

Research paper

Evaluation of Immunogenic Response of Recombinant Nucleocapsid Protein of SARS-CoV-2

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ABSTRACT

The outbreak of SARS-CoV-2 posed a great peril to humans worldwide. One critical requirement for controlling this disease is a rapid and reliable diagnostic procedure. RT-PCR is the widely used test for detecting SARS-CoV-2 but this method has several drawbacks. An automated ELISA platform has a greater testing ability as compared to PCR assays. Nucleocapsid (N) is a highly immunogenic protein, which is expressed abundantly during infection, and it has fewer mutations as compared to the other structural proteins of SARS-CoV-2. In this study, N-protein was PCR amplified, cloned, and expressed in *E. coli* BL21-CodonPlus-RIPL cells. The protein, expressed as inclusion bodies, was solubilized in 8 M tris-urea buffer (pH 6.8) and purified through Ni²⁺ affinity chromatography. Validation of the protein against 319 COVID-19 patient plasma samples by using quantitative ELISA, showed a sensitivity of 30.7% with a specificity of 98%. 3-D modeling and C-PORT analysis were performed, which showed that the epitopes were generally accessible for interaction. This protein when used in combination with other epitope-rich constructs can produce results of acceptable sensitivity level for diagnosis of COVID-19.

KEYWORDS Immunogenicity, Nucleocapsid, SARS-CoV-2, Sensitivity, ELISA

INTRODUCTION

The recent outbreak and rapid spread of new lethal coronavirus SARS-CoV-2 poses a great peril to human race worldwide. Coronaviruses belong to the family of zoonotic viruses which means these viruses are capable to transfer themselves from animals to humans. Coronaviruses belong to the family *Coronaviridae* with order *Nidovirales*, and these viruses are classified into four genera α -coronaviruses, β -coronaviruses, γ -coronaviruses and δ -coronavirus. Coronaviruses have largest genome among all RNA viruses with genome ranging from 27 kilobases to 32 kilobases [2]. SARS-COV-2 belongs to genus β -coronavirus having a + sense,

single stranded RNA genome of approximately 30 kilobases and this virus genome has 5 major open reading frames for encoding structural proteins and nonstructural replicase polyproteins [1].

SARS-COV-2 is an RNA virus having a diameter of about 60-140 nanometers, this virus is transmitted from one individual to other through respiratory droplets, coughing and sneezing. This virus then spread across many countries and caused a global threat. This virus has 90 % sequence homology with bat and coronavirus and 70% sequence homology with SARS-CoV [2]. The patients that suffer from COVID-19 develop mild to moderate symptoms of

the disease, about 5 % develop acute respiratory distress syndrome, multiple organ failure and septic shock. 15 % of the patient's progress only to pneumonia. SARS-CoV-2 is somewhat different from SARS-CoV and MERS-CoV because it has high prevalence rate, unprecedented scale of infection, asymptomatic transmission, and longer incubation period [3].

The genome of SARS-CoV-2 has two untranslated regions at terminal ends i.e., 3' UTR and 5' UTR and twelve acknowledged Open Reading Frames (Boopathi et al., 2020). Among Structural proteins produced by the virus genome include envelope protein (E), nucleocapsid protein (N), spike glycoprotein (S) and membrane protein (M) are the major ones [4]. The SARS-CoV-2 genome is composed of about 30000 nucleotides and this sequence encodes 9860 amino acids [5]. Nucleocapsid protein is a vital structural protein in SARS-CoV-2 as this protein involves in virus entry in host, binding to the viral genome and forming ribonucleoprotein core. N-protein of coronaviruses have five domains i.e., N-terminus domain, C-terminus domain, LINK (disordered central linker), dimerizing domain and RBD (RNA binding domain) [6]. N-protein protein has 419 amino acids having a size of 50 kDa. N-protein is highly immunogenic as this protein expresses abundantly during infection and has fewer mutations as compared to other structural proteins of SARS-CoV-2. Thus, detection of antibodies against N protein in SARS-CoV-2 patients is also possible at an early stage [7].

