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ORIGINAL STUDY

Evaluation of the Diagnostic Performance of Nucleocapsid Antigen for Severe Acute Respiratory Syndrome Coronavirus 2 Infection

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) was identified as the aetiological agent of coronavirus disease 2019 (COVID-19). The SARS-COV-2 genome encodes four structural proteins including spike (S) protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein. N protein is one of the predominantly expressed structural proteins and has been confirmed as an ideal target for early diagnostic detection in SARS-COV-2 infection. In the current study, the diagnostic performance of N antigen in SARS-COV 2 infected individuals is evaluated in Egypt.

Patients and methods: **All human individuals' samples ($n = 106$) were collected from El Sahel Teaching Hospital, Cairo, Egypt, after informed consent of the patients. Clinical samples ($n = 86$) (nasopharyngeal and blood specimens) were collected from every patient after confirmed infection (range from 0 to 55 days from the beginning of symptoms) using reverse transcription polymerase chain reaction. In addition, 25 healthy volunteers with no signs of clinical impairment and normal chest radiographs were included as controls.**

Results: **Significant differences in liver enzymes, alanine aminotransferase ($P < 0.0001$), aspartate aminotransferase ($P < 0.0001$), also, creatinine ($P < 0.0001$), red blood cells ($P < 0.001$), hemoglobin ($P < 0.0001$), lactate dehydrogenase (LDH) ($P < 0.0001$), white blood cells ($P = 0.029$), and ferritin ($P = 0.01$), neutrophils ($P < 0.0001$), and lymphocytes, hemoglobin A1c (HbA1c), D-dimer, C reactive protein, and LDH. LDH was the most effective biomarker in distinguishing COVID from healthy individuals [area under the curve (AUC) = 0.98, sensitivity = 97%, specificity = 95%]. Followed by lymphocytes (AUC = 0.95), D-Dimer (AUC = 0.89), and C reactive protein (AUC = 0.85), then N antigen (AUC = 0.75) with sensitivity = 50% and specificity = 100%, finally ferritin (AUC = 0.61).**

Conclusion and summary: **SARS-COV-2 N antigen showed comparable diagnostic performance to the severity of COVID-19.**

Keywords: Coronavirus disease 2019, Diagnostic performance, Severe acute respiratory syndrome coronavirus 2 nucleocapsid antigen, Severe acute respiratory syndrome coronavirus 2

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1. Introduction

The severe acute respiratory syndrome coronavirus type 2 (SARS-COV-2) has spread to almost all parts of the world, disrupting the functioning of society. SARS-COV-2 has been identified as the causative agent of the coronavirus disease 2019 (COVID-19) caused by the coronavirus (Kevadiya et al., 2021). Worldwide, the SARS-COV-2 poses an enormous threat to human health and lifestyle, causing more than 159 million cases of infection and more than 3.3 million deaths. Most viruses that cause respiratory and gastrointestinal diseases are coronaviruses (COV) (Pal & Banerjee, 2020). Through the collaborative efforts of scientists from around the world, various diagnostic techniques have been developed to aid in the clinical diagnosis of SARS-COV-2 (Chan et al., 2020; Corman et al., 2020; Konrad et al., 2020; Reusken et al., 2020).

The current gold standard for determining SARS-COV-2 infection is nucleic acid analysis from sputum or throat swabs. A surrogate marker is the detection of specific antibodies in the serum (To et al., 2020). Nucleic acid testing is significantly affected by sampling, shipping, and other procedural steps. Clinical diagnosis is greatly complicated by the fact that many results are inconsistently positive or negative and the overall positivity rate is not particularly significant (Abduljalil, 2020; Qu et al., 2020; Zainol Rashid et al., 2020). There is a great need to find another marker that can be detected earlier and more easily, as early detection of infection is difficult, making it difficult to stop the source of the disease, making it difficult to stop the spread of SARS-COV infections.

