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Laboratory procedures and reference ranges for COVID-19 for diverse patient populations: Review

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***Abstract*---**Background: Reliable and precise laboratory testing for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is crucial in managing Coronavirus Disease 2019 (COVID-19). It assists in making informed clinical decisions for infection control in healthcare settings and identifying asymptomatic individuals. This facilitates appropriate interventions, timely quarantines, and helps slow the spread of the epidemic. Laboratory tests can detect the genetic material of SARS-CoV-2 in samples and identify specific anti-viral antibodies in blood or serum. Point-of-care diagnostics (POCD) advancements have expedited clinical decision-making and strategic planning for nationwide preventive actions. Aim of Work: This review aims to provide a concise overview and comparison of existing Point-of-Care Diagnostic (POCD) methods and those currently in development. These methods, including quantitative reverse transcription PCR (RT-qPCR), serology immunoassays (SIAs), and protein microarray method (PMM), are designed for diagnosing COVID-19, offering options for both conventional and rapid testing. Methods: The review evaluates the performance and utility of quantitative RT-qPCR, serology immunoassays (SIAs), and protein microarray method (PMM) in diagnosing COVID-19. It compares the sensitivity, specificity, turnaround time, and ease of use of these POCD methods. Additionally, it discusses the potential impact of these methods on clinical decision-making and epidemic control strategies. Results: Quantitative RT-qPCR, SIAs, and PMM have shown varying levels of sensitivity and specificity in diagnosing COVID-19. RT-qPCR is considered the gold standard for detecting viral RNA, while SIAs and PMM offer insights into antibody responses. The review highlights the

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advantages and limitations of each method, emphasizing the importance of choosing the most appropriate test based on the clinical scenario and testing requirements. Conclusion: In conclusion, the review underscores the significance of reliable and rapid diagnostic testing for managing COVID-19. POCD methods such as RT-qPCR, SIAs, and PMM play a crucial role in early detection, infection control, and epidemic management. By understanding the strengths and limitations of these diagnostic tools, healthcare professionals can make informed decisions to effectively combat the spread of SARS-CoV-2 and mitigate the impact of COVID-19.

***Keywords*---**COVID-19, SARS-CoV-2, In Vitro Diagnostic Testing, RT-qPCR, Serology Immunoassays.

Introduction

COVID-19, a disease caused by the new coronavirus SARS-CoV-2 (formerly known as 2019-nCoV), originated in China and has since spread globally [1,2]. On January 30, 2020, the World Health Organization (WHO) declared the COVID-19 outbreak a worldwide public health emergency. By March 2020, the situation had worsened and evolved into a full-blown pandemic. As of 13 June 2020, Johns Hopkins University documented a total of 7,600,000 infections and approximately 427,000 fatalities [3]. Due to the fast spread of the COVID-19 pandemic and the limited capacity for laboratory-based molecular testing, new point-of-care (POC) diagnostic assays have been developed. These tests are scalable and provide quick results, making them convenient for use outside of laboratory settings. Furthermore, the need to expand COVID-19 testing has been unequivocally recognized as a crucial component of the global anti-coronavirus strategy.

The diagnostic sensitivity, specificity, and accuracy in detecting suspected COVID-19 infection, as determined by controlled testing and performance data in clinical settings, are crucial for preventing the spread of coronavirus epidemics. Inaccurate and unvalidated tests may fail to identify individuals with an ongoing illness or may erroneously classify COVID-19-negative people as positive, impeding treatment endeavors. The diagnostic laboratory and point-of-care tests (POCTs) utilized to identify SARS-CoV-2 primarily consist of reference tests based on the molecular technique known as real-time quantitative reverse transcriptase polymerase chain reaction assay (RT-qPCR), as well as serological tests that detect antibodies and antigens, serving auxiliary functions. Currently, the World Health Organization (WHO) protocols recommend using molecular quantitative reverse transcription PCR (RT-qPCR) testing of respiratory tract samples as the sole method for identifying and confirming COVID-19 cases. These methods have been thoroughly evaluated for their quality and safety. However, according to existing scientific evidence, the World Health Organization (WHO) suggests using point-of-care (POC) immunodiagnostic tests for research reasons only. These tests should not be used in clinical decision-making or patient care until they have been completely validated and there is supporting data available. Nevertheless, they may be valuable in epidemiological studies or illness monitoring and continue to progress as an essential stage in the development of a COVID-19 vaccine in the future. During periods of high demand for hospital services, doctors, governments, and health services need a speedy, sensitive, and cost-effective diagnostic test to efficiently triage patients for COVID-19 therapy and hospital admissions. Hence, the significance of an authorized and dependable diagnostic test in the COVID-19 care route cannot be overstated [4].

