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COVID-19 LABORATORY DIAGNOSIS: CURRENT PROBLEMS AND DIFFICULTIES

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Abstract:

Over the past few months, the COVID-19 outbreak has had a significant effect on clinical microbiology laboratories. This opinion discusses the difficulties that currently exist for diagnosing infections brought on by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). For a timely and precise molecular diagnosis of COVID-19 during the preanalytical stage, the appropriate respiratory tract samples must be collected at the appropriate time from the appropriate anatomic location. It takes the right precautions to ensure the safety of the laboratory personnel and to yield accurate test results. While antibody-based methods are being introduced as supplementary tools, real-time reverse transcription-PCR (RT-PCR) tests continue to be the preferred molecular test for the etiologic diagnosis of SARS-CoV-2 infection at the analytical stage. Testing results should be carefully analyzed in the postanalytical stage using both molecular and serological findings. Ultimately, rapid and accurate diagnosis and monitoring of SARS-CoV-2 infections will be made possible by random-access, integrated devices that are scalable and accessible at the point of care. These devices will also play a major role in helping to contain the outbreak.

KEYWORDS: molecular testing, serology, specimen type, COVID-19, SARS-CoV-2.

Introduction:

The significance of the laboratory diagnosis of infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is brought into sharp relief by the identification by U.S. public health officials of presumed COVID-19 cases thought to be related to community transmission of this infection (1–5). As of right now, the CDC advises practitioners to coordinate laboratory testing for COVID-19 with their local public health authority and/or the CDC. The real-time reverse transcription-PCR (RT-PCR) test (6–8), which was created for the diagnosis of

SARS-CoV (9, 10), is the recommended testing technique. Cultures of viruses are not advised. Preanalytical difficulties are current concerns for the laboratory diagnosis of COVID-19 that public health authorities, clinical microbiology laboratories, and clinicians need to be aware of. (i) The initial collection of respiratory tract specimens for COVID-19 pneumonia diagnosis and screening. Patients with COVID-19 have shown significant viral loads in their upper and lower respiratory tracts within 5–6 days of the onset of symptoms (11–14). For the purpose of screening or diagnosing an early infection, a nasopharyngeal (NP) or oropharyngeal (OP) swab is frequently advised (9, 12, 15). Because it is safer for the operator and the patient to use, a single NP swab has emerged as the recommended option. Since NP swabs typically reach the appropriate region of the nasal cavity for testing, they have an inbuilt quality control. According to Wang et al.'s recent publication, during the COVID-19 outbreak in China, OP swabs (n = 398) were utilized far more frequently than nasal swabs (n = 8); yet, the SARS-CoV-2

Only 32% of OP swabs had RNA, a much lower percentage than the 63% of nasal swabs that had RNA (16). Under normal circumstances, collecting and testing nasal and OP swabs separately or together in a single aliquot of viral transport medium might be an appealing option. However, institutions also need to take into account the potential stress that this pandemic places on national and international supply chains. To maintain supplies of FF swabs and/or transport medium, this makes restricting testing with NP swabs a great idea. But as our knowledge of the respiratory and oral contact modes of transmission grows, we might discover that the OP route is a suitable way to obtain a sufficient sample of individuals presenting with pharyngitis as their primary symptom.

Testing for COVID-19 Outside of the Nasopharyngeal Swab

Deep insertion of the swab into the nasal cavity is necessary for the appropriate collection of an NP swab specimen. Patients will probably flinch, which indicates that the swab found its target. Swabs should be rotated three times and held in place for ten seconds. Swabs ought to have covered handles made of synthetic nylon and harmless fibers like polyester (17). Theoretically, there could be a risk of spreading SARS-CoV-2 when collecting an NP/OP swab specimen, especially if airborne transmission is shown during the ongoing COVID-19 epidemic study (18). In the event that personal protective equipment (PPE) is unavailable for use, alternate methods for gathering upper respiratory tract specimens will be required (18). A self-collected saliva specimen (19–22) is an alternate method for obtaining an upper respiratory tract specimen to assess individuals with suspected COVID-19 pneumonia.

In the event that swabs become limited, the Food and Drug Administration (FDA) has approved alternative non-focked swabs and transport media under an emergency use authorization (EUA). However, there are currently no head-to-head comparisons available. Swabs should be collected and then quickly sent to the clinical microbiology laboratory using viral (universal) transport medium, preferably in a refrigerated environment (17). However, it should be emphasized that in some instances, saliva, NPs, and OPs may fail to detect early

infection, and that in cases of later infection, the low respiratory tract may now serve as the primary site of reproduction. It could be necessary to do additional testing or collect lower respiratory tract specimens. Furthermore, it's important to screen out other respiratory viral diseases including influenza and respiratory syncytial viruses. The main distinction between clinical and analytical sensitivities—that is, the capacity of a test to determine a patient's overall infection status vs the assay's capacity to identify a pathogen when it is present in a clinical specimen—is largely highlighted by COVID-19.

