Research in Molecular Medicine

Assessing N-terminal Region of Pneumococcal Choline-binding Protein D as a Vaccine Candidate Targeting *Streptococcus pneumoniae*

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ABSTRACT

Background: *Streptococcus pneumoniae* is a major cause of pneumonia and meningitis, particularly in children under five years of age. The limited serotype coverage and high cost of pneumococcal conjugate vaccines highlight the need for affordable, serotype-independent vaccine candidates. Choline-binding protein D (CbpD), a surface protein involved in pneumococcal virulence, represents a promising antigenic target. This study aimed to evaluate the immunogenic potential of the N-terminal fragment of CbpD (FCbpD) from *S. pneumoniae* serotype 19F as a candidate for protein-based pneumococcal vaccines.

Materials and Methods: The *fcbpD* gene encoding amino acids 51–353 was cloned into the pET28a vector and expressed in *Escherichia coli* BL21 (DE3). Bioinformatics analyses were performed to assess protein stability, antigenicity, and epitope prediction. The recombinant protein was purified under denaturing conditions and refolded. BALB/c mice were immunized with FCbpD in combination with flagellin as an adjuvant, and specific IgG responses were measured by ELISA.

Results: Sequence analysis demonstrated high conservation (>94%) of FCbpD among major invasive pneumococcal serotypes. Bioinformatics tools confirmed its stability and antigenicity, with three conformational B-cell epitopes identified. The recombinant protein was successfully expressed (~34 kDa) and purified. Immunization with FCbpD plus flagellin induced a significant increase in specific IgG levels compared to control groups ($P<0.05$), indicating strong immunogenicity.

Conclusion: FCbpD is a conserved and immunogenic antigen capable of inducing a specific antibody response, supporting its potential as a serotype-independent candidate for pneumococcal vaccine development

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Introduction

Streptococcus pneumoniae is an encapsulated, gram-positive bacterium that causes invasive diseases such as sepsis, meningitis, and pneumonia in humans [1, 2]. *S. pneumoniae* is a major factor in the mortality of children under 5 years of age. The pneumococcal infections cause a worldwide annual death of 317300 cases in children under 5, as estimated by the World Health Organization (WHO). More than 98 serotypes of *S. pneumoniae* were identified based on the composition of their capsular polysaccharides. However, 21 pneumococcal serotypes, including 19F, 23F, 14, 6A, 6B, 5, 1, 19A, 9V, 18C, 2, 3, 4, 8, 7F, 12A, 12F, 9A, 45, 46, 15B, are common causes of invasive pneumococcal disease. The pneumococcal serotypes 1, 5, 6A, 6B, 14, 19F, and 23F are the most significant serotypes responsible for invasive pneumococcal disease in children [3, 4].

Antibiotic treatments were ineffective against pneumococcal infections due to the emergence of multidrug-resistant strains [5]. Pneumococcal conjugate vaccines (PCVs) are preventive measures against pneumococcal disease. These vaccines are composed of pneumococcal capsular polysaccharides chemically conjugated to immunogenic carrier proteins [3, 4, 1]. The manufacturing of PCVs involves complex, multi-step processes and is costly, limiting their implementation in developing countries. Moreover, the implementation of PCVs has been followed by the emergence of non-vaccine serotypes of pneumococci, prompting the development of PCVs to target a larger number of pneumococcal serotypes. Therefore, many efforts have been made towards developing serotype-independent vaccines against pneumococci based on antigenic proteins [6].

The pneumococcal choline-binding protein D (CbpD) is a cell surface protein enabling the bacterium to colonize the host and is involved in the invasion process of *S. pneumoniae* [2, 7, 8]. The full-length CbpD has been demonstrated to be lethal to *Escherichia coli*. A fragment of CbpD (FCbpD) that lacks the choline-binding domain was successfully expressed in *E. coli* [9]. The antibody-mediated immune response is necessary for protection against pneumococcal infections [10]. A FCbpD from *S. pneumoniae* Taiwan 19F-14, at its N-terminus and comprising amino acids 51-353, encompasses B-cell epitopes as revealed by bioinformatics analysis [11].

Furthermore, flagellin of *Salmonella* enteritidis provokes immune responses to co-administered antigens and is considered an effective adjuvant [12]. In this study, we assessed the potential of FCbpD from *S. pneumoniae* 82218, a serotype 19F pneumococcus, as a vaccine candidate against *S. pneumoniae*. Therefore, the gene encoding the FCbpD protein was cloned into *E. coli*. The nucleotide sequence was determined, and the conservation of the corresponding amino acid sequence in the major invasive pneumococcal serotypes was investigated. The protein was expressed intracellularly in *E. coli*, and its solubility was evaluated. A suitable purification procedure for the FCbpD protein was developed, and the resulting recombinant protein was evaluated for antibody response in laboratory mice, using recombinant flagellin from *S. Enteritidis* as the adjuvant. The schematic workflow of this study is depicted in Figure 1. The result of this study is anticipated to be useful for the development of serotype-independent proteinaceous vaccines against pneumococci.

Materials and Methods

Bacterial strain and growth conditions

S. pneumoniae 82218 was used to prepare the genomic DNA. It was cultured on tryptic soy agar containing 5% defibrinated sheep blood, and incubated at 37 ˚C and 5% CO_2 for 18 h. The colonies were then transferred to Todd-Hewitt broth and incubated at 37 ˚C and 5% CO_2 until the desired optical density at 600 nm (OD_{600}) was achieved. *E. coli* BL21 (DE3) was used for cloning and expression studies, and it was cultivated in Luria-Bertani (LB) broth at 37 ˚C.