The nucleocapsid (N) protein was identified in the serum of SARS-COV patients on the first day of infection by ELISA using monoclonal antibodies directed against it (Liu et al., 2020). Furthermore, comparing the ability to identify SARS-COV-specific IgG, SARS-COV-2 RNA, and Protein N in the early stages of infection showed that N Protein has a significantly higher detection efficiency than the other two antibody generations monoclonal Antibodies against the N protein and the production of recombinant produced high purity N protein to detect N-specific antibodies in the host are the two main methods that researchers are focusing on (Bai et al., 2021). N protein is an essential target for the development of diagnostics and vaccines because it can trigger humoral and cellular immune responses following infection. Furthermore, one significant COVID-19 diagnostic marker is the N protein. Electrochemical immunosensors were utilized by Fabiani et al. (2021) to identify SARS-CoV-2 S and N proteins in saliva, even at concentrations as low as 19 and 8 ng/ml, respectively. A double digital enzyme-linked immunosorbent test (dELISA), based on a single-molecule array and designed to mimic the detection of the spike protein (S-RBD) and N protein, was created by Cai et al. (2021) and is incredibly sensitive, quick, and accurate. It exhibits super sensitivity and a high signal-to-noise ratio, both of which serve to increase COVID-19 diagnosis accuracy. This N protein serum level-based detection technique can reliably identify COVID-19 patients who test positive for the virus from healthy, uninfected people (Wu et al., 2023).

In light of the above, this research evaluated the positivity rate of N protein serum for the early detection of SARS-COV-2 infection in patients who had SARS-COV-2 infections.

2. Material and methods

2.1. Patients

All case samples ($n = 88$) were obtained from Al Sahel Teaching Hospital, Cairo, Egypt, after receiving the patient's written consent. Reverse transcription polymerase chain reaction RT-PCR was utilized to verify clinical samples from patients (range: 0–55 days after onset of symptoms). In addition, 20 healthy volunteers with normal chest radiographs and no clinical impairment will be used as controls. This study was approved from the General Authority for Hospitals and Educational Institutes with code HS000115.

2.2. Biochemical tests

All patients had their blood drawn following a 12 h fast. Using an automated biochemistry analyzer, routine laboratory tests such as the lipid profile, kidney, liver, international normalized ratio (INR), ferritin, fasting blood glucose (FBG), postprandial blood glucose (PPBG), hemoglobin A1c (HbA1c), D-dimers, and C reactive protein (CRP) were performed (Cobas C 111, automated biochemistry analyzer, Japan). Also, erythrocyte sedimentation rate (ESR) was recorded from laboratory data. An automatic hematology analyzer was used to measure the whole blood count (BC-2800, Mindray instruments, China).

2.3. Severity score

Two respiratory and critical care doctors who were consistently anonymized from the clinical data

evaluated and assessed the computed tomography (CT) scans independently. These CT scans were done by Toshiba equipment. Based on the previously mentioned parameters, the CT severity score was assessed. The proportion of the area affected was determined for each of the five lung lobes. It could have a lobe score of 0 (none), 1 (minimal, 1−25%), 2 mild (26−50%), 3 moderates (51−75%), and 4 severe (76−100%). The five lobe ratings were added up to create a CT severity score. The overall score is between 0 and 20 (Chen et al., 2020).

2.4. Assessment of human SARS-COV-2 nucleocapsid protein levels

Human SARS-COV-2 N protein levels were measured by ELISA by Lifespan Biosciences in China. A sandwich ELISA method was used to set up these tests. The micro-ELISA plate included in this kit has been pre-coated with an antibody specific to the SARS-COV-2 N protein. 100 µl samples (or standards) were added to the ELISA plate and the plate was incubated at 37 °C for 1 h then 100 µl of detection antibodies specific for the biotinylated SARS-COV-2 N protein was added to the wells of a micro-ELISA plate. The COV-2 N protein would bind to the specific antibody, so each well of the microplate would receive a single application of 100 µl horseradish peroxidase (HRP), which could then react. During washing free parts are removed. A substrate solution is poured into each well. Only the wells containing SARS-COV-2 N protein, biotinylated detection antibody, and avidin-HRP conjugate are stained blue. The enzyme-substrate process can be stopped by adding a stop solution, which causes yellowing. The optical density (OD) was calculated by spectrophotometry at 450 nm. The amount of SARS-COV-2 N protein was directly proportional to the OD value. The standard curve was then used to calculate the concentration of SARS-COV-2 N protein in the samples.

2.5. Statistical analysis

The statistical analysis was performed using IBM SPSS 26 (IBM Corp., 2019), while data collection and cleaning were performed using Excel 365. IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp.) was the program utilized. Descriptive statistics were used to analyze, summarize, and present the data. For evaluating hypotheses, we used Mann−Whitney tests and χ^2 tests with a significance threshold of 5%. Stepwise multivariate discriminant analysis and Receiver operating characteristic (ROC) curves were then used to assess the COVID-19 diagnosis and severity to ascertain the independent discriminative value of the variables. The score was derived from the combination of the independent criteria. The greatest discrimination score was obtained by merging the most discriminatory independent components into a logistic regression model. The best cutoff values for COVID-19 and severity diagnosis were found using the ROC curves. Common indicators of the variables were calculated, as well as performance scores.

