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Novel Vaccines against *Streptococcus pneumoniae* Based on the Immunoprotective B-cell Epitope Region of Pneumococcal Choline Binding Protein D and *Salmonella* Enteritidis Flagellin

Shirin Tarahomjoo[1,*], Soheila Ghaderi[2]

[1]Division of Genomics and Genetic Engineering, Department of Biotechnology and Central Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj 31975/148, Iran
[2]Division of Central Laboratory, Department of Biotechnology and Central Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj 31975/148, Iran
*Corresponding author: starahomjoo@gmail.com

Abstract Pneumococcal conjugate vaccines (PCVs) were constructed through chemical conjugation of pneumococcal capsules to immunogenic carrier proteins. The PCVs implementation in developing countries was prevented by their high manufacturing costs. This issue can be overcome by development of protein based vaccines against pneumococci. Antibody responses are necessary for protection against *S. pneumoniae*. Choline binding protein D (CBPD) was already identified as a pneumococcal surface protein able to elicit protection against *S. pneumoniae* and its most protective B-cell epitope region (MIBR) was determined. MIBR was highly conserved in common pneumococcal serotypes. Whole antigens are not as potent as epitope based vaccines and B-cell epitope based vaccines are more effective than whole antigen based vaccines in the prevention of infections. Bacterial flagellins are effective adjuvants that signal via Toll like receptor 5 (TLR5). The TLR5 binding site of flagellin located in the D1 domain and its proper conformation is critical for TLR5 recognition of flagellin. In the present study, therefore, we aim to design effective chimeric vaccines against pneumococci based on MIBR and flagellin of *Salmonella* Enteritidis (FliC) using bioinformatics tools. FliC was joined to MIBR at N-terminus (CFH), C-terminus (FCH) and the D3 domain (D3Gly202, D3Thr275). All of the constructs were immunoprotective regarding the VaxiJen score (0.8). The codon optimization for constructs was done using OPTIMIZER. Analysis of the mRNA secondary structures using Mfold tool revealed no stable hairpins at 5' ends of constructs and thus the antigens can be expressed appropriately. SCRATCH results indicated that the antigens can be expressed in the soluble form in *Escherichia coli* at more than 80% probability. The 3D models of antigens resulted from I-TASSER indicated the presence of alpha helix, beta sheet, turn, coil, and 3_{10} helix as the protein structural elements. Superimposing 3D models of D1 domains of antigens with the D1 domain of FliC using FATCAT indicated no change in the D1 conformation. Therefore, FliC can exert its adjuvant effects in these constructs through TLR5 signaling. Inserting MIBR in Gly202 of FliC enhanced the protein beta sheet content remarkably, which can result in appropriate thermostability of the antigen. Our results, therefore, demonstrated that D3Gly202 is a suitable vaccine candidate, which can elicit protection against common *S. pneumoniae* serotypes causing invasive pneumococcal disease in children less than 5 years of age.

Keywords: *Computational design, Flagellin, Pneumococcal conjugate vaccines, Protective epitope, Protein based vaccines, Streptococcus pneumoniae*

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1. Introduction

Streptococcus pneumoniae is a major cause of diseases such as meningitis, pneumoniae and sepsis mainly in children less than 5 years of age [1,2]. World Health Organization (WHO) reported that 476000 annual deaths among children less than 5 years of age were caused by pneumococcal infections [3]. Pneumococcal vaccines have been used to protect against pneumococcal infections. The capsular polysaccharides of pneumococci are main antigenic components of these vaccines. However, the capsules are weakly immunogenic in children and are not able to induce immune memory. Pneumococcal conjugate vaccines (PCVs) are then constructed through chemical

conjugation of pneumococcal capsules to immunogenic carrier proteins. The conjugation process increases the antibody response and induces the immune memory. WHO recommends the inclusion of PCVs in national immunization programs for children. However, the PCVs production requires multistep processes and the elicited protection is serotype dependent. These vaccines include conjugated capsules of multiple pneumococcal serotypes. Therefore, the vaccine manufacturing costs are high and limit PCVs implementation in national immunization programs of developing countries [1,2,3].

