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The Accuracy of Enzyme-Linked Immunosorbent Assay in the Diagnosis of COVID-19 in Iranian Children

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Abstract

Background

The present study aimed to evaluate the accuracy of enzyme-linked immunosorbent assay (ELISA) compared to the real-time reverse transcription-polymerase chain reaction (rRT-PCR) in the diagnosis of coronavirus disease 2019 (COVID-19) in Iranian children.

Materials and Methods

In this cross-sectional study, 90 children under 15 years of age were randomly selected from suspects of COVID-19 referred to the Tabriz Children Hospital, the main pediatric COVID-19 diagnostic center of Tabriz, from May 21, 2020 to June 21, 2020. Blood and nasopharyngeal samples were taken simultaneously at the referring time. The diagnostic accuracy of ELISA-based IgM and IgG antibody tests for COVID-19 were compared with the rRT-PCR.

Results

The calculated sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio, overall diagnostic accuracy, and diagnostic odds ratio were 0.5745, 0.9767, 0.9643, 0.6774, 24.66, 0.4357, 0.7667, and 56.60 for IgM; and 0.6170, 0.9302, 0.9355, 0.6897, 8.84, 0.4117, 0.7667, and 21.47 for IgG, respectively.

Conclusion

Due to the lower sensitivity of antibody detection-based serological tests compared to rRT-PCR, they cannot be considered as initial and reliable tests for the diagnosis of COVID-19. It can be suggested that the serological tests be only used as complementary tests to rRT-PCR or for monitoring the immune response of children with COVID-19.

Key Words: Children, COVID-19, Diagnosis, Real-time RT-PCR, SARS-CoV-2.

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1- INTRODUCTION

December of 2019 was the emergence of a new coronavirus which soon caused a pandemic disease (1). The spread of this pathogenic virus which mainly affects the respiratory system, was so quick that induced almost all countries to struggle for prevention and treatment (2). The World Health Organization (WHO) designated Coronavirus disease 2019 (COVID-19) as the official name of this new infectious disease on February 11, 2020 (3). On the same date, the International Committee on Taxonomy of Viruses (ICTV) also changed the name of the virus from "Novel Coronavirus 2019" (nCoV-2019), as its initial name, to "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) (4). SARS-CoV-2 is a single-stranded RNA virus belonging to beta-coronaviruses (5).

Coronaviruses constitute a large family of enveloped viruses and a subset of Coronaviridae ranging from the common cold viruses to the causes of more severe diseases such as Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), and COVID-19 (1, 6). COVID-19 is a highly contagious disease that can spread from person to person usually through close respiratory droplets. It has been estimated that the disease can be transmitted from each infected person to about 2.8-3.8 individuals (7, 8). Almost 81% of the patients especially children with COVID-19 have mild symptoms and can be recovered at home. In 14% of cases, a person may show severe symptoms including pneumonia and shortness of breath. In 5% of cases, the patient's condition worsens that is associated with respiratory failure, infectious shock, multiple organ failure, disseminated intravascular coagulation (DIC), and even death (9). The diagnosis of COVID-19 in children often relies on experimental results. The most commonly used samples for laboratory testing are nasopharyngeal and oropharyngeal swaps but, sputum, or aspiration of the lower respiratory tract may also be taken (10). Real-time reverse transcription-polymerase chain reaction (rRT-PCR) nowadays is considered as the confirmatory and gold standard test for diagnosis of COVID-19 which is based on the molecular detection of the virus genome in the patient sample. Some other diagnostic approaches such as serological methods have also been recently introduced. The serological tests are mainly based on the detection of specific antibodies against the SARS-CoV-2 in the peripheral blood of patients. The accuracy of these methods in diagnosis of COVID-19 in children has not yet been completely determined. Therefore, the present study aimed to evaluate and compare the results of the serological and molecular methods in the diagnosis of COVID-19 in Iranian children.

