

Immunoinformatics-driven design and *in silico* evaluation of a multi-epitope vaccine candidate against *Orientia tsutsugamushi* causing scrub typhus

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Received 25 May 2026; revised 23 June 2026

Despite decades of research, an effective vaccine against *Orientia tsutsugamushi* remains unavailable owing to the pathogen's high antigenic variability, intracellular persistence, and poor cross-protection between strains. This study was conducted to develop a conserved multi-epitope vaccine candidate against the 56kDa type specific antigen (TSA56) of *O. tsutsugamushi* through a reverse vaccinology and immunoinformatic strategy. Seven TSA56 protein sequences collected from the UniProtKB database were evaluated for their antigenicity, allergenicity, and toxicity. Chimeric vaccine candidates consisting of the conserved CTL, HTL, and B-cell epitopes, which showed immunogenic potential and good safety parameters, were constructed. Finalization of the construct exhibited good antigenic activity, no allergenicity, structural stability, and good physicochemical properties. The structural validation resulted in the finding that 95.5% of residues occupied the favoured Ramachandran regions, implying a stable tertiary structure. From molecular docking results, the molecule interacted stably with the Toll-like receptor 2 (TLR2) with the lowest docking energy of -1334.7. Normal mode analysis using iMODS validated the stability and coordinated molecular motions of the vaccine-TLR2 complex, further supporting its immunological potential. Prediction on immune system response indicated good humoral responses in terms of high levels of IgM and IgG antibodies with creation of immunological memory. Codon optimization and *in silico* cloning were done to confirm the applicability of the construct for expression in *E. coli*.

Keywords: Epitope prediction, Multi-epitope vaccine, Immunoinformatics, Reverse vaccinology, TLR2 docking

Scrub typhus is a relatively new and underreported zoonotic infectious disease, which is caused by *Orientia tsutsugamushi*, an obligate intracellular Gram-negative bacterium that enters the human body *via* the bite of infected larval trombiculid mites, popularly known as chiggers¹. *Scrub typhus* is geographically prevalent in the Asia-Pacific zone, often termed the “tsutsugamushi triangle,” which ranges from northern Japan and eastern Russia to northern Australia and Pakistan. Over the past few decades, *scrub typhus* has acquired great public health significance owing to the spread of the disease geographically, increase in the rate of disease occurrence, occurrence of periodic epidemics, and involvement of various life-threatening complications such as acute respiratory distress syndrome, hepatitis, myocarditis, acute renal injury, meningoencephalitis, and multi-organ dysfunction syndrome². Delayed detection and lack of knowledge often result in increased

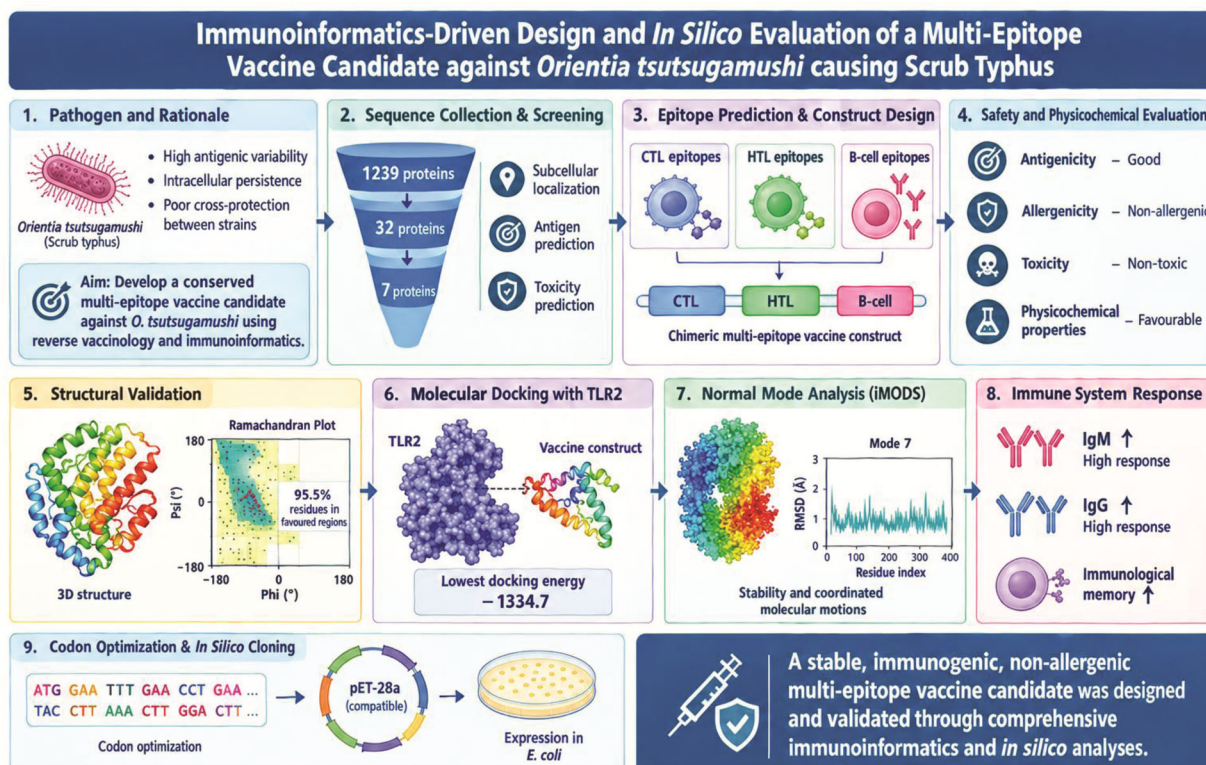
morbidity and mortality among patients, especially in remote areas where rapid diagnostic centres are not easily accessible.

There has been a significant rise in the incidence of *scrub typhus* over the last decade in India, particularly among the southern states of Tamil Nadu, Kerala, Karnataka, and Andhra Pradesh³. Climatic factors, agriculture-related risk, poor sanitary standards, and greater contact with the natural habitat infested with mites have played a role in increasing the incidence of this disease. From the clinical point of view, *scrub typhus* is characterized by acute fever, where its symptoms may overlap with other tropical diseases like dengue, malaria, leptospirosis, typhoid, *etc.*, making it difficult to differentiate and treat promptly⁴. Despite the efficacy of doxycycline and azithromycin in the treatment of *scrub typhus*, there remain issues of late diagnosis, drug resistance, and repeated attacks that pose problems in disease management⁵. Hence, development of prophylactic vaccines for *scrub typhus* has emerged as an essential area of research.

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Graphical abstract

Notwithstanding extensive research efforts, no vaccine for *O. tsutsugamushi* has been successfully licensed to date. One of the important challenges associated with the development of an *O. tsutsugamushi* vaccine is the tremendous variability of circulating strains in their genetic and antigenic characteristics⁶. The 56 kDa type-specific antigen (TSA56), which is one of the major outer membrane proteins of *O. tsutsugamushi* involved in bacteria binding and invasion and host cell infection as well as recognition by the immune system, includes multiple variable regions responsible for the strain specificity and poor cross-protection ability. As a result, traditional vaccines based on killed whole bacteria and antigen-based approaches have been rather inefficient in providing reliable protection from heterologous strains. Moreover, the intracellular localization of *O. tsutsugamushi* requires activation of both types of immune response for successful pathogen elimination⁷.

Emerging trends in computational biology, genomics, and immunoinformatics have significantly enhanced the design of epitope-based vaccines using reverse vaccinology strategies. While traditional methods of vaccine discovery depend heavily on pathogen culturing and experimentation for antigen

screening, reverse vaccinology makes it possible to quickly identify vaccine targets based on the information contained in the genome and proteome sequences⁸. Immunoinformatics approaches make it possible to predict potential CTL, HTL, and B-cell epitopes that can elicit a specific immune response while avoiding allergenicity and toxicity. Multi-epitope vaccine design has become an attractive approach to vaccine production since it involves designing vaccines comprising several epitopes from different variants of the same antigenic target⁹.

