Nucleic Acid Amplification Techniques in SARS-CoV-2 Detection

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The urgent need for accurate COVID-19 diagnostics has led to the development of various SARS-CoV-2 detection technologies. Real-time reverse transcriptase polymerase chain reaction (RT-qPCR) remains a reliable viral gene detection technique, while other molecular methods, including nucleic acid amplification techniques (NAATs) and isothermal amplification techniques, provide diverse and effective approaches. Serological assays, detecting antibodies in response to viral infection, are crucial for disease surveillance. Saliva-based immunoassays show promise for surveillance purposes. The efficiency of SARS-CoV-2 antibody detection varies, with IgM indicating recent exposure and IgG offering prolonged detectability. Various rapid tests, including lateral-flow immunoassays, present opportunities for quick diagnosis, but their clinical significance requires validation through further studies.

Keywords: SARS-CoV-2 ; COVID-19 ; molecular detection methods

1. Reverse Transcriptase Loop-Mediated Isothermal Amplification (RT-LAMP)

Loop-mediated isothermal amplification (LAMP), a widely adopted amplification method developed by Notomi et al. in 2000 [1], has gained popularity in recent times. When applied to SARS-CoV-2 amplification, LAMP necessitates modifications, incorporating Bst DNA polymerase, nucleotides, reverse transcriptase, and a specific set of primers, two outer primers, two inner primers, and two loop primers. This modified method, known as RT-LAMP, operates at a temperature range of 60–65 °C and amplifies the target sequence in just 1 h with high specificity, similar to standard RT-PCR. Remarkably, LAMP can generate 10^9 DNA copies within this short timeframe [1].

Considering that the SARS-CoV-2 virus's nucleic acid is RNA, reverse transcription is used to generate cDNA for RT-LAMP. Following that, the LAMP primers bind to their complementary target sequence in the cDNA, resulting in the formation of dumbbell-shaped DNAs. The Bst DNA polymerase, with its intrinsic strand displacement activity, amplifies the dumbbell-shaped DNA, yielding products of various sizes by the end of the reaction. The results obtained from the RT-LAMP reaction can be interpreted using turbidity, colorimetric assays, or fluorescence measurements [2][3][4].

Amaral et al. found that RT-LAMP demonstrates 100% sensitivity and 96.1% specificity in detecting fewer than 100 viral genome copies of SARS-CoV-2 in just 30 min. Additionally, they introduced a brand new colorimetric detection technique based on the interaction of divalent zinc (Zn^{2+}) with the complex metric indicator murexide (MX). In the presence of Zn^{2+}, murexide is yellow, and when Zn^{2+} is absent, it is pink [5].

Notably, because isothermal amplification occurs at approximately 62–65 °C, RT-LAMP eliminates the need for expensive temperature change instruments. This feature distinguishes LAMP as a method that has the potential to be adapted for use in portable devices. An incubation period of 30–60 min is required for the detection of SARS-CoV-2 using the RT-LAMP method [6]. Reverse transcription and LAMP can be combined in a single reaction due to the versatility of RT-LAMP.

RT-LAMP has several advantages for the amplification of nucleic acids, such as reduced time for amplification, resistance to inhibitory substances in clinical specimens, and compatibility with a broad variety of commercially accessible reagents. This technique is employed for RNA template amplification, exhibiting strong strand displacement activity and temperature endurance, in addition to being quicker and requiring fewer resources [7]. Later developments, including extending two-loop primers, have significantly lowered the time for initial LAMP reaction [8].

The versatility of RT-LAMP extends beyond SARS-CoV-2, with applications in pathogen identification for MERS-CoV, West Nile virus, Zika virus, yellow fever virus, Ebola virus, and various other pathogens [9][10][11][12][13][14][15]. The effectiveness of RT-LAMP in identifying SARS-CoV-2 was assessed in a recent study by El-Kafrawy et al. in 2022. The

researchers improved the RT-LAMP technique by contrasting two fluorescence amplification mixtures and various reaction periods. Subsequently, the outcomes were contrasted with those acquired using conventional real-time RT-PCR. To perform RT-LAMP on samples diluted 1:4 in water treated with diethylpyrocarbonate (DEPC), several sets of singleplex and multiplex LAMP primers targeting the N, S, and ORF1ab genes were used. The mixture was incubated at 65 °C with a real-time PCR 7500. Remarkably, this methodology showed 100% agreement with the industry standard protocol for SARS-CoV-2 nucleic acid detection. The researchers suggest that the test is a good fit for POC detection in public hospitals and medical centers [16].

