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Advances in Laboratory Diagnosis of COVID-19

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Abstract: *COVID-19 outbreak is the fastest spreading, most widely infected, and most difficult to prevent and control major health emergency since the founding of New China. Early diagnosis and timely control of suspected cases are key to containing the spread of the epidemic. Currently, the main laboratory detection methods include virus isolation and culture, real-time RT-PCR (rRT-PCR), genome sequencing, isothermal amplification, CRISPR/CAS technique, gene chip, and antigen-antibody detection. In this paper, we review the above detection methods and compare their advantages and disadvantages to provide a reference for the diagnosis of COVID-19.*

Keywords: *COVID-19; SARS-CoV-2; Virus isolation; Nucleic acid detection; Serological detection; Rapid diagnostic tests*

1. Introduction

The infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known as coronavirus pneumonia (COVID-19), was first identified in Wuhan, China, in December 2019, and the outbreak spread rapidly worldwide[1]. According to the World Health Organization, approximately 300 million people worldwide are infected with SARS-CoV-2 and 5.5 million have died from COVID-19[2]. Laboratory testing for SARS-CoV-2 is essential for diagnosing patients with COVID-19 and reducing the spread of the disease[3]. This paper reviews the progress of research on laboratory methods for the detection of SARS-CoV-2.

2. Pathogenic characteristics of SARS-CoV-2

SARS-CoV-2 belongs to the genus β-coronavirus, which is 60-140 nm in diameter, round or oval in shape, and consists of coronal, envelope, single-stranded RNA[4]. The nucleocapsid protein (N) wraps the genomic RNA to form a helical nucleocapsid. The membrane (M) protein is located within the cell membrane and the envelope (E), membrane (M) and spike (S) proteins form the viral envelope[5]. The genomic sequence of SARS-CoV-2 is approximately 30kb in length and is arranged in the order 5'-ORF1ab-S-ORF3a-E-M-ORF6-ORF7a-ORF8-N-ORF10[6].Based on whole-genome analysis sequencing analysis, SARS-CoV-2 is similar to bat-derived SARS-CoV[7]. SARS-CoV-2 infection is significantly less lethal (3.2%) than SARS-CoV (10%)[8], but SARS-CoV-2 is more infectious than SARS-CoV[9].

3. Laboratory testing methods

Based on the characteristics of SARS-CoV-2, several detection methods have been developed, see Table 1.

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Table 1: Comparison of laboratory methods for the detection of SARS-CoV-2.

Testing methods	Testing time	Sensitivity	Specificity	Advantages	Disadvantages	Applications
Virus isolation and culture	4-6d	High	High	High accuracy, Gold Standard	Time consuming, Complex methodology, High cost, High risk of infection	Virus structure and sequence identification
Whole-genome sequencing	4-20h	High	High	High accuracy	time-consuming and High equipment requirements	virus tracing. genetic evolutionary analysis. The study of viral mutations
RT-PCR technology	1.5-2h	High	High	It takes less time. easier to operate. Good repeatability	Allegation problem with false negatives	Widely used in clinical mass testing
Isothermal nucleic acid amplification technology	1.5h	High	High	Simple equipment, Quick to operate	False positives, high primer design requirements	Relevant studies are available. Not yet applied in the clinic
CRISPR/Cas technology	40min	High	High	Targeted properties, Low cost	Has an off-target effect	Relevant studies are available. Not yet applied in the clinic
Gene chip technology	1.5h	High	High	Fast, accurate	High cost Low sensitivity	Detection of known viruses and mutant strains
Antibody detection	20min	Medium	Medium	Fast, Good repeatability	High rate of false positives High rate of false negatives	Rapid screening of large populations
Antigen detection	20min	Low	High	Safe, fast	High rate of false positives High rate of false negatives	Rapid screening of large populations
Biosensors technology	1-20min	Medium	Medium	Fast, High accuracy	susceptible to environmental, background diseases	Relevant studies are available. Not yet applied in the clinic

