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Application of newly developed SARS-CoV2 serology test along with real-time PCR for early detection in health care workers and on-time plasma donation

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ABSTRACT

Background: As the daily number of coronavirus infection disease 19 (COVID19) patients increases, the necessity of early diagnosis becomes more obvious. In this respect, we aimed to develop a serological test for specifically detecting anti-SARS-CoV2 antibodies.

Methods: We collected serum and saliva samples from 609 individuals who work at TBZMED affiliated hospitals in Tabriz, Iran, from April to June of 2020. Real-time PCR technique was used to detect SARS-CoV-2 genome using specific primers. An enzyme linked immunosorbent assay (ELISA) test was designed based on virus nucleocapsid (N), spike (S) and its receptor binding domain (RBD) protein, and the collected sera were subjected to IgM and/or IgG analysis.

Result: Real-time PCR results showed that 66 people were infected with the SARS-CoV-2. Our designed ELISA kit showed 93.75% and 98% of sensitivity and specificity, respectively. In this study, 5.74% of participants had specific IgG against RBD, whereas the percentage for IgM positive individuals was 5.58%. Approximately the same results were observed for S protein. The number of positive participants for NP increased further, and the results of this antigen showed 7.38% for IgG and 7.06% for IgM.

Conclusion: The ELISA test beside real-time PCR could provide a reliable serologic profile for the status of the disease progress and early detection of individuals. More importantly, it possesses the potential to identify the best candidates for plasma donation according to the antibody titers.

Abbreviations: PCR, polymerase chain reaction; ELISA, Enzyme Linked Immune-Sorbent Assay; RBD, receptor binding domain; S, spike protein; N, nucleocapsid protein; SARS-CoV-2, Severe Acute Respiratory Syndrome caused by Coronavirus-2.

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1. Introduction

By late 2019, an outbreak of severe acute respiratory corona virus2 (SARS-CoV2), as a new member of coronavirus family, was announced in Wuhan, China. World health organization (WHO) named the new disease coronavirus infection disease 19 (COVID19). The disease disseminated faster than expected and created a pandemic, which becomes a serious challenge for the health care systems across the globe. The most important clinical features of COVID19 are fever, cough, and myalgia/fatigue which overlap with the symptoms of many other viral infections such as influenza (Fathizadeh et al., 2020; Huang et al., 2020). Unfortunately, a number of infected cases remain subclinical or asymptomatic, creating a major obstacle in the way of disease containment.

Currently, the main step for the in vitro diagnosis of the disease is carrying out real-time polymerase chain reaction (RT-PCR) on the virus nucleic acid. In this regard, there exist limitations such as the possibility of the presence of errors in each step of sample preparation, the time-consuming process, the requirement for well-trained staff, and expensive materials/equipment which can ultimately lead to false negative reports. Moreover, in SARS infection, the positivity rate of this test was between 50% and 79%, further emphasizing the urgent need for simpler, cheaper, and faster methods for the early detection of COVID19 (Yam et al., 2003; Liu et al., 2020; Long et al., 2020; Ozma et al., 2020).

In an immunologically healthy individual, the humoral immune system stands in front of SARS-CoV2 by producing vast, specific neutralizing antibodies of IgM/IgG classes against various antigenic parts of the virus (Liu et al., 2020; Mahmooodpoor and Nader, 2020). In some studies, the nucleocapsid protein (NP) of the virus particle was declared highly immunogenic and overexpressed during the infection. The roles of this protein involve viral genome packaging into a whole virion and the suppression of viral RNA silencer, both of which help spread the disease (Narayanan et al., 2003; Cui et al., 2015). Other parts of the corona virus also have immunogenic properties and cause the production of neutralizing and protective antibodies in people infected with the virus, which include the spike protein and its receptor binding domain (RBD) region (Amanat et al., 2020). Spike protein and its RBD section mediate the binding of the virus to host cells through the interaction with human cell receptors named angiotensin converting enzyme 2 (ACE2) (Khodadadi et al., 2020; Letko et al., 2020; Walls et al., 2020).

According to different studies, IgM levels raise during the first week following disease onset, and IgG levels elevate up to more than 40 fold a week later. This pattern of seroconversion has been reported even in RT-PCR negative cases, which points out the importance of serologic assessment of SARS-CoV2-specific antibodies (Dong et al., 2020; Du et al., 2020; Long et al., 2020a).

