OPEN

Review of Viral Testing (Polymerase Chain Reaction) and Antibody/Serology Testing for Severe Acute Respiratory Syndrome-Coronavirus-2 for the Intensivist

Michael P. Motley, BS[1]; Elliott Bennett-Guerrero, MD[2]; Bettina C. Fries, MD[3]; Eric D. Spitzer, MD, PhD[4]

Objective: As the severe acute respiratory syndrome-coronavirus-2 pandemic develops, assays to detect the virus and infection caused by it are needed for diagnosis and management. To describe to clinicians how each assay is performed, what each assay detects, and the benefits and limitations of each assay.

Data Sources: Published literature and internet.

Study Selection: As well done, relevant and recent as possible.

Data Extraction: Sources were read to extract data from them.

Data Synthesis: Was synthesized by all coauthors.

Conclusions: Available assays test for current or previous severe acute respiratory syndrome-coronavirus-2 infection. Nucleic acid assays such as quantitative, or real-time, polymerase chain reaction and loop-mediated isothermal amplification are ideal for acute diagnosis with polymerase chain reaction testing remaining the "gold standard" to diagnose acute infection by severe acute respiratory syndrome-coronavirus-2, specifically the presence of viral RNA. Assays that detect serum antibodies can theoretically diagnose both acute and remote infection but require time for the patient to develop immunity and may detect nonspecific antibodies. Antibody assays that quantitatively measure neutralizing antibodies are needed to test efficacy of convalescent plasma therapy but are more specialized.

[1]Departments of Medicine, Infectious Disease Division, Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY.

[2]Department of Anesthesiology, Stony Brook University, Stony Brook, NY.

[3]Division of Infectious Diseases, Department of Veterans Affairs, Northport VA Medical Center, Northport, NY.

[4]Department of Pathology, Stony Brook University, Stony Brook, NY.

Crit Care Expl 2020; 2:e0154

DOI: 10.1097/CCE.0000000000000154

Key Words: molecular diagnostic techniques; neutralizing antibodies; real-time polymerase chain reaction; serologic tests; severe acute respiratory syndrome-coronavirus-2

The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (coronavirus disease 2019 [COVID-19]) pandemic has led to the rapid development of many diagnostic tests. The initial focus of these tests was on molecular tests that detect viral RNA. These are relatively straightforward in their design and interpretation since the amplification of unique nucleic acid sequences provides intrinsic sensitivity and specificity. Due to heavy demand for molecular tests and limited availability of reagents and test kits, molecular testing has focused on symptomatic patients in a variety of healthcare facilities (e.g., hospitals, clinics, and nursing homes). Serological assays currently undergoing development and deployment can complement molecular testing, providing information not only about acute infection but also recovery from infection and prevalence of infection in the community. Nevertheless, these assays can be difficult to interpret because of potential cross-reactions with other coronaviruses as well as the variable magnitude and timing of the immune response to SARS-CoV-2. In addition, rapid immunoassays and routine enzyme-linked immunosorbent assay (ELISA) assays do not necessarily detect neutralizing antibodies that correlate with protective immunity.

SARS-COV-2 BACKGROUND

SARS-CoV-2 is a member of the coronavirus family, which consists of enveloped, single-stranded, positive-sense RNA viruses. Seven viruses in this family infect humans, and these fall into two genera: Alphacoronavirus and Betacoronavirus (1). SARS-CoV-2 belongs to the Sarbecovirus subgenus of the Betacoronavirus genus, that also includes a number of bat-specific SARS-related (SARSr) coronaviruses that share 96-78% of its genetic identity, as well as

SARS-CoV (responsible for the 2002/2003 China outbreak) which shares 80% identity (1, 2). In contrast, MERS-CoV, (responsible for the 2012 Middle-East Outbreak and 2015 Korean Outbreak), and human coronaviruses HKU1 and OC43 (which commonly cause milder upper respiratory illness) belong to other Betacoronavirus subgenera (Merbecovirus and Embecovirus, respectively) and are less related to SARS-CoV-2. The remaining two human coronaviruses, NL63 and 229E, are Alphacoronaviruses that are more distantly related to SARS-CoV-2, though like HKU1 and OC43, they are widely present in the community and cause cold-like illnesses (1–3).

SARS-CoV-2, like SARS-CoV, primarily infects pneumocytes within the host's lungs. This tropism is due to the virus' ability to bind angiotensin-converting enzyme 2 (ACE2) receptors using the transmembrane Spike (S) protein (**Fig. 1*A***). The S protein, found on the surface of the virus particle (virion), is one of four structural proteins (spike, nucleocapsid, membrane, and envelope) found in all coronaviruses and is responsible for both the binding of the host receptor and the fusion of the virion with the host membrane. Structurally, the S protein exists on the virion surface as a homotrimer comprised of three identical polypeptide chains. Each chain contains two subunits, S1 and S2. Subunit S1 makes up the majority of the S protein surface area, and also includes the receptor binding domain (RBD), a small stretch of amino acids that allow SARS-CoV-2 to bind to the ACE2 receptor (2). The RBD is the least genetically conserved portion of the S1 subunit, sharing only 73% similarity with SARS-CoV, and 21%–25% similarity to other human coronavirus S1 subunits (2, 4, 5). These genetic differences at the RBD dictate the virus's receptor specificity. Nearly all human coronaviruses other than SARS-CoV-2 and SARS-CoV bind glycoproteins or proteases other than ACE2 (although the distant relative NL63, appears to have independently evolved ACE2 specificity) (3, 6).

