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Recombinant highly antigenic truncated fusion-based protein as a diagnostic antigen for anti-SARS-CoV-2 nucleocapsid antibody ELISA

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ABSTRACT

Among the main structural protein of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), nucleocapsid phosphoprotein (NP) exhibits high immunogenicity and is the most abundant viral protein produced and shed during infection. Detection of antibodies against NP may help assess the number of individuals exposed to SARS-COV-2 or vaccinated against it. Based on these findings and other structural and antigenic evaluations, we designed a recombinant truncated fusion NP-based protein for application in an immunoassay for detecting immunoglobulins in patients who have recovered from COVID-19. In this research, we aligned the NPs from SARS-CoV and SARS-CoV-2 and selected highly antigenic parts of the SARS-CoV-2 sequences based on in-silico studies. The protein was expressed under optimum conditions in the bacterial host BL21 and purified by nickel immobilized metal affinity chromatography. Moreover, the purity level was assessed by SDS-PAGE and Western blotting whereas the folding of the protein was evaluated by circular dichroism. Ultimately, we used the purified recombinant protein in ELISA development in which 42 samples from convalescent patients were compared with 20 samples of the past 2019 patients who had attended laboratories for various clinical check-ups. The sensitivity and specificity were determined as 71% and 90%, respectively, in the optimum cut-off point measured by the receiver operating characteristic curve.

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has four main structural proteins including spike (S), nucleocapsid phosphoprotein (NP), membrane protein (MP), and envelope protein (EP). S is a major glycoprotein that contains a receptor-binding domain (RBD) responsible for recognizing and binding angiotensin-converting enzyme 2 receptor (ACE2), and is regarded as the common target for neutralizing antibodies and vaccine development. MP is the most abundant protein on the complete SARS-CoV-2 virion particle whereas EP is the smallest major structural protein of SARS-CoV-2 and it is involved in viral assembly, virion release, and pathogenesis [1]. NP is the most abundant viral phosphoprotein produced and shed during SARS-CoV-2 infection. NP exhibits high immunogenicity and can be detected in serum and other fluid samples during the first two weeks of infection (with peak viral shedding observed around ten days after infection) [1]. NP can be detected via sandwich immunoassays owing to its large size (a 46 kDa protein composed of 419 amino acids).

Based on alignments and other studies [2], the SARS-CoV NP and SARS-CoV-2 NP sequences are highly conserved with more than 90% identity (Fig. 1). The N-terminal region of SARS-CoV-2 NP consists mostly of positively charged amino acids responsible for RNA binding. A lysine-rich region is present between the residues 373 and 390 at the C terminus, which is predicted to be a nuclear localization signal. The essential sequence for the oligomerization of NP has been identified to be residues 343–402 [3]. Although the main role of NP is packaging the viral genome into the long, flexible, and helical ribonucleoprotein (RNP) complexes, it has other important roles; for instance, NP plays a role in modulating the host cell cycle by regulating cyclin-CDK activity which leads to the arrest of S phase or inhibiting the synthesis of type-1 interferon (1FN) resulting in enhanced viral pathogenesis via immune system interference [4–6].

NP is a highly positively charged protein which facilitates its binding to nucleic acids, alongside being one of the main causes of mounting immune responses [7,8]. This property makes NP a suitable protein for immunoassays and also a candidate for vaccine development [9–11].

Serology tests complement molecular techniques for diagnostic purposes (e.g., diagnosis of asymptomatic patients) and epidemiological
applications (e.g., seroprevalence studies). In the era of COVID-19 vaccination, serological tests should be widely utilized to evaluate the efficiency of any given vaccine in vaccinated people, and immunity in those infected (or merely infected) beside vaccination. Currently, the number of people who have received COVID-19 vaccination is increasing. The most commonly utilized COVID-19 vaccines are based on generating protective neutralizing antibodies against the S protein (even though vaccination with the inactive virus is also widely used). To distinguish between vaccination-related immunized people (vaccinated with S-based vaccines) from those who have recovered from SARS-CoV-2 infection, S-based ELISA should be combined with reliable NP-based immunoassays. Moreover, most SARS-CoV-2 new variants carry mutations/deletions in the viral S gene [12]. Based on these findings and other structural and antigenic studies, an NP truncated-based protein was designed for detecting the immunoglobulins in patients who have recovered from COVID-19.

