors must investigate and correct the processes from which these errors arose.

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

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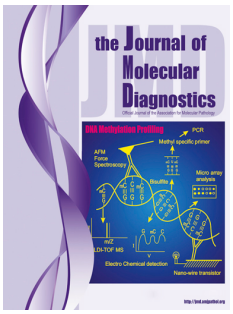

Prediction of false positive SARS-CoV-2 molecular results in a high-throughput open platform system

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Prediction of false positive SARS-CoV-2 molecular results in a high-throughput open platform system

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Running header: Predicting false positive SARS-CoV-2 results

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Abstract:

Widespread high-throughput testing for identification of SARS-CoV-2 infection by RT-PCR has been a foundation in the response to the COVID-19 pandemic. Quality assurance metrics for these RT-PCR tests are still evolving as testing becomes widely implemented. As testing increases, it is important to understand performance characteristics and the errors associated with these tests. Here, we investigate a high-throughput, laboratory developed SARS-CoV-2 RT-PCR assay to determine if modeling can generate quality control metrics that identify false positive (FP) results due to contamination. This study reviewed repeated clinical samples focusing on positive samples that test negative upon re-extraction and PCR, likely representing false positives. To identify and predict false positive samples, we constructed machine learning derived models based on the extraction methodology used. These models identified variables associated with false positive results across all methodologies, with sensitivities for predicting FP results ranging between 67-100%. Application of the models to all results predicted a total FP rate of 0.08% across all samples, or 2.3% of positive results, similar to reports for other RT-PCR tests for RNA viruses. These models can predict quality control parameters, enabling laboratories to generate decision trees that reduce interpretation errors, allow for automated reflex testing of samples with a high FP probability, improve workflow efficiency and increase diagnostic accuracy for patient care.

Introduction

The COVID-19 pandemic created the need to rapidly implement high-throughput, widespread testing in the United States. The primary method for detecting SARS-CoV-2, the RNA virus responsible for COVID-19, is reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is currently widely used and is the gold-standard for the diagnosis of many infectious diseases[1]. RT-PCR has become the predominate diagnostic modality for viral disease as results are rapidly returned, it demonstrates high specificity and sensitivity and it is relatively inexpensive[2]. However, as COVID-19 cases spread, it was quickly apparent that the need for testing outpaced health departments and clinical laboratories ability to provide testing. Thus, a multitude of RT-PCR and transcription mediated amplification (TMA) assays testing for SARS-CoV-2 have received emergency use authorization (EUA) from the FDA that include both closed platform and high-throughput, open-platform reactions (FDA, https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas, last accessed 3/4/2021). Indeed, approximately 336 million SARS-CoV-2 tests have been performed in the US, with an increased need in current infectivity hot-spots (CDC, https://covid.cdc.gov/covid-data-tracker/#cases_totalcases, last accessed 3/4/2021). Due to the rapid development and implementation of these assays, robust universal rules for interpretation and quality assurance of results have not been well-defined in the clinical setting.

Guidelines for SARS-CoV-2 testing have been released by working groups and mainly focus on clinical scenarios of when to employ testing[3]. Many SARS-CoV-2 clinical tests receiving EUA only have interpretation guidelines for persons under investigation (PUI), or those patients with an increased pretest probability of COVID-19 disease. Most of these same tests have not been validated as a screening technique for mildly symptomatic or asymptomatic patients, even though they are widely used in this manner. Testing of asymptomatic patients is a cornerstone for combating the spread of SARS-CoV-2 because transmission can be facilitated

by patients with minimally or pre-symptomatic infections[4]. As these tests have not been fully studied in the setting of minimally symptomatic individuals, there is a potential for resultant errors. Attributing a positive SARS-CoV-2 result to an asymptomatic patient has an impact on the mental health, physical health and socioeconomic wellbeing of that patient. A positive result also has wide-ranging impacts on infection control measures in the healthcare system and the community, such as isolation procedures in the hospital, closure of schools and daycares, and the halting of nursing home visits. In a low prevalence population with low pre-test probability, there is increased concern that a positive result is an error and represents a false-positive result. Additionally, a false positive SARS-CoV-2 result (FP, defined as a non-reproducible result on repeat extraction and RT-PCR) in a patient with significant symptoms due to other causes, such as a congestive heart failure patient with acutely worsening shortness of breath and cough, could lead to improper medical management. Thus, there is a need to understand error rates of SARS-CoV-2 assays to reduce the risk of false results. External quality assessments (EQAs) are traditionally performed on molecular assays by providing clinical laboratories with positive and negative samples and determining FP and false negative (FN) rates from these blinded tests. FN results due to poor nasopharyngeal sampling or changes in anatomical viral replication have been identified and discussed elsewhere[5]. However, a recent study analyzing EQAs for RNA virus detection found FP rates ranged from 0% up to 16.7%, with a median of 2.3%[6]. Therefore, it is highly likely a small portion of SARS-CoV-2 RT-PCR positive results are FPs and should be investigated.

As the need for SARS-CoV-2 testing was increasing, shortages of testing supplies developed. Thus, our clinical lab established a high-throughput, open platform SARS-CoV-2 assay using the CDC developed primers and probes that could be easily adapted for multiple extraction methodologies[7]. To study FP results across multiple extraction systems, clinical samples with concern for contamination or those with low viral load, as defined by late cycle threshold (Ct), underwent repeat extraction and testing from the primary sample. In general, FP

results from PCR may be due to testing the wrong sample (due to mislabeling, sample mix-up, or reporting errors), cross-reactivity of the PCR assay, or contamination. Contamination is of particular concern in an open platform assay when aerosolization from human or machine handling can cause the transfer of target genomic material between wells, especially when there are true positive (TP, defined as reproducible results on repeat extraction and RT-PCR) samples that can exhibit as much a 10^6 range in virus quantity. Results of initial and repeat testing were compared to determine if there were discordant results on repeat. Initially positive samples that were discordant were considered FP, while concordant samples were considered TP. Next, machine learning derived models were generated taking into account the relative viral load of the index sample, the extraction methodology and relative viral load of surrounding positive wells. These models were used as a clinical decision support tool to identify wells with a high probability of being a FP and to identify technical contributors to FP samples. Modeling identified FP samples that could be predicted across different extraction methodologies, with each model identifying different variables used to make FP predictions. From these models we can identify potential technical improvements as well as identify samples with a high probability of FP results to act as an adjunct for laboratory clinical decision making and initiate automated sample re-extraction and repeat.

Materials and Methods

SARS-CoV-2 PCR testing

Detection of SARS-CoV-2 RNA by RT-PCR has been detailed previously[7]. Individual RT-PCR reactions were performed in a 384-well plate using the United States Centers for Disease Control-designed primers and probes specific for the N1, N2 regions of the SARS-CoV-2 virus and human RNase P (RP). Nucleic acid extractions were performed using: a) manual, column-based methods [either Qiagen QIAamp Viral RNA Mini Kit (Qiagen, Gemantown, MD) or Macherey Nagel Nucelospin RNA Virus kit (Macherey Nagel, Bethlehem, PA)] run in batches of

16 samples, b) the semi-automated 16-sample throughput Promega Maxwell RSC Viral Total Nucleic Acid kit (Promega, Madison, WI) on the Maxwell RSC instrument, c) the automated 96-sample throughput MagMAX Viral and Pathogen Nucleic Acid Isolation kit on the Thermo KingFisher Flex instrument (Thermo Fisher Scientific, Waltham, MA) or d) the automated 96-sample throughput Zymo Quick-DNA/RNA Viral Mag Bead kit (Zymo Research, Irvine, CA) on the Tecan Fluent instrument (Tecan Group, Männedorf, Switzerland). Note that the Maxwell RSC viral protocol has multiple manual pipetting steps of individual samples and was considered a manual protocol for modelling purposes. Extractions were performed per manufacturer's protocols except as noted with the following modifications. For the Qiagen extraction, modifications included use of 100μl of primary patient specimen and an elution volume of 50μl. For the Macherey Nagel extraction, modifications included use of 100μl of primary patient specimen and use of 650μl of lysed sample loaded onto extraction columns. For extraction using Qiagen, Macherey Nagel or Promega kits, batches of 16 primary nasopharyngeal samples were inactivated in a biosafety hood and extracted in parallel within a biosafety cabinet (BSC) or on the Maxwell instrument and transferred to individually-capped, 2D-barcoded tubes arranged in two rows of 8 samples for transport to the physically separate PCR setup facility; there samples were opened with automated instrumentation and transferred into 96-well plates (combining six extraction batches of 16 samples) using automation-assisted pipetting instruments. Automation-assisted pipetting instruments were then used to aliquot a 384-well PCR assay plate from the 96-well sample master plates. For the automated extraction processes using KingFisher or Zymo kits, racks of 93 primary nasopharyngeal samples plus three controls were manually transferred in a BSC into 96 well plates for viral inactivation, and then placed on the automated RNA extraction instrument to complete the procedure. Following completion of automated extraction, plates were sealed and transferred to the physically separate PCR setup facility; there, 96-well plates were unsealed and used for 384-well PCR

assay plate set up as above. The assay limit of detection is between 500-1600 viral copies per milliliter with minor variation between extraction methods. 500 viral copies correlated to Ct values of between 37-37.5 across 20 replicates performed using the EUA protocols (Data not shown).

For studies on follow up testing after a predicted or repeated FP result, PCR results were performed by one of four tests: the CDC N1/N2 PCR test described above, Xpert Xpress SARS-CoV-2 PCR assay (Cepheid, Sunnyvale, CA), Aptima SARS-CoV-2 TMA assay (Hologic, Marlborough, MA), or Simplexa SARS-CoV-2 PCR assay (DiaSorin, Cypress, CA). All tests were performed as per manufacturer instructions.

Results Interpretation

Results were initially interpreted by an automated algorithm following CDC and FDA recommendations as follows:

N1 AND N2 Ct <= 40	Positive for SARS-CoV-2
ONLY N1 OR N2 Ct <= 40	Inconclusive for SARS-CoV-2
N1 AND N2 Ct Undetected AND RP Ct <= 38	Negative for SARS-CoV-2
Undetected AND RP Ct > 40	Invalid

Pathologists reviewed all auto-generated positive, inconclusive and invalid results and multicomponent plots to confirm data quality and result interpretation; this review determined the need for repeat analysis based on data quality or plate layouts indicating proximity of other positive samples. Inconclusive results with multicomponent plots demonstrating exponential amplification above a positive threshold (normalized reporter value >0.5) with no concern for contamination were not repeated and resulted as positive as per FDA recommendations. Positive or inconclusive samples with concern for contamination were re-extracted and repeated. Open platform testing can be more prone to contamination of viral products due to aerosolization, plate seal removal or pipettor drag during extraction or PCR setup[8]. To combat the risk of FP results in this open-platform LDT, samples underwent repeat extraction and PCR

if there was concern for contamination from surrounding wells as indicated by a low relative viral load (RVL; see below for calculation) in the index well and high RVL in surrounding wells. Low RVL was defined at approximately less than 0.015 (-1.80 after $\log_{10}$ transformation), which correlates with a N1/N2 Ct values of 34 and an RP Ct value of 28. RP Ct of 28 was selected as it was approximately the average value, and the N1/N2 Ct 34 value was empirically as it was approximately 50-fold less abundant than RP. Extraction methodology was also taken into account when selecting wells for repeat as manual and automated extractions empirically demonstrated different patterns of potential contamination in early quality control analyses (see Supplemental Figure S1). Inconclusive samples with poor amplification identified by multicomponent plot review were re-extracted and repeated. Negative samples with RP Ct between 38-40 are re-extracted once to confirm the negative result. All invalid results were repeated once.

Data preparation

Multiple datasets were generated from the analysis of the raw PCR data. The raw dataset was first parsed for technically valid runs with no errors. Next, RVL was calculated for all autoscored positive and inconclusive samples (as defined by the interpretation algorithm described above) using the following formula: $(2^{(RP\ Ct-\ N1\ Ct)} + 2^{(RP\ Ct-\ N2\ Ct)})/2$. This formula provides a normalized relative value of the amount of viral RNA in comparison to total human nucleic acid within each sample and was adapted from previous reports showing normalization was needed for reliable estimation of viral load across multiple samplings[9]. The formula used was adapted in order to incorporate both the N1 and N2 measurements as well as transform the Ct data to a linear scale. Next, geographical parameters were calculated that included east-west RVL, north-south RVL, surrounding RVL and diagonal RVL (Supplemental Figure S1). For manually extracted samples, the extraction batch RVL of 16 samples was calculated by summing the individual RVLs for all positive samples in that batch, as these samples were handled simultaneously in a BSC throughout the entire extraction procedure. The Tecan platform had

specific plastic consumables with 16 compartments containing 6 pipette tips in a compartment (3 horizontal, 2 vertical); the protocol included tip re-usage to minimize utilization of scarce plastic consumables and these compartments were noted to contribute to contamination in experimental optimization and validation experiments. For Zymo/Tecan extracted samples, horizontal row RVL, vertical row RVL, and Tecan 6-pack compartment RVL were calculated to account for movement of the automated pipetting heads across reagent and sample plates onboard the instrument. An example of geographical plate calculations are illustrated in Supplemental Figure S1. Some variables were derived from multiple wells (example: east-west) with the RVLs of the wells summed to generate the final variable. For mathematical purposes, undetected Ct values were assigned a value of 46 as this was one cycle higher than the total number of 45 PCR cycles performed. Finally, a fold-change variable was generated by calculating the sum of the surrounding wells RVL and dividing it by the index well RVL. We defined this parsed and calculated data as the cleaned dataset. From the cleaned dataset, four new parallel data sets were generated that included the: 1) primary dataset, 2) final resulting dataset, 3) repeated dataset, and 4) modeling dataset.

For the primary dataset, samples were grouped based on their unique sample identification number and parsed for only the first PCR result auto-called using the interpretation algorithm described above. Thus, the primary dataset represents the preliminary test result generated by automated result classification without technologist or pathologist review for data quality and contamination potential before any human-determined repeat analysis was performed. For the final resulting dataset, samples were grouped based on their unique sample identification number and parsed for the final pathologist-reviewed result, which included the final result for samples subjected to repeat extraction and analysis. To generate the repeated dataset, the cleaned dataset was parsed and filtered specifically for samples that were repeated following re-extraction and linked by the unique sample identification number. Samples that underwent repeat PCR without repeated extraction were excluded from the analysis, as these

were mostly due to PCR reaction anomalies unrelated to concern for contamination. To generate the modeling dataset from the repeated dataset, we reclassified any result initially auto-called as inconclusive in the primary dataset to positive, because the modeling approach needed a binary dependent variable for the result (positive or negative). No other result interpretations were modified. Repeated sample pairs in the modeling dataset were then parsed based on the results interpretations for true positive (TP) status (positive primary, positive repeat) which was assigned a value of 1 and for false positive (FP) status (positive primary, negative repeat) which was assigned a value of 0.

Modeling

All RVL calculated variables in the modeling dataset underwent further $\log_{10}$ transformation for modeling and graphical representation. Variables with a value of 0 were assigned the smallest value for that variable from the repeat dataset prior to $\log_{10}$ transformation. Samples were separated into either manual or automatic extraction and modeling was performed on centered and scaled data. FPs were predicted using a gradient boosting machine (GBM) model using the *caret* (v6.0-86)[10] package with a 10-fold repeated cross validation. Partial dependence plots were generated using the *pdp* package (v0.7.0)[11]. To generate confusion matrix tables, cut off values for the probabilities were set to 0.5. For analysis of the final resulting data set, FP events were defined as a probability of less than or equal to 0.15 for manual, 0.1 for automated-KingFisher extraction and 0.25 for automated-Tecan extraction. Data analysis was performed in R (v4.0.2); code is available at https://github.com/NelsonAC-UMN-Lab/COVID (last accessed 6/2/2021). Statistical testing and graphical representation were performed using GraphPad Prism version 8.4.3 for MacOS, GraphPad Software (San Diego, California) or using FlowJo Software for MacOS Version *10.7.1*. Ashland, OR: Becton, Dickinson and Company; 2019.

Regulatory Statement and Data Availability

Utilization of clinical test results for the purposes of test validation and quality improvement was reviewed and approved by the Institutional Review Board (STUDY00009560 and STUDY00010603). The repeated dataset used for modeling is included (Supplemental Table S1).

