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Perspective

COVID-19 Antibody Tests and Their Limitations

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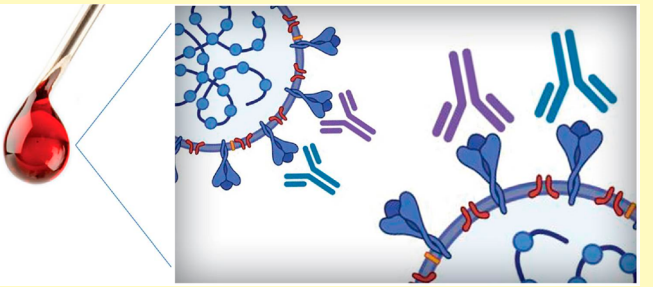
ABSTRACT: COVID-19, caused by the SARS-CoV-2 virus, has developed into a global health crisis, causing over 2 million deaths and changing people's daily life the world over. Current mainstream diagnostic methods in the laboratory include nucleic acid PCR tests and direct viral antigen tests for detecting active infections, and indirect human antibody tests specific to SARS-CoV-2 to detect prior exposure. In this Perspective, we briefly describe the PCR and antigen tests and then focus mainly on existing antibody tests and their limitations including inaccuracies and possible causes of unreliability. False negatives in antibody immunoassays can arise from assay formats, selection of viral antigens and antibody types, diagnostic testing windows, individual variance, and fluctuation in antibody levels. Reasons for false positives in antibody immunoassays mainly involve antibody cross-reactivity from other viruses, as well as autoimmune disease. The spectrum bias has an effect on both the false negatives and false positives. For assay developers, not only improvement of assay formats but also selection of viral antigens and isotopes of human antibodies need to be carefully considered to improve sensitivity and specificity. For clinicians, the factors influencing the accuracy of assays must be kept in mind to test patients using currently imperfect but available tests with smart tactics and realistic interpretation of the test results.



KEYWORDS: *COVID-19, SARS-CoV-2, antibody, false positive, false negative*

The coronavirus disease 2019 (COVID-19) was first identified in December 2019 in Wuhan, China, and rapidly spread across the globe to cause a pandemic. The World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern on 30 January 2020 and a pandemic on 11 March. As of 27 January 2021, more than 100 million cases of COVID-19 have been reported in more than 188 countries and territories, resulting in more than 2 million deaths, according to the Center for Systems Science and Engineering at Johns Hopkins University.

COVID-19 is caused by the novel severe acute respiratory syndrome−coronavirus 2 (SARS-CoV-2),[1−3] which is an enveloped virus with a positive-sense, single-stranded RNA genome, containing four main structural proteins known as spike (S), envelope (E), membrane (M), and nucleocapsid (N), along with nonstructural open reading frames, named ORF1a/b, ORF3, ORF6, ORF7a/b, ORF8, and ORF9b (Figure 1). The ORF1a/b comprises 15 nonstructural proteins (NSP1−10, 12−16) including RNA-dependent RNA polymerase (RdRp, NSP12).[4] The trimeric S protein is composed of a highly conserved C-terminal S2 subunit and a less conserved N-terminal S1 subunit. There is a receptor-binding domain (RBD) on S1 subunit, which mediates coronavirus entering host cells.[5] The dimeric N protein has two distinct RNA-binding domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), which is thought to bind with viral RNA genome probably by electrostatic interactions.[6]

In this Perspective, we briefly describe the main PCR and antigen tests designed to detect active COVID-19 disease. We then turn to our main focus to antibody assays and arrays, driven by the many reports of inaccuracies in antibody assays in the popular press. We describe existing antibody tests and their limitations including false positives and negatives and the many possible causes of unreliability.

CURRENT DIAGNOSTIC METHODS FOR COVID-19

As of 27 January 2021, there are at least 420 nucleic acid tests (NATs) including 4 next generation sequencing (NGS)-based detection, 179 immunoassays for antigens, 432 immunoassays for antibodies including 8 for neutralizing antibodies, and 1 immunoassay for simultaneous antigen and IgM/IgG that are either commercially available or in development for the diagnosis of COVID-19, according to Foundation for Innovative New Diagnostics (FIND) (https://www.finddx.org/covid-19/pipeline/), a WHO collaborating center for laboratory strengthening and diagnostic technology evaluation. Besides the above main-stream assays, there are also other tests, such as one immunoassay for cytokines for research use only, one test for white blood cell morphology and ratios, and one test for breath

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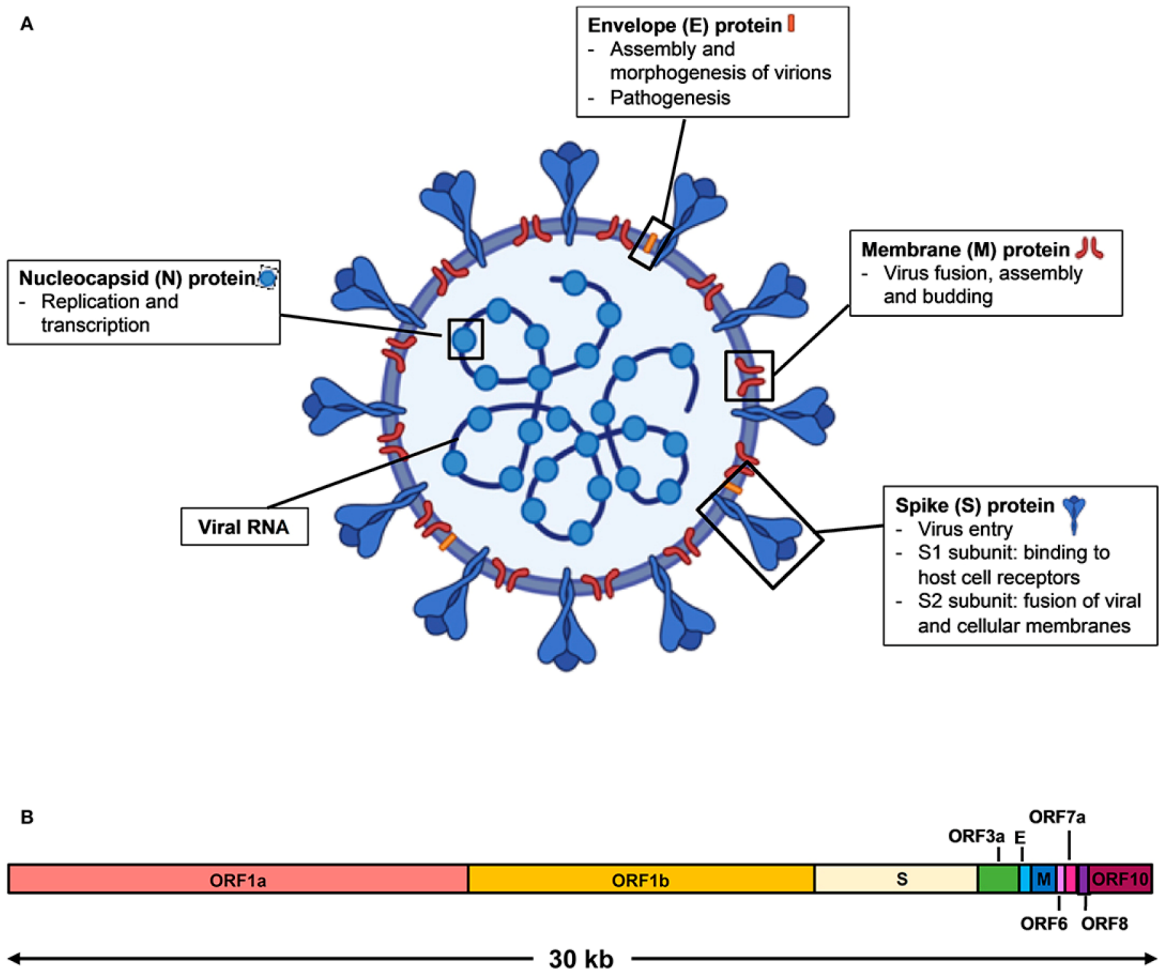

Figure 1. Schematic diagram of SARS-CoV-2 virus structure (A) and genome organization (B). Reprinted with permission from ref 7. Copyright (2020) Frontiers Media S.A.

volatile organic compounds in development (https://www.finddx.org/covid-19/pipeline/).

NATs. Currently, NATs or molecular biology tests, as the most quickly established laboratory diagnostic method in a novel viral pandemic, are the gold standard clinical diagnostic methods for COVID-19 detection. Many types of NATs have been developed to detect the unique genetic material of SARS-CoV-2 in specimens, including reverse transcription polymerase chain reaction (RT-PCR),[8−12] reverse transcription loop-mediated isothermal amplification (RT-LAMP),[13−18] droplet digital PCR (ddPCR),[19−21] CRISPR related technology,[22−25] sequencing,[26−28] and biosensors.[29−33] To date, primers-probe sets for NATs have been designed to target the ORF1a,[13,17,34] ORF1b,[8] ORF1ab,[9,16] Nsp2,[35] Nsp3,[18] RdRp,[10,11,14,36−39] S,[2,16,18,36,38,40] E,[10,11,41] M,[40] and N[8−10,12,15,17,18,38,42] genes. WHO listed a summary of available protocols of molecular assays to diagnose COVID-19.[43]

However, NATs have reportedly suffered from a high false-negative rate,[44,45] which was estimated to drop from 38% (confidence interval, CI, 18% to 65%) on the day of symptom onset to 20% (CI, 12% to 30%) 3 days post symptom onset (DPSO) and then to increase to 66% (CI, 54% to 77%) 16 DPSO.[46] The results may be influenced by improper sample types,[47,48] sampling time,[49−51] viral mutation,[52,53] interindividual variance,[54,55] intraindividual fluctuation,[56,57] inadequate handling, improper storage, and transportation of samples.[58] Moreover, the detection window for NATs is narrow in accordance with the fact that the positive rate of NAT, especially in upper respiratory tract specimens, declined significantly during the immunological phase of illness.[46] In addition, the overall throughput of available RNA tests is highly limited by their nature of requiring high workload, skilled personnel for testing and sample collection, special reagent kits, costly centralized infrastructure, and professional biosafety level (BSL)-2 lab.[59] Different from conventional RT-PCR relying on thermal cycling which takes a long turnover time from 4 h up to 3 days,[60,61] novel assays, such as Sherlock CRISPR SARS-CoV-2 kit (Sherlock Biosciences), SARS-CoV-2 RNA DETECTR Assay (Mammoth Biosciences), and the ID Now technology (Abbott Diagnostics) based on isothermal amplification, can give results in 1 h or even several minutes. Readers are directed to previous reviews[62−73] for more discussion on this topic.

