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Føns, Sofie; Krogfelt, Karen A.

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PRIORITY PAPER

How can we interpret SARS-CoV-2 antibody test results?

Sofie Føns[†] and Karen A. Krogfelt^{*,‡}

Roskilde University, Department of Science and Environment, Universitetsvej 1, Roskilde, Denmark

*Corresponding author: Department of Science and Environment, Roskilde University, Universitetsvej 1, 28A.1, DK-4000 Roskilde, Denmark. Tel/Fax: +45 46743269; E-mail: karenak@ruc.dk

One sentence summary: Antibody testing for SARS-CoV-2 is widespread despite poor test validation and limited knowledge on antibody responses, which makes it crucial to understand both the potential, limitations and interpretation of serology.

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[†]Sofie Føns, <http://orcid.org/0000-0003-0149-5909>

[‡]Karen A. Krogfelt, <https://orcid.org/0000-0001-7536-3453>

ABSTRACT

Since the outbreak of COVID-19, the world has raced to understand and accurately diagnose infection caused by SARS-CoV-2. Today, hundreds of commercial antibody tests are on the market despite often lacking proper validation and with unsatisfactory sensitivity and/or specificity. In addition, many questions related to the humoral response remain unresolved, although research is carried out at an unprecedented speed. Despite the shortcomings, serological assays have an important part to play in combating the pandemic by aiding in diagnosis and sero-epidemiological studies. However, careful attention must be paid to the application of serology and the interpretation of serological data—especially in low prevalence regions, both at an individual and at a population level. In this article, we argue that serological results are often misinterpreted, and in the eagerness to be first, methodological rigor is often taking a backseat.

Keywords: coronavirus infections; serology; seroepidemiologic studies; antibodies; diagnosis; serologic tests

INTRODUCTION

Since the outbreak of COVID-19 in Wuhan in December 2019, the virus has, as of October 10th 2020, spread globally with 36 616 555 confirmed cases and 1063 429 deaths worldwide (World Health Organization Coronavirus disease 2020). Following the release of viral genome sequences of SARS-CoV-2 in January (Zhang 2020), molecular detection kits for real-time RT-PCR were soon developed and became the gold standard for diagnosing COVID-19 by confirming the presence of SARS-CoV-2 RNA. The tests have high specificities but varying sensitivities, mostly due to sampling difficulties, including choice of specimen, and timing of peak viral load, which can lead to false-negative results. Before long, however, companies, institutions and research laboratories started flooding the market with serological kits for

detection of past (or present) SARS-CoV-2 infection. As of 10th of October 2020, the Foundation for Innovative New Diagnostics lists 342 commercial immunoassays for detecting antibodies (Foundation for Innovative New Diagnostics SARS-CoV-2 diagnostic pipeline 2020), but only 49 have currently been granted an Emergency Use Authorization by the FDA (FDA 2020). The majority of these tests fall within two categories: either a qualitative, rapid immunochromatographic assay (15–20 min), or a slower semi-quantitative enzyme-linked immunoassay (ELISA)/chemiluminescent immunoassay (CLIA) (a few hours). Most commonly, they detect IgM, IgG or both antibodies, but some detect total antibody or IgA.

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Thorough validation is needed to facilitate the potential of serology testing

Serology testing is a powerful way to monitor the progression of the pandemic by seroprevalence studies and as a tool in diagnostics. For accurate diagnosis of COVID-19, serology can be a great supplement to molecular detection. Serology is powerful further into the course of the disease, when the virus has been eliminated or exists in small numbers, as suggested in a number of publications indicating antibody testing to surpass PCR sensitivity 5–8 days after symptom onset (Guo et al. 2020; Yong et al. 2020; Zhao et al. 2020). However, in order to accurately use serology for diagnostics or estimates of spread of infection in society, extensive validation is needed. Many of the available tests are of dubious quality, where especially the low specificity is of concern.

Many manufacturers have not made their test validation available and there are no standards to employ that make it possible to compare the performance across tests and to make the tests fully quantitative. Immunoassays vary on not only which antibody they measure but also the antigen used, source of the antigens, specimen type and the secondary antibody conjugate, which influence the test performance (Haselmann et al. 2020; Kontou et al. 2020; Schnurra et al. 2020). The need for test harmonization is highlighted by the increasing number of studies published that compare the head-to-head performance of immunoassays (GeurtsvanKessel et al. 2020; Haritshoej et al. 2020; Jääskeläinen et al. 2020; Lassaunière et al. 2020; Schnurra et al. 2020; Whitman et al. 2020), often showing some discrepancy. Those studies have used pre-pandemic sera, some of which were samples from patients with respiratory virus infections, as it is essential to be able to discriminate between the e.g. 'common cold' coronaviruses and SARS-CoV-2 to avoid false positives.