The development of protein based vaccines against pneumococci offers a more affordable protective strategy against pneumococcal infections. [4]. Cell surface proteins are key factors in infective processes of pathogens and have extensively been evaluated as vaccine candidates [5,6]. Several pneumococcal surface proteins such as pneumococcal surface protein A, pneumococcal surface protein C, pneumococcal surface adhesin A, pilus proteins and histidine triad proteins were able to induce protection against *S. pneumoniae* [7]. We already identified choline binding protein D (CBPD), a pneumococcal surface protein as a suitable candidate for eliciting protection against *S. pneumoniae*. The candidate selection was based on the protein ability to elicit immunoprotection, the absence of autoimmunity induction and the amino acid sequence conservancy in pneumococcal strains [8]. Antibody responses are essential for protection against pneumococcal infections [7]. Antibodies bind specifically to a continuous amino acid sequence known as the linear B-cell epitope or to a folded structure composed of discontinuous amino acids known as the conformational B-cell epitopes. Therefore, the analysis of protective B-cell epitopes in vaccine candidates is necessary to develop effective vaccines against pneumococci [9]. The majority of B-cell epitopes are conformational. Nevertheless, the identification of linear B-cell epitopes has shown promising results for selection of the vaccine constituents [10,11]. Whole antigens are not as potent as epitope based vaccines and B-cell epitope based vaccines are more effective than whole antigen based vaccines in the prevention of infections [12,13]. Compared with conventional laboratory methods, computational approaches offer the ability to undertake rapid and comprehensive epitope assessments at much lower costs [14]. Simultaneous applications of B-cell epitope prediction tools enhance the epitope prediction accuracy [15]. We already determined B-cell epitope regions of CBPD by simultaneous applications of widely used bioinformatics tools [16]. Bacterial flagellins, the structural subunit of flagellar filaments, are effective adjuvants that signal via Toll like receptor 5 (TLR5). Structural analysis of these proteins revealed a boomerang shaped protein with four major domains. The vertical arm is constructed from domains D0 and D1, which are well conserved. The horizontal arm of the protein comprised of domains D2 and D3, which are highly variable in sequence. The TLR5 binding site of flagellin located in the D1 domain and its proper conformation is critical for TLR5 recognition of flagellin [17]. In the present study, we aim to design effective vaccines against pneumococci comprising fusion proteins linking the identified B-cell epitope region of CBPD to flagellin of *Salmonella* Enteritidis (FliC) as built in adjuvant. The appropriate fusion strategy was then investigated through evaluating immunological, transcriptional, and structural properties of designed constructs and the suitable antigen for inclusion in the vaccine was determined.

2. Methods

The immunoprotective abilities of antigens were evaluated using VaxiJen [18]. The codon optimization and calculations of the codon adaptation index (CAI) were performed using the OPTIMIZER online tool. *Escherichia coli* K-12 is the basic strain for the construction of *E. coli* based expression hosts. Therefore, the codon usage table of *E. coli* K-12 obtained from Kazusa codon usage database was used for the codon optimization [19]. Prediction of the mRNA secondary structure was carried out using Mfold web server [20]. Moreover, the probability of the protein over-expression in *E. coli* in the soluble form was calculated using the SCRATCH protein prediction tool [21]. I-TASSER web server was used for de novo prediction of the protein tertiary structure from the amino acid sequence [22]. The quality of 3D models was examined using ProSA online tool [23]. YASARA was used to visualize the protein tertiary structure and to determine the protein secondary structure constituents. Superimposing protein structures was carried out using the FATCAT program [24]. Ellipro was used to assess the solvent accessibility of amino acid residues [25].

