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Comparative evaluation of SARS-CoV-2 IgG assays against nucleocapsid and spike antigens

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Abstract.
BACKGROUND: There are few studies to compare antibody response against anti-spike (S) and anti- nucleoprotein (N) SARS-CoV-2.
OBJECTIVE: The aim of this study was to evaluate the IgG antibody production against S and N antigens of the virus and their correlation with the time and severity of the disease.
METHODS: The IgG antibodies against S and N antigens of SARS-CoV-2 in serum specimens of 72 symptomatic patients who tested real-time reverse transcription polymerase chain reaction positive for SARS-CoV-2 were detected using the ELISA technique. Different antibody response was compared and the correlation with the time from disease onset and the severity was evaluated.
RESULTS: Forty-eight of 72 (67%) patients tested positive for anti-SARS-CoV-2 antibodies, while 24 (33%) did not have detectable antibodies. Comparison of antibody levels for N and S antibodies showed that they correlate with each other well ($r = 0.81$; $P < 0.001$). However, sensitivity of anti-S SARS-CoV-2 IgG and anti-N SARS-CoV-2 IgG was 30% and 60%, during the first 7 days after symptom onset ($r = 0.53$; $P = 0.111$), but increased to 73% and 68% at more than 1-week post symptom onset ($r = 0.89$, $P = 0.111$), respectively. Cases with positive IgG response showed a decreased $CD8^+$ T cells percentage compared to the negative IgG groups (26 ± 14 vs. 58 ± 32, $p = 0.066$ in anti-N IgG group and 28 ± 15 vs. 60 ± 45, $p = 0.004$ in anti-S IgG group, respectively).
CONCLUSION: Nearly one-third of the confirmed COVID-19 patients had negative serology results. Lower percent positivity at early time points after symptom onset (less than 1 week) was seen using anti-S SARS-COV-2 IgG kit compare to the anti-N SARS-CoV-2 IgG; therefore, clinicians should interpret negative serology results of especially anti-S SARS-CoV-2 IgG with caution.

Keywords: SARS-CoV-2, antibody response, spike, nucleoprotein, lymphocyte subsets

1. Background

SARS-CoV-2 and its related disease COVID-19 is associated with significant morbidity and mortality globally [1,2]. According to the latest report of World Health Organization (WHO), more than 60 million people be-

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ing infected, with 1,420,306 deaths as of November 27, 2020. Although the fatality rate of SARS-CoV-2 is lower than those of other coronaviruses that caused disasters in the past, its higher infectivity rate makes it worse [3], probably make it as one of the biggest health and economic burden of the last 100 years [4].

Since there are no specific therapeutic drugs or vaccines for COVID-19, early detection of cases with SARS-CoV-2 infection is crucial to decrease the risk of infecting a larger population [5]. There are a number of important unanswered questions yet. First, it is uncertain how long antibodies persist after infection [6]. Second, SARS-CoV-2 serologic test could really be used in the clinical practice or not [7]; and the third, there are currently no studies which demonstrated that antibodies are protective against reinfection in humans [8,9].

Serological tests typically detect antibodies against spike protein (S) and/or nucleoprotein (N), the most immunogenic proteins of SARS-CoV-2. The S protein, consisting of a S2 and a S1 subunit is present on the envelope of SARS-CoV-2 and help the virus to connect to the human cells using the Angiotensin-converting enzyme 2 (ACE2) receptor [8]. Since anti-S protein antibodies have been shown to possess neutralizing effects *in vitro*, it has been suggested that detection of antibodies against S protein could provide a better indication of an effective immune response [10,11].

There are few studies to evaluate the SARS-CoV-2 IgG assays, and lymphocyte subsets comprehensively in COVID-19 patients with different disease severity [12], and antibody response against anti-S and anti-N SARS-CoV-2.

The aim of this study was to determine the antibody response against SARS-CoV-2 S and N protein using ELISAs for the detection of IgG and the presumptive correlation with level of lymphocyte subsets in COVID-19 patients.