An additional concern is the potential batch-to-batch variation between tests, which leads to the need for repeated validation for each batch used. In Denmark, the study of seroprevalence among blood donors had to be halted, as a new batch of the IgM/IgG Antibody to SARS-CoV-2 lateral flow test from Livzon Diagnostics showed remarkably lower sensitivity than previous batches (Leverance af antistoftest 2020).

What do sensitivity and specificity tell us? Interpreting an individual test result

The high number of antibody tests on the market each has a different sensitivity and specificity. A highly sensitive test should capture all true positive results, whereas a highly specific test should rule out all true negative results. In reality, none of the tests are both 100% sensitive and specific, hence the importance of validating the test before use to know the test characteristics. The test results from a population-based serology survey can then be adjusted for the imperfect test quality. One concern related to validation is what kind of samples were used as positive controls. Do they reflect the population being surveyed? If not, we might underestimate the seroprevalence. The positive control samples are from PCR-confirmed COVID-19 patients, but they might not represent the full clinical spectrum or the different age groups. It is still not known whether children in general have a different pattern of antibody generation compared to adults with COVID-19, and in addition, the severity of disease affects the antibody response, thus samples of asymptomatic ought to be included in the validation.

A general trend seems like that the rapid tests tend to have lower sensitivity than the semi-quantitative tests (Kontou et al. 2020), thus underestimating the true rate of seroconversion in those tested. An advantage of the rapid tests is their speed and ease of use that does not require a laboratory. However, they do depend on the operator to interpret whether they are positive or not, typically by the visualization of a red line, which can result in borderline cases.

Despite the wide spread of SARS-CoV-2, most areas around the world still have an overall low seroprevalence, which potentiates the problem of false positives when deploying antibody tests. Even in a hard-hit country like Spain, findings from perhaps the most extensive population-based sero-epidemiological study to this day, suggests that only 5% of the population had antibodies against SARS-CoV-2 (Pollán et al. 2020).

But how does the seroprevalence impact the interpretation of an individual test result? Let us take an example. In Denmark, a study among 20 640 blood donors showed an adjusted seroprevalence of 1.9% (Erikstrup et al. 2020). The sensitivity of the test was estimated to be 82.6% and specificity 99.5%. These figures result in a negative predictive value of 99.7% and a positive predictive value of 76.2%. Given a negative result as a blood donor, the probability that the result is right is almost 100%. Is the result of your test positive, the probability that the result is correct is only about three-quarters. Are you, as an individual, much better off knowing your antibody status than before? Probably not. If you live in an area with low seroprevalence and you feel healthy, the chances of you having had COVID-19 was small anyway, whereas a positive result has an almost 25% chance of being false. On top of that, we still do not know whether a positive antibody test is associated with protection from future COVID-19 infection and we also do not know for how long the antibodies last, so in fact you should not act any differently than if you had a negative result.

The issue of a low positive predictive value is potentiated the lower the seroprevalence, and thus underscores the challenges of accurately assessing one's antibody status in areas so far spared from big outbreaks of SARS-CoV-2—despite using a test with a seemingly high specificity. An alternative approach to increase the positive predictive value is to focus testing on individuals with an elevated likelihood of previous exposure to SARS-CoV-2 e.g. a history of COVID-19-like illness, or employ a second test with different design characteristics (e.g. antibody format or antigen) if the first test was positive [(CDC Information for Laboratories about Coronavirus (COVID-19) 2020; Hicks et al. 2020)]. As previously mentioned, further into the course of the disease, serology testing is likely more sensitive than molecular methods, and integration of different testing methods could help ensure correct and timely diagnosis of COVID-19. In certain areas without access to advanced laboratories, rapid antigen testing, although typically less sensitive than RT-PCR, could also be a relevant alternative e.g. for screening (CDC Information for Laboratories about Coronavirus (COVID-19) 2020).