Some recent attempts have been made in the development of vaccines against *O. tsutsugamushi* by immunoinformatics¹⁰. But most of these studies used a single strain, limited methods of epitope prediction, and were unable to conduct structural and immune simulation analyses. Further, not much work has been done to predict conserved epitopes from different forms of TSA56 to increase the potential of cross-protection. Lack of receptor docking, immune simulation, and recombinant protein expression analysis further hampered some computational vaccine design studies. Thus, there is still a gap in developing a robust platform that can help identify conserved epitopes with good immunological and structural properties.

Toll-Like Receptors (TLRs), especially TLR2, are fundamental to innate immune response to bacterial lipoproteins and subsequent stimulation of downstream inflammatory pathways. The relationship between the vaccine design and TLR2 is known to contribute to increased antigenicity, cytokine production, and activation of the adaptive immune system¹¹. As such, molecular docking studies of vaccine–TLR relationships have emerged as a significant aspect of computational vaccine evaluation. Additional considerations include immune modelling and codon optimization.

Given the rising incidence of *scrub typhus* disease and the lack of an effective vaccine, a need exists to develop improved vaccines that are safer and effective against various serotypes. In this context, the current study has been designed and conducted to formulate and predict the efficacy of a conserved multi-epitope vaccine using TSA56 of *Orientia tsutsugamushi*. TSA56 sequences from various strains were screened, followed by the identification of conserved T cell, HTL, and B-cell epitopes with strong antigenicity and safety aspects. The identified epitopes were incorporated into a vaccine construct consisting of a β -defensin adjuvant with suitable peptide linkers. The vaccine construct was analyzed using various *in silico* tools, including physicochemical parameters, 3D

modelling, molecular docking to TLR2 receptor, normal mode analysis, immune prediction, codon optimization, and cloning (Fig. 1).

Materials and Methods

Retrieval of target proteins and proteome data

Protein sequences that encode the TSA (Type-Specific Antigen) of 56 kDa from *Orientia tsutsugamushi* were identified in the UniProtKB database¹². Proteins that were representative of multiple strains and diverse antigens were chosen to identify immunologically conserved regions that could form the basis of an effective vaccine. The protein sequences chosen were those that were surface proteins or virulence factors which have key roles in the pathogenesis of the organism. A priority was set for identifying proteins that came from strains prevalent in certain endemic areas, especially South India.

Screening of proteins for vaccine suitability

The protein sequences obtained were analyzed to assess the appropriateness of such sequences as vaccines on the basis of antigenicity, allergenicity, toxicity, and subcellular localization. Subcellular localization was predicted by the use of the PSORTb v3.0.3 server to detect extracellular and outer

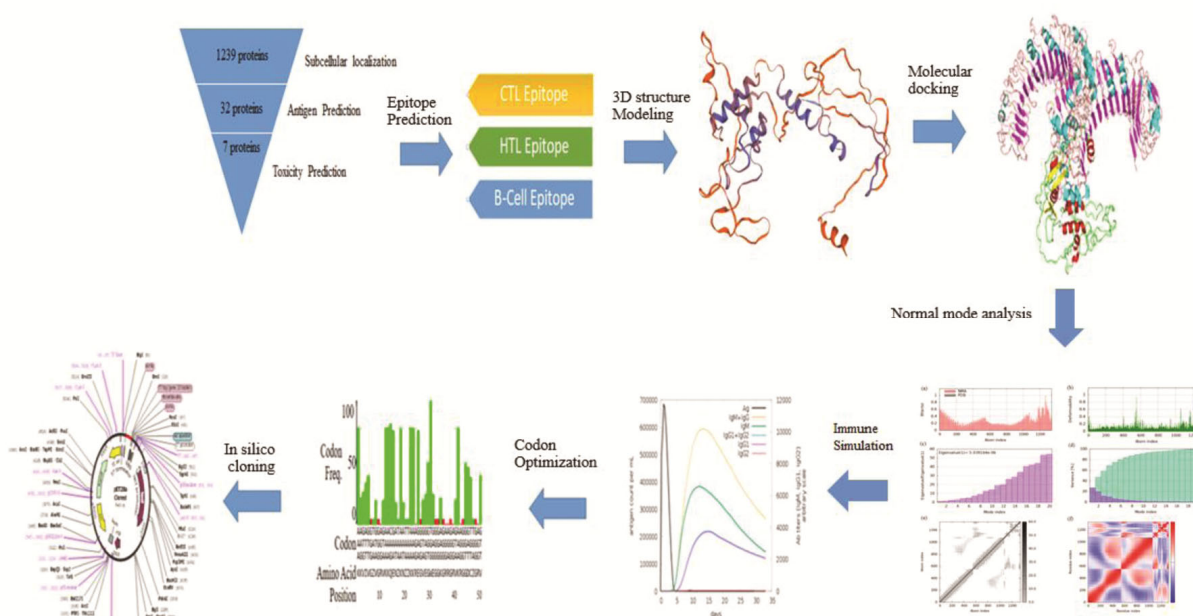


Fig. 1 — Overall workflow of the immunoinformatics-based multi-epitope vaccine design strategy against *Orientia tsutsugamushi*. The schematic representation illustrates the sequential computational pipeline employed in the study, including retrieval of TSA56 protein sequences, antigenicity screening, prediction of CTL, HTL, and B-cell epitopes, epitope filtering based on immunological safety, vaccine construct assembly, structural validation, molecular docking with TLR2, normal mode analysis, immune simulation, codon optimization, and *in silico* cloning analysis

membrane proteins that can be easily exposed to immune defense mechanisms¹³. Antigenicity prediction was done through the use of VaxiJen v2.0 server with a cut-off value of 0.4¹⁴. The proteins whose values exceeded the 0.4 limit were regarded as possible antigens. Predictions of allergenicity were done using the AllerTOP v2.0 server while toxicity predictions were conducted through the ToxinPred server^{15,16}.

Prediction and selection of CTL epitopes

Cytotoxic T lymphocyte (CTL) epitopes were predicted from the shortlisted proteins using the NetMHCpan 4.1 server¹⁷. Prediction was performed against the HLA-A*02:01 allele due to its widespread prevalence and immunological significance. Nine-amino-acid-long peptides exhibiting strong binding affinity were selected as candidate CTL epitopes. The shortlisted epitopes were further evaluated for antigenicity using VaxiJen v2.0, allergenicity using AllerTOP v2.0, and toxicity using ToxinPred. Only epitopes with high antigenicity and favourable safety profiles were retained for inclusion in the vaccine construct.

Prediction and selection of HTL epitopes

Helper T lymphocyte (HTL) epitopes were predicted using the NetMHCIIpan 4.0 server against the HLA-DRB1*01:01 allele. Epitopes demonstrating strong MHC class II binding affinity were selected for further analysis. The selected HTL epitopes were screened for antigenicity, allergenicity, and toxicity using the same computational tools employed for CTL epitope analysis. HTL epitopes capable of inducing robust CD4+ T-cell responses were prioritized to enhance cytokine production, immune regulation, and immunological memory.

Prediction of B-cell epitopes

Linear B-cell epitopes were identified using the BepiPred 3.0 prediction server¹⁸. Surface accessibility, hydrophilicity, flexibility, and antigenic propensity were considered during epitope selection. Predicted B-cell epitopes were subsequently evaluated for antigenicity, allergenicity, and toxicity to ensure their suitability for humoral immune activation. Epitopes with high antigenicity and non-toxic, non-allergenic characteristics were selected for incorporation into the final vaccine construct.

Epitope filtering and population coverage analysis

Each CTL, HTL, and B-cell epitope that made it to the list was individually analyzed for its efficacy and

safety from an immune perspective. Population coverage analysis was carried out using the IEDB population coverage calculator to estimate the extent of distribution and epitope recognition for the selected T-cell epitopes within the Indian population¹⁹. This analysis involved assessing the frequency of HLA alleles that correlated with the epitopes in question.

