The Laboratory Diagnosis of COVID-19-- Frequently-Asked Questions

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ABSTRACT

Diagnostic testing has played and will continue to play a major role in the COVID-19 pandemic. The ability to detect the SARS-CoV-2 coronavirus in respiratory secretions is essential to determine when an individual is infected and potentially infectious to others. Viral detection is used for the identification, management and isolation of individual patients. Viral detection is also used to determine when the virus has entered a community and how rapidly it is spreading. As communities attempt to re-open following periods of shutdown, the detection of both SARS-CoV-2 and specific antibodies recognizing the virus will become increasingly important as a means to assess infection and immunity in individuals and communities. Here we discuss questions commonly asked by clinicians about COVID-19 diagnostic testing.

"Testing is our way out."

- Paul Romer and Rajiv Shah, *Wall Street Journal,* 7 May 2020

From observations in tens of thousands of patients around the world, we now know that the usual incubation period of COVID-19 is 5 days, and the vast majority of infected patients who develop symptoms will do so within 10-14 days (1, 2). Viral shedding from the respiratory tract usually begins about 3 days prior to the onset of symptoms and declines once a patient becomes symptomatic (figure 1) (3). Detection of specific antibodies typically begins during the first week of symptoms, and many patients seroconvert by the end of the second week after symptoms begin, with nearly all patients becoming seropositive by the end of the third week (4–8). Some patients remain asymptomatic despite viral shedding, and asymptomatic or pre-symptomatic individuals make an important contribution to SARS-CoV-2 transmission (9–11). It is believed that most asymptomatic individuals will seroconvert as well, although more data are needed. The majority of patients with COVID-19 recover uneventfully, but some progress to develop pneumonia with hypoxemia, systemic inflammation and a hypercoagulable state (12). Viral shedding may persist in some patients while resolving in others despite the progression of pulmonary and other organ system involvement. The purpose of this brief review is to help clinicians to understand the appropriate use and limitations of diagnostic testing in COVID-19.

SARS-COV-2 DETECTION BY PCR

The SARS-CoV-2 coronavirus is an enveloped positive-stranded RNA virus whose genome contains around 30,000 nucleotides and 15 genes. Several of these genes have been used as primer/probe targets for diagnostic RT-PCR assays, including the E (envelope), N (nucleocapsid), RdRp (RNA-dependent RNA polymerase), nsp10 (non-structural protein 10) and nsp14 (non-structural protein 14, exoribonuclease) genes. Like other RNA viruses, SARS-CoV-2 is subject to mutation, although the proofreading function of Nsp14 limits the rate of nucleotide misincorporation (13). Sequence variants can result in reduced recognition by the individual primer-probe sets used in PCR-based assays (14).

How accurate are RT-PCR tests? A number of commercial assays as well as laboratory-developed tests are now available to detect SARS-CoV-2 from clinical samples by RT-PCR under emergency use authorizations from the FDA (15). The studies conducted by the test manufacturers of these assays describing their analytical accuracy are updated at the in-vitro diagnostic EUA site (16). In general these assays have high analytical sensitivity with an estimated limit of detection ranging from 100-1000 copies, and very high specificity (17, 18). In other words, these tests are highly accurate, with the exception of the Abbott ID NOW point-of-care assay, which is reported to have lower sensitivity (19). Therefore the choice of a specific testing platform may be primarily made on the basis of factors such as cost, test volume, staffing needs and turnaround time.