Enzyme linked immunosorbent assay (ELISA) is a specific, sensitive, rapid, simple, and cheap test, which has been employed in different studies as an indicator of disease status. This test becomes even more valuable in individuals who are in close contact to infected ones, especially the employees of health care systems with a subclinical disease (Amanat et al., 2020; Khan et al., 2020).

This study was aimed at evaluating the COVID19 IgM/IgG designated ELISA kit, assessing the presence and/or rate of COVID19 specific IgM/IgG antibodies in employees of hospitals as a sample of individuals with close contact to patients, in order to distinguish the healthy, infected, and carriers from each other. Besides, the verification of appropriate cases for the convalescent plasma therapy could be achieved.

2. Material & methods

2.1. Sampling

A total number of 609 employees working at different hospitals affiliated with Tabriz University of medical sciences in Tabriz city, Iran, between the ages of 24–60 were enrolled in this study, during April to June of 2020. A written consent form was signed by all the individuals, and the approval of the study was performed by the ethics committee of Tabriz University of medical sciences. In brief, 8 ml blood sample was taken from each person and the serum was separated by centrifugation. The isolated serum was either immediately taken for further assessment of SARS-CoV2 specific IgM and IgG levels, or stored in –20°C. 100 age and sex matched previously banked-serum samples since early 2019, before the SARS-CoV2 first breakage applied as negative controls. Also, esophageal and pharyngeal saliva samples were taken to assay SARS-CoV2 infection using real-time PCR.

2.2. Real-time PCR

In order to evaluate the infection of people with SARS-CoV-2 and also to confirm the results of ELISA, real-time PCR technique was used (using 3 SARS-CoV-2 specific primers). The genes and sequences of the primers used for real-time PCR are listed in Table 1. HEK-293 T cells (~1 × 107 cells) as positive control and study population cultured saliva samples were used to total RNA extraction using TRIzol as described previously (Won et al., 2020), and finally, the ratio of 260 nm/280 nm and 260 nm/230 nm wavelengths were calculated using the Nano-drop system to evaluate RNA purity and concentration. Complementary DNA (cDNA) was synthesized with random hexamer primers and oligo (dT) by using M-MLV (H-) Revert Aid Reverse Transcriptase kit (Thermo Fisher, MA). Real-time PCR was carried out to measure the expression of SARS-CoV-2 RdRP, S, and N genes in study population.

2.3. ELISA

SARS-CoV2 specific IgM and IgG serum ELISA kits were received from Florian Krammer et al. established tests (Amanat et al., 2020). Briefly, for the coating step, 2 μg/ml of SARS-CoV2 NP (MyBioSource, San Diego, CA.), S Protein, and RBD domain (Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA) in PBS buffer were coated in each well of ELISA separately in 4°C overnight. The next day, coating ingredients were washed 5 times and a blocking solution (3% non-fat milk prepared in PBS with 0.1% Tween 20 (TPBS)) was added in wells for 1 h at room temperature (RT). After removal of the blocking solution, the wells were washed 5 times. To prevent any residual virus risk, the serum samples were preheated (56°C for 1 h) before usage. Then 100 μl of each heat-treated serum (1:100 diluted) was added to the prepared wells (Rosa-Fraile et al., 2004; Hu et al., 2020). In order to obtain optimal dilution titer of sera, 20 non-infected person sera as negative control and 20 SARS-CoV-2 infected patients sera as the case group were used in 1:10, 1:50, 1:100, 1:500, and 1:1000 dilution. The results of titration and selection of optimum dilution titer of sera were showed Fig. 1. The incubation time was 2 h at RT. Next, the wells were washed 5 times. For each sample, three replicates were included. In the end and for IgM evaluation, 100 μl of horse radish peroxidase (HRP)-conjugated goat anti-human IgM antibody (1:3000 diluted, Razi Biotech, Kermanshah, Iran) was added to the related wells. In a similar manner, IgG levels were detected by adding HRP-conjugated-goat anti-human IgG antibody (1:10,000 diluted, Razi Biotech, Kermanshah, Iran). Each of the ELISA plates were incubated for 1 h at RT, which was followed by a fifth washing. At last 100 μl of TMB-Buffer solution was added, and the reaction was ultimately terminated by hydrochloride acid 3 M. The wells were read by ELISA plate reader at A450, and the background optical density was measured at 490 nm. To determine the cutoff point, the mean OD of 100 clinically-negative individual sera was calculated and the mean OD + 3*standard deviation (SD) (cutoff = μ + 3*SD; μ = 1, μ – mean OD of negative controls, and f = 3) was set as the cut-off point (Lardeux et al., 2016). It is worth to mention that, stored frozen-samples as negative controls and the fresh serum samples taken from the test population were all treated with a
same procedure before subjecting to ELISA. The cutoff values were determined as 0.21 for IgG and 0.315 for IgM, and values under these amounts were accounted as negative. As controls for the efficacy of the designed kit (specificity and sensitivity), qualitative human corona virus antibody IgG (HCoV-HKU-IgG) ELISA kit and Coronavirus COVID-19 IgM ELISA assay kit were used in parallel.

