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Review Article

General Evaluation of Covid-19 Diagnosis Methods

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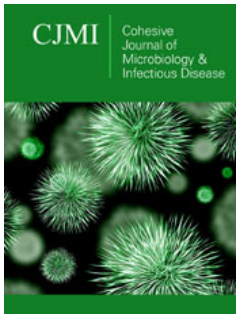
ISSN: 2578-0190



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Submission: February 07, 2022
Published: February 24, 2022

Volume 5 - Issue 5

How to cite this article: Yakup Artik, Nevra Pelin Cesur. General Evaluation of Covid-19 Diagnosis Methods. Cohesive J Microbiol Infect Dis. 5(5). CJMI. 000621. 2022. DOI: 10.31031/CJMI.2022.05.000621

Abstract

After the first case has appeared in China, the disease, COVID-19, continues to spread an omnipresent threat to global health. The reliable, fast, and effective diagnosis methods are important to control the pandemic. Today, the real-time Reverse Transcriptase (RT-PCR) test is the main diagnostic practice as a gold standard method for accurate diagnosis of this disease. On the other side, serological assays are easy to be implemented for disease screening. However, the actual limitations of these methods are required skilled personnel and specialized infrastructure. Thus, in this direction, there is an urgency to be implanted an appropriate method for the diagnosis of COVID-19 disease. Nowadays, the Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) technique is preferred because of the portable, easily implanted, and effective for diagnosis. In this assay, three main diagnosis methods (RT-PCR, serological tests, and RT-LAMP) are evaluated s within the scope of the COVID-19 diagnosis.

Keywords: SARS-CoV-2; COVID-19 disease; COVID-19 diagnosis methods

Introduction

Throughout history people have been faced with many various pandemics such as cholera, plague, Japanese Encephalitis, and Acute Encephalitis Syndrome can affect a disproportionately large number of societies, populations, or regions at the same time. Especially, recently coronavirus family members have been dominantly affected all over the world [1]. Since the early 21 century, three types of coronaviruses have been taken a role which syndrome coronavirus (SARS-CoV), which emerged in Guangdong, China, infected 8098 people and 29 countries in 2003, while the Middle East respiratory syndrome coronavirus emerged in Saudi Arabia in 2012 and spread to 27 different countries. On the other hand, nowadays a new member of coronavirus has been raised called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) first reported in the Wuhan city of China in December 2019. Globally, as of 5:30pm CET, 4 February 2022, there have been 386.548.962 confirmed cases of COVID-19, including 5.705.754 deaths, reported to WHO. As of 1 February 2022, a total of 10.040.768.270 vaccine doses have been administered [2]. It has led to a global pandemic worldwide [3]. Coronavirus family members are positively enveloped single-stranded RNA viruses that are divided into four genera as beta, alpha, gamma, and delta coronaviruses. SARS-CoV-2 is a beta strain and only affects mammals. It mainly consists of a 30kb genome with 14 Open Reading Frames (ORF) encoded by a Spike protein (S), Nucleocapsid protein (N), a Small Membrane protein (SM), an additional Membrane glycoprotein (M), and membrane glycoprotein (HE). The spike protein is the actual moiety for binding of the specific host receptor called Angiotensin Converting Enzyme-2 (ACE2) as shown in Figure 1; [4]. When the spike protein binds to the receptor, it splits into two major subunits: first, an amino terminal subunit (S1) and a carboxyl terminal subunit (S2) by host furin-like proteases. The genetic sequence of SARS-CoV-2 shares similarity 79.5% of the genome with SARS-CoV and 96.2% with RaTG13, a short RNA-dependent RNA polymerase (RdRp) region originating from CoV bats. However, the actual source of the virus has been described also yet. In general, person-

to-person droplets, contaminated materials, and direct contact of the virus are the main causes of virus transmission [5]. Fever, back pain, loss of taste/smell, cough and diarrhea are the main symptoms of the disease. Multiple organ system and respiratory failure due to cytokine storm is also described in severe cases of COVID-19. Asymptomatic cases have also been detected, with positive Polymerase Chain Reaction (PCR) results depending on their viral load representing the range of 8% to 80%. The incubation period is important for the clinical detection of the disease and has been updated as 6.4 days with recent studies [6].

