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ORIGINAL ARTICLE

Evaluation of COVID-19 antibody response with using three different tests

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ABSTRACT

Background and Objectives: In this study, the performance of three different commercial antibody assays for COVID-19 was examined and parameters affecting the antibody response were investigated. The correlation of patients' chest CT results, procalcitonin, CRP, and D-dimer levels with the antibody response were retrospectively evaluated.
Materials and Methods: COVID-19 antibodies were detected by three commercially available assays in each patient. Two of the assays were rapid immunochromatographic tests and - one was an ELISA-based IgG assay. SARS-CoV-2 RNA was tested by "COVID-19 RT-qPCR Detection Kit" using nasopharyngeal swab samples. The results of antibody tests were compared with each other, RT-qPCR, Biochemical parameters and chest CT findings.
Results: RT-qPCR was positive in 46.6% (41/88) of the evaluated patients among which 77.3% (68/88) were healthcare workers. Seventeen (41.4%) of viral RNA positive patients had a positive antibody result with at least two assays. Both of the rapid immunochromatographic tests had identical sensitivity of 36.6% and specificity of 100%, compared to RT-qPCR assay; while the sensitivity of the ELISA based Euroimmune test was 43.9%, and the specificity was 95.7%. The sensitivity and specificity of the immunochromatographic tests were 75% and 100% respectively, compared to ELISA test result. There was a correlation between antibody positivity and old age and male gender. The presence of typical chest CT findings increased the antibody positivity 13.62 times. Antibody positivity was also increased with the decrease in Ct value of the PCR assay. There was no significant relationship between the biochemical parameters (CRP, D-dimer and procalcitonin values) and the antibody or RT-qPCR results.
Conclusion: There was a correlation between antibody response and male gender, older age, presence of symptoms, typical chest CT findings and low PCR-Ct value.

Keywords: SARS-CoV-2; Diagnosis; Serology; Antibodies

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INTRODUCTION

A new coronavirus, SARS-CoV-2, causing acute respiratory disease (COVID-19) was detected in China, in December 2019 (1, 2). World Health Organization (WHO) declared the outbreak a pandemic on March 11, 2020. Until now, SARS-CoV-2 has been infected >60 million people in more than 180 countries with a death rate of 2.3% (3).

Virological diagnosis of SARS-CoV-2 consists ofnucleic acid tests (NAT) and viral antigen tests that detect the virus directly, and serologic assays detecting the immune response of the host (4). Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) test is the gold standard to detect the acute infection. Nevertheless, several pre-analytical and analytical factors such as poor specimen quality, insufficient viral load, use of inadequate assay could cause false negative test results (5).

There are several commercial assays available which detect IgM, IgG, IgA or total antibodies against SARS-CoV-2. Antibody response to the infection depends on the host. For SARS-CoV-2, seropositivity generally occurs at 7-11 days after the exposure to the virus. However, it may take longer in some patients (6). Thus, antibody tests are used as evidence of past infection. Some studies, on the other hand, have demonstrated that IgM and IgG can be detected as early as five days after the onset of the infection and serologic assays in addition to NAT are recommended in patients with clinical suspicion of COVID-19 despite of negative RT-qPCR results (7). Serological testing is relatively easier and faster than NAT, and can be conducted in many basic laboratories. Because the tests are carried out using serum and/or plasma samples, it poses a lower biosafety risk for health care workers. Three indications of COVID-19 antibody tests that are described in the guidelines are: a) assessment of patients with negative molecular diagnostic tests and with high clinical suspicion whose onset of symptoms started more than two weeks ago, b) assessment of multisystem inflammatory syndrome in children, c) seroprevalencestudies (8).

Antibody tests have been developed by many manufacturers due to increasing demand and they have been introduced to the market rapidly with limited validation studies on clinical samples. There is a need for studies that evaluate the performance and accuracy of these tests in the field.

In the present study, three different commercially available COVID-19 antibody assays are compared. The purpose was to determine the performances of the assays and to investigate the impact of SARS-CoV-2 RT-qPCR, computed tomography (CT) of the chest and biochemical parameters to antibody response.