Naturally, the latter takes into account a number of additional variables, such as the location and technique of specimen collection, as well as the organism burden according to anatomic region, disease severity, and symptom onset time (and the variation of these variables from person to person). If a patient exhibits the clinical signs of viral pneumonia, has a possible history of exposure, and/or has radiographic results (chest CT or MRI scans) consistent with COVID-19 pneumonia, repeat testing may be very crucial. How a single undiscovered result should affect decisions about social separation and patient quarantine is equally difficult to understand, especially when the patients are medical professionals (including clinical laboratory workers). In such cases, serology—which was covered in the postanalytical section—may be useful.

Fecal testing and lower respiratory samples for the diagnosis of late-stage COVID-19 pneumonia:

Monitoring and late identification of patients with severe COVID-19 pneumonia. Lower respiratory tract specimens have produced the largest viral loads for COVID-19 diagnosis, hence sputum collection or bronchoalveolar lavage should be the preferred method (18, 23). A recent study found that, although it did not compare or assess the results from NP swabs, samples from bronchoalveolar lavage (BAL) fluid produced the highest SARS-CoV-2 RNA rate (16). It may be necessary to perform both emergency intubation and respiratory isolation in a negative-pressure environment for patients who arrive with severe pneumonia and acute respiratory distress syndrome. During the intubation process, a lower respiratory tract sputum specimen should ideally be obtained. Alternatively, after intubation, sputum and/or bronchoalveolar lavage fluid specimens might be obtained (9, 11).

However, late in their clinical course, several patients with COVID-19 pneumonia have shown delayed shedding from the respiratory tract (4, 18) and significant viral RNA levels of SARS-CoV-2 in fecal material (24, 25). Patients with severe novel coronavirus infections have previously shown signs of enteric involvement (9, 26–32). SARS coronavirus was identified from stool cultures in four of these trials (26, 28, 31). In a different investigation, electron microscopy was used to show that the SARS coronavirus was present inside enterocytes (30). Therefore, rectal swab and real-time RT-PCR may be the best methods for detecting SARS-

CoV-2 in advanced COVID-19 cases, in addition to direct respiratory sample (9, 26–28, 30-32).

Safety precautions for handling specimens in preparation for PCR analysis and testing. 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, processing of respiratory specimens should be done in a class II biological safety cabinet (6, 9, 10). Before performing real-time RT-PCR, the material should be placed in lysis buffer beneath this BSL-2 cabinet for nucleic acid extraction. An inactivating agent based on guanidinium and a nondenaturing detergent should be present in the lysis buffer. It is true that the buffers found in popular commercial extraction platforms, such Qiagen EZ1 and bioMérieux easyMAG, contain detergents and guanidium, which can render any live coronavirus inactive (33–35).

Options for Point-of-Care Testing and Things to Think About When Diagnosing COVID-19:

when available and compliant with local regulatory standards for SARS-CoV-2 testing, self-enclosed systems that integrate nucleic acid extraction, amplification, and detection, like ID NOW (Abbott, San Diego, CA) (37, 38), cobas Liat (Roche Molecular Systems, Pleasanton, CA), and GeneXpert (Cepheid, Sunnyvale, CA) (39), will be highly helpful. The clinical specimen in viral transport medium is placed inside a sealed cartridge located in a class II biosafety cabinet and sealed after that. Numerous random-access sealed devices can be used for point-of-care testing at nearby clinics and hospitals that lack biosafety cabinets. In this case, the specimen collector could transfer the specimen directly into detection cartridges at the bedside or in a location without a class II biosafety cabinet by donning the appropriate protective gear (splash guard/goggles, mask, gloves, and disposable laboratory coat). The closed cartridge could then be securely placed on an instrument for testing. Nonetheless, it is important to prevent spillage of transport solution during the transfer to these cartridge-based tests, and in the event that they do happen, suitable decontamination should be carried out.

analytical problems. (i) Assay choice. Rapid SARS-CoV-2 antigen or antibody detection is now possible with the development of immunoassays. High-throughput immunoanalyzer versions of these quick point-of-care immunoassays are also being developed for population-level screening, albeit they are typically lateral flow assays. These lateral flow techniques have been developed to detect COVID-19 antibodies (IgM and IgG) or antigens like the SARS-CoV-2 virus.

Based on the experience with this technology for influenza (Flu) viruses, rapid antigen lateral flow tests would potentially offer the advantage of a quick time to result and low-cost detection of SARS-CoV-2, but are expected to suffer from poor sensitivity early in infection (40–44).

Several fast antigen assays as well as monoclonal antibodies targeted specifically against SARS-CoV-2 are being developed (45). Given the fluctuating viral loads in COVID-19 patients, there is worry that low infectious burden or sampling variability could cause antigen detection to miss instances.