2.4. Statistical analysis

Chi-square test was used to evaluating anti-coronavirus 2019 RBD, SP, and NP antibodies in participated population based on multiple comparison parameters. The gathered data were introduced to graph pad prism version 7. P value under 0.05 was considered as statistically significant.

3. Results

3.1. Demographics of enrolled cases

Among the tested cases, 373 cases were male (61.25%) and 236 cases were female (38.75%). The median age, weight, and BMI were 41.9, 74.2, and 26.5 respectively. The most important underlying comorbidities were diabetes, heart-related diseases, and hashimoto thyroiditis. Detailed features of the tested individuals are summarized in Table 2.

Due to the manifestation of clinical symptoms, real-time RT-PCR and CT-scan of the enrolled individuals, the test population were divided to positive or negative for COVID19 and subjected for further analysis in IgM/IgG ELISA confirmation.

3.2. Real-time PCR analysis of participated population

Real-time PCR results using specific primers showed that 66 people were infected with the SARS-CoV-2, including 42 out of 373 men and 24 out of 236 women. Real-time PCR results for RdRP, S, and N genes are shown in detail in Table 3.

3.3. Confirmation of the performance of the designed viral-specific IgM/ IgG antibodies ELISA assay

Confirmation of sensitivity and specificity of the anti SARS-CoV2 IgM/IgG ELISA assay was described as follows. Among the 32 definitely confirmed positive cases by clinically accepted diagnostic criteria (SARS-COV2 PCR test and CT-scan), in total, 30 samples were positive.

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Table 1

| Target gene | Primer name | Primer pairs (5′–3′) | Size (bp) | Reference |
|-------------|-------------|----------------------|-----------|-----------|
| RdRP        | SARS-CoV-2_IBS_RdRP2 | Forward: AGAATAGAGCTCGACCGTA, Reverse: CTCTCTAGTGCCGGCTATT | 101 | 17 |
| S           | SARS-CoV-2_IBS_S2    | Forward: GCTGGTGGCTCAGCTTTATA, Reverse: AGGGTCAAGTGCACTGCTA | 107 |
| N           | SARS-CoV-2_IBS_N1    | Forward: CAATGCTGCAATCGTGCTAC, Reverse: GTTGGGAGCTACCTGIGAGG | 117 |

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Table 2

| Age    | Weight | BMI       | Underlying disease | Underlying disease in family |
|--------|--------|-----------|--------------------|-----------------------------|
| Min    | Max    | Mean ± SD | Min-Max            | Mean ± SD                   |
| Men    | 28-59  | 42.4 ± 5.3 | 18.3-38.8          | 25 ± 4.6                    |
| Women  | 24-60  | 41.1 ± 6.8 | 18.3-33.4          | 29 ± 6.8                    |
| Total  | 24-60  | 41.9 ± 6.4 | 18.3-38.8          | 54 ± 6.6                    |

BMI: Body Mass Index.

Table 3

| Number of positive results for SARS-CoV-2 infection using Real-Time PCR. |
|-----------------------------|-----------------|-----------------|
| Real-time PCR               | RdRP | S | N  |
| Men (373)                   | 42   | 42 | 43 |
| Women (236)                 | 24   | 24 | 24 |
| Total (609)                 | 66   | 66 | 67 |

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Fig. 1. Reactivity of control and SARS-CoV-2 convalescent sera to different coronavirus 2019 surface antigens. A. Reactivity to RBD domain, Spike Protein, and Nucleocapsid Protein of coronavirus 2019 in 20 SARS-CoV-2 infected patient’s serum compared to 20 negative controls sera (negative control: blue line; RBD: red line; SP: green line; NP: purple). B. The same experiment data were showed but graphed as area under the curve (AUC) to get a better quantiative impression. Student’s t-test was done as statistics test using Graphpad Prism 8. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
for each or both of the antibodies. This data showed that the sensitivity of our developed ELISA kit was 93.75%. On the other hand, for specificity validation, 100 serum samples (age and sex matched with the test subjects) that were stored before the emergence of the COVID19 pandemic were tested as negative controls. 98 out of 100 negative controls were negative for anti-SARS-CoV2 IgG/IgM by our developed ELISA kits, thus the specificity of the test was equal to 98%.

