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CLINICAL UPDATE

COVID-19 antibody testing: From hype to immunological reality

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The potential role for serological tests in the current COVID-19 pandemic has generated very considerable recent interest across many sectors worldwide, *inter alia* pathologists seeking additional weapons for their armoury of diagnostic tests; epidemiologists seeking tools to gain seroprevalence data that will inform improved models of the spread of disease; research scientists seeking tools to study the natural history of COVID-19 disease; vaccine developers seeking tools to assess vaccine efficacy in clinical trials; and companies and governments seeking tools to aid return-to-work decision-making. However, much of the local debate to date has centred on questions surrounding whether regulatory approval processes are limiting access to serological tests, and has not paused to consider the intrinsically limiting impact of underlying fundamental biology and immunology on where and how different COVID-19 serological tests can usefully be deployed in the response to the current pandemic. We review, from an immunological perspective, recent experimental evidence on the time-dependency of adaptive immune responses following SARS-CoV-2 infection and the impact of this on the sensitivity and specificity of COVID-19 antibody tests made at different time points post infection. We interpret this scientific evidence in terms of mooted clinical applications for current COVID-19 antibody tests in identifying acute infections, in confirming recent or past infections at the individual and population level, and in detecting re-infection and protective immunity. We conclude with guidance on where current COVID-19 antibody tests can make a genuine impact in the pandemic.

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In recent months, as South Africa (SA) approaches the peak of SARS-CoV-2 infections, there has been increasing debate on the potential clinical and public health utility of serological tests in the COVID-19 pandemic. This discussion – which has recently played out vocally in the literature and in the media[1-3] – has arisen in part due to well-documented limitations in the availability and turnaround times of polymerase chain reaction (PCR)-based tests, and in part due to the clamour for access to tools that may best gauge the true prevalence of SARS-CoV-2 infection in SA, and the possibility of identifying people who may now be immune to future SARS-CoV-2 infection. More than 370 COVID-19 serological tests are now commercially available or in development worldwide,[4] many of which have been put forward for a potential market in SA. Although one such test has recently been approved for local use, the question remains: why has there not been more rapid local regulatory endorsement and widespread adoption of serological testing in SA as a useful tool in the current pandemic? It can be explained in terms of two fundamental factors: our evolving understanding of the dynamics of adaptive immune responses to SARS-CoV-2 infection, and the ingredients required for a good serological test. We will focus on some of the complexities involved in considering assay performance and how best to use COVID-19 antibody testing in the SA pandemic.

Recent research

Impact of time-dependent adaptive immune responses following SARS-CoV-2 infection

As described in Box 1, most COVID-19 serological tests measure pathogen-specific antibody responses following acute viral infection. Key emerging immunity in SARS-CoV-2-infected people that underpins serological testing is highlighted in Fig. 1.

- Antibody responses can target multiple viral antigens and epitopes, and these may differ between individuals. Dominant antibody responses to SARS-CoV-2 appear to be directed to epitopes within either spike (S) protein or the nucleocapsid (N) protein, although antibodies against envelope and membrane proteins can be detected.
- The antibodies that develop in response to infection can be either binding or neutralising (Fig. 1A). Neutralising antibodies interfere with the interaction between virus (the S protein) and host cell (angiotensin-converting enzyme 2 receptors), preventing viral entry, or can be involved in antibody-dependent cell-mediated phagocytosis and/or cytotoxicity through Fc receptor-dependent pathways. In contrast, non-neutralising antibodies bind specifically to virions, but do not interfere with their infectivity. The S protein is the target of many serological assays, which detect both binding and neutralising antibodies but do not currently give an indication

Box 1. Distinguishing serological tests for COVID-19

COVID-19 antigen tests. Use immobilised antibodies to directly detect SARS-CoV-2 virus or viral antigens in a sample. In principle, a good direct antigen test could be a functional alternative to polymerase chain reaction-based testing that would work from onset of symptoms and at point of care (POC) in low-resource settings. However, in practice, developing these tests has not proved straightforward, owing to challenges in identifying and validating the pairs of antibodies that are required to provide high affinity and specificity detection of low levels of antigen.