What sample types are suitable for testing? The standard sample is a nasopharyngeal (NP) swab, which is obtained by a trained health care provider. Properly obtaining an NP swab sample is uncomfortable for the patient and may provoke coughing, so it is recommended that the health care provider wear appropriate personal protective equipment (PPE) when obtaining an NP swab. Limitations in the availability of PPE and testing supplies, in addition to the operational difficulty of scaling NP swab collections for asymptomatic screening, have led to the evaluation of alternative samples including nasal swabs, mid-turbinate swabs, oropharyngeal swabs, and saliva. Saliva is particularly attractive as it requires neither swabs nor transport media, although collection and processing of saliva presents other challenges (20). The comparability of these specimen types for a qualitative test depends on the viral load present at the time of infection. Oropharyngeal swabs have shown less sensitivity compared to nasopharyngeal and nasal swabs (21). Nasal swabs and nasopharyngeal swabs may be comparable in sensitivity, but more studies are needed to compare these specimens across different patient populations. New specimen types for an EUA test must receive a specific amendment for that specimen type before they can be reported. Some studies have indicated that self-collected samples are comparable in sensitivity to those collected by health care providers, which can obviate the need to use PPE for testing (22–25). Although sputum and bronchoalveolar lavage samples may have higher viral loads and can therefore provide greater test sensitivity than upper respiratory samples (26, 27), particularly at later stages of illness, they entail a higher risk of aerosol generation or require an invasive procedure, so these sample types are obtained more selectively.

Does a negative specimen rule-out COVID-19? No. It is important to recognize that a negative PCR test cannot rule-out COVID-19. Although PCR assays have high analytical sensitivity, clinical sensitivity is not as high because some patients with COVID-19 do not have high levels of virus detectable in the upper respiratory tract. This may be because of suboptimal sampling technique, because a patient is incubating an infection or is already clearing the virus, or because viral replication is predominantly occurring at other sites, such as the lower respiratory tract. In fact, studies have demonstrated that some patients with COVID-19 and characteristic pulmonary infiltrates on CT scan have negative upper respiratory tract PCR tests (28, 29). This may be a consequence of the variable distribution of the ACE2 viral receptor protein in the respiratory tract (30). Interestingly, viral tropism for the lower respiratory tract was also seen during the H1N1 influenza epidemic (31).

Is it worth repeating a test after a patient has tested negative? In view of the less than ideal sensitivity of an NP swab to detect SARS-CoV-2 infection, it may be useful to repeat testing in a patient in whom the clinical suspicion is high (32). In our experience, the yield of repeat testing from the same source is low (33). However, the yield from repeat testing may be substantial in higher prevalence settings (34).

How long will infected patients remain positive? Patients with mild COVID-19 typically stop shedding culturable virus after about one week from symptom onset, but can continue to have detectable viral RNA in their respiratory tracts for longer periods of time (35, 36). More severely ill patients will remain PCR-positive for longer, sometimes extending for weeks-to-months (37). SARS-CoV-2 RNA can also be found in stool samples and typically continues to be detectable in the feces for weeks (38). Evidence indicates that human intestinal epithelial cells can support replication of

the virus (39), and SARS-CoV-2 has been cultured from stool samples (40). Fecal-oral transmission of SARS-CoV-2 has not been demonstrated, but there is concern that fecal shedding might contribute to the spread of infection.

Does a positive specimen mean that a patient is infectious? Not necessarily. Although viral nucleic acids can be detected during convalescence (35), culturable virus is believed to represent a better correlate of infectivity (41). However, SARS-CoV-2 culture requires a BSL-3 facility and there are no authorized clinical assays utilizing SARS-CoV-2 viral culture at this time, so clinical monitoring is dependent on RNA detection.

What does it mean when a patient with COVID-19 becomes PCR-positive after initially becoming negative? Two negative PCR assays at least 24 hours apart are commonly used as a criterion to discontinue isolation (42). However, a number of patients will revert to PCR-positivity after two negative samples (43). This may reflect fluctuations in the quantity of viral RNA shedding during recovery. When monitoring for viral load as inferred by real-time PCR cycle threshold (Ct), cases that have reverted to positivity consistently exhibit high Ct values indicative of a low viral load (44). There is presently little evidence of true virological and clinical relapse, and the prognosis for these patients appears to be good.

Is quantitative PCR (viral load) useful? There is some correlation between illness severity and viral load on presentation, as inferred by the real-time PCR cycle threshold (Ct) (37, 45). However, an isolated viral load estimate is of limited prognostic value, and even asymptomatic individuals may have high viral loads (46). Viral load trends may have greater value and might help to inform decisions to initiate a trial of antiviral therapy, but viral loads typically decline regardless of the clinical course (3, 6, 47).

