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Review Article

Serological and RT-PCR diagnosis approaches for COVID-19

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Abstract

The Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, caused COVID-19, is a pandemic disease and recently become dangerous to humans in all over the world. There is no efficient medicine for treatment of infected patients, and thousands of people are dying. Real Time-Polymerase Chain Reaction (RT-PCR) is a gold standard assay for COVID-19 diagnosis. Currently, RT-PCR assay is the routine method for detecting COVID-19, and several specific primers for different genes of SARS-CoV-2 are used. However, RT-PCR is not a perfect test, and its reliability depends on the sampling quality and accuracy of the equipment. Mutation occurrence in the virus genome and impurity in extracted RNA samples of the virus might lead to false results. Many efforts are carried out to detect SARS-CoV-2 according to various serological methods such as Enzyme-Linked Immunosorbent Assay (ELISA), colloidal gold-based, and chemiluminescence assays. Finally, the combination of molecular methods like RT-PCR and serological assays such as ELISA can improve the validity and reliability of aforementioned tests. Here, we summarize the results of some molecular and serologic tests for SARS-CoV-2 detection.

Keywords: Diagnosis, Biotechnology, Bioproducts, COVID-19, SARS-CoV-2, ELISA

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Introduction

In December 2019, an outbreak of pneumonia-like infection disease was originated at Wuhan city of China (1). Coronavirus (CoVs) was the cause of this infection, which was called by the International Committee on Taxonomy of Viruses (ICTV) SARS-CoV-2 (2) .The COVID-19 is the main danger for universal human health. The emergence of this new infection in China caused global consideration and was named a pandemic by the World Health Organization (WHO) (1). Until now, the outbreak of COVID-19 is propagating, and the agent virus is considered a severe hazard for human health since there is no effective medicine for curing the infected patients, every day the number of patients is rising in thousands in the world (3).

2. Virology

There are several diagnostic methods for detecting COVID-19, such as molecular tests, serologic based methods, and computed tomography (CT) scan. First specimens of SARS-CoV-2 were isolated from the broncho-alveolar lavage fluid (BALF) of three infected persons in Wuhan, China (4). Sequencing and phylogenetic analysis showed that SARS-CoV-2 is a positive-stranded RNA virus belonging to β-Coronaviruses (β-CoVs) family (4,5). Then, the newly discovered β-CoV was called "SARS-CoV-2" by ICTV. The Coronaviruses have enveloped viruses that have a genome that contains positive-sense single-stranded RNA. This family of viruses can infect the respiratory system, digestive system, and nervous system (6,7). According to the genotyping and serology information, there are four subfamilies for CoVs: α, β, γ, and δ. α- and β subfamilies cause infections in the human body (6,7). SARS-CoV and MERS-CoV are classified in the β subfamily (6). SARS-CoV-2 possesses a single-strand RNA genome in size 29.9 kb (8) and has nucleocapsid and β-CoVs. The nucleocapsid (N) protein is the main component of the nucleocapsid. The virus has a phospholipid bilayer envelope on its surface that contains spike (S) glycoprotein, hemagglutinin-esterase (HE), membrane (M) protein, and envelope (E) protein (9). There are 50 and 30 terminal sequences (265 nt at the 50 terminal and 229 nt at the 30 terminal regions) in the genome of SARS-CoV-2 and other β subfamily members. The genome has a reading frame order, including 50-replicase open reading frame (ORF) 1ab-S-envelope(E)-membrane(M)-N-30. It is anticipated that the length of SARS-CoV-2 genes including S, ORF3a, E, M, and N are 3822, 828, 228, 669, and 1260 nt, respectively. The genome of SARS-CoV-2 has an ORF8 gene with 366 nt in length, which resides between the M and N ORF genes and SARS-CoV (9). It is found that the SARS-CoV-2 virus could bondto the angiotensin-converting enzyme 2 (ACE2) and SARS-CoV (10). SARS-CoV-2 and ACE2 receptor interactions are necessary for cell infection. Bats are the main host for SARS-CoV (11), but the virus can infect other species via adaptation to various ACE2 variants (12). It is found that CD26 (Dipeptidyl peptidase 4, DPP4) can be a target for the S1 domain of the MERS-CoV spike protein due to co-purification by CD26 from Huh-7 cells lysates (13). MERS-CoV interacts with the DPP4 receptor of different species, which results in infection of various species and cross-infections (14). Knowing about virus and receptor interaction and proteolysis can open new horizons to predict possible cross-infections between animal and human.