COVID-19 antibody tests. These are currently more commonly available than direct antigen tests and come in different formats, including the simple and relatively cheap, qualitative lateral flow assays (otherwise known as 'rapid antibody tests') that can be used at POC or in field settings, and more accurate, quantitative lab-based enzyme-linked immunosorbent assays. These tests use immobilised SARS-CoV-2 antigens which allow the detection of circulating antibodies in plasma, serum or whole blood. Different COVID-19 antibody tests typically detect immunoglobulin G (IgG) and/or IgM antibodies to either the SARS-CoV-2 spike (S) protein (in either full-length trimeric form, or the isolated S1 or receptor binding domains), or the nucleocapsid (N) protein:

- The S protein is intuitively attractive for use in serological tests since it is directly involved in angiotensin-converting enzyme 2 receptor binding during viral entry, which therefore makes it a target for the host response – mounting either neutralising or binding antibodies that target epitope regions in spike.
- However, in the SARS-CoV-1 outbreak of 2003, a significantly higher frequency of infected individuals had anti-N compared with anti-S antibodies,[5] potentially making assays based on the N protein attractive for seroprevalence studies in the current pandemic.

While many manufacturers do not disclose the precise identity of the target viral antigens used in their COVID-19 antibody tests, it is clear that at least some have engineered the target viral antigens to optimise performance. It is therefore important to appreciate that not all COVID-19 antibody tests are equivalent, especially where the antigen used is key to its clinical performance and utility.

of function. Lab-based cellular assays are therefore required to evaluate whether anti-SARS-CoV-2 antibodies in individual samples have Fc effector function and/or neutralising activity[6,7] and reflect the differing types of antibodies involved in protection. However, rapid point-of-care antibody tests do not discriminate between binding and neutralising function and therefore may not be used to measure protective immunity.

- A primary antibody response to any new viral infection takes time to develop and is never static, with antibody titres rising over a period of several weeks post onset of symptoms and then starting to wane. Fig. 1B depicts the initial peak responses of immunoglobulin M (IgM), followed by IgG and IgA isotypes. Responses to differing viral proteins seem to have very similar timing.[8] Notably, seroconversion following SARS-CoV-2 infection is detectable in <50% of symptomatic individuals 5 - 7 days post symptom onset, even using highly sensitive immunofluorescence assays, which coincides with when the majority of infected individuals become least infectious.[9]
- Although IgM or IgG is detectable in 90% of infected individuals 14 - 16 days post symptom onset, at this stage almost none of these individuals are infectious (i.e. shedding live virus).[10]
- The magnitude of individual antibody responses following SARS-CoV-2 infection may vary 100-fold, with disease severity emerging as a strong predictor of higher titres.[10] Further research is required to dissect out the relevance of other known factors on antibody responses, such as age, gender and immunosuppression, e.g. with HIV co-infection.
- Antibody responses to SARS-CoV-2 can wane rapidly during the weeks following infection, especially in mild or asymptomatic cases. Although longitudinal studies beyond 100 days are ongoing, recent studies have demonstrated rapid waning of all isotypes of antiviral antibodies, with longer persistence of detectable IgG following severe COVID-19 disease (3 months) compared with mild disease (1 month) (Fig. 1C).[8,10] The implications of this finding for interpretation of serological test data are discussed further below.
- Emerging data also suggest an apparent disconnect in some individuals who mount cytotoxic T-lymphocyte responses to SARS-CoV-2, but not a corresponding detectable antibody response.[11] Such individuals may represent those who do not seroconvert following what was probably mild infection or whose T-cell response is robust enough to clear infection prior to seroconversion. Further research is needed to determine whether such individuals possess a genetic underpinning of innate or adaptive immunity mechanisms that confers resistance to infection without seroconversion and how this relates to future risk of re-infection, disease severity and viral transmission.

Evaluating the sensitivity and specificity of COVID-19 antibody tests: What constitutes a worthwhile test?

