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Review Article

Laboratory diagnosis of COVID-19 in China: A review of challenging cases and analysis

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Abstract Since the initial emergence of coronavirus disease 2019 (COVID-19) in Wuhan, Hubei province, China, a rapid spread of the disease occurred around the world, rising to become an international global health concern at pandemic level. In the face of this medical challenge threatening humans, the development of rapid and accurate methods for early screening and diagnosis of COVID-19 became crucial to containing the emerging public health threat, and prevent further spread within the population. Despite the large number of COVID-19 confirmed cases in China, some problematic cases with inconsistent laboratory testing results, were reported. Specifically, a high false-negative rate of 41% on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection by real-time reverse transcription-polymerase chain reaction (qRT-PCR) assays was observed in China. Although serological testing has been applied worldwide as a complementary method to help identify SARS-CoV-2, several

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limitations on its use have been reported in China. Therefore, the use of both qRT-PCR and serological testing in the diagnosis of COVID-19 in China and elsewhere, presented considerable challenges, but when used in combination, can be valuable tools in the fight against COVID-19. In this review, we give an overview of the advantages and disadvantages of different molecular techniques for SARS-CoV-2 detection that are currently used in several labs, including qRT-PCR, gene sequencing, loop-mediated isothermal amplification (LAMP), nucleic acid mass spectrometry (MS), and gene editing technique based on clustered regularly interspaced short palindromic repeats (CRISPR/Cas13) system. Then we mainly review and analyze some causes of false-negative qRT-PCR results, and how to resolve some of the diagnostic dilemma.
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Introduction

Soon after coronavirus disease 2019 (COVID-19) emerged in China at the beginning of 2020, the Chinese government immediately implemented strong measures to contain the outbreak. With great efforts, the COVID-19 cases have stabilized in China as a whole to date, albeit a small number of imported cases that intermittently emerge. However, a rapid epidemic began to spread around the world from April to date. As of 21st August 2020 (6:48pm CEST), there had been a total of 22,536,278 confirmed cases worldwide, with the largest cumulative number of COVID-19 confirmed cases (n = 5,477,305) in the United States of America (USA), followed by Brazil (n = 3,456,652), and India (n = 2.905,823).[1]

Some challenging cases of COVID-19 diagnosis were encountered in China and elsewhere, involving inconsistent laboratory testing results, mainly caused by false-negative real-time reverse transcription-polymerase chain reaction (qRT-PCR) detection. In this review, we summarize and discuss some possible causes of false-negative results, including how to resolve the diagnostic dilemma. We also review and discuss the advantages and disadvantages of the different lab assays for diagnosing COVID-19, including different molecular techniques and serological assays, and the value of combining qRT-PCR assays with serological testing. In brief, it is crucial to select appropriate diagnostic methods according to the phase of infection, or to use a combination of different methods and other clinical parameters in confirming the infection status of individuals.

SARS-CoV-2 etiological characteristics and genome organization

There are four genera under the subfamily coronavirus (CoVs),[2] including α, β, γ, and δ. SARS-CoV-2 belongs to the β CoV genus, the seventh member of the family of CoVs possessing a single-stranded,[2,3] positive-sense RNA genome. The genome of the SARS-CoV-2 virus consists of about 29,000 bases.[2,4] Studies show that there are at least 12 coding regions, including open reading frames (ORF) 1 ab, S, 3, E, M, 7, 8, 9, 10b, N, 13, and 14.[4,5] Among them, ORF 1 ab is the region of *RdRp* gene which codes for RNA polymerase and is responsible for viral nucleic acid replication.[6] The structural proteins include spike (S), crucially associated with virus transmission capacity, binding to angiotensin-converting enzyme 2 (ACE2) receptors on the cell surface to get into the host cell[4]; an envelope protein (E), responsible for the formation of virus envelopes and virus particles; membrane protein (M), responsible for membrane proteins encoded, and; nucleocapsid (N), recognition with the host RNA of virus genome.[4] These functional proteins play an essential role in genome maintenance and virus replication. After this, several accessory proteins also help in virus replication, including ORF3, ORF6, ORF7a, ORF7b, ORF8, and ORF9b.[2] The amplication fragments and loci of genes coding these proteins are shown in Fig. 1.

