Review Article

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Serological and Molecular Tests for COVID-19: A Recent Update

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ABSTRACT

The COVID-19 pandemic is probably the most devastating worldwide challenge in recent century. COVID-19 leads to a mild to severe respiratory disease and affects different organs and has become a global concern since December 2019. Meanwhile, molecular biology and diagnostic laboratories played an essential role in diagnosis of the disease by introducing serological and molecular tests. Molecular-based techniques are reliable detection tools for SARS-CoV-2 and used for diagnosis of patients especially in the early stage of the disease. While, serological assays are considered as additional tools to verify the asymptomatic infections, tracing previous contacts of individuals, vaccine efficacy, and study the seroprevalance. The average time of the appearance of anti-SARS-CoV-2 antibodies in the patient's serum is 3-6 days after the onset of symptoms for both IgM and IgA and 10-18 days for IgG. Following the outbreak of COVID-19, FDA has approved and authorized a series of serological laboratory tests for early diagnosis. Serological assays have low-cost and provide fast results but have poor sensitivity in the early stage of the viral infection. Although the serological tests may not play an important role in the active case of COVID-19, it could be effective to determine the immunity of health care workers, and confirm late COVID-19 cases during the outbreak. In this review, we compared various laboratory diagnostic assays for COVID-19.

Keywords: COVID-19, Laboratory diagnosis tests, PCR, SARS-CoV-2, Serological assays

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INTRODUCTION

Novel coronavirus disease 2019 (COVID-19) is a newly emerged disease caused by SARS-CoV-2 (Severe acute respiratory syndrome coronavirus-2), a positive-sense, single-stranded RNA virus, that causes a moderate to severe respiratory disease (1). COVID-19 has been getting universal concern since December 2019 and has overburdened public healthcare systems, as well as severe economic and social distress around the world (2). The disease started initially from Wuhan city in China, but now has spread to more than 200 countries with 117 million confirmed cases and 2.6 million deaths up to March 9, 2021 (3). The most well-known clinical and paraclinical symptoms are fever, normal or reduced leukocyte counts, nonproductive cough, multiple organ failure، pneumonia, diarrhea, and dyspnea, but the leading cause of death in patients is acute respiratory distress syndrome (ARDS) triggered by cytokine storm (4-6). The disease is transmitted via respiratory droplets generated directly from infected individuals or asymptomatic carriers (7).

SARS-CoV-2 is a phospholipid bilayer enclosed particle with a nucleoprotein within a matrix protein capsid (8). The genome codes various types of structural proteins, including S protein (spike glycoprotein trimmer), E protein (envelope), N protein (nucleocapsid), M protein (membrane), and HE (hemagglutinin-esterase) dimer (found in certain Corona-viruses) (8-10).

As early detection and quick treatment of the patients minimize the number of prospective outbreaks, COVID-19 laboratory diagnostic tests are the most crucial factor in restricting the pandemic (11). Unfortunately, sufficient testing capacity for COVID-19 is currently inaccessible in some regions. Therefore, in the current situation, preventing the spread of the disease could be the main challenge.

In the early stages of the disease outbreak, numerous countries have used various diagnostic methods and testing techniques, depending on the availability of facilities and equipment (12-14). Present World Health Organization (WHO) guidelines have prompted the development of more integrated diagnostic methods. This review article aims to evaluate the current COVID-19 laboratory tests and to compare the effectiveness and significance of various diagnostic methods.

SARS-COV-2 TESTING APPROVED BY FDA REGULATIONS

Since false positive and false negative results can lead to the disease transmission, all laboratory detection tests used for the detection of the diseases should be validated before being used. On February 4, 2020, the FDA (Food and Drug Administration) determined that there is an urgent need to approve a series of laboratory tests to detect of SARS-CoV-2 in the current situation in the United States. In accordance with this determination, the FDA issued the Emergency Use Authorization (EUA) for COVID-19 in vitro diagnostic tests during an emergency situation when there are no available, appropriate, and accepted alternatives. The EUA method allowed molecular and serological diagnostic tests to be validated, developed, and offered for clinical use within a limited period of time rather than years or longer. In this regard, FDA has approved 281 tests under EUAs, including 219 molecular-based tests, 56 serology-based tests, and 6 antigen tests (a list of the approved kits is available at: https://www.who.int/diagnostics_laboratory/200602_imdrf_collated_table_02_june_2020.pdf ?ua=1).

SEROLOGY-BASED SARS-COV-2 TESTING

While molecular techniques such as NGS and PCR play a substantial role in the detection of viral infections, there is an urgent necessity to verify the asymptomatic infections, tracing individuals retrospective

contacts, precise determination of the rate of casualties, evaluate the efficacy of the vaccine, characterization of the disease prevalence and spread, and evaluation of herd immunity(15-17). COVID-19 serological tests may clarify whether or not a person has been exposed to infection and has acquired immunity against virus (17, 18). Based on Guo et al. the mean time of appearance of anti-COVID-19 antibodies in the patient's serum is 3-6 days after the beginning of the symptoms for both IgM and IgA and 10-18 days for IgG (19). These ELISA-based methods are used to detect three types of antibodies in patient's sera, including total antibodies, IgM, and IgG (20, 21). Using the cell-expressed recombinant Receptor Binding Domain (RBD) of S protein and the HRP-conjugated antigen, an ELISA for antibody was evolved on the basis of dual-antigens sandwich ELISA (22, 23). The same HRP-conjugated antigen was used in the μ-chain capture ELISA to detect IgM antibodies (24). An indirect ELISA kit was used to measure the IgG antibodies on the basis of a recombinant nucleocapsid protein (25). Numerous studies have indicated that one to two weeks after the onset of clinical manifestations, the vast majority of COVID-19 patients might develop a protective immune response by producing specific anti-SARS-CoV-2 IgG and IgM antibodies (26-30). Whether or not these antibodies will have ongoing neutralizing activity against SARS-CoV-2 is still unknown (31). Serological tests could be a relatively simple and cost-effective way to identify or measure the different classes of antibodies. These tests may indicate whether or not a person has been infected by SARS-CoV-2 and has developed protective antibodies to prevent future re-infection (Figure 1) (20, 26, 27).