Diagnostic tests for COVID-19 are divided into two main groups: serological tests that involve detection of immunoglobulins against SARS-CoV-2 and molecular tests that involve detection of virus RNA [8]. Clinical diagnosis of COVID-19 is based mainly on nucleic acid detection through PCR, computerized tomography scan, clinical manifestation, blood culture and enzyme linked immunosorbent assay using

either antigens or antibodies IgG/IgM [9]. The current study is designed to achieve a rapid and reliable serodiagnostic method by selecting an epitope rich region for N-protein of SARS-Cov-2 [10,11].

MATERIALS AND METHODS

Bacterial Strains and Plasmid

Two strains of *E. coli* were used in this research, DH5 α for cloning and BL21-Codon plus-RIPL for expression analysis. pET28a (+) bought from Novagen, Madison, USA was expression vector. DNA purification kit was obtained from Vivantis Technologies, Malaysia. LB media contrived by Millar was used for bacterial growth. Chemical reagents like restriction enzymes (*Nhe*I and *Sac*I), *Taq* buffers, MgCl₂, dNTPs, DNA and protein markers, DNA ligase, ligase buffer and *Taq* polymerase were bought from Fermentas, a part of Thermo Fisher Scientific.

PCR Amplification

The potential epitopic rich immunogenic fragments of N-protein were retrieved from immune epitope database (IEDB). Nucleotide sequence of N-protein was retrieved from NCBI, GenBank and codon optimized sequences of selected N-fragments Nucleo-pUC57) were synthesized from Macrogen, South Korea. Gene specific primers of N-fragments was designed by OligoCalc [12], NEB Cutter [13], and Primer3 [14]. The N- fragment was amplified from gene cassette (Nucleo-pUC57) as template using NucFull F as forward primer (5'AAGGGCTAGCATGTCTGATAATG G-3') and NucFull R (5'-AGAGCTCTTAGGCCTGA GTTG-3') as a reverse primer. Suitable zero cutter restriction sites *Nhe*1(GCTAGC) and *Sac*1 (GAGCTC) were used in forward and reverse primer. The DNA of Nucleo-pUC57 gene cassette was transformed into competent cell of DH5 α through heat shock method. The transformed colonies of Nucleo-pUC57 were confirmed through

colony PCR and restriction digestion. The optimized condition for PCR was as follows; 95 °C for 5 minutes followed by 25 cycles of 95 °C for 45 seconds, 57 °C for 45 seconds 72 °C for 60 seconds and finally 72 °C for 10 minutes.

Cloning of Nucleocapsid Fragment in Expression Vector

Cell stocks of *E. coli* strain DH5 α carrying pET28a (+) (5369bp) plasmid stored at -80°C was streaked on a LB-agar plate having antibiotic kanamycin (25 μ g/ml) and incubated at 37°C for 16 hours. Plasmid was isolated through alkaline lysis method. The purified pET28a (+) vector was analyzed on 1% agarose gel and then stored at -20 °C. The Nucleo-pUC57 and pET28a (+) vector acts as an expression vector was restricted by using enzymes *Nhe*I and *Sac*I. The restricted gene and restricted plasmid were purified by using GeneJET™ Gel Extraction Kit. The purified restricted fragments of N-protein and expression vector were ligated according to the optimized protocol of Thermo scientific ligation kit. Ligation mixture was transformed in *E. coli* DH5 α cells and transformation was spread on LB agar plate having kanamycin acting as a selectable marker. Success of transformation was measured by doing colony PCR. Recombinant plasmid was extracted from PCR positive colonies by using alkaline lysis method. The positive colonies through colony PCR and restriction analysis, positive colonies were preserved and stored at -80 °C for future use.

Expression of Nucleocapsid Protein

For the expression of N-protein, positive colonies of Nucleo-pET28a BL21 (DE3) was streaked on 25 μ g/ml kanamycin having LB agar plate and incubated. A colony was picked from the streaked plate and inoculated in 20 ml LB broth media for each fragment (with 25 μ g/ml kanamycin) and incubated overnight in shaking incubator at 37 °C. Then 3% culture was inoculated from this flask into 50ml

autoclaved LB media having selectable marker (kanamycin antibiotic) and placed in shaking incubator for almost 1.5 hour at 37 °C till OD600 become 0.5 to 0.6. The protein expression of the nucleocapsid was find out by using 15 % SDS-PAGE.