DNA manipulation

Genomic DNA from *S. pneumoniae* was extracted using the method described by Saito and Miura [13]. Briefly, a 10 mL culture was grown until an OD_{600} of 0.5 was achieved. The cells were then pelleted and resuspended in 200 µL of TE buffer (pH 8.0) containing 25% w/v sucrose, 60 mM ethylenediaminetetraacetic acid (EDTA), 1.6% Sodium Dodecyl Sulfate (SDS), and 12.5 µg of proteinase K. The suspension was incubated at 37 ˚C for 16 h to obtain a clear lysate. Subsequently, the cellular debris was removed by centrifugation at 3500 g for 5 min. The supernatant was extracted with phenol and further purified by successive extractions with chloroform: isoamyl alcohol (24:1 v/v). DNA was then precipitated with absolute ethanol.

The primers for amplification of fcbpD were designed using the Primer3plus software [14] based on the gene sequence encoding CbpD of *S. pneumoniae* Taiwan 19F-14 (Accession ACO22665) (Table 1). The *fcbpD*

Table 1. Primers used in this study

Primer Designation	Primer Sequence
fcbpD-F	ATCGAATTCGGGGATGATTATCCTGCTTATTATAAAAATGG
fcbpD-R	GAGAGACTCGAGTTAACCGACAGTTGAACTATTATTGGATCC

gene was amplified by PCR from the genomic DNA of *S. pneumoniae* using *fcbpD*-F and *fcbpD*-R as forward and reverse primers, respectively. The PCR product was inserted into the pET28a vector at the *EcoR*I and *Xho*I sites. The obtained plasmid was designated pFCBPD, in which FCbpD was fused to a hexa-histidine tag to facilitate purification of the recombinant protein by metal-affinity chromatography. Nucleotide sequencing was performed to determine the *fcbpD* sequence and verify the construct. The nucleotide sequence of *fcbpD* was used to determine the amino acid sequence of FCbpD using the Genetyx software (Genetyx Co., Japan). The ProtParam software was used to determine the physicochemical properties of FCbpD based on its amino acid sequence [15].

The full-length CbpD amino acid sequences of invasive pneumococcal serotypes were retrieved from NCBI (Protein database) under accession numbers WP_000698581.1 (serotype 5), CFP94161.1 (serotype 6B), WP_000698574.1 (serotype 1), CIV33058.1 (serotype 23F), CGF24516.1 (serotype 6A), and COR13694.1 (serotype 14). Then, the amino acid sequences of their FCbpD fragments were compared with the FCbpD (serotype 19F) sequence obtained in our study using Clustal Omega and the SIM tool [16, 17].

Tertiary structure of FCbpD and conformational epitopes

The three-dimensional structure of the FCbpD protein was predicted by I-TASSER using its amino acid sequence [18]. The ProSA software was used to evaluate the tertiary structure [19]. YASARA was used to determine the protein's secondary structure and to visualize its tertiary structure. The Ellipro online software was used to predict conformational B-cell epitopes of FCbpD based on its tertiary structure [20].

Expression studies

E. coli cells harboring the pFCBPD vector were cultivated in the LB broth containing kanamycin (25 µg/mL) at 37 ˚C while shaking at 150 rpm for 16 h. The cells were then collected by centrifugation and transferred to fresh LB broth supplemented with kanamycin. The *E. coli* cells were further grown at 30 ˚C with agitation at 150 rpm until an OD_{600} of 0.5 was reached. The expression of FCbpD was induced by adding Isopropyl-β-D-thiogalactoside (IPTG) to the culture to a final concentration of 1 mM. The cells were incubated for an additional 4.5 h and then centrifuged. Protein expression was assessed by resolving whole-cell extracts using 10% SDS-PAGE, followed by coomassie brilliant blue R-250 (CBB) staining. Western blotting was performed using an anti-His tag antibody conjugated to HRP (Qiagen, Germany) to verify protein expression.

The expression of flagellin using *E. coli* cells harboring both pETflic and pKJE7 vectors was already described by Bakhtiarvand et al. [21]. The pETflic vector harbors the flagellin-encoding gene, and the pKJE7 vector contains the genes encoding the molecular chaperones. The cells were cultured in LB including kanamycin (25 µg/mL) and chloramphenicol (20 µg/mL) for 16 h as mentioned above. The recombinant *E. coli* cells were then pelleted by centrifugation and transferred to fresh LB containing the antibiotics. The induction of the molecular chaperones encoded by the pKJE7 vector was carried out by adding L-arabinose to a final concentration of 0.5 mg/mL. The cells were incubated at 37 ˚C with shaking at 150 rpm. IPTG (1 mM) was added to the culture at an OD_{600} of 0.5 to induce flagellin expression. Cell incubation was continued for 4 h. The cells were then collected by centrifugation and used to purify flagellin [21].

Solubility analysis of the recombinant protein

Following expression of FCbpD in *E. coli*, the cells were suspended in lysis buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, 1 mg/mL lysozyme) and incubated on ice for 30 min. The cells were then lysed by sonication, and the soluble FCbpD-containing supernatant was separated from inclusion bodies by centrifugation. The soluble fraction and the inclusion bodies were subjected to 10% SDS-PAGE, and the protein bands were visualized by CBB staining. The gel was scanned using the G3110 scanner (HP, USA), and densitometric analysis was performed with GelAnalyzer 23.1.1 to determine the ratio of target protein quantity in the soluble fraction to that in the inclusion body.

Purification of recombinant proteins

Recombinant proteins were purified using metal affinity chromatography based on the interaction of a nickel chelate column (Ni-NTA superflow column) (Qiagen, Germany) and the histidine tag. For purification of flagellin and FCbpD under native conditions, cells from a 50-mL culture were sonicated to obtain the soluble cellular fraction as described above.

The soluble fraction was applied to the Ni-NTA column, which was already equilibrated with the lysis buffer without lysozyme (binding buffer). The column was washed with the wash buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole), and the intended proteins were then eluted with the elution buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole). The proteins were dialyzed against the PBS buffer (pH 7.2) using a dialysis tube (3.5 kDa) (Sigma, USA).