Construction of the Multi-epitope vaccine

The final multi-epitope vaccine sequence was created by combining the CTL, HTL, and B cell epitopes together to form a chimeric sequence. To increase its immunogenicity, the β -defensin was added as an adjuvant at the N-terminal end of the multi-epitope sequence. The adjuvant was coupled with the epitope sequence using an EAAAK linker that would ensure structural stiffness and avoid any undesirable reactions. The CTL epitopes were coupled together using AAY linkers to allow for proteosomal processing and presentation on MHC class I molecules. On the other hand, the HTL epitopes were coupled together using GPMPG linkers to aid in their presentation *via* MHC class II molecules. B cell epitopes were coupled using KK linkers.

Physicochemical property analysis and solubility prediction

The physicochemical properties of the designed vaccine construct were analyzed using the ExPASy ProtParam tool. Parameters including molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were evaluated to determine protein stability and biochemical characteristics. Solubility prediction was performed using the Protein-Sol server to assess the likelihood of efficient recombinant expression and purification.

Secondary and tertiary structure prediction

The secondary structure prediction of the vaccine was done through the PSIPRED web server to determine the presence of the α -helix, β -sheet, turns, and random coils. The vaccine tertiary structure was predicted through the Swiss Model web server by means of homology modeling and threading methodology²⁰. The constructed 3D model was further validated through Ramachandran plot to assess the stereochemical quality of the molecule. Other parameters that were used for model validation include RMSD, MolProbity score, ERRAT score, and Verify3D analysis.

Molecular docking with immune receptors

For the evaluation of the interaction between the vaccine structure and the host's innate immune receptors, molecular docking study was done with Toll-like receptor 2 (TLR2). The structure of TLR2 was obtained from the Protein Data Bank (PDB), having the accession code 2Z7X²¹. The modelled vaccine structure was used as the ligand for the docking experiment. Protein-protein docking study was done using the ClusPro web server that utilizes FFT-based rigid body docking method and cluster-based energy ranking approach²². The docked complexes were ranked according to cluster size, weighted energy scores, and binding stability.

Normal mode analysis of docking complex

The stability of docking complexes was examined using Normal mode analysis (NMA) by the iMOD server (<https://imods.chaconlab.org>) to compute the collective movements and deformations of the macromolecules using their internal coordinates²³. Because NMA is known to be less computationally costly than all-atom MD simulations, it can quickly anticipate large-scale movements while using less processing resources²⁴. It should be mentioned that NMA focuses on low-frequency modes, which essentially define protein flexibility and stability, as opposed to the traditional MD simulation, which takes temperature and solvent effects into account over an extended period of time²⁵. However, it should be noted that because solvent and atomic effects are not fully considered, NMA, as implemented by the iMOD server, only provides an approximate approximation of the MD simulations. However, NMA is a widely recognized and reliable technique for first examination of the dynamic stability of protein-ligand or protein vaccination complexes²⁶. This method, which considered a number of factors such as the mobility of NMA, deformability, B-factor, eigenvalue, variance, covariance map, and elastic network, was used to examine the stability of the complex.

Immune simulation analysis

The immunogenicity potential of the created vaccine design was determined using C-ImmSim server; this is an agent-based immune simulation system. The protein sequence of the created vaccine design was submitted to the server²⁷. Predicted immune reactions included antigen elimination, antibodies formation, cytokine responses, and memory cell formation. Simulations were conducted to examine the primary, secondary, and

tertiary immune response after several exposures to the antigen. Analysis was performed on the simulated data to determine the ability of the vaccine to provide long-lasting humoral and cell-mediated immunity.

Codon optimization and *in silico* cloning

To enable efficient expression through recombinant techniques, the amino acid sequence for the vaccine was reverse engineered by codon optimizing the sequence for the expression system of *Escherichia coli* using the Java Codon Adaptation Tool (JCat). Analysis of the parameters used for codon optimization involved the Codon Adaptation Index (CAI) and GC content. Restriction sites for NdeI and XhoI were included in the optimized sequence of the gene²⁸. The optimized sequence was then cloned into the vector pET-28a(+) using SnapGene software for recombinant protein expression systems.

Result and Discussion

Retrieval of target proteins and proteome data

A total of seven protein sequences corresponding to the 56 kDa type-specific antigen (TSA) of *Orientia tsutsugamushi* were retrieved from the UniProtKB database. The selected proteins included UniProt IDs Q02938, P22940, P37915, P37918, P37916, P37917, and P37919, representing different strains of *O. tsutsugamushi*. The sequence lengths ranged from 521 to 532 amino acids, showing that the strains are structurally similar even though they're known to be antigenically variable (Suppl. Table 1). Because the 56 kDa TSA is a major surface protein and a key player in host cell attachment and immune recognition, it made a perfect target for designing epitope-based vaccines. Earlier research shows this antigen helps in triggering both humoral and cellular immune responses, making it a good candidate for multi-epitope vaccine development. Finding multiple strain-specific sequences helped in spotting conserved immunogenic regions that could provide wider protection. With the big genetic differences between circulating *O. tsutsugamushi* strains, using several TSA variants upped the chances of finding epitopes with immunogenic potential across different strains.

Screening of proteins for vaccine suitability

The retrieved TSA proteins had a computer check to see how antigenic, allergenic, and toxic they are. For antigenicity, which uses VaxiJen v2.0, all seven proteins showed high antigenic potential, scoring between 0.7281 and 0.7925. This easily passes the

Table 1 — Shortlisted CTL epitopes for the final vaccine construct

Sequence	Protein ID	Start Position	Antigenicity	Allergenicity	Toxicity
KLYADLVTT	Q02938	443	0.5268	Non Allergen	Non Toxic
YLRNISA EV	P22940, Q02938	95	0.5723	Non Allergen	Non Toxic
HMMVNPVLL	Q02938, P37916	201	1.0292	Non Allergen	Non Toxic
KLTPPQPTI	P22940	133	0.9272	Non Allergen	Non Toxic
	P37915	132			
	P37918	127			
	P37916	133			
	P37917	129			
	P37919	130			
TIMPISIAV	P37915	139	0.8694	Non Allergen	Non Toxic
RLNFQIPQV	P37917	317	1.7555	Non Allergen	Non Toxic
YLMNVKAEV	P37917	94	0.6587	Non Allergen	Non Toxic
ALNEQIIQL	P37919	353	0.6461	Non Allergen	Non Toxic

threshold of 0.4, with P22940 topping the list at 0.7925. That suggests it would be great at triggering protective immune responses. When checked for allergenicity *via* AllerTOP v2.0 and toxicity through ToxinPred, all proteins came back safe – not allergenic and not toxic. This combo of good immunological features and safety suggests the proteins could work well for vaccine development. Since surface-associated proteins interact directly with parts of the host's immune system, they're ideal for designing epitope-based vaccines. So these protein candidates, being both strongly antigenic and safe, were picked for more analyses down the line. Scoring highly on antigenicity while dodging allergenicity is key for vaccine design, minimizing harmful immune reactions and boosting protection. The findings match earlier reports showing that TSA proteins from *O. tsutsugamushi* are very effective at sparking immune responses.

Prediction and selection of CTL epitopes

The NetMHCpan 4.1 server predicted that CTL epitopes have a strong binding affinity toward the HLA-A*02:01 allele. Some of these showed great antigenicity and good safety features, making them ideal for triggering cytotoxic T-cell responses. Among the chosen epitopes, HMMVNPVLL led with the highest antigenicity score at 1.0292, while RLNFQIPQV had the second-highest at 1.7555. Following closely were KLTPPQPTI with 0.9272, and TIMPISIAV at 0.8694. Also, all picked epitopes were safe; they weren't toxic nor allergenic (Table 1).

