Abstract

The landscape of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) diagnostic testing is rapidly evolving. While serology testing has limited diagnostic capacity for acute infection, its role in providing population-based information on positivity rates and informing evidence-based decision making for public health recommendations is increasing. With the global availability of vaccines, there is increasing pressure on clinical laboratories to provide antibody screening and result interpretation for vaccinated and non-vaccinated individuals. Here we present the most up-to-date data on SARS-CoV-2 antibody timelines, including the longevity of antibodies, and the production and detection of neutralizing antibodies. Additionally, we provide practical guidance for clinical microbiology laboratories to both verify commercial serology assays and choose appropriate testing algorithms for their local populations.

Keywords: COVID-19, SARS-CoV-2, serological testing, serology algorithms

Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019 resulted in a rapid global development of molecular, antigenic and serological assays for the diagnosis of acute infection and identification of past infection. While molecular testing is widely accepted as the gold standard for diagnosis of acute infection, the role of serology is limited to special clinical cases as an adjunct for diagnosis (1). The SARS-CoV-2 antibodies are not reliably detected fewer than 7–21 days post symptom onset, making their utility in diagnosis of acute infection of limited value (2–4). However, in cases such as multiple inflammatory syndrome in adults (MIS-A) or in children (MIS-C), a positive result on a serological assay can help guide clinical management in the absence of a positive molecular test result or when molecular testing is unavailable, and positive serology results have been included as part of the clinical case definition for MIS-C (5). Outside these very specific clinical scenarios, the role of serology assays is to examine population-based prevalence rates of SARS-CoV-2, and to help inform public health decisions.

To improve the utility of serology testing, an international standard is required to allow direct comparison of assays between laboratories. Population-based studies linking quantitative serology results to clinical outcomes will be needed to help determine what level of antibody may correlate with immunity to infection. Such approaches would be similar to what has been done with other viruses (e.g. rubella), where an international standard is used to calibrate assays and a quantitative serological immunoglobulin G (IgG) is used to determine immunity (more than 10 IU/ml), or
susceptibility (less than 10 IU/ml) to infection, based on correlation with clinical outcomes (6,7). Multiple quantitative serological assays have been submitted to accreditation agencies globally, including the Food and Drug Administration (FDA), Health Canada and European Council (Conseil européen); however, to date in North America, there remains a gap in approved quantitative assays that have a correlation with immunity from infection (i.e. neutralizing antibodies). While this will not impact clinical decision making on an individual level, this remains a critical gap in the interpretation and utility of SARS-CoV-2 serology testing for serosurveillance studies.

Here we examine the current knowledge of serological testing, discuss assay limitations, describe how clinical laboratories can both validate these assays and implement appropriate algorithms for local patient populations, discuss the role for differentiating antibodies derived from natural infection versus those that are vaccine-derived and consider options for detection of neutralizing antibodies.

**Seroconversion timelines**

The antibody response to SARS-CoV-2 is relatively well studied; however, there is substantial variability in seroconversion timelines given the heterogeneity amongst populations studied (i.e. disease severity, age, presence of comorbidities, etc.), serologic tests used and serologic markers analyzed. In general, the overall range of seroconversion regardless of the type of antibody, is estimated to be between four and 14 days post-onset of symptoms (2,8,9). An early study evaluated seroconversion rates in 173 patients and reported median seroconversion times for total antibody (Ab), immunoglobulin M (IgM), and IgG that were 11, 12 and 14 days, respectively. However, the authors reported that fewer than 40% of patients had detectable antibodies within one week of onset of illness and this rose to 100% (total Ab), 94% (IgM) and 80% (IgG) by day 15 in the same patients (10). In contrast, another study reported the seroconversion rate of immunoglobulin A (IgA) was similar to IgM, with a median seroconversion of five days (IQR 3–6) after symptom onset compared with 14 days for IgG (11). Despite earlier detection of IgM in those studies, the time course for IgM and IgG seroconversion rates are similar for SARS-CoV-2 compared with other infectious diseases, where IgM preceded IgG by weeks. Indeed, both IgG and IgM detection signals were found to plateau six days after the first positive serology test among 285 coronavirus disease 2019 (COVID-19) patients (9). Thus, while studies agree that seroconversion occurs within 4–14 days of symptom onset and that IgG and IgM seroconversion dynamics are similar, the considerable variability leads to poor sensitivity of antibody testing for diagnosis of acute COVID-19, which has been well documented in the literature (1,2,12).