For purification under denaturing conditions, the cell pellet from a 50-mL culture was resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH 8.0) at 5 ml/(g wet weight) for lysis. The suspension was stirred at room temperature until it became clear. The supernatant was collected by centrifugation (the cleared lysate) and applied to the Ni-NTA column. The column was washed with buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, pH 6.3). The target protein was eluted from the column by Buffer D (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH 5.9) followed by Buffer E (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH 4.5). The refolding of the purified protein was carried out through overnight dialysis with a dialysis tube (3.5 kDa) in PBS at 4 ˚C.

The purified proteins were subjected to 10% SDS-PAGE, and the protein bands were visualized using the CBB staining. The protein purification was further confirmed by Western blotting using the anti-His tag antibody conjugated to HRP.

Immunization of mice

FCbpD was used as the antigen, and flagellin served as the adjuvant. BALB/c female mice aged 5–6 weeks were randomly divided into 4 groups (n=5). The mice were inoculated subcutaneously on days 0, 14, 28, and 42 using 50 µg FCbpD admixed with 50 µg flagellin. Three control groups were established, in which mice received 50 µg FCbpD alone, 50 µg flagellin alone, or PBS, following the same protocol as the study group. The injections were carried out at a volume of 100 µL. Two weeks after the last immunization, the mice's sera were collected for further analysis.

Assessing the anti-FCbpD specific IgG response

The anti-FCbpD IgG levels in the mice sera were assessed using the indirect enzyme-linked immunosorbent assay (ELISA) [22]. The ELISA plates (Nunc, USA) were coated using 100 µL of purified FCbpD (1 µg/well) and subsequently incubated overnight at 4 ˚C. The wells were filled with the blocking buffer (2% BSA in PBS containing 0.05% Tween 20) and incubated at 37 ˚C for 2 h. Subsequently, 100 µL of diluted antisera (1:100) was added to the wells. The goat anti-mouse IgG conjugated to HRP (Abcam, USA) was applied to the wells at a dilution of 1:10000, followed by incubation at 37 ˚C for 1.5h. The 3', 5, 5'-tetramethylbenzidine (TMB) substrate was then added to the wells, and the plate was kept at room temperature in the dark for 30 min. The absorbance of each well was measured using an ELISA reader (BioTek, USA) at 450 nm. The data were expressed as Mean±SD. The statistical analysis of the ELISA data was performed using a 1-way analysis of variance (ANOVA) followed by the Tukey test. $P<0.05$ were considered significant for pairwise comparisons.

Results and Discussion

Physicochemical characteristics, antigenicity, and conservation of FCbpD

The CbpD protein is implicated in promoting nasopharyngeal colonization by *S. pneumoniae* [9]. It is composed of a cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain at the N-terminus, which is followed by two bacterial Src homology 3 (SH3) domains and a choline binding domain at the C-terminus [11]. The full-length CbpD protein was shown to be lethal to *E. coli* cells. However, a FCbpD devoid of the choline-binding domain was successfully cloned in *E. coli* [9]. Therefore, the FCbpD protein used in this study does not contain the choline-binding domain. The *fcbpd* gene was amplified by PCR from the genomic DNA of a serotype 19F pneumococcus (*S. pneumoniae* 82218) (Figure 2a). The PCR product was observed at the expected size of 909 bp. It was then incorporated into the pET28a vector (pFCBPD) and transformed into *E. coli*. The recombinant *E. coli* was successfully identified by the colony PCR (Figure 2b). The pFCBPD plasmid insert was subjected to nucleotide sequencing. ProtParam analyzed the amino acid sequence corresponding to the obtained nucleotide sequence to determine the physicochemical properties of FCbpD (Table 2). The ProtParam analysis showed an average molecular weight of 34.141 for FCbpD. Moreover, its isoelectric point was 6.73. The in vivo half-life of FCbpD in *E. coli* was estimated to be

Table 2. Physicochemical properties of a fragment of FCbpD estimated by protparam

Physicochemical Property	FCbpD
Mwt (kDa)	34.141
pI	6.73
Half-life in *E. coli*	>10h
II	38.02
Aliphatic index	59.83
GRAVY score	-0.71

Table 3. Immunoprotection of proteins determined by vaxijen

Protein	VaxiJen Score
FCbpD	0.6406
CbpD	0.6128

FCbpD: A fragment of choline-binding protein D.

over 10 h. The in vivo half-life was calculated using the N-end rule, which correlates the in vivo half-life of proteins with the nature of the amino-terminal residue [23]. The comparison of several stable and unstable proteins revealed that the occurrence of specific dipeptides in unstable proteins was notably different from that in stable proteins. Therefore, an instability weight was assigned to each dipeptide, and the sum of these values, normalized to the protein length, was defined as the instability index (II). An II value less than 40 indicates protein stability [24]. The II value of FCbpD was lower than 40 (Table 1), indicating the protein's stability. The grand average of hydropathicity (GRAVY) score is a measure of the protein hydropathicity [25]. The negative GRAVY score of FCbpD indicates that the protein is hydrophilic and can interact with molecules of water. Moreover, the VaxiJen score for FCbpD was higher than that for CbpD (Table 3), suggesting that FCbpD is more effective at eliciting immunoprotection than CbpD. The amino acid sequence of FCbpD was highly conserved across pneumococci of the most significant serotypes causing invasive pneumococcal disease in children less than 5 years of age (Figure 3). The amino acid sequence homology of FCbpD in these serotypes was over 94% (Table 4). These results demonstrated that FCbpD can be used to develop a serotype-independent vaccine against pneumococci.

Table 4. Amino acid sequence conservation of FCbpD in most invasive pneumococcal serotypes

Pneumococcal Serotype	Amino Acid Sequence Homology (%)
1	98
5	94.6
6A	99.3
6B	97.7
14	99.3
23F	98.7
19F	100

FCbpD: A fragment of choline-binding protein D.