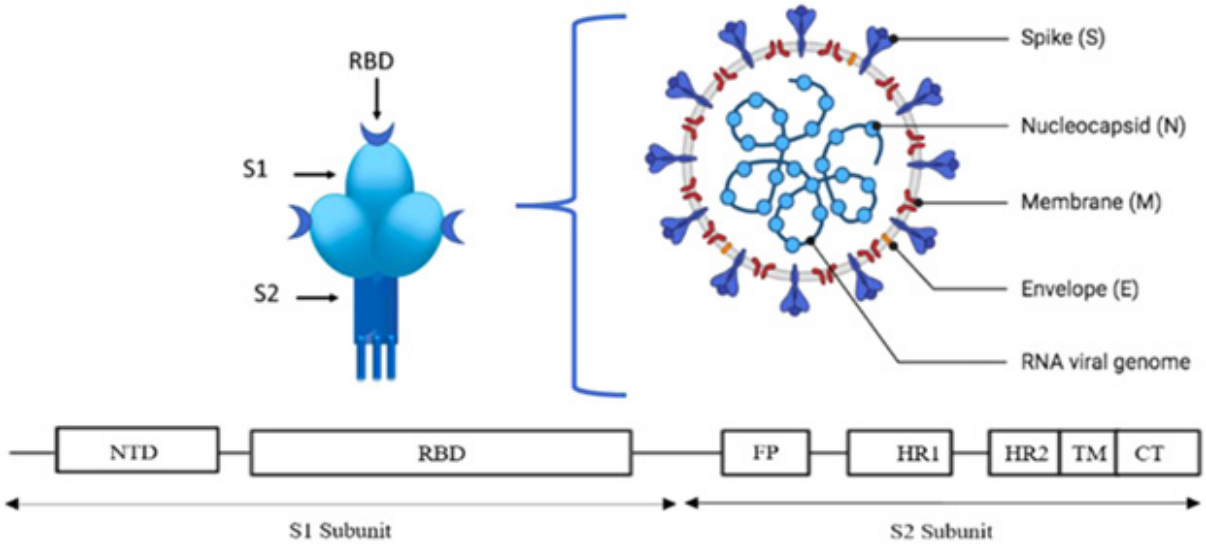

Figure 1: Structural form of SARS-CoV-2 [7].

