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Investigating the diagnostic and prognostic value of anti-SARS-CoV-2 Spike IgG/IgM ELISA tests in patients infected with coronavirus Delta variant

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SUMMARY

Aim: This study aimed to investigate the diagnostic and prognostic value of anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike IgG/IgM antibodies in patients infected with coronavirus Delta variant.
Methods: This analytical observational study included 270 unvaccinated patients (aged ≥18 years) diagnosed with coronavirus disease 2019 (COVID-19) Delta variant who referred to Emergency Department of our hospital. The serum levels of anti-SARS-CoV-2 Spike IgG and IgM were measured by indirect ELISA. Main measured outcomes included anti-SARS-CoV-2 Spike IgG and IgM, chest computed tomography (CT) severity score, clinical and laboratory findings which were prospectively evaluated throughout the study period.
Results: The IgM levels in critical patients were significantly higher than non-critical patients ($p<0.05$). But the mean level of IgG in critical patients was not significantly different from its level in non-critical patients ($p>0.05$). However, a significant positive correlation was observed between the levels of both antibodies and chest CT severity score ($p<0.0001$); this implies that their levels may reflect the degree of lung involvement. The IgM level on 15^{th}-16^{th} days after symptoms onset was significantly associated with the hazard of death even after adjusting for all other factors (adjusted HR (95%CI):1.28(1.014_1.63), $p=0.03$), whereas IgG was not ($p>0.05$). The survival probability among patients with IgM level ≥8.67 RU/ml (34.2%) was significantly lower than those with IgM level <8.67 RU/ml (99.5%, $p=0.0001$).
Conclusions: Anti-SARS-CoV-2 Spike IgM antibody was significantly associated with the disease severity and risk of death in unvaccinated patients infected with coronavirus Delta variant. However, further large-scale investigations on diverse infected populations are required to precisely determine the diagnostic/prognostic value of these antibodies.

Keywords: SARS-CoV-2, IgM, IgG, diagnostic, prognostic value

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INTRODUCTION

Given the serious threat and harms of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic to health care systems and people worldwide, identifying the cost-effec-

tive diagnostic and prognostic methods is vital for timely treatment and control of coronavirus disease 2019 (COVID-19) pandemic [1, 2]. To date, several diagnostic approaches approved by Food and Drug Administration (FDA) have been used for screening symptomatic and asymptomatic individuals, including RNA-based methods such as real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), imaging technologies such as chest computed tomography (CT), and serological testing of antibodies (IgM and IgG)-based enzyme-linked immunosorbent assay [2-4].

Some unexplained phenomena in COVID-19 patients obliges us to precisely understand the changes in immune response in different stages of infection. For example, sudden deterioration and unexpected death in some patients who previously were in stable condition and or convalescent patients with more than one negative result in nucleic acid test [5]. The key role of the host's immune response in fighting with SARS-CoV-2 has been previously recognized. The histological evidences have revealed a bilateral diffuse alveolar damage and mononuclear inflammatory cell infiltrate in the lungs of patients who died of COVID-19 [6].

Spike proteins of SARS-CoV-2 has been prioritized as potential antigens to development of serological tests due to their importance for host receptor binding, high immunogenic activity, and their ability to detect immune responses of vaccinated individuals [7]. Spike-specific IgM and IgG antibodies found in the same serum samples of COVID-19 patients imply that S-specific IgG antibodies can be found almost simultaneously with S-specific IgM [8]. The levels of anti-SARS-CoV-2 antibodies reflect humoral immune response patterns for SARS-CoV-2 infection and may be useful for diagnosis of COVID-19 as well [9]. Hence, this preliminary study aimed at evaluating the probable diagnostic and prognostic value of anti-SARS-CoV-2 Spike IgG/IgM antibodies in unvaccinated patients infected with coronavirus Delta variant.

PATIENTS AND METHODS

Study design

In this analytical observational study, clinical and laboratory characteristics of 270 patients infected with coronavirus Delta variant who had referred to the Emergency Department of Razi Hospital of Ahvaz, Iran, before general COVID-19 vaccination (March-August 2021) were prospectively evaluated. This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Ethical Code: IR.AJUMS.REC.1399.535). The local institutional ethics committee of study center oversaw the proceedings and documentation. All patients signed the informed consent prior to enrollment.

Patients characteristics

Eligible voluntary patients (aged 18 years or older) who showed the specified symptoms of COVID-19 and the positive results of SARS-CoV-2 RT-PCR test and CT scan were recruited in this study. But, patients with non-completed documentation, those who had rheumatoid arthritis and were vaccinated against COVID-19, as well as those who died before completing the tests were excluded from the study.

Considering the statistician's idea and a sensitivity of 96% for SARS-CoV-2 ELISA against spike protein previously optimized by Freeman et al., the sample size was calculated using the following formula [α=0.05; $Z\alpha/_2$=1.96; d or accuracy of estimate=0.05; P=0.96]:

$N=Z^2\ \alpha/_2 \times P(1-P=61$, but by considering 20% drop out rate, N=73 cases or more for each studied group [10].