Does age affect viral load? Older patients tend to have higher viral loads (6), just as they are at greater risk for more severe or critical illness (48, 49).

How should indeterminate or inconclusive results be interpreted? Inconclusive results generally imply that only one of two PCR targets was detected, and confirmation with a different assay that detects alternative targets is recommended. Given the high specificity of the PCR assays, most inconclusive results will ultimately be confirmed as positive. Inconclusive or Indeterminate results could also indicate that the internal controls failed and may indicate a technical issue, such as the presence of a PCR-inhibitory substance in the sample. Different labs and tests may use different terminology, so it is worth contacting a given clinical laboratory to determine how they report low positive results.

SEROLOGY

Coronaviruses contain four structural proteins: the immunodominant receptor-binding spike (S) protein, the nucleocapsid (N) protein, the envelope (E) protein and the membrane (M) protein. Diagnostic platforms used for the detection of specific antibodies to SARS-CoV-2 proteins include rapid diagnostic tests (RDT) such as lateral flow assays (LFA), enzyme-linked immunosorbent assays (ELISA), neutralization assays and chemiluminescent immunoassays (50). Only neutralization assays can provide information regarding the ability of antibodies to inhibit viral growth.

How accurate are serologic tests? The performance of various serologic tests is more variable than the RT-PCR assays for SARS-CoV-2 (8, 51–53). This is particularly important because the positive or negative predictive value of a test is dependent not only on the intrinsic test accuracy but also on the prevalence of disease in the population. An insensitive test will have a poor ability to exclude the presence of disease when prevalence is high, but more germane to COVID-19, a test with low specificity will have a poor ability to indicate the presence of disease when the prevalence is low. When only a few percent of the population have immunity to SARS-CoV-2, as is presently the case in most regions, a positive result from a serologic test with low specificity will be more likely to represent a false-positive.

Do serologic tests for SARS-CoV-2 cross-react with other coronaviruses? There are four kinds of human coronavirus that cause mild-to-moderate seasonal respiratory tract infections: 229E, NL63, OC43 and HKU1. Cross-reactivity with antibodies to seasonal coronaviruses is a theoretical concern for a SARS-CoV-2 serologic test, but for most of the commercial assays evaluated thus far, this does not seem to be the case (54, 55). To rule-out cross reactivity, a new assay should be tested against a panel of serum samples that pre-date the emergence of SARS-CoV-2, ideally more than 500, to

accurately ascertain specificity. Cross-reactivity between SARS-CoV-1 or MERS-CoV and SARS-CoV-2 is more likely (56), but should be a limited concern.

Are serologic tests useful to diagnose acute COVID-19? Although the primary use of serologic tests is to determine prior exposure to SARS-CoV-2, the detection of specific antibodies may support the diagnosis of COVID-19 in a patient with a high clinical suspicion but negative PCR tests (57–59).

How soon do antibodies develop? IgM and IgG directed against SARS-CoV-2 may appear as early as 3-6 days after the onset of symptoms. By three weeks, nearly all patients have seroconverted, and the antibodies persist for at least two months, with IgG showing greater persistence (4–8, 59).

How long do antibody responses last? The duration of antibody responses to SARS-CoV2 is unknown. Antibody responses to the common respiratory coronaviruses decay after a few years (56), and it is suspected that immunity to SARS-CoV-2 will be similar.

Does a negative specimen mean a patient has not had COVID-19 or lacks immunity to SARS-CoV-2? Antibody responses have been observed in nearly all patients with COVID-19, although it is possible that some very mild or asymptomatic infections, or infections in immunocompromised patients, may not result in seroconversion (5, 60).