Molecular diagnosis for COVID-19 confirmation

Real-time reverse transcription-polymerase chain reaction (qRT-PCR). In many countries, the preferred testing method for COVID-19 confirmation is the qRT-PCR assay which is regarded as the 'Golden' standard for virus infection confirmation. According to *Diagnosis & Treatment Scheme for Coronavirus Disease 2019 (*7th Ed*) in Chinese*, suspected COVID-19 cases are laboratory-confirmed by positive detection of SARS-CoV-2 RNA by qRT-PCR testing. qRT-PCR testing offers several advantages in the diagnosis of COVID-19. As opposed to serology testing, qRT-PCR testing is much more valuable in the early phase of infection. Firstly, qRT-PCR results are generally available within a few hours, and the testing is easy to perform on a large scale, and with low cost per sample. However, high false-negative rates of SARS-CoV-2 detection have been reported in China (41%).[7]

Common qRT-PCR amplification fragments and loci of SARS-CoV-2 are shown in Fig. 1. Different countries have selected different targets and designed different primers for qRT-PCR assays. The available primer and probe sequences designed by different countries are summarized in Table 1, including COVID-19 infection confirmatory tests for different qRT-PCR assays.

Viral genome sequencing. According to *Diagnosis & Treatment Scheme for Coronavirus Disease 2019 (*7th Ed*),* a COVID-19 diagnosis can also be confirmed by detection of a partial or whole genome sequence of the virus which is

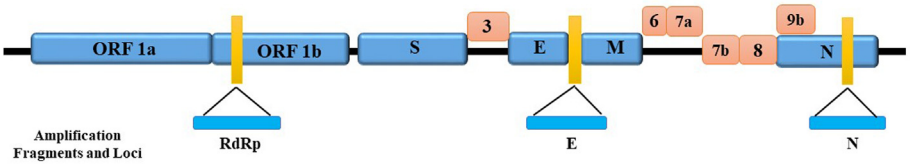

Figure 1. SARS-CoV-2 genome organization and common amplification loci by qRT-PCR. Common functional proteins in SARS-CoV-2 (blue box), such as ORF 1 ab, S, E, M, N,[3,4] and *RdRp, E* and *N* genes are selected as targets for qRT-PCR detection; accessory proteins coding regions (pink box), such as ORF3, ORF6, ORF7a, ORF7b, ORF8 and ORF9b.[2]

highly homologous with known SARS-CoV-2 strain. This is especially valuable in cases when only one SARS-CoV-2 gene target is detected for the known βCoVs by qRT-PCR. For example, Wang et al. have developed a nanopore target sequencing (NTS) method targeting 11 viral regions that is able to detect as few as 10 viral copies/mL within 1 h of sequencing.[8] In addition, next generation sequencing (NGS) also played an important role in studying the origin of SARS-CoV-2 and was very valuable in the early stages of COVID-19 outbreak in China. Based on phylogenetic analysis, SARS-CoV-2 is closely related (with 88% sequence identity) to bat-SL-CoVZC45 and bat-SL-CoVZXC21,[9] and most closely related (with 96.3% of sequence similarity) to bat-CoV RaTG13, all detected in bats.[10] However, it is not very closely related to SARS-CoV and MERS-CoV, with about 79% and 50% sequence similarity, respectively.[9]

Molecular sequencing is also used to study the evolution of SARS-CoV-2 and monitoring the virus variability. For example, in Guangdong province (China), 53 genomes from COVID-19 confirmed cases were generated by using both meta-genomic sequencing and multiplex PCR amplification followed by nanopore sequencing, to study the genetic diversity, evolution, and epidemiology of SARS-CoV-2 in China.[9] The 53 genome sequences from Guangdong province, and some viral genome sequences from other cities in China and other countries, were scattered throughout the phylogenetic tree, suggesting that most of the 53 cases were imported from different regions rather than locally transmitted.[9] Therefore, molecular sequencing can help investigators identify a native or imported species in order to evaluate if the large-scale surveillance and intervention measures implemented are effective.

Although NGS is used mostly for identification of new viral species, and understanding the impact of genetic variability to viral evolution,[11] it can also be used to detect SARS-CoV-2 in samples with low viral load. Notably, studying the evolution and transmission patterns of SARS-CoV-2 after it emerges in a new population is crucial for implementing effective measures in infection control and prevention.[9] However, NGS is currently impractical for routine use in diagnosing COVID-19 infection due to some limitations. The high cost and long testing cycles for NGS means that it is not suitable for clinical routines and thus is not available in most clinical labs.[12] Besides, all sequence-based methods are susceptible to nucleotide substitution, which can affect the oligonucleotide hybridization efficiency and result in false-negative results.[11]

