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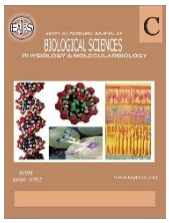

Evaluation of the Diagnostic Performances of Spike Antigen for Diagnosis of Coronavirus 2019 Disease in Egyptian Patients

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ABSTRACT

The Coronavirus Disease 2019 (COVID-19) has created serious risks to human health and public safety. Hence, there is a pressing demand for uncomplicated and precise diagnostic assays to ensure accurate identification of the infection. SARS-CoV-2, like other coronaviruses, is classified as a single-stranded, positive-sense RNA virus with four key structural proteins: the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The spike protein holds significant importance in early diagnosis of SARS-COV-2 infection due to its role in viral attachment, fusion, and cellular entry. In the current study, the diagnostic performance of SARS-CoV-2 Spike Protein S1 RBD was evaluated. Clinical samples (n =75) (nasopharyngeal and blood specimens) were collected from confirmed infected patients using reverse transcription polymerase chain reaction RT-PCR. In addition, 25 healthy participants were included as controls. Routine laboratory markers were evaluated for all participants and the SARS-CoV-2 Spike Protein S1 RBD was determined using sandwich ELISA for all participants. The result showed that the spike protein level demonstrated a statistically significant difference ($p < 0.0001$) in COVID-19 patients compared to healthy participants. Furthermore, the variables D-Dimer and CRP demonstrated a statistically significant difference ($p < 0.0001$) with the existence of COVID-19. In contrast, the analysis revealed no differences regarding LDH, body mass index, or gender among the studied groups. In addition, there was a significant positive correlation between D-dimer ($r = 0.57$, $p<0.0001$) and spike antigen. Spike antigen was the most effective biomarker in distinguishing COVID from healthy participants, with an AUC of 0.99, sensitivity of 98%, and specificity of 94%. Additionally, D-dimer has a sensitivity of 93%, a specificity of 92%, and an AUC of 0.96, and CRP has a sensitivity of 87%, a specificity of 80%, AUC=0.88. And finally, ferritin has a sensitivity of 61%, a specificity of 64%, and an AUC of 0.64. SARS-COV-2.Spike antigen can be used as a suitable diagnostic test for identifying COVID-19 infection with higher sensitivity and specificity.

INTRODUCTION

In Wuhan, China, an unidentified upper respiratory tract infection, later identified as COVID-19, rapidly spread and was declared a pandemic by the World Health Organization on March 11, 2020. Recent statistics indicate that the expected mortality rate resulting from the global epidemic exceeds six million people. Egypt, among African nations, announced the first COVID-19 case in February 2020 and reported 516,000 cases in September 2024. The primary goal of healthcare throughout the pandemic was to efficiently manage the propagation of the virus. Consequently, ensuring an early and exact diagnosis is critical to achieving this goal **(Lee *et al.*, 2010)**. When the epidemic was just starting, the primary approaches used for identifying the disease were CT scans and the presence of symptoms like fever, dry cough, exhaustion and shortness of breath. Chest CT scans are a quick and easy way to identify early COVID-19 infection with excellent sensitivity, allowing for early diagnosis and patient tracking of illness progression. Furthermore, it is essential to evaluate the progression of the disease. However, CT does not accurately distinguish between COVID-19, community-acquired pneumonia, and other lung disease presentations and needs large facilities that are only found in hospitals, which limits its use for the screening of the COVID-19 infection(Mohamadian *et al.*, 2021). The standard technique employed to detect COVID-19 was RT-PCR assay. Although the assay is extensively recognized and employed as the preferred benchmark molecular test, it exhibits certain limitations. A drawback of the assay is the possibility of producing false negative results. In addition, the assay necessitates the involvement of skilled experts, expensive equipment, and a lab facility equipped with a biosafety level 2 cabinet (Xu *et al.*, 2020; Younes *et al.*, 2020). Consequently, there is an ongoing need for novel techniques to improve COVID-19 diagnostic precision. Serological tests are immunoassays that specifically target the nucleocapsid (N) or spike (S) protein. These viral proteins are detected using certain antibodies that hook to the S or N proteins, enabling the full virus or fragment to be captured. The significance of spike protein lies in its role in virus attachment, fusion, and entry into cells (Tai *et al.*, 2020). Moreover, spike protein is a key COVID-19 diagnostic marker. According to a study conducted by (Fabiani *et al.*, 2021), electrochemical immuno sensors were employed to identify S and N proteins in the saliva of COVID-19 patients. Also, a quick ultrasensitive digital enzyme-linked immunosorbent test (dELISA) was developed by (Cai *et al.*, 2021) to detect the spike and N proteins utilizing a single-molecule array. This test was highly sensitive, rapid, precise, and specific for detecting spike and N-protein while minimizing interference from other blood proteins. The spike assay demonstrated a good and repeatable recovery rate in serum samples, which serves to increase COVID-19 diagnosis accuracy. The present work employed sandwich ELISA to identify the existence of the SARS-CoV-2 spike RBD (recombinant receptor-binding domain antigen) in serum samples and evaluate the diagnostic accuracy of the spike antigen in detecting coronavirus 2019.

