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In-Silico Design of a Novel Multi-Epitope Fimbriae Vaccine against Non-typhoidal *Salmonella*

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ABSTRACT

Introduction: The most widespread gastrointestinal infection globally is attributed to non-typhoidal *Salmonella* (NTS). Given the rising prevalence of antibiotic-resistant strains and the absence of commercially available vaccines, there is a crucial need for research and development of new vaccines against NTS. The purpose of the present study was to design a multi-epitope vaccine targeting non-typhoidal *salmonella* serovars (*Salmonella typhimurium* and *Salmonella enteritidis*) based on fimbriae protein using an immunoinformatics approach. **Methods:** The sequences of the fimbriae protein were obtained and the predicted epitopes for B and T lymphocytes were identified. The epitopes' antigenic features, non-allergenicity, and non-toxicity were investigated, and the vaccine model was constructed. The characteristics of the vaccine were determined, and its effectiveness was evaluated through docking, molecular dynamics simulation involving the interaction between the vaccine and immune receptors, and immunological simulation. The vaccine was then optimized for cloning. **Results:** B and T lymphocyte-targeting vaccine construct was developed by selecting twelve epitopes. Immunoinformatics analyses predicted that the constructed vaccine is reliable and safe, hydrophilic, and exhibits stability under diverse temperatures and conditions. Additionally, it displayed the capability to bind to immune receptors TLR4, HLA-C, and HLA-DRB1. Moreover, this vaccine candidate stimulates antibody response and memory B and T cell activation after repeated injection. **Conclusion:** This study introduced the primary results of a novel multi-epitope vaccine against NTS based on adhesion protein. *In-silico* methods predicted that the proposed vaccine can potentially elicit an immune response and be expressed in the prokaryotic system.

INTRODUCTION

Food-borne gastrointestinal infections have spread due to population expansion and lifestyle changes. Non-typhoidal Salmonellosis, caused by *Salmonella enterica* serovar *typhimurium* and *enteritidis*, is a common gastrointestinal infection [1]. It is estimated that 93 million people worldwide suffer from intestinal infections, and 155,000 people die annually due to such infections [2]. Among them, children and the elderly are the most sensitive hosts [3]. *Salmonella* infection begins with an adhesive protein, known as fimbriae, attachment to intestinal epithelial cells and penetration into these cells. The bacteria are then placed inside vacuoles of the cell membrane to hide from the immune system [4, 5]. Fimbriae have an important role in *Salmonella* pathogenesis as a virulence factor [5]. This protein could stimulate T cell response [6]. Thus, fimbriae are a suitable candidate for vaccine development. Clinical reports show that salmonellosis is usually associated with symptoms such as heartache, diarrhea, fever, and, in some cases, septicemia. However, this infection is often self-limiting in mild cases, and antibiotics are administered if treatment is required [7].

Moreover, *Salmonella* infection is difficult to control due to its tolerance to environmental stress, widespread dispersion in the environment, and resistance to various antibiotics [8]. Studies have shown that *Salmonella* can suppress antibody responses and cause chronic infections [9]. Therefore, the control of *salmonella* infections is facing challenges that show the importance of preventing the onset of infection. As vaccine development is an effective strategy to prevent most infections, many efforts have been made in the field of vaccine development for non-typhoidal

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Salmonella (NTS); however, no commercial vaccine has been yet provided. Live attenuated, subunit, recombinant, and outer membrane vesicle vaccines have been considered as NTS vaccine candidates while no recent study has been done on bacterial fimbria for the vaccine development [10-13]. On the other hand, the vaccine production process requires a lot of time and money, and their effectiveness will not be determined until they are tested into the host. Therefore, immunoinformatics knowledge is presently used to minimize costs and increase the effectiveness of vaccines. Despite multiple *in-silico* vaccine studies conducted on the entire genome and outer membrane proteins of NTS [7, 10, 14], more research is necessary to evaluate the fimbriae as a component of a potential NTS vaccine candidate. Therefore in this study, the fimbriae protein of *Salmonella typhimurium* and *Salmonella enteritidis* (ST and SE) were targeted for the first time for designing a multi-epitope NTS vaccine.

MATERIALS AND METHODS

Fimbriae Protein Sequence Retrieval

The fimbriae protein ST (Accession No.: AKG31746.1 and KYF09125.1) and SE (Accession No.: QGK61610.1 and TYG30745.1) amino acid sequences were obtained from the NCBI protein database www.ncbi.nlm.nih.gov. Additionally, the similarity among fimbriae proteins in distinct strains of NTS was evaluated using BLAST tools (BLAST: https://blast.ncbi.nlm.nih.gov).

Prediction of T cell Epitope, CTL Epitopes Immunogenicity and Linear B Cell Epitope

In intracellular bacterial infections such as *Salmonella*, cytotoxic T lymphocytes (CTL) and helper T lymphocytes (HTL) are essential in removing infected cells [15]. For this purpose, the IEDB server http://tools.iedb.org/main/tcell with SMM and SMM-align prediction methods were used for MHC-I/II, respectively. To predict CTL and HTL activating epitopes by default, 52 alleles of MHC-I and 27 alleles of MHC-II were selected in the human population. The length of amino acids was designated as 9-mers for MHC-I and 15-mers for MHC-II. Ultimately, epitopes with an affinity $IC_{50} < 500$ nM were chosen for further analysis [16]. The immunogenicity of CTL epitopes was assessed using the IEDB server http://tools.iedb.org/immunogenicity/. Epitopes exhibiting positive scores were subsequently chosen for further consideration [17]. Antibodies are vital in neutralizing fimbriae binding to host cells. Hence, identifying B lymphocyte-activating epitopes is necessary to stimulate antibody production. The ABCpred server was utilized to predict linear B cell epitopes, with a 16 amino acid length and a threshold set at 0.51. https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html [18].

Antigenicity, Allergenicity and Toxicity Prediction

For a vaccine design, the epitopes must be antigenic, non-allergenic, and non-toxic. To determine antigenicity with a threshold limit of ≥ 0.4, the VaxiJen-v2 server http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html was used. The AllerTOP v.2.0 server http://www.ddg-pharmfac.net/AllerTOP/ evaluated the allergenicity of epitopes based on similarity to known allergen epitopes. Additionally, the ToxinPred server https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php was used for the assessment of the possible toxicity of epitopes [19].