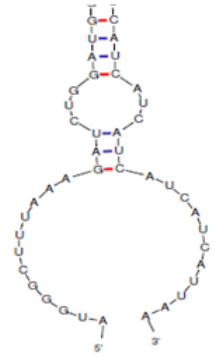
3. Results and Discussion

3.1. Design of Chimeric Vaccines

The incidence of invasive pneumococcal disease (IPD) is frequently used as a measure of the incidence of severe pneumococcal disease. Pneumococcal serotypes 1, 5, 6A, 6B, 14, 19F and 23F are common causes of IPD globally in children less than 5 years of age [3]. Bepipred and BCPreds are widely used bioinformatics tools for identification of linear B-cell epitopes. The CBTope program is used for the prediction of conformational B-cell epitopes using amino acid sequences [15]. We already identified linear B-cell epitope regions in CBPD from serotype 19F *S. pneumoniae* using Bepipred. Furthurmore, conformational B-cell epitope regions of CBPD were identified by CBTope [16]. The identified B-cell epitope regions overlapped linear B-cell epitopes identified by BCPreds. The B-cell epitope region containing amino acid residues 172-195 was the most probable immunoprotective B-cell epitope region (MIBR) regarding the VaxiJen score. Moreover, The MIBR amino acid sequence was highly conserved (more than 91% amino acid sequence match) among the common pneumococcal serotypes (unpublished results). VaxiJen was developed to classify antigens solely based on their physicochemical properties without the need for sequence alignments. A higher VaxiJen score refers to a higher probability for the immunoprotective ability. FliC was fused to the identified MIBR of CBPD at N-terminus (CFH), C-terminus (FCH) and the D3 domain (D3Gly202,

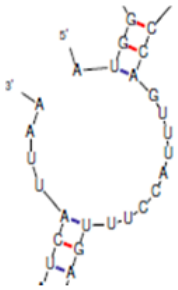
D3Thr275). The insertion sites of D3 domain containing Glycine 202 and Threonie 275 were located in loop regions of FliC. A histidine tag was introduced at the C-terminus of constructs. VaxiJen scores of all of the constructs were about 0.8. Therefore, all of them were immunoprotective.

A



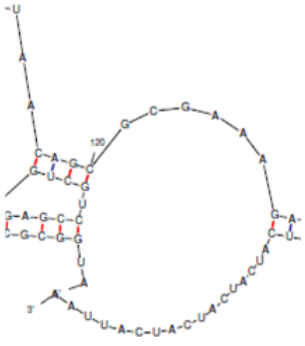
Structural element	δG	Information
External loop	-1.90	23 ss bases & 1 closing helices.
Stack	-2.40	External closing pair is G_{13}-C_{1612}
Stack	-1.10	External closing pair is A_{14}-U_{1611}

B



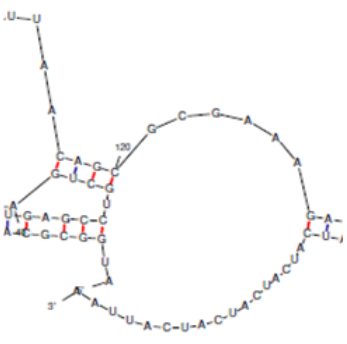
Structural element	δG	Information
Helix	-3.40	2 base pairs.
Stack	-2.10	External closing pair is U_2-A_{1016}
Stack	-3.30	External closing pair is G_3-C_{1015}
Helix	-5.40	3 base pairs.
Multi-loop	3.00	External closing pair is G_4-C_{1014} 2 ss bases & 3 closing helices.
Stack	-2.40	External closing pair is C5-G153
Stack	-3.40	External closing pair is G6-C152
Stack	-2.10	External closing pair is C7-G151

C



Structural element	δG	Information
Helix	-4.20	3 base pairs.
Stack	-3.30	External closing pair is G_3-C_{34}
Stack	-3.40	External closing pair is G_4-C_{33}
Helix	-6.70	3 base pairs.
Interior loop	-0.10	External closing pair is C_5-G_{32}
Stack	-2.10	External closing pair is C_7-G_{30}
Stack	-2.10	External closing pair is A_8-U_{29}
Stack	-3.30	External closing pair is G_9-C_{28}
Stack	-2.50	External closing pair is G_{10}-C_{27}

