



Laboratory Diagnosis of COVID-19: Current Issues and Challenges

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ABSTRACT The COVID-19 outbreak has had a major impact on clinical microbiology laboratories in the past several months. This commentary covers current issues and challenges for the laboratory diagnosis of infections caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In the preanalytical stage, collecting the proper respiratory tract specimen at the right time from the right anatomic site is essential for a prompt and accurate molecular diagnosis of COVID-19. Appropriate measures are required to keep laboratory staff safe while producing reliable test results. In the analytic stage, real-time reverse transcription-PCR (RT-PCR) assays remain the molecular test of choice for the etiologic diagnosis of SARS-CoV-2 infection while antibody-based techniques are being introduced as supplemental tools. In the postanalytical stage, testing results should be carefully interpreted using both molecular and serological findings. Finally, random-access, integrated devices available at the point of care with scalable capacities will facilitate the rapid and accurate diagnosis and monitoring of SARS-CoV-2 infections and greatly assist in the control of this outbreak.

KEYWORDS COVID-19, SARS-CoV-2, specimen type, molecular testing, serology, result interpretation

The identification by U.S. public health officials of presumptive COVID-19 cases believed to be due to community transmission of this infection brings into sharp focus the importance of the laboratory diagnosis of infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1–5). The current recommendations for laboratory diagnosis of COVID-19 from the CDC are that clinicians coordinate this testing with local public health authorities and/or the CDC. The preferred testing method is the real-time reverse transcription-PCR (RT-PCR) test (6–8) similar to that developed for the diagnosis of SARS-CoV (9, 10). Viral cultures are not recommended. This commentary addresses current issues for the laboratory diagnosis of COVID-19 that must be understood by clinicians, clinical microbiology laboratories, and public health authorities.

Preanalytical issues. (i) Initial respiratory tract specimen collection for diagnosis and screening of patients with COVID-19 pneumonia. Within 5 to 6 days of the onset of symptoms, patients with COVID-19 have demonstrated high viral loads in their upper and lower respiratory tracts (11–14). A nasopharyngeal (NP) swab and/or an oropharyngeal (OP) swab are often recommended for screening or diagnosis of early infection (9, 12, 15). A single NP swab has become the preferred swab as it is tolerated better by the patient and is safer to the operator. NP swabs have an inherent quality control in that they usually reach the correct area to be tested in the nasal cavity. Wang et al. have just reported that OP swabs ($n = 398$) were used much more frequently than nasal swabs ($n = 8$) in China during the COVID-19 outbreak; however, the SARS-CoV-2

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RNA was detected in only 32% of OP swabs, which was significantly lower than the level in nasal swabs (63%) (16). While collection/testing of both nasal and OP swabs, either as independent specimens or together within a single aliquot of viral transport medium, might be an attractive option under normal circumstances, institutions must also consider the potential stress that this pandemic places on national/international supply chains. In this light, another excellent reason to limit testing with NP swabs is to prolong supplies of flocked swabs and/or transport media. However, as we understand more about respiratory and oral contact routes of transmission, we may learn that patients with pharyngitis as a dominant initial presenting symptom can be adequately sampled via the OP route.

In order to properly obtain an NP swab specimen, the swab must be inserted deeply into the nasal cavity. Patients will likely flinch, but that means the swab has hit the target. Swabs should be kept in place for 10 s while being twirled three times. Swabs should have flocked nontoxic synthetic fibers, such as polyester, as well as synthetic nylon handles (17). Collecting an NP/OP swab specimen may carry a theoretical risk of transmitting SARS-CoV-2, particularly if airborne transmission is demonstrated as the investigation of the COVID-19 outbreak continues (18). If personal protective equipment (PPE) cannot be utilized due to scarcity of such PPE, other means of collecting upper respiratory tract specimens will be needed (18). One alternative option for collecting an upper respiratory tract specimen to evaluate patients with suspected COVID-19 pneumonia is a self-collected saliva specimen (19–22). Should the supply of swabs become scarce, other nonflocked swabs and transport media have been cleared equivalently by the Food and Drug Administration (FDA) under an emergency use authorization (EUA), but head-to-head comparisons are lacking currently.

