In silico analysis for identifying potential vaccine candidates against Staphylococcus aureus

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Purpose: Staphylococcus aureus is one of the most important causes of nosocomial and community-acquired infections. The increasing incidence of multiple antibiotic-resistant S. aureus strains and the emergence of vancomycin resistant S. aureus strains have placed renewed interest on alternative means of prevention and control of infection. S. aureus produces a variety of virulence factors, so a multi-subunit vaccine will be more successful for preventing S. aureus infections than a mono-subunit vaccine.

Materials and Methods: We selected three important virulence factors of S. aureus, clumping factor A (ClfA), iron-regulated surface determinant (IsdB), and gamma hemolysin (Hlg) that are potential candidates for vaccine development. We designed synthetic genes encoding the clfA, isdB, and hlg and used bioinformatics tools to predict structure of the synthetic construct and its stabilities. VaxiJen analysis of the protein showed a high antigenicity. Linear and conformational B-cell epitopes were identified.

Results: The proteins encoded by these genes were useful as vaccine candidates against S. aureus infections.

Conclusion: In silico tools are highly suited to study, design, and evaluate vaccine strategies.

Keywords: Computer simulation, Staphylococcus aureus, Vaccines

Introduction

Staphylococcus aureus is a gram-positive bacterium, capable of causing infections in humans, including a variety of skin infections, as well as a variety of systemic infections such as bacteremia, osteomyelitis, ventilator-associated pneumonia, intravenous catheter-associated infections [1]. In the past two decades, a significant increase in the incidence of methicillin-resistant Staphylococcus aureus has shown in many countries in worldwide [2]. The high prevalence in multidrug-resistant S. aureus and the emergence of vancomycin resistant isolates, have led researchers to look for alternative treatment approaches, such as vaccines and therapeutic antibodies. S. aureus produces several virulence factors, so efforts to develop effective vaccines against this organism have been largely unsuccessful [3]. In this regard, many antigens have been evaluated in the search to find a vaccine with the potential to protect against staphylococcal diseases.

Clumping factor A (ClfA), a fibrinogen binding surface protein, is one of the major virulence factors which can induce clumping of S. aureus in blood plasma [4]. ClfA is an attractive target for active and passive immunization studies, because it can induce
strong immune responses in rabbit and rat models of infective endocarditis and in a mouse model of septic arthritis [5]. There is a signal sequence for Sec-dependent secretion at the N terminus of ClfA structure, whereas the C terminus contains an LP XTG motif. Previous studies have localized the Fg-binding activity of ClfA to the N-terminal A region of this protein [6]. Iron surface determinant B (IsdB) is an iron-sequestering protein that is expressed in diverse S. aureus clinical isolates [7]. The biological role of IsdB has been demonstrated in numerous studies, suggesting that IsdB is a major virulence factor of S. aureus [7]. Previous studies have identified IsdB as a candidate vaccine against S. aureus infections [8]. Immunological studies on IsdB binding regions determined that amino acids residues 130 to 454 of IsdB protein are required for binding, and monoclonal antibodies (MAbs) bind to this portion effectively [9]. S. aureus also produces the gamma hemolysin toxin, which functions as two component toxin in the disruption and lysis of erythrocytes and leukocytes [10]. Gamma hemolysin locus (Hlg) contains three open reading frames, h1ga, h1gb, and h1gc genes. The three encoded proteins are all translated with a single sequence [10]. IgG antibody levels against gamma hemolysin B (HlgB) were higher in S. aureus infected patients than in healthy individuals [11]. Gamma hemolysin is produced by more than 99% of S. aureus strains [12]. As elucidated above, ClfA, IsdB, and Hlg play key roles in the pathogenesis of S. aureus infections. Therefore, in the present study, we designed a novel multi-subunit antigen that provides a suitable and safe vaccine candidate against S. aureus infections. Advanced analysis for vaccine development, such as in silico tools and bioinformatics database can be applied for vaccine development against many bacterial infections [13].

