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Original Article

Study of the Immune Response of COVID-19 Patients in Kirkuk Province

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Abstract

Background: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel coronavirus causing coronavirus disease 2019 (COVID-19); it is diagnosed based on clinical signs and laboratory detection methods such as polymerase chain reaction (PCR) and serological techniques. **Objective:** The objective of the study is to use other diagnostic methods that support the PCR method of diagnosis for COVID-19. **Materials and Methods:** The study included 90 COVID-19 patients and 26 control group. Nasopharyngeal swabs were collected from the suspected patients with COVID-19 infection for the detection of the RNA virus by PCR technique. If the PCR was positive, the serum samples were collected and used for the quantitative detection of SARS-CoV-2 S1 (IgM, IgG) by using enzyme linked immunosorbent assay. **Results:** The result of this study showed that in a total of 116 participants, there was a significant difference between IgM and IgG reactivity (±) and the number of PCR-positive and negative individuals with P value <0.0001 and P value = 0.003, respectively. In addition, a significant increase in the levels of IgM and IgG ($P \leq 0.0001$ for IgM and $P \leq 0.0001$ for IgG) was recorded in patients compared with healthy control. Moreover, a significant correlation between IgM level with $P = 0.0018$ and the onset of symptoms as well as positive correlation was noticed between IgG concentration and the onset of symptoms ($P = 0.0272$). **Conclusion:** The study concluded that antibodies developed against COVID-19 infection could appear at early stages of the infection without the confirmation of real time polymerase chain reaction, and this could be a beneficial tool for early screening of suspected as well as asymptomatic individuals.

Keywords: COVID-19, ELISA, PCR

Introduction

The coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The first report of the SARS-CoV-2 was in December 2019 in Wuhan, China.[1,2] Coronaviruses have mutations on their outer surface that resemble crowns, hence the name.[3,4] It belongs to the subfamily Orthocoronavirinae within the Coronaviridae family. The Orthocoronavirnae subfamily was divided into four genera, based on phylogeny bunching. Alpha and beta coronaviruses are only found in mammals.[5] The clinical specimens that are used to diagnose coronaviruses involve nasal secretions, blood and sputum, and bronchoalveolar lavage. Although the polymerase chain reaction (PCR) technique is the gold standard for identifying COVID-19, there are additional laboratory diagnostic procedures that can be performed.[6] The immune systems are in charge of defending the body against outside agents that can impair the functions of healthy cells. One of the most significant foreign substances that enter the human body is a virus, which triggers the immune system to produce antibodies against them.[7] Clinical laboratories commonly use serum-specific IgM and IgG detection to offer information about the virus infection time course. During infection, IgM antibodies are created as a first line of defense, and these antibodies are utilized to assess the acute phase of infection because they suggest recent

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exposure. IgG antibodies, on the other hand, are produced thereafter to offer long-term immunity and immunological memory.[8] Serological studies look for antibodies to various viral proteins and can assist in measuring COVID-19 disease prevalence in asymptomatic infections and reveal whether a person has been exposed to this virus before or had a past infection[9] and are recommended when diagnosing patients who have considerable epidemiological evidence of COVID-19 infection but a negative real time polymerase chain reaction (RT-PCR) test, as well as when diagnosing patients more than 7 days after the onset of symptoms and clinical evidence of COVID-19 infection.[10-12] The RT-PCR detection of viral nucleic acid is one of the most established laboratory diagnostic procedures and is a specific type of PCR that is used to identify RNA, and it is presently being used to detect SARS-CoV-2. Because it directly detects the presence of viral RNA, this type of test is widely employed as a frontline test for COVID-19.[13] The RT-PCR technology involves the extraction of RNA from clinical specimens using RNA isolation kits.[14] Following this, the synthesis of complementary DNA takes place, which is used as a template.[15] The PCR amplification uses specific primers and probes dedicated to the viral genomes (RdRp) as well as the N gene of SARS-CoV-2 virus.[16] The PCR machine then uses thermo-cycling conditions of increasing and decreasing temperature to allow the magnification of the targets in a multiplex manner.[17] However, the PCR approach is still a diagnostic procedure that requires laboratories and specialized equipment, which are not readily available in sufficient quantities to handle high numbers of disease cases, particularly during peak periods.[18] The aim of the study is to use other diagnostic methods that support the PCR method of diagnosis for COVID-19 and can be used in special studies to follow up on the epidemiology of the pandemic.