Procedures

The routine laboratory tests [*e.g.*, complete blood count, coagulation profile, fasting blood sugar (FBS), albumin, lactate dehydrogenase (LDH), blood urea nitrogen (BUN), etc.] were performed within 2 hours after blood sampling and within 24 hours after admission. These tests were done using standard kits (Merck KGaA, Darmstadt, Germany). Patients' sera were collected and stored at −80 °C until use for serological tests.

Patients were followed up to date of discharge and/or death in hospital by a nurse along with two investigators. Their demographic, clinical, and laboratory characteristics were collected from medical records on a daily basis and recorded in data sheet for statistical analysis. Also, all COVID-19 patients were classified into three groups [*i.e.*, patients with mild, moderate, and severe COVID-19 infection] based on the WHO severity criteria explained in our previous study [11].

T imaging and its analysis

The initial and secondary CT scans were performed for all patients on 5th-7th day and 28th-30th day after symptoms onset, respectively. A 16-channel CT scanner (Canon Medical Aquilion CT system) with tube voltage 120 kV, current 50 mA, rotation time 0.5 s, slice thickness 5 mm, and matrix 512 × 512 was applied. The scanning area was from the apex to the base of lung.

The chest CT scan scoring system suggested by Pan et al was applied to this research [12]. Depending on the percentage of lobe involvement, a range of 0 to 5 points was considered for each of the 5 lung lobes, and a total score was obtained by summing the scores of five lobe, which ranged from 0 to 25. CT severity scoring per each of five lobes was as follows: 0, no involvement (0%); 1, minimal involvement (< 5%); 2, mild involvement (5-25%); 3, moderate involvement (26-50%); 4, severe involvement (51-75%); 5, critically ill (> 75%).

Serology tests

To detect the anti-SARS-CoV-2 antibodies (IgG and IgM) in patients' sera, the 96 wells human ELISA Kits (Shanghai Zhijiang Biotechnology Co., China) were utilized. An *in vitro* indirect ELISA was applied for the quantitative measurement of antibodies against Spike protein of Delta variant based on manufacturer's instructions. To detect IgG, 100 µL of each diluted serum (1:101), standards, positive and negative controls were added in duplicate into a 96-well microplate coated with the SARS-CoV-2 Delta spike variant protein, and incubated at 37°C for 1 hour. Standards set contains IgG antibody against Spike virus antigen with concentrations of 0, 5, 10, 25, 50, 100 in a buffer containing protein as a stabilizer and thimerosal as a preservative. After washing, 100 µL of secondary antibody against human IgG conjugated with peroxidase enzyme was poured into wells and incubated at 37°C for 30 minutes. After second washing, 100 µL of the chromogen substrate was added to the wells and incubated at 37°C in darkness for 15 minutes. Eventually, the reaction was terminated by adding a stop solution into the wells, and the optical density (OD) of each well was measured using an ELISA reader set at 450 nm. A calibration curve was obtained from the point-by-point intersection of the mean absorbance of the standards and their known concentration. The concentration of each serum sample was obtained by using the intersection point of mean absorbance with the curve on the horizontal axis. For IgM, the 96-well microplate of ELISA kit was coated with Delta N and Spike S1 receptor binding domain (RBD) proteins.

Statistical analysis

SPSS software version 26 (SPSS Inc., Chicago, Ill., USA) was used for statistical analysis. Based on the nature (categorical and continuous) and normality of data, chi-squared/Fisher's exact test, independent samples t test, and analysis of variance (ANOVA) were used to comparative analysis. Association of the levels of antibodies with chest CT score was evaluated through multivariate linear regression analysis. The optimal cutoff points of antibodies were detected by receiver operator characteristic (ROC) curve and a cox model was adjusted to determine their prognostic value in identifying the survival probability in COVID-19 patients. A P-value <0.05 was considered as statistically significant.

RESULTS

Association of clinical and laboratory characteristics with severity of disease

From a total of 270 recruited COVID-19 patients, 7 cases were excluded from the study due to incomplete data. Respectively, 137 men (52.1%) and 126 women (47.9%) with mean age 56.5±16.4 and 50.4±16.1 years were completely analyzed. As shown in Table 1, there was no correlation between the gender and severity of infection caused by Delta variant (p=0.88). But the severe infection was significantly prevalent in older patients (p<0.05).

Respectively, hypertension and cardiovascular diseases (40.3%) and diabetes (26.2%) were the most prevalent underlying diseases among the COVID-19 patients, followed by urinary tract infection (UTI) and other kidney diseases (8%), respiratory disorders (5%), and tumors or other neurological disorders (3.8%). The rate of underlying diseases among patients with moderate to severe infection was significantly higher than those with mild infection (p=0.02, Table 1). Also, a significant positive correlation was found between the severity of infection and length of hospitalization (P: 0.0001). Totally, 26 cases (9.8%) died, and all of whom had severe infection.

