SARS-CoV-2 Antibody Testing: Where Are We Now?

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Background

On January 10th, 2020, the sequence to a novel coronavirus, SARS-CoV-2, was uploaded to Genbank.[1] At the time, it was associated with what was reported as a small outbreak of an atypical pneumonia in the Wuhan province of China.[2] The first confirmed case of this mysterious virus in the United States was reported by the Centers for Disease Control & Prevention (CDC) on January, 21, 2020.[3] On January 30, the World Health Organization (WHO) declared a public health emergency, and one day later, so did the United States.[4,5] By February 11, the WHO introduced the name of this disease: COVID-19, caused by the SARS-CoV-2 virus. Just over 1 month after the first cases had been reported, there were more than 42,000 cases in China and hundreds of suspected cases in 24 other countries.[6] On March 11, the WHO declared COVID-19 a pandemic,[4] and it was becoming increasingly clear that the world was on the brink of a public health crisis the likes of which has not been seen in any of our lifetimes.

The scope of human life lost, and devastation on many levels, is impossible to understate. At the time of this writing there have been more than 2.5 million COVID-19 deaths worldwide and more than 500,000 in the United States.[7] The economic and societal repercussions are still unfolding, and the pandemic is not over. The sacrifices and accommodations to daily life surely did have—and continue to have—an impact on slowing the spread of the SARS-CoV-2 virus and the COVID-19 disease it causes. But as the pandemic unfurled in unprecedented ways, so did the response by the medical and scientific community. Governments, private companies, educational institutions, and philanthropists joined forces in massive collaborative efforts to meet the challenges of the pandemic, all with common goals of understanding the SARS-CoV-2 virus, finding effective treatments, and developing accurate diagnostic strategies in the face of this fast-moving foe.[8,9] We need only look at the remarkable vaccine development to understand the fruits of such labor.[10,11]

We can also look to the development and distribution of diagnostic tests as a mark of achievement. Assays for SARS-CoV-2 became available to laboratories for this emerging analyte with astonishing speed and variety. The first report of a reverse transcriptase real-time polymerase chain reaction (RT-PCR) test developed with the intent of mass production was published on January 23, 2020.[12] By February 2, the WHO had 250,000 test kits delivered.[13] The CDC was granted a U.S. Food & Drug Administration Emergency Use Authorization (FDA-EUA) for an RT-PCR test on February 4 and had shipments to all 50 states the next day.[14,15] By the end of that month, there were more than 14 nucleic acid amplification tests (NAATs) commercially available.[13] This number soon skyrocketed, and NAATs quickly became the gold standard for COVID-19 diagnosis.[16] However, NAATs do have some performance issues, particularly false negative results, mostly because of difficulty in nasopharyngeal specimen collection and viral load. In addition, supply has not always been able to keep up with demand for NAATs. Antigen tests have emerged as well, but their lack of sensitivity relegates them to screening tools. Although detecting acute infection is of paramount importance, both the molecular and antigen methods have a limited detection window and cannot detect past infection.[17,18] And there are some questions they cannot answer. Early in the pandemic, little was known about the rate of transmission, asymptomatic infection, or the immunologic response to the novel virus.[19] The need to broaden testing strategies to include antibody testing (also known as serology testing) was clear, and commercially available tests followed before long.

The first SARS-CoV-2 antibody test was granted EUA on April 1, 2020.[20] Unlike NAATs, used as a primary diagnostic tool, antibody tests detect the humoral response to SARS-CoV-2 and are not to be used as a sole mode of diagnosis. They confirm past infection. As such, the FDA initially only suggested but did not require EUA for SARS-CoV-2 antibody tests.[21] Within the first week of April, there were at least 70 SARS-CoV-2 antibody tests on the market without such authorization. With them came false claims of diagnostic capabilities and false claims of FDA approval.[22] On May 5, amidst a slew of performance concerns and inappropriate marketing claims, the FDA tightened the reins on antibody tests and required EUA approval for their use.[23] But reports of underperformance persisted, and public trust was breached. In response, in-

dependent evaluations began in a collaborative effort with the FDA, the CDC, the National Institutes of Health (NIH), the National Cancer Institute (NCI), and the Biomedical Advanced Research and Development Authority (BARDA).[24] On June 19, the FDA created a "Do Not Use" list for SARS-CoV-2 antibody tests.[25] To date, more than 100 antibody tests have been removed from the market.

With better regulation and more rigorous evaluations, the list of FDA-EUA tests grew. Clinicians and laboratories still had to navigate the implementation of testing from a rapidly developed and densely populated market. Confusion and concern around performance, what the results mean, what tests can be used for, and what they say about immune status have persisted. It is now just past 1 year since the start of the COVID-19 pandemic, and much has been learned, but SARS-CoV-2 diagnostics is still a complex topic. This article focuses on 1 facet of SARS-CoV-2 testing: antibody testing. It is written from the perspective of clinical laboratory science and for the enrichment of knowledge thereof. With so many tests available, and so much research produced at breakneck speed, it is important to look back at what we have learned and to understand the SARS-CoV-2 antibody tests available, how they should and should not be used, what their limitations are, what questions they answer, and what questions remain.