MATERIALS AND METHODS

Ethical clearance. The study was approved by the Ethics Committee of the Faculty (date: 15.06.2020, no: 2020/13-26) and authorized by the Turkish Ministry of Health (date: 22.05.2020, no: 72292585-00.99-E.42637).

Study population. The total of 129 patients with clinical suspicion of COVID-19, were tested for SARS-CoV-2by RT-Qpcr and antibody assays between March 31, 2020 and May 17, 2020. Serum/plasma samples were collected from the patients for antibody tests. Eighty-eight patients who tested for SARS-CoV-2 antibodies after ≥14 days post-PCR (14-43 days, 25.45 days in average) were included in the study.

COVID-19 antibodies detected by three commercially available assays in each patient. Two of the assays were rapid immunochromatographic tests (Hotgen, Hotgen Biotech, Beijing, Chinaand Turklab, Izmir, Turkey), and the last one was an ELISA-based IgG assay (Euroimmun Medizinische Labordiagnostika, Lubeck, Germany).

Real time RT-qPCR. SARS-CoV-2 RNA was tested by “COVID-19 RT-qPCR Detection Kit” (Biospeedy, Ankara, Turkey) using nasopharyngeal swab samples. The assay was specific to SARS-CoV-2 and did not cross-react with other coronaviruses or respiratory viruses. It was CE-IVD certified and approved by FDA for emergency use at a later date. The target region was the viral RNA-dependent RNA polymerase (RbRp) gene fragment and with an internal control targeting human ribonuclease P (RNase P) gene. Samples with a “cycle threshold” (Ct) value less than 40 were accepted as viral RNA positive. Extraction of viral RNA was carried out in accordance with the producer’s instructions using "Viral Nucleic Acid Isolation Kit" (Bio-speedy, Ankara, Turkey). Ct value of viral target amplification and viral target Ct/internal control Ct ratio were also included in the analyses.

Participation to the QCMD external quality control program for SARS-CoV-2 RNA (ref code: CVOP20) with the used kit resulted with a perfect score.

Immunochromatographic tests. Hotgen antibody test detects total antibodies, whereas, Turklab test differentiates IgM and IgG on the same cassette. A recombinant antigen was used on the test line of Hotgen kit and a goat-based polyclonal anti-human IgG was on the control line. In the Hotgen test, 10 uL serum was pipetted to the cassette, followed by three drops of buffer solution and the result was interpreted after an incubation of 15 minutes. The presence of only the control line was accepted as negative result while the presence of control and test lines together showed the presence of total antibody in the sample. In the Turklab kit, recombinant viral nucleocapsid antigen was on the test line and chicken-based IgY on the control line. After the 10 uL serum was transferred to the cassette, two drops of buffer solution were added and the result was evaluated after 15-minute incubation by reading the control, IgM and IgG lines.

ELİSA (enzyme-linked immunosorbent assays). Euroimmun "Anti-SARS-CoV-2 IgG ELISA" assay detects IgG antibodies against recombinant SARS-CoV-2 spike protein subunit 1 (S1) antigen semi-quantitativelyin serum samples. Samples were added to the antigen coated wells after 1:101 dilution and the test was done according to the manufacturer's instructions. Positive and negative controls and calibrator were used in each study. Validation was done by values specific to the kit lot. The calculated absorbance value was evaluated as negative if it was < 0.8, and positive if it was ≥0.8.

Biochemical analyzes and computed tomography. In addition, procalcitonin, CRP and D-dimer levels and chest computed tomography (CT) results were evaluated for each patient. The biochemical tests were done with ADVIA Centaur B·R·A·H·M·S PCT, Beckman Coulter and Siemens BCS XP systems, respectively. The chest CT scan findings were reported as "typical, atypical, indeterminate, negative" in accordance with the classification recommended for COVID-19 by "Radiological Society of North America" (RSNA) (9). Only the typical CT findings were accepted as the proof for COVID-19.

