CORE DIAGNOSTICS

Antibody testing for SARS-CoV-2 infection, quantitative determination, response to vaccines and viral variability

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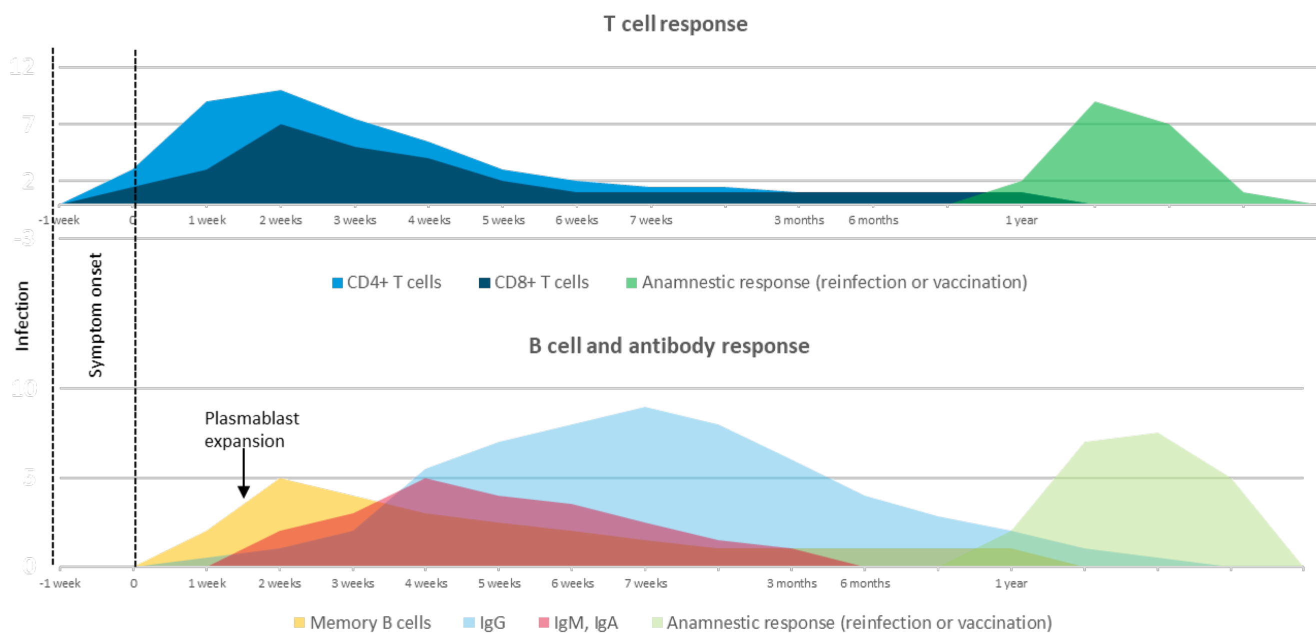
1. Immunological Response to SARS-COV-2

Coronavirus disease 2019 (COVID-19) is caused by SARS-CoV-2, a positive-sense single-stranded RNA virus belonging to the Coronaviridae family. SARS-CoV-2 is the third highly pathogenic agent of this family to affect humans after SARS-CoV in 2003 and MERS-CoV in 2012. SARS-CoV-2 has resulted in more than 3.5 million reported deaths, as of the end of May 2021; although SARS-CoV-2 has a much lower case-fatality than SARS-CoV and MERS, due to much greater transmissibility, has resulted in much greater mortality overall (1). SARS-CoV-2 gains entry into the host cells in the respiratory tract by linking the angiotensin-converting enzyme 2 (ACE2) receptor through a specific portion (receptor-binding domain, RBD) of the Spike 1 (bulb) portion of the viral membrane. SARS-CoV-2 binding to the ACE2 receptor has >20 times the affinity to the ACE2 receptor than SARS S protein, which can explain its rapid transmission worldwide (2).