Although the average systolic blood pressure (BP) in severely infected patients was significantly

Table 1 - Comparison of demographic, clinical, and para-clinical characteristics between three groups of COVID-19 patients stratified based on the disease severity.

Variables	*Severe COVID-19 [n=43 (16.35%)]*	*Moderate COVID-19 [n=58 (22%)]*	*Mild COVID-19 [n=162 (61.65%)]*	*Total (n=263)*	*P Value*
Gender, n %					
Male	21 (48.8%)	30 (51.7%)	86 (53.1%)	137 (52.1%)	0.88
Female	22 (51.2%)	28 (48.3%)	76 (46.9%)	126 (47.9%)	
Age (Year), mean ±SD	60.2±16.1	50.2±15.6	53±16.6	53.6±16.5	0.009
Male	65.8±2.7	55.9±2.5	54.4±1.8	56.5±16.4	<0.05
Female	54.7±3.7	44.1±2.8	51.5±1.8	50.4±16.1	
Underlying diseases, n (%)	31 (72.1%)	44 (75.8%)	92 (56.8%)	167 (63.5%)	0.02
Chest CT Severity Score on 5^{th}-7^{th} day after symptoms onset, mean±SD	17.5±3	14.4±4.1	12.4±4.3	13.6±4.5	0.0001
Chest CT Severity Score on 28^{th}-30^{th} day after symptoms onset, mean±SD	15.1±3.4	12.7±4	10±4.4	11.1±4.5	0.0001
ICU admission, n (%)	37 (86%)	12 (20.7%)	0	49 (18.6%)	0.0001
LOS (day), mean ±SD	12.4±7.9	8.8±3.2	4.1±1.2	6.5±4.8	0.0001
Mortality rate, n (%)	26 (9.8%)	0	0	26 (9.8 %)	0.0001
Blood pressure					
Systolic BP (mm Hg), mean±SD	130.2±17.5	129.9±17.7	122±16.2	125.1±17.1	0.001
Diastolic BP (mm Hg), mean±SD	76.6±9.3	83.2±13.3	78.4±8.7	79.2±10.2	0.002
Normal BP, n (%)	21 (48.8%)	37 (63.8%)	90 (55.5%)	148 (56.3%)	0.3
Elevated BP, n (%)	11 (25.6%)	14 (24.1%)	24 (14.8%)	49 (18.6%)	0.13
Stage 1 hypertension, n (%)	5 (11.6%)	4 (6.9%)	31 (19.2%)	40 (15.2%)	0.06
Stage 2 hypertension, n (%)	6 (14%)	3 (5.2%)	17 (10.5%)	26 (9.9%)	0.3
Respiratory rate (BPM), mean±SD	27.4±8.4	28.2±9.9	25.2±4.8	26.2±7	0.08
Oxygen saturation (%), mean±SD	80.3±19.1	91.1±4.9	93.4±4.5	90.5±10	0.0001
WBC (cells/µL), mean±SD	9720.9±5656.8	8261±3848.7	8969.1±8587.3	8935.9±7339.6	0.61
Haemoglobin (g/dL), mean±SD	11.8±1.9	12.6±1.8	12.5±2.2	12.4±2	0.08
Haematocrit (%), mean±SD	36±5.7	38.1±4.7	37.7±6.2	37.5±5.8	0.14
Platelets (cells/µL), mean±SD	193.1±70.1	197.7±59.7	210.1±75	204.6±71.2	0.27
Lymphocytes (cells/µL), mean±SD	1094.9±1357.3	1378.2±1175.4	1783.8±4410.2	1581.8±3552.6	0.47
Neutrophils (cells/µL), mean±SD	8176.2±5187.3	6628.8±3279.5	6559.3±3847.5	6839±4012.7	0.06
Albumin (g/dL), mean±SD	3.8±0.7	4.4±2.8	4.1±0.5	4.1±1.4	0.11
FBS (mg/dL), mean±SD	158.3±87	134.1±56.6	182.8±126.9	168.1±110.5	0.01
LDH (IU/L)	873.6±707.6	740.6±351.6	756.9±865.5	772.4±754.7	0.62
BUN (mg/dL), mean±SD	30.2±23.2	23.3±20.2	21.7±19.8	23.4±20.6	0.05
Creatinine (mg/dL), mean±SD	1.7±2	1.3±0.64	1.7±2.4	1.6±2.1	0.46
Na (mEq/L), mean±SD	134.2±14.2	137.9±3.9	135.7±4.6	136±7	0.03
K (mmol/L), mean±SD	4.4±0.8	4.2±0.4	4.2±0.6	4.3±0.6	0.16
Calcium (mg/dL), mean±SD	8±1.9	8.5±0.75	8.7±0.6	8.5±1	0.07
Magnesium (mEq/L), mean±SD	2.3±0.3	2.2±0.4	2.2±0.4	2.2±0.4	0.16
Direct bilirubin (mg/dL), mean±SD	0.23±0.07	0.36±0.5	0.24±0.1	0.27±0.2	0.09
AST (U/L), mean±SD	48.1±28.4	63.3±53.9	51.7±29.2	53.7±36.2	0.07

