

ORIGINAL RESEARCH ARTICLE

Reverse vaccinology approach for the design of a transmission-blocking vaccine by targeting the malaria vector *Anopheles*

Haitham Al-Madhagi* 

Biochemical Technology Program, Faculty of Applied Sciences, Thamar University, Dhamar, Yemen

Abstract

According to the latest update from WHO, approximately 263 million cases of malaria were reported globally. The mosquito species *Anopheles gambiae* is the primary vector transmitting the malaria-causing pathogen *Plasmodium* spp. Scientists have recently focused on developing a transmission-blocking vaccine (TBV) to combat malaria by targeting vector homeostasis rather than specific *Plasmodium* species, which vary in their genomes and drug susceptibilities. To create this TBV, three salivary proteins (gSG6, D7, and AgTRIO) were selected as potential antigens. From these proteins, epitopes recognized by cytotoxic T cells (18), helper T cells (30), and B cells (10) were selected. These potentially positive antigens were then combined with mycobacterial tuberculosis heparin-binding hemagglutinin using suitable peptide linkers. The resulting vaccine sequence underwent prediction of biochemical properties and secondary and tertiary structure. The best model was generated using the I-TASSER platform, refined, validated, and docked to Toll-like receptor-4 (TLR-4) via the InterEvDock 2 server. The consensus docked complex demonstrated stability, mobility, and flexibility, as assessed through the Internal Coordinates Normal Mode Analysis Server and Groningen Machine for Chemical Simulations dynamics simulations. Furthermore, the vaccine exhibited a well-defined three-dimensional structure and strong binding to TLR-4 through multiple bonds. Molecular mechanics/generalized Born surface area yielded a ΔG of $-28.5 \text{ kcal mol}^{-1}$; however, stable binding was not achieved during the 100 ns molecular dynamics simulation, as indicated by a root mean square deviation of 1.25 nm. Following *in silico* simulation of vaccination, the vaccine was predicted to significantly induce robust B-cell and T-cell responses, antibody production (both IgM and IgG), and interferon- γ secretion. Importantly, this multi-epitope malaria TBV demonstrated predicted immunogenic potency without evidence of allergenicity or toxicity, suggesting its potential to limit the transmission of multidrug-resistant *Plasmodium* strains. However, these computational predictions require validation through *in vitro* and *in vivo* studies.

Keywords: Malaria; *Plasmodium*; Transmission-blocking vaccine; Vaccine; Immunoinformatics

*Corresponding author:

Haitham Al-Madhagi
(bio.haitham@tu.edu.ye)

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1. Introduction

Malaria is one of the leading health problems worldwide. The latest report of WHO states that “globally in 2023, there were an estimated 263 million malaria cases and 597,000 malaria deaths in 83 countries, with Africa being home to 94% of total malaria cases.”¹ Owing to the COVID-19 outbreak, malaria incidence and mortality have received less attention, potentially undermining malaria control efforts.² Accordingly, the epidemiology of imported malaria has changed, with a reduction in travel-related infections during the pandemic because of worldwide restrictions on population movements.³ Nevertheless, increased infection and transmission have been reported among individuals returning from malaria-endemic hotspots.⁴ Therefore, monitoring the spread of *Plasmodium* spp. following importation into malaria-free or low-transmission areas is important. This is particularly relevant because *Anopheles* spp. can contribute to malaria transmission through three major epidemiological processes: expansion into new geographical areas, emergence of new malaria cases in previously unaffected regions, and nosocomial transmission associated with infected patients.⁵

The female *Anopheles* mosquito (primarily *Anopheles gambiae*) plays a pivotal role in malaria transmission by acquiring gametocytes during a blood meal, subsequently transmitting sporozoites to another host. Therefore, a principal strategy for malaria control is to interrupt this transmission cycle. The gametocyte stage is critical for the continued transmission and propagation of malaria.⁶ Moreover, the transition from asexual development to sexual differentiation represents a crucial juncture in the parasite's life cycle, during which the parasite can adapt its strategy to optimize its fitness. The parasite may increase gametocyte production to enhance the likelihood of transmission or favor the replication of asexual parasites to improve its survival in the host. Gametocytes that are not ingested by mosquitoes cannot revert to asexual forms, resulting in the loss of the parasite's investment in transmission.^{7,8}

Among the five *Plasmodium* species capable of causing malaria, *Plasmodium falciparum* is the most virulent and lethal, accounting for a substantial proportion of severe malaria cases and malaria-related deaths, particularly in sub-Saharan Africa. In contrast, *Plasmodium vivax* generally causes less severe disease than *P. falciparum* but has a considerably wider geographical distribution.⁹ The clinical manifestations of malaria primarily result from the asexual parasite stages in human erythrocytes, whereas transmission to mosquitoes occurs via the sexual stages, known as gametocytes. Therefore, effective control of *P. falciparum* transmission requires targeting both the

asexual and sexual stages in the human host. However, eradication of *P. vivax* is particularly challenging because dormant liver-stage hypnozoites can reactivate and cause relapses, potentially maintaining a reservoir of infection and transmission for months after the initial infection.¹⁰

The current standard therapy for malaria involves anti-malarial agents, such as artemisinin-based combination therapies, which have demonstrated high efficacy in treating the disease. However, the emergence and spread of drug-resistant *Plasmodium* strains have underscored the need to prioritize the development of effective malaria vaccines. Recent reviews have highlighted several vaccination strategies aimed at preventing disease onset and progression or reducing parasite transmissibility. These approaches include using irradiated sporozoites to stimulate an immune response against the malaria parasite, targeting the parasite's sexual stages to prevent disease transmission, and genetically attenuating the parasite to reduce virulence for use as a vaccine. While there are no licensed products for these approaches yet, it is crucial to acknowledge the enormous scientific and technical challenges encountered in developing vaccines against such a complex eukaryotic parasite. Therefore, continuing research in this area is critical to ensure that effective vaccines are developed to combat drug-resistant strains of malaria.^{11–14}

As several countries transition from malaria control toward elimination, the focus has shifted from the diagnosis and treatment of overt cases to a more comprehensive understanding of the transmission reservoir, including infections that are asymptomatic. Conventional measures and interventions, such as insecticide-treated bed nets, anti-vector spraying, and parasite screening and treatment, have played important roles in reducing malaria transmission. Accumulating evidence suggests that *Plasmodium* parasites can alter their allocation of resources toward transmission in response to changing environmental conditions, thereby influencing their persistence and spread within host populations.^{15,16}

Transmission-blocking vaccines (TBVs) represent a promising, valuable strategy for malaria control and elimination, aiming to interrupt *Plasmodium* transmission through the *Anopheles* vector during blood feeding. TBVs induce antibodies that target the parasite's maturation in the mosquito after it feeds on a vaccinated individual, thereby disrupting the parasite's life cycle. Although TBVs may not entirely prevent malaria infection in vaccinated individuals, they can reduce the proportion of infected mosquitoes and consequently, community-level malaria transmission. TBVs therefore have considerable potential for malaria elimination, and several promising TBV

candidates have progressed to clinical trials. However, their development remains challenging because of factors such as limited immunogenicity or high reactogenicity in humans. Understanding the prevalence and intensity of *Plasmodium* infection in naturally blood-fed wild mosquitoes is important for assessing the potential field effectiveness of TBVs and the level of transmission-blocking required. Therefore, TBVs warrant continued investigation as an important component of integrated malaria elimination strategies.^{17–19} Accordingly, this study aimed to design a multi-epitope vaccine targeting salivary proteins of *A. gambiae* using a reverse vaccinology approach, with the goal of generating an immune response against the malaria vector and potentially reducing its capacity to support *Plasmodium* transmission.

2. Methods

2.1. Study design

This was a multi-step reverse vaccinology approach that employed different servers and programs to design a malarial TBV. The workflow summary is illustrated in Figure 1.

2.2. Selection of antigens

Given that *A. gambiae* injects saliva into the host during blood feeding, its salivary proteins were evaluated for their potential as vaccine antigens. Previous studies have detected gSG6, D7, and AgTRIO proteins in the blood of malaria-infected individuals and demonstrated their important roles in blood feeding and the modulation of host inflammatory responses.^{20,21} Therefore, these proteins were selected as sources for epitope identification and subsequent vaccine construction. The amino acid sequences of D7 (UniProt accession Q9BIH3), gSG6 (UniProt accession C1K7K2) and AgTRIO (GenBank accession AAL68795.1) were retrieved using their respective accession numbers.

2.3. Prediction of antigenicity and compartmentalization

The subcellular localization of the selected proteins was predicted using the BUSCA server (<http://busca.biocomp.unibo.it/>).²² The antigenicity of the selected proteins was subsequently evaluated using Vaxijen 2 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>)²³, with a

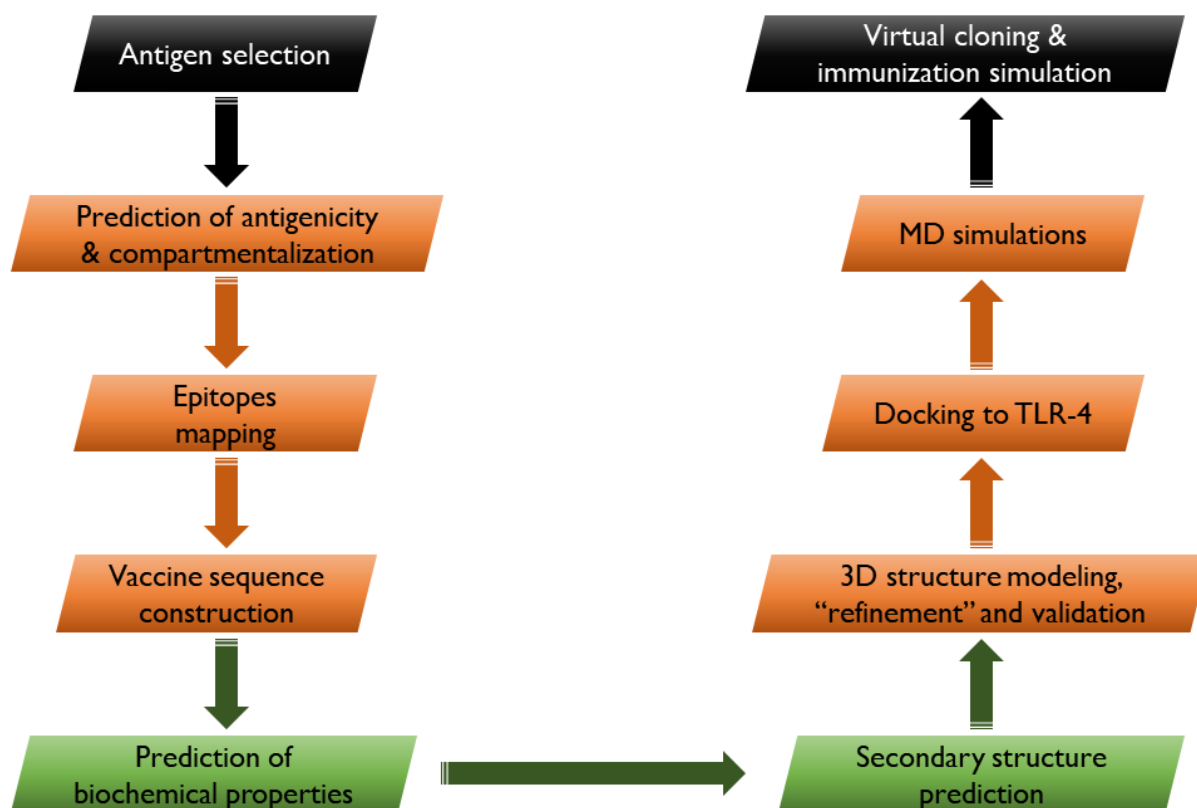


Figure 1. The adopted methodology for TBV design using a reverse vaccinology approach
Abbreviations: 3D: Three-dimensional; MD: Molecular dynamics; TBV: Transmission-blocking vaccine; TLR-4: Toll-like receptor 4.

threshold of 0.4. The protein sequences in FASTA format were submitted as input to both servers for analysis.

2.4. Epitope mapping

Following confirmation of the subcellular localization and antigenic potential of the selected proteins, the corresponding immune epitopes were identified from these protein sequences. T-cell cytotoxic (T_C) epitopes were predicted using the NetCTL 1.2 server (<https://services.healthtech.dtu.dk/services/NetCTL-1.2/>)²⁴ with a cut-off value of 0.75. T-helper (T_H) epitopes were identified using the major histocompatibility complex (MHC)-II prediction tool of the Immune Epitope Database (<http://tools.iedb.org/mhcii/>)²⁵, with epitopes having a percentile rank of <1.5 selected for further analysis. The predicted T_H epitopes were subsequently evaluated for their potential to induce interferon-gamma (IFN- γ) responses using the IFNepitope tool (<https://webs.iitd.edu.in/raghava/ifnepitope/predict.php>).²⁶ B-cell epitopes were predicted using the BepiPred 2 web server (<https://services.healthtech.dtu.dk/services/BepiPred-2.0/>).²⁷ The reported area under the curve for this prediction tool was 0.62, indicating relatively limited classification performance.

2.5. Screening of potential allergenicity and toxicity

The potential toxicity of the identified epitopes was evaluated using the ToxIBTL tool (<https://server.weigroup.net/ToxIBTL/Server.html>).²⁸ Potential allergenicity was subsequently assessed using the AllerCatPro 2.0 web server (<https://allercatpro.bii.a-star.edu.sg/>),²⁹ with the default parameters applied.

2.6. Vaccine construction

The selected epitopes were assembled into a multi-epitope vaccine incorporating the mycobacterial heparin-binding

hemagglutinin adhesin (UniProt accession A5TZK3) as an adjuvant. This adjuvant has previously been reported to induce potent immune responses.³⁰ The selected epitopes were connected using different peptide linkers: EAAAK was used to link the adjuvant to the T_C epitopes, GP GPG to connect the T_C epitopes, AAY to link the T_H epitopes, and KK to connect the B-cell epitopes, as illustrated in Figure 2. These linkers have been reported to enhance the immunogenicity and structural stability of multi-epitope vaccine constructs.³¹

2.7. Cross-reactivity

The vaccine construct was assessed for potential similarity to human self-antigens using the BLASTp tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>),³² with the default parameters.