3. Pathogenesis

COVID-19 has some symptoms such as fever, cough, myalgia, dyspnea, reduced leukocyte numbers, and pneumonia radiograph (16), as same as SARS and MERS (17). Thus, although the mechanism of SARS-CoV-2 infection is not clear, the comparison with related pathogenesis mechanisms in SARS-CoV and MERS-CoV can help predict the infection mechanism in COVID-19.

3.1. Virus entrance and infection

S protein is the major factor for virus entrance into the target cells (18). In SARS-CoV-2, S glycoprotein interacts with ACE2 receptor (19) and SARS-CoV S protein (20). It is indicated that fusion of cell membrane and virus envelope cause SARS-CoV entrance to host cell (21). It is shown that the S protein of SARS-CoV is cleaved at the 20th residue position, and this proteolysis process accelerates the fusion of virus and cell membrane, which results in infection (22). Furthermore, the entrance mechanism is based on the clathrin-dependent and –independent endocytosis (23,24). Since the virus infects the host cell, two types of proteins, including structural and polyproteins, are produced by the RNA genome of virus translation, then the RNA genome replicates (25). The translated proteins of the virus envelope reside in the endoplasmic reticulum (ER) or Golgi, which finally form the virus envelope, and the nucleocapsid proteins combine with RNA segments. Finally, viral proteins containing vesicles release from ER and Golgi systems and fuse with cell membrane for virus budding from the cell (18).

3.2. Presenting of coronavirus antigens

When the host cell was infected by the virus, its peptides could bind to major histocompatibility complex

I (MHC I), known as human leukocyte antigen (HLA), and present to the antigen-presenting cells (APCs) and virus-specific cytotoxic T cells (CTLs), which are major components of the immune response to virus infection. Thus, learning about SARS-CoV-2 antigen-presenting can help understand the mechanism of COVID-19, leading to design an efficient vaccine for it. Awkwardly, the above mentioned mechanism is not clear for SARS-CoV-2, and just a comparison with SARS-CoV and MERS-CoV mechanisms could be useful. Presenting of SARS-CoV antigen peptides is chiefly dependent on MHC I molecules (26), although MHC II can help in presentation. It is indicated that some polymorphisms of HLA are correlated to the susceptibility of SARS-CoV, including HLA-B*4601, HLA-B*0703, HLA-DR B1*1202 (27), and HLA-Cw*0801 (28), while the others such as the HLA-DR0301, HLA-Cw1502, and HLA-A*0201 alleles have an association with the protection against to the susceptibility of SARS (29). Some MHC II molecules, including HLA-DRB1*11:01 and HLA-DQB1*02:0, are related to MERS' susceptibility (30). Moreover, polymorphisms of the MBL (mannose-binding lectin) gene are correlated with the risk of SARS infection (31).

3.3. Humoral and cellular immunity

Presentation of viral antigens on infected cells can elicit humoral and cellular immune responses, motivating virus-specific B and T cells. Production of IgM and IgG antibodies rises in response to SARS infection and other severe viral infections. Specific IgM is not detected in SARS patients after 12 weeks, but IgG is detectable for a long time in the serum of infected person, which shows the protecting role of IgG (32), and the SARS-specific IgG antibodies chiefly are S- and N-specific (18). There are numerous studies about humoral immune responses against CoVs compared to cellular immune responses. It is shown that $CD4^+$ and $CD8^+$ T cell counts in the serum of SARS-CoV-2 infected persons considerably is decreased, while they are fully activated due to great extents of HLA-DR (CD4 3.47%) and CD38 (CD8 39.4%) double-positive fractions (33). Furthermore, the severe responses in SARS-CoV infected persons are related to the acute reduction of $CD4^+$ T and $CD8^+$ T cells. Even after removing antigens from serum, $CD4^+$ and $CD8^+$ memory T cells would remain according to the DTH response and production of IFN-g (34). It is indicated that after six years of infection, SARS-CoV specific memory T cells could be detectable in 14 of 23 cured persons (35). Also, specific $CD8^+$ CTLs can eradicate MERS-CoV from mice models (36). The above mentioned data can be beneficial for designing an efficient vaccine for SARS-CoV-2.

