ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
August 2021; 20(4):394-401.
Doi: 10.18502/ijaai.v20i4.6949

Diagnosis of COVID-19 by Serology in Admitted Patients with Negative RT-PCR Assay

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Received: 10 October 2020; Received in revised form: 15 April 2021; Accepted: 19 April 2021

ABSTRACT

Considering the increasing prevalence and burden of coronavirus disease 2019 (COVID-19) disease and false-negative results in routine reverse transcription-polymerase chain reaction (RT-PCR) tests, additional diagnostic methods are needed to diagnose active cases of this disease.

This prospective study was conducted on patients, in whom clinical and radiological symptoms/signs were in favor of COVID-19 while their first PCR test was negative. Later on, a second RT-PCR was performed and serological evaluation was carried out and results were compared with each other.

Out of 707 patients who had been referred to the hospital and were clinically and radiologically suspicious of disease, 137 patients with negative RT-PCR tests entered the study. RT-PCR assay became positive for the second time in 45 (32.8%). Anti-COVID-19 IgM and IgG antibodies were positive in 83 (60.6%) and 86 (62.8%) patients, respectively. Finally, it was determined that serological test was diagnostic in 73% of patients and the diagnostic yield of serology was significantly higher after the first week of illness (54.8% in the first week and 88% after that). Taking advantage of both serological tests and RT-PCR helps in diagnosing 83.9% of cases.

Based on the present study, the serology may be useful as a complementary test and in parallel to RT-PCR assay for diagnosis of COVID-19 among admitted symptomatic cases.

Keywords: Antibodies; COVID-19; Diagnosis; Reverse transcription polymerase chain reaction; Serology

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by an emerging strain of coronavirus- severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)- the first time was reported from China in December 2019,[1] and rapidly spread through the world led to a pandemic.[2] The most common presentations of COVID-19 consist of fever, fatigue, dry cough, myalgia, and diarrhea; and in the severe cases dyspnea,[3] which all are nonspecific and common among many infectious and non-infectious diseases. Thus, there is not any clinical characteristic for the disease, and confirmation by paraclinical studies is necessary. Although radiologic studies especially computed tomography (CT) scan of the lungs has good sensitivity, a large portion of COVID-19 cases do not have lower respiratory involvement. On the other hand, radiological patterns of lung involvement are not specific for COVID-19.[4]

At present, a definite diagnosis of COVID-19 among symptomatic cases is dependent on a molecular assay for the detection of SARS-CoV-2 RNA, and reverse transcriptase-polymerase chain reaction (RT-PCR) is the gold standard.[5] Although RT-PCR is very specific, it has some important limitations: 1) sensitivity is not enough and range from 32 to 93% dependent on the source of samples,[6] 2) it is an expensive test 3) it is time-consuming and, 4) it needs expert persons and technical devices. False-negative results of RT-PCR are more common in both the early and late phases of the illness when the viral load in the respiratory samples is lower. Incorrect sampling, improper handling, and technical errors are the other causes of false-negative results. [5] Thus, negative RT-PCR does not exclude active COVID-19 disease,[7] and a complementary diagnostic tool is necessary for these cases.

Serologic studies historically were used for the diagnosis of many infectious diseases especially viral infections.[8] They are cheaper than RT-PCR and widely available. Concerning these facts, antibody detection is a promising tool for confirmation of active infection of SARS-CoV-2 among RT-PCR negative cases, but for confirmation of this proposal, a better understanding of the dynamics of antibody production in COVID-19 is necessary.

In this study, we evaluate the performance of serology as a complementary test for diagnosis of COVID-19 among symptomatic cases with negative first RT-PCR assay and compare it with the results of the second RT-PCR assay.

MATERIALS AND METHODS

This prospective study was conducted at the National Research Institute of Tuberculosis and Lung diseases (NRITLD) at Masih Daneshvari Hospital, Tehran, Iran. The Ethics Committee of NRITLD approved the protocol of this study (approval number: IR.SBMU.NRITLD.REC.1399.116). Also, informed consent was obtained from all participants.