Serology is an indirect indicator of infection that is best used in retrospect since it gauges the host's reaction to an infection. Serological techniques are evolving quickly and have shown promise in confirming results beyond COVID-19 (25). In the past, serology played a significant part in understanding the epidemiology of SARS (46) and other coronavirus epidemics (47). Undoubtedly, rapid lateral flow assays for both IgM and IgG antibodies will be crucial in the COVID-19 pandemic and should enable the determination of the total mortality, the fundamental reproduction number, the involvement of asymptomatic infections, and the burden of infection. Apart from diagnosing and confirming late COVID-19 cases or assessing the immunity of healthcare personnel as the outbreak advances, serology detection is unlikely to be involved in active case management due to the notoriously nonspecific nature of IgM responses and the weeks needed to develop specific IgG responses. It is not advised to use cell culture for diagnostics.

Real-Time RT-PCR Assays for SARS-CoV-2 Detection: Going Beyond Deep Sequencing

Choosing an assay for SARS-CoV-2 molecular detection. An important factor in the first identification of SARS-CoV-2 was the use of random-amplification deep-sequencing techniques (48–52). Although they are currently unfeasible for diagnosing COVID-19, deep sequencing molecular techniques like metagenomic next-generation sequencing and next-generation sequencing will remain necessary to identify future mutations of SARS-CoV-2. Real-time RT-PCR tests, especially those developed in the United States, are the mainstay of molecular diagnostics being developed for the diagnosis of COVID-19. Hong Kong University (21, 55), the Charité Institute of Virology in Berlin, Germany (7, 54), and the Centers for Disease Control and Prevention (53). Various alternative molecular techniques are being developed and assessed globally. These include tests based on CRISPR (clustered regularly interspaced short palindromic repeats) and loop-mediated isothermal amplification, as well as multi-plex isothermal amplification followed by microarray detection (56).

choosing a target for real-time RT-PCR experiments. For molecular testing, a real-time RT-PCR technique is advised (6, 8–10). Real-time RT-PCR assays have the significant benefit of simultaneous amplification and analysis in a closed system, which reduces the possibility of false-positive results brought on by contaminated amplification products. Human respiratory and intestinal infections are caused by several coronaviruses (8, 57). SARS-like bat coronaviruses, such as SARS-CoV and SARS-CoV-2, are among these coronaviruses and form a distinct clade under the subgenus Sarbecovirus (57, 58). The positive-sense, single-stranded RNA genome of

coronaviruses has a variety of molecular targets that can be employed in PCR tests (6, 7, 57, 58). Genes encoding envelope glycoproteins spike (S), envelope (E), transmembrane (M), helicase (Hel), and nucleocapsid (N) are among those that encode structural proteins (57–59). Requirements for viral replication include species-specific accessory genes in addition to genes encoding structural proteins.

These comprise open reading frame 1a (ORF1a) and ORF1b, hemagglutinin-esterase (HE), and RNA-dependent RNA polymerase (RdRp) (7, 53–55, 57, 58). Regarding nucleocapsid protein targets, the CDC in the US suggests N1 and N2 (53) and the WHO suggests first-line screening using an E gene assay, then a confirmatory assay utilizing the RdRp gene (7). Three new real-time RT-PCR assays that target the S, N, and RdRp/Hel genes of SARS-CoV-2 have recently been developed and analyzed by Chan et al. The COVID-19-RdRp/Hel assay was the most sensitive and specific of all, with the lowest limit of detection in vitro (59). However, since the viral genes are present in equal copy quantities, assay performance is typically determined by the reagent design rather than the target itself, it is possible that well-optimized targets would originate from a variety of viral genomic regions.
At least two molecular targets should be included in the assay to prevent genetic drift of SARS-CoV-2 and possible cross-reaction with other endemic coronaviruses. Many of these molecular targets have been employed by researchers worldwide for real-time RT-PCR studies. The two-target assay seemed to be working well, thus the CDC in the US chose two loci in the nucleocapsid gene (53). For initial real-time RT-PCR testing, one study used two sequence sections (open reading frame 1b and a nucleocapsid protein) that are highly conserved across sarbecoviruses (6). An other work conducted in Hong Kong, China, employed two targets for their RT-PCR assay: the open reading frame 1b was used for confirmation after the nucleocapsid was used for screening (55). Two molecular targets have been chosen in Germany: RNA-dependent RNA polymerase and envelope (7). When the book was being prepared, a number of molecular devices had gotten urgent approval in China (8). There is currently no proof that any one of the sequence areas being employed provides a special benefit for clinical diagnostic testing. Ideally, the design would incorporate a minimum of one conserved region and one specific region to counteract the impact of genetic drift, particularly as the virus adapts to new populations.
Regulatory concerns have impeded the development and application of laboratory-developed molecular diagnostics for COVID-19 diagnosis in the United States. The FDA released new guidelines on February 29, 2020, allowing laboratories to create and use COVID-19 molecular diagnostic assays without first needing an EUA. After validation, laboratories have 15 business days to submit an EAU to the FDA. Additionally, the specimen types (such as saliva, oropharyngeal, or nasopharyngeal) that will be used in clinical settings must be included in the validation.

