



Microbiological Diagnosis of COVID-19

COVID-19'un Mikrobiyolojik Tanısı

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ABSTRACT

Various tests are used to detect the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) virus causing Coronavirus disease-19 (COVID-19) disease. Today, the realtime (RT) -PCR test combined with the reverse-transcription reaction is the gold standard method used to diagnose SARS-CoV-2. This method is referred to as quantitative realtime PCR (RT-qPCR) because it determines not only the presence of SARS-CoV-2 but also the amount of virus in the specimen. Due to the use of virus-specific primers, the specificity of the tests is considered to be 100%. For this test, swab samples taken from the upper respiratory tract such as nasopharyngeal and throat, samples from the lower respiratory tract areas such as sputum and bronchoalveolar lavage fluid, rectal swab, feces, serum and urine samples are preferred. Correct use of personal protective equipment (PPE) by healthcare professionals during sampling and testing is important. Rapid antigen tests used in addition to RT-qPCR test for the diagnosis of SARS-CoV-2 are advantageous due to the theoretical rapid result time and low cost, but the sensitivity of this method is known to be very low. Virus detection in cell cultures can be used to detect SARS-CoV-2, but it is not for routine diagnostic because the results take a long time, require labor, and expertise. Serological tests are frequently used in the diagnosis and follow-up of this disease. These are mainly ELISA, CLIA, immunofluorescence test (IFA), western blot (WB), protein microarray (microarray) and neutralization. ELISA based immunoglobulin (Ig)M and IgG antibody tests have more than 95% specificity in the diagnosis of COVID-19.

Keywords: COVID-19, PCR, antibody, antigen

ÖZ

Koronavirüs hastalığı-19 (COVID-19) hastalığına neden olan şiddetli akut solunum yolu enfeksiyonu CoV-2 (SARS-CoV-2) virüsünü tespit etmek amacıyla çeşitli testler kullanılmaktadır. Günümüzde revers-transkripsiyon tepkimesiyle birleştirilmiş Realtime (RT)-polimeraz zincir reaksiyonu (PZR) testi SARS-CoV-2'yi teşhis etmek için kullanılan altın standart yöntemdir. Bu yöntem, örnekte sadece SARS-CoV-2'nin varlığını değil aynı zamanda virüs miktarında belirlediği için kantitatif PZR (RT-qPZR) olarak anılmaktadır. Virüse özgül primerlerin kullanılması sebebiyle testlerin özgüllüğü %100 olarak kabul edilmektedir. Bu test için nazofarengeal ve boğaz gibi üst solunum yollarından alınan sürüntü örnekleri, balgam ve bronkoalveoler lavaj sıvısı gibi alt solunum yollarına ait alanlardan alınan örnekler, rektal sürüntü, dışkı, serum ve idrar örnekleri tercih edilmektedir. Örnek alma ve test çalışılması sırasında kişisel koruyucu ekipmanların sağlık çalışanları tarafından doğru kullanımı önemlidir. SARS-CoV-2 tanısı için RT-qPZR testine ilave olarak kullanılan hızlı antijen testleri teorik olarak hızlı sonuçlanma zamanı ve düşük maliyetli olmasından dolayı avantaj sağlar ancak bu yöntemin duyarlılığının oldukça düşük olduğu bilinmektedir. Hücre kültürü SARS-CoV-2 tespit etmek amacıyla kullanılabilir ancak sonuçların uzun zaman alması, emek gerektirmesi ve uzmanlık isteyen bir yöntem olması nedeniyle tanı amaçlı ve rutin olarak gerçekleştirilmez. Serolojik testler bu hastalığın tanısında ve takibinde sıklıkla kullanılmaktadır. Bunlar esas olarak ELISA, kemilüminesans testi (CLIA), immüno Floresan testi (IFA), western blot (WB), protein mikroarray (mikrodizi) ve nötralizasyondur. ELISA bazlı immünoglobulin (Ig)M ve IgG antikor testlerinin, COVID-19 tanısında %95'ten fazla özgüllüğe sahip olduğu belirtilmektedir. SARS-CoV-2 ile enfekte olan hastalarda en erken 7-11 gün sonra antikor cevabı geliştiği için hastalığın akut tanısında yararlı değildir. Virüs nötralizasyon testleri antikorların işlevselliğini ortaya koyması nedeniyle diğer serolojik testlere göre daha anlamlı sonuçlar ortaya koymaktadır.