3.1. Virus isolation and culture

Virus isolation and culture are the "gold standard" for the early identification of SARS-CoV-2 . Zhu et al. obtained the first clinical isolate of the SARS-CoV-2 virus and observed the cells using transmission electron microscopy[10]. In this study, alveolar lavage fluid from a COVID-19 patient was inoculated into human airway epithelial cells. After incubation and washing, whole-genome sequencing was performed and infected cells were prepared for electron microscopic observation. Other research groups have isolated SARS-CoV-2, used other cell lines (Vero and LLCMK2 cell lines), identified virulent strains by RT-PCR, and used electron microscopy to observe the ultrastructure of the virus and study molecular interactions in infected cells[11]. Virus isolation and culture can identify the typical structure of coronaviruses and provide gene sequences and variant sequences of virulent strains, contributing to the study of the biological characteristics of pathogens, infection mechanisms, therapeutic drugs, vaccines, and rapid detection reagents. Due to its time-consuming method, it requires specific equipment and a high level of biosecurity[12], Electron microscopy is expensive and requires specialist image analysis, so virus isolation and culture are not suitable for early clinical detection.

3.2. Nucleic acid detection

3.2.1. Whole-genome sequencing

Whole-genome sequencing of viruses is fundamental to the study of virus evolution and mutation and is one of the methods used to detect SARS-CoV-2 in the early stages of the outbreak[13]. The main sequencing technologies are high-throughput sequencing and nanopore analysis. High-throughput sequencing technology sequences the genes of specimens to analyze the homology of different viral strains for virus tracing and virus variation studies; nanopore analysis technology measures molecular sequences by analyzing the interruption of current when molecules pass through nanopores[14]. The first SARS-COV-2 genome sequence was accurately obtained by whole-genome sequencing[15]. According to WHO, 104 strains of SARS-CoV-2 have been isolated and sequenced using Illumina and nanopore technology[16]. New sequencing methods, such as 3rd generation sequencing, are fast, with fast and accurate data available in real-time from 2nd generation sequencing data sets, allowing nucleic acid sequences to be determined without PCR amplification[17]. Among them is GenomeDetective, an Internet-based software application that allows rapid and accurate identification of isolated SARS-CoV-2 sequences[18].

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The advantages of whole-genome sequencing are high throughput, short time, and high accuracy, which can help identify and classify new coronavirus strains by collecting information on new strains to track viral mutations[16]. However, it is not suitable for rapid testing of large populations due to the disadvantages of high cost, high volume of data analysis, and low clinical efficiency.

3.2.2. RT-PCR technology

RT-PCR is now the "gold standard" for COVID-19 diagnosis, providing accurate detection of individual copies of the viral genome and viral RNA[19].TaqMan probes were used to detect three regions of conserved sequence: 1) the RdRP gene in the ORF1ab region, 2) the E gene, and 3) the N gene[20]. Confirmation of SARS-CoV-2 infection requires: positive for two SARS-CoV-2 targets (ORF1ab, N) and positive for a single target in both sampling tests[21].

RT-PCR has the advantages of quantitative analysis of viral RNA, short detection time, and high efficiency, and is widely used in clinical practice. chan et al. designed new primers and probes for the PCR detection of RdRp/helicase (Hel) and S and N genes with high detection sensitivity[22]. Yip et al. developed an Nsp2 RT-PCR assay with similar sensitivity to the RdRp/Hel assay[23]. However, RT-PCR has shown drawbacks in large sample screening tests, with frequent false negatives due to inappropriate sample collection, non-standardized RNA extraction, and premature assessment. Repeat sample collection is recommended[24], perform quality control and integrate clinical evaluation with test data for comprehensive analysis[25].

3.2.3. d-PCR technology

d-PCR is an innovative technique built on the RT-PCR method and is one of the most accurate methods available for detecting viral infections. Digital PCR has been widely used for mutation analysis, viral load detection, microbiological studies, copy number variation analysis, single-cell analysis, analysis of liquid biopsy samples, and detection of low expression targets[26-28]. The d-PCR-based ddPCR utilizes the principles of sample micro-segmentation and DNA hyperdilution on a solid vector or through an aqueous-oil emulsion of the reaction mixture. The detection process is similar to RT-PCR in that viral RNA is extracted, processed by ddPCR, the target DNA contained in each droplet is amplified, and the amplified droplet is read by a droplet reader to detect the fluorescence emission signal[29].

