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Expression, purification and immunogenicity analyses of receptor binding domain protein of severe acute respiratory syndrome coronavirus 2 from delta variant

Liqing Liu, Hong Chen, Ping Liu, Xiongfeng Ke, Jingjing Song, Ying Fang, Lingbao Kong, Xiu Xin*

Institute of Pathogenic Microbiology, College of Biological Science and Engineering, and Nanchang Key Laboratory of Animal Virus and Genetic Engineering, Jiangxi Agricultural University, Nanchang, China.

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the COVID-19 pandemic. The receptor binding domain (RBD), located at the spike protein of SARS-CoV-2, contains most of the neutralizing epitopes during viral infection and is an ideal antigen for vaccine development. In this study, bioinformatic analysis of the amino acid sequence data of SARS-CoV-2 RBD protein for the better understanding of molecular characteristics was performed. The *SARS-CoV-2 RBD* gene was inserted into pET-28a vector, and efficiently expressed in *E. coli* system. Then, the recombinant proteins (RBD monomer and RBD dimer protein) were purified as antigen for animal immunization. Furthermore, the results showed that the recombinant proteins (RBD monomer and RBD dimer protein) had adequate immunogenicity to stimulate specific antibodies against the corresponding protein in immunized mice. Taken together, the results of this study revealed that RBD protein had a high immunogenicity. This study might have implications for future development of SARS-CoV-2 detection.

Introduction

The coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a serious threat to the global economy and public health security and it can produce multiple organ dysfunction syndrome or death.[1] The current emergence of novel coronavirus pneumonia is the third serious outbreak of beta coronavirus in humans in the last two decades, the first two being severe acute respiratory syndrome (SARS) in 2003 and Middle-East respiratory syndrome in 2013.[2,3] The main tools for dealing with the disease pandemic are safe and effective vaccines that allow humans to achieve herd immunity.[4] Furthermore, rapid and highly specific detection of COVID-19 infection is essential for managing virus transmission networks which allows timely detection of new mutations and viral variants that finally safeguard people health.[5]

The SARS-CoV-2 is an enveloped virus with a single-stranded, positive-sense RNA genome which belongs to the beta-coronavirus genus of the family *Coronaviridae*, closely related to SARS-CoV.[6] The genome is enclosed by viral nucleocapsid proteins as a large ribonucleoprotein complex and is surrounded by an envelope membrane with lipids and viral proteins spike (S), membrane and envelope.[7]

The S protein on the surface of the virus is a trimeric class I fusion protein. It is one of the most variable and probably the most significant part of the CoV genome.[8] The trimeric S protein on viral envelope specifically binds to a cellular receptor, angiotensin-converting enzyme-2 (ACE2) for entry into susceptible cells and thus initiates virus infection.[9] In addition, the S protein consists of two subunits, S1 and S2. The S1 fragment contains the receptor binding domain (RBD) and the S2 fragment contains the fusion peptide which are responsible for receptor binding and cell fusion, respectively.[10] The SARS-CoV-2 shares a similar host cellular entry mechanism with SARS-CoV. The SARS-CoV-2 has an extremely broad host range including cats, dogs, tigers, african lions, and so on.[11]

The most potently neutralizing antibodies to SARS-CoV-2 targets the spike RBD. Mutations in the RBD have emerged among SARS-CoV-2 variants and SARS-CoV-2 delta variant became the primarily responsible for a wave of transmission in India in 2021.[12] In particular, the delta variant also displays a series of unique physiological

***Correspondence:**

Xiu Xin. PhD
Institute of Pathogenic Microbiology, College of Biological Science and Engineering, and Nanchang Key Laboratory of Animal Virus and Genetic Engineering, Jiangxi Agricultural University, Nanchang, China
E-mail: xinxiujiangxi@sina.com

characteristics and clinical features compared to other SARS-CoV-2 variants such as high viral load, strong transmissibility and resistance against existing monoclonal antibodies therapy.[13]

Here, we evaluated the general immunogenicity of RBD protein of SARS-CoV-2 from delta variant in a mouse model. The recombinant proteins receptor binding domain delta monomer as RBD (DM), and receptor binding domain delta dimer as RBD (DD) were expressed in *E. coli*-BL21 (DE3) and explored for their ability to induce immune responses. We found that the RBD (DM) and RBD (DD) proteins were able to induce an efficient antibody response in immunized mice which can provide new theoretical support for the study of SARS-CoV-2 from delta variant.

