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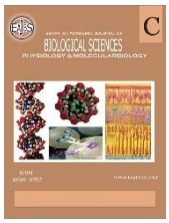

Evaluation of the Diagnostic Performance of Toll-Like Receptors 4 and 9 as Reliable Markers for Corona Virus Disease-19

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ABSTRACT

Respiratory, multiorgan, and heart failure are the causes of mortality from COVID-19 so this pandemic has increased interest in the biochemical processes that explain the complex interactions between infectious pathogens and human hosts. The virus's entry activates the immune system, including various toll-like receptor (TLR) pathways that result in the secretion of proinflammatory cytokines. This study aims to find meaningful COVID-19 biomarkers outside of conventional biochemical examinations. **Patients and Methods:** This study registered 100 people (n = 100), including 75 COVID-19 patients receiving care at the El Sahel Teaching Hospital and 25 healthy controls. We used RT-PCR, computed tomography (CT) scans, and biochemical routine profile tests like d-dimer, c-reactive protein (CRP), ferritin, and lactate dehydrogenase (LDH) to confirm that the COVID-19 virus was real. We measured toll-like receptor-4 (TLR-4) and toll-like receptor-9 (TLR-9) using the sandwich enzyme-linked immunosorbent assay (sandwich ELISA) method. **Results:** The results showed that there were statically significant differences in WBC, HCT, neutrophil, lymphocyte, monocyte, HbA1C, FBS, PPBS, and also COVID-19 profile biochemical tests except LDH. In the cases of TLR-4 and TLR-9, a significant difference appeared among the studied groups. We observed a highly significant correlation in the case of TLR-9 with ferritin and CRP. This study used ROC curves to measure the diagnostic accuracy of COVID-19. This study allowed for the accurate diagnosis of COVID-19 patients, with an AUC of 0.87 in the case of TLR-4 and 0.94 in the case of TLR-9. The ROC curve determined the cutoff points, and we measured the sensitivity and specificity of both TLR4 and TLR9 to be 80% and 79% for TLR-4, and 92% and 100% for TLR-9. **Conclusion and Summary:** TLR-4 and TLR-9 are effective biomarkers to distinguish between healthy and those with COVID-19 with acceptable sensitivity and specificity.

INTRODUCTION

The appearance of the coronavirus disease 2019 (COVID-19) in late 2019 has presented a worldwide health hazard, resulting in a continuing pandemic across numerous nations and territories (WHO, 2020; Rodriguez-Morales *et al.,* 2020). The World Health Organization (WHO) publicly recognized SARS-COV-2 as a pandemic on 30 January 2020. In March 2020, WHO further announced that it had become a global pandemic (WHO, 2021 a). However, significant efforts are underway to assess a particular treatment and other initiatives are being pursued to create vaccines (Thanh *et al.,* 2020). When the epidemic was just starting, the primary methods used for identifying the disease were CT scans and the presence of symptoms like fever, dry cough, exhaustion, and shortness of breath. The utilization of a Chest CT scan can be beneficial in the identification and tracking of complications related to COVID-19, as well as in predicting the progression of the disease. However, it is important to note that this method has limitations due to its relatively expensive cost and the significant amount of ionizing radiation that patients are exposed to during the procedure (Mehmet *et al.,* 2023). RT-PCR is considered the most dependable process for detecting COVID-19 (Gao, 2020; Xiao et al., 2020). However, it has drawbacks such as being time-consuming, often producing incorrect negative results for low viral loads, and restricting the ability to track the progression of the disease (Dai *et al.,* 2020; Mai, 2024). Besides RT-PCR, serology tests have great value as they can assess the immune response (Peeling *et al.*, 2020), track the advancement of the disease, and ascertain the duration of immunological protection after recovering from COVID-19. An inherent drawback of serologic testing is its incapacity to definitively exclude the possibility of cross-reactivity with antibodies resulting from other coronaviruses (Brandi *et al.,* 2020).

The role of physiotherapy in treating COVID-19 is crucial. Physiotherapists are commonly found in acute hospital wards and ICUs worldwide. Specifically, cardiorespiratory physiotherapy targets acute and chronic respiratory issues to enhance physical recovery post-illness. Research by Thomas *et al.* (2020) confirms the potential of physiotherapy in managing hospitalized COVID-19 patients, indicating its benefits in respiratory treatment and physical rehabilitation.

Toll-like receptors (TLRs) are a group of ten innate immune receptors that are crucial in activating innate immunity. They indirectly activate the adaptive immune system by promoting the indication of co-stimulatory molecules that aid in the stimulation of B and T cells (Akira and Takeda, 2004; Tianhao *et al.,* 2022). TLRs also regulate cytokine expression by identifying pathogen-associated molecular patterns (PAMPs), as they are a subgroup of pattern-recognition receptors (PRRs) (Hedayat *et al.,* 2011; Debnath *et al.,* 2020). TLR-4 is located in the cell membrane, whereas TLR-9 is present in endosomes (Stefania *et al.,* 2023). TLRs have a dual function in infections by viruses. They act as a signaling pathway that stimulates the interferon regulatory factor (IRF) in addition to the nuclear factor and both of them have a role in the production of type 1 interferon (IFN) and pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, and tumor necrosis factor-α. Additionally, TLRs can cause macrophage activation syndrome by initiating the Janus kinase transducers (JAK/STAT) pathway.