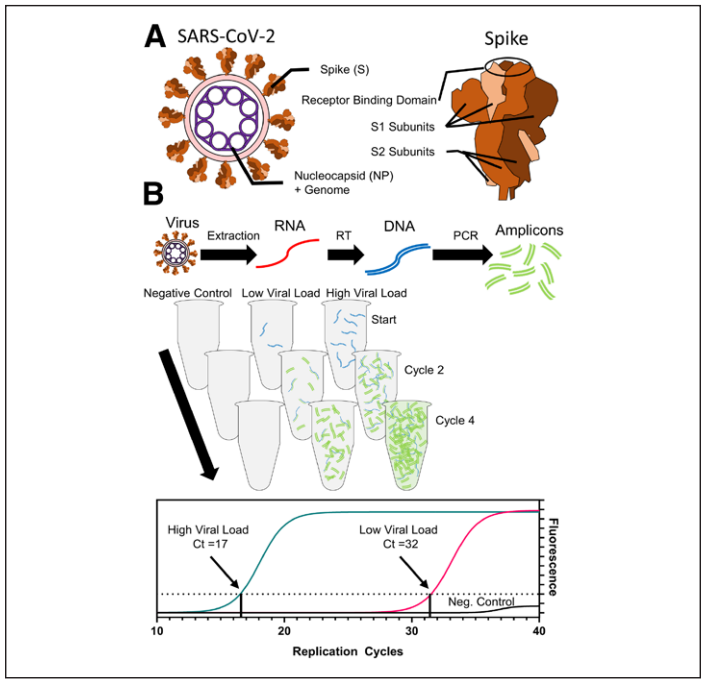

Figure 1. Severe acute respiratory syndrome-coronavirus-2 (SARS-Cov-2) structure and quantitative, or real-time, polymerase chain reaction (qPCR) overview. **A**, Rough structure of the SARS-CoV-2 virion (*Left*) and spike protein (*Right*), with relevant structural proteins and subunits labeled. **B**, Outline of qPCR. (*Top*) The general workflow of qPCR, from isolation of the virion, extraction of the RNA (*red*), reverse-transcription into DNA (*blue*), and amplification of DNA regions into amplicons (*green*). (*Middle*) Simulation of a qPCR reaction with a negative control, low viral sample, and high viral sample, demonstrating that as cycle number increases, differences between viral copy numbers are exaggerated exponentially. (*Bottom*) Example of a plotted qPCR graph, with the high viral load sample (teal) reaching the C_t threshold at replication cycle 17 ($C_t = 17$), the low viral load (*pink*) reaching C_t at cycle 32 ($C_t = 32$), and the control sample (black) failing to reach threshold.

The S2 subunit tethers the S protein to the virion membrane and includes the machinery required for virus cell fusion (5). As these features are located deep within the S protein core, S2 is more conserved than S1 (90% similarity with SARS-CoV S2, and 35%–43% similarity with the other coronavirus S2s).

Due to its location on the surface of the virus and its physiologic importance, the coronavirus S protein is predictably immunogenic. Previous studies of SARS-CoV serum responses demonstrate that convalescent sera contain high titers of antibodies that bind to the S protein (7). Some of these antibodies were also shown to protect cells from infection by live SARS-CoV in neutralization assays (explained below) (8). New studies focused on the current pandemic have also demonstrated that sera of previously infected SARS-CoV-2 patients react to SARS-CoV-2 S protein, with some sera also cross-reacting with the SARS-CoV S protein (5, 9).

Serum from patients infected with coronaviruses also exhibit high reactivity against another structural protein, the Nucleocapsid (N protein or NP) (5, 7, 8). N protein, which exhibits high homology between SARS-CoV and SARS-CoV-2 (90%) binds and stabilizes the viral genome (5). While abundant, it is found exclusively within the virion. Nevertheless, anti-N antibodies are highly prevalent postinfection, possibly generated by the presentation of digested virus protein by macrophages and other antigen-presenting cells to B cells (10). These antibodies may not provide direct protection from infection, but N protein is historically easier to produce than S-protein for use in assays. Anti-N antibodies can be used to detect prior infection, as their titers correlate with antibody titers to S-protein much like antibodies against Hepatitis-B Virus (HBV) core antigen correlate with those against HBV surface antigen (5). Furthermore, test vaccines against SARS-CoV have shown N antigens to be potent inducers of CD8 cytotoxic T cells that can recognize and kill infected cells that present NP complexed to MHCI, and antibodies to N may correlate with the development of such a response (11).

Despite also being present on the surface, the remaining structural proteins, E and M, are small and poorly exposed; natural serum responses show few antibodies to these antigens (7).

DIAGNOSES OF SARS-COV-2 BY QPCR

The SARS-CoV-2 virus was first characterized by the sequencing of its viral genome, providing the requisite information to develop quantitative, or real-time, polymerase chain reaction (qPCR) tests for the virus (1, 2, 12). Using cDNA reverse-transcribed from viral RNA extracted from the virus, qPCR, like PCR, replicates a specific region, or amplicon, of the viral genome (**Fig. 1B**). Through the use of fluorescent probes and detection steps between replication

cycles, qPCR allows quantitation of the amount of viral RNA (viral load) in a sample. As DNA is synthesized exponentially during PCR, the fluorescence also increases exponentially. The thermocycler instrument reports a Ct (cycle threshold), which is the *number of replication cycles* that are required to produce a fluorescent signal that exceeds a baseline. Samples that contain a large starting amount of viral RNA require fewer cycles to produce a detectable fluorescent signal (and therefore have a lower Ct). The Ct has a simple negative linear correlation with the logarithm of the number of viral copies in the original sample. This relationship can be used to quantify the amount of viral RNA in a specimen; however, the assays must then include additional standards containing known concentrations of viral RNA. Another type of diagnostic test that uses primer-directed replication, loop-mediated isothermal amplification (LAMP) provides point-of-care qualitative results, but the lack of discrete replication cycles does not allow accurate quantitation, and these assays typically have a higher limit of detection than qPCR (13, 14).