Kinetics of SARS-CoV-2 antibody response

By using an ELISA or other semi-quantitative tests, testing COVID-19 cases can potentially reveal something about the kinetics of the antibody response. Despite often being considered a marker of acute infection, IgM does not consistently appear before its IgG counterpart, which hinders its use as a marker of acute or recent infection. A similar trend for IgM was found among studies of SARS-CoV (Meyer, Drosten and Müller

2014), but it could partly be due to differences in testing sensitivities. The median seroconversion reported in several studies falls between 9 and 14 days post symptom onset (Grzelak et al. 2020; Long et al. 2020; Lou et al. 2020; Qu et al. 2020; Zhao et al. 2020), which emphasizes the importance of timing when testing for antibodies. One important point to make is the variability in the antibody response with some patients seroconverting within a few days post symptom onset, and others taking weeks to do so, thus testing too early will miss some cases. Testing for total antibodies appears to be more sensitive and thus detectable a little earlier than IgM or IgG alone (Harritshøj et al. 2020; Lassaunière et al. 2020; Lou et al. 2020; Zhao et al. 2020). IgA specific tests are rare, but some studies report a potential use of IgA as an early diagnostic marker (Dahlke et al. 2020; Ma et al. 2020).

Two large studies found that IgG antibodies persisted for at least three to four months after symptom onset (Gudbjartsson et al. 2020; Iyer et al. 2020), although other studies have observed a gradual decline within the first couple of months (Long et al. 2020; Perreault et al. 2020; Wang et al. 2020). Quantitative measurement of antibody titers also makes it possible to look for correlation to severity status of COVID-19 patients (PCR confirmed), with a range of studies finding higher titers among severe cases (Liu et al. 2020; Long et al. 2020; Qu et al. 2020; Salazar et al. 2020), however, the causality is still unclear. Is it because of higher viral load? Is it because the virus has successfully invaded and colonized the host? Or is the immune response detrimental?

One overall problem though is the lack of proper longitudinal studies, although with time passing since the outbreak of the pandemic more studies are surfacing (Iyer et al. 2020; Perreault et al. 2020; Wang et al. 2020). Most serology studies to this date are retrospective or cross-sectional, and those of longitudinal character often have few patients and/or few sequential samples, which limit their use for accurately answering outstanding issues regarding antibody kinetics.

Comparisons of molecular testing followed by antibody testing show that most individuals with symptoms seroconvert and that PCR testing can be positive up to a month after symptom recovery (Wajnberg et al. 2020). However, neither the clinical features nor the immune responses of asymptomatic cases have been well described yet. So far, most studies have focused on hospitalized, PCR confirmed COVID-19 patients and their antibody response. But some people may fail to mount a detectable antibody response altogether. A small study found that asymptomatic cases may have a weaker immune response to the virus and that the antibodies may diminish sooner than for symptomatic cases with a reduction in neutralizing antibodies after eight weeks (Long et al. 2020).

It is still unclear what antigen(s) are preferred in antibody assays

An important aspect to discuss is the impact of antigens in serological testing. By far the most common antigens to use are the structural proteins nucleocapsid (N) and spike (S) protein, which are also the most immunogenic. The N protein is the most abundant protein; it is small and can readily be expressed in e.g. *E. coli*. On the other hand, the trimeric spike protein extrude from the surface and the S1 subunit is used for receptor binding through the individually folded receptor binding domain (RBD), which is likely a primary target for neutralizing antibodies (Wrapp et al. 2020). The S protein is heavily glycosylated and is therefore typically expressed in mammalian cells. Many antibody kits make use of only one antigen, which opens for the possibility that

some individuals might not have a strong antibody response towards that particular antigen. Other kits only use part of the S protein, e.g. the RBD, again possibly introducing a selection bias. The use of recombinant antigens leads to less biosafety needed, it is more standardized and perhaps cross-reactivity can be avoided if only specific epitopes on the viral proteins are used.

A few research groups have developed peptide or protein microarrays, which could help establish the level of cross-reactivity between antigens and which antigens elicit the strongest response (Jiang et al. 2020). However, peptide microarrays come with a risk of false negatives if the antibodies only recognize conformational epitopes instead of linear. The N protein, like the S2 subunit of the spike, is more conserved across coronaviruses, which may increase the risk of cross-reactivity. One study found that seroconversion occurred in average two days earlier for assays detecting total Ig or IgG anti-N than for IgG anti-S (Van Elslande et al. 2020), however another study found more patients had earlier seropositivity for anti-RBD (To et al. 2020). Studies comparing the use of different antigens point in different directions with some concluding that N is preferred, others that S1 subunit or RBD is the more specific and sensitive choice (GeurtsvanKessel et al. 2020; Jiang et al. 2020; Liu et al. 2020; Ma et al. 2020; Schnurra et al. 2020; To et al. 2020).

Correlates of protection—are we any wiser 10 months into the pandemic?