These CTL epitopes are crucial for activating CD8+ T lymphocytes, key players in wiping out intracellular pathogens like *O. tsutsugamushi*. Since the bacteria hide inside host cells to evade the immune system, having epitopes that spark powerful cytotoxic reactions is super important. That a few epitopes, like KLTPPQPTI, were found across many strains raises hopes for a broader shield against different bug types. Some candidates didn't make the cut due to lower or negative antigenicity scores, so they were left out. Only keeping those that would safely boost immunity made for a solid vaccine design. In the end, the strong antigenicity of the best picks shows they could really amp up cellular immunity and clear the pathogen effectively.

Prediction and selection of HTL epitopes

The HTL epitopes picked for HLA-DRB1*01:01 showed great safety and effectiveness. Among them, YGDIDGKSV had the top score at 1.9874, followed by FKLTPPQPT at 1.9247, then MTIAPGFRA at 1.0591, and YAGIGAGLA at 0.9992 (Table 2). Plus, all of these were deemed non-toxic and non-allergenic. HTL epitopes play a big role in getting CD4+ T-cells to respond. This revs up the adaptive immune system by activating B cells and cytotoxic T cells *via* cytokine release. Including strong HTL epitopes helps create a lasting immune memory, giving us protection over the long haul. Also, finding that FKLTPPQPT shows up in several TSA variants hints at a key spot that causes a broad immune reaction. Since these HTL epitopes trigger crucial

Table 2 — Selected HTL epitopes for the final vaccine construct

Sequence	Protein ID	Start Position	Antigenicity	Allergenicity	Toxicity
FADIAGINV	Q02938	275	0.9364	Non-toxic	Non-allergen
	P22940	267			
	P37916	275			
MTIAPGFRA	Q02938	78	1.0591	Non-toxic	Non-allergen
	P22940	78			
	P37915	77			
	P37918	78			
	P37916	78			
	P37919	78			
FKLTPPQPT	Q02938	129	1.9247	Non-toxic	Non-allergen
	P22940	129			
	P37915	128			
	P37916	129			
	P37919	126			
YLRNISAEV	Q02938	92	0.5723	Non-toxic	Non-allergen
	P22940	95			
	P37916	92			
YVDLEGSYM	P37915	495	0.8402	Non-toxic	Non-allergen
FARVAGIEV	P37918	266	0.5276	Non-toxic	Non-allergen
YAGIGAGLA	P37918	451	0.9992	Non-toxic	Non-allergen
FQIQQAQQQ	P37919	320	0.9353	Non-toxic	Non-allergen
VTAQEAAAA	P37919	337	0.9930	Non-toxic	Non-allergen
YGDIDGKSV	P37919	456	1.9874	Non-toxic	Non-allergen

Table 3 — B-cell epitopes prediction for *scrub typhus* vaccine construct

Sequence	Protein ID	Start Position	Antigenicity	Allergenicity	Toxicity
GGMITGAESTRLDSTDSE	Q02938	25	1.3449	Non Allergen	Non Toxic
GKKHLSLTG	P22940	23			
VMYLRNISAEVEVGKGEV	Q02938	62	1.6154	Non Allergen	Non Toxic
DSKGEIKADSGGGTD					
PNQTSAASTRSLRLNDEQRAA	P37915	120	1.1065	Non Allergen	Non Toxic
DAEGKKHLSLTNG	P37915	30	1.4225	Non Allergen	Non Toxic
	P37918	28			
LLNITQGAPNVNPRPRQN	P37919	150	0.5952	Non Allergen	Non Toxic
LNILDHD					

cytokines like IFN- γ and IL-2, they're vital for handling infections from intracellular bacteria. So, finding such high-quality HTL epitopes makes the new vaccine design really strong immunologically.

Prediction of B-cell epitopes

Linear B-cell epitope prediction found several regions that can trigger humoral immune responses. These selected epitopes scored between 0.5952 and 1.6154 for antigenicity and were also predicted to be safe, with no toxicity or allergenic risks (Table 3). One

sequence, VMYLRNISAEVEVGKGEVDSKGEIKA DSGGGTD, stood out with the highest score of 1.6154, meaning it's really good at inducing antibodies. B-cell epitopes kick off the antibody-mediated immune response, crucial for stopping pathogens and preventing bacteria from spreading. Adding many strong B-cell epitopes in the vaccine means we're more likely to create effective neutralizing antibodies against *O. tsutsugamushi* surface proteins. Plus, some areas, like YLRNISAEV, overlap with predicted T-cell

Table 4 — Population coverage analysis of the selected CTL and HTL epitopes performed using the IEDB Population Coverage tool.

Population	Coverage (%)	Average Hit	PC90
World	95.77	20.63	10.23
Europe	98.59	25.31	13.91
North America	96.07	21.0	9.89
Southeast Asia	84.69	12.57	5.22
North Africa	81.43	12.77	4.31
South America	70.71	9.74	2.73

epitopes. This overlap is beneficial because it could boost immune system teamwork, making the vaccine way more effective overall.

Epitope filtering and population coverage analysis

The IEDB Population Coverage tool was used to conduct population coverage study in order to assess the global applicability of the chosen CTL and HTL epitopes (Table 4). The predicted CTL epitopes and the matching HLA class I alleles were included in the study (HLA-A*02:01, HLA-A*24:02, HLA-A*01:01, HLA-A*03:01, HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, and HLA-B*35:01) and HTL epitopes with representative HLA class II alleles (HLA-DRB1*01:01, HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB1*11:01, and HLA-DRB1*15:01). Both class I and class II MHC epitopes covered 95.77% of the world's population, indicating that this vaccine design can elicit an immunological response in a large number of individuals worldwide. For each individual, averages of 20.63 epitope-HLA combinations were found. Nonetheless, the PC90 value, which indicates the bare minimum of epitope-HLA pairings that 90% of the population can identify, was 10.23. Europe has the highest population coverage (98.59%), followed by North America (96.07%), Southeast Asia (84.69%), and North Africa (81.43%), according to regional study. South America, on the other hand, has the lowest but significant coverage (70.71%). Europe, North America, Southeast Asia, North Africa, and South America all had average hit values of 25.31, 21.0, 12.57, 12.77, and 9.74, respectively. Similarly, the PC90 values were 13.91, 9.89, 5.22, 4.31, and 2.73 for Europe, North America, Southeast Asia, North Africa, and South America (Fig. 2). This implies that the identified T cell epitopes can be exploited to provide broad coverage throughout geographically different communities and are well-represented by HLA alleles. This is because both endemic and non-endemic areas have comparatively high coverage values. Consequently, the created multi-epitope vaccination can be applied broadly.

Multi-epitope vaccine construct design

The last vaccine piece was put together using chosen CTL, HTL, and B-cell bits along with a β -defensin booster. To keep things stable, make the epitopes easier to process, and prevent any awkward interactions, we added peptide connectors like EAAAK, AAY, GP GPG, and KK. We used β -defensin because it fires up the body's natural defenses and makes the vaccine work better. The EAAAK connector made the structure stronger at the spots where the booster attaches. Meanwhile, the AAY and GP GPG links helped present antigens smoothly *via* the MHC Class I and II routes. Lastly, the KK links made B-cell targets more flexible and easy for antibodies to lock onto (Fig. 3). In all, this setup can really tickle both cellular and liquid-based immune responses. They carefully planned it to keep unwanted immune reactions low while enhancing the chances of spotting and fighting off real threats.

Physicochemical property and solubility evaluation

The ExPASy ProtParam tool showed that the vaccine construct has some really favourable properties. It has an isoelectric point of 5.97, meaning it's slightly acidic. With an instability index of 31.27, it's considered stable. And because it has an aliphatic index of 79.05, it should hold up well at high temperatures. Additionally, the GRAVY value came out to be -0.248, showing that the construct is hydrophilic, which helps with solubility and how easy antigens can be accessed. Further analysis from Protein-Sol gave a solubility score of 0.484, adding more proof that this thing is great for making lots of recombinant proteins. All of these positive traits mean the vaccine construct could work well on a large scale, both for making lots of it and then getting it purified afterward.

