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REVIEW

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Laboratory testing of SARS-CoV, MERS-CoV, and SARS-CoV-2 (2019-nCoV): Current status, challenges, and countermeasures

Ying Yan[1,2] | **Le Chang**[1,2] | **Lunan Wang**[1,2,3]

[1]National Center for Clinical Laboratories, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Sciences, Beijing, China

[2]Beijing Engineering Research Center of Laboratory Medicine, Beijing Hospital, Beijing, China

[3]Graduate School, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China

Correspondence
Lunan Wang, No.1 Dahua Road, Beijing 100730, China.
Email: lunan99@163.com

Summary

Emerging and reemerging infectious diseases are global public concerns. With the outbreak of unknown pneumonia in Wuhan, China in December 2019, a new coronavirus, SARS-CoV-2 has been attracting tremendous attention. Rapid and accurate laboratory testing of SARS-CoV-2 is essential for early discovery, early reporting, early quarantine, early treatment, and cutting off epidemic transmission. The genome structure, transmission, and pathogenesis of SARS-CoV-2 are basically similar to SARS-CoV and MERS-CoV, the other two beta-CoVs of medical importance. During the SARS-CoV and MERS-CoV epidemics, a variety of molecular and serological diagnostic assays were established and should be referred to for SARS-CoV-2. In this review, by summarizing the articles and guidelines about specimen collection, nucleic acid tests (NAT) and serological tests for SARS-CoV, MERS-CoV, and SARS-CoV-2, several suggestions are put forward to improve the laboratory testing of SARS-CoV-2. In summary, for NAT: collecting stool and blood samples at later periods of illness to improve the positive rate if lower respiratory tract specimens are unavailable; increasing template volume to raise the sensitivity of detection; putting samples in reagents containing guanidine salt to inactivate virus as well as protect RNA; setting proper positive, negative and inhibition controls to ensure high-quality results; simultaneously amplifying human RNase P gene to avoid false-negative results. For antibody test, diverse assays targeting different antigens, and collecting paired samples are needed.

KEYWORDS

MERS-CoV, nucleic acid testing, SARS-CoV, SARS-CoV-2 (2019-nCoV), serological testing, specimen collection

Abbreviations: +ssRNA, single-stranded positive-sense RNA; ACE2, angiotensin converting enzyme 2; CDC, Centers for Disease Control and Prevention; CLIA, chemiluminescence assay; CoV, coronavirus; CRISPR, clustered regularly interspaced short palindromic repeats; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HDA, helicase-dependent amplification; ICU, intensive care unit; IFA, immunofluorescence assay; LAMP, loop-mediated isothermal amplification; LOD, limit of detection; LRT, lower respiratory tract; MERS-CoV, Middle East respiratory syndrome coronavirus; NASBA, nucleic acid sequence-based amplification; NAT, nucleic acid tests; NP swab, nasopharyngeal swab; NP, nucleocapsid protein; OP swab, oropharyngeal swab; POCT, point of care testing; RCA, rolling-circle amplification; RdRp, RNA-dependent RNA polymerase; RPA, isothermal recombinase polymerase amplification; rRT-PCR, real time reverse transcription-polymerase chain reaction; SARS-CoV, severe acute respiratory syndrome coronavirus; SARSr-CoV, SARS-related-CoV; SDA, strand displacement amplification; SHERLOCK, Specific High Sensitivity Enzymatic Reporter UnLOCKing; TMA, transcription-mediated amplification; upE, upstream of the E protein gene; URT, upper respiratory tract; UTR, untranslated region; VLPs, virus-like particles; WB, western blot; WHO, World Health Organization.

1 | INTRODUCTION

In 2002 and 2003, severe acute respiratory syndrome coronavirus (SARS-CoV) started in China and overspread in 29 countries worldwide, affecting more than 8000 people. Ten years later, Middle East respiratory syndrome coronavirus (MERS-CoV) outbroke in Arabian Peninsula and overspread mainly in the Middle East, affecting more than 2000 people.[1] These two outbreaks demonstrated the high transmissibility and pathogenicity of emerging coronaviruses (CoVs). Since late December 2019, pneumonia of unknown cause that started in Wuhan, Hubei, China has been attracting tremendous attention. The pathogen of this unexplained pneumonia was isolated from human airway epithelial cells and identified as a novel CoV, named SARS-CoV-2.[2,3] The World Health Organization (WHO) has declared the SARS-CoV-2 a public health emergency of international concern on 5 February 2020 and more recently, a pandemic. By 18 March 2020, WHO confirmed a total of 191 127 cases of SARS-CoV-2 infection and 7807 deaths worldwide. Among them, 91 845 confirmed cases were distributed in Western Pacific region and 74 760 were in European region.[4] Rapid and accurate diagnosis of the causative viral pathogen is essential for early discovery, early reporting, early quarantine, early treatment, and cutting off epidemic transmission.

