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Contriving a novel multi-epitope subunit vaccine from *Plasmodium falciparum* vaccine candidates against malaria

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ABSTRACT

In this study, immunoinformatics strategies were used to design a subunit vaccine against malaria from immunogenic regions of three *Plasmodium falciparum* surface antigens; liver stage antigen 3-C (V750-K1433), merozoite surface antigen 180 truncate-4 (A805-P1093), and merozoite surface protein 10 region 1 (D29-N188). A multi-epitope subunit vaccine construct (VC) was designed from immunodominant B- and T-cell epitopes followed by structure prediction, evaluation, and validation. Toll-like receptors (TLRs) 2 and 4 were docked with the VC. Their complexes' molecular dynamics, immune stimulation, codon optimization, and *in silico* cloning of the VC were simulated. The VC is a 49.2 kDa antigenic and nonallergenic protein, comprised of 26% α-helix, 7% β-strand, 66% coil. The immune simulation test showed that the vaccine could provoke adaptive immune responses, and molecular docking tests showed that it interacts strongly with TLR-2 (−945.1 kcal/mol) and TLR-4 (−919.8 kcal/mol) to form complexes of high stability that hardly deform. The guanine-cytosine content and codon adaptation index of the VC were 42.94 and 0.99 after codon optimization. *Escherichia coli* pET-28a(+) was identified as the best vector for optimal gene expression. In conclusion, the study reveals that the VC shows promising results in neutralizing *falciparum* malaria.

INTRODUCTION

Malaria is a deadly tropical disease caused by an infection of the protozoan *Plasmodium*, and it continues to be the most significant human parasitic illness in the world. It is a vector-borne illness. It spreads through the bites of female Anopheles mosquitoes infected with *Plasmodium* parasites [1]. Over the last century, despite the increased research efforts and control measures to drive down the malaria burden globally, eradication strategies and interventions have only been fairly successful. The parasite has co-evolved with new interventions, and eradication remains ongoing [2]. *Plasmodium* species have developed resistance to all known classes of antimalarial compounds and drugs which is one of the major challenges in the fight against malaria [3], in addition to inadequate research funding, healthcare professionals, facilities, research efforts in malaria-endemic regions, and limited knowledge about naturally acquired immunity to malaria [4,5].

Vaccination is an economical and successful solution to stop infectious diseases [6–10]. Advances have been made in blood-stage vaccine development, as they have reached clinical trials; however, they were unsuccessful in controlling human malaria infection on the field [11–13]. Antigen polymorphism, redundancy, parasite immune evasion, and low effectiveness of vaccine candidates have greatly hindered the rapid development of a licensed vaccine to neutralize malaria [14–18]. Furthermore, increasing insecticide resistance and asymptomatic infections have also been major setbacks [19,20], and cases of resurgence and increased malaria deaths have

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been reported in South American nations [21]. Vaccination has been identified as a critical therapeutic option for eliminating malaria; hence, it is a priority for sustained, substantial, and cost-effective control [22–24]. The only available licensed vaccine against malaria, RTS, S/AS01 shows low to modest efficacy in thwarting malaria caused by *Plasmodium falciparum*, which is enough to prevent clinical malaria but not enough for the global eradication of malaria [1]. Several other vaccines are currently under development, including the R21/Matrix-M (with 77% efficacy in preliminary trials), *P. falciparum* sporozoite, and the self-amplifying RNA vaccine. However, no vaccine with 100% efficacy remains available [25–27].

Post-genomic era vaccine development often involves preliminary computational analysis using bioinformatics tools to predict protective antigens rather than experimental studies with the pathogen, as in conventional vaccinology. Antigen characterization using *in silico* strategies and bioinformatics tools is crucial to the protein-based vaccine design and development [28]. Reverse vaccinology is named so because the vaccine discovery process employs computational methods to analyze genomic data instead of wet lab experimental studies with a pathogen, as with conventional vaccinology [29]. This technique has gained increasing global popularity and usage by research groups in the last 20 years for vetting the whole genome for vaccine antigens against several pathogens including *Leishmania* [30], *Lassa* fever [31], Dengue [32], Schistosomiasis [33], and *P. falciparum* [34], among others. This strategy is effective as it saves cost and time, and decrease the risk of failure compared to conventional vaccinology [35].

The World Health Organization set the goal to eradicate malaria in 1948, and efforts have been intensified to this end. Correspondingly, malaria-related fatalities progressively decreased, but this progress stalled due to the COVID-19 pandemic and attributed to the interference with malaria services and diagnostic practices [36]. In 2021, the number of malaria cases worldwide was recorded to be 241 million, with 627,000 related deaths [37]. In light of the available data and the current state of global malaria burden, this research aimed to develop a potent multi-epitope blood stage subunit vaccine candidate against *P. falciparum* by integrating epitopes from the most immunogenic regions of three merozoite surface proteins namely, liver-stage antigen 3 (LSA3-C), merozoite surface antigen 180 (MSA 180), and merozoite surface antigen 10 (MSA 10).