Results

Testing with our laboratory-developed RT-PCR assay for detection of SARS-CoV-2 viral RNA began on March 22, 2020. By August 12th, 2020, 206,445 samples were resulted with an overall 3.5% positive rate (Table 1, left-primary and right-final resulting datasets, see *Materials and Methods* for description of data generation). Results are composed of samples from both symptomatic and asymptomatic patients. The primary results generated prior to repeat of any samples demonstrated 1.4% invalid and 1.0% inconclusive results; however, repeat testing and pathologist review resolved most of these cases and a much smaller number of samples remained inconclusive (0.1%) and invalid (0.4%) (Table 1). Early in this time period, sample RNA was extracted using a manual column-based method or a low-throughput magnetic bead-based method for sets of 16 samples. Later, automated 96-well plate-based methods for RNA extraction were validated and used regularly. Manual extraction, based on extraction sets of 16 samples, was still performed daily on a smaller number of samples left over after plate-based RNA extraction. In total, three distinct extraction workflows were used for these clinical samples: manual extraction, automated KingFisher-based extractions and automated Tecan-based extractions. Daily test volume increased as the amount of automation was increased (Figure 1A). By August, up to 6,000 tests were performed daily. As testing volume increased, the prevalence of positive SARS-CoV-2 tests decreased from ~10-20% positivity to ~1.0-1.5% by early July, but then began to increase to ~5% in late July (Figure 1B). Validation, performance and comparison studies for the SARS-CoV-2 RT-PCR test are described elsewhere[7]. By employing this high-throughput, open platform LDT RT-PCR assay with different extraction

methods, testing was able to be rapidly scaled to account for increasing community needs with fewer supply bottlenecks associated with some proprietary, closed platform approaches of detecting SARS-CoV-2.

Of the total samples assayed (36,692 manual, 169,564 automated, 189 with no extraction method), 3,311 samples were re-extracted, underwent subsequent PCR and resulted (Table 2, repeated dataset). Samples that underwent automated extraction using either the Tecan or KingFisher methods are combined here to compare manual vs automated methods. On retrospective data review, extraction methods for 54 repeated samples (4 positive samples, 1 sample with no reaction, 46 not detected) were not identified. These samples were removed from modeling analysis but are present in the total data set. The most common reason samples were repeated following automated extraction were invalid primary results while the most common reasons samples were repeated following manual extraction was inconclusive primary results (Table 2). Analysis comparing TP and FP results demonstrated significantly more FP events when initial manual extraction methods were used (Fischer's exact test, $P<0.0001$) (Figure 2A, B, Table 3). Thus, FP events are identified in the SARS-CoV-2 PCR assay and are generated at different rates across extraction methodologies.

We hypothesized that samples with initial positive/inconclusive results returning negative results on repeat are likely FP events due to contamination; however, some samples could represent an initial TP and repeat FN due to poor sample quality or inconsistent detection of low-concentration SARS-CoV-2 RNA. We used our data to evaluate for these two possibilities. First, if the FP events were associated with poor sample collection, the FPs would demonstrate reduced sample quality and reduced ability to detect viral RNA. The Ct values of the human internal positive control (RNaseP, RP) were compared across TP and FP samples within the same extraction type, finding no difference (One-way ANOVA, manual comparison $P>0.99$, automated comparison $P=0.93$) (Figure 2C). This suggested the extent of sampling and/or sample integrity was not different between TP and FP samples. To explore the possibility of the

inability to identify inadequate sample collection due to minimal difference between RP Ct values, all measured samples were compared. RP Ct values were found to be normally distributed with a mean of 28.1 and standard deviation of 3.0 (*Data not shown*), demonstrating ~95% of samples were between 34 and 22 Ct. Thus, there is a 2^{12} difference in RP transcript quantity and is likely enough difference between samples to identify poor quality specimens based on RP Ct value. Next, we hypothesized that the identification of FP results was due to samples with a viral concentration below this assay's LOD. The limit of detection (LOD) for this assay is defined as the viral RNA quantity at which 95% (19 of 20) of replicates for positive samples are identified, which was associated with average Ct values of ~37.5. As the viral concentration falls below the LOD, the frequency of replicates producing a positive result will also decrease. To test this hypothesis, the RVL of all TP and FP samples in the repeated dataset were compared, finding no significant difference between the manual extraction (One-way ANOVA, manual comparison P=0.71), but a trend towards lower RVL for FP in the automated extractions (One-way ANOVA, P=0.11) (Figure 2D). This does not rule out that samples with low viral load are contributing to the initially positive SARS-CoV-2 results that repeat negative, but it does suggest that low viral concentration is not clearly the main contributor to non-reproducible results and justifies detailed study of potential mechanisms driving contamination.

Sample contamination occurs in either a reproducible or a probabilistic manner. Technical methodology may introduce the risk for reproducible contamination, as most technicians/technologists, and all automated machines, perform tasks in an ordered fashion (i.e. pipetting of samples left to right or starting at well A1 and going to well A12). Stochastic contamination can occur due to aerosolization of RNA or amplicon to surrounding wells and has been shown to occur in plate-based bacterial 16s RNA sequencing[12]. Even with stochastic contamination, there was still reproducible patterns of contamination in the 16s sequencing as abutting wells were more likely to exchange material than distant wells[12]. To investigate

reproducible errors and potential sources of contamination in this open-platform assay, we set out to predict samples that had a high-likelihood of being FP in order to identify the samples for repeat. Our basic assumptions were that wells directly surrounding a sample were most likely to contribute to contamination and that the relative difference in viral concentration between samples would impact the probability of a FP result. Due to technical differences across the three extraction methods, we hypothesized that certain directional relationships between neighboring samples could be more prominent contributors to the manner of contamination. Based on these assumptions, we defined 12 different variables (Supplemental Table S2 and Supplemental Figure S1) for analysis and modeled data from each of the three extraction methodologies separately. The models' predictions showed a positive predictive rate of 87%, 93% and 65% for detecting FPs using the manual, automated-KingFisher and automated-Tecan extraction methods, respectively (Figure 3A, B, C). Sensitivity of the models for the three extraction methods were 96%, 100% and 67%, while the specificity was only 36%, 90% and 66%, respectively (Figure 3A, B, C). Identification of the top variables contributing to the models' predictions demonstrated that N2 Ct value contributed substantially in all three models, while unique extraction-specific values such as the extraction batch RVL (manual), and the horizontal and Tecan shared pipette RVLs (automated-Tecan) were important in their respective methods (Figure 3 D, E, F). These results demonstrate the extraction methodologies have different variables associated with prediction of FPs, indicating that contamination is produced in a method-dependent fashion and that models need to be generated individually when applying this QC methodology to other assays.

We further studied the data used to generate the manual extraction model, as the manual method may be most broadly applicable to other laboratories and potentially the most likely to demonstrate reproducible error. We first focused on the top three variables contributing to the manual model: N2 Ct, extraction batch RVL and the calculated fold-change of the measured well to the surrounding well RVL. Comparison of these three variables showed FPs

exhibited higher values across all variables when compared to TPs (Figure 4A, B, C). These results support our hypothesis about potential sources of inter-sample contamination. First, the presence of multiple positive samples, particularly those with higher viral loads within a single manual extraction batch (n=16 samples) are associated with FPs. Second, samples with higher or undetected viral N2 Ct values are more likely to be FPs. Lastly, large differences in the collective viral load of all adjacent samples surrounding an index well, compared to the index well itself, is more likely to identify a FP sample. However, none of these three variables were able to fully separate TP and FP samples, indicating the need for a more nuanced model.

Next, we investigated whether more specific physical relationships between positive samples and the surrounding wells impacted the final result. First, we analyzed the association of more specific geographical variables relating the RVLs of neighboring samples on east-west, north-south and diagonal axes to the incidence of FP and TP results (Figure 4 D, E, F). The east-west RVL was higher in FP events, while the north-south and diagonal RVLs were not significantly different between TP and FP (Figure 4 D, E, F). Reasons for these directional differences associated with FP results are not certain but may suggest that the process of aliquoting into and out of 2-D barcoded tubes for sample transfer to PCR setup may be a source for technical improvement based on our quality review of this specific workflow. We also tabulated whether the wells surrounding TP and FP events did or did not contain a positive sample, irrespective of the relative viral load (Figure 4 G, H, I). Indeed, the presence of positive samples in the east-west, north-south or diagonal wells increased the likelihood a sample was FP, suggesting the mere presence of a positive well of any RVL may be able to contaminate an adjacent well. Interestingly though, a proportion of FPs demonstrated no positive samples in the immediately neighboring wells, suggesting contamination may also occur from outside the directly surrounding samples during processing. To determine how many FP and TP samples were adjacent to a positive well, we correlated the RVL and fold change measurements for manually extracted repeated samples (Figure 4J). Samples without surrounding positive wells

were represented at the lowest RVL values (within rectangle gate), while those with positive adjacent wells were above this line of samples (within oval gate). Analysis revealed 40% of TP results did not have a surrounding positive sample, while 10% of FP did not have a surrounding positive well (Figure 4J). Analysis of the RVL and fold change values for the full manually extracted data set with FP predictions showed 6% of samples had empty surrounding wells, while 44% of TP samples had directly adjacent empty wells (lower box) (Figure 4K). Thus, the model predicts samples of low RVL with adjacent samples of high RVL are more likely to be predicted FP, while samples of high RVL without adjacent positive samples are more likely to be TP.

Next, the manual extraction model was interrogated to understand how the model variables predicted FP samples. First, the FP probability of manually extracted repeated samples was evaluated as a function of N1 and N2 Ct values (Figure 5A, B). Samples with N1 and N2 Ct values less than ~38 typically repeated as positive when extracted and measured again, while values greater than ~38 were more likely to not be detected upon repeat. To compare the experimental and model data, one-dimensional partial dependency plots were generated for variables in the model (Figure 5C-G). Partial dependency plots show the model's probability of predicting a FP sample as a function of the input variable. The N2 Ct (Figure 5D) and extraction batch RVL (Figure 5E) variables both show approximately 20-30% increases in the model FP probability at higher values. N1 Ct (Figure 5B), fold change (Figure 5F) and RVL (Figure 5G) also show increases in the probability of a FP sample at higher values, but the model prediction only changes minimally over the variable range. Comparison of the experimental data and model reveal the model assigns increased likelihood of FP results to a Ct value of greater than 38 for both N1 and N2 and that even though the experimental N1 and N2 data look similar, N2 carries more weight in predicting FP than N1 in the model. Next, two-dimensional partial dependency plots were created to investigate how two variables interacted within the prediction models (Figure 5H). As expected, low N1 and N2 Ct values were more

likely to be TP, while samples with high N1/N2 Ct values are predicted FP. High extraction batch RVL values led the model to predict the sample as FP, even at low N2 Ct values, suggesting that more caution is necessary in QC review as more positive samples are batch-handled in a BSC simultaneously. Lastly, samples with low fold change were less likely to be FP, especially at N2 Ct values less than ~38. This analysis better defines the interaction of variables that can be used to discriminate manually extracted TP and FP samples. As well, samples with a summed extraction batch RVL greater than 10^5 or a change of 10-fold compared to the positive surrounding wells were more likely to be FP.

Lastly, the three models were used to identify potential FP results in the final resulted dataset that were not identified for repeat extraction by pathologist review. The probability threshold for identifying a FP in the models was increased such that we maximized accuracy of FP predictions. Using this new probability threshold, the models predicted an additional 170 samples concerning for a FP result (0.08% of total results, 2.3% of positive/inconclusive results) that had not been identified for repeat. FP samples identified by retesting or modeling may represent low-viral titer samples in the pre-symptomatic or early asymptomatic phase of infection that will progress and become TP results. To address this possibility, manually extracted FP samples (retested and predicted) were investigated to determine if the patient received a follow up test within the following 14 days. 14 days was selected to balance the following considerations: allowance for progression of the viral illness, reduction the likelihood of new infection and to maximize the sample number for assessment. 16 patients were identified that had a second test within 14 days, finding 11 of these patients (69%) were negative on retesting. Thus, these models can be used as a decision-support tool when identifying samples needing repeat due to concerns for contamination. Given that the model misidentified predictions in the pathologist-selected repeat dataset used for training, complementary pathologist or technologist review of the PCR data still serves an essential role in identifying FP events for the SARS-CoV-2 RT-PCR assay.

Discussion

Using data generated from repeated samples on an LDT high throughput test, we developed models to predict false positive SARS-CoV-2 PCR results and generate QC metrics for individual extraction methodologies. Employment of these models and identified variables will be used to improve the assay, as well as support clinical decision making when performing data quality review of results. Specifically, the manual extraction model identified high relative viral loads within each 16-sample extraction batch as a potential source of contamination. As well, the presence of a positive result in the surrounding wells, particularly in the east-west well, helped to identify FP results. From these findings, the protocol and technique can be altered in an attempt to reduce contamination during specific steps of processing. Lastly, we showed that an additional 2.3% (n=170) of positive and inconclusive samples had a high likelihood of a FP result but were signed out as positive without re-extraction and repeat PCR. This highlights the importance of providing computational decision-support methods to pathologists or technologists performing QC review of data for molecular viral testing.

Analysis and identification of the variables likely contributing to FP results include the extraction batch RVL in the manual extraction model, as well as individual sample N2 and calculated fold change values in all models. Specifically, the proposed QC metrics to identify FP manually extracted samples are: 1) N2 Ct value (>38), 2) Extraction batch RVL (>10^5) and 3) Fold-change (>10). These three variables demonstrated the largest contribution to predicting FPs and demonstrated cut-off values that could be employed quickly for manual evaluation of specimen result quality. One important caveat of this work is that these models only inform the FP events generated using the modeled assay workflow and is not directly applicable without modification to other SARS-CoV-2 assays. However, use of this modeling framework to identify technical components contributing to FP results can be applicable to all open platform RT-PCR assays. Monitoring of the variables identified above were incorporated into our lab's standard workflows for this assay and was applied to the analysis of a newly developed extraction-free

SARS-CoV-2 RT-PCR assay using the CDC N1/N2/RP targets. Use of a Ct cutoff and fold change variable were employed to identify samples to automatically repeat using an orthogonal testing method, increasing turnaround time and accuracy of the assay.

Identification of extraction batch RVL as a potential predictor of FP results points to contamination that is hypothesized to be caused by aerosolization of samples during multiple manual extraction handling steps. This finding allows for specific protocol modifications and standardization of technique among operators to improve results and reduce contamination rates. In all models, N2 Ct values were identified as a predictor of FP results, while high N1 Ct values did not show the same ability to differentiate TP and FP results. This may point to differences in the efficiency of detecting N1 and N2 targets and may be a finding applicable to assays using this primer and probe set. For automated-Tecan samples, the model was less accurate at discriminating TP and FP results. Several hypotheses could be made for this finding. First, it is possible any of the models are over-fit and identifying insignificant differences within the repeated data that can distinguish the two groups but has no real-world applicability. Recursive feature elimination of variables was performed in an attempt to avoid overfitting, but no variables were identified for removal (*Data not shown*). Second, a complex set of interactions could be driving FP events that are not easily identifiable using single or two variable analysis as shown using partial dependency plots. Lastly, the results of the automated extraction model may point to only stochastic FP generation with little to no reproducible sources of error and may be due to mechanical differences in the specific performance of the different automated systems utilized in our laboratory. Nevertheless, this analysis shows the importance of generating a model to potentially identify and mitigate sources of error, allowing for improvement of technique and reduction of erroneous results across multiple methodologies.

Many of the results flagged by the models as potential FPs are composed of samples with higher Ct values. This is partially due to the conversion of inconclusive to positive results for modeling purposes. This finding is also due to the assumptions made about TP events and

the selection of repeats generated for the training data set: that it is unlikely a sample with high viral load is FP, while a sample with low viral RNA concentration is potentially explainable by contamination. Because most of the training data and therefore predictions are approaching the LOD, it is difficult to confidently distinguish between low viral titer and contamination. Follow up testing of patients with predicted and re-extracted FP samples shows minimal conversion to a TP result over the next 14 days, suggesting that the algorithm identifies contamination and not just early infection. Distinguishing between these two events may have different utility in certain clinical situations. For example, missing a TP with low viral load when performing contact tracing or during pre-hospital admission to a non-isolation ward could have severe consequences as patients in these clinical situations may be in a pre-symptomatic phase with low viral titers and could be at high risk to spread SARS-CoV-2 as their viral titer increases. Conversely, over-calling FP results when monitoring health care workers could lead to unnecessary staffing shortages in critical service areas. Further, FP results for pre-hospital or pre-surgical admissions could confound patient placement and immediate management of emergent non-COVID-19 health issues, leading to sub-optimal patient care and inefficient utilization of healthcare resources. These complexities stress the importance of clinical correlation in the interpretation of SARS-CoV-2 test results and highlight the major need for sufficient sample collection supplies and analytic reagents to offer subsequent sampling to patients with inconclusive, low viral load results.