After collection, swabs should be placed in viral (universal) transport medium for rapid transportation to the clinical microbiology laboratory, ideally under refrigerated conditions (17). It should be noted, however, that in some cases, saliva/NPs/OPs may miss early infection and that in later infection, the main site of replication may have shifted to the low respiratory tract. Repeated testing or obtaining lower respiratory tract specimens may be required. Moreover, other respiratory viral pathogens such as influenza and respiratory syncytial viruses must be ruled out. In many ways, COVID-19 highlights the key difference between analytic and clinical sensitivities, that is, the ability of an assay to detect a pathogen when it is present in a clinical specimen versus the ability of a test to identify a patient's overall infected status. The latter, of course, reflects various other factors that include the specimen site and method of collection, in conjunction with the burden of organism as a function of anatomic location, disease severity, and time symptomatic (and variability of these factors from individual to individual). Repeated testing may be particularly important if a patient has a clinical picture of viral pneumonia, a potential exposure history, and/or radiographic findings (chest computed tomography [CT] or magnetic resonance imaging [MRI] scan) consistent with COVID-19 pneumonia. Equally challenging are how the results of a single undetected result should impact decisions regarding patient quarantine and social distancing, in particular when the patients themselves are health care providers (including clinical laboratory staff). Serology, as discussed in the postanalytical section, may assist in such situations.

(ii) Late detection and monitoring of patients with severe COVID-19 pneumonia. Ideally, sputum sampling or bronchoalveolar lavage should be used for collecting lower respiratory tract specimens as they have yielded the highest viral loads for the diagnosis of COVID-19 (18, 23). A recent study revealed that samples bronchoalveolar lavage (BAL) fluid yielded the highest SARS-CoV-2 RNA rate although this study did not compare/evaluate results from NP swabs (16). Patients who present with severe pneumonia and acute respiratory distress syndrome may require emergent intubation as well as respiratory isolation in a negative-pressure room. If possible, a lower respiratory tract sputum specimen should be collected during the intubation procedure. Alternatively, sputum and/or bronchoalveolar lavage fluid specimens can be collected after intubation (9, 11).

However, some patients with COVID-19 pneumonia have demonstrated high viral RNA loads of SARS-CoV-2 in fecal material (24, 25) as well as delayed shedding from the respiratory tract (4, 18) late in their clinical course. Enteric involvement previously has been seen in patients with severe novel coronavirus infections (9, 26–32). In four such studies, SARS coronavirus was isolated from stool cultures (26, 28, 31). In another study, SARS coronavirus was demonstrated inside enterocytes by electron microscopy (30). Thus, aside from direct respiratory sampling, the preferred method for detecting SARS-CoV-2 in advanced COVID-19 cases may be a rectal swab and real-time RT-PCR (9, 26–28, 30–32).

(iii) Safety measures for specimen processing for PCR processing and testing.

Processing of respiratory specimens should be done in a class II biological safety cabinet (6, 9, 10), although some laboratories would argue that biosafety level three (BSL-3) work procedures should be used and that the safety cabinet should be in a negative-pressure room within the laboratory such as that used for mycobacterial cultures. For nucleic acid extraction before real-time RT-PCR is performed, the specimen should be transferred to lysis buffer under this BSL-2 cabinet. The lysis buffer should contain a guanidinium-based inactivating agent as well as a nondenaturing detergent. Indeed, the buffers included in common commercial extraction platforms, such as the bioMérieux easyMAG or Qiagen EZ1, do contain guanidium/detergents and are able to inactivate any viable coronavirus (33–35). Similarly, universal transport medium that includes guanidinium salt is available from Merlin Biomedical (Xiamen, China) (http://www.chinamerlin.com/en/index.php?p=products_show&id=166&s_id=&c_id=68&lanmu=2). Because this test is a reverse transcription method, the saliva/swabs used to collect the clinical specimens should be quickly added to lysis buffer to disinfect the specimen as well as to stop degradation of the coronavirus RNA (6, 9, 10). The clinical specimens/swabs should not be heated to 56°C for 30 min as evidence suggests that this process may also degrade the coronavirus RNA even as it inactivates viable coronavirus (9, 36).