Materials and Methods

Main reagents, plasmid and experimental animals. *BamH*I and *Xho*I restriction endonuclease enzymes, T4 DNA ligase and Taq DNA polymerase, were produced by TaKaRa (Beijing, China). The 8.00 - 180 kDa protein standard Marker, DL 15 k and DL 2 k DNA Marker were purchased from Yeasen Biotechnology (Shanghai, China). Freund's complete and Freund's incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer were purchased from TransGen Biotech (Beijing, China). Anti-His tag antibody antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies and electrochemiluminescence detection kit were bought from Proteintech (Wuhan, China). Competent cells of *E. coli*-DH5α, DE3 and prokaryotic expression vector pET-28a were obtained from EMD Biosciences (Novagen, Beijing, China). The RBD dimer sequences of SARS-CoV-2 from delta variant were codon optimized, synthesized and cloned into pET-28a vectors to generate pET-28a-delta-RBD (DD) recombinant plasmid by Beijing Qingke Biological Co. Ltd. (Beijing, China). Female C57BL/6J mice aged 5 weeks were obtained from the Laboratory Animal Science Center of Nanchang University. Jiangxi Agricultural University's Animal Care and User Committee, as well as Laboratory Animal Ethics Committee gave their approval for the mouse research, which was carried out under accordance with their approvals (Reference No. JXAC20180046).

Bioinformatics analysis. Based on the amino acid sequences of RBD (DM) and RBD (DD) protein, protparam software was employed to predict the basic physical and chemical properties (https://web.expasy.org/cgi-bin/protpa RAM/protparam). Protein B-cell epitopes were predicted using the predictors of Bepipredmon the IEDB website (http://tools.immuneepitope.org/main/). Swiss model was used to predict the three-dimensional structure of protein (https://swissmodel.expasy.org/).

Plasmid construction. A DNA fragment encoding RBD domain of spike protein was amplified from pET-28a-delta-RBD (DD) by polymerase chain reaction (PCR) using specific primers [forward primer: 5′-CGCGGATCCATGAGAGTCCAAC (*BamHI*)-3′, reverse primer: 5′-CCGCTCGAGGAAATTGACACATTTG (*XhoI*)-3′.], digested with *BamHI* and *XhoI* restriction enzymes and cloned into pET-28a prokaryotic expression vector at the digestion sites to generate pET-28a-delta-RBD (DM) recombinant plasmid. Finally, the inserted gene fragments of constructed plasmids were confirmed by PCR identification, double restriction enzyme digestion and sequencing.

Expression and purification of the recombinant fusion proteins. The DE3 cells were transformed by validated recombinant plasmids pET-28a-delta-RBD (DM), and pET-28a-delta-RBD (DD). Transformed cells were grown at 37.00 ˚C on Luria-Bertani medium. Protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Then, the three key factors affecting the expression of recombinant proteins-induction time, induction temperature and induction agent concentration were optimized. The expression of the target protein was analyzed by 12.00% SDS-PAGE gel, and the optimal expression conditions of the RBD (DM) and RBD (DD) proteins were examined. After induction, expression levels were assessed in induced cell pellets, supernatant and sediment by 12.00% SDS-PAGE gel to confirm the expression form of the target protein. Pellets from IPTG-induced DE3 cells were resuspended in 8.00 M urea at 4.00 ˚C overnight. Subsequently, followed by centrifugation, the supernatant was sterilized by filtration through 0.22 μm filters. Then, the samples of the obtained proteins were applied to Ni-NTA affinity chromatography column. Purified proteins were eluted with buffers containing different concentrations of imidazole. Protein samples were stored at – 80.00 ˚C for further analysis.