The most important aspect of controlling pandemics around the world is understanding the SARS-CoV-2 genome mutations. Recently, numerous variants of SARS-CoV-2 have been identified. Therefore, rapid and reliable detection of SARS-CoV-2 is crucial to control the rate of virus spreading. Creating adequate awareness and preparation against the virus is the main effective factor in order to prevent the transmission of the virus. Especially many patients show asymptomatic infection and are the most common carriers of the disease. These patients contribute to the spread of infection. The first step to control the spread of virus infection is the diagnosis of the disease. From this point of view, there are four different strategies: q-RT-PCR, serological tests, RT-LAMP and Point of Care (POC) respectively. In this review, we evaluate the diagnosis of COVID-19 disease to understand which method is most effective to control the pandemic [7].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The basis of molecular diagnosis is identification and amplification of viral genetic material from a sample of suspect individuals. This identification is done by RT-PCR. A set of protocols has been published by World Health Organization (WHO) that includes designing primers that bind to specific areas of the viral genome and subsequently amplify them. Once the viral RNA and nucleocapsid gene of SARS-CoV-2 were identified, they were used as a template for the design of oligonucleotide primers and binary hydrolysis probes for the in vitro qualitative detection of RNA using the RT-PCR technique. Its mechanism of action includes isolation and purification of RNA extracted from the upper and lower respiratory regions. These are then reverse transcribed into cDNA and then subjected to devices with specific software for their subsequent amplification. This process provides suitable conditions for the binding of probes between the forward and reverse primers. However, during the extension phase of the PCR cycle, these probes are degraded by the 5′ nuclease activity of a reporter dye and a quencher dye that cleaves Taq polymerase. This separation produces a fluorescent signal, and with each cycle, more and more reporter dye cleavages occur, thereby increasing the fluorescence intensity. The intensity of each cycle is measured via a PCR detection software system. Along with the test samples, the human RNase P (RP) gene is also targeted by a set of primers and probes as a control during the PCR run. The result interpretation of RT-PCR should always be done by professional experts [8]. Different countries have adopted different viral targets for the molecular-based detection of this pathogen. For example, China relied mainly on the identification of the ORF1ab and N gene; The USA has identified three targets in the N gene of the virus; Germany focused on the RdRP, E and N gene for detection; Paris Pasteur Institute targets 2 regions within the RdRP gene; Thailand mainly used the N gene; Hong Kong has developed a diagnostic test based on the identification of the ORF1b-nsp14 and N gene; spike protein (S protein) targeted by the National Institute of Infectious Diseases of Japan [9].

Serological Tests

Blood-based tests are serological tests used to determine whether a person has an infection. Antibodies are used to detect disease. Antibodies (IgM and IgG) are specific for an antigen in the blood. In principle, the immune system recognizes these antigens of an infected person as a foreign element, and specific antibodies can be created in the body to fight the infection. Therefore, these antibodies can act as labels for the disease, usually produced after the second week of virus infection. Although IgM antibodies can be detected after 10-20 days, IgG is detected after 20 days of SARS-CoV-2 infection [10]. An antibody is a specific protein produced within the host cell in response to an infection. Therefore, an antibody test identifies the presence of antibodies in the sample that may develop as part of the immune response if a person has been previously exposed to the viral pathogen. There are four different serological assays commonly used in laboratories for the detection of SARS-CoV-2 infection; neutralization test, Enzyme-Linked Immunosorbent (ELISA) qualitative detection, chemiluminescent immunoassay qualitative detection and rapid diagnostic determinations [11]. The neutralization technique identifies by the presence of antibodies in the patient's serum based on their ability to block the replication of SARS-CoV-2 in Vero E6 cell lines. However, detection of antibodies specific to virus proteins that are not primarily involved in replication can be missed with this approach [11].

On the other hand, SARS-CoV-2 identifies the presence of antibodies through the formation of the colored product obtained after binding of secondary labeled antibodies to the primary antigen-antibody complex by ELISA. In this viral detection process, the plate is usually coated with viral specific protein such as S and then loaded with a blood serum sample. If antibodies are present, they bind to the viral antigen forming an antigen-antibody complex. Secondary antibodies labeled with fluorochrome, or substrates are then added to this plate, which recognizes the preformed antigen-antibody complex and produces a detectable color change or fluoresces through a chemical reaction [12]. In the chemiluminescent method, it identifies the presence of antibodies through the formation of luminescence activity obtained by a chemical reaction of secondary labeled antibodies binding to the primary antigen-antibody complex. On the other hand, information about whether the growth of the virus can be inhibited by these antibodies is not fully provided. Also known as Chemiluminescent Immunoassay (CLIA), the method is a modified version of ELISA where luminescence is measured for the detection of pathogens. It is a quantitative test that can measure the number of IgG, IgM, and IgA antibodies. This test allows patient samples to be mixed with viral specific proteins. The formation of the antigen-antibody complex is detected by the binding of another secondary antibody, which then undergoes a chemical reaction to produce light. The amount of light emitted is then calculated to quantify the number of antibodies present in the sample [13].