2. Methods

2.1. Protein design

NPs from SARS-CoV and SARS-CoV-2 were aligned (Fig. 1A) and particular parts of the SARS-CoV-2 sequence were selected based on in-silico studies. In detail, the antigenic epitopes of NP were predicted using the Antibody Epitope Prediction tool from IEDB Analysis Resource (https://tools.immuneepitope.org/bcell/) (Fig. 2).

The truncated protein was selected in a way that it had the highest in-silico prediction of antigenicity even though it did not have the maximum in-silico surface accessibility. Residues 149–173, 204–232, and 370–394 were selected and fused in duplicate form using an alpha helix linker (AAEAAAKA) with a His-tag designed in the N-terminus of the whole fusion protein for purification and characterization proposes. The resultant protein’s theoretical pI and molecular weight were estimated by ProtParam (https://www.expasy.org/) whereas the secondary structure was predicted by the SCRATCH server (http://scratch.proteomics.ics.uci.edu/).

2.2. Protein cloning, expression, purification, and analysis

The pET28a(+) plasmid containing the coding sequence of the designed protein was ordered for synthesis and subsequent subcloning by the GENERAY Biotechnology Company (China). The recombinant plasmid was then transformed into competent Escherichia coli (E. coli) BL21 cells as a host for protein expression. Colony PCR using the universal T7 primers was employed to confirm the transformation step. The bacteria harboring the recombinant plasmids were cultured in 500 mL Luria Bertani (LB) broth, and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to them following which they were incubated at 37 °C for 4 h. Finally, the bacterial cells were harvested and centrifuged at 3000×g for 10 min. The resulting pellet which contained the recombinant protein was lysed using lysis buffer (100 mM NaH₂PO₄, 10 mM Tris(hydroxymethyl) amino methane, 0.2 M NaCl, 10 mM Imidazole, 8 M urea; pH = 8.2), followed by centrifugation at 10,000×g for 20 min, and then the resulting supernatant was loaded onto a nickel ion affinity column for His-tag-based protein purification. Fractions related to the purification of the recombinant protein by nickel column chromatography were analyzed by SDS-PAGE under reducing conditions. The resulting gel was stained with Coomassie brilliant blue G-250 for 3 h. Ultimately, de-staining was carried out with 20% methanol and 10% glacial acetic acid solution. The purified protein was analyzed by Western blotting using anti-polyHistidine– Peroxidase antibody (Sigma Aldrich, Merck KGaA, Darmstadt, Germany; cat. No.: A7058).

2.3. Circular dichroism (CD) spectroscopy

The purified protein CD spectrum was recorded at room temperature, under 2000–2700 psi pressure of nitrogen by Jasco J810 CD spectrometer (Jasco Inc., MD, United States). The spectra in the far-UV region (190–240 nm) were recorded by loading a protein concentration of 0.2 mg/mL onto a 0.5 mm quartz cuvette.

The molar ellipticity (θ) and related wavelength (λ) were obtained and analyzed, and then the protein’s secondary structure content was estimated using the Yang. jwr software [13].

2.4. Human serum samples

The experimental protocol was carried out according to the ethical guidelines of Tarbiat Modares University (Code of ethics: 1399.015). 42 positive samples were obtained from the clinics samples whose IgG results were positive by the commercial ELISA kit routinely used in the national vaccine efficacy program and also from those who had a PCR-positive SARS-CoV-2 infection in the past month. 20 serum samples collected in 2018 (before COVID-19 emergence) were used as negative control.