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Variables	*Severe COVID-19 [n=43 (16.35%)]*	*Moderate COVID-19 [n=58 (22%)]*	*Mild COVID-19 [n=162 (61.65%)]*	*Total (n=263)*	*P Value*
ALT (U/L), mean±SD	35.8±22.1	52.2±79.2	40±26.3	42±43.6	0.11
ALP (IU/L), mean±SD	229.1±147.4	192.7±80.5	206.8±104.4	207.4±108.2	0.24
ESR (mm/hr), mean±SD	53.6±32.5	53.4±21.1	49.7±27.1	51.1±26.8	0.53
CRP (mg/L), mean±SD	47.3±31.8	44.8±29.9	46.6±33	46.4±32.1	0.9
PT (Second), mean±SD	13.3±2.7	12.9±1.1	12.9±1.4	12.9±1.7	0.3
PTT (Second), mean±SD	34.5±8	34.5±4.6	34.8±6.17238	34.7±6.2	0.92
INR, mean±SD	1.2±0.4	1.1±0.2	1.1±0.2	1.1±0.3	0.26
Anti-Spike IgG antibody concentration on 15-16th day after symptoms onset (RU/mL), mean±SD	9.3±3.6	8.7±3.3	8.4±2.7	8.6±3	0.24
Anti-Spike IgG antibody concentration on 28-30th day after symptoms onset (RU/mL), mean±SD	7.4±3.5	7.9±3.4	7.6±2.9	7.6±3.1	0.74
Anti-Spike IgM antibody concentration on 15-16th day after symptoms onset (RU/mL), mean±SD	10±2.2	5.7±0.8	5.1±0.9	6±2.1	0.0001
Anti-Spike IgM antibody concentration on 28-30th day after symptoms onset (RU/mL), mean±SD	2.4±0.6	0.1±0.4	0.05±0.3	0.2±0.7	0.0001

Notes: CU: Intensive care unit; LOS: Length of in-hospital stay; BP: blood pressure; BPM: breaths per minute; FBS: Fasting Blood Sugar; LDH: Lactate dehydrogenase; BUN: Blood Urea Nitrogen; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; PT: Prothrombin time; PTT: Partial Thromboplastin Time; INR: International normalized ratio.

higher than other groups, overall, the frequency of cases with elevated BP and/or hypertension was not significantly different between the three groups of patients (p>0.05). Also, the average respiratory rate was not significantly different between the three groups (p=0.08). Nevertheless, the mean (SD) level of oxygen saturation (%) in severely infected patients was significantly lower than those with mild to moderate infection (p=0.0001, Table 1).

No significant difference was found between the three groups in terms of the mean levels of white blood cells (WBCs), haemoglobin, haematocrit, platelet count, lymphocyte count, neutrophil count, albumin, LDH, BUN, creatinine, blood potassium, calcium, magnesium, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), prothrombin time (PT), partial thromboplastin time (PTT), international normalized ratio (INR), and anti-Spike IgG antibody (p>0.05). But the mean levels of anti-Spike IgM antibody in severely infected patients were significantly higher than that in mildly to moderately infected patients (p<0.05, Table 1).

Overall, the mean levels of both antibodies were significantly declined after 28_30 days of symptoms onset (p=0.0001).

Association of anti-SARS-CoV-2 Spike IgM/IgG antibody levels with Chest CT Severity Score

As shown in Figure 1, a significant positive correlation was found between the levels of anti-SARS-CoV-2 Spike IgG and IgM antibodies and chest CT severity score taken at 5-7th and/or 28-30th days after symptoms onset (p<0.0001). The results of multivariable linear regression analysis indicated that anti-Spike antibodies, particularly IgM, were independently associated with chest CT severity score (p<0.05, Table 2 and 3).

Prognostic value of anti_SARS-CoV-2 Spike IgG/IgM ELISA tests for mortality

The univariate and multivariate Cox models by considering all significant clinical and laboratory markers were adjusted to validate the predictive value of anti-SARS-CoV-2 Spike antibodies. Every non-significant marker with variance inflation factor (VIF) >5 was deleted from the multivariate model to cor-