Statistical analyses. Statistical analyses were performed with "IBM SPSS Statistics" (version 25.0, Chicago) software. While evaluating antibody test results, each assay was assessed separately, and then samples reactive with at least two assays were considered as antibody positive for further analysis. The relationship between antibody results and gender, chest CT findings was examined with chi-squared test, while the association of age, biochemical parameters, RT-qPCR Ct value was investigated by *t*-test and Mann-Whitney U test. Cohen's kappa was used to evaluate agreements between antibody test results and RT-qPCR, chest CT and biochemical findings. The Kappa value (κ)of >0.80 indicated perfect, 0.60-0.80 good, 0.60-0.40 moderate and 0.40-0.20 fair agreement. Logistical regression analysis was used for independent variables predicting RT-qPCR and antibody results. The sensitivities and specificities of the antibody tests were determined according to the result of RT-qPCR, and additionally, performances of immunochromatographic tests were evaluated against to the ELISA test.

RESULTS

Antibody testing was performed on 129 of these patients, among which, 88 cases that had at least 14 days (14-43 days) after the PCR evaluation were included in the study.

Study group consisted of 48.9% (43/88) males and 51.1% (45/88) females, with a mean age of 37 ± 12.74 (median: 35). Majority (68/88, 77.3%) of the patients were health care workers, while the others were from COVID-19 outpatient clinic (n:9, 10.2%), emergency department (:10, 11.4%) and anesthesiology intensive care unit (n:1, 1.13%).

SARS-CoV-2 RNA was positive by RT-qPCR in 41/88 (46.6%) of the patients. PCR Ct values were between 9.42 - 40.00. A total of 17 (17/41, 41.4%) patients with a positive PCR assay had anti-COVID-19 antibodies that were detected by at least two of the three assays used in the study, whereas in one patient, only Euroimmun-IgG ELISA test was positive. Among the 47 viral RNA negative patients, 45 (95.7%) were antibody negative while two patients were antibody positive only with Euroimmun test (Table 1). Patients who had positive result with theTurklab test, both IgM and IgG lines were reactive.

Antibody positivity in male gender was significantly high erwith Hotgen (p: 0.010) and Euroimmun

tests (p:0.042), while no such gender effect was observed with Turklab assay (p:0.162). The mean age of antibody positive patients was significantly higher than those with negative result (Table 2). The mean age was 43.47 ± 15.89 (p:0.042) and 46.73 ± 20.42 (p:0.032) in Hotgen and Turklab positive cases, respectively. Although a similar finding was detected with Euroimmun test, the difference was not statistically significant (p:0.075).

When at least two positive results by different assays were taken as the indication of confirmed antibody positive status, gender was not a significant factor (p:0.060), while the mean age was significantly higher in antibody positive cases (p:0.047) (Table 2).

All of the confirmed antibody positive patients (n:17) were also viral RNA positive. Patients who had a PCR-Ct value≤18.92 ± 7.87 (p:<0.000) and viral Ct/internal control Ct ratio ≤1.01 ± 0.47 had significantly higher rate of antibody positivity (Fig. 1).

When RT-qPCR was accepted as the reference, the sensitivity and specificity of the Hotgen and Turklab antibody tests were identical, 36.6% and 100%, respectively. Each test detected 15 antibody positive samples, 13 of which were the same patients. In addition, each test detected 2 more positive samples. All antibody positive patients detected by immunochromatographic tests were also reactive by ELISA and RT-qPCR assays. The sensitivity of the Euroimmun test was 43.9% and specificity was 95.7% compared to PCR assay. Euroimmun antibody test was positive in two patients with a negative PCR result. One of them had a Euroimmun IgG S/CO value of 2.04 while the thoracic CT and biochemical parameters were normal. The other patient had a S/CO value of 1.48, and there was no request for thoracic CT or biochemical tests.

When RT-qPCR was the reference, the positive predictive value (PPV) was 100% for Hotgen and Turklab and 90% for Euroimmun; the negative predictive value (NPV) was 64.4% for Hotgenand Turklab and 66.2% for Euroimmun. When detection with at least two tests was accepted as the confirmed antibody positivity, sensitivity of the antibody detection was 41.5% and specificity was 100%, PPV was 100%, and NPV was 66.2%. When Euroimmun ELISA result was the reference, sensitivity of both Hotgen and Turklab was 75%, specificity was 100%, PPV was 100% and NPV was 93.15%.