Materials and Methods

Study setting and period

The study was carried out at the Shifaa 14 Hospital in Kirkuk city during the period between December 2021 and February 2022.

Sample collection and processing

The samples were randomly collected from 90 patients and 26 controls, the healthy group, taking in consideration the time of symptom onset in the patient group. Nasopharyngeal swabs were collected from all patients who were suspected to have COVID-19 infection. All swabs were placed in viral transport media and submitted for viral RNA extraction. All extraction procedures were performed according to the manufacturer's instructions (Kogenebiotech, Korea). Following this step, the eluted RNA generated from the extraction process was introduced to the master mix protocol; this involves mixing 18.3 µL of RT-PCR reaction buffer and 1.7 µL of RT-PCR enzyme (Aehealth, United Kingdom).

Table 1: Thermal cycling setting of PCR technology

Steps	Temperature	Duration	Cycle
1	50°C	10 min	1
2	95°C	3 min	1
3	95°C	5 s	45
	60°C	35 s or 20 s	

The master mix was loaded to each dedicated PCR tube at 20 mL/well, and 20 mL of eluted RNA was loaded to each PCR tube. Next, the tubes were admitted to RT-PCR machine (Sacace Sacyclers 96, Italy). The thermo-cycling condition is displayed in Table 1. The result interpretation considered FAM channel for ORF1ab gene and VIC channel for N gene. A positive result was obtained if Ct value was ≤38 and the amplification curve was S-shaped, whereas if Ct > 40 in the FAM channel, then it is considered as negative. Some of the PCR amplification curves are shown in Figure 1. If the PCR was positive, venous blood was extracted and centrifuged to obtain serum, which will be utilized for the detection of quantitative SARS-CoV-2 IgM and IgG antibodies (Abs) via enzyme linked immunosorbent assay (ELISA) antibodies assay (AESKULISA Diagnostics, Germany) according to the manufacture instruction. The interpretation of ELISA results (IgG and IgM) was performed according to a cutoff value of ≥10 for positive result and less than 10 for negative results.

Statistical analysis

The statistical analysis was performed using Graph Pad Prism software version 5 (Graph Pad Prism Software Inc., San Diego, CA) and used the Mann–Whitney T test and chi-square test. A P value ≤ 0.05 was considered significant.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee in the Kirkuk Directorate of Health, Iraq according to the document number (42463) on 9/12/2021.

Results

The study was conducted in Kirkuk city and enrolled 90 patients confirmed with RT-PCR for SARS-CoV-2 and in addition 26 healthy subjects. The data obtained from RT-PCR are illustrated in the graph below [Figure 1].

The current study illustrated that the highest levels of IgM (mean = 14.53) and IgG (mean = 17.22) were recorded in patients with COVID-19 compared with healthy control [IgM (7.76) and IgG (9.07)] with significant differences between the tested and control groups (P value ≤ 0.0001