Form and Function of SARS-CoV-2

Before discussing the humoral response, it is helpful to have a basic understanding of the SARS-CoV-2 virus. Coronaviruses are a large family that infect a range of mammalian and avian hosts. In humans, disease is typically mild, but some, like SARS-CoV-1 and MERS-COV, have more severe pathology. The SARS-CoV-2 virus is uniquely devastating, not just in the severity of the COVID-19 disease but in how infectious it is and the novel challenge it is to our immune system. The SARS-CoV-2 virus is a betacoronavirus. Members of this family are known to jump from zoonotic to human hosts. The closest relative of SARS-CoV-2 is BatCoV RaTG13, found in horseshoe bats.[26] This relationship sparked the theory that the virus made a zoonotic leap in street markets where horseshoe bats are sold. However, at 96% homology with SARS-CoV-2, this bat source theory remains a possibility but not a certainty.[27] In fact, SARS-CoV-2 shares considerable homology with many other coronaviruses as well.[28] However, small changes in this virus's RNA have given rise to striking changes in transmission and pathogenesis.

The SARS-CoV-2 genome is 29,881 bp, encoding 4 structural proteins—spike (S), envelope (E), membrane (M), and nucleocapsid (N)—and several nonstructural proteins (open reading frames, proteases, accessory proteins).[1] The genome forms a single strand of positive-sense RNA contained within a nucleocapsid, enveloped by a membrane from which abundant spike proteins protrude. As in other coronaviruses, the SARS-CoV-2 S protein is composed of a short intravirion domain, an anchoring transmembrane domain, and an n-terminus stalk with 2 subunits, S1 and S2.[29] It assembles into trimers that give the "corona"-like motif seen by electron microscopy. Heavy glycosylation of the S protein may initially shield the SARS-CoV-2 virus from immune surveillance; however, this camouflage is not sufficient because the S protein is highly immunogenic.[30,31] The S2 subunit is a conserved region, facilitating fusion. The most distal S1 subunit contains the receptor binding domain (RBD), which is where

the virus directly binds to host cells—angiotensin-converting enzyme 2 (ACE2) receptors in the case of SARS-CoV-2—triggering conformational changes that facilitate entry into the cell. The key to what makes SARS-CoV-2 both novel and pathogenic lies in this region of the S protein. The S1 subunit of SARS-CoV-2 is unique, particularly at the RBD, in that it has more binding residues and a higher affinity for the ACE2 receptor than previously seen, making the SARS-CoV-2 virus both novel and distinctly pathogenic.[32]

Antibody Targets in SARS-CoV-2 Testing

To develop antibody tests, the question of which SARS-CoV-2 antigens elicit an immune response had to be answered. Phage display libraries, epitope maps, and peptide arrays were quickly assembled. Across multiple platforms, antibodies against the RBD, S, and N proteins emerged as the most potently immunogenic, selective, and widely produced by those patients infected with COVID-19.[33-36] Although exact epitopes may differ, virtually all commercially available tests—and all EUA assays—rely on detecting antibodies to these proteins, either separately or in combination.[37]

Many test platforms focus on the RBD of the S1 subunit. Some target the S1 subunit or the S protein as a whole. Antibodies against these antigens correlate with viral neutralization and indicate the sensitivity and specificity needed for clinical utility.[38-40] Some serologic assays include antibodies directed against the S2 subunit in their detection schema (eg, Liaison, DiaSorin).[37] Although the S2 subunit is immunogenic, it does not make a good target by itself. The S2 subunit is highly conserved among human coronaviruses (HCovs), and IgG against the S2 subunit is common in those unexposed to SARS-CoV-2.[41] This potential for cross-reactivity excludes the S2 subunit as a stand-alone target.[42,43]

The N protein that forms the RNA-housing nucleocapsid also elicits potent activation of the immune system.[44] Like the S2 subunit, the N protein is highly conserved across HCovs.[45] However, it does make an attractive target for serologic testing because the immune response during and directly after COVID-19 infection is so pronounced.[46] Clearly established cutoff values for detection and minimal cross-reactivity have been shown across multiple platforms (eg, Abbott, Bio-Rad).[37] Some studies have suggested that the detection of N antibodies is more sensitive than for those that target the S protein.[47-49]

When considering which test platform is suitable, it is essential to understand the goal of serologic testing. For example, both the Pfizer and Moderna vaccines elicit an immune response against the S protein. If evaluating vaccine response is a goal, then a test that targets S protein antibodies must be used. If, on the other hand, a population is being studied for infection rates, especially a population that may include persons who have been vaccinated, then a test that targets N protein antibodies would be more informative.

Antibody Dynamics

Analysis of plasma from those with COVID-19 confirmed an early consensus around targeting antibodies against the S, RBD, and N proteins of SARS-CoV-2 for assay development. However, an understanding of antibody dynamics was not immediately clear. Early studies often consisted of small specimen sizes and lacked the kind of longitudinal data that only time could provide. Heterogeneity in the intensity and duration of antibody production—a feature that continues to be observed—

contributed to conflicting and unclear information regarding antibody dynamics.[50,51] At the time of this writing, 1 year out from the beginning of the COVID-19 pandemic and many studies later, some uncertainty remains, but much has been revealed.

Antibody response in those with moderate to severe symptoms is the most studied and the most clearly characterized. The IgA, IgM, and IgG binding antibodies against the S, RBD, and N antigens, along with neutralizing antibodies (nAbs), are produced by the vast majority of those with symptomatic COVID-19.[52-55] Some studies have reported IgA and IgM as early detection markers,[46,50,56] whereas many others have reported that IgG appears almost simultaneously.[51,52,54,57,58] Explanations for this distinctive feature include a lengthy asymptomatic period or a slow buildup to detectable levels of IgA and IgM, but a definitive answer has yet to be found. It has also been reported that IgG production is delayed in some severely ill patients, whereas other studies have found that some severely ill patients have a delayed IgM response.[51,59] More study is needed to investigate these discrepant findings, but perhaps heterogeneity in early antibody response is itself the underlying feature.