The correlation between three antibody tests and RT-qPCR results was evaluated with Kappa analysis

Table 1. Data of the study

Patients number, n (%)	Antibody Assays			Real time RT-PCR	CT Results (n)				D-dimer		Biochemical Results (n) CRP		Procalcitonin			
	Hotgen	Turklab	Euroimmun	Bioksen	Typical	Atypical	Negative	Indeterminate	Normal	High	Normal	High	Normal	Local infection	Systemic infection	Severe sepsis	Septic shock
45 (51.3)	N	N	N	N	1	2	15	2	19	4	18	7	18	3	1	-	-
23 (26.1)	N	N	N	P	-	-	16	-	14	1	14	2	11	1	-	-	-
13 (14.8)	P	P	P	P	3	-	2	4	5	4	4	6	6	2	1	-	-
1 (1.13)	N	N	P	P	-	-	-	-	-	-	-	-	-	-	-	-	-
2 (2.3)	N	N	P	N	-	-	2	-	1	-	1		1	-	-	-	-
2 (2.3)	N	P	P	P	-	-	1	1	2	-	1	1	-	1	-	-	-
2 (2.3)	P	N	P	P	-	-	2	-	2	-	1	1	2	-	-	-	-
Total: 88					4	2	38	7	43	9	39	17	38	7	2	-	-
					51				52		56			47			

N: Negative
P: Positive
D-dimer: Normal: 0-0.55 ug/mL (FEU), Yüksek: >0.55
CRP: Normal: 0.2-5 mg/L, Yüksek: >5
Procalsitonin: Normal: 0-0.05 ng/mL, local infection: 0.05-0.5 ng/mL, systemic infection:0.5-2 ng/mL, severe sepsis: 2-10 ng/mL, septic shock: >10 ng/mL

Table 2. Relation between gender, age and antibody response

		Antibody negative	Antibody positive	Positive percentage (%)	p
Sex					
Hotgen					
	Male	31	12	27.9	0.010
	Female	42	3	6.7	
Turklab					
	Male	33	10	23.3	0.162
	Female	40	5	11.1	
Euroimmun					
	Male	29	14	32.6	0.042
	Female	39	6	13.3	
≥2 Antibody assay positive					
	Male	31	12	27.9	0.060
	Female	40	5	11.1	
Age					
Hotgen	mean ± SD	36.23 ± 11.75	43.47 ±15.89		0.042
Turklab	mean ± SD	35.56 ± 9.67	46.73 ± 20.42		0.032
Euroimmun	mean ± SD	35.65 ± 9.88	43.65 ± 18.63		0.075
≥2 Antibody assay positive					
	mean ± SD	35.61 ± 9.76	45.24 ± 19.65		0.047