MATERIALS AND METHODS

1-Patients:

The current study was conducted on 25 healthy participants and 75 patients diagnosed with COVID-19. Reverse transcription polymerase chain reaction (RT-PCR) was utilized to verify clinical samples from participants. The participants were chosen from Sahel Teaching Hospital, which relates to the General Organization for Teaching Hospitals and Institutes (GOTHI). A comprehensive medical history and clinical examination were conducted for all participants. The study included adult males and females over 21 years old who were confirmed by a positive PCR test on swab specimens and had clinical symptoms. While excluding patients under the age of 21, individuals with bacterial infections, incomplete data, and pregnant women.

Healthy participants were selected based on negative swabs for the molecular detection of the virus and the lack of symptoms related to COVID-19.

2-Samples:

Clinical samples (n=75) (blood and nasopharyngeal specimens) were obtained ranging from 0-55 days from the beginning of symptoms. Blood samples will be centrifuged to obtain clinical samples and stored at −80°C until assayed. In addition, twenty-five healthy individuals with normal chest radiographs and no indications of clinical deterioration served as controls. Blood samples were collected from all participants after a 12-hour fasting period and then divided into four separate Tubes. The primary cohort was employed exclusively for the purpose of ELISA analysis. The second tube without anticoagulant and the sera were then separated and analyzed for both standard and potential indicators. The third was transferred into EDTA tubes in order to perform a comprehensive blood count and assess the erythrocyte sedimentation rate (ESR). Finally, the fourth was transferred into sodium citrate tubes in order to perform the D-dimer test. Samples for the PCR test were taken from the nasopharynx and oropharynx.

3-Laboratory Investigations:

Biochemical analysis including serum alanine aminotransferase (ALT); serum aspartate aminotransferase (AST); lactate dehydrogenase (LDH); blood sugar levels during fasting (FBS); postprandial glucose test (PPBS); average blood sugar levels over the past 3 months (HbA1C); and D-dimer were determined using an automated biochemistry analyzer (Olympus AU400; Diagnostic Systems Group of Olympus America Inc.). Serum ferritin was measured using the ELISA technique with the corresponding kits (ab260058; Abcam; England and EIA-1872; DRG International, Inc., USA). A complete blood count was determined using an automated hematology analyzer (Phoenix NCC-3300; Neo Media Bioscience Technology; Bulevar Svetog Cara Konstantina 3) The International Normalized Ratio (INR) was measured using a semi-automated coagulation analyzer (KC1 Delta; Tcoag; Ireland). Additional parameters are assessed including ESR and CRP.

4-COVID-19 Detection Using RT-PCR Method:

The nasopharyngeal and oropharyngeal samples were stored at a temperature range of 2–25 °C until they were analyzed. The detection of COVID-19 using the RT-PCR technique was conducted using three different kits: 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). The Cobas® SARS-CoV-2 test kit consists of various components and substances.

5-Assessment of SARS-CoV-2 Spike Protein S1 RBD Protein Levels:

Serum samples were kept at a temperature of -80°C until they were analyzed and then rapidly thawed before the measurement. SARS-CoV-2 Spike Protein S1 RBD protein was determined with an ELISA kit (bioassay technology laboratory; Cat No. E-EL-E605, Zhejiang, China) by ELISA conducted on a SUNRISE microplate reader, Serial No. 802345678120. The kit employs the Sandwich-ELISA concept. The plate has been pre-coated with a SARS-CoV-2 S1 RBD-specific antibody. Wells were incubated at 37 °C for 90 minutes after the addition of 100 µL of samples (or standards). Following the removal of the liquid, 100 µL of biotinylated detection antibody, which is specific for SARS-CoV-2 Spike Protein S1 RBD, was introduced into the wells of the plate and incubated for 1 hour at a temperature of 37°C. Free components were washed away. Then 100 µL of Avidin-horseradish peroxidase (HRP) conjugate was applied sequentially to each well of the microplate and incubated for 30 minutes at a temperature of 37°C. Free components were washed away. 90 µL of substrate solution was added to each well and incubated for 15 minutes at a temperature of 37 °C. The blue coloration was observed exclusively in the wells that contained SARS-CoV-2 S1 RBD, biotinylated detection

antibody, and Avidin-HRP conjugate. The enzyme-substrate reaction was halted by the introduction of 50 μL of stop solution, resulting in yellow coloration. Measurement of the optical density (OD) is conducted using spectrophotometry at a specific wavelength of 450 ±2 nm. The optical density (OD) value exhibited a direct correlation with the concentration of SARS-CoV-2 S1RBD. The concentration of SARS-CoV-2 S1RBD in the samples was calculated by comparing the OD of the samples to the standard curve.