2 | OVERVIEW OF CORONAVIRUSES

CoVs are enveloped single-stranded positive-sense RNA (+ssRNA) viruses that can infect respiratory, gastrointestinal, hepatic, central nervous systems of humans, other mammals and birds.[5] CoVs belong to the subfamily *Coronavirinae* in the family *Coronaviridae*.[6] This subfamily consists of four genera-alpha, beta, gamma, and delta-CoVs.[7] Seven CoVs are known to cause respiratory diseases in humans. Among them, Alpha-CoVs HCoV-NL63, HCoV-229E, and beta-CoVs HCoV-OC43, HKU1 can induce mild upper respiratory disease in immunocompetent individuals, while the other three CoVs SARS-CoV, MERS-CoV, and SARS-CoV-2 belonging to beta-CoVs are more pathogenic.[6,8] The genome of a typical CoV contains a 5′ untranslated region (UTR), a conserved replicase domain (ORF 1ab), four genes S, E, M, and N to encode structural proteins spike, envelope, membrane, and nucleocapsid proteins, a 3′ UTR, and several unidentified nonstructural ORFs (Figure 1).[6,9]

Person-to-person spread of SARS-CoV, MERS-CoV, and SARS-CoV-2 mainly occurs via respiratory droplets produced when an infect person coughs or sneezes.[10] Fever was the most prevalent symptom that occurred in 86% to 97% SARS-CoV-2 infected patients, followed by dry cough (59%-76%), fatigue (34-68%), and dyspnea (20%-40%).[11] The median time from first symptom to dyspnea was only 5 days.[12] Apart from acute respiratory syndrome, some other organ dysfunctions, including gastrointestinal symptoms, hepatic dysfunction, splenic atrophy, seizures, and lymphadenopathy have been found in SARS and MERS.[13-16] Since SARS-CoV-2 shares the same receptor angiotensin converting enzyme 2 (ACE2) with SARS-CoV,[17,18] it is reasonable to speculate that these organ dysfunctions may also be found in SARS-CoV-2 infected patients. Actually, a retrospective, observational study showed that in 52 critically ill adult patients who were admitted to the intensive care unit (ICU), 15 (29%) had acute kidney injury, 12 (23%) cardiac injury, and 15 (29%) liver dysfunction.[19] The most severe sequelae after rehabilitation from SARS were femoral head necrosis and pulmonary fibrosis. High-dose steroid pulse treatment was used to suppress inflammation, which caused subchondral osteonecrosis in about 5% of SARS patients.[20] The risk of osteonecrosis was 0.6% for patients receiving less than 3 g and 13% for patients receiving more than 3 g prednisolone-equivalent dose.[20] In addition, the use of high dose of hydrocortisone or methylprednisolone for an extended duration was shown to be a significant risk factor for osteonecrosis.[21] In order to suppress inflammation without causing osteonecrosis in SARS-CoV-2 infected patients, Chinese National Health Commission recommended that glucocorticoid should

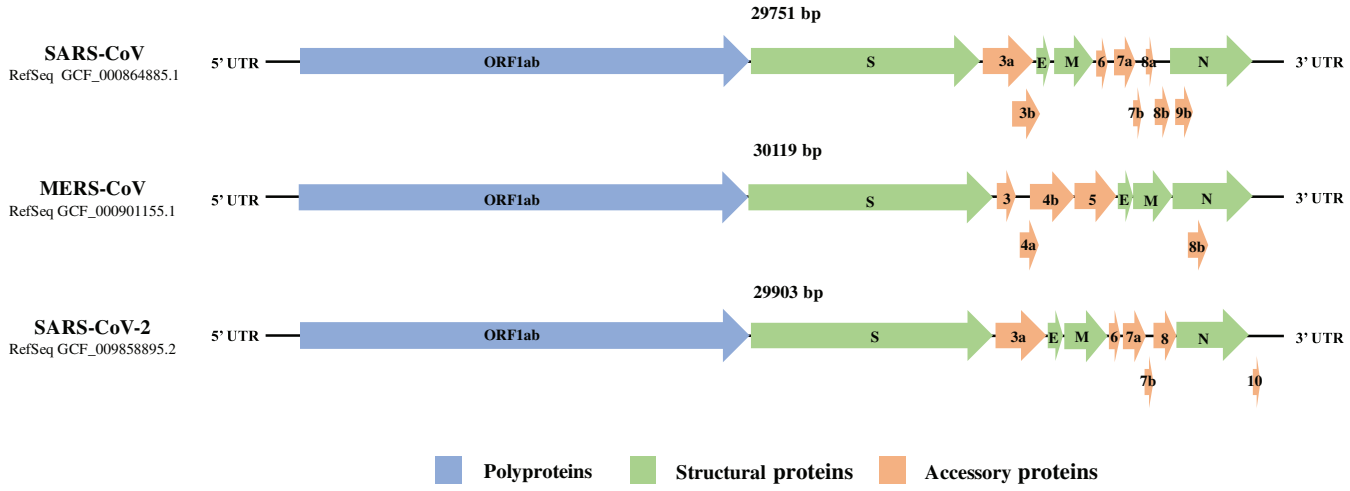

FIGURE 1 Genome structures of SARS-CoV, MERS-CoV, and SARS-CoV-2. All three CoVs contain a conserved replicase domain (ORF 1ab) (blue). The structural genes (green) S, E, M and N are common features to all CoVs, they encode the structural proteins spike, envelope, membrane, and nucleocapsid, respectively. The accessory genes (orange) are unique to different CoVs

be used in a short period (3-5 days) as appropriate, and the dose should not exceed 1 to 2 mg/kg/d of methylprednisolone.[22] Moreover, it should be considered that glucocorticoid treatment could delay clearance of viral RNA because of immunosuppression.

