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# Update to COVID-19 serologic testing : FAQs and caveats

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### INTRODUCTION

As of early January 2022, almost 2 years after the first cases of Coronavirus disease 2019 (COVID-19) appeared, a PubMed search for "COVID antibody" revealed more than 14,000 publications. Such tremendous explosion of knowledge is striking, but it takes considerable reading and critical thinking to identify the meaningful knowledge. Regarding serology testing, the influx of data have prompted an immense amount of discussion regarding its potential use for COVID-19 as a diagnostic tool to assess potential immunity.

It is important to highlight that serologic testing has never been routinely used to diagnose infections with respiratory viruses such as influenza, parainfluenzae, respiratory syncytial viruses, adenoviruses, or metapneumovirus or for diagnosing the severe acute respiratory syndrome, Middle East respiratory syndrome, and H1N1 influenza during those epidemics. However, the pandemic status of COVID-19 combined with the initial shortage of nucleic acid detection kits has raised the prospect of resorting to serology as an alternative method to detect the virus, so it is relevant to ask how useful it may be for diagnosis and predicting immunity to reinfection. The answer to those questions is not very, according to the Infectious Diseases Society of America, United States Food and Drug Administration (FDA), and Centers for Disease Control and Prevention (CDC), which have issued clear statements against the clinical use of COVID-19 serologic testing to assess immunity after vaccination, to diagnose SARS-CoV-2 infection in place of virologic testing, or to detect evidence of past infection.[1-3]

The following addresses some common questions regarding serologic testing for COVID-19.

The statements and opinions expressed in COVID-19 Curbside Consults are based on experience and the available literature as of the date posted. While we try to regularly update this content, any offered recommendations cannot be substituted for the clinical judgment of clinicians caring for individual patients.

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### IS IGM SEROLOGY RELIABLE FOR DIAGNOSING ACUTE COVID-19?

Antibodies, also known as immunoglobulins, belong to five different classes of which IgM, IgA, and IgG are important in immune response to infectious agents. Classically, IgM appears first and then due to class-switching, IgA and IgG appear, with IgG having much higher affinity against the antigens than IgM. Studies show that the appearance of detectable IgM antibodies after infection with SARS-CoV-2 is delayed for 10 to 14 days after the onset of symptoms, resulting in abysmal sensitivity ranging from 17% to 50%.[4,5] Note that this is not days after exposure or infection but days after the onset of clinical symptoms. Unfortunately, the results may not be clinically useful because COVID-19 often progresses quickly within the first 7 to 10 days.[6] Thus, by the time of serologic diagnosis, patients could be critically ill with septic shock or multiorgan failure, or they could have inadvertently infected innumerable contacts. The clinical bottom line is that serology testing is NOT useful either diagnostically or from an infection control and public health perspective.

#### Testing problems with IgM

IgM tests, in general, have an inherent predisposition to false-positive results mainly owing to their lower avidity than IgG antibodies. Viruses as distantly related as the Dengue virus have been reported to cause false-positive IgM results in COVID-19 serologic tests.[7] Antinuclear antibodies are another cause for false-positivity for which elevated titers are relatively common in patients over age 50, which is the median age for more severe COVID-19. False-positive results also have been documented in serum from patients with influenza or influenza vaccine recipients. Influenza vaccine recipients constitute a large population who may have overlapping signs and symptoms of influenza and COVID-19. Other potential causes for false-positives include recent infection with influenza virus, syphilis, herpes simplex virus, human metapneumovirus, parvovirus, rheumatoid factor, enterovirus, rhinoviruses, parainfluenza viruses, *Chlamydophila pneumoniae*, *Streptococcus pneumoniae*,

respiratory syncytial viruses, acute Epstein Barr virus or cytomegalovirus infection, human immunodeficiency virus, hepatitis B virus, *Toxoplasma*, pregnancy, adenoviruses, malaria, *Trypanosoma*, helminthic infections, and most importantly, reinfection with common coronaviruses (OC43, NL63, HKU1, and 229E), the latter co-circulate with SARS-CoV-2.