This investigation seeks to assess the feasibility of diagnosing COVID-19 by utilizing TLR-4 and TLR-9 as immune receptors that escalate in response to viral load. The objective is to develop a precise diagnostic estimation model that can aid clinicians in identifying individuals infected with COVID-19. This will be achieved through the application of stepwise multivariate discriminant analysis.

MATERIALS AND METHODS

1-Patients:

There were 100 participants in this research (n=100) who were categorized into

two groups: 75 infected patients and 25 healthy persons. The participants were taken from El Sahel Teaching Hospital, which is connected with the General Organization for Teaching Hospitals and Institutes (GOTHI). The diagnosis of COVID-19 was made by analyzing CT scans and RT-PCR to detect the genetic material of the SARS-CoV-2 virus in samples taken from the nose and throat and performing COVID-19 profile assays (including D dimer, CRP, and ferritin) on blood samples. In severe cases of suspected or confirmed COVID-19, where patients experience exudative consolidation, excessive mucus production, and difficulty clearing secretions, respiratory physiotherapy interventions in hospital wards or ICUs may be necessary. For moderate cases, physiotherapy plays a role in offering exercises, mobilization, and rehabilitation to aid in the recovery of critically ill COVID-19 survivors, facilitating their return to normal functioning at home. Mild cases typically do not require hospitalization but necessitate medical care and medication at home.

The study included adult males and females over 22 years old. The study selected patients who had received a diagnosis of virus infection, confirmed by a positive PCR test on swab specimens, and had clinical symptoms. Exclusions were made for patients under the age of 22, individuals with bacterial infection, incomplete data, and pregnant women. Healthy people were selected when molecular swabs showed negative results for detecting the virus and the lack of symptoms related to COVID-19.

The blood samples from all participants were separated into four groups. The first group was used for ELISA testing. The second group was collected without the use of an anticoagulant, and the sera were separated and tested for routine and candidate markers. The third group was withdrawn into EDTA tubes for a complete blood count besides erythrocyte sedimentation rate (ESR) measurement while finally the last group was withdrawn into sodium citrate tubes. PCR tests were conducted using samples taken from the nasopharynx and oropharynx by swabs.

2-Laboratory Investigations:

Multiple tests were conducted on patient blood samples, including a complete blood count using an automated hematology analyzer (Phoenix NCC-3300; Neomedia Bioscience Technology, Bulevar Svetog Cara Konstantina). Liver function tests, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH), fasting blood sugar levels (FBS), postprandial glucose test (PPBS), average blood sugar levels over the past 3 months (HbA1C), and D-dimer, were analyzed using an automated biochemistry analyzer (Olympus AU 400; Diagnostic Systems Group of Olympus America Inc). The levels of inflammation biomarkers, specifically serum ferritin and C-reactive protein (CRP), were measured using the ELISA technique with the corresponding kits (ab260058; Abcam; England and EIA-1872; DRG International, Inc., USA). The International Normalized Ratio (INR) was measured using a semi-automated coagulation analyzer (KC1 Delta; Tcoag; Ireland). The measurement of the erythrocyte sedimentation rate (ESR) was also conducted.

3-COVID-19 Detection by Using RT-PCR:

The PCR test was conducted using the cobas® 6800 SARS-CoV-2 system from Roche Molecular Systems in Branchburg, NJ. Three different kits were utilized: the cobas® SARS-CoV-2 test kit (P/N 09175431190), the cobas® SARS-CoV-2 Control kit (P/N 09175440190), and the cobas® Buffer Negative Control kit (P/N 07002238190).

4-Detection Levels of TLR-4 and TLR-9 in Patient'S Serum:

To measure the concentration of TLR-4 (ng/mL) and TLR-9 (ng/L) in serum samples, we used plates that were already coated with TLR9 and TLR4 antibodies. This allowed the TLRs in the samples to attach to the antibodies when they were added. The used kits for TLR-4 and TLR-9 were (bioassay technology laboratory; Cat No: E0356Hu, Zhejiang, China) and (bioassay technology laboratory; Cat No: E0346Hu, Zhejiang, China) respectively. In both kits,

40 µl of the sample was introduced into the sample wells following the addition of 50 µl of the standard to the standard wells. Prior to introducing 50 µl of streptavidin-HRP to the sample and standard wells, except the blank control wells, 10 µl of anti-TLR4 antibody was applied to the TLR4 ELISA kit and 10 µl of anti-TLR9 antibody was applied to the TLR9 ELISA kit. The plates were well blended, hermetically sealed, and thereafter placed in an incubator set at a temperature of 37°C for 60 minutes. Following the removal of the sealers, the plates were washed five times using a wash buffer. For each washing cycle, the wells were submerged in 300 µl of wash buffer for a duration of 30 to 60 seconds, after which the plates were dried using paper towels. Each well was administered 50 µl of substrate solution A, followed by 50 µl of substrate solution B. The plates were subsequently sealed using fresh sealers and located in a dark for 10 minutes at a temperature of 37 °C. Each well was supplemented with 50 µl of stop solution, causing an instantaneous change in color from blue to yellow. After applying the stop solution, the optical density (OD value) of each well was promptly determined via a microplate reader set to 450 nm within 10 minutes (biotech 800TS microplate reader, Santa Clara, California). An optical density (OD) value exhibited a direct correlation with the concentration of TLR4 (ng/mL) and TLR9 (ng/L). The concentration of each sample was detected using the standard curve.