Table 5. Elements of FcbpD secondary structure determined by YASARA

Secondary Structure Element	Element (%)
α-helix	2
β-sheet	23.8
Turn	17.2
Coil	57.1

FCbpD: A fragment of choline-binding protein D.

Structures of FCBPD and conformational b-cell epitopes

The tertiary structure of FCbpD was predicted by iTASSER (Figure 4a). The z-score of the protein 3D model was -2.74, within the typical range for proteins of similar size (Figure 4b). This result indicates the model's suitability. Analysis of the secondary structure of FCbpD using YASARA revealed that it comprised alpha-helices, beta-sheets, turns, and coils (Table 5).

Ellipro predicts conformational B-cell epitopes from protein 3D structures. It first determines protein surface patches and then identifies B-cell epitopes in regions of the protein protruding from its globular surface. It assigns a score (protrusion index) to each B-cell epitope, which is the average of the scores of its constituent amino acids. The B-cell epitope score indicates the proportion of protein atoms projecting from the molecule's bulk and implicated in the antibody binding [20]. Ellipro predicted three conformational B-cell epitopes for FCbpD at regions comprising amino acids 283-353, 51-201, and 202-275, with lengths ranging from 26 to 111 amino acids (Table 6, Figures 5a, 5b and 5c). Most B-cell epitopes are conformational, and these epitopes constitute 90% of the B-cell epitopes of native proteins [26]. The presence of conformational B-cell epitopes in FCbpD demonstrates its potential to stimulate the immune response against pneumococci.

Expression and purification of recombinant FCbpD

The FCbpD protein was expressed intracellularly in *E. coli* using the T7-lac promoter. The recombinant protein was observed at 34 kDa, as expected (Figure 6a). The solubility analysis revealed that FCbpD was expressed in both the soluble cellular fraction and inclusion bodies (Figure 6b). The soluble FCbpD protein accounted for 36.3% of the total FCbpD expressed. When protein purification was performed under native conditions from the soluble cellular fraction, the purified protein had a molecular weight of less than 30 kDa (Figure 6c). The small molecular weight of the purified protein is attributed to proteolytic degradation of FCbpD during purifica-

Table 6. Conformational B-cell epitopes of a fragment of FCbpD predicted by Ellipro

Epitope No.	Residues	No. of Residues	Score
1	A:V283, A:A285, A:T286, A:V287, A:I288, A:V297, A:H298, A:Y299, A:D300, A:Q301, A:I302, A:W310, A:L311, A:S312, A:Y313, A:T314, A:A315, A:Y316, A:N317, A:G318, A:S319, A:R320, A:R321, A:Y322, A:Q324, A:L325, A:E326, A:G327, A:V328, A:T329, A:S330, A:S331, A:Q332, A:N333, A:Y334, A:Q335, A:N336, A:Q337, A:S338, A:G339, A:N340, A:I341, A:S342, A:S343, A:Y344, A:G345, A:S346, A:N347, A:N348, A:S349, A:S350, A:T351, A:V352, A:G353	54	0.725
2	A:G51, A:D52, A:D53, A:Y54, A:P55, A:A56, A:Y57, A:K59, A:N60, A:G61, A:S62, A:Q63, A:E64, A:I65, A:D66, A:Q67, A:W68, A:R69, A:M70, A:Y71, A:S72, A:R73, A:Q74, A:C75, A:F78, A:A80, A:N85, A:V86, A:N87, A:G88, A:F89, A:E90, A:I91, A:P92, A:A98, A:N99, A:E100, A:W101, A:G102, A:H103, A:R104, A:R106, A:E108, A:G109, A:Y110, A:R111, A:V112, A:D113, A:N114, A:T115, A:P116, A:T117, A:G130, A:H131, A:V132, A:A133, A:W134, A:S136, A:N137, A:V138, A:M139, A:G140, A:D141, A:Q142, A:I143, A:E144, A:Y150, A:G151, A:Y152, A:T153, A:E154, A:S155, A:Y156, A:N157, A:K158, A:R159, A:I160, A:I161, A:K162, A:A163, A:N164, A:T165, A:M166, A:K173, A:D174, A:L175, A:D176, A:G178, A:S179, A:V180, A:G181, A:N182, A:S183, A:Q184, A:S185, A:S186, A:T187, A:S188, A:T189, A:G190, A:G191, A:T192, A:H193, A:Y194, A:F195, A:K196, A:T197, A:K198, A:S199, A:A200, A:I201	111	0.631
3	A:K202, A:T203, A:E204, A:P205, A:L206, A:V207, A:S208, A:A209, A:T210, A:V211, A:I212, A:D213, A:Y214, A:A251, A:V252, A:N253, A:K254, A:N255, A:P256, A:L257, A:G258, A:N259, A:K272, A:T273, A:K274, A:S275	26	0.582

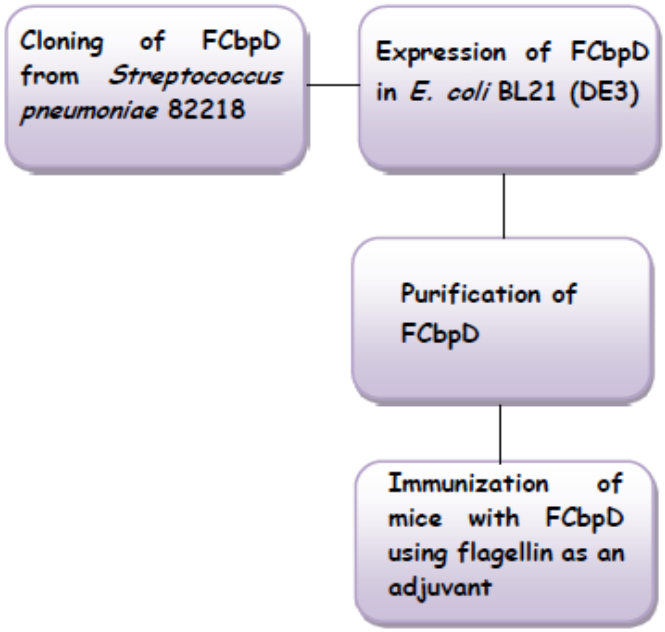

Figure 1. Schematic flowchart of this study for assessment of FCbpD as a vaccine candidate targeting *S. pneumoniae*
FCbpD: A fragment of choline-binding protein D.