Analysis of Population Coverage

The analysis of population coverage and MHC restriction for the predicted epitopes in different regions was performed using default consensus methods (ANN, SMM, and Combinatorial Library) with the IEDB population coverage tool http://tools.iedb.org/population/. The server output provided a summary of the distribution of epitopes by calculating the overall population density in a specific area [14].

Construction of Multi-epitope Vaccine

The design of a vaccine structure involves integrating epitopes that are antigenic, immunogenic, non-toxic, and non-allergenic. This was achieved by incorporating GPGPG linkers for B cell and HTL epitopes and AAY linkers for CTL epitopes. In addition, the immunogenicity of the vaccine will be improved using P9WHE3 as an adjuvant. The P9WHE3 sequence was retrieved from the database https://www.uniprot.org/uniprotkb/P9WHE3/entry and connected to the vaccine construct through the EAAAK linker [20, 21].

Prediction of Physical and Chemical Properties of Vaccine Structure

The ProtParam online server https://web.expasy.org/protparam was employed to predict the physical and chemical properties of the vaccine structure. These properties include half-life, molecular weight (Mw), stability, aliphatic index, theoretical isoelectric point (pI), and the GRAVY value (indicating the hydrophilicity or hydrophobicity of side chains) [22]. For the assessment of solubility, the Protein Sol database https://protein-sol.manchester.ac.uk was utilized. Since any number above 0.45 indicates higher solubility than the average soluble *E. coli* protein, the experimental dataset threshold was set at 0.45 [23].

Prediction of Secondary and Tertiary Structures of the Vaccine

The PSIPRED Property online tool was employed to generate the secondary structure of the vaccine http://bioinf.cs.ucl.ac.uk/psipred/. This server contains two feed-forward neural networks designed to accurately predict transmembrane topology, transmembrane helix, folds, and domains [24]. The SPOMA online tool https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html was employed to determine the percentage of alpha helix, beta sheet, and coil structures within the vaccine [25] By combining SOPMA with a neural networks method (PHD), the prediction covers 82.2% of residues, accounting for 74% of co-predicted amino acids [25]. The I-TASSER server https://zhanglab.ccmb.med.Umich.Edu/I-TASSER was used to predict the tertiary structure [19]. I-TASSER employs the sequence-to-structure-to-function paradigm, creating three-dimensional (3D) atomic models through a combination of multiple threading alignments and iterative structural assembly simulations. The confidence score (C-score) serves as a metric for assessing the accuracy of the 3D model, typically ranging between -5 and 2. A higher C-score indicates better model quality [26].

Tertiary Vaccine Structure Refinement and Validation

The Galaxy Refine server was utilized to refine the tertiary vaccine structure http://galaxy.seoklab.org. This server works

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based on molecular dynamics simulation to obtain the overall structure by reconstructing and repacking the side chains [27]. Then the validation of the refined structure was assessed using the PROCHECK server https://saves.mbi.ucla.edu/. The outcomes encompass the Ramachandran plot, illustrating the energy distribution of each amino acid across favored, allowed, and disallowed regions. Additionally, it provides the percentage of amino acids within the allowed and non-allowed regions, serving as a determinant of the quality of the 3D model structure [28]. Moreover, to enhance validation, the ProSA online tool and ERRAT were utilized.

Discontinuous B cell Epitope Prediction

Since over 90% of B cell epitopes are discontinuous, the Ellipro server http://tools.iedb.org/ellipro was used to predict these epitopes. This server calculates an ellipsoid score for each residue based on the vaccine's tertiary structure, and this score is defined as a PI (Protrusion Index) value. The threshold parameters were conFig.d to default settings, with a minimum residue score of 0.5 and a maximum distance of 6 Å [29, 30].

Docking of Vaccines with Immune System Receptors and Molecular Dynamics Simulation

To control *Salmonella* infection, the innate and adaptive responses must cooperate. Since *Salmonella* suppresses MHC expression and T cell activation [31] and Toll-Like Receptor (TLR) play a particular role in the activation of innate immune responses, the possibility of binding the vaccine model with TLR4 (PDB ID: 3fxi), HLA-C (PDB Id: 5XS3) and HLA-DRB1 (PDB Id: 4MDJ) were investigated by molecular docking method in ClusPro 2.0 server https://cluspro.bu.edu/login.php [19]. Epitopes 'YHLDNGAGGR' and 'FRLSVIDVDNNTPVK' were docked with HLA-C and HLA-DRB1, respectively. Molecular dynamics simulation for assessing the stability and physical movements of the vaccine-TLR-4 docked complex was conducted using the iMODS online server http://imods.Chaconlab.org.

This server utilizes normal mode analysis (NMA) in internal (dihedral) coordinates to predict the collective motions of proteins. iMODS calculates key parameters such as deformability, B-factor, eigenvalues, variance, covariance map, and elastic network for the vaccine-receptor complex [32-34].

Immune Simulation for Vaccine

To evaluate the immune response profile in the *in-silico* model, the amino acid sequence of the vaccine was submitted to the C-ImmSim server https://kraken.iac.rm.cnr.it/C-IMMSIM/. All simulation parameters were chosen as default. A total of three injections were administered at time steps 1, 84, and 168, each separated by four-week intervals, resulting in a total of 1050 simulation steps. The C-ImmSim service, utilizing the position-specific scoring matrix (PSSM) and machine learning methods, assesses both humoral and cellular immunity to the vaccine model. The C-ImmSim determines the real-life immune response by stimulating three different tissues of mammals, including Bone marrow, Thymus, and tertiary lymphatic organs [14].

Vaccine Optimization and *in-silico* Cloning

To achieve the best protein expression in *E. coli* strain K12, the vaccine model was optimized in Java Codon Adaptation Tool (JCat) http://www.JCat.de/. Protein expression levels are calculated using the codon adaptation index (CAI > 0.8) and the GC content (30 to 70%). The refined vaccine sequence was subsequently inserted into the *E. coli* plasmid vector pET-28a (+), positioned between the *Eco*RI and *Bam*HI restriction sites. The SnapGene software was employed to validate the expression of the vaccine.