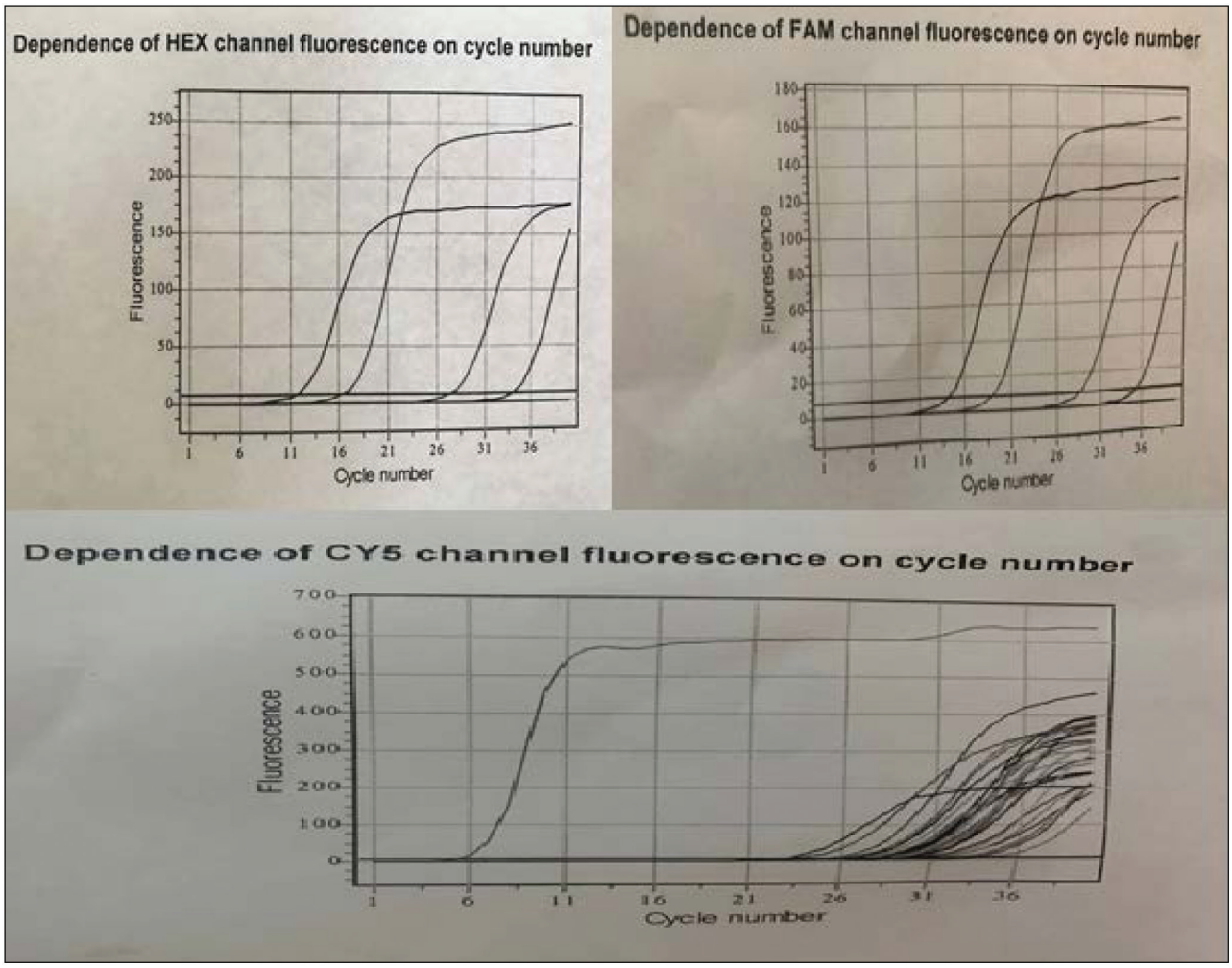

Figure 1: Multiplex RT-PCR amplification curves obtained from SARS-CoV-2 positive patients illustrating FAM channel for ORF1ab, VIC channel for N gene, and Cy5 for internal control

for IgM and $P \leq 0.0001$ for IgG) when Mann–Whitney test was applied [Figure 2].

The data in Table 2 showed a comparison between IgM reactivity (±) and the number of PCR-positive and -negative individuals in a total of 116 persons [90 PCR-confirmed COVID-19-positive patients (77.59%) and 26 controls (22.41%)]; the IgM positive were only 51 (43.97%), whereas 65 (56.03%) participants were negative using Qi square test, which indicated a significant difference with $P \leq 0.0001$ between them.

Equally, Table 3 showed the IgG reactivity (±) compared with PCR results in all groups via Qi square test; there was a significant difference between them with $P = 0.003$, with a total number of test individuals of 116, of which 90 (77.59%) were PCR-confirmed COVID-19-positive patients and 26 (22.41%) were COVID-19-negative people. The IgG results were positive in 69 (59.48%) participants and were negative in 47 (40.52%).