2- MATERIALS AND METHODS

2-1. Study design and population

In the present cross-sectional study, 90 children under 15 years of age were randomly selected from suspects of COVID-19 referred to the Tabriz Children Hospital, the main pediatric COVID-19 diagnostic center of Tabriz, Tabriz, Iran, from May 21, 2020 to June 21, 2020. These samples had at least a criterion such as a history of contact with COVID-19 patients, fever, dry cough, shortness of breath, ground-glass opacities (GGO), and consolidation in the chest computed tomography (CT) scan, a decrease in the number of lymphocytes and white blood cells (WBC) in the complete blood count (CBC). They were included in the study after obtaining informed consent from their parents. The demographic characteristics of the patients were recorded in questionnaire forms.

2-2. Samples collection

Blood samples and nasopharyngeal and oropharyngeal swabs were taken simultaneously at the referring time. Blood samples were centrifuged, serum separation and distribution were done, and then they were kept at -70 ℃ before the tests. Swap samples were immediately placed in sterile vials containing 3 ml of viral transport media (VTM) including Hank's balanced salt solution (pH 7.4) containing BSA (1%), amphotericin (15 µg/mL), penicillin G (100 units/mL), and streptomycin (50 µg/mL). The samples were then heat-inactivated at 56 ℃ for 30 minutes. Due to the possibility of aerosol formation, the samples were allowed to reach room temperature before further experiments. If the samples were not used immediately, they were kept at 4 °C.

2-3. Laboratory measurements

2-3-1. Molecular assay

Total viral RNA was extracted from the swap samples by a commercial kit, FastPure Viral DNA/RNA Mini Kit (Vazyme Biotech Co., Nanjing, China), according to the manufacturer's instructions. A commercial kit, LightMix Sarbeco V E-gene plus EAV control (Roche, Germany) was used to detect the SARS-CoV-2 genome in the samples using the rRT-PCR according to the kit protocol.

2-3-2. Serological assay

After the sample collection, serum anti-SARS-CoV-2 IgM and IgG antibodies were evaluated using the enzyme-linked immunosorbent assay (ELISA) with commercial kits (Pishtaz Teb Diagnostics, Tehran, Iran) according to the manufacturer's protocol. Briefly, for evaluation of serum IgG, 100 µL of controls and diluted serum samples (1:100) were added into the 96-well microplate (coated with N protein), and then incubated for 30 minutes at 37°C. After washing cycle (5x), 100 µL secondary antibody (against human IgG) labeled with conjugated enzyme was added into the wells and then incubated for 30 minutes at 37℃. After the second washing step, 100 µL chromogen-substrate was added into the wells and incubated for 15 minutes at 37℃ in darkness. Finally, 100 µL stop solution was added to the wells to terminate the reaction. A microplate reader at 450 nm measured the optical density (OD) of each well with a 630 nm filter as a reference wavelength within 30 minutes. The ratio of OD to the cutoff value (OD of the blank well+0.15) was considered as cutoff-index (CI). A CI more than 1.1 was considered positive, and below than 0.9 was considered as a negative result. Samples with a CI between 0.9 and 1.1 were considered suspicious and had to be retested with fresh samples after a few days. For the IgM evaluation, the dilution factor was changed (1:50), and the samples were also mixed (1:1) with an assay buffer before adding into the wells. The cutoff value was also modified (OD of the blank well +0.25).

2-4. Ethical consideration

The children were included in the study after obtaining informed consent from their parents. The entire process was done with personal protection equipment in a biosafety level 2 (BSL 2) laboratory. The ethics committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1399.247) approved the study.

2-5. Data Analyses

The statistical analyses were performed using SPSS software version 16.0. The quantitative variables such as age were shown as mean ± standard deviation (SD), and compared by independent samples t-test between COVID-positive and COVID-negative patients. The qualitative data such as gender, and the results of serological and molecular tests were recorded in percentages and compared by Chi-Square test. The diagnostic values of ELISA-

based serological tests for COVID-19 were evaluated in comparison with the rRT-PCR as the gold standard test. True positive, false positive, true negative, and false negative values were calculated by the crosstabs. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR-), overall diagnostic accuracy (ODA), and diagnostic odds ratio (DOR) were also calculated based on the formulas presented in Table 2 (11). A p-value of less than 0.05 ($p<0.05$) was considered statistically significant.

