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REVIEW ARTICLE

Approaching the Interpretation of Discordances in SARS-CoV-2 Testing

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The coronavirus disease 2019 pandemic has upended life throughout the globe. Appropriate emphasis has been placed on developing effective therapies and vaccines to curb the pandemic. While awaiting such countermeasures, mitigation efforts coupled with robust testing remain essential to controlling spread of the disease. In particular, serological testing plays a critical role in providing important diagnostic, prognostic, and therapeutic information. However, this information is only useful if the results can be accurately interpreted. This pandemic placed clinical testing laboratories and requesting physicians in a precarious position because we are actively learning about the disease and how to interpret serological results. Having developed robust assays to detect antibodies generated against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and serving the hardest-hit areas within the New York City epicenter, we found 3 types of discordances in SARS-CoV-2 test results that challenge interpretation. Using representative clinical vignettes, these interpretation dilemmas are highlighted, along with suggested approaches to resolve such cases.

Keywords. place holder 1; place holder 2; place holder 3.

The coronavirus disease 2019 (COVID-19) pandemic is affecting every corner of the globe. Fifteen months after initial cases were reported in Wuhan, China, in December 2019 [1], the global number of cases exceeded 116 million, with >2 500 000 deaths (World Health Organization). The United States was hit particularly hard, accounting for ~25% of all cases and deaths while constituting only ~5% of the global population. New York City became the initial epicenter of the American outbreak, reaching >6300 daily hospitalizations in early April 2020. In addition to mitigation efforts, a critical aspect of controlling local outbreaks includes implementing robust testing schemes [2].

Soon after, the genome sequence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of COVID-19, was available [3], multiple testing paradigms were developed to detect SARS-CoV-2 infection [4]. Initially, these assays focused on detecting viral RNA [4–7], the gold standard for active infection. However, this genome sequence also allowed cloning and purification of viral proteins, thereby enabling development of serological testing [8]. Serological assays provide essential information about infectious diseases, including (1) identifying acute, subacute, and prior infections through detecting pathogen-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) isotypes, respectively, to enable determination of true disease prevalence [9]; (2) providing insights into host immune responses to infection [10]; (3) quantifying antibody titers in recovered patients to identify convalescent plasma donors [11]; and (4) enabling isolation of neutralizing antibodies to produce therapeutic monoclonal antibodies [12–15]. Therefore, serological assays provide important diagnostic, prognostic, and therapeutic value.

There are many types of serological assays, including lateral flow, enzyme-linked immunosorbent assay (ELISA), immunoblotting, and functional readouts, such as viral neutralization assays [16]. Discordances can exist between assays that utilize different techniques and assays that use the same approach [9, 17, 18]. As 2 leading academic institutions in New York City, serving the hardest-hit areas within the initial US epicenter, we independently developed and clinically validated ELISAs to measure IgM, IgG, and immunoglobulin A (IgA) antispike antibodies (Albert Einstein College of Medicine [Einstein]) [19] and IgM and IgG antispike antibodies and IgG antinucleocapsid antibodies (Columbia University Irving Medical Center [CUIMC]) [20]. These assays performed similarly, with sensitivities of up to 91% and 93% and specificities of 99% and 96% for the Einstein and CUIMC assays, respectively.

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Cross-validations comparing these platforms were highly concordant. However, both institutions encountered several cases yielding challenging, difficult-to-interpret results. We categorized such cases based on the following types of result discordances: (1) antibodies recognizing nucleocapsid vs spike antigens; (2) nucleic acid testing vs serology; and (3) differences between household members. Representative clinical vignettes of each type of discordance are presented herein, along with a discussion of how they might be interpreted.