Plaque Reduction Neutralization Test (PRNT)

PRNT is a serological approach that utilizes a specific antibody's ability to prevent (neutralize) the virus from forming plaques in a cell monolayer (32-34). In this assay, a predetermined volume of virus must be combined with dilutions of the patient serum, followed by adding this mixture to the cell line cells (Figure 2A) (35, 36).

The number of plaques formed after a few days should be used to determine the PFU (plaque-forming units) concentration by. Vital dyes, such as neutral red, should be added to each plate to determine and count the number of plaques (32, 37-39). Depending on the virus type, the PFU is measured by fluorescent antibodies, microscopic observations, or specific dyes that react with the infected cell (40).

Interpretation is typically based on 70% neutralization, which defines as the last serum dilution capable of inhibiting 70% of the total plaques. For working with a live

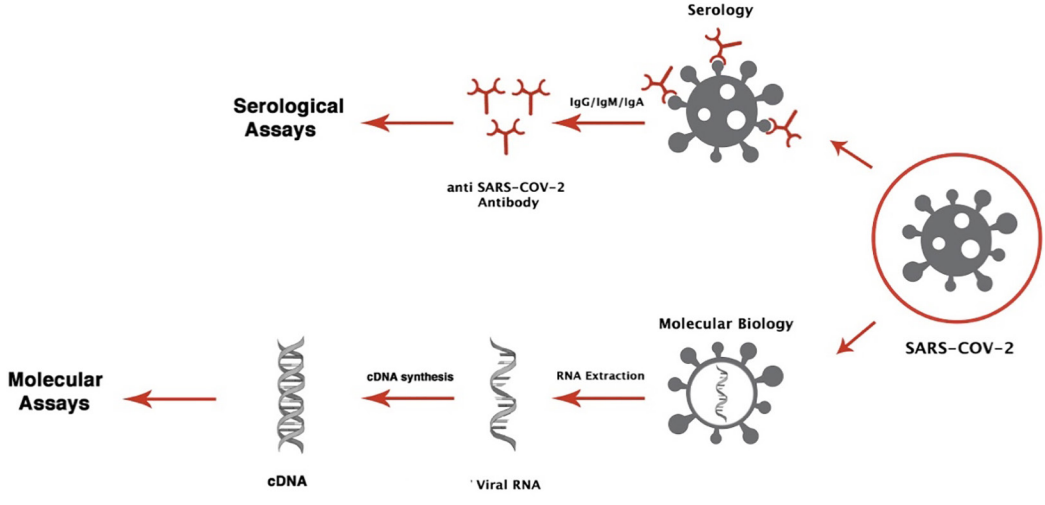

Figure 1. Diagnostics tests of SARS-CoV-2.

virus, laboratories must have a biosafety level 3 (BSL3) (41-43).

Microneutralization Test (MNT)

The microneutralization test is a basic immunology method in virology, epidemiology, and vaccine assessment (44, 45). MNT is based on the direct observation of the degree of cytopathic effects under an inverted microscope. In this method, virus-damaged cells can be easily differentiated from fully undamaged cells, but partially damaged cells are difficult to determine (38, 46). It is therefore difficult to evaluate a titration curve to scale a neutralizing antibody's strength with serially diluted testing antibodies. According to Manenti et al., the colorimetric cytopathic effect-based MNT could be utilized as a reliable clinical test method for vaccine and epidemiological studies in SARS-CoV-2 infection.

For the MNT evaluation, two different methods of readout could be used including objective (spectrophotometric), and subjective (using an inverted optical microscope) (47). Figure 2B shows the schematic steps of the MNT. No need to use BSL3 labs and uses microtiter plates in combination with ELISA to detect virus-infected cells are the advantages of MNT (48, 49).

Surrogate Viral Neutralization Test (SVNT)

The two main limitations in performing conventional virus neutralization assays are the need for BSL3 and live pathogens. Surrogate viral neutralization test (SVNT) is a method, that detects neutralizing antibodies (NAbs) in serum without the involvement of an active virus, organism, or cells. In this assay, ACE2 (Angiotensin-converting enzyme 2) receptor from the host and purified receptor-binding domains of S protein are used (50, 51). In an ELISA plate well (Figure 2C), SVNT is designed to simulate the interaction between host antibodies and virus S protein (52, 53). Specific neutralizing