**Antibody longevity**

The length of time that antibody responses persists, and possibly confer protection from reinfection, is pivotal to understanding SARS-CoV-2 infection dynamics (detailed timeline for antibody detection can be seen in (9)). Using sequential serum samples from 65 patients and 31 seropositive healthcare workers, Seow et al. (13) showed seroconversion of IgM, IgG and IgA occurred in more than 95% of cases when sampled equal to or greater than eight days post-symptom onset. Neutralizing antibody kinetics were consistent with other acute viral infections (13), with an initial peak at 3–4 weeks (magnitude of peak dependent on disease severity) followed by declining neutralizing antibody titres. Interestingly, patients with a high peak infectivity dose maintained neutralizing antibody titres longer compared with patients with a lower peak infectivity dose (13). Neutralizing antibody decline occurred simultaneously with declines in IgG titres against SARS-CoV-2 spike (S) glycoprotein and receptor-binding domain (RBD), as well as IgM and IgA binding to S glycoprotein and RBD. In patients with mild to moderate disease, IgG antibody titres were found to be stable for up to five months, with a significant correlation between anti-spike binding titres and neutralization (14).

Additional studies using plaque reduction neutralization test (PRNT)/microneutralization assays in combination with anti-spike enzyme-linked immunosorbent assay (ELISA) procedures have further validated the findings of longitudinal neutralization antibody duration (15,16). Previously observed declines in antibody titres during the first few months after infection is expected as short-lived plasma cells are depleted; however, when these are replaced by long lived antibody secreting cells, neutralizing antibodies will persist for several months in most individuals (17).

Interestingly, longitudinal antibody longevity studies in serum and saliva showed that IgM and IgA levels rapidly declined following peak levels (18), while IgG remained relatively stable in both biofluids (up to 105 days post symptom onset). Similarly, others found that 92.3% of patients (N=427) remained IgG positive 3–6 months post symptom onset (19). While most evidence suggests that IgM and IgA antibody levels drop significantly compared with IgG isotypes, in patients with a remote history of COVID-19, they appear to play a key role in the initial neutralizing antibody response. Serum IgA was shown to contribute to virus neutralization up to one month following symptom onset (20), while IgM was shown to neutralize SARS-CoV-2 in both pseudoviral particle and wild type virus assays (21).

Lessons on antibody longevity for SARS-CoV-2 may be learned by studying other human coronaviruses; a strategy that has been reviewed extensively elsewhere (22). A recent systematic review highlighted the kinetics, protection correlations and antibody association with disease severity among human coronaviruses. In general, antibody responses to other human coronaviruses, such as SARS-CoV, Middle Eastern respiratory syndrome coronavirus (MERS-CoV) and seasonal coronaviruses, are present for one year after infection; in some cases, antibodies may persist for longer (23). Moreover, other evidence suggests that serum antibody titres remained relatively high two years
after SARS-CoV infection, and up to 55% of patients had detectable antibodies at three years post infection (24). However, no detectable anti-SARS-CoV antibodies were observed in patients six years post infection (25). Similarly, antibodies against MERS-CoV have been shown to persist for approximately three years (26), although the persistence of anti-MERS-CoV antibodies depended on disease severity, as patients with subclinical or mild disease had low or undetectable levels of antibodies two years after infection (27). In contrast, seroprevalence studies of human coronavirus (HCoV)-229E and HCoV-OC43 suggested that antibody titres wane significantly one year after infection (22). The persistence of anti-SARS-CoV-2 antibody response remains to be seen although given the association of the antibody signal and disease severity (13), it is likely that persistence of antibodies will correlate with disease severity.

**Impact of disease severity and age**

Given that many SARS-CoV-2 infections are subclinical or asymptomatic, it is critically important that the antibody response (including titres, seroconversion and time to seronegativity) in these patient populations be well understood. It is now widely recognized that titres of SARS-CoV-2 antibodies are positively associated with clinical severity of disease. Zhao et al., (10) first reported that a significantly higher titre of total antibody was independently associated with a worse clinical classification (p=0.006) at 2-weeks post illness onset. A high total antibody titre was hypothesized to be a risk factor for critical illness and that it may even be used as a surrogate marker for worse clinical prognosis. A similar observation was made in 289 COVID-19 patients clinically categorized as having mild, moderate or severe infection. Patients with a severe infection had significantly higher levels of S1-specific IgA and IgG compared with those with a mild infection (28). Moreover, S1-specific IgG was detectable after two weeks in only 20% of patients in the mild group compared with 100% of patients in the severe and moderate groups.