Lysis of *E. coli* Cells by Ultrasonication

The induced *E. coli* cells were harvested through ultra-centrifugation and resuspended separately in lysis buffer (50 mM Tris-Cl buffer having pH 6.8) keeping a ratio 1:5 (1 ml of lysis buffer in pellet of 50 ml of induced media). The mixtures were placed on ice for 50 minutes and Cells were then lysed by sonication for 30 mins, one cycle of sonication consist of 10 seconds pulse, 30 second rest at 40% amplitude. After that, the sonicated mix was pelleted down at 8000 rpm for 10 minutes and preserved at -20°C. 1 ml of total cell protein sample was taken in separate Eppendorf tube after sonication and rest of the sample was centrifuged at 8000 rpm for 10 minutes.

Ni⁺² Affinity Chromatography

The tube having Ni⁺² charged chromatography resin was inverted for mixing the slurry. Equilibration buffer was prepared by using Tris-cl having final concentration of 20 mM, sodium chloride having a final concentration of 0.5 M, imidazole having final concentration of 5 mM and urea having final concentration of 8 M. Then add protein sample dissolved in 8 M urea and collect this flow through in separate falcon tube after that the column was washed with 10 ml of wash buffer (pH 6.8). Wash buffer was prepared by using tris-Cl having final concentration of 20 mM, sodium chloride having a final concentration of 0.5 M, imidazole having final concentration of 20 mM and urea having final concentration of 8 M. Then 3 ml of elution buffers of 50 mM,100 mM,150 mM,200 mM,250 mM,300 mM and 500 mM imidazole were passed to elute

our desired protein. Elution buffers also have 8M urea. The 15 % SDS gel was done to confirm which fraction has our desired protein. For removing urea from desired protein sample, fractions having desired protein were pooled in dialysis tube and subjected to dialysis for removing urea.

Dialysis tube was first placed in 6 M urea buffer at 4 oC under continuous stirring for 8 hours after that dialysis tube was removed and shifted to 4 M urea buffer and kept at 4 °C under continuous stirring for 8 hours, then dialysis tube was removed and shifted to 2 M urea buffer and kept at 4 °C under continuous stirring for 8 hours and at the end dialysis tube was removed and shifted to 0 M urea (distilled water) and kept at 4 °C under continuous stirring for 8 hours to remove urea completely.

Immunogenic Validation

For immunogenic validation, Nucleocapsid was confirmed against plasma samples of COVID-19 patients. The ELISA protocol was used according to the optimized protocol reported our lab. The readings were recorded on an ELISA reader (HUMAREADER plus, human GMBH) by a protocol reported earlier [15, 16].

Molecular Modeling

The 3D model of nucleocapsid protein was predicted through online Alpha Fold Protein Structure Database developed by EMBL-EBI and DeepMind (<https://alphafold.ebi.ac.uk/>) [17, 18]. The protein-protein interface residues interactions were predicted through online freely available algorithms CPORT (<https://alcazar.science.uu.nl/services/CPORT/>) [19]. The active and passive residues predicted through CPORT algorithms are highlighted in red (active) and green (passive) [20].

RESULTS

Selection of the Immunogenic Fragments

Nucleocapsid protein was targeted for Serodiagnostic due to its immunogenicity and conserved nature. It has 20 epitopes as

predicted by using the Bepipred-1.0 tool. The nucleocapsid fragments contains 419 amino acids along with twenty highly active epitopic fragments started from (40 to 54), (44 to 61), (160 to 174), (160 to 177), (160 to 181), (161 to 170), (164 to 169), (168 to 185), (176 to 191), (180 to 187), (268 to 286), (292 to 308), (294 to 301), (306 to 322), (353 to 369), (355 to 374), (361 to 375), (362 to 369), (382 to 389) and (394 to 399) respectively. The epitopic amino acids in nucleocapsid fragment was bold in below sequences. The schematic diagram showing epitopes of N-protein shown in Figure 1.