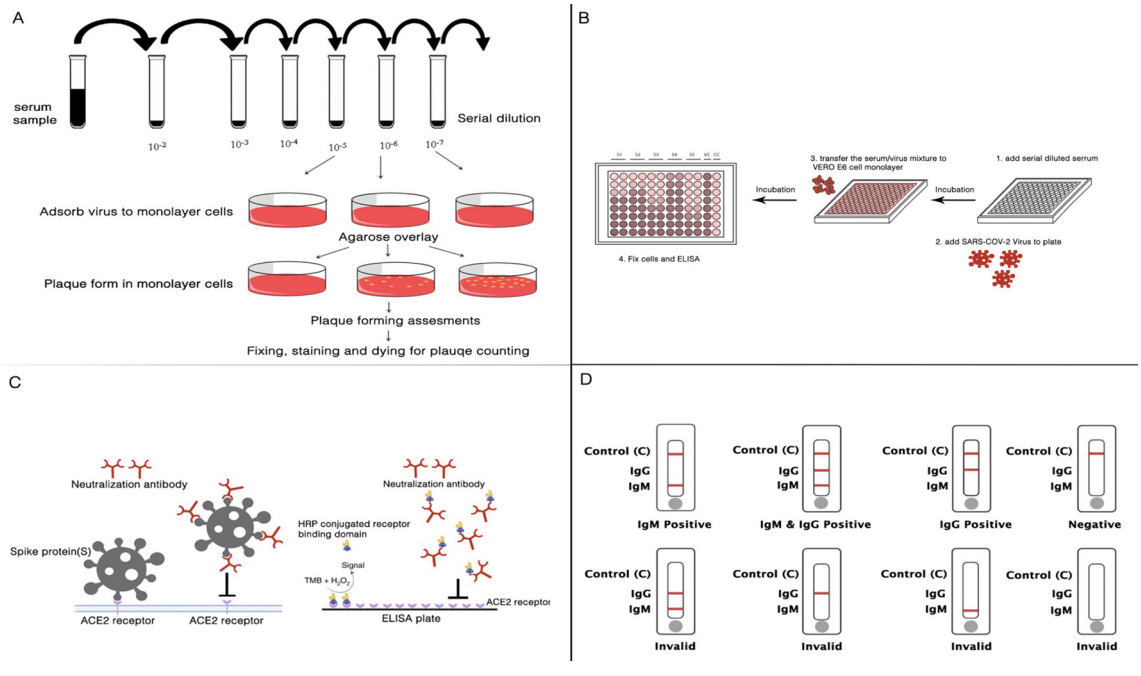

Figure 2. A. Plaque reduction neutralization (PRNT) test steps, B.MNT assay schematic steps Schematic steps of MNT assay, C. SVNT assay, D. Rapid tests based on chromatographic immunoassay. IgM+: Two lines appear in the cassette, in the IgM and in the control (C) regions. IgM+ and IgG+: three lines appear in the cassette, in the IgM, IgG, and in the C regions. IgG+: Two lines appear in the cassette, in the IgG and in the C regions. Negative: just one line in the C region appears. If C region fails to appear, the test result will be invalid even if IgM region or, IgG region or both have lines. If we have the negative results in this test, it means that the individual may not be patient or the patient may be in the early onset of disease (IgM below the detectable concentration).

antibodies in the patient serum can neutralize/ block the interaction between ACE2 and receptor-binding domains from SARS-CoV-2 S protein. In SVNT, horseradish peroxidase (HRP) conjugated receptor binding domain can be attached to RBD of S protein (ACE2 receptors) as well as to the immunodominant neutralizing antibodies in the serum patients in a species- and isotype- independent manner. A BSL2 laboratory is required to establish SVNT (50, 54, 55).

Rapid Diagnostic test (RDT)

The uncontrollable Covid-19 outbreak convinced scientific managers that early virus detection is crucial in controlling the virus's pandemics. Rapid tests are a group of paper-based immunoassays that are designed to be used when a preliminary screening test result is required. They are simple, inexpensive, fast, intuitive, and portable. According to the Bosch™ company researchers, the Coronavirus antibody rapid diagnostic test is used for the qualitative detection of IgG and IgM antibodies in human plasma, sera, or whole blood specimens (Figure 2D) (56, 57). This test consists of two components: the analyser and test cartridges. In each of the cartridges, there are biological components used to demonstrate whether a sample contains SARS-CoV-2 or other pathogens. Reliable results are ready in less than 2.5 hours (38, 58, 59). These types of rapid tests are based on chromatographic immunoassay and only a few microliters of serum, plasma, or whole blood are used for the qualitative assay of IgG and IgM antibodies to COVID-19 (38, 60, 61). The results are generated within 5–30 minutes (62-65). Based on the findings of different studies, the time it takes for different classes of immunoglobulins to emerge may vary from person to person. Padoan et al. concluded that IgG and IgM would appear about a week after the onset of clinical manifestations. In addition, Guo and his colleagues found that the average time for the detection of IgM antibody in patients was reported to be 5 days and IgG antibody was reported to be 14 after the onset of disease (19) (16). Even though all COVID 19 patients seem to produce anti-SARS-CoV-2 IgG within 12-14 days after the beginning of the disease, IgM are only found in <90% of patients (66). In the report published by Wolfel et al., IgG and IgM seroconversion was seen one week after the onset of symptoms in more than 50% of patients (15, 35). Another study conducted by Lippi et al. has proved that two weeks after the onset of symptoms, the serum level of specific IgM against SARS-CoV-2 remains high in 100% of COVID-19 patients (67). Another research conducted by Jin et al. yielded similar findings. In their study, the positive effects of IgG and IgM antibodies against SARS-CoV-2 were observed to be 95% and 50%, respectively (68). In addition, according to Du et al., the rate of detectable IgG and IgM antibodies against SARS- CoV-2 in patients is 100% and 78%, respectively (69). Pan et al., also found that within 15 days from the onset of clinical symptoms, the positivity rate for IgG and IgM antibodies is about 97% and 74%, respectively (70).

COVID-19 ELISA

SARS-CoV-2 specific antibodies were detected in patient serum or plasma samples using an indirect immune-enzyme assay. During the COVID-19 pandemic, ELISA became one of the most valuable screening tests particularly for population screening. This method is based on the reaction of recombinant antigens such as SARS-CoV-2 nucleocapsid protein, S1 protein, and S protein RBD which is a ACE2 receptor coated on the polystyrene surface (33, 71). If antiviral antibodies against mentioned glycoproteins are present in the patient sera, the antibody may bind specifically to the target protein, and this complex may be detected by a tracer enzyme-labeled anti-human globulin based on colorimetric readout (72). A standard curve is used to determine the antiviral antibody titer using the obtained optical density (OD) (Figure 3). This technique can detect IgG, IgM, or total