Currently (as of May 16, 2020), the FDA has granted Emergency Use Authorization (EUA) for SARS-CoV-2 molecular tests to 61 test kit manufacturers and commercial laboratories and 28 laboratory-developed tests authorized for use by the singular developing laboratory. While the initial EUA molecular assays, such as the one from Centers for Disease Control and Prevention , used manual protocols, many of the subsequent commercial assays tend to use individual cartridges that contain all of the reaction components (e.g., Cepheid Xpert) or are performed on large robotic platforms that can process more than a 1,000 specimens per day (e.g., Roche 6800/8800). Cartridge-based assays offer rapid turnaround time (within 60 min), require less space, and are simpler to deploy, with the trade-off of greater cost per test and lower throughput. Instruments that use cartridge-type molecular tests, such as the Cepheid Xpert, Biofire Film Array, and Genmark ePlex, are available in many smaller hospitals and can also be used for "syndromic" panels to detect a range of respiratory and gastrointestinal pathogens. Larger health systems may use multiple types of analyzers to meet their particular needs. They may use rapid low-throughput assays for the testing of urgent or emergent admissions, as well as high-throughput instruments for less time-critical and more cost-efficient testing for inpatient units and outpatient settings where 6-24-hour turnaround times are more acceptable.

False-positive results for molecular tests are uncommon because of intrinsic designs and rigorous quality control guidelines. The fidelity of base pairing makes qPCR and other amplification techniques highly specific, distinguishing SAR-CoV-2 from other coronaviruses. Additionally, the EUA application process requires that a SARS-CoV-2 molecular test not cross-react with 20 commonly encountered respiratory pathogens and additionally have greater than 95% negative percent agreement (analogous to specificity when there is no established reference method) with at least 30 specimens that tested negative using another EUA test. In a recent study comparing four SARS-CoV-2 PCR assays (with a range of sensitivities), the negative percent agreement ranged from 96% to 100% (15). Nevertheless, improper sample handling and contamination of reagents have the ability to create such false positives.

The clinical sensitivity of SARS-CoV-2 PCR tests (the ability to detect infection) using nasopharyngeal or other upper respiratory specimens is not well-defined since a positive PCR test is the gold standard for diagnosis in most published studies. While the analytical sensitivity or limit of detection (LOD) for a EUA molecular test under ideal conditions can vary between 50 and 1000 viral copies/mL (15), the clinical sensitivity depends also on factors related to collection of sample, such as specimen type SARS-CoV-2 presence in the upper airways is less than that of the lower airways, and nasopharyngeal swabs, nasal swabs, and oropharyngeal swabs differ greatly in their ability to detect virus (16–19). One report found that 73% of 353 of patients who tested positive by nasopharyngeal swab tested negative by simultaneous oropharyngeal swab (16), and until recently nasopharyngeal swab has been the preferred specimen to collect. Sputum samples and pulmonary lavage have been shown to have higher sensitivity than both nasal and oropharyngeal swabs, though the number of such samples tested has been small (19–21). These lower respiratory specimens may be useful for detecting SARS-CoV-2 in severely ill patients. However, this type of testing is generally limited, at present, to large academic institutions and reference laboratories that have carried out the necessary validation experiments that must be performed before routinely testing these specimen types. Furthermore, collection of these specimens can introduce additional risk to clinicians. There is increasing evidence that molecular tests run on saliva may be also be a suitable and more easily obtained specimen (22)

Timing of specimen collection is also crucial to clinical sensitivity. Early in the course of infection, viral loads are high, typically in the range of 10,000 to several million copies/mL in upper respiratory specimens, resulting in low Ct readouts. This is the case of both clinical disease and asymptomatic infection, as symptoms of COVID-19 tend to result from the immune response to the infection. Over a few days, these high viral loads can decrease by a 1,000-fold or more (23). Drops in viral load during late acute illness may be inconsequential for more sensitive assays under ideal collection conditions, but may be missed if the collection method recovers too little RNA, or the test cannot detect enough. This is especially true for tests using nasal or oropharyngeal swabs, which can see significant drops in sensitivity after a week of symptoms (24, 25) Large studies comparing clinical sensitivity of diagnostic tests to well-defined clinical and laboratory criteria defining COVID-19 disease are needed.

Another diagnostic challenge is the interpretation of persistently positive PCR tests in convalescent patients. It is not uncommon to have a positive PCR test (often with a low viral load) 4–6 weeks after the resolution of symptoms (24, 26). PCR and related tests only detect genetic material, including remnants of dead virus, and do not necessarily indicate active infection by replicating virus. Indeed, a small study using viral culture suggests that patients with protracted qPCR positivity may not be infectious (27).