Similar to other contagious illnesses, the RT-qPCR technique and serological tests are appropriate for the in vitro diagnostics (IVD) of individuals who are suspected of being infected with SARS-CoV-2. The RT-qPCR approach, which is done in real time, is particularly useful in the early phases of viral infection when the virus replicates rapidly. This technique allows for immediate identification of the pathogen's genetic material. Serological approaches provide an alternative way to diagnose infection by identifying particular antibodies in the blood serum that target viral proteins generated in response to SARS-CoV-2. Serological in vitro diagnostic (IVD) assays identify antibodies that neutralize the virus [5]. These tests are specifically employed when the immune response against the SARS-CoV-2 virus is already occurring. These in vitro diagnostic methods may ascertain the rate at which antibodies against the virus are generated, potentially impacting the detection of individuals who have already acquired immunity. It is not recommended to utilize the findings of serological in vitro diagnostic (IVD) testing as a means of diagnosing or ruling out SARS-CoV-2 infection, or determining one's infection status [6].

The expeditious identification of the whole genomic composition of SARS-CoV-2 in the early stages of the pandemic facilitated the development of precise primers and standardized laboratory procedures for COVID-19. The methodology for the first RT-qPCR test, targeting the RNA-dependent RNA polymerase (RdRp), envelope (E), and nucleocapsid (N) of SARS-CoV-2, was released in late January 2020. The new coronavirus SARS-CoV-2 has a receptor-binding domain (RBD) that bears resemblance to the RBD found in the 'original' SARS-CoV, which initially appeared in 2002. The functionally significant open reading frames (ORFs) 1a and 1b, along with other key structural proteins such as the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, are intricately connected and well documented. Previous investigations indicate that the M and E proteins play a vital role in the assembly of the virus. On the other hand, the S protein is important for its ability to interact and attach to host cells. This is made possible by the RBD of the S protein, which allows it to engage with angiotensin-converting enzyme 2 (ACE2). The viral particles possess a S protein on their surface, which has been seen to exhibit a high level of immunogenicity. The N protein, a crucial component of SARS-CoV-2, plays a vital role in several processes. It is primarily responsible for transcribing and replicating the viral RNA, packaging the enclosed genetic material into virions, and interacting with the cell cycle of host cells. Furthermore, coronaviruses possess the N protein, which has a significant capacity to provoke an immune response and is highly produced during viral infection (Figure 1). The S/N proteins are being considered as possible antigens for serodiagnosis of COVID-19, similar to other diagnostic approaches used for identifying SARS illness, which rely on S/N proteins [7].

Aim of Work

This review aims to provide a concise overview and comparison of existing Point-of-Care Diagnostic (POCD) methods and those currently in development. These methods, including quantitative reverse transcription PCR (RT-qPCR), serology immunoassays (SIAs), and protein microarray method (PMM), are designed for diagnosing COVID-19, offering options for both conventional and rapid testing.

Molecular RT-qPCR Assay Reference for Verified COVID-19 Diagnosis

Currently, there is a global effort to develop new techniques that will streamline and expedite the identification of the novel coronavirus. Currently, there are around 200 genetic tests available for commercial use, with other firms awaiting the completion of the necessary procedures and the issuance of certificates by the Food and Drug Administration (FDA) or In Vitro Diagnostics (IVD). It is important to mention that a significant proportion of the tests offered are only given with the Research Use Only (RUO) certificate. However, it is crucial to note that this certificate does not indicate anything about the quality or validity of the test. Products certified as IVD must undergo more stringent testing and adhere to the ISO13485 standard. Furthermore, IVD reagents must adhere to specific local regulations, such as the requirement for CE marking in Europe. This necessitates compliance with Directive 98/79/EC of the European Parliament and Council, which was established on October 27, 1998, and focuses on in vitro diagnostic medical devices. Compliance involves a comprehensive evaluation process, including the validation of clinical samples.