In the period of study from 21 May to 21 July 2020, a total of 137 admitted symptomatic cases suspected to COVID-19 with compatible lung imaging but negative first RT-PCR assay were recruited. RT-PCR assay was performed on upper respiratory specimens mostly oropharyngeal samples. Recently, Bastos et al, demonstrated that the sensitivity of Saliva sampling is similar to nasopharyngeal swabs and is replaceable for SARS-CoV-2 testing.[9] Nucleic acid was extracted from the samples with the QIA symphony system (QIAGEN, Hilden, Germany) and SARS-CoV-2 RNA was detected using primer and probe sequences for screening and confirmation based on the sequence discussed by Corman et al.[10] For all of them, the second specimen of RT-PCR assay was sent and a concomitantly serologic study was performed. Finally, other than the purpose of this study some patients may have more than two RT-PCR samples.

For every patient, one blood sample was collected. The serum immunoglobulin (Ig) M and IgG antibodies against SARS-CoV-2 were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (PishtazTeb Diagnostics Company, Iran, catalog no. PT-SARS-CoV-2. IgM-96 and catalog no. PT-SARS-CoV-2. IgG-96). The test principle was based on an indirect ELISA method using microwells coated with a certain amount of nucleocapsid antigen of the SARS-CoV-2 virus. Then the diluted serum was added and allowed to react with solid-phase antigens. After incubation at room temperature, the

washing solution was used to remove unbound antibodies, and then the secondary conjugated antibody (anti-human IgM and IgG horseradish peroxidase conjugate) was added into the wells. After another incubation and wash, a solution of chromogen-substrate was added and incubated for 15 minutes. Finally, a stop solution was added to terminate the reaction, and the optical density (OD) of each well was measured by spectrophotometry at 450 nm. The ratio of OD to the cutoff value was used to calculate the cut-off index (COI) for each test, those higher than 1.1 were considered as positive. The cut-off value was OD of negative control plus 0.25 for IgM and 0.15 for IgG. The sensitivity claimed by the manufacturer was 79.4% for IgM and 94.1% for IgG detection and specificity was 97.3% and 98.3% respectively.

At rest, oxygen saturation on room air was used to determine the severity of the disease. The patients with O_2 saturation equal to or less than 93% were categorized as cases with severe disease.[11,12] The diagnostic yield of serology was calculated as the frequency of positive anti-COVID-19 IgM and/or IgG and was compared with the results of the 2nd RT-PCR assay. Also, the potential correlation between the diagnostic utility of antibody response and other factors consisting of age, sex, disease severity, and duration of symptoms were investigated.

All data were analyzed using SPSS (Version 16.0; SPSS Inc., Chicago, IL). The categorical variables were compared using the chi-square. T-test or Mann-Whitney U test was used for normally and non-normally distributed continuous variables respectively. A *p*-value of less than 0.05 was considered statistically significant. The receiver operating characteristic (ROC) curve was performed to show from which day is proper to perform a serologic study for the diagnosis of COVID-19.

RESULTS

In the period of study, 707 cases clinically and radiologically suspected to COVID-19 were admitted. Among them, the result of RT-PCR was negative for 143 patients. Six RT-PCR negative cases were excluded from the study due to the establishment of another diagnosis. Finally, the results of the second RT-PCR and serologic assays were investigated for all 137 patients with a wide range of disease severity from mild to a critical state. The mean age of cases was 55.6±17.5 years, 79of them (57.7%) were male and others were female.45.3% of cases were in the 1st week of illness, 45.9% in the 2nd week, and 8.8% in the 3rd week. The Median of symptom duration before admission, first RT-PCR assay, second RT-PCR assay, and the serologic study was 8, 9, 12, and 13 days respectively. 94 cases (68.6%) had severe disease.