5-Statistical Analysis:

Data was gathered utilizing Microsoft Excel 365©, while IBM SPSS 26 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, USA, NY: IBM Corp) was employed for statistical analysis. Descriptive statistics were conducted to analyze, condense, and present the data. The hypothesis was evaluated by the Chi-square and Mann-Whitney tests at a significance level of 5%. The study's outcomes were expressed as the mean ± standard deviation (SD) or median. Statistical significance was attributed to P values below 0.05. The correlation between TLR-4 also TLR-9 and other laboratory data was measured using Pearson or Spearman correlations. Subsequently, the independent discriminative value of the components for detecting the virus was assessed using receiver operating curves (ROC) and stepwise multivariate discriminant analysis. ROC curves were employed to identify the optimal threshold values for identifying the most accurate COVID-19 diagnosis. Additionally, the area under the curve (AUC) was calculated to compare the diagnostic efficacy of each parameter.

RESULTS

1-Levels of Laboratory Data Among Studied Groups:

The demographic information of the groups being studied is shown in Table 1. COVID-19 patients had elevated levels of white blood cell count, platelet count, ALT, AST, CRP, Ferritin, d-dimer, and a more extended INR. Figure (1), demonstrated that the levels of TLR-4 and TLR-9 were increased in those infected patients.

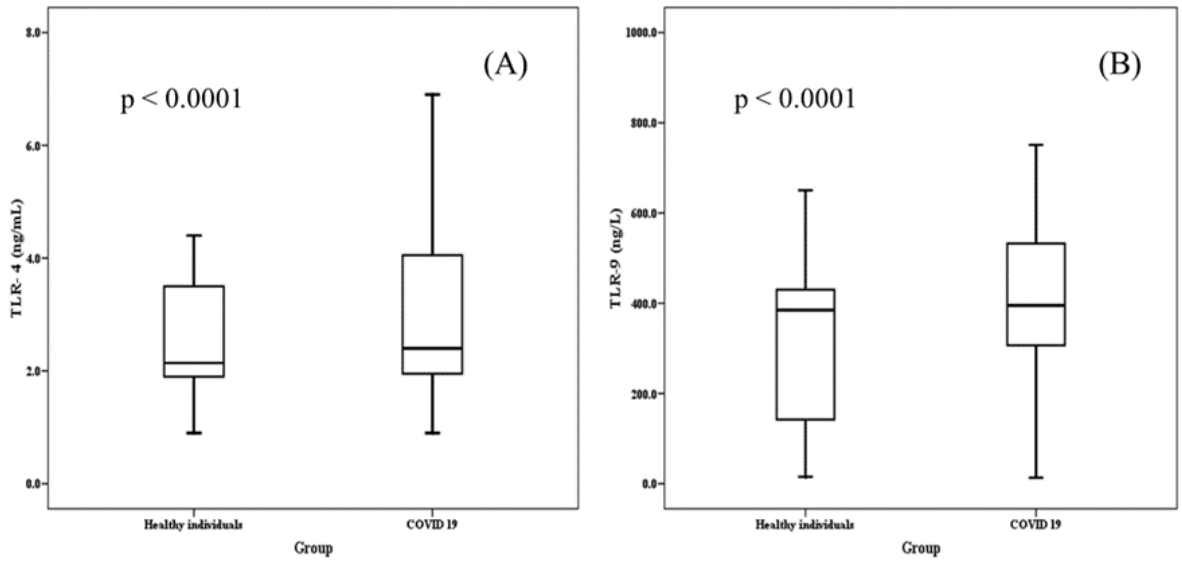

Fig. (1) Levels of TLR-4 and TLR-9 among studied. **(A)** showed elevated levels of TLR-4 in COVID-19 patients than in healthy individuals while, **(B)** showed elevated levels of TLR-9 infected patients than in healthy people.

A significant difference was observed among the compared groups regarding (WBC, HCT, neutrophil, lymphocyte, HbA1C, PPBS, ALT, D dimer, and CRP. The observed differences were very significant for the variables WBC, HCT, neutrophil, lymphocyte, HbA1C, PPBS, ALT, D dimer, and CRP, with a p-value of less than 0.0001, as indicated in Table (1). On the other hand, there was no significant difference among the groups under investigation in age, BMI, Hb, RBC, monocyte, ferritin, ESR 1, ESR 2, INR, and LDH.