The mechanisms of the host immune response to SARS-CoV-2 have been elucidated to a large extent. Both humoral and cellular adaptive immune response are elicited and their kinetics over the course of a natural infection has been described (3), as summarized in Figure 1. The T cell response begins during the incubation phase and leads to the stimulation of plasma cells that expand and differentiate. Initial Ab responses are made by short-lived plasmablasts that develop in extrafollicular sites, while the subsequent development of high-affinity and persistent Abs involves maturation and the expansion of B cells in germinal centers. Those centers harbor both memory B cells and plasmablasts, the latter usually short-lived but some persist as long-lived plasma cells (4). Beginning a few days following infection and up to four weeks following, antibodies of different classes are detectable in serum or plasma (5, 6, 7, 8). Unlike other viral infections, the appearance of specific IgM, IgA and IgG is usually separated only by a few days, and quite often is concomitant (6). However, the persistence of IgM (and IgA) is limited as most infected persons will not have detectable titers after 3-4 months from the onset of infection (7). Thus, testing for the specific IgM response allows to determine a 'recent' infection, while looking for the specific IgG response enables to evaluate the acquired humoral immune response for a long period after the infection.

Figure 1: Kinetics of the adaptive cellular and humoral immune response to SARS-CoV-2. Modified from: D.S. Stephens, M.J. McElrath. JAMA 2020; 324: 1279-1281



2. Virus Specific Antibody Targets

The detection profile of viral particles and humoral response for SARS-CoV-2 has been well studied over the last year and two immunodominant proteins have emerged for this virus: the Nucleocapsid protein (N) which binds to the RNA of the virus and the spike protein (S) which gives it its Corona or "crown-like" look. Both N and S stimulate an early and strong humoral response (5, 6, 7, 8), and therefore the diagnostic assays.

2.1 BINDING ANTIBODIES

Specific antibodies of the different classes binding to N antigens are usually detectable within 2-3 weeks after disease onset following a positive result for SARS-CoV-2 RNA (or antigen) (5, 6, 7). Differences in seroconversion

following infection can be associated with disease severity as mild or asymptomatic infections may test positive for antibodies later following infection. Differences in seroconversion timing are also linked to the immune status of the patient (5, 6, 9) and immunocompromised persons may develop a delayed or less robust antibody response (5, 6, 9). Some reports indicate that subjects with a mild infection develop a robust cellular immune response, with levels of both T and B memory cells rising and maintaining a good functional activity for a long time after recovery (10). Levels of anti-N antibodies rise for several weeks following infection but are more likely to decline over time during convalescence. Van Elslande et al have estimated the half-life of anti-N antibodies by linear regression to be about a month and a half, with a likelihood of 50% of individuals testing negative after 201 days from a first positive result (11). Antibodies towards S-derived antigens, and more specifically against RBD, behave differently: they may appear very early, like anti-N (8), but remain detectable for a much longer period of time following natural infection, with an estimated half-life of almost seven months and a likelihood for 50% of individuals testing negative only after more than two years (11). Another report (12) showed anti-RBD antibodies to be still detectable in 96% of 393 convalescent patients after 11-13 months from onset (Figure 2). The different decay of those two antibody specificities allows for the design of a diagnostic workflow in which an initial testing for anti-S, followed by testing for anti-N, will help distinguish between a natural and a vaccine immune response with a high likelihood of a correct classification (13) (Figure 3).

Figure 2: Anti-RBD positivity rates (ARCHITECT SARS-CoV-2 IgG II Quant) after 1 and 11-13 months from onset. Data from: F. Gallais et al. medRxiv preprint doi: https://doi.org/10.1101/2021.05.07.21256823