## IS IGG SEROLOGY TESTING AN OPTION FOR DIAGNOSING ACUTE OR CONVALESCENT COVID-19?

IgG seroconversion is delayed after the onset of symptoms (more than 35 days in some cases), but typically occurs in 2 to 3 weeks, at which time it can be detected if the test specificity is high.[5] Commercially available serologic assays of IgG require validation with an authentic (ie, using live virus) plaque-reduction neutralization test (PRNT). Unfortunately, this is not done either when tests are developed by the manufacturers or when they are validated by clinical laboratories prior to routine clinical use.

In brief, PRNT requires mixing live viruses with serially diluted serum followed by cell culture to view the cytopathic effect be it measured at 50% or higher endpoint (the higher the percentage, the more specificity and less sensitivity would be). PRNT is a functional assay that requires significant expertise and a biosafety level 3 facility (not available in hospitals and most commercial and reference laboratories), and it is not amenable to automation; however, it is necessary to be included when any new assay is being validated. Ideally, this test should be done by manufacturers prior to the FDA submission. For laboratory-developed tests, however, the onus is on the laboratory to ensure PRNT is done on-site or in collaboration with a reference laboratory that has PRNT capability. Additionally, PRNT needs to be done head-to-head against other known coronaviruses, particularly those that are commonly acquired in the community (eg, 229E, OC43, NL63, HKU1), which have always been detected using nucleic acid amplification tests. Thus far, virtually none of the published studies or commercially available kits have documentation of such validation.

That said, PRNT has limitations. Previous exposure to common coronaviruses may lead to an early and high-titer humoral immune response to SARS-CoV-2, which is reminiscent of the original antigenic sin phenomenon.[8] As time elapses, however, the humoral response may become more specific to SARS-CoV-2. Studies have shown greater than 90% seroprevalence of common coronaviruses in the United States. Interestingly, Wölfe[14] and others[9] reported finding a significant degree of serologic cross-reactivity between SARS-CoV-2 and common coronaviruses. Further, IgG responses were much stronger and appeared earlier than IgM responses, providing additional support to the original antigenic sin. It seems that exposure to SARS-CoV-2 triggers previous memory response to all common coronaviruses. Based on the current information, it is not clear which target provides the best specificity, but specificity should increase over time as the immune response becomes more fine-tuned. This, however, will be well beyond the recovery time and, thus, of no use for routine diagnostic purposes.

The recently developed pseudoneutralization assays that use vesicular stomatitis virus or lentiviruses, despite acceptable statistical correlation (marginally) with PRNT, have three main limitations.

- First, they are not FDA authorized or commercially available and are typically laboratory-developed. They require tremendous expertise to develop, maintain, and interpret; therefore, their availability is very limited.
- Second, they are limited to whatever spike gene is transduced during development. The vast majority use the ancestral (wild type) SARS-CoV-2 spike protein, so they are not easily amenable to emerging variants.
- Third, they work based only on the antigen-antibody binding principle in which antigen has a fixed structure with no or much less flexibility, co-receptor (eg, lectins) involvement or the like, than the authentic PRNT.

As a result, the titers measured by these pseudoneutralization assays may not necessarily reflect antibody protection in vivo, and only if one assumes that neutralizing antibody is the correlate of protection.

On the IgG side, false-positives using both manual and instrument-based assays as well as point-of-care testing kits have been observed in serum samples, similar to false-positives noted for IgM. Finally, even if IgG is used with a highly specific assay for diagnosing acute COVID-19, it takes several weeks to see a minimum 4-fold rise in antibody levels, which would be too late to be of clinical use.