Does a positive antibody test mean that a patient is immune? It is likely that the detection of specific antibodies in a patient with a history of COVID-19-like illness will be indicative of at least some degree of immunity (61). Experimental animals re-challenged with pathogenic coronaviruses exhibit resistance to re-infection (62). However, a quantitative cutoff of antibody titer that correlates with protective immunity is undefined. As with other viruses, it is possible that a low titer of antibodies is not protective. Also, it is not presently known whether neutralizing antibodies are the primary mechanism of immune protection. In fact, higher antibody titers are observed in patients with more severe illness (5, 6, 51). As patients with mild COVID-19 may recover despite low antibody titers, and patients with severe COVID-19 have persistent illness despite the development of high antibody titers, one may question whether neutralizing antibodies are in fact protective. The reported therapeutic benefits of convalescent plasma might be due to constituents other than neutralizing antibodies (63). Moreover, the development of neutralizing antibodies is accompanied by T cell responses (64, 65), which may contribute to protection.

Are quantitative serologies helpful? Because SARS-CoV-2 is a new human pathogen, pre-existing adaptive immunity is non-existent, so acute and convalescent titers are not required to establish the diagnosis of COVID-19. Until specific cutoffs are identified as a correlate of protective immunity, qualitative serologic results are sufficient to provide clinical guidance. However, reporting of quantitative serological read-outs could offer clinicians more information about the potential for false positives and false negatives for values near the positivity threshold.

Is there a correlation between age and antibody titer? Older age correlates with a higher likelihood of severe illness from COVID-19 and with the development of higher antibody titers (59), perhaps due to a higher antigen load.

OTHER DIAGNOSTIC TESTS

Is point-of-care testing available? Affordable point-of-care (POC) diagnostics for SARS-CoV-2 could facilitate the widespread testing and contact tracing strategies proposed for post-pandemic wave containment (66). However, performance characteristics and usability are critical parameters for these tests, as they are typically deployed without the quality assurance apparatus of a high complexity laboratory. A POC antigen detection assay was recently authorized by the FDA, but is known to be less sensitive than PCR, so its clinical role has yet to be defined. Negative results using this assay should be confirmed by a more sensitive method in most instances. Other assay technologies, including a CRISPR-based nucleic acid detection system (67), may be utilized in POC formats in the future if appropriate sensitivity can be achieved.

Are biomarkers useful? A variety of biomarkers, including lymphocyte count, neutrophil-to-lymphocyte ratio, CRP, troponin T, D-dimer, LDH, procalcitonin, IL-6 and ferritin are predictive of disease progression and mortality in COVID-19 (table 1) (68, 69). These laboratory tests play a vital role in identifying patients at risk for complications and to guide treatment interventions.

CONCLUSIONS

Used appropriately, laboratory tests to detect SARS-CoV-2 and specific antibody responses to SARS-CoV-2 can be invaluable in guiding both patient care and public health decisions. However, these tests have their limitations and should always be interpreted in concert with epidemiological and clinical information.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the extraordinary and dedicated staff of the clinical laboratories at the University of Washington.

Financial support. This work was supported by departmental funds from the Department of Laboratory Medicine, University of Washington School of Medicine, Seattle.

Potential conflicts of interest. F.C.F. has received grants, personal fees, and nonfinancial support from BioFire and Cepheid; personal fees from the Infectious Diseases Society of America; non-financial support from Luminex; and grants and nonfinancial support from EliTech, outside the submitted work. A.L.G. has received personal fees from Abbott Molecular, outside the submitted work. S.N. is an employee of LabCorp.

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Figure 1. Typical Course of Virus, Antibody and Biomarker Detection in Patients with Mild or Severe COVID-19. Adapted from (70).

Table 1. Laboratory Biomarkers of COVID-19 Progression. Worsening of biomarkers is predictive of disease progression and can inform decisions to initiate antiviral, antiinflammatory, anticoagulant or supportive treatment interventions. Adapted from (68, 69).

HEMATOLOGIC	**⇩ Lymphocyte count** **⇧ Neutrophil count** **⇩ Platelet count**
BIOCHEMICAL	**⇩ Albumin** **⇧ Creatinine** **⇧ Lactate dehydrogenase** **⇧ Cardiac troponin** **⇧ B-type natriuretic peptide** **⇩ O_2 saturation**
INFLAMMATORY	**⇧ CRP** **⇧ Ferritin** **⇧ Procalcitonin** **⇧ IL-6**
COAGULATION	**⇧ D-Dimer**

Figure 1