2.8. Calculation of biochemical properties

The amino acid sequence of the constructed vaccine was submitted to the ProtParam online tool (<https://web.expasy.org/protparam/>)³³ to determine its physicochemical properties, including molecular weight, isoelectric point (pI), net charge, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). Additionally, the solubility of the vaccine construct was evaluated using the Protein-Sol tool (<https://protein-sol.manchester.ac.uk/>).³⁴

2.9. Prediction of vaccine secondary structure

The secondary structure of the vaccine construct was subsequently predicted using the NetSurfP 3.0 web server (<https://services.healthtech.dtu.dk/services/NetSurfP-3.0/>).³⁵ This server provides comprehensive information on key structural characteristics, including the secondary structure adopted by each amino acid residue,



Figure 2. The strategy employed to construct the multi-epitope TBV using the appropriate adjuvant and peptide linkers
Abbreviations: TBV: Transmission-blocking vaccine; T_C : T-cell cytotoxic; T_H : T-helper.

relative surface accessibility, and the predicted degree of structural disorder.

2.10. Modeling, refinement, and validation of the vaccine structure

The FASTA sequence of the constructed vaccine was submitted to the I-TASSER platform (<https://zhanggroup.org/I-TASSER/>)³⁶ for tertiary structure prediction, with all parameters set to their default values. This platform ranks the modeled structures by their confidence score (C-score), which reflects the estimated quality of the predicted three-dimensional (3D) structure; higher C-scores indicate greater confidence in the model. The generated model was subsequently refined using the DeepRefiner online tool (<http://watson.cse.eng.auburn.edu/DeepRefiner/index.php>).³⁷ The ResNet deep-learning model and Adventurous refinement mode were selected, followed by post-refinement analysis. Finally, the refined structure was validated using the Ramachandran plot generated by PROCHECK (<https://saves.mbi.ucla.edu/>)³⁸ and the residue error plot provided by DeepRefiner.

2.11. Protein–protein docking and molecular mechanics/generalized Born surface area computation

The refined and validated 3D vaccine model was docked against the innate immune system receptor Toll-like receptor-4 (TLR-4; Protein Data Bank [PDB] ID: 3FXI) using the InterEvDock 2 platform (<https://bioserv.rpbs.univ-paris-diderot.fr/services/InterEvDock2/>).³⁹ This platform integrates three docking approaches—SOAP_PP, FRODOCK 2, and the main InterEvDock 2 algorithm—and ranks the top 10 docked models by docking score. The consensus approach combines the outputs of the three algorithms to improve the reliability of the predicted protein–protein interactions. Unlike some other docking platforms, InterEvDock 2 reports docking scores with positive values, with higher scores indicating more favorable predicted interactions. Furthermore, the protein–protein interactions within the top-ranked vaccine–TLR-4 complex were analyzed using LigPlot+ software.⁴⁰ The binding free energy of the vaccine–TLR-4 complex was then computed using the HAWKDOCK platform, employing the molecular mechanics/generalized Born surface area (MM/GBSA) (<http://cadd.zju.edu.cn/hawkdock/>)⁴¹ approach.

2.12. Molecular dynamics simulations

The next step involved molecular dynamics (MD) simulations using the WebGro web server (<https://simlab.uams.edu/>).⁴² GROMOS96 43a1 force field was selected, with TIP4P as the water model. The system was placed in

a triclinic simulation box and supplemented with 0.15 M potassium chloride. Energy minimization was performed using the steepest-descent algorithm for 1,000 steps, followed by equilibration at constant number of particles, volume, and temperature, and at constant number of particles, pressure, and temperature, held at 300 K and 1 bar, respectively. A 100 ns MD simulation was subsequently performed to evaluate the relative mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (Rg), and solvent accessible surface area (SASA).

The conformational changes and dynamic stability of the TBV–TLR-4 complex were further investigated using normal mode analyses (NMA) implemented in the Internal Coordinates Normal Mode Analysis Server (iMODS) (<https://imods.iqfr.csic.es/>)⁴³ to assess the biophysical attributes of the TBV–TLR-4 complex. The iMODS server is a widely used and customizable platform that provides coarse-grained analysis of protein dynamics based on C-alpha atoms and dihedral coordinates, enabling assessment of collective motions associated with protein domain dynamics. The B-factor was analyzed to estimate the amplitude of atomic displacement within the TBV–TLR-4 complex. Eigenvalues were also evaluated to determine the relative impact of each deformation movement in the motion of interacting proteins within the TBV–TLR-4 complex. Lower eigenvalues generally indicate greater structural stability, while higher values suggest the opposite. Variance plots and covariance matrices were examined to characterize molecular flexibility, the amplitude of fluctuations associated with individual modes, and overall conformational change within the TBV–TLR-4 complex. For this analysis, the docked TBV–TLR-4 complexes were prepared in PDB format and submitted to the iMODS server.

2.13. Disulfide engineering

The vaccine construct was further optimized through disulfide engineering to enhance its structural stability and promote proper folding. This modification was performed using the computational Disulfide by Design 2 web server (<http://cptweb.cpt.wayne.edu/DbD2/>).⁴⁴ This strategic modification was intended to strengthen intramolecular interactions, thereby improving the overall robustness and functional integrity of the designed immunogen.

2.14. Virtual cloning and RNA secondary structure prediction

The virtual cloning process was performed using the SnapGene software (GSL Biotech), a widely used molecular biology platform for designing and analyzing recombinant DNA constructs, available at <http://snapgene.com/>. Specifically, the amino acid sequence of the vaccine

construct was first reverse-translated into its corresponding DNA sequence using the backtranseq tool (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/)⁴⁵, available through the European Bioinformatics Institute (EBI) web services. Subsequently, the DNA sequence was codon-optimized for expression in the target host using the JCat tool (<http://www.jcat.de/>).⁴⁶ The optimized DNA sequence was then virtually cloned into the pET-28a(+) expression vector using the restriction enzymes XhoI and SamI. The pET-28a(+) vector is widely used for recombinant protein expression in *Escherichia coli* and facilitates the production and subsequent purification of recombinant proteins through its His-tag sequence. For prediction of the RNA secondary structure of the vaccine construct, the RNAfold server was used⁴⁷ (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). The predicted structure was evaluated based on the minimal free energy (MFE) of folding and the partition function.

2.15. Immunization simulation

The present study also employed computational immune simulation (C-ImmSim) (<https://kraken.iac.rm.cnr.it/C-IMMSIM/>)⁴⁸ to evaluate the immunological response elicited by the designed vaccine. C-ImmSim is a widely used computational tool that simulates the natural immune environment and predicts immune responses involving B cells, helper T lymphocytes, cytotoxic T lymphocytes, immunoglobulins, cytokines, and other immune parameters. The simulation volume and number of simulation steps were set to 10 and 100, respectively, while all other parameters were maintained at their default settings. C-ImmSim was used to assess the predicted immunogenicity of the vaccine construct

and to characterize its potential to elicit a broad and sustained immune response. This approach is a standard methodology in immunology research, allowing for the evaluation of the immunogenic potential of vaccine candidates in a controlled and reproducible manner.

2.16. Population coverage of the vaccine

The predicted epitopes and their corresponding human leukocyte antigen (HLA) alleles were submitted to the IEDB Population Coverage tool (<http://tools.iedb.org/population/>)²⁵ to estimate the potential population coverage of the vaccine at both global and African regional levels. The selected MHC alleles included HLA-A30, HLA-B15, HLA-C07, HLA-B58, and HLA-DRB1*03, which were selected based on their reported distribution among African populations.^{49,50}

3. Results

3.1. Antigen selection and characterization

Three salivary proteins of *A. gambiae*, namely D7, gSG6 and AgTRIO, were selected as potential antigens for epitope identification. Their predicted antigenicity scores were 0.5011, 0.5528, and 0.4797, respectively, all exceeding the predefined antigenicity threshold of 0.4. Furthermore, all three proteins were predicted to localize to the extracellular space, supporting their potential secretion in mosquito saliva. The predicted subcellular localization and antigenicity profiles are presented in Table 1.

3.2. Epitope mapping

Following the confirmation of the antigenicity of the selected salivary proteins, epitope mapping was performed

Table 1. The selected vector salivary proteins along with the sequence, antigenicity, and subcellular localization

Protein name	Protein sequence	Antigenicity	Localization
D7	MIVPRVLLFILLELFVQATQAFKALDPEEAWVYERCHEDHLPSPGNRETYLKT WKFWKLEPNDVTHCYVKCTLAGLQMYDEKTNFKPETVPVQHEAYKSFTF VESSKVNELQQALSSLNAGSGSCAEVFNAYLPVHNKYIGVSRKIYHGTVDVSAK IYEAKPEIKKQEEFFAYCAKKALGANGKEGYKKIRDYELADSAEFRNAMDCVF RGFRYMDDSGLKVDEVVRDFNLINKSDLEPEVRSVLASCTGTHAYDYSSCLLNS SYKEDFRNAFYFHELRSANYGYLAMGKVYEGPEKVKEELKKLNY	0.5011 (probable antigen)	Extracellular space
gSG6	MAIRVELLAMVLLPLLLLESVPPYAAAEKVWVDRDKVYCGHLDCTRVATFKG ERFCTLCDTRHFCECKETREPLPYMYACPGTEPCQSSDRLGSCSKSMHDLVLCR IDQAFLEQ	0.5528 (probable antigen)	Extracellular space
AgTRIO	MCRGLSAVILLISLSAQLHVVGEEAPKPEKEICGLKVGRLLDSVKGWLSVSQQ EKCPNLKYCENKIQADQYNLPLTCIRWRSQNPASPAGSLGGKDVVSKIDAAMA NFKTLFEPMKADLAKLEEEVKRQVLDAAWKALEPLQKEAYRSTLASGRIERAVFYS FMEMGDNVRLDNFYQANVEELLKYAWALPMHKKQSRMYDLIGQLVQSSKSPM LQTLHAYELATVVNPELENRENLLNDQVQLRDNLKYKNSFATLVSIARHFPDHF TLRQRLFKLPDGSKPGADTLNIVNFIAQLPSDELRLSSIDLLQLSLTAENGLTVQDP EYVYRLSQLAHAMPSLVDVKAHPDLQQSVDLMAKFNTPIDGKTLQYQNGISPS SSVAA	0.4071 (probable antigen)	Extracellular space

to identify T_C, T_H, and B-cell epitopes.

(i) Cytotoxic T-cell epitopes

Cytotoxic T-cell epitopes were predicted from all three selected proteins and subsequently screened for antigenicity, allergenicity, and toxicity. Only epitopes that demonstrated high predicted antigenicity and no predicted allergenicity or toxicity were considered suitable for inclusion in the vaccine construct. The length of all candidate T_C epitopes was restricted to eight amino acids. Although several epitopes met these selection criteria, only two epitopes from each protein were selected for presentation in Table 2.

(ii) T-helper epitopes

Similarly, T_H epitopes were extracted and predicted from the three precursor proteins and evaluated for potential antigenicity, allergenicity, and toxicity. For each protein, two 15-mer T_H-cell epitopes exhibiting strong antigenicity and no predicted allergenicity or toxicity were selected. The selected epitopes were further assessed for their potential to induce IFN- γ responses. Several epitopes fulfilled all the predefined selection criteria, and the selected candidates are summarized in Table 3.

(iii) B-cell epitopes

Unlike the T_C- and T_H-cell epitopes, which were restricted to 8-mers and 15-mers, respectively, the lengths of the B-cell epitopes were not restricted. Consequently, the predicted

epitopes varied considerably in length, ranging from 7 to 32 amino acids, with epitope 1 comprising 32 amino acids, while the others were short, with only seven amino acids. All selected candidates demonstrated strong predicted antigenicity and no significant predicted allergenicity or toxicity. Epitope 6 was excluded because it did not meet the predefined selection criteria. The potential B-cell epitope candidates are presented in Table 4.

It is noteworthy that a total of 10 B-cell epitopes, 18 T_C-cell epitopes, and 30 T_H-cell epitopes were identified; however, only the highest-performing candidates that met the predefined selection criteria were selected for further analysis.

3.3. Biochemical characteristics

The vaccine construct comprised 491 amino acids and had a predicted molecular weight of 54,003.61 Da. Its theoretical pI was 8.29, indicating a slightly alkaline character, consistent with the higher number of positively charged residues (Arg + Lys = 76) than negatively charged residues (Asp + Glu = 73). The predicted half-life varied by expression system: 30 h in mammalian reticulocytes *in vitro*, > 20 h in yeast *in vivo*, and > 10 h in *E. coli in vivo*. These estimates suggest a relatively short protein half-life across the tested expression systems. The calculated instability index was 35, indicating that the protein construct is predicted to be stable. The aliphatic

Table 2. The predicted cytotoxic T-cell epitopes along with the potential antigenicity, allergenicity, and toxicity

Epitope	Antigenicity	Allergenicity	Toxicity
FILLELFV	1.9417 (probable antigen)	No significant hit	Non-toxic
LLFILLEL	1.9548 (probable antigen)	No significant hit	Non-toxic
VLLPLLLL	2.7479 (probable antigen)	No significant hit	Non-toxic
MVLLPLLL	2.5760 (probable antigen)	No significant hit	Non-toxic
LLKYAWAL	2.0730 (probable antigen)	No significant hit	Non-toxic
SLSAQLHV	1.5608 (probable antigen)	No significant hit	Non-toxic

Table 3. The predicted T-helper epitopes along with the potential antigenicity, allergenicity, and toxicity

Epitope	Antigenicity	Allergenicity	Toxicity	IFN- γ
KIRDYELADSAEFRN	0.8975 (probable antigen)	No significant hit	Non-toxic	+
GFRYMDDSGLVDEV	0.8618 (probable antigen)	No significant hit	Non-toxic	+
EKVWVDRDKVYCGHL	0.7736 (probable antigen)	No significant hit	Non-toxic	+
KVWVDRDKVYCGHLD	0.9782 (probable antigen)	No significant hit	Non-toxic	+
NDQVVQLRDNLKNS	1.2400 (probable antigen)	No significant hit	Non-toxic	+
QKEAYRSTLASGRIE	1.3099 (probable antigen)	No significant hit	Non-toxic	+

Abbreviation: IFN- γ : Interferon-gamma.