Direct Antigen Tests. These tests may not be as reliable as NATs, due to clinical performance influenced not only by sample types, sampling time, inadequate handling, improper storage, and transportation but also by cross-reactivity in the immunoassay. In addition, direct antigen tests have a similar narrow detection window to NATs. Rapid antigen tests such as lateral-flow immunoassays (LFIA) or immunochromatographic (ICG) assays[74−77] and microfluidic immunoassays,[78] which have the advantage of low cost, short turnaround time, and convenience without the need of sophisticated instruments, have been developed to detect the virus in respiratory samples. But these tests alone are not recommended for an initial COVID-19 diagnosis because their poor clinical or diagnostic sensitivity, i.e. the ratio of true positive/(true positive + false negative), have been reported as 30.2% (32/106),[76] 50% (47/94),[75] and 57.6% (76/132).[74] A chemiluminescence immunoassay (CLIA) for antigen showed a clinical sensitivity of only 55.2% (173/313).[79]

An ultrasensitive enzyme-linked immunosorbent assay (ELISA) coupled with thio-nicotinamide adenine dinucleotide (NAD) cycling was reported with a limit of detection (LOD) of 2.3×10^{-18} mol/

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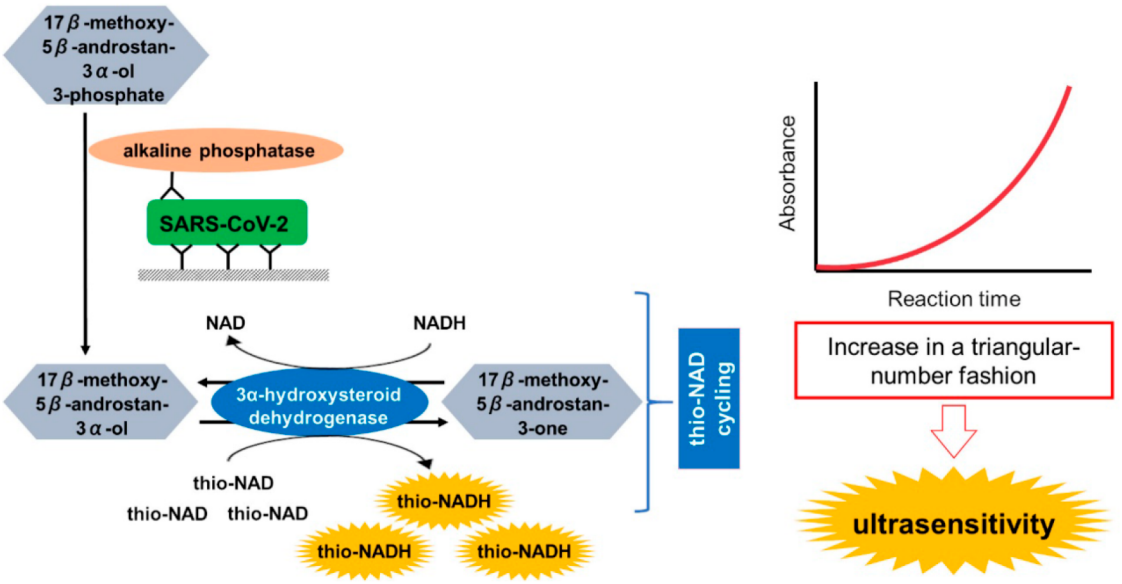

Figure 2. Ultrasensitive ELISA coupled with Thio-NAD cycling for SARS-CoV-2. Reprinted with permission from ref 80. Copyright (2020) MDPI (Basel, Switzerland).

assay for SARS-CoV-2 S protein.[80] Thio-NAD cycling in this strategy is achieved using alkaline phosphatase, androsterone derivative (17β-methoxy-5β-androstan-3α-ol 3-phosphate), and 3α-hydroxysteroid dehydrogenase (3α-HSD) and its coenzymes (NADH and thio-NAD). During this cycling reaction, thio-NADH accumulates in a triangular-number fashion, which is measured at an absorbance of 405 nm (Figure 2). This ultrasensitive method may play a significant role if it can be commercialized, but its clinical specificity, i.e. the ratio of true negative/(true negative + false positive), needs to be independently validated with enough samples outside the lab.

A portable, ultrarapid (3 min), ultrasensitive (LOD of 1 fg/mL) cell-based biosensor was developed for the detection of the SARS-CoV-2 S1 spike protein antigen.[81] The binding of the SARS-CoV-2 S1 protein to its specific antibody, which is engineered on the membrane of Vero cell by electroinserting, results in a change of bioelectric properties of the cell measured by a bioelectric recognition assay. The biosensor can be further coupled with a portable read-out device operated via smartphone or tablet into a ready-to-use platform, which can be potentially applied for the mass screening of SARS-CoV-2 surface antigens without prior sample processing. Manufacturing of engineered cells may be a limitation.

A highly sensitive label-free field-effect transistor biosensor device was developed where SARS-CoV-2 spike antibody was conjugated onto a graphene sheet via 1-pyrenebutyric acid *N*-hydroxysuccinimide ester.[82] SARS-CoV-2 spike protein was detected based on conductance changes after antigen−antibody binding. LODs were reported as 1 fg/mL in phosphate-buffered saline and 100 fg/mL in clinical transport medium for SARSCoV-2 spike protein, 16 pfu/mL in culture medium, and 242 copies/mL in clinical samples for SARS-CoV-2 virus. No sample pretreatment or labeling was required for assays. Cross-reactivity was only evaluated with MERS-CoV antigen, and the clinical performance needs to be validated further. An electrochemical immunosensor combining magnetic beads with carbon black-based screen-printed electrodes was developed for rapid detection (30 min) of SARS-CoV-2 S or N protein.[83] The LODs in untreated saliva were reported as 19 ng/mL for S protein and 8 ng/mL for N protein. Preliminary assessment showed an agreement in 22/24 samples with RT-PCR and no cross-reactivity with seasonal influenza virus A (H1N1) and 2009 influenza virus pH1N1. Further validation is needed.

ANTIBODY TESTS

Although they do not confirm the presence of active virus, antibody tests have some impressive advantages in comparison with NATs and antigen tests. These include a much longer detection window, operator ease and safety to collect blood rather than respiratory samples, stability of human antibodies compared to viral RNA during sample collection, preparation, transport and storage,[84] and more uniform distribution of antibodies in blood than virus in respiratory samples which may cause false negative results in NATs.[85] Antibodies can also be detected successfully in saliva in addition to blood.[86] Also, these tests do not require BSL-2 laboratories.

Antibody tests can play a supplementary but indispensable role in (1) diagnosis of suspected cases with negative viral RNA test or past COVID-19 infection;[64,87,88] (2) surveillance and epidemiological assessment at a population level[89−91] from which the true case fatality rate can be determined and according to which medical resource can be distributed; (3) monitoring immune responses to assess the course, degree, and durability of immunity;[61,92−94] (4) identifying potential convalescent plasma donors;[95,96] (5) therapeutic antibody development and evaluation;[97−100] (6) vaccine development and evaluation;[87,101,102] and (7) contact tracing to figure out the subsequent chains of events and define clusters of cases.[103]

Both neutralizing and binding antibodies can be targeted for detection. The former bind to a specific part of a pathogen and have been observed in a laboratory setting to decrease SARS-CoV-2 viral infection of cells. The latter, binding or non-neutralizing antibodies, such as immunoglobulins (Ig), bind specifically to the pathogens but do not interfere with their infectivity. Binding antibodies signal the presence of a pathogen in the body, while neutralizing antibodies block the entry of a pathogen into a cell. IgM is considered an indicator of early stage infection, while IgG is an indicator of current or prior infection.[87] IgA and IgM can persist in the body for about 2 months while IgG can last for more than 3 months.[93,104] Although some individuals maintained neutralizing antibody titers >1000 at >60 DPSO, others had detectable neutralizing

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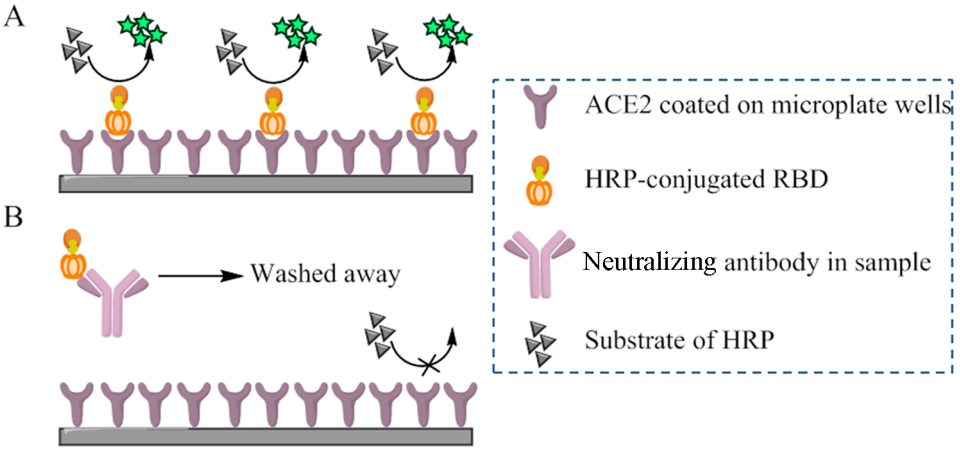

Figure 3. Principle of the SARS-CoV-2 sVNT without (A) and with (B) neutralizing antibody.