Real Time RT-qPCR genetic diagnostics are designed to detect and distinguish SARS-CoV-2 in samples obtained from individuals exhibiting symptoms of COVID-19. These tests work by identifying specific RNA sequences that are exclusive to SARS-CoV-2. The virus's genetic material is obtained from various specimens (such as sputum, tracheal aspirate, bronchoalveolar lavage, swabs from the nasopharynx and pharynx, blood, urine, or stool) and then amplified using the real-time polymerase chain reaction (PCR) technique. This amplification process involves reverse transcription (RT) and is detected using fluorescent reporter probes that are specific to the SARS-CoV-2 virus [8,9].

Currently, the global market offers RT-qPCR assays designed to identify ORF1ab, E, N, or S gene sequences, either alone or in different combinations. The tests vary in terms of their sensitivity, stability, and examination duration. The test methodology is intricate and expensive, mostly appropriate for large, centralized diagnostic facilities. The duration of tests typically ranges from 4 to 6 hours. However, due to logistical constraints related to transporting clinical specimens, the maximum execution time is 24 hours [10].

As per the guidelines provided by the World Health Organization (WHO), RT-qPCR assays should have the capability to identify three specific genes in a single reaction: E gene, N gene, and RdRP gene. This enables us to identify viruses belonging to the beta-coronavirus group by detecting the E gene. Additionally, it allows us to specifically identify the SARS-CoV-2 virus by detecting the N gene, RdRP, and ORF1ab. This strategy ensures both redundancy in identifying infections and minimizes the possibility of false negative findings by targeting several SARS-CoV-2 markers. This minimizes the likelihood of receiving questionable outcomes, in which case the need for verification arises [11].

The implementation of the RT-qPCR technology follows a rigorous and time-consuming standard process. Therefore, scientists continuously endeavor to develop more contemporary adaptations of the RT-qPCR assays, aiming to reduce the time needed for analysis [12,13]. Bosch Healthcare Solutions has unveiled a rapid test that might potentially provide findings within 2.5 hours. This testing kit is designed for the Vivalytic platform and is known for its speed. The PCR test is completely automated and requires just the insertion of a swab into a cartridge, which is then examined by the machine. This versatile platform for molecular laboratory diagnostics allows for testing of numerous samples and use of multiple analytical techniques. The whole process may be automated and completed quickly. Regrettably, the technology has not yet been made accessible to several healthcare facilities, and the diagnostic cartridges are only designed for the particular analyzing apparatus [14].

BioMaxima has developed a test that offers significant benefits such as a brief waiting period, with findings obtainable in only 2 hours. Furthermore, the test has a high analytical sensitivity, with a minimum detection limit of ≥10 RNA copies per reaction, which surpasses that of other similar methods. The molecular test kit's reagents must possess significantly improved stability to provide safe transportation and room temperature storage. In contrast, many other tests on the market need freezing and transportation with dry ice. Storing these reagents under settings that differ from the recommended ones, even for a brief period, is dangerous and may lead to the production of inaccurate negative test findings [15].

The most recent advancements in molecular testing, pioneered by Oxford University experts, exhibit heightened sensitivity compared to prior iterations. This breakthrough enables the examination of patients at earlier stages, facilitating prompt and efficient containment of the coronavirus. This novel diagnostic test for SARS-CoV-2 yields rapid results within 30 minutes, which is three times faster than the fastest current methods that focus on viral RNA and provide results in 1.5 to 2 hours. Additionally, this test only requires the use of relatively simple technical devices. In addition to these benefits, the scientists involved in the test's creation assert that it could potentially identify individuals with early-stage coronavirus infections more effectively than current methods. Furthermore, the test's results can be easily interpreted without the need for specialized equipment, making it accessible to a broader range of healthcare facilities and professionals. The FDA has just approved this rapid molecular test, which incorporates a diagnostic technology that has shown excellent performance in identifying several illnesses, such as Hepatitis C Virus (HCV) and influenza, in recent years [16].