Structural modelling and validation

The secondary structure prediction showed that the vaccine construct was made up of 44.87% alpha-helices, 21.67% beta-strands, 26.24% random coils, and 7.22% beta-turns. This balanced mix suggests it's flexible and stable enough to present its epitopes effectively (Fig. 4). When we looked at the tertiary structure modelling and validation, everything pointed towards a reliable structure. The Ramachandran plot showed that 95.5% of residues fell in the favoured regions, signaling excellent quality. Other tests like RMSD (0.327), GDT-HA score (0.98), MolProbity score (1.75), ERRAT score (92.43%), and Verify3D

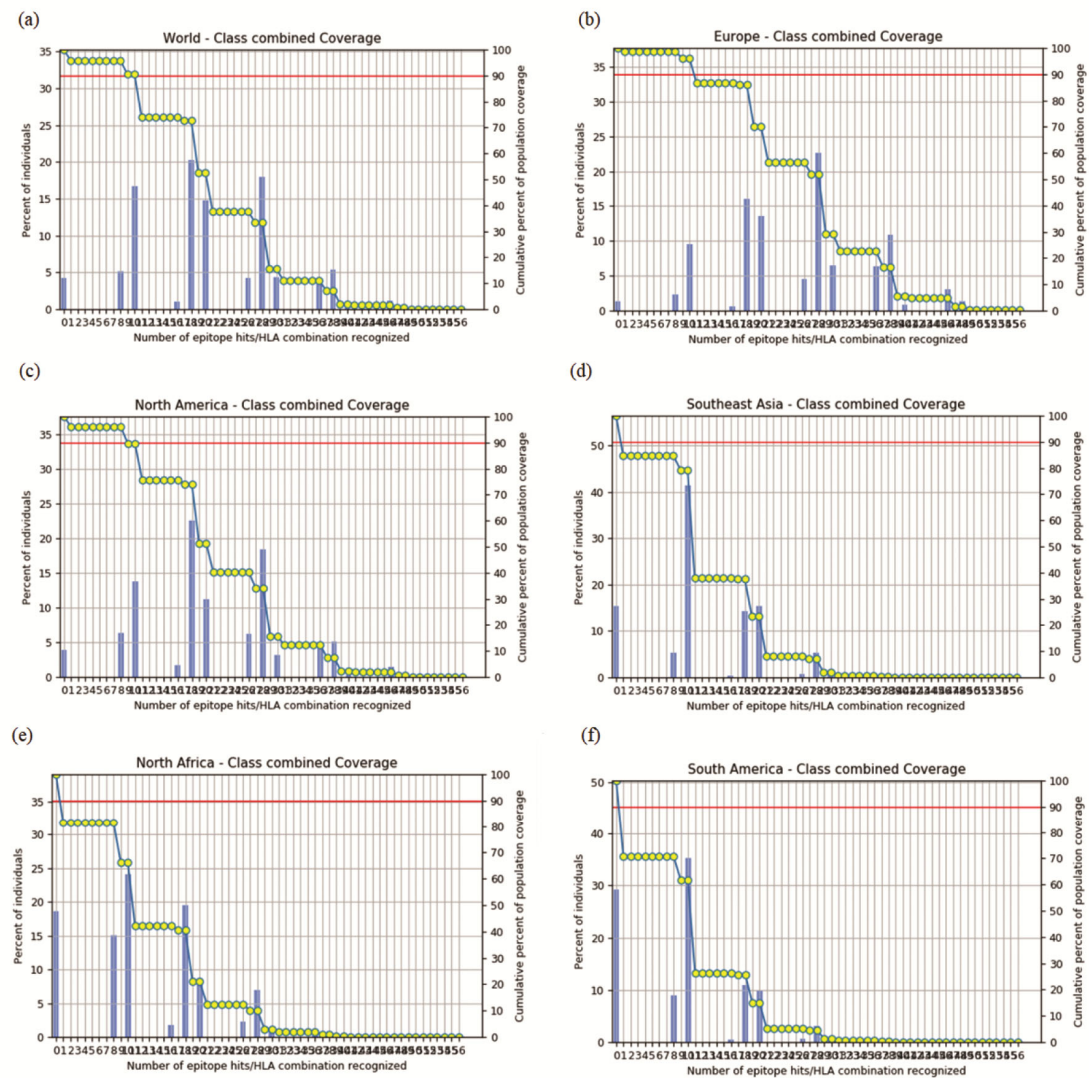


Fig. 2 — Population coverage analysis of the selected CTL and HTL epitopes performed using the IEDB Population Coverage tool. The combined HLA class I and class II epitope set demonstrated population coverage of 95.77%, 98.59%, 96.07%, 84.69%, 81.43%, and 70.71% in the World (a), Europe (b), North America (c), Southeast Asia (d), North Africa (e) and South America (f) populations, respectively. The histograms represent the percentage of individuals recognizing a given number of epitope-HLA combinations, while the cumulative curve indicates the overall population coverage

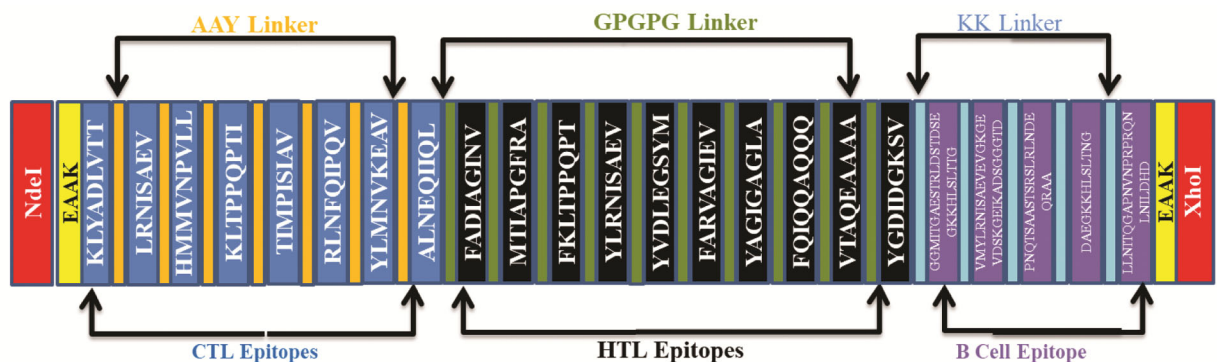


Fig. 3 — Schematic representation of the finalized multi-epitope vaccine construct

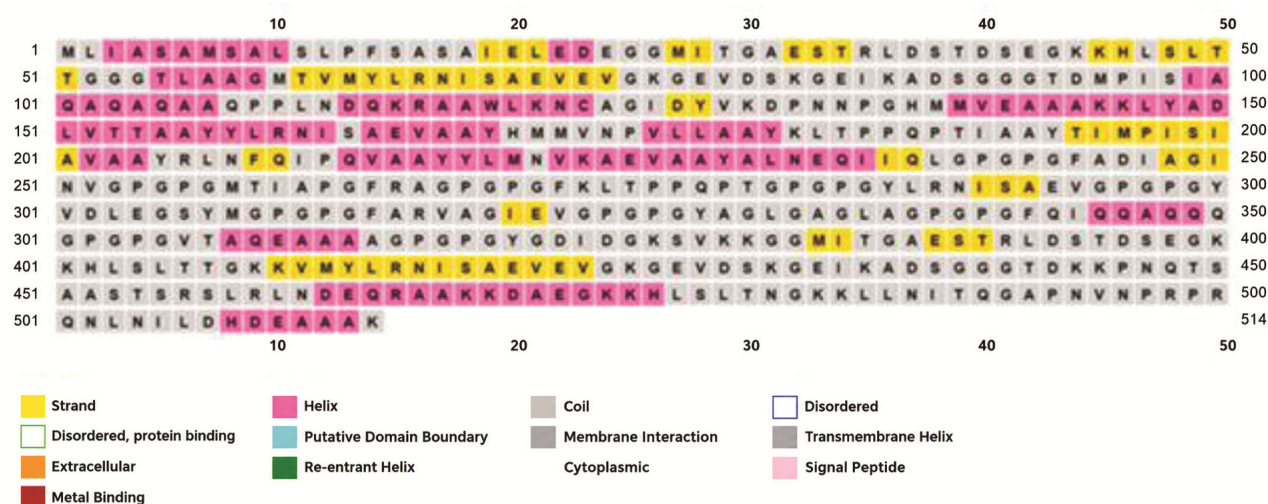


Fig. 4 — Predicted secondary structure of the multi-epitope vaccine construct against *scrub typhus*