Discordance Between Antispike and Antinucleocapsid Antibodies

A 30-year-old male, with a medical history of cutaneous marginal zone lymphoma treated with 5 cycles of rituximab (1050 mg/dose), presented to the emergency department with 2 weeks of fatigue and fever (T_{max} 102.4), 1 week of loose stools and cough, a 30-lb weight loss over 1 week, and increasing shortness of breath. He was also tachycardic and hypoxic (O_2 saturation of 90%). Diffuse patchy opacities on chest x-ray suggested multifocal bacterial and/or viral pneumonia. A nasopharyngeal swab for SARS-CoV-2 reverse transcription polymerase chain reaction (RT-PCR) was positive. He was admitted, placed on 2 L of oxygen, and started on a 5-day course of hydroxychloroquine/azithromycin along with a short course of piperacillin-tazobactam and vancomycin. His hospital course was complicated by acute renal failure, but he recovered without requiring intubation or hemodialysis and was discharged after 10 days. A serum sample collected 5 days after presentation (~3 weeks after symptom onset) was tested for SARS-CoV-2 antibodies using a laboratory-developed ELISA and was positive for antinucleocapsid IgG, but negative for antispike IgG and IgM antibodies.

The spike and nucleocapsid proteins are 2 of the 4 main structural proteins of SARS-CoV-2. Spike is a glycoprotein on the virion surface, which binds the human angiotensin-converting enzyme 2 (ACE2) receptor and mediates viral entry into host cells [21, 22]. Nucleocapsid protein resides inside the viral envelope and encapsulates the RNA genome [23]. Because SARS-CoV-1 spike protein and nucleoprotein are highly immunogenic [24, 25], their orthologs became obvious targets for antibody testing when SARS-CoV-2 emerged. But how are results to be interpreted when someone has antibodies against 1 protein but not the other?

Because SARS-CoV-2 serological tests are widely utilized, it is increasingly important to understand the breadth and diversity of testing platforms, the caveats for interpretation, and how to reconcile discordant results when using different testing paradigms. As of August 2020, 33 serological methods have received emergency use authorization (EUA) from the Food and Drug Administration (FDA) [26]. These tests utilize various antibody detection approaches, including ELISA, magnetic chemiluminescence enzyme immunoassay (MCLIA), immunofluorescence, and lateral flow assays; they also measure different immunoglobulin isotypes, including IgM, IgG, and/or IgA. Among the 18 high-throughput tests, differences remain depending on which viral proteins are targeted. Spike is the most common target, used in 11 tests. However, nucleocapsid is used in tests developed by vendors (eg, Roche, Abbott) that supply reagents to many clinical laboratories, including many national reference laboratories. Therefore, knowing which antigen is detected and which immunoglobulin isotype is measured is critical for interpreting results, particularly as many clinical laboratories utilize multiple platforms. This is particularly relevant with the widespread administration of vaccines targeting the spike protein. Patients vaccinated with spike antigen–based vaccines will only be positive in assays that test for antispike antibodies and will lack antibodies that recognize the nucleocapsid antigen unless they become infected by SARS-CoV-2 and develop an immune response to that infection.

In our experience, discordant results between antibodies against the spike and nucleocapsid proteins are not infrequent. Indeed, clinical testing of >5000 individuals, which were a mix of PCR-confirmed, suspected, and asymptomatic cases, found that 12% of these patients had antibodies that recognize only 1 of these proteins (data not shown). However, the explanation is not simply due to differences in assay sensitivity; indeed, sensitivities for antispike and antinucleocapsid assays are comparable overall, not only with the CUIMC ELISA, but also with most published methods [27]. Therefore, what are plausible explanations for patients to exhibit only antinucleocapsid antibodies, but not antispike antibodies?

Four human coronaviruses (hCoVs) cause seasonal upper respiratory tract infections: OC43, HKU1, NL63, and 229E. One explanation for only finding antinucleocapsid antibodies may be due to cross-reactivity between SARS-CoV-2 and these seasonal coronaviruses. The spike protein is the most divergent antigen among hCoVs, whereas the nucleocapsid is reasonably conserved [28, 29]. Therefore, spike protein–based tests are considered more specific [9, 28]. However, the magnitude of a humoral response depends greatly on antigen dose, and the nucleocapsid is the most abundant viral protein. Thus, nucleocapsid-specific assays may have some advantage in detecting antibodies early during infection when the number of infected cells is low and the amount of all expressed viral proteins is limited [30, 31].