Table 4. The predicted B-cell epitopes along with the potential antigenicity, allergenicity, and toxicity

Epitope	Antigenicity	Allergenicity	Toxicity
DEKTNTFKPETVPVQHEAYKSFTVESSKVNE	0.5955 (probable antigen)	No significant hit	Non-toxic
KKALGANGKEGYKKIRDYELADSAEFR	0.5296 (probable antigen)	No significant hit	Non-toxic
SVKEDFR	1.6709 (probable antigen)	No significant hit	Non-toxic
CPGTEPCQSSDRLGSCSKSMH	0.8552 (probable antigen)	No significant hit	Non-toxic
WRSQNPASPAGSLGGKD	0.9707 (probable antigen)	No significant hit	Non-toxic
AKLEEEVKR	1.0855 (probable antigen)	No significant hit	Toxic
MEMGDNVRLDNYFQPA	0.8059 (probable antigen)	No significant hit	Non-toxic
AENGLTVQDPEY	1.3392 (probable antigen)	No significant hit	Non-toxic
LVDVKAHPDLQQSVDD	0.6334 (probable antigen)	No significant hit	Non-toxic
NTPIDGKTLQYQNIGISPSSS	0.6964 (probable antigen)	No significant hit	Non-toxic

index and GRAVY were 82.99 and -0.502 , respectively, suggesting good thermostability and a slightly hydrophilic character. Furthermore, the predicted solubility score was 0.6, which was higher than the average solubility score of *E. coli* proteins (0.4; Figure 3), suggesting favorable solubility characteristics that may facilitate recovery of the recombinant vaccine protein following expression.

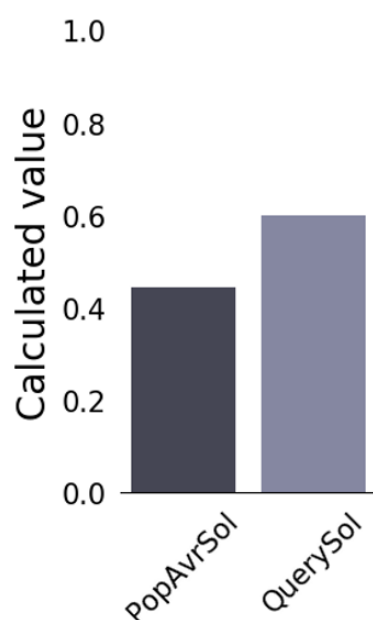


Figure 3. The computed solubility of the constructed vaccine shows a slight elevation compared to the average proteins in the expression system

3.4. Cross-reactivity

The potential immunogenicity of the vaccine construct against human self-antigens was also assessed. The

analysis revealed no significant sequence cross-reactivity with human self-antigens, suggesting a low likelihood of unintended interactions and supporting the predicted safety profile of the vaccine construct.

3.5. Secondary structure

The amino acid sequence of the constructed vaccine was submitted to the NetSurfP-3.0 server for secondary structure prediction. The NetSurfP-3.0 output indicated that the constructed vaccine was predominantly α -helical, interspersed with coil regions. Notably, no β -sheets were predicted in the secondary structure of the constructed vaccine. These findings were consistent with the predicted tertiary 3D structure of the vaccine generated using the I-TASSER server, in which 18 α -helices occupied most of the vaccine 3D structure, with short stretches of coil regions. Furthermore, most of the predicted disordered regions of the protein were located within the coil regions, consistent with the modeled vaccine structure. Overall, the agreement between the secondary- and tertiary-structure predictions supports the predicted structural organization of the constructed vaccine. The secondary structure of the constructed TBV is shown in Figure 4.

3.6. Three-dimensional structure modeling, refinement, and validation

The Ramachandran plot was used to assess the quality of the refined model. The percentage of amino acid residues in the favored region increased from 82.4% to 88.7%, indicating improved overall 3D structural quality. Moreover, the proportion of residues in the additionally allowed region decreased from 13.2% before refinement to 8.5% after refinement. The refined model also exhibited improved values for several other quality parameters, including a MolProbity score of 1.751, a Rosetta free energy of -764.698 , and a predicted global quality score of

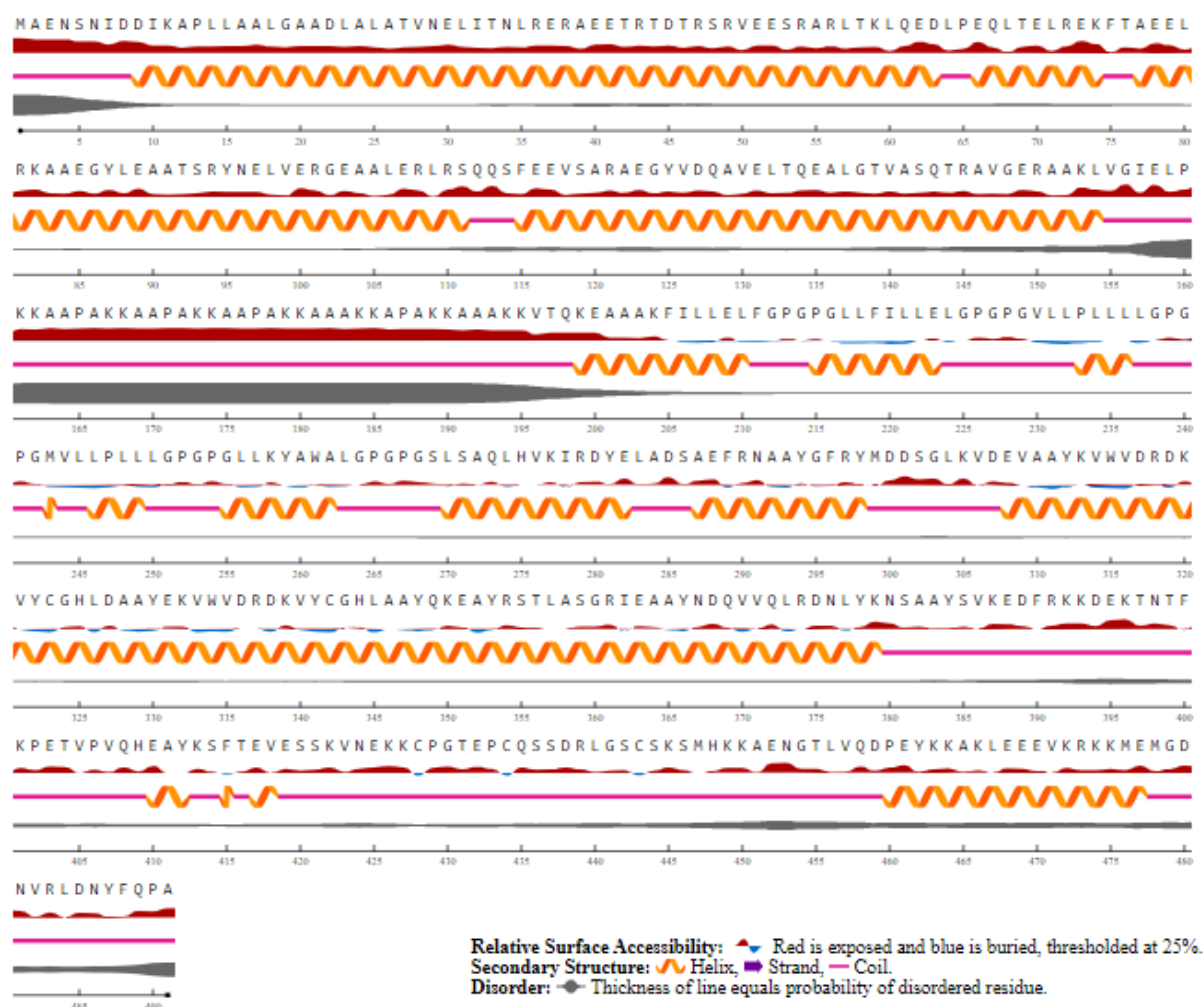


Figure 4. Secondary structure of the designed vaccine predicted using the NetSurfP 3.0 server. The predicted structure is predominantly composed of α -helices interspersed with coil regions.

0.117, collectively supporting its improved quality. Among the DeepRefiner outputs, Model 1 demonstrated the best overall structural quality. The predicted local quality of this model is shown in Figure 5. Minor fluctuations (residue errors) were observed at residues 175–180 and 435–440; however, the overall model quality was considered excellent.

3.7. Docking

The InterEvDock 2 server provided docking scores generated by three protein–protein docking algorithms and their corresponding scoring functions: InterEvDock 2, SOAP_PP, and FRODOCK2. The docking scores generated by InterEvDock 2 ranged from 21.78 to 24.13 kcal/mol. In contrast, the top 10 models generated by SOAP_PP and

FRODOCK2 exhibited score ranges of –38,050 to 2,763.78 kcal/mol and –37,782.34 to 2,363.63 kcal/mol. Among the predicted complexes, the server identified IES1 from InterEvDock 2 as the best-docked model with the highest docking score (24.13 kcal/mol). Moreover, its docked pose was highly similar to that of Complex_SOAP_PP1 generated by SOAP_PP (Table 5). Therefore, IES1 was selected for subsequent protein–protein interaction analysis and MD simulations.

3.8. Protein–protein interaction

Cys 29, Pro 28, Val 30, Gln 39, Met 41, Glu 42, Asn 44, and Phe 63 of TLR-4 engaged in hydrophobic interactions with the constructed vaccine. Moreover, Leu 43, Glu 608, and Gly 617 of TLR-4 formed three hydrogen bonds with

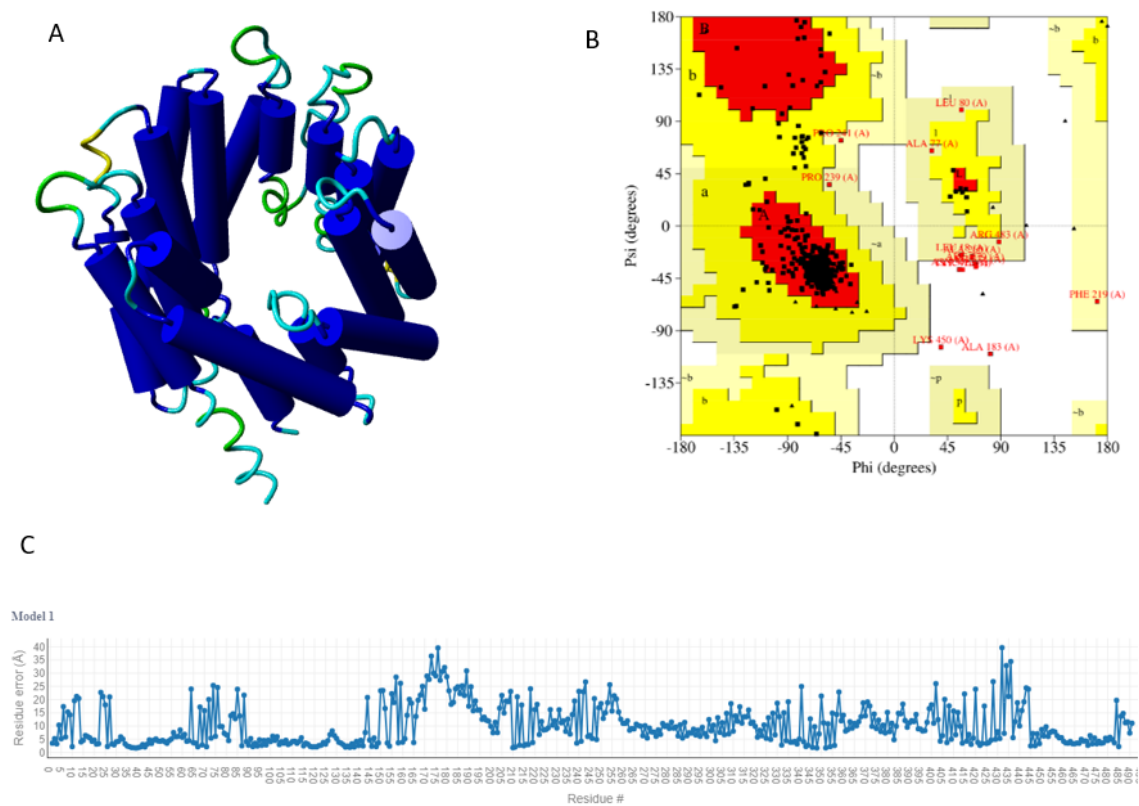


Figure 5. Predicted tertiary structure of the designed vaccine construct (A), Ramachandran plot (B), and residue error plot (C). The tertiary structure was consistent with the predicted secondary structure, while the majority of amino acids were located in the favored region of the Ramachandran plot.