The impact of disease severity on seroconversion kinetics and their relationship to neutralizing properties in serum is not well understood. When compared to patients with mild symptoms, those with severe symptoms had a significantly faster time to IgG seroconversion (median 22 versus 11 days, respectively) with approximately 10% of patients with mild symptoms never seroconverting (29). Detectable IgG levels were still observed more than 75 days post symptom onset in patients who had seroconverted. Interestingly, even at 90 days post-symptom onset a small number (N=3) of patients developed total antibody levels below the limit of detection of commercial assays yet still had a detectable neutralizing response (titre range of 8–48). This observation is in direct contrast to that reported previously, where sera from 20% of discharged patients had no neutralizing properties despite sera from 100% of patients showing seroconversion (30). Furthermore, in a small study, the development of IgM in patients with severe disease was delayed (N=6; eight days) compared with mild disease (N=39; six days) (31). Jiang et al. (32) evaluated rates of seroconversion in non-severely ill patients with COVID-19, as well as asymptomatic patients, concluding that different IgM/IgG kinetics exist depending on the severity of the disease. Indeed, the authors reported that IgG seroconversion occurred among 94% of symptomatic and 85% of asymptomatic patients while IgM seroconversion occurred in 74% of symptomatic patients and only 31% of asymptomatic patients (p<0.001). Interestingly, the authors also reported that the median time to seroconversion (IgM or IgG) among the asymptomatic group was significantly shorter compared with the symptomatic group (median seven days from first positive polymerase chain reaction assay vs. 14 days; p<0.001).

While it is generally accepted that antibodies persist for longer periods in severe cases of COVID-19, there is considerable variation among studies even when normalized for the clinical severity. For example, IgG in mild to moderate disease appears to persist anywhere from 3–5 months (14,33). A study evaluating symptomatic and asymptomatic patients with COVID-19 associated pneumonia (apparent or subtle on radiography) found antibodies were more frequently detected among symptomatic patients (100% vs. 71%; measured at two and five months post symptom onset) (34). Furthermore, titres decreased significantly between the two time points. Another study comparing IgG and neutralizing antibody levels in asymptomatic versus symptomatic patients showed 40% of the asymptomatic patients became seronegative in the early convalescent phase (2–3 months post symptom onset) compared with only 12.9% of symptomatic patients (35). Interestingly, Choe et al. (36) also reported that neutralizing antibody titre correlated with severity of disease, suggesting that patients with severe disease may be more protected from reinfection compared with patients with subclinical or asymptomatic infection. It should be noted that to date, there are only a few documented cases of reinfection (37–40), which suggests that either other immune mechanisms (such as the T cell-mediated response) may contribute to protection against SARS-CoV-2 reinfection, or that, as a function of the short time that SARS-CoV-2 has been circulating and combined with implementation of public health restrictions, sufficient time has not yet passed for re-infection to be detected. Additionally, as most of these early studies examined the level of anti-nucleocapsid antibodies, association of high levels of antibody with poorer clinical outcome may be related to the production of anti-nucleocapsid rather than anti-spike antibodies.

Although there are less data on the relationship between age and the antibody response in COVID-19, recent evidence suggests there may be distinct antibody responses in children and adults. It has been shown that unlike adults, who produced robust levels of anti-S and anti-nucleocapsid (N) antibodies, children produced less anti-N and neutralizing antibodies (41).
The clinical significance of this is unknown, although it is interesting considering disease severity is positively associated with age.

The large variability in how the antibody response was measured (i.e., different assays, platforms, methods, and antigenic targets) makes it difficult to make direct comparisons between studies. Despite this limitation, it is clear that not all infected individuals will mount an antibody response and that the level of antibodies may wane over time. This has significant implications for the interpretation of antibody testing for diagnostic purposes, especially for use as a surrogate marker of immunity to SARS-CoV-2. At best, anti-SARS-CoV-2 antibodies have good positive predictive agreement for neutralizing properties; however, the negative predictive agreement is poor.