Moreover, self-enclosed systems integrating nucleic acid extraction, amplification, and detection such as ID NOW (Abbott, San Diego, CA) (37, 38), cobas Liat (Roche Molecular Systems, Pleasanton, CA), and GeneXpert (Cepheid, Sunnyvale, CA) (39), when available and meeting local regulatory requirements for SARS-CoV-2 testing, will be very useful. Once the clinical specimen in viral transport medium is transferred into a cartridge in a class II biosafety cabinet, the cartridge is sealed. Many of these random-access sealed devices are suitable for point-of-care testing for local hospitals and clinics without biosafety cabinets. In this situation, the specimen collector in appropriate protective gear (splash guard/goggles, mask, gloves, and disposable laboratory coat) could directly transfer the specimen into detection cartridges at bedside or in a location without a class II biosafety cabinet, and the closed cartridge could be safely placed on an instrument for testing. However, spills of transport solution during transfer to these cartridge-based tests should be avoided, and if they occur, decontamination should be performed as appropriate.

Analytical issues. (i) Assay selection. Immunoassays have been developed for rapid detection of SARS-CoV-2 antigens or antibodies. These rapid point-of-care immunoassays are generally lateral flow assays, but high-throughput immunoanalyzer versions are also in development for population-level screening. Such lateral flow assays have been developed for detecting antigens such as the SARS-CoV-2 virus or for detecting antibodies (IgM and IgG) against COVID-19.

Rapid antigen lateral flow assays would theoretically provide the advantage of a fast time to result and low-cost detection of SARS-CoV-2 but are likely to suffer from poor sensitivity early in infection, based on the experience with this method for influenza (Flu) viruses (40–44). Monoclonal antibodies specifically against SARS-CoV-2 have been under development, and several rapid antigen assays are being developed (45). There is concern that, given the variability of viral loads in COVID-19 patients, antigen detection may miss cases due to low infectious burden or sampling variability.

Serology measures the host response to infection and is an indirect measure of infection that is best utilized retrospectively. Serological methods are rapidly being developed and have proven to be useful in confirming past COVID-19 (25). Serology previously has had an important role in the epidemiology of SARS (46) and other coronavirus outbreaks (47). Rapid lateral flow assays for both IgM and IgG antibodies undoubtedly will play an important role in the COVID-19 outbreak and should allow the burden of infection, the role of asymptomatic infections, the basic reproduction number, and the overall mortality to be determined. However, IgM responses are notoriously nonspecific, and given the weeks required to develop specific IgG responses, serology detection is not likely to play a role in active case management except to diagnose/confirm late COVID-19 cases or to determine the immunity of health care workers as the outbreak progresses. Cell culture is not recommended for diagnostic purposes.

(ii) Assay selection for molecular detection of SARS-CoV-2. Random-amplification deep-sequencing methods played a major role in the initial identification of SARS-CoV-2 (48–52). Deep sequencing molecular methods such as next-generation sequencing and metagenomic next-generation sequencing will continue to be needed to determine future mutations of SARS-CoV-2 but are currently impractical for diagnosing COVID-19. Most of the molecular diagnostics being developed for the diagnosis of COVID-19 involve real-time RT-PCR assays, including those from the U.S. Centers for Disease Control and Prevention (53), Charité Institute of Virology in Berlin, Germany (7, 54), and Hong Kong University (21, 55). Other molecular methods are being developed and evaluated worldwide and include loop-mediated isothermal amplification, multiplex isothermal amplification followed by microarray detection, and CRISPR (clustered regularly interspaced short palindromic repeats)-based assays (56).