Often when the detection of an antibody response towards SARS-CoV-2 is discussed, it is assumed that reactivity correlates with neutralization, and that neutralization equals immunity (or confers some level of protection), which like WHO warned in April, is too early to say. There are different assays commonly employed when testing for neutralization. Plaque reduction neutralization test (PRNT) is considered the gold standard; however, like the cytopathic effect-based microneutralization (MN) assay, it makes use of cultivated live virus that requires a biosafety lab level 3 (BSL-3). Instead, many researchers make use of pseudotyped neutralization assays, which can be handled in a BSL-2 lab. Pseudotyped virus neutralization assays have been used for many types of viruses, however, few SARS-CoV-2 studies have examined its correlation to other neutralization assays like PRNT or MN (Grzelak et al. 2020). A series of studies have reported a correlation between detecting antibodies or antibody titers to neutralizing ability (GeurtsvanKessel et al. 2020; Grzelak et al. 2020; Jääskeläinen et al. 2020; Salazar et al. 2020; To et al. 2020; Wu et al. 2020), but binding is not always predictive of neutralization (Crisuolo et al. 2020; Manenti et al. 2020).

Despite the uncertainty of the role of neutralizing antibodies and the waning of protection, we can probably draw on our knowledge from other viral infections. We can likely expect that we are either immune against reinfection for months or perhaps even a couple of years, or that having encountered the virus before at least will help clear the virus faster the next time around with possibly fewer symptoms. From the SARS epidemic back in 2003 we know that high antibody levels are maintained for at least 16 months before declining significantly (Liu et al. 2006), but one study found that some patients still had detectable neutralizing antibodies 17 years later (Anderson et al. 2020). The humoral response is not the only level of protection, so studies on the cellular immunity are also warranted. In a study by Braun et al., they found that 83% of COVID-19 patients as well as 34% of healthy donors had SARS-CoV-2 spike protein-reactive CD4⁺ T-cells, albeit at lower frequencies among

the healthy donors (Braun *et al.* 2020). It was speculated that this might correlate to some protection if you have had a common cold from coronaviruses. Other studies have similarly observed T-cell reactivity against SARS-CoV-2 in unexposed people, but the source and clinical relevance remain unknown (Sette and Crotty 2020). Single cell transcriptomic analysis has helped shed light on the remarkable heterogeneity in the SARS-CoV-2 reactive CD4 + T cell response among patients with subsets of T-cells correlating to disease severity and antibody levels (Meckiff *et al.* 2020).

Recently, confirmed cases of reinfection have been reported in various countries (Gupta *et al.* 2020; Tillett *et al.* 2020; To *et al.* 2020), however, this is not necessarily a big concern nor unexpected. Waning antibody levels, a poorly developed immune response to SARS-CoV-2 from the first infection or genetic changes in the viral surface antigens could be the explanation. These reinfection cases may be outliers, or reinfection may be more common for other infections as well than we know due to less scrutiny compared to SARS-CoV-2. It is important to note though that a decline in antibody levels after a few months since symptom onset is normal and does not rule out the longevity of protection, as it is also conferred by memory cells.

Literature on COVID-19 is exploding, but warrants a word of caution

On a final note, it is challenging to stay aware of all the literature relating to SARS-CoV-2 serology. The number of publications is exploding and preprints are being released at an unprecedented speed. As of October 11th 2020, the preprint servers medRxiv and bioRxiv contain 9456 articles related to COVID-19/SARS-CoV-2 (medRxiv COVID-19 2020). On one hand, such a unified response by the scientific community is remarkable, on the other hand, it is becoming increasingly difficult for proper science to stand out and some studies and manuscripts are likely rushed.

Additionally, in the eagerness of making preliminary results readily accessible, the outcome of many of the earliest seroprevalence studies were reported in the press before a scientific (albeit not necessarily peer-reviewed) article was released. That is problematic since such results might influence public policy and public opinion before the scientific community has had the chance to scrutinize the results and methods. Often these studies were based on convenience sampling with a selected group and/or had a low participation rate, and thus they were not representative for the general population. However, the results from large serological studies like the Spanish ENE-COVID are now appearing (Pollán *et al.* 2020).

CONCLUSION

We have a lot left to learn about the antibody response to SARS-CoV-2 infection and how this knowledge can guide us in our efforts to combat the pandemic. When using serological assays, careful consideration must be placed on the testing strategy with focus on maximizing specificity and consequently positive predictive value, since the overall prevalence of antibodies in most populations is still low.

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