3.4. Cytokine profile in COVID-19 infection

It is demonstrated that Acute Respiratory Distress Syndrome (ARDS) is the major cause of COVID-19 mortality. ARDS is the most prevalent clinical-pathological sign of SARS-CoV-2, SARS-CoV, and MERS-CoV infections (33). ARDS causes an increase in the cytokine burst, results in systemic inflammation due to high concentrations of pro-inflammatory cytokines (IFN-a, IFN-g, IL-1b, IL-6, IL-12, IL-18, IL-33, TNF-a, TGFb, etc.) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, etc.) which is released via immune system cells in SARS infection (16, 37-39). The elevated levels of cytokines can stimulate the immune system against the body, leading to ARDS and organ trauma, resulting in mortality in acute SARS-CoV- 2 infected patients and SARS and MERS infections (33).

3.5. Evasion of coronavirus from the immune system

SARS-CoV and MERS-CoV could use several waysto evade the immune system. Pathogen-associated molecular patterns (PAMPs) that are present in the structure of many microbes are detected by pattern recognition receptors (PRRs). Though SARS-CoV and MERS-CoV could produce double-membrane vesicles that replicate their dsRNA inside them, and cells PRRs cannot identify their patterns (40). IFN-I (IFN-α and IFN-β) can be protective in SARS and MERS infected patients, but this pathway is suspended in infected mice (41,42). MERS-CoV has an accessory protein called 4a, which directly interacts with dsRNA and can block the IFN releasing at the level of MDA5 activation (43). Also, ORF4a, ORF4b, ORF5, and membrane proteins of MERS-CoV could prevent the transportation of IFN

regulatory factor 3 (IRF3) to the nucleus and IFN b promoter activation (44). Coronavirus can effect on presenting of antigens. For instance, MERS-CoV can suppress antigen presentation-related genes in infected cells (45). Thus, preventing the immune system perturbation by SARS-CoV-2 could be a significant aim for designing anti-COVID-19 drugs.

4. Serological tests

Molecular tests considering their costs, require instruments and experts with sufficient knowledge to perform the test, limiting SARS-CoV-2 diagnosis in all laboratories (2). According to WHO guidance and considering the high transmission of the query infection, which is respiratory, safety precautions while sampling and working during molecular tests are more important than other assays (3). Regarding the risks of working with COVID-19 samples, safety protocol alongside using special masks (N95), face shield, and gowns are required to minimize the spread of infection and reducing the risk of failure (4). Given these conditions, serological tests can be beneficial considering their ease of use, less need for advanced equipments, and skilled labors. Using a blood sample, antibodies against the SARS-CoV-2 antigen can be detected, which indicates the passive infection with the SARS-CoV-2 virus (5). In serological assays, usually, two kinds of antibodies, called IgM and IgG, are measured to check whether the infection has happened or not (6). Compared to RT-PCR, which is the most used test for SARS-CoV-2 RNA detection, the serological tests can reduce the false-negative results (7). Molecular tests such as PCR can be disturbed by external factors, including sampling and sample preparation, instruments, test operator, environmental space, and internal factors such as a mutation in the virus genome (8, 9). Researches show that serological assays in parallel molecular assays can generate more reliable results (10). Since the new coronavirus outbreak, various companies and research teams studied antibody response against the SARS-CoV-2 virus and developed a serological platform for the recognition of SARS-CoV-2 infection. According to Johns Hopkins' Center for Health Security report, companies that developed serological platforms for COVID-19 diagnosis are listed in Table 1. In a study, Juanjuan Zhao *et al*. obtained samples from 173 patients during their hospitalization. Through total antibody results, IgM, and IgG tests on blood samples, they showed a typical antibody response against acute SARS-CoV-2 infection. Seroconversion rate and antibody levels went in an upward direction during the first two weeks. After 15 days, the presence of antibodies increased to 100.0% (Ab), 94.3% (IgM) and 79.8% (IgG). They indicated that serological assays are a crucial supplement to molecular tests (11). Another study was applied to profile early antibody response to SARS-CoV-2 infection by Li Guo *et al.* They used an ELISA-based method with the recombinant viral nucleocapsid protein on 208 plasma samples, including 82 confirmed and 58 suspicious (NAAT negative but with typical manifestation) samples to assess the IgA, IgM, and IgG levels. They indicated serological methods are more efficient than RT-PCR 5.5 days from the onset of symptoms(12). An ELISA-based assay was developed in another study to detect SARS-CoV-2 neutralizing, spike- and nucleocapsid-specific antibodies, and they also showed the higher sensitivity of IgA rather than IgG through comparing two commercial kits (13). Another study was applied to evaluate the serological hallmarks of infection with SARS-CoV-2 from the exposure and post symptoms onset by Bin Lou *et al.* All enrolled patient's positive samples (80) were confirmed to be infected with SARS-CoV-2 by RT-PCR assays on positive samples. The total antibody, IgM, and IgG assays applied using three serological methods, including ELISA, colloidal-gold lateral-flow immunoassay (LFIA), chemiluminescence microparticle immunoassay (CMIA). Based on their results, the seroconversion rate for Ab, IgM, and IgG were 98.8%, 93.8%, and 93.8%, respectively (14).