2. Methods

This study was performed at the Masih Daneshvari Hospital, Tehran, Iran and approved by the local ethics committee (approval number: IR.SBMU.MSP.REC. 1399.260).

Seventy-two symptomatic patients who tested real-time reverse transcription polymerase chain reaction (RT -PCR) was positive for SARS-CoV-2 in nasopharyngeal swab samples and admitted to the infectious disease ward were recruited into the study. The presence of SARS-CoV-2 was detected as previously described [5]. Demographic data, laboratory parameters, and clinical severity during the hospitalization period were retrieved from patient records. The COVID-19 patients were classified into moderate, severe, and critical groups [12]. For the purpose of this study, at rest oxygen saturation (O_2 sat) and respiratory rate were used for severity classification. Patients with pulmonary infiltration in chest imaging and O_2 sat more than 93% with ambient air were classified as moderate group and patients with O_2 sat $\leqslant$ 93% or a respiratory rate of more than 30 breaths/min were categorized as severe group. The patients, who need noninvasive or mechanical ventilation; and the patients with shock, or who need intensive care management, were classified as critical cases.

Days of symptoms were recorded based on first day of onset of COVID-19 symptoms, as documented by managing clinicians. In addition, we collected COVID-19 patients who have detected lymphocyte subsets and SARS-CoV-2 antibodies during the same day.

2.1. SARS-CoV-2 antibody detection

For every patient, one blood sample was collected. The serum IgG antibodies against N and S antigens of SARS-CoV-2 were measured according to the manufacturers' instructions using the enzyme-linked immunosorbent assay (ELISA) kits supplied by Pishtaz Teb Diagnostics Company, and EUROIMMUN anti-SARS-CoV-2 assay kits.

2.2. Flow cytometry analysis

The percentages and absolute counts of total T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, and NK cells were determined by using phycoerythrin conjugated anti-human CD4, CD19, CD56 antibodies; anti-human CD8 and CD16 allophycocyanin conjugated antibodies; and fluorescein sothiocyanate conjugated antibody for $CD3^+$ T cells according to the manufacturer's (PharMingen) instructions. A FACSCalibur^{TM} flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for cell analysis.

2.3. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Measurement data were tested for normality. Data that confirmed normality were expressed as mean $\pm$ standard deviation (*SD*), and t-test was used for comparison between groups. Median and interquartile range

(IQR) were used for noncompliant data. The comparison between multiple groups, Kruskal-Wallis test was used for pairwise comparison between groups. Pearson correlation tests were also performed. A two-sided P value of less than 0.05 was considered statistically significant.

3. Results

A total of 72 patients with COVID-19 admitted to the Masih Daneshvari Hospital, Tehran, Iran were enrolled in the study. The majority of the patients with RT-PCR-confirmed SARS-CoV-2 were female (57%, $n = 41$), and the median age was 60 years (IQR: 45–68 years). Forty-eight (67%) of the patients had $\geqslant 1$ risk factor, including heart disease, chronic lung disease, diabetes mellitus, and hypertension. The cases were classified into three groups, moderate (11 cases, 15%), severe (27 cases, 38%), and critical (34 cases, 47%).

A total of 72 plasma samples were collected during the hospitalization and tested for antibodies against SARS-CoV-2 S and N antigens. Forty-eight of 72 (67%) patients tested positive for anti-SARS-CoV-2 Abs with either Pishtaz Teb or EUROIMMUN anti-SARS-CoV-2 Assay, while 24 (33%) did not have detectable antibodies. Although the number of serology positive cases using Pishtaz Teb or EUROIMMUN anti-SARS-CoV-2 kits was similar, 3 cases had positive anti-N SARS-CoV-2 IgG and negative anti-S SARS-CoV-2 IgG tests. On the other hand, 3 cases showed detectable anti-S SARS-CoV-2 IgG tests, while the negative anti-N SARS-CoV-2 IgG was found.

