Molecular and serological assays for SARS-CoV-2: insights from genome and clinical characteristics

Running head: molecular and serological assays for SARS-CoV-2

Authors

Jiping Shi[1,2], Dongsheng Han[1,3], Runling Zhang[1,3], Jinming Li[1,3,4]*, Rui Zhang[1,2,3,4]*

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

[2] Peking University Fifth School of Clinical Medicine, Beijing Hospital, Beijing, P.R. China;

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

[4] Beijing Engineering Research Center of Laboratory Medicine, Beijing, P.R. China.

*** Correspondence**

Rui Zhang, Ph.D., Jinming Li, Ph.D., National Center for Clinical Laboratories, Beijing Hospital, No.1 Dahua Road, Dongdan, Beijing, 100730, P.R. China. Tel: 86-10-58115053; Fax: 86-10-65212064; e-mail: ruizhang@nccl.org.cn (Rui Zhang), jml@nccl.org.cn (Jinming Li).

Keywords

SARS-CoV-2, molecular assays, serological assays, application, quality assurance.

Nonstandard Abbreviations

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CDC, Centers for

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Disease Control and Prevention; COVID-19, corona virus disease 2019; WHO, World Health Organization; RT-PCR, reverse transcription real-time PCR; UTRs, untranslated regions; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; Hel, helicase; RBD, receptor-binding domain; BALF, bronchoalveolar lavage fluid; FDA, Food and Drug Administration; EUA, Emergency Use Authorizations; NMPA, National Medical Products Administration; QCMs, quality control materials; CMIA, chemiluminescent microparticle immunoassay; LFI, lateral flow immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescent immunoassay.

Abstract

BACKGROUND: The ongoing outbreak of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has posed a challenge for worldwide public health. A reliable laboratory assay is essential both to confirm suspected patients and to exclude patients infected with other respiratory viruses, thereby facilitating the control of global outbreak scenarios.

CONTENT: In this review, we focus on the genomic, transmission and clinical characteristics of SARS-CoV-2, and comprehensively summarize the principles and related details of assays for SARS-CoV-2. We also explore the quality assurance measures for these assays.

SUMMARY: SARS-CoV-2 has some unique gene sequences and specific transmission and clinical features that can inform the conduct of molecular and serological assays in many aspects, including the design of primers, the selection of specimens and testing strategies at different disease stages. Appropriate quality assurance measures for molecular and serological assays are needed to maintain testing proficiency. Because serological assays have the potential to identify later stages of the infection and to confirm highly suspected cases with negative molecular assay results, a combination of these two assays is needed to achieve a reliable capacity to detect SARS-CoV-2.

Introduction

The emergence of pathogenic coronaviruses has been a global public health challenge in recent years. On January 7, 2020, the China Centers for Disease Control and Prevention (CDC) officially announced the outbreak of a novel pneumonia caused by a pathogenic coronavirus in Wuhan, China. Subsequently, this novel coronavirus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and its associated clinical syndrome was named corona virus disease 2019 (COVID-19) *(1)*. SARS-CoV-2 is wreaking havoc in 215 countries, yielding a total of more than 4,000,000 confirmed cases and 250,000 deaths throughout the world *(2)*, and has been declared a public health emergency of international concern by the World Health Organization (WHO) *(3, 4)*.

The ability to reliably diagnose SARS-CoV-2 would facilitate the identification of infected patients and the therapy of COVID-19 *(5)*, so development of a reliable assay has been one of the foremost matters for public health and clinical interventions. Nucleic acid assays by reverse transcription real-time PCR (RT-PCR) are the main approach to diagnose infections, and repeated serological antibody testing over time represents another valuable approach. For molecular and serological assays, the design of reagents, selection of specimens, and detection strategies in different stages are determined by the characteristics of SARS-CoV-2. Therefore, elucidating the unique regions in the genome as well as the specific transmission and clinical characteristics of SARS-CoV-2 is key to detection. Negative assay results with some current molecular assays seen in clinical cases highly suspected of COVID-19 have lessened the clinical

acceptance of these assays in favor of radiologic assays in some cases *(6)*. The reasons for negative results in such cases may involve low viral loads in specimens and unverified reagents and inadequate testing operations, so the quality assurance of reagents and testing is indispensable. Consequently, a thorough examination of the characteristics of the virus, recent assays and related quality assurance measures is needed and should help facilitate the diagnosis of SARS-CoV-2.

In this review, we first focus on the current knowledge regarding the genomic, transmission and clinical characteristics of SARS-CoV-2, and summarize insights gained from these characteristics for the development of molecular and serological assays. As some testing reagents have been approved for immediate use without substantial clinical validation, we also analyzed quality assurance measures needed for these assays to assure the accuracy of results. We believe that a comprehensive summary of the assays and quality assurance systems is important for virus detection, thereby facilitating better control of the epidemic.

Genome and Phylogeny

GENOME OF SARS-COV-2

SARS-CoV-2 is a linear, single-stranded, positive-sense RNA virus, and the whole viral genome is approximately 29903 nt (GenBank, MN908947.3) in length *(7, 8)*. The virus genome contains two flanking untranslated regions (UTRs) and a long open reading frame (ORF), and has the following arrangement: 5'-replicase (ORF1ab)-structural proteins [Spike (S)-Envelope (E)-Membrane (M)-Nucleocapsid (N)]−3' *(9)* (**Fig. 1A**). The ORF regions, which account for approximately 2/3 of the genome,

encode nonstructural proteins *(10)*. These nonstructural proteins can be cleaved by viral 3C-like protease and papain-like protease to form viral RNA-dependent RNA polymerase (RdRp) and helicase (Hel), which guide viral genome replication, transcription and translation *(11)*. The 3'-end of the genome encodes four structural proteins, including spike, envelope, membrane, and nucleocapsid proteins, as well as accessory proteins *(12)* (**Fig. 1B**). Notably, as a positive-stranded RNA virus, SARS-CoV-2 has a high mutation rate due to the lack of proofreading activity of polymerases *(13)*.

HOMOLOGY, AND PHYLOGENY OF SARS-COV-2

The homology and differences in genome between SARS-CoV-2 and other bat-related viruses are pivotal for laboratory assays. Sequencing of the genomes of these viruses has revealed that SARS-CoV-2 is closely related to bat-SL-CoVZC45 and bat-SL-CoVZXC21 with a similarity of 88% *(14)*. The notorious SARS-CoV and MERS-CoV (responsible for the original SARS outbreak in 2002-2003 and the Middle East respiratory syndrome in 2012-2014, respectively) have similarities of approximately 79% and 88% to SARS-CoV-2, respectively (**Table 1**) *(14, 15)*. Compared with bat-SL-CoVZC45 and bat-SL-CoVZXC21, the E gene is the most conserved region in SARS-CoV-2, with a similarity >93%, followed by the M and N gene regions; the sequence identity of ORF1a (approximately 90%) is greater than that of ORF1b (approximately 86%); notably, the S gene exhibits the lowest similarity, at approximately 75% (**Table 1**) *(14)*. Phylogenetic analysis indicates that SARS-CoV-2 clusters with members of the sarbecovirus subtype of betacoronaviruses, and the

relationships between these pathogenic viruses have been revealed previously *(8, 14)* (**Fig. 1C**). In summary, these sequence similarity studies revealed the genetic differences of SARS-CoV-2 from other bat-related viruses and can be used to suggest the targeted genes that should be chosen for molecular assays.