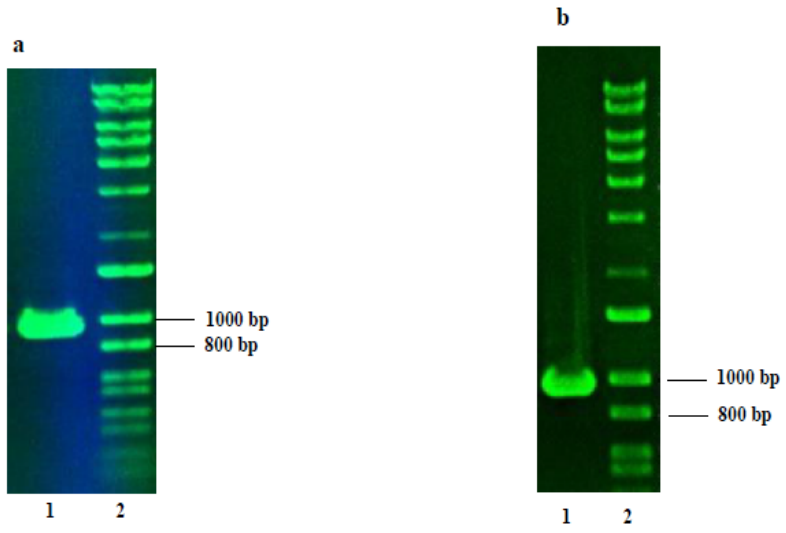

Figure 2. a) PCR amplification of fcbpD from the genomic DNA of *S. pneumoniae* 82218 (Lane 1, PCR product; Lane 2, marker); b) Colony PCR product for identification of *E. coli* harboring the pFCBPD vector (Lane 1, PCR product; Lane 2, DNA marker)
FCbpD: A fragment of choline-binding protein D.
Note: The marker band sizes were indicated in the figure.

```
WP_000698581.1(5)    GDDYPAYYKN  60
CFP94161.1(6B)       GDDYPAYYKN  60
WP_000698574.1(1)    GDDYPAYYKN  60
CIV33058.1(23F)      GDDYPAYYKN  60
SSI82218(19F)        GDDYPAYYKN  60
CGF24516.1(6A)       GDDYPAYYKN  60
COR13694.1(14)       GDDYPAYYKN  60
                     **********

WP_000698581.1       GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEMPAAYGNANEWGHRARREGYRVDNTPTIGS       120
CFP94161.1           GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
WP_000698574.1       GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
CIV33058.1           GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
SSI82218             GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
CGF24516.1           GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
COR13694.1           GSQEIDQWRMYSRQCTSFVAFRLSNVNGFEIPAAYGNANEWGHRARREGYRVDNTPTIGS       120
                     ****************************:***********************************

WP_000698581.1       ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRVVKANTMTGFIHFKDLDGGSV       180
CFP94161.1           ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRVIKANTMTGFIHFKDLNGGSV       180
WP_000698574.1       ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRVIKANTMTGFIHFKDLDSGSV       180
CIV33058.1           ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRIIKANTMTGFIHFKDLDGGSV       180
SSI82218             ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRIIKANTMTGFIHFKDLDGGSV       180
CGF24516.1           ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRIIKANTMTGFIHFKDLDGGSV       180
COR13694.1           ITWSTAGTYGHVAWVSNVMGDQIEIEEYNYGYTESYNKRIIKANTMTGFIHFKDLDGGSV       180
                     ************************************::**************:.***

WP_000698581.1       GNSQSSASTGGTHYFKTKSAIKTEPLVSA------------------------------       209
CFP94161.1           GNSQSSTSTGGTHYFKTKSAIKTEPLVSGTVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
WP_000698574.1       GNSQSSASTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
CIV33058.1           GNSQSSTSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
SSI82218             GNSQSSTSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
CGF24516.1           GNSQSSTSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
COR13694.1           GNSQSSTSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYDQILEKDGYKWLSYTAY       240
                     ******:*******************.******************************

WP_000698581.1       ---------------------------------------------TVIDYYYPGEKVHYD       224
CFP94161.1           NGSYRYVQLEAVNKNPLGHSVLSSTGGTHYFKTKSAIKTEPLVSGTVIDYYYPGEKVHYD       300
WP_000698574.1       NGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKIKSAIKTEPLVSATVIDYYYPGEKVHYD       300