2.1 | Specimens

Appropriate specimen collection and processing is the first and very important step for laboratory diagnosis. Specimen types of patients with respiratory virus infection are diverse. Viral RNA could be detected in upper respiratory tract (URT), lower respiratory tract (LRT), stool, blood, and urine of SARS-CoV, MERS-CoV, and SARS-CoV-2 infected persons. Among them, URT specimens, as well as available LRT specimens in severely affected patients must be collected for diagnosis and should be tested repeatedly if the first testing is negative in patients with epidemiological history and suspected symptoms of SARS-CoV-2 infection. It was reported that SARS-CoV-2 RNA could be detected in stool, blood, or urine even if it could not be detected in URT specimens,[23,24] so collecting stool, blood or urine samples if condition permits is helpful to improve positive rate when LRT specimens are unavailable. To demonstrate viral clearance during treatment, samples should also be collected and tested repeatedly. WHO recommended that the frequency of specimen collection should be at least every 2 to 4 days until there are two consecutive negative results in a clinically recovered patient at least 24 hours apart.[25] Also, Chinese National Health Commission and the US Centers for Disease Control and Prevention (CDC) recommended that negative results of real time reverse transcription-polymerase chain reaction (rRT-PCR) testing for SARS-CoV-2 from at least two sequential respiratory tract specimens collected at least 24 hours apart can be considered to discontinue transmission-based precautions.[25,26]

The quality of specimens can be affected by several steps including operation of specimen collection, transport, and storage. For URT and sputum samples, sample quality depends greatly on the operation of the collectors. Firstly, synthetic fiber swabs with plastic shafts and sterile container were recommended to use when collecting, while calcium alginate swabs or swabs with wooden shafts should not be used as they may contain substances that inactivate viruses and inhibit PCR testing.[27] Secondly, to get enough virus infected cells, swabs must be inserted deep enough. In detail, nasopharyngeal (NP) swab must be inserted through the nares parallel to the palate, and oropharyngeal (OP) swab needs to be inserted into posterior pharynx and tonsillar areas.[28,29] Several studies have reported that combined NP and OP specimens could increase the sensitivity of detecting respiratory viruses.[30-32] As seen in Table 1, although using different RT-PCR methods targeting different genes, the threshold cycle (CT) value of NP and OP swabs was much lower than NP or OP swab alone, suggesting that in accordance with other respiratory viruses, combining NP and OP swabs together could increase viral load and the sensitivity of SARS-CoV-2 RNA detection. Sputum is often confused with saliva. Although SARS-CoV-2 could be detected in saliva,[33] sputum is preferred by several guidelines.[27,29,34] Patients should cough deeply so that sputum rather than oral secretions is collected. Dehydration may lessen the fluid in the lungs and make it hard to produce sputum, hence drinking water before collection can increase availability. To avoid contamination, all types of samples must be transported in sterile containers. To ensure sample stability, storage condition should be strictly controlled, which is summarized with other key points of specimen collection in Table 2.

After specimen collection, different methods should be used to process specimens for different purposes. For isolation and culture of virus, centrifuging samples to remove cellular debris, and then inoculating the supernatant on human airway epithelial cells, Vero E6 cells or Huh-7 cells. It took about 96 hours for SARS-CoV-2 to be successfully cultured in human airway epithelial cells, and took about 6 days to be cultured in Vero E6 or Huh-7 cells.[10,22] The isolation and culture should be conducted at BSL-3, and laboratory workers should wear protective equipment, including disposable gloves, solid front or wrap-around gowns, scrub suits, or coveralls with sleeves that fully cover the forearms, head coverings, shoe covers or dedicated shoes, eye protection, and respiratory protection.[35] Inactivation of viruses without reducing detection efficiency is required for testing RNA of high pathogenic CoVs at BSL-2 and protecting experimenters from infection. Trizol, Trizol LS (Life Technologies), and buffer AVL (Qiagen) have been standard methodology for purifying and extracting viral RNA for years. Guanidine salt in these agents can inhibit nuclease, thereby ensuring viral RNA is not degraded. Viral RNA in samples placed in buffer AVL was stable for at least 48 hours at 32°C, and at least 35 days at either 4°C or −20°C.[36] In addition, the powerful denaturing activity of guanidine isothiocyanate in these reagents could denature and dissolve protein, thus effectively inactivating enveloped viruses. The phenol component of Trizol could also disrupt membranes and denature proteins. These reagents were shown to inactivate alphaviruses, flaviviruses, filoviruses, bunyaviruses, and ebola virus.[37-39] Kumar, et al. confirmed that AVL, Trizol and Trizol LS could completely inactivate MERS-CoV within 10 minutes' room temperature incubation.[40] Thus, although many methods have been verified to effectively inactivate SARS-CoV and MERS-CoV,[41] we suggest handling samples in these reagents, as well as other virus retention reagents containing guanidine salt would be an effective way to inactivate and stabilize viruses, without affecting subsequent molecular testing of SARS-CoV-2. Since CoVs are sensitive to heat, heating inactivation of samples could effectively inactive virus if SARS-CoV-2 antibody needs to be tested at BSL-2. However, it should be noticed that heat inactivation could significantly interfere with the levels of antibodies, and might cause false-negative results. It was reported that the anti-SARS-CoV-2 IgM levels of all samples decreased by an average level of 53.56%, and the IgG levels were decreased in 64.71% samples by an average level of 49.54% after heating at 56°C for 30 minutes.[42]

It has been reported that viral loads of SARS-CoV and MERS-CoV in respiratory specimens always peak in the second week after symptom onset, and viral loads in LRT specimens were higher than in URT specimens.[43-45] As seen in Table 3, positive rate of SARS-CoV and MERS-CoV RNA in LRT specimens was about 100% at the first

2 weeks of illness, while it remained relatively lower in URT specimens. So that LRT specimens are preferred to test if available. Now, several studies have shown the viral kinetics of SARS-CoV-2 in respiratory specimens. Zhang, et al. reported that only 50% of OP swabs were positive with a CT value of 32.1 (30.9-32.75) at first sampling after symptom, while after 5 days, only 25% of oral swabs were positive with a CT value of 26.95 (25.98-27.75).[23] As seen in Tables 1 and 3, differently to SARS-CoV and MERS-CoV, SARS-CoV-2 viral loads in respiratory specimens often peak in the first week of illness and decrease thereafter.