3- RESULTS

The mean age of children was 10.29 ± 2.49 years consisting of 52 (57.8%) boys and 38 (42.2%) girls. Based on the rRT-PCR results, among 90 studied children, 47 (52.2%) individuals were COVID-positive, and 43 (47.8%) children were COVID-negative. As presented in **Table.1**, the mean age of COVID-Positive children was 10.36 ± 2.45 year, and mean age of COVID-Negative group was 10.21 ± 2.56 ($p=0.774$). Number of boys was more than girls in both groups, but both groups were not significantly different in terms of sex ratio ($p= 0.357$). Results of the ELISA indicated that 27 (57.45%) children of COVID-positive group and only one child (2.33%) of COVID-negatives were positive in terms of IgM. However, 29 (61.70%) cases from COVID-positive patients, and 3 children (6.98%) from COVID-negatives were positive in terms of IgG (**Table.1**).

Therefore, as presented in **Table.2**, values of true positive, false positive, true negative, and false negative were 27, 1, 42, and 20 for IgM; and 29, 3, 40, and 18 for IgG, respectively. Furthermore, sensitivity, specificity, PPV, NPV, LR+, LR-, ODA, and DOR were calculated based on the formulas depicted in **Table.3**, which included 0.5745 (57.45%), 0.9767 (97.67%), 0.9643 (96.43%), 0.6774 (67.74%), 24.66, 0.4357, 0.7667 (76.67%), and 56.60 for IgM; and 0.6170 (61.70%), 0.9302 (93.02%), 0.9355 (93.55%), 0.6897 (68.97%), 8.84, 0.4117, 0.7667 (76.67%), and 21.47 for IgG, respectively.

Table-1: The demographic and antibody test characteristics of COVID-Positive and COVID-Negative children.

Parameter	COVID-Positive n=47	COVID-Negative n=43	**P**-value
Age (year)	10.36 ± 2.45	10.21 ± 2.56	0.774
Gender (F/M)	22/25	16/27	0.357
IgM-Positive (n, %)	27 (57.45)	1 (2.33)	<0.001
IgG-Positive (n, %)	29 (61.70)	3 (6.98)	<0.001

COVID-19, coronavirus disease 2019; IgG, Immunoglobulin G; IgM, Immunoglobulin M.

Table-2: The values of true positive, false positive, true negative, and false negative of anti-SARS-CoV-2 IgM and IgG antibody tests.

Immunoglobulin type	COVID-Positive (rRT-PCR; n)	COVID-Negative (rRT-PCR; n)
IgM-Positive (n)	TP = 27	FP = 1
IgM-Negative (n)	FN = 20	TN = 42
IgG-Positive (n)	TP = 29	FP = 3
IgG-Negative (n)	FN = 18	TN = 40

Ig G: Immunoglobulin G; IgM: Immunoglobulin M; FN: False negative; FP: False positive; rRT-PCR: Real-time reverse transcription-polymerase chain reaction; TN: true negative; TP: true positive.

Table-3. Diagnostic accuracy measures of anti-SARS-CoV-2 IgM and IgG antibody tests.

Parameters	Formula	SARS-CoV-2 IgM	SARS-CoV-2 IgG
Sensitivity	TP/(TP + FN)	0.5745	0.6170
Specificity	TN/(TN + FP)	0.9767	0.9302
PPV	TP/(TP + FP)	0.9643	0.9355
NPV	TN/(TN + FN)	0.6774	0.6897
LR+	Sensitivity/ (1- Specificity)	24.66	8.84
LR-	(1 - sensitivity)/specificity	0.4357	0.4117
ODA	(TP + TN)/ (TP + FP + TN + FN)	0.7667	0.7667
DOR	LRP/LRN	56.60	21.47

IgG, Immunoglobulin G; IgM, Immunoglobulin M; PPV, Positive Predictive Value; NPV, Negative Predictive Value; LR+, Positive Likelihood Ratio; LR-, Negative Likelihood Ratio; ODA, Overall diagnostic Accuracy; Diagnostic Odds Ratio; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