Briefly, three putative antigenic determinants of ClfA, IsdB, and Hlg, were bound together by two hydrophobic linkers. Then, chimeric protein structure was analyzed through an in silico approach. The results are discussed in the following paragraphs.

Materials and Methods

Sequences, databases and structure design

Related sequences for clfA, isdB, and hlg genes were obtained from publicly available sequence databases, primarily from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using ClustalW software of European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/clustalw2), to find a common fragment in all the sequences. Antigenic sequences within the genes (clfA, isdB, and hlg) were selected and fused together by hydrophobic linkers to design a new synthetic chimeric gene. Online database for gene optimization was used to optimize the chimeric gene (http://www.jcat.de/) [14]. The chimeric gene was designed for cloning and expression in Escherichia coli. Vaxijen server was used to predict the immunogenicity of each protein alone and in combination with each other (http://www.jenner.ac.uk/VaxiJen) [15].

Prediction of mRNA secondary structure

The analysis of the messenger RNA secondary structure of chimeric gene was performed by mfold software. The portal for the mfold web server is http://www.bioinfo.rpi.edu/applications/mfold [16].

The physico-chemical parameters

The physico-chemical parameters of the chimeric protein including molecular weight, extinction coefficient, half-life, instability index, theoretical isoelectric point (pI), grand average of hydropathy (GRAVY) and total number of positive and negative residues were obtained using the Expasy ProtParam (http://us.expasy.org/tools/protparam.html/) [17].

Secondary and tertiary structure prediction

The protein secondary structure prediction was performed by Garnier-Osguthorpe-Robson (GOR) secondary structure prediction server [18]. I-TASSER ab initio online software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the three-dimensional structure [19]. The structure was validated to see the quality of the resulting stereochemistry of structure by Ramachandran plot in PROCHECK software (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK).

Prediction of antigenic B-cell epitopes

The amino acid sequence of chimeric protein was analyzed using the software based on B-cell epitope prediction algorithms to predict continuous and discontinuous B-cell epitopes. The first, chimeric protein was analyzed for continuous B-cell epitopes using Bcepred (http://www.imtech.res.in/raghava/bcepred/) [20]. Discotope server we then used (http://www.cbs.dtu.dk/services/DiscoTope) [21] to predict discontinuous B-cell epitopes from three-dimensional protein structures.
Results

Sequences, databases, and structure design
The residues 500-559 in the N-terminal region of ClfA, have been reported to be involved in polyclonal antibodies generated against a ClfA recombinant protein. The amino acids residues 130 to 454 of IsdB protein are required for binding, and MAb bind to this portion effectively. For the third fragment, the amino acid residues 26 to 76 of Hlg protein were selected. Based on sequences compared by ClustalW, N-terminal A region of ClfA (59 amino acids), IsdB (324 amino acids), and Hlg (50 amino acids) it was shown that these three parts were highly conserved among different strains of S. aureus. Linkers consisting of EAAAKEAAAKEAAAK repeats and DPRVPSS repeats were used to separate these three domains from each other. It was shown that helix formation can be stabilized by using these linkers between the three domains. The BamHI and HindIII restriction sites for cloning in prokaryotic vectors were successfully introduced at the N- and C-terminal of sequence, respectively. Schematic diagram of protein domain structures with linker’s sites designed is shown in Fig. 1. Codon bias and GC content were calculated in both wild type and chimeric genes. The codon adaptation index (CAI) of chimeric gene was 0.99, while that of wild type gene was only 0.60.

The GC content was reduced from 45.56% to 45.06%, so it is expected an increase in the mRNA stability of the chimeric gene (Fig. 2). Antigen index by VaxiJen server for ClfA, IsdB, Hlg, and chimeric gene was 0.60, 0.47, 0.58, and 0.58, respectively.

mRNA structure prediction
The minimum free energy for secondary structures formed by RNA molecules was appointed. All 28 structural elements obtained in this investigation showed RNA folding. ΔG of the best predicted structure was \(-336.80\) kcal/mol (Fig. 3A) and the first nucleotides at 5’ did not have a hairpin or pseudoknot (Fig. 3B). In native mRNA, first nucleotides didn’t also construct pseudoknot and the ΔG of the structure was \(-279.30\) kcal/mol (Table 1).