Interpreting Results of the COVID-19 Test: Difficulties Beyond Positive/Negative Findings

problems with postanalysis. (i) Analysis of the molecular data. In the US, a case is first deemed to be laboratory confirmed if both of the two targets in the CDC assay (nucleocapsid proteins N1 and N2) test positive (53). A test is considered positive if the cycle threshold (CT) value is less than 40, and negative if the CT value is 40 or higher. If only one of the two nucleocapsid proteins (N1 or N2) has a CT value of less than 40, it is considered indeterminant and needs to be confirmed with retesting (53). Positive results for two or more targets are currently regarded as positive in China for assays with three targets (60). Viral loads ascertained by real-time RT-PCR tests should not currently be utilized to estimate COVID-19 severity or to track therapy response, despite certain connections having been found (11–13, 61, 62). On the other hand, transmissibility may be indicated by low CT values that indicate large viral loads (18, 63).
(ii) The infectivity and cure tests. whether it comes to determining whether to remove patients from isolation and discharge them, monitoring them after their COVID-19 pneumonia has resolved may also be crucial. Discharged patients have a higher risk of spreading the coronavirus to others if they continue to shed the virus (27). As a result, in certain situations, self-quarantine for up to one month has been advised. More research is necessary to determine if NP and OP swabs are enough for the test of infectivity or the test of cure (64). Since SARS-CoV-1 was cultured from stool during the 2002–2003 SARS outbreak (26, 28, 31), and SARS-CoV-2 was cultured from stool during the COVID-19 outbreak (16), one method of testing for cure has been to show two consecutive negative real-time RT-PCR tests from rectal swabs. Therefore, a rectal sample that tests positive for real-time PCR indicates that the patient may continue to be contagious by excreting viable SARS-CoV-2 in their stools (16, 24–28, 30-32). Nevertheless, despite significant virus RNA concentrations, a relatively recent research on 20 consecutive COVID-19 patients revealed that no infectious virus was identified from stool samples (14). It is yet unknown how the recovery of live virus from the same samples correlates with RT-PCR positivity in the feces.

COVID-19 serology. Four structural proteins are present in members of the coronavirus family: the spike (S), membrane (M], envelope (E), and nucleocapsid (N) proteins. It seems that two of these proteins are significant antigenic sites for the creation of COVID-19 detection serological tests. Finding serum antibodies against S proteins from the coronavirus spike has been the main goal of serological techniques (47). The coronavirus envelope spike is in charge of receptor fusion and binding, as well as host identification.
Final thoughts. The ongoing, unparalleled COVID-19 pandemic has brought attention to how crucial it is to diagnose human coronavirus infections in a lab setting in order to stop the infection's spread and treat people who have a serious condition. This discussion has covered the most recent problems with SARS-CoV-2 testing. For early diagnosis or screening, for instance, an NP swab is preferred over an OP swab due to its higher diagnostic yields, better patient tolerance, and operator safety. To boost sensitivity, an NP swab and an OP swab must be used in tandem, although this needs twice as many swabs. Self-collected saliva or nasal washes could be utilized as an alternate specimen type for epidemiological screening and for the "worried well,"

or asymptomatic individuals without a history of exposure who want to be tested to make sure they are not infected, in the event that NP swabs become rare.

Conclusion:

Hospitalized patients would therefore be the only ones to receive NP swabs; if the patient tests negative, deep sputum or BAL fluid samples may need to be taken. It is crucial to comprehend the significance of retesting or using bronchoscopy in individuals with severe sickness in the event that the initial screening test yields negative results. Rectal swabs' potential use in diagnosing patients with late infections or as a test of infectivity or cure is still understudied, but it urgently needs to be addressed. The necessity of extensive screening and testing using molecular and/or serological testing to ascertain the actual death rate and other epidemiological markers is equally underappreciated. Ultimately, it is imperative that integrated, random-access, point-of-care molecular devices are developed quickly in order to provide precise diagnosis of SARS-CoV-2 infections. Real-time patient treatment and infection control decisions will heavily depend on these short-turnaround-time (STAT) diagnostics, particularly in situations where respiratory isolation resources are limited and other less contagious forms of pneumonia are prevalent. These assays are quick, easy, and safe to employ in neighborhood clinics and hospitals that are in charge of identifying and caring for such individuals and already have the necessary equipment.

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