2.5. Protein-related IgG ELISA of human samples

The purified recombinant protein was used for the development of an ELISA-based immunoassay. 96-well plates were coated with 1 μg/well of the recombinant protein and incubated overnight at 4 °C in 0.05% bicarbonate buffer (pH = 9.6) as coating buffer. Then, the plates were washed twice with phosphate-buffered saline (PBS) containing 0.5%
 Tween 20 (PBST) and once with PBS. Skimmed milk (5%) in PBS was used as the blocking agent (300 μl/well) for 90 min at room temperature (RT) and then subsequent washing steps were carried out. Some negative and positive serum samples were assayed randomly by the above mentioned method to find the negative sample with the lowest absorbance rate as well as the positive sample with the highest absorbance rate. The mentioned samples were diluted with a ratio of 1:10 to 1:2500 using PBS. Next, the dilution ratio in which the highest difference between the positive control and negative control was observed was used for the analysis of the remainder of the samples.

50 μl of each sample was loaded onto each well of a 96-well ELISA plate. The plates were incubated at RT for 75 min and then they were washed again. HRP-conjugated goat anti-human antibodies (with a concentration of 1:10,000 in PBST) were used to probe the human antibodies against our protein as they were incubated for 75 min at RT. Ultimately, 50 μL of 3, 30, 5, 50-Tetramethylbenzidine (TMB) was added for color development as the plates were incubated for 10 min. The reaction was stopped using 50 μL hydrochloric acid (1 N). The absorbance was read at 450 nm and 630 nm as the baseline to compare the negative and positive samples using an ELISA reader.

2.6. Diagnostic performance of IgG ELISA

The receiver-operating characteristic (ROC) was used to define the cut-off value that distinguishes positive results from negative results by the related sensitivity and specificity. The significance of the difference between the negative control and IgG positive samples was calculated by the independent sample T-test. The IBM® SPSS® Statistics was used to perform this analysis.

3. Results

3.1. Expression, purification and analysis

The non-his-tagged proteins were removed by increasing the imidazole molarity in the washing buffer up to 100 mM and our desired protein was yielded at this point up to 200 mM of imidazole. The resulting protein was analyzed by SDS-PAGE and Western blotting which confirmed the presence of our protein (Fig. 3).

3.2. Secondary structure prediction and estimation

As shown in Table 1, the secondary structure predicted for the recombinant truncated NP was 26% α-helix, 6% β-strands, and 68% coils. On the other hand, the CD spectrum showed no β-strands; however, 29% α-helix, and 71% other secondary structures were estimated by CD spectrum. Overall, both methods of secondary structure prediction are similar indicating turns and coils.

3.3. ELISA

The negative sample with the lowest absorbance signal and the positive sample with the highest absorbance signal in the random assay showed differences at all the examined dilution ratios. However, the dilution ratio of 1:100 was selected as the proper dilution ratio for the further steps of the experiments (Fig. 4).

3.4. Diagnostic performance of IgG ELISA

The sensitivity and specificity of SARS-CoV-2 ELISA for IgG antibodies were tested with confirmed COVID-19 patients’ sera (group A) and healthy individuals’ sera (group B) by analyzing the ROC curve. The area under the curve is approximately 0.85. Moreover, the ELISA results showed that out of 42 confirmed COVID-19 patients’ sera, 30 sera were IgG-positive. The results also demonstrated that out of the 20 healthy persons’ sera, 18 sera were IgG-negative in our ELISA-based test, implying a reasonable level of specificity. There was a significant difference in the results between the negative control and IgG-positive samples (P = 0.001) (Fig. 5).
4. Discussion

Since the COVID-19 pandemic, numerous countries have suffered from the complications of SARS-CoV-2 infection. During this period, many studies were conducted on the prevention, diagnosis, and treatment of COVID-19. In the meantime, diagnosis plays an essential role in controlling this disease [14]. In this regard, diagnostic tools that are faster, cheaper, and more accurate are more desirable [15]. Since the ELISA technique has most of the mentioned features, this technique is welcomed in clinical laboratories for assessing the possible infection and recoveries after infection, and also for the assessment of the immune system’s response to inactivated vaccines such as Sinopharm, BIV1-CoViran, and FAKHRAVAC [16,17]. NP has the most antigenic properties alongside being the most shed protein into the body fluids; therefore, it has attracted the attention of researchers investigating in this field. With the introduction of various vaccines, which are mainly based on the SARS-CoV-2 S protein, it is possible to differentiate between vaccinated

| Table 1 | Prediction and estimation of the secondary structures of the recombinant NP by SCRATCH and CD spectroscopy, respectively. |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Secondary structure | α-helix (%) | β-strand (%) | Others (%) |
| SCRATCH prediction | 26 | 6 | 68 |
| CD Estimation | 29 | 0 | 71 |