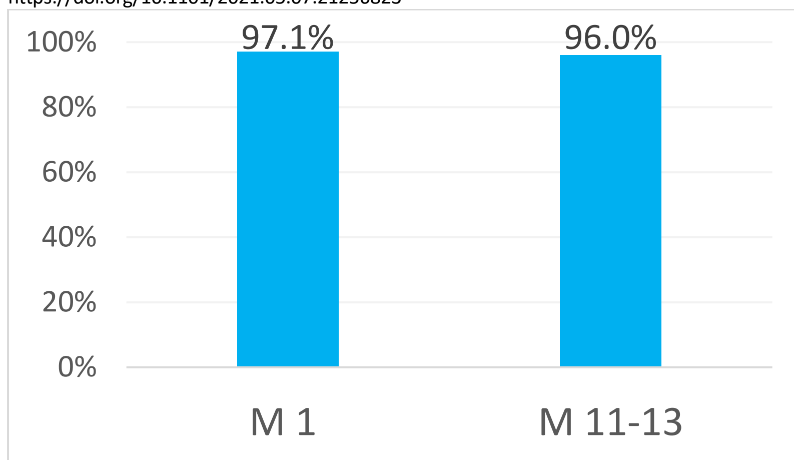
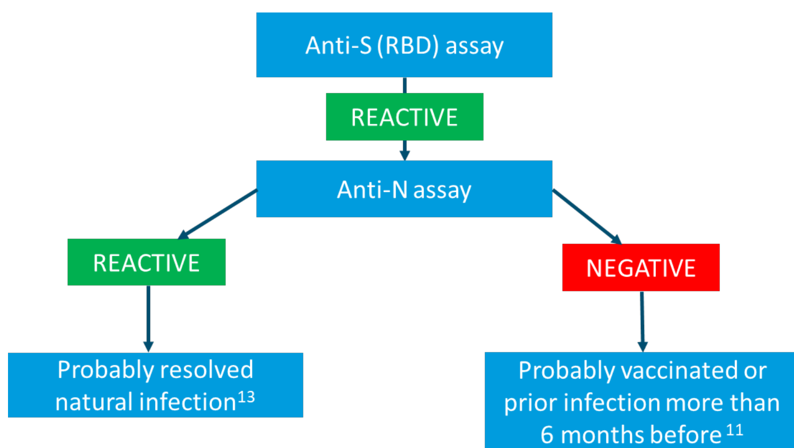


Figure 3: Proposed workflow for SARS-CoV-2 antibody testing. Although the specificity of both antibody assay is very high, the positive predictive value in low-prevalence populations will diminish. In such instances, an initial approach that combines two different assays as af first step may be considered. Test results must be considered along with other clinical data available.



2.2 NEUTRALIZING ANTIBODIES

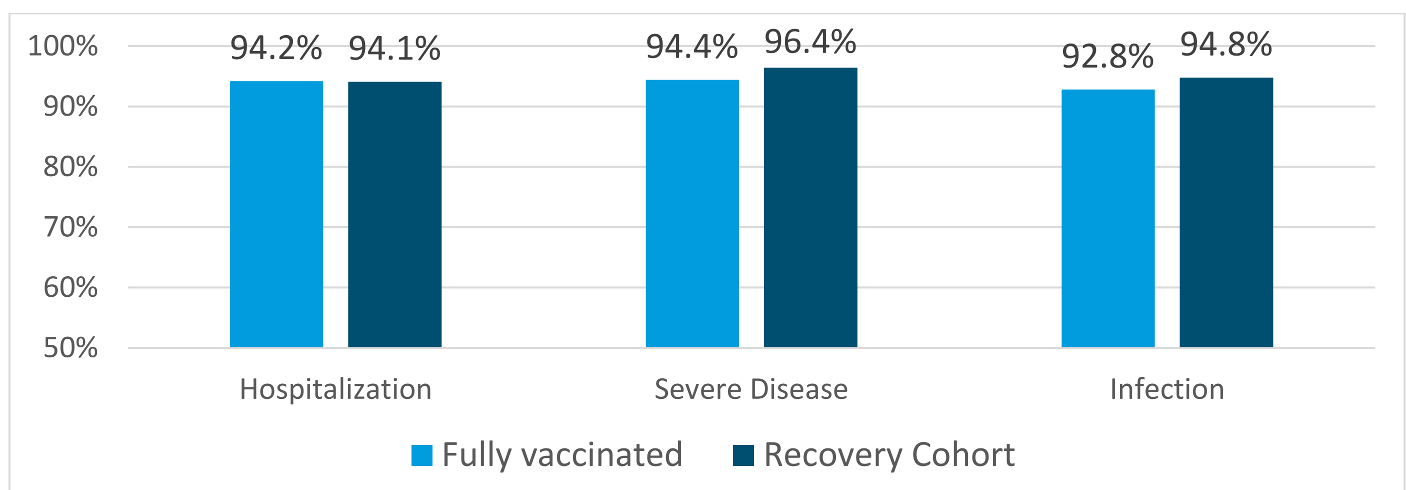
Those are antibodies that effectively inhibit the linkage of the virus to the host cell receptor and represent only a small portion of all clones stimulated after SARS-CoV-2 infection (14). A neutralizing antibody response develops very early after infection, often concomitantly with binding antibodies (6, 15, 16) and great majority of those antibodies are targeting the RBD region (17), not surprisingly since that portion of Spike 1 links the ACE2 receptor. A neutralizing antibody response may be evaluated by classical viral neutralization methods, that require BSL3 laboratory facilities and skilled personnel, or by pseudoviral neutralization for which BSL2 is sufficient. In both