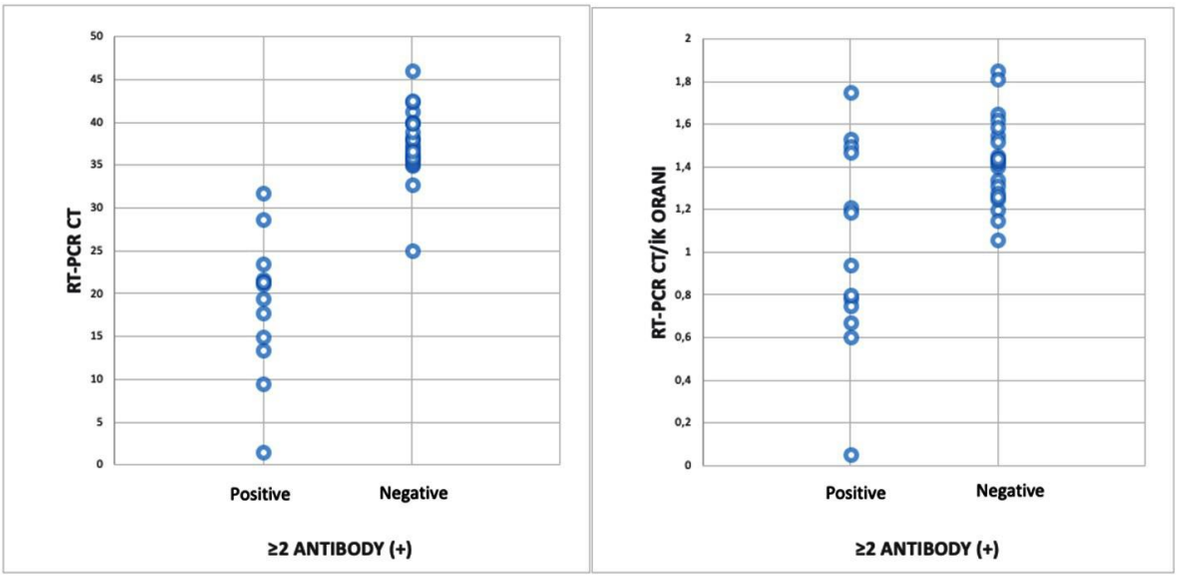

Fig. 1. Relation between antibody positivity and RT-qPCR Ct value and viral Ct/IC Ct ratio

and a low-moderate level of agreement was observed for each of them. When antibody tests were compared to each other, perfect agreement was observed between Hotgen-Turklab and Turklab-Euroimmun pairs and a good level of agreement was detected between Hotgen-Euroimmun.

Regarding biochemical markers, data were available for procalcitonin in 53.4% (47/88), for D-dimerin 59% (52/88), for CRP in 63.6% (56/88) of the patients. Chest CT scan results was available in 58% (51/88) of the cases (Table 1). Among the 17 antibody positive patients with at least two tests, three had a typical chest CT findings for COVID-19, while only one patient negative for viral RNA and antibodies had

a typical CT scan result (p: 0.007). No significant relation was detected between CRP, D-dimer and procalciton in values and the presence of viral antibodies or RNA (Table 3).

Independent variables effecting the antibody and PCR results were evaluated with logistic regression analysis. The presence of typical CT finding increased positive antibody results13-fold (Table 4), while male gender increased the positive PCR results 4-fold (Table 5).

Predictive PCR-Ct value for antibody positivity was 32.24 (92.86% sensitivity, 100% specificity) which determined by ROC analysis. The area under the curve was (AUC): 0.994 (p<0.001) (Table 6, Fig. 2).

Clinical data of 40 PCR positive patients tested for antibody were evaluated for the presence of symptoms (fever, headache, sore throat, cough, shortness of breath, anosmia/ageusia, muscular pain, diarrhea). There was at least one symptom in 16 of the 17 patients (94%) who had positive antibody results with ≥2 tests while symptoms were detected in 13/23 (56.5%) antibody negative cases (p: 0.01). All 23 viral RNA positive patients that were antibody negative by all three assays were outpatients and had high Ct values (range 35-40).

Table 3. Relation between CRP, D-dimer, procalcitonin values and positive antibody test result detected by ≥2 assays

	Antibody Negative	Antibody Positive	P
CRP (n:56)			
Mean ± SD	7.65 ± 19.91	16.55 ± 31.15	0.098
D-Dimer (n:52)			
Mean ± SD	0.44 ± 0.70	0.80 ± 1.02	0.091
Procalcitonin (n:47)			
Mean ± SD	0.06 ± 0.17	0.09 ± 0,21	0.149

Table 4. Assessment of the independent variables that effect antibody results by logistic regression analysis

Independent Variables	OR	%95 GA	p
Age			
Continuous	1.067	0.960-1.185	0.228
Sex			
Female/Male	6.817	0.703-66.149	0.098
CT finding			
+/-	13.642	1.504-123.77	0.020
CRP			
<5 mg/L / >5 mg/L	6.361	0.627-64.580	0.118

OR: Odds Ratio

Table 5. Assessment of the independent variables that effect RT-qPCR results bylogistic regression analysis

