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Serotype Independent Vaccine Design against *Streptococcus pneumoniae* Based on B-cell Epitopes of Autolysin, Zinc Binding Lipoprotein and Plasmid Stabilization Protein

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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*. Autolysin, zinc binding lipoprotein (ZBL), and plasmid stabilization protein (PSP) were already identified as pneumococcal surface proteins able to elicit protection against *S. pneumoniae* serotype 19F and their most probable immunoprotective B-cell epitope regions (MIBRs) were determined. MIBRs were fully conserved in the most common pneumococcal serotypes causing invasive pneumococcal disease in children. Whole antigens are not as potent as epitope based vaccines and every epitope in a multi epitope based vaccine can individually induce a protective immune response against the pathogen. Thus better immunoprotection can be achieved by multi epitope based vaccines. In the present study, therefore, we aim to design a chimerical vaccine against pneumococci based on the identified MIBRs using immunoinformatic tools. These regions were joined together using the $(EAAAK)_4$ linker. The MIBRs orders affected the immunoprotective ability of the fusion protein as estimated by VaxiJen tool. The fusion protein consisting of MIBRs of autolysin, PSP and ZBL respectively (APZ) showed the highest probability for eliciting immunoprotection and was used for further study. The codon optimization was done for APZ using OPTIMIZER. Analysis of the mRNA secondary structure using Mfold tool revealed no stable hairpin at the 5' end and thus the antigen can be expressed appropriately. The 3D model of the antigen resulted from I-TASSER indicated the presence of alpha helix, turn, and coil as the protein structural elements. Analyzing physicochemical properties of the chimerical antigen using ProtParam showed that the fusion protein was stable and its half life in *Escherichia coli* was more than 10 h. Considering the GRAVY score, the chimerical antigen possessed a hydrophilic nature and it can be expressed in the soluble form in *E. coli* at 92.2% probability. These results demonstrated that the chimerical antigen composed of conserved MIBRs is a suitable vaccine candidate, which can elicit protection against common pneumococcal serotypes.

Keywords: *computational design, 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]. Twenty one pneumococcal serotypes including 19F, 14, 6B, 1, 23F, 5, 6A, 19A, 9V, 18C, 2, 4, 7F, 12F, 3, 12A, 8, 46, 15B, 45 and 9A are the most common pneumococcal serotypes causing invasive pneumococcal disease in children worldwide [2].

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 a multistep process 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. Cell surface proteins are key factors in infection processes of pathogens and have extensively been evaluated as vaccine candidates [4,5]. We already identified three pneumococcal surface proteins including autolysin, zinc binding lipoprotein (ZBL), and plasmid stabilization protein (PSP) as suitable candidates for eliciting protection against *S. pneumoniae* serotype 19F. The candidate selection was based on the protein ability to elicit immunoprotection, the absence of autoimmunity induction and the amino acid sequence conservancy in serotype 19F pneumococcal strains [6]. 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 [8]. 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 [9,10]. Whole antigens are not as potent as epitope based vaccines and usually a few epitopes are sufficient to elicit protective immune responses. As a result, B-cell epitope based vaccines are more effective than whole antigen based vaccines in the prevention of infections. In addition, every epitope in a multi epitope based vaccine can individually induce a protective immune response against the pathogen. Hence, more efficient immunoprotection can be achieved against infections by multi epitope based vaccines [11,12].

Compared with conventional laboratory methods, computational approaches offer the ability to undertake rapid and comprehensive epitope assessments at much lower costs [13]. Simultaneous applications of B-cell epitope prediction tools enhance the epitope prediction accuracy. Bepipred and BCPreds are widely used as 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 [14]. We already determined the most probable protective B-cell epitope regions (MIBRs) of autolysin, ZBL, and PSP by simultaneous applications of results of these three programs [15]. In the present study, we used the identified MIBRs to design a chimerical vaccine against pneumococci. The immunological, physicochemical, and structural properties of the chimerical antigen were evaluated using immunoinformatic tools to elucidate its appropriateness as a protein based vaccine against pneumococci.

2. Methods

The immunoprotective abilities of antigens were evaluated using VaxiJen [16]. Required pneumococcal protein sequences were retrieved from NCBI. The SIM tool was used to determine homology percentages of amino acid sequences. Amino acid sequences were aligned using Clustal Omega to analyze the B-cell epitope regions conservancy.