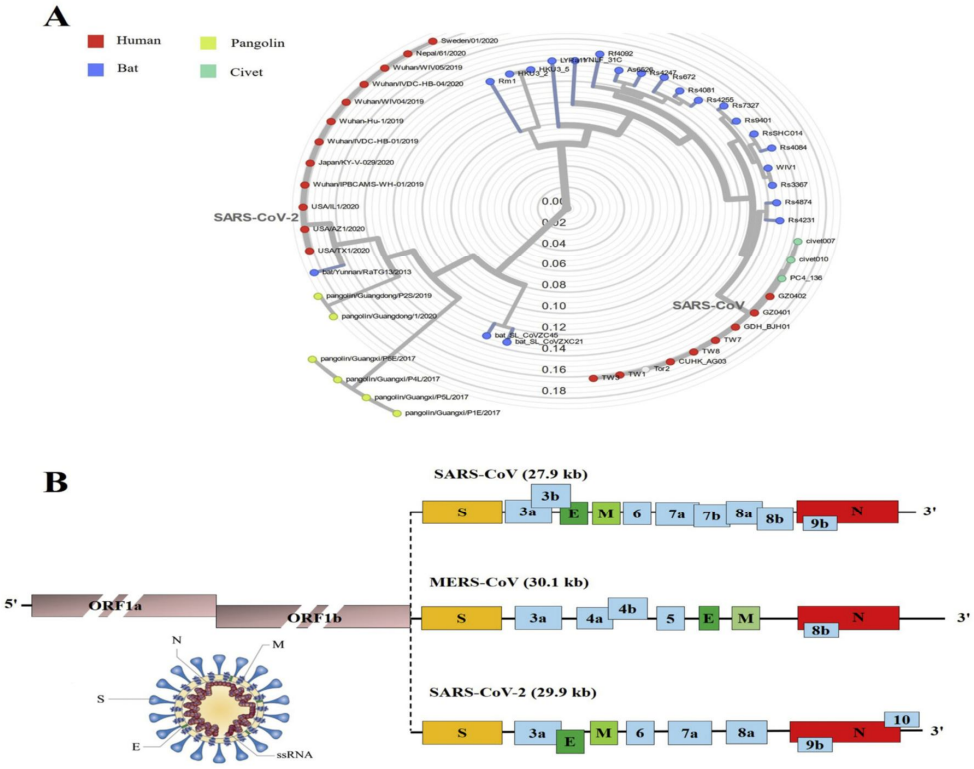

Fig. 1. The phylogenetic tree of SARS-like coronaviruses completes genome sequences and genome of SARS-CoV, MERS-CoV, and SARS-CoV- 2.

(A) This phylogeny shows the evolution of SARS-like b-coronaviruses, including samples from human (n ¼ 20), bat (n ¼ 22), civet (n ¼ 3), and pangolin (n ¼ 6). The phylogenetic tree of complete genome sequences of coronaviruses was obtained and analyzed with Nextstrain (https://github.com/blab/sars-like-cov).

Coronaviruses form enveloped and spherical particles of 100e160 nm in diameter. They contain a positive-sense single-stranded RNA (ssRNA) genome of 26e32 kb in size. In SARS-CoV, MERS-CoV, and SARS-CoV-2, the 50-terminal two-thirds of the genome ORF1a/b encodes polyproteins, which form the viral replicase transcriptase complex. The other ORFs on the one-third of the genome encode four main structural proteins: spike (S), envelope (E), nucleocapsid (N), and membrane (M) proteins, as well as several accessory proteins.

Table 1. List of companies that developed serological platforms for evaluation of antibody response in patients with SARS-CoV-2 infection. The information of the table obtained according to Johns Hopkins' Center for Health Security report.