Currently, this LDT defines inconclusive results as amplification and detection of only one of two targets. It is unknown if inconclusive results are at increased risk of being a FP. Tests receiving EUA approach inconclusive results differently: some classifying them as negative, some classifying them as positive, or some leaving it to the discretion of the laboratory (FDA, https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas, last accessed 3/4/2021). These EUA tests also have different Ct cut-off values for the respective positives, inconclusive and

negatives. Some SARS-CoV-2 tests allow positives to be assigned with only one target amplifying at a Ct of 45, while others are negative at Cts of greater than 37. The sensitivity and specificity of the tests change with the alteration of Ct cutoff values and PCR efficiency, while the clinical purpose of the test should reflect how the Ct value thresholds are set[13]. Currently, SARS-CoV-2 testing focuses on increasing the sensitivity of the test and identifying any amount of viral RNA present in a sample, even if there is a concurrent increase in FPs. If instead the goal were to identify patients at risk for spreading infection, reducing the positive Ct cutoff would be more practical. Recent works have found that samples with Cts >35 or viral load $<10^5$ copies were unable to generate recoverable, culturable virus[14,15]. However, these results need to be interpreted cautiously as the studies were performed *in vitro* and Ct values have been shown to be assay dependent[14,15]. To address the questions of Ct values, transmissibility and test sensitivity thresholds, proficiency testing and standardized samples may be useful. A recent SARS-CoV-2 proficiency test has shown 97% consensus for samples with ~5,000 copies/ml and negative samples[16], but correlating virus quantity with culture recoverability may be a more useful approach for clinical labs. Nonetheless, until these studies are performed, inconclusive results are difficult to interpret. In this study, inconclusive results that underwent repeat were coded as positive in training datasets for the purpose of modeling. When initially inconclusive results were reviewed in the repeat dataset, 59% (409 of 690) were FP, while 41% (281 of 690) were TP. In contrast, review of initially positive results in the repeat dataset showed 35% (55 of 158) were FP, while 65% (103 of 158) were TP (Fischer's Exact test, P<0.0001, *Data not shown*) demonstrating that inconclusive samples were found more frequently to be FP. Nonetheless, given the overall frequency of TP results in the initially inconclusive samples for this LDT assay, it would be inappropriate to simply call inconclusive results negative because it would result in the misdiagnosis of reproducible, positive samples. Based on these results, we propose two options for follow up testing of a positive sample with high probability of a FP result. First, report the result as inconclusive and request that the patient is sampled and measured

again in 48-72 hours to identify patients with low viral load early in infection. Second, if the patient cannot be tested again, perform repeat analysis of the primary sample either on the same platform or optimally on an orthogonal method. Clinical scenario will dictate the follow up testing options, but this is a reasonable laboratory approach until more robust data is available to calibrate assay thresholds with clinical infectivity.

Finally, generation of these models will lead to improvement in multiple aspects of the test. First, it allows the laboratory to identify aspects of the technique that are reproducibly generating errors. Technical modifications will allow for remediation of these issues and comparison of pre- and post-modification results will reveal the effect on error rates. Second, model implementation can create automatic FP flags to improve workflow efficiency in triaging technical repeats, likely decreasing result turnaround times. Third, modeling can support clinical decision making and provide increased ability to identify potential errors. Anatomic pathology and radiology have begun to employ artificial intelligence (AI) for image analysis as an adjunct diagnostic tool, but AI has not been widely used for molecular diagnostics clinical decision support[17,18]. For SARS-CoV-2 testing, we propose to use this method as a clinical decision support AI to flag results that have a high FP probability. Of note, some of the models had poor positive predictive values (i.e., automated-Tecan), and would be unlikely used as an AI decision support. However, this same model could still be used to identify potential reproducible contamination issues. This metric could be incorporated into the clinical decision process and inform the reviewer if the sample should be repeated. This model may enforce confirmation bias while training the reviewer to miss other errors, but future studies will be needed to determine how the adjunct tool alters clinical decision making. Nonetheless, this tool will be helpful for technique troubleshooting and clinical decision-making support and can be adapted to a wide variety of molecular diagnostic applications.

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Author Contributions

RJM analyzed and interpreted the data and wrote the manuscript. NP contributed to data analysis and interpretation and edited the manuscript. MS contributed to acquisition and interpretation of data and discussed design of the manuscript. JD contributed to acquisition and interpretation of data and discussed design of the manuscript. KBB contributed to study conception and design, data acquisition, and edited the manuscript. PF, ABK and BT contributed to interpretation of data and edited the manuscript. SY contributed to study conception and design and edited the manuscript. ACN contributed to study conception and design, interpreted data and wrote the manuscript.

ACN is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Overview of testing during time of study. (A) Number of samples measured for SARS-CoV-2 as a function of time. (B) Frequency of positive SARS-CoV-2 samples as a function of time. Bars represent a single day of tests.

Figure 2. False positive results are not explained by sample quality or relative viral load. (A) Frequency of repeated manually extracted samples that were true positive (TP) (black) and false positive (FP) (grey). (B) Frequency of repeated automatically extracted samples that were TP (black) and FP (grey). (C) RP cycle threshold (Ct) values for repeated TP and FP samples that were initially manually or automatically extracted (Tukey box and whisker One-way ANVOA with multiple comparisons, ns=No significance) (D) Relative viral load (RVL) of repeated TP and FP samples that were initially manually or automatically extracted (Tukey box and whisker One-way ANVOA with multiple comparisons).

Figure 3. Machine learning derived models identify FP samples. (A-C) Confusion matrix from machine learning derived models of predicting FP events of (A) manually, (B) automated-KingFisher or (C) automated-Tecan extracted samples. Bar graph of the variable contribution to the models generated for the (D) manual, (E) automated-KingFisher and (F) automated-Tecan extraction methods. For each model, individual variables used in the modeling were normalized to the maximal contributor variable.

Figure 4. Identification of variables contributing to FPs in manual extraction. (A) Scatter plot of $\log_{10}$ transformed extraction batch RVL of TP and FP samples (samples represented by a single point, two-tailed Student's T-test, bar at median). (B) Scatter plot of N2 Ct values of TP and FP samples (samples represented by a single point, two-tailed Student's T-test, bar at median). (C)

Scatter plot of log_{10} transformed fold change of TP and FP samples (samples represented by a single point, two-tailed Student's T-test, bar at median). Scatter plot of log_{10} transformed (D) east-west, (E) north-south and (F) diagonal RVL of TP and FP samples (samples represented by a single point, two-tailed Student's T-test, bar at median). Enumeration of TP (black) and FP (grey) samples with (+) and without (-) a positive sample present in the (G) east-west, (H) north-south or (I) diagonal positions (for each bar graph an individual Fischer's exact test was performed for the unique geographical data and the P-value is noted within the graph). (J) Scatter plots of RVL by fold change for manually extracted samples that were repeated due to concern for FP results (points represent single sample), with gates showing the frequency of samples with positive surrounding wells (high fold-change and low RVL, oval) and negative surrounding wells (low fold-change and range of RVL-rectangle) . (K) Scatter plots of RVL by fold change for manually extracted samples that were predicted to be FP (left) or TP (right) (points represent single sample) with gates showing the frequency of samples with positive surrounding wells (high fold-change and low RVL, oval) and negative surrounding wells (low fold-change and range of RVL-rectangle). * P≤ 0.05, ** P ≤ 0.01, **** P ≤ 0.0001..

Figure 5. Identification of variables used for predicting FPs in the manual extraction model. FP probability as a function of (A) N1 Ct and (B) N2 Ct. The points represent the FP probability between that point and the next lowest Ct value point normalized to the total frequency of FP events. Partial dependency plots showing the probability of predicting a FP event in the manual extraction model for (C) N1 Ct, (D) N2 Ct, (E) Extraction batch RVL, (F) Fold change, and (G) RVL as a function of variable values. (H) Partial dependency heatmap plots show the probability of predicting a FP event (red =low probability, blue = high probability) in the model as a function of N1 Ct vs N2 Ct (left), Extraction batch RVL vs N2 Ct (middle) and fold change vs N2 Ct (right).

Table 1. Summarized primary and final SARS-CoV-2 results

Result	Primary dataset* n(%)	Final resulting dataset* n(%)
Not Detected	194706 (94.3)	198153 (96.0)
Positive SARS-CoV-2	6727 (3.3)	7273 (3.5)
Invalid	2894 (1.4)	777 (0.4)
Inconclusive	2103 (1.0)	202 (0.1)
QNS	14 (0.006)	35 (0.02)
No Reaction	1 (0.0005)	5 (0.002)
Total	206445	206445

QNS= Quantity not sufficient

*See *Materials and Methods* for description of dataset generation

Table 2. Summarized results of SARS-CoV-2 repeat dataset

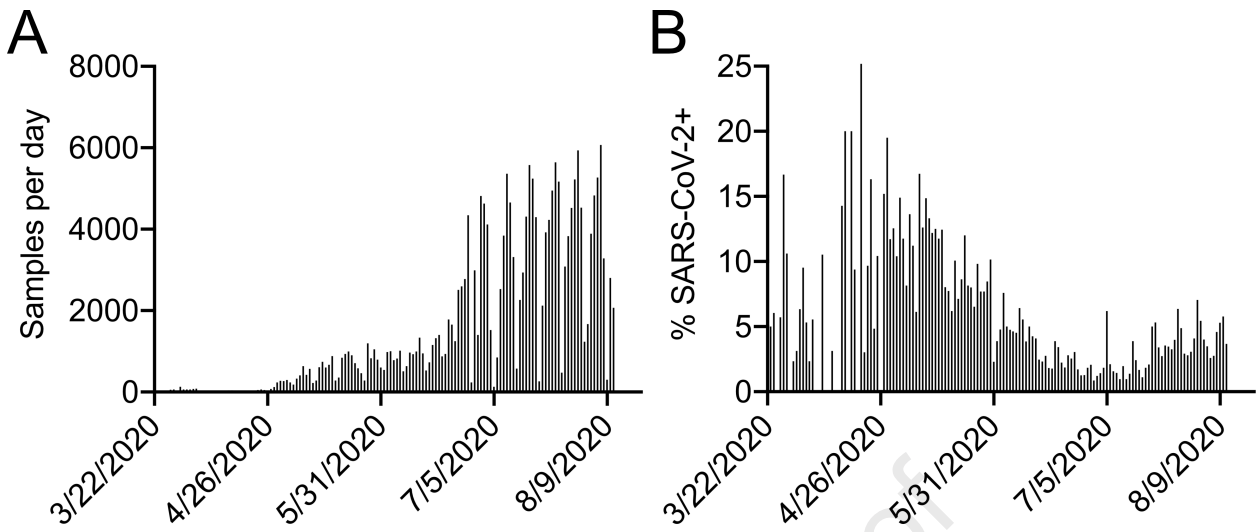
Results	Manual n(%)	Automated n(%)
Inconclusive→Not Detected	93 (40.0)	321 (10.4)
Invalid→Not Detected	39 (16.7)	1785 (58.0)
Not Detected→Not Detected	37 (15.9)	187 (6.1)
Inconclusive→Positive SARS-CoV-2	21 (9.0)	244 (7.9)
Invalid→Invalid	14 (6.0)	287 (9.3)
Positive SARS-CoV-2→Not Detected	13 (5.6)	35 (1.1)
Positive SARS-CoV-2→Positive SARS-CoV-2	11 (4.7)	84 (2.7)
Invalid→Positive SARS-CoV-2	2 (0.9)	41 (1.3)
Not Detected→Inconclusive	1 (0.4)	3 (0.1)
Positive SARS-CoV-2→Inconclusive	1 (0.4)	2 (0.06)
Inconclusive→Inconclusive	1 (0.4)	33 (1.1)
Invalid→QNS	-	21 (0.7)
Not Detected→Positive SARS-CoV-2	-	4 (0.1)
Not Detected→Invalid	-	5 (0.2)
Invalid→Inconclusive	-	21 (0.7)
Inconclusive→Invaild	-	4 (0.1)
Positive SARS-CoV-2→Invalid	-	1 (0.03)
Total	233	3078
All samples ran (206445)*	36692	169564

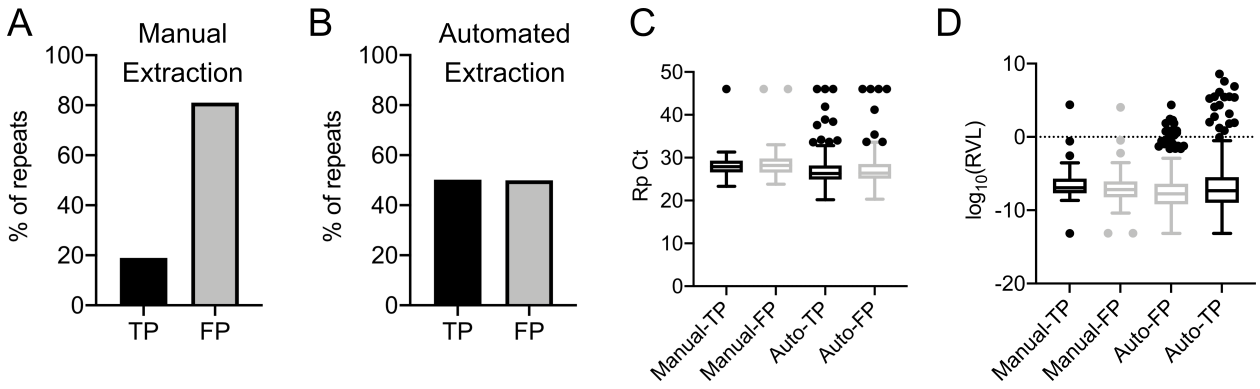
- = Not identified in this extraction method, *= 189 samples were not coded for extraction method, QNS= Quantity not sufficient

Table 3. Summarized results of SARS-CoV-2 modeling dataset

Results	Manual n(%)	Automated n(%)
Positive SARS-CoV-2→Not Detected	98 (42.0)	330 (10.7)
Not Detected→Not Detected	66 (28.3)	246 (8.0)
Invalid→Not Detected	25 (10.7)	1718 (55.8)
Positive SARS-CoV-2→Positive SARS-CoV-2	24 (10.3)	343 (11.1)
Invalid→Invalid	11 (4.7)	317 (10.3)
Not Detected→Positive SARS-CoV-2	7 (3.0)	32 (1.0)
Invalid→Positive SARS-CoV-2	1 (0.4)	79 (2.6)
Not Detected→Invalid	1 (0.4)	5 (0.2)
Positive SARS-CoV-2→Invalid	-	8 (0.3)
Total	233	3078

- = Not identified in this extraction method





A

Predicted data	Repeat data: Manual	False Positives	True Positives
	False Positives	103	16
	True Positives	4	9

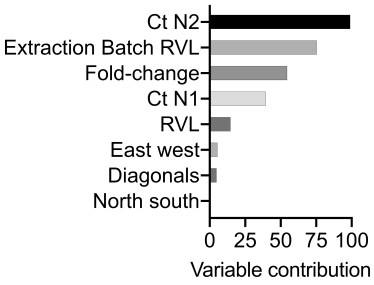
B

Predicted data	Repeat data: Automated-KingFisher	False Positives	True Positives
	False Positives	41	3
	True Positives	0	27

C

Predicted data	Repeat data: Automated-Tecan	False Positives	True Positives
	False Positives	214	112
	True Positives	102	217

D



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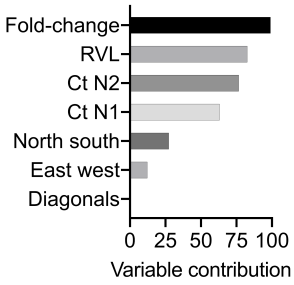

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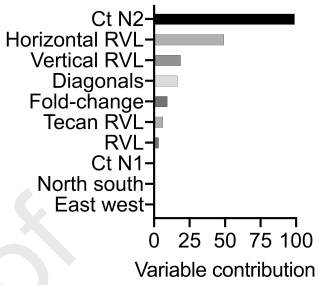