Regardless of which isotype is detected first, antibody levels of IgA, IgM, and IgG are low in the first week of infection. Median seroconversion appears by days 7 to 12 postsymptom onset (PSO), with virtually 100% seroconversion by days 14 to 15 (**FIGURE 1**). The IgA and IgM levels gradually rise until approximately week 3 PSO, then decline.[52,53,57] Furthermore, IgG rises sharply in early infection at levels much higher than IgA and IgM, peaks at approximately week 5, and stays elevated for at least 7 weeks PSO.[50-52] Neutralizing antibodies correlate strongly with levels of binding IgG against the S and RBD antigens and moderately with anti-N IgG.[52,60,61] This development is encouraging, although not established, proof of durable immunity. Disease severity does correlate with the robustness of antibody response in the vast majority of patients; however, a correlation with clinical outcome has not been established.[50,57,60]

FIGURE 1. SARS-CoV-2 RNA levels peak approximately 1 week from exposure, then decline, becoming undetectable in most by approximately day 14. Median seroconversion occurs just after the first week PSO and reaches nearly 100% by day 14. IgM may or may not appear before IgG and IgA, but its half-life is the shortest, becoming undetectable by approximately 4 weeks PSO for many. IgG levels rise the highest and stay elevated for an extended period of time. PSO, postsymptom onset.
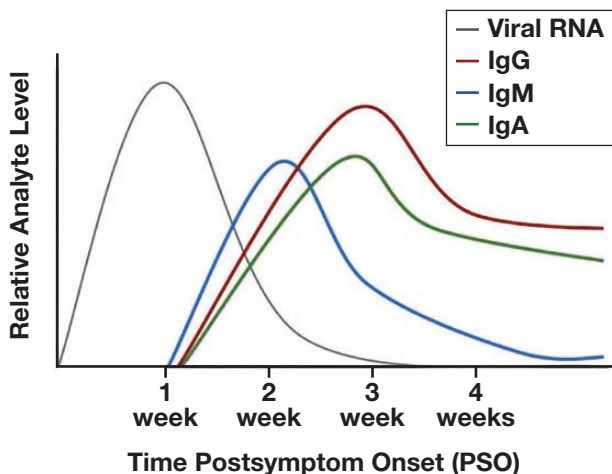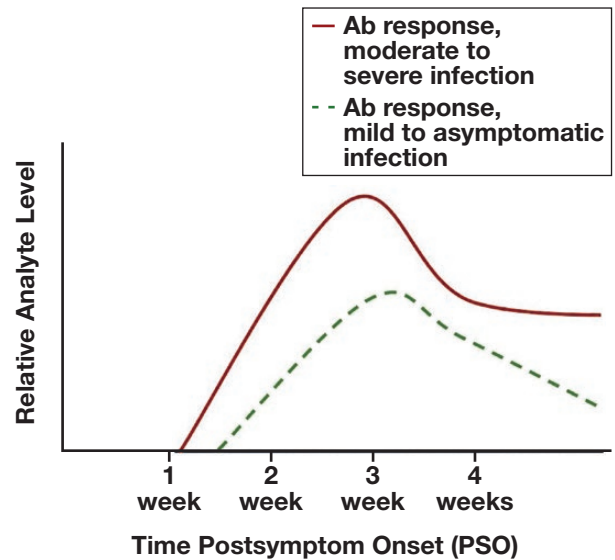

FIGURE 2. Moderate to severe infections elicit a markedly more robust immune response than mild to asymptomatic infections. Less is known about the duration of circulating antibodies in mild and asymptomatic infection, and whether or not an immune response correlates with lasting immunity is unknown at this time. PSO, postsymptom onset.

Antibody dynamics of mild to asymptomatic infection are less understood. Even the most recent studies include small specimen sizes and substantial heterogeneity. That said, 1 reproducible finding is that mild to asymptomatic SARS-CoV-2 infections do produce an antibody response, albeit less pronounced than moderate or severe infections (**FIGURE 2**).[50,62-64] The level of IgM seems to be short-lived or undetectable in a mild to asymptomatic infection, but IgG and total antibody levels are often detectable for weeks after exposure.[54,65] Seronegativity in moderate to severe illness is rare, but rates are unknown in mild to asymptomatic infection. This detail is something to consider when using antibody tests for epidemiological study. Neutralizing antibody production has been reported with mild infection, and some evidence supports nAb production in asymptomatic infection.[62] However, little is known about the prevalence and duration of nAb production in people with mild and asymptomatic infection at this time, and it is not known if or how the immune response relates to durable immunity.[66]

The groundwork for developing and implementing commercially available antibody tests would not have been possible without first characterizing SARS-CoV-2 antibody dynamics. All applications, from seroprevalence studies to convalescent plasma collection, rely on this basic understanding. A substantial body of research to this end emerged rapidly, and the work continues. Longitudinal studies that extend further and further from the time of symptom onset are underway, and a clearer picture of their duration will soon be realized. Measuring antibody titers may not help predict disease outcome, but they can help gauge severity and monitor disease progression. The relationship to antibody production, particularly nAbs, points to durable immunity, although this question remains a topic of intense interest. The immune response to SARS-CoV-2 varies substantially depending on disease severity, and heterogeneity, even within severity stratifications, seems to be a hallmark of the disease. Larger studies, with more participants, especially those with mild to asymptomatic infection, will deepen our understanding of antibody dynamics.