Loop-mediated isothermal amplification (LAMP). Loop-mediated isothermal amplification (LAMP) was developed as a rapid, accurate, and cheaper molecular technique to amplify the target sequence at a single reaction temperature instead of the sophisticated thermal cycling equipment required in qRT-PCR testing. The LAMP method has some advantages that makes it useful for point-of-care (POC) testing.[13,14] First, the amount of viral nucleic acid produced is much higher than in the qRT-PCR assay, and a negative or positive result can be visually differentiated by using a colorimetric change without requiring a machine to read the results. In addition, LAMP results are available in 1 h, and there is no requirement for expensive reagents or specialized equipment, making it useful for POC diagnosis in remote clinical facilities without sufficient laboratory capacity. Moreover, some studies have demonstrated that the LAMP assay has higher sensitivity and specificity compared to qRT-PCR assays as it utilizes six primers to identify multiple regions on the target in a single reaction.[15,16] In Saudi Arabia, Kashir and Yaqinuddin demonstrated the effectiveness of LAMP in the detection of SARS-CoV-2 in samples with very low viral load. Besides, cross-reactivity of RT-LAMP assays with other human coronaviruses was not demonstrated in a Korean research study.[13] However, LAMP assays also have some limitations. Kashir and Yaqinuddin indicated that a complex primer design system of the LAMP assay may limit the choice of target sites and resolution or specificity. Besides, unlike the qRT-PCR technique, the LAMP technique is still in the development stage, so there is a lack of relevant literature on performance evaluation.

Clustered regularly interspaced short palindromic repeats (CRISPR-Cas). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas)-based nucleic acid detection technology can be used for site-specific modifications and gene editing in microorganisms.[17] A research group from China developed the CRISPR/Cas13 system, using two guide RNAs (gRNAs) to identify *S* and *ORF 1 ab* gene of the SARS-CoV-2 genome.[17] If SARS-CoV-2 is present in the sample, each of the two gRNAs will recognize its associated *S* and *ORF1ab* gene, and then guide Cas13 to cleave the two targets.[17] Finally, bands from the cleaved SARS-CoV-2 RNA can be visualized. Thus if the visualized bands are available, it means the presence of specific targets in the sample, thus achieving the purpose of detecting SARS-CoV-2.[17] This method has been shown to consistently detect SARS-CoV-2 RNA of between 10 and 100 copies per μL of input, and can be completed within 40 min by visually reading the detection result from a lateral flow dipstick.[18] Hou et al. also evaluated the diagnostic performance of 'CRISPR-nCoV' for SARS-CoV-2 RNA detection, and reported a 100%

Table 1 Summary of available SARS-CoV-2 qRT-PCR assays.

Institution	Gene target	Forward Primer (5′-3′)	Reverse Primer (3′-5′)	Probe (5′-3′)	Application
China. CDC[a]	*ORF1ab*	CCCTGTGGGTTTTACACTTAA	ACGATTGTGCATCAGCTGA	FAM-CCGTCTGCGGTATGTGGA AAGGTTATGG-BHQ1	A positive detection of SARS-CoV-2 is considered, if both ORF1ab and N gene assays are positive in the same sample; if only one assay is positive, repeat testing is recommended, and if confirmed, this is also considered a positive SARS-CoV-2 case.
	N	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG	FAM-TTGCTGCTGCTTGACA GATT-TAMRA	
WHO (Germany)[b]	*E*	ACAGGTACGTTAATAGTT AATAGCGT	ATATTGCAGCAGTACGCACACA	FAM-ACACTAGCCATCCTTAC TGCGCTTCG-BBQ	The *E* gene assay is used as the first-line screening tool, followed by confirmatory testing with *RdRp* gene assay and additional confirmatory analysis by *N* gene assay.
	RdRp	GTGARATGGTCATGTGTGGCGG	CARATGTTAAASACACTAT TAGCATA	**P 1:** FAM-CCAGGTGGWAC RTCATCMGGTGATGC-BBQ **P2:** FAM-CAGGTGGAACCTCATCA GGAGATGC-BBQ	
	N[c]	CACATTGGCACCCGCAATC	GAGGAACGAGAAGAGGCTTG	FAM-ACTTCCTCAAGGAACAA CATTGCCA-BBQ	
U.S. CDC[d]	*N1*	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGT TGAATCTG	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	Two monoplex assays (N1, N2) were designed for specific detection of SARS-CoV-2. A positive detection of SARS-CoV-2 is considered if both assays are positive; whereas if only one assay is positive, the result is unconvinced, repeat testing is recommended.
	N2	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	
The University of Hong Kong[e]	*ORF1b-nsp*	TGGGGYTTTACRGGTAACCT	AACRCGCTTAACAAAGCACTC	FAM-TAGTTGTGATGCWATCATGA CTAG-TAMRA	The *N* gene assay is recommended as a screening assay and the *ORF1b* assay as a confirmatory one.
	N	TAATCAGACAAGGAACTGATTA	CGAAGGTGTGACTTCCATG	FAM-GCAAATTGTGCAATT TGCGG-TAMRA	
Thailand[b]	*N*	CGTTTGGTGGACCCTCAGAT	CCCCACTGCGTTCTCCATT	FAM-CAACTGGCAGTAACCA-BQH1	None