SEROLOGY TESTING FOR DIAGNOSIS

While qPCR of nasopharyngeal swabs remains the gold standard of testing acute cases, laboratory tests that detect antiviral

antibodies (often referred to as serological tests) can be an important adjunct to molecular assays amidst the current pandemic. Serological assays may detect the later stages of infection, when they may be less detectable by some PCR tests (24, 25). Two studies showed that antibody testing can augment qPCR in improving overall sensitivity after day 7 of symptoms (24, 25), "although maximal sensitivity is not achieved until 14 days after symptom onset" (28). Immunoglobulin (Ig) M titers against SARS-CoV-2 may be present in as short as a few days from symptom onset, and IgG titers may exist for weeks or longer (24). Some serological assays can also be performed with a minimum amount of blood derived from a simple finger prick. Finally, serological assays can identify patients who have already recovered from disease. If current reports hold true that cases of re-infection are few, such tests could potentially allow preferential deployment of previously exposed healthcare workers to operate in high risk settings, though the correlation between antibodies and protection remains controversial (see below). Convalescent patients also hold the potential to be donors for passive immunization, or serum-transfer therapies, and such assays can be valuable screening tools for this purpose (29) However, care should be taken when using serological assays in immunocompromised patients, as some may not mount a humoral immune response capable of generating antibodies.

The standard type of assay for detecting antiviral antibodies in serum is an indirect ELISA (**Fig. 2*A***) (7, 30) which involves initially adding sera or dilutions of sera to wells of a plastic plate containing prebound viral antigens. These antigens bind only the antigen-specific antibodies found within the serum and retain them when the plate is washed. The antigen-bound antibody is later detected with an enzyme-linked secondary antibody, which binds to general host antibody, or to host antibody of a specific isotype (e.g., IgG, IgM). The plate is then developed with a colorimetric substrate, which the enzyme on the secondary antibody cleaves to cause a color accumulation proportional to the amount of antibody bound. During early ELISA development, many dilutions of the same serum are used to examine the gradient of color intensity (optical density, OD), and arbitrary OD cutoffs are used to determine the titer, or minimum dilution factor, needed for the OD to "exceed" background signal. However, as these assays become perfected for diagnostic use, they must be calibrated to provide accurate "positive" and "negative" results. ELISA-type assays can also be adapted to run on the surface of paramagnetic beads rather than plastic plates. Such technologies are suitable for processing in automated high throughput commercial instruments that also use sensitive chemiluminescent detection systems. Additionally, as monoclonal antibodies specific to SARS-Cov-2 are developed, the format of the ELISA can be flipped to allow direct detection of antigens from nasopharyngeal specimens; this has recently been accomplished by Quidel, which has received EUA of its antigen sandwich ELISA test (https://www.fda.gov/media/137885/download) with a clinical sensitivity of 80% relative to qPCR. The specifics of antigen tests are not covered in this review.

Many commercial tests have adapted the principles of the ELISA into rapid and simple immunochromatographic assays, such as lateral-flow assays (LFA) (31). These assays can be single or multistep,

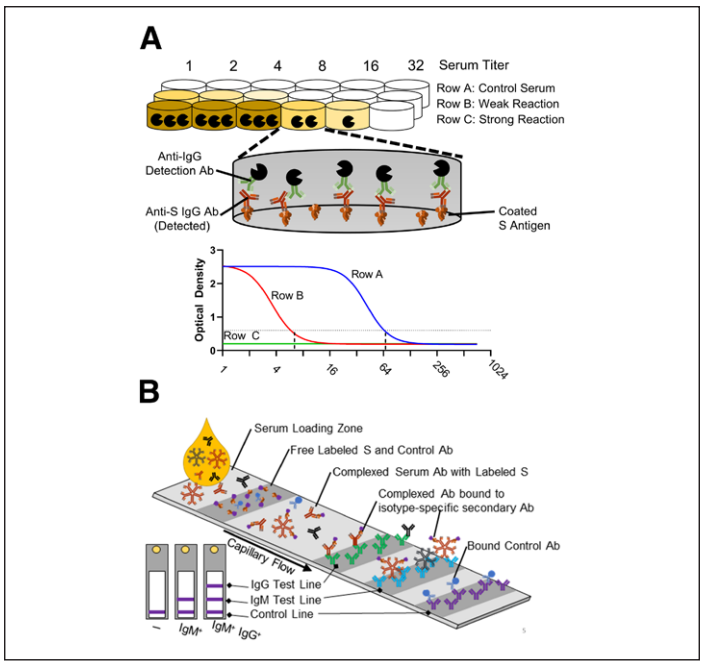

Figure 2. Examples of enzyme-linked immunosorbent assay (ELISA) and lateral flow assays (LFAs) for serum antibody detection against spike protein. **A**, An indirect ELISA detecting immunoglobulin (Ig) G antibodies (Ab) against anti-severe acute respiratory syndrome-coronavirus-2 (SARS-Cov-2) S protein (*red*). (*Top*) Rows of wells coated with SARS-Cov-2 S protein (the antigen) are filled with various dilutions of serum containing anti-S antibodies (*red*). After washing, the wells are incubated with IgG-specific detection antibody (*green*) that is linked to an enzyme (*black*), which bind anti-S antibodies, forming a "sandwich". Finally, a developing solution is added, and wells with reactive IgG antibodies accumulate color (*gold*). Using a different detection antibody, this assay can also be used to detect IgM antibodies (not pictured). (*Bottom*) The intensity in color after the final step of ELISA is read and plotted in a semilog graph. Strongly reactive sera (*Row A*) demonstrate maximum signal at lower titers, while weakly reactive sera (*Row B*) show less signal with fewer dilutions. The dotted lines indicate the test titer value according to an arbitrary threshold above the background signal (*Row C*). **B**, A simple single-channel LFA, such as that developed by Cellex, that uses antigen as the detection molecule. Serum is added to the left Loading Zone, and all antibodies in the serum, either reactive to the S antigen (*red*) or not (*black*), move right to left by capillary action. Reactive Abs pick up detector-labeled (*purple/red*) S protein (which forms top of the "sandwich") as they travel to the test lines. IgG antibodies bind to the IgG test-line, which contains immobilized anti-human-IgG antibodies (*green*). IgM antibodies bind to the IgM test line, which contains immobilized anti-IgM antibodies (*blue*). When anti-S antibodies complexed with detector-labeled S-protein bind to the correct appropriate test line, the "sandwich" is completed, and the detector causes a color change at that line. Control detection antibodies that join the serum antibodies during loading travel with the serum and bind species-specific secondary antibodies at the far end of the strip, causing a color change that indicates the assay is finished and results can be read. Other assays examining one isotype per channel may swap the top and bottom components of the "sandwich", such that the detector is bound to free isotype-specific antibodies, while the antigen is immobilized at the test lines.