Anahtar Sözcükler: COVID-19, PZR, antikor, antijen

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Introduction

Coronaviruses (CoV) are a large family of viruses that can cause mild, self-limiting infections such as the common cold, common in the community, to more serious infections such as Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS). Based on the Latin meaning of these protrusions “corona” (crown), these viruses are named as CoV (crowned virus) (1).

This review summarizes all the laboratory diagnostic methods available in the management of COVID-19 and the latest scientific publications on this topic. Our aim includes the most up-to-date information showing the sample types taken in the tests performed for direct and indirect laboratory diagnosis of the virus, and the situations that need to be considered during the sample collection and transport phase.

Sample Types, Collection and Transport Media Alternatives

The rapid laboratory diagnosis of Coronavirus disease-19 (COVID-19) viral pneumonias caused by SARS-CoV-2 includes the application of correct test methods and the taking of the appropriate sample from the patient at the right time. SARS-CoV-2s can be detected from samples taken from both upper respiratory tract (URT) such as nasopharyngeal (NP) and throat, and lower respiratory tract (LRT) sites such as sputum and bronchoalveolar lavage fluid (BAL) (2). In addition, there are also publications stating that saliva taken from the URT can also be used in diagnosis (3). The collection of sputum, and especially BAL, by bronchoscopy creates an increased biological safety risk for healthcare workers through the generation of aerosol droplets. The correct use of personal protective equipment (PPE) by healthcare workers is important (4). Bronchoscopy is a highly technical procedure that requires well trained personnel. Upper respiratory specimens are easy to collect, facilitating access to testing for patients with mild symptoms and in resource-limited settings (4-6). Serum samples are another source for the detection of SARS-CoV-2. However, only 15% of patients hospitalized with viral pneumonia have detectable viral RNA in their serum (7). NP swab sample is most frequently preferred for the detection of SARS-CoV-2 by molecular methods [polymerase chain reaction (PCR)]. In addition, URT specimens such as oropharyngeal (OP), middle concha, or anterior nostrils may be accepted. Swabs with aluminum or plastic shafts are preferred. Swabs containing calcium alginate, wood or cotton are not recommended as they may contain ingredients that inhibit the PCR test (8). LRT samples such as sputum, endotracheal aspirates, and BAL have higher sensitivity than URT samples such as NP swabs. Even if the test result is negative in URT samples, the test must be repeated from the samples taken from the LRT especially in cases of severe progressive disease. Ideally, sputum or BAL are the recommended specimen types to demonstrate the highest viral load. In cases of severe pneumonia or ARDS, it is useful to take an LRT sample during intubation. Sputum and/or BAL samples can also be obtained after intubation. A high level of viral load was also found in the stool in cases with pneumonia. Serum and urine are usually negative for the presence of viral nucleic acid regardless of disease severity. (9,10).

SARS-CoV-2 has been shown in enterocytes and isolated from faecal cultures. For this reason, it will be beneficial to study real time (RT)-PCR in rectal samples as well as respiratory samples (11).

RNA positivity is at the highest level 7-10 days after the onset of symptoms from URT areas and it is recommended to take samples during this period for diagnosis. In patients with asymptomatic or mild symptoms, it is recommended to take both nasopharynx and oropharynx swabs together to increase sensitivity. These can be taken on the same viral transport medium (VTM). Since the RNA positivity continues for 3 weeks from the onset of the disease, it is recommended to take LRT samples from patients with severe symptoms, productive cough and intubated patients during this period. About two weeks after the onset of symptoms in the stool, the RNA is permanently detectable. Other samples such as urine and blood can be collected if necessary. Blood samples can be considered for serological investigations (3,12).