Although evidence is still emerging, a difference in the rate of decay of anti-SARS-CoV-2 antibodies might provide another explanation for detecting antibodies against 1 protein but not the other. Recently, the half-life of antibodies to the spike protein, specifically to the receptor binding domain, were analyzed. Spike-specific antibodies rapidly declined in patients with mild COVID-19 symptoms who recovered from the disease, with a half-life of 36 days [32]. However, no equivalent reports gauged the decay rate of nucleocapsid-specific antibodies after recovery from COVID-19; nonetheless, following SARS-CoV-1

infection, antibodies to the nucleocapsid are more long-lasting than those to other structural proteins [33].

As human leukocyte antigens (HLAs) orchestrate immune responses and are associated with the magnitude of antibody response to several viruses, including hepatitis B and influenza [34, 35], host genetic variability may contribute to the discordant antibody response between spike and nucleocapsid proteins. Indeed, in silico analyses of viral peptide–MHC class I binding affinity revealed that the capacity to present SARS-CoV-2 viral peptides varies significantly between HLA molecules [36]. These differences could impact the activation of the cellular immune response and antibody response to SARS-CoV-2, as well as disease severity [37].

Another possible explanation relates to the presence of underlying comorbidities. While validating our laboratory-developed ELISAs, we found that patients who were immunocompromised or had a recent history of cancer (such as the patient described above who recently received rituximab to treat his lymphoma) were particularly enriched in the group of patients who only had antibodies against the nucleocapsid antigen. How an immunocompromised state affects antibody responses to 1 but not all viral proteins remains unclear. However, underlying malignancies and immunomodulatory therapies are associated with altered or delayed antibody responses to SARS-COV-2 [38, 39].

Discordance Between Viral Nucleic Acid and Serological Testing

A 23-year-old male with no significant medical history experienced mild symptoms and tested positive by RT-PCR for SARS-CoV-2 during screening in New Rochelle, the site of the initial outbreak in the greater New York City area. He did not require oxygen support and isolated at home. After identification as a potential convalescent plasma donor, a serum sample was collected 34 days after symptom onset to screen for anti-SARS-CoV-2 antibodies. The patient was negative for antispike IgA and IgG antibodies by ELISA.

Given the recent importance placed on serological tests as a measure of immune protection, a negative SARS-CoV-2 antibody result, despite previous diagnosis by RT-PCR, can confuse clinicians and patients. The pretest probability is relatively high that the patient had COVID-19 based on a history of having symptoms and being from a high-risk area. Therefore, it is unlikely that the initial RT-PCR result was a false positive. It is important to consider conditions that could explain the negative serological result in an individual who had COVID-19, including the test type, disease severity, and timing of sample collection.

Here, an antispike IgG and IgA ELISA was used to analyze the patient's serum. As discussed above, some reports indicated earlier seroconversion of antinucleocapsid, as compared with antispike, antibodies. Given that most individuals seroconvert for IgG by 14–20 days after symptom onset, regardless of assay (and antigen) utilized [10, 40], a negative result at 34 days could be considered unlikely. However, although most studies evaluated time to seroconversion for acutely ill, hospitalized patients, this patient had a mild symptomatic illness not requiring hospitalization or oxygen supplementation, and individuals with mild disease have been shown to have lower overall antibody responses in several recent studies [10, 41–44]. It is also possible that this individual had a robust humoral immune response that rapidly waned. Indeed, some studies observed a decline in antibodies in the first 3 months after infection; nonetheless, most individuals continue to have detectable antibodies during this period [32, 45, 46]. Additionally, in 2 studies on convalescent patients, individuals with no or extremely low levels of anti-SARS-CoV-2 antibody on initial testing were mostly positive when retested at a later time, though a small number failed to seroconvert [47, 48]. Depending on test sensitivity, low levels of antibody may not be sufficient to provide a positive result on a qualitative or semiquantitative test, and factors that can result in lower antibody responses should be considered. An adult patient with a positive RT-PCR result and mild disease who initially tests negative for anti-SARS-CoV-2 antibodies warrants a second test, potentially using nucleocapsid as the target antigen. A similar sequential testing approach was deployed when screening health care workers, with equivocal results [49].

