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COVID-19 detection methods: from the clinic to the laboratory

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Abstract:

Despite the post-epidemic period of COVID-19, systematic summary studies of SARS-CoV-2 are still important to cope with future outbreaks of new coronaviruses. In this paper, we reviewed many different methods for detecting SARS-CoV-2 virus, including nucleic acid, antibody, and antigen detection. The RT-PCR is one kind of the gold standard for SARS-CoV-2 diagnosis and has high specificity and sensitivity. The T-PCR technique also has some limitations due to its own basic principles, such as the possibility of false negatives or false positives. The article also discusses the importance of combining antigen and antibody detection with RT-PCR to complement RT-PCR in diagnosis. In addition, the article describes emerging detection technologies and the latest progress in SARS-CoV-2 detection. Overall, we provide a piece of comprehensive and valuable information for understanding SARS-CoV-2 detection methods, which is important for guiding public health practice and responding to possible new virus outbreaks.

Keywords:SARS-CoV-2; Methodology; Detection; RT-PCR

Introduction

In December 2019, SARS-CoV-2 made its first outbreak in Wuhan, China[1-3]. Given its wide-spreading intensity in the population, the WHO has designated it as a public health emergency of world concern. Since the first cases were reported in December 2019, this virus has caused 760 million infections and 6.9 million deaths globally at least as of 2023, when WHO declared the end of the emergency phase of COVID-19[4]. SARS-CoV-2 virus can cause the upper and lower respiratory tracts infections, commonly presenting with symptoms including cough and fever, which can progress to pneumonia. SARS-CoV-2 can also affect the nervous system, skin, and gastrointestinal tract, occasionally manifesting in atypical symptoms like visual or taste impairment[3, 5, 6]. Most patients exhibit mild infections, and approximately 20-40% remain asymptomatic[6, 7]. However, some patients develop severe complications, including thromboembolic events and acute respiratory distress syndrome[3]. Furthermore, SARS-CoV-2 has had a complex and heavy impact on people's mental health. and the global economy, exacerbating social disparities and a crisis of confidence. Nevertheless, the pandemic has led to a substantial increase in public health awareness,

strengthening of public health systems, and enhancement of emergency response capabilities. Governments have also accumulated valuable experience in managing outbreaks, providing crucial insights for future public health incidents[6]. Nevertheless, the pandemic has led to a substantial increase in public health awareness, strengthening of public health systems, and enhancement of emergency response capabilities. Governments have also accumulated valuable experience in managing outbreaks, providing crucial insights for future public health incidents[6].

Presently, according to the evolutionary results of whole genome sequences, coronaviruses are classified into α, β, γ, and δ genera, and β-coronaviruses could be subdivided into four subgroups(terms as A.B, C, D). The SARS-2 virus belongs to the genus β and is the 7_{th} coronavirus, which could infect homo sapiens.[8] The complete genome of the SARS-CoV-2 virus is approximately 30kb, containing a ssRNA with an envelope, typically circular or oval, 60-140 nm in diameter, and encoding approximately 27 proteins[9].

The SARS-CoV-2 genome includes two-thirds of the 5' region encoding the orf1ab polyprotein, and one-third of the 3′ region encoding structural proteins.[8-10] The structural proteins comprise envelope (E) proteins, membrane (M) proteins (also be terms as matrix proteins), nucleocapsid (N) proteins, hemagglutinin esterase (HE) proteins, and spike (S) glycoproteins. While the N proteins form a compound with the viral genome RNA to create the E and M proteins. The two functional subunits of the S proteins, which are the main surface glycoproteins, S1 and S2, are expressed and are essential for receptor binding and membrane fusion. For most host cell infections, SARS-CoV-2 utilizes the RBD on their surface S proteins to recognize and bind to the host receptor ACE2, thereby facilitating infection.[10-12]

SARS-CoV-2, being single-stranded RNA viruses, are prone to high rates of mutation during replication due to their lack of a proofreading mechanism[10]. Recombination events can take place when human hosts are concurrently infected with diverse subtypes or daughter branches of the virus, thereby producing recombinant virus strains. Particular mutations or recombinations can influence virus. For example, amino acid alterations in S protein can heighten binding affinity to ACE2 receptors and boost its intracellular replication and spread. Moreover, certain mutations in the S protein may increase immune evasion, potentially reducing the efficacy of vaccines and diminishing cross-protection between different viral sub-lineages, which could precipitate reinfections[9, 10].

Acquired immunity, a specific mechanism for viral clearance (Figure 1). When SARS-CoV-2 infection, cellular immunity is activated when naive T cells, stimulated by antigens, express diverse cytokine receptors, differentiate, proliferate, and differentiate into functional T cells[13]. These functional T cells then re-encounter target cells displaying specific antigens and exert a cytotoxic effect. Humoral immunity, on the other hand, is mediated by specific B cells and constitutes a humoral immune response against thymus-independent antigens, primarily through the production of the virus-specific antibodies that confer immunological protection[13]. After SARS-CoV-2 infection, B cells are activated and produce IgM antibodies. After undergoing latency, logarithmic, plateau, and decline phases, when the same antigen re-invades the organism, a rapid and efficient specific memory response is triggered, characterized by the production of high-affinity IgM, a swift increase in serum antibody concentration, and a high antibody titer[14]. These antibodies, secreted in the mucous membranes and blood, neutralize pathogens by binding and inactivating antigens[13, 14]. Current evidence indicates that The adaptive immune response will gradually take over 1-2 weeks post-infection. The humoral immune response is primarily mediated by virus-specific antibodies targeting viral proteins(also terms as antigen), such as the S and N proteins, while the cellular immune response is directed against a broader spectrum of structural and nonstructural viral components.[12, 15, 16]