Samples collected for SARS-CoV-2 laboratory testing should be kept in a refrigerator (2-8 °C) for up to 72 hours; If it exceeds 72 hours, it should be stored frozen at -70 °C or below (3,13).

The Centers for Disease Control and Prevention (CDC) has published a procedure for laboratories to create their own viral transport media (VTM); other solutions that can be used in the absence of VTM are phosphate buffered saline, liquid Amies, and saline (14,15).

Sample packaging and in-house transport primary container must be closed with a screw cap. The container must be plastic. The outer surface of the primary container should be disinfected with 70% ethanol, placed in a sealed bag and placed in a secondary container prior to shipping. The secondary container must be leak proof and impact resistant and labeled as it contains infectious material. Pneumatic system should not be used for transportation (16-18). The triple packaging system should be used for transportation to the outer center (17-19). Aerosol generating processes must be carried out in a level II biosafety cabinet (BSC-II). Samples where nucleic acid extraction or inactivation has been performed in BSC can be processed outside of the BSC in accordance with standard precautions. Attention should be paid to cross contamination during nucleic acid extraction. If an automated nucleic acid extraction system is not used, procedures must be performed at Class II or higher BSC. After the processes are finished or when sample contamination occurs, the bench should be disinfected with appropriate disinfectants (70% ethanol, 2% glutaraldehyde, sodium hypochlorite [0.05%; 500 ppm] etc.) (19-22). The CDC recommends testing for 3 groups: inpatients with symptoms associated with COVID-19, symptomatic individuals at risk of poor prognosis of the clinical process, and individuals with a history of travel to the affected area or having contact with suspected/certain COVID-19 patients within 14 days. The CDC currently does not recommend testing for asymptomatic individuals (17).

Cell Culture

Isolation of SARS-CoV-2 in cell culture is not performed routinely for diagnostic purposes, as the results take a long time, requires effort and expertise. SARS-CoV-2 can primarily be produced in cell lines such as Vero monkey cells and LLC-MK2, but in suspected cases, cell culture-based diagnosis should not be performed in routine diagnostic laboratories for biosafety reasons. However, virus isolation in cell cultures is used to support the development of vaccines and therapeutic agents (23,24).

Rapid Antigen Tests

Rapid antigen tests are theoretically advantageous due to the rapid result time and low cost detection of SARS-CoV-2s, however, when the experiences with this method in influenza (Flu) viruses are evaluated, it can be stated that the sensitivity is quite low. Cases may be missed due to severe variation in viral load of patients (3,25). The development of an accurate, fast, early and simple fluorescence immunochromatographic method for the detection of the SARS-CoV-2 nucleocapsid protein in the NP swab for the diagnosis of COVID-19 has been reported (3). The inclusion of colloidal gold-based immunoglobulin G (IgG) as the detection reagent has been reported to be an approach that may increase the sensitivity of rapid antigen tests for respiratory viruses (26). The use of rapid bedside tests in suspicious cases will enable effective patient triage and lead to the correct use of limited quarantine facilities (3).

It can be expected that these tests will find wide use in small clinics or hospitals that do not have molecular methods or in screening before RT-PCR and become the recommended tests in guidelines.

Molecular Tests (Viral RNA Tests-Nucleic Amplification Tests)

Nucleic acid amplification tests (NAT) that detect viral RNA are used in the direct detection of SARS-CoV-2. The most important issue in these tests is that the viral RNA is present in the sample collected (27). Currently, the RT-PCR test combined with the reverse-transcription reaction is the gold standard method used to diagnose SARS-CoV-2. This method is referred to as quantitative-PCR (RT-qPCR) as it determines not only the presence of SARS-CoV-2 in the sample but also the amount of virus. Like almost all laboratory tests, the RT-qPCR method can give false positive or false negative results due to problems with sample collection and transport, RNA extraction and enzyme inhibitors (28). RT-PCR protocols used in the detection of SARS-CoV-2 RNA are provided on the CDC and website of World Health Organization (WHO) (29).