Furthermore, testing requires a minimum of 2 blood draws (acute and convalescent), posing additional infection control challenges. It is also important to note that a significant proportion of asymptomatic and mild cases never seroconvert, and a higher proportion lose the antibody titers during early convalescence.[10,11]

### Additional concerns

Another pivotal point is the often neglected concept of positive predictive value, particularly when the pretest probability (in this case, seroprevalence) is low. As populations lose antibody levels over time and seroprevalence dwindles, PPV also drops precipitously leading to very unreliable results. This can easily happen with seroprevalence of less 5% and specificity of less than 100%. In an elegant study out of Scotland done before the advent of COVID-19 vaccines,[12] PRNT showed neutralizing antibody positivity of 2.8% and 1.3% in primary care and pediatric patients, respectively. They then determined the specificity of 5 different commercial serologic products that had received emergency use authorization from the FDA. The calculated specificities were 99.1%, 98%, 95.4%, 98.3%, and 97.4%. Based on those, the positive predictive values were 75%, 56.3%, 38.5%, 62.5%, and 52.6%, respectively. This shows that even with slightly lower than 100% specificity, positive predictive values can drop, yielding an unreliable result.

To further confound matters, on the regulatory side, the FDA unfortunately relaxed criteria for test approval and marketing on March 16, 2020. Manufacturers then flooded the market with test kits, some based on questionable data, before the FDA changed its policy in late April 2020. The FDA later admitted to this mistake in a published perspective, although they still put the onus on the laboratories that offer the tests.[13] In the post-COVID-19 vaccine era, although pre-test probability (seroprevalence to spike or its domains) has artificially gone up, such tests are obviously useless for diagnosing infections as antibodies can very well be due to vaccination.

Last but not least, and as an exception, serology testing could be used to help diagnose multisystem inflammatory syndrome in children if SARS-CoV-2 RNA tests are negative and there are no other alternative explanations for the clinical condition.[14] Such results, however, must be interpreted with extreme caution, especially before intravenous immunoglobulin therapy is given

## IS IGG SEROLOGY RELIABLE FOR EVALUATING LACK OF INFECTIVITY OR IMMUNITY TO REINFECTION?

The short answer to both questions is no. Patients with a positive IgG result may still be sick and can shed the virus in their respiratory secretions or stool. Upper respiratory samples can remain positive for viral RNA for several weeks after onset, when patients typically have IgG antibodies.[5] Viral shedding in stools has been reported for up to 47 days after infection, which speaks against authentic neutralizing capacity of tissue-transudated IgG and in situ-produced secretory IgA antibodies.[15] Thus, having circulating neutralizing antibodies may not ensure lack of infectivity.

### Reinfection immunity

After the first wave of the pandemic, reinfection case reports flooded the literature, casting doubt on possible long-term immunity consistent with what is known about common coronaviruses.[16,17] In several reports, individuals had high-titer antibodies detected either by authentic PRNT or commercial kits that detect IgG to receptor-binding domain of the spike protein shortly before or at the same time as the onset of the reinfection episode. This was also the case with post-vaccination breakthrough infections.[18,19] The clinical point is that the presence of neutralizing antibodies does not mean protection.

Regarding COVID-19, the correlate of protection is not known yet, although it has been established for many other viral diseases. For example, the correlate of protection for hepatitis B virus infection is anti-surface antigen antibody level at or very close to 10 mIU/mL, a level routinely used for occupational health purposes. For COVID-19, the correlate of protection has to be inferred from randomized controlled trials, which are ongoing. It is also necessary to standardize serological assays as it would allow unbiased comparisons across the studies. Therefore, determination of the immune status of individuals, including healthcare workers, to SARS-CoV-2 infection cannot be established at this time using serology.[1,2]

### Additional complications

To further confound matters, an individual can be infected and become sick with common coronaviruses during any season and sometimes several times during a season. This suggests that immunity to some coronaviruses is short-lived, and lingering IgG antibodies from previous seasons does not mean an individual is necessarily immune to infection with the same coronaviruses. Furthermore, cell-mediated immunity (typically mediated through $CD8^+$ T cells) plays a pivotal role. In a well-designed study by Oberhardt and colleagues, $CD8^+$ T cells were shown to play a role in protection against COVID-19 when neutralizing antibodies were not present.[20] It is important to highlight that due to convenience in collecting blood and in performing serology, the common misconception remains that the correlate of protection is neutral-

izing antibodies, but since other accompanying immune parameters are seldom measured, their role is under-recognized. In fact, in patients with multiple sclerosis who had been on anti-CD20 therapy, it was found that despite very low seroconversion rate and compromised circulating follicular helper T cell responses, they had "augmented" SARS-CoV-2-specific $CD8^+$ T cells induction while preserving Th1 cell priming compared with healthy adults.[21]