(iii) Target selection for real-time RT-PCR assays. A real-time RT-PCR method is recommended for molecular testing (6, 8–10). A major advantage of real-time RT-PCR assays is that amplification and analysis are done simultaneously in a closed system to minimize false-positive results associated with amplification product contamination. There are a number of coronaviruses that cause respiratory and intestinal infections in humans (8, 57). Among these coronaviruses are a group of SARS-like bat coronaviruses, including both SARS-CoV and SARS-CoV-2, that comprise a unique clade under the subgenus *Sarbecovirus* (57, 58). Coronaviruses have a number of molecular targets within their positive-sense, single-stranded RNA genome that can be used for PCR assays (6, 7, 57, 58). These include genes encoding structural proteins, including envelope glycoproteins spike (S), envelope (E), transmembrane (M), helicase (Hel), and nucleocapsid (N) (57–59). In addition to the genes that encode structural proteins, there are species-specific accessory genes that are required for viral replication. These include RNA-dependent RNA polymerase (RdRp), hemagglutinin-esterase (HE), and open reading frame 1a (ORF1a) and ORF1b (7, 53–55, 57, 58). In the United States, the CDC recommends two nucleocapsid protein targets (N1 and N2) (53) while WHO recommends first-line screening with an E gene assay followed by a confirmatory assay using the RdRp gene (7). Chan et al. have just developed and compared the performance of three novel real-time RT-PCR assays targeting the RdRp/Hel, S, and N genes of SARS-CoV-2. Among them, the COVID-19-RdRp/Hel assay had the lowest limit of detection *in vitro* and higher sensitivity and specificity (59). However, it is likely that well-optimized targets will arise from a number of viral genomic locations since assay performance is usually dictated by the reagent design, not the target itself, since the viral genes are present in equal copy numbers.

To avoid potential cross-reaction with other endemic coronaviruses as well as potential genetic drift of SARS-CoV-2, at least two molecular targets should be included in the assay. Various investigators in different countries have used a number of these molecular targets for real-time RT-PCR assays. In the United States, the CDC has selected two loci in the nucleocapsid gene as the two-target assay appears to be performing well (53). One study utilized two sequence regions (open reading frame 1b and a nucleocapsid protein) that are highly conserved among sarbecoviruses for initial real-

time RT-PCR testing (6). Another study in Hong Kong, China, used two targets for its RT-PCR assay; the first used the nucleocapsid for screening followed by confirmation by the open reading frame 1b (55). In Germany, two molecular targets (envelope and RNA-dependent RNA polymerase) have been selected (7). In China, at the time of manuscript preparation, several molecular devices had received urgent approval (8). To date, there has been no indication that any one of the sequence regions used offers a unique advantage for clinical diagnostic testing. However, the ideal design would include at least one conserved region and one specific region to mitigate against the effects of genetic drift, especially as the virus evolves within new populations.

In the United States, regulatory issues have complicated the development and implementation of laboratory-developed molecular tests for the diagnosis of COVID-19. On 29 February 2020, the FDA issued new guidance for laboratories to be able to develop and implement COVID-19 molecular diagnostic tests prior to obtaining EUA. Laboratories are required to submit an EAU to the FDA within 15 business days after validation. Moreover, the validation must include the specimen types (e.g., nasopharyngeal, oropharyngeal, or saliva) that are to be used clinically. Although these new regulatory burdens did not prohibit the development of molecular laboratory testing for the diagnosis of COVID-19, they did create a lot of extra work. At the time of writing, the U.S. FDA had granted quite a few EUAs (<https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#coronavirus2019>; accessed 28 March 2020).

Postanalytical issues. (i) Interpretation of molecular results. In the United States, initially if both of two targets in the CDC assay (nucleocapsid proteins N1 and N2) test positive, a case is considered to be laboratory confirmed (53). A cycle threshold (C_T) value of less than 40 is defined as a positive test, while a C_T value of 40 or more is defined as a negative test. A C_T value of <40 for only one of the two nucleocapsid protein (N1 and N2) is defined as indeterminate and requires confirmation by retesting (53). Currently, in China for the assays with three targets, positives for two or more targets are considered positive (60). Although some correlations have been revealed, viral loads determined by real-time RT-PCR assays should not be used yet to indicate COVID-19 severity or to monitor therapeutic response (11–13, 61, 62). However, low C_T values indicating high viral loads may be used as an indication of transmissibility (18, 63).