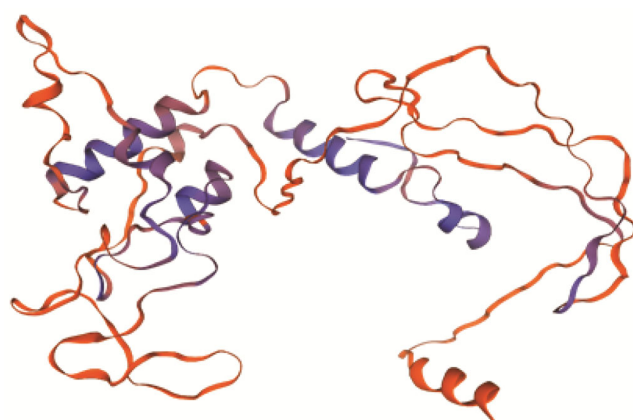


Fig. 5 — Three-dimensional structural model of the designed multi-epitope vaccine construct

score (81.73%) all added more support for how accurate and sturdy the model is (Fig. 5). Proper folding is a key to keeping epitopes accessible and making sure immune receptors can recognize them. Given the robust validation results from this study, it looks like the construct has the stability needed for those important immunological interactions.

Molecular docking with TLR2 receptor

The molecular docking analysis of the vaccine construct with Toll-like receptor 2 (TLR2) showed some strong and stable binding interactions. Many docking clusters had favourable weighted energy scores, meaning the receptor engaged efficiently. Specifically, Cluster 4 had the lowest energy score at -1334.7, indicating the strongest binding affinity, based on (Table 5). TLR2 plays a key role in detecting bacterial parts and setting off downstream inflammatory signals. The solid interactions between the vaccine and TLR2

Table 5 — Models Score of protein-protein interaction

Cluster	Members	Representative	Weighted Score
0	70	Center	-1034.6
		Lowest Energy	-1152.0
1	57	Center	-1065.0
		Lowest Energy	-1097.2
2	51	Center	-964.8
		Lowest Energy	-1120.0
3	47	Center	-1151.9
		Lowest Energy	-1183.8
4	45	Center	-1017.0
		Lowest Energy	-1334.7

hint at the potential to spark innate immune responses, which are crucial for presenting antigens and boosting adaptive immunity. Furthermore, the stable docking shapes seen across various clusters support how well the vaccine candidate fits and its likely ability to stimulate the immune system.

Normal mode analysis of docking complex

Normal Mode Analysis (NMA) was performed using the iMODS server to evaluate the flexibility and collective motions of the protein structure. The NMA B-factor profile revealed heterogeneous mobility along the structure, with increased fluctuations observed in several regions, particularly toward the higher atom-index positions (Fig. 6a). The deformability profile exhibited several pronounced peaks, suggesting the presence of flexible or hinge-like regions capable of accommodating conformational changes (Fig. 6b). The first normal mode displayed an eigenvalue of 5.039164×10^{-6} ,

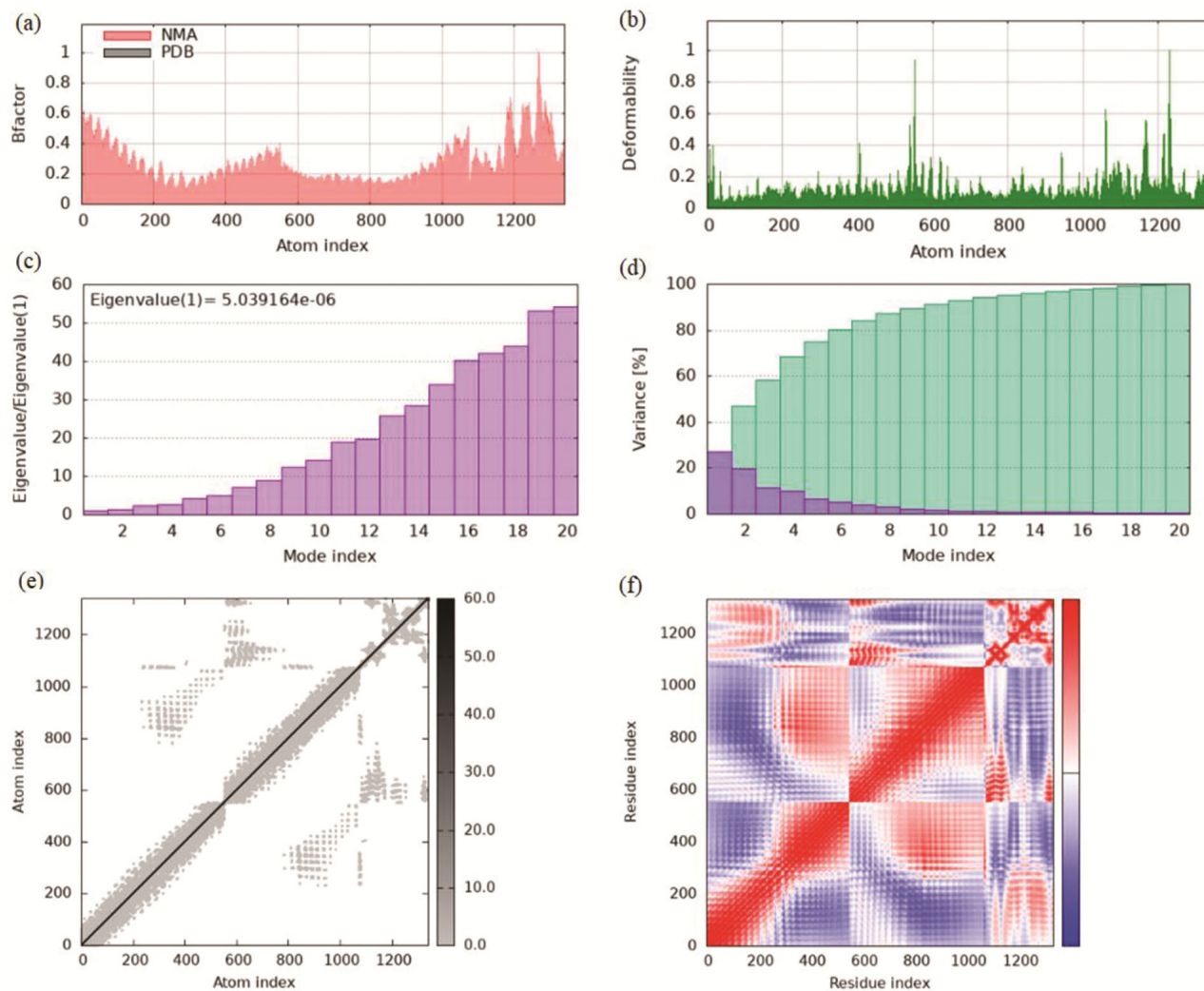


Fig. 6 — Normal mode analysis of the TLR-2–vaccine complex using the iMODS server showing (a) NMA mobility and B-factor profile, (b) deformability plot, (c) eigenvalue distribution, (d) individual and cumulative variance, (e) covariance matrix depicting correlated, anti-correlated and uncorrelated residue motions, and (f) elastic network model illustrating interatomic stiffness within the complex

indicating a relatively low modal stiffness and a potentially significant contribution of low-frequency collective motion (Fig. 6c). The variance profile demonstrated that the initial normal modes contributed substantially to the overall equilibrium motion (Fig. 6d). The covariance map showed both correlated and anti-correlated residue motions, indicating coordinated movements between different structural regions (Fig. 6e). The elastic network analysis further demonstrated heterogeneous residue connectivity and varying interaction stiffness throughout the structure (Fig. 6f). Collectively, these results suggest that the protein possesses a structured but flexible architecture capable of undergoing coordinated conformational changes.