but all involve added serum and reactants traveling across a linear membrane by capillary action, traveling through different detection regions. This eliminates the need for separate washing steps and permits multiplexing to allow detection of more than one antibody isotype in a single test. In one type of single-step LFA (**Fig. 2B**) (32), serum antibodies first flow through an area where those specific to the virus pickup free viral antigen that are conjugated to a detection molecule, such as colloidal gold. These coupled antibodies, as well as the nonreactive ones, then pass over an area

with discrete bands of immobilized secondary antibodies that capture sera antibodies of specific isotypes. Presence of the detection molecule at a particular band, which causes a color change, indicates that sera antibodies of that particular isotype also successfully bind antigen (**Fig. 3**). Alternatively, LFAs with multiple channels may contain viral antigen immobilized on a surface, which then captures antiviral antibodies complexed to a labeled conjugate antibody. While most immunochromatographic assays provide only qualitative results, some of them incorporate additional components to allow for increased sensitivity, specificity, or semiquantitative estimates of antibody levels. Immunochromatographic assays are ideal for point of care assays because they are easy to use and commonly do not require additional large equipment, though they may suffer from decreased performance compared to enzyme-linked immunosorbent assay (ELISA) (discussed below).

When designing any binding assay, selection of which antigen to test is crucial. This selection should consider a variety of factors, including antigen availability, specificity, and concentration. As mentioned above, antigens such as M and E proteins that are small or less-exposed may prove poor antigens to test immunity for SARS-CoV-2. Next, testing with an antigen with conserved epitopes may cause cross-reactivity, where antibodies specific to a previous infection, such as SARS-CoV, test positive on a test for SARS-CoV-2 (5, 24). Such cross-reactivity may not matter in the case of the current pandemic as SARS-CoV is not prevalent amongst most patients. Nevertheless, if the protein or subunit used as the antigen is so conserved that it shares similarity with distant but more endemic viruses such as HKU or OC43, antibodies against these viruses could theoretically cause false positive results and limit the specificity of the assay (31, 33) Such concerns have led some assays to use specific subunits, such as S1 or the RBD itself as the antigen, as these are most specific and might limit cross-reactivity. Regardless of the antigen used, extensive validation, with proper controls, is always required to ensure the functional use of any assay.

Once an assay is developed, it must then be tested and calibrated in the clinical setting. Determining a diagnostic cutoff can be challenging because over time, humans are exposed to numerous infectious agents that may exhibit varying degrees of antigenic similarity. Many commercial infectious disease serological assays use a "reactive" control that is validated by comparing individuals known to have infection with an uninfected control group. This requires use of a reference method to distinguish the two populations. In a quantitative assay, the OD or signal of the patient's serum is compared with the signal of the "reactive" control to generate an index; index values greater than 1 are then interpreted as "reactive" (or "positive"). Because of the novelty of SARS-CoV-2, and the lack of an official gold standard to reference these tests, diagnostic cutoffs for these assays are likely to evolve. However, over time as these assays are used and compared with clinical progression, more accurate cutoffs will eventually be calibrated.