[a] The assay was established as a Chinese official protocol and published in *Technical Guide for Prevention and Control of Coronavirus Disease 2019 in Medical Institutions*. 5th Ed *(in Chinese)*.[44]

[b] The assay was originally proposed by the Charité-Universitätsmedizin Berlin Institute of Virology,[59] and then endorsed by WHO[60]; The Thailand's official assay was also published in the WHO document.[60]

[c] The *N* assay was recommended as an additional confirmation of COVID-19 infection.[59]

[d] The assay was established as a U.S official protocol and published in *2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes.*[61]

[e] The assay was designed by The University of Hong Kong (HKU), School of Public Health and published in *Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR*.[62]

SARS-CoV-2, severe acute respiratory syndrome coronavirus; qRT-PCR, real-time reverse transcription polymerase chain reaction; ORF, open reading frames; *RdRp*, RNA-dependent RNA polymerase gene; *N*, nucleocapsid protein gene; *E*, envelope protein gene. CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.
Note.

sensitivity in 52 samples.[18] Given the rapidity, simplicity, higher sensitivity and specificity of CRISPR-nCoV compared to PCR-based methods, the prospect of CRISPR/Cas-based SARS-CoV-2 detection looks very promising.[49] However, this technique is still in the exploratory and research stage, and needs to be further evaluated by more tests.

Nucleic acid mass spectrometry (MS). A powerful new method for rapid identification of emerging diseases has been recently described, and is based on polymerase chain reaction (PCR) to amplify nucleic acid targets from large groupings of organisms, using electrospray ionization mass spectrometry (ESI-MS) for precise mass measurements of PCR products and characterization of base composition to identify organisms in a sample.[19] During the last decade, MS has successfully been used for molecular diagnosis of viral infections.[20] Sampath et al. demonstrated that this method could identify and differentiate between SARS and other known CoVs, including the human CoV 229E and OC43.[19] The method has high-throughput capabilities of automated analysis of more than 1500 PCR reactions per day, with a detection sensitivity of 1 PFU/mL.[19] Thus it is useful in the surveillance of viral infections, and boost rapid identification of known or emerging pathogens.[21] At present, Darui Biotech Company in China has developed a nucleic acid MS with a capacity of simultaneously detecting more than 20 pathogens (including SARS-COV-2), but requiring professional personnel to operate.[17]

Analysis of challenging cases inconsistent with clinical testing

Despite the significant increase in the number of laboratory-confirmed cases, and the identification of common clinical characteristics in the diagnosis of COVID-19, some rather odd or difficult cases have been reported in China and elsewhere, with inconsistent clinical laboratory testing results and/or clinical symptoms. These problematic or odd cases mainly involved some asymptomatic or clinically mild cases, with no typical COVID-19 radiological indications or defined clinical symptoms, but with positive detection of SARS-CoV-2 RNA. Conversely, some suspect cases with typical viral pneumonia radiological features of COVID-19, but with negative detection of SARS-CoV-2 RNA, were also reported in China.

Some studies have reported that asymptomatic cases are common in younger and middle-aged populations without underlying diseases.[22] Besides, more studies showed that a large number of asymptomatic cases were medical staff.[22,23] Thus qRT-PCR testing plays a crucial role in screening for high-risk populations, close contact tracing, and longitudinal surveillance, to better prevent and early control this epidemic.[23]

On the contrary, there have also been some odd cases in which qRT-PCR detection for COVID-19 is negative, but with highly suspicious clinical symptoms and radiologic findings consistent with the disease. Although, detection of viral nucleic acid is regarded as the 'Golden' standard for virus infection confirmation, a negative result cannot exclude COVID-19 due to possible false-negative results. To date, many cases of suspected false-negative detection of SARS-CoV-2 RNA have been reported in several hospitals both in China and elsewhere. These false-negative cases present challenges for prevention and control of the COVID-19 pandemic, especially when the testing result plays a crucial role in determining whether the patient receives continual medical care and isolation, or is discharged.[24] Given the high infectious potential of COVID-19, it would be ideal to treat these false-negative cases as positive, but due to limited space in the hospital, this might present another challenge.