6-Statistical Analysis:

Microsoft Excel 365[3] was utilized for the process of data collection and cleaning, whereas IBM SPSS 26 (IBM Corp. Released 2019) was employed for statistical analysis. IBM SPSS Statistics for Windows, Version 26.0, produced by IBM Corp., based in Armonk, USA, NY, was used for data analysis in this study. Descriptive statistics were employed to analyze, synthesize, and present the data. In the context of hypothesis testing, the Mann-Whitney U test and the Chi-square test were utilized, with both tests being run at a significance level of 5%. The correlation between SARS-CoV-2 Spike Protein S1 RBD and other laboratory data was measured using Pearson or Spearman correlations. The variable's diagnostic effectiveness was assessed by creating a ROC curve and then calculating the AUC. Receiver operating characteristic (ROC) curves were employed to determine the optimal threshold values for the diagnosis of COVID-19. The quantification of diagnostic indicators was conducted using percentage values.

RESULTS

1-Routine Markers Levels Among The Studied Groups:

The analytical and clinical data for the two groups being studied are given in Table 1. No statistically significant differences were seen across groups concerning BMI, hb, RBC and LDH. The variables WBC, HCT, lymphocyte, neutrophil, HbA1C, ALT, AST, PPBS, D-dimer and CRP exhibited a significant difference ($p< 0.0001$) with the existence of COVID-19. The spike antigen level demonstrated a statistically significant elevation ($p < 0.0001$) in those diagnosed with COVID-19 in comparison to healthy participants as shown in Figure (1).

Table 1: Levels of COVID-19 routine markers among the studied group.

Variables	Healthy (n=25)	COVID (n=75)	p-value
Male count (%)	12(48%)	44(59%)	0.35
Female count (%)	13(52%)	31(41%)	0.35
Age (Years)	43.6±11.6	45.7±13.4	0.4
BMI (Kg/m^2)	26.7±4.9	28.5±4.6	0.1
RBC ($10^6/\mu L$)	3.8±0.5	3.9±0.4	0.5
Hb (g/dL)	11.2±1.3	11.3±1.4	1
HCT (%)	41.9±4.9	34.2±3.7	< 0.0001
MCV (fl)	67.8±1.8	67.7±3.3	0.88
MCH (pg)	31.2±1.8	30.2±3.5	0.19
MCHC (g/dL)	29.5±2.6	30.8±2.7	0.03
WBC ($10^3/\mu L$)	6.7 (4.8 - 8.6)	3.1 (2.5 - 4.5)	<0.0001
Neutrophils (%)	54.7±4.1	68.8±3.6	<0.0001
Lymphocytes (%)	40.6±3.5	24.2±4.2	<0.0001
Monocytes (%)	5(4 – 6.5)	7 (5 - 9)	<0.0001
Platelets ($10^3/\mu L$)	221.3±45.9	273.8±80.3	0.002
ESR 1hr (mm/hr)	11 (7 - 12)	12 (8 - 20)	0.09
ESR 2hr (mm/hr)	23 (18 - 27.5)	33 (19.5 - 45)	0.03
INR	1.1±0.1	1.2±0.2	< 0.0001
D-dimer (ng/mL)	0.34 (0.26 - 0.39)	1.05 (0.8 - 1.7)	< 0.0001
CRP (mg/L)	3.6 (2.9 - 4.55)	12 (8.9 - 24)	< 0.0001
LDH (U/L)	181.5±29.9	189.9±26.5	0.188
Ferritin (ng/mL)	134.5 (124 - 144.3)	200 (73.8 - 400)	0.04
HbA1c (%)	3.6±0.5	6.5±0.9	< 0.0001
FBG (mg/dL)	94.3±10.1	102.3±12.9	0.006
PPBG (mg/dL)	109.1±10.8	121.7±14.1	< 0.0001
ALT (U/L)	22.4±5.5	30.1±5.9	<0.0001
AST(U/L)	23.8±4.5	32.4±8.6	<0.0001
Spike antigen(ng/ml)	0.97(0.27-3.7)	20.3(16.8-22.9)	< 0.0001

CRP, C reactive protein; ESR, erythrocyte sedimentation rate; FBG, fasting blood glucose; ALT, alanine transaminase; AST, aspartate transferase; HbA1c, hemoglobin A1c; HCT, hematocrit; INR, international normalized ratio; LDH, lactate dehydrogenase; PPBG, postprandial blood glucose; RBC, red blood cell; WBC, white blood cell.