Immunoblotting. The lysate of uninduced bacterial, induced bacterial and the purified recombinant protein were subjected to 12.00% SDS-PAGE electrophoresis, then, transferred to polyvinylidene fluoride membranes. After protein transfer, the membranes were blocked with 10.00% non-fat milk at 37.00 ˚C for 1 hr. After washing with Tris-buffered saline supplemented with Tween-20 (TBST), the membranes were incubated overnight at 4.00 ˚C with the anti-His monoclonal antibody (diluted 1:5,000) as the primary antibody. The next day, after washing with TBST, the membranes were incubated for 1 hr at 37.00 ˚C with the HRP-conjugated goat anti-mouse antibody (diluted 1:2,000) as the secondary antibody. Finally, following washing with TBST, the electrochemiluminescence solution was added to the membranes. After that the chemiluminescent, exposure imaging development and the data were converted for further analyses.

Animal immunization. The immunogenicity of RBD (DM) and RBD (DD) proteins were assessed in a C57BL/6J mice model. Specific pathogen-free 5-week-old female C57BL/6J mice were randomly divided into the RBD (DM), RBD (DD) and the negative control groups (n = 5). For immunization of mice, the purified RBD (DM), RBD (DD) recombinant proteins and sterilized phosphate-buffered saline solution were mixed with an equal volume of complete Freud's adjuvant, respectively. In the presence of incomplete Freund's adjuvant, animals were immunized at one-week interval with two booster vaccinations in total. The tail-tip blood was centrifuged to obtain serum and stored at – 80.00 ˚C for further analyses.

Enzyme-linked immunosorbent assay (ELISA) assays for immune serum analysis. The ELISA plates were incubated overnight at 4.00 ˚C with 250 ng per well of the purified recombinant protein. After washing with phosphate-buffered saline with Tween-20 (PBST), the ELISA plates were blocked with 5.00% non-fat milk at 37.00 ˚C for 1 hr. Following washing with PBST, sera samples from immune mice as the primary antibody with different dilutions (two-fold serial dilutions, starting from 1:100) were added into wells and incubated at 37.00 ˚C for 2 hr. After an extensive washing process, HRP-conjugated goat anti-mouse antibody (1:5,000) as the secondary antibody were added and incubated at 37.00 ˚C for 1 hr. After washing the plates with PBST buffer, 3,3',5,5'-tetramethyl-benzidine substrate was added to the ELISA plates and incubated at 37.00 ˚C for 45 min. Finally, the reaction was stopped by adding 2.00 M H_2SO_4 and the absorbance of each well was measured at 450 nm using a micro-plate reader (Bio-Rad, Hercules, USA).

Results

Bioinformatics analysis and comparison. Amino acids of RBD (DM) and RBD (DD) of SARS-CoV-2 from delta variant are shown in Table 1. Results indicated that the RBD (DM) and RBD (DD) gene encoded 223 and 446 amino acids, respectively. Using a threshold value 0.50 for Bepipred, 11 potential B cell epitopes were found in RBD (DM) protein (Fig. 1A). The amino acid residues of B cell antigen epitope are shown in Figure 1B. Using a threshold value 0.50 for Bepipred, 18 potential B cell epitopes were found in RBD (DD) protein (Figs. 1D and 1E). In addition, the three-dimensional structure of RBD (DM) and RBD (DD) proteins are shown in Figures 1C and 1F. Overall, these results suggested that the delta-RBD (DM) and delta-RBD (DD) proteins might have the potential to induce immune responses.

Plasmid construction. Recombinant plasmid map of pET-28a-delta-RBD (DD) is shown in Figure 2A. Through bacterial PCR, an obvious band with size corresponding to 1,338 bp was detected (Fig. 2B). And by double enzyme digestion, a 5,369 bp band (pET-28a fragment) and a 1,338 bp RBD (DD) fragment were identified (Fig. 3A). Sequencing showed that the plasmids above were constructed successfully. The RBD (DM) fragments was amplified from pET-28a-delta-RBD (DD) and inserted into pET-28a vector (Fig. 3B). By PCR amplification, the fragment of 669 bp was identified by 1.00% agarose gel electrophoresis which was in accordance with the predicted size (Fig. 3C). The positive clone after plasmid transformation was identified by bacterial PCR (Fig. 3D). The recombinant plasmid was further confirmed by enzyme digestion with *BamHI* and *XhoI*, which resulted in a 5,369 bp band (pET-28a fragment) and a 669 bp RBD (DM) fragment (Fig. 3E). Therefore, the cloning of RBD monomer in the pET-28a vector was confirmed.