Causes of false-negative molecular diagnosis of COVID-19

Some possible causes of false-negative COVID-19 results are discussed below. First, the level of virus shedding differs in different parts of the body as the infection progresses. Thus low viral load levels in different samples and time periods of illness could result in false-negative detection of SARS-CoV-2 RNA, especially for discharged patients. SARS-CoV-2 RNA has been detected in the oral cavity-associated specimens during early infection, and in anal swabs during late infection.[25] In a study by Wu et al. involving 74 patients, viral shedding in the throat (throat swabs) was detected at a mean of 16.7 days, in comparison to a later appearance of viral RNA in fecal samples with a prolonged viral clearance for a mean of 27.9 days.[26] Wang et al. also found a longer duration of viral shedding in throat/nasal swabs for over 72 days after onset of illness.[27] In addition, a study from Germany showed that shedding of viral RNA in the sputum could outlast the end of symptoms (over three weeks) in six of nine patients.[28] As for nasopharyngeal swabs, a study showed that in about 53% of cases, viral clearance was achieved 21 days after onset of symptoms.[29] In short, if the sampling time is out of sync with the viral shedding dynamics at different anatomic sites, or the viral load is below the qRT-PCR detectable limit during the viral shedding, this will increase the possibility of false-negative results of qRT-PCR tests in the samples.

Fig. 2 shows a general relationship between viral load kinetics of SARS-CoV-2 from upper respiratory tract (URT) and the course of COVID-19 infection. He et al. suggested that viral shedding might begin 2—3 days in the URT before onset of symptoms (Fig. 2).[30] Then the viral load (in throat swabs) peaks during the first week of illness and gradually decreases in the second week (Fig. 2), and the researchers supposed that infectiousness peaked on or before symptom onset, as per data obtained from 23 patients.[30] However, a research study in Germany indicated that viral shedding in pharyngeal swabs reached a peak in the first week of symptomatic presentation.[28] Feng et al. reported on a case from China with fever and patchy ground-glass opacity on chest CT on admission, but with four negative sequential qRT-PCR results on the pharyngeal swabs.[31] It was not until the fifth day of admission, that the fifth qRT-PCR test was positive. This case indicates that the four negative serial qRT-PCR testing results were possibly false-negatives. One possible reason is that although the virus had already started shedding in his pharyngeal site before or after admission, it was not detected until day 5 due to the low viral load below the detectable limit of the qRT-PCR assay. In Korea, a similar case was reported in a patient with a fever who had SARS-CoV-2 detected from a mixed specimen of nasopharyngeal and oropharyngeal swabs on day 2 of symptom onset.[32] However, the viral load started to decline

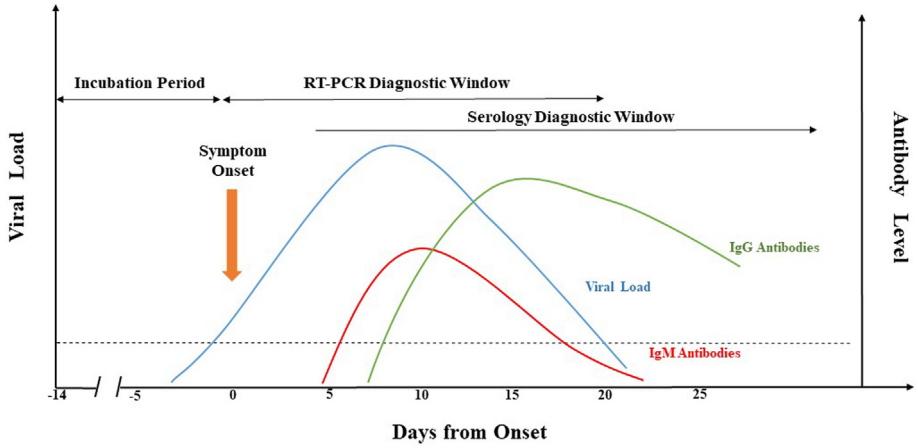

Figure 2. A general overview of the relationship between the viral load in URT specimens and the clinical course of COVID-19 infection, and estimation of antibody levels during COVID-19 infection.

from day 7, and viral RNA was undetectable by qRT-PCR for two successive days from day 15 in spite of the ongoing infection, suggesting that viral load kinetics, sampling time and duration of the illness, can have an influence on qRT-PCR results.[32]

Multiple COVID-19 cases which were SARS-CoV-2 positive by qRT-PCR assays in the respiratory tract swabs after patients had been discharged from hospital, have become highly controversial in China.[33] Zhou et al. reported a case who met the criteria for hospital discharge but was tested positive for SARS-CoV2 again 10 days after discharge.[34] Thus a longer observation period should be considered for discharged patients.