D



Structural element	δG	Information
Helix	-4.20	3 base pairs.
Stack	-3.30	External closing pair is G_3-C_{34}
Stack	-3.40	External closing pair is G_4-C_{33}
Helix	-6.70	3 base pairs.
Interior loop	-0.10	External closing pair is C_5-G_{32}
Stack	-2.10	External closing pair is C_7-G_{30}
Stack	-2.10	External closing pair is A_8-U_{29}
Stack	-3.30	External closing pair is G_9-C_{28}
Stack	-2.50	External closing pair is G_{10}-C_{27}

Figure 1. The mRNA secondary structures and free energy details at 5' ends of chimeric vaccines predicted by Mfold. A, CFH; B, FCH; C, D3Gly202; D3Thr75

Adapting the gene codon usage to the host codon usage can enhance the expression level of the encoded protein. The codon optimization includes altering target gene codons so that they reflect the codon usage of the host more closely without changing the amino acid sequence of the encoded protein [19]. Results of OPTIMIZER showed that CAIs of native genes of all the constructs were 0.65. However, the codon optimized gene sequences showed a CAI of 1. CAI measures the similarity between the codon usage of a gene and the codon usage of the reference set of genes. Its value is in the range of 0-1 [19]. Therefore, the codon usage of the optimized gene sequence was the same as the codon usage of *E. coli* reference group of genes and the optimized gene can be expressed at suitable levels in *E. coli*. GC contents of native genes of all the constructs were increased on average from 44.45% to 55.85% upon codon optimization. The GC content of the gene affects its codon usage [26]. The gene expression level correlates inversely with the stability of mRNA hairpin structures close to the 5' end of mRNA [27]. The 5' ends of the optimized genes mRNA did not constrain any stable hairpins as predicted by Mfold (Figure 1). Therefore the designed vaccines are expected to be expressed at appropriate levels.

E. coli is a popular host for expression of recombinant proteins owing to its superior properties including the ease of genetic manipulation and the high expression level. However, *E. coli* is often not able to fold foreign proteins properly and inclusion bodies are then formed. The protein recovery from inclusion bodies requires a multistep complex process, which can lead to a significant loss in the final protein yield [28,29]. SCRATCH was used for predicting the probability of the protein expression in the soluble form in *E. coli*. Solubility probabilities of CFH, FCH, D3Gly202 and D3Thr275 were 0.838, 0.824, 0.838 and 0.835 respectively. Therefore, the majority of proteins is expressed in the soluble form in *E. coli* and can then be recovered from the soluble cellular fraction instead of inclusion bodies.

3.2. Structures of chimeric Vaccines

The tertiary structures of designed vaccines were predicted using I-TASSER. I-TASSER predicts 5 models for each construct (data not shown). The most confident models given for CFH, FCH, D3Gly202, and D3Thr275 (Figure 2) had confidence scores (C-score) of -0.43, -0.99, -0.57, and -0.75 respectively. C-score is in the range of -5 to 2 and a higher C-score indicates a model with a higher confidence [22]. ProSA was used to check the quality of antigens 3D models. The obtained z-scores for the 3D models of CFH, FCH, D3Gly202, and D3Thr275 were -4.1, -3.6, -5.13, and -5.33 respectively and these scores were within the range of typical scores of native proteins at the similar sizes (data not shown). Alpha helix, beta sheet, turn, coil and 3_{10} helix were the structural elements of designed vaccines (Table 1). The percentages of structural elements in antigens secondary structures were determined using YASARA. Fusion of MIBR to the N-terminus of FliC resulted in enhancing the protein beta sheet content. Whereas joining MIBR to the C-terminus of FliC decreased the beta sheet content. D3Gly202 showed the highest beta sheet content between the designed vaccines and the lowest beta sheet content was observed in the D3Thr275 structure. It was shown that the increase in the protein beta sheet content was much more significant in hyperthermophilic proteins compared with moderately thermophilic ones. In addition, enhancing the beta sheet content increased the protein thermostability [27]. Therefore, D3Gly202 is expected to have the highest thermostability among designed vaccines.