Transmission and Clinical Characteristics

TRANSMISSION OF SARS-COV-2

SARS-CoV-2 likely originated from the Chinese horseshoe bat *(14)*, and the initial patients were infected mainly by wild animals *(14)*, while the subsequent cases were infected by these patients *(16)*; sustained human-to-human transmission brought about a global pandemic. As is typical of respiratory viruses, the main spread routes of SARS-CoV-2 include intimate contact and respiratory droplets *(17)*. Moreover, SARS-CoV-2 has been detected in feces and urine *(18, 19)*, indicating the risk of fecal-oral transmission, and aerosol or contact propagation caused by excreta pollution *(20)*. The virus is rampant not only in the respiratory system but also in other organs, which can be explained by the distribution of virus affinity receptors. The interaction of the receptor-binding domain (RBD) with receptors of host cells involves the S1 and S2 subunits-mediated receptor binding and membrane fusion, respectively *(21)*. SARS-CoV-2 has been proven to enter and infect the host cells as mediated by binding to ACE2 receptors (**Fig. 2A**) *(22)*. ACE2 receptors are abundantly expressed in the respiratory system; however, they are more highly expressed in the lower respiratory tract than in the upper respiratory tract, with the expression being the highest in alveolar epithelial cells *(23)*. In addition, ACE2 receptors are also highly present in the epithelial

cells of the oral mucosa, tongue, small intestine, heart, and kidney (**Fig. 2B**) *(23, 24)*. Therefore, SARS-CoV-2 infects the respiratory system initially, and virus replication in alveoli causes alveolar vascular rupture as the disease progresses; then, the virus may spread throughout the body through blood, infecting other organs expressing ACE2 receptors *(25)*.

CLINICAL CHARACTERISTICS OF INFECTED PATIENTS

After infection of SARS-CoV-2, the general incubation period is 1 to 14 days, usually 3-7 days *(26)*. The main clinical symptoms of infected patients involve fever, cough, expectoration, headache, myalgia or fatigue, dyspnea, diarrhea, nausea and vomiting *(27)*; gastrointestinal symptoms have been reported in 10% of cases, a higher proportion than found in other coronaviruses *(28)*; however, some infected patients do not present with typical symptoms and thus represent asymptomatic infections *(29)*.

For laboratory testing, the viral loads in different stages, populations, and organs are discrepant. In most patients, the average days from onset to the early, progressive, and recovery stages are 4 (range 2 -6), 12 (range 7 -19), and 20 (range 10 -33) days, respectively *(30)*. The virus rapidly replicates in the first few days *(31)*, reaching a peak (approximately 10^4-10^7 copies/ml) in the early or progressive stages, and then declines with the viral load mostly lower than 10^4 copies/ml in the recovery stage. (**Fig. 3**) *(32-34)*. The total viral shedding time is approximately 20 days *(34)*. Therefore, the viral load in the early and progressive stages is significantly higher than that in the recovery stage *(30)*. Of different populations examined, the viral load in elderly patients was reported to be the highest *(35)*. Viral RNA has been detected most frequently from

nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage fluid (BALF), saliva, anal swabs, and serum specimens *(31, 36)*. Generally, the viral load was 6-fold higher in respiratory tract specimens (approximately 4.33×10^4 RNA copies/ml, including nasopharyngeal aspirates/swabs, throat swabs, saliva, and sputum) than in non-respiratory specimens (approximately 7.06×10^3 RNA copies/ml, including plasma, urine, and feces/rectal swabs) *(37)*. For different specimens from the respiratory tract, the viral load detected in sputum was higher than that detected in the nose and throat *(30, 34)*. In one study, the viral load in sputum was reported to be 7.52×10^5 copies/ml, approximately 10-fold higher than that of the pharyngeal swab, which was 7.99×10^4 copies/ml, while the load in a nasal swab was 1.69×10^5 copies/ml *(32)*.

Several results of virus detection by RT-PCR were found to be unconvincing in clinical use. For instance, a child was found to be virus positive based on stool specimens for at least 9 days, but the respiratory tract specimens were still negative according to RT-PCR *(38)*; additionally, some clinically highly suspected patients had negative results for the virus in oral swabs *(39)*. Hence, one single nucleic acid test of a single sample may miss an infected patient.

Molecular Assays

CONSIDERATIONS FOR THE DEVELOPMENT OF MOLECULAR ASSAYS

The diagnosis of SARS-CoV-2 infection in clinical laboratories worldwide mainly depends on the detection of the viral nucleic acids by RT-PCR *(40)*. For RT-PCR assays, what specific genes are selected as the target region is crucial. The E gene of SARS-CoV-2 has been shown to be highly similar to that of other coronaviruses, a fact that

can serve as the first-line screening tool *(41)*; in addition, SARS-CoV-2 has low homology with other bat-related viruses in the ORF1b (involving RdRp), N and S genes *(10, 41, 42)*, which are relatively specific genes worth targeting. In clinical RT-PCR protocols, single-target and multi-target assays are commonly employed. The former refers to the use of one set of primers and related probes to target a single gene region of virus in the RT-PCR system, whereas the latter uses multiple sets of primers and their related probes, such as reagents targeting the ORF1ab and N genes in China and those that target the N gene in Thailand *(43)*. Since SARS-CoV-2 is a single-stranded RNA that is prone to mutation, increasing the number of specific targets in the RT-PCR system will increase the testing sensitivity. However, if the primers are not specifically designed, multiple targets in the PCR system could interfere with each other, reducing the amplification efficiency and clinical sensitivity *(44)*. In summary, selecting an appropriate number of targets for RT-PCR protocols depends on the detection purpose, the specific primer sequence, and the results of performance verification.

