

SARS-CoV-2 Molecular Diagnostics in China



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KEYWORDS

• SARS-CoV-2 • Molecular diagnosis • Real-time PCR • Serologic assays

KEY POINTS

- Primers designed to target various RNA sequences within different genes of SARS-CoV-2 affect the sensitivity.
- Molecular techniques and serological assays widely used in China have the advantages and disadvantages of these techniques.
- Immunoassays have been developed for detection of COVID-19 but still as a complementary identification assay.

INTRODUCTION

Since the outbreak of coronavirus disease 2019 (COVID-19), the number of infected people has been increasing rapidly worldwide.^{1,2} As of February 22, 2022, more than 420 million confirmed cases of COVID-19 and over 5.8 million deaths worldwide had been reported.³ With effective prevention and control strategies, China won a significant early victory against COVID-19, and now mainly focuses on preventing the transmission of imported COVID-19.⁴ One of the successful strategies in China is rapid and extensive detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) to decrease the risk of transmission by rapidly enabling isolation and contact tracing.

SARS-CoV-2 is a positive-sense, single-stranded RNA virus, and the whole viral genome is approximately 29,903 nt (GenBank, MN908947.3) in length.^{5,6} SARS-CoV-2 consists of at least 12 coding regions, including open reading frames (ORF) 1 ab, S, 3, E, M, 7, 8, 9, 10b, N, 13, and 14.^{6,7} Orf1ab and orf1a genes are located at the 5'-end of the genome, which encode pp1ab and pp1a proteins, respectively. The 3'-end of the genome encodes 4 structural proteins including spike, envelope, membrane, and nucleocapsid proteins, as well as accessory proteins. Genomic sequencing revealed that SARS-CoV-2 was closely related to bat-SL-CoVZC45 and

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bat-CoV RaTG13 with a similarity of 88% and 96.3%, respectively,^{8,9} whereas only shared about 79% and 50% sequences with SARS-CoV and MERS-CoV.¹⁰

According to Diagnosis & Treatment Scheme for Coronavirus Disease 2019 (7th Edition) in China, 3 methods have been used for the diagnosis of SARS-CoV-2 infection, including detection of positive SARS-CoV-2 nucleic acids by reverse transcription-polymerase chain reaction (RT-PCR), viral gene sequencing to detect known SARS-CoV-2 sequences, and the identification of positive SARS-CoV-2-specific IgM and IgG antibodies in serum.¹¹ Numerous commercial kits for SARS-CoV-2 have been developed and used in the battle against COVID-19. As of November 20, 2020, a total of 51 approved kits for SARS-CoV-2 had been approved by the National Medical Products Administration of China (NMPA), including 24 that detect nucleic acids (), 25 kits that detect antibodies , and 2 kits targeting antigens .¹² High-throughput sequencing, RT-PCR, RT-loop-mediated isothermal amplification (RT-LAMP) have been widely used for SARS-CoV-2 nucleic acid detection,¹³⁻¹⁵ and RT-PCR is recommended in the guideline for the COVID-19 diagnosis and treatment program in China.¹⁶ The serologic assays mainly include lateral flow immunoassay (LFIA), chemiluminescence immunoassay (CLIA), or enzyme-linked immunosorbent assay (ELISA), used to detect antibodies produced by individuals exposed to SARS-CoV-2. Some LFIA-based antigen detection kits have been developed recently.

This review summarizes the molecular techniques and serologic assays widely used in China and discusses the advantages and disadvantages of these techniques. In brief, it is crucial to select appropriate diagnostic methods or combine different methods and other clinical parameters to confirm the SARS-CoV-2 infection status of individuals.