**How to implement testing in the clinical laboratory**

Validation of antibody tests for infectious disease often depends on studies aimed to calculate the diagnostic sensitivity and specificity (i.e., correlating assay response to true positives and true negatives). In the case of SARS-CoV-2, this is inherently difficult for several reasons. Evidence suggests that humoral response and resultant seropositivity are affected by the severity of infection (presence/absence of detectable antibody), the time since symptom onset (antibody longevity) and the antigen target (antibody isotypes and viral protein recognition). Moreover, because the sensitivity of ribonucleic acid (RNA) testing is dependent on the time of sample collection in disease and the quality of sample, discordant serology results are often difficult to interpret for most clinical laboratories that do not have access to neutralization assays. Therefore, it is recommended that clinical laboratories offer assays that have undergone extensive review by a governing body such as Health Canada or the FDA, or by published peer-reviewed performance analyses when available. The requirement is on the clinical laboratory to ensure serology assays are used as intended and meet local accreditation standards.

The complexity and scope of internal verification studies required for laboratories to offer serology testing is highly dependent on the method of choice. Many clinical laboratories will choose to offer automated chemiluminescent immunoassay methods using existing instrumentation and infrastructure that are Health Canada or FDA emergency use authorization approved. When using these validated methodologies, with provided performance characteristics, a modified method verification may be acceptable with reduced rigour of testing to ensure the assay is fit for purpose. However, modifications to a validated method, such as using a different specimen type like a dried blood spot or altering the manufacturer’s cut-off, requires complete method validation prior to patient testing (considerations for verification and validation are shown in Table 1). In the case of a laboratory-developed test, a complete method validation is required. A detailed flow diagram depicting additional possible scenarios for method validation and verification was published previously (42).

| Table 1: Minimal requirements for validation and verification of qualitative assays |
|--------------------------------------------------|-----------------------------------------------|
| **Item**                                   | **Verification**                                                                 |
| Requirements                | Assay methodology and reagents must be unchanged from the manufacturer’s instructions |
| Purpose                          | Laboratory validates all performance characteristics in their laboratory and all sample types to be used |
| Sample number                  | A statistically significant number of samples (generally 50 minimum) must be used in the evaluation process to cover the full range of expected results for the intended use |
| Statistical analyses           | A statistical correlation with existing validated methods or comparisons with known outcomes (“gold standard”) are required for qualitative methods. % CV, SD and 95% CI are recommended |
| Calculations                   | All performance characteristics must be tested |
| **Sensitivity/specificity:** Minimum of 20 samples (10 positive, 10 negative), or a recommended 100 samples: 50 samples valid for the method that are positive for SARS-CoV-2 RNA and 50 negative samples valid for the method that are negative for SARS-CoV-2 RNA OR that have been tested by a validated comparator immunoassay and were positive (N=50) or negative (N=50) for SARS-CoV-2 antibody. Apply the binary classification test (“Test outcome vs. condition”) to determine both characteristics. Determine CI%. |

Recommended 100 samples: 50 positive and 50 negative run over 5–10 days must be used, and cover the full range of expected results.

All performance characteristics must be tested
Verification and validation are required to ensure the accuracy and reliability of diagnostic tests. Verification is the process of confirming the performance of a test by comparing the results to a known standard, while validation is the process of establishing the characteristics of the test, including its sensitivity, specificity, and precision.