2. Nucleic Acid Sequence-Based Amplification (NASBA)

This approach requires fewer cycles than RT-qPCR and LAMP and can produce a large copy number in 1.5–2.0 h using a two-stage isothermal amplification experiment. This technique's stability, sensitivity, and specificity in detecting single-stranded RNA (ssRNA) make it highly promising for POC applications, especially in low-resource situations [17]. NASBA was successfully used to detect SARS-CoV during previous epidemics, with real-time monitoring made possible using fluorochromes in the reaction. A modified version called Multiplex RT-NASBA could simultaneously identify several viral infections. Xing et al. [18] analyzed 614 clinical samples from patients with respiratory tract infections using the RTisochipTM-W system. Of them, 201 were preclinical, 25 were clinically diagnosed, and 14 were COVID-19 positive. In a single run, this system can analyze sixteen samples in ninety minutes.

3. Recombinase Polymerase Amplification (RPA)

Results are obtained in 10 to 15 min using an isothermal approach that amplifies DNA sequences at 37 to 42 °C. In this diagnostic method, the recombinase enzyme first interacts with primers to generate a recombinase–primer complex. The primer looks for its complementary sequence and resides in the target site while the recombinase breaks down the target strand. A single-stranded DNA-binding protein stabilizes the other strand while DNA polymerase lengthens the broken strand. Future rounds of extension can still use the recombinase [19].

RT-RPA is a more advanced version of RPA designed for coronavirus detection that includes a reverse transcription step. Lau et al. created an RT-RPA procedure in which the endpoint can be determined in 15–20 min using SYBR green or a lateral flow strip technique. According to their results, RT-RPA has a 100% specificity and 98% sensitivity for detecting 7.659 C/μL [20]. Similar RT-RPA protocols were built by other groups as well, such as Wang et al., 2021 [21].

A method for quick diagnosis and POC testing called microfluidic integrated lateral flow recombinase polymerase (MI-IF-RPA) was created by Liu et al. Extracted RNA was loaded into the chip's inlet holes with the RT-RPA reaction buffer and primers specific to the SARS-CoV-2 N gene. Afterward, the chip was incubated at 42 °C for 15 min. The results were analyzed using the lateral flow assay, where two bands indicate a positive sample and one band indicates a negative sample. The chip displayed 100% specificity and 97% sensitivity, and it exhibited no cross-reactivity with other viruses such as coronavirus OC43, respiratory syncytial, or influenza. This suggests that an RT-RPA assay based on chips could be created [22].

4. CRISPR-Based Detection Technique

The method known as clustered regularly interspaced short palindromic repeats (CRISPR) may identify any type of nucleic acid present in a sample, including RNA and DNA. It has several advantages compared to conventional diagnostic methods. This system offers a fast turnaround time, excellent sensitivity and specificity, and an affordable and dependable diagnostic platform. Additionally, by removing the requirement for thermocycler applications, CRISPR facilitates POC testing [23][24].

Viral load is often used in traditional approaches to assess findings; low viral load individuals are regarded as negative. In contrast, the CRISPR technique relies on identifying the viral genome, which minimizes the possibility of false negative results and permits early infection identification. Furthermore, this technology can distinguish between mutant strains within the population because it is intended to produce guide RNA (gRNA) unique to a conserved area of the virus genome.

Broughton et al. created the SARS-CoV-2 DNA endonuclease-targeted CRISPR trans reporter (DETECTR), a CRISPR-based detection method. The procedure begins with RNA extraction from the collected sample, followed by reverse transcription to produce cDNA. Isothermal amplification techniques are then used to amplify the cDNA, with primers

designed to assess the amplification of the SARS-CoV-2, N, and E genes. A lateral flow assay, facilitated by a FAM-biotinylated reporter molecule, can yield results in 30–40 min [25].

Hou et al. compared metagenomic sequencing and RT-PCR to a CRISPR-based diagnostic method. They created CRISPR-COVID, a fast detection test that uses RT-RPA to amplify the SARS-CoV-2 ORF1a gene. Notably, this method takes only 40 min to complete, which is a significant improvement over the 20–24 hours required for RT-PCR analysis and metagenomic sequencing. The fluorescence readout determines the assay results, which reveal a SARS-CoV-2 specificity of 100% [26].