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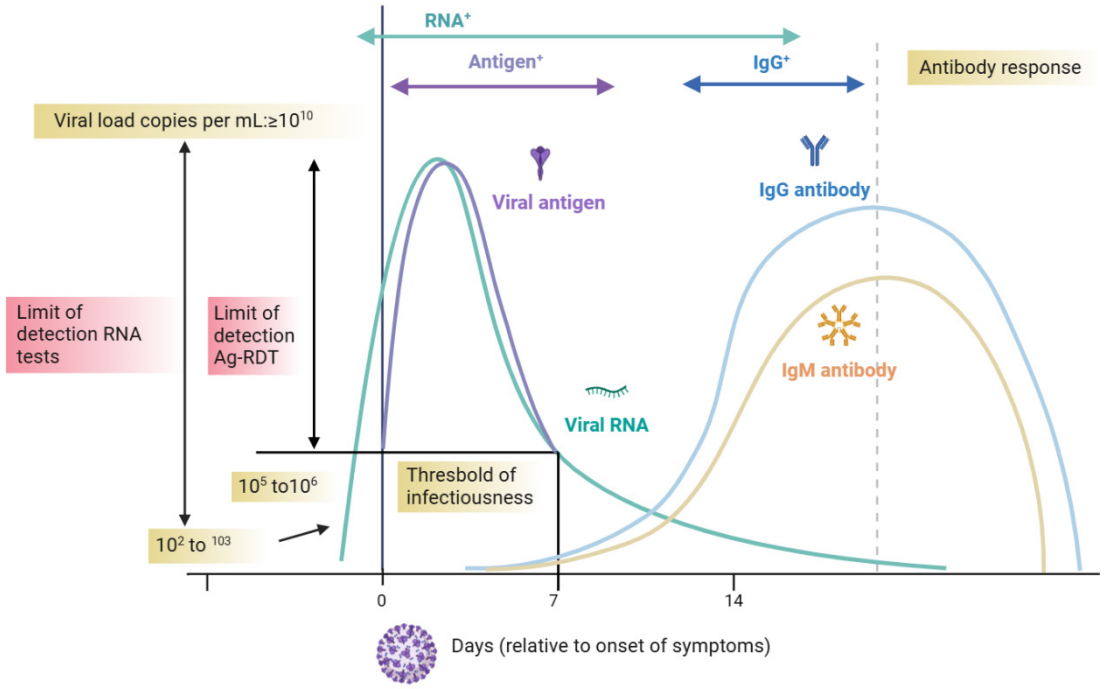

Figure 1. Timeline of different diagnostic modalities to detect COVID-19-corresponding host responses

The optimal time frame for the use of antigenic or molecular to confirm SARS-CoV-2-infected individuals was determined based on the lower limit of viral detection by molecular and antigenic tests. Nucleic acid testing modalities enable the earliest detection of viral infection (X-color curve in the figure). The antigenic assay provides a quick and easy way to detect new coronavirus infections (shown as an orange curve in the figure). Ag-RDT = antigenic apoptosis detection test. Created in BioRender.

Although WHO declared the emergency phase of COVID-19 to be over in May 2023, a systematic review is needed for new coronaviruses. This review focuses on nucleic acid assays (RT-PCR), antibody assays, and antigen assays, along with an extensive overview of emerging detection technologies and recent advances. The aim is to provide additional information for responding to possible new outbreaks in the future.