3. Results

3.1. Levels of routine markers in different studied groups

No significant differences were found between the study groups in terms of age, hypochromic microcytic anemia (MCHC), platelet, and monocyte count, as shown in Table 1. However, there were significant differences in alanine aminotransferase (ALT) ($P < 0.0001$), aspartate aminotransferase (AST) ($P < 0.0001$) that measured as a routine liver profile test, creatinine ($P < 0.0001$), red blood cell (RBC) activity ($P < 0.001$), hemoglobin (Hb) ($P < 0.0001$), lactate dehydrogenase (LDH) ($P < 0.0001$), White blood cell (WBC) ($P = 0.029$), and ferritin ($P < 0.01$), neutrophils ($P < 0.0001$), and lymphocytes, HbA1C, D-dimers, CRP and LDH show significant differences as shown in Fig. 1.

3.2. Level of nucleocapsid COVID antigen in relation with severity score

The level of N COVID antigen was increased with the increase in severity of the disease (Fig. 2).

3.3. Correlation

N COVID antigen was negatively correlated with both lymphocytes and WBCs ($r = -0.52$, $P < 0.0001$; $r = -0.66$, $P < 0.0001$, respectively), while it showed a significant positive correlation with D-Dimer, LDH, HbA1C, and severity score ($r = 0.56$, $P < 0.0001$; $r = 0.6$, $P < 0.0001$; $r = 0.34$, $P < 0.0001$; $r = 0.73$, $P < 0.0001$, respectively).

3.4. Diagnostic performance

The diagnostic accuracy of potential markers to detect COVID was assessed using ROC curves. LDH was the most effective biomarker in distinguishing COVID from heathy individuals

Table 1. Demographic and laboratory features of the studied groups.

Variables	Healthy (n = 20)	COVID (n = 88)	P value
Male count (%)	8 (40)	53 (60.5)	
Female count (%)	12 (60)	35 (39.5)	
Age (years)	42.9 ± 1.4	48.0 ± 1.4	= 0.1
BMI (kg/m^2)	33.5 ± 1.2	28.1 ± 0.5	<0.0001
Hb (g/dl)	13.0 ± 0.2	11.2 ± 0.2	<0.0001
RBC (10^6/μl)	4.9 ± 0.1	3.9 ± 0.1	<0.0001
HCT (%)	37.2 ± 0.8	34.3 ± 0.4	<0.003
MCV (fl)	77.0 ± 1.0	67.7 ± 0.3	<0.0001
MCH (pg)	28.2 ± 0.8	30.6 ± 0.4	<0.004
MCHC (g/dl)	31.5 ± 0.5	30.9 ± 0.3	= 0.37
WBC (10^3/μl)	8.5 ± 0.5	3.5 ± 0.1	<0.0001
Neutrophils (%)	58.1 ± 0.9	68.9 ± 0.4	<0.0001
Lymphocytes (%)	33.9 ± 1.1	23.9 ± 0.5	<0.0001
Monocytes (%)	6.2 ± 0.2	7.3 ± 0.3	= 0.08
Platelets (10^3/μl)	228.6 ± 8.3	295.8 ± 23.0	= 0.16
ESR 1 h (mm/hr)	6.7 ± 0.5	16.8 ± 1.4	<0.0001
ESR 2 h (mm/hr)	14.3 ± 1.0	39.0 ± 3.1	<0.0001
INR	0.8 ± 0.0	1.2 ± 0.0	<0.0001
D-dimer (ng/ml)	0.2 ± 0.0	1.2 ± 0.1	<0.0001
CRP (mg/l)	5.7 ± 0.5	17.2 ± 1.8	= 0.002
LDH (U/L)	136.4 ± 4.5	189.7 ± 2.6	<0.0001
Ferritin (ng/ml)	122.9 ± 1.7	241.0 ± 21.6	= 0.01
HbA1C (%)	4.7 ± 0.1	6.1 ± 0.1	<0.0001
FBG (mg/dl)	100.6 ± 2.0	102.1 ± 1.3	= 0.6
PPBG (mg/dl)	120.6 ± 2.7	122.29 ± 1.481	= 0.6
GOT (U/L)	20.9 ± 1.2	32.3 ± 0.9	<0.0001
GPT (U/L)	23.0 ± 2.0	30.1 ± 0.6	<0.0001
SARS-CoV-2 N-protein (ng/ml)	0.0 ± 0.0	3.0 ± 0.5	= 0.005

CRP, C reactive protein; ESR, erythrocyte sedimentation rate; FBG, fasting blood glucose; GOT, glutamate oxaloacetate transaminase; GPT, glutamic-pyruvic transaminase; HbA1c, hemoglobin A1c; HCT, hematocrit; INR, international normalized ratio; LDH, lactate dehydrogenase; PPBG, postprandial blood glucose; RBC, red blood cell; WBC, white blood cell.