Cloning of Nucleocapsid Fragment in Expression Vector

Nucleo-pUC57 recombinant plasmid was transformed into DH5α cells of *E. coli*, colony PCR was performed to confirm the success of transformation. The restricted fragments were ligated transformed into DH5α competent cells. The positive clones were confirmed through PCR and restriction digestion. The sample were analyzed on 1% agarose gel and results are shown in Figures 2 and 3.

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1      40      44      54
MSDNGPQNQRNAPRITFGGPSDSTGSNQNNGERSGARSQRRPQGLPNNNTASWFTALT
61
QHGKEDLKFPGRGQVPINTNSSPDDQIGYYRRATRRIRGGDGKMKDLSPRWYFYLYGT
160      174
GPEAGLPYGANKDGIHWVATEGALNTPKDHIGTRNPANNAIVLQLPQGTTLPKGFYA
177      181
EGSRGGSQASSRSSSRNSSRNSTPGSSRGTSARMAGNGGDAALALLLDRLNQLE
268      286
SKMSGKGQQQGGQVTVTKKSAAEASKKPRQKRTATKAYNVTQAFRRGPEQTQGNFG
292      317
DQELIRGGTDYKHWPQIAQFAPSSAFFGMSRIGMEVTPSGTWLTYTGAIKLDDKDP
353      375      381      389      394      399
NFKDQVILNKHIDAYKTFPPTEPKKDKKKKADETQALPQRQKKQQTVTLLPAADL
419
DDFSKQLQSMSSADSTQA

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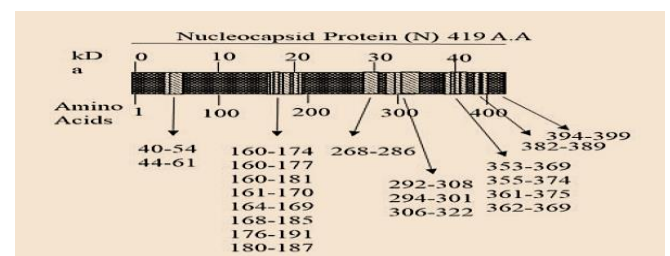


Figure 1: Schematic diagram showing epitopes of nucleocapsid protein of SARSCoV-2

Expression and Purification

Nucleocapsid protein was expressed and analyzed on SDS-PAGE. The size of the protein was 49 kDa. The expression level of N-protein was approximately 20%. The protein was insoluble, so it was solubilized in 8 M Tris-Urea buffers having pH 6.8 and purified by using Ni^{+2} affinity chromatography. The purity of the protein was >90% was shown in Figure 4.

Immunogenic Validation

For immunogenic validation, N-protein was confirmed against 319 plasma samples of COVID-19 patients. The sensitivity of plasma samples against N-protein was 30.7% and specificity was 98%. The detection potential was estimated by using the software GraphPad Prism 5 and represented in the form of an ROC (Receiver Operator Characteristic) curve as shown in Figure 5. The AUC value showing the detection potential of Nucleocapsid was 0.8364.

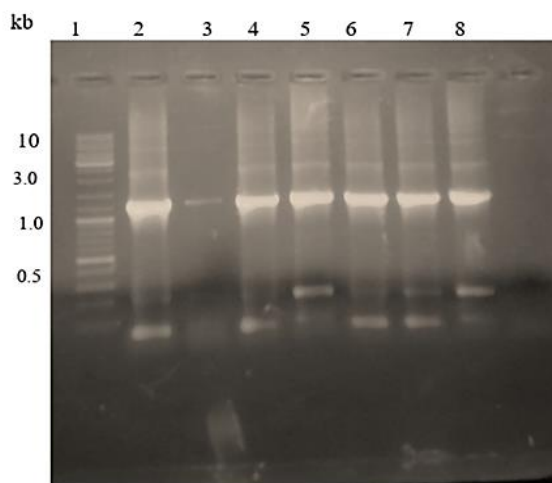


Figure 2: 1% agarose gel analysis of colony PCR of *E. coli* DH5 α cells transformed with recombinant plasmid (pET28a harboring nucleocapsid gene) Lane 1: DNA Ladder, Lane 2-8 shows positive colony PCR of selected colonies.