REVERSE TRANSCRIPTION—POLYMERASE CHAIN REACTION

Nucleic acid detection is an important diagnostic tool for the clinical diagnosis, segregation, rehabilitation, and discharge of patients, and was also applied as the “gold standard” for the detection of SARS-CoV-2 infection in the early stage of the epidemic. Currently, numerous primers are designed to target various RNA sequences within 6 genes of SARS-CoV-2 including ORF1a/b, ORF1b-nsp14 (50-UTR), RdRp (RNA-dependent RNA polymerase), S, E, N1/N2/N3, and RdRp/Hel (RNA-dependent RNA polymerase/helicase).¹⁷ The Chinese Center for Disease Control and Prevention (CDC) recommends the use of primers and fluorescent probes targeting SARS-CoV-2 ORF1ab and nucleocapsid protein (N) gene regions.¹⁸ The CDC in America recommends two nucleocapsid targets (N1, N2,), whereas Europe recommends initial screening with E gene followed by confirmation targeting the RdRp.^{19,20} SARS-CoV-2 has low homology with other bat-related viruses in the ORF1b (involving RdRp), N, and S genes, which are relatively specific genes worth targeting.^{21,22} Recent clinical evaluations have further demonstrated that the N1, N2, and E gene detection assays have better performance than the RdRp and N3 detection assays.²³ More recently, Chan and colleagues designed novel primers and probes for real-time RT-PCR detection of RdRp/Helicase (Hel), S and N genes, which was more sensitive than assays targeting other genes.²⁴

At the start of the epidemic in China, RT-PCR kits were developed rapidly and had the earliest clinical application; however, the sensitivity of RT-PCR results was only 30% to 50%.²⁵ This is due to a variety of factors, including low viral loads in specimens such as throat swabs and other respiratory samples, samples not being properly preserved, and the technology itself, which would be affected by virus mutation and PCR inhibitor.^{25,26} Viral loads of respiratory tract specimens are highest in bronchoalveolar lavage fluid (BALF), followed by sputum, nasal swabs, and pharyngeal swabs;

however, in clinical application, nasopharyngeal and oropharyngeal swabs served as the main sample types for clinical testing due to ease of sampling. Because of the limited sensitivity of RT-PCR, a negative result from an oral-nasopharyngeal swab was not sufficient for hospital discharge in China.^{27,28} Virus inactivation before testing should also be considered to cause false-negative results. Thermal inactivation of samples at 56°C for 30 min was recommended to ensure biosafety for laboratory personnel before SARS-CoV-2 RNA detection.²⁹ However, approximately half of the weakly positive samples were RT-PCR negative after thermal inactivation of SARS-CoV-2 at 56°C for 45 min in parallel testing.³⁰

A series of assays have been approved by the NMPA with Emergency Use Authorization in response to COVID-19 infection; however, the analytical performance claimed in the corresponding instructions by manufacturers has not been thoroughly validated. In clinical applications, the differences in nucleic acid extraction methods, RT-PCR processes, personnel, or equipment lead to variations in testing results among different laboratories.³¹ Nucleic acid extraction is one of the most critical steps for nucleic acid detection to ensure the reliability of molecular diagnosis.³² In China, various nucleic acid extraction methods were applied by the laboratories. Among these methods, manual column-based, manual magnetic bead-based, automated column-based, and automated magnetic bead-based methods accounted for 21.3% (198/931), 15.3% (142/931), 1.5% (14/931), and 51.7% (481/931), respectively.³³ For each positive sample of external quality assessment, the percentage agreement of the laboratories using magnetic bead-based extraction method was higher than those using column-based extraction method.³³

False-negative results could potentially arise from mutations occurring in the primer and probe-target regions in the SARS-CoV-2 genome.³⁴ As RNA viruses have a high degree of genetic variability, mismatches between primers and target sequences caused by mutations can lead to poor detection performance. The results should be validated with different primer sets against the same gene and combined with patient history and other clinical data to accurately determine SARS-CoV-2 infection status.³⁵

HIGH-THROUGHPUT SEQUENCING

In the early stage of the epidemic, the metagenomics next-generation sequencing (mNGS) was used to identify and analyze the genome of SARS-CoV-2 within 5 days by the Chinese CDC. The phylogenetic analysis of these genomes showed that the similarity between the genomic sequence of SARS-CoV-2 and SARS or bat-derived strains were 79% and 88%, respectively.⁶ The first mNGS system related to the ultra-high-throughput sequencer DNBSEQ-T7, with the supporting analysis software and nucleic acid detection kits, has been approved by the NMPA, which can identify and diagnose coronaviruses, including SARS-CoV-2 and other infectious respiratory pathogens, and enable rapid detection of viral sequences.³⁶ Nanopore sequencing is a third-generation genome sequencing technology providing real-time analysis and rapid insights, which does not require enzymes to amplify samples and directly performs full-length sequencing of SARS-CoV-2 and additional respiratory viruses within a few hours.^{37,38} However, NGS is currently impractical for routine use in most clinical laboratories for the diagnosis of SARS-CoV-2 infection due to some limitations, such as the high cost and long testing cycles.