CIV33058.1           NGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKTKSAIKTEPLASGTVIDYYYPGEKVHYD       300
SSI82218             NGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYD       300
CGF24516.1           NGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYD       300
COR13694.1           NGSYRYVQLEAVNKNPLGNSVLSSTGGTHYFKTKSAIKTEPLVSATVIDYYYPGEKVHYD       300
                     *******************************************.***************

WP_000698581.1       QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSHSSSTVG       284
CFP94161.1           QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
WP_000698574.1       QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
CIV33058.1           QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
SSI82218             QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
CGF24516.1           QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
COR13694.1           QILEKDGYKWLSYTAYNGSRRYIQLEGVTSSQNYQNQSGNISSYGSNNSSTVG       360
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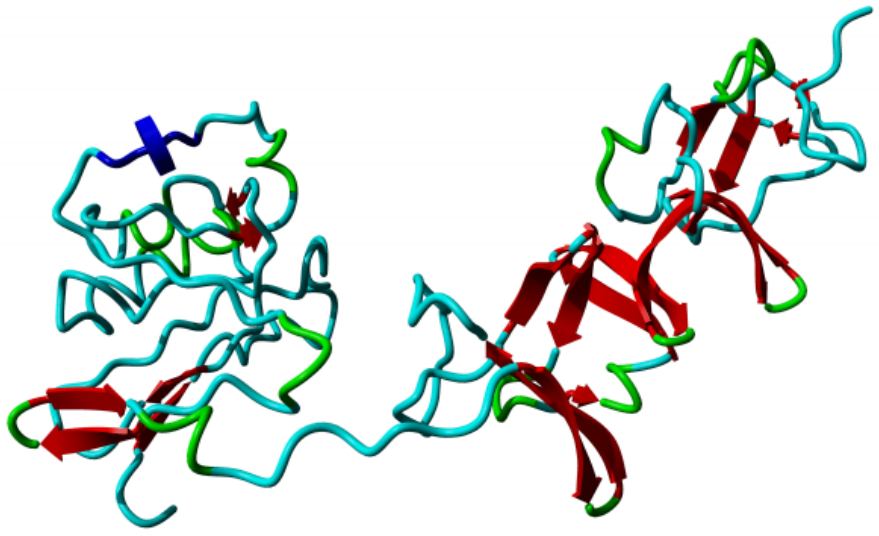
Figure 3. Analyzing the amino acid sequence conservation of FCbpD in invasive pneumococcal serotypes using clustal omega
FCbpD: A fragment of choline-binding protein D.

tion from the soluble fraction. However, the recombinant protein was successfully purified under denaturing conditions at the expected molecular weight and refolded via gradual urea depletion from the protein preparation by dialysis (Figure 6d). The inclusion body proteins are highly stable and resistant to proteolytic degradation. Moreover, the proteolytic activity of *E. coli* proteases is inhibited by the urea used for the protein purification under denaturing conditions [27]. The recombinant flagellin intended for use as an adjuvant was purified under



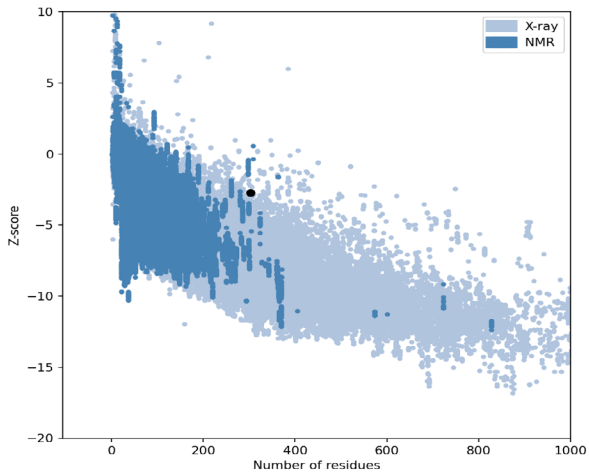


Figure 4. Tertiary structure of FCbpD obtained from iTASSER

a) The Z-score plot of FCbpD obtained from ProSA, b) The Z-score of FCbpD shown as a black circle in the figure FCbpD: A fragment of choline-binding protein D.

native conditions from the soluble cellular fraction of *E. coli* cells harboring pETflic and pKJE7 (Figure 7).

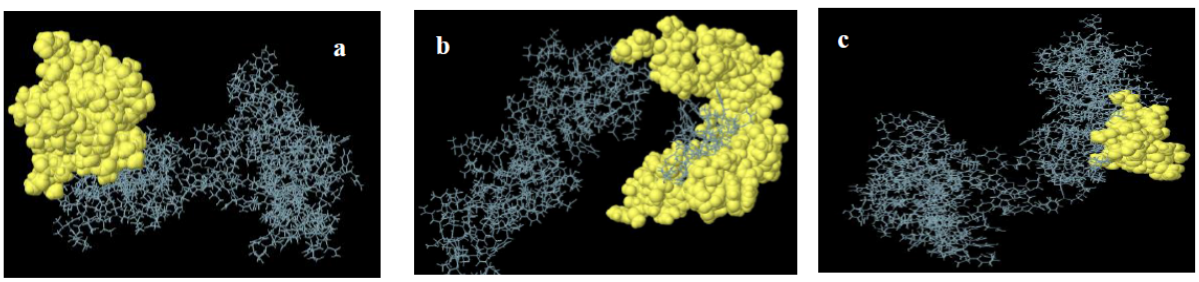


Figure 5. Conformational B-cell epitopes of FCbpD predicted by Ellipro

a) Epitope1, b) Epitope 2, c) Epitope 3 the amino acid residues constituting the epitopes were indicated in Table 6 FCbpD: A fragment of choline-binding protein D.

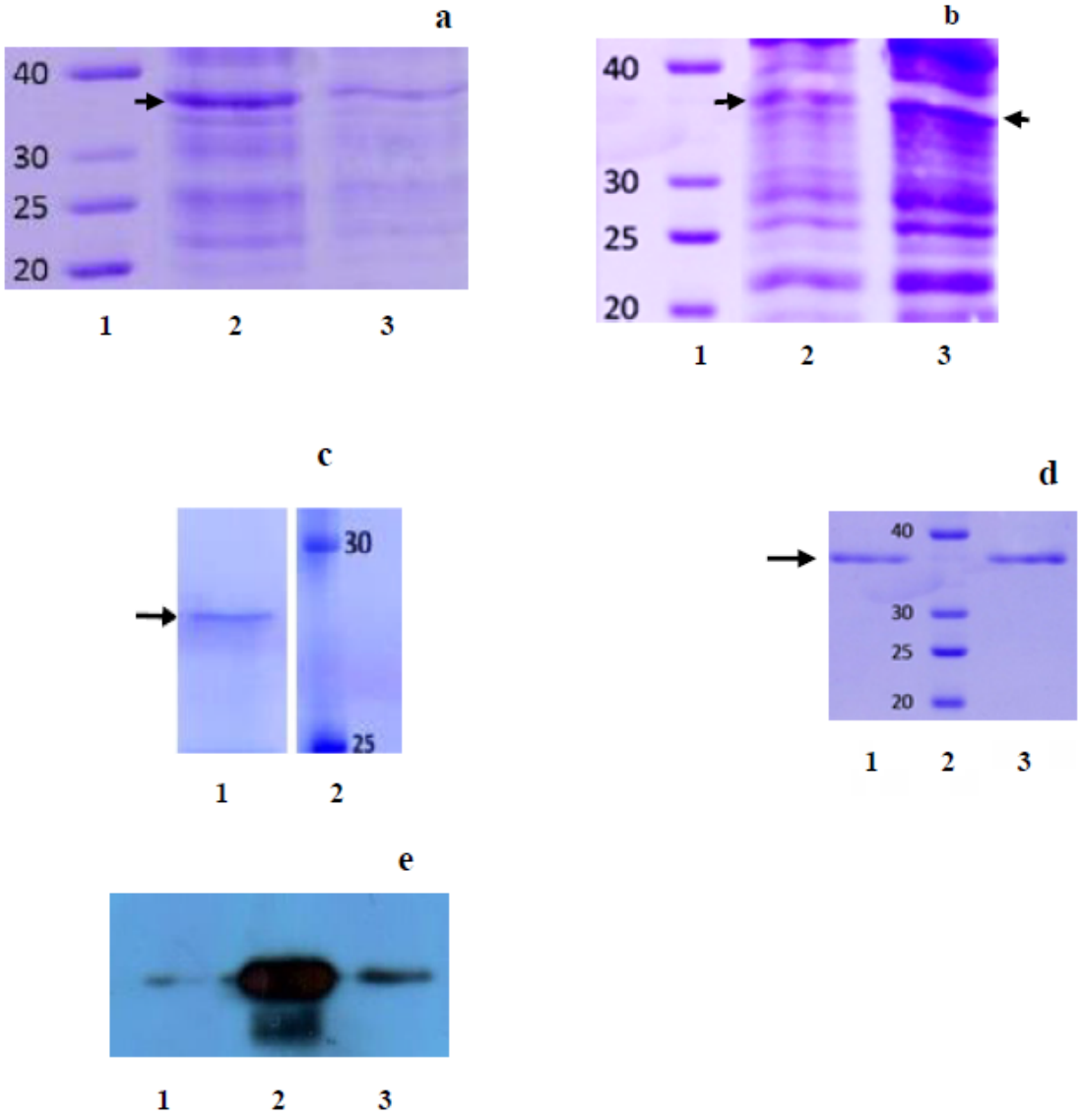


Figure 6. Expression, solubility analysis, and purification of FCbpD

Note: a) The FCbpD expression in *E. coli* after 4.5 h of IPTG induction (lane 2), and at the onset of the IPTG induction (lane 3), lane 1, protein marker; b) Solubility analysis of recombinant FCbpD, lane 2, FCbpD in soluble cellular fraction, lane 3, FCbpD in inclusion bodies, lane 1, protein marker; c) FCbpD purification from the soluble cellular fraction under native conditions; Lane 1, the purification product (low molecular weight band), lane 2, protein marker; d) FCbpD purification under denaturing conditions followed by dialysis, lane 1, the purified FCbpD after dialysis, lane 2, protein marker, lane 3, FCbpD purified under denaturing conditions (the protein bands in parts a-d were visualized by CBB staining following 10% SDS-PAGE); e) The Western blot of FCbpD, lane 1, FCbpD expressed in *E. coli* at the onset of the IPTG induction; lane 2, FCbpD expressed in *E. coli* after 4.5 h of the IPTG induction, lane 3, the FCbpD protein purified under denaturing conditions followed by dialysis.