METHODOLOGY

A systematic flow chart was followed stepwise to identify immunodominant B- and T-cell epitopes and the design of a subunit vaccine via immunoinformatics techniques. A subsequent biophysical study was performed using integrated docking, immune simulation, and molecular docking. Finally, codon optimization was executed to ensure optimal expression in the microbial host (Fig. 1).

Amino acid sequence retrieval

To retrieve the primary sequence of liver stage antigen 3, MSA 180, and merozoite surface protein 10, the PlasmoDB [38] server was employed. However, only the most immunogenic regions of these proteins–(LSA3-C; V750-K1433), merozoite surface antigen 180 truncate-4 (MSA 180-T4; A805-P1093), and merozoite surface protein 10 region 1 (MSP10 R1; D29-N188) were further exploited for the vaccine construct (VC), following results from prior studies [39–41].

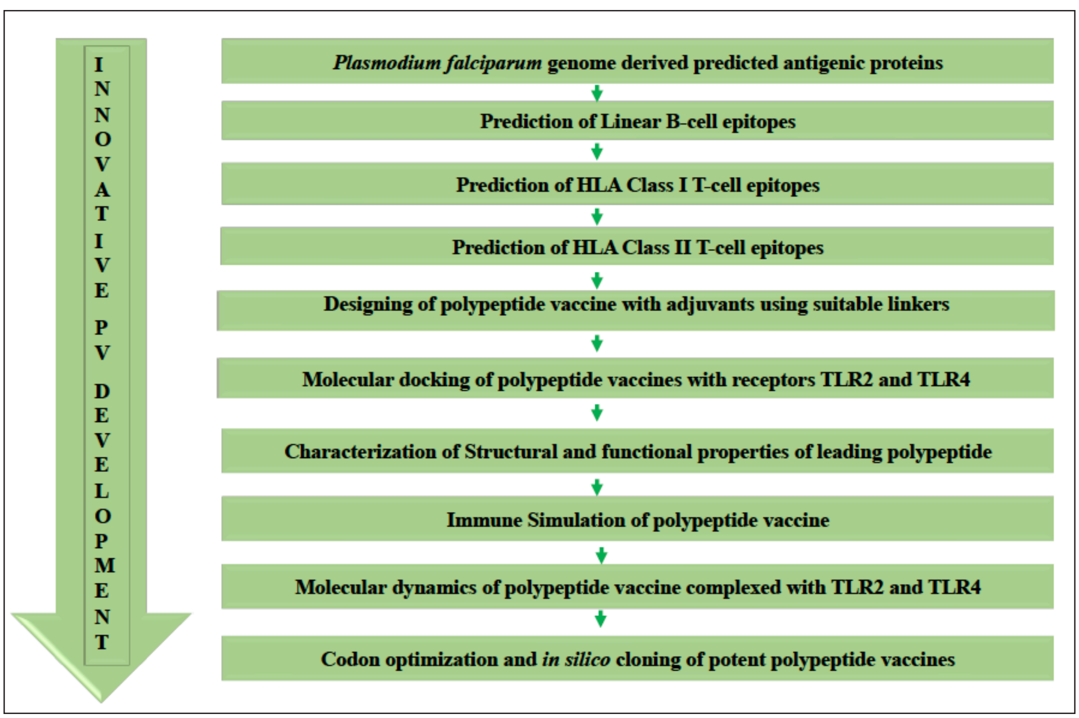

Figure 1. Methodology flow chart showing the step-by-step procedures used.

Forecast of B-cell epitopes

Using default parameters for both, two servers were used to forecast the B-cell epitopes, including ABCpred [42] and BepiPred 2.0 [43]. Matching epitopes forecasted by both servers were selected. Furthermore, finalized epitopes were vetted for antigenicity (VaxiJen 2.0 server) [44], allergenicity (AllergenFP v.1.0) server [45], toxicity (ToxinPred server) [46], and conservancy (Protein Blast - NCBI). For conservancy, the epitopes were screened against *Homo sapien*s proteome (taxid: 9606), with nonhomology indicated by e-value scores ≥0.05 [47].

Forecast of CD4+ and CD8+ T-cell epitopes

The NetMHCIIpan 4.0 server [48] was utilized to identify helper T lymphocyte (HTL) epitopes with a threshold of 1% (strong binding) and 5% (weak binding). Twelve (12) major histocompatibility complex (MHC) supertypes available on the NetCTL 1.2 server were screened to predict CD8+ epitopes, with default parameters [49,50]. Also, the predicted epitopes were screened for antigenicity (VaxiJen 2.0 server) and immunogenicity (IEDB server), set at a threshold of 0.4 and default parameters respectively.