### 3.4. Serological detection of viral specific IgM and IgG antibodies in studied population

609 collected sera were tested for the possible existence of IgM/IgG antibodies against SARS-CoV2 NP, S Protein, and RBD antibodies by our novel ELISA kit. In men, positive results of IgG and IgM ELISA tests for the RBD were 22 and 17, respectively, while the results for women in this protein were as following 13 and 17. A total of 35 subjects tested positive for anti-RBD IgG, whereas positive individuals for the IgM was 34. The ELISA test for S protein showed similar results in the study population with a very small increase in comparison with the RBD, with 37 and 35 positive cases for IgG and IgM, respectively. Although the NP ELISA test showed a similar pattern, there was a significant increase compared to the RBD ELISA results. The number of positive individuals for IgG and IgM in the NP ELISA test was 45 and 43, respectively. The detailed percentage of IgM/IgG levels between the tested subjects are summarized in Table 4. There were no significant differences in results of anti-RBD, SP, and NP antibodies in studied population using multiple comparisons. The detailed results of these comparisons were showed in Table 5.

### 4. Discussion

Since the beginning of the COVID19 pandemic, the application of different diagnostic tools has been questioned globally. Frequently, serology tests are used for the evaluation and screening of viral infections, even though their application in COVID19 has not been clarified. In this regard, we showed that our developed anti-SARS-CoV2-specific IgM and/or IgG ELISA kit, has an accepted specificity and sensitivity, which could prove accommodating in the evaluation of the COVID19 prevalence through the recognition of asymptomatic individuals in addition to the potential verification of the best plasma donors for further plasma convalescent therapy.

Like many other viral infections, when the immune system is exposed to SARS-CoV2, the level of IgM antibodies raises rapidly at first. Following the decrease and disappearance of IgM, IgG increases and stays in high titers longer than IgM (Liu et al., 2020b). In parallel with our study, Shengdian and Shangen et al. showed that in a population of 238 hospitalized COVID19 patients, serum IgM and/or IgG antibody positive cases were significantly higher than viral nucleic acid positive individuals. Moreover, they explained that the antiviral antibody rates, unlike viral RNA test, were elevated through the time; antibody responses showed a peak on day 11 while viral RNA dropped dramatically. They interpreted their findings by proposing that the real-time RT-PCR and IgM/IgG ELISA tests should be applied together (Liu et al., 2020a). Cheng lian and Zhen et al. demonstrated a comparative study and suggested the application of serological tests as well as viral nucleic acid tests, as the false negative results of the molecular diagnosis of COVID19 could be reduced in this manner (Liu et al., 2020b).

Krammer et al developed an ELISA kit for the detection of seroconversion in COVID19-infected individuals and declared the possibility of detection even 3 days post disease onset. Moreover, they suggested that as the ELISA assay could be handled in biosafety level2, provides antibody subtyping, and is scale-convertible, it should be considered as a more reliable test in managing COVID19 diagnosis and therapy (Amanat et al., 2020). The investigation of Qin and al-long et al. for-antibody response to SARS-CoV2 infected patients in a multi-center study population revealed that patients become 100% positive for IgG near day 20 of disease onset. Also, day 13 was declared as the median seroconversion day for both IgM and IgG. They proposed that the IgM and IgG should be detected at the first phase of disease. Thus, the serology test can act as an absolute help for the identification of suspected or close-contact individuals (Long et al., 2020a). According to the mentioned studies, the median day of seroconversion and a follow-up test in positive cases are needed for better interpreting the results.