Expression and purification of recombinant fusion proteins. The pET-28a-delta-RBD (DM) and pET-28a-delta-RBD (DD) plasmids were transformed into DE3 cells and the relative molecular weight of the RBD (DM) and RBD (DD) proteins were 26.00 kDa and 50.00 kDa, respectively. The results showed that the expression level of RBD (DM) protein was the highest at IPTG concentration of 0.60 mM (Fig. 4A), induction time of 16 hr (Fig. 4B) and a temperature of 22.00 ˚C (Fig. 4C). Also, the recombinant protein was predominantly expressed in the form of inclusion bodies, expressed in sediments (Fig. 4D). After separation, washing of the inclusion bodies, and dissolving in 8.00 M urea, the recombinant proteins RBD (DM) were collected at high purity at the concentration of 100 mmol mL^{-1} imidazole (Fig. 4E). Immunoblotting results showed that both in induced sample and purified protein were specifically detected, but not in the uninduced sample (Fig. 4F).

Table 1. Amino acid composition analysis.

Amino acids	RBD (DM)		RBD (DD)	
	Number	Percentage	Number	Percentage
Asparagine	21	9.40%	42	9.40%
Valine	20	9.00%	40	9.00%
Serine	17	7.60%	34	7.60%
Phenylalanine	16	7.20%	32	7.20%
Tyrosine	15	6.70%	30	6.70%
Glycine	15	6.70%	30	6.70%
Proline	13	5.80%	26	5.80%
Lysine	13	5.80%	26	5.80%
Leucine	13	5.80%	26	5.80%
Arginine	12	5.40%	24	5.40%
Threonine	12	5.40%	24	5.40%
Alanine	12	5.40%	24	5.40%
Cysteine	9	4.00%	18	4.00%
Asparagine	9	4.00%	18	4.00%
Isoleucine	9	4.00%	18	4.00%
Glutamine	7	3.10%	14	3.10%
Glutamic Acid	7	3.10%	14	3.10%
Tryptophan	2	0.90%	4	0.90%
Histidine	1	0.40%	2	0.40%
Pyrrolysine	0	0.00%	0	0.00%
Selenocysteine	0	0.00%	0	0.00%
Methionine	0	0.00%	0	0.00%