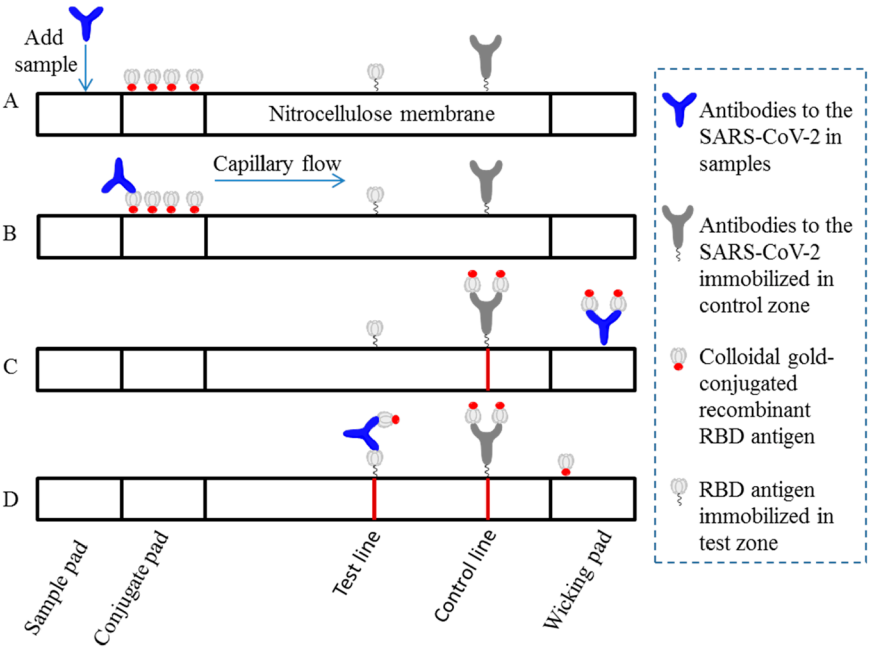

Figure 4. Principles of WANTAI SARS-CoV-2 Ab Rapid Test.

antibody titers over only a relatively short period (less than 40 DPSO).[104]

Neutralization Assays. These are standard methods for coronavirus serology in blood serum[105,106] and can function as reference methods to evaluate the diagnostic performance of binding antibody tests.[107] Plaque reduction neutralization tests (PRNT) take advantage of virus−antibody interactions in a test tube or microtiter plate to measure antibody effects on viral infectivity in virus-susceptible cells. Briefly, serial dilutions of serum sample or antibody solution to be tested are incubated with a standardized amount of virus. The resulting immune complexes are then added to the virus-susceptible cell monolayer. Then the cells are covered with a semisolid medium that prevents the virus from spreading indiscriminately. After several days for incubation, plaques can be visualized by fluorescent antibodies or specific dyes. PRNT end-point titers are expressed as the reciprocal of the last serum dilution showing the desired percent reduction in plaque counts.[108,109] Although PRNT is considered the "gold standard" for detecting and measuring neutralizing antibodies, intensive labor and time (3−7 days) as well as not being readily amenable to automate makes it difficult to use on a large scale.

Microneutralization (MN) assays usually detect the viral antigens in virus-infected cells in microtiter plates in combination with an ELISA, which can yield results within two days. Briefly, serially diluted sera are preincubated with a standardized amount of virus prior to the addition of host cells. After an overnight incubation, the cells are fixed in the microtiter plate and the presence of viral antigens in infected cells is detected by ELISA. The detection of viral antigens indicates the absence of neutralizing antibodies at that serum dilution.[110,111] MN assays measure neutralizing antibodies in an automated, high-throughput, and more objective way.[112]

But both PRNT and MN tests usually require viral culture growth that needs to be conducted in a BSL-3 laboratory. The pseudovirus neutralization assay, in which the SARS-CoV-2 protein is grafted onto harmless viruses or virus-like particles, is safer and more high-throughput and can be done in BSL-2 facilities.[113,114]

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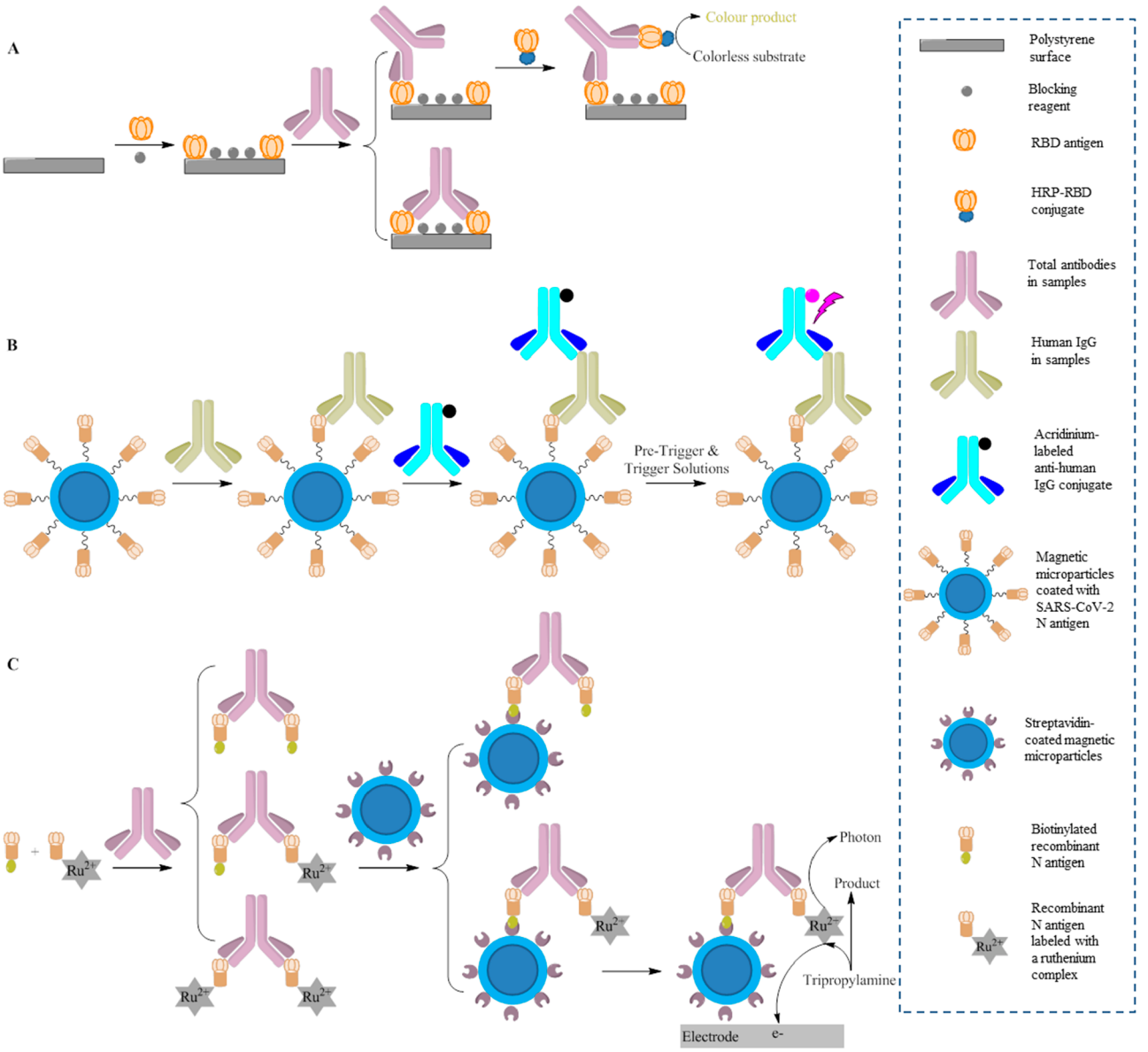

Figure 5. Principles of WANTAI SARS-CoV-2 Ab ELISA (A), Abbott SARS-CoV-2 IgG assay (B), and Elecsys Anti-SARS-CoV-2 ECL immunoassay (C).

Recently, a surrogate virus neutralization test (sVNT) without the need for any live virus or cells that can be completed in 1−2 h in a BSL-2 laboratory was developed.[115] Briefly, anti-SARS-CoV-2 neutralizing antibodies block horseradish peroxidase (HRP)-conjugated receptor binding domain (RBD) protein from binding to the angiotensin-converting enzyme 2 (ACE2) protein precoated on an ELISA plate (Figure 3). It reportedly achieves 100% (200/200) specificity and 98% (49/50) −98.9% (173/175) sensitivity at the final serum dilution of 1:20. Its commercial product, i.e. the cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript USA Inc.), has been given Emergency Use Authorization (EUA) by the US FDA recently (https://www.fda.gov/media/143583/download). However, its clinical performance still requires independent third-party assessment. Another question is whether the interaction between binding antibodies and HRP-conjugated RBD may also block HRP-conjugated RBD from binding to ACE2, which may cause potential false positives in neutralization assays.