On the other hand, some patients tested positive for SARS-CoV-2 RNA in their fecal samples for nearly 5 weeks after hospital discharge, but with consecutive respiratory samples being negative, possibly due to extended duration of viral shedding in faeces.[26] A study by Wu et al. reported on 2 cases with detection of viral RNA in the fecal samples for 33 continuous days after testing negative in respiratory tract samples, and with positive SARS-CoV-2 RNA in their fecal samples for 47 days after first onset of symptoms.[26] Notably, live virus isolation from fecal samples has rarely been successful in mild cases, mainly due to low viral load.[28] Therefore, despite the presence of SARS-CoV-2 RNA in the fecal samples, further research is needed to determine the infectivity potential of these patients. In summary, it is suggested that follow-up testing be done on discharged patients with prolonged viral shedding, using fresh fecal samples at specific time points, and to extend the follow-up period for discharged patients through testing of respiratory tract swabs, to minimize potential transmission of COVID-19.[26] Additionally, multi-site and different time point specimen collection may be used to minimize the incidence of false-negative detection of SARS-CoV-2 RNA due to viral shedding dynamics.

Second, the quality of samples at different phases of infection also plays a role in the detection of SARS-CoV-2 nucleic acid, and hence in the incidence of false negative detection of COVID-19. For example, two highly suspected cases were reported in China where there was no viral RNA detected in the URT specimens but was positive in BALF.[35] Furthermore, a patient was reported from Switzerland who had a 2-day history of dyspnea and a 6-day history of fever (39 °C) with suspect chest imaging features, but with two false-negative results of nasopharyngeal and oral swabs by qRT-PCR assays. Finally, the patient was confirmed COVID-19 positive by SARS-CoV-2 RNA detection in a BALF sample.[36] In Thailand, a patient with persistent fever, tested continually negative for SARS-CoV-2 RNA in nasopharyngeal and oropharyngeal samples up to day 5.[37] On day 8, a BALF sample tested positive for SARS-CoV-2 RNA by the qRT-PCR assay.[37]

It was unclear why these patients' URT specimens tested consecutively negative for SARS-CoV-2 RNA. Some possible causes include improper collection or handling of specimens, and low viral load due to diminished viral shedding in URT specimens. Another possible explanation is the relatively lower sensitivity of nasopharyngeal and oral swab qRT-PCR assays for SARS-CoV-2 RNA, ranging from 56% to 83%, in comparison to lower respiratory tract (LRT) specimens.[36] Although BALF specimens increase the detection rates of COVID-19, their collection requires a suction device and a skilled operator, and is also painful for the patients, so they are not convenient for routine laboratory diagnosis of SARS-CoV-2 RNA.[35]

Yang et al. revealed that save for BALF, sputum was the best specimen for laboratory diagnosis of COVID-19, followed by nasal swabs which were most recommended, with detection rates ranging from 74.4% to 88.9% and 53.6%–73.3%, respectively, for both severe and mild cases during the first 14 days after onset of illness.[35] However, COVID-19 patients always present a dry cough, with only 28% able to produce sputum for diagnostic evaluation.[38] In most studies of respiratory virus infections, nasopharyngeal or throat swabs are normally used for viral load monitoring. However, the collection of nasopharyngeal swabs is an invasive procedure; it is uncomfortable for the patient and poses a risk of transmission of the virus to the healthcare workers from coughing and sneezing.[38] Previous studies have also demonstrated a relatively low SARS-CoV-2 RNA detection rate in throat swabs (collected $\geq$ 8 days), especially in samples from mild cases, and thus throat swabs are not recommended to limit the incidence of false-negative

results.[35] Compared with nasopharyngeal swabs, saliva is much more acceptable to patients and is safer for healthcare workers to collect.[39] A previous study has shown that saliva has a high and consistent coronavirus detection rate of >90% with nasopharyngeal specimens.[38] Hence, if the clinical, laboratory and radiological features are highly suspicious for COVID-19, but with negative qRT-PCR tests on URT specimens, performing qRT-PCR assays on LRT specimens might improve the detection rate of SARS-CoV-2 in specimens such as sputum and BALF. Thus for challenging COVID-19 cases, different types of samples are recommended from a patient for combination testing to reduce the incidence of false-negative results.