Table 1 shows the results of RT-PCR and serologic tests among patients under study.

There was not any correlation between age, sex, the severity of the disease, second RT-PCR conversion, and diagnosis of COVID-19 with the serologic study. On the other hand, the diagnostic yield of serology and the rate of IgM and IgG antibody detection were significantly higher in patients with a longer duration between symptom initiation and admission. (p<0.014, 0.001, and 0.015, respectively).

Table 2 shows the correlation between serology and other factors.

We divided the patients into two groups, the first group who were admitted during the first week of their illness and the second who were admitted later. The rate of IgM detection, IgG detection, and diagnostic serology was 40.3%, 48.4%, and 54.8% in the first week and 77.3%, 74.7%, and 88% later respectively, all of them were statistically significant (p-values were<0.001, 0.002, and<0.001 respectively). The second RT-PCR assay was positive for 32.8% of cases in the first week of illness and 34.7% for patients with a longer duration of symptoms, without statistically significant difference.

ROC curve analysis was conducted to find the best time to add the serologic assay to RT-PCR testing (Figure 1). The area under the ROC curve (AUC) was 0.545 (95% confidence interval [CI], 0.433–0.657) for the 4th day, 0.616 (95% CI, 0.503–0.728) for the 5th day, and significantly increases to 0.682 (95% CI, 0.574–0.790) for the 6th day.

Table 1. The pattern of RT-PCR and serologic studies among 137 cases suspected to COVID-19 with negative 1st RT-PCR assay

Parameter	N (%)
Sensitivity of IgM	83 (60.6)
Sensitivity of IgG	86 (62.8)
Positive IgM / negative IgG	13 (9.5)
Negative IgM / positive IgG	16 (11.7)
Positive both IgM & IgG	70 (51.1)
Sensitivity of 2nd RT-PCR	45 (32.8)
Any positive RT-PCR	51 (37.2)
The final method of COVID-19 diagnosis*	
IgM	83 (60.6)
IgG (negative IgM)	17 (12.4)
IgM and/or IgG	100 (73)
2nd RT-PCR (negative IgM & IgG)	12 (8.8)
3rd or later RT-PCR#	3 (2.2)
Combination of RT-PCR & serology	115 (83.9)
Clinical diagnosis (negative tests)	22 (16.1)

* Serologic assays considered before 2nd RT-PCR for diagnosis confirmation. # Not performed for all cases

IgM: anti-COVID-19 immunoglobulin M, IgG: anti-COVID-19 immunoglobulin G, RT-PCR: real-time polymerase chain reaction

Table 2. Correlation between serology and other factors

Parameter	IgM			IgG			Diagnostic serology		
	neg	pos	*p**	neg	pos	*p*	no	yes	*p*
Age (years)	58.6±20.1	53.5±15.3	0.068	57.8±19.3	54.2±16.2	0.244	58.8±20.4	54.3±19.2	0.181
Gender (%)									
Male	30 (38)	49 (62)	0.687	26 (32.9)	53 (67.1)	0.223	22 (27.8)	57 (72.2)	0.796
Female	24 (41.4)	34 (58.6)		25 (43.1)	33 (56.9)		15 (25.9)	43 (74.1)	
Symptom duration (days)	7.6±5.7	9.7± 3.8	**0.001**	7.6± 5.0	9.6± 4.4	**0.015**	7.2± 5.4	9.5± 4.3	**0.014**
Severity (O2 sat)									
>93%	17 (31.5)	26 (31.3)	0.985	16 (31.4)	27 (31.4)	0.998	10 (27)	33 (33)	0.504
≤93%	37 (68.5)	57 (68.7)		35 (68.6)	59 (68.6)		27 (73)	67 (67)	

* *p*-value less than 0.05 considered significant.