Currently, many RT-PCR protocols for SARS-CoV-2 have been approved by the U.S Food and Drug Administration (FDA) Emergency Use Authorizations (EUA) or National Medical Products Administration (NMPA), or were listed on the WHO website (**Table 2**). For instance, in the RT-PCR protocol for SARS-CoV-2 developed in Germany, primers targeting the E gene can screen all bat-related coronaviruses, and primers for the RdRp and N genes were specifically used to confirm the presence of SARS-CoV-2 *(45)*. Furthermore, performance verification results revealed that the assay based on RdRp gene had a higher analytical sensitivity than those based on N and

E genes *(41)*. In the United States, the CDC has published their RT-PCR protocols for SARS-CoV-2, which are based on three primers (N1, N2, and N3) for the N gene *(43, 46)*. Moreover, the high-throughput Cobas 6800 assay by Roche Diagnostics targets the ORF1a and E genes, and is capable of offering fast and reliable results. The specific primers for ORF1a confirm SARS-CoV-2 infection, and primers for the conserved E genes are used for all sarbecovirus detection *(47, 48)*. In China, reagents mainly target the ORF1ab and N genes according to the China CDC *(49)*. Although protocols based on different primers have been developed and approved quickly, the lack of a thorough performance validation of and comparison between these protocols is considered a key gap. Recently, a study compared two protocols developed by Germany and CDC, respectively *(45, 46)*. The results revealed that the analytical sensitivity and specificity of the N1, RdRp (modified by the study), and E assays were higher than those of the other assays *(50)*. In summary, the characteristics of the SARS-CoV-2 genome can guide the design of targeted gene regions and performance verification for molecular assays.

INSIGHTS FOR THE APPLICATION OF MOLECULAR ASSAYS

First, diagnosis of the infection mainly depends on positive viral nucleic acid test results and not on clinical symptoms *(51)*; asymptomatic infections that yield positive viral nucleic acid results may be potential sources of infection *(29, 52)*. Second, the selection of specimens for molecular assays is crucial. Viral loads of respiratory tract specimens are highest in BALF, followed by the sputum, nasal swabs, and pharyngeal swabs; when situation permits, sample selection should be prioritized in the same order

to prevent misdiagnosis caused by insufficient viral load (**Fig. 2C**) *(53, 54)*. Recent studies have confirmed the validity of this selection priority. The positive detection rate of nasopharyngeal swabs in 14 of 18 patients was higher than that of oropharyngeal swabs, and the PCR Ct values of the former were also lower *(34)*. Nasal and pharyngeal swab samples exhibited a lower positive rate compared with BALF and sputum samples *(55)*. In addition, a recent study evaluated the positive detection rate of 1070 different specimens collected from 205 infected patients, and the results revealed that the positive rate of BALF was highest (14/15, 93%), followed by sputum (75/104, 72%), nasal swabs (5/8, 63%), throat swabs (126/39,32%), stool (44/53, 29%), blood (3/307,1%), and urine (0/72, 0%) *(53)*. Therefore, if multiple sampling methods can be adopted for patients, specimens with high positive rates, such as BALF or sputum, should be selected preferentially.

Third, a negative result from an oral-nasopharyngeal swab is not sufficient for a hospital discharge *(56)*. In clinical application, collecting BALF requires complicated procedures and is not suitable for all patients; and many patients have nonproductive cough, so the feasibility of sputum sampling is low. Therefore, nasopharyngeal and oropharyngeal swabs serve as the main sample types for clinical testing; however, these samples may yield negative results on molecular assays due to low viral loads, leading to misdiagnosis. Even if results from nasopharyngeal or oropharyngeal swab are negative, the virus may harbor in the gut or the recovered patient may still be a virus carrier *(57)*. Consequently, negative testing results should be combined with the lack of clinical symptoms to properly guide discharge, and a certain period of observation is

essential. Fourth, sampling of different sites in suspected persons or repeatedly sampling at different infected stages may prevent false negative results from insufficient viral loads. For instance, virus positivity in stool specimens but negativity in respiratory tract specimens can suggest an infection *(38)*. Some patients with positive chest CT findings showed negative molecular results from mouth swabs, and after repeated testing from mouth swabs, they were all finally confirmed to have infections *(39)*.

Quality Assurance of Molecular Assays

In the diagnosis of suspected cases by RT-PCR, several undesirable conditions may occur: (1) false positive: a specimen that does not contain SARS-CoV-2 tests positive for the virus; (2) false negative: a specimen containing a sufficient quantity of SARS-CoV-2 tests negative for the virus; or (3) a specimen not containing sufficient SARS-CoV-2 tests negative for the virus, a result that may not be consistent with highly suspected results from radiography. For instance, in the diagnosis of a group of suspected patients, the positive rates from the RT-PCR of throat swabs and chest CT imaging were different, approximately 59% (601/1014) and 88% (888/1014), respectively *(6)*. Although the CT test can yield some false positives, a large number of highly suspected cases as determined by CT cannot be confirmed by RT-PCR and may lead to diagnostic confusion with possible serious outcomes. In summary, these three discrepant scenarios can mislead diagnosis and clinical management. Quality assurance would help improve the accuracy and reliability of SARS-CoV-2 molecular assays, especially in the face of this highly contagious virus; hence, robust quality assurance

should be implemented in each clinical laboratory.

FALSE POSITIVE

False positive results are mainly caused by cross-contamination between specimens or residual contamination from a prior laboratory amplification, and the cross-reaction of other viruses due to nonspecific primers. Solutions include keeping the clinical laboratory environment clean, and standardizing the procedures of specimen transportation and detection to prevent contamination *(43)*. Several negative quality control materials (QCMs) should be randomly placed among clinical samples daily for analysis, a method that can be effective in identifying systemic problems that lead to false positive results (**Fig. 2C**) *(58)*. Finally, using primers that target the unique genomic regions of SARS-CoV-2 can specifically detect the virus and reduce cross-reactivities.

FALSE NEGATIVE

False negative results are mainly due to unreliable detection reagents and nonstandard testing operations. Some assays target two or more regions of the viral genome for detection; however, the sensitivity of reagents in different regions may be different, or competition may occur between these different targets *(44)*, which results in false negatives. Since RNA viruses have strong genetic variability *(13)*, mismatches between primers and target sequences caused by mutations can lead to poor detection performance and false negatives *(59)*. Inactivation before testing should also be considered, and common approaches include thermal and chemical inactivation. High temperature can denature viral structural proteins to reduce their invasiveness; however,

studies found that approximately half of the weakly positive samples (7/15, 46.7%) were RT-PCR negative after thermal inactivation of SARS-CoV-2 at 56°C for 45 min in at least one parallel testing *(61)*. The reason might be that released RNA from lysed viruses is degraded due to the breakage of chemical bonds caused by high temperature *(60, 61)*, and the degrees of degradation increased with temperature elevation *(60)*. Compared with thermal inactivation, inactivation by guanidinium lysis had a smaller impact on RT-PCR results, with fewer false negatives (2/15/, 13.3%) *(62)*. Currently, the instructions of most kits do not contain inactivation information, and several kits approved in China indicate that samples should be inactivated by thermal inactivation at 56°C for 30 min (immunofluorescence, Anbio) or chemical inactivation based on guanidine buffer (RT-PCR, Wuhan Easydiagnosis) *(63, 64)*. Additionally, the CDC suggested conducting chemical inactivation of samples based on external lysis buffer in kits in American laboratories *(46)*.