instances, the ability to neutralize the virus in the classical viral neutralization methods is measured by plaque reduction neutralization titer (PRNT) as the end dilution titer of patients' sera able to inhibit virus growth by 50% (ID50) or 90% (ID90) (18). Since those techniques are quite cumbersome and do not allow for rapid processing of many samples, surrogate neutralization methods based on a competitive blocking of antibodies directed to RBD have been proposed (19). Neutralizing antibodies, like binding antibodies, will decay over time after a natural infection and different patterns have been described, from an absence of neutralizing response to a persistence modeled up to several years (20). The same applies after vaccination: a recent observation (21) showed the persistence of binding and neutralizing antibodies for up to seven months after the administration of a first dose of an mRNA vaccine but with a progressive decrease in levels, more evident for neutralizing antibodies and linked to the older age of the recipients. Similar results have been described by Khoury et al (22), who described the decay of neutralizing titer in vaccinated subjects over the first 3-4 months after vaccination to be at least as rapid as the decay observed in convalescent subjects. From those observations, a practical question emerges: which neutralization levels may be considered sufficient to prevent SARS-CoV-2 infection and disease? The answer is still unclear: first, different neutralization methods will yield different results and a harmonization of such manual methods is difficult to achieve (17, 23, 24). Second, modelling the decay of neutralization titer over the first 250 days after immunization predicts a significant loss in protection from SARS-CoV-2 infection will occur, although protection from severe disease should be largely retained (22) as the protective effect of neutralizing antibodies in humans has been demonstrated (25, 26) and it has been recently shown that neutralization level is highly predictive of immune protection, and provide an evidence-based model of SARS-CoV-2 immune protection that will assist in developing vaccine strategies to control the future trajectory of the pandemic (26). However, since neutralization titers against some SARS-CoV-2 variants of concern are reduced compared to the vaccine strain (26, 27), a weak neutralizing activity at medium or low titers, such as less than 1:80 at ID50 (28) or lower than 1:40 at ID90, (29) will not always guarantee an adequate protection to COVID-19 disease and even less to SARS-CoV-2 infection (22).

2.3 CORRELATION BETWEEN BINDING AND NEUTRALIZING ANTIBODIES

Commercially available antibody assays for SARS-CoV-2 detect binding antibodies, and as we have seen only a limited subset of those have neutralizing capacity (14). Many studies have addressed the issue of 'correlating' binding and neutralizing antibody response. Since the latter is due to antibodies directed to Spike epitopes, and mostly to RBD (17), correlation is higher when only anti-S binding antibodies are measured. However, a fair to strong correlation has been reported also between anti-N antibodies and viral neutralization (6, 24). This phenomenon may be explained by the concomitant rise and fall of the two different types of antibodies, as outlined both in studies on the early antibody response (6, 11) and in the convalescent phase (15). The agreement between anti-RBD binding antibodies and neutralizing antibodies is biologically more meaningful and has been demonstrated by several experiments that showed, quite predictably, a good correlation (17, 28, 30). However, a correlation factor will not guarantee the same clinical utility and safety as having a defined positive predictive value (PPV) for a strong neutralizing response according to specific binding antibody levels. This PPV approach has been used during the validation of Abbott SARS-CoV-2 IgG II Quant assays and allowed the identification of specific thresholds that have a 95% likelihood to correspond to a strong neutralizing activity, i.e., a 1:250 ID50 dilution by the Broad Institute PRNT assay (31, 32). This high predictive value helps in the follow up of vaccinated individuals as well as in the convalescent phase of a natural infection, since those two situations have demonstrated similar effectiveness in preventing a 'de novo' SARS-CoV-2 related disease (33, 34) (Figure 4).