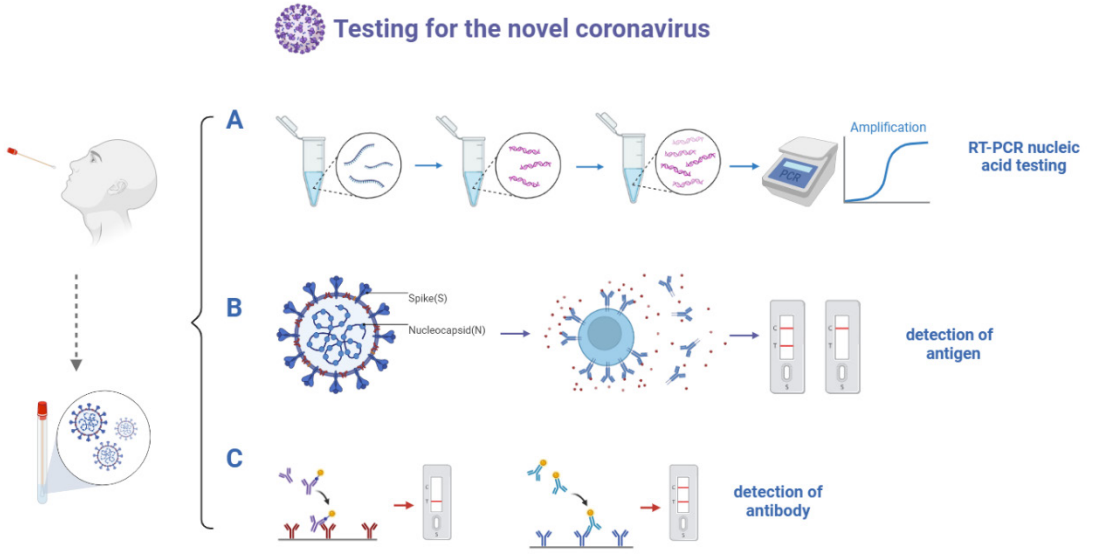

Figure 2. Methods to SARS-CoV-2 detection

To detect the SARS-CoV-2 virus, specimens from patients who may be infected are used. Quantitative real-time PCR, is used to detect viral RNA. Primers specific to any number of target areas in a virus's genome are used to extract, reverse-transcribe, and amplify viral RNA from a lysis virus using qPCR. Detection of viral antigens is often used as a rapid identification of specific viral proteins from lysed viruses. Antibody assays are generally used in late-stage infections and retrospective studies of the presence or absence of a history of infection, especially to assess the body's immune response. Created in BioRender.

1.RT-PCR detection of viral genes is the gold standard for virus detection

RT-PCR was recognized as the gold standard for detecting SARS-CoV-2 infection (Figure 2.A), which is renowned for its high specificity and sensitivity. And this technology is capable of generating results in a high-throughput and short time, which is usual a few hours[17, 18]. The procedure entails two primary steps(Figure 3): 1. Reverse transcription: RNA is transcribed into DNA in this stage. Utilizing reverse transcriptase, cDNA is generated by synthesizing the RNA template into the complementary DNA; 2. PCR amplification: cDNA samples are then amplified through the PCR, employing gene-specific primers and fluorescent probes. The cDNA serves as a template for PCR amplification, facilitated by DNA polymerase and primers, resulting in exponential growth and amplification of the cDNA into a quantity sufficient for detection[17-19].

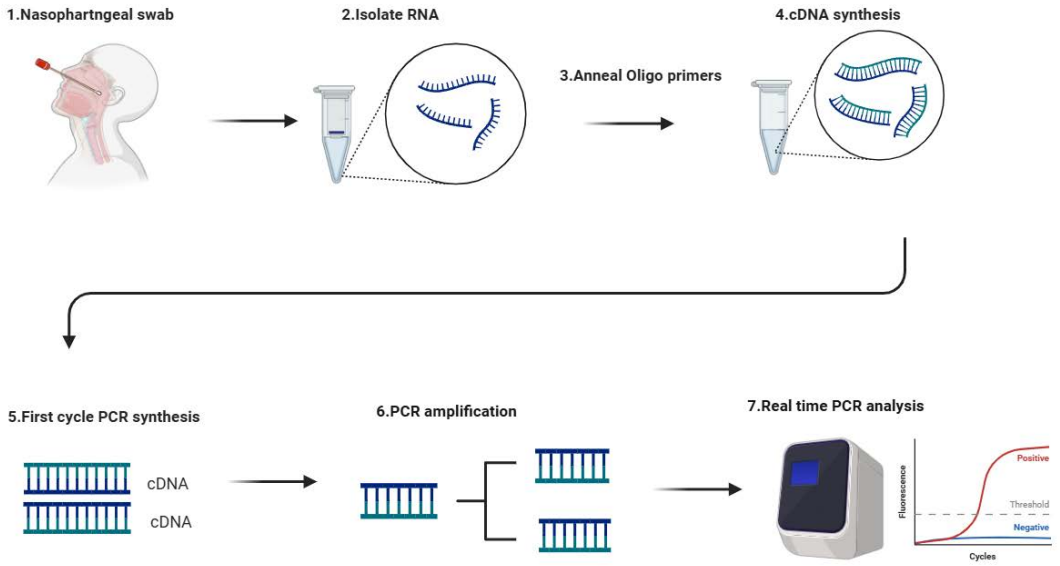

Figure 3. The mechanism of RT-PCR detection for viral genes

Among the SARS-CoV-2 assays, the nucleic acid assay is one of the most commonly used.1. Specimens from potentially SARS-CoV-2-infected individuals are obtained by nasopharyngeal swab, oral swab, or nasal swab.2. The viral RNA is extracted.3. Annealing oligo primers are added.4. Reverse transcription of the RNA into cDNA is performed using reverse transcriptase, which breaks down the RNA template.5. The second DNA strand is completed by RT (or Taq polymerase) activity of the DNA polymerase. And then the Taq polymerase to complete the synthesis of the second DNA strand.6. The newly generated DNA serves as a template.7. A quencher is used to mask the fluorescence on the probe.8. When DNA polymerase hydrolyzes the probe after extension of the forward primer, the fluorophore is released during the extension phase. Real-time thermal cycling then records the fluorescence emission after excitation. The dsDNA is doubled during each PCR amplification cycle, resulting in a proportional increase in the total fluorescence signal. Created in BioRender.

RT-PCR is the method of first choice for detecting SARS-CoV-2 virus in clinical practice[5, 17, 20-22]. It directly measures parts of the viral genome, as opposed to secondary biomarkers like antigens or antibodies. The collection of suitable specimens from the anatomical location of infection is necessary for the accurate identification of any infectious disease. The gold standard for respiratory viruses including the SARS-CoV-2 virus specimens is obtained from the upper respiratory tract using the nasopharyngeal swabs[18]. Moreover, the validity of other collection methods, including mid-nasal tube swabs, anterior nostril sampling, oropharyngeal swab sampling, or saliva and gargle, has also been established. NP swabs are optimal for upper respiratory viruses, while body fluids and endotracheal secretions have been utilized for lower respiratory infections[19].