The limit of detection of different assays varies, and viral loads in specimens below the limit of detection will also lead to a negative result. Insufficient virus in a specimen is caused mainly by incorrect sampling sites or sampling techniques and improper sampling time, such as sampling at the later stages of progression. To solve this problem, specimens should be obtained in order of priority, sampling at different infection stages as possible, or performing detection from a variety of samples (including swabs, stool, and blood) *(54)*.

In conclusion, rapid optimization of testing kit quality and standard operating procedures are top priorities for solving the issue of false negatives *(65)*. First, robust

performance verification of assays is required. Second, choosing guanidine hydrochloride buffer instead of thermal inactivation if inactivation is required will exhibit a smaller impact on RT-PCR results *(49, 62)*. Additionally, it is desirable that laboratorians standardize the procedure of nucleic acid extraction and testing to avoid false negatives *(46)*. Third, positive quality control materials, such as synthetic SARS-CoV-2 RNA or stocks from positive specimens, should be used in the detection of clinical specimens (**Fig. 2C**), and laboratories should participate in external quality assessment to improve testing proficiency *(58)*. Finally, RT-PCR and serological assays can be combined in the progressive and recovery stages to reduce false negatives.

Serological Dynamic Antibody Assays

PRINCIPLE OF SEROLOGICAL ASSAYS

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. The titer of secretory IgA indicates mucosal immune responses against SARS-CoV-2. IgM indicates the acute infectious stage, while IgG represents middle and later stages of infection or previous infection. The temporal dynamics of antibodies against SARS-CoV-2 presented in different studies may be slightly discrepant, and IgA has been reported less often in this regard than IgM and IgG. In one study, IgA and IgM were both detectable at the 5th day (median), while IgG appeared on the 14th day (median) *(66)*; in another study, the median seroconversion times for IgM and IgG were reported to be day 12 and day 14 *(67)* (**Fig. 3**), respectively. Subsequently, the antibody levels increased rapidly; the IgA level was reported to not increase after day 21; the IgM level

did not increase significantly after about day 15, and was present from day 10 to day 30; and the IgG level reached a plateau by day 21 where it persisted *(66)*. In view of these time courses, the serological analysis of IgM and IgG has the potential to determine the infection stages and assess disease epidemiology.

Currently, some antibody test kits have been applied in research or approved for clinical applications, involving mainly applications for IgM and IgG (**Table 3**) *(49)*. The detection targets include one or several specific antibodies or total antibodies *(49, 67)*. The antigens also vary in reagents, including RBD, S1, S and N proteins *(66, 68)*, and synthetic or other amino acid-like antigens have also been used *(69)*. In clinical practice, the specificity of antibody assays based on the RBD antigen can reach 90% *(67)*, and the cross-reaction between SARS-CoV-2 and SARS-CoV is limited *(70).* In the detection based on S antigens, the S1 antigen has a specificity of 100% for coronaviruses other than SARS-CoV and is more specific than the S antigen because the latter has cross-reactivity with MERS-CoV *(71)*. This phenomenon can be explained by the fact that the S2 subunit is more conserved than the S1 subunit. In addition to SARS-CoV, cross-reactivity with MERS-CoV was also found in assays based on the N protein *(66, 71)*. Therefore, RBD and S1 are more specific antigens for antibody assays than the S and N antigens. Some synthetic or other similar antigens were reported to exhibit a better performance and cannot be compared easily because of the difference in synthetic sequences *(69, 71)*.

APPLICATION AND QUALITY ASSURANCE OF SEROLOGICAL ASSAYS

Antibodies represent host humoral responses against SARS-CoV-2, indicating the

status of infection. Therefore, serological antibody assays can be used in the diagnosis of suspected cases either in combination with molecular testing or for additional testing in suspected cases with negative nucleic acid results (**Fig. 2**) *(67)*; moreover, the levels of IgG and IgM against the S and N proteins of SARS-CoV-2 were found to be correlated with virus neutralization titer *(35)*, and a higher titer of antibody was independently associated with a worse clinical classification *(67)*; these data suggest the potential value of antibody titers for evaluating the prognosis and recovery of patients. Since antibody titer in most patients increases 10 days after symptom onset (**Fig. 3**), sampling during the progressive and recovery periods is more effective (**Fig. 3**) *(49)*. In summary, serological antibody testing enables analysis of the dynamics of infections with SARS-CoV-2; more importantly, it has lower operational requirements and can reduce the risk of medical staff exposure due to respiratory sampling.

Despite the advantages of serological assays, the testing performance of antibody assays should also be considered. In an analysis of 397 and 128 blood samples from SARS-CoV-2 infected and uninfected individuals, IgM/IgG assays had a sensitivity and specificity of 88.66% and 90.63%, respectively *(68)*. Therefore, some situations that lead to false positives and false negatives still require attention. Cross-reactivity with other subtypes of coronaviruses may be a threat. Antibody assays are susceptible to the influence of endogenous interferents, including rheumatoid factors, heterophilic antibodies and complements *(72, 73)*, as well as exogenous factors, such as specimen hemolysis, yielding false positive results *(74)*. Enhancing the specificity of the antigen peptide of the reagents to reduce the cross-reactivity with other viruses, and diluting the

specimens and changing the enzyme-labeled antibodies can reduce the incidence of false positives. In addition, a combination of antibody assays, clinical symptoms and molecular results should be used for diagnosis to minimize false positives. False negative results also require attention. There are specific testing windows for serological antibody detection; IgA and IgM usually last a short time, and IgG may be produced in the later period, so sampling at unsuitable stages may lead to false negatives *(68)*. Differences in individual immune response and antibody production also may lead to false negative results. Therefore, at least two serology results at different time points combined with negative RT-PCR result are helpful to rule out false negatives (**Fig. 2C**). Testing strategy is also important; the combined IgM-IgG test was reported to have better practicality and sensitivity than tests for only IgM or IgG *(68)*. In addition, some laboratories will inactivate samples before nucleic acid testing, but it is controversial whether to inactivate samples before antibody testing *(64)*. The instructions of IgM/IgG detection kits (magnetic particle luminescence, Bioscience) indicate that serum specimens should be thermally inactivated at 56 °C for 45 ± 5 min; some kits (colloidal gold, LINZON, and colloidal gold, Shanghai Superchip) indicate that the inactivation of the samples at 56°C for 30 min has no significant effect on the results. However, another two detection kits for IgG (colloidal gold, Wondfo) and IgM (colloidal gold, Hecin) specify not to use samples with thermal inactivation; other instructions of antibody detection kits approved by the NMPA or available in the world do not include any inactivation information. In theory, thermal inactivation may affect antibody testing as the antibodies are proteins, but the impact may be specific to the kits and their testing

principle. Additional research on the effect of inactivation on antibody testing should be conducted in the future.