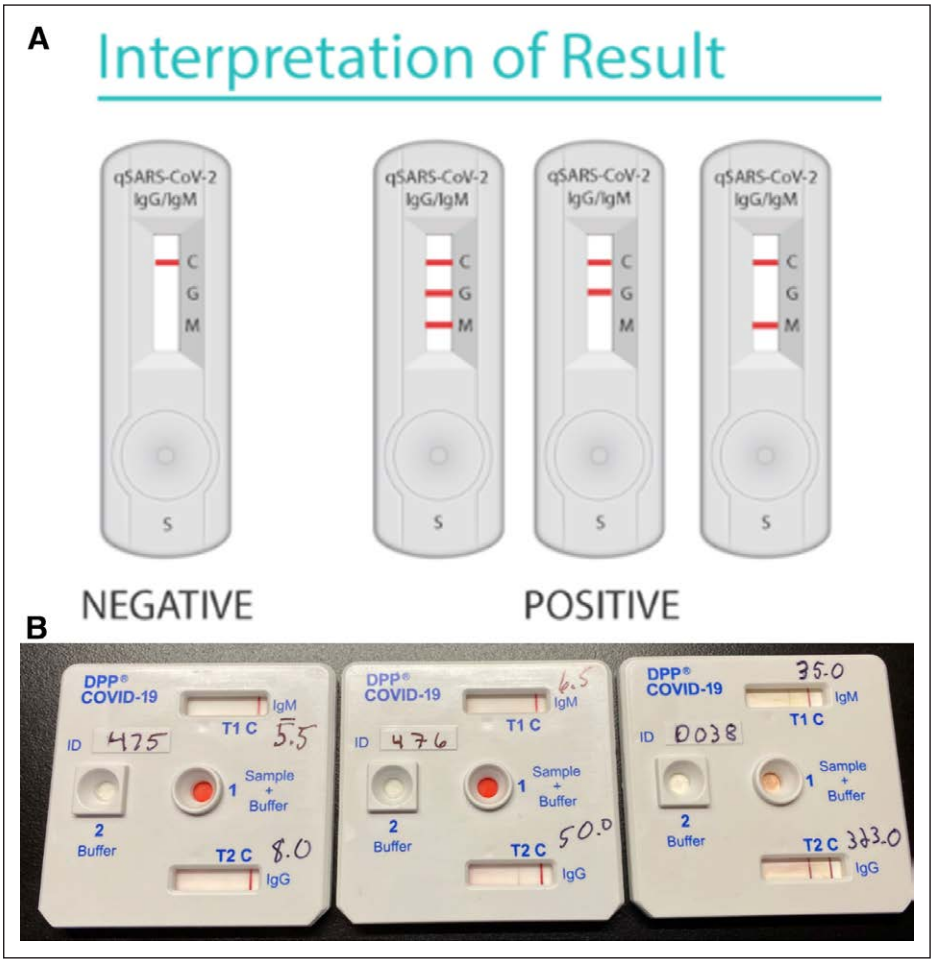

Figure 3. Images of lateral flow assays (LFA, *Top*) and rapid immunochromatographic test (*below*). (**A**) A single channel LFA, as used by Cellex. (**B**) A multichannel immunochromatographic test produced by ChemBio. Plates for three separate individuals are shown. The test on the left shows only control bands for immunoglobulin (Ig) M (*top*) and IgG (*bottom*), that is, a "nonreactive" or negative test for antibodies. The test in the middle shows a weak band for IgG to the left of the control band. The test on the right shows a weak band for IgM (*top*) and a strong band for IgG (*bottom*). COVID-19 = coronavirus disease 2019.

As of May 16, 2020, the FDA has granted Emergency Use Authorization for 12 tests that detect antibodies (IgM/IgG or IgG only) against SARS-CoV-2. This group includes 10 kit manufacturers or reference laboratories, one academic laboratory, and a state health department. In addition, nearly 200 manufacturers had notified the FDA that they have validated and are offering serology tests as set forth in Section IV.D of the FDA's "Emergency Policy for Diagnostic Tests for COVID-2019" (https://www.fda.gov/medical-devices/emergency-situations-medical-devices/faqs-diagnostic-testing-sars-cov-2). Only 6 of these assays have been listed as "FDA Authorized"; the others (many of which are rapid-type assays) have been listed as "Not FDA Authorized," pending submission of validation data to the FDA for an EUA application. There is little

published data describing the performance characteristics of most of these assays; however, the FDA has begun publishing reported measures of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for serology tests that have an EUA (https://www.fda.gov/medical-devices/emergency-situations-medical-devices/eua-authorized-serology-test-performance). One recent study evaluated 10 LFAs and two ELISAs that had not yet been given EUA by the FDA (33). Among specimens from SARS-CoV-2 RT-PCR-positive individuals, the percentage of seropositive specimens increased with time interval, peaking at 82%–100% in samples taken >20 days after symptom onset. Test specificity ranged from 84% to 100%% in pre-COVID-19 specimens. The majority demonstrated a specificity greater than 95% but only three had a specificity >99% (33).

It should be noted that despite high apparent sensitivities and specificities, the current low prevalence of SARS-CoV-2 has significant effects on the PPV of these tests. For example, if a test has 95% sensitivity and 99% specificity, and 5% of the population has had a COVID-19 infection, the PPV would be 83%; if the specificity is instead 95%, the PPV drops to 50%.

SEROLOGY TESTING FOR CONVALESCENT PLASMA USE

While antibody tests can accurately detect viral exposure, they may be less helpful in identifying or detecting the presence of neutralizing antibodies that can prevent the virus from infecting host cells and therefore confer immunity. For example, while anti-NP antibodies may correlate with protection against infection, as well as with the presence of anti-S antibodies, assays that detect SARS-CoV-2 anti-NP antibodies cannot directly detect neutralizing antibodies, which most likely bind S protein (34). In addition, the S protein of SARS-CoV-2 and other coronaviruses can flip between open and closed conformations, and their RBDs can be masked by the presence of glycan sugars surrounding them (6, 35). Such features can cause the infected host to synthesize decoy antibodies that bind the S protein, but do not affect infectivity. As a result, the presence of binding antibody and protection against infection is not always correlated. Indeed, one study found a negative correlation, where high antibodies against SARS-CoV-2 were found in the sickest patients (25). Therefore, testing of antibody correlates of protection against SARS-CoV-2 and other viruses requires more informative tests, namely the use of viral inhibition assays.

In a basic viral inhibition assay, also known as a plaque reduction neutralization test (PRNT), monolayers of cultured cells are exposed to live virus mixed with sera (**Fig. 4*A***) (30). After an incubation period, the cells are checked for signs on infection, such as the presence of multinucleated syncytia on microscopy (8), or the expression of viral proteins on the infected host surface, which can be identified with fluorescent detection antibodies (30). The numbers of infected cells in plates exposed to virus and sera are then compared with that of plates exposed to virus alone, with the hope that plates exposed to virus with sera have fewer infections. Such assays can also use dilutions of sera to quantify the minimum titer that can inhibit the number of infected cells by a certain percentage, such as 50% (**Fig. 4*B***). These minimum titers are then reported as the inhibitory dose, or ID (e.g., ID50).