Binding Antibody Tests. These are usually in the form of sandwich immunoassays and are used more widely than the neutralization assays. Various binding antibody assays have been developed to detect immunoglobulins IgA, IgM, and IgG in blood against immunogenic proteins of SARS-CoV-2, including ELISA,[2,105,106,116−118] LFIA or ICG assay,[61,119−127] CLIA with enzyme[128,129] or nonenzyme labels,[92,130] electrochemiluminescence immunoassay (ECLIA),[131] fluorescence immunoassay (FIA),[86] protein microarrays,[132−135] biosensors,[136,137] and immunofluorescence assays (IFA).[138−140]

As an example of lateral flow sensing, the WANTAI SARS-CoV-2 Ab Rapid Test (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.) employs a chromatographic lateral flow sensor device in a cassette format. Briefly, colloidal gold conjugated recombinant receptor binding domain (RBD) antigens of SARS-CoV-2 are dry-immobilized at the end of a

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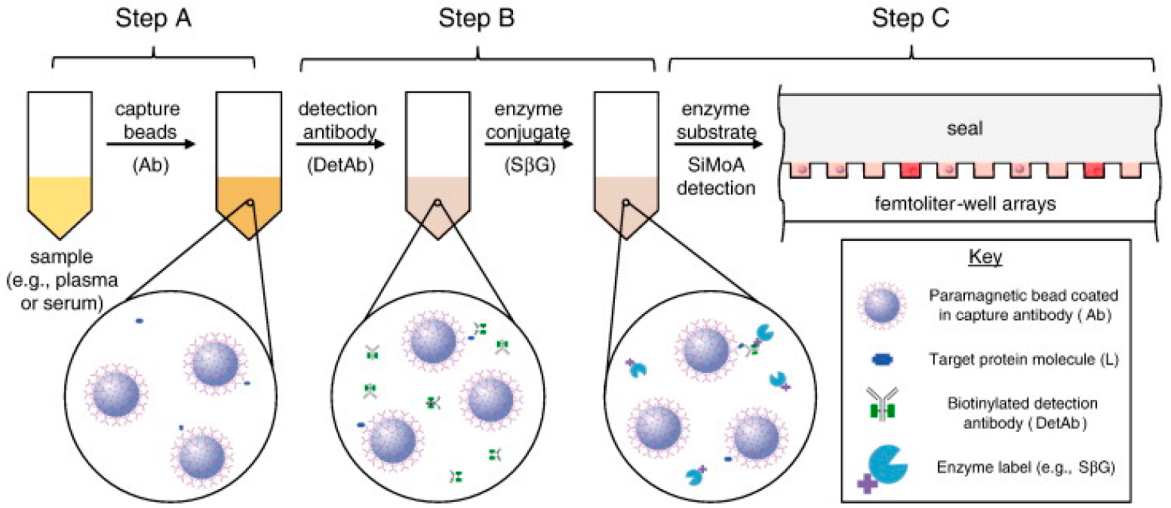

Figure 6. Digital ELISA (Simoa) based on the detection of single immunocomplexes in arrays of femtoliter wells. Reprinted with permission from ref 155. Copyright (2020) Elsevier B.V.

nitrocellulose membrane strip. After the sample is added, SARS-CoV-2 antibodies migrate driven by capillary action and bind with the gold-conjugated antigens. The complexes continue to migrate along the strip until reaching the Test Zone where they are captured by the SARS-CoV-2 RBD antigens to generate a visible red line due to the aggregated gold particles. Unbound gold-conjugated particles continue to migrate until the Control Zone where they are captured by antibodies to induce the control red line, which indicates the validity of the sensing (Figure 4). However, the antigen-binding sites of antibodies in samples may be occupied by the gold conjugated recombinant RBD antigens and thus cannot be captured by the RBD antigen immobilized in the Test Zone, which may lead to false negative results (Figure 4C). This rapid test was reported with a sensitivity of 97.5% (78/80) and specificity of 95.2% (199/209) by Lou et al.,[61] but poor sensitivities of 4% (2/49), 52% (12/23), and 65% (13/20) were also reported using samples from three medical institutions.[141] Besides the general colloidal gold nanoparticles, Eu(III) fluorescent microspheres[142] and quantum dots[143] have also been used in immunochromatographic assays to detect SARS-CoV-2 specific antibodies.

WANTAI SARS-CoV-2 Ab ELISA (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.) is a novel commercial ELISA kit developed in polystyrene microwell strips based on a double-antigen sandwich immunoassay. Briefly, the antibodies in a patient's serum or plasma samples are captured by recombinant RBD antigen of SARS-CoV-2 immobilized in polystyrene microwells. Then HRP-labeled recombinant RBD antigen conjugate is added and bound to the antibody−protein complex inside the wells. After removing the unbound reagents by washing, colorless substrate reagent solution is added and catalyzed by HRP into a blue product, which turns yellow and is detected after the reaction is stopped with sulfuric acid (Figure 5A). However, the antigen-binding sites of antibodies in samples may be occupied only by the RBD antigen immobilized in polystyrene microwells and thus could not bind with HRP-RBD conjugate, which may reduce the sensitivity (Figure 5A). This assay was validated with a sensitivity of 97.5% (78/80)[61] and 98% (98/100)[144] and specificity of 100% (300/300),[61] but in another report the sensitivity reached only 62% (59/95) in total patients and 79% (38/48) in patients with at least 7 days of symptoms.[145]

A two-step chemiluminescent microparticle immunoassay (CMIA) also called magnetic particle-based chemiluminescent immunoassay (MCLIA), i.e. the Abbott SARS-CoV-2 IgG assay (Abbott Diagnostics), was given EUA by the FDA for qualitative detection of IgG in human serum or plasma against the SARS-CoV-2 N protein. Briefly, antibodies to SARS-CoV-2 in the sample are captured by paramagnetic microparticles coated with SARS-CoV-2 N antigen, which are then bound by acridinium-labeled antihuman IgG. After adding Pre-Trigger and Trigger Solutions, chemiluminescence is generated and measured by ARCHITECT i1000SR and i2000SR measurement systems, or other authorized instruments (Figure 5B). The sensitivity of this assay from the estimated day of symptom onset for 125 patients was 96.9% (95% confidence interval [CI], 89.5% to 99.5%) at 14 days, and 100% (95.1% to 100%) at 17 days.[146] But 42% (217/511) positive samples detected by this kit were negative by an in-house ELISA,[147] and insufficient positive rates of 8.8% for <7 DPSO, 40.5% during 7−13 DPSO, 81.0% during 14−20 DPSO, and 84.4% for ≥21 DPSO were also reported.[148]

The Elecsys Anti-SARS-CoV-2 ECL immunoassay (Roche Diagnostics) utilizing a double-antigen sandwich test principle on Cobas E analyzers (Roche Diagnostics) was authorized as EUA by the FDA for in vitro qualitative detection of total antibodies to SARS-CoV-2 N protein in human serum and plasma.[131] Briefly, sample, biotinylated SARS-CoV-2-specific recombinant N antigen and SARS-CoV-2-specific recombinant N antigen labeled with ruthenium(II) tris(2,2′-bipyridyl) complex are mixed to form a sandwich complex, which then binds to streptavidin-coated magnetic microparticles via interaction of biotin and streptavidin. This reaction mixture is magnetically captured onto the surface of an electrode in the measuring cell. After removal of unbound substances and addition of coreactant, application of a voltage to the electrode induces chemiluminescent emission that is measured by a photomultiplier (Figure 5C). In some cases, the antigen-binding sites of antibodies in samples may be occupied either only by the biotinylated N antigen or only by the ruthenium−N antigen complex, which may decrease sensitivity (Figure 5C). This ECL immunoassay was validated with a sensitivity of

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99.5% (184/185) after 14 days post-PCR confirmation and specificity of 99.8% (10432/10453) while insufficient positivity rates were separately reported as 10.0% <7 DPSO, 37.8% during 7−13 DPSO, 85.7% during 14−20 DPSO, 90.6% ≥21 DPSO,[148] 46.0% (17/37) during 1−10 DPSO, 79.0% (30/38) for >10 DPSO,[149] 68.8% for <15 DPSO, 85.7% during 16−20 DPSO, and 88.9% for >20 DPSO.[150]

An ultrasensitive Single Molecule Array (Simoa), also known as digital ELISA, was used to detect IgG, IgM, and IgA simultaneously.[151] Briefly, four viral targets (S, S1, RBD, and N) are covalently immobilized on four types of 2.7 μm carboxylated paramagnetic beads encoded with four dyes (absorbed at 488, 647, 700, and 750 nm), respectively. IgG, IgM, or IgA in human samples are captured by the antigen-conjugated beads. After washing, beads are introduced to biotinylated antihuman immunoglobulin antibodies and streptavidin-β-galactosidase (enzymatic probe) in sequence. After washing, the beads are resuspended in resorufin β-d-galactopyranoside (substrate of enzymatic probe) and loaded into femtoliter-volume well arrays on the Simoa HD-X Analyzer (Quanterix). The femtoliter-volume wells are designed to hold only a single bead. After isolating the beads in the femtoliter-volume wells, the microwell array is sealed with oil and imaged in five optical channels. A sensitivity of 99% and specificity of 99% for the validation set were reported at both early and late stages using the models created on the training set. This Simoa assay relies on the special arrays of tiny wells about 50 fL (4.5 μm diameter and 3.25 μm depth)[152] or 40 fL (4.25 μm diameter and 3.25 μm depth)[153,154] designed to hold only a single bead of 2.7 μm diameter (Figure 6). A drawback of this assay is that the measuring Simoa instrument (Quanterix) costs more than $200,000.