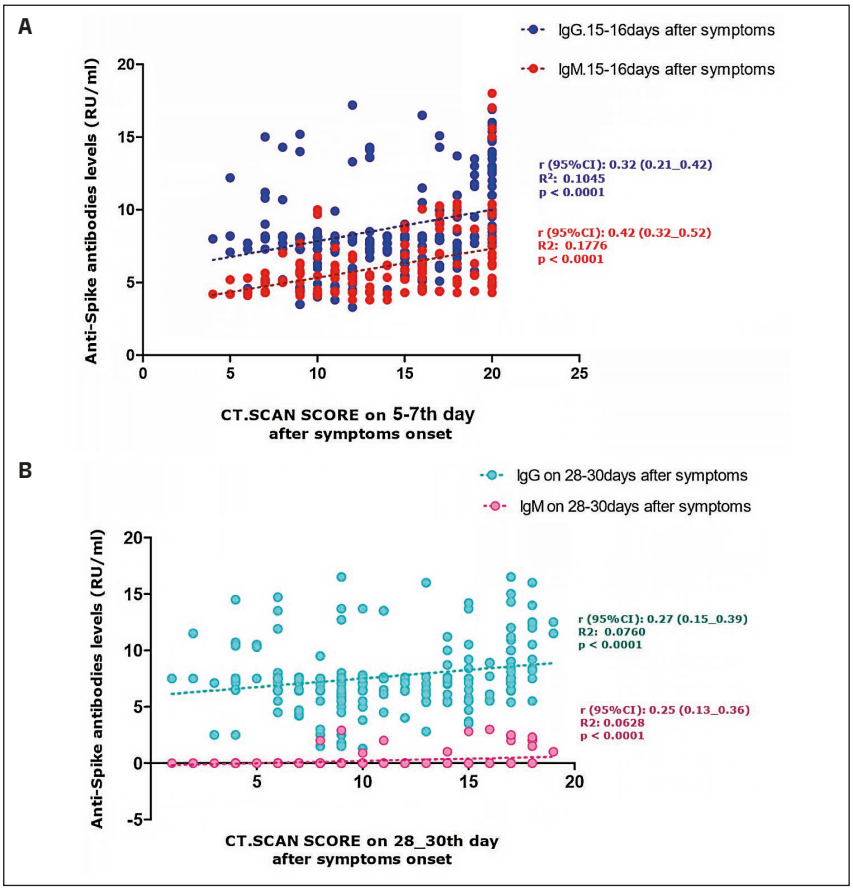

Figure 1 - Pearson positive correlation between Anti_ SARS-CoV-2 Spike IgM/IgG antibodies concentration in serum (RU/ml) on 15th-16th day (A) and/or 28th-30th day after symptoms onset (B) and chest CT intensity score taken at 5th-7th and 28th-30th days after symptoms onset.

Table 2 - Univariate and multivariate linear regression analysis to determine the independent factors associated with Chest CT Severity Score on 5-7th day after symptoms onset.

Variables	*Univariate*			*Collinearity Statistics*	*Multivariate*		
	β	*95% CI for B*	*P Value*	*VIF*	β	*95% CI for B*	*P Value*
Age	-0.01	-0.037-0.03	0.84	–	–	–	–
Gender (M:1, F:0)	-0.02	-1.32-0.88	0.69	–	–	–	–
Underlying disease (No: 0, Yes: 1)	0.11	-0.09-2.18	0.07	–	–	–	–
ICU admission (No: 0, Yes: 1)	0.28	1.9-4.62	0.0001	2.094	–0.05	–2.52-1.43	0.58
Respiratory rate	0.17	0.03-0.18	0.006	1.026	0.21	0.04-0.18	0.001
Blood pressure (Normal: 0, Elevated: 1, Stage 1 hypertension: 2, Stage 2 hypertension: 3)	-0.08	-0.9-0.16	0.17	–	–	–	–
Oxygen saturation	-0.22	-0.16-0.03	0.003	1.785	0.13	–0.01-0.13	0.11
WBC	0.2	0	0.001	2.449	0.07	0	0.48
Haemoglobin	-0.03	-0.33-0.20	0.64	–	–	–	–
Haematocrit	-0.005	-0.09-0.09	0.93	–	–	–	–
Platelets	-0.01	-0.009-0.007	0.84	–	–	–	–

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Variables	*Univariate*			*Collinearity Statistics*	*Multivariate*		
	β	*95% CI for B*	*P Value*	*VIF*	β	*95% CI for B*	*P Value*
Lymphocytes	0.11	0	0.07	–	–	–	–
Neutrophils	0.22	0	0.0001	3.160	0.06	0	0.56
Albumin	-0.04	-0.52-0.25	0.5	–	–	–	–
FBS	-0.17	-0.01--0.002	0.006	1.117	-0.1	-0.01-0.001	0.13
LDH	0.23	0.001-0.002	0.0001	1.132	0.13	0-0.001	0.06
BUN	0.13	0.001-0.054	0.04	1.350	-0.06	-0.04-0.02	0.4
Creatinine	0.004	-0.27-0.3	0.94	–	–	–	–
Na	0.09	-0.02-0.23	0.11	–	–	–	–
K	0.01	-0.87-1.04	0.86	–	–	–	–
Calcium	-0.05	-0.86-0.32	0.37	–	–	–	–
Magnesium	0.11	-0.08-2.75	0.06	–	–	–	–
Direct Bilirubin	0.04	-1.39-2.96	0.48	–	–	–	–
AST	0.1	-0.002-0.03	0.1	–	–	–	–
ALT	0.02	-0.01-0.01	0.68	–	–	–	–
ALP	0.15	0.001-0.01	0.01	1.430	0.03	-0.005-0.007	0.64
ESR	-0.007	-0.02-0.02	0.9	–	–	–	–
CRP	0.04	-0.01-0.02	0.52	–	–	–	–
PT	0.005	-0.31-0.34	0.93	–	–	–	–
PTT	0.04	-0.06-0.12	0.54	–	–	–	–
INR	-0.01	-2.28-1.77	0.8	–	–	–	–
Anti-Spike IgG antibody concentration on 15^{th}-16^{th} day after symptoms onset	0.32	0.31-0.65	0.0001	1.207	0.22	0.12-0.51	0.002
Anti-Spike IgM antibody concentration on 15^{th}-16^{th} day after symptoms onset	0.42	0.66-1.13	0.0001	2.837	0.4	0.36-1.20	0.0001