Table 5. Protein–protein docking between TBV and TLR-4 using InterEvDock 2, SOAP_PP, and FRODOCK 2 algorithms

InterEvScore models	IES score (higher is better)	SOAP_PP models	SOAP_PP score (lower is better)	FRODOCK2 models	FRODOCK2 score (higher is better)
Complex_IES1	24.13	Complex_SOAP_PP1	−38,050.00	Complex_FRODOCK1	2,763.78
Complex_IES2	23.54	Complex_SOAP_PP2	−38,018.63	Complex_FRODOCK2	2,671.43
Complex_IES3	23.11	Complex_SOAP_PP3	−37,969.51	Complex_FRODOCK3	2,639.26
Complex_IES4	22.73	Complex_SOAP_PP4	−37,902.55	Complex_FRODOCK4	2,623.85
Complex_IES5	22.73	Complex_SOAP_PP5	−37,886.23	Complex_FRODOCK5	2,584.62
Complex_IES6	22.5	Complex_SOAP_PP6	−37,829.75	Complex_FRODOCK6	2,556.25
Complex_IES7	22.02	Complex_SOAP_PP7	−37,809.5	Complex_FRODOCK7	2,407.38
Complex_IES8	21.87	Complex_SOAP_PP8	−37,808.93	Complex_FRODOCK8	2,389.82
Complex_IES9	21.78	Complex_SOAP_PP9	−37,784.38	Complex_FRODOCK9	2,372.19
Complex_IES10	21.78	Complex_SOAP_PP10	−37,782.34	Complex_FRODOCK10	2,363.63

Abbreviations: TBV: Transmission-blocking vaccine; TLR-4: Toll-like receptor 4.

Tyr 294 and Lys 190 of the vaccine. Glu 27 of TLR4, a negatively charged amino acid, formed a salt bridge with the oppositely charged Arg 298 residue of the vaccine (Figure 6). These multiple intermolecular interactions, including hydrophobic contacts, hydrogen bonds, and salt-bridge formation, suggest a favorable theoretical interaction between the constructed vaccine and TLR4. Such interactions may support the vaccine's potential to engage TLR4 and contribute to immune activation; however, experimental validation would be required to confirm this effect. Additionally, the calculated total free binding energy of the vaccine–TLR-4 complex was -28.5 kcal/mol. Table 6 summarizes the MM/GBSA free binding energy of the complex.

3.9. Molecular dynamics simulations

Molecular dynamics simulations using the Groningen Machine for Chemical Simulations (GROMACS) were performed for approximately 100 ns to assess the stability

of the constructed vaccine. The results of the GROMACS MD simulations are presented in Figure 7. RMSD reflects the deviation of the protein backbone from its initial conformation throughout the simulation, with lower RMSD values generally indicating greater structural stability. The vaccine protein remained relatively stable, with RMSD values not exceeding 1.25 nm by the end of the MD simulation period. However, the trajectory did not reach a clearly defined equilibrium plateau during the simulation period, suggesting that the vaccine construct may have undergone ongoing conformational adjustments.

The vaccine protein exhibited multiple peaks in the RMSF profile. These peaks were predominantly associated with connecting loop regions, particularly around residues 100, 250, and 400, which link the helices distributed throughout the vaccine construct, consistent with the secondary structure analysis.

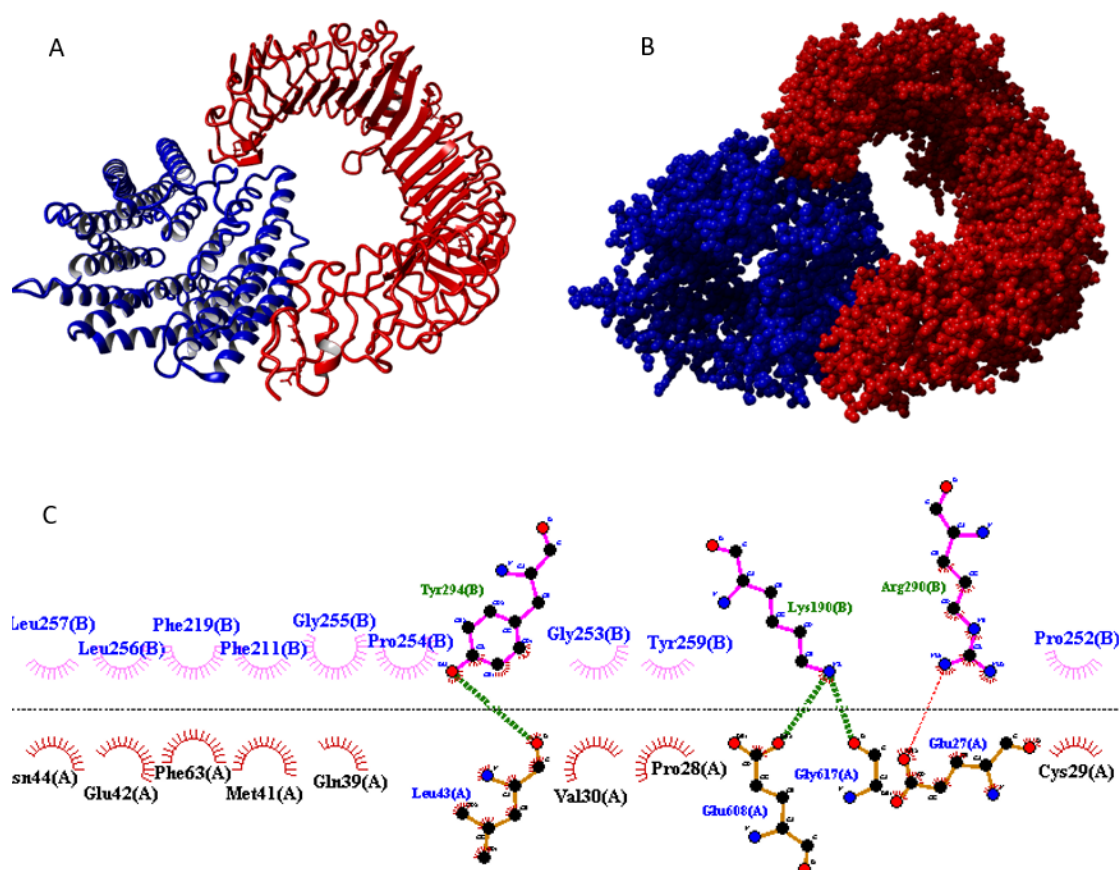


Figure 6. Interaction of the best-docked TBV–TLR-4 complex shown in cartoon representation (A) and ball representation (B). The detailed interface residues are shown in (C). The TBV binds favorably to the active site of the TLR-4 receptor, forming multiple hydrogen bonds and hydrophobic interactions at the protein–protein interface.

Abbreviations: TBV: Transmission-blocking vaccine; TLR-4: Toll-like receptor 4.

Table 6. Computation of free binding energy of the vaccine-TLR-4 ensemble

Energy	Value (kcal/mol)
Van der Waals	-76.63
Electrostatic potentials	-1,160.98
Generalized born	1,218.48
Solvation energy	-9.12
Total	-28.25

Abbreviation: TLR-4: Toll-like receptor 4.

The stability and conformational behavior of the vaccine protein were further assessed using Rg and SASA values. Rg reflects changes in the compactness of the protein structure over time, whereas SASA represents the

surface area accessible to solvent molecules. The Rg profile of the vaccine structure showed fluctuations over the 100-ns simulation, decreasing from approximately 2.63 nm at the beginning to approximately 2.52 nm toward the end, with reduced fluctuations in the later stages. This pattern suggests a gradual transition towards a more compact conformation of the vaccine construct, which was further supported by the SASA profile.

The deformability plot illustrates the regions of the interacting proteins that are most susceptible to structural deformation. The deformability profiles of TLR-4 and the constructed TBV showed relatively limited deformation, particularly around residues near position 600 of TLR-4 and residues 750–850 of the TBV. These amino acid residues fluctuate because they are hotspot regions at the

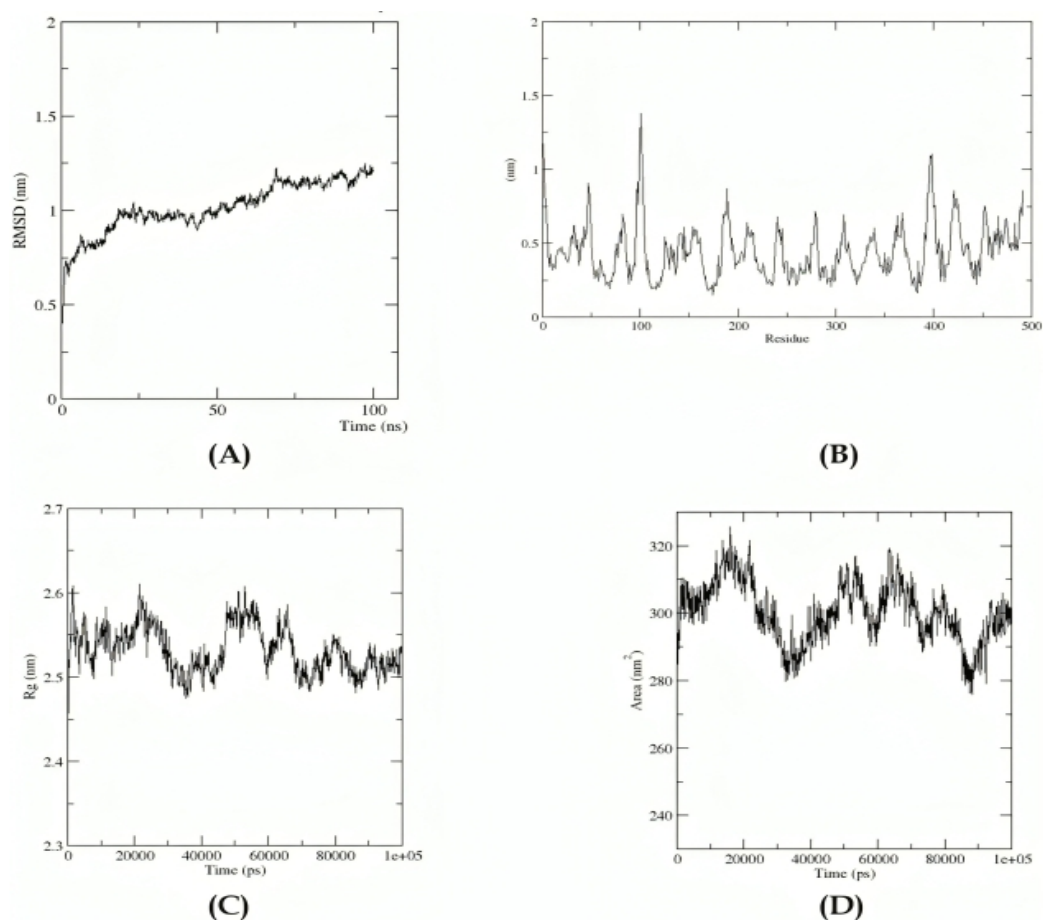


Figure 7. GROMACS MD simulation of the vaccine construct for 100 ns, showing RMSD (A), RMSF (B), Rg (C), and SASA (D). The vaccine construct exhibited relatively high fluctuations in RMSD and Rg throughout the simulation, with no clearly defined equilibrium plateau, indicating ongoing conformational adjustments during the 100-ns simulation.

Abbreviations: GROMACS: Groningen Machine for Chemical Simulations; MD: Molecular dynamics; Rg: Radius of gyration; RMSD: Root-mean-square deviation; RMSF: Root-mean-square fluctuation; SASA: Solvent-accessible surface area.

interface between the docked proteins. The B-factor plot indicates the overall mobility of the protein residues upon complex formation. In this study, the TBV–TLR-4 complex exhibited high stability as reflected by extremely low B-factor values, indicating that conformational changes upon binding were confined. A low eigenvalue indicates protein deformation due to underlying fluctuations. The eigenvalue obtained from the NMA was 7.63×10^{-6} , indicating a very low deformational energy and a good stability profile of the TBV–TLR-4 complex. Moreover, the structural integrity and protein flexibility of the TBV–TLR-4 complex were assessed by examining the variance (%) contributed by individual normal modes. The complex exhibited greater flexibility, as reflected by the elevated variance values shown in Figure 8. The observed flexibility did not appear to substantially disrupt the complex's overall structural organization. Collectively, the NMA results support the structural stability of the predicted TBV–TLR-4 complex and suggest that the vaccine construct can maintain favorable interactions with the TLR-4 receptor despite local conformational fluctuations, highlighting the potential of the constructed vaccine to elicit a strong inflammatory and immune response.

3.10. Immunization simulation

The vaccine construct protein sequence was submitted to the C-ImmSim server to predict the potential immune

response following vaccination. The simulation showed that antigen levels peak during the first four days, followed by a rise in antibody levels. Both IgM and IgG responses were predicted, with a subsequent class-switching response characterized by a greater increase in IgM than IgG. The overall immunoglobulin response peaked approximately two weeks after vaccination and gradually declined thereafter (Figure 9A). IgM-secreting B cells constituted the B-cell population, while memory B cells showed a marked increase during the first week following vaccination (Figure 9B). Similarly, the constructed vaccine elicited strong predicted responses of both total and memory T_H cells (Figure 9C). The development of memory B- and T-cell populations suggests the potential for immunological memory following vaccination. Regarding T_C cells, the simulated response showed a sharp increase, reaching an initial peak at approximately three weeks, followed by a second peak after approximately one month (Figure 9D). This finding may reflect the characteristics and assumptions of the simulated immune-response model rather than a definitive absence of intracellular immune involvement. Among the innate immune-cell populations, dendritic cells and macrophages exhibited fluctuating responses throughout the simulation. Most dendritic cells remained in a resting state, whereas a greater proportion of macrophages were predicted to be in an active state (Figure 9E,F). The number of natural killer

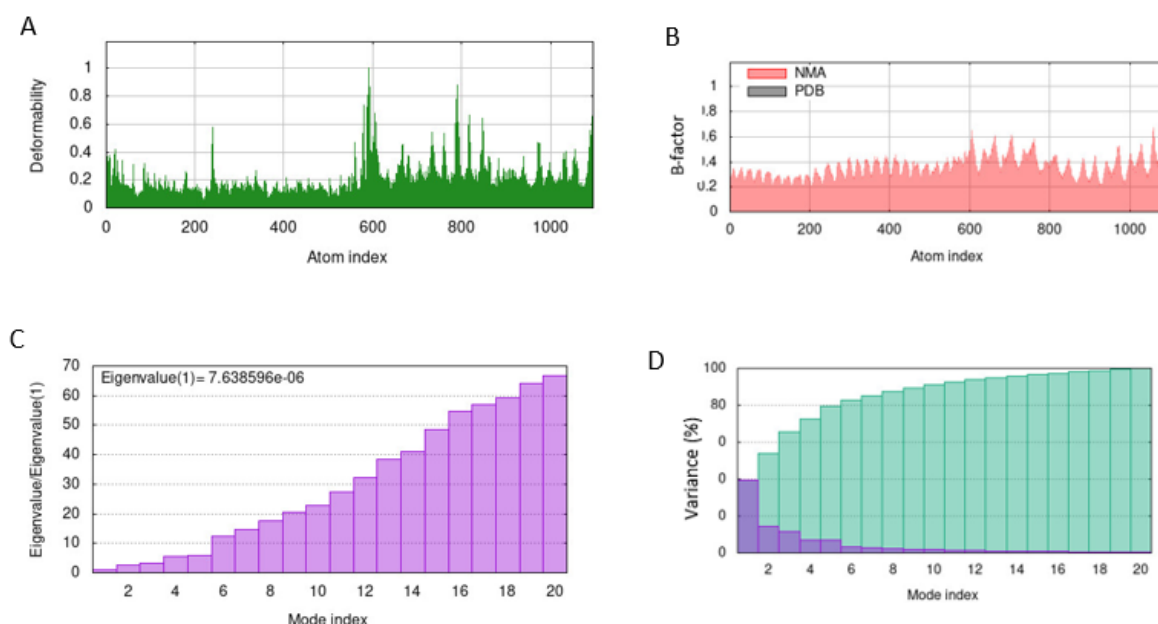


Figure 8. Molecular dynamics simulation of the best docked complex performed using the iMODS, showing the deformability profile (A), B-factor (B), eigenvalue (C), and variance (%) of the normal modes (D).