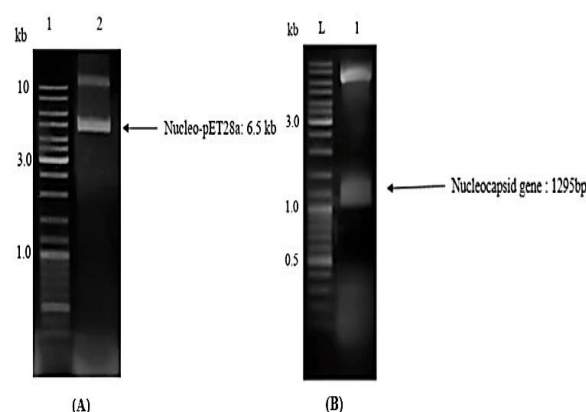


Figure 3: 1% agarose gel analysis of intact and restricted Nucleo-pET28a. (A) 1% Agarose gel analysis of Plasmid isolation of Nucleo-pET28a. Lane 1: DNA Ladder, Lane 2: Nucleo-pET28a plasmid. (B): Double digestion of recombinant plasmid (pET28a harboring Nucleocapsid gene). Lane L: DNA ladder, Lane 1: double digestion of recombinant plasmid (pET28a harboring nucleocapsid gene) with Nhe1 and Sac1.

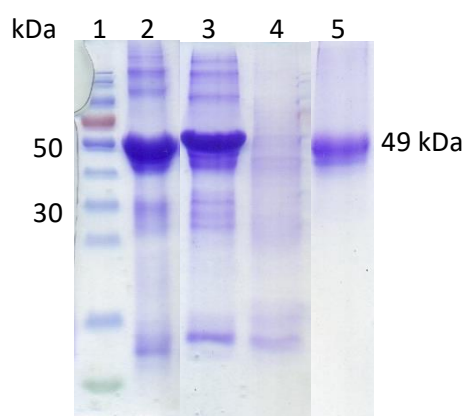


Figure 4: SDS-PAGE analysis of the total proteins and their fractions expressed in *E. coli* transformed with pET-N. Lanes 1: protein markers; 2: total proteins of the induced cells; 3: insoluble lysate fraction; 4, soluble fraction; 5: purified protein.

Molecular Modeling of Nucleocapsid Protein

3-Dimensional structure of N-protein was predicted by AlphaFold2, and C-PORT analysis was performed to predict solvent accessibility. PyMOL was used for the visualization of 3-D structures [18]. The 3D modeling and C-PORT analysis were

performed, which showed that the epitopes were generally accessible for interaction. Moreover, the CPORT analysis further confirm the interacting, supportive and non-supportive residues of the nucleocapsid fragments (Fig: -7C). All the interacting amino acids of nucleocapsid protein were highlighted in red colour of the CPORT surface models (Fig: -7D). The 3D structure of nucleocapsid and CPORT analysis were shown in Figure 6.

DISCUSSION

A global emergency caused by a novel coronavirus known as SARS-CoV-2 and its infection rate was so high. To control this pandemic, it is important to diagnose it efficiently. RT-PCR, LAMP assay, and antibody-based detection methods are employed to diagnose SARS-CoV-2A number of research institutes tried to develop Serodiagnostic tests for the detection of antibodies with good analytical specificity and quantitative assessment as well. A team of U.S. scientists designed a monoclonal antibody assay for quantitative serodiagnostic of COVID-19 [21-22]. They selected the receptor binding domain, S1 domain of spike protein, full spike protein,

recombinant spike proteins of human coronaviruses, and nucleocapsid protein and designed four monoclonal antibodies for use in ELISA. By using these monoclonal antibodies quantitative analysis of infection can also be done.

A rapid diagnostic test GICA (colloidal gold immunochromatography assay) was development because of their high specificity for IgM and IgG antibodies [23].

Nucleocapsid protein is a vital structural protein in SARS-CoV-2, and it is also highly immunogenic so its detection in SARS-CoV-2 patients is also possible. The nucleocapsid gene has a size of 1295 bp which in turn encodes a protein having size of a 49 kDa. The functions of this protein involve entering the host, binding to the viral genome, and forming a ribonucleoprotein core. It has 20 reported epitopes so; it is an excellent candidate for serodiagnosis [24].