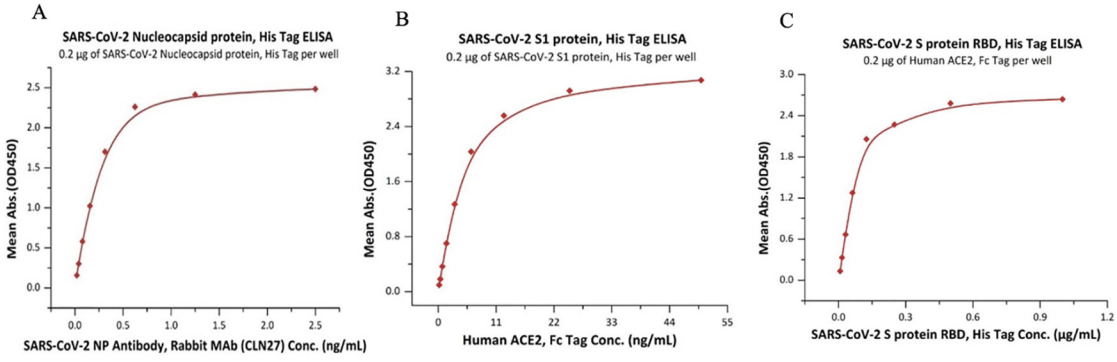

Figure 3. Standard curve used for determining the concentration of SARS-CoV-2 nucleocapsid protein (A), S1 protein (B), and S protein RBD, and (C) specific antibodies in the serum/plasma samples.

antibody in the patient serum and may be the most accurate way to assess the functional antibodies' existence at various stages of the disease (33, 73-76). ELISA has acceptable sensitivity specially after 6-10 days after the beginning of symptoms. According to Okba et al., the sensitivity of the recombinant nucleocapsid protein-based ELISA for IgM detection seems to be significantly lower than that of the recombinant S Protein-based ELISA (33). Depending on the type of kit, the sensitivity of the IgM and IgG tests varies (studies have reported different sensitivities of different kits). According to Hou et al., during the first week after the onset of disease, the IgM level increased and peaked within two weeks and then decreased to near-background levels in almost all patients. After one week, the titer of IgG was detectable and remained high for a long period. They also showed that patients with severe status had higher IgM levels compare to the mild one, while IgG levels in critical cases were lower than mild and severe ones because of compromised immune response or a high disease activity. More detailed studies showed that in in dead cases, IgM and IgG were undetectable during the disease course or IgM levels remained high while in recovered patients, IgM levels decreased rapidly. (77, 7). Antibody response can be lower in patients taking immunosuppressive drugs or in those who are immunocompromised. Alternatively, the virus may be present in the early stages of the infection, when the immune system is still upregulating antibody production. For these reasons, a negative IgG and IgM antibodies responses do not rule out a SARS-CoV-2 infection, nor do they provide information about possible future protection (33, 63, 65-67).

xMAP® SARS-CoV-2 Multi-Antigen IgG Assay

The xMAP® SARS-CoV-2 antibody test is a bead-based, highly sensitive multiplex assay that detects the existence of antibodies against three distinct SARS-CoV-2 antigens in patient plasma or serum samples, including the S1 subunit of the S protein, the RBD of the S protein, and the nucleocapsid protein (Figure 4) (78-80). This method can assess up to 96 samples in less than 3 hours, and the kit is compatible with MAGPIX®, Luminex® 200™, and FLEXMAP 3D® instruments. In this method in the first place, serum or plasma samples are mixed with beads and incubated for 60 minutes before being added to detection antibody mix. After incubation, the re-suspended sample must be transferred to the mentioned instruments for analysis (62, 81-84). This technique is also used to determine the presence of a virus antigen using specific bead-coated antibodies.

Indirect Immunofluorescence Assay (IFA)

An indirect immunofluorescent assay, Vero E6 cells infected with the inactivated SARS- CoV2 strain could be used as the antigen. The specificity of the assay for IgG, IgA, and IgM is 96.3%, 100%, and 98.6%,

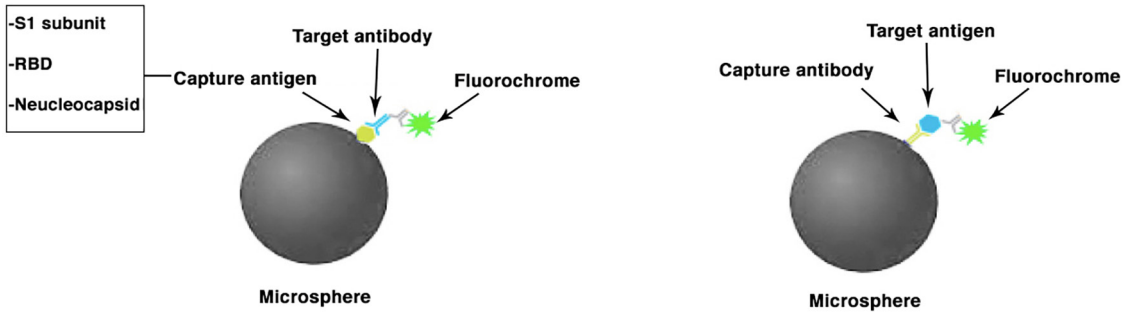

Figure 4. The xMAP multiplexing technology supports all 3 types of structural SARS COV-2 glycoproteins based on capture sandwich, indirect, or competitive methods.

respectively. Research shows that the IFA is a beneficial test for the screening of SARS-CoV-2 exposure especially at the population levels (25, 85).

MOLECULAR BASED TECHNIQUES FOR DETECTING SARS-COV-2

Up to the present, several molecular assays have been performed to detecting SARS-CoV-2 nucleic acid in patient samples. In these methods, applying high accuracy procedures for nucleic acid extraction from the patients' sample is of utmost importance. In this regard, considering the viral load in the affected individuals, as well as sampling in precise time, have a critical role in decreasing false negative molecular assessment outcomes. Within the first week after the beginning of the symptoms, higher viral loads in patients with COVID-19 are discovered, especially in the upper and lower respiratory tracts (35, 86-88). In the early stages of infection, sampling through oropharyngeal (OP) and/or nasopharyngeal (NP) swab are suggested (87, 89, 90). Although the OP swab was frequently used during the China COVID-19 outbreak, the low levels of virus RNA were detected in the OP swab compared to NP swab (91). Sampling of bronchoalveolar lavage or sputum was utilized to detect the highest viral loads in severe forms of COVID-19 (91-93). Furthermore, patients with COVID-19 pneumonia have high SARS-CoV-2 RNA loads in their feces (94, 95). As a result, detecting RNA in severe COVID-19 patients could be done using a rectal swab (89, 96-101).