Fig. 3. A: SDS-PAGE of the fractions related to the purification of the recombinant protein by nickel column chromatography. Left lane: protein marker; lanes 1, 2, 3, and 4: the elution of washing by 200, 150, 100, and 60 mM imidazole, respectively; lane 5: the elution of the lysed bacteria after passing through the column; lane 6: the lysed bacteria before loading onto the column. B: Ponceau S staining of the transferred proteins to the nitrocellulose membrane. C: The Western blotting of the his-tagged recombinant protein.

Fig. 4. The descending titer of serum in a negative and positive sample in the ratios of 1:10 to 1:2500 with the highest difference being observed at 1:100 ratio.

Fig. 5. A: OD_{450 nm} relating to the SARS-CoV-2 IgG-negative and IgG-positive serum sample results used for ROC curve analysis. The dashed line shows the cut-off threshold determined by ROC curve analysis. B: ROC curve.
individuals and those who have recovered from COVID-19 by simply measuring antibodies against NP. Based on these characteristics, an antibody-based assay was developed using a minimal amount of NP which could give the highest accuracy and reproducibility.

In this study, a fusion truncated NP-based protein, which showed high antigenicity following in-silico studies, was cloned into an expression vector (Fig. 2). The expression vector was transformed into the bacterial host E. coli BL21 and then protein expression was induced. Next, the expressed protein was purified using nickel ion affinity column. The secondary structure estimated by the CD spectrum analysis showed high similarity with that of in-silico prediction. Finally, the applicability of this protein was assessed in an antibody-based ELISA detection setting. The difference in optical densities (OD) between the positive and negative control samples was apparent. Moreover, the sensitivity and specificity of this developed ELISA-based test were 71% and 90%, respectively. These are comparable with the same parameters reported by other researchers that used the same platform [18,19].

Antigen detection tests commonly rely on antibodies against NP to detect exposure to SARS-CoV-2. However, antibodies against NP and S are used alternatively and in a combinatorial fashion in serological tests. This might result from the fact that antibodies against both N and S are present in the sera of convalescent individuals following their exposure to SARS-CoV-2. Therefore, antibody-dependent serological diagnostic tests are inclined to use both NP and S as diagnostic antigens.

Considering the sensitivity and specificity of the available tests, it is safe to say that lateral flow assays can compete with ELISA with privileges of simplicity and the ability to give fast results [20]. However, it should be noted that some variable factors, such as sampling, render lateral flow assays unable to be a standalone diagnostic method for COVID-19 [21]. The modified NP protein used in this study was smaller, and designed to support production and purification of the recombinant protein, yet still function like the total NP reported in similar platforms. Furthermore, our results are almost consistent with those reported by other investigations which used the whole NP in ELISA [20,22,23]. Differences between the modified NP in this study and the full NP might be due to poorly accessible sites and other in-silico factors such as B-cell epitopes (Fig. 2). Another limitation of this study is that antibodies generated to other Coronaviruses were not assessed; however, note, NP is highly conserved among coronaviruses (Fig. 1A). Finally, S-based antibody detection methods have more antigenic change in comparison with that of NP [24,25].

5. Conclusions

In this study, an ELISA-based diagnostic test was developed to detect SARS-CoV-2 NP-specific IgG antibodies by using a truncated fusion NP expressed in a bacterial host as an abundant and highly immunogenic target. The results showed a significant difference between the negative and positive IgG samples, and also the sensitivity and specificity of this developed diagnostic test were determined as 71% and 90%, respectively.

Author statement

Abbasali Salarifar: Conceptualization, Data curation, Formal Analysis, acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. Saeed Khallil: Methodology, Conceptualization. Mohammad Javad Rasaei: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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