Figure 4: Protective effect of vaccination and natural infection. Data from Y. Goldberg et al, medRxiv preprint doi: https://doi.org/10.1101/2021.04.20.21255670



2.4 CELLULAR IMMUNE RESPONSE

Several studies have highlighted the importance of cellular immunity and cellular memory (B and T cells) for the pathogenesis of COVID-19, recovery from disease and infection and provision of long-lasting protection to SARS-CoV-2 (10). However, the direct measurement of B and T cell memory cells is a much more elaborate and costly endeavor than the assessment of humoral immunity, and the presence of either of those cell types may be quickly verified by the anamnestic Ab response usually elicited in short order (1-2 days) upon activation of the immune system (3).

3. Quantitative Determination of SARS-COV-2 Specific Antibodies

Most available SARS-CoV-2 antibody assays provide a qualitative result, that may be used in a semi-quantitative fashion to help 'measure' antibody levels in different populations and timeframes after infection. While this approach has been extensively used, a carefully designed quantitative approach is preferred for multiple purposes: a) carrying out seroprevalence studies that shall be most useful to determine the real prevalence of SARS-CoV-2 infection since several studies have demonstrated that prevalence of infection is underestimated by confirmed COVID-19 cases, especially among younger people (23); b) assessing the different host humoral response to a natural infection in relationship to demographic factors (age, gender, ethnicity), clinical factors (severity of disease, concomitant or preexisting conditions) and immunological factors (coinfections, immunosuppression, immunocompromised patients); c) monitoring of antibody levels over time, either on single individuals or on specific populations; d) evaluating the antibody response after vaccination. For these purposes, quantitative antibody assays need to provide a good sensitivity and specificity, a very good precision, enabling to accurately establish variations over time and across populations, and most of all an extended dynamic range (8). Antibody levels show a huge variation, spanning from non-detectable to low positive levels in immunocompromised and in subjects infected a long time (5-6 months) before testing to the very high levels that are attained at the peak of a natural infection, or even higher levels after a complete vaccination course (35). Therefore, assays with a limited dynamic range will bear the inconvenience of needing dilution to deliver a quantitative result in any clinical setting. Ideally, having a standardization of SARS-CoV-2 antibody tests will be helpful and the WHO approached this by setting up and validating a first standard preparation (36). Unfortunately, this standard has a low concentration that does not cover the complete measuring interval needed in cases of a robust humoral response. Furthermore, even if the adoption of an international standard shall reduce interlaboratory variations (37) and the arbitrary units (AU) currently used by commercial assays may be converted to BAU (for the Abbott SARS-CoV-2 IgG II Quant assays the conversion is 1 BAU/mL = 0.142*AU/mL), the levels expressed by different assays will still diverge due to the polyclonal nature of the standard; the benefit of employing the new units to harmonize values and find common thresholds for correlates to protection appears very limited at the moment (31).

4. Antibody Response to COVID-19 Vaccines

Currently, several options on SARS-CoV-2 vaccines are available, including: 1) those based on mRNA vaccines that use lipid nanoparticles to protect and deliver the mRNA coding for Spike antigen and effectively express the immunogen, 2) viral vector vaccines that encode the viral gene of interest into one of several well-characterized vectors, such as adenovirus and vesicular stomatitis virus (VSV), and 3) the more 'classic' inactivated virus vaccines (38). All vaccine demonstrated a good to excellent efficacy in preventing or reducing COVID-19 disease, hospital admission and admission to intensive therapy, and fatal outcomes (36). Efficacy has been correlated with both anti-S antibody levels and neutralizing antibody activity (39), suggesting that immune correlates of protection can be used as surrogate endpoints for vaccine efficacy. Experimental data on a nonhuman primate model of SARS-CoV-2 infection that replicates key features of human infection (40) have demonstrated that an mRNA vaccine (mRNA-1273) encoding the prefusion-stabilized S protein encapsulated in a lipid nanoparticle elicited a strong mucosal and circulating antibody responses in a dose-dependent manner. Viral replication was significantly reduced in bronchoalveolar lavages and nasal swabs following SARS-CoV-2 challenge in vaccinated animals and was most strongly correlated with levels of anti-S antibody binding and neutralizing activity.

