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ORIGINAL ARTICLE

Diagnostic potential of new linear epitopes derived from the N-terminal domain of the SARS-CoV-2 Glycoprotein S

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ABSTRACT

Background and Objectives: The aim of this study was to assess the effectiveness of a new linear epitope from the N-terminal domain (NTD) of the SARS-CoV-2 S protein in the diagnosis of COVID-19.
Materials and Methods: Serum samples from patients were confirmed to have COVID-19 by means of RT-PCR. The linear epitope sequence of the NTD was amplified by RT-PCR, inserted into an expression vector, and produced in *Escherichi coli* (DE3) pLysS. Subsequently, the recombinant proteins were purified and refolded. The interaction between the purified protein and the antibodies in COVID-19 patient sera was evaluated using ELISA.
Results: Sequencing verified that the N-terminal linear epitope was successfully cloned into the PET-22b vector with a 6His-tag at the C-terminal end. The presence of a 25 kDa band on SDS-PAGE indicated the successful purification of the recombinant protein using Ni-NTA chromatography. The results of ELISA showed that the NTD linear epitope had strong sensitivity (88%) and specificity (96%) for identifying viral infection in COVID-19 patients' blood samples.
Conclusion: The findings of this study demonstrated that the NTD linear epitopes of the SARS-CoV-2 spike protein exhibit significant sensitivity and specificity for the diagnosis of COVID-19 infection using serological techniques. However, further evaluations involving larger sample sizes across diverse ethnic populations is essential.

Keywords: COVID-19; Epitopes; Enzyme-linked immunosorbent assay; Protein purification; Serologic diagnosis

INTRODUCTION

The outbreak of COVID-19 in 2019 has presented the health system with noteworthy and unprecedented challenges (1, 2). This disease is caused by a newly discovered coronavirus that shares similarities with the SARS-CoV, the virus responsible for severe acute respiratory syndrome. The International Committee

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on Taxonomy of Viruses (ICTV) Coronavirus Study Group (CSG) designated this new virus as SARS-CoV-2 (3). Data obtained from the World Health Organization (WHO) indicates that there will be more than 774 million patients and seven million deaths by January 2024 (4). Coronaviruses (CoVs) are a group of RNA viruses with positive-sense genetic material that are enclosed by an envelope (5). The fusion of viral and cellular membranes aids in the entry of the virus into the host cell leading to subsequent infection. The integration of membranes is facilitated by a viral transmembrane glycoprotein known as S protein, which is found in the viral envelope, and its corresponding receptor is on the membrane of the host cells (6-8).

Various methods have been developed for diagnosis of COVID-19 infections including molecular and serological tests. The nucleic acid test is regarded as the gold standard for virus detection, particularly effective for early diagnosis. Nevertheless, the sensitivity of nucleic acid detection is often inadequate due to the limited quantity of viral nucleic acid present in the respiratory tract. Consequently, serological screening has gained widespread adoption, offering advantages such as straightforward procedures, reduced costs, and quicker detection times. However, the performance of serological test are mainly attributed to the types of antigens. Several viral proteins have been attempted for serodiagnosis. Antigen-detecting rapid diagnostic tests have not been confirmed by the WHO for patient care. Various other reports regarding the performance of the commercially available rapid antigen tests have been published from Germany (5), Iran (9), Italy (10) Spain (11, 12), Thailand (13) and the USA (14). All these results indicated that the sensitivity was decent (70.6%-100%) and the specificity was high (96%-100%).

The surface localization of glycoprotein S not only enables its integration with the cell membrane but also makes it a direct target for the host immune responses, particularly neutralizing antibodies (15). Given its crucial roles in viral pathogenesis and stimulation of both humoral and cellular immune responses in the host (8), protein S represents a primary target for vaccine design, antiviral therapy and infection diagnosis (16).

The S protein is synthesized as a polyprotein precursor consisting of 1273 amino acids in the rough endoplasmic reticulum (RER) (17). The unprocessed precursor contains an N-terminal endoplasmic reticulum (ER) signal sequence that directs the glycoprotein S to the RER membrane and is removed by cellular signal peptidases in the ER lumen (18, 19). A membrane sequence located at the C-terminal of the S protein prevents its diffusion in the ER lumen and subsequent secretion from the infected cell (20, 21).