Types of Testing

There are currently more immunoassays on the market for SARS-CoV-2 testing than for any other pathogen.[67] The rapid development of hundreds of tests is both impressive and cause for scrutiny. Performance concerns and fraudulent claims undermined the credibility of SARS-CoV-2 antibody testing as a whole, but after underregulated and oversaturated beginnings, a clear menu of test methodologies with strong performance characteristics has emerged. That said, the menu is still large and diverse. At the time of this writing there are 65 SARS-CoV-2 antibody tests with EUA,[37] and many more are available on the global market. There is no gold standard for antibody tests, and different methodologies fulfill different testing needs. With such a broad range of tests available, the focus in this report is on tests that are available in the United States with FDA-EUA. The major types of SARS-CoV-2 antibody tests commercially available are lateral flow immunoassays (LFIAs), enzyme-linked immunosorbent assays (ELISAs), and chemiluminescent immunoassays (CLIAs). Although they are not routinely used in clinical laboratories, neutralization assays are discussed here as well. For each test, the principle, characteristics, usage, advantages, and limitations are addressed.

LFIAs

The LFIAs are a simple, qualitative method for detecting antibodies against the SARS-CoV-2 virus. Whole blood, serum, or plasma is applied to a membrane, and the specimen moves by capillary action laterally along a test strip. When the specimen reaches a conjugate pad, antibodies of interest bind to reporter-tagged antigens. The conjugate complexes move down the strip until they are captured by a test region. Tagged control antibodies get carried along as well. If the specimen has the antibodies of interest, then capture antibodies embedded in the test region will bind with the complex and a colorimetric or fluorescent signal will be produced. If the test is valid, then a control line will be present.[68] Studies have shown that SARS-CoV-2 LFIAs target antibodies against epitopes of the S or N protein, or both. Most LFIAs detect a combination of IgM and IgG. Some detect total antibodies or IgG alone.[37]

The LFIAs are rapid tests, with turnaround times (TATs) of less than 30 minutes. They are simple to perform, require a small amount of specimen, are relatively inexpensive, and can be scalable for mass testing. The demand for LFIAs for point-of-care (POC) testing has been strong, but of the 19 LFIAs granted EUA, only 2 are Clinical Laboratory Improvement Amendments (CLIA) waived and approved for POC testing. Both CLIA waived assays are validated for finger-stick specimens; the rest require phlebotomy and are considered to be of moderate to high complexity.[37]

Specificity of the SARS-CoV-2 antibody LFIA >95% has been widely reported, but independent evaluations of sensitivity vary considerably. Meta-analysis of commercially available LFIA has found pooled sensitivity to be between 66% and 76%.[69,70] Difficulty in reading and interpreting faintly positive tests and inconsistent reproducibility have also been reported.[71] Cross-reactivity remains a concern because large-scale studies evaluating cross-reactivity with other viruses, particularly other betacoronaviruses, are lacking.

In general, LFIAs underperform in comparison to manufacturer evaluations, but their ease of use and economical production mean that demand for SARS-CoV-2 LFIA testing is high. Great caution must be applied to this demand.[72] The LFIAs simply determine prior exposure to SARS-CoV-2, and the accuracy of this determination should be viewed with scrutiny. Contact tracing, knowledge of serostatus, and aid in diagnostics are all potential uses for LFIAs, but it is important to recognize potential shortcomings and not to overestimate what individual results mean. Where SARS-CoV-2 LFIA testing can have an important role is in serosurveillance and epidemiological study.[73] According to the CDC, the prevalence of SARS-CoV-2 remains largely unknown.[74] The LFIAs offer a simple, economical option that can be applied to large-scale testing and by minimally trained personnel. It is likely that they will be instrumental in population serosurveillance, especially in underserved areas. The CDC, FDA, and independent organizations are continuing evaluations, which will be important moving forward.

ELISAs

Although many variations exist, the basic principle for SARS-CoV-2 ELISA testing begins with coating microtiter wells with a SARS-CoV-2 antigen of interest, allowing antibodies in serially diluted patient specimens to bind with the antigens, followed by the addition of an enzyme-conjugated reporter antibody that produces a signal when substrate is added. The signal can be colorimetric or fluorescent (the latter are described as enzyme-linked fluorometric assays). These sensitive assays can be used for qualitative or semi-quantitative analysis. Researchers and clinical laboratories use ELISAs widely for SARS-CoV-2 antibody testing.

Currently there are 19 SARS-CoV-2 ELISA tests with EUA. Detection includes IgM, IgG, a combination, or total antibody. Most ELISAs detect antibodies directed at epitopes on the S protein. Two detect antibodies against the N protein, and 2 detect both. Specificity is widely found to be >95%. Pooled meta-analysis has described sensitivity for IgM or IgG at approximately 85% and combination IgM/IgG sensitivity at >90%.[69,70]

The ELISAs are moderate- to high-complexity tests. They require skilled personnel in a certified laboratory for clinical use. They are much more costly and labor-intensive than LFIAs, and TATs are on the order of 1 to 4 hours. A standard microtiter plate can run batches of 96 specimens, and high-throughput platforms are available. Much of what we know about SARS-CoV-2 antibody dynamics and the determination of which antigen and antibody isotypes yield the best sensitivity and specificity comes from ELISA testing.[46,49,57,75-77] Quantitative tests may be used to monitor disease progression, and the sensitivity of qualitative tests outperforms LFIAs when serostatus is being established. Their moderate to high throughput makes ELISA tests a good candidate for high-volume testing, including serosurveillance, and epidemiological studies. They may also play a role in monitoring vaccine response.[78]