Compared to RT-PCR, d-PCR has higher sensitivity, specificity, accuracy, and stability; and can accurately assess SARS-CoV-2 viral load and diagnose asymptomatic patients[30]. However, d-PCR is more suitable for the detection of specimens with low viral loads and is still in the research phase and not widely used in clinical practice.

3.2.4. Isothermal nucleic acid amplification technology

Isothermal nucleic acid amplification technology is easy and convenient to operate without changing the temperature during amplification and enables rapid on-site diagnosis of SARS-CoV-2[31]. Current techniques include loop-mediated isothermal amplification (LAMP) and Penn PAMP. The LAMP technique is based on isothermal conditions of 60℃-65℃ and relies on the synthesis of self-looping strand-substituting DNA by Bst DNA polymerase[32]. Yan et al. developed RT-LAMP assays for ORF1ab and S genes with LODs of 20 copies/reaction and 200 copies/reaction, respectively[33]. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription rolling polymerase amplification (RT-RPA) may be the most promising alternatives to PCR, with advantages in terms of specificity, sensitivity, reaction efficiency, and product yield. the Penn-RAMP technique performs an initial reaction with an external LAMP primer, which then triggers the next highly accurate LAMP reaction. The first stage uses F3 and B3 external primers, while the other four RAMP primers are further mixed in the second stage. This "nested" concept greatly increases the sensitivity of LAMP by a factor of approximately 10-100 compared to normal LAMP[34].

Although both techniques are sensitive, they are prone to false-positive results due to non-specific isothermal amplification and require a high level of primer set design, skilled operators, and a dedicated laboratory to perform the assay.

3.2.5. CRISPR/Cas technology

CRISPR/Cas is a prokaryotic immune system that generates resistance to nucleic acids from external plasmids or phages. Bacteria recognize target DNA and RNA by CRISPR RNA and Cas and cleave the invading foreign nucleic acids. Studies have shown that CRISPR and Cas, primarily Cas12a and Cas13, are used to detect specific nucleic acids in a sample by binding to RNA or DNA targets specified by the guide RNA sequence, respectively, and then indiscriminately shearing the DNA/RNA probe containing

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the fluorescent signal to generate an amplification signal[35].CRISPR/Cas-based diagnostic platforms include the SHERLOCK assay platform[36], Using Cas13a nuclease activity, the first step is RT-LAMP, in which viral RNA is reverse transcribed into cDNA and amplified by strand displacement DNA polymerase (LAMP), and the second step includes RNA transcription and side-branch cleavage by CRISPR/13a, enabling targeting of specific SARS-CoV-2 sequences, a platform with faster detection times and 100% sensitivity and specificity for the diagnosis of covid-19 compared to RT-PCR[37, 38]. Curti et al. developed an ultra-sensitive, rapid, and portable CRISPR/Cas12a assay for the effective detection of SARS-CoV2-RNA[39].

CRISPR/Cas-based assays are more sensitive and specific, less expensive to analyze, and require less time. However, its sensitivity and specificity need further clinical validation due to the lack of clinical test samples.