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 [17]. Prediction of mRNA secondary structures were carried out using Mfold web server [18]. Physicochemical properties of proteins were evaluated using PROTPARAM based on the amino acid composition [19]. Moreover, the probability of the protein over-expression in *E. coli* in the soluble form was calculated using the PROSOII tool [20]. The YASARA program was used to determine the protein secondary structure constituents. I-TASSER web server was used for de novo prediction of the protein tertiary structure from the amino acid sequence and the quality of the 3D model was assessed using ProSA online tool [21,22]. The stereochemical quality of the tertiary model was examined using the Ramachandran plot on the Cambridge RAMPAGE server. YASARA was used to visualize the protein tertiary structure.

3. Results and Discussion

3.1. MIBRs Conservation in Common Pneumococcal Serotypes

We already identified MIBRs of autolysin, ZBL, and PSP from serotype 19F *S. pneumoniae* [15]. The amino acid sequences of these proteins from other common pneumococcal serotypes including 14, 6B, 1, 23F, 5, 6A, 19A, 9V, 18C, 2, 4, 7F, 12F, 3, 8, 46, 15B, 45 and 9A were retrieved from NCBI. However, we did not find any amino acid sequences for these proteins from serotype 12A *S. pneumoniae*. The SIM tool analysis revealed that the identified MIBR of autolysin was present in autolysins of the pneumococcal serotypes at more than 93% amino acid sequence match. In addition, MIBR of ZBL was present in ZBLs of the pneumococcal serotypes at more than 97% amino acid sequence match. MIBR of PSP was available in PSPs of the pneumococcal serotypes at more than 98% amino acid sequence match. Amino acid sequence alignments using Clustal Omega demonstrated that the identified MIBRs were fully conserved in the pneumococcal serotypes (Figure 1 - Figure 3). Therefore, the identified MIBRs can elicit protection against the common pneumococcal serotypes.

3.2. Design of Chimerical Vaccine

The chimerical vaccine was composed of MIBRs of autolysin, ZBL, and PSP joining together using the $(EAAAK)_4$ linker. A histidine tag was introduced at the C-terminus of the construct. MIBRs orders affected the protective ability of the chimerical vaccine as indicated by VaxiJen scores (Table 1). 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 [16]. The chimerical antigen containing MIBRs of autolysin, PSP, and ZBL respectively (APZ) had the highest VaxiJen score and was used for further analysis.

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CIW56807 (14)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CIS99374 (6B)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CIT07703 (1)        FIQSADGTGWYYLKPDGTLVDKPDFTVEP
CGF65840 (23F)      FIQSADGTGWYYLKPDGTLADKPEFTVEP
CIU11224 (5)        FIQSADGTGWYYLKPDGTLADKPEFTVEP
CJR67591 (6A)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CYJ81225 (19A)      FIQSADGTGWYYLKPDGTLADKPEFTVEP
CJN80488 (9V)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CIT79207 (18C)      FIQSADGTGWYYLKPDGTLADKPEFTVEP
CEV52024 (2)        FIQSADGTGWYYLKPDGTLADKPEFTVEP
CIP53137 (4)        FIQSADGTGWYYLKPDGTMADKPDFTVEP
CIT14838 (7F)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CYL89407 (12F)      FIQSADGTGWYYLKPDGTLADKPEFTVEP
CEV72468 (3)        FIQSADGTGWYYLKPDGTLADKPEFTVEP
CYK24891 (8)        FIQSADGTGWYYLKPDGTLADKPEFTVEP
COP97599 (46)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CYM09340 (15B)      FVQSADGTGWYYLKPDGTLADKPDFTVEP
COB52764 (45)       FIQSADGTGWYYLKPDGTLADKPEFTVEP
CMZ24647 (9A)       FIQSADGTGWYYLKPDGSMADKPEFTVEP
WP_000405240 (19F)  FIQSADGTGWYYLKPDGTLADKPEFTVEP
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Figure 1. Amino acid sequence alignments of autolysin MIBRs using Clustal Omega**.** Accession numbers of autolysins and the pneumococcal serotypes were given besides sequences of MIBRs. * indicated identical amino acids in alignments and other symbols showed different amino acids in alignments