A multiplex fluorescence immunoassay (FIA) aided with magnetic microparticles was developed to detect IgG, IgA, and IgM against SARS-CoV-2. Briefly, SARS-CoV-2 RBD and ectodomain (ECD) protein containing the S1 and S2 subunits of the S protein, S1, S2, and N proteins along with SARS-CoV N antigen and human coronavirus-229E ECD antigen are individually coupled to magnetic microparticles in microplate wells. The antibodies in saliva or serum are captured by the antigen on the microparticles. After incubation, R-phycoerythrin-labeled antihuman IgG, IgA, or IgM is added to form the sandwich complex. Finally, the median fluorescence intensity of each bead set is measured.[86] Within the multiplex SARS-CoV-2 panel, the salivary anti-N protein IgG response resulted in the highest sensitivity (100% [28/28]) for detecting prior SARS-CoV-2 infection (≥10 DPSO). The salivary anti-RBD IgG response resulted in 100% (134/134) specificity.[86] Of note, variance was observed using the same kind of antigen from different suppliers in this report. The sensitivity of an optimized antigen−antibody set still needs to be evaluated with sufficient samples.

A protein microarray or immunoblot technology was developed for quantitative simultaneous antibody detection against multiple SARS-CoV-2 antigens.[133] Briefly, ca. 3.5 nm seed gold nanoparticles are deposited with sciFLEXARRAYER S3 spotter (Scienion AG) into an array of 170 spots on a chip. The S1, S2, and N antigens of SARS-CoV-2 and E and M antigens of SARS-CoV are printed onto the gold surface functionalized with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxy succinimide. A set of control spots are designed to correct for variations in temperature, nonspecific binding, and variations in the illumination field. Diluted sera are passed over the chip to capture IgG, IgM, or IgA against each viral protein, and then the immuneturbidimetric antihuman antibodies are added to complete a sandwich assay. Changes in brightness of the spots are detected with a video camera at each incubation step which is measured as the area under the curve (i.e., using the time course of sensor response) and converted into a quantified response. However, the combined sensitivity of 79% (76/96) and specificity of 70% (16/23) of this technology showed poor performance in a real-world evaluation.[133]

A simple, rapid, and inexpensive colorimetric paper-based ELISA was developed to detect the IgG specific to SARS-CoV-2 nucleocapsid antigen.[156] Briefly, chromatography filter paper is sandwiched with laminate films with holes and then coated with the recombinant SARS-CoV-2 nucleocapsid antigen to capture the SARS-CoV-2 antibody in the sample. After adding sample, horseradish peroxidase (HRP) conjugated antihuman IgG and 3,3′,5,5′-tetramethylthe benzidine substrate are added successively to facilitate a naked-eye readout. Requiring only a few microliters of sample, this assay can be completed within 30 min with a LOD of 9.0 ng/μL (0.112 IU/mL) and thus has the potential to be developed into a point-of-care diagnostic device.

An opto-microfluidic sensing platform with gold nanospikes was developed to detect the antibodies specific to the SARS-CoV-2 spike protein.[136] The antigen−antibody binding can be read out by the wavelength shift of localized surface plasmon resonance peak of gold nanostructures caused by the local refractive index change in 30 min with an LOD of about 0.5 pM. This label-free point-of-care test may complement standard serological assays after validation. The gold nanospikes covered glass substrate in the microfluidic chip needs to be fabricated by electrodeposition.

An electrochemical immunosensor combining an aerosol jet nanoprinted reduced-graphene-oxide-coated 3D electrode in a microfluidic device was developed to detect antibodies specific to SARS-CoV-2 within seconds.[137] Briefly, gold micropillar array electrodes, fabricated by aerosol jet nanoparticle 3D-printing, are functionalized by nanoflakes of reduced graphene oxide, which are in turn decorated with viral antigens. The functionalized electrode is then integrated with a microfluidic device to form a standard electrochemical cell. The binding of specific antibodies in samples to the antigens on the 3D electrode surface can be read out by the impedance change of the electrical circuit via electrochemical impedance spectroscopy (EIS). Antibodies to SARS-CoV-2 spike S1 protein and its receptor-binding-domain (RBD) can be detected with LODs of 2.8 pM and 16.9 pM, respectively, and read by a smartphone-based user interface. This sensor can be regenerated within a minute for reuse by eluting the antibodies from the antigens with a low-pH solution, but the complex 3D electrode may limit massive applications.

Serum antibodies against SARS-CoV-2 can also be analyzed by immunofluorescence assays (IFA). Briefly, Vero cells infected with SARS-CoV-2 virus are transferred onto a microscope slide and fixed. Patient samples are diluted and loaded onto the slide for incubation. Antibodies are visualized with fluorescein-conjugated antihuman IgM or IgG antibodies under a fluorescence microscope.[138] An in-house developed IFA was reported with a sensitivity of 76.5% (13/17) during 5−9 DPSO and 100% (16/16) during 10−18 DPSO and a specificity of 100% (19/19).[139] A whole spike-based IFA was even used as a reference method to assess the diagnostic

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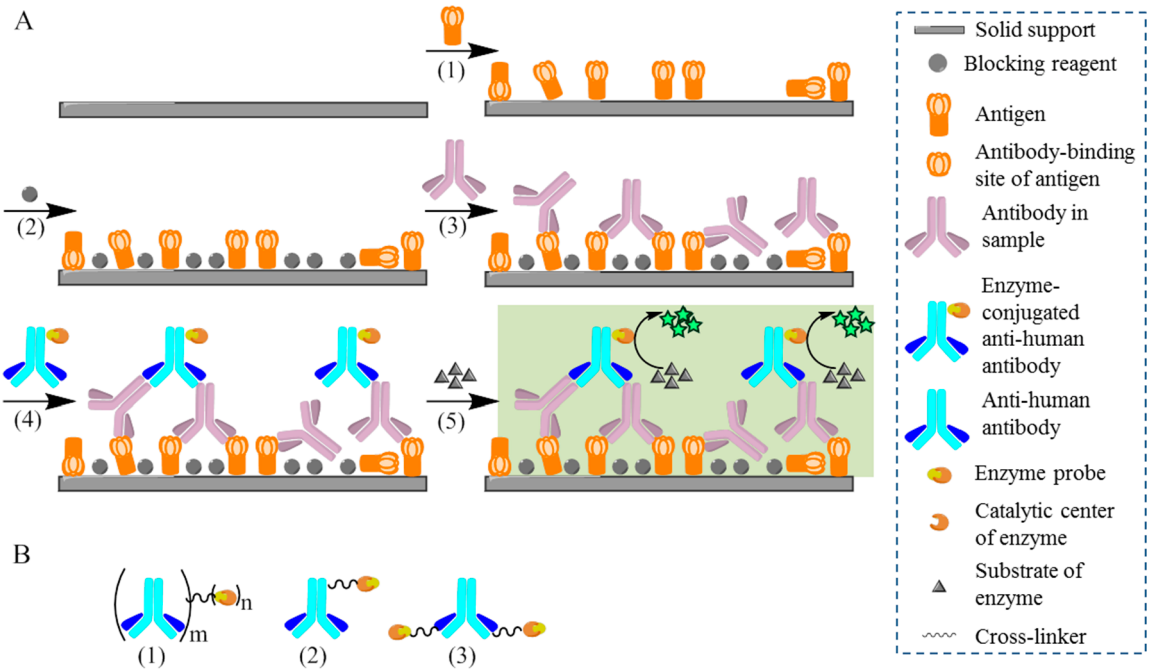

Figure 7. Schematic diagram of indirect ELISA with nonuniform immobilization of capture proteins on substrate support (A) and nonuniform enzyme probe-antibody conjugates (B).

accuracy of the Euroimmun SARS-CoV-2 IgG and IgA immunoassay.[140] But IFA needs to be manually performed by personnel experienced with the fluorescence microscope, which limits usefulness and throughput.

Although many immunoassays have been developed to detect specific antibodies against SARS-CoV-2, antibody assays struggle to give a definitive result, which is considered one of the biggest challenges with immunoassays.[157] The performance of COVID-19 serological assays usually show a wide diversity in clinical performance in different scenarios, as summarized by Ghaffari et al.[158] Moreover, the inherent inaccuracy of all serological tests for antibodies is a big difficulty that may inevitably lead to misclassifications even when the best methodologies, most reliable reagents, and stringent internal and external quality controls are used.[159] Because the antibodies are part of the body's immune response to exposure and not from the virus itself, such testing cannot be used for diagnosis of infection, according to the FDA.[160] On the other hand, China required passengers bound for China via direct flights to test negative for both nucleic acid and IgM antibodies against SARS-CoV-2 (http://www.china-embassy.org/eng/notices/t1828184.htm). In addition to the inaccuracies described above, antibody tests cannot reliably detect the presence of infection during the early stages of disease due to the lag in antibody production.[7,66] For example, only 38.3%−64.1% of total antibody,[61,161] 28.7%−33.3% of IgM,[61,161,162] and 19.1%−47.8% of IgG[61,161,162] were detected in the first week after symptom onset. Besides, the lack of detection limit (LOD) in antibody tests due to lack of antibody standards limits the direct comparison of analytical sensitivity between different immunoassays.

REASONS FOR FALSE NEGATIVES IN ANTIBODY IMMUNOASSAYS

False negatives of antibody tests may result from poor sensitivity or inadequate antibody levels in the specimen. The former is mainly influenced by the assay formats, antigens to target, test antibody quality, and isotypes of antibodies to be detected. The factors impacting the latter include sampling time and diagnostic testing windows which depend on the antibody response dynamics, as well as individual factors.