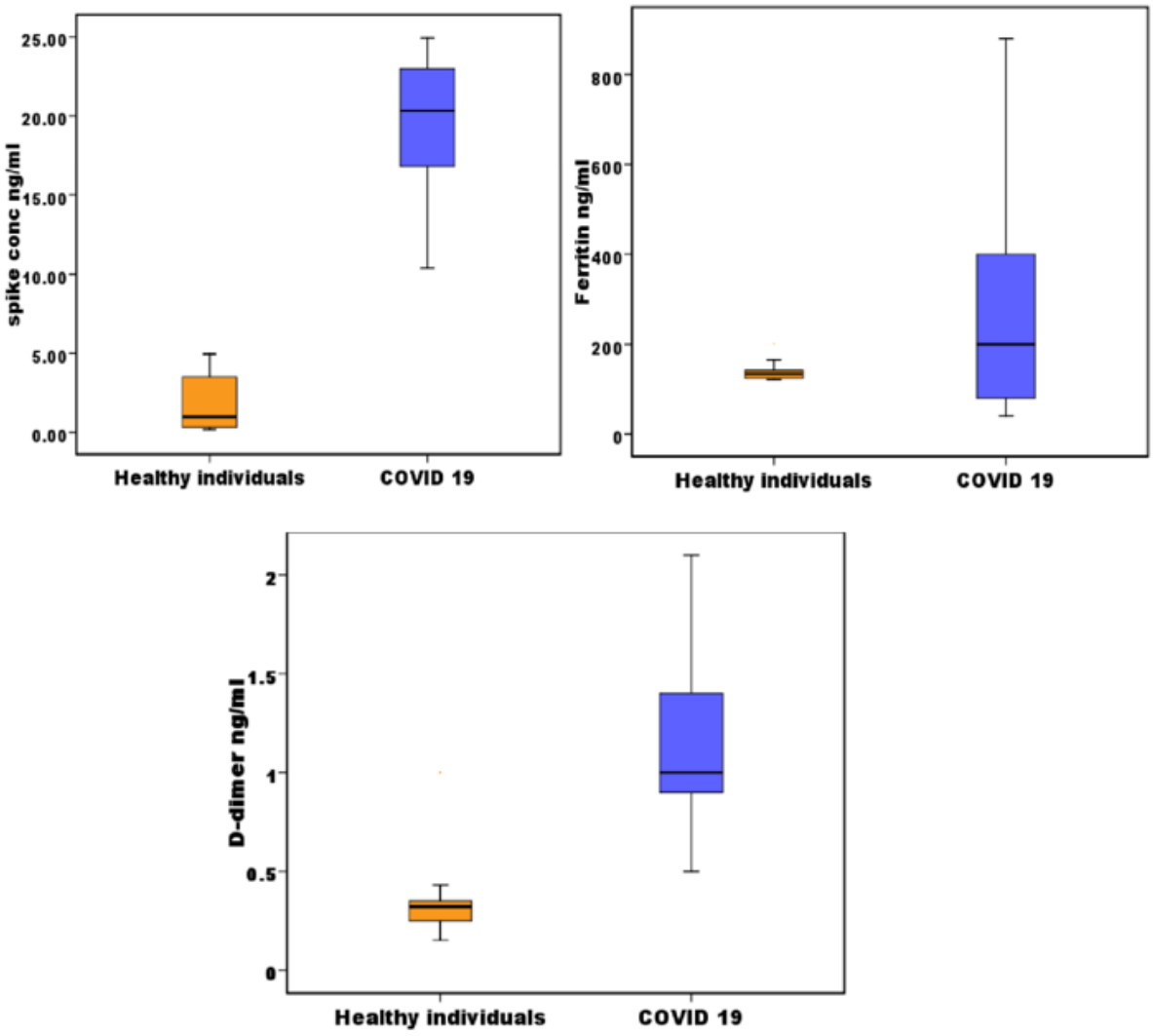

Fig 1: Levels of spike antigen, D-dimer, CRP and ferritin among studied groups**.**

2-Correlation Between Spike Antigen And Laboratory Findings:

Table 2, demonstrated that there was a statistically significant positive correlation between spike antigen and Hb-A1C, Neutrophil, and D-dimer ($r = 0.74$, $p<0.0001$; $r = 0.71$, $p<0.0001$; $r = 0.57$, $p<0.0001$, respectively). Conversely, there was a statistically significant negative correlation between spike and lymphocytes ($r = -0.76$, $p<0.0001$). On the other hand, there wasn't any statistically significant correlation ($r = 0.00$) observed between spike antigen and LDH (Fig.2).