Evidence showed that viral RNA could be detected in blood and feces,[33,46] raising the possibilities of blood transmission and oral-fecal transmission. A study of 2134 SARS-CoV infected specimens showed that the rate of viral shedding in feces was low in the first 5 days of illness (up to 28%), but rose gradually to peak at around 70% at 9 to 14 days with very high titers, even higher than in nasopharyngeal aspirates.[44] Other studies also showed that the positive rate for stool specimens peaked at Weeks 2 and 3, with a higher diagnostic yield than pooled throat and nasal swabs, and nasopharyngeal aspirate specimens.[47-49] MERS-CoV was detectable in stools in the second week after illness.[50] Different from SARS-CoV, only 14.6% of stool samples yielded viral RNA.[51] Recently, viral RNA of SARS-CoV-2 was reported in stools, and the virus was isolated. The kinetics of viral loads in stools are still not very clear. Data showed that SARS-CoV-2 RNA can be detected in stools of 53% (9/17) confirmed cases at the first 2 weeks of illness, with viral loads ranging from 550 to 1.21×10^5 copies/ml.[52] Another study showed that 25% SARS-CoV-2 infected patients had detectable viral RNA with a CT value of

TABLE 1 Viral kinetics (CT value or log10 copies/mL) of SARS-CoV-2 in respiratory specimens

	NP swab		OP swab		NP and OP swabs[46]		sputum[46]		Saliva[33] Log10 copies/mL
Days of illness	n = 17[110]	n = 1[111]	n = 17[110]	n = 1[111]	n = 1	n = 1	n = 1	n = 1	n = 12
0	31	—	26	—	—	—	—	—	5.5
1	—	—	—	—	—	—	—	—	6.4
2	21	—	40	—	25.05	—	—	—	6.5
3	32	—	34	—	28.33	—	27.52	—	6.15
4	32	18—20	36	21—22	—	—	—	—	6.5
5	27	—	35	—	32.15	—	22.05	—	2.6
6	—	—	36	—	28.26	—	30.99	—	4.9
7	34	23–24	36	32—33	28.56	—	24.92	—	5
8	35	—	—	—	29.66	—	27.36	—	2.5
9	40	—	40	—	30.38	—	ud	—	0
10	36	—	39	—	33.4	—	29.7	—	0
11	38	33—34	34	36—40	35.09	—	29.96	—	2
12	37	37—40	39	ud	32.2	—	32.5	—	—
13	36	—	37	—	ud	—	32.63	—	—
14	40	—	36	—	33.27	35.43	ud	32.29	—
15	39	—	39	—	ud	26.89	ud	—	—
16	40	—	40	—	ud	ud	ud	—	—
17	39	—	40	—	—	32.61	—	—	—
18	40	—	40	—	—	ud	—	—	—
19	40	—	40	—	—	—	—	—	—
20	40	—	40	—	—	ud	—	ud	—
21	38	—	40	—	—	ud	—	—	—
22	—	—	—	—	—	ud	—	ud	—
23	—	—	—	—	—	ud	—	ud	—
24	—	—	—	—	—	ud	—	ud	—
25	—	—	—	—	—	36.69	—	ud	—
26	—	—	—		—	ud	—	ud	—

Note: Reference 110 targets N and ORF1b gene, Reference 111 targets N gene. Reference 46 targets RdRp gene and E gene, while only the results of RdRp gene are summarized in this table. Reference 33 targets S gene. Raw data were not published in References 110 and 33, so the CT values and viral loads are estimated from figures. The finding of undetectable in swabs in between CT values of 26.89 and 32.61 might be caused by the poor quality of specimen collected in D16, which was not discussed in the original article. Time indicated in Reference 33 is the time after hospitalization.
Abbreviations: NP swab, nasopharyngeal swab; OP swab, oropharyngeal swab; ud, undetected.

TABLE 2 Key points of specimen collection

Specimen type	Purpose of collection	Collection materials	Transport and storage	Guarantee of sample stability, and inactivation without influencing NAT test	Avoid false negative results of NAT caused by specimen quality
Nasopharyngeal and oropharyngeal swabs	Collect for diagnosis	Synthetic fiber swabs with plastic shafts, sterile container	1. If could test in 24 hours, store at 4°C. Otherwise store at −70°C 2. Serum could be store at 4°C for 3 days, and stored at ≤−20°C for long-stem preservation 3. If long-distance transport is required, use dry ice or other refrigeration methods 4. Avoid repeated freezing and thawing	Virus retention medium containing guanidine salt	1. Set positive, negative, and inhibition control when extraction and amplification 2. Test human RNase P gene simultaneously
Sputum	Collect for diagnosis if produced	Sterile container			
Bronchoalveolar lavage	Collect in severe patients for diagnosis	Sterile container			
Endotracheal aspirate, and nasopharyngeal aspirate		Sterile container			
Blood	NAT test for improve diagnosis rate, monitor viremia, epidemiological surveillance	Vacuum blood collection tubes with EDTA anticoagulant			
Serum	Collect 2 samples acute and convalescent for antibody test to monitor seroconversion, epidemiological surveillance	Anticoagulant-free blood collection vacuum tubes			
Stool	Improve diagnosis rate when LRT specimens are unavailable	Sterile container			
Urine	Improve diagnosis rate when LRT specimens are unavailable	Sterile container			

Note: "Collection materials" and "Transport and storage" reference the guidelines of 2019-nCoV laboratory testing delivered by WHO and Chinese National Health Commission.[29,34]
Abbreviation: LRT, lower respiratory tract.