RESULTS

T Lymphocyte Epitopes

Amino acid sequences were blasted to determine the similarity of fimbriae protein between *Salmonella* strains and serovars. The analysis results indicated a homology of more than 99.72% in the selected fimbriae (AKG31746.1, KYF09125.1, QGK61610.1, and TYG30745.1) with different strains of ST and SE and other *Salmonella* serovars. Based on the IEDB results, 4830, 4900, 4872, and 4928 epitopes were found for CTL lymphocytes, and 5085, 5160, 5130, and 5190 predicted epitopes were determined for HTL lymphocytes, respectively. The epitopes with the highest binding affinity, antigenicity score, non-allergenicity, non-toxicity, and immunogenicity were chosen for further investigation (Tables 1 and 2).

Table 1. The fimbriae epitope candidates for cytotoxic T lymphocyte and their immunogenic characteristics.

Protein	Allele	Start	End	Epitope	IC_{50}	Antigenicity Score	Allergenicity	Toxicity	Immunogenicity score
S. typhimurium AKG31746.1	HLA-C*05:01	312	320	HLDNGAGGR	3.63	2.5872	Non-allergen	Non-toxic	0.11892
S. typhimurium KYF09125.1	HLA-C*03:03	174	182	ASTLNIFPY	10.73	0.8993	Non-allergen	Non-toxic	0.21274
S. entritidis QGK61610.1	HLA-C*03:03	173	181	PASTFDILP	10.98	1.0547	Non-allergen	Non-toxic	0.22608
S. enteritidis TYG30745.1	HLA-C*03:03	260	268	FDAPLAIEF	12.12	1.2155	Non-allergen	Non-toxic	0.19839

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Table 2. The candidate fimbriae epitopes for helper T lymphocyte and their immunogenic characteristics.

Protein	Allele	Start	End	Prediction sequence	IC_{50}	Antigenicity Score	Allergenicity	Toxicity
S. Typhimurium AKG31746.1	HLA-DRB1*01:01	187	201	PTVPFRPLTVSAFNL	47	0.9670	Non-allergen	Non-toxic
S. Typhimurium KYF09125.1	HLA-DRB1*01:01	196	210	VPFKPLPVSAFNLRF	32	2.2812	Non-allergen	Non-toxic
S. Enteritidis QGK61610.1	HLA-DRB1*07:01	65	79	IPFSYTCITAINTYS	22	0.7254	Non-allergen	Non-toxic
S. Entritidis TYG30745.1	HLA-DRB1*13:02	294	308	FRLSVIDVDNNTPVK	37	0.8356	Non-allergen	Non-toxic

Epitopes of Linear B Lymphocyte

The ABCpred server predicted linear B lymphocyte epitopes for ST and SE fimbriae proteins. The results were shown 20, 19, 24, and 22 epitopes for AKG31746.1, KYF09125.1, QGK61610.1, and TYG30745.1 accession numbers, respectively. High binding score epitopes (> 0.51) with 16 amino acids are shown in Table 3.

Table 3. The fimbriae epitope candidates for B lymphocytes and their immunogenic characteristics.

Protein Accession No	Epitope sequence	Start position	Score	Antigenicity score	Allergenicity	Toxicity
S. Typhimurium AKG31746.1	QEGSLTPVTFTWRDIQ	103	0.9	0.7234	Non-allergen	Non-toxic
S. Typhimurium KYF09125.1	TQPGISVGPPTVPFKP	185	0.87	0.7471	Non-allergen	Non-toxic
S. Entritidis QGK61610.1	APLAIEFRTNGLTLAD	258	0.88	0.7693	Non-allergen	Non-toxic
S. Entritidis TYG30745.1	GLSVEPPTVPFRPLTV	188	0.84	1.0874	Non-allergen	Non-toxic

Population Coverage Analysis

The world population coverage analysis revealed that the selected T cell epitopes covered 100% and 95.85% of MHC-I and MHC-II, respectively. The percentage of this coverage was also shown for each area (Fig. 1).

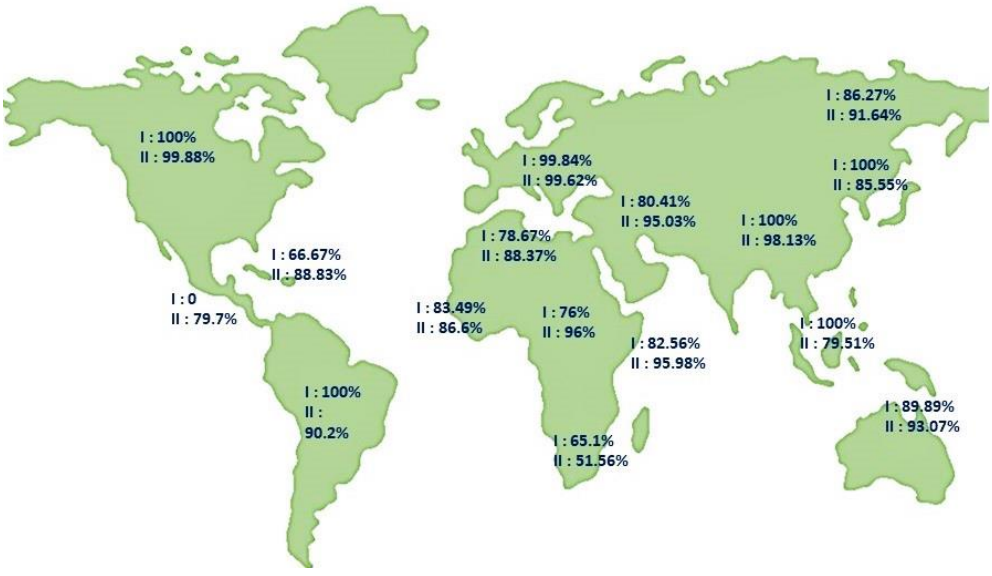

Fig. 1. The population coverage percentage of selected epitopes in the world. I: HLA-I and II: HLA-II.