Table 3 - Univariate and multivariate linear regression analysis to determine the independent factors associated with Chest CT Severity Score on 28th-30th day after symptoms onset.

Variables	*Univariate*			*Collinearity statistics*	*Multivariate*		
	β	*95% CI for B*	*P Value*	*VIF*	β	*95% CI for B*	*P Value*
ICU admission (No: 0, Yes: 1)	0.2	1.03-4.52	0.002	2.14	0.03	-1.83-2.72	0.7
Respiratory rate	0.25	0.08-0.25	0.0001	1.03	0.19	0.05-0.2	0.001
Oxygen saturation	-0.04	-0.19-0.1	0.57	–	–	–	–
WBC	0.17	0	0.007	2.42	0.05	0	0.54
Neutrophils	0.2	0	0.005	2.78	0.03	0	0.76
FBS	-0.18	-0.01-0.002	0.005	1.07	-0.16	-0.01-0.002	0.008
LDH	0.20	0.001-0.002	0.001	1.06	0.18	0-0.002	0.002
BUN	0.09	-0.007-0.05	0.14	–	–	–	–
ALP	0.14	0.001-0.01	0.03	1.29	0.07	-0.002-0.009	0.25

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Variables	Univariate			Collinearity statistics	Multivariate		
	β	95% CI for B	P Value	VIF	β	95% CI for B	P Value
Anti-Spike IgG antibody concentration on 28th-30th day after symptoms onset	0.22	0.14-0.51	0.0001	1.09	0.2	0.11-0.46	0.001
Anti-Spike IgM antibody concentration on 28th-30th day after symptoms onset	0.26	0.95-2.58	0.0001	2.11	0.22	0.38-2.54	0.008

Table 4 - Univariate and multivariate Cox model by considering all significant clinical and laboratory markers to validate the predictive value of anti-SARS-CoV-2 Spike antibodies.

Variables	Univariate		Collinearity Statistics	Multivariate analysis with all significant variables	
	HR (95% CI)	p-value	VIF	Adjusted HR (95% CI)	p-value
Age	1.05 (1.02-1.08)	0.001	1.4	1.06 (0.98_1.14)	0.1
Gender (M:1, F:0)	1.72 (0.74-3.98)	0.2	–	–	–
Underlying disease (No: 0, Yes: 1)	1.47 (0.54-3.99)	0.44	–	–	–
ICU admission (No: 0, Yes: 1)	6.71 (2.37-19)	0.0001	2	1.43 (0.26-7.83)	0.7
Respiratory rate	1 (0.96-1.06)	0.53	–	–	–
Blood pressure (Normal: 0, Elevated: 1, Stage 1 hypertension: 2, Stage 2 hypertension: 3)	1.03 (0.72-1.47)	0.87	–	–	–
Oxygen saturation	0.94 (0.92-0.97)	0.0001	1.8	0.96 (0.92-1)	0.05
WBC	1 (1-1)	0.5	–	–	–
Haemoglobin	0.92 (0.76-1.12)	0.43	–	–	–
Haematocrit	0.97 (0.90-1.04)	0.42	–	–	–
Platelets	0.99 (0.98-1)	0.07	–	–	–
Lymphocytes	1 (1-1)	0.32	–	–	–
Neutrophils	1 (1-1)	0.05	–	–	–
Albumin	0.93 (0.66-1.31)	0.7	–	–	–
FBS	1 (0.99-1)	0.95	–	–	–
LDH	1 (1-1)	0.07	–	–	–
BUN	1.02 (1-1.03)	0.03	1.4	1.03 (0.99-1.07)	0.1
Creatinine	1.11 (0.96-1.3)	0.15	-	-	-
Na	0.96 (0.94-0.98)	0.0001	1	0.85 (0.73-0.98)	0.03
K	2.04 (1.32-3.15)	0.001	1.6	0.75 (0.22-2.54)	0.64
Calcium	0.71 (0.59-0.84)	0.0001	1.15	0.74 (0.58-0.96)	0.02
Magnesium	0.31 (0.09-1.07)	0.06	–	–	–
Direct bilirubin	0.27 (0.004-19.36)	0.55	–	–	–
AST	1 (0.99-1)	0.96	–	–	–
ALT	1 (0.99-1)	0.95	–	–	–
ALP	1 (1-1)	0.06	–	–	–
ESR	0.99 (0.98-1)	0.5	–	–	–