In this study nucleocapsid protein was amplified and cloned in pET28a (+) by using specific restriction sites (*NheI* and *SacI*). The recombinant plasmid was

also developed for the detection of SARS-CoV-2. Six recombinant constructs of spike and N-protein were designed comprising rN1, rN, and rN2, and rS-RBD, rS, and rS-RBD-mFc. These optimized proteins were applied on a strip to detect antibodies. Among these three recombinant proteins, only two were selected for GICA strip

expressed in *E. coli* BL21-CodonPlus-RIPL cells. The Expression of protein was optimized, and optimized expression was seen at 0.5 mM IPTG concentration. Protein was expressed as inclusion bodies. Therefore, the protein was solubilized in 8 M Tris-Urea buffers having pH 6.8 and purified by using Ni^{+2} affinity chromatography. This protein was confirmed against 319 COVID-19 patient plasma samples by using quantitative ELISA with a sensitivity of 30.7% and specificity of 98%. 3-D modeling and C-PORT analysis were performed, which showed that the epitopes were generally accessible for interaction. This protein can be used in combination with other epitope-rich constructs of spike protein for better detection of COVID-19.

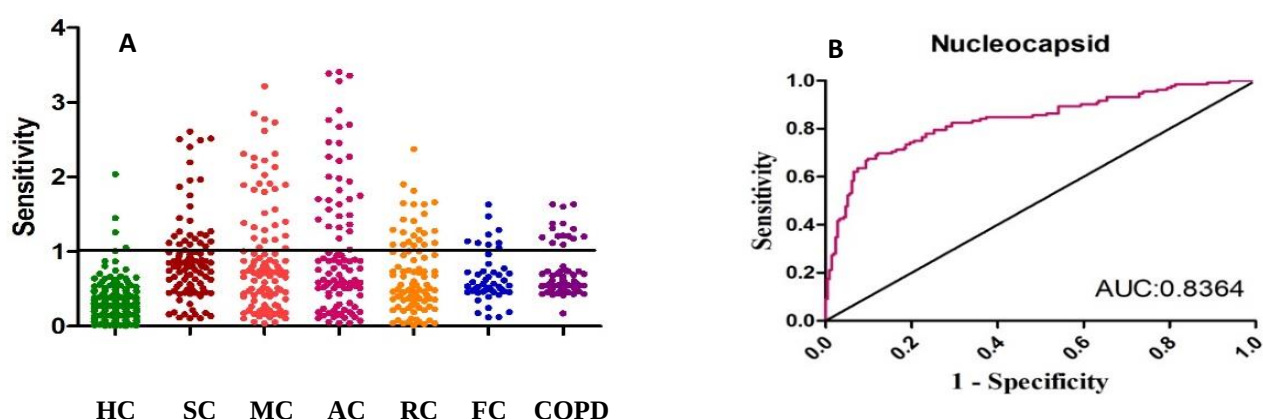


Figure 5: Scatter dot plot of normalized OD450/630 showing reactivity of plasma sample from severe covid, mild covid asymptomatic, recovered, follow up, and COPD subject groups for N protein. **B:** ROC curves showing AUC values for the plasma samples from the COVID-19 patients for the N-protein.

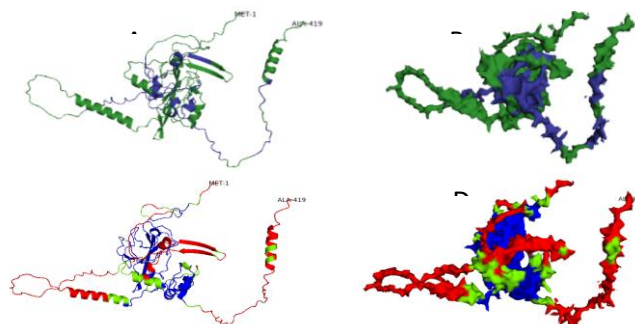


Figure 6: 3D structure of nucleocapsid Protein (A). 3D ribbon structure and surface model of nucleocapsid fragment obtained through Alpha Fold2 (A and B, respectively). The N & C termini in the ribbon structures show the residues in those positions. The 3D structure obtained through CPORT analysis (C and D) show the molecular regions in red, green, and blue as interacting, supportive and non-supportive, respectively.

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