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However, there are limitations to sampling, and the sampling process must be conducted under professional supervision. After sampling, pre-treatment steps such as thermal lysis or guanidine inactivation prior to RNA extraction and amplification or testing are first required to ensure that the sample is non-infectious[23]. Any preprocessing changes made to the original specimen should be carefully examined to prevent any negative effects on subsequent assays (such as RNA or antigen stability). Additionally, the real-time RT-PCR sample procedure consists of sample lysis, viral RNA purification, synthetize cDNA, sequence amplification and detection, and result analysis. Most of the RT-PCR kits currently on the market for SARS-CoV-2 testing consist of a reverse transcription amplification enzyme, two or three sets of primers and probes for amplifying specific regions of the viral genome, and reagents for negative and positive controls..[8] RT-PCR kits can be utilized for infected samples from all parts of the body, and the interpretation of the results is the same, despite differences in the corresponding primers, probe sequences, etc., among the kits. The test is deemed "invalid" if every gene is negative. "SARS-CoV-2 positive" is the test result if all viral N genes are present as positive. Retesting of the material is required when only one of the target viral genes is present as positive. "SARS-CoV-2 negative" is the test result when all viral N genes are present as negative. [18]

The sensitivity of real-time RT-PCR was also influenced by the operator, including the primers design, the probe sequence and its target of viral gene sequence, the reagents, the instrument, and its operating parameters. 10^2 to 10^3 genomic copies per milliliter is the lower limit of detection. For real-time RT-PCR, a variety of target genes have been used, but those encoding S, N, ORF1ab, and E have been used most frequently. One of the accurate primer-probe sets for identifying the N and open reading frame(ORF)-1 genes, respectively, were found to be "2019-nCoV_N2", "N3" and "ORF1ab". The E gene primer/probe combination reported by Corman et al. and the CDC-defined N2 primer-probe set were the most accurate primer-probe set. Except for the RdRp-SARSr set from Charité, Germany, which is less sensitive under the specified experimental conditions.[8]

In the initial stages of an outbreak, RT-PCR adopts a two- or multi-gene detection strategy to ascertain assay specificity. Many commercial methods still use assays that use two or more targets, however, some laboratories switched to single-target assays to improve efficiency and streamline workflow when new crown outbreaks developed and disease frequency rose. Dual-target tests, in which no less than two viral genes are identified concurrently, are still the most common option in the majority of cases, even though new mutations have affected some gene targets. [24]

RT-PCR is a rapid, reliable, highly automated, and fully traceable method, making it the most trustworthy method for SARS-CoV-2 detection today, despite several remaining limitations. The demand for detection is escalating rapidly. For high-throughput instruments, larger sample pool depths are required to achieve higher efficiencies, but at the expense of a corresponding decrease in sensitivity and the potential for false-negative results[19]. Only a small percentage of the specimens with lower virus loads usually go unnoticed when frequency is low, increasing detection capacity and lowering testing costs. Because many shares need to be handled in situations where disease incidence is high, the benefits of sharing are lost. However, because the virus is progressively cleared during the recovery process and eventually disappears completely, this approach does not provide data on patients who recover from SARS-CoV-2. Similarly, patients in the early stages of viral infection may not have a "positive" response to the assay due to the low viral load in their bodies, resulting in not enough virus on the swab. Consequently, the combination of antigen and antibody detection is crucial for complementing RT-PCR in diagnostic protocols.[18]

2.Antigen detection is a simple and efficient method for virus detection

Antigens increase significantly in concentration after viral infection of the organism, especially during periods of massive viral replication in the bodyAntigens are substances that stimulate and elicit an immune response(Figure 2.B), which in turn neutralizes pathogens and confers protection to the host organism. Therefore, we can detect actively replicating viruses in SARS-CoV-2 infections based on this characterization using an antigenic detection approach [18]. Unlike RT-PCR, which targets RNA, the antigen assay focuses on identifing SARS-CoV-2 proteins, primarily the S and N proteins. During sample collection, antigen detection commonly entails the use of nasopharyngeal swabs or nasal swabs[12]. High-throughput antigen-based assays can be conducted using enzyme-linked immunosorbent assays (ELISAs), which was also a high sensitivity and throughput method, or on LFA strips (lateral flow type assays), known for their rapid results. Additionally, enzyme immunoassay (EIA) techniques such as chemiluminescent immunoassays (CLIAs) can be performed on semi-automatic or fully automatic instruments.

Antigen testing is used in a wide range of laboratory and clinical settings, both as a high-throughput analysis and as a rapid test, which capable of being interpreted visually or through instrumental means[18]. Unlike polymerase chain reaction (PCR)-based methods, antigenic assays directly

identify viral antigens (e.g., S, M or N protein) or viruses themselves. Similar to PCR-based assays, antigen testing can only indicate that an individual is virally infected and does not reflect an individual's history of infection. Since antigens emerge before antibodies and are targeted earlier in the infection course, they may offer more reliable indicators than antibody tests.[8] Antigen testing is less expensive and easier to perform than nucleic acid testing, with results in 15-20 minutes. [18]. However, antigen testing is less precise than nucleic acid testing; it demonstrates the highest sensitivity for specimens harboring infectious viruses with a Ct value below 25, displaying a strong correlation with the presence of infectious viruses, and is less sensitive and challenging to detect when viral loads are low or in asymptomatic cases. The lower limit of detection for antigenic testing is 10^5-10^6 copies per milliliter, and clinical data suggest that individuals with viral loads below 10^6 copies per milliliter are unlikely to transmit the virus, rendering the antigen test a valuable rapid sorting tool for swiftly pinpointing infected individuals who are most apt to spread the virus.