(AUC = 0.98, sensitivity = 97%, specificity = 95%). Followed by lymphocytes (AUC = 0.95), D-Dimer (AUC = 0.89), and CRP (AUC = 0.85), then N antigen (AUC = 0.75) with sensitivity = 50% and specificity = 100%, finally ferritin (AUC = 0.61) (Fig. 3) (Table 2).

4. Discussion

To stop the spread of diseases, rapid detection is crucial. The creation of numerous SARS-COV-2 diagnostic assays has been aided by the availability of the entire genomic sequence of the virus. During the pandemic, RT-PCR has been utilized as a quick

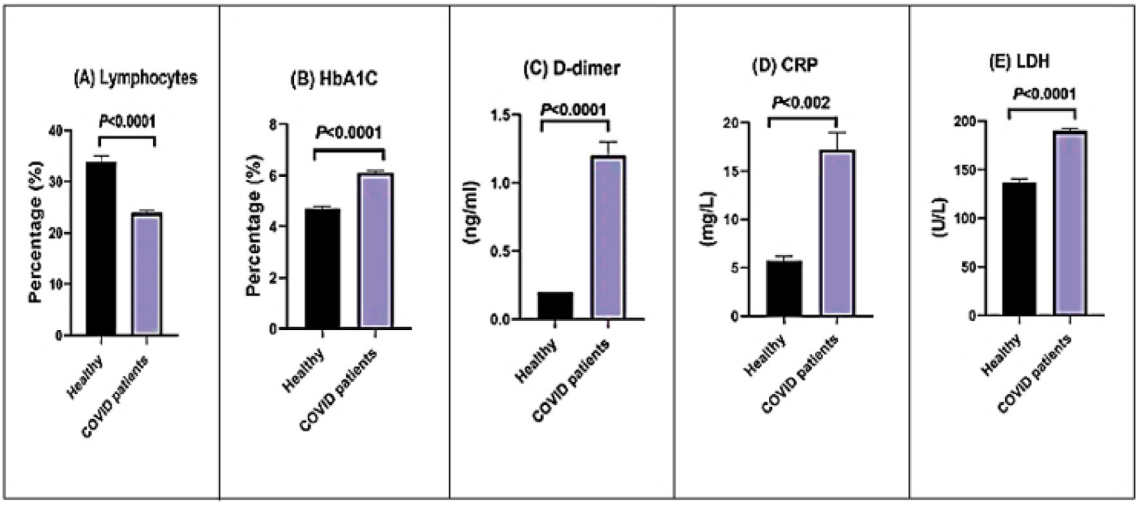

Fig. 1. Laboratory features of the studied groups. CRP, C reactive protein; COVID, coronavirus disease; LDH, lactate dehydrogenase.

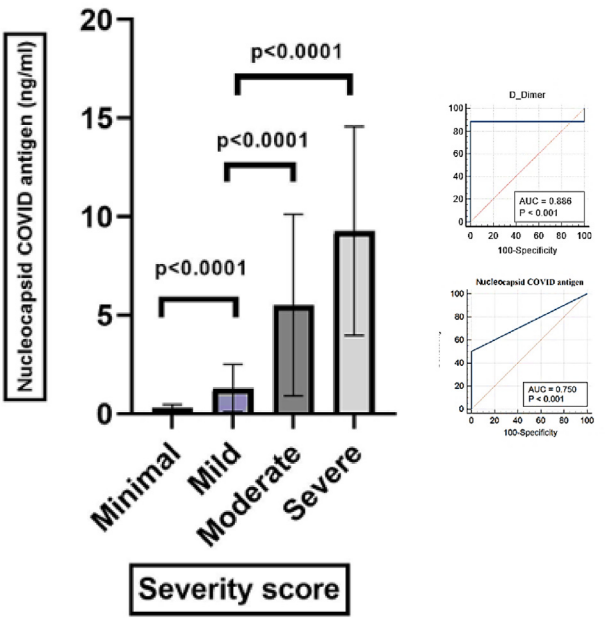

Fig. 2. Level of nucleocapsid coronavirus disease antigen in relation with severity score.

diagnostic test (Asselah et al., 2021). However, the sensitivity of viral RNA testing varies according to the time between exposure and testing, which may result in false-negative results (Woloshin et al., 2020). As a result, serological tests are receiving more attention from laboratories.