Designing of polypeptide subunit vaccine

The finalized B- and T-cell epitopes were integrated using several linkers, including the EAAAK (L1), AAY (L2), GPGPG (L3), and KK (L4) linkers. The adjuvant (ADV) RS09 was included at the N-terminals of the VC and is joined to the CD8+ epitopes by the EAAAK linker. The CD8+ epitopes were intralinked through the AAY linker and interlinked with the $CD4^+$ epitopes by the GPGPG linker, which also intralinks the $CD4^+$ epitopes. KK linkers interlink the $CD4^+$ and B-cell epitopes and intralink the B-cell epitopes. The final VC comprises a His-tag (HHHHHH) at the C-terminal and is arranged ADV-CTL epitopes-HTL epitopes-B-cell epitopes–6X His tag.

Secondary and tertiary structure prediction, evaluation, and validation

Psipred [51] and RaptorX [52] servers were employed to forecast the secondary organization of the multi-epitope construct. Robetta [53], which creates three-dimensional (3D) models by employing ab initio and comparative modeling simulations, was used to predict the constructs' tertiary structure [54]. Pymol 2.5 visualization software was used to envision the protein's 3D organization. Subsequent refinement to improve the 3D model was evaluated using the GalaxyWEB [55] server.

The ProSA-web server [56] and Saves v.6.0 [57] server were employed to ascertain the quality of the 3D models. The *Z*-scores of each protein solved experimentally using data from X-ray and nuclear magnetic resonance sources are presented in a plot by ProSA. It determines whether the *z*-score of the 3D model of the query protein falls within the normal range for scores for native proteins of comparable size. The Ramachandran plot examines the model's phi/psi distribution and C-beta deviation to determine the backbone (C-alpha) geometry [58].

Conformational B-cell epitopes

The VCs tertiary structure was put in the Ellipro server, which forecasts and visualizes discontinuous B-cell epitopes [59].

Structural and functional characterization of vaccine construct

The ExPASy-ProtParam tool [60] was used to estimate the physicochemical parameters of the VC. Furthermore, the antigenic and allergenic properties were predicted.

Molecular docking and immune simulation of vaccine construct

Toll-like receptors (TLR)-2 and -4 were selected as effective immunological targets for provoking the innate immune system. The ClusPro 2.0.php server was employed in accessing the interaction between the TLRs (receptor) and VC (ligand) [61,62]. The C-IMMSIM web server, which uses machine learning techniques and a position-specific scoring matrix, was utilized to determine the immune profile of the VC for 3 injections administered 4 weeks apart [63,64]. Default parameters were set with 1, 84, and 170 specified time steps [65].

Molecular dynamics (MD) simulation of vaccine construct-TLR complexes

The iMODS server [66] predicts the MD, which uses the normal mode analysis (NMA) in internal coordinates to delineate the collective protein motions.

Codon optimization and *in silico* cloning of vaccine construct

To forecast a viable option for the expression and isolation of the VC, the JCat server [67] was used to improve the construct's nucleotide sequence to commonly utilized codons of the *Escherichia coli* K 12 strain to enable optimal protein production by the expression host. Also, *in silico* cloning requires the SnapGene software to incorporate restriction sites (EcoRI and BamHI), after which the genetic sequence is integrated into the vector pET-28a(+).

RESULTS AND DISCUSSION

Proteins that encrust the extracellular surface of merozoite and are released from its specialized secretory apical organelles are considered potential vaccine candidates for the erythrocytic stage malaria [68]. Robust experimental evidence supports the importance of these surface proteins to host cell invasion, parasite growth, and survival and as potential preventive measures against *P. falciparum* malaria in humans [11,40,69–75]. In this study, three experimentally validated vaccine candidates against *falciparum* malaria were selected for further downstream immunoinformatics analysis.

The LSA3-C, MSA 180-T4, and MSP10 R1 are regions of blood-stage *P. falciparum* proteins that possess characteristics which have been experimentally validated to be malaria vaccine candidates, and this makes them attractive targets for developing a sub-unit vaccine [39–41]. Liver-stage antigen 3 (PF3D7_0220000) is a ~175kDa protein expressed in liver and blood stages on sporozoites surface, infected hepatocytes, and blood-stage merozoites, and several research groups have reported it to be a viable pre-erythrocytic and blood stage vaccine target [39,76–78]. Furthermore, LSA3 was reported to be highly conserved following genomic sequence analysis of 20 *P. falciparum* clinical isolates from diverse geographical regions and localized in the parasitophorous vacuole during the ring-stage following merozoite ingress [78,79]. Previous studies on the full-length and discrete regions of LSA3-C have reported its viability as a pre-erythrocytic and blood-stage vaccine candidate in addition to being the most immunologic antigen segment [39,78,79].