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Multi-epitope Vaccine Construction

The GPGPG linker connected the candidate epitopes for B cell and HTL, followed by the linking of CTL epitopes using AAY linker. Also, at the N terminal side of the multi-epitope vaccine, adjuvant P9WHE3 was attached by EAAAK linkers (Fig. 2). The final vaccine sequence was blasted and it was determined that the vaccine construct was not similar to the human proteome

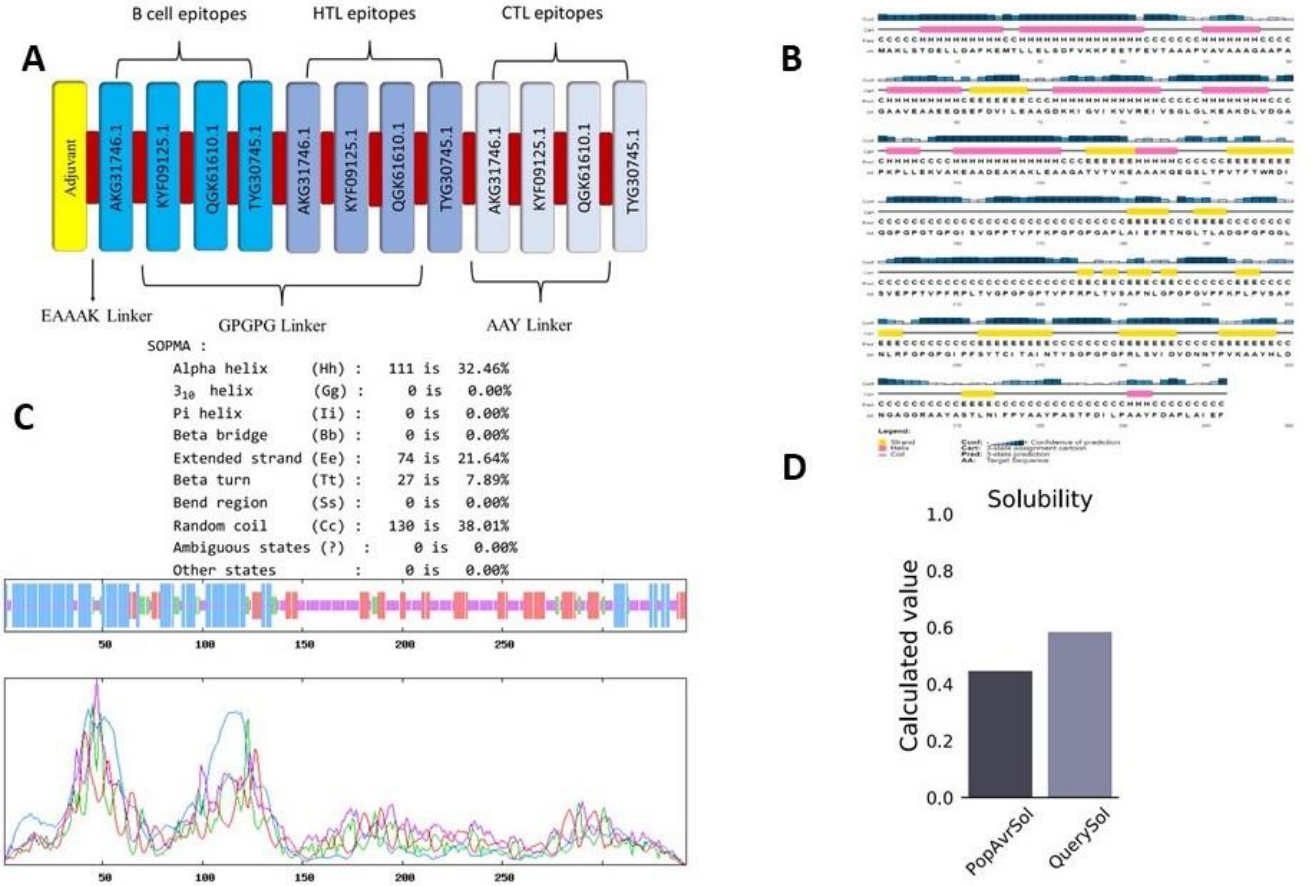

Fig. 2. (A) The final structure of a multi-epitope vaccine with 342 amino acids. The epitopes of B cell, HTL and CTL were bound by GPGPG and AYY linkers respectively and the adjuvant (P9WHE3) sequence was linked by EAAAK linker at the N terminal of the vaccine. **(B)** The prediction of the secondary structure of vaccine constructs. **(C)** The analysis of secondary structure in the SOPMA tool shows that the vaccine contains 32.46% alpha-helix, 21.64% extended strands, 7.89% beta-sheet and 38.01% random coils. **(D)** The solubility of the vaccine construct was calculated to be 0.58.

Physicochemical Characteristics of the Vaccine Structure

The final structure of the vaccine contains 342 amino acids, with a molecular weight of 35.09 kDa, an isoelectric point (pI) of 4.8, and an instability index of 31.47 (> 40: instability), which indicated an acidic and stable structure vaccine. Furthermore, the aliphatic index and Grand average of hydropathicity (GRAVY) score were determined to be 85.41 and 0.095, respectively. These values indicated the thermostability and hydrophilicity of the vaccine structure. The estimated half-life of the construct was 30 h in mammals, over 20 h in yeast, and exceeding 10 h in *E. coli*. The solubility score of the vaccine was determined to be 0.58, suggesting the protein solubility upon expression (Fig. 2).

Secondary and Tertiary Structures, Refinement, and Vaccine Validation

The secondary structure prediction of the vaccine showed 38.01% random coil, 32.46% alpha helix, and 7.89% beta sheets. The high percentage of random coils indicates the greater flexibility of the vaccine structure (Fig. 2). Based on the modeling of the tertiary structure by the I-TASSER server, the best model with the highest C score (-3.77) was chosen. This model exhibited a TM score of 0.31 ± 0.10 and a root-mean-square deviation (RMSD) score of 15.9 ± 3.2 Å (Fig. 3). GalaxyRefne serves to increase the consistency of the vaccine model. This server produced 5 refinement models, among which the best significant model included GDT-HA (0.9276), RMSD (0.489), MolProbity (2.320), clash value (15.3), Poor rotamers (0.0) and Rama value (86.5) (Fig. 3). The validation of the refined vaccine model was performed using RAMPAGE web server and Ramachandran plot analysis. The outcomes revealed that 82.4% of the residues were in the most favored region, 13% were in the additional allowed region, 1.5% were in the generously allowed region, and 3.1% were in the disallowed region. Furthermore, both ERRAT and ProSA-web assessments indicated that the overall quality factor of the refined vaccine model was 65.99% and -7.14 (Fig. 3). Taken together, all results have validated the tertiary vaccine model.