Conclusion

The SARS-CoV-2 epidemic is spreading worldwide. Accurate diagnostic assays can offer a robust way for the timely identification of infected individuals, which is key to preventing retransmission of SARS-CoV-2. RT-PCR is widely employed in the molecular diagnosis of SARS-CoV-2 infection in laboratories, and dynamic serological antibody assays are supplementary methods. Both methods should be designed according to the characteristics of SARS-CoV-2. Gene regions including ORF1ab, N, and S genes are commonly targeted regions for RT-PCR *(43)*; and RBD, S1, S, and N proteins or synthetic antigens can be used to detect antibodies in serological assays. BALF typically has the highest viral load, followed by sputum, nasal swabs, and pharyngeal swabs, suggesting the ideal priority order for selecting specimens *(53)*. Viral nucleic acid and antibody concentrations fluctuate in different infection stages, suggesting infection status may be determined using a combination of molecular and serological assays *(67)*. In the incubation period and early infection stage, respiratory tract specimens should be used for molecular detection; in progressive stages, both the molecular and serological assays are useful for diagnosis; in the recovery stage, serology assays can be used to determine infection and recovery (**Fig. 3**). For RT-PCR results inconsistent with clinical picture, sampling in different organs or repeatedly sampling at different stages may prevent negative results due to insufficient viral loads. Notably, quality assurance of assays is essential for the reliable detection of SARS-

CoV-2, and each laboratory should conduct quality assurance measures to improve testing proficiency *(58)*.

Full understanding of the genomic characteristics, transmission and clinical features of SARS-CoV-2 will lead to better molecular and serological assays, and provide clinical testing personnel information to improve the accuracy of SARS-CoV-2 testing.

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Fig. 1. The genome structure and phylogenetics of SARS-CoV-2. (A) The genome structure of SARS-CoV-2, which is arranged as follows: 5'-replicase (ORF1ab)-structural proteins [Spike-Envelope -Membrane -Nucleocapsid]−3'. **(B)** A sketch diagram of the E, S, M, and N proteins of SARS-CoV-2. **(C)** Phylogenetic analysis reveals that SARS-CoV-2 belongs to the sarbecovirus subtype of betacoronavirus and is closely related to bat-SL-CoVZC45 and bat-SL-CoVZXC21. UTRs, untranslated regions; ORF: open reading frame.

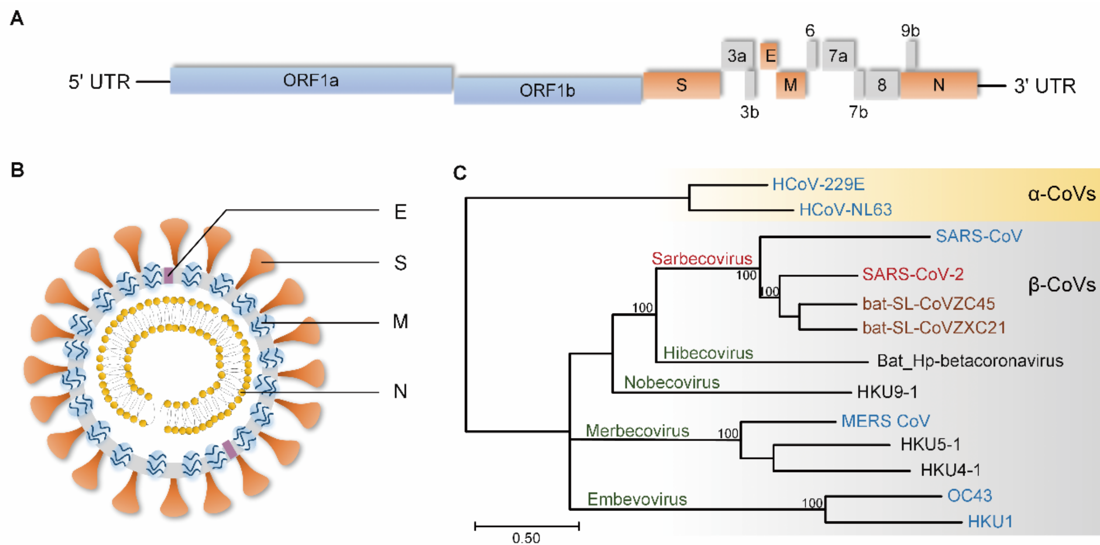


Fig. 2. Schematic overview of physiologic, genetic, specimen collection and quality control factors influencing assays for SARS-CoV-2. **(A)** The interaction of RBD and ACE2 when SARS-CoV-2 invades human body, and the process of virus invading the lung. **(B)** Genomic and clinical characteristics of SARS-CoV-2. SARS-CoV-2 has specific gene regions, and RBD, S1, S, and N proteins induce specific antibodies. Viral load varies across different ages, infection stages and organs. In elderly patients, the viral load is high. Different organs have different distributions of the ACE2 receptors thereby yielding different viral loads. **(C)** Specific characteristics influence the design and performance of assays. In molecular assays, S, ORF1ab, N, and E genes are targeted regions to detect SARS-CoV-2; BALF is the most preferred specimen, followed by sputum, nasal swabs, pharyngeal swabs, and stool/anal swabs. With appropriate quality assurance, molecular assays can be used to diagnose infected patients. In addition, antibody dynamics revealed by serological assays serve as complements to molecular assays, especially for suspected patients with negative molecular assay results. RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; BALF, bronchoalveolar lavage fluid; RT-PCR, reverse transcription real-time PCR; ORF: open reading frame.