The physico-chemical parameters
The average molecular weight of chimeric protein was calculated 53.2 kDa. Isoelectric point (pl) was defined as the pH at which the surface of protein is covered with charge but net charge of the protein is zero. Acidity of the protein was indi-

![Fig. 1. Schematic representation of Staphylococcus aureus antigenic construct consists of clfA, isdB, and hlg genes bound together by appropriate linkers for expression in Escherichia coli.](image)

![Fig. 2. Analysis of wild type and optimized chimeric gene based on codon usage.](image)

![Fig. 3. (A) Prediction of RNA secondary structure of chimeric gene using mfold algorithm. (B) Predicted structure has no hairpin and pseudoknot at 5’ site of mRNA.](image)

| Structure          | Free energy (kcal/mol) | Base pair                              |
|--------------------|------------------------|----------------------------------------|
| Helix              | -6.70                  | 4 Base pairs                           |
| Hairpin loop       | 4.20                   | Closing pair, G834-C846                 |
| Stack              | -2.40                  | External closing pair, U4-A803          |
| Stack              | -3.30                  | External closing pair, C5-G808          |
| Stack              | -2.10                  | External closing pair, C4-G807          |

![Table 1. Free energy details related to 5’ end of chimeric gene mRNA structure by mfold web server](image)
cated by pI value (pI<7.75).

Extinction coefficient of chimeric protein at 280 nm was 59,250/M/cm. The biocomputed half-life was greater than 10 hours. Expasy ProtParam classifies the chimeric protein as stable protein with instability index, 30.11. Aliphatic index and GRAVY of chimeric protein was 67.94 and -0.806, respectively.

**Secondary and tertiary structure prediction**

The chimeric protein of secondary structure was obtained by online software. In order to validate our secondary structure prediction method, first the clfA, isdB, and hlg were used as test sequences. We obtained predicted structural elements using software GOR IV (http://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html). The results show that, structural contents of protein include extended strand, random coil, and alpha helix.

Composition of secondary structure predicted for chimeric protein was 29.25% alpha helix, 25.81% extended strand, and 44.95% random coil (Table 2). Analysis of the amino acid composition demonstrated two regions with a low sequence complexity. These regions have linker sequences. The secondary structure prediction of the protein is shown in Fig. 4. Comparative and *ab initio* modeling of the synthetic sequence was used to get the chimeric protein three-dimensional models. The three-dimensional modeled structure for protein was generated by I-TASSER software. Results of tertiary structure of the fusion protein construction using I-TASSER showed a protein with three main domains linked together with linkers (Fig. 5). The confidence score (C-score) of models predicted by I-TASSER was -0.22. C-score is usually in the range of -5 to 2 and the higher the value of C-score, the higher the model confidence. In addition, the template modeling (TM)-score and root-mean-square deviation (RMSD) for this model was 0.68±0.12 and 7.7±4.3, respectively.

Analysis of chimeric protein by using the Ramachandran plot showed that 75.3% of amino acid residues from the structure modeled by I-TASSER were incorporated into the favored regions of the plot. Apart from that, 14.6% of residues were in allowed regions of the plot and 10.1% in outlier region (Fig. 6).

**Prediction of B-cell epitopes**

The chimeric protein of continuous B-cell epitopes was predicted by Bcepred software. All 20-mers in B-cell epitopes having Bcepreds cutoff score>0.8 were selected (Table 3). Another selection for the chimeric protein of continuous B-cell epitopes was performed based on single characters including hydrophilicity, antigenicity, flexibility, accessibility, polarity, and exposed surface, as shown in Table 4. The results of this investigation included peptides and their corre-

| Sequence | Alpha helix | Extended strand | Random coil |
|----------|-------------|-----------------|-------------|
| clfA | 0.00 | 37.29 | 62.71 |
| isdB | 31.23 | 26.73 | 42.04 |
| hlg | 20.00 | 26.00 | 54.00 |
| Chimeric gene | 29.25 | 25.81 | 44.95 |

Values are presented as percentage.