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Variables	*Univariate*		*Collinearity Statistics*	*Multivariate analysis with all significant variables*	
	HR (95% CI)	*p-value*	*VIF*	*Adjusted HR (95% CI)*	*p-value*
CRP	1.017 (1-1.03)	0.007	1	1.02 (0.99-1.057)	0.2
PT	1.23 (1.05-1.45)	0.008	7.7	–	–
PTT	1.06 (1-1.13)	0.05	–	–	–
INR	4.35 (1.85-10.24)	0.001	7.9	–	–
Anti-Spike IgG antibody level on 15th-16th day after symptoms onset	1.07 (0.95-1.21)	0.25	–	–	–
Anti-Spike IgG antibody level on 28th-30th day after symptoms onset	1.63 (0.25-10.84)	0.61	–	–	–
Anti-Spike IgM antibody level on 15th-16th day after symptoms onset	1.58 (1.4-1.8)	0.0001	3	1.28 (1.014-1.63)	0.03
Anti-Spike IgM antibody level on 28th-30th day after symptoms onset	0	0.6	–	–	–

rect the multicollinearity. As shown in Table 4, the IgM level on 15th-16th day after symptoms onset was significantly associated with the hazard of death even after adjusting for all other factors (adjusted hazard ratio (HR) [95% CI]: 1.28 [1.014-1.63], p=0.03), whereas the IgG level was not (p>0.05).

Based on a ROC analysis, the anti-Spike IgM antibody level on 15th-16th day after symptoms onset with an area below the curve of 96.4 % and a cut-off point of 8.67 RU/ml showed a good efficiency in predicting the survival probability in the studied population (95% CI: 0.94 to 0.98; specificity: 94.5%, sensitivity: 96%; positive predictive value (PPV): 65.7%, negative predictive value (NPV): 99.5%, P: 0.0001). The survival probability in the patients with the anti-Spike IgM level higher than or equal to 8.67 RU/mL (34.2%) was significantly lower than in those with IgM level <8.67 RU/mL (99.5%) (log-rank p=0.0001), and also vice versa for mortality rate (*i.e.*, 65.8% vs 0.5%).

DISCUSSION

Over the recent three years, the COVID-19 epidemic has been the main cause of high mortality worldwide [13]. So, it's necessary to discover valid, rapid and cost-effective diagnostic and prognostic methods for screening critically ill patients infected with SARS-CoV-2 and timely prevention of adverse outcomes [14]. The S1 subunit of SARS-CoV-2 spike protein contains the RBD with a critical neutralizing domain which could induce a high-potency neutralizing antibody response; this highly purified RBD is the essential antigen used in a set of IgA, IgM, and IgG diagnostic ELISA kits [15].

In the present study, the prognostic value of the anti-SARS-CoV-2 IgG/IgM ELISA kits coated with Delta N and S1 RBD proteins was investigated. Our results showed a correlation between the levels of anti-Spike IgG measured at 15th-16th day and 28th-30th day after symptoms onset and severity of infection, but not as much as IgM. The levels of anti-Spike IgM in the severely infected patients were significantly higher than in non-severe patients. Such a significant positive correlation between these antibodies and chest CT severity score implies that their levels may reflect the degree of lung involvement. It should be considered that the classification of patients based on the disease severity was not adjusted only using the chest CT scan score, but also a set of vital, clinical, and laboratory manifestations titled "WHO severity criteria" [11] was considered.

Lei et al. (2021) reported that the levels of IgG responses against most of non-structural proteins upon admission day were significantly associated with the severity of infection, and even IgG antibodies had a notable predictive potency for patient's death [16]. By contrast, our results indicated that the anti-Spike IgG level could not independently predict the patient's death, in spite of its positive correlation with the chest CT severity score. But the anti-Spike IgM antibody showed a significant asso-

ciation with mortality risk. This inconsistency between our results and the results of Lei et al.'s study [16] may be due to the use of different types of antigens (structural and non-structural proteins), antibody measurement techniques, different time of antibody measurement, and clinical and immunological differences between different study populations.