### Table 1: Minimal requirements for validation and verification of qualitative assays (continued)

| Item                                | Verification                                                                                     | Validation                                                                                      |
|-------------------------------------|-----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Calculations (continued)            | **Precision**: is defined as the closeness of agreement between independent test results obtained under conditions of the assay run (includes repeatability and reproducibility). Minimum of 20 samples: 10 replicates each of one positive and one negative sample; 10 RNA positive and 10 RNA negative samples. Create aliquots and freeze all aliquots necessary for testing to avoid freeze-thaw variability between repeats. If comparing to another immunoassay, a range of S/CO values within the samples would be desirable. **Repeatability**: Assay 10 replicates of the positive and negative samples in a single run. Determine the SD and % CV for the S/CO values. **Reproducibility**: Assay the 20 specimen aliquots on three different days. Determine the SD and % CV for the S/CO values for each specimen. | **Diagnostic (clinical) sensitivity is defined as the percentage of individuals with the target condition (as determined by the diagnostic accuracy criteria) whose test values are positive. Diagnostic (clinical) specificity is defined by the percentage of individuals without the target condition (as determined by the diagnostic accuracy criteria) whose test values are negative.** **Target specificity (cross-reactivity) ensures the test is specific only for the analyte of interest. This is determined by testing other pathogens within the same family or disease group.** Positive predictive value is defined as the percentage of individuals with a positive test result who have the target condition (as determined by the diagnostic accuracy criteria). Consider evaluating parameters as a function of the population prevalence. Negative predictive value is defined as the percentage of subjects with a negative test result who do not have the target condition (as determined by the diagnostic accuracy criteria). Consider evaluating parameters as a function of the population prevalence. **Precision**: Create aliquots and freeze all aliquots necessary for testing to avoid freeze-thaw variability between repeats. If comparing to another immunoassay, a range of S/CO values within the samples would be desirable. **Repeatability**: Assay 10 replicates of the positive and negative samples in a single run. Determine the SD and % CV for the S/CO values. **Reproducibility**: Assay the 20 specimen aliquots on three different days. Determine the SD and % CV for the S/CO values for each specimen. **Normal values**: 120 specimens should be run to establish normal range of values for local testing population. |
| Other considerations                 | When possible, laboratories should consider using multiple operators to perform verification, particularly when result interpretation is required. If the assay documentation does not include a full validation report or incomplete performance characteristics as recognized by technical organizations, then a user laboratory validation is required. | When possible, laboratories should consider using multiple operators to perform validation, particularly when result interpretation is required. |

Abbreviations: CI, confidence interval; CV, coefficient of variation; RNA, ribonucleic acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S/CO, signal to cut-off ratio; SD, standard deviation

Method verifications are likely to be the most common form of method evaluation performed by clinical laboratories at present. At minimum, clinical laboratories should verify manufacturer claims by assessing the diagnostic sensitivity and specificity, assay precision (reproducibility), cross-reactivity of non-SARS-CoV-2 antibodies and interfering substances (such as hemoglobin, lipids or biotin) commonly found in their patient population. A summary of the minimum suggested sample sizes for establishing diagnostic sensitivity and specificity are provided in Table 2. The clinical laboratory must verify the assay performance using statistical correlations and comparisons based on manufacturer claims. Careful scrutiny is required in the case of discrepant results, and arbitrator testing can be performed by external laboratories performing the same methodology. If verification specimens are sourced locally, patient history may be considered to reconcile test performance and to further characterize the testing population (outpatient, hospitalized or intensive care unit, and timing of sample collections). Furthermore, it may be prudent to interrogate the signal obtained from the assay in expected positives that may be in the equivocal range because some patient antibody titres may have waned over time, and assay cut-offs were assigned with only limited samples. A particular challenge during verification is resolving suspected false positive serologic results given the variable disease prevalence (43). Potential approaches to resolve discrepancies include testing for another assay or target antigen, reviewing the clinical history of the patient case, including time since symptom onset and contact tracing, or reviewing prior SARS-CoV-2 RNA testing results.
Table 2: Theoretical number of samples required for establishing diagnostic sensitivity and specificity estimates by error margin and confidence interval

| Estimated sensitivity or specificity | Estimate with 2% error | Estimate with 5% error |
|-------------------------------------|------------------------|------------------------|
|                                     | Confidence             | Confidence             |
|                                     | 90%  | 95%  | 99%  | 90%  | 95%  | 99%  |
| 90%                                 | 610  | 864  | 1,493| 98   | 138  | 239  |
| 92%                                 | 466  | 707  | 1,221| 75   | 113  | 195  |
| 94%                                 | 382  | 542  | 935  | 61   | 87   | 150  |
| 95%                                 | 372  | 456  | 788  | 60   | 73   | 126  |
| 96%                                 | 260  | 369  | 637  | 42   | 59   | 102  |
| 97%                                 | 197  | 279  | 483  | 32   | 45   | 77   |
| 98%                                 | 133  | 188  | 325  | 21   | 30   | 52   |
| 99%                                 | 67   | 95   | 164  | 11   | 15   | 26   |

Consideration of orthogonal testing algorithms for severe acute respiratory syndrome coronavirus 2 serology