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ACB91389 (14)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CIS80688 (6B)       LKQTTDQEGPAIEPEKAEGTKTVQNGYFEDAAV
CYJ18336 (1)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CIV40182 (23F)      LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CGE92770 (5)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CTM03618 (6A)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CGF58250 (19A)      LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CJN37956 (9V)       LKQTTDQEGPAIEPEKAEDTKTVPNGYFEDAAV
CYL89341 (18C)      LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CEV49157 (2)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CEW33207 (4)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CIR20644 (7F)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CYL82783 (12F)      LKQTTDQEGPAIEPEKAEDTKTVPNGYFEDAAV
CEV72048 (3)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CIP13677 (8)        LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
COP78223 (46)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CIR46457 (15B)      LKQTTDQEGPAIEPEKAEGTKTVQNGYFEDAAV
CON12282 (45)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
CMZ27921 (9A)       LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
WP_000724068 (19F)  LKQTTDQEGPAIEPEKAEDTKTVQNGYFEDAAV
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Figure 2. Amino acid sequence alignments of ZBL MIBRs using Clustal Omega**.** Accession numbers of ZBLs and the pneumococcal serotypes were given besides sequences of MIBRs. * indicated identical amino acids in alignments and the space showed different amino acids in alignments

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CIW52344 (14)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CEY90865 (6B)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIS79081 (1)        KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIV22281 (23F)      KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQINDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIU16151 (5)        KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CGF29401 (6A)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CYK11415 (19A)      KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIT49374 (9V)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CJR69921 (18C)      KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CEV50364 (2)        KIVTNNLSDKNAKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIP49052 (4)        KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIQ94844 (7F)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
COJ08935 (12F)      KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIT02691 (3)        KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
COB19615 (8)        KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
COP56736 (46)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CIR32168 (15B)      KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNREFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
COM50295 (45)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
CMZ06186 (9A)       KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
WP_000834749 (19F)  KIVTNNLSDKNEKEKNKEEKQSNSNNVIDSNQKNGEFNSSKDNRQMNDKIDNKQDNKTEEVNYKIVGDGRETENHIN
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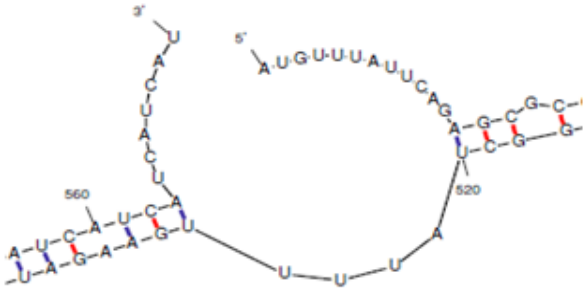
Figure 3. Amino acid sequence alignments of PSP MIBRs using Clustal Omega**.** Accession numbers of PSPs and the pneumococcal serotypes were given besides sequences of MIBRs. * indicated identical amino acids in alignments. The space and**:** showed different amino acids in alignments

Table 1. Effect of MIBRs orders on chimerical vaccine immunoprotection

Chimerical vaccine construct						VaxiJen score
$MIBR_{PSP}$	**$(EAAAK)_4$**	**$MIBR_{Autolysin}$**	**$(EAAAK)_4$**	**$MIBR_{ZBL}$**	**His tag**	1.2140
$MIBR_{PSP}$	**$(EAAAK)_4$**	**$MIBR_{ZBL}$**	**$(EAAAK)_4$**	**$MIBR_{Autolysin}$**	**His tag**	1.1891
$MIBR_{Autolysin}$	**$EAAAK)_4$**	**$MIBR_{PSP}$**	**$(EAAAK)_4$**	**$MIBR_{ZBL}$**	**His tag**	1.2216



Stack	-2.10	External closing pair is A_{13}-U_{520}
Stack	-3.40	External closing pair is G_{14}-C_{519}
Helix	-5.50	3 base pairs.
Bulge loop	0.50	External closing pair is C_{15}-G_{518}
Stack	-2.40	External closing pair is C_{17}-G_{517}

Figure 4. The mRNA secondary structure and free energy details at the 5' end of chimerical vaccine predicted by Mfold

Adapting the gene codon usage to the host codon usage can enhance the expression level of the encoded protein. The codon optimization includes altering the 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 [17]. Results of OPTIMIZER showed that CAI of native chimerical *apz* gene was 0.673. However, the codon optimized *apz* gene sequence 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 [17]. Therefore, the codon usage of the optimized *apz* 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*. The GC content of the native *apz* was increased from 30.9% to 49.6% upon codon optimization. The GC content of the gene affects its codon usage [23].

The gene expression level correlates inversely with the stability of mRNA hairpin structures close to the 5' end of mRNA [24]. The 5' end of the optimized *apz* mRNA did not constrain any stable hairpins as predicted by Mfold (Figure 4). Therefore the chimerical vaccine is expected to be expressed at appropriate levels.