Since the start of the COVID-19 pandemic, numerous articles have been published comparing claimed and real-world clinical performance of antibody tests in seroprevalence studies. Typically, independent real-life performance appears less impressive than manufacturer-reported data, perhaps owing to patient selection bias towards hospitalised patients with severe disease during test development. A recent meta-analysis of 40 studies on COVID-19 serological tests reported in preprints or peer-reviewed publications since January 2020 reinforces this, finding that the average sensitivity of enzyme-linked immunosorbent assays (ELISAs) or lateral flow assays (LFAs) measuring IgM or IgG was ~66% and ~84%, respectively, while average specificity of ELISAs and LFAs ranged from 96% to >99%, with a high risk of patient selection bias being found in the majority of studies.[12] It is important also to appreciate that ~7 - 14 days after SARS-CoV-2 infection, an IgM response will occur that can then switch to IgA and IgG in most people after 2 - 3 weeks of infection. The sensitivity of antibody testing is therefore dependent on gaining samples during different window periods of infection. If the window of testing for IgM, IgA or IgG is at the onset of symptoms, this would bias the sensitivity to IgM. More commonly, as it is easier to accrue samples, if testing is performed 2 - 3 weeks after symptoms (and possibly 4 weeks after initial infection), this would bias sensitivity away from IgM and towards IgG (Fig. 1).

This time-dependency of class-specific antibody responses is reflected by meta-analyses showing that in the first week post onset of symptoms (POS), clinical sensitivity of IgG-specific ELISAs and LFAs is typically poor (~24% and ~13%, respectively), improving to 65% and 50%, respectively, at week 2 and to 82% and 80%, respectively, at week 3.[12] In addition, new evidence is emerging that low-titre antibody responses in convalescent individuals can