Within the trans-Golgi network, the S glycoprotein of SARS-CoV-2 undergoes proteolytic cleavage by cellular furin or furin-like proteases at the S1/S2 cleavage site, generating an S1 surface subunit that binds the virus to the host cell's surface receptor and the transmembrane S2 subunit (8, 22, 23), subunit consists of 672 amino acids and is divided into four domains: N-terminal domain (NTD) (sequence 14-305), C-terminal domain (CTD) and two subdomains SD1 and SD2 (24).

NTD-specific neutralizing antibodies are capable of targeting the S protein in both its prefusion and post-fusion conformations (25). Additionally, the apparent accessibility of this region in the published structures of the S-trimer of SARS-CoV-2, coupled with their high sequence conservation among coronaviruses (CoVs), suggests that these regions are promising immunogenic candidates for the design of vaccines aimed at eliciting a robust neutralizing antibody response against SARS-CoV-2 (26).

In this study, we present a newly designed epitope antigen based on the NTD domain of SARS-CoV-2, aiming to elucidate its potential as a target for diagnostic applications.

MATERIALS AND METHODS

Patients and sera. This study involved 100 participants, 50 individuals diagnosed with COVID-19 via positive RT-PCR test results (COVID-19 group) and 50 healthy subjects who provided serum samples prior to the COVID-19 pandemic (at least six months prior to the outbreak of Covod-19 pandemic). The study was thoroughly explained to all participants, and written consent was obtained from each subject prior to the sampling process.

Identification of linear epitopes of target protein and primer design. The amino acid sequence of protein S was retrieved from the NCBI database and used for the identification of linear and discontinues epitopes using IEDB database and Ellipro software.

Upon inputting the target protein sequence into the designated "submit" area of the program, the linear epitopes of the specified protein were retrieved in a tabular format based on the provided scores. The region of interest was chosen based on the epitopes with the highest score.

Then, using Gene Runner software, forward and reverse primers were designed based on the nucleotide sequence of linear epitopes of NTD, and sites for restriction enzymes were predicted on the 5' end of the primers.

SARS-CoV-2 RNA extraction. Trizol method was used to extract viral RNA from the clinical samples. Briefly, 500 µl of Trizol solution was added to the samples, and mixed for 5 min. After adding chloroform and centrifugation at 12000 rpm for 5 minutes, the aqueous phase containing RNA was separated and mixed with three volume of ethylic alcohol. Then, the RNA was precipitated by centrifugation, washed with 70% ethanol and dissolved in DEPS treated water after drying.

SARS-CoV-2 cDNA synthesis. cDNA synthesis was performed using the SinaClone first strand cDNA synthesis kit according to the manufacturer's protocols. Briefly, in the first step, a mixture consisting of 1 µg total RNA (template), 1 µl primer, 1 µl dntp mix, and up to 10 µl DEPS-treated water was incubated at 70°C for 5 minutes. In the second step, a mix was prepared by combining 4µl 5x M-MULV buffer, 1 µl M-MULV reverse transcriptase, 0.5 µl RNase inhibitor, and up to 10 µl DEPS-treated water. The products from the previous step were added to this mix, and the reaction was incubated at 50°C for 50 minutes. The reaction was then terminated by incubating at 70°C for 15 minutes.

PCR amplification and cloning of the NTD linear epitope fragment. The synthesized cDNA was amplified using specific primers in the PCR process. The 20 microliters PCR reaction contained PCR buffer with a concentration of 1x, 2 mM magnesium chloride, 0.5 µM primers, 0.2 mM dNTPs, 1 unit of Taq DNA polymerase, and 1 microliter of extracted cDNA. The temperature cycling program consisted of 5 minutes incubation at 95°C, 31 cycles of incubation at 94°C for 1 minute, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds with a final extension for 5 minutes at 72°C.

The PCR product and vector pET22b were digested by NdeI and Xho1 restriction enzymes and then ligated together by using T4 DNA ligase at 4°C overnight.

Transformation of recombinant vector into *E. coli* BL21. Bacterial transformation was performed to introduce the recombinant plasmid into *E. coli* BL21 (DE3) pLysS strain. The transformed bacteria were then plated on LB agar containing 100 µg/ml ampicillin. After incubation, colonies that contained the recombinant plasmid were identified using PCR with primers specific to the target gene. Positive colonies were selected and transferred to a master plate for further use in subsequent experiments.

Expression of the recombinant protein in *E. coli*. From the confirmed colonies obtained in the previous step, few positive colonies were transferred into a broth medium supplemented with ampicillin and incubated until reaching the appropriate turbidity (OD 590=0.5). Subsequently, this starter culture was transferred to a fresh medium containing ampicillin to enable large scale expression. Once the culture reached the desired OD of 0.5, the culture was induced by IPTG at a concentration of 0.3 mM.