Abbreviations: iMODS: Internal Coordinates Normal Mode Analysis Server; NMA: Normal mode analysis; PDB: Protein Data Bank.

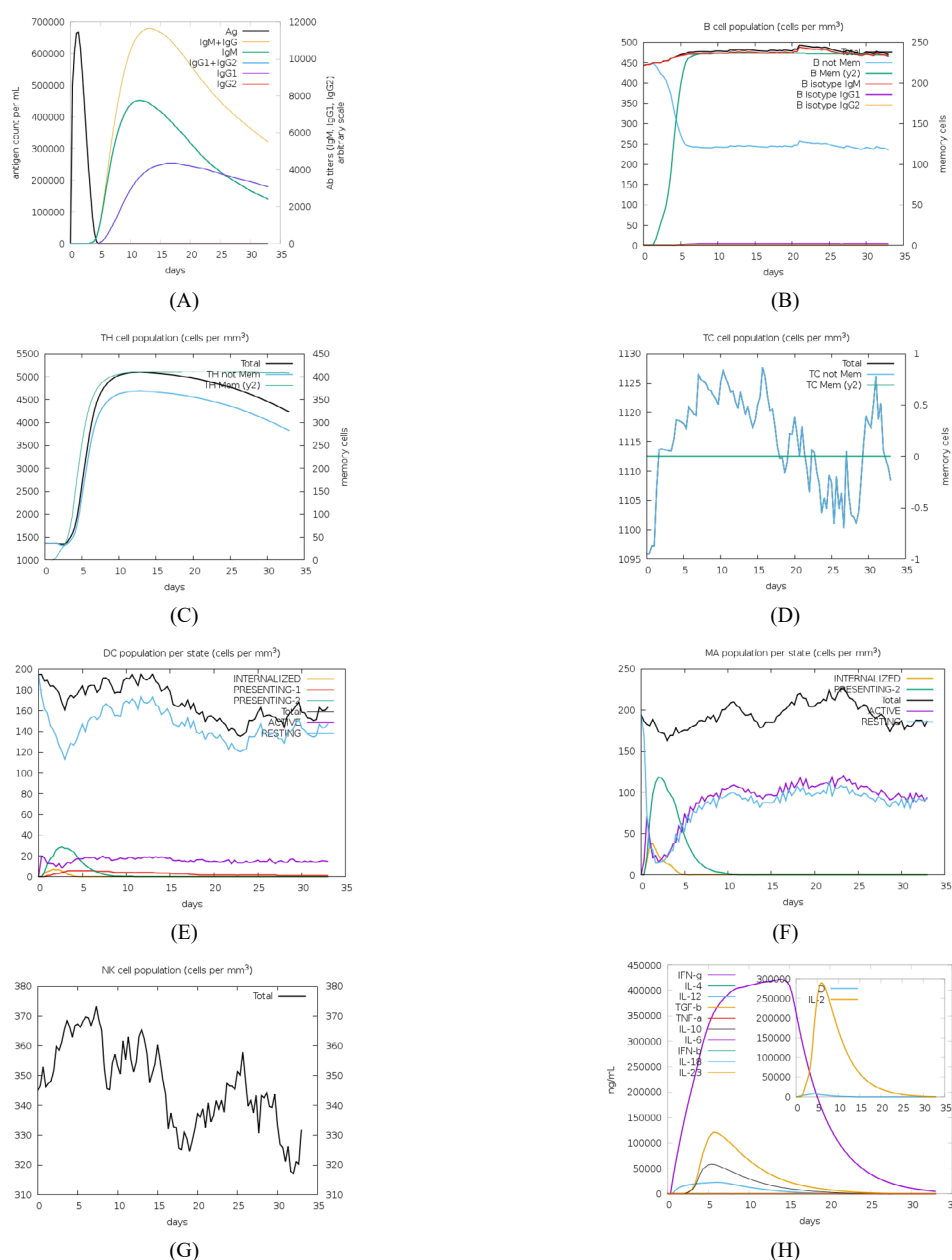


Figure 9. Simulated immune response to a designed TBV targeting malaria transmission by *Anopheles gambiae*. The figure depicts the predicted temporal dynamics of Ag exposure and humoral and cellular immune responses following TBV vaccination. (A) Simulated Ag levels together with the corresponding Ab responses, including IgM + IgG, IgM, IgG1 + IgG2, IgG1, and IgG2, showing the induction and subsequent decline of Ag-specific Abs over time. (B) B-cell population dynamics, showing total, non-Mem, Mem, and isotype-specific B-cell populations and illustrating the development and persistence of the humoral immune response. (C) TH-cell dynamics, including total, non-Mem, and Mem TH-cell populations. (D) TC dynamics, including total, non-Mem, and Mem TC populations. (E) DC dynamics, including total cells and cells in internalized, presenting-1, presenting-2, active, and resting states, representing Ag processing and presentation. (F) MA population dynamics, including total cells and cells in internalized, presenting-2, active, and resting states, demonstrating the simulated innate immune response and MA activation following vaccination. (G) NK-cell population dynamics over the simulation period. (H) Simulated cytokine profiles, including IFN- γ , IL-4, IL-12, TGF- β , TNF- α , IL-10, IL-6, IFN- β , IL-18, and IL-23, with IL-2 shown in the inset, illustrating the temporal cytokine environment associated with the vaccine-induced immune response. Collectively, the panels show the coordinated development of Ag-specific humoral and cellular immune responses, APC dynamics, innate immune responses, and cytokine changes induced by the designed TBV candidate.

Abbreviations: Ab: Antibody; Ag: Antigen; APC: Antigen-presenting cell; B: B cell; DC: Dendritic cell; IFN: Interferon; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IL: Interleukin; MA: Macrophage; Mem: Memory; NK: Natural killer; TBV: Transmission-blocking vaccine; TC: Cytotoxic T cell; TGF: Transforming growth factor; T_H: T-helper cell; TNF: Tumor necrosis factor.

cells gradually decreased after the first week following vaccination (Figure 9G). Among the cytokines assessed, IFN- γ showed a pronounced predicted increase following vaccination (Figure 9H).

3.11. Disulfide engineering

Upon submission of the vaccine PDB file to the Disulfide by Design 2 server, 53 potential residue pairs suitable for disulfide-bond engineering were identified, each associated with a unique χ^3 value and energy score. The top-10 candidate residue pairs were ALA24–GLU468, MET1–GLU3, SER385–VAL407, SER119–ALA175, PRO215–LEU218, LEU224–ALA262, ILE278–ASP336, TYR412–GLY441, ALA284–ALA287, and ILE220–LEU256. The predicted free energy values of these candidate pairs ranged from 4.92 to 6.87 kcal/mol, whereas the corresponding χ^3 values ranged from 102.13° to 112.45°. These candidate residue pairs were considered potential sites for introducing disulfide bonds to enhance the structural stability of the vaccine construct. A comparison between the engineered and original vaccine constructs is presented in Figure 10.

3.12. Virtual cloning

The primary structure (amino acid sequence) of the constructed vaccine was back-translated into a DNA sequence, which was subsequently optimized for cloning. The codon adaptation index (CAI) value of the sequence before optimization was 0.27, denoting a low probability of expression owing to the use of less abundant codons. The guanine–cytosine (GC) content was 67.8%, reflecting a high GC content that could increase the melting temperature required to separate the two DNA strands, potentially

hindering the expression and production processes (Figure 11). Following codon optimization, the CAI value increased to 0.97, while the GC content decreased to 51.3%, indicating a high potential for expression of the vaccine construct.

In addition, the predicted secondary structure of the vaccine RNA exhibited a stable conformation, with an MFE of -481.55 kcal/mol, as shown in Figure 11C.

Restriction and fragment cloning were performed to insert the vaccine construct (1,503 bp) into the pET-28a(+) plasmid (5,369 bp), generating a recombinant plasmid with a total length of 6,845 bp. The inserted sequence was engineered with an HQ tag (HQQHQHQ) to facilitate subsequent purification by metal affinity chromatography. The vaccine construct was excised and cloned between the XhoI and SalI restriction sites (Figure 12). The resulting recombinant plasmid was therefore ready for subsequent *in vitro* expression and validation. The overall plasmid cloning workflow is illustrated in Figure 13.

3.13. Population coverage

The vaccine construct was predicted through immunoinformatics analysis to provide 40.27% global population coverage. The average hit was 1.11, while the PC90 value was 0.17, indicating that the vaccine construct could achieve broad global coverage, with at least one allele predicted to respond to at least one epitope in the vaccine ensemble. The predicted global population coverage of the vaccine is depicted in Figure 14. When assessed specifically across Africa, the vaccine was predicted to cover an average of 34.18% of the African population, with the highest coverage observed in Central Africa (55%).

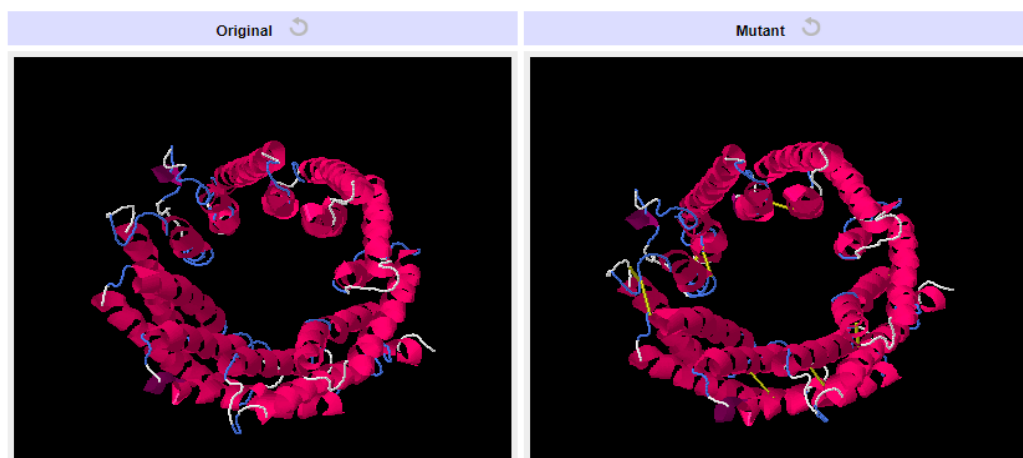


Figure 10. Disulfide engineering of the vaccine construct, showing the original version (Left) and the mutated version (Right). Disulfide bridges are shown in yellow.