![Graphical results for secondary structure prediction of chimeric protein. Purple, red, and blue colors indicate extended strand, coil, and helix, respectively.](image-url)
corresponding threshold scores. The higher the threshold score means the higher the specificity and binding affinity.

Furthermore, the results show no epitope at linker sites between different domains (amino acid 60 to 75 and amino acid 410 to 417). The Discotope server used to predict the chimeric protein of discontinuous B-cell epitopes showed 90 B-cell epitope residues out of 113 total residues identified (Table 5).

**Table 3. B-Cell epitopes from full length proteins using Bcepred**

| Position | Epitope                                      | Bcepred score |
|----------|----------------------------------------------|---------------|
| 46       | EQDEPEPGEIPEPEAAAKE                          | 1             |
| 25       | NEVAFNGSGGODKPV                             | 0.999         |
| 349      | TISDKNTRTIIPYEG                             | 0.977         |
| 99       | EMKKKDGTQQFYHYASSV                              | 0.985         |
| 405      | DKSHPVPSSGKTPVS                              | 0.984         |
| 292      | DTKVYVYEVENNESMMVDTF                         | 0.972         |
| 78       | IKPAKDKDHASPNRID                             | 0.948         |
| 430      | VTLYKTATADSKFKISOQ                           | 0.947         |
| 327      | MVMETNDDYWKOMWEGQR                           | 0.924         |
| 181      | IVSTHFIKKEKEYDYTLME                         | 0.912         |
| 225      | APYKKAKTLERQVYNOKIQ                          | 0.863         |
| 202      | AQIPYNSADKFKTEEDYKAE                        | 0.764         |

**Discussion**

In the past decade, increased levels of *S. aureus* strains resistant to antimicrobial agents have become an important problem in medical centers around the world. No vaccine currently exists to prevent *S. aureus* infections. So, it is urgently needed to search for finding an effective vaccine in order to prevent and reduce *S. aureus* infections, especially individuals who are at high risk for *S. aureus* infections such as patients hospitalized for long periods, undergoing surgery, suffering from chronic illnesses and dialysis [7]. Over the past several decades, different virulence factors have been tested as vaccines to prevent staphylococcal infections, including alpha hemolysin, PVL, ClfA, fibrinogen binding protein, enolase (laminin binding protein), and protein A. The main problem for designing an effective vaccine for *S. aureus* is that this organism can produce several potential virulence factors. But this problem can be solved using multi-component vaccines. Many studies have also demonstrated that a multi-subunit vaccine are more successful in preventing *S. aureus* infections than a mono-subunit vaccine [22]. The most important step is to select suitable candidate antigens for vaccine design. Appropriate candidate antigens should be expressed by the majority of clinical *S. aureus* isolates and generate strong immune response in animal models [8].

In present study, we selected Hlg, ClfA, and IsdB proteins that play an important role in *S. aureus* infections and are produced by more than 99% of *S. aureus* strains. For designing
our chimeric vaccine as a chimeric protein, we selected epitopes from residues 500-559 of ClfA, amino acid residues 130 to 454 of IsdB, and the first 50 amino acid residues after Hlg signal peptide, because polyclonal antibodies generated against these regions. The constructed fusion proteins require suitable linkers to bind protein domains. In order to separate the different domains of our chimeric protein, linkers consisting of EAAAK repeats and DPRVPSS repeats expected to form a monomeric hydrophobic α-helix and random coil were designed. Our successful experience in using these linkers in chimeric gene has shown that it could lead to logically acceptable results [23]. These linkers could significantly control the distance and reduce the interference between the domains. Codon optimization has a great advantage in designing synthetic genes. We were performed codon optimization to enhance recombinant protein production in E. coli. CAI on the optimized gene sequence was 0.99 compared to that of the wild type gene, which was only 0.60, indicating that the optimized gene sequence could be expressed well. VaxiJen server also showed that chimeric gene was immunogenic. Messenger RNA secondary structure has a major role in the expression of proteins, such as the regulation of