Several studies have evaluated both the antibody response and cellular immunity after vaccination. The humoral response to mRNA or viral vector vaccines has been studied with the Abbott SARS-CoV-2 IgG II Quant assays and reported in multiple papers (8, 41, 42, 43, 44, 45, 46). Immunocompetent individuals usually develop high anti-RBD levels after having received both vaccine doses, with a lower but still valid response in elderly people and in males (Figure 5) (46). A diminished response, both in frequency of anti-RBD positivity and in antibody levels, has been reported in subjects with a prior immune compromised status (47, 48) and this may suggest that for 'special' groups a different vaccination schedule and/or further doses may be needed to achieve a protective response. On the other side, several investigators pointed out that subjects that have been immunized after a prior natural exposure reach higher levels of both binding and neutralizing antibodies and a stronger T-cell response after a

single vaccine dose (41, 42, 45, 46), indicating that a second dose of vaccine may be unnecessary or avoidable in previously infected individuals.

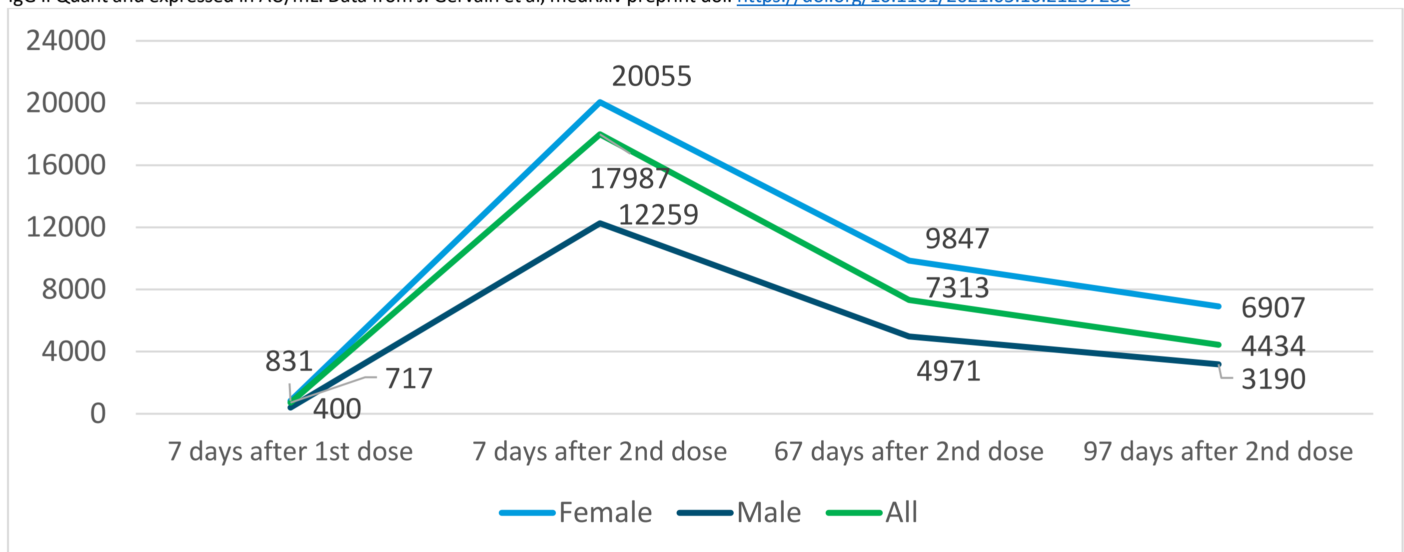


Figure 5: Monitoring of anti-RBD levels in 49 health care workers vaccinated with two doses of an mRNA vaccine. Levels are determined by ARCHITECT SARS-CoV-2 IgG II Quant and expressed in AU/mL. Data from J. Gervain et al, medRxiv preprint doi: https://doi.org/10.1101/2021.05.16.21257288

Finally, two modelling studies have demonstrated that serology can be useful prior to vaccine administration. Bubar et al (49) have demonstrated that limiting vaccination to seronegative individuals by employing an antibody test with 96% sensitivity and 99% specificity – both below the claims of the Abbott SARS-CoV-2 IgG II Quant assays – would warrant a higher efficacy in reducing infections, death and years of life lost in all age groups. Ayoub et al (50) have indicated that gains from prioritization by antibody status were largest in settings where the proportion of the population already infected at the start of vaccination was 30-60%, but advantages appeared also at lower prevalence. Besides gaining time on immunity coverage, this strategy will also result in the need of half the number of vaccine doses administered to prevent a single infection. For a vaccine that only protects against disease and not infection, vaccine impact was reduced by half but the relative gains from using antibody status to prioritize vaccination recipients were similar.