There are various methods to detect SARS-CoV-2, including gold nanoparticle-based biosensors. These biosensors have been widely used in different scientific fields including medical care, biology, chemistry, and physics. They serve as signal amplifiers and resonance light scattering for viral detection. Moitra and colleagues [27] have developed a technique that can detect COVID-19 in just 10 min. The approach is relatively simple, and it involves the use of plasmonic gold nanoparticles that change color in the presence of the virus, hence making the test positive. This method detects viral RNA on the first day of infection, and the RNA can be extracted from the sample within 10 min. The test uses small gold particles to detect specific proteins. When coupled with the virus gene sequence, the gold nanoparticles change the color of the liquid reagent from purple to blue. This test's accuracy is determined by its ability to detect the virus, which many commercially available test methods cannot achieve until several days after infection. The authors claim that this test's development and use are significantly cheaper than laboratory tests since it does not require laboratory equipment or qualified personnel to administer and examine [27].

- **B. Serological assays**

In disease surveillance, detecting antibodies against a virus in infected patients is an important diagnostic approach. Even though RT-qPCR is the most widely used technology for detecting active SARS-CoV-2 infections, viral RNA becomes practically undetectable 14 days after sickness, and false negative results are possible due to inappropriate viral sample handling. These challenges underline the importance of developing simple test kits based on the detection of human antibodies generated in response to viral infection. The main principle behind antibody-based immunodiagnosis is to identify antibodies (IgG and IgM) produced in response to viral infection and/or viral antigen using an enzyme-linked immunosorbent assay (ELISA) [28].

According to research, antigen-specific antibodies can be discovered in a patient after 3 to 6 days, with IgG being detectable later in an infection [29]. These tests are excellent for use in resource-constrained labs since they can offer information on both ongoing and historical infections. They can also be used in disease surveillance programs to improve the understanding of the infection rate in the community. While serological assays can detect both ongoing and past infections, they are most efficient in verifying SARS-CoV-2-specific antibody responses to detect past infections [29].

- **Detection of SARS-CoV-2 antibodies**

The production of antibodies against SARS-CoV-2 is the first line of defense against infection. By day 7, neutralizing antibodies can be found in as many as 50% of infected people, and by day 14, all infected people have them. For SARS-CoV-2 diagnostics, serological investigations provide an alternative to RT-qPCR, and the combination of serological testing and real-time PCR greatly increases the rates of positive virus identification. IgG antibodies suggest previous SARS-CoV-2 exposure, whereas IgM antibodies indicate recent viral infection. As a result, immunodiagnostic assays are essential for assisting in the creation of COVID-19 vaccines and for determining the level of infection in people who are not actively infected [30].

In most people, IgM levels rise during the first week after SARS-CoV-2 infection, peak after two weeks, and subsequently decline to near-normal levels, owing to the significant demand for quick tests in identifying COVID-19 infections [31]. After a week, IgG is visible and stays raised for a long time—up to 48 days at times—possibly offering protection against reinfection. After infection, IgA responses usually start to manifest 4–10 days later. In addition to IgG and IgM, serum IgA is used as a diagnostic predictor [32][33]. Differential target antigens have an impact on the range of SARS-CoV-2 antibodies. Antibody titres may drop seven days following infection [31].

According to current research, antibodies specific to SARS-CoV-2 have been discovered in saliva [30]. To evaluate the variations in antibody levels between sera and saliva, multiplex SARS-CoV-2 antibody immunoassays were used. Parallel compartmental humoral immune responses are suggested by antibodies in saliva that match those in sera [30]. Salivary IgA is correlated with the severity of COVID-19 disease, and a fast immunoassay was developed in parallel using the BreviTest platform technology to measure salivary IgA [20].

It is interesting to note that low IgA levels can suggest herd immunity even in cases when no viral exposure has been documented [34]. Antibodies specific to SARS-CoV-2, especially those found in saliva, may be detected for surveillance purposes. Still up for debate, though, are the best antigens to use in serological testing. Although the viral S protein is a good option, it is still unclear which precise area of the S protein should be targeted. As an alternative, different S protein isoforms, such as those seen in different strains, could be used to guarantee the repeatability of the assay [35].

Testing time might range from 13 min (Abbott) to 45 min (Cepheid Xpert Xpress) to receive results [36]. Two colloidal gold immunoassays (VivaDiag COVID-19 IgG-IgM based and Assay Genie rapid POC kit), two lateral-flow immunoassays (SureScreen rapid test cassette and BioMedomics rapid test), and one time-resolved fluorescence immunoassay (Goldsite diagnostics kit) are available.