Antigen testing has yielded discordant results in terms of the sensitivity and specificity in detecting SARS-CoV-2 variants, with notable variations between self-testing and the environmental conditions as well as the sample types employed in testing, specifically those collected by healthcare professionals. Ag-RDTs may experience a further decline in sensitivity due to increased hybrid immunity and the presence of mucosal antibodies[19, 24]. In light of the low sensitivity of antigenic detection for SARS-CoV-2, research has commenced exploring the implementation of innovative sensor and biosensor technologies to enhance sensitivity. Multiple studies have employed electronic and electrochemical assistance to develop rapid and sensitive diagnostic devices for the detection of SARS-CoV-2, such as field effect transistors and electrochemical sensors, which are expected to improve the sensitivity of antigen detection, though further optimization is still needed.[8, 12] It has been proposed in literature that repeated testing could increase the likelihood of identifying SARS-CoV-2 infections and improve sensitivity. However, the potential for repeated testing and false-negative results in the acute care setting presents additional challenges, particularly when decisions need to be made promptly or shortly after reporting. The quest for enhanced sensitivity has been a focal point in antigen detection research.[12] Certainly, highly sensitive and selective antigen testing will significantly alter the global spread of viruses and hold great significance for public health.

3.Antibody detection is an essential tool for assessing immunization status and epidemiological investigations

Antibodies are highly specific(Figure 2.C), and there are five main classes present in the human body: IgM, IgD, IgG, IgA, and IgE. IgM and IgG are the immunoglobulins commonly utilized in antibody testing for SARS-CoV-2[7, 16, 24]. IgM has demonstrated utility in detecting early immune responses. IgM is the antibody produced in the early stages of infection, while IgG is the antibody produced in the middle and later stages of infection and is also the most abundant antibody in the serum. Antibody testing involves measuring virus-specific IgG and IgM concentrations in a blood sample to determine whether the body is defending itself against a pathogen, such as an infectious virus, or whether it is promoting infection. [8] Common antibody detection methods include lateral flow assays (LFA) and ELISA, as well as automated high-throughput instrumentation for CLIAs, lateral flow immunoassays (LFIAs), and other techniques capable of delivering rapid results. LFA is an assay based on the antigen-antibody reaction, representing a rapid test for detecting a target in a sample, relying on the specific binding or hybridization between antigen and antibody under the principles of capillary chromatography.[18]

When performing the SARS-CoV-2 antibody test, the test sample is placed in a sample pad. Subsequently, capillary action drives the sample towards the binding pad, which is impregnated with colloidal gold-labeled antibodies specifically targeting the antigen of interest. As the gold-labeled antibody encounters and binds to the target antigen, the complex continues to migrate through the device, reaching the Test Line on the nitrocellulose membrane. At this point, the antigen-bound colloidal gold-labeled antibody interacts and binds to the antibody that has been pre-immobilized on the Test Line. Following this interaction, the sample progresses further to the Control Line[18]. Here, any remaining labeled antibody in the sample binds and is captured by the antibody immobilized on the Control Line. If the test is used to detect only one antibody and the result is negative, a stripe should appear on the control line. If the test is positive, two lines appear on the control line as well as on the detection line. For tests assessing both antibody types and yielding a negative result, a single stripe should again be present at the control line. However, if both antibodies are detected as positive, three stripes will be eviden[8]

ELISA, a versatile technique for both qualitative and quantitative analysis, involves the immobilization of soluble antigens or antibodies onto a solid-phase carrier, typically polystyrene, followed by the execution of an immune response leveraging the specific binding affinity between antigens and antibodies. The 96-well plate could be employed configuration for this assay[12, 20]. The SARS-CoV-2 antigen is initially coated onto the wells of a plastic microplate. Patient serum is then added, and the plate is

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incubated and washed to eliminate any unbound material. Next, a solution containing a labeled secondary human antibody is introduced, enabling binding if the corresponding antibody is present in the sample; the absence of this antibody results in no binding[12]. The plate is again incubated and washed to remove any excess antibodies. Finally, after the enzyme conjugate, frequently horseradish peroxidase, binds to the relevant antigen or antibody, the presence of an immune reaction is ascertained through the color reaction of the added substrate. The intensity of this color reaction is positively correlated with the amount of the corresponding antigen or antibody in the specimen, so test results can be interpreted based on the color development.[18, 20]

LFA, falling under the category of dry chemical detection technology, is adaptable to naked-eye observation or employment of simple optical electronic readers, facilitating real-time rapid testing in the field, inclusive of self-testing; whereas ELISA, being an immunosorbent assay, is predominantly conducted in laboratory settings, necessitating a certain level of equipment and trained operators. Studies have shown that IgM is produced relatively early after SARS-CoV-2 infection. Because of the early appearance of antibodies, IgM antibody testing can be used in conjunction with nucleic acid testing to improve case detection and contact tracing in late-stage patients.[25, 26]. However, ELISA is not suitable for early diagnosis, as patients do not develop IgM and IgG antibodies in their serum until 7-14 days post-infection[18, 26]. Nevertheless, the ELISA method, when combined with nucleic acid detection, can serve as the primary method for the laboratory diagnosis of SARS-CoV-2, offering a robust experimental tool and scientific basis for future vaccine research against this virus.