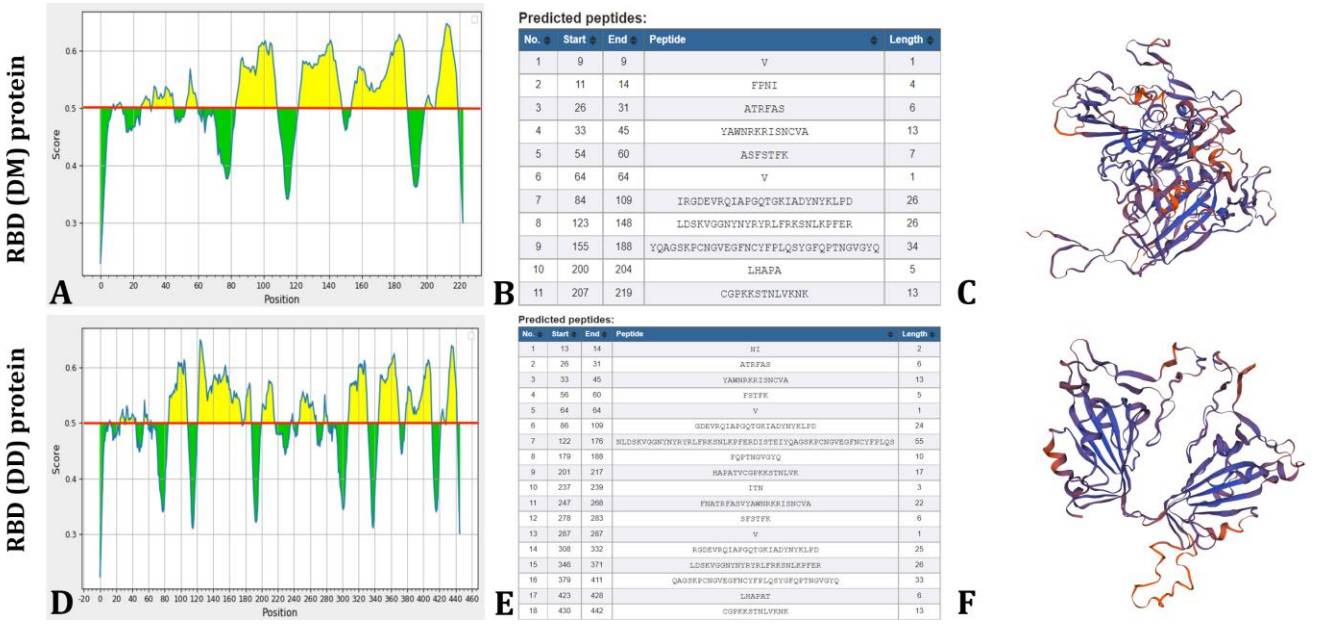

Fig. 1. A and **D)** Graphical representation of the antigenic epitopes of protein confirmation with BepiPred with the threshold value ≥ 0.50, **B** and **E)** Predictable peptides of protein, **C** and **F)** three-dimensional structures of protein predicted by Swiss-model.

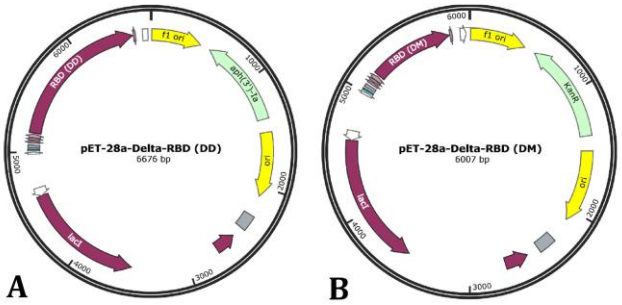

Fig. 2. Recombinant plasmid map of **A)** pET-28a-Delta-RBD (DD), and **B)** pET-28a-Delta-RBD (DM).

We optimized the temperature, time, and concentration of IPTG for RBD (DD) protein expression. Results showed that the optimal condition of RBD (DD) protein expression was 0.80 mM for IPTG (Fig. 4G), 14 hr for induction time (Fig. 4H), and 27.00 ˚C for temperature (Fig. 4I). It was mainly expressed in the form of inclusion bodies (Fig. 4J). The recombinant proteins RBD (DD) were collected at high purity at the concentration of 100 mmol mL^{-1} imidazole (Fig. 4K). Immunoblotting assays with the commercial anti-His tag antibody detected the predicted size in IPTG-induced bacterial lysate samples and purified protein, but not in un-induced sample (Fig. 4L). These results showed that the expression and purification of the SARS-CoV-2 RBD (monomer and dimer) using *E. coli* were successful.

RBD (DM) and RBD (DD) immunogenicity in mice. The sera were obtained from immunized mice in different weeks and the serum titers of antibody were further detected by ELISA. Results showed that the titer of specific antibodies in the experimental group was gradually increased (Fig. 5). Five weeks after immunization, the average titer reached 1:204,800 (Fig. 5A) indicating that