Abbreviation: IgM: anti-COVID-19 immunoglobulin M, IgG: anti-COVID-19 immunoglobulin G

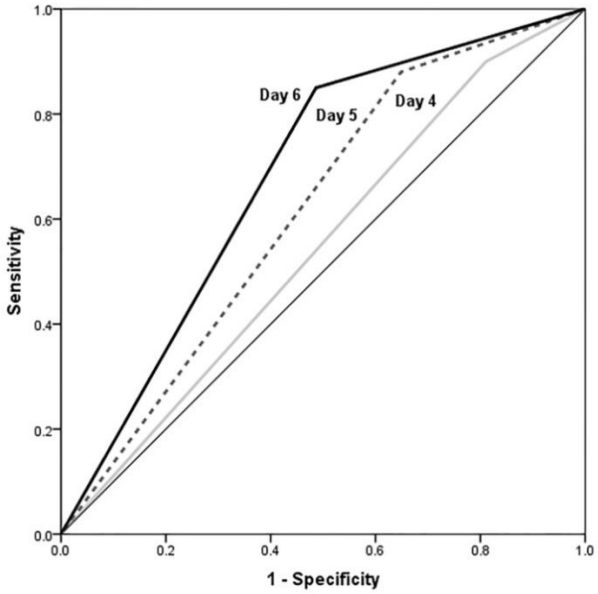

Figure 1. Receiver operating characteristic (ROC) curve analysis for sensitivity and specificity of the date of positive immunoglobulin M (IgM) on the diagnosis of Coronavirus disease 2019 (COVID-19)

DISCUSSION

Successful control of SARS-COV are widely available and include several methods for antibody detection such as ELISA, and chemiluminescence microparticle immunoassay (CMIA).[19] These tests have been designed for the detection of antibodies against the spike protein (SP) or the nucleoprotein (NP) of SARS-CoV-2.[19] Detection of anti-SP especially anti-receptor-binding domain (RBD) antibodies is more sensitive[20,21] and more specific than the detection of anti-NP antibodies for diagnosis of COVID-19due to less cross-reaction with other coronaviruses.[22,23]

In a systematic review and meta-analysis a pooled sensitivity of ELISAs measuring IgG or IgM antibodies was 84.3% and of CLIAs was 97.8%. Pooled specificities ranged from 96.6% to 99.7%.[24] Due to acceptable sensitivity, some proposed a combination or hybrid approach, performing the serologic assay as a complementary to RT-PCR for diagnosis of COVID-19.[5, 21, 25] Few published studies evaluated the efficacy of this approach. Wang conducted a retrospective study on 141 cases and compared the detection rate of the RT-PCR with a combination of both methods. They used CMIA for the detection of antibodies against RBD. The sensitivity of the 1st RT-PCR was 39.7% and it rose to 62.4%, 86.7%, and finally to 92.2% by 2nd, 3rd, and more testing. With the combination of the results of RT-PCR and serology, the sensitivity was raised to 98.6%.[15] The higher sensitivity they found in comparison to our study may be due to the difference in the technique of the serologic assay and the design of the study. In another study, Gue et al, showed an increase in detection rate from 51.9% by a single RT-PCR assay to 98.6% by combining RT-PCR with IgM ELISA assay using recombinant NP as coating antigens.[18] Zhao and coworkers evaluated the performance of a combined approach among 173 confirmed cases of COVID-19 by real-time RT-PCR. They showed combining RT-PCR and antibody assay significantly improved the detection rate (p<0.001), even in the first week of the illness (p=0.007).[26]

Another consideration about the performance of serologic assay is the interval between symptom onset and sampling.[16] Most of the studies showed the sensitivity of RT-PCR decreases and the sensitivity of serologic assays increases with time since the onset of symptoms.[24] In the Zhao et al. study, the sensitivity of

antibody detection raised gradually since day 8 and finally surpassing RT-PCR.[26] The results of our study showed the performance of serologic assay is higher from the sixth day of illness, very close to the results found by Gue et al,[18] which was 5.5 days. So it may be rational to use a hybrid approach in the second week of illness for suspected inpatient cases of COVID-19 with a negative first RT-PCR test.[17]