Real-Time PCR (RT-PCR)

Real-time PCR is recommended for COVID-19 diagnosis as a gold standard assay. The most prominent feature of RT-PCR is the simultaneous amplification and analysis, which could minimize false positive results. Specific primers are used to amplify the SARS-CoV-2 RNA sequences (102). Highly expressed or conserved sequences, such as those encoding structural proteins like nucleocapsid (N), envelope (E), helicase (Hel), and Spike glycoproteins (S), as well as non-structural genes like RNA-dependent RNA polymerase (RdRp), open reading frame 1a or 1b (ORF 1a/b), and hemagglutinin-esterase (HE), could be considered as the most favorite targets for the SARS-CoV-2 Real-Time PCR (103-108).

When compared to other genes used in several European laboratories, RdRp sequence targeting showed a higher sensitivity (105, 109). Besides, detecting N1 and N2 nucleocapsid proteins is recommended in the USA (106). However, WHO proposes initial screening with the evaluation of the E gene, accompanied by a test for the RdRp gene (105).

To minimize the risk of cross-reaction with other coronaviruses, the assay should target at least two sequences. In the USA, CDC recommended two loci in the nucleocapsid gene (N1 and N2). A positive result is defined as a CT value of less than 40, while a CT

value of 40 or more is reported as a negative result. On the other hand. Retesting should be done if a CT value of 40 is found for one of the N1 or N2 nucleocapsid proteins (106).

Targeting ORF1b and one nucleocapsid gene were performed in one study (110). In China, the nucleocapsid gene was chosen for the initial screening, which was confirmed by analyzing ORF1b (108). In Germany, RdRp and E genes were chosen for detecting SARS-CoV-2 (105). Although the exact targets are not clearly defined now, it is recommended that two preserved loci be selected to mitigate the effects of genetic drift or cross-reaction with other coronaviruses.

Recently, one study designed a novel RT-PCR with high sensitivity and specificity by targeting the special sequence for RdRp/Hel (helicase/hemagglutinin-esterase or HE) (111). This method has a low cross-reactivity with other SARS-COV viruses and a high sensitivity, even in low viral loads, especially in saliva, plasma, or the upper respiratory tract samples (111).

Importantly, positive real-time PCR in stool samples was shown in COVID-19 patients. So, the consecutive negative RT-PCR result of rectal swabs should be considered before a patient is discharged (91). However, one study on 20 COVID-19 patients showed negative results from stool samples (35). Therefore, further research is required to determine the association between stool RT-PCR results and patient recovery.

Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)

Another investigation, known as RT-LAMP, is identified for detecting viral RNA. This assessment is considered as a fast method for amplifying the target DNA in 30 minutes (112). RT-LAMP has been established to detect a number of pathogens including bacteria, viruses, and malaria (113-116). This method showed a high sensitivity for detecting SARS-CoV-2 precisely at the low copy numbers (<10 copies per reaction) (117). The RT-LAMP has a high specificity and 4 or 6 primers are used to bind the specific targets on DNA (118). Until now, only a few RT-LAMP based molecular assays have been utilized for SARS-CoV-2 RNA. Huang et al. developed a rapid and one-step RT-LAMP for SARS-COV-2 detection that can be performed in less than 20 minutes at a constant 65°C (119). Accordingly, Lamb et al. reported a rapid RT-LAMP with high specificity and accuracy in COVID-19 patients (120). Abbott Diagnostics developed the ID NOW COVID-19 test, which was performed in 13 minutes or less to detect SARS-CoV-2 viral RNA in upper respiratory tract. RT-LAMP is consisting of three main steps. The primary invasive reaction is the first step, in which target DNA is hybridized with two different types of probes (upstream and downstream) and forming flaps due to the effect of an endonuclease enzyme. In the second step, secondary invasive reaction, the flaps formed from the previous step are attached to the hairpin probe and lead to the enzymatic breakdown of a part of the hairpin probe. In the last step, called nanoparticle hybridization, when the target is present in the test tube, the cleaved hairpin probe is unable to trigger the aggregation of gold nanoparticles that have been added to the tube, leading to the reddish color of the reaction. When the target is absent, the cleaved hairpin probe remains intact, leading to the aggregation of gold nanoparticles and then the reaction becomes colorless. Jiang et al. developed a single-step and accurate RT-LAMP which validate in a significant number of clinical samples, including 213 patients who were negative and 47 patients who were positive (121). Another study has described a high accuracy RT-LAMP called isothermal LAMP-based method for COVID-19 (iLACO) that targeted ORF1ab in 248 COVID-19 patient samples (122).

Transcription-Mediated Amplification (TMA)

TMA assay is a single-tube technique that uses two enzymes, RNA polymerase and

reverse transcriptase, to detect DNA or RNA more accurately than RT-PCR (120). In line with this, Hologic's Panther Fusion platform has the potential to perform TMA and RT-PCR. This principle has a high throughput and can screen other respiratory viruses with symptoms similar to those of COVID-19. Gorzalski et al., found that TMA has a higher sensitivity than RT-PCR for detecting SARS-COV-2 in a limit-of-detection analysis (LoD). It has been suggested that freezing and thawing samples before TMA assay has a negative impact on COVID-19 samples (123). In comparison to RT-PCR, this approach is easier to use and has higher sensitivity and throughput testing.

CRISPR Array

The CRISPR/Cas system is known as a prokaryotic immune system that eliminates foreign pathogenic DNA. The CRISPR/Cas-based methods have been developed as a promising treatment for a variety of diseases, including genetic disorders and cancers (124, 125). In this method, several Cas enzymes, along with a guide RNA, are used for cleavage of the target sequence. Cas9 is considered an efficient enzyme in gene editing strategies, while Cas12 and Cas13 were used to diagnose diseases (126, 127).