Immune simulation analysis

The immune simulation results depicted in Figure 7 show how the vaccine antigen interacts with immunoglobulins during the immune response. When you first get exposed, there's a quick jump in antigen concentration. It then drops as your immune system kicks into gear. During the primary response, IgM antibodies appear first, showing that the antigen has been spotted. With later exposures, IgG antibody levels spike, pointing to a powerful secondary and tertiary response. This response has greater specificity and affinity too. The antigen levels drop fast after boosters, likely due to efficient clearance *via* antigen-antibody complexes. Also, the steady high IgG levels mean that immunological memory is forming,

essential for lasting protection. Altogether, these findings show that the multi-epitope vaccine triggers a strong, adaptable humoral immune response that effectively neutralizes antigens.

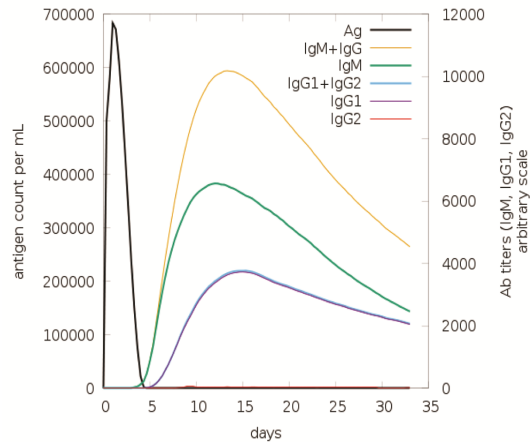


Fig. 7 — Antigen clearance and immune response dynamics predicted through immune simulation analysis

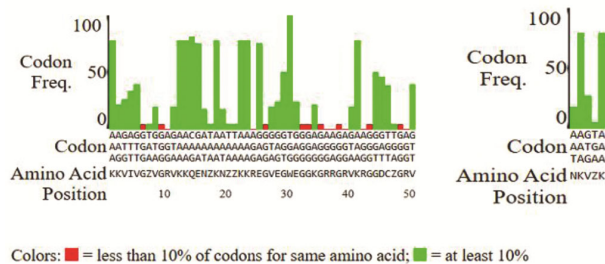


Fig. 8 — Codon optimization analysis of the vaccine construct for *Escherichia coli* expression

Immune simulation studies showed strong humoral immune responses after vaccine administration. During the primary response phase, there was a big increase in IgM levels. After that, IgG levels went way up for secondary and tertiary exposures. This means that immunological memory and long-term adaptive immunity were successfully established. The antigen concentration dropped quickly after each booster, suggesting efficient antigen clearance thanks to antigen-antibody interactions. Plus, the sustained IgG responses show the creation of memory B cells that offer long-term protection against future infections. So, all these results together prove that the vaccine can create strong and lasting immune responses using both primary and memory immune processes.

Codon optimization and *in silico* cloning

The codon optimization showed a high Codon Adaptation Index, close to 1.0, and the GC content was just right too—between 30% and 70%. This means the sequence should work great in the *Escherichia coli* system. Plus, it supports efficient transcription and translation without any problematic elements getting in the way (Fig. 8). When we used *in silico* cloning into the pET-28a(+) vector, we found that the optimized vaccine gene inserted perfectly between the NdeI and XhoI sites. The orientation and reading frame were correct, with the vector's His-tag sequence still working (Fig. 9). This setup makes things

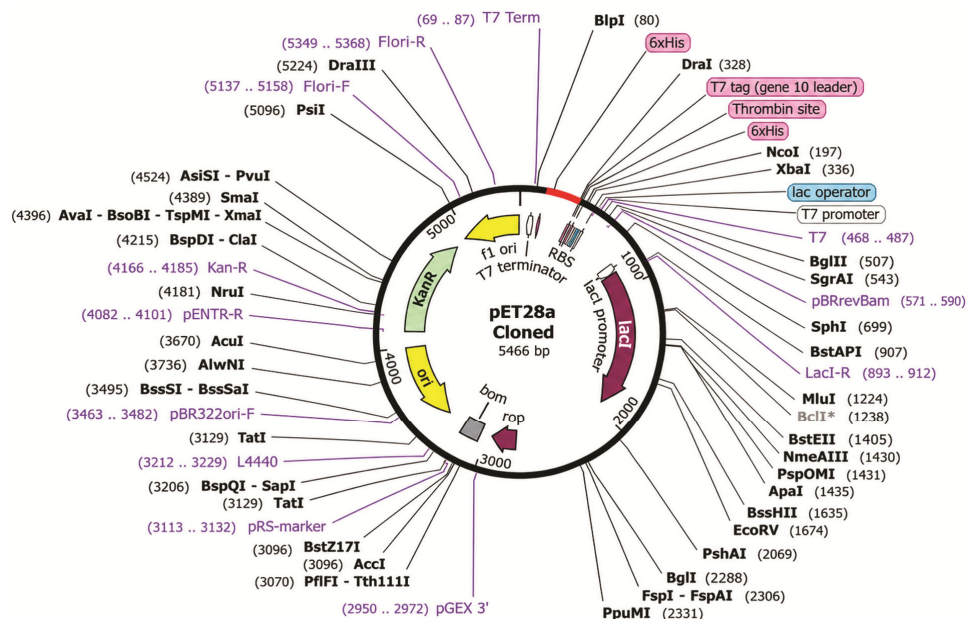


Fig. 9 — *In silico* cloning of the optimized vaccine gene into the pET-28a(+) expression vector

perfect for future protein purification and production steps. All these results show that our vaccine design is ready for real-world expression studies.

Discussion

The current study has adopted a combined approach involving reverse vaccinology and immunoinformatics to develop a multi-epitope vaccine candidate that targets the 56 kDa type-specific antigen (TSA56) of *Orientia tsutsugamushi*, which causes *scrub typhus* disease. The rising prevalence of *scrub typhus* along with the absence of a commercialized vaccine and high antigenic variability among different strains calls for new methods of vaccine development. Traditional strategies used to develop vaccines against *O. tsutsugamushi* have been found to be less effective since immunity developed against a particular strain does not provide broad cross-protection against other strains.

TSA56 was chosen as the vaccine candidate due to its prominent position as an immunogenic outer membrane protein associated with bacterial adhesion, cell entry, and recognition. TSA56 has been shown to be an important molecule in triggering both cell-mediated and antibody-dependent responses to infection²⁹. Nevertheless, TSA56 contains hypervariable segments that cause significant antigenic variation and create considerable obstacles in vaccine development. In order to counteract this problem, the current study included seven TSA56 protein variants originating from various strains of *Orientia tsutsugamushi*. The involvement of multiple strains allowed the detection of common epitopes in TSA56 proteins.

The antigenic screen showed that all the identified TSA56 proteins were highly immunogenic in nature, and the results of allergenicity and toxicity test showed that they are safe to use as a vaccine component. Surface-associated proteins are believed to be excellent candidates for vaccines because they are easily accessible to host's immune system and play an important role in interaction between pathogens and hosts. This is in agreement with the fact that TSA56 proteins are highly immunogenic in nature. Moreover, the filtration of epitopes on the basis of antigenicity, allergenicity, and toxicity would result in safer vaccine candidates.

A successful immune response to intracellular pathogens like *O. tsutsugamushi* needs the coordination between cell-mediated and humoral immune responses. Therefore, this vaccine formulation included CTL, HTL, and B-cell epitopes. The selected CTL epitopes were found to have good antigenic properties along with excellent HLA binding properties that show promise for inducing CD8+ T-cell responses to kill infected cells. Among the selected epitopes, RLNFQIPQV and HMMVNPVLL showed exceptionally high antigenicity. Cell-mediated cytotoxic T-cell responses are vital in case of *scrub typhus* as the bacterium resides and multiplies inside host cells.