In general, laboratories should strive to use serological assays with manufacturer-claimed sensitivity of greater than 95% and specificity of greater than 99.5% (1). Many of the SARS-CoV-2 specific antibody assays currently approved for use in North America meet these performance criteria when used alone (44–48). However, when testing is performed in low prevalence populations or in patients with a low pre-test probability of disease the positive predictive value or post-test odds will be unacceptably low despite excellent specificity. Therefore, laboratory professionals may adopt an orthogonal testing strategy to improve the specificity and positive predictive values of serologic test interpretation for SARS-CoV-2 (49–51). In orthogonal testing, samples that test positive or equivocal on an initial test are re-tested using a second test to confirm or refute the result (1). Orthogonal testing strategies have been recommended by public health authorities in North America in low prevalence populations (1,52), and detailed protocols are available (53). Most of the orthogonal testing approaches for SARS-CoV-2 antibodies involve two independent tests, each with unique assay design characteristics such as antigen type (54–56) or assay formats (57). However, these approaches may have practical limitations in terms of implementation because the specific epitope targeted in each manufacturers assay is unknown or not provided (58). Improvements in sensitivity can also be accomplished in orthogonal testing approaches by reducing cut-off values for commercial high-throughput automated SARS-CoV-2 assays (59); however, alteration of manufacturer recommendations requires full validation of the new cut-off values prior to clinical use.

When users are considering either a single serological testing algorithm or a two-step (orthogonal) testing algorithm, they must consider the reason for testing, the intended use of the data generated and the expected prevalence of SARS-CoV-2 in the population of interest. For example, use of serology for special clinical case testing (e.g. in MIS-C cases) or in seroepidemiological studies, requires high assay sensitivity and high negative predictive value in early infection (more than two weeks) and late convalescence, respectively. Indeed, in these scenarios reporting true cases should be the priority; although false positives are not desirable, they can be tolerated more so than false negatives. For example, in seroepidemiological studies, a sensitive assay with a good positive predictive value is critical to identify true cases to provide robust population level estimates of seropositivity. In contrast, when screening potential convalescent plasma donors, the presence of SARS-CoV-2 antibody is critical (60), and a positive predictive value more than 99% should be required. Because high negative predictive value is less important in this scenario, orthogonal testing is recommended in both low and high population prevalence levels (56). These examples illustrate the need for careful consideration of orthogonal testing strategies that are tailored to the intended use of the serological data. As a result of these different scenarios, SARS-CoV-2 antibody testing strategies will vary based on site-specific requirements.

Serological assays to determine severe acute respiratory syndrome coronavirus 2 antibody neutralization potency

To better understand and characterize SARS-CoV-2 immunity after natural infection or vaccination, functional assays such as virus neutralizing tests are required. The previous/current gold standard methodologies to detect and quantify SARS-CoV-2 neutralizing antibodies have used cell-culture-based infection assays, which block viral entry into cells in vitro. These live-virus assays use wild type SARS-CoV-2 virus incubated with dilutions of a patient’s sera or plasma. The mixture is then added to susceptible cells to determine if the sera inhibits or neutralizes the cytopathological effect or plaque reduction is observed. PRNT provide a means to quantitate neutralization titres associated with an individual’s clinical specimen. However, live virus assays require biosafety level-3 containment, are labour-intensive and due to the biologic variation associated with these assays they can be difficult to standardize (61–63).

Neutralization assays that use pseudotyped viruses, such as the vesicular stomatitis virus or lentivirus-based systems that incorporate SARS-CoV-2 spike protein, can be used in biosafety level-2 laboratories (64). An example of a basic procedure for establishing a pseudotype assay using lentivirus particles involves transfecting a “packaging” cell line, such as HEK 293T cells, with a number of plasmids to produce safe, non-replicative viral particles expressing the spike protein. Transfected plasmids include a reporter-expressing plasmid, a plasmid encoding the SARS-CoV-2 spike and a number of plasmids encoding lentiviral proteins required for assembling viral particles. The transfected cells produce the pseudotype viruses, which can then be used...
to infect permissive cells expressing the SARS-CoV-2 receptor, angiotensin-converting enzyme 2 (ACE2), to measure a decrease in cytopathological effect, via the reporter signal, or by plaque reduction in the presence of patient serum.