Table 2: Correlation between laboratory data and spike antigen.

Variables	Correlation	P-value
LDH(U/L)	0.00	0.99
Ferritin(ng/mL)	0.05	0.64
FBS (mg/dL)	0.07	0.53
MCH(pg)	0.08	0.44
MCV(fl)	-0.02	0.84
RBCs(10^6/µL)	-0.11	0.92
ESR_1hr(mm/hr)	0.11	0.29
MCHC(g/dL)	0.13	0.22
CRP (mg/L)	0.24	0.02
PPBS (mg/dL)	0.23	0.03
ESR_2hr(mm/hr)	0.15	0.15
Monocytes%	0.32	<0.0001
AST(U/L)	0.34	0.001
WBCs(10^3/µL)	-0.28	0.1
ALT(U/L)	0.44	<0.0001
INR	0.48	<0.0001
HCT%	-0.55	<0.0001
D-dimer(ng/mL)	0.57	<0.0001
Hb(g/dL)	0.65	0.55
PLTs(10^3/µL)	0.39	<0.0001
Neutrophil%	0.71	<0.0001
Hb A1C%	0.74	<0.0001
Lymphocytes%	-0.76	<0.0001

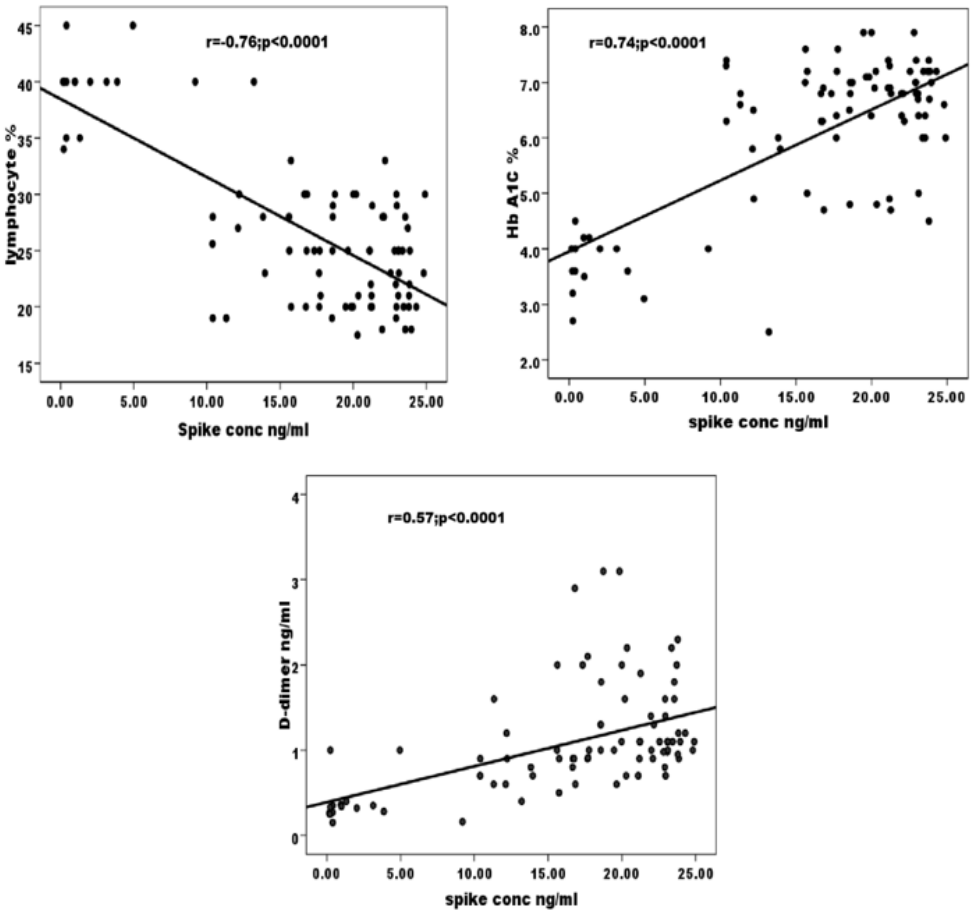

Fig 2: Correlation between levels of spike antigen and HBA1C, D-dimer, Lymphocyte.