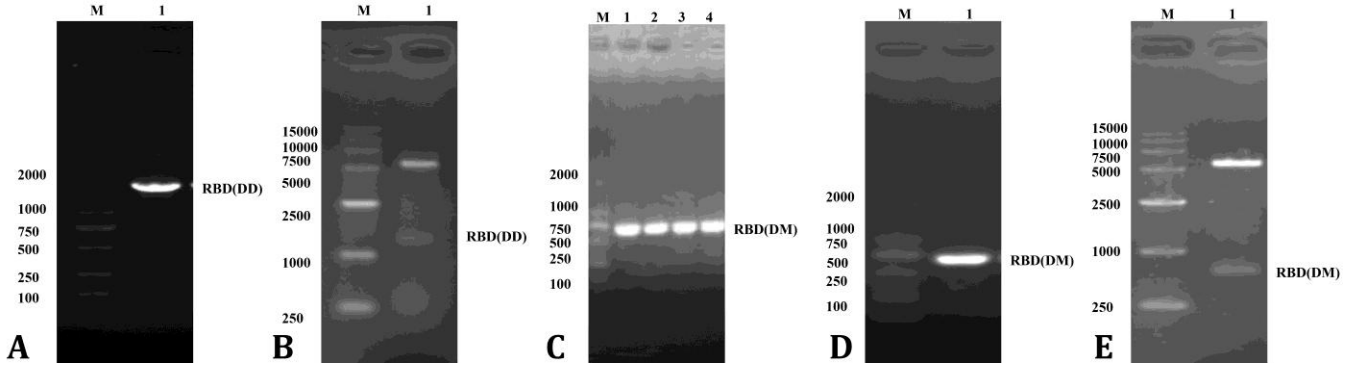

Fig. 3. Verification of recombinant plasmid. **A)** The identification of positive clone after plasmid transformation by bacterial polymerase chain reaction (PCR). **B)** Recombinant plasmid pET-28a-delta-RBD (DD) was identified by double enzyme digestion. **C)** The amplicons of RBD (DM) by PCR. Lanes 1 - 4 were different repetitions. **D)** The identification of positive clone after plasmid transformation by bacterial PCR. **E)** Recombinant plasmid pET-28a-delta-RBD (DM) was identified by double enzyme digestion.

RBD (DM) protein induced potent humoral immunity in immunized mice. After the first boost immunization with RBD (DD) protein, the serum titer of the mice was increased to 1:102,400 at the fourth week which was higher than RBD (DM) protein induced serum titer (1:3,200) at the fourth week. Five weeks after immunization, the average titer reached 1:204,800 (Fig. 5B) indicating that RBD (DD) protein also induced excellent humoral immunity in immunized mice. Overall, the monovalent and bivalent antigens based on the recombinant RBD showed excellent immunogenicity by ELISA and the RBD (DD) protein was more effective in immunization compared to the RBD (DM) protein.