Assay Formats. Limitations exist in different assay formats for detecting antibodies as discussed in the "Antibody tests" section. LFIAs, like the other POC serological tests, usually have a lower diagnostic performance compared with laboratory tests[163] partly because they test a smaller volume of blood in a less controlled environment.[164] Their performance may vary in the routine testing laboratory in comparison with the performance stated by the manufacturers.[89,141,145,165] Therefore, "clinical validation of the diagnostic performance of rapid tests for COVID-19 in real-life should be carried out by comparison with a gold standard test in a sufficiently large number of target population subjects before introducing them into the routine as a stand-alone diagnostic test."[165] Taking into consideration the inaccuracy of POC tests and their large effect on the epidemic dynamics of COVID-19, Gray et al. claimed that "No test is better than a bad test."[166]

Albeit laboratory tests, including ELISA, CLIA, and ECLIA, basically have better accuracy based on the reliable instrumentation manipulated by skilled laboratory personnel in more stringent internal quality controls, false negative results may still be observed. Reasons for unreliability include, but are not limited to, accumulated errors from multiple steps involved, nonuniform immobilization of proteins reagents on substrate support, nonuniform probe−protein conjugates, and background noise from unreacted protein reagent residues. To illustrate, we discuss these issues below using the fundamental indirect ELISA as an example.

Multiple Steps Involved. Regarding the indirect ELISA antibody test, the immunoassay usually involves (1) immobilization of viral antigen (capture protein) in wells of a 96-well plate by either physical adsorption or covalent cross-linking

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followed by washing; (2) blocking the nonspecific binding sites of the substrate support with a protein blocking agent such as bovine serum albumin or casein followed by washing; (3) incubating the plate wells with sample followed by washing; (4) binding antispecies antibody (for detecting) labeled with enzyme probe followed by washing; and (5) triggering the signal after loading the reactant substrate for the enzyme probe that usually provides a color (Figure 7A). The accumulated errors from every step will contribute to the ultimate unreliability of results.

Nonuniform Immobilization of Capture Proteins on Substrate Support. Protein immobilization in random orientation on a solid support by either physical adsorption or covalent cross-linkage may cause an inconsistent immobilized quantity of capture proteins binding sites due to steric hindrance caused by neighboring proteins especially at high surface concentration and substantial loss of affinity due to shielding of active binding sites of the proteins.[167] This may in turn influence the binding between the antibodies in the sample and enzyme-labeled antispecies antibodies (Figure 7A), but this problem would occur mainly at the upper part of the dynamic range, not at low antibody concentrations. Although impressive progress in oriented protein immobilization has been achieved,[168,169] many challenges still exist in the fields of materials, chemistry, biology, and physics to make this strategy simple, versatile, efficient, stable, and economical.[170] Also, the full benefits of this strategy on sensitivity and detection limits are uncertain.[171] In addition, other issues, such as denaturation, distance between coupled proteins and the support surface, loading capacity, nonspecific binding, and distribution homogeneity, also influence the results of the assay.[167,170]

Nonuniform Probe−Protein Conjugates. Probes in conjugates usually include the enzyme probe in ELISA and CLIA, nonenzyme probes in CLIA and FIA, and ruthenium complex probe in ECLIA, which are tagged with either the antispecies monoclonal antibodies for individual isotype of immunoglobulin or antigens for total antibodies. In the indirect ELISA, enzymes are covalently conjugated to monoclonal antibodies either directly by reactive groups on both the enzymes and antibodies or indirectly via homo- or heterobifunctional reagents after introduction of reactive groups (e.g., thiol or maleimide groups). However, enzyme−antibody conjugation may result in irreproducible labeling efficiency with heterogeneous enzyme/antibody molar ratios (Figure 7B-1) along with different reactive sites of conjugation, although extensive purification can reduce the heterogeneity. In some cases, the conjugation may possibly impair the catalytic activity of the enzyme (Figure 7B-2) and antigen-binding activity of the antibody (Figure 7B-3) due to steric hindrance or cross-linking the active group which is essential for the function of protein. Further, polymerization of enzymes or antibodies happens to different extents during conjugation, which may increase the nonspecific binding of enzyme-labeled conjugates in quantitative enzyme immunoassay.[172] These problems also exist in indirect labeling using biotin−avidin systems. Strategies for site-specific modification of proteins for selective labeling with defined stoichiometry[173−177] have been developed, but they are in general more time-consuming and complex than a classical chemical conjugation approach.

Background Noise from Unreacted Protein Reagents Residue. Usually the unreacted protein reagents can be easily eliminated during the washing step. In some assays, multifunctional polymer is coated onto the solid support to provide abundant functional groups for achieving high protein loading capability, to adjust surface properties of the substrate supporter to preserve the native conformation of the attached protein and to prevent nonspecific adsorption.[170] However, the polymers may change from the linear form to cross-linked gel after reacting with cross-linker reagents such as glutaraldehyde in the covalent immobilization. Then the unreacted protein reagents might not be thoroughly eliminated from the gel network by the conventional washing buffer, hence resulting in an unacceptable level of background noise originated from the substrate.[178]

Antigens Used. To date, N and S proteins as well as their subunits have been used for developing antibody assays (Table S1). The sensitivity of the ELISA for IgM against S was significantly higher than against N.[116] Both IgG and IgM against RBD by ELISA were more sensitive than against N.[179] Among the prokaryotically expressed recombinant N, N1, and N2 proteins and eukaryotically expressed recombinant S1, S-RBD, and S-RBD-mFc spike proteins, S1 and S-RBD-mFc showed the highest ELISA titers to detect IgM and IgG.[120] But the anti-N IgG in the magnetic-bead-based fluorescence immunoassay resulted in the highest sensitivity for detecting prior SARS-CoV-2 infection in saliva among the antigens such as ectodomain containing the S1 and S2 subunit, S1, S2, RBD, and N.[86] In a comprehensive study, N was more sensitive to target than S and RBD for both IgG and IgM detection while S was more sensitive than RBD and N for IgA detection.[104] In addition, cumulated data suggested that anti-S humoral responses were enriched among mild COVID-19 patients, whereas anti-N humoral responses were elevated in the severe cases.[94,180,181]

The reactivity of COVID-19 sera is, in general, stronger against the full-length S protein than against the RBD, which may reflect the higher number of epitopes on the much larger S protein.[117] However, S protein is more difficult to express into prokaryotic cells in its full length protein than the S1 subunit or RBD.[182] A neutralizing human antibody binds to the N-terminal domain (NTD) rather than RBD of the S protein of SARS-CoV-2,[183] suggesting that the immunoassay targeting the S1 subunit including NTD should be more sensitive than that targeting only RBD.

In addition, false negatives may result from denaturation of recombinant viral proteins which cannot be correctly recognized by patients' antibodies,[182] considering that SARS-CoV-2 S protein is less stable than SARS-CoV S protein.[184] Even the same kind of protein from different suppliers has resulted in varied performance for antibody tests.[86] A comprehensive list of reported performance of immunoassays for binding antibodies is given in Table S1.

Isotypes of Antibodies. Disparities in sensitivity were found between different tests due to different isotopes of antibodies to be detected.[185] A higher sensitivity for IgG than IgM was reported in some reports,[92,130,162,179,186] while in another report anti-S IgM was more sensitive than anti-S IgG.[116] Both IgM and IgG were reported to be less sensitive than total antibody[61,161] or IgA.[105,187] Low rates of isolated IgM antibody detection were reported in a majority of studies, which, according to Infantino et al., could be false negatives due to low antibody concentrations or their short lifetime.[182] IgM was considered unlikely to play the primary role in COVID-19 antibody testing by Bohn et al.[64] due to traditional specificity challenges associated with high false-positive rates.[188] Similarly, the specificity of IgA against SARS-CoV-2

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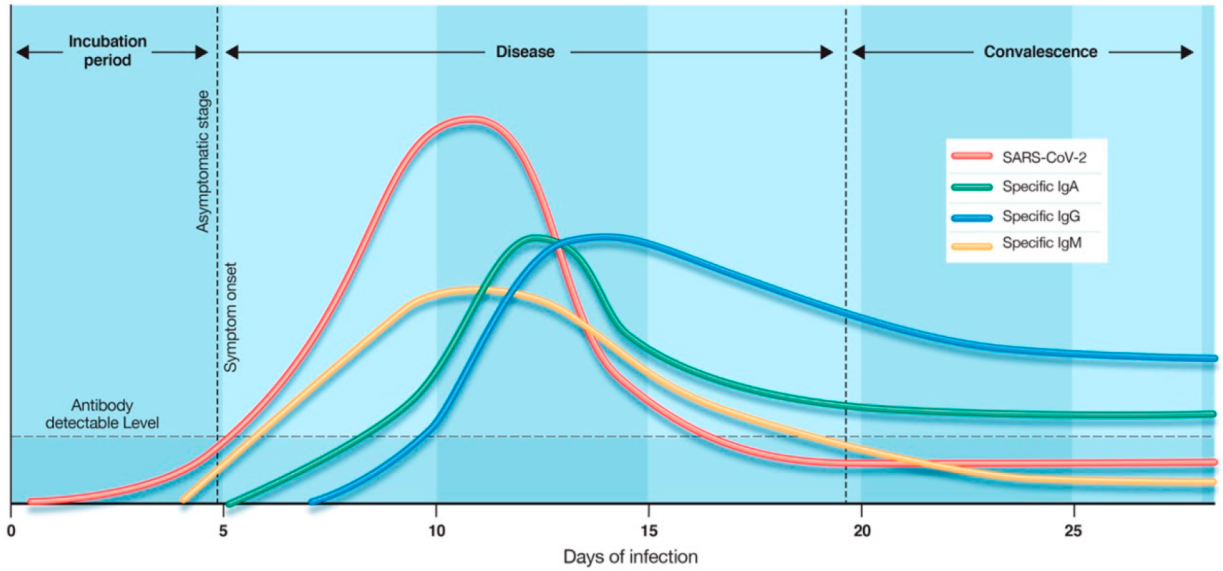