Currently, the world health organization doesn't recommend serology as a routine tool for the diagnosis of current infection with COVID-19.[27] The infectious diseases society of America (IDSA) recommends considering the time from symptom onset for interpretation of the results of a serologic study among symptomatic patients. Although the IDSA panel suggests against the use of IgM antibody assays for the diagnosis of COVID-19 during the first two weeks of illness, they suggest IgG antibody testing in symptomatic highly suspected patients and repeatedly negative RT-PCR assay.[28] Also, the 7th edition of the China national guideline-2 needs an accurate, rapid, and preferably non-expensive diagnostic method. This study supported the advantage of the combination of RT-PCR and serology for the detection of SARS-COV-2 with a high degree of sensitivity, as a useful tool for accurate and timely diagnosis of suspected patients. If the patients with positive IgM or IgG antibodies are considered as COVID-19 cases, a combination of serology and RT-PCR can lead to the diagnosis of 83.9% of cases. The serologic study alone was diagnostic for 100 among 137 cases (sensitivity as 73%). On the other hand, the 2nd RT-PCR assay was diagnostic for 32.8% of patients without considering serologic studies and 8.8% among patients with negative results of serology (both IgM and IgG antibodies). The combination method was more useful from the 6th day of symptom onset.

The sensitivity of RT-PCR for the detection of SARS-CoV-2 from respiratory samples has been estimated from 32 to 90%.[13] When the first RT-PCR assay is negative, the common approach in patients with clinic and imaging compatible with COVID-19 is repeating the test sometimes three to five times to get a positive result.[14] Even after multiple RT-PCR assays, all results may be negative, it was 16.1% in our study and 7.8% in Wang's study.[15]

The pattern of antibody response to SARS-CoV-2 is complex and highly variable, and any scenario is predictable (for example: missing IgM, presence of IgG before IgM, and delayed appearance of IgM).[16] Older studies showed the presence of IgM and IgG antibodies, about 6 days and 8 days after infection with SARS-CoV respectively. Because SARS-CoV-2 belongs to the same family of viruses, it can be expected that the time of the seroconversion is similar for COVID-19.[5] Xiang et al, reported the presence of specific IgM and IgG antibodies against SARS-CoV-2 on the fourth day after symptom onset.[17] In a recent study by Guo et al, the median duration of IgM and IgG antibody detection was 5 days and 14 days after symptom onset respectively.[18]

Many serologic tests for the detection of SARS-CoV-19 infection have been developed in recent months. They for COVID-19, recommends serological testing as a supplementary tool for the diagnosis of COVID-19.[29]

Two points of our work are the prospective nature and specific design to evaluate the efficacy of a combination of RT-PCR and serology in the diagnosis of admitted cases suspected of COVID-19. Also, our study has a limitation. Most cases (68.6%) under our study had severe disease (O_2sat≤93). So our findings are relevant to admitted cases that are suspected of COVID-19 and cannot be generalized to outpatient cases.

One of the limitations of the present study was the sampling method, which was mostly an oropharyngeal sample. Although nasopharyngeal sampling has more chance of virus detection, it needs special swaps which not available everywhere, especially in the first few months of the pandemic outbreak in Iran.

Our study showed that antibody assessment can be helpful among patients with undetectable levels of SARS-CoV-2 RNA in their respiratory samples. This evidence supports the usage of a combination of molecular and serological tests for the accurate diagnosis of COVID-19. For symptomatic suspected cases of COVID-19 with negative first RT-PCR assay, especially after the sixth day of symptom onset serologic study is suggested as a complementary tool in parallel with the repetition of molecular tests.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the National Institute of Tuberculosis and Lung Disease (NRITLD), Masih Daneshvari Hospital colleagues who helped us in the survey.

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