The N protein is one of the primary immunogens among the four structural proteins of the COVs (Meyer et al., 2014). Numerous serological investigations have demonstrated that N caused substantial antibody responses in hosts (Qu et al., 2020; Sun et al., 2020). In individuals with PCR-positive results, it was discovered that the N protein was detected more frequently than the S protein (Okba et al., 2020). Since the test for specific antibodies against SARS-COV-2 in the serum will not produce a positive result until around 7 days after infection or later, it is difficult to detect the illness at an early stage. In light of this, it is imperative to look into the diagnostic value of SARS-COV-2 proteins in the early stages of SARS-COV-2 infection. Several studies have evaluated the serum N protein level in SARS-COV-2 infected patients and looked at the correlation with the serum N protein antibody level using a commercial kit (Bai et al., 2021).

The sensitivity of RT-PCR for nasopharyngeal aspirate or throat swab specimens is 73.3% [95% confidence interval (Cl): 68.1–78.0%], for sputum specimens, it is 97.2% (95% Cl: 90.3–99.7%), for saliva specimens it is 62.3% (95% Cl: 54.5–69.6%), and for blood specimens, it is 7.3% (95% Cl:

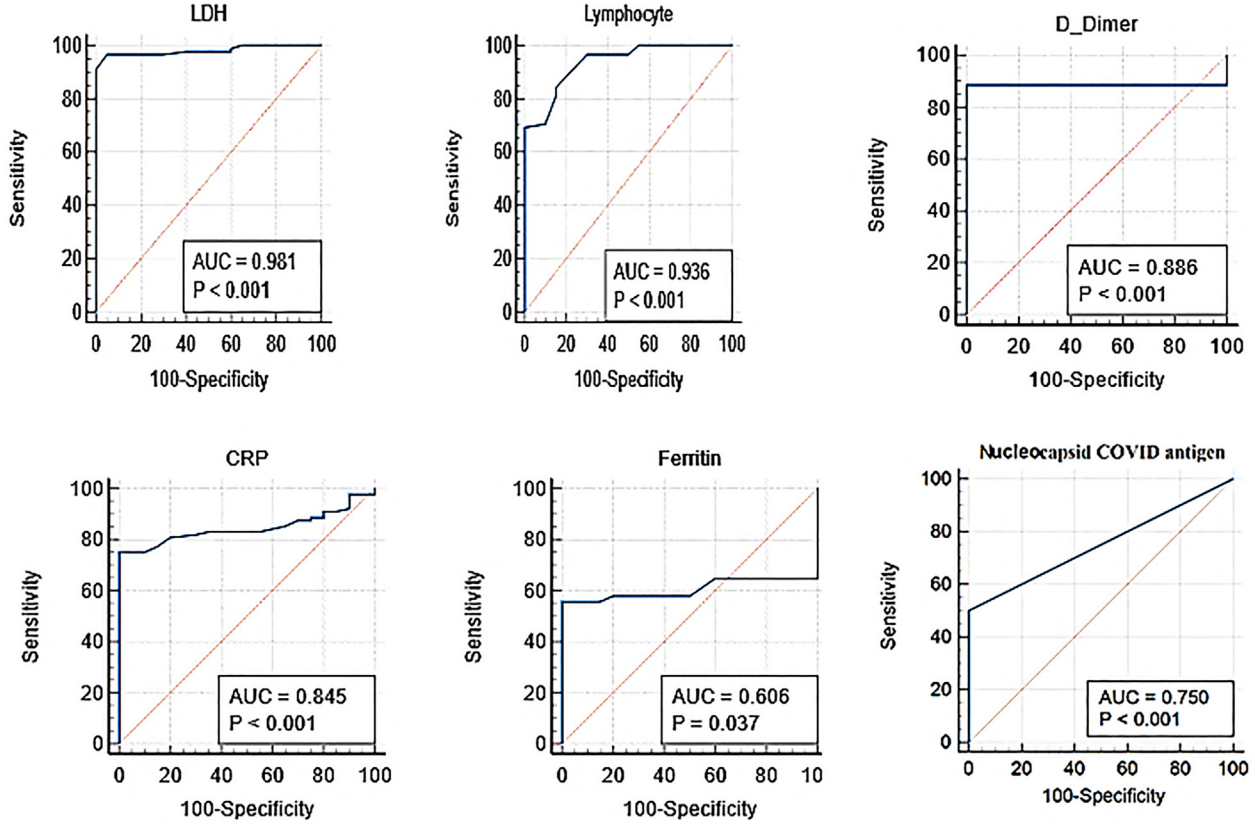

Fig. 3. Level of nucleocapsid coronavirus disease antigen in relation with severity score.