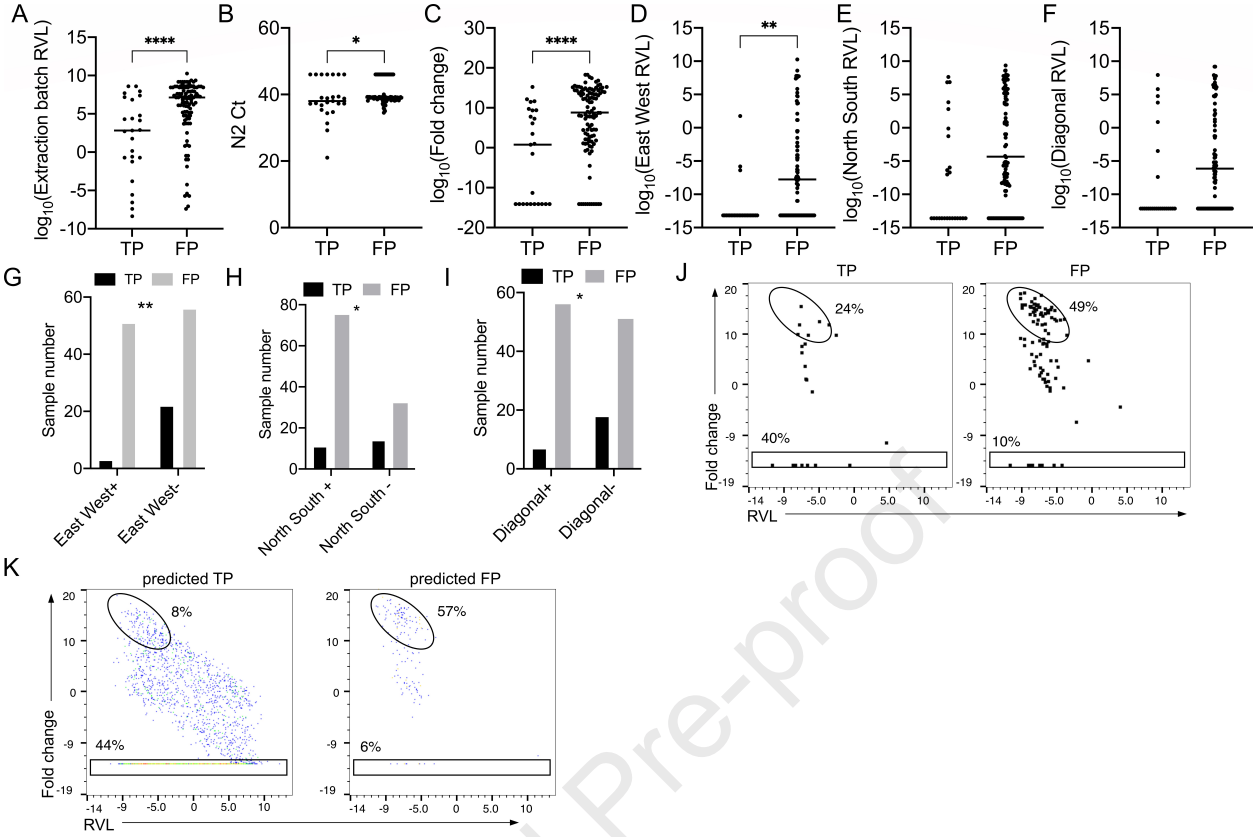

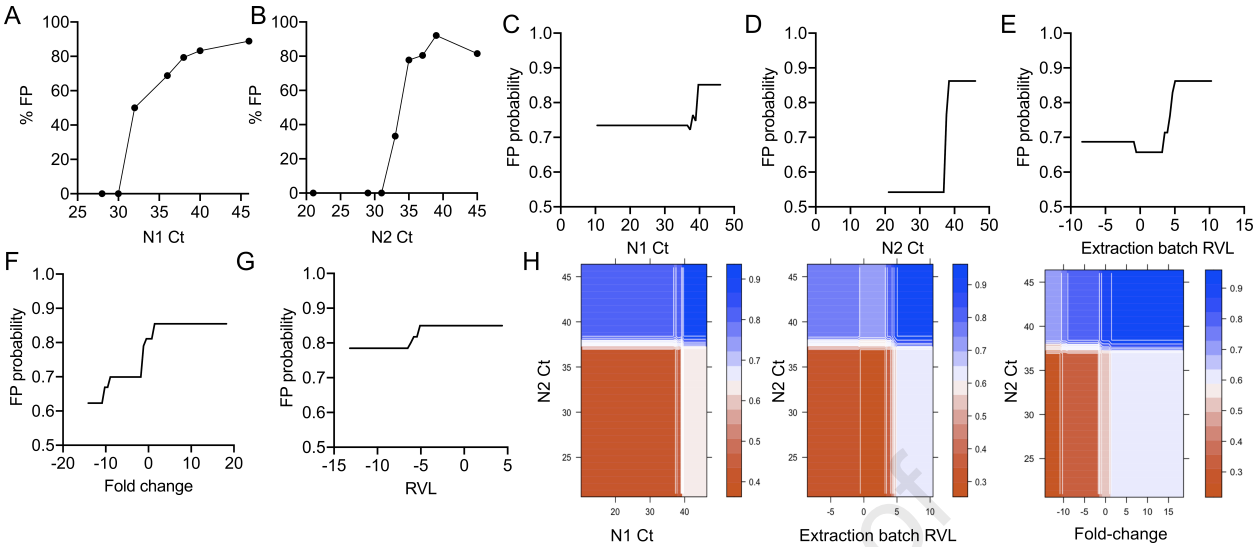

College of American Pathologists (CAP) Microbiology Committee Perspective: Caution Must Be Used in Interpreting the Cycle Threshold (Ct) Value

To the Editor—We read with great interest the article by Magleby and colleagues entitled "Impact of SARS-CoV-2 Viral Load on Risk of Intubation and Mortality Among Hospitalized Patients with Coronavirus Disease 2019" [1]. This article adds to the growing body of work on using the polymerase chain reaction (PCR) cycle threshold (Ct)-value associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA detection in clinical specimens as a prognostic indicator and to establish criteria for active infection and transmissibility. Although we recognize the importance of studying laboratory results and their relevance to care of patients with coronavirus disease 2019 (COVID-19), we wish to inform your readers of potential caveats that must be considered when applying published findings regarding Ct-values to their own patients' results.

1) Specimen collection method, specimen source, transport media type and volume, duration from specimen collection to analysis, and days from infection to specimen collection can all impact the amount of viral RNA that could be detectable by an assay, and these variables are reflected in the Ct values.
2) No quantitative SARS-CoV-2 assays have received Emergency Use Authorization (EUA) by the Food and Drug Administration (FDA). Additionally, no international, commutable standardized reference material is currently available, which would be needed for validation of quantitative assays that generate comparable results across manufacturers and laboratories. Although specimens with lower Ct-values generally have more viral RNA than specimens with higher Ct-values, the quantitation and precision associated with those differences in Ct-values have not been determined.
3) Only traditional real-time PCR assays produce a Ct-value. Some diagnostic assays used to detect SARS-CoV-2 RNA use isothermal amplification methods, which do not produce a Ct-value. Other PCR platforms use nested PCR, which is not designed for quantitative interpretation.
4) Ct-values can vary significantly between and within methods. The College of American Pathologists (CAP) recently surveyed more than 700 laboratories using proficiency testing material produced from the same batch (Figure 1). The median Ct-values reported by the instruments for different FDA EUA methods varied by as much as 14 cycles. Within a single test performed on the same instrument, the difference in the median Ct-values for different targets was as high as 3.0 cycles. Finally, within a single gene target for a single method, up to 12.0 cycle differences were seen across all laboratories. The assay and gene target used by Magleby et al, ORF1a detected by the Roche cobas system, differed by approximately 6.0 cycles across all laboratories responding to the survey. Many clinical laboratories are using multiple tests that assess different gene targets for SARS-CoV-2 and are performing testing on different platforms. This adds to the potential variability of Ct-values produced by a single laboratory.

The ongoing shortage of commercial testing reagents presents a major obstacle to conducting large research studies comparing testing platforms. We thus believe that data from the CAP proficiency testing survey

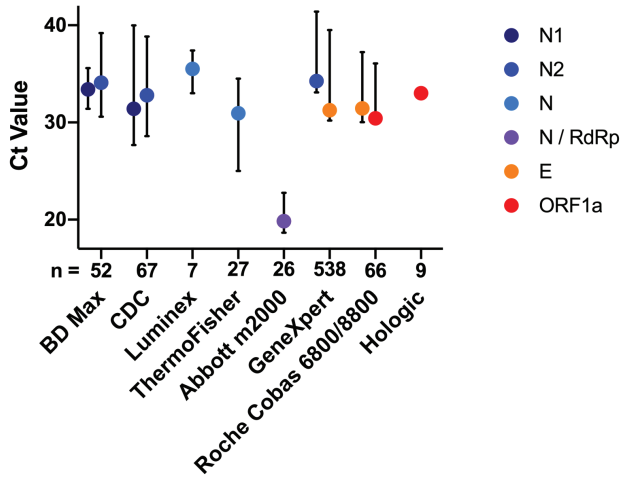

Figure 1. Ct values for gene targets and manufacturers for the same batch of testing material. Median Ct values (*filled circles*) and the range of Ct values from low to high (*whiskers*) are shown. The number of survey respondents using each method is indicated below the *x*-axis. Of note, the material used for the PT Survey did not contain all gene targets in use by commercial assays, and Ct values entered by laboratories under "Miscellaneous" were not incorporated into the data. Data from the users of the Cepheid GeneXpert and GeneXpert Xpress System were combined into a single category for the purposes of this visualization, as both systems employ the same test cartridge and there was likely misreporting between these 2 categories by survey participants. The Hologic category only includes values from the Panther Fusion SARS-CoV-2 assay, as the Hologic Aptima assay does not produce Ct values. Abbreviations: Ct, cycle threshold; PT, proficiency testing; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

are extremely valuable in advancing our understanding of Ct-value commutability in SARS-CoV-2 molecular testing. If healthcare providers and researchers attempt to employ Ct-values as a component of their patient assessment, we caution them to consider the points described in this letter.

Notes

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Potential conflicts of interest. N. W. A. reports being on the Scientific Advisory Board of Diasorin Molecular; F. S. N. reports honoraria from Abbott Molecular; D. R. reports receiving research funding on novel antibacterial agents through Shionogi Inc., Tetraphase pharmaceuticals Inc., and VenatoRx pharmaceuticals; grants from BD, Roche, BioFire, and OpGen; Advisory Board for Luminex; and speaker fees for BD; R. C. S. reports honoraria from BioFire. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Reliability of E gene Cycle Threshold (Ct) Values in Interpreting Duration of COVID-19 Infection

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Introduction:

- There is growing interest in using cycle threshold (Ct) values generated by real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays to determine contagiousness and timing of an individual's COVID-19 infection.
- Based on current guidelines, COVID-19 infected individuals are considered contagious within the first 10 days of symptom onset.
- Previous literature has suggested that COVID-19 infected individuals are not contagious when Ct value > 33.
- We sought to determine the correlation between E gene Ct values and duration of COVID-19 symptoms.

Methods:

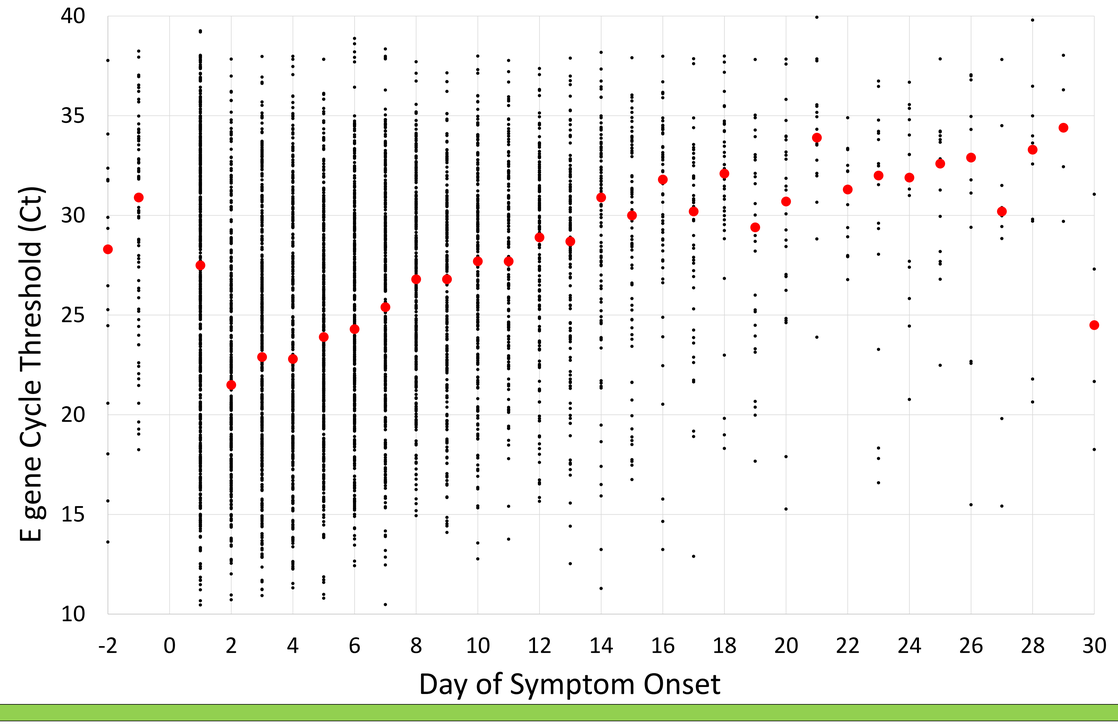
- Provincial data of positive COVID-19 cases from the Alberta Health Services Public Health and Alberta Precision Laboratory databases were linked and analyzed.
- Databases included positive COVID-19 cases from March 1 to May 31, 2020.
- Symptom duration and status at the time of collection was determined during case investigation by Public Health.
- Only E gene Ct values from our laboratory's lab developed test (LDT) were included.
- Specimens included nasal swabs, throat swabs, nasopharyngeal swabs and endotracheal tube aspirates.

Table 1. Patient Characteristics and Associated Median E gene Cycle Threshold (Ct). N = 5,756.

Characteristic			Median E gene Ct (mean)
Gender	Male	52.9%	26.7 (26.4)
	Female	47.1%	26.5 (26.2)
Age	Mean age (median, range)	42.5 (42, 0.08 – 10.5)	-
	Age ≥65	12.1%	**23.6 (24.1)**
	Age <65	87.9%	26.8 (26.6)
City of collection	High River	10.2%	26.1 (25.7)
	Calgary	63.5%	26.3 (26.2)
	Edmonton	9.6%	25.5 (25.4)
	Other	16.7%	N/A
Location of collection	Community	88.8%	**27.3 (26.9)**
	Emergency room	5.3%	22.6 (23.4)
	Inpatient	2.9%	24.7 (24.9)
	Nursing home	3.0%	22.3 (23.9)
Specimen type	Endotracheal Tube Aspirate	1.1%	22.0 (23.6)
	Nasal	8.1%	22.3 (23.1)
	Nasopharyngeal	19.1%	23.6 (23.8)
	Throat	71.6%	**27.8 (27.7)**
Symptoms	Asymptomatic	787 (13.7%)	29.9 (29.4)
	Pre-symptomatic*	92 (1.6%)	30.4 (29.4)
	Symptoms ≤ 7 days)	3107 (54.0%)	24.2 (24.5)
	Symptoms ≥ 7 days)	1770 (30.7%)	28.6 (27.9)

*Defined as symptoms starting within 2 days after swab collection

Figure 1. Scatter Plot of E gene Ct value per Symptom Onset Day. Red dots represent median E gene Ct values. From day 2-14, $R^2 = 0.970$, $p<0.001$.



Results:

- There were 7,974 positive COVID-19 cases observed in Alberta during March 1 to May 31.
- 5,756/7,974 had symptom onset provided and were performed using the LDT.
- Adults age ≥65 had lower Ct values than those aged <65 ($p<0.001$).
- Community patients had higher Ct values than patients in ER, inpatients or nursing home residents ($p<0.001$).
- Throat samples had the highest Ct values among specimen types ($p<0.001$)
- From day 2 – 14, there was a linear trend towards higher median Ct values with increasing days of symptom onset ($R^2 = 0.970$, $p<0.001$, Figure 1).
- However, Ct values ranged widely, regardless of symptom onset.
  - 25% and 10% of individuals with symptoms ≤ 7 days had Ct value >29.1 and >32.8, respectively.
  - 25% and 10% of individuals with symptom onset > 7 days had Ct value <24.3 and <20.3, respectively.