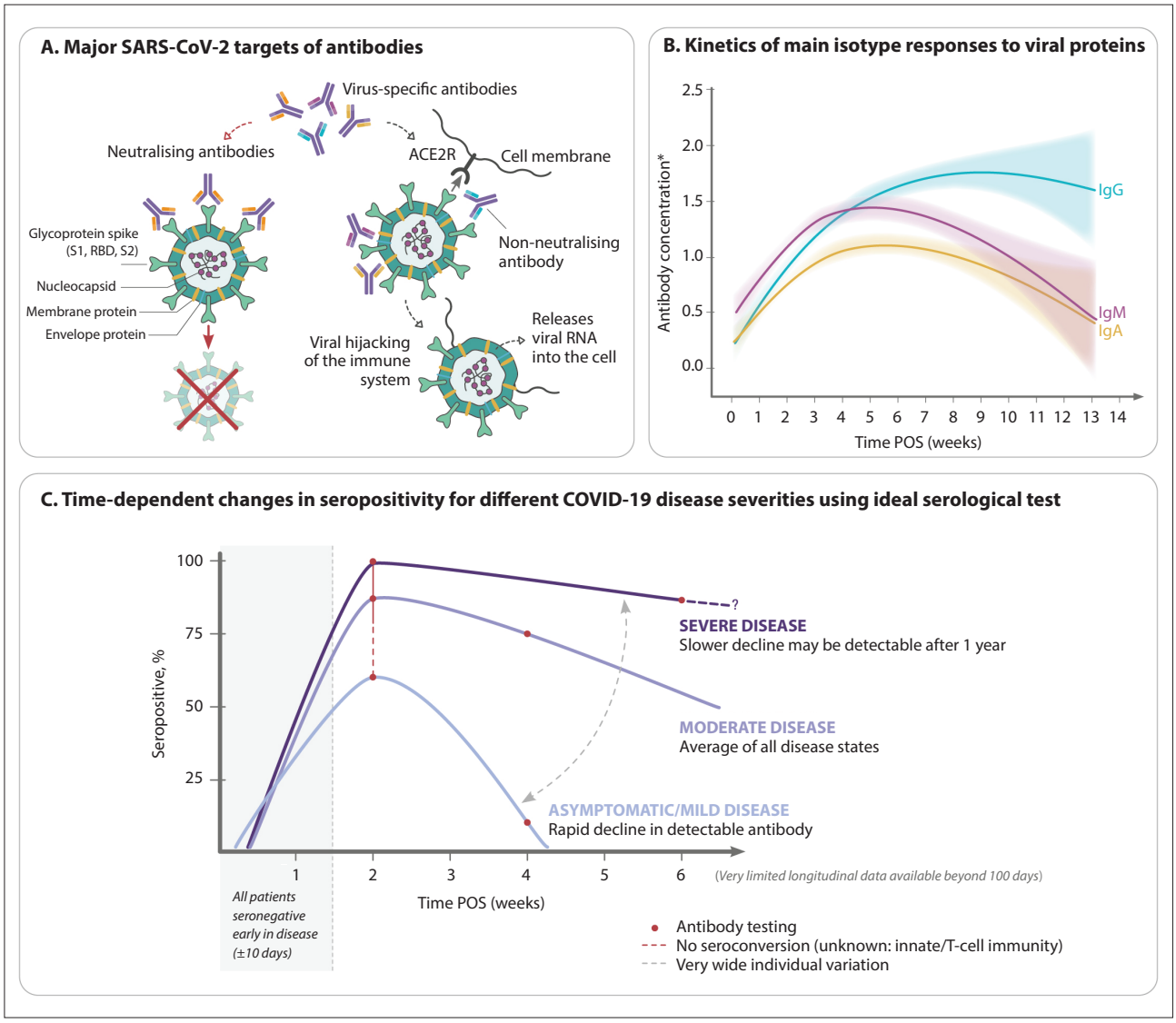

Fig. 1. Emerging biology of antibody responses to SARS-COV-2 infection and relevance to serology. Cross-sectional and limited longitudinal studies of antibody responses to SARS-CoV-2 infection are published or in pre-print. The major SARS-CoV-2 viral proteins targeted by antibodies are shown in (A), several of which are targets of available serological assays. Targeted antibodies may be functional or non-functional, and this is not necessarily evident from serological test outputs. Different isotype responses against viral epitopes have differing kinetics, shown in (B), adapted using published enzyme-linked immunosorbent assay data from Seow et al.;[8] *IgM and IgG are the major isotypes targeted in current serology. Antibody kinetics and adaptive responses to different COVID-19 disease severity will determine the best possible performance of an ideal serological test, and this is modelled in (C) given our current understanding. (ACE2R = angiotensin-converting enzyme 2 receptor; S1 and S2 = spike protein 1 and 2; RBD = receptor-binding domain of the spike protein; POS = point of symptom onset; *Arbitrary units (optical density at 1:50 dilution – this is not an absolute scale, since it depends on the precise assay set-up).)*

wane more quickly than expected. One recent report showed that the percentage of PCR-positive, hospitalised COVID-19 cases with potent neutralising antibody titres dropped from 60% at 3 weeks POS to 17% at 65 days POS.[8] Similarly, another recent article reported that virus-specific IgG-positive proportions for symptomatic and asymptomatic individuals in their cohort were 84% and 81%, respectively, in the acute phase, but dropped to 73% and 49%, respectively, in the early convalescent phase (defined as 8 weeks after discharge from hospital).[10]

Our own recent studies on a cross-sectional SA cohort of convalescent COVID-19 cases (including asymptomatic, mild and severe cases; samples collected 3 - 4 weeks post first positive PCR test) identified virus-specific IgG titres of varying magnitudes in ~65% of individuals (Blackburn *et al.*, unpublished data). These findings suggest that some individuals in this cohort may never have generated a virus-specific IgG response, while in others the response may have waned very quickly, with no simple correlation with disease severity being observed. Longitudinal data are key to the interpretation of such variable antibody responses.

Why is all of this important? To avoid the detection of *false negatives*, we need to know the performance characteristics for measuring all antibody isotype responses to SARS-CoV-2. This not only speaks to the quality of detection, but also to knowledge about when the testing kits can be used at a given stage of infection and with what sensitivity. This is also a balancing act with specificity – which needs to be high enough to avoid *false-positive* responses due to cross-reactivity with conserved antigens across the coronavirus family. High sensitivity and specificity will be of critical importance for seroprevalence studies, as well as for studies that aim to determine the extent and durability of immunity in the SA population; research

is therefore urgently needed on longitudinal cohorts in SA in order to be able to model these effects more accurately. Furthermore, it is also critical to evaluate these serological tests thoroughly prior to implementation and to adhere to scientific rigour in the approval process.

Discussion

From our knowledge of how immunity to acute viral infections develops and the range of individual test characteristics described above, the clinical application of serological assays needs to be considered carefully depending on the question being asked.

Can we identify acute infections?