Antibody assays, despite their limitations, are frequently employed as a complementary tool to molecular assays. The current applicability of antibody detection methods is limited. The most prominent issue is cross-reactivity[12], which refers to antibody bind to different antigens sharing the same or similar epitopes, potentially resulting in decreased specificity. The paramount challenge posed by cross-reactivity is the likelihood of generating false positive results and potential non-specificity. Additionally, cross-reactivity can cause a delayed seroconversion, implying that patients may not exhibit IgM and IgG antibodies in their serum until 7-14 days after infection, thereby contributing to false negative outcomes. Moreover, distinguishing between natural infection and vaccine-induced immunity may not be feasible. However, antibody testing facilitates late or retrospective diagnosis and, when coupled with nucleic acid testing, can enhance diagnostic accuracy and more effectively screen asymptomatic patients. Further research is warranted to advance our understanding of antibody testing for SARS-CoV-2, particularly in vaccine immunization.[12, 18]

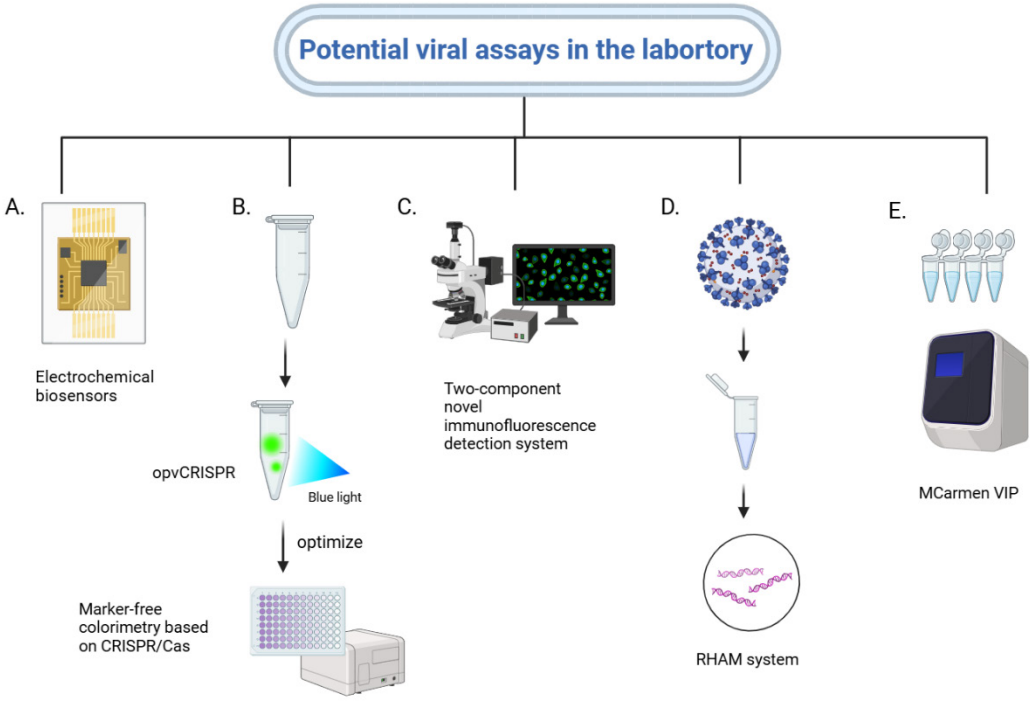

Figure 4. Potential viral assays in the laboratory development stage

For different application scenarios, there are several promising viral detection methods in the laboratory develop-

ment stage. Figure A shows an E-biosensor-based method. The principle is briefly described as being used to detect S proteins captured on an electrochemical biosensor(E-biosensor), and then the interaction between virus and redox probe is analyzed. This method enables highly sensitive and specific at 1.02×10^6 TCID50/ml. B demonstrates an experimental method combining reverse transcription loop-mediated isothermal amplification (RT-LAMP) and Cas12a excision for near single-molecule detection in 45 minutes. C is a two-component immunofluorescence detection system that employs a biotinylated antibody targeting cross-strain N and a fluorescent protein. This method allows assessment of a vaccine's ability to establish B cell response compared to conventional assays. The developed two-component assay system is high sensitivity, fewer stages of analysis, lower cost, and the flexibility to be modified to detect other pathogens in addition to SARS-CoV-2 with appropriate antibodies. D is based on the RHAM System (RNase HII-assisted amplification), a quick, sensitive, and specific molecular diagnostic platform that combines RNase HII-mediated fluorescence reporter genes with LAMP-mediated exponential amplification. The RHAM System allows for the amplification and visualization of the target at 5×10^2 copies/mL, generating positive signals in less than 15 minutes. The positive signal is within 15 minutes. This method eliminates the need for RNA extraction, provides rapid and accurate detection of target viruses, is non-cross-reactive with other common respiratory viruses, and has high sensitivity and specificity. E is a platform called mCARMEN VIP that combines CRISPR-based diagnostics with microfluidics. The technology utilizes the specific recognition properties of the CRISPR-Cas system to design specific crRNAs that selectively target and bind to specific viral RNA sequences. By using multiple crRNAs at the same time, multiple viruses or virus variants can be detected. Created in BioRender.