A 55-year-old male presented with shortness of breath and fever up to 103.5°F for 9 days, with no improvement on outpatient treatment with azithromycin and nonsteroidal anti-inflammatory drugs. A chest x-ray demonstrated bilateral infiltrates, and a chest computed tomography (CT) scan demonstrated bilateral airspace consolidations in the lower lobes of the lungs with diffuse nodules and ground glass opacities in all lobes. Tests for other viral and bacterial infections, including a viral respiratory panel, were negative. RT-PCR of nasopharyngeal swabs for SARS-CoV-2 was negative 3 times. Due to a high clinical suspicion for COVID-19, a serum sample was collected and serological testing was positive for IgG, IgM, and IgA antispike antibodies. The patient was intubated due to severe hypoxic respiratory failure, but later succumbed with multiorgan failure 25 days after symptom onset.

Although the current gold standard for SARS-CoV-2 diagnosis is RT-PCR, a negative result, even in a patient with high clinical suspicion, is not rare. RT-PCR testing can have false-negative rates as high as 54%, though this is highly variable depending on the study and the test utilized [50]. As with serological testing, timing of sample collection is important and is intimately related to the kinetics of virus replication. Based on a meta-analysis of 7 studies, the false-negative rate for RT-PCR is highest before symptom onset (<5 days postexposure), is lowest ~8 days postinfection, and then steadily increases [51]. Importantly, repeat testing increases diagnostic sensitivity, even when conducted on the same day as the initial test [7], although retesting remained negative in this patient. In addition to

analytical errors in conducting RT-PCR testing, pre-analytical variables, including timing and method of sample collection, sample handling, and specimen type, also affect diagnostic performance [52]. Indeed, bronchoalveolar lavage fluid and sputum had higher sensitivity for viral RNA detection than the commonly collected nasopharyngeal swab specimens [53], thus providing a potential reason for this patient's multiple negative RT-PCR results. One possible explanation of this phenomenon may include the presence of viral particles only in the lower respiratory tract vs the upper respiratory tract. In this scenario, a patient would presumably still mount an immune response without detectable virus in the upper respiratory tract.

Given these issues with RT-PCR, adjunct diagnostic tests are useful to increase diagnostic sensitivity. CT imaging was adopted early in the pandemic, given reports of characteristic shared features in infected individuals [54] and increased sensitivity when combined with RT-PCR [55–57]. Serological testing is a potentially more accessible and cost-effective adjunct for diagnosis. There is growing evidence for positive serology in suspected patients with negative RT-PCR results [42, 58], and testing schemes combining molecular and serological testing increase the sensitivity for diagnosing disease [10, 59]. Although RT-PCR is more useful for detecting infection early, before an adequate antibody response occurs, serological testing is more sensitive at later time points, especially >15 days after symptom onset, when most infected individuals have seroconverted [10, 42]. The high sensitivity of serological tests later in the disease course allows for a high positive predictive value for diagnosis in the context of high seroprevalence (>90% in areas like New York City with >20% seropositivity) [19, 60].

Household Discordance

A 9-year-old male in a family of 4 presented with a mild cough and fever in March 2020. The patient had an uneventful course and fully recovered with supportive care at home. During this period, the patient's brother was in constant contact with the patient, and the pair were said to be "all over each other all the time." Both the mother and father were involved in caring for the patient, and all 4 members quarantined in the same household and did not practice social distancing at home. Of note, the father had a mild cold in January 2020 that tested positive for coronavirus HKU1. Serological testing was performed on the entire family ~6 weeks after the onset on the patient's symptoms. The patient tested positive for antispike IgM and IgG, as well as antinucleocapsid IgG. The father tested positive for antinucleocapsid IgG and negative for antispike antibodies. Both the mother and brother tested negative for all these antibodies.