Company	Platform type	Query anibody
Cellex Inc.	RDT	IgM and IgG against nucleocapsid protein
ChemBio	RDT	IgM and IgG against nucleocapsid protein
Ortho-Clinical Diagnostics, Inc.	ELISA	IgG
Mount Sinai	ELISA	IgG

Autobio Diagnostics Co. Ltd. (jointly with Hardy Diagnostics)	RDT	IgM and IgG
DiaSorin Inc.	ELISA	IgG
Bio-Rad	Modified-ELISA	IgM, IgG, IgA
Roche	electro-chemiluminescence immunoassay (ECLIA)	IgM, IgG
Euroimmun AG	ELISA	IgG
Wadsworth Center, New York State Department of Health	Microsphere immunoassay	IgG, IgM, and IgA
Siemens Healthcare Diagnostics Inc.	CLIA	total antibody

5. RT-PCR test

One of the main and reliable used diagnostic tests for detecting RNA or DNA pathogens is rRT- PCR, which is rapid and specific (15). It contains several parts as follow; virus sampling by nasopharyngeal swabs (the plastic and aluminum swabs are preferred), virus nucleic acids extraction, and the rRT- PCR kits designed to detect the virus by primer and target probes in less than 40 cycle thresholds (16). Two enzymes are needed to convert the virus RNA to cDNA during RT-PCR; at first, used a reverse transcriptase enzyme to make cDNA, and second Taq polymerase enzyme is recommended to extend the amount of cDNA by simple PCR, which is better to do in one step. Most diagnostic methods are fluorescence-based quantitative RT-PCR (RT-qPCR) which sre designed in one or two-step (2). Multiple target genes and primers were manufactured for rRT-PCR technique, which are related to virus RNA sequences variation, including the envelope (E), nucleocapsid (N), spike (S), RNA-dependent RNA polymerase (RdRp), and ORF1 genes. In different protocols, various target genes were recommended; the WHO guideline suggested that the E gene for screening and the RdRp region of the orf1b gene is to confirm the test (17). During the amplification of DNA, the 5' nuclease activity Taq polymerase leads to the reporter were disconnected from the quencher dye and generating a fluorescent signal in every PCR cycle, and this fluorescent signal is read by quantitative RT- PCR machine. The RT-q PCR is done in one or two steps, every method shows advantage and disadvantages, for instance, the two-step method is more sensitive and more flexible; however, one step is more rapid and decreasing the cross-contamination between RT and real-time PCR (6). The quality of this molecular assay is directly related to the laboratory facilities and very skilled lab operators. Thus, the sensitivity and accuracy of the rRT- PCR is not exactly complete. One of the main disadvantages of rRT- PCR is that in some cases, the results of rRT- PCR may indicate false negative or false positive related to various reasons. The RNA extraction and quality of throat swabs sampling require a more reliable diagnosis; also, as an advantage of this diagnostic method, rRT- PCR assay does not need to access live virus (18). The existence of mutation in probe targets could be considered as one of the reasons for the rRT- PCR false-negative result.So it is recommended that the most conserved parts of the virus chose as a detecting sequence so eliminated any mistake for binding to primers sequence can reduce the false-negative results. One way to decrease the false reporting results is to prepare various types of samples such as stool and blood besides respiratory specimen (19). In some cases, the results of chest CT images indicate the opacity and virus infection; however, their rRT- PCR results are false-negative, so these patients need to repeat the rRT-PCR test. Combine these two diagnostic methods is recommended to access a more accurate results (20, 21). Another diagnostic test that helps decline false-negative results of rRT- PCR is the

serological test, which relies on detecting the antibodies against virus proteins in a patient blood sample (18).

Conclusion

The outbreak of recently appeared coronavirus called SARS-CoV-2 is a global health threat. Since there is no specific and efficient drug or vaccine for COVID19 treatment, diagnostic methods and detection techniques with high sensitivity and specificity are required to prevent the virus outbreak. Though, RT-PCR shows false results and is not a perfect test, RT-PCR is still the gold standard test for the detection of SARS-CoV-2, which is based on the RNA genome of the virus. A lot of researches were done on serological diagnosis of COVID19 worldwide, indicating promising results. It is concluded that a combination of genome-based methods and serological assays could increase the reliability of the diagnostic testing for SARS-CoV-2.

Consent of interests

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