CLIAs

The newest antibody detection technology on the market, CLIAs are similar in principle to ELISAs in many respects (eg, direct, indirect, and sandwich formats), but they rely on light emission to detect analytes. The general principle of SARS-CoV-2 CLIA testing begins with an antigen-coated solid phase, such as a polystyrene well, or more commonly paramagnetic beads. The patient specimen is added and allowed to bind to the antigen, and then an enzyme-conjugated antihuman antibody is added that binds to that complex. A substrate containing a luminescent, like luminol, initializes an oxidation reaction. As the luminescent

goes from an excited to a ground state, a photon is emitted. The photons are measured by a luminometer. The amount of antibody present in the specimen correlates to the amount of light produced by the reaction.[79]

Currently there are 25 CLIAs with EUA. Eighteen detect antibodies against epitopes on the S protein, 3 detect antibodies against the N protein, and 4 detect both S and N antibodies. Most detect IgM or IgG, 3 detect a combination of IgM and IgG, and fo4ur are pan-Ig assays.[37] Like ELISAs, CLIAs can be qualitative or quantitative and are useful for fulfilling many of the same testing needs. However, CLIAs are the most sensitive platform available. Pooled meta-analysis indicates >95% specificity and >96% sensitivity.[69,70] Another advantage is that many CLIA platforms are high-throughput and several are fully automated, offering excellent scalability for mass testing.

The CLIAs are not rapid tests. The TATs are approximately 1 hour, but automation makes testing less labor-intensive than most ELISAs. They are moderate- to high-complexity tests, requiring skilled personnel and a certified laboratory. A major disadvantage is cost. Platforms for CLIAs are the most expensive of any immunoassay platforms, although an argument can be made that the greater productivity allowed by these platforms offsets the cost of operation for high-volume settings.[79] For laboratories that have the means, CLIAs make an excellent platform for monitoring disease progression, serosurveillance, epidemiologic study, and monitoring vaccine response.[80] Another important use of CLIAs is convalescent plasma production, used by the American Red Cross to screen all donations and to select candidates for convalescent plasma donation.[81] Whatever the application, CLIAs' superior sensitivity offers richer information about the length of antibody response and better detection of antibodies at low levels.

Neutralization Assays

The aforementioned tests detect binding antibodies, but their detection does not necessarily indicate a humoral response capable of overcoming a virus. A better measure of this response is through the detection of nAbs. Neutralization assays use live virus or a pseudovirus to assess protective immunity. Patient serum is incubated with the live virus, and the mixture is inoculated onto a cell culture. Neutralization is measured by how much viral growth is inhibited, as compared to controls to which no patient serum is added.[82] Live SARS-CoV-2 viral neutralization assays require a biosafety level (BSL) 3 laboratory; pseudovirus tests require a BSL2. Tests are labor-intensive and take 3 to 4 days for results. They are expensive and high-complexity tests, not feasible for large-scale testing or for most clinical laboratories. However, antibody tests like ELISAs and CLIAs may be able to act as a surrogate. Many studies have reported that binding IgG levels, particularly to the RBD domain of the S protein, strongly correlate with nAbs.[54,83-85] Research continues to investigate this correlation, and it is likely that ELISA and CLIA testing can be an alternative to the costly and time-consuming neutralization assays. The FDA has given EUA clearance to 1 ELISA described as a neutralization assay because it measures the blocking of RBD binding to the ACE2 receptor protein by antibodies present in patient serum.[86] Although it is not a traditional neutralization assay, it is a good example of the ongoing evolution of antibody testing and of the roles that antibody testing can fulfill.

Role of Antibody Testing

Antibody tests are indirect; they measure the immune response to SARS-CoV-2 rather than directly detecting the virus. As such, they are not a primary diagnostic tool. However, antibody tests can complement diagnosis, especially in patients in whom there has been a delay between symptom onset and testing or when clinical presentation indicates COVID-19 but NAAT tests are negative.[87] As the virus is cleared, it drops below the limits of NAAT detection. After 14 days PSO, the ability of NAATs to detect SARS-CoV-2 infection drops to approximately 50% (**FIGURE 3**). By day 30, most who have contracted COVID-19 will be NAAT-negative.[88,89] Conversely, seroconversion—and reliable antibody testing—occurs by day 14 PSO. Thus, antibody testing can be a useful adjunct to diagnosis when NAAT is negative but clinical presentation indicates COVID-19. Some suggest combining NAAT and antibody testing for optimal diagnostic accuracy, especially as variants emerge.[89] After diagnosis, clinicians may use antibody testing to monitor the duration and magnitude of patients' antibody response as part of disease course management and to predict when the virus has been cleared.

Arguably, antibody tests are the most important tool for surveillance and epidemiologic studies.[90-92] They indicate past infection long after the infection has cleared, and specimen collection is more reliable than nasopharyngeal swabs. There is a great need for accurate and sensitive testing on a large scale for monitoring outbreaks and establishing actual population prevalence. Because of the limitations of NAAT, seroprevalence provides a more accurate measure of true infection rates. The CDC, NIH, and WHO have massive seroprevalence study efforts underway, and smaller-scale efforts by academic and clinical institutions continue to build our body of knowledge. For population studies, especially where prevalence is expected to be low, choosing a test with high specificity is key to minimizing false positives. The CDC recommends using tests with specificities of 99.5% or above.[93] And at this stage in test development, we do have the means to execute studies with this performance goal.