TABLE 3 Viral kinetics of SARS-CoV, MERS-CoV, and SARS-CoV-2

Sample type	Time of illness	SARS-CoV	MERS-CoV	SARS-CoV-2	
URT	Week 1	32%, 5.36[①43]; 58%, 4.18[①44]; 39%[②44]	75.5%, 4.5[③51]; 25%, 5[③45]; 33.3%, 5[⑤45]	5.3[③52]; 31.5 CT[⑤110]; 36 CT[③110] 50%, 32.1 CT[③23]; 100%, 30.5 CT[⑤112]	
	Week 2	68%, 7.28[①43]; 60%, 5.25[①44]; 32%[②44]; 42.3%, 5.8[①49]	45.8%, 3.5[③51]; 88.2%, 5.5[③45]; 23.5%, 4.5[⑤45]	3.8[③52]; 37 CT[⑤110]; 38 CT[③110]; 25%, 31.65 CT[③23]; 94.4%, 31.95 CT[⑤112]	
	Week 3	4.99[①43]; 39%, 4.77[①44]; 25%[②44]	0[③51]; 27.3%, 4.5[③45]; 5.88%, 5.5[⑤45]	40 CT[⑤110]; 38 CT[③110]; 69.2%, 33.05 CT[⑤112]	
LRT	Week 1	100%[44]	100%, 8[51]; 100%, 6[④45]	6.1[⑥52]	
	Week 2	100%[44]	93%, 7[51]; 100%, 6.8[④45]	5.8[⑥52]	
	Week 3	67%[44]	66.7%, 5[51]; 100%, 6.3[④45]	N/A	
Stool	Week 1	47%, 6.52[44]	16.7%, 4.5[51]	25%, 31.65 CT[③23]; 75%, 28.2 CT[112]	53%, 2.74-5.08[52]
	Week 2	97%[43]; 70%, 7.95[44]; 87.2%, 7.0[49]	14.3%, 4[51]	37.5%, 26.5 CT[③23]; 42.9%, 30.3 CT[112]	
	Week 3	54%, 5.33[44]	0[51]	N/A	
Blood	Week 1	33%[44]; 50%, 2.37[53]	48.1%, 4[51]; 10%, 4.8[45]	0[112]	40%[23]
	Week 2	25%[44]; 50%, 2.59[53] 41.5%, 2.7[49]	25%, 3[51]; 29.4%, 4.2[45]	25%, 33.15 CT[112]	
	Week 3	33%[44]; 25%, 2.18[53]	0[51]; 17.6%, 4.7[45]	N/A	
Urine	Week 1	33%[44]	9%, 2[51]	0[112]; 6.9%[24]	
	Week 2	25%[44] 28.8%, 4.4[49]	0[51]		
	Week 3	14%[44]	0[51]		

Note: Data are expressed as "positive rate, viral load (log10 copies/mL or CT value)," which are converted directly from raw data or estimated from figures. Respiratory sample type:[①]NP aspirates, [②]Other URT specimens consisted of throat and nasal swabs, throat swabs, nasopharyngeal swabs, and nasal swabs, [③]throat swabs, [④]sputum or tracheal aspirate, [⑤]nasopharyngeal swabs, [⑥]Sputum. Time indicated in References 51 and 23 is the time of diagnosis, NOT the time of illness.

31.65 (27.53-33.23) in anal swabs at first sampling, while 5 days later, 37.5% patients could test viral RNA positive in anal swabs with a CT value of 26.5 (24.22-29.38).[23] More importantly, SARS-CoV-2 RNA can be found in anal swabs even if it cannot be detected in oral swabs.[23] Thus, it might be an optional way to improve the diagnosis rate of SARS-CoV-2 infection by testing stool samples when LRT specimens are unavailable.

Blood samples can be collected for both nucleic acid testing and serological testing. Testing of virus from blood samples could be an effective way to monitor viremia. SARS-CoV RNA was detected in 50% of plasma and 78% of serum samples during the first week of illness.[53] High-SARS-CoV viral load in serum correlated with oxygen desaturation, mechanical ventilation, and death.[49] About 33% of serum samples yielded MERS-CoV RNA at initial diagnosis, and were associated with a worse clinical course.[51,54] Patients with detectable SARS-CoV-2 RNA in the blood progressed to severe symptom stage, indicating a strong correlation of serum viral RNA with the disease severity.[55] In keeping with SARS-CoV and MERS-CoV,[53,56] plasma/serum SARS-CoV-2 quantification could also represent a potentially useful early diagnostic and prognostic tool.

2.2 | Nucleic acid tests

Specific primers and standard operating procedures for nucleic acid tests (NAT) could be established as soon as the complete genome of the virus was sequenced, making NAT the optimal method for diagnosis. Current NAT tests for RNA viruses mainly include RT-PCR, alternative isothermal amplification methods, and CRISPR-Cas13a based Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) system.[57]

Due to its simplicity, easy methodology and extensively validated standard operating procedure, RT-PCR is now the preferred and most widely used method for NAT. RT-PCR assays targeting ORF 1a, ORF 1b, S gene, N gene of SARS-CoV can detect <10 genome equivalents.[58,59] Assays targeting M gene and 3′UTR also showed high sensitivity.[60,61] Three rRT-PCR assays for routine detection of MERS-CoV have been developed and recommended by WHO.[62] The primers target upstream of the E protein gene (upE), the ORF 1b, and the ORF 1a. Among them, sensitivity for upE target was 3.4 copies per reaction or 291 copies/mL of sample, which was considered highly sensitive and recommended for screening.[63] The ORF 1a assay was considered of equal sensitivity with the upE assay, while the ORF 1b assay was considered less sensitive than the ORF 1a assay.[63,64] An alternative approach involved in two rRT-PCR assays targeting the MERS-CoV N gene with a sensitivity of ≤10 copies/reaction, which can complement upE and ORF 1a assays for screening and confirmation has also been published and authorized for emergency use as an in vitro diagnostic test for MERS-CoV by the US Food and Drug Administration (FDA).[65]