FCbpD: A fragment of choline-binding protein D.

Specific total IgG response induced by recombinant FCbpD

After administration of the mixture of FCbpD and flagellin, FCbpD or the flagellin protein alone, and PBS to mice, the levels of specific total IgG induced against FCbpD in the mice sera were measured using the indirect ELISA method two weeks after the last injection (on day 56). Our results indicated a significant increase ($P<0.05$) in antibody levels in the FCbpD+flagellin group compared with the control groups, including FCbpD alone, flagellin alone, and the PBS group (Figure 8). These results demonstrated FCbpD's ability to elicit an antibody response in mice and the effectiveness of flagellin as an

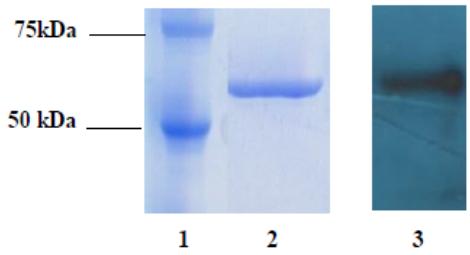


Figure 7. The purified recombinant flagellin used as the adjuvant

Note: Lane 1, protein marker; lane 2, the purified recombinant flagellin visualized by CBB staining after performing 10% SDS-PAGE; lane 3, Western blot of the purified flagellin.

FCbpD: A fragment of choline-binding protein D.

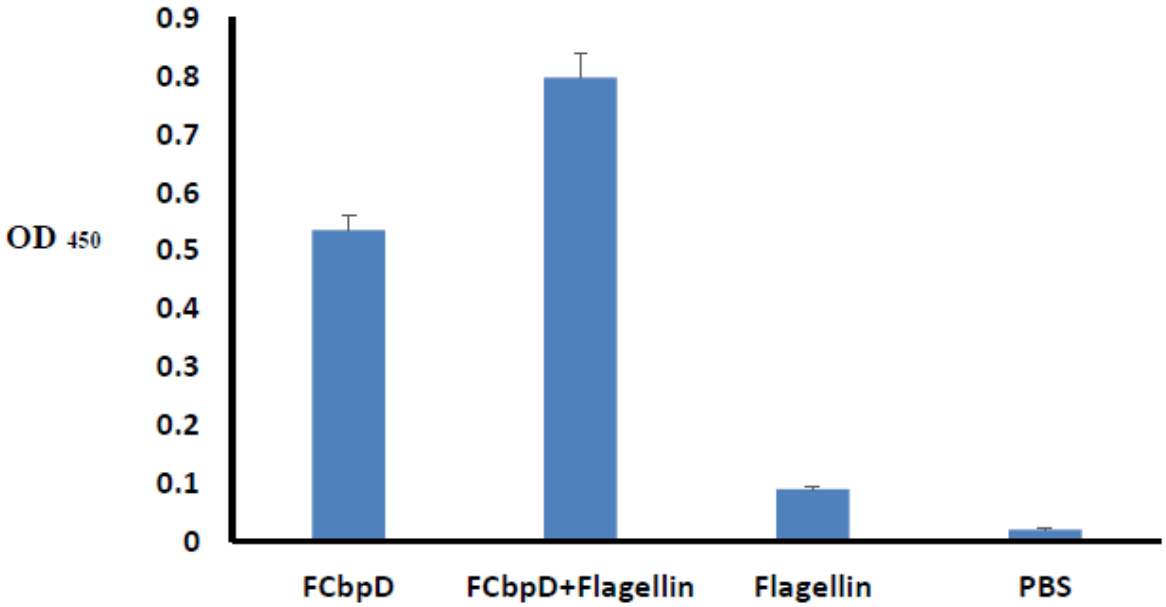


Figure 8. ELISA for assessing the specific total IgG induced in the sera by the recombinant FCbpD

FCbpD: A fragment of choline-binding protein D.

adjuvant. Moreover, given the least increase in the ELISA signal of vaccinated mice compared to control mice (0.263 units of OD450) and a standard deviation of 0.04 from our results, the effect size (d) was calculated to be 6.575. With a significance level of α=0.05 and a total sample size of 20 mice across 4 groups, the study power, calculated using G*Power software, exceeded 99.999%. Therefore, the statistical power of this study is deemed adequate. In addition, we did not observe any adverse events in any of the mouse groups.

It was shown that *Salmonella* flagellin can protect mice against *S. pneumoniae* by stimulating innate immunity. However, when a mutant flagellin unable to activate toll-like receptor 5 (TLR5) was used, the protective effect was not observed [28]. In this study, we also employed *Salmonella* flagellin's innate immune-activating function to elicit a specific adaptive immune response against pneumococcal FCbpD, thereby protecting mice against *S. pneumoniae*.

The bacterial flagellins activate receptors of the innate immune system, such as TLR5, which triggers the production of innate immune effectors, such as pro-inflammatory cytokines, thereby enhancing adaptive immune responses to co-administered antigens. Therefore, bacterial flagellins have been effectively used as adjuvants in various vaccines