3.3. Structures of Chimerical Vaccine

Results of the secondary structure prediction using YASARA indicated that the alpha helix, turn and coil are the structural elements of APZ (Table 2). The tertiary structure of the chimerical vaccine was predicted using I-TASSER. I-TASSER predicts 5 models for the chimerical vaccine (data not shown). The most confident model given for APZ (Figure 5) had confidence scores (C-score) of -3.61. C-score is in the range of -5 to 2 and a higher C-score indicates a model with a higher confidence [21]. The quality of the 3D model given for APZ was checked using ProSA and the z-score was within the range of typical scores of native proteins at the similar sizes (Figure 6). Therefore APZ had features, which were characteristics of native structures. In the Ramachandran plot (Figure 7), 92.6% of the amino acid residues (favorable region: 74.5% and allowed region: 18.1%) occupied the desired space. These results indicated the appropriate structural stability of the chimerical vaccine.

Table 2. Percentages of APZ secondary structure elements estimated using YASARA

Secondary structure element	APZ
Alpha helix	51.6%
Beta sheet	0%
Turn	14.7%
Coil	33.7%

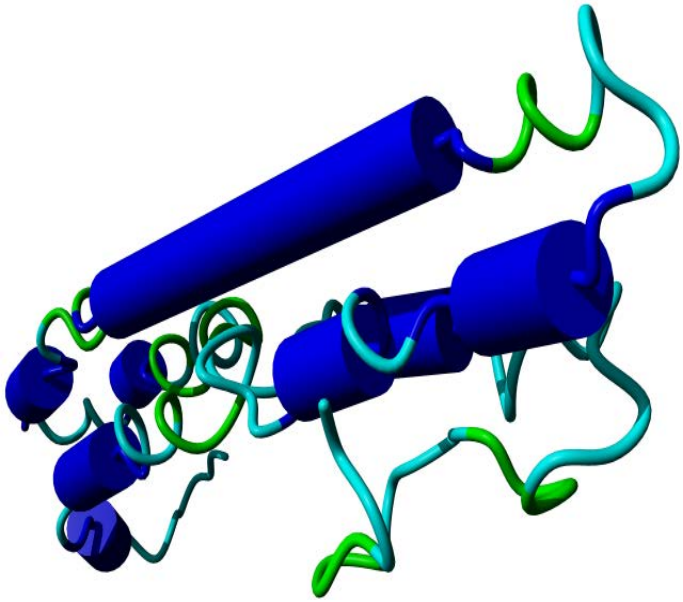


Figure 5. Tertiary structure of chimerical vaccine obtained using I-TASSER. Alpha helix was shown as cylinder. Coils and turns were shown in light blue and green respectively

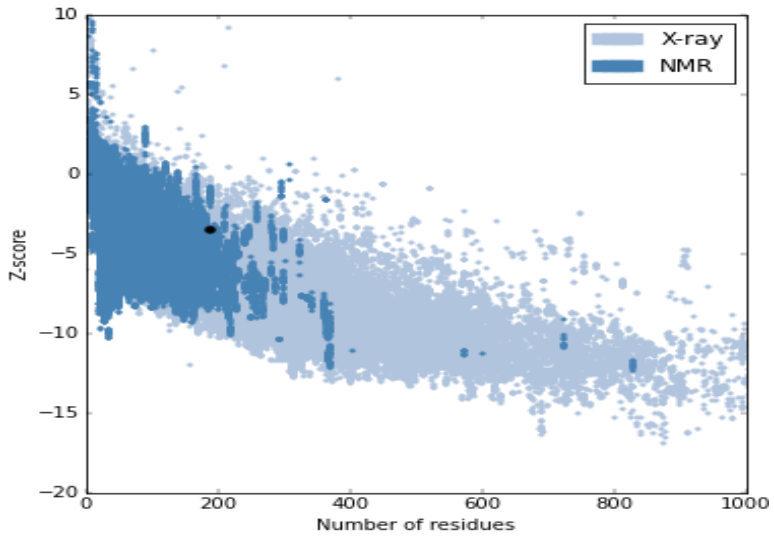


Figure 6. Z-score plot of chimerical vaccine obtained using ProSA. The Z-score of APZ was shown as a large black dot indicated by an arrow