Table 1. Comparison of laboratory findings among studied groups:

Variables	Healthy	COVID - 19	P-Value
Age (years)	44.7±10.6	46.6±12.4	0.5
BMI (Kg/m^2)	27±4.5	24.5±6.6	0.08
Hb (g /dl)	12.5±1.9	12.5±1.9	0.9
RBC (10^2/L)	4.5±0.8	4.1±1.2	0.12
WBC (10^9/L)	6.7 (4.9 - 8.5)	3.1 (2.6 - 4.4)	< 0.0001
HCT (%)	45±5.6	31.3±4.7	< 0.0001
PLT (10^9/L)	221.4±45.8	273.9±80.2	0.002
Neutrophil (× 10^3 /mm^3)	59.8±3.9	66.8±4.6	< 0.0001
Lymphocyte (×10^3/mm^3)	44.6±5.5	20.2±2.1	< 0.0001
Monocyte (× 10^3 /mm^3)	5.5 (5 - 7)	7 (5 - 9)	0.01
Hb A1C (mmol/mol)	2.9±1.1	7.5±0.9	< 0.0001
FBS (mg/dL)	95.3±8.5	101.5±8.7	0.002
PPBS (mg/dL)	105.1±12	125.7±13.5	< 0.0001
ALT (U/L)	20.4±5	32.4±6.4	< 0.0001
AST(U/L)	25±4.2	30.3±8.7	0.004
Ferritin (ng/mL)	134.5 (124 - 144.25)	200 (73.8 - 400)	0.04
D Dimer (ng/mL)	0.34 (0.26 - 0.39)	1.05 (0.9 - 1.6)	< 0.0001
CRP (mg/dL)	3.6 (2.1 - 4.45)	12 (8.75 - 23)	< 0.0001
ESR 1 (mm/hr.)	11 (7 - 12)	12 (8 - 20)	0.09
ESR 2 (mm/hr.)	23 (18 - 27.5)	34 (18.5 - 45)	0.03
INR	1.2±0.09	1.4±0.4	0.015
LDH (U/L)	180.1±30.2	190.5±26.5	0.1

2-levels of TLR-4 and TLR-9 Among Studied Groups:

The levels of candidate markers demonstrated a significant difference among groups, with a P-value < 0.0001 for both TLR4 and TLR9 Table (2).

3-Correlation Between Laboratory Data and TLR-4:

Table (3), demonstrated that there wasn't any statistically significant correlation observed between TLR-4 and any of the factors.

Table 2: Levels of TLR-4 and TLR-9 among studied groups:

Variables	Healthy	COVID-19	P-Value
TLR-4 (ng/mL)	2.1 (1.9 - 3.6)	2.4 (1.9 - 4.1)	< 0.0001
TLR-9 (ng/L)	384.8 (115 - 440.6)	395.3 (303.7 - 542.8)	< 0.0001

Table 3: Correlations between TLR-4 and laboratory data:

variable	Correlation (r)	p-value
Lymphocyte (× 10^3 /mm^3)	-0.12	0.3
BMI (Kg/m^2)	-0.1	0.4
Hb (g /dl)	-0.1	0.3
PLT (10^9/L)	-0.07	0.5
PPBS (mg/dL)	-0.06	0.5
FBS (mg/dL)	-0.05	0.6
Monocyte (× 10^3 /mm^3)	-0.02	0.8
RBC (10^2/L)	-0.01	0.9
ALT (U/L)	-0.01	0.9
LDH (U/L)	0.01	0.9
ESR-2 (mm/hr.)	0.02	0.8
Age (years)	0.02	0.6
CRP (ng/mL)	0.05	0.7
INR	0.05	0.8
ESR-1 (mm/hr.)	0.08	0.4
AST (U/L)	0.1	0.5
WBC (10^9/L)	0.11	0.3
D Dimer (ng/mL)	0.11	0.3
Neutrophil (× 10^3 /mm^3)	0.13	0.3
Hb A1C (mmol/mol)	0.14	0.2
Ferritin (ng/mL)	0.2	0.2
HCT (%)	0.2	0.1

4-Correlation between TLR-9 and Laboratory Findings:

Table (4), demonstrated that there was no significant correlation between TLR-9 and all variables, except for ferritin and CRP. A significant correlation was seen in the case of CRP and ferritin, with P-values of 0.09 (r = 0.2) and 0.009 (r = 0.3) respectively **(**Fig.2).