Independent Variables	OR	%95 GA	p
Age			
Continuous	0.999	0.925-1.079	0.978
Sex			
Female/Male	4.725	1.272-22.463	0.048
CT finding			
+/-	4.750	0.746-30.250	0.099
CRP			
<5 mg/L / >5 mg/L	1.201	0.164-8.815	0.857

OR: Odds Ratio

Table 6. Calculation of RT-qPCR cycle threshold (Ct) value in predicting the antibody positivity by ROC analysis

RT-qPCR Ct value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
30.195	92.31	95.83	92.31	95.83
32.245	92.86	100	100	95.83
33.86	86.67	100	100	91.67

PPV: positive predictive value, NPV negative predictive value

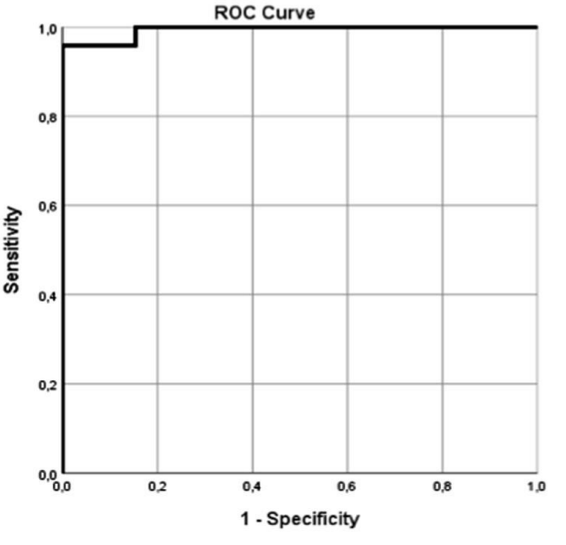

Fig. 2. ROC curve of RT-qPCR Ct value in predicting the positive antibody result

DISCUSSION

Our study shows that 41.4% of the PCR positive patients were antibody positive with ≥2 assays, ≥14 days after the PCR test. The sensitivity of the immunochromatographic card tests and ELISA in comparison to RT-qPCR were 36.6% and 43.9%; specificity was 100% and 95.7% respectively. The sensitivity of the ELISA test was higher than the immunochromatographic card tests as expected. The sensitivity of the immunochromatographic tests in comparison to ELISA was 75% and specificity was 100%. The high specificity of immunochromatographic tests makes a positive result significant, while low sensitivity shows that a negative result cannot exclude previous infection. As far as we know, this is the first report evaluating the immunochromatographic tests used in this study. In similar studies comparing antibody assays, specificity is almost always higher than sensitivity of the test. Sensitivity is affected by antibody isotypes, the antigen, test method, severity of the infection and the number of days passed after the onset of the infection or the detection of viral RNA. In a study comparing the performances of six different rapid immunochromatographic tests with ELISA and NAT, the sensitivity of the tests was between 10% and 55%, which was increased in patients who had been having the symptoms for at least seven days with a CRP level greater than 100 mg/L (10). It was underlined that the negative results in the rapid tests could not be used to exclude COVID-19 infection due to their limited sensitivity but they could help the management of cases in selected groups with their high specificity (10). In another study, diagnostic performance of seven rapid immunochromatographic tests and Euroimmun ELISA was evaluated on the 14^{th} to 25^{th} day after the onset of the symptoms. Sensitivity for IgG in rapid tests was 92.1-100%, and specificity was 90.3-99%. In the same group, sensitivity of Euroimmun IgG ELISA revealed a lower sensitivity (89.5%) than some rapid tests which was explained as a slower seroconversion detected with ELISA. To support this hypothesis, the researchers stated that IgG was detected in nine patients with all of the rapid tests on the early days of the infection, while Euroimmun IgG ELISA was negative. IgM detection of rapid immunochromatographic tests showed a great variability while not providing an improvement for the diagnostic performance (11). In our study, IgM and IgG were both positive in all of the antibody positive samples, therefore the value of detecting IgM could not be evaluated separately.