The data displayed in Table 4 indicate that anti-SARS-CoV-2-specific IgM and IgG antibodies were not detectable in the very early days of infection (from day 0 to day 3). Anti-SARS-CoV-2-specific IgM antibody was recorded from day 4 onward; the IgM antibody titers increased over time, peaking at approximately day 20 and then began to decline, whereas anti-SARS-CoV-2-specific IgG antibody was noticed from day 7 onward, peaking at approximately day 25. The sensitivity recording for both IgM and IgG ELISA was 43.96% and 59.48%, respectively.

When the patients were grouped depending on the time of disease onset, the data demonstrated moderate positive significant correlation between IgM concentration ($P = 0.0018$ with r = 0.3252) and the onset of symptoms using Spearman correlation test [Figure 3]. Likewise, weak positive correlation was found between IgG concentration and the onset of symptoms ($P = 0.0272$ with r = 2328) as illustrated in Figure 4.

The antibodies generated against COVID-19 in both PCR-positive patients and healthy controls were arranged as follows: in the patient group, 49 (54%) developed both IgM+/IgG+ Abs, whereas in the control group, neither of

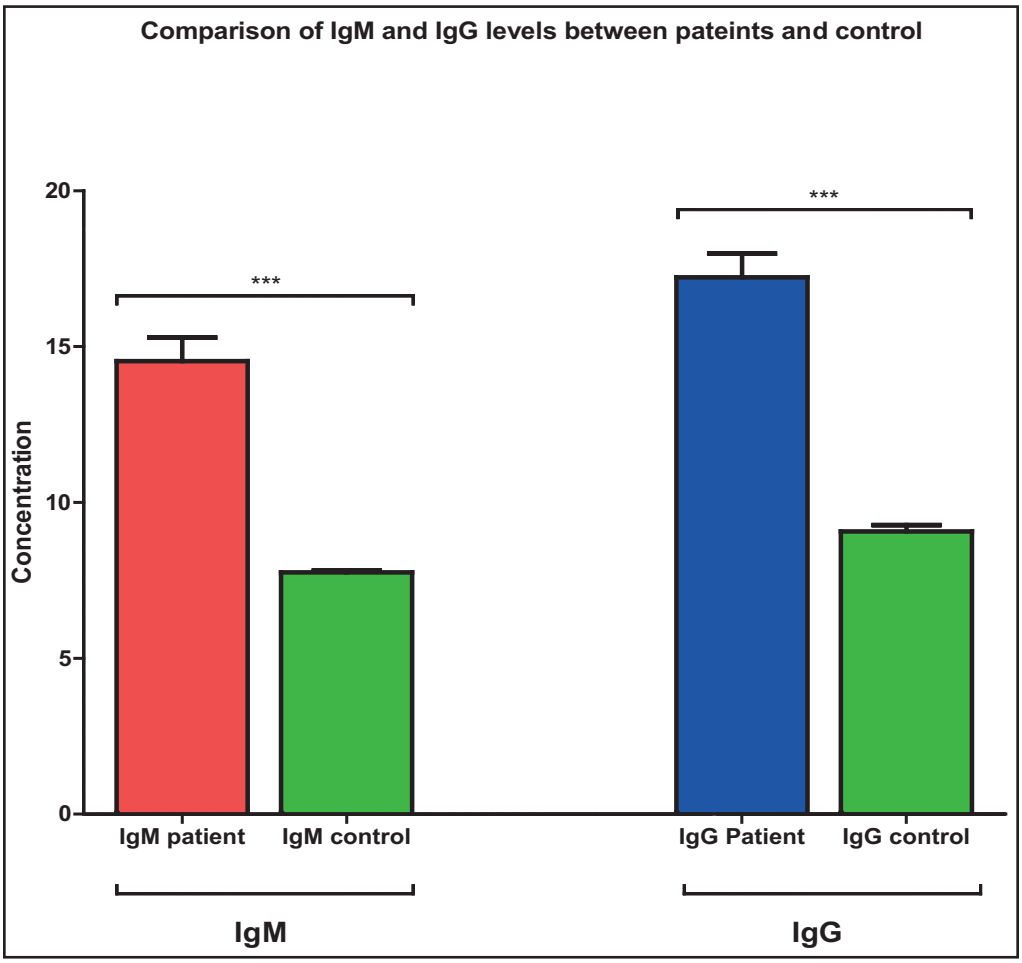

Figure 2: Comparison of IgM and IgG levels between patients and the control group (*P* value = 0.0001 for IgM and *P* value = 0.0001 for IgG)