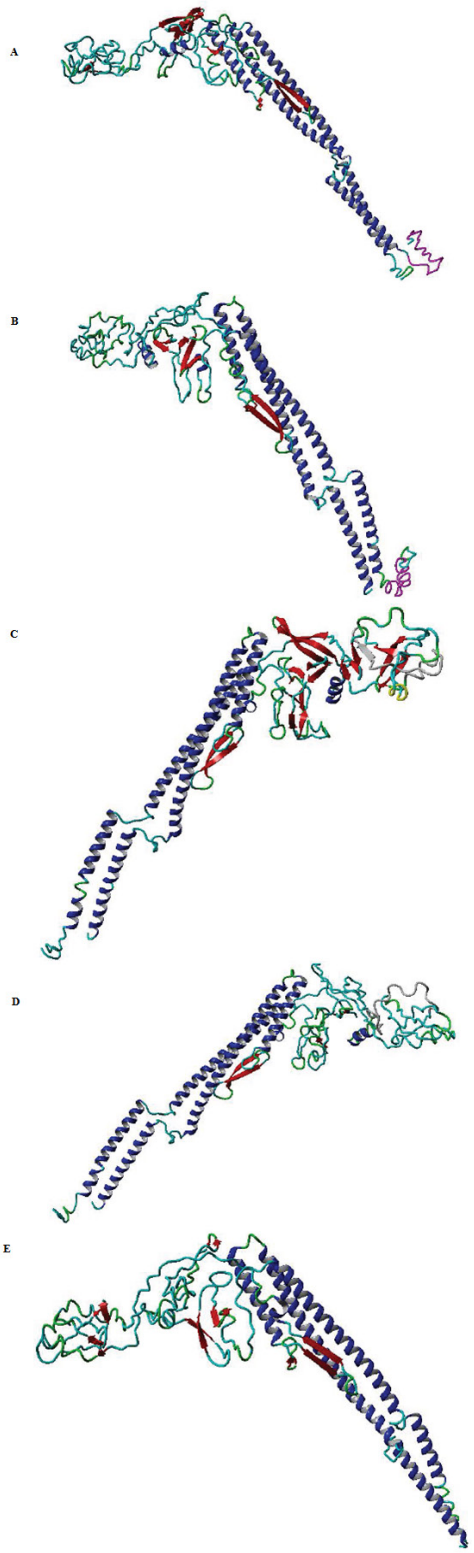


Figure 2. 3D models of chimeric vaccines. A, CFH; B, FCH; C, D3Gly202; D, D3Thr275; E, Flagellin. MIBR of CBPD was shown in pink color in A and B. This region was shown in Gray color in C and D

Table 1. Percentages of secondary structure elements of chimeric vaccines

Secondary structure element	CFH	FCH	D3Gly202	D3Thr275	FliC
Alpha helix	37.2	37.8	37.8	38.2	36.6
Beta sheet	9.8	6.1	17.4	3.2	7.1
Turn	10.9	15	14.5	15	17
Coil	42	41.1	29.3	43.6	39.2
3_{10} helix	0	0	0.9	0	0

The TLR5 binding region of FliC resides in the D1 domain and its appropriate conformation is necessary for TLR5 recognition of FliC. Therefore, we compared the D1 structures in designed vaccines with that of FliC by superimposing them using FATCAT program. FATCAT results indicated that the D1 structure of D3Gly202 was significantly similar to the D1 structure of FliC (Figure 3). The D1 structures of D3Thr275, CFH and FCH were also significantly similar to the D1 structure of FliC (data not shown). Our results indicated that the conformation of the D1 domain of designed vaccines was not different from that of FliC and thus FliC can exert its adjuvant effects in these constructs via TLR5 signaling.