Table 4. Epitopes predicted in chimeric protein by different parameters based on Bcepred software

| Prediction parameter | Epitope sequence |
|----------------------|------------------|
| Hydrophilicity       | NNGSSGSGDIDK, VPEQDPDEPGEE, EAAAEKAAAEAAAKRE, IDKDHASPN, EMKDKDGTQQ, DSKPEIE, EVYEGDK, SYDVKD | |
| Flexibility          | VAFNNGSS, PAIKDOHASP, IDFMEMKKKD, VIFTDSE, IFSEVSSNGT, THFNKNE, AEYKKEK, ONQOPTN, RTISDOK | |
| Accessibility        | SSVKPAR, FTTSDKPEI, SGQVWRFEVYEGDKQLPKI, SYDVMKYV, NGTAKV, STHFNKEEKDYTM | |
| Tums                 | GYNSNII, VAFNNGSS, STSFHNKE, ETTNDYYW | |
| Exposed surface      | PEQDPEP, KEAAAEKAAKnPAIKDKHQSA, IDKMEMKKDGTQQ, VYEGDKKLPIK, HFNKMKEKYDYT, DFKKTEYDKAEK, AYVKAKTLE, LNKKDDKLPKIIKAEYKKKLEDT, VQFTNPKE, NDDYWWK, SKDACKNTR, TKAINTKD, VQKDIVDK, VDKSKEKDT | |
| Polarity             | DEPEGPDEP, KEAAAEKAAKnPAIKDKHQSA, IDKMEMKKDGTQQ, VYEGDKKLPIK, HFNKMKEKYDYT, DFKKTEYDKAEK, AYVKAKTLE, LNKKDDKLPKIIKAEYKKKLEDT, VQFTNPKE, NDDYWWK, SKDACKNTR, TKAINTKD, VQKDIVDK, VDKSKEKDT | |
| Antigenic propensity | GIDKPVQVEQPD, KLPKIVSYSYITKV, IFFSVS, VIKVSSTHF, QDITKYWYESVE, TFVPHKIP, TIIFFPYVE, IVKHVAKTLE, QYHVIRVKDD, KITPVSVVKKVD | |

Table 5. Conformational B-cell epitopes from full length proteins using DiscoTope server

| Start-end position | Start-end | Start-end | Start-end | Start-end | Start-end | Start-end |
|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| 4-288              | 12-305    | 5-320     | 19-334    | 12-348    | 25-361    | 3-382     |
| 10-289             | 10-307    | 17-321    | 10-335    | 15-349    | 34-362    | 3-382     |
| 11-290             | 23-309    | 10-322    | 25-333    | 18-350    | 8-364     | 8-383     |
| 14-191             | 2-310     | 1-323     | 33-337    | 11-351    | 20-362    | 1-384     |
| 6-292              | 22-311    | 11-324    | 13-338    | 10-352    | 4-367     | 14-385    |
| 10-294             | 30-312    | 14-325    | 14-339    | 11-353    | 1-366     | 1-384     |
| 17-296             | 19-313    | 21-325    | 31-340    | 1-354     | 7-370     | 13-387    |
| 6-299              | 16-314    | 25-329    | 9-341     | 1-355     | 8-373     | 4-388     |
| 7-300              | 10-315    | 32-329    | 12-343    | 12-356    | 7-375     | 23-389    |
| 1-301              | 31-316    | 15-330    | 1-344     | 9-357     | 8-377     | 9-390     |
| 8-302              | 17-317    | 33-331    | 10-345    | 11-358    | 23-378    | 8-397     |
| 8-303              | 21-318    | 11-332    | 7-346     | 19-359    | 5-379     | 11-400    |
| 2-304              | 16-319    | 15-333    | 30-347    | 13-360    | 9-380     |
gene expression that is highly dependent on forming stable structures by nucleotide pairing in 5’ terminus [24]. We used mfold software to predict RNA secondary structure. The data from mRNA prediction showed that the mRNA was stable enough for efficient translation in the host. 5’ Untranslated regions (5’-UTRs) of mRNAs play important roles in increasing the translation rates, half-lives, numbers of transcripts, and upregulation after heat shock. In our investigation, the first nucleotides at 5’ of mRNAs did not show a hairpin or pseudoknot and the folding free energies of 5’-UTRs were higher than other genomic regions. Therefore, with these 5’-UTRs of mRNAs, we can expect to increase the translation rates of chimeric protein in the host.