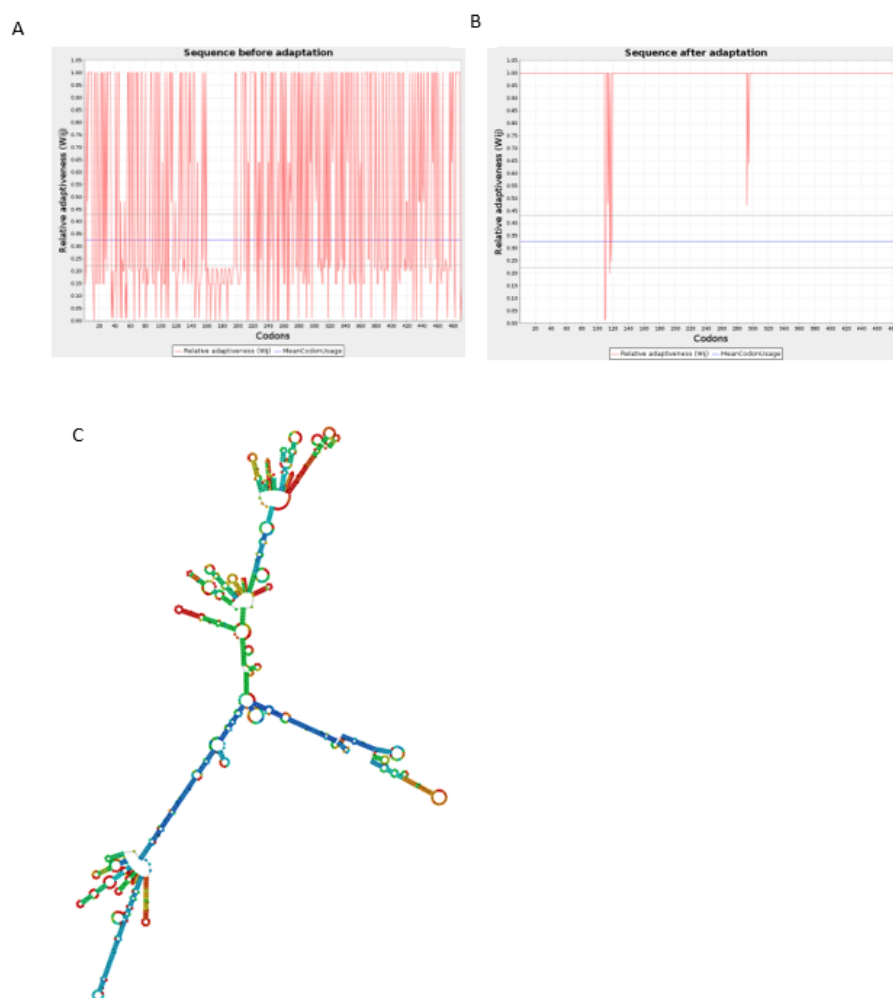


Figure 11. Codon optimization of the back-translated sequence of the designed vaccine (A, B) and the secondary structure of the enhanced RNA sequence of the vaccine (C)

4. Discussion

Immunoinformatics and reverse vaccinology are two promising approaches for vaccine design against a wide range of pathogens, relying primarily on *in silico* techniques. Immunoinformatics involves the use of computational tools to predict and analyze immunological properties of proteins, including potential epitopes recognized by the immune system and allergenicity. By identifying potential epitopes, immunoinformatics can facilitate the design of vaccines that elicit a strong and targeted immune response against the specific pathogens. Reverse vaccinology, on the other hand, uses bioinformatics approaches to identify potential vaccine candidates from genomic or proteomic data. This approach has been particularly

successful in identifying novel vaccine candidates against bacterial pathogens. Collectively, these approaches have the potential to accelerate vaccine development while reducing the time and costs associated with conventional vaccine development strategies.^{51,52}

Malaria is a complex disease that affects millions of people worldwide, and there are currently two approved malaria vaccines. RTS,S/AS01E, a malaria candidate vaccine, has demonstrated efficacy in clinical trials and real-world settings. TBVs represent another approach to reducing malaria transmission. By stimulating the production of antibodies that inhibit parasite maturation within mosquitoes after they fed on vaccinated individuals, TBVs aim to disrupt the parasite life cycle. Recent research has identified several promising TBV candidates that have

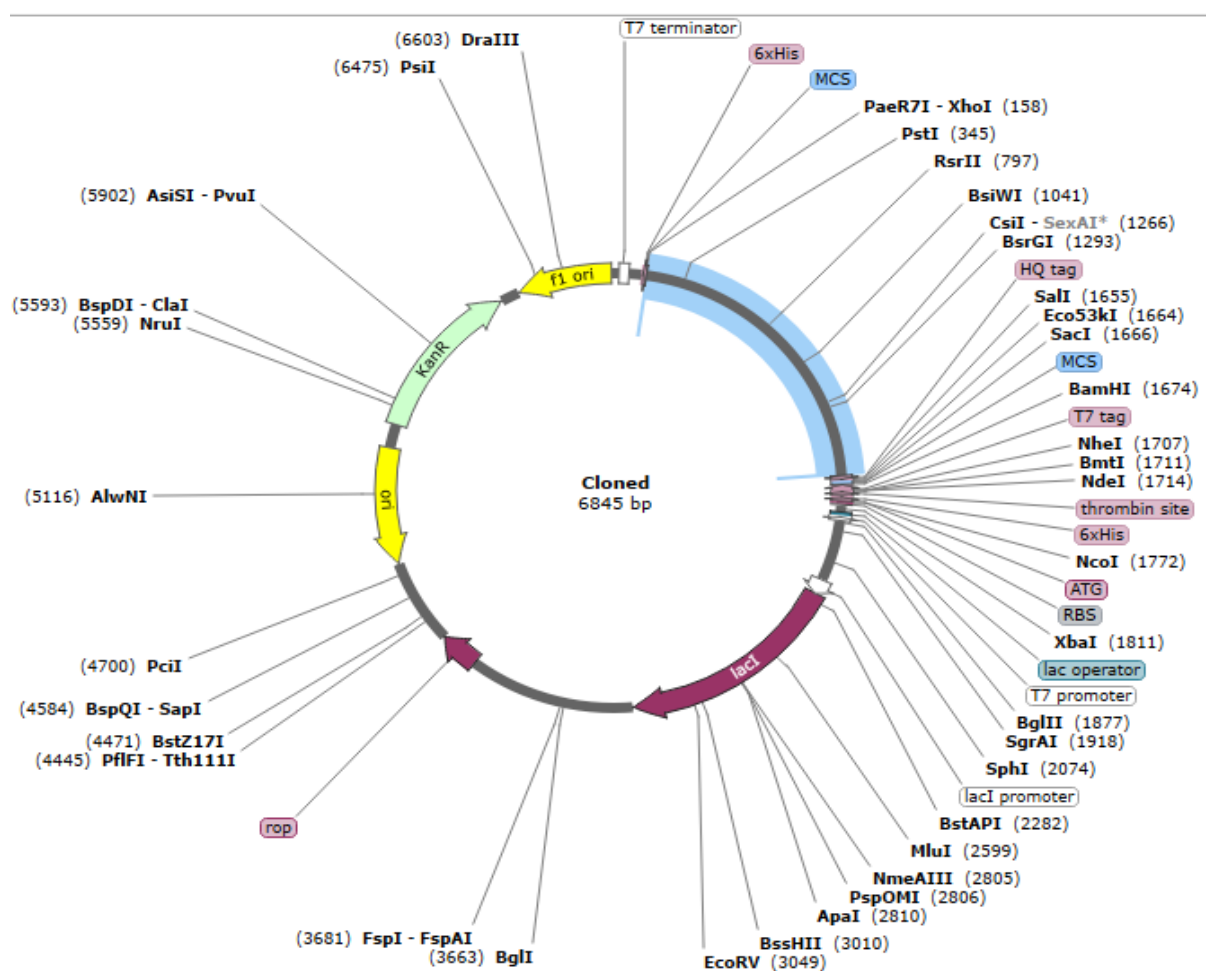


Figure 12. pET-28a(+) plasmid containing the cloned vaccine sequence (highlighted in blue)

progressed to clinical trials following successful preclinical evaluation. However, the development of TBVs remains challenging because of factors such as low immunogenicity or high reactivity in humans. Despite considerable progress in malaria vaccine development, the emergence of *Plasmodium* variants against which existing vaccines may provide limited protection remains a major challenge.^{53–55}

Antibody-dependent enhancement in malaria occurs when antibodies unintentionally promote *P. falciparum* invasion of red blood cells through interactions involving complement receptor 1, potentially reducing vaccine effectiveness.⁵⁶ Vector–host cross-reactivity refers to immune responses targeting shared antigens among different *Plasmodium* species. Such cross-reactivity may broaden protection but also cause off-target effects that influence vaccine outcomes. For example, cross-reactive antibodies and T-cell responses have been observed

between *P. falciparum* and *P. vivax*.^{57,58} Balancing these factors is therefore crucial for developing malaria vaccines that provide strong, broad, and safe immunity without enhancing infection.

Three salivary proteins of the malarial vector *A. gambiae* were selected: AgTRIO, D7, and gSG6. D7 protein and salivary apyrase are distinct salivary proteins with different functions. D7 protein binds and scavenges host hemostatic and inflammatory mediators, including leukotrienes, thromboxane, and serotonin, while salivary apyrase inhibits platelet aggregation. On the other hand, gSG6 is involved in blood feeding, although its precise role remains incompletely understood.⁵⁹ The antigenicity and extracellular secretion of these proteins into saliva were evaluated using Vaxijen 2 and BUSCA, respectively. Several T_H1, T_H2, and B-cell epitopes were subsequently identified from these antigens, with only potentially antigenic, non-

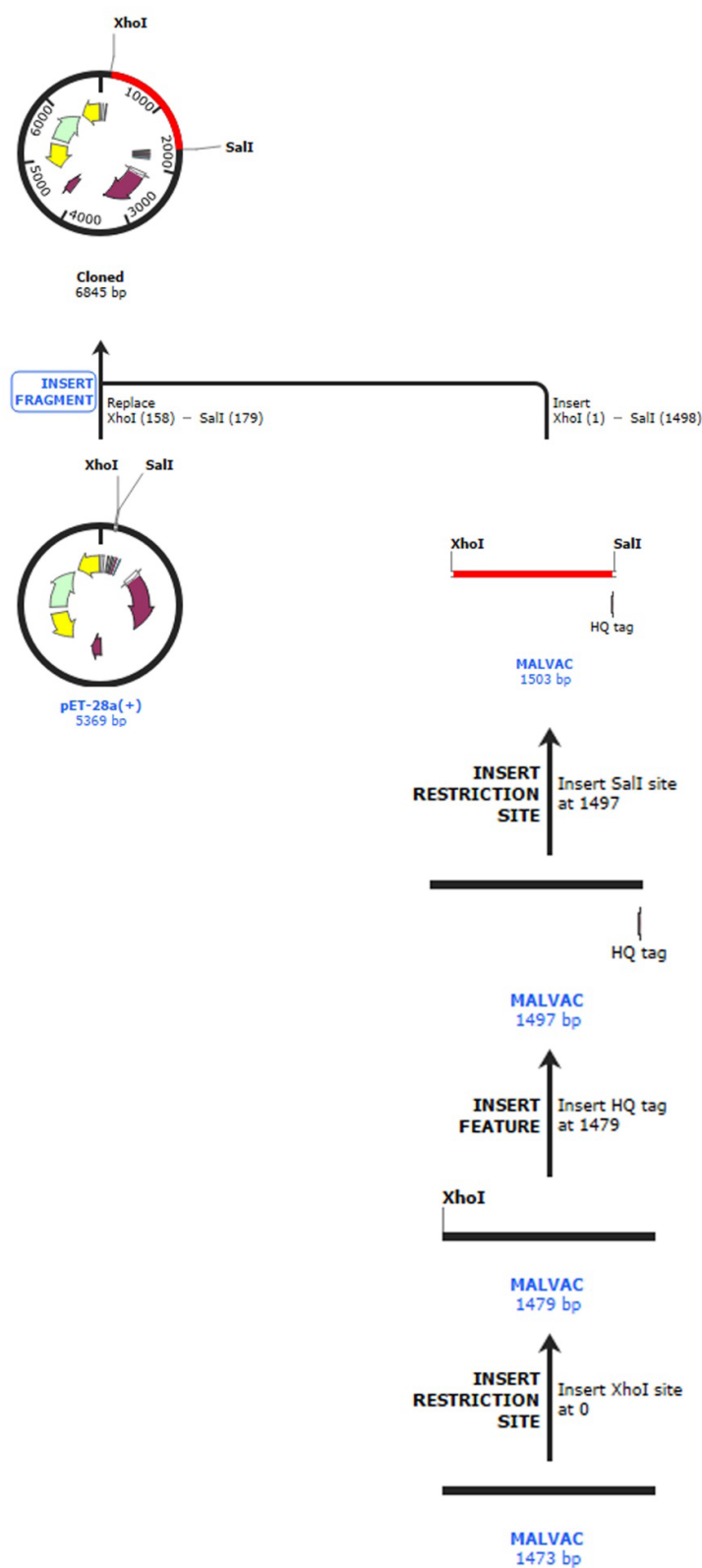


Figure 13. Flowchart illustrating the steps involved in the virtual cloning process
Abbreviation: MALVAC: Malarial vaccine.

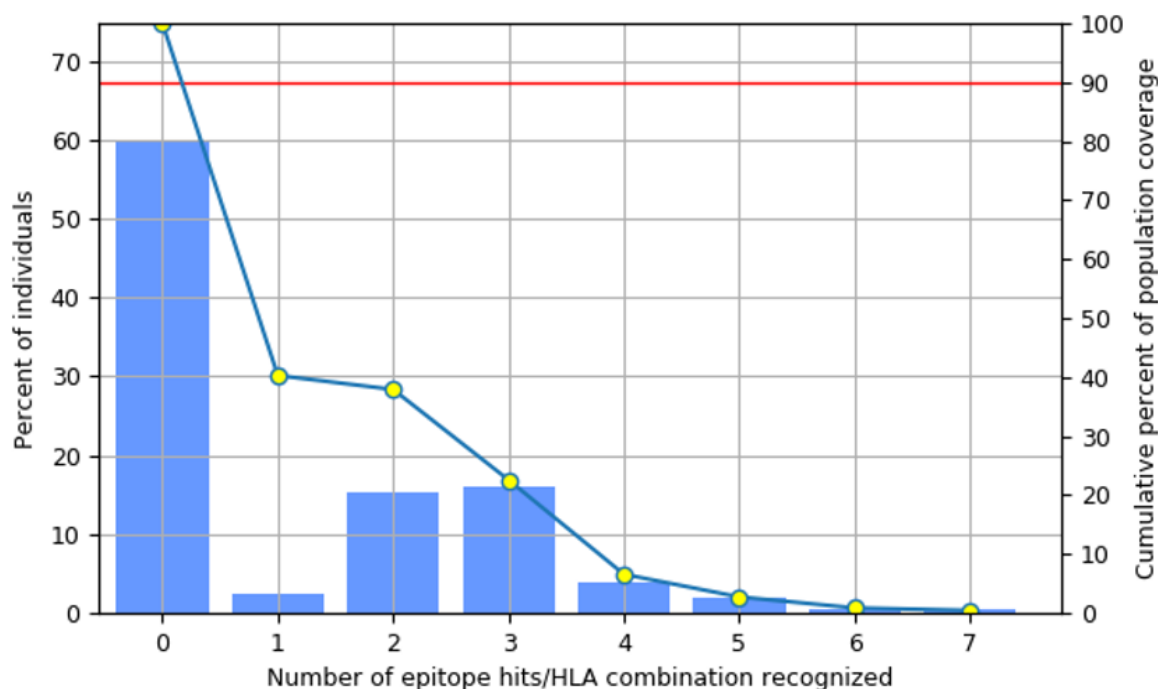


Figure 14. Predicted worldwide population coverage of the epitopes included in the vaccine ensemble
Abbreviation: HLA: Human leukocyte antigen.