3.2.6. Other nucleic acid detection technologies

In addition to the nucleic acid detection methods described above, new nucleic acid detection technologies are emerging, such as gene chip technology and nucleic acid mass spectrometry. Gene chip is a rapid, high-through put COVID-19 detection method based on the principle that reverse transcription of coronavirus RNA produces cDNA labeled with specific probes[40], coronavirus RNA templates produce complementary DNA, specific probes label reverse transcription, and hybridize the labeled target to the probe chip. Currently, a thermostatic amplification microarray-based kit has been developed for the detection of six respiratory viral nucleic acid tests, which can detect novel coronaviruses, influenza A viruses, influenza B viruses, and other related viruses. The kit is designed for the simultaneous detection of multiple nucleic acid target genes at high throughput[41]. Gene microarrays can analyze a large number of samples at once, but their application is limited by the high risk of false positives, the high cost of the technology, the low sensitivity of the assay, and the complexity of the analysis of the results. Nucleic acid mass spectrometry is a new type of soft ionization mass spectrometry technique with high sensitivity, high throughput, simple operation, and simultaneous detection of multiple pathogens, which is suitable for the identification of pathogens of respiratory infectious diseases[42]. Currently, nucleic acid mass spectrometry kits for the simultaneous detection of more than 20 pathogens (including SARS-CoV-2) have been developed in China. Xin Wenwen et al. developed a method combining multiplex PCR with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to simultaneously detect and differentiate seven human coronaviruses. This method has high specificity and sensitivity, providing a novel method for rapid, high-throughput, and highly accurate detection of new coronaviruses[43]. These new assays have high technical requirements such as instrumentation and personnel qualifications and have not yet been widely used in clinical practice.

3.3. Serological detection

3.3.1. Antibody detection

Antibody testing can be used as an indirect serological method for the detection of SARS-CoV-2. IgM antibodies appear positive around day 4 of COVID-19 cases, and the positivity rate reaches 100% by day 8, and IgM starts to disappear after 28 days, while IgG antibody positivity rate starts to increase[44]. Antibody detection is performed by colloidal gold[45], enzyme-linked immunosorbent assay (ELISA), chemiluminescence[46] and other methods. The SARS-CoV-2 IgM/IgG antibody test kit approved by the State Drug Administration for clinical use can detect IgM and IgG simultaneously, which improves the accuracy and sensitivity of the test and the operation method is relatively simple[47]. The neutralization assay (NA) is a test for the loss of pathogenicity of a virus or toxin to a susceptible animal after binding to a corresponding antibody and is highly specific[48]. Due to the antigenic cross-reactivity between SARS-CoV-2 and other subtypes of coronaviruses[49], the long window period for antibody production, and the small number of antibodies produced by immunocompromised patients, there is still a certain amount of false-positive and false-negative rates in antibody testing.

3.3.2. Antigen detection

In SARS-Cov-2, the N and S proteins are the major antigens, and antibodies against these two proteins may persist in the sera of SARS patients for 30 weeks[50]. Rapid detection methods based on SARS-Cov-2 antigen include immunofluorescence, enzyme-linked immunoassay, and immunochromatography[51]. In addition to the common methods, a SARS-CoV-2 coronavirus nucleocapsid antigen detection half-band lateral flow (HSLF) assay has been developed with better clinical sensitivity[52]. The antigen test has high sensitivity and specificity, however, there is a lack of sufficient clinical samples and experimental confirmation. Serological methods are indirect methods for detecting SARS-CoV-2 and

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cannot be used as a diagnostic basis for a single serological test result[53].

3.4. Rapid diagnostic tests

Recent years have seen the development of rapid diagnostic tests (RDTs), such as urine tests for human chorionic gonadotropin and immunodeficiency virus, which have reduced the reliance on laboratory infrastructure[54]. Rapid diagnostic tests based on POCT technology continue to evolve, with the advantage of being time- and space-independent and applying small portable instruments to enable timely and rapid testing in the field[55]. 1000s of molecular and antigen-based immunoassays for the detection of SARS-CoV-2, including at least 400 RDTs, are now available worldwide[56]. POCT-based assays are divided into nucleic acid kits and antigen-antibody kits, with CRISPR technology combined with lateral flow chromatography falling into the nucleic acid POCT category and others such as Abbott's IDNow COVID-19 capable of detecting nucleic acids within 17 min-1.5 h[57]. The antibody-based POCT test can provide results within minutes and is easy to use and suitable for rapid screening of large volumes of specimens. Several SARS-CoV-2 antibody-based rapid test kits have already been approved for marketing. The antibody detection method is mainly used to detect SARS-CoV-2 indirectly by detecting IgM or IgG in patients through colloidal gold or chemiluminescence assays, etc. Academician Zhong Nanshan's team has developed a combined SARS-CoV-2 IgM/IgG antibody detection reagent, which can be tested in a drop of blood and the results can be obtained in about 15 minutes. The sensitivity of the test was 88.66% and the specificity of the test was 90.63%, as confirmed by a multicenter evaluation of clinical specimens[58]. The antigen detection kits are designed to detect viral antigens including N and S proteins in plasma[59]. They are developed as specific POCT products by immunocolloidal gold, immunofluorescence chromatography, and high throughput chemiluminescence. Our team has been involved in the development of a new coronavirus antigen detection kit (latex method) from Beijing Goldwolf. Although these methods need to be further validated, POCT products are convenient, rapid, safe, and highly sensitive, and can be used as a complementary diagnostic tool for patients with negative viral nucleic acid tests to improve clinical diagnosis, as well as a rapid screening tool during periods of mass movement and gathering of people, such as the resumption of work, production, and schooling across the country.