3-Diagnostic Performance Of Spike Antigen And Laboratory Findings In The Detection of COVID-19:

The spike antigen's diagnostic effectiveness was evaluated using ROC curves, yielding a value of 0.99. The sensitivity and specificity of the spike antigen were found to be 98% and 94%, respectively. The positive predictive value (PPV) was determined to be 99%, while the negative predictive value (NPV) was found to be 94%. The test achieved an overall accuracy of 98% and the spike cut-off was 10.4ng/ml. For D-dimer (AUC = 0.96, sensitivity = 93%, specificity = 92%), CRP (AUC=0.88, sensitivity=87%, specificity=80%), and finally ferritin (AUC=0.64, sensitivity=61%, specificity=64%) (Table 3) and (Fig. 3).

Table 3: Diagnostic performance of laboratory findings in COVID-19:

Biomarker	(AUC)	Sensitivity	Specificity	PPV	NPV	Accuracy
Spike antigen(ng/mL)	0.99	98%	94%	99%	94%	98%
D-dimer(ng/mL)	0.96	93%	92%	97%	82%	93%
CRP (mg/L)	0.88	87%	80%	93%	67%	85%
Ferritin(ng/mL)	0.64	61%	64%	83.6%	36%	62%

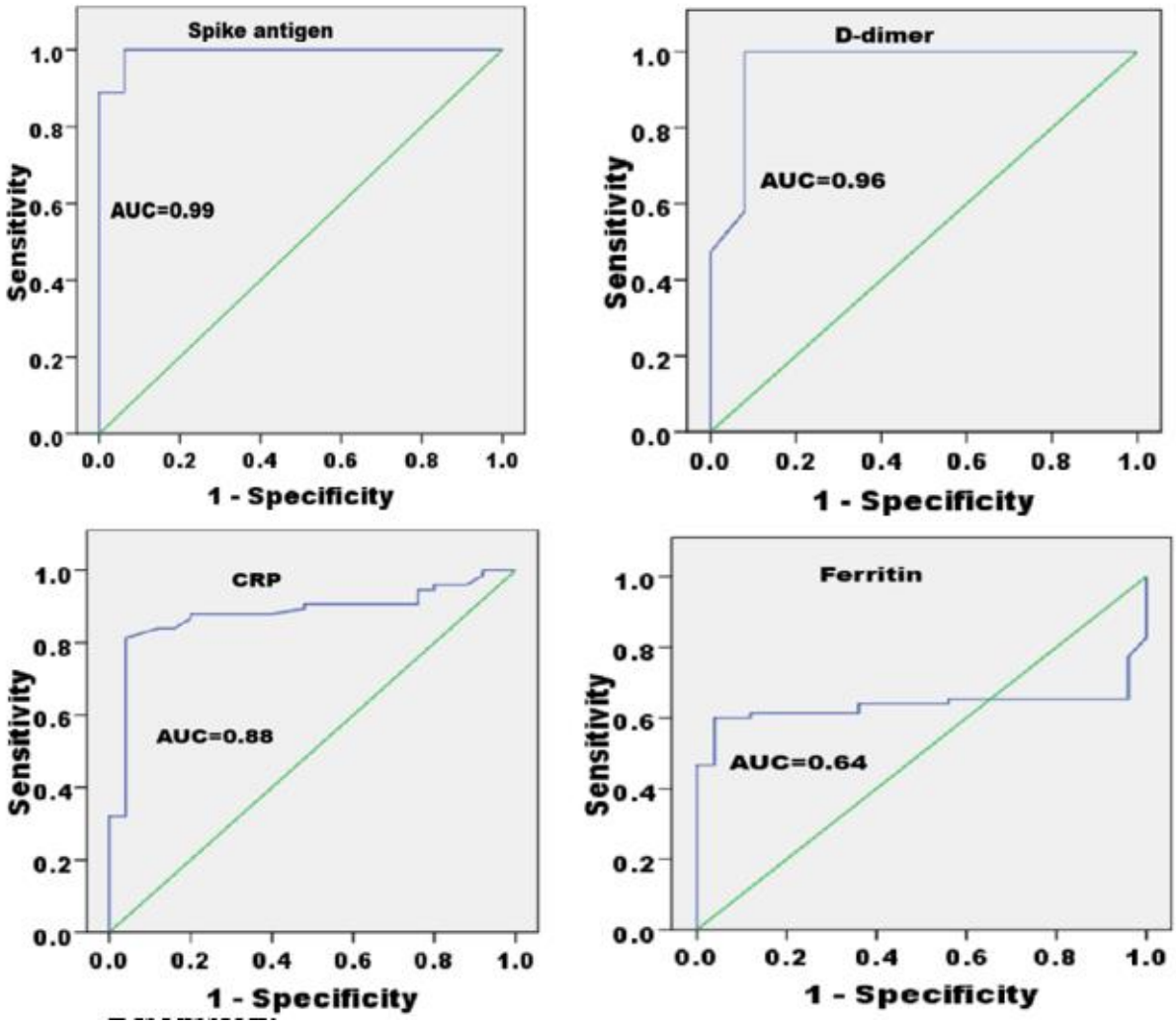

Fig 3: Diagonal segment of the ROC curves for Spike antigen, D dimer, CRP and ferritin

DISCUSSION

The coronavirus disease 2019 (COVID-19) continues to spread globally. So, quick, uncomplicated, and precise diagnostic methods are desperately needed. Moreover, over the two last years, the re-emergence of Flu-A/B and HRSV infections in the wake of the pandemic highlighted the need for differential diagnosis in detecting and distinguishing SARS-CoV-2, Flu-A/B, or HRSV patients in the coming season (Luštrek *et al.,* 2024).

The typical laboratory diagnosis for COVID-19 infection is RT-PCR assays. This assay is the most popular and frequently employed as the preferred, highest-quality molecular test with excellent specificity and sensitivity (Wagatsuma *et al.,* 2005). Even though the PCR test is superior in terms of the limit of detection (LOD) it has certain limitations, as summarized in the following: (1) Lack of specificity (frequently leads to inaccurate positive and negative outcomes). (2) PCR techniques are complicated and consume time; (3) the test needs professional, skilled individuals, expensive equipment, and a biosafety level 2 cabinet-equipped lab (Chu *et al.,* 2020; D'Cruz *et al.,* 2020; Hirotsu *et al.,* 2020). Moreover, sensitivity for PCR was 72% for Bronchoalveolar specimens, 63% for nasal swabs, 32% for pharyngeal swabs, 62.3% for saliva specimens; and 7.3% for blood specimens (Sethuraman *et al.,* 2020). As a result, an alternative approach is urgently