The mean duration from onset of symptoms to perform anti-N and anti-S IgG test was close between negative and positive anti-N IgG groups (17.0 $\pm$ 8.8 vs. 17.31 $\pm$ 10.7 days) and negative and positive anti-S IgG groups (15.7 $\pm$ 9.4 vs. 17.7 $\pm$ 10.4 days), respectively.

In the current study, sensitivity of anti-S SARS-CoV-2 IgG and anti-N SARS-CoV-2 IgG was 30% and 60%, respectively during the first 7 days after symptom onset, but increased to 73% and 68% at more than 1-week post symptom onset (Table 1).

The median level of anti-S SARS-CoV-2 IgG during the first week after onset of symptoms was 0.45 (IQR: 0.29–2.9) that was significantly lower than the observed anti-S SARS-CoV-2 IgG in groups who were sampled after 1 week from onset of symptoms (9.0; IQR: 0.6–13.6, p value $= 0.004$); while no significant difference was found in the level of anti-N SARS-CoV-2 IgG in groups during the first week and after 1 week from onset of symptoms (10. (IQR: 0.2–20.8) vs. 14.2 (IQR: 0.4–25.6); p value $= 0.34$).

The median level of both anti-S SARS-CoV-2 IgG and anti-N SARS-CoV-2 IgG in severe and critical illness patients were not differ significantly compare to those in moderate course of disease ($p = 0.46$ and $p = 0.21$, respectively).

Comparison of antibody levels for N and S antibodies showed that they correlate with each other well ($r = 0.81$; $P < 0.001$). Among the RT-PCR-positive patient samples collected > 14 days after onset of symptoms, seropositive N antibodies were detected in 24 out of 34 samples, yielding a sensitivity of 63%. A similar analysis of the spike antibody in samples collected > 14 days after onset of symptoms showed a slightly higher sensitivity of 66% (25 of 38) ($r = 0.94$; $P < 0.001$), while their correlation among samples collected < 14 days after onset of symptoms was lower ($r = 0.66$; $P < 0.001$).

Lower percent positivity at early time points after symptom onset (less than 1 week) was seen using anti-S SARS-COV-2 IgG kit compare to the anti-N SARS-CoV-2 IgG ($r = 0.53$; $P = 0.111$), while anti-S SARS-COV-2 IgG in samples collected > 7 days after onset of symptoms showed a slightly higher sensitivity compare to the anti-N SARS-COV-2 IgG kit (73% versus 68%, respectively; $r = 0.89$, $P = 0.111$).

Further, compared to the negative anti-N and anti-S IgG group, the neutrophil counts were lower in the anti-N IgG positive group (6.3 $\pm$ 1.0 vs. 19.9 $\pm$ 9.7, $p < 0.001$) and anti-S IgG positive group (6.4 $\pm$ 1.61 vs. 9.4 $\pm$ 6.01, $p = 0.005$), respectively; while the counts of total WBC and lymphocyte were not significantly differ in negative and positive anti-N or anti-S IgG groups.

The antibody levels and lymphocyte subsets of 14 COVID-19 patients were evaluated and cases with positive IgG response showed a decreased CD8 cell percentage compared to the negative IgG groups (26 $\pm$ 14 vs. 58 $\pm$ 32, $p = 0.066$ in anti-N IgG group and 28 $\pm$ 15 vs. 60 $\pm$ 45, $p = 0.004$ in anti-S IgG group, respectively). No significant differences were found between antibody levels and other lymphocyte subsets.