Another important neglected issue is the fact that different studies have used different antigenic parts of the SARS-CoV2 virus for developing ELISA kits. Finding the best immunostimulatory part of the virus particle by precisely observing the studies in this field will be helpful both in the development of ELISA kits and in future vaccine creation. Virus neutralization and host protection, or on the other hand, acute inflammation and antibody dependent enhancement (ADE) of a given antibody are determined by multiple factors, which consist of the concentration, affinity, specificity and isotype of the antibody. Anti-N and anti-S IgG are similarly provoked in immunized mice by viral vector vaccines encoding SARS-CoV nucleocapsid (N) and S protein, respectively. Nevertheless, upon re-challenge, significantly upregulated secretion of pro-inflammatory cytokines, enhanced lung infiltration of eosinophils and neutrophils and more severe pathology of the lungs are observed in N-protein immunized mice (Yasui et al., 2008). Conversely, other in vivo experiments demonstrate that protective antibody responses are conferred by subunit or peptide vaccines which lay their

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### Table 4

Serological information of the participants for coronavirus19 surface antigens including RBD, S protein, and NP.

| RBD (IgG (%)) | S protein (IgG (%)) | N protein (IgG (%)) |
|--------------|-------------------|-------------------|
| Men | 22 | 17 | 23 | 18 | 28 | 25 |
| Women | 13 | 17 | 14 | 17 | 17 | 18 |
| Total | 35 | 34 | 37 | 35 | 45 | 43 |

| RBD (IgM (%)) | S protein (IgM (%)) | N protein (IgM (%)) |
|--------------|-------------------|-------------------|
| Men | 5.8% | 4.55% | 6.16% | 4.82% | 7.23% | 6.7% |
| Women | 5.5% | 7.2% | 5.93% | 7.2% | 2% | 7.62% |
| Total | 5.74% | 5.58% | 6.07% | 5.74% | 7.38% | 7.06% |

### Table 5

Serological evaluation of the participants for coronavirus19 surface antigens including RBD, S protein, and NP based on multiple comparisons.

| Target | Comparison parameters | Variables | p value |
|--------|-----------------------|-----------|---------|
| IgG RBD vs SP vs NP | Men (N = 373) | 0.6349 |
| Women (N = 236) | 0.7298 |
| Men & women (N = 609) | 0.4644 |
| IgM RBD vs SP vs NP | Men (N = 373) | 0.3665 |
| Women (N = 236) | 0.9795 |
| Men & women (N = 609) | 0.4995 |
| IgG | Men (N = 373) vs Women (N = 236) | RBD | 0.8405 |
| SP | 0.9062 |
| IgM | Men vs women (N = 609) | RBD | 0.1659 |
| SP | 0.8891 |
| Men (N = 373) | IgG vs IgM | RBD | 0.4108 |
| SP | 0.4218 |
| NP | 0.6643 |
| Women (N = 236) | IgG vs IgM | RBD | 0.4504 |
| SP | 0.5574 |
| NP | 0.8606 |
| Men & women (N = 609) | IgG vs IgM | RBD | 0.9014 |
| SP | 0.8080 |
| NP | 0.8248 |

RBD: Receptor Binding Protein; S protein: Spike protein; N protein: Nucleocapsid protein; p < 0.05 was considered as statistically significant.
focus on the antibody response against particular epitopes of the RBD of the S protein (Du et al., 2009).

Seroconversion does not occur in some patients of COVID-19. In a recent study, Takuya et al. indicated that immunity against SARS-CoV-2 infection is higher than the tests show. They investigated SARS-CoV-2 specific T cell subsets and serological tests in asymptomatic or mild COVID-19 individuals and observed no seroconversion in some of these patients (Sekine et al., 2020). Adding to this, it has been shown that, almost 5 days after disease onset the IgM and IgA antibody become positive (Sterlin et al., 2020) and following that to after 20 days IgG get present (Long et al., 2020a; Long et al., 2020b). Of interest, severe -ill female COVID-19 patients produced IgG earlier and even in high titers (Sep, 2017; Zhao et al., 2020). The exact duration of SARS-CoV2 specific antibody existence in plasma is still unclear but it was known that specific antibodies against the other members of beta-corona viruses drop in 6–12 months (Chan et al., 2005). According to the knowledge we gained from previous infection to SARS and MERS, 600 ml plasma donation in 14 days intervals up to 6 months showed no significant decrease in antibody titer (Salazar et al., 2020). There is different ongoing convalescent therapy clinical trials across the globe for finding the best donors and therapy-time (www.clinicaltrial.gov). Since the going convalescent therapy clinical trials across the globe for finding decrease in antibody titers (Salazar et al., 2020). There is different on-antibody existence in plasma is still unclear but it was known that (Sep, 2017; Zhao et al., 2020). The exact duration of SARS-COV2 specific infection is higher than the tests show. They investigated SARS-CoV-2 recent study, Takuya et al. indicated that immunity against SARS-CoV-2

Declaration of competing interest

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