required to overcome these limitations. Unlike PCR-based approaches, serological screening relies on antigen detection to detect viral components (i.e., spike protein, M protein, or released N protein) or the virus itself without the need for thermal amplification. Furthermore, it is more precise than those depending on antibody detection since antigen precedes antibodies, appear in the early stages of infection, and are target-specific. Antigen detection tests using spikes or nucleocapsid have been devised and reported (Jiang *et al.,* 2020; Premkumar *et al.,* 2020). The spike is trimeric with an alcove form, with an S1 head and S2 stalk in each unit and covers the outside of the virus, which permits it to attach itself to the host cell membrane's ACE2 receptors. Additionally, this mechanism facilitates the fusion of the viral membrane with the host cell. (Huang *et al.,* 2020; Shang *et al.,* 2020) RBD which is located in the S1 head region, interacts with the receptor present on the cellular membrane, triggering cell entrance(Walls *et al.,* 2020).ELISA assays depending on spike have been demonstrated to be more specific than nucleocapsid, due to false-positive results with nucleocapsid. (Yamaoka *et al.,* 2020).

Compared with other studies evaluating COVID-19 infection, spike antigen testing in our study performed with similar sensitivity and specificity to (Hirotsu *et al.,* 2021) When nasopharyngeal swab samples are utilized using the LUMIPULSE antigen test, (Porte *et al.,* 2020) Reported sensitivity of 93.9% and specificity of 100% for individuals who developed symptoms within a week when applying a luminescent immune chromatographic antigen test for COVID-19 and (Park *et al.,* 2023) Stated that sensitivity and specificity of 90.9% and 99.5%, respectively using an oil- and beads-free single molecule assay with digital immuno-RCA. On the other hand (Kivrane *et al.,* 2022) Reported that the developed LFA assay showed potential for SARS-CoV-2 identification in saliva samples with 26.5% sensitivity and 58.1% specificity. Moreover (Barlev-Gross *et al.,* 2021) Reported that sensitivity was 66% and specificity was 99% when using the TRF ELISA spike assay and (Mertens *et al.,* 2020) Reported that sensitivity was 57.6% and specificity was 99.5%. In addition, there is a quick detection method that combines nanozyme and enzymatic chemiluminescence immunoassays with a lateral flow strip dedicated spike protein, despite all other assays utilized the nucleocapsid. (D. Liu *et al.,* 2021).