Figure 8. Time kinetics of antibody response in COVID-19. Reprinted with permission from ref 158. Copyright (2020) MDPI (Basel, Switzerland).

was reported to be lower than IgG.[105] Detection of IgG against SARS-CoV-2, in contrast to IgM and IgA, was considered by Theel et al. to have a larger role to play during this pandemic,[189] a view also supported by other reports.[140,190,191] Isho et al. further pointed out that the sensitivity/specificity characteristics of of IgA and IgM were lower than those of the IgG assays in part because IgA and IgM responses waned more rapidly in patients.[93] Apparently, IgG is a longer lasting antibody associated with potential viral neutralizing activity.[189,192]

Antibody Response Dynamics. The diagnostic testing window is perhaps one of the most important factors impacting test sensitivity (Figure 8). Great heterogeneity in the time of detecting antibodies after symptom onset and large variance of antibodies levels in different patients have been observed (Table S2), which creates challenges for serological testing. The immunoassay results were negative for 7.2%−12.4% of individuals with positive PCR COVID-19 tests after more than 14 days in a prevalence study of SARS-CoV-2 in Spain.[89] Further, there is also a possible failure of some severely affected patients to generate the antibody response.[187]

Seroconversion Time. The classical immune response to viruses generally involves IgM production first after a few days of infection, often accompanied by emergence of IgA, and then followed by a shift to IgG production.[64,87,193] In COVID-19, current evidence is conflicting between some groups concluding IgM is produced first,[92,187] while others suggest IgM and IgG production occur simultaneously.[86,130] In one report, seroconversion of IgM was found to occur at the same time, or earlier, or later than that of IgG in different patients.[162] Total antibodies[61] and IgA[86] specific to the SARS-CoV-2 were reported to appear several days before IgG and IgM. Accumulating data suggest that seroconversion of total antibody, IgA, IgM, and IgG occurs as early as 1,[187] 1−2,[61,161] 1−4,[61,92,161,162,181,187,194] and 1−4[61,92,161,162,181,187,194] DPSO, with a median time of 9−11,[61,161] 5[187] or 13,[195] 8−14,[61,161,162,195−197] and 8−14[61,161,162,187,195−197] DPSO, respectively (Table S2). In addition, the seroconversion from 45 patients whose exposure time was determined occurred in a median time of 15 days after exposure for total antibody, 18 days for IgM, and 20 days for IgG.[61] However, some infected individuals did not seroconvert or their antibody titers waned within short periods of time following initial production.[117,161,162,198,199]

Peaking Time of Antibody Titer. The levels of total antibody, IgA, IgM, and IgG increased rapidly after 6−8 DPSO.[61,130,200] Cumulative data suggested that the average time to reach the highest titer was about 2 weeks for total antibodies,[61] 2−3 weeks for IgA,[93,187,200] 2−3 weeks for IgM,[92,130,181,187,197,200] and 3−4 weeks for IgG[92,93,116,130,162,181,187,197,200] since symptom onset (Table S2). Both IgG and IgM levels reached a plateau in 6 days after the first seroconversion.[162]

Time of Highest Positive Rate. The positive rates peaked 15−21 days for total antibody,[161] ≥12 days for IgA,[200] 15−22 days[61,130,162,196,200] for IgM, and ≥12 days for IgG[130,162,196,200] after symptom onset (Table S2). The proportion of positive patients seemed to decrease more than 50 days after a positive RT-PCR result.[185] The cumulative positive rate of different antibodies during a certain period of post symptom onset is varied in different reports. Usually, the more patients that are enrolled, the longer period that is needed to observe a higher cumulative positive rate, which is expected due to interindividual differences. For example, the cumulative positive rate reached 100% (80/80) on day 16[61] or 99% (172/173) on day 25[161] for total antibody, 74% (28/38) in the third week,[181] 100% (80/80) on day 21[61] or 99% (172/173) on day 30[161] for IgM, and 100% (38/38) in the third week,[181] 97% (78/80) on day 29,[61] or 99% (172/173) on day 35[161] for IgG after symptom onset. The cumulative positive rates for total antibody, IgM, and IgG were 100%, 94.2%, and 96.7%, respectively, on day 37 post virus exposure from 45 patients whose exposure time was determined.[61]

Dynamic Comparison between Viral Load and Antibody Response. It was estimated that the detection rate of IgM overtook that of PCR tests for throat swabs after 5.5 days postsymptom onset.[187] The sensitivity of total antibody, IgM, and IgG detected by a commercial ELISA kit overtook that of

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Table 1. Cross-Reactivity of Antibodies against Other Viruses with SARS-CoV-2

	Antigens of SARS-CoV-2				
Virus	N	S	S1	RBD	S2
SARS	+ for IgG[105,187]	+ for IgA[214] + for IgG[105,212−214]	50% (1/2)−100% (3/3) for IgA, 100% (3/3, 7/7) for IgG[105]	+ for IgG[105,212,215]	+ for IgG[212,216]
MERS	14% (1/7) for IgG[105]	+ for IgA[214] + for IgG[105,214]			+ for IgG[216]
HKU1		+ for IgA/M/G[209] + for IgA/G[214]			+ for IgG[216]
OC43		+ for IgM/G[209] + for IgA/G[214]			+ for IgG[216,222]
NL63		+ for IgA/M/G[209]			+ for IgG[216]
229E		+ for IgA/M/G[209]			+ for IgG[216]
Alpha1					+ for IgG[216]
Dengue			17% (16/95) for IgA, 5% (5/95) for IgG[217]		
Hepatitis B	8% (1/13) for total antibody[218]				

the PCR tests for respiratory tract samples on days 9, 10, and 10 after symptom onset,[161] which is consistent with another report.[61] The sensitivity of antibody assays (4/8 for IgM and 7/8 for IgG) by a commercial LFIA kit overtook that of RNA testing (3/8 for sputum RNA and 2/8 for throat swabs RNA) after the second week of disease onset.[201] The sensitivity of combined antibodies (IgG and/or IgM) by a commercial ELISA kit overtook that of RNA testing for the pharyngeal swab on day 11.[202]

Individual Factors. Both interindividual variance and intraindividual fluctuation in antibody levels have been observed in the antibody response.[93,161,162,181,197,200] Longitudinal analysis of IgG identified 2−8.5%[198] or 22%[199] of COVID-19 cases who did not seroconvert even weeks after infection. Antibody tests may also miss infections among people who are immunocompromised and do not produce antibodies[203] due to HIV infection or immunosuppressive drugs.[88] In addition, cumulative data suggest that antibody levels were correlated with the severity of COVID-19. Neutralizing antibody,[204] total antibodies,[161,204] IgA,[195] IgM,[205] and IgG titers[92,162,181,195,204,205] in severe COVID-19 groups were higher than those in the nonsevere groups. Some cases of asymptomatic carriers were reported to be seronegative.[206,207] Oppositely, it was also claimed that there is no strong association between seroconversion and disease severity.[206]

REASONS FOR FALSE POSITIVES IN ANTIBODY IMMUNOASSAYS

False positive detection in antibody immunoassays may result from imperfect specificity of methods which is often due to antibody cross-reactivity as well as contamination of samples or reagents.

Cross-reactivity. In general, antibody tests face the challenge of interference from billions of other endogenous antibodies in samples.[159,208] For example, pre-existing IgG cross-reactive with SARS-CoV-2 S and N proteins were detected in about 10% of healthy individuals who were uninfected and unexposed to the SARS-CoV-2 using flow cytometry and ELISA.[209] A common concern in serological testing for COVID-19 is cross-reactivity with other pathogens, which may give false-positive results.[7,87,88] It is thought that pre-exposure of high or low pathogenic human coronaviruses generates cross-reactive antibodies toward SARS-CoV-2.[210,211] Potential cross-reactivity of antibodies against SARS-CoV-2 was detected toward the SARS-CoV,[105,187,212−216] MERS-CoV,[105,214,216] HCoV-HKU1,[209,214,216] HCoV-OC43,[209,214,216] HCoV-NL63,[209,216] HCoV-229E[209,216] and HCoV-Alpha1[216] as well as Dengue virus[217] and Hepatitis B[218] (Table 1). In fact, more than 90% of adults have antibodies to the common circulating coronaviruses (HCoV-HKU1, HCoV-OC43, HCoV-NL63, and HCoV-229E)[99] and are susceptible to cross-reactivity in COVID-19 antibody tests, even if homology of SARS-CoV-2 is lower with these strains.[64,189,219] Although anti-SARS-CoV antibodies were reported to bind cross-reactively to the S,[212] S1, RBD[105,212,215] and N proteins[105,187] of SARS-CoV-2, this cross-reaction is of less significance because there has been no SARS case report since 2004 and the number of infections with SARS-CoV was limited to 8096 worldwide according to WHO (https://www.who.int/publications/m/item/summary-of-probable-sars-cases-with-onset-of-illness-from-1-november-2002-to-31-july-2003).