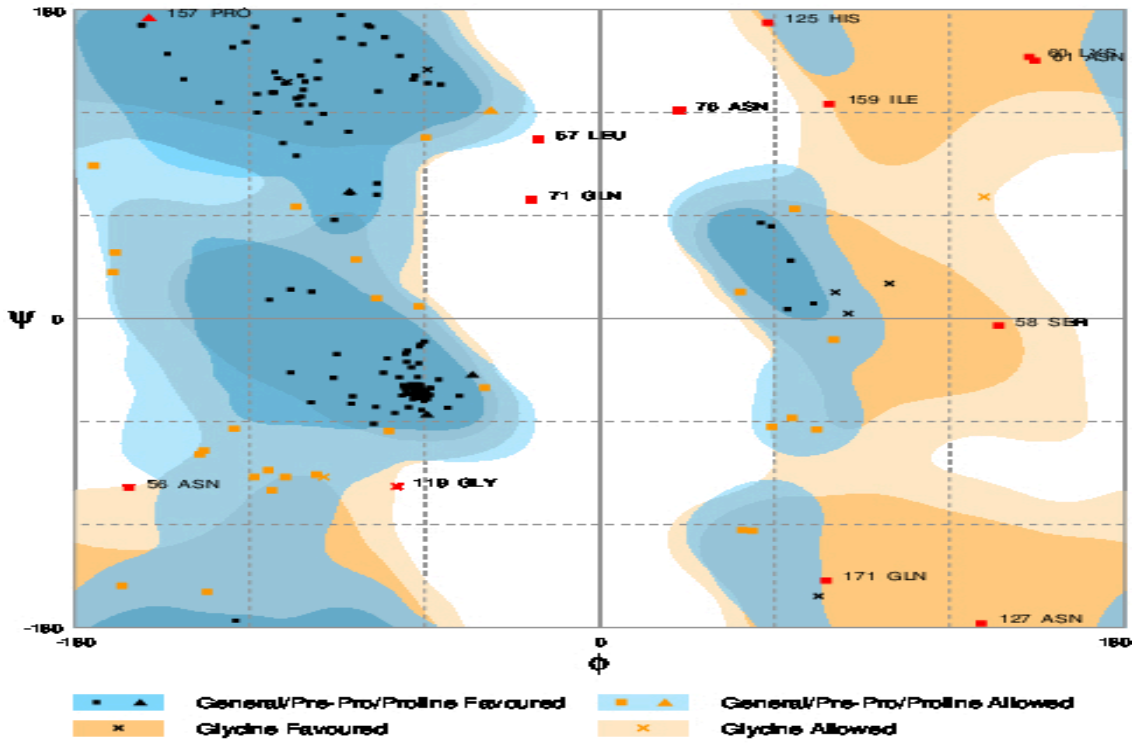


Figure 7. Ramachandran plot of chimerical vaccine tertiary structure

3.4. Physicochemical Properties of Chimerical Vaccine

PROTPARAM analysis revealed that the average molecular weight (MW) of APZ was 20.88 kDa and the isoelectric points (pI) of the chimerical antigen was 5.35 (Table 3). The estimated half life of the antigen in *E. coli* was more than 10 h. The instability index (II) provides an estimate of the protein stability in a test tube. Proteins with II values lower than 40 are considered as stable [19]. The II value of APZ was less than 40 that indicated the antigen stability. The grand average of hydropathicity (GRAVY) score is used to evaluate the hydropathical character of proteins. A negative GRAVY score indicates a hydrophilic protein [19]. The negative GRAVY score of APZ therefore indicated that it can interact with water molecules.

Table 3. Physicochemical properties of chimerical vaccine calculated by Protparam

Protein	Mwt	pI	Half life in *E. coli*	Half life in yeast	Half life in mammalian reticulocyte	II	AI	GRAVY score
APZ	20.879 kDa	5.35	> 10 h	> 20 h	30 h	35.56	49.05	- 1.267

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 [25,26]. PROSOII was used to assess the chance of a protein to be soluble upon heterologous expression in *E. coli* based on its amino acid sequence composition. The solubility probability of APZ was 0.922. Therefore, the majority of the protein is expressed in the soluble form in *E. coli* and can then be recovered from the soluble cellular fraction instead of inclusion bodies.

4. Conclusions

The chimerical vaccine designed in this study can elicit protection against *S. pneumoniae* independent of the serotype. It can be expressed at appropriate levels in *E. coli* in the soluble form and is stable. Therefore, APZ is a suitable proteinaceous vaccine candidate against *S. pneumoniae*.

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