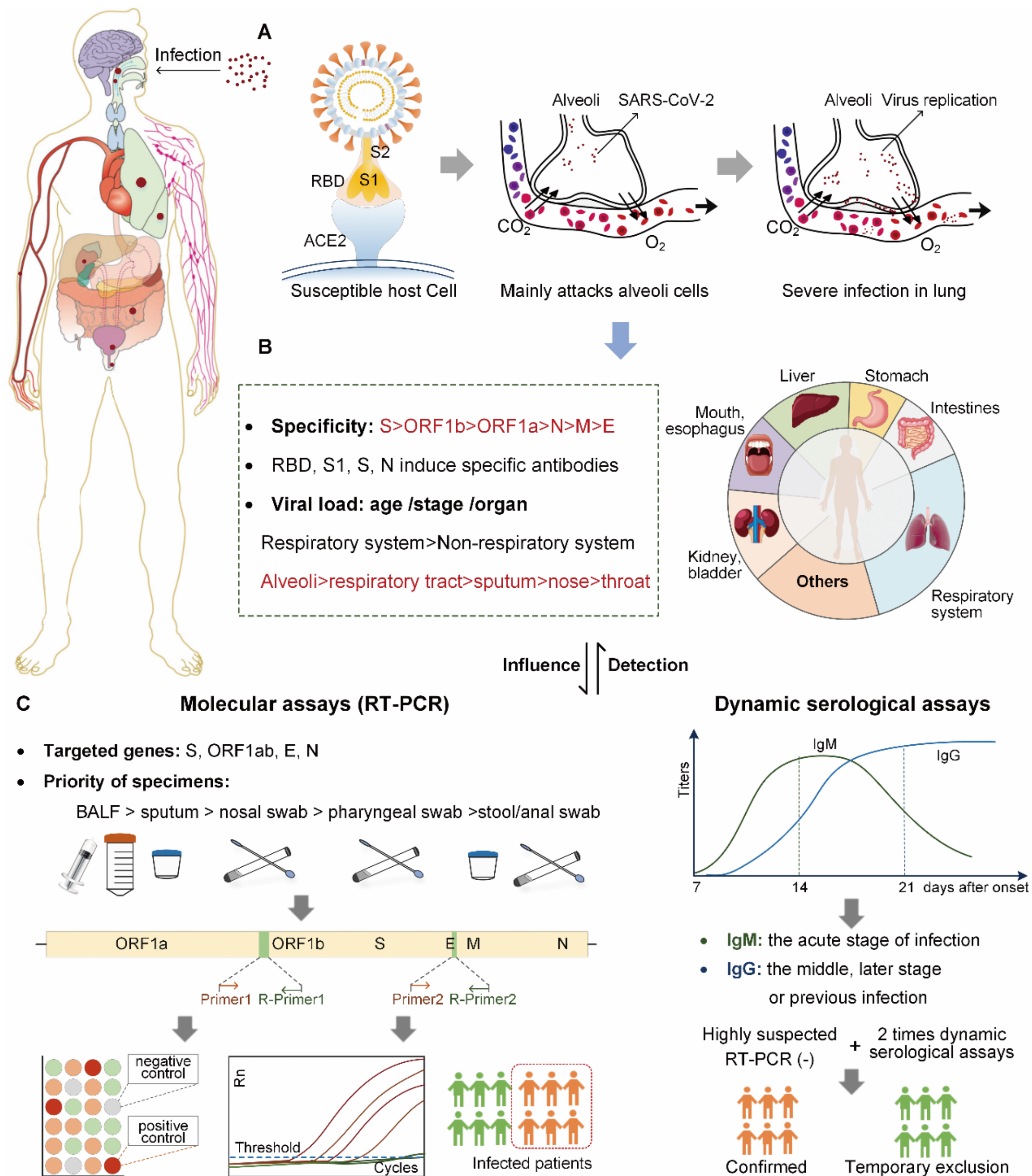
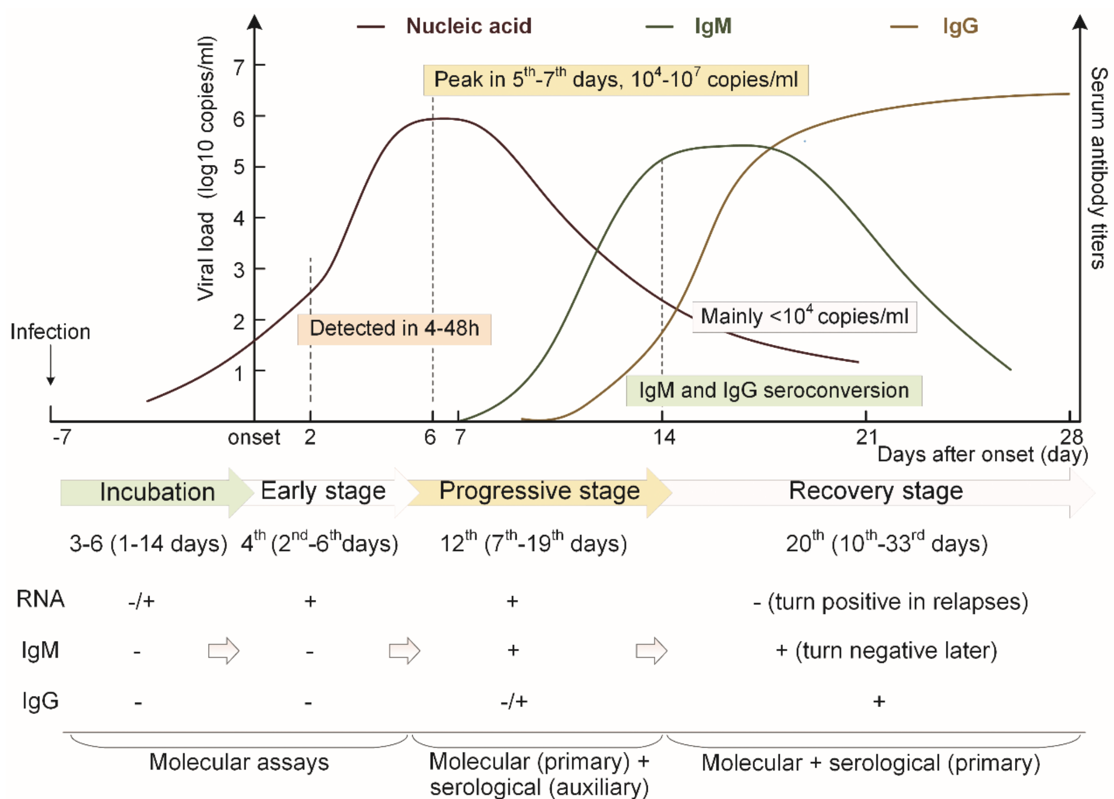


Fig. 3. The temporal dynamics of nucleic acid and antibodies in SARS-CoV-2 patients. In most patients, the infection mainly involves the incubation period, early, progressive and recovery stages. Viral nucleic acids can be detected 4-48h after onset, mainly peaking on the 5th to 7th days with viral loads of 10^4 to 10^7 copies/ml, then decline with the viral load mostly lower than 10^4 copies/ml in the recovery stage, and the total shedding time is approximately 20 days. IgM can be detectable in the 5th and

12th day (median) after onset reported in two studies, respectively; IgG seroconversion occurs approximately on the 14th day after onset, then IgM and IgG rapidly increase on recovery stages. Subsequently, IgM gradually decreases over a duration from day 10 to day 30, while IgG persists. In each infectious stage, nucleic acids, IgM, and IgG exhibit varied concentrations, suggesting testing strategies combining molecular and serological assays can be used to reveal infection status and stages.