ISOTHERMAL AMPLIFICATION ASSAYS

Isothermal amplification of nucleic acid is a method for the rapid and efficient accumulation of nucleic acid at a specific constant temperature. RT-LAMP has been

introduced to detect SARS-CoV-2 with a series of 4 target-specific primers targeting 6-different regions of the genome in a combined LAMP and reverse transcription-based methodology.³⁹ RT-LAMP showed a high degree of specificity (99.5%), sensitivity (91.4%) compared with RT-qPCR for identification of SARS-CoV-2.⁴⁰ Currently, point-of-care testing (POCT) of SARS-CoV-2 in nasal swabs using RT-LAMP from Abbott Diagnostics has been approved by US FDA. However, it is restricted to one sample per run.⁴¹ Relevant products have been also approved by the NMPA in China as a potential POCT method in airports, community clinics, and hospitals. The limitations of RT-LAMP assays are that the technology is more complicated than RT-PCR and involves multiple pairs of primers, limiting the choice of target sites and resolution or specificity.⁴²

CRISPR-BASED NEWLY DEVELOPED METHODS

The CRISPR/Cas is a gene-editing toolbox, a combination of guide RNA (CRISPR RNA or crRNA) and Cas enzyme complex for detecting various target sequences and being applied in diagnostic microbiology and biomedicine. Recently, CRISPR has been developed for the detection of SARS-Cov-2 in China and the clinical sensitivity and specificity are comparable to RT-qPCR.^{43,44} Owing to yield rapid read-outs and sensitive results of CRISPR, which is suitable candidates for simple POCT when coupled with lateral flow readouts.

ANTIBODY DETECTION ASSAYS

Serologic IgM/IgG antibody detection is suggested as a complementary identification assay to indirectly confirm SARS-CoV-2 infection. Briefly, the detection of specific antibodies can provide serologic evidence for infection and help confirm the diagnosis in patients with negative nucleic acid tests but high clinical suspicion.⁴⁵ In SARS-CoV-2 infection, RBD, S, and N proteins serve as the main antigens to stimulate the immune response of the body, producing IgA, IgM, and IgG antibodies. Particularly, the S1 subunit was more specific than S2 in detecting the SARS-CoV-2-specific antibodies.⁴⁶ The seroconversion of specific IgM and IgG antibodies against SARS-CoV-2 typically turn positive in the second or third week after symptom onset, but IgA and IgM were both detectable at the 5th day (median), whereas IgG appeared on the 14th day (median) in another study.⁴⁷ It has been reported that IgM peaks around 15 to 21 days after infection then slowly began to decline, whereas IgG peaks at 22 to 39 days and lasts for a longer time.⁴⁸ IgM and IgG antibodies convert to negative around 36 days and over 50 days, respectively.⁴⁷

Currently, immunoassays have been developed for the detection of COVID-19 infection in serum, plasma, and whole blood. Among these strategies, LFIA based on gold particles, up-converting phosphor, or quantum dot fluorescence, along with CLIA and ELISA, are the most promising approaches. The LFIA is user-friendly, cheap, and easily mass-produced, and the main advantage of LFIA is its potential usage for POCT. The diagnostic performance of 7 IgG/IgM LFIA kits has been evaluated for detecting SARS-CoV-2 antibodies in COVID-19 patients, and the specificity was $\geq 90.3\%$ for IgG, $\geq 91.3\%$ for IgM, and $\geq 97.1\%$ for the combination IgM and IgG. The sensitivity 14 to 25 days after onset of symptoms of the IgG LFIA was $\geq 92.1\%$.⁴⁹ Another study has reported that the sensitivity of the NG-Test was estimated to be 85% (95% confidence interval [CI] 71.9%-92.3%) and the specificity 98.3% (95% CI 95.0%-100.0%) for both IgG and IgM when compared with the ELISA Wantai Immunoassay.⁵⁰ Another meta-analysis reported the sensitivity/specificity of CLIA, ELISA, and LFIA were 92% (95% CI: 86%-95%)/99% (CI: 97%-99%), 86% (CI:

82%-89%)/99% (CI: 98%-100%), and 78% (CI: 71%-83%)/98% (95% CI: 96%-99%), respectively.⁵¹ CLIA platforms are widely used as serologic techniques for the quantitative detection of specific antigens or antibodies, which are believed to be promising emerging methods for SARS-CoV-2 antibody detection.

Serologic testing also has some limitations. The slow antibody response to SARS-CoV-2 virus indicates that they cannot be helpful in the early stages of infection. Thus, serologic testing alone cannot be used for diagnosis or exclusion of SARS-CoV-2 infection. Furthermore, it is also not suitable for general population screening.⁵² False-positive detection of IgM and IgG antibodies has been described, mainly associated with the manufacturer-determined cut-off values of the kit. A weak positive result near the cut-off value is likely to be a false positive.⁵³ Another reason for false-positive results is interfering substances in plasma samples including interferon, rheumatoid factors and nonspecific antibodies.⁵⁴ In addition, potential cross-reactivity of SARS-CoV-2 antibodies with antibodies generated by other coronaviruses could also result in false-positive results.⁵⁵

ANTIGEN-BASED ASSAYS FOR SARS-CoV-2

Recently, 2 antigen-based kits for rapid SARS-CoV-2 detection have been approved by the NMPA of China. These antigen-based detection kits were developed based on LFIA using the double antibody sandwich method.³⁶ With RT-PCR assay as the reference standard, the sensitivity, specificity, and percentage agreement of nucleocapsid protein antigen testing by the fluorescence immunochromatographic assay was 75.6% (95% CI, 69.0–81.3), 100% (95% CI, 91.1–100), and 80.5% (95% CI, 75.1–84.9) respectively, suggesting high specificity and relatively high sensitivity in SARS-CoV-2 diagnosis in the early phase of infection.⁵⁶ Although antigen tests may detect virus early in infection, they may have lower sensitivity compared with nucleic acid amplification tests and may cross-react with other coronaviruses.⁵⁷

SUMMARY

Although the application of mNGS technology played an important role in detecting the pathogen SARS-CoV-2 in the early stage of the epidemic in Wuhan, RT-PCR is still the gold standard for the diagnosis of COVID-19 and plays an essential role in patient management as well as infection control. POCT molecular testing platforms such as Qiagen's, BioFire's Filmarray and Cepheid's GeneXpert can deliver fast, safe, simple, and accurate molecular detection of pathogens such as COVID-19, which are believed to be promising emerging methods for SARS-CoV-2 detection.

SARS-CoV-2 has evolved during the past two years, and the viruses have displayed a large number of genetic variations. This may cause mismatches between primers, probes, and target sequences, and lead to reduced detection performance and false-negative results. To prevent this, RT-PCR primer sets should be updated according to the genetic variants in SARS-CoV-2 genomic sequences.

In the future, novel epidemics or pandemics may be inevitable. There are various types of pneumonia-related pathogens, including SARS-CoV-2, SARS-CoV, influenza virus, parainfluenza virus, adenovirus, respiratory syncytial virus, rhinovirus, mycoplasma, and Chlamydia. It is necessary to focus on the development of detection technologies and supporting reagents that can simultaneously rapidly detect dozens of pathogens, while still being the high-throughput and low-cost differential diagnostic technologies.

CLINICS CARE POINTS

- RT-PCR is recommended in the guidelines for the COVID-19 diagnosis and treatment program in China due to simple operation, high throughput screening and super sensitivity.
- Serological IgM/IgG antibody detection is suggested as a complementary identification assay to indirectly confirm SARS-CoV-2 infection due to detect not the pathogen itself and the slow antibody response.

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