(ii) Test of cure and test of infectivity. Monitoring patients with resolution of COVID-19 pneumonia may also be important in terms of when they should be released from isolation and discharged. If discharged patients are still shedding viable coronavirus, they are likely to infect other people (27). Therefore, self-quarantine for up to 1 month has been recommended in some cases. NP and OP swabs may not be sufficient for either test of cure or test of infectivity (64), but this needs further investigation. One approach to test of cure has been to demonstrate two consecutive negative real-time RT-PCR tests from rectal swabs; this suggestion is based on the fact that SARS-CoV-1 was cultured from stool during the 2002–2003 SARS outbreak (26, 28, 31), and SARS-CoV-2 has been cultured from stool during the COVID-19 outbreak (16). Thus, a rectal swab that is positive by real-time PCR testing suggests that this patient may be shedding viable SARS-CoV-2 in their stools, thereby remaining infectious (16, 24–28, 30–32). However, a very recent study on 20 serial COVID-19 patients indicated that infectious virus was not isolated from stool samples in spite of high virus RNA concentrations (14). The correlation of RT-PCR positivity in stool with recovery of live virus from the same samples remains to be fully investigated.

(iii) Serology of COVID-19. Members of the coronavirus family have four structural proteins: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. Two of these proteins appear to be important antigenic sites for the development of serological assays to detect COVID-19. Serological methods have focused on detecting serum antibodies against S proteins from the coronavirus spike (47). The coronavirus envelope spike is responsible for receptor binding and fusion and determines host

tropism and transmission capability (57, 58). S proteins are determined by the S gene and are functionally divided into two subunits (S1 and S2). The S1 domain is responsible for receptor binding while the S2 domain is responsible for fusion. SARS-CoV and SARS-CoV-2 bind to human angiotensin-converting enzyme 2, which is found on human respiratory cells, renal cells, and gastrointestinal cells (57, 65, 66). The other protein that appears to be an important antigenic site for the development of serological assays to detect COVID-19 is the N protein, which is a structural component of the helical nucleocapsid. The N protein plays an important role in viral pathogenesis, replication, and RNA packaging. Antibodies to the N protein are frequently detected in COVID-19 patients (67, 68), suggesting that the N protein may be one of the immunodominant antigens in the early diagnosis of COVID-19 (69).

As mentioned above, rapid lateral flow assays for antibodies (IgM and IgG) produced during COVID-19 have been developed (70). Seroconversion occurred after 7 days of symptomatic infection in 50% of patients (14 days in all) but was not followed by a rapid decline in viral load (14). Serological methods, when available, will play an important role in the epidemiology of COVID-19 and in determining the immune status of asymptomatic patients but are unlikely to play any role in screening or for the diagnosis of early infections (14, 67, 68). However, serology may be useful for confirming the diagnosis of COVID-19 (25).

Concluding remarks. The ongoing, unprecedented outbreak of COVID-19 globally has emphasized the importance of the laboratory diagnosis of human coronavirus infections in order to limit the spread as well as to appropriately treat those patients who have a serious infection. This commentary has addressed current issues regarding such testing for SARS-CoV-2. For example, an NP rather than OP swab is recommended for early diagnosis or screening because it provides higher diagnostic yields, is better tolerated by the patient, and is safer for the operator. An NP swab can be combined with an OP swab to increase sensitivity but requires twice the number of swabs. Should the NP swabs become scarce, self-collected saliva or nasal washes could be used as an alternative specimen type for epidemiological screening and for the “worried well,” who are asymptomatic persons with no exposure history who wish to be tested just to be sure they are not infected. NP swabs would then be reserved for hospitalized patients; those who test negative may need deep sputum or BAL fluid samples collected. The importance of repeated testing or the use of bronchoscopy in patients with severe illness should the first screening test be negative must be understood. The role of rectal swabs in testing patients with late infection or as a test of infectivity/cure is currently not well studied but needs urgent attention. Equally unappreciated is the need for broad screening/testing with molecular testing and/or serological testing in order to determine the true mortality rate as well as other epidemiological markers. Finally, the importance of rapid development of integrated, random-access, point-of-care molecular devices for the accurate diagnosis of SARS-CoV-2 infections cannot be overemphasized. These short-turnaround-time (STAT) tests will be very important for real-time patient management and infection control decisions, especially when other less infectious forms of pneumonia are present and respiratory isolate resources are scarce. These assays are safe, simple, and fast and can be used in local clinics and hospitals that already have the needed instruments and that are responsible for identifying and treating such patients.

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