Several commercial and in-house assays that detect SARS-CoV-2 RNA have been developed. Chu, et al reported two one-step quantitative rRT-PCR assays targeting ORF1b and N gene that could detect SARS-CoV-2 < 10 copies/reaction, while the N gene assay was about 10 times more sensitive than the ORF1b gene assay in detecting positive clinical specimens.[11] It might be possible that clinical samples contain infected cells expressing subgenomic mRNA, resulting in more N gene copies.[66] Corman, et al. developed assays targeting E gene and RNA-dependent RNA polymerase (RdRp) gene that obtained best sensitivity with limit of detection (LOD) of 5.2 and 3.8 copies/reaction, respectively, while N gene assay was slightly less sensitive.[67] Furthermore, when using other basic RT-PCR reagents (Taqman Fast Virus 1-step Master Mix), another lab obtained the sensitivity of 3.2 copies/reaction for E gene assay and 3.7 copies/reaction for RdRp gene assay.[67] Some groups from different countries shared their protocols of in-house developed molecular assays. In summary, Chinese CDC recommended primers and probes targeting ORF1ab and N gene.[68] In Germany, Charité recommended E gene assay as first line screening assay with technical LOD of 5.2 copies/reaction, and RdRp gene assay as confirmatory assay with technical LOD of 3.8 copies/reaction.[67] Scientists from Hong Kong University recommended the N gene RT-PCR as a screening assay and the ORF1b assay as a confirmatory one.[69] Ministry of Public Health of Thailand recommended assay targeting N gene.[70] National Institute of Infectious Diseases of Japan recommended nested RT-PCR assay targeting ORF1a and S gene, as well as RT-PCR targeting N gene.[71] The US CDC has developed assays including three pairs of primers targeting the N gene of SARS-CoV-2, and authorized emergency use by the US FDA.[72] Details of these assays are summarized in Table 4. Most of the in-house assays, as well as commercial kits are designed to detect two or three regions of SARS-CoV-2 genome. The different regions are amplified at the same time. Chinese National Health Commission recommended that both ORF1ab and N should be tested positive for a positive result. If only one region is positive, the result needs to be re-tested.[73] The US CDC recommended that all the N region targets should be detectable for a positive result, or be undetectable for a negative result. If only one target is positive, the result is inconclusive and need to be re-tested.[74] So, it is important that all the two or three regions tested should be positive to identify a positive case.

RT-PCR relies on sophisticated equipment that it may not be present in resource-limited regions. These limitations gave birth to alternative isothermal amplification methods, such as transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), isothermal recombinase polymerase amplification (RPA), and rolling-circle amplification (RCA).[75-80] These alternative isothermal amplification methods are simple, rapid, specific and sensitive which only need a heating block or water bath capable of maintaining a constant temperature.[81,82] Chantratita, et al developed a real time NASBA assay that could detect as little as one copy/reaction of SARS-CoV RNA.[83] Compared to RT-PCR, a RT-LAMP assay of SARS-CoV showed 100-fold-greater sensitivity, with a detection limit of 0.01 PFU.[84] In another study, detection rate of the RT-LAMP assay was lower than the RT-PCR assay for samples isolated from patients within the first 3 days of disease onset, and was similar to RT-PCR when detecting specimens sampled from patients with more than

TABLE 4 In-house assays for detecting SARS-CoV-2

Country	Institute	Gene target	Primers and probes	Sensitivity
China	China CDC	ORF1ab	F: CCCTGTGGGTTTTACACTTAA	N/A
			R: ACGATTGTGCATCAGCTGA	
			P:FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	
		N	F:GGGGAACTTCTCCTGCTAGAAT	
			R: CAGACATTTTGCTCTCAAGCTG	
			P: FAM-TTGCTGCTGCTTGACAGATT-TAMRA	
Germany	Charité	E	F:ACAGGTACGTTAATAGTTAATAGCGT	5.2 copies/reaction screening assay
			R:ATATTGCAGCAGTACGCACACA	
			P:FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	
		RdRp	F:GTGARATGGTCATGTGTGGCGG	3.8 copies/reaction confirmatory assay
			R:CARATGTTAAASACACTATTAGCATA	
			P2:FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	
			P1:FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	
China	HKU	N	F: TAATCAGACAAGGAACTGATTA	2^{-4}-2000 TCID50/reaction screening assay
			R: CGAAGGTGTGACTTCCATG	
			P: FAM-GCAAATTGTGCAATTTGCGG-TAMRA	
		ORF1b	F: TGGGGYTTTACRGGTAACCT	2^{-4}-2000 TCID50/reaction confirmatory assay
			R: AACRCGCTTAACAAAGCACTC	
			P:FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA	
Japan	National Institute of Infectious Diseases	ORF1a	1st-F:TTCGGATGCTCGAACTGCACC	Nested RT-PCR
			1st-R:CTTTACCAGCACGTGCTAGAAGG	
			2nd-F:CTCGAACTGCACCTCATGG	
			2nd-R:CAGAAGTTGTTATCGACATAGC	
			Seq-F:ACCTCATGGTCATGTTATGG	
			Seq-R:GACATAGCGAGTGTATGCC	
		S	1st-F:TTGGCAAAATTCAAGACTCACTTT	
			1st-R:TGTGGTTCATAAAAATTCCTTTGTG	
			2nd-F:CTCAAGACTCACTTTCTTCCAC	
			2nd-R:ATTTGAAACAAAGACACCTTCAC	
			Seq-F:AAGACTCACTTTCTTCCACAG	
			Seq-R:CAAAGACACCTTCACGAGG	
		N	F:AAATTTTGGGGACCAGGAAC	Five copies/reaction Real-time RT-PCR
			R:TGGCAGCTGTGTAGGTCAAC	
			P:FAM-ATGTCGCGCATTGGCATGGA-BHQ	