RNA detection may be conducted using RT-qPCR or reverse transcription–loop amplification (RT–LAMP). A typical RT-qPCR test takes an average of 90 to 120 minutes to analyze a group of samples, but LAMP may be finished in about 30 minutes. Indirect isothermal amplification, often known as LAMP, is a rapid DNA amplification process used for detecting pathogens like viruses or bacteria. The LAMP reaction typically occurs at a consistent temperature, allowing for the amplification of the target DNA within a 30-minute timeframe. The LAMP approach employs either four or six primers to bind to six distinct sections of the target DNA, resulting in a very high level of specificity. Due to its RNA composition and length of around 30 kb, SARS-CoV-2 may be rapidly identified by combining a single reverse transcriptase (RT) reaction with LAMP. This approach eliminates the need for purifying cDNA from reverse transcriptase, resulting in a considerable reduction in reaction time. It is important to mention that a one-step RT-qPCR approach, like-wise, does not need cDNA purification, since it is a single-stage reaction. Oxford University's team has created four sets of LAMP starters, each including six starters, specifically designed to target the viral RNA of SARS-CoV-2 in the ORF1ab, S gene, and N gene areas. To interpret the data, a colorimetric approach has been used. This method allows the amplification of viral RNA to be read visually, without the need for costly equipment. Furthermore, the method's sensitivity may detect as low as 80 copies of viral RNA per 1 mL in the sample. It also allows for a single step procedure without the need for separate RNA extraction, enabling direct amplification of RNA from the sample [17,18].

Utilizing rapid molecular testing may be very advantageous in Emergency Departments and admission rooms for conducting exams. Point-of-care tests (POCTs) are necessary to expedite the process of making clinical choices and reduce the burden on centralized testing facilities [16]. Point-of-care testing (POCT) refers to the immediate delivery of test results in patient care settings, such as hospitals, urgent care clinics, and emergency rooms. This eliminates the need for costly and time-consuming laboratory procedures.

In addition, the American firm Cepheid has created a rapid test that they claim can be completed in 45 minutes, whereas the Dutch pharmacists who control Qiagen have devised a test that takes 1 hour. The US Food and Drug Administration has granted an emergency permission for the use of Cepheid's point-of-care COVID-19 diagnostics, Xpert Xpress SARS-CoV-2, as stated by the FDA. The test was specifically developed to rapidly identify the presence of the new coronavirus in about 45 minutes. It utilizes samples taken from a nasopharyngeal swab, nasal wash, or nasal aspirate. The Xpert Xpress SARS-CoV-2 test cartridge is a point-of-care test (POCT) designed specifically for identifying the genetic material of the SARS-CoV-2 virus. It uses a technique called reverse transcription quantitative polymerase chain reaction (RT-qPCR) and does not rely on the use of additional chemicals or substances. On the contrary, the GeneXpert System requires the test to be conducted in a laboratory that is authorized by CLIA (Clinical Laboratory Improvement Amendments) or in certain patient care settings [19].

It is crucial to recognize that RT-qPCR assays also have certain limitations. They are very beneficial when there are favorable outcomes, but their diagnostic usefulness seems to be lower in circumstances when COVID-19 has to be excluded. A negative RT-qPCR test result does not definitively imply that an individual has not acquired an infectious illness. Other individual considerations, such as exposure risk and possible laboratory mistakes, must also be taken into account. False negative findings may arise due to inadequate collection, transportation, or handling of the sample, or due to poor extraction of nucleic acid from clinical materials [9]. Additionally, the occurrence of false positive findings might be attributed to situations where the sample includes substances that hinder the amplification process or an inadequate quantity of viral molecules.

Conversely, a false positive outcome might occur as a consequence of sample cross-contamination during handling or preparation, or between samples from different patients. In addition, the impact of vaccinations, antiviral medications, antibiotics, chemotherapeutic agents, or immunosuppressive drugs has not been accounted for. Furthermore, the set/kit does not specifically target disorders caused by other bacterial or viral infections. Negative findings do not definitively rule out infection and should not be the only criterion for determining patients' therapy. The test results obtained from using the kit are intended only for therapeutic reasons. When diagnosing and treating patients, it is important to take into account their symptoms, medical history, reaction to therapy, and other laboratory testing [9]. The sensitivity of this molecular approach might vary depending on the kind of material collected (broncho-alveolar lavage, sputum, nasal swabs, or throat swabs). Furthermore, the accuracy of the method relies on the stage of infection, the pace at which SARS-CoV-2 multiplies, and the level of clearance. It is noteworthy that the reported accuracies of RT-qPCR are greater when using SARS-CoV-2-specific primers and doing in vitro validation in tightly controlled laboratory environments [20].