A clinical study will be required to assess clinical significance. SARS-CoV-2 IgG (Abbott) sensitivity in N-based immunoassays can reach 100%. In the case of S-based immunoassays, the Liaison SARS-CoV-2 S1/S2 IgG and the combined S- and N-based platform COVID-19 VIRCLIA IgG MONOTEST showed comparable sensitivity. The neutralization test for plaque reduction had a sensitivity of 93.3%. To assess specificity, all assays, except for the enzyme-linked immunosorbent assay (IgG) (EUROIMMUN), produced at least one positive result for the negative SARS-CoV-2 antigen control. This most likely suggests that assay sensitivity has changed significantly over time and between testing platforms [37].

Despite being the gold standard for immunoglobulin-based detection, the plaque reduction neutralization test has some limitations, including a restricted number of sample analyses and the requirement of a biosafety level 3 laboratory. There is a strong association between the plaque reduction neutralization test and the titres produced by these assays. Antibody tests are currently used mostly for epidemiological testing [37][38].

- **C. SARS-CoV-2 Antigens**

The N and S proteins are the primary immunogens in SARS-CoV [39]. One recent fast diagnostic test that used novel antigens demonstrated increased sensitivity and specificity in patients who were symptomatic and had a high viral load [40]. In contrast, a quick method that used a fluorescent immune chromatographic assay to find the N protein demonstrated maximum sensitivity only in the early stages of infection [41]. According to a mass spectrometry study, gargle solution samples from COVID-19 patients included the N protein [42].

For almost 80 years, mass spectrometry has been recognized as one of the most sensitive procedures in the literature. Although a genetic probe can detect the presence of virus RNA in a biological sample, mass spectrometry can confirm the presence of the generated protein, and immunofluorescence can then emphasize the iconographic aspects of the contacts. Spectral counting is a semi-quantitative evaluation in terms of absent or low protein and/or peptide presence [43]. In vitro, bacterial cultures can be evaluated for increased or decreased protein concentrations during infection, as well as the expansion of viral particle peptide numbers. Compared to statistical analysis and immunofluorescence microscopy in samples, this method allows data to be cross-referenced, with each serving as a control for the other. Mass spectrometry can detect the presence of D-amino acids in peptides. As a result, the immunofluorescence microscopy technique can be integrated with the spectral counting of viral peptides in bacterial cultures using mass spectrometry, as well as identifying D-amino acids within viral peptides in bacterial cultures and patient blood. Previous studies [44][45] have described the genetic aspect of viral replication in bacterial cultures, interactions by electron microscopy and immunofluorescence by light microscopy, evidence of the nitrogen isotope N15 introduced into 30-day bacterial cultures, and presence in viral proteins after another seven days, all of which serve as the foundation for detailed analysis of genetic–molecular aspects [46].

According to Petrillo et al.'s study, the use of immunofluorescence and spectral counting in mass spectrometry seems to aid in identifying viral proteins in tested samples. This method also enables the detection of various viral proteins produced during bacterial growth in an in vitro investigation. It is critical to highlight the bacteriophage behavior of SARS-CoV-2, as well as other viruses. Increasing signal intensity by fluorescence microscopy with fluorescent antibodies in fractions of bacterial cultures containing the virus under a study conducted for up to 30 days appears to be increasingly crucial for ignoring potential bacteriophage behavior.

Furthermore, 73.6% of COVID-19 patients with confirmed cases had their urine samples test positive for the N protein using a fluorescence immunological chromatographic assay [41]. The S protein is thought to be more suited for detection during the recovery phase since it manifests later in the infection [47]. A microplate reader makes it easy to perform an ultrasensitive antigen test tailored for the S protein [48].

In addition to established methods for detecting the SARS-CoV-2 coronavirus nucleocapsid antigen, a half-strip lateral flow (HSLF) assay has been developed. This assay has higher clinical sensitivity than standard serology assays, with an LOD of 3.03 ng/mL for the commercially available Genscript N protein [49]. Another novel strategy uses a nanozyme-based chemiluminescent paper assay that can be used with common cell phones and has a LOD of 0.1 ng/mL for the SARS-CoV-2 recombinant spike antigen [50]. An antibody's specificity in identifying the target is matched when using a particular nucleotide aptamer against the N protein for antigen detection. However, it offers the potential for heightened sensitivity and greater flexibility in developing assays for various purposes [51].

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