Recently, a few studies have proposed the efficacy of CRISPR/Cas-based methods for the rapid detection of SARS-CoV-2. Zhang et al. described a high-sensitivity CRISPR/Cas13-based nucleic acid detection (SHERLOCK) for rapid SARS-CoV-2 detection (128, 129). Spike glycoproteins and ORF1a sequences in the SARS-CoV-2 genome were targeted.

Accordingly, Sherlock Biosciences developed SHERLOCK method, a CRISPR/Cas-based strategy that uses Cas13a that targets the S gene and Orf1ab gene (129). Mammoth Biosciences reported a method by means of Cas12- based method for detecting of the E and N sequences of SARS-CoV-2, followed by isothermal target amplification. These tests are low-cost and have a great ability for point-of-care diagnosis (130).

Rolling Circle Amplification (RCA)

RCA is a nucleic acid replication procedure that is carried out in isothermal conditions with minimal false positive results, that are common in PCR-based tests. The efficiency of this method in detecting SARS-CoV-2 has yet to be set on. A study by Wang et al. described an RCA-based assay for detection of SARS-CoV-2 in the sputum samples from seven SARS patients obtained on days 5 to 13, after the onset of illness. The RCA strategy was proposed as a highly efficient, rapid, and sensitive method for the detection of SARS-CoV-2 (131).

Microarray

For detecting mutations associated with SARS-COV, genome microarray is an efficient procedure. With 100% accuracy, this method has been performed to trace up to 24 single nucleotide polymorphisms (SNP) related to mutations in the S gene of SARS-CoV (132).

In a study conducted by Long et al., 20 samples were assayed using the microarray to detect the SARS-CoV and for identification of the genotypes of the six mutated bases, including C (nt27827) C (nt9404), C (nt22222), G (nt19838), T (nt9479), and A (nt21721) (132). Previously, a non-fluorescent low-cost array has been performed to detect the entire coronavirus genus after RT-PCR (133). Genome microarray assays appear to provide a platform for the detection of mutational variations in SARS-COV-2, which may be essential for the identification of a virus infection potency. However, no one has yet documented using this method to identify SARS-COV-2 mutations.

Proteome microarray is based on the detection of proteins in a patient's sample using fixed surface antibodies (134). The recognition of proteins by these antibodies results in the emission of a fluorescent signal that could be analyzed through the associated equipment. In a study by Wang *et al*. a peptide library containing 966 peptides representing SARS-CoV-2 proteins was generated using

proteomic microarray (135). Their study showed that anti-SARS-CoV-1 antibodies can also interact with SARS-CoV-2 proteins. IgG and IgM antibodies were also screened by microarrays in COVID-19 patients that could target M, N, S, ORF1ab, ORF7a, and ORF8 epitopes. In addition, had the immune dominant epitopes, such as N, S, and ORF3a were found in more than 80% of the patients. Their experiments showed three primary protein S epitopes for interaction with IgM antibody including 1046-GYHLM-1050, 886-WTFGA-890, and 816-SFIED-820; and six primary epitopes for IgG including 11196-SLIDL-1200, 26-PAYTN-30, 356-KRISN-360, 186-FKNLR-190, 806-LPDPSKPSKRSFIED-820, and456-FRKSN-460.

With respect to N protein, eight major epitopes were identified for IgG detection including 366-TEPKKDKKKKADET QALPQRQKKQQTVTLPA ADL-400, 66-FPRGQ-70, 166-TLPKG-170, 96-GGDGK-100, 226-RLNQL-230, 206-SPARM-210, 316-GMSRI-320, and 256-KKPRQ-260; and two major epitopes were identified for IgM detection including 386-QKKQQ-390 and 206-SPARM-210.

In another study by Poh et al., (136) used proteomic microarray to identify immunodominant B-cell epitopes were found in 25 serum samples from COVID-19 patients. A decreased ability to neutralize the SARS-CoV2-pseudovirus was found by depleting the antibodies targeted the S21P2, S14P5, and S21P2+S14P5 epitopes.

Proteomic microarray could be an effective approach for mapping the antibodies profile and characterizing the main epitopes in the production of vaccines and diagnostic tests.

NxTAG® CoV Extended Panel

NxTAG® CoV Extended Panel is a qualitative, multiplex, nucleic acid test used to detect MERS-CoV, SARS-CoV, SARS-CoV-2, and 22 other common respiratory pathogens nucleic acids in human respiratory samples designed to use on the MAGPIX instrument. To improve the accuracy of the results, this technique detects three different target sequences on the target DNA. Up to 96 samples could be assessed with this method in approximately four hours (137-139).

SPECIMEN COLLECTION FOR MOLECULAR ASSESSMENTS

It is essential to take a sample from the appropriate anatomical site at the proper moment. Swabs should be positioned at the sampling site for at least ten seconds and rotated three times before being removed. According to different researches, increased viral loads are seen in the upper and lower respiratory tracts within five to six days of the appearance of the symptoms (74). To detect or screen early SARS-CoV-2 infection, an oropharyngeal swab or nasopharyngeal swab is often recommended during this time span. Based on Wang et al. After the incidence of COVID-19 in china; nasopharyngeal swabs have been used much less frequently than oropharyngeal swabs. However, SARS-CoV-2 RNA was shown to be substantially higher in 63 percent of nasopharyngeal swabs compared with the oropharyngeal swabs (just 32%).

Consequently, combining both types of upper and lower respiratory tract swabs within a common medium could be an ideal way of detecting infection (140, 141). Before performing real-time PCR test, the RNA is isolated from the specimen using a lysis buffer consisting an inactivating agent (including guanidinium) (142). Common commercial COVID-19 extraction kits (Qiagen EZ1 or bioMérieux easyMAG) generally contain guanidium/detergents to inactivate any viable virus.