Although pseudotype virus formats alleviate biosafety limitations, they have similar drawbacks to conventional PRNTs in that they are difficult to standardize across laboratories, and the assay characteristics may vary depending on culture conditions, virus strains and cell lines used. Furthermore, these cell-based assays require highly skilled personnel, are low throughput and have suboptimal turnaround times for clinical decision making. These drawbacks make implementation of pseudotype viral assays in the clinical laboratory impractical.

Recently, several ELISA-based surrogate neutralization assays that detect antibodies targeting the viral spike protein RBD have come to market (65–67). These more rapid assays are based on antibody-associated blockage of the interaction between the spike RBD and the ACE2 receptor. The procedure by Abe et al. (67) uses immobilized ACE2 and soluble biotinylated RBD (which exhibited increased sensitivity to other reagent configurations), and provides a direct comparison with conventional ELISAs (detecting antibodies that bind RBD) in a plate format.

A number of commercial assays using a similar ELISA-based platform have also become available (e.g. GenScript cPass, Cayman SARS-CoV-2 antibody ELISA) that indirectly and semi-quantitatively measure the neutralizing capability of SARS-CoV-2 antibodies. The GenScript surrogate virus neutralization test (sVNT) kit has been compared with conventional cell-based neutralization assays (68–70) and displayed good specificity, and comparable sensitivity to virus culture-based assays, but demonstrated somewhat reduced efficacy in identifying samples with a lower level of virus neutralization potency. This observation may be explained by the specific detection of antibodies targeting the RBD in the assay, as non-RBD neutralizing antibodies are not targeted in the sVNT assays. It should also be noted that unlike functional PRNT or cell-based assays, surrogates may detect non-neutralizing antibodies in some samples. However, the advantage of both commercial and non-commercial ELISA-based sVNT assays is the ease of use and the potential for automation and standardization. Moreover, with appropriate validations, these may be incorporated into conventional virus neutralizing testing algorithms and offer an important tool to assess neutralization in clinical specimens.

Additional standardization is underway to compare cell-based neutralization assays with surrogate tests utilizing pseudotype viruses or ELISA-based competitive binding assays (71). Good correlation was observed between a modified ELISA-based surrogate assay with a conventional PRNT and spike pseudotyped viral vector-based platforms (67). Antibody titres between TCID50 neutralization tests and lentiviral/vesicular stomatitis virus pseudotype assays correlated well (71). It should be noted that although cell-based neutralization assays, such as PRNTs or micro-neutralization tests, are the reference standards for detection and quantification of neutralizing antibodies in clinical specimens, the biological nature of these test may lead to some variability in titres and sensitivity when comparing inter-laboratory results (72). The utilization of proficiency panels made up of well pedigreed control and patient sera/plasma samples facilitates standardization between laboratories.

Commercially available, high-throughput serological assays that measure the binding of antibodies to various viral antigens have been directly compared to neutralization antibody titres in patient samples using PRNT or sVNT assays (73–75). The results from these comparisons show that commercial serology assays are sensitive for the detection of total antibodies but are less robust at predicting the neutralization titre relative to conventional (e.g. PRNT) or sVNT assays. Furthermore, the reliability of commercial high-throughput platforms that are specific for spike/RBD protein antigens to infer neutralization titre equivalents may depend upon time frames for specimen collection and the specific antibody induction responses of an individual. However, more recent studies have described a strong correlation between anti-RBD antibody concentrations and spike/RBD-ACE2 inhibiting antibody titers (r>0.86, p<0.001) (76,77), with the possibility of anti-nucleocapsid antibodies (signal to cut-off greater than or equal to 5.0) serving as a surrogate for screening of high neutralizing antibody titer plasma (greater than or equal to 160) (78).