723 **Table 1. Sequence similarity between SARS-CoV-2 and other bat-like coronavirus.**

Coronavirus	Nucleotide similarity (%)								S protein amino acid similarity (%)	Reference
	Whole genome	ORF1a [a]	ORF1b	ORF1ab	S	N	M	E		
bat-SL-CoVZC45	87.6-88.1, 89.1	90.7-91.0	86.1	88.9	75.2-77.8, 84.0	91.1	93.4	98.7	80.0 (S), 70.0 (S1), 99.0 (S2)	*(10, 14, 15, 22)*
bat-SL-CoVZXC21	87.5-88.0	90.3-90.9	86.1-86.2	88.7,	74.7-77.1	91.2	93.4	98.7	70.0 (S1), 99.0 (S2)	*(10, 14, 15)*
SARS-CoV	79.0-79.7, 82.0	75.4-76.0	86.2-86.3	79.5	72.7-73.4	87.7-88.1	85.1-85.4	93.5-94.7	76.0 (S)	*(9, 10, 14, 15)*
Bat SARr-CoV RaTG13	96.3	96.0	97.3	-	93.1	96.9	95.5	99.6	-	*(10, 75)*
BtCoV/4991 GeneBank KP876546	-	-	98.7 (370 nt of RdRp)	-	-	-	-	-	-	*(15)*

[a] ORF, open reading frame; RdRp, RNA-dependent RNA polymerase.

727 **Table 2. Molecular assays to diagnose SARS-CoV-2.**

Organization (listed alphabetically)	Date issued	Manufacturer (reagent) [a]	Principle	Targeted gene	Limit of detection
FDA [b] EUA, WHO	02/04/2020	CDC	RT-PCR	N1, N2	10^3-$10^{3.5}$ copies/ml
FDA EUA	02/29/2020	Wadsworth Center, New York State Department of Public Health's	RT-PCR	N1, N2	0.5 copies/reaction
	03/12/2020	Roche Molecular Systems (Cobas)	RT-PCR	ORF1ab, E	0.009 $TCID_{50}$/ml (ORF1ab), 0.003 $TCID_{50}$/ml (E)
	03/13/2020	Thermo Fisher Scientific (TaqPath)	RT-PCR	ORF1ab, N, S	10 GE/reaction
	03/16/2020	Hologic (Panther Fusion)	RT-PCR	ORF1ab (2 regions)	0.01 $TCID_{50}$/ml
	03/16/2020	Laboratory Corporation of America	RT-PCR	N1, N2, N3	6.3×10^3 copies/ml
	03/17/2020	Quidel Corporation (Lyra)	RT-PCR	ORF1ab	800 copies/ml
	03/17/2020	Quest Diagnostics Infectious Disease	RT-PCR	N1, N3	136 copies/ml
	03/18/2020	Abbott Molecular (RealTime)	RT-PCR	RdRp, N	100 copies/ml
	03/19/2020	DiaSorin Molecular LLC (Simplexa)	RT-PCR	ORF1ab, S	500 copies/ml (nasopharyngeal swab), 242 copies/ml (nasal swab), 1.2×10^3 copies/ml (BALF)
	03/19/2020	GenMark Diagnostics	PCR, electrochemistry	NA	1×10^5 copies/ml

	03/20/2020	Primerdesign	RT-PCR	ORF1ab	330 copies/ml
	03/20/2020	Cepheid (Xpert)	RT-PCR	N2, E	250 copies/ml
	03/23/2020	BioFire Defense	RT-PCR	ORF1ab (2 primers), ORF8	330 copies/ml
	03/23/2020	Mesa Biotech (Accula)	RT-PCR	N	200 copies/reaction
	03/24/2020	PerkinElmer	RT-PCR	ORF1ab, N	9.3 copies/ml (ORF1ab), 30.5 copies/ml (N)
	03/25/2020	Avellino Lab USA	RT-PCR	N1, N3	5.5×10^4 copies/ml
FDA EUA, NMPA	03/26/2020	BGI Genomics	RT-PCR	ORF1ab	150 copies/ml (throat swab), 100 copies/mL (BALF)
FDA EUA	03/27/2020	Abbott Diagnostics Scarborough Abbott (ID NOW)	Isothermal amplification	RdRp	125 GE/ml
	03/27/2020	Luminex Molecular Diagnostics (NxTAG)	RT-PCR, hybridization	ORF1ab, N, E	5×10^3 GE/ml
	03/30/2020	NeuMoDx Molecular	RT-PCR	Nsp2, N	150 copies/ml
	03/30/2020	QIAGEN GmbH (QIAstat-Dx)	RT-PCR	RdRp, N	500 copies/ml
	04/01/2020	Ipsum Diagnostics (COV-19 IDx)	RT-PCR	N	8.5×10^3 copies/ml
	04/02/2020	Becton, Dickinson & Company (BioGX)	RT-PCR	N1, N2	40 GE/mL
	04/03/2020	Luminex Corporation (ARIES)	RT-PCR	ORF1ab, N	7.5×10^4 GE/mL
	04/03/2020	ScienCell Research Laboratories	RT-PCR	N1, N2	$10^{3.5}$ copies/ml

	04/03/2020	Co-Diagno (Logix Smart)	RT-PCR	RdRp	4.3×10^3 copies/ml
	04/06/2020	Gnomegen	RT-PCR	N1, N2	8 copies/reaction
	04/07/2020	InBios International (Smart Detect)	RT-PCR	RdRp, N, E	12.5 GE/reaction, 1100 GE/ml
	04/08/2020	DiaCarta (QuantiVirus)	RT-PCR	ORF1ab, N, E	100-200 copies/ml
	04/10/2020	Atila BioSystems (iAMP)	RT-PCR	ORF1ab, N	4×10^3 copies/ml
FDA EUA, NMPA	04/14/2020	Maccura Biotechnology	RT-PCR	ORF1ab, N, E	1×10^3 copies/ml
FDA EUA	04/16/2020	GenoSensor	RT-PCR	ORF1ab, N, E	1×10^3 copies/ml
	04/16/2020	KorvaLabs (Curative-Korva)	RT-PCR	N1, N2	200 copies/ml.
	04/17/2020	Fosun Pharma USA	RT-PCR	ORF1ab, N, E	300 copies/ml
	04/18/2020	OSANG Healthcare (GeneFinder)	RT-PCR	ORF1ab	500 copies/ml
	04/20/2020	Trax Management Services (PhoenixDx)	RT-PCR	RdRp, E	100 copies/ml
	04/21/2020	Seegene (Allplex)	RT-PCR	RdRp, N, E	$1.3\text{-}4.2\times10^3$ copies/ml
	04/22/2020	altona Diagnostics GmbH (RealStar)	RT-PCR	E, S	0.1 PFU/mL
	04/23/2020	SD Biosensor	RT-PCR	RdRp, E	125-500 copies/ml
	04/27/2020	SEASUN BIOMATERIALS (U-TOP)	RT-PCR	ORF1ab, N	10 copies/reaction, 1×10^3 copies/ml
	04/29/2020	Rheonix	RT-PCR	N1	625 GE/mL
	04/29/2020	LabGenomics	RT-PCR	RdRp, E	2×10^3 copies/ml