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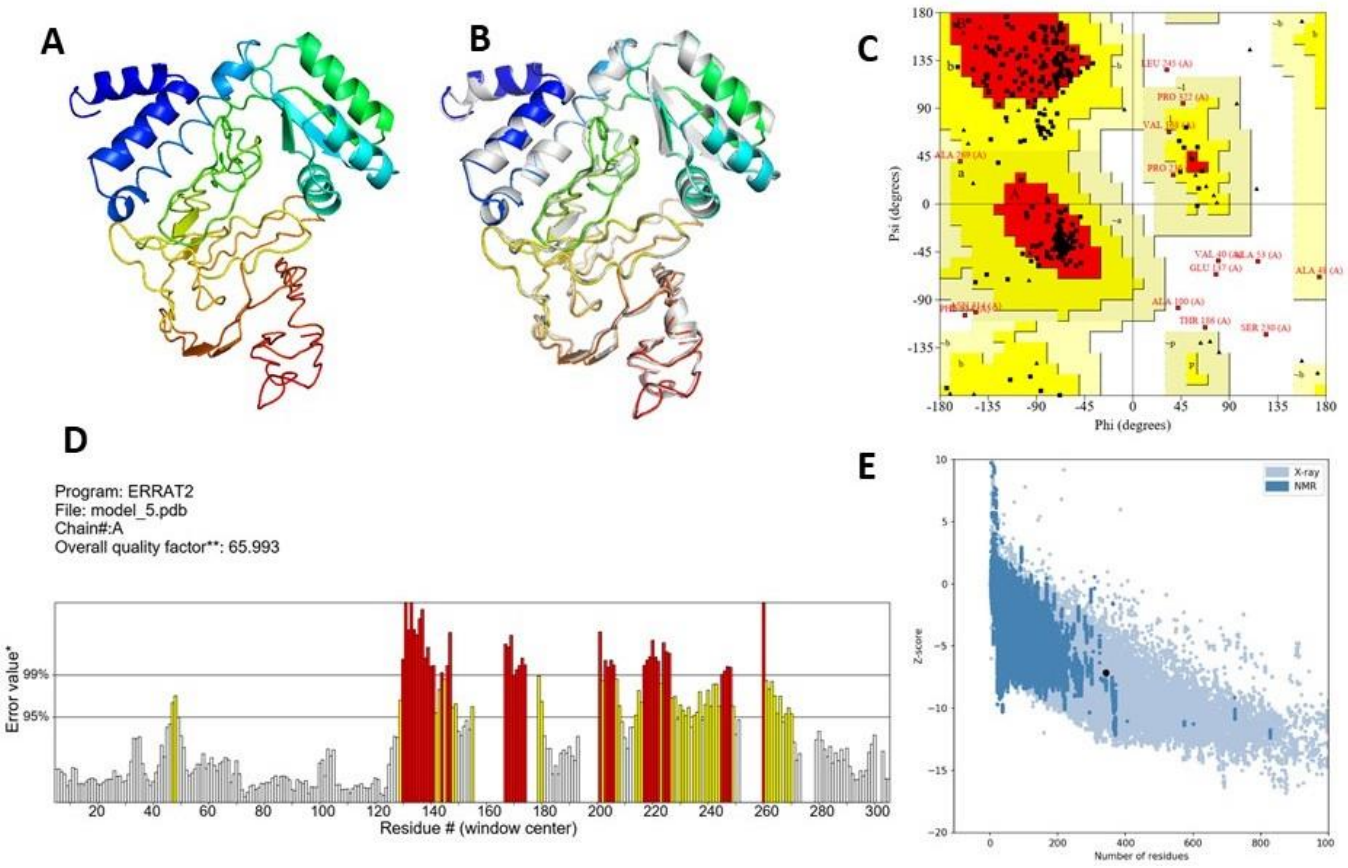
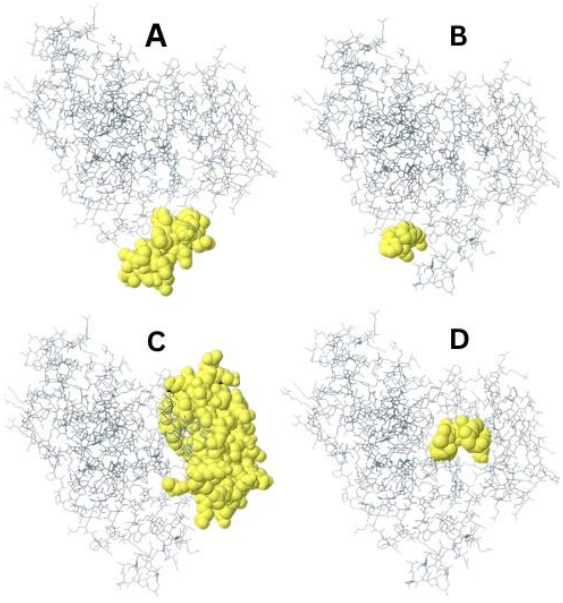


Fig. 3. The tertiary structure, refinement and validation of vaccine. **(A)** The three-dimensional structure of vaccine. **(B)** The 3-D structure of the vaccine after refinement. **(C)** Ramachardan plot indicating 82.4% of the residues were in the most favored region, 13% were in the additional allowed region, 1.5% were in the generously allowed region and 3.1% were in the disallowed region. **(D)** The ERRAT quality factor was 65.99%. **(E)** ProSA-web, with a Z score of -7.14.

Discontinuous B Cell Epitopes

According to the ElliPro server results, a total of 206 residues were distributed across seven discontinuous B-cell epitopes, with scores spanning from 0.504 to 0.848. The epitope sizes ranged from 3 to 104, and a score value ≥ 0.635 was chosen as the threshold for discontinuous epitopes (Fig. 4).



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E

No.	Residues	Number of residues	Score
1	A:M1, A:A2, A:K3, A:L4, A:S5, A:T6, A:D7, A:E8, A:L9, A:L10, A:D11, A:A12, A:F13, A:K14, A:E15, A:M16, A:T17, A:E20	18	0.848
2	A:F28, A:E30, A:T31, A:F32	4	0.767
3	A:A50, A:V54, A:E55, A:A56, A:A57, A:E58, A:E59, A:Q60, A:S61, A:E62, A:F63, A:D64, A:E68, A:A69, A:A70, A:G71, A:D72, A:K73, A:K74, A:I75, A:G76, A:V77, A:I78, A:K79, A:V80, A:R82, A:E83, A:I84, A:V85, A:S86, A:G87, A:L88, A:G89, A:L90, A:A100, A:L104, A:L105, A:V108, A:A109, A:K110, A:E111, A:A112, A:A113, A:D114, A:E115, A:A116, A:K117, A:A118, A:K119, A:L120, A:E121, A:A122, A:A123, A:G124, A:A125, A:T126, A:V127, A:T128, A:V129, A:K130, A:E131, A:A132, A:A133, A:A134, A:K135	65	0.666
4	A:E137, A:G138, A:S139, A:L140, A:T141, A:P142	6	0.635

Fig. 4. (A-D). The discontinuous B cell epitopes of multi-epitope vaccine (Yellow area). **(E)** The residues and score (score value ≥ 0.635) of discontinuous B cell epitopes.