When we were analyzed the physico-chemical parameters of our chimeric gene by ProtParam software, the pI value (pI<7.75) showed acidic nature of the protein. Extinction coefficient of chimeric protein at 280 nm was high. Expasy ProtParam classifies the chimeric protein as stable on the basis of instability index (instability index, 30.11). One of the most significant problems in designing a recombinant protein is to have functional properties of the protein sequence. This problem is usually solved by accurate three-dimensional structure of the protein. Our result showed that ab initio I-TASSER software could predict the folds and good resolution model for our chimeric protein. Both RMSD and TM-score were used to evaluate the predicted models. Expected TM-score of 0.68 ± 0.12 validates the accuracy of model.

A correct topology model usually has a TM-score more than 0.5. Z-score and C-score also show its confidence. Both measure the deviation of the total energy of the structure with respect to an energy distribution derived from random conformations and overall model quality are determined by Z-score [19]. Native proteins with Z-scores outside a range characteristic indicate erroneous structures. Our chimeric structure revealed a protein with full stability based on Ramachandran plot predictions. In Ramachandran plot analysis, nearly, a negligible 2% of the residues were found to be in outlier region that could probably be due to the presence of chimeric junctions.

A crucial step in the development of bacterial vaccine based on recombinant protein is to identify B-cell epitopes. B-cell epitopes are specific regions of an antigenic surface protein. There are two different types of epitopes: continuous (linear) epitopes and discontinuous (conformational) epitopes. Bioinformatics analysis for reliably predicting continuous B-cell epitopes are very useful. In this study, we have identified continuous B-cell epitopes of the chimeric protein based on several different methods such as antigenicity accessibility, hydrophobicity, secondary structure and flexibility analysis. The analysis of all continuous B-cell epitopes was performed completely to obtain good results, without any missing. The epitopes located on protein surface could interact easily with antibodies, and they were generally flexible. Another important step in immunological studies is to identify discontinuous epitopes that are essential for antibody-antigen interaction. DiscoTope software recognized 90 conformational epitopes for B cells. DiscoTope uses tertiary structure which seems to be more reliable than other software.

In summary, we have combined several bioinformatics techniques for immunogenicity prediction of chimeric protein. Our data indicates that epitopes of our chimeric protein, designed from ClfA, IsdB, and Hlg of S. aureus could induce B-cell-mediated immune responses successfully. Therefore, our chimeric protein is suggested as a vaccine candidate against S. aureus infections. As the next steps to complete the initial studies on vaccine development, were recommend in vitro synthesis and in vivo experimental studies to test the efficacy of the chimeric protein.

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References

1. Spellberg B, Daum R. Development of a vaccine against Staphylococcus aureus. Semin Immunopathol 2012;34: 335-48.
2. Stranger-Jones YK, Bae T, Schneewind O. Vaccine assembly from surface proteins of Staphylococcus aureus. Proc Natl Acad Sci U S A 2006;103:16942-7.
3. Adhikari RP, Karauzum H, Sarwar J, et al. Novel structurally designed vaccine for S. aureus alpha-hemolysin: protection against bacteremia and pneumonia. PLoS One 2012;7:e38567.
4. Hall AE, Domanski PJ, Patel PR, et al. Characterization of
a protective monoclonal antibody recognizing Staphylococcus aureus MSCRAMM protein clumping factor A. Infect Immun 2003;71:6864-70.