NMPA	01/26/2020	Shanghai ZJ Bio-Tech	RT-PCR	RdRp, N, E	1×10^3 copies/ml
	01/26/2020	Shanghai GeneoDx Biotech	RT-PCR	ORF1ab, N	500copies/ml
	01/26/2020	BGI Biotech	Combinatorial Probe-anchor Ligation Sequencing	NA	NA
	01/28/2020	DAAN GENE	RT-PCR	ORF1ab, N	500 copies/ml
	01/28/2020	Sansure Biotech	RT-PCR	ORF1ab, N	200 copies/ml
	01/31/2020	Shanghai BioGerm Medical Biotech	RT-PCR	ORF1ab, N	1×10^3 copies/ml
	02/22/2020	CapitalBioPro	Isothermal amplification	N, S	15 copies/reaction
	02/27/2020	Beijing Applied Biological Technologies	RT-PCR	ORF1ab, N	200 copies /ml
	03/12/2020	Wuhan Easydiagnosis Biomedicine	RT-PCR	ORF1ab, N	500 copies /ml
	03/16/2020	Ustar Biotechnologies (Hangzhou)	Isothermal amplification, RT-PCR	ORF1ab, N	1×10^3 copies/ml
	03/24/2020	Anbio (Xiamen)	Hybridization capture, immunofluorescence	ORF1ab, N, E	500 copies/ml
	03/24/2020	FOSUN PHARMA	RT-PCR	ORF1ab, N, E	300 copies/ml
	03/26/2020	Rendu Biotechnology	Targeted hybridization, isothermal amplification	ORF1ab	250 copies/ml
	03/31/2020	Wuhan ZHONGZHI	Isothermal amplification, chromatography	ORF1ab, E	1×10^3 copies/ml
	03/31/2020	Wuhan ZHONGZHI	Double amplification	ORF1ab, E	100 copies/ml

	04/03/2020	Beijing KingHawk Pharmaceutical	RT-PCR	ORF1ab, N	500 copies/ml
	04/16/2020	BioPerfectus technologies	RT-PCR	ORF1ab, N	350 copies/ml
WHO	01/24/2020	China CDC	RT-PCR	ORF1ab, N	NA
	01/2020	China HongKong	RT-PCR	ORF1b-Nsp14, N	NA
	01/2020	France	RT-PCR	RdRp, E	NA
	01/17/2020	Germany	RT-PCR	RdRp, E	3.8 copies/reaction (RdRp), 5.2 copies/reaction (E)
	01/2020	Japan	RT-PCR	Multiple targets, S	5 copies/reaction
	01/23/2020	Thailand	RT-PCR	N	NA

[a] The molecular reagents approved by FDA EUA and NMPA as of April 30, and assays in different countries/regions presented on the WHO website are included in the table; [b] FDA, Food and Drug Administration; EUA, Emergency Use Authorizations; WHO, World Health Organization; CDC, Centers for Disease Control and Prevention; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; NMPA, National Medical Products Administration; BALF, bronchoalveolar lavage fluid.

732 **Table 3. Serological antibody assays to detect SARS-CoV-2.**

Organization (listed alphabetically)	Date issued	Manufacturer (reagent) [a]	Principle	Targeted antibody	Positive agreement (%) [b]	Negative agreement (%)
FDA [c] EUA	04/01/2020	Cellex	LFI	IgM/IgG	93.8	96.4
	04/14/2020	Ortho Clinical Diagnostics	Immunometric technique	Total (IgM/IgG)	83.3	100
	04/14/2020	Chembio Diagnostic System (DPP)	Immunochromatographic test	IgM/IgG	93.5 (total), 77.4 (IgM), 87.1 (IgG)	95.9 (total), 100 (IgM), 95.9 (IgG)
	04/15/2020	Mount Sinai Laboratory	ELISA	IgG	92.0	100
	04/24/2020	Autobio Diagnostics	LFI	IgM/IgG	88.2	99.0
	04/24/2020	DiaSorin (LIAISON)	CLIA	IgG	25.0 (<5th), 89.8 (6th-14th), 97.6 (>15th)	-
	04/24/2020	Ortho-Clinical Diagnostics	CLIA	IgG	87.5	100
	04/26/2020	Abbott Laboratories	CMIA	IgG	25.0 (3rd-7th), 86.4 (8th-13th), 100 (>14th)	99.6
	04/29/2020	Bio-Rad Laboratories	ELISA	Total (IgM/IgA/IgG)	92.2 (total), 100 (serum), 83.3 (plasma)	99.6 (total), 99.5 (serum), 100 (plasma)
	04/30/2020	Wadsworth Center , New York State Department of Health	Microsphere immunoassay	Total (IgM/IgA/IgG)	79.3 (> day 20), 88.0 (> day 25)	-
NMPA	02/22/2020	Wondfo Biotech	Colloidal gold	IgG	86.4	99.6
	02/22/2020	Innovita Biological Technology	Colloidal gold	IgM/IgG	87.3	100
	03/01/2020	Bioscience	Magnetic particle chemiluminescence	IgM/IgG	94.3 (total), 88.3 (IgM), 87.2 (IgG)	99.5 (total), 99.5 (IgM), 99.3 (IgG)
	03/06/2020	Xiamen Innodx Biotech	CMIA	Total	80.3 (total), 41.5 (<7th),	98.1

			(IgM/IgG)	85.5 (8th-14th), 92.5 (>14th)	
03/11/2020	Guangdong Hecin-Scientific	Colloidal gold	IgM	91.3	98.3
03/13/2020	Nanjing Vazyme Medical	Colloidal gold	IgM/IgG	91.5	97.0
03/14/2020	LIVZON	Colloidal gold	IgM/IgG	90.6 (total), 79.9(IgM), 84.3 (IgG)	99.2 (total), 99.7 (IgM), 99.4 (IgG)
04/10/2020	Dynamiker Biotechnology	Magnetic particle chemiluminescence	IgM/IgG	94.2 (total), 89.1 (IgM), 89.8 (IgG)	99.5 (total), 99.7 (IgM), 99.7 (IgG)
04/10/2020	Shanghai Superchip	Colloidal gold	Total (IgM/IgG)	87.4	98.3

[a] The serological reagents approved by FDA EUA and NMPA as of April 30 are included in the table; [b] The positive and negative agreement (%) data are derived from the instructions of reagents, which are determined by comparing the results of each serological assay with RT-PCR results; [c] FDA, Food and Drug Administration; EUA, Emergency Use Authorizations; CMIA, chemiluminescent microparticle immunoassay; LFI, lateral flow immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescent immunoassay; NMPA, National Medical Products Administration.