Irrespective of test accuracy, the kinetics of antibody response make the use of serology for the diagnosis of acute infection very limited. In severe disease, antibody responses are likely to appear after treatment decisions need to be made, even in situations where PCR-based testing is a false negative. Emerging data around infectivity in mild disease suggest that serology results are likely to be positive only after individuals are no longer infectious.[9] Multisystem-inflammatory syndrome in children[13] may be the one exception, where linking the inflammatory picture to a recent SARS-CoV-2 infection through detection of particular IgM responses may aid diagnosis and management.

Can we confirm recent or past infections?

Individuals. There is undoubtedly a major role for serological assays in SARS-CoV-2 infection, but owing to both the varied immune response between people and variable test performance characteristics, applications are not so straightforward at the individual level. The majority of PCR-positive patients with SARS-CoV-2 infection produce a measurable antibody response, irrespective of disease severity. As discussed above, this may be detectable in the first 2 weeks after infection, but only in any reliable way >2 weeks following the start of symptoms.[12] Importantly, however, the rapid waning of antibody titres during convalescence means that a negative result does not imply absence of prior infection. This may have implications for individuals interacting with employers or insurers requiring evidence of infection, as well as people wishing to return to interacting with vulnerable family members or other communities after an unconfirmed but suspected SARS-CoV-2-related respiratory tract infection. For individual diagnosis, ensuring that serological testing is performed in the best time window after an acute illness (possible between 3 and 6 weeks) and using a test optimised for sensitivity (thereby reducing false-negative results) is critical to get right.

Population. This is undoubtedly the major role for serological assays in SARS-CoV-2. Serological assays may have several public health applications, and we have already seen a number of recent seroprevalence publications from countries with substantial outbreaks. Such reports typically suggest seroprevalence to be orders of magnitude (5 - 50-fold) higher than PCR-proven cases: for example, one report estimated that up to 23% of individuals in New York City may have been infected,[14] while other reports found an average seroprevalence of 5% in Spain, with a higher prevalence around Madrid (>10%) and a lower prevalence in coastal areas (<3%).[15] In all cases, however, these reports suggest a very large remaining vulnerable population. A key issue again here is that with waning of antibody titres, such cross-sectional studies are only really able to estimate what the prevalence was 1 - 3 months earlier, and would underestimate prevalence the longer the elapsed time since infections and outbreaks occurred. Further research is therefore required to understand the full spectrum of the innate and adaptive immune responses to SARS-CoV-2 as a function of time, in order to more accurately model the effect of waning antibody levels or absence of seroconversion on estimations of past infections in a given population. This would in turn allow the population-level susceptibility (currently assumed by models to be 100%) to be determined from actual data and would also affect calculation of the effective reproductive rate of the virus. For population-level serological testing, optimising the testing strategy for specificity is required, to minimise false positives in low-prevalence settings (Box 2), and a two-test strategy may need to be implemented for all positive cases to reduce the impact of type 1 errors on the seroprevalence data.

Can we detect re-infection and protective immunity?

Humoral responses to other beta-coronaviruses vary, with neutralising antibody responses to SARS-CoV-1 detectable in certain individuals with severe disease several years after infection.[16] In contrast, re-infection with 'common cold' alpha- and beta-coronaviruses is known to occur every few years, with waning humoral immunity.[17] It is currently unknown what the variable and waning titres of antibodies against SARS-CoV-2 detected in serological assays might mean for re-infection or protective immunity as the pandemic unfolds in SA, or indeed the implications of this for the durability of vaccine-induced immunity. It is important to note, however, that rapid waning of circulating antibodies does not automatically mean that secondary antibody responses will not re-emerge upon secondary exposure – this will depend on whether or not a robust memory B-cell response has been generated as a result of the primary infection.[18] Further studies are therefore urgently required to understand whether there is a serological correlate of the development of memory B-cells, particularly those that encode neutralising antibodies.