Antibody testing for SARS-CoV-2 is used in blood donor screening and in convalescent plasma preparation.[94] Antibody testing is also used to evaluate vaccine effectiveness.[95] Both applications require sensitive methodologies. The American Red Cross, for example, is interested in quantitative data for convalescent plasma preparation and has a high test volume with routine screening. The organization relies on CLIA methodology to address these needs.[94] Monitoring vaccine response requires sensitive test methodologies, because immune response is typi-

FIGURE 3. As the SARS-CoV-2 virus is cleared, the sensitivity of RT-PCR tests diminishes. However, antibody test sensitivity is highest 2 to 3 weeks PSO. Sensitivity typically remains high for weeks after this peak and long after the window of RT-PCR clinical utility. PSO, postsymptom onset; RT-PCR, real-time polymerase chain reaction.
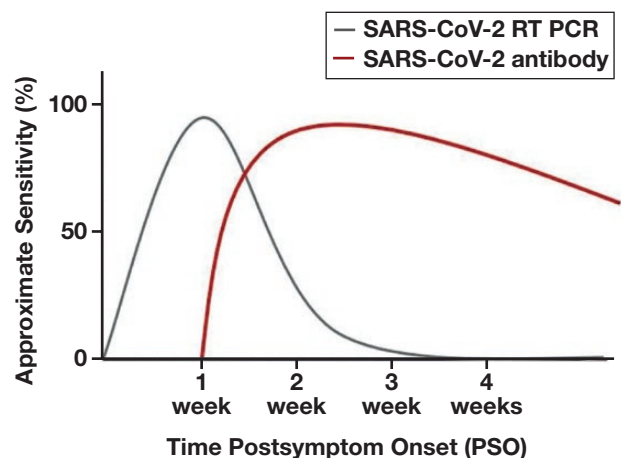
cally less pronounced than natural infection. Both CLIA and ELISA tests can be appropriate. But it is important to be mindful of which antibodies are expected from vaccine inoculation. For example, both FDA-approved mRNA vaccines utilize the S protein. Choosing a test that detects antibodies against the S protein would be necessary for evaluating vaccine efficacy. Tests that detect antibodies against the N protein would be useful in differentiating those infected by the virus vs those who gained immunity through vaccination.

Antibody testing can also play a role in contact tracing.[96,97] With the window of time that NAATs are reliably positive, it is unreasonable to use them as a sole method of contact tracing. Most commonly, LFIAs are used for this application, but any methodology could be used. There has been an interest in using antibody testing as "return-to-work passports."[98] But reinfection rates and durable immunity remain in question. At this time, the CDC advises against using antibody testing as a determinant for returning to the workplace.[93] The recommended appropriate uses of LFIAs, ELISAs, and CLIAs for patient antibody production to SARS-CoV-2 are summarized in **FIGURE 4**.

Specimen Types

Most LFIAs are validated for plasma, serum, and whole blood. Currently, 2 LFIAs with EUA are approved for finger-stick, POC testing. This number may change, because the demand for quick and accessible POC antibody testing is high. There is also a great demand for at-home specimen collection because it reduces the chances of exposure, and the ease of collection may lead to a higher number of people participating in testing. To this end, assays that rely on IgA detection in saliva and assays that can be performed from dried blood spot (DBS) specimens are being explored, but none have reached the level of performance required by the FDA, and at-home specimen collection poses its own set of issues.[99,100] One CLIA assay has EUA to perform testing from DBS, but the overwhelming specimen types acceptable for these tests are serum and plasma. Manufacturer instructions vary,

but in general, specimens may be refrigerated at 4ºC for 2 to 3 days before testing and frozen at –20ºC for 1 month.[37]

Predictive Value

Sensitivity is the ability of a test to detect a true positive. Specificity is the ability of a test to detect a true negative. Accuracy is the ability of a test to differentiate between true positive and negative specimens.[101] These terms are commonly understood by anyone working in a clinical laboratory. But SARS-CoV-2 antibody test performance is often further characterized by looking at an assay's positive predictive value (PPV) and negative predictive value (NPV). See **TABLE 1** for a definition and comparison of these diagnostic terms. These terms may be less familiar, but they are important to understand. PPV is the probability an individual positive test result represents true antibody positivity, and NPV is the probability an individual negative test result represents a true antibody negativity.[37] These values are based not only on the sensitivity and specificity of an assay but also on the prevalence of the disease in a population. Research has shown that PPVs increase with disease prevalence and that NPVs increase the lower the disease prevalence. The key to using antibody testing for large-scale prevalence studies is using tests with high PPVs. The FDA calculates NPVs and PPVs for SARS-CoV-2 antibody assays based on the assumption of 5% disease prevalence. All FDA-EUA assays have NPV >98%, but PPV varies considerably. Several LFIAs have PPV <60%; the lowest is 49.6%. The ELISA and CLIA platforms have PPVs that range from >80% to 100%. Online calculators are available, including one provided by the FDA and the *British Medical Journal* (**TABLE 1**).[37,102]