In this study, we were able to successfully identify spike proteins in blood samples from individuals diagnosed with

COVID-19. We utilized a commercially available kit, specifically the SARS-CoV-2 Spike protein S1 RBD ELISA kit (bioassay technology laboratory; Cat No. E-EL-E605, Zhejiang, China). This kit is designed for the quantitative measurement of S1RBD concentration in samples obtained from the subjects under investigation, which had been previously confirmed through polymerase chain reaction (PCR) analysis. According to our results, the exceptional sensitivity of the spike antigen plasma tests makes them potentially perfect for use as confirmatory testing in certain contexts Based on the cut-off value determined from the ROC curve our results evaluate the diagnostic performance of this ELISA kit for the detection of spike protein with a sensitivity of 98%, specificity of 94% and AUC of 0.99. The positive predictive value (PPV) was found to be 99%, while the negative predictive value (NPV) was 94% and The test achieved an overall accuracy of 98%.

The clinical diagnosis of COVID-19 could be supported by laboratory inflammatory markers, standard lab profile testing and CT which can serve as the basis for implementing infection control measures. The markers in this study were chosen based on prior research findings that have demonstrated a correlation between COVID-19 disease and various abnormalities, including reduced platelet count, lymphopenia, elevated CRP, and decreased ESR (Rodriguez *et al.,* 2020; Yang *et al.,* 2020). Additionally, elevated D-dimer, low hemoglobin, and high ferritin levels have been identified as frequently reported abnormalities in individuals with COVID-19 (Lippi & Plebani, 2020). According to (Grobler *et al.,* 2020), D-dimer tends to remain within normal ranges or exhibit a slight increase during the initial stages of COVID-19, like in our study.(Demelo-Rodríguez *et al.,* 2020) Reported that D-dimer sensitivity was 95.7 percent, 29.3 percent of specificity, and an AUC of 0.729, while our study yielded a sensitivity of 93 percent, a specificity of 92 percent, and an AUC of 0.96. CRP is an accurate indicator of disease and inflammation. Rodriguez-Morales *et al.* (2020) found that C-reactive protein (CRP) levels tend to be low in general but exhibit an elevation during acute inflammatory reactions. Moreover, its levels increase by themselves along with viral or bacterial infections. CRP is closely linked to condition severity and inflammation degree(Malik *et al.,* 2021). Our study findings agreed with (R. Liu *et al.,* 2020), that CRP raised among COVID-19 patients in comparison to the healthy control group (Jabber *et al.,* 2022)stated that the sensitivity of CRP in COVID-19 patients was 85 percent, with a specificity of 61 percent while our study had a sensitivity of 87 percent and a specificity of 80 percent.

Ferritin recently received attention as a biomarker for inflammation in COVID-19. It is regarded as a direct immune system mediator, and several data points indicated that there may be a physio-pathogenic relationship between COVID-19 and "Hyperferritinemic Syndromes. According to (Cheng *et al.,* 2020)ferritin increases with viral infection and shows active viral replication. Also, its levels are significantly greater in severe COVID-19 patients, non-survivors, and patients with long-lasting disorders. According to(Velavan & Meyer, 2020) Ferritin is typically normal, ranging from 30 to 400 μg/L in mild COVID-19 illness; moreover, with severe illness, serum ferritin levels are elevated >400 μg/L(Gómez-Pastora *et al.,* 2020). Serum ferritin levels in our study were normal, but in COVID-19 patients elevated. (Mohamed *et al.,* 2021)reported that ferritin showed a sensitivity of 54.1 percent and 69.1 percent specificity while our study had a sensitivity of 61% and 64% specificity for ferritin. The overall model that could best diagnose COVID-19 was when spike antigen was combined with D-dimer, the combination between them cleared absolute AUC, specificity and sensitivity.

Conclusion:

Finally, our finding supports that spike antigen detection can be used as a suitable screening test for identifying early

COVID-19 infection with satisfactory levels of sensitivity and specificity and could be useful in many clinical practice and research settings. The study's results are limited to a small number of COVID-19 patients and need to be verified in a larger population to ensure representativeness and avoid potential impact on test results. Verification in larger and varied cohorts could make the test more useful in clinical practice and research contexts, since the test's practicality may make its usage preferable in some settings.

Declarations:

Ethical Approval: The study obtained ethical approval from the Ethical Committee of the General Organization for Teaching Hospitals and Institutions (GOTHI) with the assigned approval number HS000115. Written consent was obtained from physicians in a sequential way in order to recruit patients and controls.

Conflict of interest: The authors declare no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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