Table 2. The diagnostic performance of different blood biomarker for severe acute respiratory syndrome coronavirus type 2.

Variables	AUC	Cut-off	*P* value	Sensitivity	Specificity	+PV	−PV	Accuracy
Ferritin	0.61	>136	0.04	56	100	100	34	55
D-Dimer	0.89	>0.49	<0.001	89	100	100	67	88
CRP	0.85	>9.5	<0.001	75	100	100	48	75
Lymphocytes	0.94	≤25.6	<0.001	69	100	100	43	69
LDH	0.98	>159	<0.001	97	95	99	86	92
Nucleocapsid COVID antigen	0.75	>0	<0.001	50	100	100	31	50

COVID, coronavirus disease; CRP, C reactive protein; LDH, lactate dehydrogenase.

4.1–11.7%). One study found that the sensitivity and specificity for antigen testing when nasopharyngeal swab samples are utilized for the evaluation using a chemiluminescent enzyme immunoassay (CLEIA) automated analyzer are 76.1% (range, 44.4–100%) and 100%, respectively (Hirotsu et al., 2021). Another research that used a different threshold value showed that saliva samples could be measured with sensitivity and specificity of 77.8 and 99.6%, respectively (Asai et al., 2021). The optimal cut off value for serum samples was used by Yokoyama et al. (2021); However, the diagnostic power of serum N antigen levels determined in COVID-19 serum samples is equal to or even better than RT-PCR or antigen testing in other sample types. Before antibody development, the specificity of SARS-COV-2 serum N protein detection was 96.84% and the sensitivity was 92% (Li et al., 2020). Also, Hofmann et al. (2022) found that this test showed a specificity of 95.9% and a sensitivity of 76.4%. A range of sensitivity (62–81.4%) and specificity (93–100%) were observed in earlier investigations that involved both hospitalized and outpatient populations. Higher sensitivity, ranging from 80 to 98.3%, and specificity, ranging from 96.8 to 100%, were observed in several studies evaluating the effectiveness of nasal N antigen testing. According to our results, the exceptional specificity of the N antigen plasma tests makes them potentially perfect for use as confirmatory testing in certain contexts, such as blood banking, pediatric or supplementary laboratory testing, point-of-care finger stick testing (Mathur et al., 2022). Based on the cut-off value determined from the ROC curve of the receptor, our results evaluate the diagnostic performance of this ELISA kit for the detection of N protein with a specificity of 100% and a sensitivity of only 50%. The kit's limitations are evident in the fact that its sensitivity was poor in the early stages of the illness but increased with time. The maximum sensitivity was observed 14 days after the onset of symptoms.

Patients with moderate and severe COVID-19 illness tended to have higher blood N antigen levels than those with mild disease, according to Yokoyama et al., 2021. This is because serum N antigen levels can forecast the COVID-19 disease's maximum severity. Before the disease deteriorated, serum N antigen levels increased. These earlier results corroborate earlier studies' hypotheses of a possible relationship between the highest illness severity and blood viral RNA levels (Eberhardt et al., 2020; Fajnzylber et al., 2020). Patients with anti-SARS-COV-2-specific antibody titers, which begin to grow around a week after the onset of symptoms, may have serum N antigen levels that are distinct from those of patients with mild and severe illness at an earlier stage (Jin et al., 2020; Long et al., 2020). Intriguingly, there was no difference in blood N antigen levels between people with mild sickness and those with severe illness. The results of this study are in line with other findings, which show that the level of N antigen rises in direct proportion to the severity of the disease. This kit may be used to distinguish between various severity levels in a meaningful way.

4.1. Conclusion

The SARS-COV-2 N antigen can be used as a useful diagnostic biomarker to detect the SARS-COV-2 infection with high specificity and comparable diagnostic performance to the severity of COVID-19. This study has certain limitations because of a small sample size and inadequate clinical markers, such as oxygen saturation, breathing, days after the onset of symptoms, etc. Therefore, future research should address the various clinical markers and use a larger sample size.

Conflicts of interest

There's no conflict of interest.

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