While traditional viral inhibition assays are prohibitively difficult for natural SARS-CoV-2, which must be handled in Biohazard Safety Level 3 conditions, these assays can be easily modified with current technologies to provide safer and easier study settings (30). The virus itself can be attenuated by genetic modification, or alternatively its proteins expressed in a less-virulent nonhuman "pseudotyped" viral vector (8). These pseudotyped virions can be used to infect nonhuman cell lines that express the correct receptor. Additionally, the packaging of genes encoding fluorophores or enzymes into these virions and cell lines can also allow the identification of infection by fluorescence or bioluminescence, which can be automated. This automation can be used through the use of plate readers that optically measure bulk signal in each cell culture well, or alternatively through flow cytometry, where individual cells in a culture mix are passed sequentially through a detector that counts the exact percentage of cells that either carry an infection marker or remain uninfected thanks to protection by serum antibodies (30, 35).

It is important to note that host factors and population heterogeneity can contribute significantly to variability in cross-reactivity and neutralization efficacy of convalescent sera. Studies in animal models using genetically-attenuated virus (35) can create antibodies that neutralize both SARS-CoV, and SARS-CoV-2, while human sera samples may only neutralize one species (4). Some antibodies may also promote antibody-mediated enhancement of infection at insufficient concentrations (36). These differences stem from the fact that host responses differ

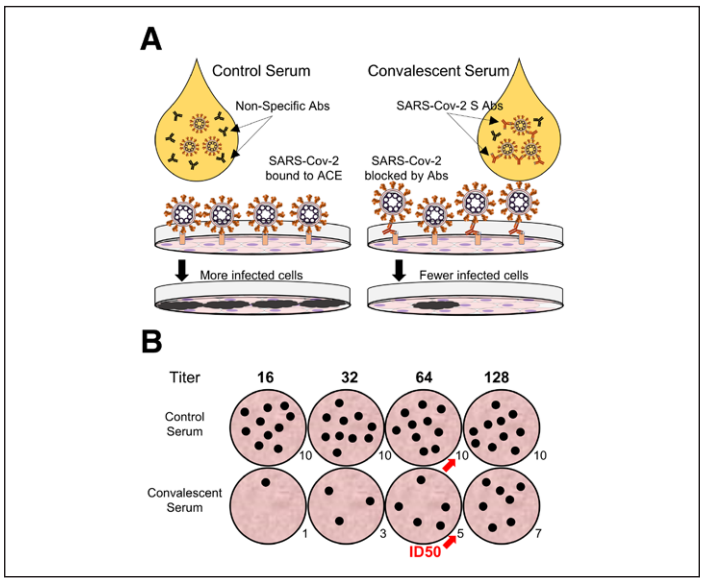

Figure 4. Example of a plaque-reduction neutralization titer assay for detection of severe acute respiratory syndrome-coronavirus-2 (SARS-Cov-2) neutralizing antibodies (Ab). **A**, Non-reactive serum from a control patient, or reactive serum from a convalescent patient is mixed with live virus and plated on monolayers of healthy cells expressing angiotensin-converting enzyme 2 (ACE2). Neutralizing antibodies against SARS-Cov-2 (*red*) bind the virus and prevent attachment to ACE2 (*orange squares*), leading to fewer infected cells (*black plaques*). **B**, Dilutions of serum are screened for reductions in the number of infected cell groups (*plaques*). *Red arrows* indicate the titer at which the patient's convalescent serum reduces the number of plaques by 50% relative to the control (ID50).

based on genetics and other factors. Patient serum responses are also polyclonal, containing antibodies that recognize several different antigens, all at different relative concentrations. As a result, randomized clinical trials to test the efficacy of convalescent plasma will be required, and testing of convalescent plasma should be done to quantify in-vitro neutralizing efficacy. Future prospective monoclonal antibodies against SARS-CoV-2 could provide a more consistent therapeutic response (37). However, such antibodies require rigorous testing, and may require combination with additional monoclonal antibodies to be efficacious. Such endeavors cannot be scaled quickly in time to meet current demand.

CONCLUSION

Nucleic acid amplification technology (PCR) assays and serological assays are both important components in current efforts to diagnose, treat, and limit the spread of SARS-Cov-2. As outlined in this review, providers should be aware of the strengths and limitations of individual assays. qPCR will remain the gold standard for diagnosing acute infection and will assist diagnoses of immunocompromised patients. Serological tests can aid in diagnosing late acute infections and will be important for diagnosing previously undiagnosed resolved infections. High-capacity ELISA-type serological assays will be essential in the coming months to assess the extent of infection in the community, information that is needed to help guide options for relaxing isolation restrictions. Lastly, neutralization assays will be vital in testing potential plasma donors and monoclonal antibodies as viable treatment options. Ultimately, all of these different assays need optimization to provide accurate results, and need to be scaled up to meet global demand.

Dr. Motley is supported by NIAID F30 AI140611. Dr. Fries is an attending at the U.S. Department of Veterans Affairs–Northport VA Medical Center, Northport, NY. She is supported by U.S. Veterans Affairs Merit Review Award 5I01 BX003741 and NIAID R01 AI127704. The contents of this review do not represent the views of VA or the United States Government. Stony Brook University received antibody test kits from ChemBio Diagnostics in exchange for providing coded laboratory samples for validation of their assay. No authors received any personal monies from this company nor are advisors or consultants.