TABLE 4 (Continued)

Country	Institute	Gene target	Primers and probes	Sensitivity
Thailand	National Institute of Health	N	F:CGTTTGGTGGACCCTCAGAT	N/A
			R:CCCCACTGCGTTCTCCATT	
			P:FAM-CAACTGGCAGTAACCA-BQH1	
United States	US CDC	N1	F:GACCCCAAAATCAGCGAAAT	N/A
			R:TCTGGTTACTGCCAGTTGAATCTG	
			P:FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	
		N2	F:TTACAAACATTGGCCGCAAA	
			R:GCGCGACATTCCGAAGAA	
			P:FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	
		N3	F:GGGAGCCTTGAATACACCAAAA	
			R:TGTAGCACGATTGCAGCATTG	
			P:FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1	

Abbreviations: N/A, Not applicable; RT-PCR, reverse transcription-polymerase chain reaction.

3 days of illness.[85] A RT-RCA assay for SARS-CoV could detect as few as five copies of SARS viral genome.[86] For MERS-CoV, a RT-RPA method targeting the N gene could detect virus RNA with a sensitivity of 10 copies/reaction. In addition, the run time of the RT-RPA was between 3 and 7 minutes for 10^7 and 10 molecules, respectively.[87] Several RT-LAMP methods have also been developed for MERS-CoV detection with a high sensitivity.[88-93] Further alternative isothermal amplification methods for detecting SARS-CoV-2 genome need to be established, and would show potential applicability for point of care testing (POCT) in resource-limited areas.

In 2002, Jansen et al. firstly discovered clustered regularly interspaced short palindromic repeats (CRISPR) system.[94] In 2003, it was confirmed for the first time that CRISPR-Cas9 can be used to edit human genes.[95] In 2017, combining RPA and CRISPR-Cas13a, SHERLOCK system was developed for NAT.[57] SHERLOCK detected viral particles down to 2 aM, and could discriminate between similar viral strains.[57] Recently, a protocol for detection of SARS-CoV-2 using SHERLOCK system has been published.[96] This method can detect SARS-CoV-2 in a range between 20 and 200 aM (10-100 copies per microliter of input) within an hour. However, this method has not yet been validated with real patient samples, so it should not be used for clinical purposes now.

Although many RT-PCR assays for the SARS-CoV-2 have been developed, NAT positive cases only account for about 50% of clinically confirmed cases. To raise the positive rate of NAT, standard collection, strict storage and transportation conditions, proper extraction and amplification procedures are needed. To increase test sensitivity, increasing the template volume and total reagent volume for a single run would be very useful. To improve test specificity, amplifying a second genome region for confirmation is suggested. Most importantly, appropriate controls should be established to ensure the results are reliable. For SARS-CoV, which are also suitable for other viruses, WHO recommended one negative control and one positive control for extraction, one water control and one positive control for a PCR run, and the patient sample spiked with a weak positive control to detect PCR inhibitory substances (inhibition control).[97] Except for these external controls, internal controls, including plasmid DNA, virus-like particles (VLPs), RNase P gene, and housekeeping genes of airway epithelial cells such as beta-actin have been used for NAT of respiratory viruses including SARS-CoV-2. Of all these kinds of internal controls, only RNase P presents in all types of SARS-CoV-2 infected samples and can control the quality of all procedures, from sample collection to amplification (Table 5). Thus, we recommend RNase P as internal control when amplifying SARS-CoV-2 RNA.

2.3 | Serological tests

Serological testing may be conducted for diagnosis in rare situations where NAT is not possible, for investigation of an ongoing outbreak, or for serological surveys, including to retrospectively assess the extent of an outbreak.[62] Although antibody seroconversion provides reliable proof of infection, serological testing lags behind the

TABLE 5 Internal control of nucleic acid test

Internal control type	Nucleic acid type	Preparation	Risk of contaminating sample RNA	Sample collection control	Extraction control	Reverse transcription control	Amplification control	Inhibition control	Competent or non-competent control
RNase P	RNA	Easy	No	√	√	√	√	√	×
Housekeeping gene of airway epithelial cells	mRNA	Easy	No	√	√	√	√	√	×
				Only applicable to respiratory specimens					
Plasmid DNA	DNA	Complex	High	×	×	×	√	√	√
Virus-like particles	RNA	More complex	High	×	√	√	√	√	√

detection of viral genome by molecular testing, making it not suitable for early diagnosis. In SARS, higher neutralizing antibody response was associated with a longer illness.[13] In most MERS patients, the levels of IgG and neutralizing antibodies were weekly and inversely correlated with LRT viral loads.[51] In addition, an early MERS-CoV antibody response was associated with reduced disease severity.[98] Thus, testing antibody is helpful for surveillance, prediction of disease outcome and epidemiological investigation, but not for early diagnosis.

A variety of serological assays were established for detecting SARS-CoV and MERS-CoV, mainly including enzyme-linked immunosorbent assay (ELISA), chemiluminescence assay (CLIA), immunofluorescence assay (IFA), western blot (WB), protein microarray, and neutralization. Of these methods, ELISA and CLIA are suitable for first line screening because of the large throughput, short processing time, and simple operating procedure, while neutralization assay is used as the gold standard for confirmation in many laboratories.