Table 4: Correlations of TLR-9 and laboratory data.

variable	Correlation (r)	p-value
HCT (%)	-0.2	0.1
ESR-2 (mm/hr.)	-0.1	0.5
PLT (10^9/L)	-0.1	0.3
Lymphocyte (× 10^3 /mm^3)	-0.1	0.2
FBS (mg/dL)	-0.03	0.8
Hb (g /dl)	-0.02	0.8
D Dimer (ng/mL)	-0.01	0.9
BMI (Kg/m^2)	0.01	0.9
LDH (U/L)	0.01	0.9
INR	0.02	0.9
ESR-1 (mm/hr.)	0.02	0.9
Age (years)	0.03	0.8
PPBS (mg/dL)	0.03	0.8
RBC (10^2/L)	0.04	0.7
Monocyte (× 10^3 /mm^3)	0.05	0.6
AST (U/L)	0.07	0.3
WBC (10^9/L)	0.1	0.3
ALT (U/L)	0.1	0.5
Neutrophil (× 10^3 /mm^3)	0.13	0.2
Hb A1C (mmol/mol)	0.14	0.2
CRP (ng/mL)	0.2	0.09
Ferritin (ng/mL)	0.3	0.009

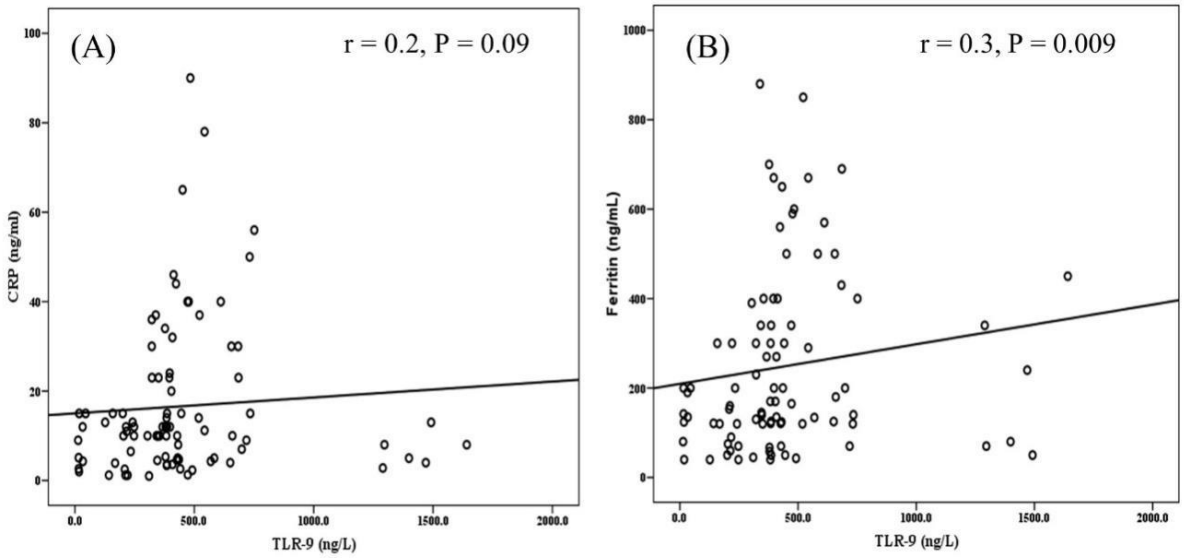

Fig. (2): Correlations between TLR-9 and laboratory data.**(A)** showed a correlation between CRP and TLR-9. **(B)** Showed a correlation between Ferritin and TLR-9.

5-Diagnostic performance of laboratory findings in the diagnosis of COVID-19:

The sensitivity in addition to the specificity of COVID-19 profile biochemical examinations (ferritin, d dimer, CRP) was assessed using ROC curves and cutoff points. For D dimer, the sensitivity was 93% and the specificity was 92%. The CRP test had 88% sensitivity and 79% specificity, however, the ferritin test had 62% sensitivity and 64% specificity (Table 5 & Fig.3). LDH results were excluded from the study since they had a non-significant value.

Table 5: Diagnostic performance of D-dimer, CRP, and Ferritin in the diagnosis of COVID19:

Variable	AU	Std. Error	P Value	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
D-dimer (ng/mL)	0.9	0.3	<0.0001	0.9	1.1
CRP (mg/dL)	0.8	0.04	<0.0001	0.8	0.9
Ferritin (ng/mL)	0.6	0.05	0.04	0.5	0.7