In COVID-19 patients, virus isolation from the rectal and anal swabs and stool specimens has been observed in 23% to 82% of adult patients (143). Positive anal swab for viral RNA or live contagious virus was also identified up to 28 days after symptoms have developed (144). In some patients, a positive

rectal swab test was seen on the early days of SARS-CoV-2 infection (145). A rectal swab can also be performed in a patient, who meets one out of the following two criteria, even in the absence of gastrointestinal symptoms: 1) Suspected cases with negative NP, OP, or sputum swap sample, 2) cases with a negative NP, OP, or sputum swap sample with close contact with the confirmed patients.

T-SPOT TEST

SARS-CoV-2 T-SPOT test is an assay based on the ELISPOT platform, which evaluates the cell-mediated immunity to SARS-CoV-2 using viral peptide pools derived from the virus. In this PBMC separation test, a specific number of washed PBMCs and the specific SARS-CoV-2 antigens are added to a plate containing the coated anti-interferon-gamma (IFN-γ) antibody. Previously in-vivo primed T cells (due to infection) will respond to the same antigens in-vitro by secreting the IFN-γ cytokine. The anti- IFN-γ antibodies coated in each well capture the mentioned cytokine secreted by the primed T cells (146). Individual IFN-γ producing T cells are counted by visualizing the footprint of each IFN-γ producing T cells left behind by IFN-γ secretion.

ACCURACY AND SENSITIVITY OF SARS-COV-2 LABORATORY TESTS

Since no laboratory assay can guarantee a 100 percent accuracy, all laboratory experiments should be compared to the gold standard to assess their sensitivity and specificity. The absence of such a specific standard test for covid-19 detecting causes it difficult to assess the specificity of the test. The accuracy and sensitivity of the covid-19 test, as well as the possibility of pre-testing or predicted possibility of disease prior to the test, affect the interpretation of the results. According to this, a positive covid-19 RT-PCR test, for instance, have a higher significance than a negative test due to high specificity and lower sensitivity of test. However, a negative RT-PCR result cannot rule-out the SARS-CoV-2 infection especially in patients with COVID-19 symptoms. According to a systematic review conducted by Rodriguez et al., the false-negative rates of RT-PCR ranged between 2% to 29% (equating to the sensitivity of 71-98%) for SARS-CoV-2 detection (147). The accuracy and sensitivity of SARS-CoV-2 molecular tests also rely on the accuracy of the sampling (viral RNA swabs) as well as the sampling site. According to Wand et al., in COVID-19 patients RT-PCR test have a high sensitivity for bronchoalveolar lavage and sputum samples (93% and 72%, respectively) compared to nasal or throat swabs (63% and 32%, respectively) (91). The degree of viral clearance or multiplication, the stage of the disease, and how gene targets are used all affect accuracy (148, 149). The accuracy of the serological-based analysis for COVID-19 is also still under investigation. Since there is no appropriate gold standard for comparing the results of corvid-19 tests to determine the exact accuracy of each test, clinical parameters such as chest radiographs, computed tomography scans, and the patients' history should be considered as well.

DISCUSSION

COVID-19 serological tests are used to track retrospective contacts of individuals, evaluate the vaccine efficacy, characterize the prevalence and spread of the disease, and evaluate herd immunity, while COVID-19 molecular tests are used to detect active disease by searching the genome of the virus in the patients' samples (71). The timetable for performing each laboratory test for COVID-19 could vary relying on the type of test and also from person to person. According to the various studies, seroconversion could occur after the viral load has reached its peak (Figure 5). Serological analyses therefore are

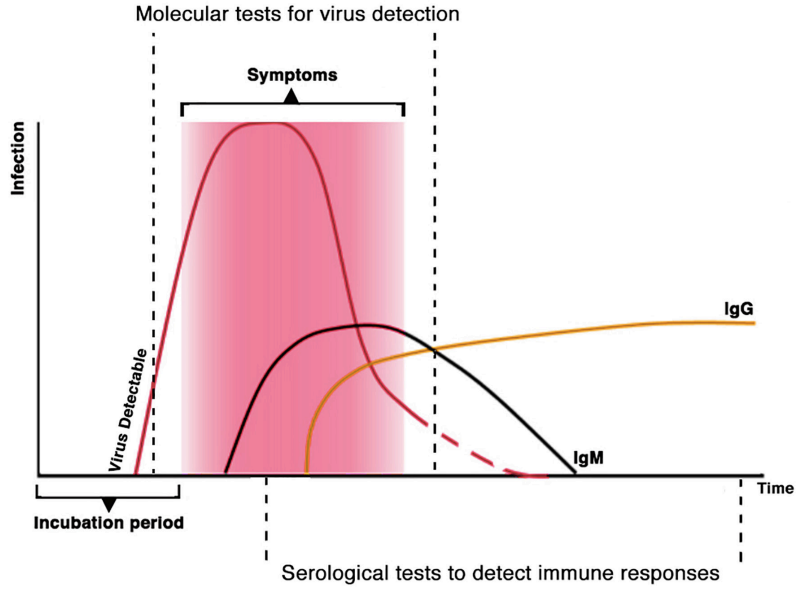

Figure 5. Molecular vs. serology and their significance in terms of COVID-19.

less efficient in the initial phases of COVID-19 (58, 88). The majority of the molecular diagnostic techniques of COVID-19 are focused on real-time PCR tests. Loop-mediated isothermal amplification, multiplex isothermal amplification, and CRISPR-based assays are among the molecular experiments being established all over the world. For an immediate and accurate molecular diagnosis, the required specimen from the respiratory tract must be collected from the appropriate anatomical site at the proper moment (141). While random-amplification next-generation sequencing (NGS) or metagenomic next-generation sequencing methods are presently unusable for detecting COVID-19, they were instrumental in the initial detection of SARS-CoV-2 and could be applied for assessing potential SARS-CoV-2 mutations (150). Based on the results of the studies, there may be different times for different classes of antibodies in individuals. As IgG and IgM antibodies rise and fall at different times, this fluctuation provides information on where the patient is in the recovery process and when exposure has occurred. Ideally, IgG and IgM assessment along with an RT-PCR, provide the maximum amount of diagnostic information (Table 1).