allergic, and non-toxic epitopes being selected. For T_H -cell epitopes, only those predicted to have potential IFN- γ -inducing capacity were considered. The selected epitopes were subsequently linked to the mycobacterial heparin-binding hemagglutinin adjuvant using different potent peptide linkers. The resulting vaccine construct was then evaluated for its biochemical characteristics and secondary and tertiary 3D structures. The vaccine construct exhibited acceptable stability and thermostability, a relatively short *in vitro* half-life, and high solubility compared with the average *E. coli* proteins. The predicted secondary and tertiary structures obtained using NetSurfP-3.0 and I-TASSER, respectively, showed strong concordance, revealing a protein architecture composed of 18 α -helices connected by coiled regions, with no β -sheets. To simulate the initial innate immune recognition of the vaccine, molecular docking between the vaccine construct and TLR-4 was performed using the InterEvDock 2 platform. The results indicated strong binding through multiple types of interactions, including hydrogen bonds, hydrophobic interactions, and salt bridges, as visualized with LigPlot+. The stability, mobility, and flexibility of the TBV-TLR-4 complex were subsequently assessed using the iMODS web server. TLR4 and TLR9 have been demonstrated to participate in signaling pathways that stimulate protective

immunity against blood-stage *Plasmodium* spp.⁶⁰ However, the docked complex did not demonstrate the expected stability, mobility, or flexibility profiles. RMSD and Rg values greater than 1.25 nm for the vaccine construct suggested relatively low structural stability, consistent with findings reported for similar immunoinformatics-based vaccine approaches.⁶¹ Following assessment of potential immune activation through TLR-4, the vaccine construct was subjected to *in silico* cloning into the pET-28a(+) plasmid for potential expression in the *E. coli* K2 strain. This plasmid has been modified, reportedly resulting in enhanced protein expression and yield in *E. coli* K2.⁶² The vaccine DNA sequence was also designed with an HQ tag, a short peptide sequence that facilitates protein isolation and purification using metal-affinity chromatography. HQ-tagged proteins can be expressed in bacterial, mammalian, insect, wheat germ, and rabbit reticulocyte systems under different conditions and purified using different materials and methods across a range of scales, from small-scale to large-scale preparations, either manually or automatically.^{63,64}

Finally, the vaccine construct was evaluated for its potential to induce an immune response following a simulated administration. The results predicted potent activation of various innate and adaptive immune

components, particularly T_H memory cells and B-cell memory cells, accompanied by the production of IgM and IgG antibodies. Moreover, the vaccine was predicted to induce a marked increase in IFN- γ secretion, which may promote inflammatory responses and facilitate the activation of B and T cells. Consequently, a proportion of these activated cells may differentiate into memory cells, potentially contributing to longer-lasting immune protection against the targeted antigens. Most importantly, the predicted production of IgM and IgG antibodies may have implications for transmission-blocking immunity. These antibodies could potentially be ingested by *A. gambiae* during blood feeding and subsequently interact with the targeted mosquito salivary proteins within the vector. Such antibody-mediated interference may impair vector functions associated with blood feeding and parasite transmission. Thus, rather than directly targeting *Plasmodium* spp., this vaccine strategy aims to reduce malaria transmission by targeting mosquito vector proteins. Regarding population coverage, the vaccine ensemble was predicted to cover more than 40% of the global population. The predicted coverage was distributed across multiple geographical regions, and this may be particularly relevant given that malaria transmission is disproportionately concentrated in sub-Saharan Africa.

Several previous studies have reported *in silico* vaccine design targeting different *P. falciparum* antigens. For instance, Maharaj and colleagues⁶⁵ reported in 2021 an immunoinformatics-based multi-epitope anti-malarial vaccine incorporating 12 CD4⁺ T-cell epitopes, 10 CD8⁺ T-cell epitopes, and one B-cell epitope. The selected epitopes were rigorously screened for antigenicity, immunogenicity, and non-allergenicity using advanced computational prediction tools. HLA binding analysis revealed that the selected epitopes exhibited broad population coverage, effectively targeting HLA alleles present in approximately 78.48% of the global population, highlighting their potential for broad immunological applicability. Pritam *et al.*⁶⁶ also employed bioinformatics approaches to investigate the *P. falciparum* genome and identified five potential protective antigens. Notably, the GDPAA, RSKMA, and EKE epitope ensembles of *P. falciparum* were predicted to contain highly versatile T-cell epitopes with potential applicability across populations in malaria-endemic regions. These epitope ensembles were therefore proposed as promising candidates for the future development of broadly effective malaria vaccines. In another study, candidate antigens (msp1, ama1, rh5, eba175, glurp, celtos, csp, lsa3, Pfsea, trap, conserved chrom3, hyp9, hyp10, phistb, surfn8.2, and surfn14.1) were evaluated as potential targets for *P. falciparum* vaccine development in sub-Saharan Africa. These antigens include targets associated with both the

pre-erythrocytic and erythrocytic stages of the parasite.⁶⁷ In addition, Atapour and his colleagues⁶⁸ described the *in silico* design of a multi-epitope vaccine targeting the blood-stage of malaria. The candidate vaccine was computationally designed against the *P. falciparum* PfGARP protein and incorporated five B-cell epitopes and five MHC class II-restricted T-cell epitopes linked by flexible peptide spacers to facilitate antigen presentation. To enhance immunogenicity, the construct was fused with flagellin, a potent TLR5 agonist, as a molecular adjuvant. *In silico* immunological simulations predicted robust adaptive immune responses, suggesting the construct's potential to induce protective immunity against malaria infection.

To the best of our knowledge, only one previous study has reported the use of mosquito salivary proteins (14 proteins) as targets for a TBV against malaria. This study selected 14 salivary proteins from the vector *Anopheles stephensi*, a species that is widely distributed across the Indian subcontinent and is also found, to a lesser extent, in the Middle East and Southeast Asia.⁶⁹ In contrast, the present study describes the design of a multi-epitope TBV targeting salivary proteins of the malarial vector *A. gambiae*, with the objective of potentially reducing malaria transmission at the vector–host interface.

The proposed multi-epitope TBV is hypothesized to operate through three interconnected mechanisms. First, antibodies generated against AgTRIO and D7 may interfere with hemostatic and host-interacting functions at the mosquito bite site—thereby restoring host platelet aggregation, vasoconstriction, and inflammatory signaling—potentially impairing mosquito blood-feeding efficiency and consequently reducing the probability of successful parasite acquisition. Second, by blocking salivary immunosuppressive factors, the vaccine may facilitate local innate immune clearance of sporozoites in the skin before hepatic invasion. Third, and most critically, antibody-mediated neutralization of these salivary proteins is hypothesized to disrupt the parasite's permissive midgut environment, interfering with blood-meal digestion, peritrophic matrix integrity, and subsequent ookinete-to-oocyst transformation, ultimately reducing sporozoite loads in the salivary glands. This multi-pronged strategy—targeting vector feeding, host immune modulation, and parasite establishment—provides a robust, species-transcendent rationale that circumvents *Plasmodium* genetic variability and drug resistance.

Designing potent vaccines is a complex and resource-intensive process that requires extensive data analysis, rigorous investigations, experimental validation, and, ultimately, appropriate animal models to assess efficacy and safety. Although *in silico* approaches for multi-epitope

vaccine design provide a powerful means of identifying candidate epitopes and potentially eliciting broad immune responses, they have several inherent limitations. One major limitation is the difficulty of predicting cross-reactivity and potential off-target immune responses with complete accuracy. Bioinformatic algorithms generally predict B-cell and T-cell epitopes based on sequence homology and structural similarity derived from known antigens. Consequently, predicted epitopes may exhibit unintended similarity to host proteins, potentially raising concerns regarding autoreactivity. Although computational tools are available for screening potential similarities against the human proteome, these approaches depend on the completeness and quality of reference databases and may not detect all relevant similarities or fully account for structural context and post-translational modifications. Another limitation is the restricted assessment of innate immune receptor interactions in the present study. Molecular docking was performed primarily against TLR-4, whereas other pattern-recognition receptors, including TLR-7 and TLR-9, may also contribute to recognition of the vaccine construct and subsequent immune activation. Therefore, the observed TLR4 interaction should not be interpreted as evidence that TLR4 represents the sole or predominant receptor responsible for vaccine-induced immune responses. Furthermore, computational approaches have limited capacity to predict cross-reactivity with environmental antigens or commensal microorganisms, which may potentially contribute to unintended inflammatory pathways or allergic responses. The considerable diversity of environmental and microbial antigens, together with the complexity of host immune regulation, makes comprehensive *in silico* assessment of these effects challenging. Therefore, although *in silico* approaches can substantially accelerate vaccine development by prioritizing promising candidate epitopes and reducing the number of constructs requiring experimental testing, their predictions should be regarded as hypothesis-generating rather than definitive evidence of efficacy or safety until these candidates are rigorously validated through extensive *in vitro* and *in vivo* assays to rule out harmful off-target immune reactions.

5. Conclusion

In conclusion, the development of a TBV that targets mosquito vector proteins rather than the *Plasmodium* antigens represents a potentially promising strategy for reducing malaria transmission. In this study, a multi-epitope TBV was designed using three salivary proteins of *A. gambiae*, namely AgTRIO, D7, and gSG6, together with mycobacterial tuberculosis heparin-binding hemagglutinin as an adjuvant. The vaccine construct exhibited strong

binding to TLR-4 and induced appropriate B-cell, T-cell, and antibody responses with no apparent allergenicity or toxicity. The availability of the pET-28a(+) plasmid containing the vaccine sequence provides a potential avenue for further *in vitro* and *in vivo* testing. Overall, this study highlights the potential of multi-epitope TBVs as a promising strategy to undermine the spread of the ongoing multi-drug-resistant *Plasmodium* spp. infections. Further *in vitro* and *in vivo* studies are required to determine whether the predicted immunogenicity, safety, and transmission-blocking effects translate into measurable biological protection. Future research should first focus on optimizing the recombinant expression and purification of the vaccine construct and confirming its structural integrity, solubility, and appropriate folding. Experimental assessments of antigenicity and immunogenicity, including enzyme-linked immunosorbent assays, should subsequently be performed to determine whether vaccination induces antibodies that recognize the targeted mosquito salivary proteins. Subsequent validation through mosquito feeding assays will determine the vaccine's ability to disrupt parasite transmission in the vector. Additionally, allele-matched HLA transgenic mouse trials could provide critical insights into human-like immune responses, ensuring the vaccine's efficacy across diverse populations. Integrating these approaches will accelerate the development of a robust TBV capable of reducing malaria transmission by *A. gambiae*.

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Conflict of interest

The author declares no conflict of interest.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

All data generated or analyzed during this study are included in this published article.

References

1. WHO. Fact sheet about malaria. 2024. Available from: <https://www.who.int/news-room/fact-sheets/detail/malaria>. Accessed July 3, 2025.
2. González-Sanz M, Berzosa P, Norman FF. Updates on Malaria Epidemiology and Prevention Strategies. *Curr Infect Dis Rep*. 2023;25(7):131-139.
doi: 10.1007/s11908-023-00805-9
3. Steffen R, Lautenschlager S, Fehr J. Travel restrictions and lockdown during the COVID-19 pandemic—impact on notified infectious diseases in Switzerland. *J Travel Med*. 2020;27(8):taaa180.
doi: 10.1093/jtm/taaa180
4. Norman FF, Treviño-Maruri B, Ruiz Giardín JM, *et al*. Trends in imported malaria during the COVID-19 pandemic, Spain (+Redivi Collaborative Network). *J Travel Med*. 2022;29(6):taac083.
doi: 10.1093/jtm/taac083
5. Choy B, Bristowe H, Khozoe B, Lampejo T. Increased imported severe *Plasmodium falciparum* malaria involving hyperparasitaemia (>10%) in a UK hospital following relaxation of COVID-19 restrictions compared to the pre-pandemic period. *J Travel Med*. 2022;29(8):taac116.
doi: 10.1093/jtm/taac116
6. Chawla J, Oberstaller J, Adams JH. Targeting Gametocytes of the Malaria Parasite *Plasmodium falciparum* in a Functional Genomics Era: Next Steps. *Pathogens*. 2021;10(3):346.
doi: 10.3390/pathogens10030346
7. Alemayehu A. Biology and epidemiology of *Plasmodium falciparum* and *Plasmodium vivax* gametocyte carriage: Implication for malaria control and elimination. *Parasite Epidemiol Control*. 2023;21:e00295.
doi: 10.1016/j.parepi.2023.e00295
8. Carter LM, Kafsack BFC, Llinás M, Mideo N, Pollitt LC, Reece SE. Stress and sex in malaria parasites: Why does commitment vary? *Evol Med Public Health*. 2013;2013(1):135-147.
doi: 10.1093/emph/eot011
9. Meibalan E, Marti M. Biology of Malaria Transmission. *Cold Spring Harb Perspect Med*. 2017;7(3):a025452.
doi: 10.1101/cshperspect.a025452
10. Oduma CO, Koepfli C. *Plasmodium falciparum* and *Plasmodium vivax* Adjust Investment in Transmission in Response to Change in Transmission Intensity: A Review of the Current State of Research. *Front Cell Infect Microbiol*. 2021;11:786317.
doi: 10.3389/fcimb.2021.786317
11. Daily JP. Malaria 2017: Update on the Clinical Literature and Management. *Curr Infect Dis Rep*. 2017;19(8):28.
doi: 10.1007/s11908-017-0583-8
12. Siddiqui AJ, Bhardwaj J, Saxena J, *et al*. A Critical Review on Human Malaria and Schistosomiasis Vaccines: Current State, Recent Advancements, and Developments. *Vaccines*. 2023;11(4):792.
doi: 10.3390/vaccines11040792
13. Draper SJ, Sack BK, King CR, *et al*. Malaria Vaccines: Recent Advances and New Horizons. *Cell Host Microbe*. 2018;24(1):43-56.
doi: 10.1016/j.chom.2018.06.008
14. Alven S, Aderibigbe B. Combination Therapy Strategies for the Treatment of Malaria. *Molecules*. 2019;24(19):3601.
doi: 10.3390/molecules24193601
15. Koepfli C, Nguiragool W, Almeida ACG de, *et al*. Identification of the asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* gametocyte reservoir under different transmission intensities. *PLOS Neglected Trop Dis*. 2021;15(8):e0009672.
doi: 10.1371/journal.pntd.0009672
16. Oduma CO, Ogolla S, Atieli H, *et al*. Increased investment in gametocytes in asymptomatic *Plasmodium falciparum* infections in the wet season. *BMC Infect Dis*. 2021;21(1):44.
doi: 10.1186/s12879-020-05761-6
17. Challenger JD, Olivera Mesa D, Da DF, *et al*. Predicting the public health impact of a malaria transmission-blocking vaccine. *Nat Commun*. 2021;12(1):1494.
doi: 10.1038/s41467-021-21775-3
18. Takashima E, Tachibana M, Morita M, Nagaoka H, Kanoi BN, Tsuboi T. Identification of Novel Malaria Transmission-Blocking Vaccine Candidates. *Front Cell Infect Microbiol*. 2021;11:805482.
doi: 10.3389/fcimb.2021.805482
19. Duffy PE. Current approaches to malaria vaccines. *Curr Opin Microbiol*. 2022;70:102227.
doi: 10.1016/j.mib.2022.102227
20. Oseno B, Marura F, Ogwang R, *et al*. Characterization of *Anopheles gambiae* D7 salivary proteins as markers of human-mosquito bite contact. *Parasites Vectors*. 2022;15(1):11.
doi: 10.1186/s13071-021-05130-5
21. Kearney EA, Agius PA, Chaumeau V, Cutts JC, Simpson JA, Fowkes FJ. *Anopheles* salivary antigens as serological biomarkers of vector exposure and malaria transmission: A systematic review with multilevel modelling. *eLife*. 2021;10:e73080.
doi: 10.7554/eLife.73080