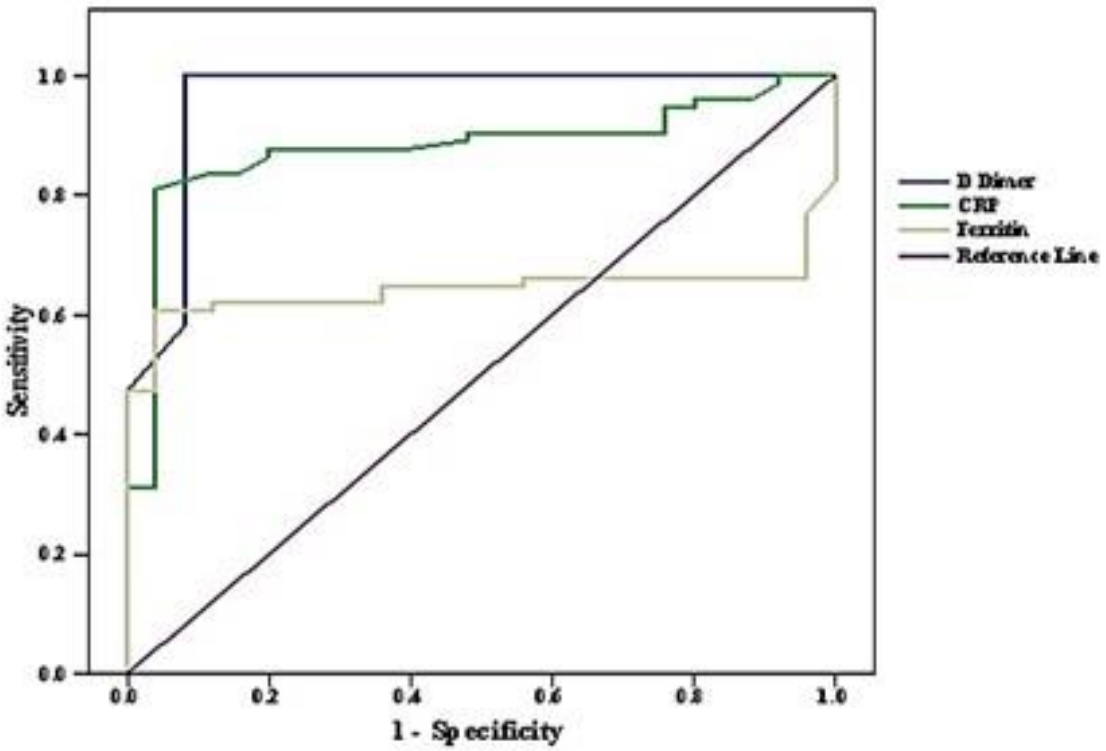

Fig (3): ROC curves of d-dimer, CRP, and ferritin
The blue line represented the ROC curve of d-dimer, the green line represented the ROC curve of CRP while yellow line represented the ROC curve of ferritin.

6-Diagnostic Performance of TLR-4 and TLR-9 in the Diagnosis of COVID-19:

The study enabled precise identification of COVID-19 patients using TLR-4 with an AUC of 0.87 and TLR-9 with an AUC of 0.94 (Table 6 & Fig. 4). The ROC curve was employed to identify the optimal cutoff point for COVID-19 diagnosis. In the instance of TLR-4, the best cutoff point was determined to be 2, while for TLR-9, it was found to be 150. Using these exact cutoff points, the TLR-4 test demonstrated a sensitivity of 80% in addition to a specificity of 79%, whereas the TLR-9 test demonstrated a sensitivity of 92% besides a specificity of 100%.

Table 6: The ROC Curve of TLR-4 and TLR-9

Variable	AU	Std. Error	P Value	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
TLR-4 (ng/mL)	0.8	0.03	<0.0001	0.89	0.99
TLR-9 (ng/L)	0.9	.04	<0.0001	0.79	0.96

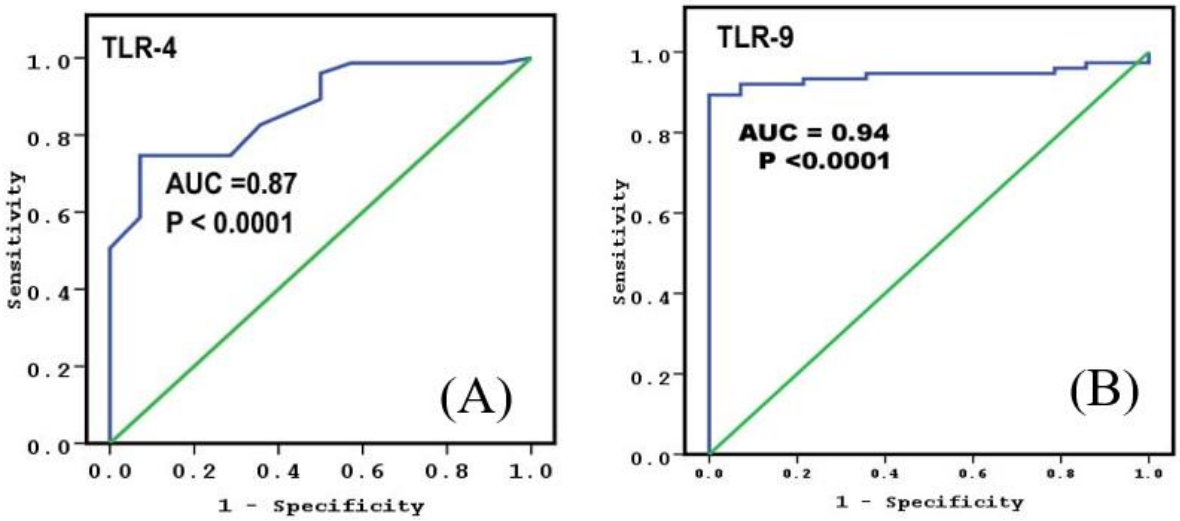

Fig. (4): ROC curve for TLR-4 and TLR-9.
Area under curve of candidate markers, **(A)**: ROC curve for TLR-9 and **(B)**: ROC curve for TLR-4