The reproductive number (R_0), a basic epidemiological measurement, represents, on average, the number of people the disease is spread to by an infected individual. R_0 describes the relative "contagiousness" of a pathogen and varies dramatically between organisms. For example, one of the most infectious agents is measles virus, with an R_0 that is normally stated as 12–18, but was reported to approach >200 in certain conditions [61], whereas the 1918 H1N1 flu was estimated to have an R_0 of ~2.0 [62]. Initial reports suggested that the R_0 for SARS-CoV-2 was 1.4–2.5 [63], with more recent estimates reaching as high as 5.7 [64]. Given the relatively high R_0 for SARS-CoV-2, how is it that no other household member, when they were constantly around one another in quarantine, was infected by this patient? This raised concerns about whether the patient was a false positive, or conversely, whether the family members were false negatives.

Like most clinical serological assays, ours was a single-point assay. A single-point assay uses a predetermined serum dilution to perform the assay. Therefore, if a patient has low titer antibodies, then the dilution used may not be able to detect such low antibody levels. The most definitive way to identify low antibody levels is to perform an antibody titer. To this end, serial serum dilutions are made and assayed to determine the titer. We performed our ELISA using serial serum dilutions to detect IgM and IgG antibodies recognizing the spike antigen and found that the other family members were true negatives, whereas the patient had a high titer. How, then, can the lack of spread in this family be explained?

Systematic retrospective studies from China provided empirical evidence that household transmission, unintuitively, is actually fairly limited [65, 66]. An initial study from 2 hospitals near Wuhan studied 105 index patients and 392 family household contacts [66]. Of these 392 household members, only 16.3% (64 members) subsequently contracted SARS-CoV-2 from the index patient. This rate was noted to be higher than that reported for SARS-CoV-1 and MERS, 2 related coronaviruses [67, 68]. Interestingly, the secondary attack rate for household members under age 18 was 4%, whereas it was 20.5% for those age >18, demonstrating more frequent transmission among adults than children. Within their cohort of 105 index patients, 14 immediately self-quarantined within their households "with masks, dining separately, and residing alone" [66], resulting in no subsequent family member becoming infected. Although this needs replication in larger studies, the initial results suggest that quarantining infected individuals within the household may significantly curb household transmission. Similarly, another retrospective analysis of contact tracing data in Guangzhou identified a household transmission rate of 17.1% [65]. This study also found a positive correlation between transmission rate of an infected adult to other household members based on age, with household members <20 years of age having a secondary attack rate of 5.2%, whereas those age >60 had a secondary attack rate of 18.4%. Overall, these studies identify a household transmission rate of ~16%–17%, with children being the least susceptible.

Household members spend significant time with each other in close proximity, particularly during quarantine, yet SARS-CoV-2 spreads to <20% of household members. This poses a conundrum of how the virus has spread so efficiently to every corner of the globe. It was noted that the spread of several infectious agents occurs from a small number of "superspreaders" who infect a disproportionate number of contacts [69, 70]. This phenomenon is described as the 20/80 rule, where 20% of infected individuals transmit the disease to 80% of infected individuals. Indeed, the 2003 SARS epidemic in Asia was thought to have been spread primarily by superspreaders [69]. Increasing evidence points to superspreading events as a prime source of spread of COVID-19 [71–74]. Therefore, local household spread, especially coupled with self-quarantining, may not represent a significant mode of COVID-19 spread.