The creation of hybrid assays is necessary to limit the number of false negative findings and achieve the ideal outcome and a reliable diagnostic gold standard. The integration of the improved RT-qPCR test with serological immunoassays, which can detect either antibodies or antigens, would provide an extra diagnostic benefit for precise and rapid COVID-19 identification in laboratory testing, particularly when the human body responds to an infectious bio-stimulant [21].

Assessment of Molecular RT-qPCR Tests for the Detection of SARS-CoV-2 RNA

Recent advancements in infection diagnostics have allowed for the simultaneous use of many approaches to efficiently identify respiratory viruses. The selection of appropriate tests relies on factors such as the specific virus being targeted, the estimated quantity of antigens, the population of patients to be tested, and the technical expertise and experience of the testing facility. Validating RT-qPCR testing is a crucial measure in the fight against the novel coronavirus. The least detectable and quantifiable quantity of analyte is a crucial metric for determining the effectiveness of the diagnostic technique. The metrics that describe such features are referred to as the limit of detection (LoD) and the limit of quantification (LoQ). LoD, or Limit of Detection, is often used synonymously with terms such as "sensitivity," "analytical sensitivity," or "detection limit" in various laboratory settings. However, it is important to note that the term "sensitivity" may be interpreted and used in several different contexts. Sensitivity and clinical specificity are distinct characteristics. Diagnostic sensitivity refers to the ratio of genuine positive outcomes to the total of true positive and true negative results. A sensitivity ratio of 100% indicates that every individual who is unwell has been correctly detected. The specificity of a test is defined as the ratio of true negative findings to the total number of true negative and false positive results. The 100% specificity indicates that every healthy individual in a certain test has been accurately recognized as being healthy and not having the illness.

Novel Serology Immunoassays for Rapid Immunodiagnostic Testing

Immunological techniques often include chemiluminescent assays to detect immunoglobulin IgG and IgM specific to SARS-CoV-2 in blood samples using an analyzer. Alternatively, immunochromatographic assessments in the form of fast point-of-care tests (POCTs) may be used, which do not need any extra equipment. Despite current research to enhance their development, it is advisable to use the techniques for detecting anti-SARS-CoV-2 antibodies during the coronavirus outbreaks [22,23]. Approximately one week after the first clinical symptoms appear, the effectiveness of molecular diagnostics (PCR) for detecting SARS-CoV-2 infections progressively decreases. This is because there is a reduction in the number of virus particles present in the respiratory tract epithelium. Patients in such instances may have misleading negative outcomes, even while the virus is still present.

Out of the many modern technologies, particular emphasis should be placed on quick lateral flow immunoassay (LFIA), commonly known as immunochromatographic assays. Although they are increasingly being used in diagnostics, lateral flow assays may not be as well regarded in the scientific community as PCR procedures or Enzyme-Linked Immunosorbent Assay (ELISA tests). The differences between tests vary depending on the kind, but their fundamental mechanism remains consistent. They use the distinctive characteristic of antibodies, which is their ability to selectively attach to a particular particle or a cluster of identical particles (antigen). Lateral Flow Immunoassays (LFIAs) provide a straightforward and cost-effective method for identifying the presence or absence of a specific component in a specimen under examination, such as detecting the presence of a virus in a blood sample. These tests may be used to examine a variety of test materials, such as whole human blood, blood plasma, serum, feces, urine, perspiration, cerebrospinal fluid, or tears [9]. The test concept relies on an immunological technique that utilizes particular antibodies, often in conjunction with colloidal gold. In this procedure, a little amount of the item being tested is placed on a nitrocellulose membrane, and it flows along the membrane due to capillary action. Once the sample is taken in by the membrane, if the test turns out to be positive, the antigen attaches to the colloidal gold complex and its corresponding antibodies. As a result, the reaction leads to the creation of a complex, which may be identified by the test. The interpretation of findings involves verifying or excluding the existence of antigens in the analyzed sample, relying on color test strips that become visible during the test [21,22].

The Ongoing Advancements in COVID-19 Diagnostics

A novel test using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas12a) has been recently developed, including visual readouts. The test has the capability to identify 10 copies of the viral gene in only 45 minutes, making it a straightforward and dependable diagnostic procedure [23-27]. Furthermore, it has a high level of sensitivity and specificity. To facilitate rapid diagnosis in hospital settings or upon admission, the test has been equipped with a single-stranded DNA (ssDNA) reporter that is tagged with a green fluorescent particle. This particle will be detached by the Cas12a protein when the nucleic acid of SARS-CoV-2 is detected in the testing system. By following this method, a distinct emission of green fluorescence will be achieved, which may be easily seen without any magnification, under illumination with light with a wavelength of 485 nm [28-36]. To achieve a high level of specificity, the test was developed using 15 crRNAs. These crRNAs are capable of differentiating between single nucleotide polymorphisms in four particular domains: ORF1a, ORF1b, N, and E genes, which are associated with other viruses linked to SARS [37].