DISCUSSION

Inadequate or delayed innate responses have been strongly linked to the failure to control initial COVID-19 infection and the progression to severe illness (Blanco-Melo *et al.*, 2020; Iwasaki *et al.*, 2020). Therefore, it is crucial to promptly diagnose the disease to help physicians in detecting the infection with the virus. The use of COVID-19 profile biochemical assays (d-dimer, CRP, ferritin, and LDH) for virus detection is widespread. Physical therapy plays a vital role in both hospitalized and non-hospitalized patients (Paz et al., 2021). For non-hospitalized patients with mild and moderate symptoms, physical therapists play a role in the enhancement of respiratory performance and evaluation of the need for hospitalization based on pulse oximetry measurements of oxygen saturation and dyspnea (SpO2). On the other hand, hospitalized patients with mild symptoms can benefit from physical therapy by preventing them from getting worse; in these situations, physical therapists also continuously evaluate if respiratory physical treatment is necessary (Arbillaga *et al.*, 2021).

The outcomes of this study indicated the levels of ferritin, CRP, and d-dimer were significantly difference in patients compared to healthy persons. The elevation levels of d-dimer were 0.34 (0.26-0.39) in healthy individuals compared to 1.05 (0.9-1.6) in patients, with a p-value of < 0.0001. Similarly, the levels of CRP were 3.6 (2.1-4.45) in healthy individuals and 12 (8.75-23) in patients, with a p-value < 0.0001. Lastly, the levels of ferritin were 134.5 (124-144.25) in healthy individuals and 200 (73.8-400) in patients, with a p-value of 0.04. The LDH data were disregarded since they had non-significant values.

COVID-related death is strongly related to hypercoagulability and an increased risk of venous thromboembolism (VTE) events. In severe situations, these variables might lead to thrombo-inflammation (Bikdeli *et al.*, 2020). Endothelial activation, a process in which the

virus enters endothelial cells through ACE receptors, is a mechanism that causes microvascular thrombosis in COVID-19 (Hunt and Levi, 2020). D-dimer is a substance that is produced when plasmin breaks down fibrin polymer, leading to the formation of deep vein thrombosis (DVT) and pulmonary embolism (van *et al.,* 2020). Therefore, increased amounts of d-dimer indicate the breakdown of fibrin. Individuals with d-dimer levels exceeding 1000 ng/ml have a 20-fold greater risk of mortality in contrast to those with lower d-dimer levels (van *et al.,* 2006). This increased risk is attributed to the aggregation of d-dimer in the alveoli and lung parenchyma, resulting in lung damage (Varga *et al.,* 2020). The sensitivity and specificity of D-dimer for detecting VTE events in 2158 COVID-19 patients were 90% (indicating high sensitivity) and 60% (reflecting low specificity), respectively (Haoting *et al.,* 2021).

CRP is a nonspecific protein made by hepatocytes during the acute-phase response (Mazen et al., 2023). It serves as an early indicator of inflammation or infections caused by viruses and bacteria (Nehring et al., 2023). CRP is triggered on cell membranes that have been stimulated, such as endothelial cells and platelets. Additionally, CRP exhibits a preference for binding to phosphocholine, a molecule that is abundantly present on the outer membrane of damaged cells. In a case-control study by Amal *et al.* which included 135 patients and 137 healthy controls, CRP was significantly higher in patients with COVID-19 disease (Amal *et al.,* 2021).

Serum ferritin is a type of protein that is produced in response to acute-phase reactions (Kappert et al., 2020; Poonkuzhi *et al.,* 2020). The existence of ferritin in case of infection can be attributed to inflammation or cellular damage, and its increased levels indicate a significant inflammatory reaction to the entrance of SARS-COVID-2 to the body, affecting iron metabolism (Jeffrey *et al.,* 2022). Based on research done by Edeas M. *et al.,* the release of ferritin from damaged intracellular storage is believed to be the underlying cause of hyperferritinemia (Edeas *et al.,* 2020). This condition is assumed to be cytokine-mediated and involves the involvement of interleukin (IL) 1b, IL-6, and IL-18 besides interferon (IFN)-γ, and macrophage-colony stimulating factors. These cytokines that promote inflammation drive liver cells to create various proteins such as CRP and ferritin. By activating pro-inflammatory pathways, ferritin can assist in the development of sickness (Ruscitti *et al.,* 2020). These observations suggest that ferritin and cytokines may have a role in a reciprocal feedback process (Jenifer *et al.,* 2020). Excessive iron can also trigger the process of fibrin polymerization (Pretorius and Kell, 2014) and cause a condition of increased blood clotting (Colafrancesco *et al.,* 2020), which may explain the development of blood clotting disorders in patients (Zhang *et al.,* 2020; Mazzeffi *et al.,* 2021). According to a meta-analysis performed in 2020 by Cheng *et al.* on 10,614 confirmed COVID-19 cases, individuals with severe COVID-19, nonsurvivors, and those with one or more chronic conditions exhibit significantly higher ferritin levels (Cheng *et al.,* 2020).