The basic issue in serological assays is the source of antigen. Once virus has been isolated from patients, cell lysate or supernatant of virus infected cells could be used for serological assays. These methods are most convenient and rapid, as they only need susceptible cell cultures and virus. However, they need to be performed in BSL-3, and proper inactivation of the virus without reducing immunogenicity of antigens is required, thus limiting the widespread use of virus-based serological testing. Furthermore, cross reaction is likely to happen because of the conserved sequence and structure between CoVs. By cloning immunogenic viral genes into prokaryotic or eukaryotic expression plasmids, transfecting the plasmids into bacterial or mammalian cells and purifying proteins, recombinant antigens can be developed to overcome the disadvantages of virus infected cells.[99] Recombinant antigens are safe and do not require BSL-3 containment. Importantly, although taking longer time and more steps for development, recombinant antigen based assays enable the selection of immunogenic and virus specific antigens to maximize both specificity and sensitivity, and more suitable for standardization.[100] In SARS-CoV and MERS-CoV, N and S protein are the major immunogenic proteins and the first choice for producing recombinant antigens. Antibodies to proteins S, 3a, N, and 9b were detected in the sera from convalescent-phase SARS patients. Among them, anti-S and anti-N were dominant and could persist in the sera of SARS patients until week 30, while only anti-S3 showed significant neutralizing activity.[101] Anti-N appeared earlier than anti-S, indicating that S protein-based assays may be preferable for use with convalescent sera.[102,103] Sera tend to react against both antigens with higher sensitivity.[102] Cross reaction also happened in assays using recombinant antigens. Moderate cross-reactivity between SARS-CoV and porcine CoVs was mediated through amino acids 120 to 208 of the N protein.[104] Bioinformatics analysis demonstrated a significant B-cell epitope overlapping the heptad repeat-2 region of S protein of SARS-CoV and HCoV-EMC,[105] which is known to harbor an epitope for broadly neutralizing antibody in the case of SARS-CoV.[106] As a result, convalescent SARS sera cross reacted with HCoV-EMC.[105] Thus, for SARS-CoV-2, specific recombinant antigens are required. Also, testing

antibodies with different assays targeting different regions of antigens could avoid false-positive results produced by cross reaction.

WHO declared that positive antibody test results indicated a previous infection with SARS-CoV. Seroconversion from negative to positive or a 4-fold rise in antibody titer from acute to convalescent serum indicated recent infection. No detection of antibody after 21 days from onset of illness seemed to indicate that no infection with SARS-CoV took place.[107] For MERS-CoV, the US CDC recommended ELISA as a screening test and microneutralization as a confirmatory test for MERS-CoV antibodies.[108] Where a patient has evidence of seroconversion in at least one screening assay and confirmation by a neutralization assay in samples ideally taken at least 14 days apart, this patient can be considered a confirmed case. A 4-fold increase in MERS-CoV antibody titer by neutralization tests in acute and convalescent serum samples performed in parallel is needed for confirmation.[62] For conducting serology of SARS-CoV-2, or broad CoV serology on paired samples (in the acute and convalescent phase), WHO recommended that the first serum sample should be collected in week 1 of illness and the second collected 3 to 4 weeks later, collecting at least 3 weeks after onset of symptoms if only a single serum sample can be collected.[34]

In SARS, IgG seroconversion was documented in 93% patients at mean of 20 days.[43] Positive antibody, neutralizing antibody titer of most patients was shown to peak between Weeks 5 and 8 after onset and to decline with a half-life of 6.4 weeks.[13] In most MERS patients, robust antibody responses developed by the third week of illness, and were delayed further in severely ill patients requiring mechanical ventilation.[51,98] An IgG and IgM ELISA test which used bat SARS-related-CoV (SARSr-CoV) Rp3 nucleocapsid protein (NP) as antigen was developed previously.[109] This SARSr-CoV NP is 92% identical to SARS-CoV-2 NP, thus this assay was used for serological testing of SARS-CoV-2.[23] Both IgM and IgG titres were relatively low or undetectable in first sampling (not first day of illness). On Day 5, an increase of viral antibodies can be seen in nearly all patients. IgM positive rate increased from 50% to 81%, whereas IgG positive rate increase from 81% to 100%.[23] This is in contrast to a relatively low detection positive rate from molecular test. Based on this result, serological testing might improve the detection positive rate. However, the clinical records of patients involved in this study were not available. Although the target patients were those who received around 10 days of medical treatments upon admission, it is not sure on which days of illness the high positive rate of IgG and IgM occurred. Diverse assays and more evidence are needed to confirm the IgG and IgM kinetics of SARS-CoV-2.

3 | CONCLUSION

With the progress of global integration, emerging and reemerging infectious diseases are becoming easier to transmit all over the world. The very important and first key to respond to outbreaks is early discovery. Laboratory testing plays the major role in early detection of infected persons, enabling recognition of the infection source and cutting off the transmission route. The results of laboratory testing are affected by various factors. To improve NAT capacity of SARS-CoV-2 RNA, we summarized the literature and guidelines and suggested that: (a) If LRT specimens are unavailable, collect stool and blood samples at later period of illness to improve the positive rate of NAT. (b) Increase template volume to raise the sensitivity of detection. (c) Put samples in reagents containing guanidine salt such as TRIZOL, TRIZOL LS, or AVL buffer to inactivate virus as well as protect RNA. (d) Set proper positive, negative and inhibition controls for extraction and amplification to ensure quality results. (e) Simultaneously amplify human RNase P gene as internal control to avoid false-negative results. At the time of writing this review, serological evidence of SARS-CoV-2 is still poor. Diverse assays targeting different antigens are needed. Also, collecting paired samples would help to monitor the kinetics and positive rates of serological testing in different periods of diseases.

CONFLICT OF INTEREST

All authors have no conflict interest to disclose.

ORCID

Lunan Wang https://orcid.org/0000-0003-3163-7891

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