Thirdly, false-negative detection of SARS-CoV-2 by qRT-PCR is possibly associated with difficulty in detecting residual virus resident in pulmonary tissues. A patient reported by Yao et al. was initially confirmed as SARS-CoV-2 positive by qRT-PCR testing on nasopharyngeal swabs.[33] Later on, it was demonstrated histopathologically that residual SARS-CoV-2 virus was present in pulmonary tissues, but with three consecutive negative results by qRT-PCR of nasopharyngeal swabs in the following days. Unfortunately, the patient died in the end.[33] This case raised the possibility that non-detection of SARS-CoV-2 in the nasopharyngeal swabs might not be fully indicative of the virus status in lung tissue. Thus detection of SARS-CoV-2 RNA in BALF and extension of quarantine or hospital discharge period, are recommended, especially for elderly patients with underlying diseases.[33]

Fourth, co-infection with other viruses may have an impact on qRT-PCR detection accuracy. Influenza A virus was one of the commonest viral pathogens causing co-infection among patients with SARS-CoV-2 infection in China.[40] Lai et al. reported on two COVID-19 cases co-infected with influenza A virus but which yielded false-negative results for SARS-CoV-2.[40] Zhao et al. reported on a COVID-19 patient with HIV-1 and HCV coinfection, who showed continuously negative SARS-CoV-2 RNA tests by qRT-PCR but with a delayed antibody response against SARS-CoV-2 in the plasma.[41] Therefore, co-detection of SARS-CoV-2 with another virus presents additional challenges in the diagnosis of COVID-19. Further research is needed to verify the influence of other viral infections on SARS-CoV-2 detection in viral co-infected patients.

Fifth, false-negative results are possibly associated with *in vitro* viral nucleic acid diagnostic kits with unstable sensitivity, and some methods of RNA extraction. Many countries have designed different SARS-CoV-2 diagnostic kits with different targets and primers for qRT-PCR assays (summarized in Table 1). Although it is commonly accepted, as per data from many studies, that *E*-gene based qRT-PCR assays have a higher diagnostic sensitivity than other targets, the specificity of *RdRp and N* gene have been shown to be higher. Actually, during the early stages of COVID-19 outbreak in China, there were a series of false-negative detection of SARS-CoV-2 RNA in some samples caused by some poor sensitivity of diagnostic kits developed in an emergency (no data available). However, the sensitivity of currently available diagnostic kits from different manufacturers has been significantly improved. In the last few months, very few reported false-negative cases were related to low or unstable sensitivity of the kits.

For highly suspected or already confirmed cases, if only one target is used for COVID-19 confirmation or follow-up diagnosis, it is important to improve the accuracy rate of qRT-PCR tests by comparing with different diagnostic kits. Some researchers from Beijing Centre for Disease Prevention and Control found that thermal inactivation might reduce the detectable amount of SARS-CoV-2 in qRT-PCR assays,[42] thereby resulting in false-negative results, and this is particularly common in the early phase of infection with low viral load in samples. Although thermal treatment of samples before RNA extraction is not recommended by WHO,[43] thermal inactivation of samples under 56 °C for 30 min is required to ensure biosafety for laboratory personnel based on the Chinese guideline.[44]

Sixth, some other causes of false-negative qRT-PCR results have been analyzed in other countries. Tahamtan and Ardebili from Iran indicated that mutations in the primer and probe target regions in the SARS-CoV-2 genome could result in false-negative qRT-PCR results. They indicated that it was possibly caused by genetic variability of SARS-CoV-2 resulting in mismatches among the primers, probes and the target sequences.[45] In fact, since the first SARS-CoV-2 genomic sequence became available, several studies have reported on a rapid genetic evolution of SARS-CoV-2 through a phylogenetic tree analysis.[46] Both natural mutation and active viral recombination are able to weaken the efficiency of oligonucleotide annealing, declining the sensitivity and specificity of qRT-PCR detection.[46] In order to avoid false-negative results due to the unknown mutation, continuous monitoring of genetic variability is necessary, and targeting multiple regions in the viral genome is crucial to SARS-CoV-2 detection.[45]

Last but not least, proper management of sample collection and storage is essential for reducing the incidence of false negative qRT-PCR detection of COVID-19. For example, if samples are collected too early or too late during an infection, this may have an effect on viral load. Furthermore, improper storage and/or transportation of specimens can result in RNA degradation leading to a false-negative result.[44] In addition, whether a standardized clinical laboratory is adequately equipped and has well trained laboratory personnel for virus detection, is also an important factor. Therefore, strengthening the professional training of laboratory operators and improving the laboratory quality management system can also reduce the incidence of false-negative results.