Although analyses of hospitalized patients have identified common features predisposing individuals to severe disease [75], the characteristics of superspreaders are not known. Similarly, characteristics that enable individuals to resist infection are also not known. One emerging idea proposes that uninfected individuals may have underlying T-cell-mediated immunity [76]. Intriguingly, SARS-CoV-2-infected individuals, and even pre-COVID-19 specimens, had evidence of CD4 and CD8 T-cell-mediated immunity against SARS-CoV-2 [76]. In particular, these T cells evoked responses against the SARS-CoV-2 nucleocapsid protein. This is significant because the nucleocapsid protein is relatively well conserved among the betacoronaviruses, including those causing the common cold. Therefore, it is enticing to speculate whether infection with other coronaviruses provides T-cell-mediated protection to SARS-CoV-2, such as in this patient's father, who had a recent HKU1 infection. In addition to the the role of the adaptive immune system, genetic and serological studies of severe cases of COVID-19 found an enrichment of loss-of-function mutations or autoantibodies that target type I interferon–related genes [77, 78], implicating the importance of the innate immune response and its interplay with the adaptive immune system to combat SARS-CoV-2.

Final Thoughts

The primary concern of patients undergoing serological testing is "Am I immune?" This concern was further amplified when certain governments, including the United States, floated the idea of issuing "Immunity Passports" to allow those with SARS-CoV-2 antibodies to travel freely, while restricting others. In addition to the ethical issues associated with such a system [79], it fundamentally misunderstands the role of serological testing. Its main clinical utility lies in identifying those who were previously infected, and it does not provide information about whether a patient is immune per se. That is, the presence of antibodies is not synonymous with protective immunity, nor is the absence of antibodies synonymous with viral susceptibility. The clinical vignettes discussed herein highlight situations where individuals may fall into such discordant categories.

Neutralizing antibodies are thought to confer immunity and seem to be specific to the SARS-CoV-2 spike protein [80]. Therefore, it is unclear whether individuals who have antinucleocapsid antibodies, but not antispike antibodies, are protected from future infection. This is particularly relevant because many commercial testing platforms only detect antinucleocapsid antibodies. Furthermore, we found that not all individuals with antispike antibodies have viral-neutralizing antibodies [81]. Because ELISAs cannot specifically identify the presence of neutralizing antibodies, no definitive conclusions can be made whether an individual who tests positive will be protected.

In contrast, a negative test does not necessarily exclude the possibility of protection. As indicated above, if the assay only detects antinucleocapsid antibodies, it is still possible that the patient has antispike antibodies, a subset of which may be neutralizing. Even if the ELISA detects antispike antibodies, most clinical assays are single-point tests, using only 1 dilution of serum or plasma to detect the presence of antibody, and call a test positive if the signal reaches a predetermined threshold. Thus, it is possible that an individual who tests negative simply has low titers that are not detected at the dilution used for the assay. This may be particularly relevant when testing is performed several months after symptom onset. Even though a normal antibody response eventually wanes, the presence of the appropriate memory B cells can still provide protection. In addition to the humoral response, T-cell-mediated immunity may provide protection. The role of T-cell immunity in SARS-CoV-2 infection is only recently beginning to be unraveled [82]. Intriguingly, unlike the humoral response, preexisting primed memory T cells that recognize SARS-CoV-2 may be present in samples from the pre-COVID-19 era [76, 83]. Thus, infection with other common human coronaviruses may extend T-cell-based protection against SARS-CoV-2. This suggests that, even in the absence of SARS-CoV-2 antibodies, some individuals may have protective T-cell-mediated immunity.

The discordances discussed herein highlight some interpretation challenges faced by clinical laboratory personnel and requesting physicians. Physicians should be aware of the details of the assay performed (eg, which antigen is used, which isotype is detected, whether the assay is a single-point assay) to interpret results properly in order to guide management, particularly with the widespread administration of spike antigen–based vaccines. Most importantly, physicians must inform patients that serological testing does not determine if they are immune, but rather provides evidence of prior infection. Viral neutralization assays and T-cell-based assays represent the next phase of testing paradigms, which will need to be clinically implemented to help answer the question of "Am I immune?"

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