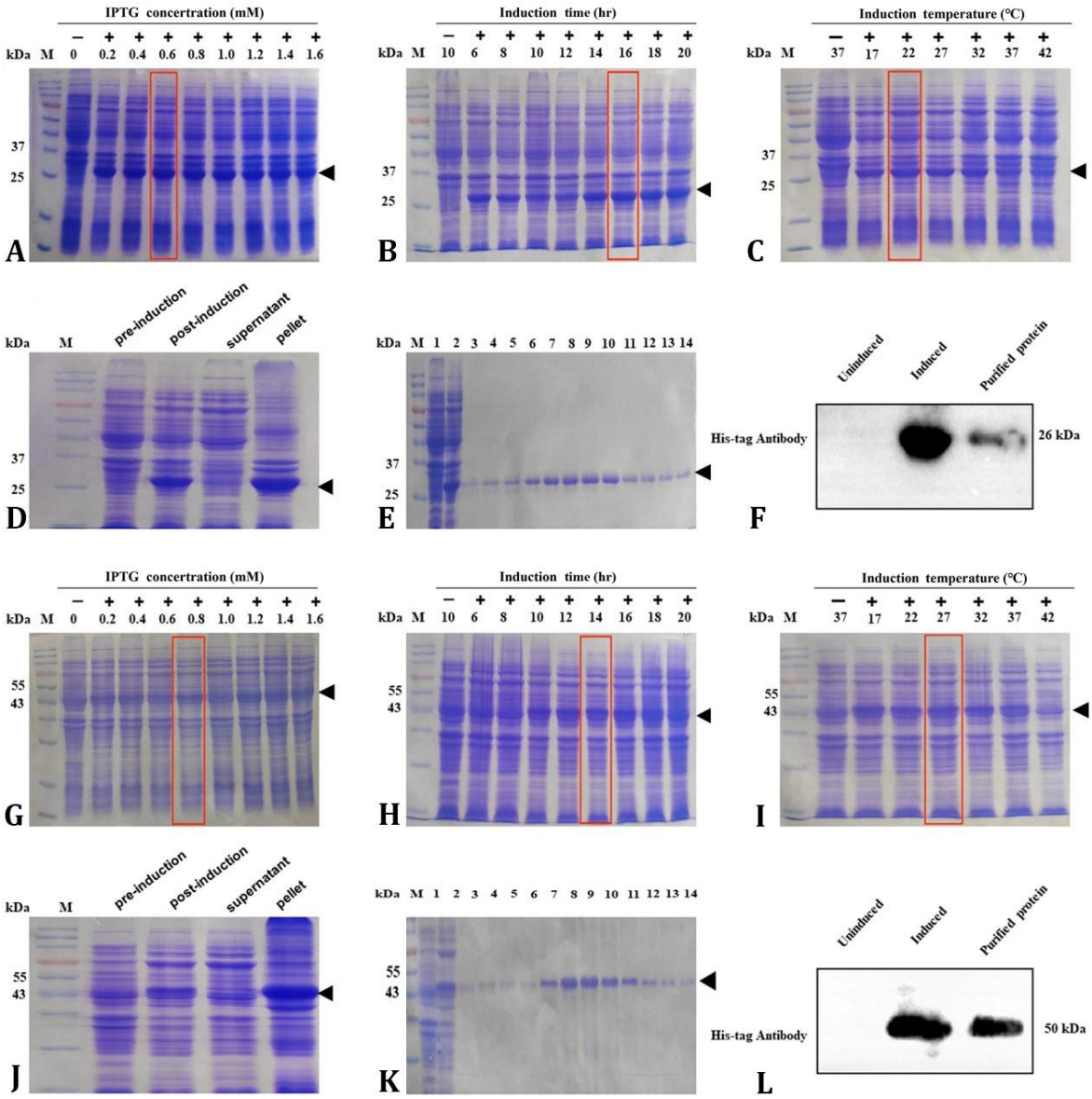

Fig. 4. Expression, purification and verification of recombinant proteins: Optimization of His-RBD (DM) expression conditions. **A)** Optimizations of isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentration, **B)** Induction time, **C)** Induction temperature, in which the bands of selected conditions were framed. **D)** His-RBD (DM) protein content as seen pre-induction, post-induction, supernatant, pellet obtained from induced cells, **E)** Purification of protein. Lane 1: Uninduced bacterial solution, lane 2: Induced bacterial solution. From lane 3 to lane 14, the protein was eluted gradually at the concentration of 100 mM imidazole. Lane 3 to lane 4: 20.00 mM, lane 5 to lane 6: 40.00 mM, lane 7 to lane 10: 100 mM, lane 11 to lane 14: 250 mM, **F)** Proteins were subjected to immunoblotting analysis using commercial anti-His tag antibody; Optimization of His-RBD (DD) protein expression conditions. **G)** Optimization of IPTG concentration, **H)** Induction time, **I)** Induction temperature, in which the bands of selected conditions are framed, **J)** His-RBD (DD) protein content as seen pre-induction, post-induction, supernatant, pellet obtained from induced cells, **K)** From lane 3 to lane 14, the protein was eluted gradually at the concentration of 100 mM imidazole. Lane 3 to lane 4: 20.00 mM, lane 5 to lane 6: 40.00 mM, lane 7 to lane 10: 100 mM, lane 11 to lane 14: 250 mM, and **L)** Proteins were subjected to immunoblotting analysis using commercial anti-His tag antibody.