Following is an example of a calculation of PPV and NPV: For a test has 90% sensitivity, 98% specificity, and 5% disease prevalence, and 1000 people are tested, one can expect 19 false-positive results and 5 false-negative results. There is a 70.3% chance that a positive test reflects an antibody-positive person (PPV) and a 99.5% chance that a negative test reflects an antibody-negative person (NPV).[103]

FIGURE 4. Uses of SARS-CoV-2 antibody testing according to test methodology. CLIA, chemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay; LFIA, lateral flow immunoassay.
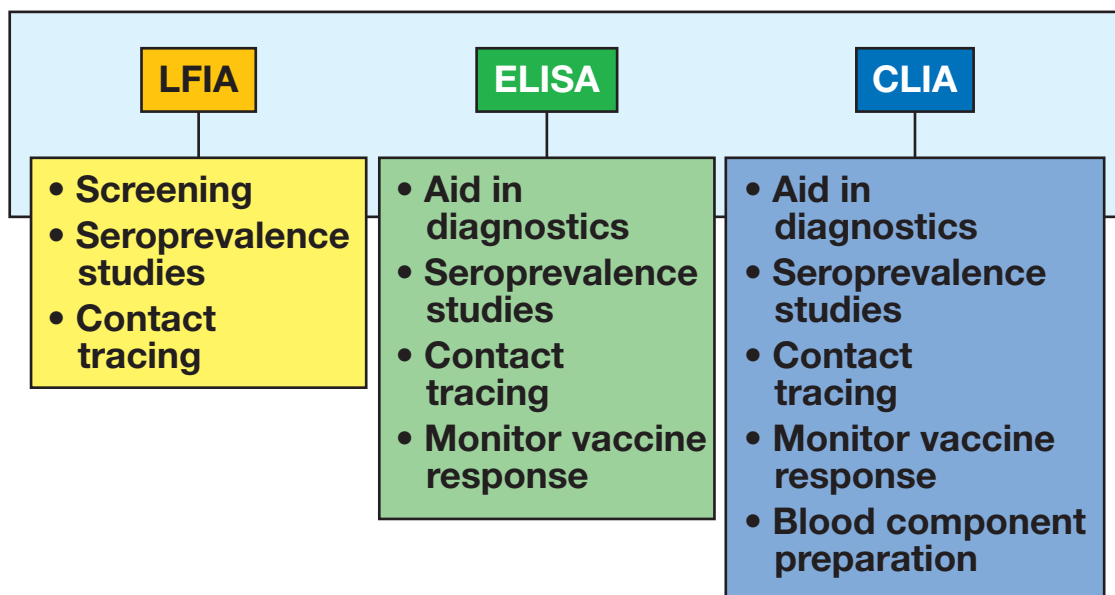
TABLE 1. Definition and Comparison of Diagnostic Terms

	Has SARS-CoV-2 Antibodies	Does Not Have SARS-CoV-2 Antibodies
Positive results	True positive (a)	False positive (b)
Negative results	False negative (c)	True negative (d)

Algorithms that use more than 1 test with differing antigenic targets are a strategy for increasing PPV for laboratories that have the resources to do so. Ideally, only tests with the highest sensitivity and specificity would be used. But for many laboratories and testing facilities, this level of testing is not feasible. For each application of SARS-CoV-2 testing, the pros and cons, including predictive value, must be balanced against the reality of the skill level of the labor force and the resources of the testing facility.

Limitations

As with any humoral response, antibodies to SARS-CoV-2 take time to build to detectable levels. Sensitivity does not reach EUA acceptable limits on any platform until 8 to 14 days PSO. Thus, SARS-CoV-2 antibody tests do not detect acute infection. Most manufacturers include sensitivity and specificity data based on days from symptom onset. However, the majority of COVID-19 infections are mild to asymptomatic. Establishing this crucial timing of collection is often not possible, especially in serosurveillance and epidemiologic studies. In addition, studies suggest that patients with mild to asymptomatic infections produce a less-robust immune response that wanes faster than that in more severely affected individuals.[62] There are a small portion of those who have been infected by SARS-CoV-2 that do not produce antibodies to the virus, and few studies have characterized the SARS-CoV-2 antibody response of immunosuppressed patients.[104,105] False negatives resulting from assay sensitivity, an inability to optimally time specimen collection, and individual immune responses are a limiting factor with SARS-CoV-2 antibody testing.

The true prevalence of COVID-19 in the U.S. population is not known at this time.[37] Prevalence can vary substantially across different populations, but overall, estimates remain low in the general population.[106,107] Antibody testing is a tool at the forefront of gathering the data necessary to make better estimates, but even assays that meet the FDA standards for sensitivity and specificity can have poor PPV in low-prevalence populations.[37] This possibility means that false positives are a limitation of SARS-CoV-2 antibody testing, particularly in low-prevalence populations. Choosing tests with high specificity mitigates this limitation, as does using 2 test algorithms.

Interfering substances can be an issue with any immunoassay. Potential interfering substances for SARS-CoV-2 immunoassays include endogenous factors like hemoglobin, triglycerides, and elevated protein and exogenous factors like acetaminophen, ascorbic acid, biotin, and hydroxychloroquine.[37] Manufacturers of EUA assays provide evaluations of known potential interfering substances. Performance above these thresholds can affect results. Patient medication history and visual evaluation of specimens help discern spurious results from such substances, but the risk cannot be eliminated.