It has been shown that neutralizing antibodies peak at 31 to 35 days after onset of COVID-19 where only 54% of the sera had $PRNT_{90}$ titer of 160 or higher. Beyond 35 days, such titers were only seen in 24% of the subjects.[22] Also, PRNT performed 8 months after vaccinations showed more than 33- and 43-fold drops in titers for mRNA-based vaccines.[23] Furthermore, SARS-CoV-2 can trigger syncytium formation among lung epithelial cells, thereby paving the way for cell-to-cell transmission of the virions. In this way, virions may continue to be infectious in a patient by escaping protection from antibody neutralization. This is efficiently done by the delta variant.[24] Additionally, lectins enhance the infection.[25] Of note, the latter 2 phenomena cannot be replicated by the current commercial and noncommercial serological assays, leading to overestimation of the so-called immunity.

### Clinical implications

To summarize, given the studies mentioned earlier plus that the correlate of protection has not been fully established, it is important to note that serological testing results, typically in the form of IgG levels against the spike protein or its domains, do not predict severe COVID-19 in the future, do not predict the need for vaccination (including one or more boosters), or the need for prophylactic or therapeutic monoclonal antibodies, especially in the context of the omicron variant emergence. Furthermore, serological testing results cannot predict the susceptibility or immunity to any variants.

## IS IGG SEROLOGY RELIABLE FOR SCREENING COVID-19 CONVALESCENT PLASMA?

Treatment of infected patients with convalescent plasma was initially deemed as a reasonable option in the absence of antivirals and therapeutic monoclonal antibodies. To that end, and after several iterations, the FDA changed the neutralizing antibody titer to greater than 250 (based on $PRNT_{50}$) and revised several commercial products with updated signal-to-cutoff ratios for each in relation to this particular clinical use. Several clinical trials were conducted based on the available commercial products using FDA-recommended cutoffs. Most of these trials used even higher signal-to-cutoff ratios to ensure presumed efficacy. As of this writing, the overwhelming majority of the clinical trials failed to show any efficacy in composite outcomes (eg, hospitalization, intensive care unit admission, mechanical ventilation, death) by 30 days after onset or admission even when convalescent plasma was given within the first few days.[26] Antibody-dependent enhancement has been shown in coronaviruses, which may potentially lead to adverse outcomes. Although this may also have implications for vaccine design (similar to that of the Dengue vaccine), it may also lead to potential adverse outcomes for convalescent plasma therapy.[27,28] Similar to monoclonal antibodies, convalescent plasma use, especially in immunocompromised patients, may lead to mutations and the emergence of new variants with a large number of mutations.[29]

## IS IGG SEROLOGY RELIABLE FOR ASSESSING HERD IMMUNITY?

At the beginning of the pandemic when there were uncertainties about serosurveillance, the answer to this question was maybe, but even then it was very dependent on the specificity of the assay. Ideally, once fine-tuned assays are available and resources allow, impact assessments will need to be done in large scale, collaborative studies performed using well-balanced and unbiased sampling approaches that include multiple age, sex, race/ethnicity, and geographic cohorts. This was not coherently done for SARS-CoV-2. In fact, studies relied on convenient sampling and usually on one commercial or an in-house developed test. As a result, such studies led to unusually high seroprevalences, overestimation of asymptomatic-to-symptomatic ratio, and low case-fatality ratios. The main issue with such overestimation would be undue relaxing of nonpharmaceutical interventions, setting the stage for more virus evolution and appearance of new variants. An excellent review is available on the best sampling approaches for unbiased serosurveys.[30]

The main reason why such serosurveys are done is to assess whether a given population has reached herd immunity. Herd immunity to SARS-CoV-2, however, is elusive.[31] There are three main reasons for this.