4.Potential viral assays in the laboratory development stage

The rapid identification and sensitive detection of disease-causing pathogens constitute pivotal measures in responding to major public health emergencies. The technologies capable of swiftly discerning the presence or absence of infection can facilitate disease control, safeguard uninfected individuals from viral exposure, and strengthen the overall management of public health incidents. Currently, although the spread and impact of the virus have been greatly reduced in many areas, there is still a need for low-cost, accurate, and rapid diagnostic methods to further minimize the impact of SARS-CoV-2 or other viruses. In this section, several cutting-edge research-level approaches proposed in the published literature for SARS-CoV-2 detection are delineated.

Hamidreza Ghaedamini et al. employed an E-biosensor-based method to detect S proteins captured on an E-biosensor(Figure 4. A), followed by an analysis of the virus's interaction with the redox probe using electrochemical impedance spectroscopy and cyclic voltammetry (CV)[27]. This approach enables the highly sensitive with a viral load of 1.02×10^6 $TCID_{50}$/mL[27].

This approach utilizes printed gold electrodes and printed carbon electrodes to develop two E-biosensors for the sensitive and selective detection of SARS-CoV-2[27]. These biosensors employ ACE2 as a bioreceptor that binds to the S protein. The immobilization of ACE2 was achieved using 1H, 1H, 2H, 2H-perfluorodecanethiol self-assembled monolayers on the SPGE surface and aromatic diazonium salts on the SPCE surface. The interaction between the captured SARS-CoV-2 and the redox probe was analyzed using electrochemical impedance spectroscopy and CV. Compared with the PFDT-modified SPGE, the aromatic diazonium salt-modified SPCE showed superior performance, displaying a clear pair of redox peaks in CV, and the redox response was linear with the logarithm of the SARS-CoV-2 concentration, with a limit of detection of 1.02×10^6 $TCID_{50}$/mL. The biosensor also exhibited significant selectivity for SARS-CoV-2 compared with the H1N1 virus[27].

Although this method is capable of achieving the desired sensitivity and specificity for SARS-CoV-2 detection, it currently requires 12 hours to analyze samples, which needs to be further optimized for scenarios that demand rapid sample detection. In conclusion, this study has developed a novel E-biosensor with high sensitivity, high selectivity, and good applicability in detecting SARS-CoV-2, highlighting its significant potential for epidemic prevention and control[27].

In recent years, many rapid detection systems have been developed. The team of Y. Wang et al. developed a visual SARS-CoV-2 detection system called "opvCRISPR," (Figure 4.B) which combines RT-LAMP and Cas12a cutting to achieve near single-molecule detection in less than 45 minutes[28]. This system is created by adding CRISPR/Cas12a reaction reagents to a cap, amplifying an RNA template using RT-LAMP, and then mixing it with Cas12a reagents for cleavage. Once the Cas12a nuclease is activated by recognizing the DNA target, it nonspecifically splits the burst fluorescent ssDNA reporter gene, producing a fluorescent signal visible to the naked eye under blue light[28]. This method has a sensitivity close to the single-molecule level, demonstrating good specificity, rapidity, reliability, and affordability. However, additional steps are still required for RNA extraction.[28]

Based on this, Kim H, Jang H, et al. proposed a CRISPR/Cas-based label-free colorimetric assay that could fur-

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ther enable cost-effective molecular diagnosis of SARS-CoV-2[29] (Figure 4. B). This technique utilizes 3,3'-diethylthiobicarbonyl iodine inserted into the double strand of the thymine adenine (TA) repeating sequence to form a dimer, which exhibits a color shift[29]. Target samples are then amplified using LAMP or recombinase polymerase amplification and subsequently placed in the CRISPR/Cas12a system. This method demonstrates high sensitivity and specificity.[29]

Alexandra Rak et al. developed a novel two-component immunofluorescence assay system based on the micro-neutralization (MN) assay(Figure 4. C), employing for the first time a biotinylated antibody agaist cross-strain N and a fluorescent protein[30]. This system was designed to assess serum-induced SARS-CoV-2 suppression in infected cell cultures, facilitating the detection of SARS-CoV-2 strains and determining infection titers of the viruses, and the titer of serum virus-neutralizing antibodies in clinical practice[30]. The system targets the detection and enumeration of coronavirus fluorescence-forming units and is composed of two consecutively used developmental components: a biotinylated antibody against the N of B.1 and EGFP-streptavidin protein. This approach allows for the estimation of the vaccine's ability to establish a cross-strain B-cell response, in comparison to traditional S-targeting tests. The two-component test system is high sensitivity, few analytical phases, low cost, and the flexibility to be modified to detect other pathogens using appropriate antibodies in addition to the SARS-CoV-2 strain.