Table 2: Qi square comparisons of IgM results with PCR-positive COVID-19 in a total of 116 participants, *P* = 0.0001

Comparison of RT-PCR with IgM	Positive		Negative		Total	*P* value
	No.	%	No.	%		
PCR	90	77.59	26	22.41	116	<0.0001
IgM	51	43.97	65	56.03	116	

Table 3: Qi square comparisons of IgG results with PCR-positive COVID-19 in a total of 116 individuals, *P* = 0.003

Comparison of RT-PCR with IgG	Positive		Negative		Total	*P* value
	No.	%	No.	%		
PCR	90	77.59	26	22.41	116	0.003
IgG	69	59.48	47	40.52	116	

them was positive, and those patients with IgM−/IgG+ were 18 (20%), and in the control group, there are only two (8%) participants. In the patient group, the number of IgM+/IgG− was only 2 (2%), and none in the control group were found to be positive. Finally, 21 (23%) of COVID-19 patients were both IgM−/IgG−, whereas in the control group, 24 (92%) were IgM−/IgG− as elucidated in Table 5.

Discussion

The study was based on the RT-PCR detection of SARS-CoV-2 in clinically ill patients who have been hospitalized for a while using the standard method of confirmation despite some limitations associated with this technique.[19] The disease has been reported to cause illness globally, and laboratories around the world collaborated to develop more information as well as diagnostic tools to control the infection.[20]

This study included 116 participants who were tested for COVID-19 RT and compared with ELISA immunoassay results for detecting SARS-CoV-2-specific IgM and IgG. Samples from COVID-19 cases obtained during the disease or convalescence stages were initially confirmed by RT-PCR. In addition, these specimens have been re-tested to obtain double confirmation and considered as true positives. Despite the fact that RT-PCR is the gold standard for confirmation, several factors including the quality of sampling and the amount of virus recovered during specimen collection, as well as the amount of RNA extracted, might affect its sensitivity.[21-23]

In the patient group, some of them had not yet developed neither IgM nor IgG as this requires 2 weeks for IgM to develop and more than 3 weeks for IgG to emerge[24]; despite the fact that all patients were PCR positive, still the exact date of onset of symptoms was not indicated accurately by some or even most of the patients.[25] Our results indicated that some of the patients were able to develop IgM Abs at around day 4 of the disease onset, and this is in agreement with Lee *et al.*, who described the development of IgM Abs after 3–6 days of infection and after 8 days for IgG Abs.[24] These results are not in parallel with other study, which reported an average of 14 days for Abs to develop against COVID-19 infection.[26]

Our data revealed that most of the patients were able to develop IgM Abs at around 8–14 days of the initial infection with COVID-19, and this is probably due to the variation of time point at which the blood was extracted from each participant, in addition to the immune status of each individual.[27] However, in the case of IgG, a positive result indicates that there might be a previous infection or the patient could be at some point of late COVID-19 infection; therefore, it is vital to differentiate between the infection stages,[22] in order to have an idea about the management options for each patient. This suggests that even in the case of having undetectable IgM/IgG Abs, the infection with COVID-19 cannot be ruled out, and re-testing for Abs screening after few days is highly advised.[22] Hence, Abs screening is highly recommended for the diagnosis of COVID-19; yet in the early stages of the disease, the diagnosis cannot relied on Abs screening as seroconversion may occur at around 20 days of infection.[27] However, some researches have pointed out to the seroconversion as an early as 4 days of the symptom onset.[28] On the other hand, patients with positive PCR for COVID-19 and negative for IgM/IgG Abs could reflect a true negative status in which Abs have not produced yet or there might be some sort of defective immune response. Nevertheless, conducting Abs screening can provide a mean of sensitive method to detect COVID-19 in those who have symptoms or asymptomatic with

Table 4: The number of IgM- and IgG-positive results according to the days of symptom onset (n = 90)

ELISA parameters in the patient group				
Time of symptom onset (days)	IgM+		IgG+	
	No.	%	No.	%
0–7	6	12	11	16
8–14	28	55	35	52
≥15	17	33	21	31
Total	51	100	67	100
IgM sensitivity = 43.96%				
IgG sensitivity = 59.48%				