Various studies reported a significant association between the disease severity of COVID-19 and higher levels of antibodies [17-21]. Zhao et al. reported that the average levels of antibodies on 12th day before symptoms onset were not significantly different between the critical and non-critical patients, but their levels in two weeks after onset were significantly higher in critical patients than non-critically ill patients. Also, older age, male gender, and high titer of antibodies are the independent factors associated with disease severity [18]. In the present population, the level of IgG antibody on 15^{th}-16^{th} day after symptoms onset was not significantly higher in critical patients than non-critically ill patients, which may indicate its weakness in differential diagnosis. But the IgM antibody levels on 15^{th}-16^{th} and/or 28^{th}-30^{th} days after symptoms onset were strongly associated with disease severity. On the other hand, both antibodies had a significant correlation with a series of laboratory factors, which implies that their levels may also reflect the patients' general health status. Some factors including older age, comorbidities, low oxygen saturation, high FBS, and hyponatremia were also associated with disease severity. Due to the use of gender homogenization in the selection of patients, the gender factor was not subjected to statistical comparative evaluation.

Additionally, the present results demonstrated that the levels of both antibodies were significantly decreased after 28-30 days of symptoms onset compared to 15^{th}-16^{th} day, which confirms the results of Mitani et al. study in this regard [22].

Moreover, the present findings showed that COVID-19 patients with IgM level ≥8.67 RU/mL were significantly at higher risk of death compared with those with IgM level <8.67 RU/mL. But IgG antibody showed not an independent strong association with the patient's death. The exact immune mechanisms that lead to such a difference in the prognostic value of these antibodies are not known. Generally, the IgM titer increases before that of IgG; nevertheless, the rise of IgM titer among symptomatic COVID-19 patients occurred almost simultaneously with IgG titer, and/or rarely IgG increased ahead of IgM [22]. Also, IgM detection tests may cause false positive results for non-specific reasons, such as cross-reaction and or rheumatoid factor (RF) in severe infections [23]. However, the high prevalence of SARS-CoV-2 and COVID-19 in our province as well as withdrawal of COVID-19 patients with rheumatoid arthritis from this study can notably lower the possibility of false positive results caused by cross-reaction with the seasonal strains of the coronavirus and/or RF. Nevertheless, this study only assessed a portion of COVID-19 infected population (unvaccinated patients infected with SARS-CoV-2 delta variant) and it does not include patients infected with other circulating viral variants and vaccinated population. Therefore, our results cannot be generalized to all COVID-19 patients, and the values of anti-SARS-CoV-2 IgG and IgM determined in this study is merely theoretical and has little practical value. Hence, further large-scale studies on diverse infected populations are required to draw more accurate results about the practical value of these serological tests.

Advantages and limitations of the study

Unlike retrospective studies, prospective assessment of patients throughout this study period allows accurately identifying the causal relationships between the variables and outcomes. Moreover, excluding the patients with rheumatoid arthritis from the study can notably reduce the possibility of false positive results caused by RF. However, daily measurement of the anti-SARS-CoV-2 Spike antibodies was not possible to precisely detect the patients' humoral immune response patterns. Furthermore, the study population merely consists of patients with SARS-CoV-2 delta variant infection and unvaccinated patients. Lack of evaluation of the value of anti-SARS-CoV-2 IgG and IgM in patients infected with other circulating viral variants, vaccinated population and or in population with prior infection is another weaknesses of the study. For this reason, the study has a purely theoretical value of scientific speculation, but has little practical applicability.

CONCLUSIONS

The levels of both antibodies, especially anti-SARS-CoV-2 Spike IgM antibody, were associated with

the chest CT severity score or disease severity. Also, anti-SARS-CoV-2 Spike IgM antibody measured on 15th-16th days after symptoms onset showed an eligible association with the risk of death. The use of anti-SARS-CoV-2 Spike antibody low-cost ELISA Kits may be convenient and affordable. However, the possibility of false positive results of antibody detection tests for non-specific reasons limits their clinical use. Also, the value of antibodies has only been investigated in patients with SARS-CoV-2 delta variant infection and unvaccinated patients, but patients infected with other circulating viral variants and vaccinated population were ignored. So, finding of this study has solely theoretical value, but has little practical applicability. Accordingly, further large-scale studies on diverse infected populations are required to precisely determine the diagnostic/prognostic value of antibodies, as well as discover novel techniques to minimize or prevent SARS-CoV-2 antibody false positives in enzyme-linked immunosorbent assays.

Ethical approval
This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. with Ethical Code: IR.AJUMS.REC.1399.535. The local institutional ethics committee of study center oversaw the proceedings and documentation.

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Declaration of conflicts of interest
The authors have disclosed no conflicts of interest.

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Mandana Pouladzadeh: Project administration, Supervision; Mofid Hosseinzadeh: Project administration, Supervision, Review & editing; Reza Khedri: Investigation; Parastoo Moradi Choghakabodi: Investigation, Conceptualization, Methodology, Validation, Writing draft, Review, Editing; Payam Amini: Data Analysis; Alireza Ghorbani Bavani: Investigation; Hossein Bahrami Moghaddam: Investigation; Babak Behmanesh: Investigation; Ali Delirrooyfard: Assistance in investigation; Alireza Sokooti: Assistance in investigation; Behnam Sheibani: Assistance in investigation.

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