Furthermore, as a quick, accurate, and sensitive molecular diagnostic tool, Zhuo Xiao and Xiaoli Liu et al. have created a novel isothermal amplification technique called the RHAM system (RNase HII-assisted amplification) that depends on LAMP-mediated exponential amplification and RNase HII-mediated fluorescent reporter genes (Figure 4.D).[31]. The RHAM system can amplify and visualize targets with high sensitivity ($5x10^2$ copies/ml) in an isothermal system, generating a positive signal in less than 15 minutes. With the aid of a standard LAMP primer set, Bst DNA polymerase amplifies the target sequence exponentially in the first reaction. Later, when the amplified product hybridizes to a fluorescent probe that contains ribonucleotides and is tagged with fluorophores and quenching groups, RNase HII recognizes and cleaves the ribonucleotide's 5' inside the DNA-probe duplex. The qPCR machine reads the enhanced fluorescent signal that results from the digested fluorescent probe dissociating and releasing the fluorescent moiety from the quenching moiety. This method does not require RNA extraction, provides rapid and accurate detection of the target, is non-cross-reactive with other common respiratory viruses, and demonstrates high sensitivity and specificity.[31]

The mCARMEN VIP (Figure 4.E) (multiplexed CRISPR-based microfluidic platform for clinical detection of SARS-CoV-2 variants) is a cost-effective virus and variant detection platform that was presented by Nicole L. Welch and Meilin Zhu et al. It combines CRISPR-based diagnostic and microfluidic technologies with a streamlined workflow for clinical use, making it easier to detect respiratory viruses and identify SARS-CoV-2 variants.[32]. This technology exploits the specific recognition properties of the CRISPR-Cas system by designing specific crRNAs (CRISPR RNAs) that can selectively target and bind to specific viral RNA sequences. Through the simultaneous use of multiple crRNAs, it is feasible to detect a wide array of viruses or viral variants. Integrating the CRISPR detection system onto a microfluidic chip enables rapid, high-throughput, and low-cost detection processes. The mCARMEN VIP platform offers rapid turnaround times comparable to RT-qPCR, is capable of detecting samples with low viral loads, and demonstrates high specificity and sensitivity. It is also easy to administer, offering high efficiency and a wide range of assays. However, in some instances, there may be cross-reactivity, potentially leading to false positive results. Although it can deliver rapid results, it may not provide as comprehensive genomic information as NGS[32]. Moreover, specialized personnel are required to interpret test results, which could limit its application in resource-constrained settings. Consequently, further improvements are still warranted.

Conclusion

Despite the unprecedented challenge posed to the public health system by the global outbreak of SARS-CoV-2, significant advancements were made in the development of diagnostic assays during the COVID-19 pandemic. As our understanding of viral load, structural coding, mutation types, and other biological characteristics has deepened, it has become crucial to balance the rapidity, affordability, sensitivity, and specificity of tests while maintaining assay accuracy. Molecular testing remains the gold standard for detecting SARS-CoV-2. Although antibody tests are not currently capable of detecting the presence of the virus, they still play a vital role in large-scale testing. Moreover, the development of antigen detection has been refined to achieve higher sensitivity. To date, the rapid and accurate detection of SARS-CoV-2 and other infectious viruses has been of paramount importance in guiding public health practices. The future development of coronavirus assays will likely focus on enhancing sensitivity and specificity, exploring rapid detection techniques, and optimizing detection processes to better meet the needs of public health prevention and control.

Detection method	Pros	Cons	Sensitivity	Specificity	Accuracy	How cheap to operate	Efficiency
RT-PCR	Preferred Gold Standad	Preferred Gold Standard	High sensitivity	High specificity	High accuracy	High accuracy	High throughput detection, suitable for mass screening
Antigen detection	It is simple, rapid, cost-effective and more efficient than PCR	It is simple, rapid, cost effective and more efficient than PCR	Less sensitive than PCR	Higher	Not as accurate as nucleic acid testing	It is easy to operate and does not require complex experimental equipment	High throughput, results within 15-20 minutes
Antibody Testing	Can detect if an individual has ever been infected with SARS-CoV-2 as a supplement to PCR	It is impossible to distinguish whether it is vaccination or a natural infection that produces antibodies, and antibody levels decline over time	There are many influencing factors and the sensitivity is lower than that of PCR	High specificity	Influenced by antibody test methods and reagents	Easy to operate	The detection speed is fast, suitable for rapid screening and epidemiological investigation.
Electrochemical biosensor	The detection speed is fast, suitable for rapid screening and epidemiological investigation.	It takes 12 hours to analyze the sample	High sensitivity	High specificity		High requirements for equipment	It takes 12 hours to analyze the sample, which is less efficient
opvCRISPR	Sensitivity is close to single molecule level and superior to PCR		It takes 12 hours to analyze the sample, which is less efficient	High specificity		More complex, higher cost	The near Single-molecule detection was achieved in less than 45 minutes
Marker-free colorimetry based on CRISPR/Cas	opvCRISPR is optimized	More complicated	High sensitivity	High specificity		High cost	

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New two-component immunofluorescence detection system	The analysis phase number is small, and it can be applied to pathogens other than SARS-CoV-2		High sensitivity	High specificity			High efficiency
RHAM System	No need to pick up RNA, no cross reaction		High sensitivity (5×10^2 copies /mL)	High specificity	Higher than PCR		Less than 15 minutes
mCARMEN VIP	Cost effective, high throughput	Cross-reactivity is present and may result in false positive results	High sensitivity	High specificity	High accuracy	Special personnel are needed to interpret test results	High Efficiency

Table 1.Detection method summary

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