4. Discussion

Our data showed that for both N and S antigens, the sensitivity was 67%, and 33% did not have detectable antibodies, so negative serological results alone cannot exclude the diagnosis of COVID-19 that is consistent with the previous report [13]. Comparison of antibody

Table 1
The sensitivity of anti-N SARS-COV-2 IgG and anti-N SARS-COV-2 IgG assays during the time

		Less than 1 week		More than 1 week		P value	Less than 2 weeks		More than 2 weeks		P value
		N	%	N	%		N	%	N	%	
Anti-N SARS-COV-2 IgG	Negative	4	40	20	32	0.720	10	29	14	37	0.62
	Positive	6	60	42	68		24	71	24	63	
Anti-S SARS-COV-2 IgG	Negative	7	70	17	27	0.013	11	32	13	34	1.0
	Positive	3	30	45	73		23	68	25	66	

levels for N and S antigens showed that they correlate with each other well ($r = 0.81$; $P < 0.001$). The sensitivity for antibody to the N protein for samples collected $\leqslant 7$ days after onset of symptoms was 60% (6 of 10). Analysis of S antibodies at this time point showed a reduced sensitivity of 30% (3 of 10). Taken together, timing of when the tests are used is important [14] and our findings indicate that detection of antibodies against the N protein is more sensitive than detection of antibodies against the S protein during the first week after symptom onset, and that N antibodies generally appear earlier than spike antibodies that is in consistent with previous report [15]. At the onset of SARS-CoV infection, B cells elicit an early response against the N protein, while antibodies against S protein could be detected after 4–8 days from the early stage of acute infection [3,16]. N protein is an internal viral protein of SARS-CoV-2 and is not a target of neutralizing antibodies, so earlier and even stronger anti-N antibody production might observe [17].

According to the previous report, although nearly 93% of exposed asymptomatic individuals had detectable T cell responses to SARS-CoV-2, only 60% of cases were seropositive [18]. In the current study, we found that 36% ($n = 20$) and 34% ($n = 19$) of the recovered patients had negative anti-N and anti-S IgG, respectively. The definite mechanism remains unclear [19].

Several studies reported that weak or non-responders for IgG antibody had higher viral clearance than strong responders and robust antibody response correlate with the severity of the disease [6,20], while in our study similar to previous reports [6,21], antibody response in severe and critical illness patients were not differ significantly compare to those in moderate course of disease. We concluded that antibody levels could not be used to predict the severity of the disease that was in consistent with previous reports.

In our study, the neutrophil counts were lower in the IgG positive group compared to the negative IgG group that is consistent with Liu et al. study [19]. Cases with positive IgG response showed a decreased CD8 cell percentage compared to the negative IgG groups (26 $\pm$ 14 vs. 58 $\pm$ 32, $p = 0.066$ in anti-N IgG group and 28 $\pm$ 15 vs. 60 $\pm$ 45, $p = 0.004$ in anti-S IgG group, respectively), while no significant differences was observed between antibody levels and the counts of other lymphocyte subsets in COVID-19 patients, which might be due to that the detection of lymphocyte subsets could not reflect the specific T cell or plasma cell levels during SARS-CoV-2 infection [12]. Our results are consistent with Zhang et al. that reported no association between antibody levels and the T cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and B cells [22].

The strength of our study includes the using the same cohort of unique, non-duplicate COVID-19 patients' sera to compare performance of anti-S and anti-N SARS-CoV-2 IgG response head-to-head. There are a number of limitations to our study. First, we only included a limited number of samples particularly for determination of lymphocyte subsets. Second, the control samples were not included for calculation of specificity. Third, we did not follow the patients for evaluating possible seroconversion. Finally, we only evaluated the diagnostic performance in patients with moderate to critical COVID-19 and did not study the antibody response in asymptomatic persons and patients with mild COVID-19.

5. Conclusion

In our study, nearly one-third of the confirmed COVID-19 patients had negative serology results. Compared to the anti-N SARS-CoV-2 IgG assay, anti-S SARS-COV-2 assay showed lower sensitivity during the first week after symptom onset; therefore, clinicians should interpret negative serology results of especially anti-S SARS-CoV-2 IgG with caution. Further investigation of patients who fail to produce detectable levels of IgG is highly recommended.

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Conflict of interest

The authors declare that they have no competing interests.

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