4- DISCUSSION

In the present study, the accuracy of ELISA-based IgM and IgG antibody tests for COVID-19 were compared with the rRT-PCR in children. The serologic tests included in the present study did not demonstrate suitable sensitivity for clinical use on children with COVID-19. Due to the acceptable sensitivity and specificity of rRT-PCR in detecting the SARS-CoV-2 genome, it has been considered as the gold standard test in COVID-19 diagnosis (12). The genome of this RNA virus has different regions (13). At the 5-terminus of the genome, orf1ab, as the largest gene, and orf1a gene express the pp1ab and pp1a proteins, respectively. They together contain 15 non-structural proteins (nsp1 to nsp10, and nsp12 to nsp16). The main structural proteins of the virus are encoded by the four structural genes including spike (S), envelope (E), membrane (M), and nucleocapsid (N) genes located at the 3-terminus of the genome. Some accessory genes (3a, 3b, p6, 7a, 7b, 8b, 9b, and orf14) are also distributed among these structural genes (13). Different regions of the SARS-CoV-2 genome such as S, E, M, N, orf1a, and orf1ab genes can be targeted for PCR-based molecular detection (14). In the present study, a 76-bp region of E gene was amplified by specific primers, and the results indicated that among 90 studied cases, 47 children (52.2 %) were positive and 43 ones (47.8%) were negative. Even though a chest CT may be also helpful in the diagnosis and management of COVID-19 (9), it was not evaluated in the present study. It is important to note that a patient suffering from COVID-19 may have a

normal chest CT at the beginning of the disease on referring time. Bernheim et al. (15) reported that 20 (56%) individuals out of 36 studied patients had normal CT up to two days after symptom onset. Also, Pan et al. (16) reported that 4 (19%) individuals out of 21 patients with normal primary CT had abnormalities in their chest CT after about 4 days of follow-up. Yang et al. (17) also found that among 149 studied patients, out of 17 (11.4%) cases with primary normal chest CT, the CT of 12 cases were still normal after ten days of follow-up, and the remaining 5 became positive after about 7 days. These results indicate that a normal chest CT cannot reject the diagnosis of COVID-19, especially at the early stages of the disease.

Therefore, although chest CT was considered the as a diagnostic criterion of COVID-19 in the fifth edition of the Diagnosis and Treatment Program of 2019 New Coronavirus Pneumonia proposed by The National Health Commission of China (18), it has been removed from the sixth version (19). Although rRT-PCR is still considered as a standard reference in the diagnosis of COVID-19, the test has also a false-negative rate. It may be due to the fact that some factors such as the disease stage, clinical condition, sample type, quality of sample collection, transfer and storage qualities of samples, accuracy of PCR steps, lab errors, type of target gene, and possible mutations of SARS-CoV-2 genome can affect the sensitivity of the rRT-PCR resulting in a false result (20, 21). Due to some advantages such as easier sampling (blood), faster turn-around time, and lower workloads, anti-SARS-CoV-2 antibodies detection-based serological tests have been recently introduced as a possible way to detect COVID-19 (20). Although the underlying mechanisms of the host immune response and antibody secretion against SARS-CoV-2 have not yet been fully understood, some recent studies (22, 23) have reported valuable results. Xiang et al. (23) evaluated the serological tests and antibody dynamics of anti-SARS-CoV-2 IgM and IgG in patients with suspects or confirmed COVID-19 within 3-40 days after the onset of symptoms. The results indicated that the antibodies were detectable as early as the fourth day after the symptom onset. In their study, the sensitivity, specificity, PPV, NPV, and the consistency rate of IgM in the confirmed patients were 77.3% (51/66), 100%, 100%, 80.0%, and 88.1%, and those of IgG were 83.3.3% (55/66), 95.0%, 94.8%, 83.8%, and 88.9 %, respectively. In patients with suspected COVID-19, they were 87.5% (21/24), 100%, 100%, 95.2%, and 96.4% for IgM, and those of IgG were 70.8% (17/24), 96.6%, 85.0%, 89.1%, and 88.1%, respectively. Different targets of the virus structure may be targeted by the immune system and related antibody response. In the present study, the N antigens (proteins) of SARS-CoV-2 coated in the wells were used by applied ELISA kit for detecting specific IgG and IgM.