5. Ganesh VK, Rivera JJ, Smeds E, et al. A structural model of the Staphylococcus aureus CIFA-fibrinogen interaction opens new avenues for the design of anti-staphylococcal therapeutics. PLoS Pathog 2008;4:e1000226.

6. Hartford OM, Wann ER, Hook M, Foster TJ. Identification of residues in the Staphylococcus aureus fibrinogen-binding MSCRAMM clumping factor A (CIFA) that are important for ligand binding. J Biol Chem 2001;276:2466-73.

7. Kuklin NA, Clark DJ, Secore S, et al. A novel Staphylococcus aureus vaccine: iron surface determinant B induces rapid antibody responses in rhesus macaques and specific increased survival in a murine S. aureus sepsis model. Infect Immun 2006;74:2215-23.

8. Schaffer AC, Lee JC. Vaccination and passive immunisation against Staphylococcus aureus. Int J Antimicrob Agents 2008;32 Suppl 1:S71-8.

9. Brown M, Kowalski R, Zorman J, et al. Selection and characterization of murine monoclonal antibodies to Staphylococcus aureus iron-regulated surface determinant B with functional activity in vitro and in vivo. Clin Vaccine Immunol 2009;16:1095-104.

10. Gouaux E, Hobaugh M, Song L. Alpha-hemolysin, gamma-hemolysin, and leukocidin from Staphylococcus aureus: distant in sequence but similar in structure. Protein Sci 1997;6:2631-5.

11. Verkaik NJ, Dauwalder O, Antri K, et al. Immunogenicity of toxins during Staphylococcus aureus infection. Clin Infect Dis 2010;50:61-8.

12. Prevost G, Cribier B, Couppie P, et al. Panton-valentine leukocidin and gamma-hemolysin from Staphylococcus aureus ATCC 49775 are encoded by distinct genetic loci and have different biological activities. Infect Immun 1995;63:4121-9.

13. Nazarian S, Mousavi Gargari SL, Rasooli I, Amani J, Bagheri S, Alorasool M. An in silico chimeric multi subunit vaccine targeting virulence factors of enterotoxigenic Escherichia coli (ETEC) with its bacterial inbuilt adjuvant. J Microbiol Methods 2012;90:36-45.

14. Grote A, Hiller K, Scheer M, et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res 2005;33:W526-31.

15. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics 2007;8:1.

16. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406-15.

17. Wilkins MR, Gasteiger E, Bairoch A, et al. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 1999;112:531-52.

18. Sen TZ, Jernigan RL, Garnier J, Kloczkowski A. GOR V server for protein secondary structure prediction. Bioinformatics 2005;21:2787-8.

19. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 2008;9:40.

20. El-Manzalawy Y, Dobbs D, Honavar V. Predicting flexible length linear B-cell epitopes. Comput Syst Bioinformatics Conf 2008;7:121-32.

21. Kringelum JV, Lundegaard C, Lund O, Nielsen M. Reliable B cell epitope predictions: impacts of method development and improved benchmarking. PLoS Comput Biol 2012;8:e1002829.

22. Schaffer AC, Lee JC. Staphylococcal vaccines and immunotherapies. Infect Dis Clin North Am 2009;23:153-71.

23. Amani J, Mousavi SL, Rafati S, Salmanian AH. In silico analysis of chimeric espA, eae and tir fragments of Escherichia coli O157:H7 for oral immunogenic applications. Theor Biol Med Model 2009;6:28.

24. Gaspar P, Moura G, Santos MA, Oliveira JL. mRNA secondary structure optimization using a correlated stem-loop prediction. Nucleic Acids Res 2013;41:e73.