Biosensors are also a rapid diagnostic tests (RDTs) technology that can be used to effectively diagnose COVID-19 infections quickly and directly by binding to receptors (antagonists) such as antibodies, nucleic acids, specific receptors, and enzymes and then transducing the electronic signal generated by the bioreceptor binding to the analyte[60]. There are different types of biosensor platforms available for diagnosing COVID-19, including electrochemical biosensors, colorimetric biosensors, fluorescence-based biosensors, surface-enhanced Raman scanning (SERS) biosensors, quartz crystal microbalance (QCM) biosensors, and localized surface plasmon resonance (LSPR). The most commonly used is electrochemical POCT for the detection of SARS-CoV-2 protein or RNA[61]. Seo et al. developed an immobilized SARS transistor biosensor for the detection of low concentrations of SARSCoV2 spiked protein (LoD, 1fg/ml), which can effectively identify anti-SARS-CoV-2 spiked antibodies[62]. Smartphones are also integrated into chip-based biosensors for imaging and signal analysis, allowing rapid detection of amplified signals in less than an hour for diagnosing viral infections[63]. Paper-based biosensors have been used for rapid in situ virus detection due to the low cost, easy availability, and biodegradability of paper[64, 65]. The advantages of biosensors are fast detection times, high sensitivity and specificity, and low cost. Although widely used, further improvements in sensitivity and specificity are still needed[66].

4. Summary and outlook

The high rate of infection and mortality from COVID-9 has made it one of the most devastating infectious diseases in history, and new variants have emerged in more than 20 countries, leading to a more rapid spread of the epidemic. Therefore, identifying the source of infection, asymptomatic individuals, infected individuals, or contaminated objects is an effective measure to contain transmission. Virus isolation is a direct method for early detection and obtaining viral strains, whole-genome sequencing can accurately characterize new genetic variants of SARS-CoV-2, RT-PCR is the gold standard for SARS-CoV-2 detection, and serological testing is convenient, direct, and fast; in addition, new genetic technologies such as thermostatic nucleic acid amplification, dPCR, CRISPR/CAS, RT-LAMP have also been used for the detection of SARS-CoV-2, with obvious advantages in terms of nucleic acid quantification and sensitivity; POCT-based biosensors can be used not only for immunosensing and genetic testing but also as an alternative method that does not require special

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biometric techniques. The above methods can improve the diagnostic capability of COVID-19 and have also led to the emergence of a large number of low-cost, high-accuracy assays. In practice, the choice of the essay should be based on the specific purpose of the test, in order to optimize and economize. It is also possible to combine several methods in order to avoid the disadvantages of a single method. In addition, the diagnosis of COVID-19 should be based on clinical and epidemiological history, etiological diagnosis, and support for the diagnosis of infection and/or its complications.

In the face of the huge market demand resulting from the expansion of the epidemic, high-throughput, autonomous, small and inexpensive, simple, rapid, and suitable for bedside or field testing are the future trends in the development of SARS-CoV-2 assays. Future research should focus on personalized medicine, using data quantification for accurate assessment of a patient's health status, allowing data analysis in a remote setting by developing smartphone applications, while enabling data storage to track a patient's health status, facilitating analysis and tracking of patient health information for real-time health monitoring in the field. As technology continues to advance, it is believed that more suitable methods for viral testing will be developed to provide more clinical options.

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