Molecular Docking with Immune Receptors and Molecular Dynamics Simulation

The results from the molecular docking of the vaccine structure with TLR-4, HLA-C, and HLA-DRB1 on Clus Pro server 2.0 demonstrate a high affinity of the selected epitopes for binding. Specifically, the binding scores were -774.1 kcal/mol for HLA-C groove and -783.9 kcal/mol for HLA-DRB1 (Fig. 5). Since TLR-4 is the innate immune sensor for recognition of *Salmonella* lipopolysaccharide and inflammatory cytokine production, binding the vaccine model to TLR-4 and stimulating innate immunity is essential for subsequent immune response. Hence, from the 30-docked models, the best model had the lowest energy value: -940.9 kcal/mol (Fig. 6). The iMOD server was utilized for molecular dynamics simulation and normal mode analysis (NMA) to assess the vaccine-TLR-4 complex stability. It was found that the eigenvalue for the TLR-4 vaccine complex was $1.761020e \times 10^{-5}$. A lower value indicates lower energy is required for the deformation of the vaccine structure. The covariance matrix shows how residues are correlated. The elastic network model uses springs to connect atoms. (Fig. 6). These findings suggest that the vaccine model exhibits stable interactions with TLR-4 and HLA molecules, which can potentially activate the innate and adaptive immune responses.

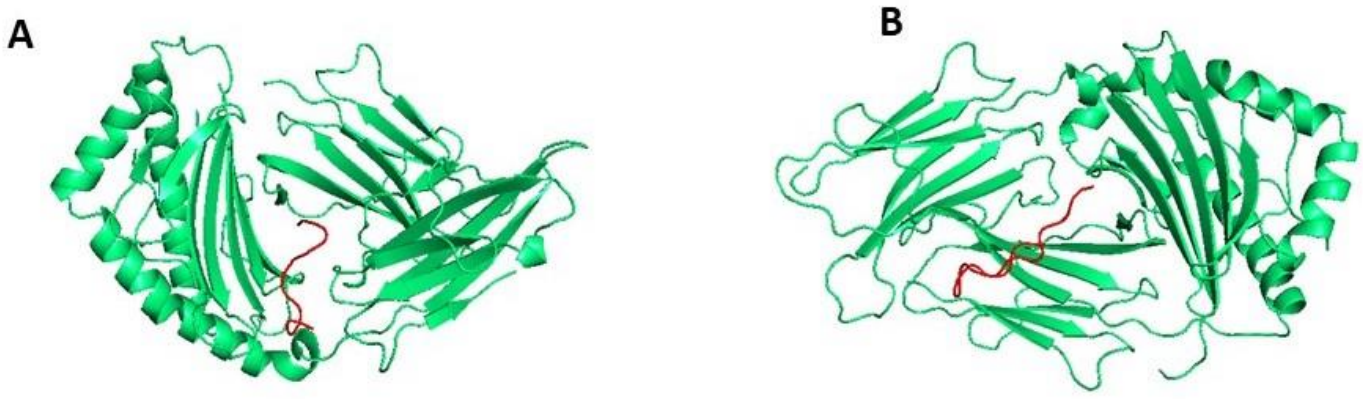

Fig. 5. The peptide-protein docking of vaccine model with HLA receptors. (**A)** YHLDNGAGGR (red color) docked with HLA-C (green color). (**B)** FRLSVIDVDNNTPVK (red color) docked with HLA- DRB1 (green color).

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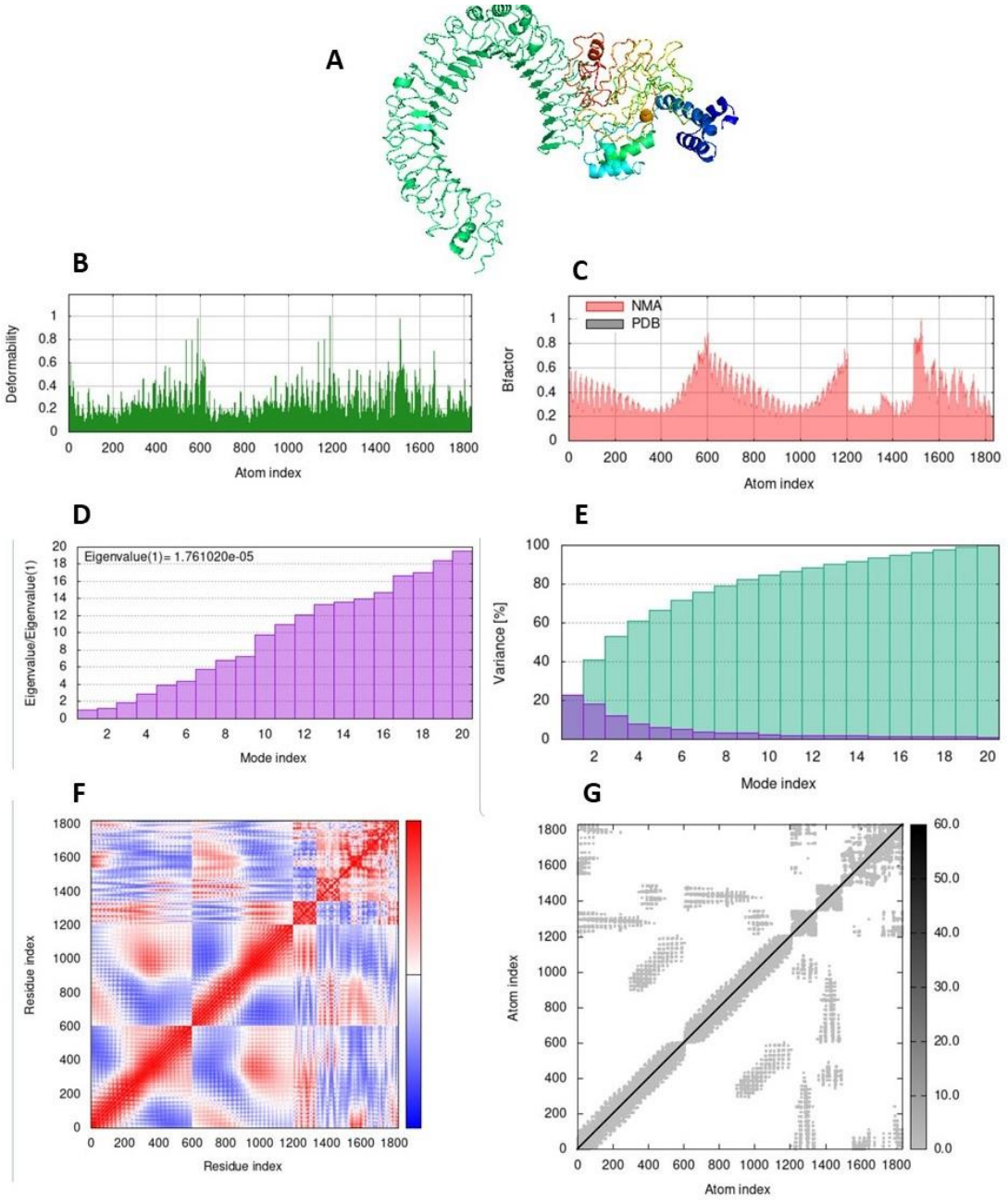