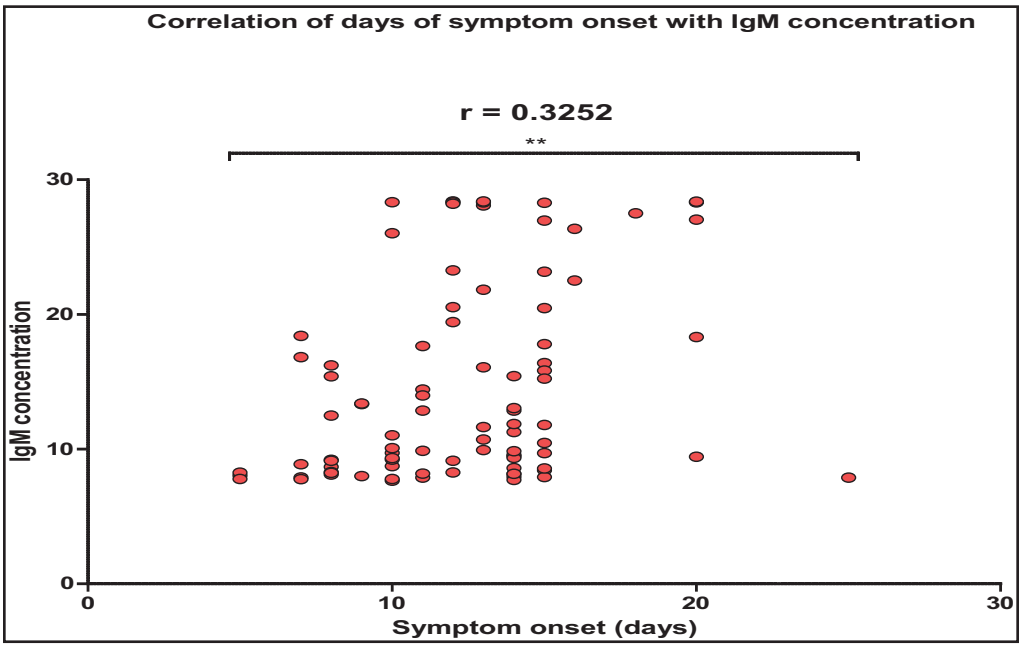

Figure 3: Correlation of days of symptoms with IgM levels (P = 0.0018)