First and foremost, as noted earlier, the immune correlate of protection against COVID-19 has not been fully established. This means that the mere presence of antibodies in an unvaccinated individual does not signify protection, as evident by the occurrence of

reinfections and breakthrough infections.

The second reason is that assays have low specificity, especially when used as a stand-alone test instead of being followed by PRNT or at least in an orthogonal approach. The latter should include 2 well-characterized assays with specificities preferably over 99%. There are 2 great examples of this in the literature published before vaccines. One is a study from Wuhan, China, in which 2 in-house developed tests were used: a receptor-binding domain-based test and a nucleoprotein-based test. Results were confirmed using an authentic PRNT. The result was a seroprevalence of 2.29% in a large population of healthy blood donors shortly after the first wave.[32] The other is a study from Japan in which they used an orthogonal approach by screening general population using 2 well-known commercial assays with results confirmed by PRNT.[33] The result was a seroprevalence of 0.1%.

These results simply tell us that with such low seroprevalences, a test with a claimed specificity of 99.8% would have a very low positive predictive value, culminating in overestimation of the seroprevalence when used as a stand-alone test. The downside is that such levels may be misinterpreted as evidence of herd immunity. For example, in a study from India that used a popular commercial assay with a claimed specificity of 99.7%, authors arrived at a seroprevalence of 54.1%.[34] This was shortly before the COVID-19 delta wave hit India, resulting in a catastrophic outcome. Given that this seroprevalence was considered to be near herd immunity, why had this still happened? The answer is a combination of using suboptimal serosurvey approaches, a lack of established correlate of protection, and the appearance of new variants. The most recent example of the latter is the emergence of omicron variant with a reinfection rate much higher than that of the prevalent beta and delta variants in South Africa, which had a pre-omicron seroprevalences as high as 59%.[35]

The third reason is that the seroprevalence is underestimated owing to waning antibody levels and sero-reversion over time. In addition, the fortunate arrival of COVID-19 vaccines has altered the seroprevalence estimates as vaccinated individuals typically should have anti-spike antibodies; therefore, serosurveys to look for evidence of natural infection should be limited to nucleocapsid-based assays that usually offer lower specificities than the receptor-binding domain-based assays. This is further confounded in individuals with past infection who are vaccinated or in individuals who experience breakthrough infection; in both scenarios, anti-spike and anti- nucleocapsid antibodies may be present.

Here is some math to consider. As of January 2022, there have been more than 59 million definite cases of COVID-19 reported in the United States. If we assume that these only constitute 20% of the total cases (based on some serosurveys), that would calculate to about 285 million cases. Taking the current United States population into account, that would give us an estimated seroprevalence of 86%. This is before including the population that has received 1 (74%) or 2 doses (60%) of the vaccines. One could assume that such a high percentage would almost equal herd immunity, but the reality of the steep rise in new cases every day is in sharp contrast to that assumption. To clarify, this is not meant to smear the tremendous success that vaccines have achieved in reducing hospitalization and death, but rather to highlight the lack of reliability in messages that serosurveys may send to us confirming the elusive nature of herd immunity.

## CONCLUSION

In the end, it is important to note that serology is a convenient measure to assess immunogenicity in vaccine trials, but it should be limited to that context — it should not be used in clinical decision-making. This notion, as stated earlier, is endorsed by both CDC and FDA and includes both immunocompetent and immunocompromised patients regardless of past COVID-19 diagnosis or vaccine history. Properly validated assays should be used for such immunogenicity trials where authentic PRNT is not readily available. It is also important to distinguish immunity from immunogenicity as two separate concepts when reading and interpreting vaccine trials results. Assessing other immune parameters such as specific $CD8^+$ T cells markers and functionality should not be abandoned for the sake of labor intensiveness or technical challenges and be instead replaced by more convenient serology. The ultimate goal is to find the correlate of immune protection, but that is not necessarily to use it clinically but to fine-tune our current vaccine arsenal to properly respond to this pandemic.

## DISCLOSURES

The author reports no relevant financial relationships which, in the context of their contributions, could be perceived as a potential conflict of interest.

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