In rapid antigen tests, the diagnosis is made to identify the presence or absence of antibodies to the virus in the patient's blood serum samples. It works on the principle of lateral flow testing, in which the antigen-antibody complex moves by capillary action across a membrane and is immobilized by capture antibodies producing a color change. Rapid Diagnostic Tests (RDT) are considered a small, portable, and inexpensive test method. They work on the principle of lateral flow testing, where samples in the form of nasal swab, saliva, or blood show color lines to identify positive or negative results. In a lateral flow assay, a membrane containing gold nanoparticle labeled antibodies (Au-Ab) and capture antibodies is in two different lines. When the patient's sample is loaded onto the membrane, it moves along the membrane by capillary action. It encounters Au-Ab first and the viral antigens bind to form a complex. This complex then advances and is captured by the capture antibodies in the second line, and its immobilization on this surface results in the production of colored lines that confirm the tests [14].

RT-LAMP

The RT-LAMP technique is a recently preferred technique, which is a fast and sensitive method used in the detection of SARS-CoV-2. Nucleic acid determinations take place over 1 hour and this easily interpretable colorimetric assay requires only a heat source. It is a fast and cost-effective simple colorimetric technique that makes it an effective solution to increase global testing capacity. It is also a single tube technology for detecting target nucleic acid sequences. Based on the RT-LAMP technique, it uses six primers, including four primers selected by combining fragments of target DNA and two additional loop primers used to amplify a particular gene region. Recently, RT-LAMP for Point of Care (POC) has been applied for many RNA virus infections. From this point of view, RT-LAMP has an important place in the diagnosis of virus infections such as SARS-CoV-2 [15].

The colorimetric sensing loop-mediated isothermal amplification (RT-LAMP) method was first proposed by Zhang et al. This technique provided results on par with existing molecular RT-qPCR assays and facilitated viral detection without being dependent on complex infrastructures. Jinzhao Song, a professor at the University of Pennsylvania, has proposed another modification of the RT-LAMP technique with the use of Recombinase Polymerase Amplification (RPA) for a two-step amplification in a single tube in 2020. Using this strategy, he also proposed the manufacture of a Point of Care (POC) device in which diagnosis can be made at home and clinical setups are not required. Such devices combine a paper-based technology with the RT-LAMP assay technique. One of the notable features of this device is its potential to integrate quarantined and self-isolated individuals on their own with a smartphone where highly sensitive, reliable and fast results can be obtained. This can be done by collecting nasal swabs, adding RT-LAMP-specific reagents to a sheet, and visualizing a color change. Therefore, it reduces visits to hospitals for checkups, and the chance of infection spreading is significantly reduced. Nucleic acid determinations take over 1 hour and typically do not require the required temperature values, unlike the traditional PCR technique of RT-LAMP, a powerful amplification method performed in an

isothermal environment [16]. By incubating the nucleic acid sample, 4 (or 6) specially designed primers, and Bst DNA polymerase in the same test tube at approximately 60 to 65 °C, simultaneous amplification and detection of nucleic acid can be completed in a single step. The primer set used for a typical RT-LAMP assay consists of two inner and two outer primers that recognize six different regions of the target DNA sequence. The Forward Inner Lining (FIP) consists of the F2 region and a complementary sequence of the F1 (F1c) region, while the Backward Inner Lining (BIP) consists of the B2 region and a complementary sequence of the B1 (B1c) region. The forward outer primer (F3) and reverse outer primer (B3) have sequences that complement the sequences of the F3c and B3c regions, respectively. These regions surround the desired amplified sequence. Primers used for any RT-LAMP assay, nucleotide base pair concentration and positions, distance between DNA regions, thermodynamics of primers, etc. should be optimized by a number of factors [17].