22. Savojardo C, Martelli PL, Fariselli P, Profiti G, Casadio R. BUSCA: an integrative web server to predict subcellular localization of proteins. *Nucleic Acids Res.* 2018;46(W1):W459-W466.
doi: 10.1093/nar/gky320
23. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinform.* 2007;8(1):4.
doi: 10.1186/1471-2105-8-4
24. Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, Nielsen M. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. *BMC Bioinform.* 2007;8:424.
doi: 10.1186/1471-2105-8-424
25. Vita R, Mahajan S, Overton JA, *et al.* The Immune Epitope Database (IEDB): 2018 update. *Nucleic Acids Res.* 2019;47(D1):D339-D343.
doi: 10.1093/nar/gky1006
26. Dhanda SK, Vir P, Raghava GP. Designing of interferon-gamma inducing MHC class-II binders. *Biology Direct.* 2013;8(1):30.
doi: 10.1186/1745-6150-8-30
27. Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res.* 2017;45(W1):W24-W29.
doi: 10.1093/nar/gkx346
28. Wei L, Ye X, Sakurai T, Mu Z, Wei L. ToxIBTL: prediction of peptide toxicity based on information bottleneck and transfer learning. *Bioinformatics.* 2022;38(6):1514-1524.
doi: 10.1093/bioinformatics/btac006
29. Nguyen MN, Krutz NL, Limviphuvadh V, Lopata AL, Gerberick GF, Maurer-Stroh S. AllerCatPro 2.0: a web server for predicting protein allergenicity potential. *Nucleic Acids Res.* 2022;50(W1):W36-W43.
doi: 10.1093/nar/gkac446
30. Menozzi FD, Reddy VM, Cayet D, *et al.* Mycobacterium tuberculosis heparin-binding haemagglutinin adhesin (HBHA) triggers receptor-mediated transcytosis without altering the integrity of tight junctions. *Microbes Infect.* 2006;8(1):1-9.
doi: 10.1016/j.micinf.2005.03.023
31. Shamriz S, Ofoghi H, Moazami N. Effect of linker length and residues on the structure and stability of a fusion protein with malaria vaccine application. *Comput Biol Med.* 2016;76:24-29.
doi: 10.1016/j.compbiomed.2016.06.015
32. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. *Nucleic Acids Res.* 2008;36(2):W5-W9.
doi: 10.1093/nar/gkn201
33. Garg VK, Avashthi H, Tiwari A, *et al.* MFPPI-multi FASTA ProtParam interface. *Bioinformatics.* 2016;12(2):74-77.
doi: 10.6026/97320630012074
34. Hebditch M, Carballo-Amador MA, Charonis S, Curtis R, Warwicker J. Protein-Sol: a web tool for predicting protein solubility from sequence. *Bioinformatics.* 2017;33(19):3098-3100.
doi: 10.1093/bioinformatics/btx345
35. Høie MH, Kiehl EN, Petersen B, *et al.* NetSurfP-3.0: accurate and fast prediction of protein structural features by protein language models and deep learning. *Nucleic Acids Res.* 2022;50(W1):W510 - W515.
doi: 10.1093/nar/gkac439
36. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. *Nat Methods.* 2015;12(1):7-8.
doi: 10.1038/nmeth.3213
37. Shuvo MH, Gulfam M, Bhattacharya D. DeepRefiner: high-accuracy protein structure refinement by deep network calibration. *Nucleic Acids Res.* 2021;49(W1):W147-W152.
doi: 10.1093/nar/gkab361
38. Laskowski RA, MacArthur MW, Thornton JM. PROCHECK: validation of protein-structure coordinates. In: *International Tables for Crystallography*. International Union of Crystallography; 2012:684-687.
doi: 10.1107/97809553602060000882
39. Quignot C, Rey J, Yu J, Tufféry P, Guerois R, Andreani J. InterEvDock2: an expanded server for protein docking using evolutionary and biological information from homology models and multimeric inputs. *Nucleic Acids Res.* 2018;46(W1):W408-W416.
doi: 10.1093/nar/gky377
40. Laskowski RA, Swindells MB. LigPlot+: Multiple Ligand-Protein Interaction Diagrams for Drug Discovery. *J Chem Inf Model.* 2011;51(10):2778-2786.
doi: 10.1021/ci200227u
41. Weng G, Wang E, Wang Z, *et al.* HawkDock: a web server to predict and analyze the protein-protein complex based on computational docking and MM/GBSA. *Nucleic Acids Res.* 2019;47(W1):W322-W330.
doi: 10.1093/nar/gkz397
42. WebGro | UAMS. Accessed February 4, 2023. <https://simlab.uams.edu/index.php>.
43. López-Blanco JR, Aliaga JI, Quintana-Ortí ES, Chacón P. iMODS: internal coordinates normal mode analysis server.

- Nucleic Acids Res.* 2014;42(W1):W271-W276.
44. Craig DB, Dombkowski AA. Disulfide by Design 2.0: a web-based tool for disulfide engineering in proteins. *BMC Bioinform.* 2013;14:346.
doi: 10.1186/1471-2105-14-346
 45. McWilliam H, Li W, Uludag M, *et al.* Analysis tool web services from the EMBL-EBI. *Nucleic Acids Res.* 2013;41(W1):W597-W600.
doi: 10.1093/nar/gkt376
 46. 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(2):W526-W531.
doi: 10.1093/nar/gki376
 47. Langdon WB, Petke J, Lorenz R. Evolving Better RNAfold Structure Prediction. In: Castelli M, Sekanina L, Zhang M, Cagnoni S, García-Sánchez P, eds. *Genetic Programming*. Vol 10781. Lecture Notes in Computer Science. Springer International Publishing; 2018:220-236.
doi: 10.1007/978-3-319-77553-1_14
 48. Rapin N, Lund O, Bernaschi M, Castiglione F. Computational Immunology Meets Bioinformatics: The Use of Prediction Tools for Molecular Binding in the Simulation of the Immune System. *PLoS ONE.* 2010;5(4):e9862.
doi: 10.1371/journal.pone.0009862
 49. Banjoko AW, Ng'uni T, Naidoo N, Ramsuran V, Hyrien O, Ndhlovu ZM. High resolution class I HLA-A, -B, and -C diversity in Eastern and Southern African populations. *Sci Rep.* 2025;15(1):23667.
doi: 10.1038/s41598-025-06704-4
 50. Janse Van Rensburg WJ, De Kock A, Bester C, Kloppers JF. HLA major allele group frequencies in a diverse population of the Free State Province, South Africa. *Heliyon.* 2021;7(4):e06850.
doi: 10.1016/j.heliyon.2021.e06850
 51. Martinelli DD. In silico vaccine design: A tutorial in immunoinformatics. *Healthc Anal.* 2022;2:100044.
doi: 10.1016/j.health.2022.100044
 52. Sharma V, Singh S, Ratnakar TS, Prajapati VK. Chapter 29 - Immunoinformatics and reverse vaccinology methods to design peptide-based vaccines. In: Tripathi T, Dubey VK, eds. *Advances in Protein Molecular and Structural Biology Methods*. Academic Press; 2022:477-487.
doi: 10.1016/B978-0-323-90264-9.00029-5
 53. Laurens MB. RTS,S/AS01 vaccine (Mosquirix™): an overview. *Hum Vaccin Immunother.* 2019;16(3):480-489.
doi: 10.1080/21645515.2019.1669415
 54. Miller LH, Duffy PE, Culleton R. Transmission-Blocking Vaccines: From Conceptualization to Realization. *Am J Trop Med Hyg.* 2022;107(3):1-2.
doi: 10.4269/ajtmh.22-0023
 55. Duffy PE, Patrick Gorres J. Malaria vaccines since 2000: progress, priorities, products. *npj Vaccines.* 2020;5(1):48.
doi: 10.1038/s41541-020-0196-3
 56. Miura K, Flores-Garcia Y, Long CA, Zavala F. Vaccines and monoclonal antibodies: new tools for malaria control. *Clin Microbiol Rev.* 2024;37:e00071-23.
doi: 10.1128/cmr.00071-23
 57. Mitran CJ, Yanow SK. The Case for Exploiting Cross-Species Epitopes in Malaria Vaccine Design. *Front Immunol.* 2020;11:335.
doi: 10.3389/fimmu.2020.00335
 58. Cao Y, Bansal GP, Merino K, Kumar N. Immunological Cross-Reactivity between Malaria Vaccine Target Antigen P48/45 in *Plasmodium vivax* and *P. falciparum* and Cross-Boosting of Immune Responses. *PLoS ONE.* 2016;11(7):e0158212.
doi: 10.1371/journal.pone.0158212
 59. Vijay S, Rawat M, Sharma A. Mass spectrometry based proteomic analysis of salivary glands of urban malaria vector *Anopheles stephensi*. *Biomed Res Int.* 2014;2014:686319.
doi: 10.1155/2014/686319
 60. Zhang Y, Zhu X, Feng Y, *et al.* TLR4 and TLR9 signals stimulate protective immunity against blood-stage *Plasmodium yoelii* infection in mice. *Exp Parasitol.* 2016;170:73-81.
doi: 10.1016/j.exppara.2016.09.003
 61. Al-Madhagi H, Kanawati A, Tahan Z. Design of multi-epitope chimeric vaccine against Monkeypox virus and SARS-CoV-2: A vaccinomics perspective. *J Cellular Molecular Medi.* 2024;28(10):e18452.
doi: 10.1111/jcmm.18452
 62. Shilling PJ, Mirzadeh K, Cumming AJ, Widesheim M, Köck Z, Daley DO. Improved designs for pET expression plasmids increase protein production yield in *Escherichia coli*. *Commun Biol.* 2020;3(1):214.
doi: 10.1038/s42003-020-0939-8
 63. Godat B, Engel L, Betz NA, Johnson TM. Methods for the purification of HQ-tagged proteins. *Methods Mol Biol.* 2008;421:151-168.
doi: 10.1007/978-1-59745-582-4_11
 64. Loughran ST, Bree RT, Walls D. Purification of Polyhistidine-Tagged Proteins. *Methods Mol Biol.* 2017;1485:275-303.
doi: 10.1007/978-1-4939-6412-3_14
 65. Maharaj L, Adeleke VT, Fatoba AJ, *et al.* Immunoinformatics approach for multi-epitope vaccine design against *P. falciparum* malaria. *Infect Genet Evol.* 2021;92:104875.
doi: 10.1016/j.meegid.2021.104875

66. Pritam M, Singh G, Swaroop S, Singh AK, Singh SP. Exploitation of reverse vaccinology and immunoinformatics as promising platform for genome-wide screening of new effective vaccine candidates against *Plasmodium falciparum*. *BMC Bioinform.* 2019;19(S13):468.
doi: 10.1186/s12859-018-2482-x
67. Ajibola O, Diop MF, Ghansah A, *et al.* In silico characterisation of putative *Plasmodium falciparum* vaccine candidates in African malaria populations. *Sci Rep.* 2021;11(1):16215.
doi: 10.1038/s41598-021-95442-4
68. Atapour A, Vosough P, Jafari S, Sarab GA. A multi-epitope vaccine designed against blood-stage of malaria: an immunoinformatic and structural approach. *Sci Rep.* 2022;12(1):11683.
doi: 10.1038/s41598-022-15956-3
69. Pandey RK, Bhatt TK, Prajapati VK. Novel Immunoinformatics Approaches to Design Multi-epitope Subunit Vaccine for Malaria by Investigating Anopheles Salivary Protein. *Sci Rep.* 2018;8(1):1125.
doi: 10.1038/s41598-018-19456-1