Fig. 6. The protein-protein docking of vaccine model with TLR-4 and molecular dynamics simulation. **(A)** Protein-protein docking of vaccine model (rainbow) with TLR-4 (green color) **(B)** Molecular dynamics simulation: deformability. **(C)** B-factor. **(D)** Eigenvalues (lower value means easier deformation). **(E)** Variance (red color: individual variances and green color: cumulative variances). **(F)** Covariance map (red: correlated, white: uncorrelated, blue: anti-correlated). **(G)** Elastic network (darker regions mean stiffer regions).

Immune Response Simulation

The results from the C-ImmSim server indicate a substantial increase in immune responses, resembling real immunological responses. Three vaccine injections caused an increase in the total B cell population, IgM and IgG response, and memory cells following decreased vaccine antigen after the third injection. The Th and CTL populations were also stimulated, and T-memory cells were detected. This vaccine stimulates IFN-γ and IL-2 production during three injections. Therefore, these findings confirmed the vaccine model's putative antigenic and immunogenic properties (Fig. 7).

Vaccine Optimization and *in-silico* Cloning

The JCat online tool optimized the amino acid sequence of the vaccine to 1026 bp linear DNA. The CAI-value was calculated to be 0.96, and the GC content was 55.8%, indicating that the vaccine had potentially strong expression in *E. coli* K12. After that, the optimized vaccine sequence was cloned into plasmid pET-28a (+) (data not shown).

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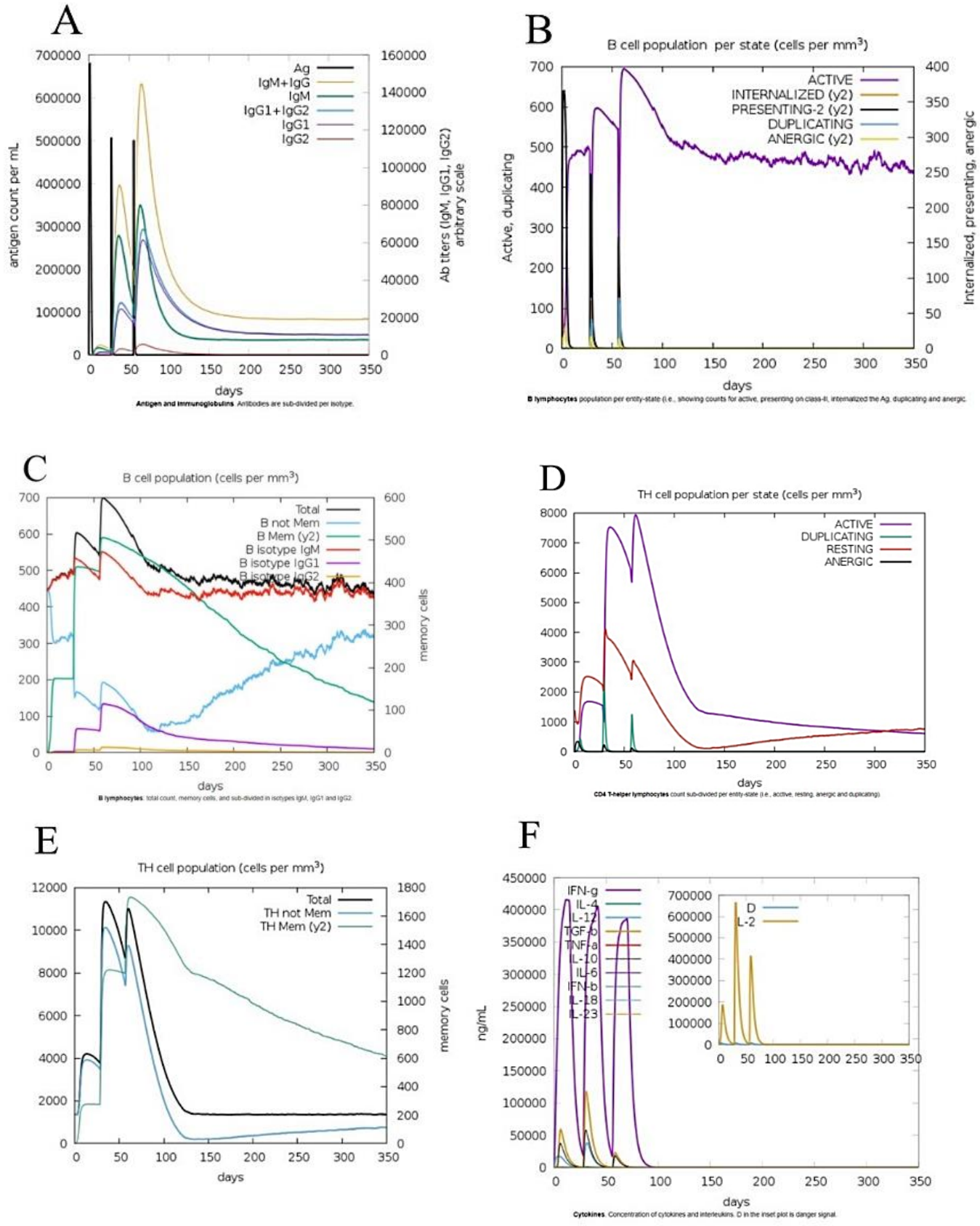

Fig. 7. *In-silico* simulation of the immune system. (A) Increased antibodies response (IgM and IgG: cream peak) and decreased antigen (black peak) after the repeated injection (B) Activation of B cell population (purple peak) (C) Increased memory B cells (green peak) (D) T cells activation after the second injection (purple peak) (E) Increased memory Th cells after the third injection (green peak). (F) Increased IFN-γ (purple peak) and IL-2 (cream peak) in response to the vaccine.