Discussion:

- While there was a linear trend towards increasing Ct values with duration of symptom onset at time of specimen collection, there is a wide enough variation such that a Ct value alone cannot reliably predict an individual's date of symptom onset or contagiousness.

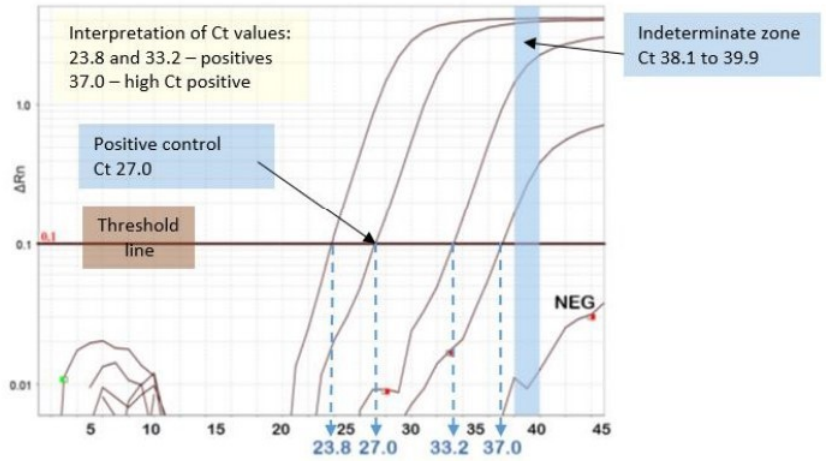
Contact: William Stokes at William.Stokes@ahs.ca

PCR and Ct values in COVID-19 testing – Frequently Asked Questions and Important Considerations

Canadian Public Health Laboratory Network (CPHLN)
Respiratory Virus Infections Working Group

What is a cycle threshold (Ct) value? Most tests that detect the RNA or ribonucleic acid (RNA) or genetic fingerprint of the virus that causes COVID-19 (e.g. a polymerase chain reaction, or PCR) do so through a process where specific bits of the genetic fingerprint are amplified using a temperature cycling reaction that repeats up to 45 times (called amplification cycles). The amount of genetic material doubles after each cycle (Figure 1). The number of amplificationcycles required to create enough copies of the viral RNA to be detected is called the cycle threshold or Ctvalue. The more RNA that is present in the patient sample, the fewer cycles are required for the signal toreach the detection threshold (low Ct value). The fewer RNA present in the clinical sample, the more cycles are required. So a low Ct value corresponds to a high viral load, while a high Ct value corresponds to a low viral load.

Figure 1: An Example of a Real-Time PCR Amplification Curve



Note: This amplification curve is presented on a logarithmic scale. Curves can also be viewed on a linear scale, which will look different but does not change the Ct interpretation. Not all commercial real-time PCR assays provide Ct values or amplification curves for viewing by the user. In addition, some molecular assays are based on other technologies (e.g. flow cytometry), and hence, do not provide Ct values.

Source: Public Health Ontario: An Overview of Cycle Threshold Values and their Role in SARS-CoV-2 Real-Time PCR TestInterpretation

How are Ct values used? The fewer amplification cycles it takes to pass this threshold (a low Ct value) the more virus is likely to be present in the initial sample. The more cycles required to amplify the viral genes above the threshold (a high Ct value) suggests a lower amount of virus present in the initial sample.There can be up to 45 total number of cycles for many molecular tests. The Ct value is the cut-off that calls a test positive, which is defined by the manufacturer of the test or the laboratory during the validation process to make sure that the PCR test is correctly detecting the presence of the virus and not false signals. In certain circumstances, such as patients with compromised immune systems or need to be retested following recovery from COVID-19, Ct values can be used to monitor changes in the amount of virus present in a person's samples over time. This can be complex and typically requires consultation between health care providers and laboratory specialists.

Does a certain Ct value predict who is infectious? A person is deemed infectious if they shed virus particles that are intact and able to go on to infect others. PCR tests cannot distinguish viral genomic material coming from intact viral particles in persons who are infectious or viral particle fragments that are present in individuals who have recovered. A frequent question is whether Ct values can help determine whether an individual is infectious or not. It is not possible to directly translate a Ct value into degree or duration of infectiousness. There is good evidence that when more than 35 cycles are required to detect virus, the virus concentration is so low that it is unlikely to grow the virus in the laboratory. However, the cells used in the laboratory to grow the virus are different from the cells in the back of the throat and nose (nasopharynx) or the lungs in people. So just because one can't grow the virus in a laboratory that does not mean that it won't transmit. Many believe that with low viral RNA copy numbers (high Ct value) the virus is not likely to be transmitted. A recent study which followed patients who were symptomatic but did not require hospitalization showed that those with higher viral loads (lower Cts) infected a higher proportion of their immediate contacts. But we do not know how much virus is actually required to cause an infection in someone and there are other important factors that may influence infectiousness, including the health of the person exposed and the type of exposure that has happened.

Important factors to consider in interpreting Ct values:

1) *Ct values will depend on the stage of infection* – Between exposure to the virus and symptom onset (e.g.. incubation or pre-symptomatic period), the amount of virus in a person's sample can be initially too low to be detectable (negative). A person with an initially negative result may progress to give a test with a high Ct value i.e. >30 (low viral load), then to a lower Ct value (increased viral load) dramatically within a couple of days. Laboratories across the country have seen many cases where the person is tested early during their course of infection and the initial sample had a very high Ct value ~35 (low virus RNA concentration) and the following day the Ct was ~14 (high virus RNA concentration).

2) *Ct values are affected by the type of the sample taken from the person* - Nasopharyngeal swabs (those that go deep into the nose to swab the back of the upper throat) are the most sensitive specimen type for people who do not need admission to hospital; throat/nasal swabs, and gargles/saliva may not have as much virus in them (so they would give a positive test with a higher Ct value). In people where COVID-19 has infected their lungs, these samples from the nose/throat can be negative and a deeper sample like sputum is needed to detect the virus. In addition, the type of swabs used for collecting samples may also influence the Ct value.

3) *Ct values are affected by the quality of the sample taken from the person* - The quality of the sample collected is very important. If you don't get the best possible sample, less virus will be in it and this can lead to a sample with an artificially high Ct value in a person who could have a lot of virus in their system.

4) *Ct values cannot be compared between different PCR tests* - There is no standard yet to be able to compare one test to another so the Ct range can greatly differ by the type of test used, that may use different signal detection methods. In fact, even when testing identical samples using different PCR tests, the results can differ by up to 8 Ct values (e.g. from 22 to 30). This has been observed in the laboratories from different jurisdictions (e.g. ON, BC and SK).

5) *The genetic finger print of the virus can be picked up long after the virus is no longer infectious* – PCR can be positive for over 100 days or more after infection, usually with tests that have high Ct values but in most cases are unlikely to transmit to others beyond 10 days post symptom onset. This finding has been considered in the Infection Prevention and Control (IPAC) and public health practice that recommends patient isolation based on symptom onset, disease severity and the presence of any underlying, immunocompromising conditions instead of on PCR results alone both in some healthcare facilities and more so in the community setting.

6) *The impact of new variants on Ct values is not clear* – Our current tests can detect the new COVID-19 variants of concern (VOCs) - B.1.1.7 (first detected in the United Kingdom), B.1.351 (first detected in South Africa) and P.1 (first detected in Brazil). It has been documented that B.1.1.7 and B.1.351 variants are more infectious, and patients with B.1.1.7 infections have lower Ct values (higher viral loads) compared with those infected with the originally circulating (non-variant) SARS-CoV-2 virus. B.1.351 and P.1 are undergoing further study. We are closely following the VOC-positive samples in Canada to better understand the impact of these variants on our laboratory tests.

Key Points and Recommendations:

1. Ct values can sometimes be used by practitioners, in combination with clinical and epidemiologic information, to make judgment-based decisions. Ct values should not be used alone to make concrete clinical or public health decisions.

2. Not all nucleic acid amplification assays produce Ct values or an equivalent proxy measure of viral 'RNA load'.

3. High Ct values are not yet proven to be able to declare someone non-infectious, only that they are less likely to be infectious.

4. As a result, it is not recommended that Ct values be routinely clinically reported with SARS-CoV-2 RT-PCR results.

5. If a laboratory chooses to routinely report Ct values, it is recommended that clear language regarding uncertainty in interpretation and which authorities may need to be consulted for decision making be included in the report.

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Centers for Disease Control and Prevention

COVID-19

Frequently Asked Questions about Coronavirus (COVID-19) for Laboratories

Updated Mar. 10, 2021 Print

What are you looking for?

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Accessing Laboratory Testing

How do clinicians get access to SARS-CoV-2 viral testing?

Clinicians can access laboratory tests for SARS-CoV-2, the virus that causes COVID-19, through clinical laboratories performing tests authorized or intended to be authorized by the U.S. Food and Drug Administration (FDA) under an Emergency Use Authorization (EUA). Clinicians should consult with the laboratories that routinely perform their diagnostic services to see how best to access SARS-CoV-2 testing.

Clinicians also can access viral testing through their state public health departments. The Association of Public Health Laboratories (APHL) provides a list of available public health laboratory testing locations.

For a list of COVID-19 EUAs, see FDA's COVID-19 Emergency Use Authorizations for Medical Devices .

Where do laboratories get access to reagents and materials to perform viral testing for SARS-CoV-2?

Public health laboratories can access test kits and extraction materials for SARS-CoV-2 testing through the International Reagent Resource (IRR) . The IRR supports state and local public health laboratories, as well as other qualified laboratories participating in public health surveillance and studies.

CDC's real-time reverse transcription polymerase chain reaction (RT-PCR) test to detect SARS-CoV-2 in upper and lower respiratory specimens received an Emergency Use Authorization (EUA) from FDA on February 4, 2020, and is distributed by IRR. CDC's new multiplex assay, which detects influenza A, influenza B, and SARS-CoV-2 simultaneously, received an EUA from FDA on July 2, 2020, and is also being distributed through IRR. IRR also provides several additional commercially produced assays that have received an EUA from FDA to detect SARS-CoV-2 viral RNA in respiratory samples.

Clinical and commercial laboratories conducting SARS-CoV-2 viral testing can acquire test reagents from commercial reagent manufacturers that have received EUA from FDA. Commercial labs can get reagents for CDC's 2019-nCoV Real-Time RT-PCR from qualified sources listed in the instructions for use . A list of commercially available reagents for

use with the multiplex assay is not currently available. However, CDC has shared the primers and probes sequences, so other laboratories and companies may manufacture their own reagents. Genomic RNA material for validation purposes

Can laboratories use specimen collection devices other than those listed in the manufacturer's instructions or EUA (e.g., swabs) for SARS-CoV-2 testing?

According to FDA, when one entity establishes equivalent performance between parallel testing of the same specimens with the new and original components (including viral transport media [VTM]), and FDA's review of the validation data indicates that it could be applicable to modifications of other tests with an authorized EUA, FDA will post this information on its website so that other laboratories can refer to the validation for their testing. Then, other laboratories do not need to conduct their own bridging study for the same modification. For additional information regarding FDA's policy for modification, see FDA's frequently asked questions website.

I can't find swabs or media for SARS-CoV-2 testing. What are my options?

The US Department of Health and Human Services (HHS) is directly managing allocation of swabs and media, including viral transport medium (VTM), based on state and territory testing plans that were submitted in response to the Coronavirus Aid, Relief, and Economic Security (CARES) Act requirements. Allocations were predetermined to maximize state and territory testing using a data-driven algorithm based on population, high incidence areas, and COVID-19 Task Force's directives. Currently, HHS is distributing the following swabs: nasopharyngeal (NP), nasal, foam, and poly swabs. HHS is distributing the following media: saline, phosphate buffered saline solution (PBS), and VTM. For specific swab or medium requests, delivery site changes, or other related requests, contact COVID19TestSupplies@hhs.gov.

Public health and clinical laboratories can also produce their own VTM if it is unavailable for purchase. In response to VTM shortages, CDC posted a standard operating procedure for the preparation of VTM. Saline is also an acceptable transport medium for some COVID-19 viral assays, including the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Check the Instructions for Use to see which transport medium is acceptable.

Are pathologists able to sign out cases remotely during the COVID-19 public health emergency?

CMS has indicated that it will allow laboratories to use temporary testing sites for remote review and reporting of laboratory data, slides, and images if specific criteria are met. Please refer to this CMS Memorandum for additional information.

I cannot obtain the materials I need to perform CDC's 2019-nCoV Real-Time RT-PCR Diagnostic Panel test. What should I do?

The U.S. Food and Drug Administration approved several amendments to this test's Emergency Use Authorization to allow state public health laboratories and others the flexibility to use additional extraction methods and extraction instruments with the CDC 2019-nCoV rRT-PCR Diagnostic Panel.

Can we still order CDC's first viral test for SARS-CoV-2, or is the multiplex assay replacing it?

This new test is designed for use at CDC-supported public health laboratories at state and local levels, where it will supplement and streamline surveillance for flu and COVID-19. The use of this specialized test will be focused on public health surveillance efforts and will not replace any COVID-19 tests currently used in commercial laboratories, hospitals, clinics, and other healthcare settings.

CDC's first viral test for SARS-CoV-2 (the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (ER-34)) will still be available for qualified laboratories to order through the International Reagent Resource (IRR) external icon . The new multiplex assay can also be ordered through the IRR. Check the IRR website for details.

For additional questions, please visit: Clinical Questions about COVID-19: Questions and Answers: Testing, Diagnosis, and Notification

General Guidance and Regulatory Requirements

Under what circumstances should laboratories use either a SARS-CoV-2 viral or serology (antibody) test that has received EUA from FDA?

FDA has authorized EUAs for both viral and antibody tests for COVID-19. Viral (nucleic acid and antigen) tests are used to diagnose the presence of SARS-CoV-2 infections. In contrast, antibody tests can detect IgG, IgA, and IgM antibodies from an immune response to SARS-CoV-2.

Whenever possible, laboratories should rely on viral tests to diagnose the presence of SARS-CoV-2 infections. However, a negative result from viral testing does not rule out COVID-19.

Most of the PCR-based tests that use two or more targets are likely to have high specificity (few false positives). However, there is some variation in the stated sensitivity of the different assays, and sensitivity is highly dependent on the stage of the disease. For this reason, negative results should always be interpreted in the context of the exposure history and symptoms of the patient.

Results from antibody testing should not be used to diagnose or exclude SARS-CoV-2 infections or to inform infection status. Negative results from antibody testing do not rule out SARS-CoV-2 infections, particularly for those individuals who have been exposed to the virus and are still within the estimated incubation period. Until the performance characteristics of antibody tests have been evaluated, it is possible that positive results from such testing may be due to past or present infections with a coronavirus other than SARS-CoV-2.

If a laboratory initially uses antibody testing for diagnostic purposes, follow-up testing using a viral test should be performed. Read more:

- Important Information on the Use of Serological (Antibody) Tests for COVID-19: FDA Letter to Healthcare Providers
- FDA EUA Authorized Serology Test Performance

Where can I find additional CDC guidance about laboratory testing?

CDC has published the following interim guidelines and updates them regularly:

- Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens for COVID-19
- Overview of Testing for SARS-CoV-2 (for healthcare professionals)
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with COVID-19
- CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use
- CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay Instructions for Use

My facility would like to begin SARS-CoV-2 testing. Do we need a Clinical Laboratory Improvement Amendments (CLIA) certificate? Can my facility be granted a waiver from the CLIA certification requirements so that I can begin testing immediately?

requirements so that I can begin testing immediately?

Before conducting SARS-CoV-2 viral testing, a laboratory must be CLIA-certified and meet applicable regulatory requirements. The Centers for Medicare and Medicaid Services (CMS) does not have the authority to grant waivers of exceptions that are not established in a statute or regulation. For additional information, please refer to the FAQs on the CMS website: CMS Coronavirus Information .

What is the CLIA test complexity categorization of SARS-CoV-2 tests that do not have an EUA?