The results of serological tests indicated that 27 (57.45%), and 29 (61.70%) children out of 47 confirmed patients by rRT-PCR were positive in terms of IgM and IgG, respectively. The difference in serological and molecular results in the present study may be because swab and blood sampling were performed simultaneously within a week of the symptoms onset. This period (one week) may not be enough for an effective immune response and detectable antibody secretion (seroconversion) in some patients. In a study by Long et al. (22), the positive rates of IgM and IgG were about 40% and 55%, respectively about a week after symptom onset, and they reached their maximum values about three weeks after symptom onset. It was also found in the present study that among 43 children with negative rRT-PCR test, one (2.33%) and three (6.98%) individuals were positive in terms of IgM and IgG,

respectively. In these individuals, the negative result of the molecular test might be false due to the mentioned factors affecting the sensitivity of rRT-PCR, or due to the removal of the virus by the host immune system before the sampling. During an immune response against a pathogen, IgM is usually detectable earlier than IgG in the body, but according to results of the present study as well as studies by Xiang et al. (23), and Long et al. (22), IgM and IgG, both were detectable in more than half of patients within the first week after symptom onset. Moreover, this immune response against SARS-CoV-2 seems faster than SARS and other types of coronavirus pneumonia (23). In the present study, the calculated sensitivity, specificity, PPV, NPV, LR+, LR-, ODA, and DOR were 57.45%, 97.67%, 96.43%, 67.74%, 24.66, 0.4357, 76.67%, and 56.60 for IgM; and those of IgG were 61.70%, 93.02%, 93.55%, 68.97%, 8.84, 0.4117, 76.67%, and 21.47.

Sensitivity (positivity in disease), estimates the probability of getting a positive test result in subjects with the disease. Therefore, it associates with the ability of a test to identify the ill. Specificity (negativity in health) estimates the probability of getting a negative test result in a healthy individual. Therefore, it correlates to the ability of a test to identify the healthy. PPV determines the probability of being ill for an individual with a positive test result. NPV estimates the probability of not having a disease for an individual with a negative result. LR+ describes how many times more likely a positive test result occurs in patients than in healthy individuals. The farther LR+ is from 1, the stronger the evidence for the presence of the disease. If LR+ is equal to 1, the test could not identify the ill from the healthy. LR- defines how much less likely the negative test result is to occur in a patient than in a healthy person. LR- is usually less than 1, because it is less likely that a negative test result occurs in subjects with than in individuals without the disease. ODA is represented as the proportion of correctly classified individuals among all cases. It can be affected by disease prevalence. With the same sensitivity and specificity, ODA of a distinct test increases as the disease prevalence decreases; and eventually, DOR can be applied for a comprehensive estimation of the discriminative power of diagnostic approaches and for comparison of diagnostic accuracies between two or more tests. Overall, these diagnostic accuracy evaluations estimate how well a test detects the target condition of interest. They determine the agreement between an index test (ELISA), and a reference standard (rRT-PCR) for the ability to diagnose a target disease (COVID-19) (24, 25). The differences between the results of these diagnostic accuracy measures in the present study and the results of Xiang et al. (23) may be due to use of different serological kits. Also, in Xiang et al. (23) study the diagnostic values of serological tests were evaluated 13 days after symptom onset in adults, but in the present study to precise evaluation of the serological test and its diagnostic value, serum and swap sampling were done simultaneously from children at the referring time within a week after symptom onset. It is also important to note that COVID-19 may occur with various severities in different age groups, geographical regions, and periods due to immune response variations of individuals and possible mutations of SARS-CoV-2.

4-1. Study Limitations

In the present study, the N gene of SARS-CoV-2 by the molecular assay, and the anti-SARS-CoV-2 IgM and IgG antibodies through ELISA, were targeted in the COVID-19 diagnosis. It is imperative that the other genes such as S, E, M, N, orf1a, and orf1ab, and the other antibodies such as IgA be targeted and evaluated in future

studies. The lack of a comparison between different ELISA kits can be considered as another limitation of the present study. Further studies with larger sample size are necessary for confirming the results.

5- CONCLUSION

In conclusion, although anti-SARS-CoV-2 antibody detection-based serological tests are cost-effective, faster, easier sampling, and easier to use compared to the PCR based molecular methods, due to their lower accuracy, they cannot be considered as initial and reliable tests for diagnosis of COVID-19. Given the seroconversion of specific antibodies against SARS-CoV-2 during the middle and later stages of COVID-19, it is better that serological methods be only used as complementary tests to rRT-PCR or for monitoring the immune response of the patients against SARS-CoV-2.

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7- CONFLICT OF INTEREST: None.

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