For monitoring the SARS-CoV-2 infection, developing a high-performance diagnostic test has become a top priority. Currently, the gold standard for detecting the SARS-CoV-2 is nucleic acid testing (NAT) using RT-PCR (110, 151, 152).

Molecular-based detection techniques, such as Real-Time PCR, are the fast and perfect diagnostic tools for SARS-CoV-2 and are increasingly used to diagnose suspected patients definitively and to screen the sources of COVID-19 infection and prevent its prevalence. In fact, nucleic acid detection-based Real-Time PCR is a rapid and reliable method to viral detection, particularly in patients who are just starting to show symptoms. This test with a high sensitivity and specificity considered as a "gold standard" for recognition of other RNA/DNA viruses. Real-time reverse transcriptase-PCR as a specific and simple method is currently used qualitatively and/or quantitatively for the RNA viruses detecting. However, due to the severity of the COVID-19 pandemic, RT-PCR is now used to detection of SARS-CoV-2 RNA in the samples of infected patients (153-155).

Despite the value of real-time RT-PCR, the possibility of false negative and false positive findings is a major concern. As a result, its sensitivity and precision aren't fully accurate or 100%. For example, many "suspected"

Table 1. A possible interpretation of ELISA and molecular tests that were performed for each patient. This table is based on the existing knowledge about the rise and fall of SARS-CoV-2 RNA and IgG/IgM antibodies and the correction of these level variations with the initial period of infection, the onset of symptoms, and on the recovery phase (77, 88, 163-166).

qPCR	IgM	IgG	Clinical Significance
+	-	-	Window period of infection
+	+	-	Early-stage of infection
+	+	+	Active phase of infection
+	-	+	Late or recurrent stage of infection
-	+	-	Early-stage of infection. qPCR result may be falsely negative
-	-	+	Recovered patients or may have had a past infection
-	+	+	Recover stage of infection. qPCR result may be falsely negative

cases with COVID-19 clinical symptoms and similar computed tomography (CT) images, for example, were found to be negative by real-time RT-PCR (156). Therefore, the negative result of this method should not be used as the only criterion-referred for the detection and treatment of such patients. In this way, several factors, such as genetic diversity and mutation of the virus, have been suggested to be related to the various challenges related to the COVID-19 diagnosis by RT-PCR in real time (157). So, the results obtained by real-time RT-PCR using different primers that could target various parts of the viral RNA genome can affect sequence variation and test results. In other words, mutations in the genome target regions by the designed primers and probes may lead to reduce in assay efficiency and causing false-negative result because of the mismatches developed between the primers/probes and the target sequences. The personnel expertise as well as the laboratory standardization are also drawbacks that may result in false-negative results (158, 159). Another issue that leads to false-negative findings is sampling procedures. Appropriate sample types and the optimum time at which the viral load of SARS-CoV-2 reaches its peak have yet to be determined. For example, studies have shown that sputum and nasal swabs were found to be the most accurate samples for diagnosing COVID-19. However, throat swabs were not recommended. In the patients with gastrointestinal involvement, stool specimens should be used in addition to respiratory specimens. It will be preferable to use stool samples instead of respiratory samples in the case of COVID-19 patients with gastrointestinal symptoms. Furthermore, false negative results may be caused by existing the repressors in the specimen and/or insufficient viral load as a result of improper processing, transportation, or handling (160, 161). Finally, some specimens consisting the elements that could interfere with the target nucleic acid extraction and amplification. The negative template control (NTC) and the internal control should be included in the detection of SARS-CoV-2 by Real-Time PCR to prevent the possibility of false-positive results and sample contamination. Therefore, for the accurate diagnosis of SARS-CoV-2 patients, both molecular assays such as real-time PCR and clinical features must be considered.

Although more optimization is required for increasing the sensitivity of the molecular-based methods, other approaches including multiplex isothermal amplification followed by microarray detection, and CRISPR are also being developed worldwide (162). RT-LAMP is a Real-Time PCR alternative method, which has demonstrated high sensitivity for detection of SARS-CoV-2, especially at the low copy numbers of viruses. Unlike Real-Time PCR, which requires a series of temperature changes in each cycle, RT-LAMP allows for continuous amplification at a persistent temperature, reducing the requirement for a thermal cycler (118). A few recent studies have found that the methods based on CRISPR

for the rapid recognition of SARS-CoV-2 are efficient. This approach has been suggested to target Spike glycoproteins and ORF1a sequences in the SARS-CoV-2 genome, using Cas13. For point-of-care diagnosis, these tests have attracted considerable attention. The TMA technique was recognized as a high throughput method for screening other respiratory viruses with symptoms like COVID-19. In order to SARS-CoV-2 infection diagnosis, it is critical to develop a rapid test with high accuracy. Taken together, despite many of the unresolved concerns and challenges, considerable improvement was made in the development of diagnostic tests. However, further investigations are needed to determine the sensitivity and specificity of each test in COVID-19 samples.

CONCLUSION

In COVID-19 patients, serological and molecular assays could be used to diagnose SARS-COV-2. Despite the fact that molecular tests have a high specificity and sensitivity, correct sampling time as well as viral load play a significant role in reducing false results. According to the results of serological tests, the IgM and IgG levels have shown to be notably higher in severe cases of COVID-19 compared to the patients with mild disease. COVID-19 serological assays need the elevated sensitivity to recognize the lower amounts of antibodies especially in the patients with mild disease. However, serological tests should not be used as a diagnostic criterion for protective immunity and should instead be used to define attack rates, case fatality rates, or other epidemiologic questions. Many commercial RDTs have poor sensitivity but high specificity especially in the early days (about 7-10 days) of disease onset, and according to the published data, few RDTs and commercial ELISAs have met high standards. The time at which the antibody is produced, the duration of the positivity, the rate of antibodies and the viral load may vary from person to person.

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