The RT-LAMP amplification process begins when FIP complexes with target DNA at the F2 region to form double-stranded DNA, which is in equilibrium at about 65 °C. DNA polymerase with strand-changing activity then initiates DNA synthesis from the FIP and simultaneously replaces a single strand of DNA. After this initiation step, the F3 primer then binds to the complementary F3c region and replaces the FIP-complementary helix. Due to the F1c sequence in FIP, the FIP sequence can self-anneal and form a loop structure at one end of the DNA. This strand then serves as the target for BIP-initiated DNA synthesis followed by strand displacement from B3-induced DNA synthesis. This allows the other end of the single DNA strand to form a loop structure, thus resulting in a dumbbell-like DNA structure. It acts as a template for subsequent amplification. After a dumbbell-like structure is formed, exponential amplification of the dumbbell structure is initiated and DNA polymerase initiates DNA synthesis at the F1 site. FIP also hybridizes from the F2 region to the single-loop structure, and DNA synthesis of this primer causes displacement of the F1-prepared strand and self-attachment to a loop structure. Finally, self-prepared DNA synthesis is restarted from the B1 site in the new loop, reinforcing the existing template, as well as creating a new one from the displacement of the FIP-complementary strand [18]. Because DNA can be amplified 109 times in one hour, it is possible to achieve great amplification from this iterative process.

To further increase amplification efficiency, RT-LAMP with six primers (loop primer) has also been developed. Loop primer RT-LAMP uses six instead of four primers, the forward and reverse loop primers (LF and LB) anneal to the regions between the F1/F2 and B1/B2 regions, respectively. Loop primers have been proven to enhance the RT-LAMP process, as amplification is fixed from increased starting points for DNA synthesis. RT-LAMP detects RNA rather than DNA sequences. Reverse transcriptase is added to the RT-LAMP mix to help convert viral RNA into complementary DNA (cDNA) to be used for amplification. This procedure has been of great help in the diagnosis of a large number of RNA viruses. To detect multiple pathogens in the same test tube, multiple RT-LAMP assays with more primers or unique fluorescent signals have been developed. All these iterations of the RT-LAMP procedure show how promising it is in the field of viral diagnostics. As a result, it has great potential in detecting SARS-CoV-2 [19].

Conclusion

Disaster is defined as a holistic situation of natural or man-made events that develops suddenly, requires a systematic approach to control, interrupts or stops social life, cause loss of life and property, and often cannot be overcome with local capacity. Biological disasters can be caused by humans as well as naturally occurring as infectious diseases and epidemiologically [20]. In particular, it is important to understand how the COVID-19 disease spreads. Patients are divided into as symptomatic and asymptomatic. Although patients with symptoms can be detected by various diagnostic methods, patients who do not show symptoms called asymptomatic are the main reason for the spread of the disease. For this reason, developed countries have turned face to faster tests. In this way, it is thought that they will perform a continuous scan. In the early stages of infection, the main problem with RT-PCR is false negative results. Insufficient and incorrect extraction of nucleic acids for RT-PCR results in false negative results. Therefore, chest computed tomography scanning is recommended as a complementary tool. The problem of RT-PCR with erroneous results is emerging more and more. In addition, RT-PCR requires trained medical personnel, specialized instrumentation, technical workmanship, and special chemicals or reagents. The accuracy of serological tests is also not sufficient to detect SARS-CoV-2 infection. Generally, these tests can be combined with RT-PCR based on the presence of viral RNA. The RT-LAMP technique is an alternative to traditional quantitative RT-PCR methods that do not require expensive tools to perform the reaction or interpret the results. RT-LAMP can provide higher sensitivity than RT-PCR in clinical samples. RT-LAMP is a nucleic acid amplification test like RT-PCR, which is a simple, low-cost and rapid method. This strategy can be rapidly implemented, and viability verified with clinical specimens before being made available for mass diagnostic testing at times. This approach can be used to monitor exposed individuals or potentially assist dredging efforts in the field and potential ports of entry. During the event, the development time of kits and devices gets even shorter. After all, it is an analysis system that can read both quantitatively and qualitatively.

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