5. SARS-CoV-2 Variants

Despite having a large RNA genome, SARS-CoV-2 has a good proofreading capability that limits the occurrence of mutations compared to most other RNA viruses. Nevertheless, it has been estimated that SARS-CoV-2 evolves at a rate of ~1.1 x 10 substitutions per site per year, corresponding to one substitution every ~11 days and more than 12,000 mutations have already been detected in the SARS-CoV-2 sequence, compared with the reference sequence described at the beginning of the outbreak (51). The widespread S protein mutation, D614G, has been found to increase viral infectivity (52) and has become dominant worldwide. Additionally, that mutation and other recurring ones such as E484K, N501Y and their combinations have been found in some fast-spreading CoV-2 lineages in UK, South Africa, Brazil and other countries (53). Those variants are more infectious than the original strain and may pose issues for diagnosis, treatment and prevention of SARS-CoV-2 infection, and thus have been named 'variants of concern' (VOC).

Viral neutralization studies have been performed using authentic viruses and antibodies induced either by a natural infection or by vaccination. A comprehensive review (25) has indicated that immune sera/plasma retained most of its neutralizing potency against B.1.1.7 and B.1.427/B.1.429 variants, but significantly lost neutralizing potency against B.1.351 and P.1 variants, with B.1.351 having the worst reductions. Longitudinal monitoring of emerging variants and antibody-induced immunity is then warranted, though the effectiveness of currently available vaccines shall not be seriously hampered, provided that a substantial share of the target population becomes immunized. Serological assays may also be challenged by VOC and this occurrence is actively monitored by Abbott's Research and Development team. First, 'in silico' examination of B.1.1.7, B.1.351, B.1.617, or P.1 sequences revealed no mutations that are predicted to impair performance of Abbott antibody assays targeting the nucleocapsid or RBD regions of the viral variants. Overall assessments indicate the Abbott assays as able to detect the major viral variants including B.1.1.7, B.1.351, B.1.617 and P.1. In detail, a first direct evidence has been obtained on the B.1.1.7 variant (54), where 19 out of 19 samples collected >15 days after onset from patients infected by that variant showed a positive result by the ARCHITECT and Alinity i SARS-CoV-2 IgG II Quant assays (31, 32). Another study, carried

out in France (55), showed a very good correlation (r >0.930) with a live-virus neutralization assays for variants D164G, B.1.1.7 and B.1.351 and suggested that anti-RBD levels >2.3 log_{10} AU/mL by the ARCHITECT SARS-CoV-2 IgG II Quant assays would guarantee a neutralization against D145E and B.1.1.7 and >3.0 log_{10} AU/mL against all three variants.

6. Conclusion

The newer developments of SARS-CoV-2 serological testing have brought up the possibility to determine the levels of antibody response at all stages of the infection as well as after vaccination. In the early stages the presence of SARS-CoV-2 antibodies is strongly associated with clearance of infectious virus isolation and it has been demonstrated (56) that seropositivity and viral RNA are likely more reliable markers of infectious virus suppression than subjective measure of COVID-19 symptoms. Since the IgG serological response is long-lasting the evaluation of this Ig class is very well suited for the purpose of population screening to assess the burden of infections and possibly to evaluate incidence and the efficacy of prevention measures. The value of serology in helping determine the real burden of infection is highlighted by the evidence that on average 44% of all SARS-CoV-2 infection, with a range of 20-85%, are asymptomatic (57) and therefore may be hardly detectable by symptom-based testing strategies. A recent study (58), carried out in Wales after antibody screening of 33,822 people in an area with a 2% prevalence of SARS-CoV-2 antibodies, yielded a conservative estimate of 360 cases prevented, representing a would-be reduction of 11% of all cases diagnosed. Modelling healthcare burden estimates that 24 hospitalizations, 5 intensive care admissions and 15 deaths were prevented, representing 6.37%, 11.1% and 8.19%, respectively of the actual counts during the same period. A less conservative, best-case scenario predicts a much higher number of cases prevented (2,328, i.e., an 80% reduction in would-be cases). Cost effectiveness analysis indicates 108 quality-adjusted life years (QALYs) gained, an incremental cost ratio of £2,143 per QALY gained and net monetary benefit of £6.2m that would increase to £15.9m in the less conservative scenario.