Supplementary serological testing

To resolve the limitations of qRT-PCR testing and difficult COVID-19 suspected cases, serological testing (IgM/IgG antibody detection) is suggested as a complementary identification assay.[47] The clinical significance of false-negative qRT-PCR results (related to the course of infection) combined with serological testing is summarized in Table 2. Specific IgM and IgG antibodies can be used in determining whether the patient has a recent or previous viral infection,[48] and also in quantification of SARS-CoV-2-positive cases, including asymptomatic and recovered cases.[49] For example, SARS-CoV-2 antibodies were detected in 10.1% (28/276) of asymptomatic medical staff at one hospital in China, and five of them were in close contact with confirmed COVID-19 patients, but were qRT-PCR

Table 2 Serological testing among the cases of false-negative qRT-PCR results in different clinical stages.

Stages of infection	Different tests		
	qRT-PCR	IgM	IgG
Early stage of infection (qRT-PCR result may be false-negative)[a]	−	+	−
Past infection (recover)	−	−	+
The late or recovery stage of infection (qRT-PCR result may be false-negative)[a]	−	+	+

[a] The false-negative qRT-PCR results are associated with the course of infection. qRT-PCR, real-time reverse transcription polymerase chain reaction.
Note.

negative.[50] Another study detected IgM and IgG antibodies in 84.21% and 94.74% of 19 patients with negative SARS-CoV-2 detection by qRT-PCR assays but with typical clinical symptoms, respectively.[51] This strongly suggests that serological testing can significantly reduce the risk of misdiagnosis and play a crucial role in timely diagnosis, treatment and prevention of COVID-19.[51]

However, serological testing also has some limitations, mainly the slow antibody response to SARS-CoV-2 virus means that they cannot be helpful in the early stages of infection.[49] Seroconversion is usually detectable between 5 and 7 days and 14 days after onset of symptoms.[52] Based on the U.S. FDA, the non-specific IgM antibodies to SARS-CoV-2 are detectable just a few days after initial infection,[53] but IgM levels throughout the course of infection can be rapidly declining and finally undetectable. However, IgG antibodies remain detectable for a longer period, or even when SARS-CoV-2 RNA is undetectable,[54] as described in Fig. 2. In China, Zhang et al. reported on 15 patients with relatively low or undetectable IgM and IgG titers on day 0 (the day of first sampling).[25] However, increasing antibody titers were demonstrated on the patients on day 5, and this was interpreted as a transition from early to later phase of viral infection with dynamic changes of viral presence.[25] On the contrary, there was a relatively low positive detection rate by qRT-PCR assays during the same period. Thus, serological testing alone cannot confirm or exclude COVID-19 infection.[55] For example, a negative result cannot rule out the infection because the patient may not be infected at the sampling time, as the individual may be in the 'window period' (delay in the production of antibodies), especially for those who have a history of close contact with confirmed cases. Moreover, false-positive detection of IgM and IgG antibodies have been described,[51] mainly associated with cut-off values of the kit. A weak positive result near the cut-off value is likely to be a false positive.

Another reason is that some existing interfering substances in plasma samples (including interferon, rheumatoid factors (RF) and non-specific antibodies) can lead to false-positive results. Jia et al. demonstrated differing detection results of IgM/IgG antibodies in serum samples with different RF concentrations.[56] In a total of nine serum samples with different RF concentrations, detection of IgM specific antibodies was observed at a RF concentration of $>$331 IU/mL, and both IgM and IgG test results were positive in samples with a RF concentration of 981. 2 IU/mL.[56] Additionally, potential cross-reactivity of SARS-COV-2 antibodies with antibodies generated by other coronaviruses probably also results in false-positive results.[57] For example, Lv et al. found a high frequency of cross-reactivity between S protein of SARS-CoV-2 and SARS-CoV among plasma samples from 15 COVID-19 patients.[58]

In short, although serological testing alone is not enough to confirm COVID-19 infection, combining both serological testing and molecular techniques can improve the identification rate of COVID-19. Serological testing is valuable in evaluating the overall immune response in a large scale population surveillance.[24]

Conclusion

In summary, to resolve the COVID-19 challenging cases, more comprehensive analysis and/or further evaluation of different diagnostic methods is needed. Improving the identification rates of SARS-CoV-2, including reducing the incidence of false-negative/false-positive results, still remains a considerable challenge in the laboratory diagnosis of COVID-19 in China, requiring further research. At present, vigilance is still required in China as there remains a risk that SARS-CoV-2 transmission may reignite with an increasing number of COVID-19 imported cases being reported.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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