Two of the viral RNA negative patients, one with COVID-19 symptoms, were IgG positive with Euroimmun test while rapid tests yielded negative results. The patient with symptoms tested with Rt-qPCR twice with an interval of five days and both tests were resulted negative. The antibody tests of these cases were done 20 and 22 days after the first PCR tests. It was not possible to confirm if these cases had the infection, therefore there is a possibility of a false negative PCR result or a false positive antibody reaction.

In our study, the presence of CT findings were found to be significant in predicting antibody positivity. A study conducted with immunochromatographic tests in China also concluded that chest CT with the antibody testing can be used to diagnose COVID-19 in centers that do not have a molecular laboratory (12). Another influencing factor was the age of the patient. The mean age was significantly higher in antibody-positive cases and the importance of age was particularly evident in rapid immunochromatographic tests. Male gender was also a factor that increased antibody response in Hotgen and Euroimmun tests. Although there are contradictory results about the importance of the gender in the literature, male gender, older age and severe disease were detected among the factors increasing the antibody response by many studies (13, 14).

Another finding of this study was the detection of a significantly higher rate of antibody response in people with a low PCR-Ct value. Other studies also have shown the relationship between viral load and antibody response and found that the lower the mean Ct value, the higher the ELISA index (S/CO) (15). The optimal Ct value to predict antibody response was 34 (with 93.7% sensitivity and 82.4% specificity) in the paper of Wellinghausen Net al, while it was 32.24 (with 92.86% sensitivity and 100% specificity) in our study.

In this study, 23 of the samples were viral RNA positive but antibody negative with all three assays. Although it is known that there are patients who do not develop antibodies despite PCR positivity, the frequency of such cases is different between the studies. Henry BM et al. found that 11% of the cases were antibody negative and this rate decreased as more time passed after the onset of the symptoms (16). The study suggested that optimal time to evaluate the an-

tibody response was at least 3-4 weeks after the onset of the symptoms and at least two weeks after the end of the symptoms (15). In the paper of Wajnberg et al. there were cases in which antibody response could not be detected even in 50 days (17). The severity of the disease and the type of antigen (S or N) used in the antibody assay are among the factors that influence the detection of antibody response (18, 19). In our study, presence of symptoms had an effect on antibody response, since nearly half of the patients with negative antibody result were asymptomatic while there was only one asymptomatic case in the antibody-positive group. PCR positive and antibody negative group of patients were all outpatients and 43.5% were asymptomatic. These samples had high Ct values which correlates with low viral load at least during the time of sampling. Several studies also showed lack of detectable antibody response in some infected patients since humoral immune response is dependent on the duration and magnitude of viral exposure (15). There may also be a low probability of false PCR positivity.

Lymphocyte and neutrophil count, CRP levels, ESR, procalcitonin (PCT) and D-dimer levels were found to be associated with the severity of the COVID-19 in many studies (20). Increased procalcitonin in COVID-19 generally shows the presence of an accompanying bacterial or fungal infection or severe systemic inflammatory response syndrome. In our study, there was no significant relation between CRP, D-dimer, PCT levels and antibody response.

The limitations of this study are the lack of clinical data and symptom onset dates for some of the patients. Therefore, the day of the PCR test was used as the starting point for detection of ≥14 days. On the other hand, the onset of the symptoms is sometimes subjective and it is not always possible to demonstrate its accuracy. Small sample size is another limitation of the study.

In summary, this study evaluated the antibody response in a group of patients by two rapid immunochromatographic tests and anIgG-ELISA test, ≥14 days after the RT-qPCR assay. Antibody response was detected 41.4% of the PCR positive patients. The performance of the two immunochromatographic tests, one detecting total antibody and the other capable of differentiating IgG and IgM was identical. The sensitivity of the IgG ELISA test was higher than the immunochromatographic tests. The high specificity of immunochromatographic tests made a positive result significant, while low sensitivity showed that a negative result cannot exclude previous infection. Antibody response correlated with male gender, older age, presence of typical chest CT findings and low PCR-CT value.

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