Tests for SARS-CoV-2 that are offered prior to or without an EUA have not been reviewed by FDA, are not FDA-authorized, and have not received a CLIA categorization . Thus, those tests are considered high complexity by default until they receive an EUA or other FDA review that indicates they may be performed as moderate complexity or waived tests. For more information, visit FDA COVID-19 Resources , and navigate to the section titled “General FAQs.”

When FDA authorizes emergency use for a SARS-CoV-2 point-of-care test, can that test be used in CLIA certificate-of-waiver facilities?

When the FDA grants an EUA for a point-of-care test, that test is deemed to be CLIA-waived. For the duration of the national emergency declaration for COVID-19, such tests can be performed in any CLIA-certified patient care setting with a certificate of waiver.

How do I apply for a CLIA certificate so that my testing facility can perform SARS-CoV-2 testing?

The federal CLIA program contracts with states to carry out certain oversight and recording functions of the CLIA program. The state in which the laboratory is located processes applications for CLIA certificates. After the laboratory has identified a qualified and certified laboratory director and has provided all required information on the CMS-116 application, a CLIA number will be assigned and the laboratory can begin testing if applicable CLIA requirements have been met. For additional information, please refer to the FAQs on the CMS website: CMS Coronavirus Information .

Can a laboratory without a CLIA certificate conduct surveillance testing?

Yes. If a laboratory conducts surveillance testing on a specimen without a unique identifier and the results of that testing are not returned to the individual, or to the individual’s healthcare provider, employer, etc., that laboratory does not need a CLIA certificate. Surveillance testing results may be returned in aggregate to the institution that requested the study. In such cases, surveillance testing may indicate the need to conduct additional and perhaps more targeted diagnostic testing or screening at the individual level in a CLIA-certified laboratory to improve population or setting-specific health. If at any time a facility conducting surveillance testing intends to report a patient-specific testing result, it must first obtain a CLIA certificate and meet all CLIA requirements to perform that testing.

Can my laboratory report SARS-CoV-2 variant sequencing results to the individual being tested or to state and local public health departments?

CDC and public health partners are working to detect and characterize emerging SARS-CoV-2 variants through genomic surveillance using sequencing technologies. Surveillance testing is performed on de-identified specimens, and thus results are not linked to individuals. Surveillance testing cannot be used for individual decision-making.

Laboratories that perform sequencing for the purpose of genomic surveillance (i.e., to monitor and characterize the incidence and prevalence of a particular variant at a population level) should test de-identified specimens and not link results to individuals. However, specific variant test results that can be identified through genomic surveillance testing

cannot be reported to the individual who was tested or their healthcare provider or used for individual decision-making unless that test is

- Compliant with applicable the Food and Drug Administration (FDA) laws and regulations
- Performed in a facility certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) that meets requirements to perform high complexity tests

De-identified surveillance testing results may be reported in aggregate to state or local public health departments. Patient-identified sequencing test results that indicate specific SARS-CoV-2 variants can be reported to public health departments to be used only for public health purposes. The CLIA regulations apply if the health department intends to provide that data to individual patients for medical decision-making purposes.

If a laboratory performs sequencing to identify SARS-CoV-2 variants for diagnostic or health assessment purposes and reports specific variant test results to the individual who was tested or to their healthcare provider, the laboratory needs to be CLIA-certified and in compliance with the regulations, and the SARS-CoV-2 sequencing method must be validated for diagnostic use and comply with applicable the FDA laws and regulations.

How does my laboratory assess the validity of a specimen that has been obtained through home collection?

At-home collection of specimens, both unsupervised and supervised by a medical professional, is currently available for specific tests authorized by the Food and Drug Administration. Additional authorized diagnostic tests for the detection of SARS-CoV-2 will likely have this capability as well.

There have been reports of fraudulent specimens being submitted to laboratories for testing, often as a result of unsupervised collection and travel- or work-related requirements. Laboratories should make every effort to confirm the specimen has been obtained correctly and from the individual that is being tested. Generally, the Clinical Laboratory Improvement Amendments of 1988 (CLIA) requires laboratories to ensure positive specimen identification and optimum integrity of a patient's specimen using at least two separate (distinct) or unique identifiers, such as patient's name or other unique identifier, the sex and age or date of birth of the patient, the test(s) to be performed, the specimen source, and the date and, if appropriate, the time of specimen collection.

Test Developers

Can test developers reference the Emergency Use Authorization (EUA) for CDC's diagnostic multiplex assay for flu and SARS-CoV-2 when validating or seeking authorization for a test based on the CDC design?

Yes. CDC has extended right of reference for manufacturers and clinical laboratories to cite the EUA for CDC's Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay (FDA submission number EUA201781). This means clinical laboratories and commercial manufacturers may avoid repeating studies CDC has already conducted in support of its EUA. CDC has published the primers and probes sequences, so other laboratories and companies may manufacture their own reagents. The sequences are identical to those used for the CDC kit and may be used by commercial manufacturers and clinical laboratories in the design of their own independent assays. These sequences are labeled *research use only* because the primers and probes manufactured from these sequences cannot be used under CDC's EUA. Only primer and probe sets distributed through the International Reagent Resource may be used with the assay under CDC's EUA.

Where do test developers get the genomic RNA needed to validate test performance for FDA?

Currently, genomic RNA material can be used for validation purposes in biosafety level 2 laboratories (BSL-2). Genomic RNA material is available through BEI Resources . Registration with BEI Resources is required to request SARS-CoV-2 materials. BEI Resources is prioritizing and fast-tracking all SARS-CoV-2 registrations with a 12- to 72-hour turnaround time for all SARS-CoV-2-related registrations. Please contact BEI Resources at contact@beiresources.org or 1-800 359-7370 for questions.

Developers are required to sign a material transfer agreement prior to the release of materials.

All BEI Resources reagents are provided worldwide. There is no cost for the reagents themselves. However, shipping and handling charges may apply.

Commercial sources also may have this material.

For Public Health Laboratories: If a kit to detect the virus (SAR-CoV-2) is needed, contact the International Reagent Resource

What is NIH's BEI Resources Repository?

BEI Resources Repository was established by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health to provide reagents, tools, and information for studying Category A, B, and C priority pathogens, emerging infectious disease agents, non-pathogenic microbes, and other microbiological materials of relevance to the research community including diagnostic developers. Centralizing these functions within BEI Resources facilitates access to these materials by the scientific community and ensures quality control of the reagents.

My facility created a laboratory-developed test (LDT) to detect SARS-CoV-2. We need to have the first five positive and negative specimens confirmed. Can we send these specimens to CDC?

Laboratories using an LDT to detect SARS-CoV-2 should confer with their state public health laboratory for assistance. If the state public health laboratory cannot assist, contact respvirus@cdc.gov.

Serology

Does CDC accept specimens for antibody testing?

CDC is currently performing antibody surveys to understand how COVID-19 has spread in the U.S. population. CDC is not using its antibody tests for diagnostic purposes, and thus is not accepting antibody test requests intended for COVID-19 patient diagnosis.

Will CDC submit its antibody test for an EUA?

Not at this time. CDC is using its antibody test as part of a multi-agency study to evaluate current commercially marketed antibody tests for specificity and sensitivity and to help determine how results from antibody tests could support policymaking. CDC will share information publicly on the recommended use of antibody testing as soon as enough data becomes available.

Should I test for IgG, IgM, or total immunoglobulin antibodies?

Currently, there is no identified performance advantage of assays that test for IgG or IgM antibodies compared to those that test for total immunoglobulin antibodies. Using an assay that tests for IgM antibodies may detect a more recent infection with SARS-CoV-2, but typically both IgM and IgG rise early in SARS-CoV-2 infections. IgM levels do wane earlier than IgG, and thus assays that test IgM alone may not detect prior infection. Scientists from CDC and elsewhere are continuing to investigate SARS-CoV-2 immune responses and immunoglobulin (antibody) persistence over time using either IgG or total antibodies test.

Laboratory Biosafety

How should the laboratory perform a risk assessment to identify and mitigate risks?

All laboratories should perform a site-specific and activity-specific risk assessment to identify and mitigate risks and determine if enhanced biosafety precautions are warranted based on situational needs, such as high testing volumes, and the likelihood to generate infectious droplets and aerosols. Risk assessments and mitigation measures are dependent on the procedures performed, identification of the hazards involved in the process and/or procedures, the competency level of the personnel who perform the procedures, the laboratory equipment and facility, and the resources available.

The risk assessment should identify all potential scenarios of a particular activity that could produce a negative outcome. The risk assessment should prioritize those potential negative outcomes, or risks, based on an evaluation of the likelihood and consequences of each of those identified risks. The risk assessment should determine the most appropriate control measures, and how the system will measure the effectiveness of those control measures.

For additional information, refer to the following:

- Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)
- Risk Assessment Best Practices
- World Health Organization Laboratory Biosafety Manual, 3rd
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition)

Are certified Class II biological safety cabinets (BSCs) required to process suspected or confirmed SARS-CoV-2 specimens? Should laboratory staff put procedures in place to minimize personnel exposure if there is no certified Class II BSC?

For procedures with a high likelihood to generate aerosols or droplets, use either a certified Class II Type A1 or A2 BSC or additional precautions to provide a barrier between the specimen and personnel. Examples of these additional precautions include personal protective equipment (PPE), such as a surgical mask or face shield, or other physical barriers, like a splash shield; centrifuge safety cups; and sealed centrifuge rotors to reduce the risk of exposure to laboratory personnel.

For additional information, refer to the following:

- CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)
- Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)

How should point-of-care testing (POCT) be conducted outside a traditional laboratory?

For viral testing of specimens conducted outside of a traditional clinical laboratory, such as rapid respiratory testing, use Standard Precautions to provide a barrier between the specimen and personnel during specimen manipulation.

For additional information, refer to:

- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)

If laboratory personnel collect blood or respiratory specimens directly from suspected or confirmed COVID-19 patients, what PPE should they wear?

If laboratory personnel have direct contact with suspected or confirmed COVID-19 patients, they should follow recommended PPE for health care providers while in the presence of these patients.

For additional information, refer to:

- Interim Infection Prevention and Control Recommendations for Patients with Suspected or Confirmed Coronavirus Disease 2019 (COVID-19) in Healthcare Settings
- OSHA 29 CFR 1910.1030 Bloodborne Pathogens Standard

What is the recommended biosafety level for handling suspected or confirmed SARS-CoV-2 patient specimens?

Routine viral testing of patient specimens, such as the following activities, can be handled in a BSL-2 laboratory using Standard Precautions:

- Using automated instruments and analyzers
- Staining and microscopic analysis of fixed smears
- Examination of bacterial cultures
- Pathologic examination and processing of formalin-fixed or otherwise inactivated tissues
- Molecular analysis of extracted nucleic acid preparations
- Final packaging of specimens for transport to diagnostic laboratories for additional testing. Specimens should already be in a sealed, decontaminated primary container
- Using inactivated specimens, such as specimens in nucleic acid extraction buffer
- Electron microscopic studies with glutaraldehyde-fixed grids

For additional information, refer to the following:

- CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)
- OSHA 29 CFR 1910.1030 Bloodborne Pathogens Standard

What disinfectant should personnel use to decontaminate work surfaces?

Decontaminate work surfaces and equipment with appropriate disinfectants. Use EPA-registered hospital disinfectants with label claims to be effective against SARS-CoV-2 . Follow manufacturer's recommendations for use, such as dilution, contact time, and safe handling.

For additional information, refer to the following:

- List N: Disinfectants for Coronavirus (COVID-19)

How should specimens be stored?

Store specimens at 2-8°C for up to 72 hours after collection. If a delay occurs in extraction, store specimens at -70°C or lower. Store extracted nucleic acid samples at -70°C or lower.

For additional information, refer to the following:

- Interim Infection Prevention and Control Recommendations for Patients with Suspected or Confirmed Coronavirus Disease 2019 (COVID-19) in Healthcare Settings
- CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel

How should laboratory personnel remove biohazardous waste from the laboratory or testing area for decontamination and disposal?

Handle laboratory waste from testing suspected or confirmed COVID-19 patient specimens as all other biohazardous waste in the laboratory. Currently, there is no evidence to suggest that this laboratory waste needs additional packaging or disinfection procedures.

For additional information, refer to the following:

- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition)

What are Standard Precautions?

Standard Precautions are the minimum infection prevention practices that apply to patient care, regardless of suspected or confirmed infection status of the patient, in any setting where health care is practiced. They are based on the principle that there is a possible risk of disease transmission from any patient, patient sample, or interaction with infectious material. Standard Precautions include hand hygiene and use of personal protective equipment (PPE) when indicated, in addition to practices to ensure respiratory hygiene, sharps safety, safe injection practices, and effective management of sterilization and disinfection for equipment and environmental surfaces. The exact implementation of Standard Precautions should be determined by an activity-specific risk assessment.

For additional information, refer to the following:

- 2007 Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Healthcare Settings
- CDC Isolation Precautions
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition)
- Standard Precautions for All Patient Care

What are infectious aerosols and droplets?

Aerosols and droplets containing particles that are <100 µm in diameter are not visible to the naked eye. Laboratory

workers may not be aware that such particles can be generated during many laboratory procedures and that these particles could be inhaled or could cross-contaminate work surfaces, materials, and equipment.

Infectious aerosols are small liquid or solid particles suspended in the air that contain infectious agents. They can disperse throughout the laboratory and remain infective over time and distance. These particles are of a size that may be inhaled into the lower respiratory tract (<5 μm in diameter). Examples of organisms transmitted by aerosols include spores of Aspergillus spp., Mycobacterium tuberculosis, rubeola virus (measles), and varicella-zoster virus (chickenpox).

Droplets traditionally are defined as larger infectious particles (>5 μm in diameter) that rapidly fall out of the air, contaminating gloves, the immediate work area, and the mucous membranes of the persons performing the procedure.

Examples of infectious agents that are transmitted via the droplet route include Bordetella pertussis, influenza viruses, adenovirus, Mycoplasma pneumoniae, SARS-associated coronavirus (SARS-CoV), group A streptococcus, and Neisseria meningitidis.

For additional information, refer to the following:

- WHO Laboratory Biosafety Manual, 3rd
- 2007 Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Healthcare Settings
- CDC Isolation Precautions

What procedures can generate aerosols and droplets?

Many routine laboratory procedures can potentially generate aerosols and droplets that are often undetectable. The following laboratory procedures have been associated with the generation of infectious aerosols and droplets: centrifugation, pipetting, vortexing, mixing, shaking, sonicating, removing caps, decanting liquids, preparing smears, flaming slides, aliquoting and loading specimens, loading syringes, manipulating needles, syringes or sharps, aspirating and transferring blood and body fluids, subculturing blood culture bottles, spilling specimens, and cleaning up spills.

For additional information, refer to the following:

- Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition)
- Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)

When is it appropriate to transport suspected or confirmed SARS CoV-2 specimens by pneumatic tube?

It depends on the type of specimen being transported:

- CDC recommends that respiratory specimens from patients with suspected or confirmed COVID-19 should not be transported through pneumatic tubes. At this time, this recommendation only applies to suspected or confirmed COVID-19 respiratory specimens. Examples of respiratory specimens include nasopharyngeal (NP) and oropharyngeal (OP) swabs, nasal mid-turbinate (NMT) swabs, tracheal and lower respiratory tract aspirates, bronchoalveolar lavage (BAL) specimens, and sputum.
- Based on currently available data, other types of specimens from patients with suspected or confirmed COVID-19, such as blood, urine, and feces specimens, are still acceptable to transport through pneumatic tubes.

Facilities should ensure that all personnel who transport specimens via pneumatic tubes are trained in safe handling practices, specimen management, and spill decontamination procedures.

Each facility should also evaluate its risks and determine the most appropriate biosafety measures and practices to implement.

For additional information, refer to the following:

- Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories MMWR, Supplement / Vol. 61 January 6, 2012

How should decentralized and point-of-care (POC) testing for COVID-19 diagnostic purposes be conducted outside of a traditional laboratory?

Testing sites that operate a POC diagnostic instrument must have a current Clinical Laboratory Improvement Amendments of 1988 (CLIA) certificate. During the COVID-19 public health emergency, the Centers for Medicare & Medicaid Services (CMS) will permit a laboratory to extend its existing Certificate of Waiver to operate a temporary COVID-19 testing site in an off-site location (e.g., long-term care or correctional facilities). The temporary COVID-19 testing site is only permitted to perform waived tests, consistent with the laboratory's existing CLIA certificate, and must be under the direction of the existing laboratory director.