For information regarding this article, E-mail: eric.spitzer@stonybrookmedicine.edu

REFERENCES

1. Zhu N, Zhang D, Wang W, et al; China Novel Coronavirus Investigating and Research Team: A novel coronavirus from patients with Pneumonia in China, 2019. *N Engl J Med* 2020; 382:727–733
2. Zhou P, Yang XL, Wang XG, et al: A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020; 579:270–273
3. Fehr AR, Perlman S: Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol* 2015; 1282:1–23
4. Ou X, Liu Y, Lei X, et al: Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun* 2020; 11:1620
5. Okba NMA, Muller MA, Li W, et al: Severe acute respiratory syndrome coronavirus 2-specific antibody responses in coronavirus disease 2019 patients. *Emerg Infect Dis* 2020 Apr 8. [online ahead of print].
6. Tortorici MA, Veesler D: Structural insights into coronavirus entry. *Adv Virus Res* 2019; 105:93–116
7. Wang J, Wen J, Li J, et al: Assessment of immunoreactive synthetic peptides from the structural proteins of severe acute respiratory syndrome coronavirus. *Clin Chem* 2003; 49:1989–1996
8. Zhang JS, Chen JT, Liu YX, et al: A serological survey on neutralizing antibody titer of SARS convalescent sera. *J Med Virol* 2005; 77:147–150
9. Amanat F, Stadlbauer D, Strohmeier S, et al: A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med*. 2020.
10. Junt T, Moseman EA, Iannacone M, et al: Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 2007; 450:110–114
11. Kim TW, Lee JH, Hung CF, et al: Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. *J Virol* 2004; 78:4638–4645
12. Kralik P, Ricchi M. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. *Front Microbiol* 2017; 8:108
13. Notomi T, Okayama H, Masubuchi H, et al: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000; 28:E63
14. Yan C, Cui J, Huang L, et al: Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clin Microbiol Infect* 2020; 26:773-779
15. Zhen W, Manji R, Smith E, Berry GJ: Comparison of four molecular in vitro diagnostic assays for the detection of SARS-CoV-2 in nasopharyngeal specimens. *J Clin Microbiol* 2020:JCM.00743–00720
16. Wang X, Tan L, Wang X, et al: Comparison of nasopharyngeal and oropharyngeal swabs for SARS-CoV-2 detection in 353 patients received tests with both specimens simultaneously. *Int J Infect Dis* 2020; 94:107–109
17. Yu F, Yan L, Wang N, et al: Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis* 2020;ciaa345
18. Mawaddah A, Gendeh HS, Lum SG, Marina MB: Upper respiratory tract sampling in COVID-19. *Malays J Pathol* 2020; 42:23–35
19. Wang W, Xu Y, Gao R, et al: Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA* 2020; 323:1843–1844
20. Liu R, Han H, Liu F, et al: Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin Chim Acta* 2020; 505:172–175
21. Lin C, Xiang J, Yan M, et al: Comparison of throat swabs and sputum specimens for viral nucleic acid detection in 52 cases of novel coronavirus (SARS-Cov-2)-infected pneumonia (COVID-19). *Clin Chem Lab Med* 2020.
22. Wyllie AL, Fournier J, Casanovas-Massana A, et al: Saliva is more sensitive for SARS-CoV-2 detection in COVID-19 patients than nasopharyngeal swabs. *medRxiv*. 2020.04.16.20067835
23. Zou L, Ruan F, Huang M, et al: SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med* 2020; 382:1177–1179
24. Guo L, Ren L, Yang S, et al: Profiling early humoral response to diagnose Novel Coronavirus Disease (COVID-19). *Clin Infect Dis* 2020;ciaa310
25. Zhao J, Yuan Q, Wang H, et al: Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis*. 2020;ciaa344
26. Xiao AT, Tong YX, Gao C, et al: Dynamic profile of RT-PCR findings from 301 COVID-19 patients in Wuhan, China: A descriptive study. *J Clin Virol* 2020; 127:104346
27. Wolfel R, Corman VM, Guggemos W, et al: Virological assessment of hospitalized patients with COVID-2019. *Nature* 2020; 581:465–469
28. Tang MS, Hock KG, Logsdon NM, et al: Clinical performance of Two SARS-CoV-2 serologic assays. *Clin Chem* 2020;hvaa120
29. Casadevall A, Pirofski LA: The convalescent sera option for containing COVID-19. *J Clin Invest* 2020; 130:1545–1548
30. Payne S (Ed): Methods to study viruses. *In*: Viruses. Cambridge, MA, Academic Press, 2017, pp 37–52
31. Lassaunière R, Frische A, Harboe ZB, et al: Evaluation of nine commercial SARS-C AQ3 oV-2 immunoassays. *medRxiv* 2020.04.09.20056325

32. Li Z, Yi Y, Luo X, et al: Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J Med Virol* 2020 Feb 27. [online ahead of print]
33. Whitman JD, Hiatt J, Mowery CT, et al: Test performance evaluation of SARS-CoV-2 serological assays. *medRxiv* 2020.04.25.20074856
34. Berry JD, Jones S, Drebot MA, et al: Development and characterisation of neutralising monoclonal antibody to the SARS-coronavirus. *J Virol Methods* 2004; 120:87–96
35. Walls AC, Park YJ, Tortorici MA, et al: Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 2020; 181:281–292.e6
36. Wan Y, Shang J, Sun S, et al: Molecular mechanism for antibody-dependent enhancement of Coronavirus Entry. *J Virol* 2020; 94:e02015–02019
37. Wang C, Li W, Drabek D, et al: A human monoclonal antibody blocking SARS-CoV-2 infection. *bioRxiv* 2020:2020.2003.2011.987958