Purification and refolding of the recombinant protein. After an overnight incubation, the medium was centrifuged, and the resulting pellet were dissolved in a sonication buffer containing 2 mM EDTA, 10 mM Tris and 100 mM NaH2PO4. Prior to sonication, the sample was frozen and subsequently defrosted. The sample underwent sonication for a total of 30 cycles, with each cycle comprising 30 seconds of sonication followed by a 30-second rest on ice. The lysate was subjected to centrifugation at 9000 rpm, and the soluble and insoluble fractions were analyzed using 12% SDS-PAGE. The sample was centrifuged again, and the pellet was washed in a buffer consisting of the sonication buffer and 2% Triton 100X.

For solubilization of inclusion body, the pellet was dissolved in a solubilizing buffer consisting of 1 mM EDTA, 50 mM Tris, 100 mM NaCl, 10 mM Imidazole and 8 M Urea. Initially, the pellet was mixed with 3 mL of the solubilizing buffer containing DTT. Following centrifugation, the clarified protein solution was diluted with refolding buffer composed of 25 mM Tris, 1 mM EDTA, 100 mM NaCl, 3 M Urea, 5% Glycerol, 5 mM Cysteine, 1 mM Cystine and 400 mM L-Arginine. The final solution was transferred

into a dialysis bag to remove excess materials and urea. For the purification of the recombinant protein, the Ni-NTA affinity chromatography was employed.

To purify the protein, the lysate's soluble fraction underwent filtration through the Ni-NTA column post equilibration with buffer A (300 mM NaCl, 50mM NaH2PO4, 10mM imidazole, pH 8). Subsequently, the column was rinsed with buffer B (NaH2PO4, NaCl, pH 8, with 20 mM imidazole). Following this, the protein was eluted using buffer A supplemented with imidazole (250 mM), and its purity was assessed through SDS-PAGE. The purified protein was then dialyzed against PBS (pH 7.2) and its concentration was measured at 280 nm using a Nanodrop spectrophotometer.

ELISA. One hundred microliter of purified recombinant protein with a concentration of 10 µg/ml was applied to each well for coating. Subsequently, the plate was blocked with 300 µl of blocking buffer consisting PBS-0.1% Tween 20 and incubated for one hour at RT. Following the blocking, a washing buffer composed of PBS-0.05% Twin 20 was used to wash the plate. Sera from confirmed COVID-19 patients and healthy subjects were added to the wells and incubated for one hour. After washing to remove any unbound components, the HRP conjugated anti human IgG as secondary antibody was added to wells and incubated for one hour at RT. After washing, the TMB substrate was added and the absorbance of the wells was measured at 450 nm using an ELISA reader. The cutoff value was determined to be twice the average optical density (OD) of the negative samples, resulting in a value of 0.6.

Statistical analysis. The accuracy of the assay was determined by analyzing the results of one hundred serum samples. Statistical analysis was conducted using GraphPad software to calculate the sensitivity and specificity of the ELISA results. These values were determined in accordance with the specified equations.

RESULTS

Patient information. Serum samples were obtained from individuals who underwent RT-PCR testing for COVID-19 at the Central Laboratory of East Azerbaijan Province. Among the 100 patients, 36 were male with an average age of 40 years, while 64 were female with an average age of 37 years. Among the 50 positive cases, 15 were male and 35 were female. Additionally, out of the 50 negative cases, 29 were female and 21 were male.

Predicting the linear epitopes on protein S. Ellipro, and the IEBD web server were utilized to predict the linear B-cell epitopes and discontinues epitopes (Table 1). An analysis was conducted on the results obtained from three software programs, and their findings were compared. The selection of epitope regions was based on the examination of scores and the consideration of physicochemical properties, including hydrophilicity (> 0), flexibility, surface accessibility (> 0), and antigenicity (> 0).

Construction and expression of the target gene. The sequence encoding the desired epitopes region was successfully amplified and cloned into the expression vector pET 22b using restriction digestion method (Fig. 1A) which was confirmed by sequencing. The *E. coli* BL21 strain was utilized to introduce the recombinant vector for expression. Upon induction with 0.3 mM IPTG, the cloned gene was expressed, resulting in a 24 Kd protein (Fig. 1B). Analysis of sonicated bacteria through SDS-PAGE indicated that the recombinant protein was expressed as an inclusion body.