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Vaccine Optimization and *in-silico* Cloning

The JCat online tool optimized the amino acid sequence of the vaccine to 1026 bp linear DNA. The CAI-value was calculated to be 0.96, and the GC content was 55.8%, indicating that the vaccine had potentially strong expression in *E. coli* K12. After that, the optimized vaccine sequence was cloned into plasmid pET-28a (+) (data not shown).

DISCUSSION

Vaccination has emerged as the most efficient and cost-effective strategy for controlling infectious diseases. Scientists have long been dedicated to developing a vaccine for non-typhoidal *Salmonella* (NTS). However, as of now, no effective commercial vaccine has been made available. Hence, there is a critical requirement to construct a novel vaccine that stimulates the immune system and inhibits the colonization of the bacterium in the intestine. In the present study, we used immunoinformatics methods for the first time to design an NTS vaccine based on fimbriae epitopes. Accordingly, we selected two dominant NTS serovars (SE and ST) [8] and retrieved four fimbriae protein sequences. The BLASTp analysis of these sequences shows more than 99.72% homology among SE and ST serovars. Hence, these fimbriae proteins were conserved good candidates for the vaccine design. Previous studies were based on the development of a multi-epitope vaccine involving TolA (membrane-spanning protein), outer membrane proteins, and *Salmonella* enterotoxin [7, 10, 14, 35]; however, no research on fimbriae protein has been yet conducted. In the previous study, we utilized OMPs (OmpA and OmpD) for a vaccine design [14]. OMPs are suitable vaccine candidates because they can be recognized by the immune system, are necessary for cell communication and are immunogenic and thermostable. Moreover, LPS and other bacterial components such as lipoproteins, and peptidoglycans are potential targets as NTS accine candidates.

Interestingly, the bacterium may rapidly modify its composition in response to environmental changes that can lead to a change in its immunogenicity [11, 36]. Thus, in this study, fimbriae proteins which are critical in bacterial adherence and colonization and are conserved among *Salmonella* serovars [5], were chosen for the vaccine development. The fimbriae protein epitopes (T cells and B cells) found in the current study were selected based on their capacity to pass through immunoinformatics filters, namely antigenicity, allergenicity, immunogenicity, and toxicity. Epitopes that did not pass this process were removed. The chosen epitopes were bound to each other by GPGPG and AAY linkers to construct a linear protein structure. These linkers were designed to enhance the antigen presentation and processing and they also adjusted the stiffness and flexibility of the putative protein [37, 26]. To enhance the vaccine immunogenicity, we also added P9WHE3 as an adjuvant to the N terminal of the vaccine sequence. P9WHE3 is a 50S ribosomal protein L7/L12 of *Mycobacterium tuberculosis* that is an agonist for TLR-4 [38]. The vaccine structure was comprised of 342 amino acids with a molecular weight of 35.09 kDa, making it suitable for protein purification and vaccine production [39, 40]. The constructed vaccine showed a 4.8 isoelectric point (pI) and an instability index of 31.47, confirming the vaccine structure's stability and acidic feature. The aliphatic index and GRAVY score demonstrated that it has a hydrophilic side chain.

These data showed that the vaccine model has an antigenic epitope and is a thermostable and soluble structure, similar to the Omps-based vaccines [14].

The first step of *Salmonella* infection begins with the attachment of bacteria to the host cells, followed by colonization. On the other hand, TLRs are pattern recognition receptors that recognize the attachment of proteins such as fimbriae. Previous studies have shown that TLR-4 plays a crucial role in stimulating innate immune response against NTS [41, 31]. Hence, the proposed vaccine model has the potential to bind TLR-4. Moreover, binding of T cell epitopes to MHC-I and MHC-II demonstrates that this vaccine model could activate CTL and THL cells, respectively. To investigate the vaccine potential in immune response stimulation, C-ImmSim server output indicated that this vaccine can potentially stimulate the adaptive immunity by triggering B cell and T cell responses as well as the antibody response. The antibody response is critical in preventing NTS, particularly against fimbriae proteins [5]. Furthermore, the *in-silico* results demonstrated that the vaccine model can increase IL-2 and IFN-γ levels to their maximum with three injections which were similar to our previous results [14].

Since macrophages are the target cell for *S. typhimurium* infection, IFN-γ activates the macrophages and the ensued intracellular killing of the invading *Salmonella* [42]. Also, IL-2 causes T cell proliferation, which is a positive trend in IFN-γ production and clearance of the bacteria. However, the recent study conducted by Yadav et al. indicates that natural infection of *S. typhimurium* suppresses IL-2 production and has an adverse effect on the long-term immunity [43]. Thus, based on immune simulation findings, this vaccine can potentially induce an effective immunity against NTS. The cloning and expression of multi-epitope proteins are essential phases in vaccine production.

Hence, codon optimization was done to obtain a high level of expression. The GC content of the vaccine model was in an optimum range between 30 to 70% [10], and the CAI value indicates the overexpression potential of the vaccine in *E. coli* K12.

In conclusion, due to antibiotic resistance and mortality rates, NTS species are currently a major global concern. Accordingly, this study presented a novel multi-epitope vaccine based on the fimbriae protein of NTS. The antigenicity and safety of the vaccine were confirmed by immunoinformatics analysis. Moreover, the vaccine's interaction with TLRs and HLA proteins demonstrates its capacity to stimulate the innate immune system and adaptive responses. It is envisaged to confirm the present encouraging *in-silico* results by the future experimental and challenge studies in order to prove the proposed NTS vaccine concept.

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Not applicable.

CONFLICT OF INTERESTS

The authors declare they have no conflict of interests.

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