After infection occurs, pathogen-associated molecular patterns (PAMPs) of viruses can be detected by specific host pattern recognition receptors (PRRs), like TLRs. TLRs are essential elements of the human immune system that show a significant role in resisting and eliminating the virus by augmenting the production of antibodies during COVID-19 infection (Tianhao *et al.,* 2022). Each kind of TLR has a distinct function in this process. In the present investigation, patients exhibited higher levels of TLR-4 and TLR-9 compared to healthy individuals. The results indicate that the level of TLR-4 was 2.1 (1.9-3.6) compared to 2.4 (1.9-4.1) with a p-value of less than 0.0001. Similarly, the level of TLR-9 was 384.8 (115-440.6) versus 395.3 (303.7-542.8) with a p-value <0.0001.

The activation of TLR-4 on the cell membrane occurs when certain components

of the viruses fail to completely unfold. Oxidized phospholipids, capable of stimulating TLR4, are present in case of viral lung infections like SARS-CoV-2 (Aboudounya *et al.,* 2021). TLR4 can also detect particular damage-associated molecular patterns (DAMPs) produced in the case of deceased or lysed fragments of cells after a viral infection or harm to the host tissue (Aboudounya *et al.,* 2021). There are several ways in which TLR-4 overexpression is connected to altered immune responses to viral components or stimulation of cytokine signaling (Li *et al.,* 2020).

The study carried out by Zahra et al. involved 90 patients and focused on assessing the expression of mRNA of TLR-9 in the epithelial cells of infected patients compared to control participants (Zahra *et al.,* 2022). The activation of TLR9 is initiated by the presence of DNA motifs that are rich in cytosine-phosphate-guanine (CpG) and are not methylated. These motifs are mostly present in bacterial, mitochondrial, and fetal DNA, but are seldom present in genomic DNA for adults (Hemmi et al., 2000). SARS-CoV-2 infection causes impaired functioning of mitochondria (Ajaz *et al.,* 2021), characterized by heightened production of reactive oxygen species (ROS) within the lungs (Abouhashem et al., 2020), decreased levels of calcium ions in the blood (Zhou et al., 2020), and higher amounts of mitochondrial DNA (mtDNA), which in turn activate TLR9 signaling.

The AUC values for d-dimer, CRP, and ferritin were computed as 0.96, 0.85, and 0.64, respectively, using the data in this investigation. Sensitivity and specificity were determined using ROC curves and cutoff points. The sensitivity and also specificity for d-dimer were computed as 93% and 92% respectively while for CRP were 88% and 79% respectively and lastly, for ferritin, were 62% and 64% respectively.

According to a study investigated by Ahmed N. Kaftan et al., utilizing a mix of common laboratory indicators (CRP, ferritin, D dimer, and LDH) can serve as a valuable method for diagnosing COVID-19 prior to employing RT-PCR. The conclusions of the study point out that combining the parameters of CRP + ferritin yielded an AUC of 0.77, indicating a sensitivity of 55% while the specificity was 97%. Combining ferritin and LDH resulted in an AUC of 0.83, which shows sensitivity of 65% in addition to specificity of 92%. Combining CRP and LDH yielded an AUC of 0.78, which gets on sensitivity of 56% and specificity of 98%. Combining CRP, LDH, and ferritin resulted in an AUC of 0.85, with 73% in the case of sensitivity and 88% in the case of specificity. Finally, combining CRP, LDH, ferritin, and D dimer yielded an AUC of 0.85, with a sensitivity of 75% and specificity of 87% (Ahmed *et al.,* 2021).

This study utilized the increased expression of TLR4 and TLR9, which is observed during infection, as a means to identify COVID-19. AUC for TLR-4 was 0.87, but for TLR-9 it was 0.94. The TLR-4 exhibited a sensitivity of 80% with a specificity of 79%, while the TLR-9 showed a sensitivity of 92% and a specificity of 100%. Studies are scarce on the utilization of TLR-4 and TLR-9 for diagnosing COVID-19. The most reliable biomarker for distinguishing between healthy individuals and those with COVID-19 is d-dimer, which has an AUC of 0.96. This biomarker exhibits a sensitivity of 93% and a specificity of 92%. Following d-dimer, the biomarkers TLR-9 and TLR-4, and then CRP with an AUC of 0.85 (sensitivity 88%, specificity 79%), and finally ferritin with an AUC of 0.64 (sensitivity 62%, specificity 64%), are also helpful in distinction.

Conclusion:

The TLR-4 biomarker can be utilized as an effective diagnostic tool with satisfactory levels of sensitivity and specificity. The TLR-9 can serve as a valuable marker for accurately identifying SARS-COV-2 infection with a notable level of sensitivity and specificity. This study is restricted by a little amount of sample size and a lack of adequate clinical indicators, such as respiratory rate, oxygen saturation,

and the number of days from the onset of symptoms. Subsequent investigations should use a greater number of participants in order to scrutinize the multitude of clinical signs.

Declarations:

Ethical Approval: The study received permission from the ethics committee of the General Organization for Teaching Hospitals and Institutions (GOTHI), with the assigned approval number HS000115. Prior to participating in the study, each patient provided their written informed consent.

Conflict of interests: There's no conflict of interest**.**

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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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