Purification and refolding of the recombinant protein. The inclusion bodies were extracted and dissolved in 8 M urea and subsequently refolded by dilution and dialysis methods. The Ni-NTA column chromatography process led to the purification of the protein with high purity, which was observed as a singular band of the anticipated size during SDS-PAGE analysis (Fig. 1C).

ELISA. For the evaluation of the performance of purified recombinant NTD, an ELISA method was employed. The protein was coated at a concentration of 10 µg/ml and then reacted with positive and negative sera from individuals who had been confirmed by RT-PCR. The results of this analysis using GraphPad Prism are presented in Fig. 2.

Calculating sensitivity and specificity. Among the 50 positive samples, 44 showed positive results with an optical density (OD) of 0.6 or higher, while 6 samples tested negative with an OD below 0.6. Consequently:

Table 1. Discontinues epitopes (a) and linear epitopes (b).

(a)

No.	Residues	Number of residues	Score
1	A:S32, A:S33, A:V34, A:L35, A:H36, A:S37, A:T38, A:Q39	8	0.949
2	A:Y131, A:Y132, A:H133, A:K134, A:N135, A:N136, A:K137, A:S138, A:W139, A:M140	10	0.856
3	A:R233, A:S234, A:Y235, A:L236	4	0.854
4	A:Y24, A:P26, A:D27, A:K28, A:V29, A:F30, A:R31	7	0.771
5	A:R8, A:T9, A:Q10, A:L11, A:P12, A:P13, A:A14, A:Y15, A:T16, A:N17, A:S18, A:F19, A:T20, A:R21, A:L43, A:P44, A:F45, A:F46, A:S47, A:N48, A:W51, A:H53, A:I55, A:H56, A:V57, A:S58, A:G59, A:T60, A:N61, A:G62, A:T63, A:K64, A:R65, A:D67, A:R201, A:D202, A:L203, A:P204, A:Q205, A:G206, A:F207, A:S208	42	0.639
6	A:G94, A:T95, A:T96, A:L97, A:D98, A:S99, A:K100, A:T101, A:Q102, A:C118, A:E119, A:Q121, A:F122, A:S148, A:S149, A:A150, A:N151, A:N152, A:C153, A:T154, A:F155, A:E156, A:I184, A:D185, A:G186, A:Y187, A:D215, A:L216, A:P217, A:I218, A:G219, A:I220, A:N221, A:I222, A:T223	35	0.633
7	A:K84, A:M164, A:D165, A:L166, A:E167, A:G168, A:K169, A:Q170, A:G171, A:N172, A:F173, A:K174, A:P196, A:I197, A:N198, A:L199, A:V200	17	0.603
8	A:Q1, A:C2, A:V3, A:N4, A:L5, A:T6, A:T7, A:C123, A:N124, A:D125, A:E143, A:F144, A:R145	13	0.534
9	A:D40, A:L41, A:F42	3	0.51

(b)

No.	Start	End	Peptide	Length
1	5	21	LTTRTQLPPAYTNSFTR	17
2	27	28	DK	2
3	30	30	F	1
4	60	68	TNGTKRFDN	9
5	98	99	DS	2
6	133	142	HKNNKSWMES	10
7	148	149	SS	2
8	151	151	N	1
9	158	179	VSQPFLMDLEGKQGNFKNLREF	22
10	191	208	YSKHTPINLVRDLPQGFS	18

Sensitivity = (Number of true positives) / (Number of true positives + Number of false negatives)
Sensitivity = 44 / (44 + 6) × 100 = 88%

Calculating the specificity. Among the 50 negative samples, 48 showed negative results (OD < 0.6), while 2 tested positive (OD ≥ 0.6). Therefore:
Specificity = (Number of true negatives) / (Number of true negatives + Number of false positives)
Specificity = 48 / (48 + 2) × 100 = 96%

DISCUSSION

The COVID-19 pandemic continues to pose significant challenges worldwide, with a large number of people being infected every day despite the availability of valuable vaccines. Early diagnosis is crucial