Antibodies towards the N antigen retain their value to assess infection and are especially useful to detect breakthrough infection in subjects undergoing vaccination, since almost all available vaccines do not elicit this kind of antibody response (37). Determining antibodies directed against Spike epitopes by quantitative methods brings the additional advantage of detecting a sustained antibody response over time and correlate those levels to other components of the host immune response. Table 1 reports the different combinations of virological (SARS-CoV-2 RNA and antigen) and serological methods and suggests possible interpretation criteria for those. Of note, on the serology side IgG antibodies to Nucleocapsid, IgM antibodies to Spike and IgG antibodies to Spike by quantitative methods are considered separately.

Table 1

RNA			Ag	IgG (S RBD)				IgG (N)	IgM (S RBD)	Suggested interpretation (most likely)
Pos		Neg		Pos			Neg			
high Ct	Low Ct			High	Med	Low				
	✓		-				✓	-	-	Late incubation phase or very early infection (<2 days)
	✓		+				✓	-	-	Early infection – occurring less than 2 weeks earlier
	✓		+				✓	-	+	Infectious, high antibody levels are often associated with a severe clinical course
	✓		+		✓			+	+	Infectious, if IgM and IgG are elevated with Ag positive there is strong potential for severe disease. Either IgG or IgM may be negative during the first 2-3 weeks
✓			-			✓		+	+	Late acute stage COVID-19 infection 10-14 days post symptom onset, very low likelihood of patient being infectious to others
		✓	-		✓			+	+	Late acute stage COVID-19 infection, patient no longer infectious to others
		✓	-	✓				+	+	Early convalescent stage
		✓	-				✓	-	-	No SARS-CoV-2 infection. If testing occurs after the 2nd dose of vaccine it may indicate an insufficient immune response. This is more likely to occur in elderly people and/or immunocompromised / immunosuppressed patients
		✓	-			✓		-	-	Infection occurred >6 months before. If vaccinated, possibly linked to the same factors as in the previous case
		✓	-			✓		-	+	Recipient of an mRNA or other spike protein-based vaccine. If testing occurs >21 days post second dose clinical background should be checked and cellular immunity/neutralizing antibody possibly verified
		✓	-		✓			-	-	Recipient of an mRNA or other spike protein-based vaccine if tested 14-21 days post dose 1. If tested >21 days and <3 months post dose 2 it could be advisable to confirm levels of neutralizing antibody
		✓	-	✓					-	Vaccination by spike protein-based vaccine. High likelihood of strong neutralizing response and cellular immunity
		✓	-	✓				+	-	Natural infection and / or vaccinated and naturally infected in the recent past

Different combinations of viral and host biomarkers for SARS-CoV-2 infection. The interpretations shall be considered in general terms due to the variations in individual immune response and in the performance of different assays. High, medium (Med) and low levels are indicated in general with no specific values on the purpose of this model, no definite thresholds are recommended; Pos = positive; Neg = negative; Ct = cycle thresholds by RT-PCR; S RBD = Spike Receptor Binding Domain; N = Nucleocapsid; + = positive by the assay specifications; - = negative by the assay claims.

Lastly, whenever immunity conferred by specific antibodies is fully demonstrated, measuring this specific antibody response will allow establishment of a strong correlation to protection. This will be of utmost relevance for a tailored serological diagnosis in recovered as well as in vaccinated individuals. On the former, a threshold for antibody levels will become relevant to establish the likelihood of SARS-CoV-2 infection being re-acquired – the European Centers for Disease Control have already indicated that a previous infection is likely to guarantee a good protection towards reinfection for 5-7 months (59) and higher levels of immune markers were correlated with a reduced risk of symptomatic infection (60). As the numbers of vaccinated individual grow larger, measuring antibody levels will facilitate plans for future immunization campaigns and eventually for administration of booster doses of vaccine by a personalized approach (37). In both groups, SARS-CoV-2 antibody levels with a high concordance and predictive value for a neutralizing humoral response and a strong cellular response will also allow the lifting of travel restrictions and easing, or abolishing, the other generic measures set up for transmission prevention.

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