**Detecting antibodies derived from vaccination versus natural infection**

With the roll-out of SARS-CoV-2 vaccines, the detection and quantification of vaccine-induced antibody by current commercial assays needs to be verified in relation to the detection of natural immunity following infection. A difference in the ability to detect antibodies from both natural infection and vaccination has been seen with other viral infections, including varicella zoster virus (VZV), where commercial assays can readily detect IgG antibodies in people who have had a natural infection but many commercial assays are less robust in detecting vaccine-induced antibodies (79). Multiple studies are currently ongoing to determine what SARS-CoV-2 antibodies are detected with commercial assays, and if there are variances in detection based on the type of vaccine received by an individual. The current lack of serum from vaccinated individuals makes it challenging for a clinical laboratory to incorporate appropriate serology algorithms, particularly as both vaccine and laboratory assay targets are proprietary. Furthermore, with new vaccine candidates emerging, the variability in vaccine type and antigen may limit the ability of current commercial anti-SARS-CoV-2...
The role for differentiation of antibodies derived from natural infection versus vaccine-induced antibodies does not have importance for clinical management, and there are currently no Health Canada approved anti-SARS-CoV-2 assays specific for vaccinated populations. In the first four months following vaccination, it is likely that natural versus vaccine-induced antibodies can be differentiated using a combination of anti-nucleocapsid and anti-spike assays. In Canada, for example, where only spike-based vaccines are currently being used, a positive anti-nucleocapsid could indicate natural infection, and a negative anti-nucleocapsid but positive anti-spike could indicate vaccine-induced antibodies. However, this process is not straightforward as negative anti-nucleocapsid but positive anti-spike antibody profiles have been identified in those following natural infection (anti-spike antibodies persist longer than anti-nucleocapsid (50,80)). The landscape becomes more complicated after four months or longer because anti-nucleocapsid antibodies are expected to decline among patients who have recovered following natural infection. Therefore, current assays cannot determine whether a patient had positive anti-spike antibodies and negative anti-nucleocapsid antibodies due to a history of infection or a vaccination. From a public health perspective, it is important to understand the overall immunity of a population, and whether immunity is derived from vaccine or natural infection is academic. In jurisdictions using only spike-based vaccines, the use of spike or receptor binding domain assays should be considered as the primary targets for screening to ensure both scenarios are detected in surveillance studies. Conversely, the presence of vaccination-induced antibodies may be problematic for clinical testing; for example, in the diagnosis of MIS-C. As such, clinical serology testing is not recommended for individuals who have received the COVID-19 vaccine, especially if an assay that detects anti-N antibody is not available. Better markers of immune status are needed that are simple, reproducible and robust.

The use of serology in determination of immunity is challenging. There are currently no commercially available assays to determine immune status, which can only be evaluated using PRNT assays. In the absence of a universal standard, or a surrogate marker for immunity, the detection of antibodies in serological assays, regardless of the signal strength, cannot determine with confidence whether an individual would be susceptible or immune to a subsequent challenge with SARS-CoV-2. Therefore, the value of an “immunity passport”, where individuals with detectable antibody have fewer public health restrictions, is limited and may harm public health efforts aiming to decrease the spread of infection.

Towards the end of 2020, a series of SARS-CoV-2 variants of concern have emerged within different geographic regions of the world, such as the United Kingdom, South Africa and Brazil (81). These variants include specific mutations within the spike protein (K417N, E484K, N501Y) that are shared among all independent variant lineages (82). The most pressing concern with these variants is the increased transmissibility (83) associated with them; but also troubling is the aspect of immune escape, with the potential to evade detection and thus diagnosis. These mutations have been shown to affect neutralization properties depending on whether monoclonal or polyclonal antibodies were tested (84–86). Furthermore, the P.1 variant first found circulating in Manaus, Brazil, was associated with re-infection in immunocompetent individuals (87) which presumably would make it more difficult to interpret results from serology and PRNT assays targeting wild-type virus or viral antigens.

Currently there is no recommendation for pre or post-vaccine immunity screening (88), as there is a lack of correlation between antibody detection and immunity to infection, and little work has been done to understand antibody production in immunocompromised populations. To understand what level of antibody could correlate with immunity from infection, more in-depth studies are needed, including creation of an international standard to allow comparison of antibody levels between different vaccines, assays and laboratories.

Conclusion

Implementation of SARS-CoV-2 serology in the clinical laboratory is challenging, and laboratory professionals must be aware of the limitations of these assays. There are a number of unknown factors that affect these assays, and guidelines and recommendations for their use in clinical laboratories are ever evolving. Here we present the most up-to-date testing recommendations in Canada, and provide practical guidance for laboratories to choose appropriate serological assays and employ the best testing algorithms for their local populations.

Authors’ statement

CLC — Conceptualized project, original draft, review of manuscript, final editing of manuscript
JNK, VT, JVK, JG, CO, JLR, MD, TH, DRS, AL, LJ, PNL, HW, CT, LRL, MM, JDF, AD — Conceptualized project, original draft, review of manuscript
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All authors have approved the final version sent for publication and are accountable for all aspects of the work.

Competing interests
None.
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