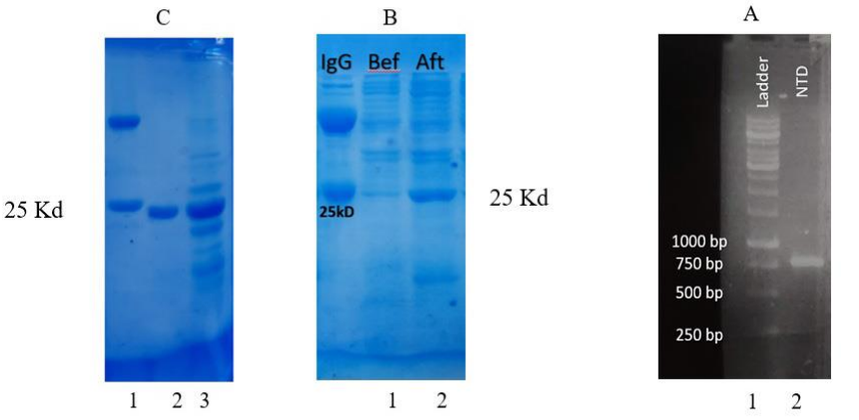

Fig. 1. A: DNA Ladder (1), PCR amplification of NTD fragment of protein S (2); B: Expression of NTD fragment of protein S in *E. coli*: Before (Bef) adding IPTG (1), After (Aft) adding IPTG (2); C: Purification of recombinant NTD fragment of protein S by Ni-NTA affinity chromatography: IgG as size marker on SDS-PAGE (1), After purification (2), Before purification (3).

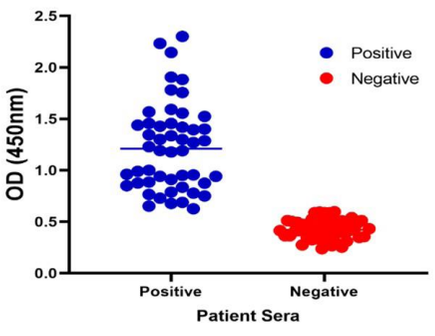

Fig. 2. The OD values of reactivity of Covid-19 positive and negative sera with protein S linear epitopes. The results above 0.6 are assumed as positive (Blue dots); the results under 0.6 are assumed as Negative (Red dots). The cut off is 0.6.

for preventing disease transmission and ensuring appropriate treatment. The current standard for diagnosing SARS-CoV-2 infection is the quantitative reverse transcription polymerase chain reaction (qRT-PCR). However, this technique is hindered by issues such as insufficient equipment availability and a notable rate of false negatives, which are often the result of inadequate sampling. To address these challenges and enhance diagnostic accuracy, researchers are increasingly turning their attention to serological methods that identify specific antibodies targeting SARS-CoV-2 (27).

The NTD region of the S glycoprotein is situated on the surface of the virus and interacts directly with the immune system. Studies have revealed that this protein plays a crucial role in stimulating the humoral immune system, resulting in the production of antibodies. Consequently, through meticulous design and the careful selection of highly immunogenic epitopes from this protein, it is feasible to improve virus detection, thereby enhancing the accuracy of diagnostic tests.

Previous research has demonstrated that patients infected with the SARS-CoV-2 virus produce specific IgG and IgM antibodies against S proteins of the virus following the onset of symptoms. These antibodies play a crucial role in the immune response against the virus and can provide valuable information about the patient's immune status and potential immunity to future infections (28-30).

In this study we identified the linear epitopes of the NTD of S protein and expressed in *E. coli*. Our results indicated high level expression of the recombinant NTD as inclusion body. Moreover, in-vitro refolding using rapid dilution with subsequent dialysis resulted in soluble protein with high stability (31, 32).

Subsequently, the reactivity of purified NTD with sera from patients infected with SARS-CoV-2 was analyzed using ELISA assay. Evaluation of 50 samples of Covid-19 patients along with 50 negative samples which were collected before Covid-19 pandemic revealed a sensitivity and specificity of 88% and 96%, respectively. Our results indicate that this linear epitope containing NTD fragment has the potential to be used in the development of ELISA-based diagnostic methods for SARS-CoV-2 and improving rapid and serological tests, which could play a crucial role in early detection, disease management and public health.

A study carried out by Roy et al. on the receptor binding domain (RBD) revealed that the specificity rates for IgG, IgA, and IgM antibodies were 99.56%, 96.55%, and 96.98%, respectively (33).

In terms of utilizing ELISA-based tests for SARS-CoV-2, a team led by Slepnev, has successfully developed a kit that demonstrates high specificity and sensitivity. The kit exhibits a specificity of 100% and a sensitivity of 84.2% (34).

CONCLUSION

The results of the present study indicated that the recombinant linear epitopes derived from NTD of SARS-CoV2 is able to detect Covid-19 specific antibodies with a good sensitivity and specificity. However, further studies are needed to confirm the sensitivity and specificity of the assay in a larger population and also investigating the potential for developing point-of-care diagnostic tests based on this epitope could be helpful.

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