The pursuit of novel diagnostic techniques is ongoing. Due to the transmission of SARS-CoV-2 through droplets, scientists have suggested using exhaled breath condensate (EBC) for diagnostic purposes. This is because the RT-qPCR tests show higher diagnostic sensitivity and specificity when performed on broncho-alveolar lavage (BAL) samples compared to sputum or nose/throat swabs. EBC has a high degree of similarity in terms of its biochemical makeup and origins when compared to BAL. Examinations with EBC are a recently developed diagnostic technique primarily used to evaluate inflammation in the respiratory system. The components of EBC accurately represent the makeup of the fluid that lines the bronchi and alveoli. EBC, or expelled breath condensate, is a concentrated mixture of tiny droplets from the lining of the lungs. It consists of a range of substances, including small ions, proteins, and organelles. It may also potentially include viruses, fungus, and bacteria. Collecting samples of the condensate is a safe and non-invasive procedure that is simple to carry out. It may be done several times and is suitable for patients with severe consequences of COVID-19. One significant benefit is that it can be conducted on young children using specialized face masks. During the examination, the patient is instructed to breathe normally for about 10-15 minutes. Then, they are asked to exhale into a device that has a cooling system. This device collects the condensation, which may be tested for the presence of SARS-CoV-2. The use of this approach seems to be warranted from a public health standpoint. The current false positive findings obtained are contributing to the ongoing global spread of the virus [38,39].

Conclusion

The discipline of laboratory diagnostics optimization is seeing rapid development during the COVID-19 pandemic. It plays a crucial role in supporting modern medicine, government decision-making, and healthcare plans. The primary objective of scientist-clinician teams is to apply the most dependable diagnostic methods. However, due to COVID-19 being a novel medical condition, there is currently insufficient evidence to establish guidelines for interpreting serological POCTs. Like many other contagious illnesses, the diagnostic efficacy of a test is not only determined by the technique of obtaining the specimen, the quality of the sample, and the equipment used. Equally crucial pre-analytical factors include the specific time at which a sample is obtained, as well as the appropriate protocol for storing and managing the sample prior to analysis, starting from the moment of collection until the testing phase.

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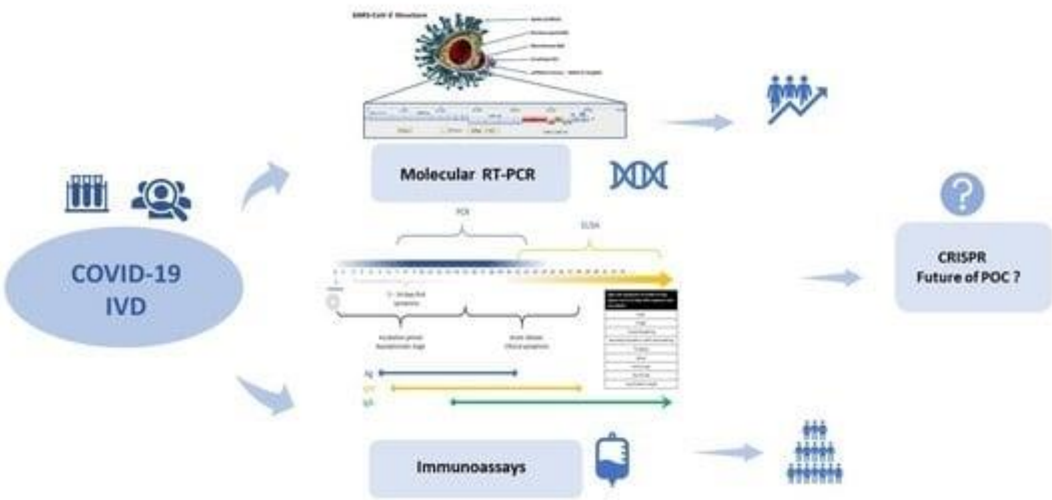

Figure 1. Diagnostic tests for COVID-19