Cross-reactivity from other antibodies must be considered when using SARS-CoV-2 antibody tests on any platform. Because of the expedited validation process and the limited availability of reference material, cross-reactivity studies for EUA SARS-CoV-2 antibody tests are scant. Although manufacturer specimen sizes are small across all FDA-EUA assays, cross-reactivity with noncoronavirus antibodies (eg, antibodies against HIV, cytomegalovirus, hepatitis B virus, and influenza; antionuclear antibodies, and rheumatoid factor) seem to pose little threat to commercially available SARS-CoV-2 antibody testing.[37] Of more concern is the potential for cross-reactivity by antibodies to viruses that share significant homology with SARS-CoV-2. Here, the data are sorely lacking. Some manufacturers have not evaluated the cross-reactivity of antibodies against other coronaviruses at all. This includes MERS, SARS-CoV-1, alpha-COV 229E, beta-COV OC43, and beta-COV HKU1. Of those that have, specimen sizes were small—often 5 or fewer. Two ELISA manufacturers have noted cross-reactivity with SARS-CoV-1,[37] but these are hardly enough data to make any broad statements about cross-reactivity. Cross-reactivity is an issue that urgently requires further evaluation.

Considering the circumstances of this novel virus, some accommodations to study design have been taken to accomplish important work. However, not all studies were conducted with the kind of rigor that avoids bias. Two large meta-analysis studies evaluated bias in SARS-CoV-2 antibody testing, and both arrived at similar findings.[69,70] Some studies from the meta-analyses showed bias in how the index test was used and some with application of the reference standard test, but these bias risks were minimal. A major risk of bias was found in the timing of the specimen collection. Cochrane et al identified this bias in 54% of studies[70] and Lisboa-Bastos et al[69] in 67% of studies. Participants were either not stratified according to time PSO, or the timing was unclear. The overwhelming and more concerning bias was found in participant selection. Cochrane et al. identified participant selection bias in 89% of the studies,[70] and Lisboa-Bastos et al[69] identified this bias in 98% of the studies. The main reason for this finding was the tendency to select hospitalized patients with COVID-19 for inclusion in the study, with little to no inclusion of patients with mild and asymptomatic infection. Bias in how assays are evaluated means that accuracy in clinical settings may be lower than expected; however, it is not always possible, especially in the midst of a pandemic, to mitigate the risks. As performance evaluation studies continue, we can expect improvement in the areas that need attention.

Conclusion

It is just past 1 year since COVID-19 was declared a pandemic in the United States. The medical and scientific community's response to meet testing needs is nothing short of astonishing. However, the urgency for the testing and rapid development of assays has not been without its problems. The flurry of antibody tests with dubious claims and subpar performance showcases the importance of oversight by the FDA and the benefit of entities like the CDC, NIH, and NCI. With their guidance, numerous independent performance evaluations of SARS-CoV-2 antibody tests have been published, and efforts on this front continue.[37,107-110] For example, the NIH developed the Rapid Acceleration of Diagnostics program, which helps with validating SARS-CoV-2 tests.[111] In addition, panels have been developed by the collaborative efforts of the FDA, CDC, NIH, NCI, and BARDA to aid the validation

process. Shortcomings like study design bias and limited cross-reactivity studies are perhaps unavoidable with such a novel and devastating virus, and developing a validation strategy takes time. But it is clear that we are moving along the trajectory of filling in the gaps that need to be filled.

Today we have a diverse test menu to choose from, many have sensitivities and specificities that exceed the FDA's requirements, and we know how to use them. The SARS-CoV-2 antibody tests may not be used as primary diagnostic tools, but they are a helpful component of diagnostics when used in conjunction with NAATs, and particularly with patients who are past 14 days PSO. They are used in convalescent plasma donor selection and contact tracing, and to monitor vaccine response. Antibody tests are invaluable to surveillance studies. In fact, the NIH just released a massive seroprevalence study (still in preprint), and more are underway.[112] A better grasp on true population prevalence will be realized with continued efforts like these. As we move into the second year of the pandemic, we can now have the testing capability and the time postpandemic outbreak to conduct the studies to answer the questions surrounding antibody dynamics at all severity stratifications, particularly mild to asymptomatic infection.

Early public confusion about antibody testing has been met with clear, transparent, and meaningful education by entities like the CDC and FDA. This guidance includes the messaging that a positive antibody test result does not confirm immunity. Preliminary studies, and the studies of related HCovs, do suggest durable immunity for some period of time; however, we still do not know the extent of immunity after infection with the SARS-CoV-2 virus. This is a question that accurate and reliable antibody tests will play a role in answering. But for now, we must not conflate a detectable antibody response with durable immunity.

Emerging variants and rising infection rates remind us that this pandemic is not over. There is concern that new strains may evade detection by molecular methods, which require precise sequence agreement for primers to bind and viral RNA to be detected. The CDC is closely monitoring variants in the United States.[113] Current molecular testing seems to be effective at detecting SARS-CoV-2 variants.[114] But should this change, antibody testing may prove instrumental in detecting outbreaks.

Regardless of the direction the virus takes us, it is clear that antibody testing has been invaluable to developing and understanding the SARS-CoV-2 virus and the COVID-19 pandemic. The questions that remain are answerable through reliable testing and diligent work. Although this is the first time in our lifetimes that a pandemic like COVID-19 has been seen, it brings to our collective attention that it may not be the last. How academic institutions, private companies, and governmental agencies have interfaced provides a kind of blueprint for challenges we may face in the future. The development and implementation of SARS-CoV-2 antibody testing has not been without its challenges, but it has been a remarkable process, one that is still in motion, and one that will continue being beneficial in moving us forward to a postpandemic world.

Acknowledgments

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