Laboratories should consider the following when using POC instruments for COVID-19 diagnostic purposes:

- Use the instrument in a location that has a current CLIA certificate.
- Perform a site-specific and activity-specific risk assessment to identify and mitigate safety risks.
- Train staff on the proper use of the instrument and ways to minimize their risk of exposure.
- Follow Standard Precautions when handling clinical specimens, including hand hygiene and the use of PPE, such as laboratory coats or gowns, gloves, and eye protection. If needed, additional precautions can be used, such as a surgical mask or face shield, or other physical barriers, such as a splash shield to work behind.
- When using patient swabs, minimize contamination of the swab stick and wrapper by widely opening the wrapper before placing the swab back into the wrapper.
- Change gloves after adding patient specimens to the instrument.
- Decontaminate the instrument after each run by using an EPA-approved disinfectant for SARS-CoV-2 and following the manufacturer's recommendations for use, including dilution, contact time, and safe handling.

For additional information, refer to:

- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)
- Fact Sheet: Guidance – Proposed Use of Point-of-Care (POC) Testing Platforms for SARS-CoV-2 (COVID-19)

What safety issues are there with PrimeStore® Molecular Transport Medium (MTM) when used with SARS-CoV-2 testing platforms?

PrimeStore® MTM transport media contains guanidine thiocyanate, which produces a dangerous chemical reaction that releases cyanide gas when exposed to bleach (sodium hypochlorite). The PrimeStore® MTM transport media being provided by state health departments is currently labeled at the bulk box level, but individual vials lack labels to warn users of the reactive ingredient.

Do NOT use PrimeStore® MTM with any Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) platforms that include a disinfecting step that uses bleach (e.g., Panther® Hologic, Panther Fusion® Systems).

In addition to its reactivity, PrimeStore® MTM may be harmful by inhalation, in contact with skin, and if swallowed. Wear appropriate personal protective equipment (PPE) as required by your laboratory protocols, including laboratory coat, safety glasses, and gloves. Dispose of product content and container in accordance with all local, regional, national, and international regulations. Untreated waste should not be disposed into the sewer unless fully compliant with all applicable requirements. See the Material Safety Data Sheet for disposal information.

What safety issues can occur when using a mixture of A549 and Mv 1 Lu cell lines (also referred to as A549/Mv 1 Lu mix or R-Mix™) for culturing respiratory viruses?

It has been shown that Mv 1 Lu cells can support low level replication of SARS-CoV, which could result in the inadvertent growth of SARS-CoV-2. Therefore, CDC recommends that laboratories **discontinue the use** of the A549/Mv 1 Lu mix (R-Mix™) or any other mixture containing Mv 1 Lu cell lines.

Based on recent publications, (Severe Acute Respiratory Syndrome Coronavirus 2 from Patient with Coronavirus Disease, United States), A549 and MDCK cells lines (which make up R-Mix Too™) do not support SARS-CoV-2 replication. As a result, R-Mix Too™ may be considered for use as an alternative for R-Mix™.

For additional information, see

- SARS-associated Coronavirus Replication in Cell Lines
- Severe Acute Respiratory Syndrome Coronavirus 2 from Patient with Coronavirus Disease, United States

Specimen Packing and Shipping

Do people packing patient specimens, isolates or cultures for transport need to be trained and competent?

For transporting patient specimens, cultures or isolates, personnel must be trained in the proper safety, packing, and shipping regulations for Division 6.2, UN 3373 Biological Substance, Category B in accordance with the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulations (DGR) . Personnel should be trained in a manner that corresponds to their function-specific responsibilities.

For additional information, refer to the following:

- Guidance on regulations for the transport of infectious substances 2019 – 2020

What specific packaging should personnel use when shipping suspected or confirmed SARS-CoV-2 patient specimens, isolates or cultures?

Pack and ship suspected or confirmed SARS-CoV-2 patient specimens, cultures or isolates as UN 3373 Biological Substance, Category B, in accordance with the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulations (DGR) :

1. A leakproof primary container.
2. A leakproof, watertight secondary packaging with absorbent material.
3. A rigid outer packaging to protect the specimens during shipment.

For additional information, refer to the following:

- IATA Dangerous Goods Regulations Packaging Instruction 650
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)
- Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition)

At what temperature should specimens be shipped?

Specimens should be shipped at 2-8°C with ice packs. If the specimen is frozen, ship overnight on dry ice. The primary receptacle and the secondary packaging should maintain their integrity at the temperature of the refrigerant used as well as the temperatures and the pressures which could result if refrigeration were lost. Packages containing dry ice should be designed and constructed so as to prevent the buildup of pressure and to allow the release of gas that could rupture the packaging.

For additional information, refer to the following:

- CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)

What information is required on the outer package for shipment of specimens with ice packs?

Ensure the outer package has been properly marked and labeled with the following:

1. Hazard labeled with UN Identification Number already on label – UN 3373
2. Biological Substance, Category B
3. Shipper's name, address, and phone number
4. Receiver's name, address, and phone number
5. Name and phone number of a responsible person is optional if it is on the airway bill

For additional information, refer to the following:

- Guidance on regulations for the transport of infectious substances 2019 – 2020
  - Dangerous Goods Documentation
  - Click on "Infectious substances" and there is an option to download the packing instructions.
- Labels for UN 3373
  - When using cold pack – Include the name and telephone number of the person who will be available during normal business hours who knows the content of the shipment (can be someone at CDC). Place the label on one side of the box and cover the label completely with clear tape (do not tape just the edges of the label).
- Schematic for packaging, UN 3373 Category B

What information is required on the outer packages for shipment of specimens with dry ice?

Ensure the outer package has been properly marked and labeled with the following:

1. Hazard labeled with UN Identification Number already on label – UN 3373
2. Biological Substance, Category B

3. Hazard Labeled with UN Identification Number- UN 1845
4. Dry Ice along with the net weight (kg) of the dry ice
5. Shipper's name and address
6. Receiver's name and address
7. Name and phone number of a responsible person.

For additional information, refer to the following:

- Guidance on regulations for the transport of infectious substances 2019 – 2020
- IATA Dangerous Goods Regulations Packaging Instruction 650
  - Packing Instructions 650 for UN 3373
  - Click on "Infectious substances" and there is an option to download the packing instructions.
- Labels for UN 3373
  - When using dry ice – Include the name and telephone number of the person who will be available during normal business hours who knows the content of the shipment (can be someone at CDC). Place the label on one side of the box and cover the label completely with clear tape (do not tape just the edges of the label).
- Schematic for packaging, UN 3373 Category B

What information is required on an overpack if used for specimen shipment?

The overpack should be marked in accordance with the packing instructions required for the outer package:

1. Hazard labeled with UN Identification Number already on the label – UN 3373
2. Biological Substance, Category B
3. Shipper's name, address, and phone number
4. Receiver's name, address, and phone number
5. Package Orientation Label
6. Marked with the word "Overpack"
7. Name and phone number of a responsible person is optional if it is on the airway bill

For additional information, refer to the following:

- IATA Dangerous Goods Regulations Packaging Instruction 650
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)

Is a shipper's declaration required? What documentation is required for shipment? What if specimens are shipped on dry ice?

A shipper's declaration is not required for UN 3373 Biological Substances, Category B shipped samples. If an Air Waybill is used, the "Nature and Quantity of Goods" box should show "UN 3373 Biological Substance, Category B" along with the number of packages. If specimens are shipped on dry ice, include UN 1845, Dry Ice, 9, along with the net weight of the dry ice. See IATA PI 650 for additional information.

For additional information, refer to the following:

- Guidance on regulations for the transport of infectious substances 2019 – 2020
- IATA Dangerous Goods Regulations Packaging Instruction 650

Is a Responsible Person required on the shipping paperwork?

Yes, a Responsible Person should be listed on the air waybill or Shipper's Declaration (if applicable).

For additional information, refer to the following:

- Guidance on regulations for the transport of infectious substances 2019 – 2020
- IATA Dangerous Goods Regulations Packaging Instruction 650

Once packaging of the samples is complete should staff members decontaminate the work area?

Decontaminate work surfaces and equipment with appropriate disinfectants. Use EPA-registered hospital disinfectants with label claims to be effective against SARS-CoV-2 . Follow manufacturer's recommendations for use, such as dilution, contact time, and safe handling.

For additional information, refer to the following:

- Interim Infection Prevention and Control Recommendations for Patients with Suspected or Confirmed Coronavirus 2019 (COVID-19) in Healthcare Settings
- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)

Specimen Types

Are stool specimens appropriate for SARS-CoV-2 viral testing?

Stool specimens do not have Emergency Use Authorization (EUA) approval and thus are not acceptable for SARS-CoV-2 viral testing. Although data are limited, they indicate that stool might not be an appropriate specimen. Stool specimens are less sensitive than respiratory specimens, and SARS-CoV-2 RNA is often detected later during COVID-19 illness. Therefore, testing stool early in illness could potentially lead to false negative SARS-CoV-2 viral test results. Also, even though viral tests have detected SARS-CoV-2 RNA in stool (i.e., a positive test), infectious virus has only been confirmed very rarely, if at all in samples. In other words, a positive SARS-CoV-2 test does not necessarily mean a patient is currently infected and can infect others.

Interpreting Results of Diagnostic Tests

What influences the likelihood of false-positive or false-negative diagnostic test results?

The likelihood of obtaining a false-positive or false-negative diagnostic test result is influenced by factors related to the testing scenario and the test being used (e.g., sensitivity and specificity of the diagnostic test). Diagnostic tests perform optimally for detecting an infection when the **pretest probability** is high. Pretest probability is the likelihood that the person being tested actually has the infection. This likelihood is based on both the proportion of people in the test population or group who have the infection at a given time (prevalence) and the clinical presentation (including symptoms and known exposure) of the person being tested. In other words, the pretest probability increases with

increasing prevalence in the population and clinical indications of illness in the person being tested. In contrast, tests

What factors have the greatest impact on false-positive rates?

Positive predictive value is the probability that a person who has a positive test result most likely has the infection. Pretest probability and test specificity have the greatest impact on false-positive rates. As the pretest probability and the specificity of the test increases, the false-positive rate decreases and the positive predictive value increases.

What factors have the greatest impact on false-negative rates?

Negative predictive value is the probability that a person who has a negative test result most likely does not have the infection. Pretest probability and test sensitivity have the greatest impact on false-negative rates. As the pretest probability decreases, the false-negative rate decreases and the negative predictive value increases. As the sensitivity of the test increases, the false-negative rate decreases and the negative predictive value increases.

What is the relationship between pretest probability and positive and negative predictive values?

Relationship between pretest probability and positive and negative predictive values

Pretest Probability*	Negative Predictive Value**	Positive Predictive Value**	Impact on Test Results
Low	High	Low	Increased likelihood of False Positives Increased likelihood of True Negatives
High	Low	High	Increased likelihood of True Positives Increased likelihood of False Negatives

*Sensitivity and specificity of tests are not affected by the pretest probability

**Predictive values are affected by the pretest probability

Do all reverse transcriptase-polymerase chain reaction (RT-PCR) diagnostic tests for SARS-CoV-2, the virus that causes COVID-19, detect the same thing?

All RT-PCR tests for SARS-CoV-2 detect genetic material from the virus. However, among the available diagnostic RT-PCR tests, the nucleic acid target within the SARS-CoV-2 genome varies.

Can a diagnostic RT-PCR test show how infectious someone is?

No. RT-PCR tests are used to identify and diagnose an active infection but cannot be used to show how infectious someone is. Get more information about when you can be around others if you had COVID-19.

What is a cycle threshold (Ct) value from a RT-PCR test?

To improve the test's ability to detect virus, an RT-PCR test creates many copies of the same genetic material from the virus in a process called amplification. The cycle threshold (Ct value) is the point at which a reaction reaches a fluorescent intensity above background levels. The Ct value indicates when the nucleic acid target is detectable in the amplification process. There is a correlation between the Ct value and the amount of viral genetic material that was present in the specimen.

Can a Ct value determine how much viral genetic material is present in an individual patient specimen?

A Ct value does not indicate how much virus is present, but only whether or not viral genetic material was detected at a defined threshold. RT-PCR tests can be either ***qualitative*** or ***quantitative***, and this affects how a Ct value is interpreted. As of October 23, 2020, all diagnostic RT-PCR tests that had received a U.S. Food and Drug Administration (FDA) Emergency Use Authorization (EUA) for SARS-CoV-2 testing were ***qualitative*** tests.

1. In a ***qualitative*** RT-PCR test, known amounts of virus are used during the development of the test to determine what Ct values are associated with positive and negative specimens. A Ct value is generated when testing a patient specimen. The Ct value is interpreted as positive or negative but cannot be used to determine how much virus is present in an individual patient specimen.
2. In a ***quantitative*** RT-PCR test, a range of known numbers of genome copies, called reference samples, are tested alongside each RT-PCR reaction. By comparing the Ct value of a patient specimen to the Ct values from the reference samples, the test can calculate the copy number of target nucleic acid. The correlation between Ct value and viral load can be used in evaluating data from groups of people in categories such as symptomatic or asymptomatic and can be applied to infer the difference in the relative amount of viral load between the two. Although a quantitative RT-PCR test can estimate the level of viral load in a population, a quantitative RT-PCR test cannot determine how much virus is present in an individual patient specimen.

Can a Ct value predict how infectious an individual with COVID-19 is?

No. Ct values should not be used to determine a patient's viral load, how infectious a person may be, or when a person can be released from isolation or quarantine.

An RT-PCR test uses multiple repeating amplification cycles to create more and more copies of the virus' genetic material. Specimens with lower amounts of virus will require more cycles to amplify that genetic material to reach an amount that can be detected, resulting in a higher Ct value. Thus, there is a correlation between the Ct value and the amount of starting viral genetic material that was present in the specimen.

For both qualitative and quantitative RT-PCR assays, the correlation between Ct values and the amount of virus in the original specimen is imperfect. It is therefore problematic to infer any relationship between an individual patient's Ct value and their viral load. Ct values can also be affected by factors other than viral load. For example, if the specimen is not collected or stored properly or the specimen is collected early during the infection, the Ct value may be higher than it would be under ideal conditions. Thus, a high Ct value could also result from factors **not** related to the amount of virus in the specimen. The correlation between Ct and viral load can be used to evaluate data from groups of people and infer the difference in the relative amount of viral load between the two groups (e.g., between symptomatic and asymptomatic individuals).

If a Ct value can be affected by factors like specimen collection, how do I know if my RT-PCR test result is accurate?

In addition to detecting SARS-CoV-2 genetic material, each RT-PCR diagnostic test also detects a small portion of a patient's genome. Detecting the patient's genetic material in the specimen confirms the quality of the specimen and the

processing steps of the test. If the patient's genetic material is detected, then we can be reasonably sure that the viral genetic material was not degraded, and the test result is accurate.

Can Ct values from different RT-PCR tests be compared?

No. For a given RT-PCR diagnostic test, the genetic material from a patient sample must be processed using a specific series of steps to produce a valid test result. However, the steps used to process the genetic material, the specific genetic target being measured, and the amount of the patient sample used varies among RT-PCR tests. Because the nucleic acid target (the pathogen of interest), platform and format differ, Ct values from different RT-PCR tests cannot be compared.

Anatomic Pathology

What are the anatomic pathology best practices to prevent COVID-19 exposure while performing procedures and processing specimens?

Manual processing of fresh unfixed specimens, including frozen sections, should be conducted in a manner that provides a barrier between the specimen and personnel during specimen manipulation. In addition, protect the mucous membranes of the eyes, nose, and mouth during procedures that are likely to generate **splashes, sprays, droplets, and aerosols.** Examples of these barriers include:

- Performing tissue dissection in a certified Class II A1 or A2 biological safety cabinet (BSC) if available
- Working behind a splash shield
- Using combinations of PPE, such as:
  - surgical mask with attached eye shield
  - surgical mask and goggles
  - mask and a face shield that fully cover the front and sides of the face
  - double gloves or mesh cut-resistant gloves
  - surgical scrubs, shoe covers, full gown, plastic apron, and hair covering
  - N95 respirators or powered air-purifying respirators (PAPRs) (the use of respiratory protection requires fit testing and appropriate training)

What precautions should clinical and non-clinical support staff take when handling specimen containers that may be contaminated with blood and body fluids?