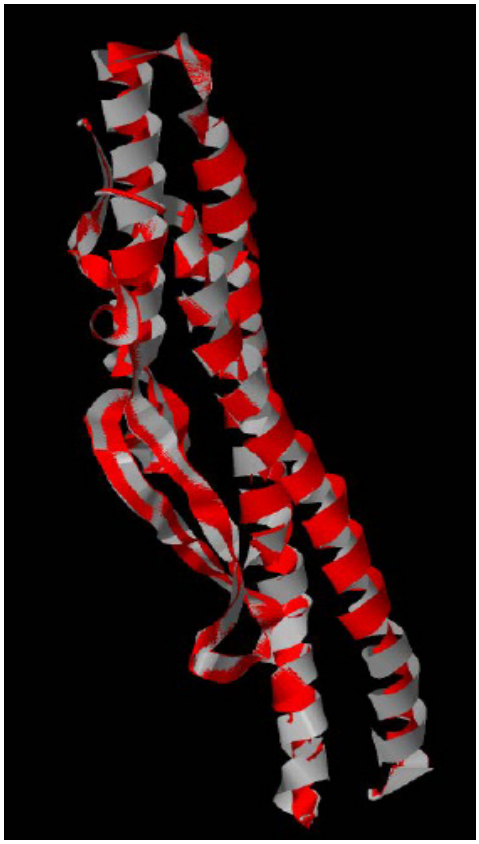


Figure 3. superimposing the D1 domain of FliC (gray) with the D1 domain of D3Gly202 (red) by FATCAT

3.3. Solvent accessibility of MIBR residues in designed constructs

B-cell epitopes should be exposed on the protein surface to be recognized by B-cells or bind to antibodies. A higher solvent accessibility for an amino acid residue indicates its better surface exposure. The solvent accessibility of MIBR amino acid residues in designed vaccines were evaluated using Ellipro. Amino acid residues with higher scores assigned by Ellipro, were associated with a better solvent accessibility. All of the amino acid residues of MIBR in fusion proteins were solvent accessible and therefore exposed on the protein surface. However, solvent accessibilities of PHE1 to GLY10 of MIBR in FCH were higher than those of MIBR in other designed vaccines (Table 2). In addition, the solvent accessibilities of GLN12 to TYR22 of MIBR in CFH were higher than those of other antigens. The variations in the solvent accessibilities of MIBR residues correlate with alterations in FliC conformations in designed constructs.

4. Conclusions

The insertion of MIBR in FliC did not alter the conformation of D1 domain, which includes the TLR5 binding region. Therefore, FliC can exert its adjuvant effects in the designed chimeric vaccines through TLR5 signaling. The MIBR insertion in GLy202 enhanced the beta sheet content remarkably, which can improve the vaccine thermostability. Moreover, D3Gly202 can elicit protection against common serotypes of *S. pneumoniae* and can be expressed at appropriate levels in *E. coli* in the soluble form. Therefore, it is a suitable proteinaceous vaccine candidate against *S. pneumoniae*.

Table 2. Ellipro scores for evaluating solvent accessibility of MIBR amino acid residues

Amino acid residue	CFH	FCH	D3Gly202	D3Thr275
PHE	0.837	0.954	0.792	0.761
LYS	0.837	0.991	0.887	0.850
ASP	0.822	0.998	0.876	0.844
LEU	0.843	0.983	0.803	0.839
ASP	0.874	0.983	0.846	0.772
GLY	0.850	0.996	0.887	0.831
GLY	0.883	0.983	0.865	0.820
SER	0.891	0.970	0.876	0.809
VAL	0.906	0.970	0.915	0.779
GLY	0.915	0.959	0.942	0.761
ASN	0.924	0.939	0.965	0.744
SER	0.939	0.946	0.935	0.692
GLN	0.948	0.946	0.898	0.779
SER	0.978	0.935	0.876	0.705
SER	0.991	0.913	0.839	0.705
THR	0.991	0.902	0.868	0.744
SER	0.983	0.913	0.820	0.685
THR	0.996	0.902	0.746	0.692
GLY	0.996	0.867	0.701	0.597
GLY	0.989	0.867	0.666	0.531
THR	0.967	0.856	0.571	0.447
HIS	0.972	0.867	0.596	0.364
TYR	0.961	0.844	0.503	0.364

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