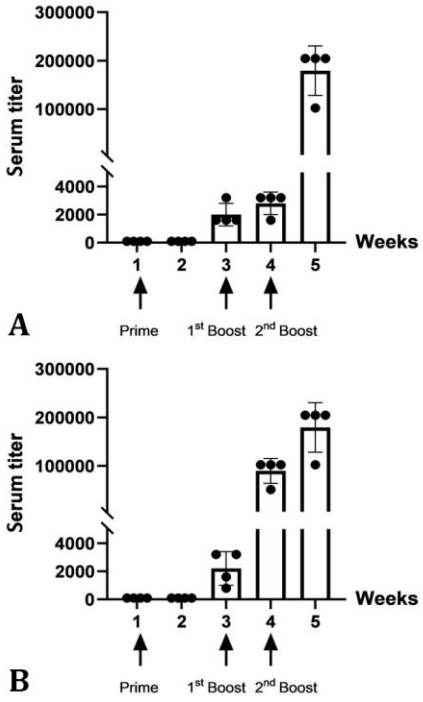

Fig. 5. Humoral responses induced by immunization of purified protein in mice. The C57BL/6J mice (5 weeks old) were immunized with **A)** purified RBD (DM) protein, and **B)** RBD (DD) protein. Antisera were collected and subjected to enzyme-linked immunosorbent assay for detecting antibody titer.

Discussion

The emergence of SARS-CoV-2 has placed a huge burden on populations around the world infecting many people and causing thousands of deaths worldwide.[14] Therefore, it is vital to find effective and safe neutralizing antibodies and vaccines against COVID-19.[15] The critical neutralizing domain is the receptor-binding domain (RBD) of the spike protein (S protein) which could lead to a highly potent neutralizing antibody response.[16] Using the RBD fragment as an antigen, the neutralizing epitopes can be better exposed. So far, mostly biochemical methods are being tested in order to prevent binding of the virus to ACE2.[17]

Researchers have made great efforts to develop more effective and safer vaccines and drugs against COVID-19 such as virus-like particle vaccines, nucleic acid vaccines, subunit and adenovirus-based vector vaccines, neutralizing antibodies, and inactivated and live-attenuated vaccines.[18] Inactivated vaccines are produced by growing SARS-CoV-2 in cell culture followed by chemical inactivation of the virus, however, their yield could be limited by the productivity of the virus in cell culture. Recombinant protein vaccines can be divided into recombinant spike-protein-based vaccines and recombinant RBD-based vaccines. Other coronavirus proteins such as the nucleo-capsid proteins are also being studied for vaccine development since they are able to generate a strong T-cell response, and their genes are frequently conserved and less prone to recombination compared to spike proteins.[19] Furthermore, these recombinant proteins can be expressed in different expression systems.

Some studies have described modified S or RBD such as S trimer and RBD dimer which were developed to generate more neutralizing antibodies than monomeric proteins.[20] The immunogenicity of RBD monomers is suboptimal, and multimeric display of RBD molecules is a potential strategy for the optimization.[21]

Various expression systems including *E. coli*, mammalian cells, insect cells, yeast and plants have been utilized to express the recombinant RBD protein.[22] In our previous studies, we successfully expressed and purified RBD monomers and dimers of prototype SARS-CoV-2 and Omicron variant in *E. coli*.[23] As the dominant viral strain in 2020, delta is well known for its strong transmission and replication ability.[24] Previous studies have shown strong immunogenicity of delta and confirmed that yeast-derived delta RBD was an effective vaccine candidate for SARS-CoV-2 and its variants.[25] In this study, we presented a prokaryotic expression system that allowed an easy, fast and cost-effective way to prepare the monomers and dimers of delta RBD protein and their antibodies. The first aim was to steadily express RBD protein of SARS-CoV-2 from delta variant in *E. coli* system. Subsequently, through optimization of expression conditions-induction time, induction temperature and induction agent concentration, RBD protein was efficiently expressed. Following the purification process, RBD protein could be collected for animal immunization. The RBD (DM) and RBD (DD) proteins both showed good immunogenicity, and the immunogenicity of RBD monomers was lower than that of RBD dimers.

In this study, a successful construction of prokaryotic expression plasmid, pET-28a-Delta-RBD (DM) and pET-28a-Delta-RBD (DD) were constructed to express recombinant protein. The present work verified the immunogenicity of purified proteins after immunizing mice suggesting that RBD (DM) and RBD (DD) proteins of SARS-CoV-2 from delta variant produced a good immune response after immunizing mice. In conclusion, our current findings shed light on the future development vaccines and ELISA diagnostic kits of SARS-CoV-2 from delta variant.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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