It is also worth reiterating that not all anti-S antibodies have high neutralising potency: a recent report showed that out of 89 patient-derived anti-RBD (receptor-binding domain of the spike protein) monoclonal antibodies tested, only 58% neutralised SARS-CoV-2 pseudovirus,[18] which means that current serological assays do not directly report on whether the detected antibody is neutralising. Therefore, no serological assay with quantitative or qualitative readouts can yet provide robust evidence that long-lasting protective immunity is present. Despite the fact that COVID-19 serological tests have provoked considerable interest from employers and governments in recent months as potential immunity passports, until more research has been done, we cannot stress too strongly the need

Box 2. The importance of test specificity in seroprevalence studies on COVID-19

Current local regulatory clinical performance targets for COVID-19 serological tests are minimum 85% sensitivity and 98% specificity.[19] To put these numbers into practical context, if the prevalence of SARS-CoV-2 infection in the target population is 1%, then for a test with these performance characteristics, 67% of all positive results would be false positives, which would severely limit the utility of the data in unbiased population-level seroprevalence studies. On 19 July 2020, there were ~360 000 polymerase chain reaction-confirmed cases in SA, equating to a known prevalence of 0.6%; even if the true prevalence is actually five times higher at 3%, then ~42% of positive results would still be false for a test meeting those minimum requirements. So specificity, not sensitivity, is absolutely key for seroprevalence studies and really needs to be close to 100% when used in a field setting, unless the true disease prevalence is much higher.

for caution to avoid over-interpretation of antibody testing results in terms of likely immunity to future infection – a view also held by the World Health Organization.[20]

More about protective immunity: Vaccines

An important future use of ELISA-based quantitative serological testing is around vaccine evaluation: whether a vaccine can elicit immunity in immunised individuals. Testing for vaccine-induced immunogenicity can take the form of measuring either binding or neutralising antibodies, the latter being the functional antibody that neutralises the virus before entry into host cells. Having an antibody test that is both sensitive and specific to the vaccine product will be important, so that vaccine-induced immunity can be measured. The holy grail of vaccine development is to induce protective immunity in immunised individuals; currently there are ~170 candidate vaccines in development,[21] with ~27 vaccines in human clinical trials. As certain products enter phase III efficacy trials,[22] we will begin to understand what constitutes protective immunity. Is it binding or neutralising antibodies or is it the T-cell arm that confers protection?[23] Whatever the protective mechanisms might be for different vaccine products, antibody responses can become important correlates of protection and their measurement will probably be used to judge the spread of protective immunity in the population. Whether measurement of binding and/or neutralising antibodies is important in evaluating vaccine-induced protective immunity remains to be seen. Hopefully the current trials underway will provide this evidence. Either way, once we have established a quantitative serological correlate of immune protection, we can start to evaluate whether herd immunity is evolving, which could assist in protecting vulnerable people in our population. We also need to note two important caveats to vaccine-induced antibodies and their measurement. First, not all antibody responses may be protective and, theoretically, non-functional antibodies may do the opposite and enhance disease by antibody-dependent enhancement.[24,25] Being able to measure vaccine-induced antibodies (including specific immunoglobulin class and subclass) along with function (neutralising) will therefore be a critical component of future vaccine serological testing. Second, distinguishing natural infection from vaccine responses will be important in the context of identifying whether a vaccine response is able to provide protection from SARS-CoV-2 infection or whether 'breakthrough' infections can occur. A serological test that can distinguish vaccine-induced from infection-induced antibody response may therefore need to be based on detection of antibodies to specific neutralising epitopes on the S protein, or to non-S proteins, such the N protein (noting that the N protein would be absent in vaccines that only target the S protein, but would be present in live attenuated or killed SARS-CoV-2 formulations), or both. However, this may come at a cost of lower specificity, as anti-N antibodies may cross-react with other coronaviruses that are in common circulation.[26,27]

In conclusion, the choice, approval and use of either ELISA or rapid antibody testing for host immunity to SARS-CoV-2 has to be met with thought and scientific rigour. It is critical to pay homage to how the host immune system interacts with acute viral infections and to become informed around the complex interplay between viral antigens, timing of infection, antibody function and testing utility. Antibody testing for SARS-CoV-2 should not be thought of as diagnostic or prognostic, but as a precious tool to evaluate population immunity to natural infection, as a valuable research tool, and as a correlate of vaccine-induced protection.

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Conflicts of interest. CMG sits on a South African Health Products Authority (SAHPRA) Expert Committee for evaluating ELISA, rapid and antigen kits. JMB consults for Sengenics Corporation (Singapore), who market protein array tools for antibody/autoantibody profiling applications.

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