Target genes specific to SARS-CoV-2 can be investigated by RT-qPCR or sequencing (30). The most common preferred example for SARS-CoV-2 specific RT-qPCR is swab taken from the nasopharynx (NF) and/or oropharynx (OP). The swab is placed in a liquid transport medium (viral transport medium-VTM). In patients with pneumonia, LRT samples such as sputum and BAL should be tested in addition to NP and oral

secretions. In detection of SARS-CoV-2, the probability of virus detection is different for each clinical sample. Virus detection rate may vary from patient to patient and during the course of the disease. For example, while nasal and OP samples are negative in pneumonia patients, LRT samples may be positive (8,31). A negative test result does not exclude the possibility of the person being infected. If the test result is positive, the result is probably correct. However, contaminating samples with viral RNA (by a laboratory worker infected with SARS-CoV-2 as a result of cross-contamination while collecting the sample) may lead to false positive results. Since viral RNA does not mean live virus, detection of viral RNA does not indicate that the patient is contagious. Considering that infectiousness may occur before symptoms begin or even without symptoms, screening asymptomatic patients may also be considered. Unfortunately, little is known about viral RNA detection in asymptomatic patients and such testing strategies are unrealistic to use available resources (32). Insufficient sample collection may cause false negativity. After sample is taken, RNA extraction is performed and then qualitative real time-PCR is applied for target detection (33). The panel developed by CDC is a real-time qPCR panel for the detection of all SARS-like betaCoV and SARS-CoV-2. Using three separate pairs of primers, the *N* gene is targeted. One primer/probe set detects all betacoronaviruses, while two sets are specific for SARS-CoV-2. If positivity is detected in all three sets, it can be reported positive for SARS-CoV-2. This panel received an emergency use authorizations (EUA) on February 4, 2020 (2,12). The most commonly used target gene regions are envelope (E), nucleocapsid (N), spike (S), RNA-dependent RNA polymerase (RdRp), and *ORF1* genes (34). Sensitivity and specificity are high in these tests. There is no cross reaction with other coronavirus strains. A cycle threshold value below 40 (Ct; "Cycle threshold") is used as the criterion for positivity (35). Ct is the minimum number of amplification cycles required to generate a fluorescent signal that can be detected in PCR. The low Ct value indicates the high amount of viral RNA in the sample. It has been stated that the Ct values obtained in cases with a generally severe course are lower than the Ct values of mild cases and that the virus release is long-term in severe cases (36). However, these data need to be supported by other additional studies. Generally, viral RNA can be detected in the NP swab of symptomatic cases on day 1 of symptoms and reaches a peak value in the first week. Positivity begins to wane towards the 3rd week and then drops to undetectable levels. However, Ct values obtained from seriously hospitalized patients are lower than the Ct values of mild cases, and PCR positivity may persist 3 weeks after the disease (36). It does not always indicate active virus presence. In some cases, viral RNA was detected even after 6 weeks after the first positive test. There are cases that are found to be positive after two consecutive negative PCR tests performed 24 hours apart. The most common mistakes include errors in the execution of the test, reinfection or reactivation. The PCR positivity process is different in samples except NP swab. PCR positivity in sputum samples can still persist even after NP samples become negative (37).

If the target gene and internal control amplification are invalid, the test should be repeated. In samples with low viral load, values close to Ct values may indicate false negative or false positive results. Therefore, if necessary, the test should be repeated from the same sample or from a new ordered sample (12). Due to the use of primers specific to the virus genome sequence, the specificity of the tests is accepted as 100%. False-negative results may occur due to unsuitableness of sampling timing (samples collected too early or too late) and inaccuracies in the sampling technique (especially inadequate sampling in NP samples). In addition, improperly processed or transported samples, the formation of viral genetic mutations, the presence of PCR inhibitors, and the application of antivirals before testing are other factors that cause false negative results. Possible false positive results are due to technical errors and reagent contamination (12,38,39). Appropriate positive, negative and inhibition controls should be used for extraction and amplification steps in order to ensure quality control of RT-PCR tests for the detection of SARS-CoV-2. Internal control primers specific for host genes such as the human *RNase P* gene should be used to avoid false negative results (40).