The use of well-conserved antigens among different coronaviruses may result in false positive results.[2,220] RBD and the S1 subunit of S protein demonstrated lower cross-reactivity than N protein and the S2 subunit of S protein between SARS-CoV-2 and common human coronaviruses.[105,221] This is expected from the amino acid sequence homology of different antigens between SARS-CoV-2 with SARS-CoV, MERS-CoV, HKU1, OC43, NL63, and 229E.[105,187]

Autoimmune Disease Antibodies. No cross-reactivity was observed between autoantibodies in autoimmune disease and antibodies against SARS-CoV-2,[223] but Vojdani and Kharrazian suggested potential antigenic cross-reactivity between SARS-CoV-2 and human tissue with a possible link to an increase in autoimmune diseases.[224] Further, SARS-CoV-2 IgM was detected in 61.1% (22/36) rheumatoid factor IgM-positive sera by ELISA and gold immunochromatography assay.[225]

Contamination of Samples or Reagents. Occasional false positive results may occur due to technical errors and reagent contamination.[226] An unknown interference in the ELISA tests for IgA and IgG against SARS-CoV-2, which, according to the authors of the study, could be in the blocking

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or coating matrix apart from the specific antigen coated, resulted in a consistent false-positive result in two HCoV-OC43 patients.[105] This may just reflect the pre-existing cross-reactive antibodies. Insufficient surface blocking and stability of the reagents may also cause false-positives in serological assays.[227]

False Negative Controls. The negative control from apparent "healthy" people with negative SARS-CoV-2 RNA or negative antibody tests against SARS-CoV-2 is unreliable in consideration of the varied incubation period and the false negative result of current tests. In detail, the laboratory RT-PCR reference standard method may misclassify samples from infected patients as false negatives that may be further wrongly used as "negative controls" to evaluate new assays. This misclassification may affect the apparent diagnostic performance of the antibody tests being evaluated.[228] Alternatively, neutralization antibody tests have also been reported to function as the reference standard method for serological assays.[107] But not all the binding antibodies are neutralizing. On the other hand, specimens prior to the COVID-19 era were also collected as negative controls, which, however, may be in doubt due to the report that SARS-CoV-2 was detected in waste waters in Barcelona as early as on March 12, 2019 [233] (https://www.ub.edu/web/ub/en/menu_eines/noticies/2020/06/042.html). Erroneous negative controls can result in underestimated specificity in the diagnostic evaluation of new assays.

SPECTRUM BIAS OR SPECTRUM EFFECT

The spectrum bias describes the variation in performance of tests for prediction, screening, and diagnosis of disease among different population subgroups.[229] The clinical performance of tests reflected in sensitivity, specificity, and likelihood ratios (or predictive values) varies with the pretest probability (or prevalence) of disease in a population[44,230] due to spectrum bias or spectrum effect[231] (eq 1 and Figure 9). This may partially explain the disagreement in clinical performance of the same commercial test from different reports. Thus, care should also be taken by researchers, clinicians, and policy makers when interpreting the test results and comparing the performance of diagnostic tests developed in different populations using different methods.[229] The other forms of diagnostic bias were also discussed by Carpenter et al., such as incorporation bias, differential verification bias, imperfect criterion standard bias, and temporal bias.[232]

$$\mathrm{PPV} = \frac{\text{sensitivity} * \text{prevalence}}{\text{sensitivity} * \text{prevalence} + (1 - \text{specificity}) * (1 - \text{prevalence})} \quad (1)$$

In this equation, PPV is the positive predictive value.

FUTURE DIRECTIONS

The number of binding epitopes on antigens and the affinity between antigens and antibodies are also important factors influencing sensitivity besides the abundance of antigens in the virus. Binding epitopes for IgM and IgG were identified on S (n = 8), N (n = 8), M (n = 5), E (n = 0), NSP1 (n = 1), NSP2 (n = 5), NSP3 (n = 7), NSP4 (n = 1), NSP5 (n = 0), NSP6 (n = 1), NSP7 (n = 0), NSP8 (n = 1), NSP9 (n = 1), NSP10 (n = 1), NSP12 (n = 5), NSP13 (n = 3), NSP14 (n = 3), NSP15 (n = 2), NSP16 (n = 1), ORF3a (n = 4), ORF6 (n = 0), ORF7a (n = 3), ORF8 (n = 1), and Orf10 (n = 0) proteins using SARS-CoV-2 proteome peptide microarrays.[135] Similarly, significant IgM and IgG antibody responses to ORF9b and NSP5 proteins were also identified.[134] These results suggest that the other proteins besides the S and N of SARS-CoV-2 may be alternative choices to be targeted for antibody detection.

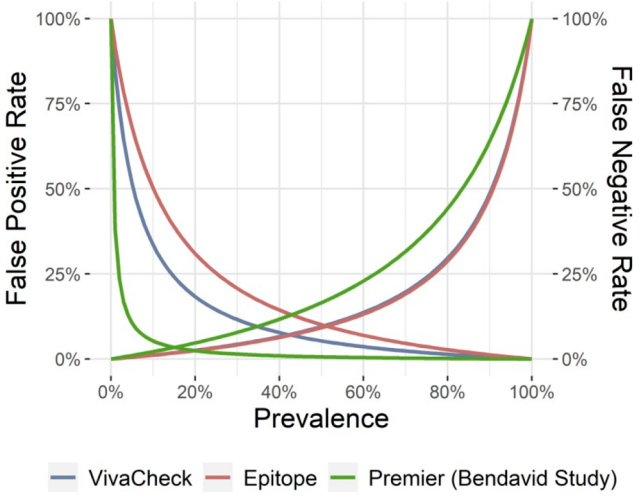

Figure 9. False positive and false negative rates as a function of pretest probability (or prevalence for surveillance studies) for serologic tests for SARS-CoV-2 antibodies. Reprinted with permission from ref 232. Copyright (2020) John Wiley & Sons, Inc.

Primary and secondary antibody cross-reactivity can often be made negligible by making the assays as sensitive as possible enabling very large sample dilution that dilutes cross-reactive interference to very low levels as well.[234,235] The specificity of immunoassays can also be improved if specific or cross-reactive epitopes are identified by epitope mapping. For example, novel antibody epitopes dominating the antigenicity of S protein in SARS-CoV-2 compared to SARS-CoV were screened using antibody epitope bioinformatic tools,[236] which may be useful to develop more specific serology tests to reduce the false positives. On the other hand, the homogeneous conserved residues at the N-terminal domain of N protein are considered as one of the main reasons for the cross-reactivity when N protein is targeted. Yamaoka et al. reported that the specificity of antibody tests improved when N-terminally truncated N protein was targeted as the antigen.[237] In addition, urea dissociation tests were confirmed to be useful for reducing SARS-CoV-2 IgM false-positive results in gold immunochromatography and ELISA because urea can be used as a substance for dissociation of antigen−antibody binding to evaluate the affinity of IgG.[225]

Antibody tests targeting multiple antigens in parallel could yield higher sensitivity and specificity than conventional tests

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based on a single antigen of SARS-CoV-2.[86,238] Using multiple antigen-based antibody signatures, Klompus et al. differentiated COVID-19 patients from healthy controls in a highly accurate manner through machine learning.[239]

More accurate diagnoses can be obtained from multiple biomarkers which may be detected in an integrated system. For instance, 31 immune biomarkers including multiple SARS-CoV-2 antigens and multiple anti-SARS-CoV-2 immunoglobulins were quantified from 70 μL of plasma sample using the ultrasensitive Simoa assay. However, it requires a specialized instrument (HD-X Analyzer (Quanterix)) that is quite expensive.[240] Simultaneous IgG/IgM/unreported antigen detection of SARS-CoV-2 was achieved on an integrated microfluidic fluorescence immunoassay system.[78] Another group quantified SARS-CoV-2 N protein, IgG, IgM, and C-reactive protein in serum and saliva using a multiplexed electrochemical graphene-based platform called SARS-CoV-2 RapidPlex.[241] However, diagnostic performances of these multiplexed assays need to be independently evaluated based on more samples in the real world.

CONCLUSIONS

Serological tests are sensitive for the late and recovery stage of infection, which is of great value not only to identify infected individuals with negative RT-PCR results but also to develop and evaluate vaccines and therapeutic antibodies. Although numerous immunoassays have been reported for diagnosing COVID-19, many of them either showed an unsatisfactory diagnostic performance or lacked stringent evaluation for their performance in the real world based on enough samples. False negatives of antibody immunoassays can arise from assay formats, antigens to target (S and N proteins as well as their subunits of SARS-CoV-2), isotypes of antibodies to detect (IgA, IgM, IgG, and total antibodies), the diagnostic testing window, interindividual variance, and intraindividual fluctuations in antibody levels. Reasons for false positives of antibody immunoassay mainly involve cross-reactivity from other viruses, and possibly autoimmune diseases such as rheumatoid factor. The spectrum bias has an effect on both the false negatives and false positives. One of the foci on current technological innovations of immunoassays is to improve sensitivity to reduce the false negatives and to improve specificity to decrease the false positives,[242] which requires assay developers to carefully consider not only the improvement of assay formats but also the selection of specific antigens and isotope of antibodies to detect. Ramdas et al. pointed out that creative use of currently imperfect but available tests with smart tactics could go a long way to reach improved accuracy and precision.[243] For clinicians, these factors influencing the accuracy must be kept in mind in testing patients and interpreting the test results realistically.

Testing alone will not stop the spread of SARS-CoV-2 but is a large part of a strategy to control it.[244] On one hand, diagnostic reasoning and managed care of COVID-19 based on laboratory tests reduces risk of systemic complications and contributes to better outcomes for infected patients. On the other hand, timely isolation of infected patients protects others from exposure to this virus. The major lessons learned from COVID-19 testing should be of significance to prepare in advance for future worldwide medical crises.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c02621.

Table S1. Performance of immunoassays for binding antibodies. Table S2. The time of detecting antibodies after symptom onset. (PDF)

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Notes

The authors declare no competing financial interest.

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