targeting infectious diseases [12]. However, in a Phase 1 clinical trial of a flagellin–adjuvanted influenza vaccine, some vaccinees experienced local and systemic symptoms ranging from mild to severe, depending on the administered dose. The severe adverse effects were self-limiting [29]. Therefore, while bacterial flagellins are powerful adjuvants, these proteins also pose safety concerns for human use, potentially leading to regulatory issues.

Bacterial flagellins induce antibody responses against themselves. Booster doses are required to elicit efficient protection by most vaccines. The repeated administration of vaccines containing bacterial flagellins as adjuvants elicits stronger antibody responses to flagellins and thereby reduces flagellins' adjuvant ability to enhance immune responses against co-administered antigens. The central hypervariable domain of bacterial flagellins is responsible for inducing antibody responses against these proteins. The development of flagellin mutants with deletions of B-cell epitopes in the hypervariable region reduces immunogenicity, thereby reducing the reactogenicity associated with flagellin–adjuvanted vaccines [12].

Conclusion

In this study, the *fcbpD* gene encoding the FCbpD protein of *S. pneumoniae* 82218, a serotype 19F pneumococcus, was cloned and sequenced. Analysis of the corresponding amino acid sequence revealed the protein's stability. Moreover, the amino acid sequence of FCbpD showed high conservation in the corresponding proteins of pneumococcal serotypes 1, 5, 6A, 6B, 14, 19F, and 23F, which are the primary serotypes implicated in invasive disease among children under 5. The conformational B-cell epitopes required for antibody induction were identified within the tertiary structure of FCbpD. This protein was expressed intracellularly in *E. coli*. The recombinant protein was expressed in both the soluble cellular fraction and inclusion bodies. However, it could not be purified at the correct molecular size from the soluble cellular fraction under native conditions. The recombinant FCbpD was successfully purified from the cleared cell lysate under denaturing conditions. It elicited a specific antibody response in mouse sera when recombinant *Salmonella* Enteritidis flagellin was used as the adjuvant. These results demonstrate the feasibility of using the recombinant FCbpD protein as a vaccine candidate against pneumococci, independent of serotype. However, in vitro analyses of the elicited antibody's functions, such as opsonophagocytic and bactericidal activities, as well as in vivo evaluations of the protection induced by FCbpD in a challenge model, are needed to complete the efficacy studies of FCbpD.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Code: SBMU.REC.1392.494). Animal experiments were conducted in accordance with the guidelines of the Ministry of Health and Medical Education of Iran for the care and use of laboratory animals.

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Authors contribution's

Methodology, investigation, data analysis, and writing the original draft: Zahra Sadeghi; Conceptualization, supervision, review and editing: Shirin Tarahomjoo.

Conflict of interest

The authors declared no conflict of interest.

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