All laboratories should perform a site- and activity-specific risk assessment and follow Standard Precautions when handling specimen containers and paper requisitions that could have been contaminated by tissue and fluid specimens. This risk assessment may suggest use of some of these mitigation strategies:

- Use face shields and/or work behind a splash guard whenever possible.
- Store human specimens in closed containers that can be decontaminated before moving them to a secure area.

Place specimen containers in closed and clearly labeled plastic bins until pick-up and disposal according to your institutional waste management policies.

What are the biosafety recommendations for performing frozen sectioning on confirmed and suspected COVID-19 patient specimens?

Avoid frozen sectioning from confirmed COVID-19 patients whenever possible. Talk with the relevant clinical and surgical teams about the clinical necessity and benefit of frozen sectioning and consider appropriate alternatives for suspected and confirmed COVID-19 cases. When frozen sectioning is unavoidable, the following are recommended, if possible:

- Receive specimens in an area apart from administrative staff
- Consider using a cryostat that has a downdraft and other safety features.
- Use cryostats in a closed room that has inward directional (negative) airflow vented directly to the outside or recirculated through a HEPA filter to avoid contaminating the rest of the surgical pathology suite.
- Provide grossing rooms with inward directional air flow.
- Reduce the number of operators to a minimum.
- Wear appropriate PPE, including but not limited to:
  - Fluid-resistant disposable double gloves and gown,
  - Fluid-resistant disposable apron,
  - Eye protection (face shield or goggles), and
  - N95 respirator or fluid-resistant surgical mask.
- Do not use freezing sprays; they are not recommended by the manufacturers of cryostat instrumentation.
- Wear cut-resistant, stainless steel mesh gloves during disassembly, cleaning, and disinfection of microtome knives.
- Collect accumulated instrument shavings and discard them as biohazardous waste.
- Follow local standard decontamination procedures of the cryostat and other surfaces. Ultraviolet lights are not a substitute for terminal cleaning of the instrument.

For additional information, refer to the following:

- Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)
- Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories

What chemical treatments inactivate SARS-CoV-2 in tissues during histopathology processing?

Human tissues submitted for permanent pathologic examination typically undergo several processing steps with chemicals that have been shown to inactivate coronaviruses:

- Studies with SARS-CoV-1 and MERS-CoV have shown that virus inactivation for these coronaviruses occurs in a time-dependent fashion with both formalin fixation and temperatures of 56°C or above.
- Alcohol at 70% concentration or higher has been shown to inactivate the virus and tissue processing typically includes a series of alcohol dehydration steps that use 70% to 100% alcohol prior to paraffin embedding.
- In addition, the final step of applying a glass or plastic coverslip to the slide provides an additional barrier between the personnel and the tissue.

For additional information, refer to the following:

- Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV
- Inactivation and safety testing of Middle East Respiratory Syndrome Coronavirus
- Practical Guide to Specimen Handling in Surgical Pathology
- Coronavirus disinfection in histopathology
- NSH-COVID-19: Novel Coronavirus Resources

Does a grossing station that draws air and fumes toward the rear of the unit offer the same protection as a biosafety cabinet?

No. Grossing stations pull formalin fumes away from the person who is doing the dissecting. In general, grossing stations are not as effective as biosafety cabinets at protecting the user from exposure to biological agents.

For additional resources related to biological safety cabinets, refer to:

- Fundamentals of Working Safely in a Biological Safety Cabinet provides free training CEU
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) (6th edition) Appendix A, Section III _Biological Safety Cabinets (page 370).

Ordering Supplies (For Public Health Laboratories)

What Is CDC's International Reagent Resource (IRR)?

The International Reagent Resource (IRR) was established by CDC to provide registered users with reagents, tools, and information for studying and detecting influenza virus and other pathogens, including SARS-CoV-2. IRR is primarily a resource used for procuring pathogen test components and assembling, qualifying, and distributing these kits for use in public health activities. This resource supports detection and characterization of pathogens, which will aid in informing interventions. By centralizing these functions within IRR, access to and use of these materials in the scientific and public health community is supported and quality control of the reagents is assured.

To assist health departments during the COVID-19 pandemic, IRR expanded from April through December to provide more products needed for viral testing, including numerous commercially produced Emergency Use Authorization (EUA) assays. IRR is managed under a CDC contract by American Type Culture Collection (ATCC).

What supplies are being distributed by IRR for testing for SARS-CoV-2?

IRR provides CDC-manufactured kits and controls associated with its EUA applications. These include:

- Influenza SARS-CoV-2 Multiplex Assay (EUA) (Catalog No. Flu SC2-EUA)
- CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel (EUA) (Catalog No. 2019-nCoVEUA-01)
- CDC Human Specimen Control (IVD) (10 x 0.5 mL) (Catalog No. KT0189)
- CDC 2019-nCoV Positive Control (EUA) (Catalog No. VTC-04)

Where can I find a complete product list of items for SARS-CoV-2 testing?

All CDC test kits associated with current EUAs will be available to order through IRR for the duration of the emergency response. Commercial reagents may be added or removed from the IRR catalog as needed to ensure equitable nationwide testing.

What is the new process for swab ordering?

The US Department of Health and Human Services (HHS) is directly managing allocation of swabs and media, including

viral transport medium (VTM), based on state and territory testing plans that were submitted in response to the Coronavirus Aid, Relief, and Economic Security (CARES) Act requirements. Allocations were predetermined to maximize state and territory testing using a data-driven algorithm based on population, high incidence areas, and COVID-19 Task Force's directives. Currently, HHS is distributing the following swabs: nasopharyngeal (NP), nasal, foam, and poly swabs. HHS is distributing the following media: saline, phosphate buffered saline solution (PBS), and VTM. For specific swab or medium requests, delivery site changes, or other related requests contact COVID19TestSupplies@hhs.gov.

Ordering Supplies (For Clinical Laboratories)

Can I register my lab or hospital with IRR?

CDC limits IRR registration and SARS-CoV-2 diagnostic reagent distribution to U.S. state and local public health laboratories validated to perform SARS-CoV-2 viral testing. During the SARS-CoV-2 pandemic, CDC will defer the decision to authorize new laboratories to the corresponding state public health laboratory.

How do I obtain reagents for the CDC EUA real-time RT-PCR assay for SARS-CoV-2?

Clinical laboratories can purchase reagents for the CDC EUA real-time RT-PCR primers and probes from Integrated DNA Technologies (IDT) or Biosearch Technologies. CDC has posted a list of approved reagents and acceptable lots on the CDC COVID-19 website. Clinical laboratories also can purchase commercially developed viral tests with an EUA from the manufacturer.

Last Updated Mar. 10, 2021

Ct Values:
What They Are and How They Can be Used

Version 1 • November 9, 2020

www.aphl.org

Introduction

There are many factors that impact the use and interpretation of Ct values that are generated during real-time PCR testing. Diagnostic laboratories should not include Ct values on laboratory reports because it could be out of compliance with laboratory regulations and they should not be used to inform patient management. In some instances, Ct values may provide information that assists in prioritizing or informing public health surveillance, contact tracing and investigations, but APHL does not yet recommend this as a routine practice. This is an area that requires further investigation and gathering of data before that step is taken. Sharing and interpreting Ct values in the context of public health surveillance or a public health investigation should always be done in consultation with jurisdictional public health laboratory staff.

What are nucleic acid amplification tests (NAATs)?

There are several different kinds of diagnostic tests that detect the nucleic acid (DNA or RNA) of a pathogen in a patient specimen. The majority of these assays work by amplifying the target nucleic acid present, but they do not tell us whether the pathogen is infectious or alive.

NAATs can be based on different types of chemical reactions, including real-time polymerase chain reaction (real-time PCR), transcription-mediated amplification (TMA), loop-mediated isothermal amplification (LAMP) or other chemistries. Different types of NAATs may be reported differently.

NAATs may be developed as multiplex assays, meaning they can detect multiple pathogen targets in one test.

What are COVID-19 Diagnostic NAATs?

There are many different NAAT-based tests to detect SARS-CoV-2 RNA for the diagnosis of COVID-19, some based on real-time PCR (e.g., the CDC Diagnostic Panel, Cepheid Xpert Xpress SARS-CoV-2, Roche Cobas SARS-CoV-2) and others based on methods like TMA (e.g., Hologic Aptima SARS-CoV-2) or LAMP. A full list can be obtained on the FDA's EUA website.

NAATs for COVID-19 diagnostic testing are generally very sensitive, meaning they can detect very low levels of viral RNA, and very specific, meaning they detect only SARS-CoV-2 RNA.

All of the commercially available diagnostic NAAT tests for COVID-19 in the US are "qualitative" tests— the test produces a qualitative result of positive or negative. The tests are NOT designed to provide a semi-quantitative or quantitative measurement of the level of viral RNA in the specimen.

What is a Ct value?

Many NAAT tests generate a number as part of the test result. For real-time PCR, this is called the Ct or "cycle threshold" value. A Ct value is defined as the number of amplification cycles required to reach a fixed background level of fluorescence at which the diagnostic result of the real-time PCR changes from negative (not detectable) to positive (detectable).

The total number of cycles required to exceed the established threshold to call a result positive is specific to that test platform, and generally ranges from about 15 to 45 cycles. Different tests calculate the Ct values differently, and different tests also count the number of cycles differently. Some tests generate the Ct value through software installed on the instrument itself, some require the operator to interpret and define the Ct value based on parameters set by the test manufacturer, while others do not generate a Ct value that is available or visible to the operator and simply provide a positive or negative test result. In addition, some tests have an established Ct "cutoff" beyond which the test result is considered negative; for others, the "cutoff" is the last cycle of the test. These parameters are determined by the test manufacturer and cannot be altered by the laboratory performing the test.

Is there variability in Ct values?

Short answer: Yes

The number of cycles required for detectable amplification of viral RNA is dependent on a long list of variables beyond simply how much viral RNA is present in a patient specimen. The relative impacts of these variables on the Ct value differs between test platforms and can vary widely. Variables that can impact Ct values include but are not limited to:

Pre-analytic Variables

- Efficiency of the collection of specimen
- Time of collection of specimen after onset of infection
- Specimen type—matrix effect
- Specimen type – level of viral RNA in different specimen types (e.g., upper vs. lower respiratory tract) can differ between specimens from the same patient at the same time
- Storage and transport conditions of specimen prior to testing
- Age of specimen

Analytic Variables

- Nucleic acid extraction efficiency
- Amount of viral RNA in the specimen
- Nature of the target RNA and design of the primer/probe sequences
- Efficiency of the real-time PCR chemistry in the assay (singleplex, multiplex)
- Method for defining/determining Ct value

I can get a quantitative test for HIV, why can't I get one for COVID-19?

Short answer: They are not currently commercially available in the US

Quantitative viral load assays are specifically designed for this purpose. They are run on specimen types that mitigate the impact of variables on the Ct value and include controls and calculations to assess viral load. For example, an HIV quantitative viral load assay is performed on a blood specimen. This specimen is homogenous and can be collected in a very standardized manner. The real-time PCR assay used to calculate viral load includes a set of controls to "standardize" the specimen (e.g., a control for specimen adequacy) and a set of standards (i.e., known dilutions of virus for calibration). Ct values of the patient specimen are compared to those of the standard curve to calculate the viral load in a standardized specimen.

This type of assay is not yet available for SARS-CoV-2. Respiratory specimens are not homogeneous and are challenging to standardize. The collection process of a respiratory specimen does not lend itself to quantifying the amount of virus present. Each swab collection is different and does not assure that the same amount of sample is collected. Quality of specimen collection is impacted by other variables including the skill of the collector, which nostril is swabbed first, or whether the patient recently ate or drank. Many COVID-19 diagnostic real-time PCR assays do not include specimen adequacy controls, and those that do still lack the standardization necessary to calculate viral load.

Cts and infectiousness—can we infer one from the other?

Short answer: No

There are a number of reasons that Ct values should not be used to determine how infectious someone is. The first relate to the nature of the available testing methods and the inherent variability of Ct values:

- The available assays are qualitative, not quantitative. Qualitative tests are not designed to provide an indication of possible infectivity.
- There are many variables that impact Ct value that are unrelated to the amount of viral RNA in a specimen (see above).
- The only method available for determining the presence of live virus in a specimen is inoculating the virus into cell culture to determine if the virus can grow there. This is a very insensitive and qualitative method, may not detect low levels of infectious virus and does not necessarily correlate with infectiousness.

There are also simply not enough data at this time to infer a correlation between detectable SARS-CoV-2 viral RNA and infectiousness. We do not know how much virus (as measured by detecting viral RNA) is needed in a respiratory specimen for a person to be able to transmit it to someone else. We also do not know what the "cutoff" is for a person to no longer be infectious (i.e., at what point the amount of virus in a person's respiratory specimen is too little for them to be able to infect others).

Do Ct values correlate with viral load?

Short Answer: Often, but not always

There is a relationship between Ct values and amount of virus in a patient specimen, but they are not equivalent. There are many variables that impact Ct values (see above). Although Ct may be used as a proxy for viral load, caution must be taken when interpreting in this manner. A high Ct value often correlates with a low viral load, but not always.

A specimen could have a very high viral load, but also a high Ct value (i.e., it took more cycles to detect the viral RNA) because the extraction was inefficient, the patient just drank something that inhibited the real-time PCR reaction, or the specimen was packaged inappropriately and reached a high temperature during transportation to the lab and the viral RNA in the specimen degraded in the heat.

Any specimen that generates a result that is defined as "positive" by the test manufacturer is considered positive. As with any diagnostic test, the result should be interpreted in the clinical context.

The process of viral replication and infection must be taken into consideration as well. If a specimen is collected very close to the time of the initial infection the viral load may be very low as the virus has not had a lot of time to replicate; a specimen collected in the coming days may have a much higher viral load. A specimen collected many days to weeks after the initial infection may have a low viral load, and viral RNA can be detectable for many weeks after infection in some patients. Limited epidemiological and culture data indicate that patients are not infectious more than 10-15 days post-onset of symptoms.

Can I compare a Ct value from one test method to another?

Short answer: No

Ct values and cutoffs are assay- and method-specific. A specimen with a Ct of 35 by one assay will not necessarily have the same Ct value by other assays. These values can vary up to two to three logs from test to test due to how the tests are designed.[1]

There can be a difference in the relative sensitivities of FDA authorized tests which may also impact Ct values. According to comparison data recently published by FDA using a standard panel, there can be as much as a 1000-fold difference between the various assays.[2]

Why don't labs report Ct values on their reports for NAATS?

Short answer: This would be a regulatory violation

All currently-available nucleic acid tests for SARS-CoV-2 are FDA-authorized as qualitative tests, and Ct values from qualitative tests should never be used to direct or inform patient management decisions. Therefore, it would be a regulatory violation for laboratories to include Ct values on patient reports.

Can Ct values be used to inform infection control decisions?

Short Answer: We need additional data

The amount of detectable viral RNA in an infected individual is quite low in the first few days after infection, then rises exponentially for several days before dropping back off. It is reasonable to conclude that this period of peak viral load is when the infected individual is most capable of transmitting the virus to others, and when their specimens will have

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[1] D. Rhoads, DR Peaper, RC She, FS Nolte, CM Wojewoda, NW Anderson, BS Pritt. College of American Pathologists microbiology committee perspective: Caution must be used in interpreting the cycle threshold (Ct) value. *Clin. Infect Dis*. (2020), 10.1093/cid/ciaa1199

[2] FDA SARS-CoV-2 Reference Panel Comparative Data webpage.

their lowest Ct values. Therefore, a positive real-time PCR test result with a low Ct value can be interpreted as being from a person with a high viral load and high chance of transmissibility. However, most individuals would be considered non-infectious by 10 days post-symptom onset, although a NAAT may still be positive with a relatively high Ct value since the assay is detecting left-over fragments of the viral RNA. Additionally, correlates between viral load and infectiousness are not completely understood, including the interpretation of viral loads in asymptomatic individuals.

Additional data on when an individual is infectious and capable of transmitting virus are needed to further inform how Ct values may be used to inform public health decision making.

Additional Notes about Diagnostic Laboratories

All laboratories that perform diagnostic testing on human specimens must adhere to state and federal regulations and always perform rigorous evaluation of a new test—in addition to ongoing monitoring—to assure that tests are performing as expected. This involves testing known positive and negative samples to ensure the test is working properly, evaluating staff to make sure they are performing the test correctly and continual assessment of results.