In the same way, the observed HTL epitopes exhibited high antigenicity and non-allergic characteristics that could contribute to their activation of CD4+ T lymphocytes. Helper T cells play an important role in the production of cytokines, as well as the development of B cells and establishment of immunological memory. The conserved HTL epitopes like FKLTPPQPT and MTIAPGFRA have been frequently observed in the various TSA56 mutants, implying the existence of conserved dominant immunogenic regions able to provide cross-reaction immunity. These findings are significant as antigenic variation among the different *O. tsutsugamushi* strains had constrained the effectiveness of other vaccines.

Moreover, adding B-cell linear epitopes provided more strength to the vaccine structure, since it helped in developing immunity through antibodies³⁰. Antibodies formed against the antigens present on the surface of the pathogen help in neutralizing the pathogen and preventing its adhesion as well as providing better immunity. The chosen epitopes showed good antigenicity and safety, making them ideal for developing humoral immunity. Moreover, certain epitopes such as YLRNISA EV were found to overlap in both T-cell and B-cell epitopes, implying that both types of immunities could be developed simultaneously.

Coverage of the population by the epitopes under consideration is important for the analysis of multi-epitopic vaccines since HLA polymorphism affects the presentation of antigens and activation of T-cells in different populations. Since there is a very high coverage of several geographical locations, it can be assumed that the selected conserved TSA56-derived epitopes are sufficiently widespread in common HLA alleles. Moreover, high average epitope-HLA

interaction frequency and PC90 values indicate the likely immunological significance of the selected epitopes³¹.

Another vital feature of the present study involves the rational design of the multi-epitope vaccine construct. β -defensin was included as the adjuvant in order to promote the activation of the innate immune system and to increase immunogenicity. There is scientific evidence on the antimicrobial and immunomodulatory effects of the β -defensins, which made these molecules potential adjuvants in vaccine development owing to their capability to activate dendritic cells³². In addition, appropriate peptide linkers were designed in order to ensure epitope stability and effective antigen processing. Specifically, the EAAAK linker ensured the stability of the adjuvant and epitope sections of the molecule, whereas the AAY and GPGPG linkers ensured the efficient processing of the CTL and HTL epitopes *via* MHC class I and II pathways, respectively.

The physicochemical study revealed that the developed vaccine candidate had ideal physicochemical properties. The lower value of the instability index implied structural stability of the protein, while the negative value of the GRAVY score indicated hydrophilicity, which was advantageous for the solubility and accessibility of the proteins. The aliphatic index also confirmed the thermal stability of the vaccine candidate, making it an ideal candidate for expression. Other vaccines with similar physicochemical properties have been proposed recently using immunoinformatics in their development process³³.

Further evidence to prove the validity of the developed vaccine was obtained from structural modelling and validation tests. The predicted tertiary structure proved to possess very good stereochemical quality since 95.5% of the residues were present in the preferred region of the Ramachandran plot. Moreover, the excellent ERRAT score, Verify3D analysis results, and poor MolProbity score demonstrated the correct structural folding without any steric clashes in the protein structure³⁴. The importance of correct structural conformation cannot be ignored when dealing with multi-epitope vaccines because misfolding may lead to improper antigen presentation and receptor binding.

Activation of innate immunity is very important for induction of an adaptive immune response to

pathogens like bacteria. Thus, molecular docking analysis was conducted to check the interaction between the designed vaccine structure and Toll-like receptor 2 (TLR2), which acts as a pathogen recognition receptor for bacteria and plays an important role in inflammatory signalling. The results of the docking analysis showed that there were some good docking scores between TLR2 and the designed vaccine. Out of all models analysed, Cluster 4 had the lowest value of weighted docking energy score, indicating a strong receptor-ligand binding affinity. Interaction between the ligand and TLR2 might help to induce an immune response and promote downstream signalling. Such interactions were observed previously in computational vaccine studies³⁵.

The normal mode analysis using iMODS gave another piece of evidence about the stability of the interaction between the vaccine construct and TLR2. While docking analysis is a static form of binding, normal mode analysis using iMODS assesses the intrinsic flexibility and coordinated movements of atoms within the protein complex. The low value of eigenvalues and correlation among residue fluctuations reveal that there is enough flexibility needed for biological interaction without losing the stability of the docked complex. Computational based analysis of stability has been extensively used in vaccine studies in order to confirm the results achieved by molecular docking analysis³⁶.

The immune system simulation study has further confirmed the ability of the proposed vaccine construct to trigger an immune response. Based on the simulation results, there is an increased level of IgM and IgG antibodies in response to the antigen injection and boosters. This implies the development of a strong humoral immune response to the vaccination process. It is also evident from the increasing levels of IgG antibody and the subsequent reduction in the antigen concentrations after repeated exposures to the pathogen, which means immunological memory, has been established effectively³⁷. Therefore, the immune response pattern obtained demonstrates the ability of the vaccine design to elicit adaptive immunity.

The feasibility of the translation of this vaccine construct was assessed through codon optimization and *in silico* cloning analysis prior to its expression in a recombinant format. The optimized sequence demonstrated a good codon usage as well as

an acceptable GC ratio for successful expression in *E. coli*. Further, successful incorporation of this sequence in pET-28a(+) plasmid confirmed its suitability for subsequent studies on protein expression. This is an important aspect of this study because efficient expression is a crucial step towards experimentation and vaccine production.

However, despite the optimistic results, there are some shortcomings of the current study that need to be mentioned. The first one is that the research was purely theoretical and, thus, needs further experimental validation by conducting *in vitro* and *in vivo* studies. The second shortcoming of the study is that only a limited number of HLA alleles were used in the analysis of population coverage of the vaccine candidates. Furthermore, only normal mode analysis using iMODS server was performed and molecular dynamics simulations were not conducted to determine the stability of the vaccine-receptor complex at physiological conditions. It is also worth mentioning that induction of cytokines and the protective efficacy of the vaccine were not validated experimentally.

In general, the current study illustrates the use of immunoinformatics and reverse vaccinology tools to develop a vaccine target against *scrub typhus* based on a rational approach. The application of conserved epitope prediction, structural analysis, immune system simulation, docking with receptors, normal mode analysis and codon optimization allowed constructing a robust computational pipeline for designing a vaccine against the antigenically diverse *O. tsutsugamushi* serotypes. The results pave the way for further experimental research towards obtaining an efficient multi-epitope vaccine against *scrub typhus*.

Conclusion

For the design of a conserved multi-epitope vaccine against *Orientia tsutsugamushi* targeting its 56 kDa Type-Specific Antigen (TSA56), this study adopted a combined immunoinformatics and reverse vaccinology strategy. Conserved CTL, HTL, and B-cell epitopes from various forms of TSA56 were found to possess strong antigenic, non-allergic, and nontoxic properties. The vaccine was predicted to have stable physiochemical and structural properties, stable TLR2 binding ability, and the capacity to induce both humoral and cellular immunity according to immune simulation experiments. Moreover, codon

optimization and *in silico* cloning showed that the vaccine could be expressed in *E. coli* cells. Though the results obtained from this study have been encouraging, the current study remains theoretical and requires experimental validation by means of *in vitro* immunological tests as well as *in vivo* animal experiments. Furthermore, inclusion of other HLA alleles along with MD simulation might help to enhance the efficacy of the vaccine and its population coverage. However, considering the encouraging findings obtained from this study, the designed vaccine construct could serve as a basis for developing a broadly applicable epitope-based vaccine against *scrub typhus*.

Acknowledgement

The authors are grateful to the Management of Sethu Institute of Technology, Pulloor, Tamilnadu, India, for the research facilities.

Conflict of interest

All authors declare no conflict of interest.

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