Serological Tests

Serological tests can be performed for diagnosis when nucleic acid tests (NAT) are not possible, or for serological investigations, including investigating an ongoing outbreak or retrospectively assessing the degree of an outbreak (41). There are various serological measurement methods for the detection of SARS-CoV and MERS-CoV. These are mainly enzyme-linked immunosorbent test (ELISA), chemiluminescence test (CLIA), immunofluorescence test (IFA), western blot (WB), protein microarray (microarray) and neutralization (39). It is stated that ELISA-based IgM and IgG antibody tests have a specificity of more than 95% in the diagnosis of COVID-19. Studying these tests when the first PCR test is performed and from two different serum samples taken 2 weeks later can further increase the diagnostic accuracy (42). Antibody response develops after 7-11 days in patients infected with SARS-CoV-2. Some patients may develop antibodies later. Therefore, antibody tests are not useful in the diagnosis of acute disease. It is not known whether individuals with SARS-CoV-2 infection are fully or partially protected from reinfection and how long protective immunity lasts (32). In monitoring SARS-CoV-2 serology from consecutive samples (in acute and recovery phase), the WHO recommends that the first serum sample be collected in the first week of the disease and the second after 3-4 weeks. If only a single serum sample is available, it is recommended that it be examined at least 3 weeks after the onset of symptoms (43). The use of serological tests in the diagnosis of acute infections is limited only when symptoms appear. Detection of the antibody response is possible after weeks. For this reason, negative results, especially in those who have been exposed to the virus recently, do not exclude SARS-CoV-2 infection. Other molecules such as rheumatoid factor, nonspecific IgM may cause a false positive result. The

similarity of the N proteins of SARS-CoV-2 and SARS-CoV is approximately 91.2%. Therefore, a cross reaction between the N protein of SARS-CoV-2 and antibodies against other human CoV may occur (44). Tests that detect NC antibodies have the highest sensitivity, as the highest antibody response is against the virus's most abundant protein, nucleocapsid (NC). Antibodies against the receptor binding region (RBD-S) of the spike protein are expected to be more specific and neutralizing. Therefore, the use of one or both antigens increases the detection sensitivity of immunoglobulin (Ig)G and IgM (9). The situations in which antibody tests are useful are listed below (32).

1. Contact tracking,
2. Serological surveillance at local, regional and national level,
3. Identification of individuals who develop an immune response against the virus,
4. Detection of the development of protective immunity,
5. Making the decision of returning to work for individuals who are at risk of being exposed to SARS-CoV-2 again, such as healthcare professionals,
6. Identification of individuals who can be donors for therapeutic and prophylactic neutralizing antibodies,
7. Do not detect the sensitivity of PCR tests,
8. Determining the true extent of the pandemic,
9. Calculation of statistics such as case mortality rate,
10. Diagnostic testing of viral RNA negative individuals presenting at the late stage of the disease.

Serological analysis, on the other hand, is important for understanding the epidemiology of asymptomatic infections and emerging SARS-CoV-2s (3).

Neutralizing Antibodies

Virus neutralization tests are tests aimed at detecting the highest serum titer in tissue culture that stops viral infection. Determination of serum titers can be made by Tissue Culture Infectious Dose 50 (TCID₅₀) or plaque assays. These methods, which are serological tests, reveal more significant results than other serological tests that detect the binding of antibodies only to antigen, since they reveal the functionality of antibodies (45). Neutralizing antibodies are a subset of antibodies produced against a virus that independently block viral entry into host cells and consist primarily of the IgG isotype (32). It is not recommended for use as a routine test method.

As a result, timely and accurate laboratory diagnosis of COVID-19 has an important place in determining life-saving and infection control strategies by slowing down the pandemic, limiting the spread of the virus, starting from patient treatment management.

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