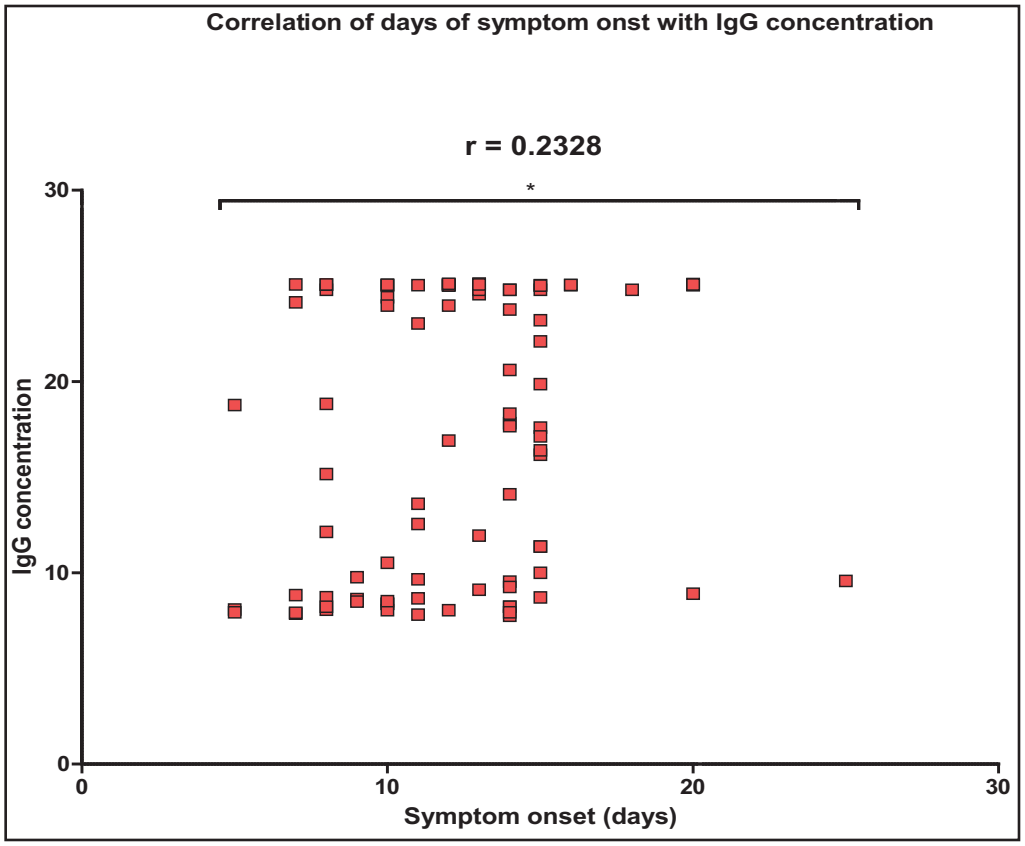

Figure 4: Correlation of days of symptoms with IgG levels ($P = 0.0272$)

Table 5: Results of IgM and IgG antibodies for COVID-19 by ELISA immunoassay

COVID-19 ELISA tests	Patients		Control		Total
	No.	%	No.	%	
IgM+, IgG+	49	54	0	0	49
IgM−, IgG+	18	20	2	8	20
IgM+, IgG−	2	2	0	0	2
IgM−, IgG−	21	23	24	92	45
Total	90	99.9	26	99.9	116

negative RT-PCR results.[29] Over that, performing Abs screening can provide a better epidemiological picture on the prevalence of infection in the community and those worker in the health sector who may or not have symptoms or in the case of lacking the facility for a confirmatory method such as RT-PCR.[29]

The present study exhibited that an elevated concentration of IgM and IgG was recorded in patients with COVID-19 compared to healthy controls, with significant ($P \leq 0.0001$) differences between the two groups. This finding is in agreement with Guo *et al.*, who reported similar results to the one reported here.[23,30] Conversely, the Abs test for SARS-CoV-2 is of crucial benefit in the case of RT-PCR negative, but it does have some drawbacks including the inability to discriminate whether the IgG level is due to past infection or is the result of seroconversion.[31,32] Over that, the low specificity for Abs to recognize spike protein of SARS-CoV-2 from other coronavirus family could mask a definitive diagnosis as they share the same spike protein in the family of corona, and cross-reactivity could yield false positive data.[33,34]

In our study, when the patients were grouped according to the time between the onset of disease and testing for Abs development, the data demonstrated a positive statistical relation between IgM and IgG levels and the time of onset of symptoms (P value = 0.0018 and 0.0272, respectively). These data are in agreement with other studies that confirmed the correlation between the onset of the symptoms with increased Abs to COVID-19 infection,[19,34] whereas it disagrees with Van Elslande *et al.* who recorded seroconversion at later days of initial infection.[35]

On the other hand, the amount of Abs generated against SARS-CoV-2 could widely vary between individuals since the infection has recently emerged, and the immune response to the disease is not fully understood.[36]

The sensitivity recording for both IgM and IgG ELISA used in this research was 43.96% and 59.48%, respectively; these findings disagree with other researches who reported

elevated sensitivity for both IgM and IgG Abs test for patients infected with COVID-19.[31,37] On the other hand, it is in conflict with Zhao *et al.* who mentioned very low sensitivity of both IgM and IgG tests when performed at early days of patient admission.[34]

The low sensitivity reported in this research for both IgM and IgG suggests that the time of sample collection was greatly varied among patients as well as some of them were hospitalized for few days to a couple of weeks since the appearance of the symptoms, and this could greatly influence the generation of Abs against the virus in addition to other cofactors such as the immune status of the patient and the coexistence of other diseases.

Conclusion

In this study, we conclude that Abs developed against COVID-19 infection could emerge at early stages of the infection, and this could offer a useful mean to early screen individuals who have symptoms or even asymptomatic with the lack of PCR confirmation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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