Plasmodium falciparum merozoite surface antigen 180 (*Pf*MSA180) is a 170kDa protein that is essential and conserved in all *Plasmodium* sp.; it is expressed on the periphery of merozoites and has been implicated in merozoite ingress and egress during the asexual blood stage of the parasite's lifecycle [41,80]. *Pf*MSA180 (PF3D7_1014100) has been reported to be critical for parasite invasion of the erythrocyte, and antibodies against the C-terminal region of *Pf*MSA180 [MSA 180 Tr-4 (A805-P1093)] abrogated merozoite invasion *in vitro* and conferred protective immunity against malaria [41]. Furthermore, the *Pf*MSA180 (PfMSA180-Tr4) C-terminal region was reported to be highly conserved across isolates, interacts with the red blood cell (RBC) surface protein-CD47, and stimulates antibodies that abrogate parasite invasion; hence, Nagaoka *et al.* [41] proposed MSA 180 Tr-4 as a potential vaccine candidate. *Pf*MSP10 (PF3D7_0620400) is an 80 kDa protein that directly interacts with *Pf*GAMA, which is key for erythrocyte invasion. The *Pf*MSP10 R1 (D29-N188) region was hypothesized to be the interacting region [40]. Also, bioinformatics genome-wide screening has predicted *Pf*MSP10 to be a putative vaccine candidate [81].

Prediction of linear B-cell epitopes

B-cells comprise one of the two main types of cells in the adaptive immune system. Their epitopes are antigenic components, which trigger the synthesis of antibodies [82]. To overcome the challenge of prediction inaccuracy, we employed two servers for better B-cell epitope mapping. The results were compared, and consistent epitopes with both servers were selected. Also, nonantigenic, allergenic, toxic, and conserved epitopes in humans were discriminated against. The top three epitopes from each vaccine candidate that passed the screening (antigenicity, allergenicity, toxicity, and conservancy) for inclusion in the VC were selected (Table 1) from the epitopes with scores ≥ 0.52 (Table S1).

CD4+ T-cell epitopes prediction

MHC II encrusts the periphery of antigen-presenting cells (APCs). These MHCs exhibit nonself-peptides to helper T-cells, which coordinates other immune responses [83]. Hence, it is essential to predict epitopes with a higher likelihood of being displayed by the MHC-II molecule. Strong interaction between the HTL epitope and HLA-DR is critical for epitope immunogenicity, and excellent HTL epitope candidates are expected to interact optimally with numerous HLA alleles [84,85]. To this end, LSA3-C, MSA-Tr4, and MSP10 R1 were subjected to CD4+ epitope prediction, and only the epitopes that can bind to three or more HLA-DR alleles were agreed upon [86]. The top three epitope sequences for each are included as shown in Tables 2 and S2.

CD8+ T-cell epitopes prediction

APCs and infected RBCs cells display peptides of pathogen origin on MHC I molecules expressed on their surface to cytotoxic T-cells, facilitating clearance and immunological memory [87]. LSA3-C, MSA-Tr4, and MSP10 R1 were screened for their MHC-1 epitopes using all 12 MHC-I supertypes available on the NetCTL1.2 server. Table 3 shows the finalized epitopes after the proteins were subjected to further screening parameters, including; antigenicity, allergenicity, immunogenicity, and conservancy predictions.

Polypeptide subunit vaccine design

The finalized B- and T-cell epitopes were integrated using suitable linkers alongside two ADVs. ADVs present a classical approach to targeted delivery of subunit vaccines and maximizing protective immunity, as protein vaccine candidates are poorly immunogenic [88]. They bridge the antigens with the APCs by projecting visibility of the otherwise non or weakly immunogenic antigen to the immune cells to trigger a better immune response [89]. RS09, a synthetic TLR4 agonist comprising seven amino acid residues [89], was incorporated as ADVs at the N-terminal of the VC [90,91]. The EAAAK linker increases

Table 1. Selected linear B-cell epitopes with their antigenicity, allergenicity, toxicity, and conservancy.

Antigen	Epitope	Position	Score	Antigenicity	Allergenicity	Toxicity	Conservancy
LSA 3-C (V750-K1433)	VEHIISGDAHIKGLEE	428	0.92	+	-	-	-
	TESIKDKEKDVSLVVE	209	0.90	+	-	-	-
	TVEISGESLENNEMDK	8	0.89	+	-	-	-
MSA-Tr4 (A805-P1093)	HKDNDSRYTDNSNKNR	35	0.91	+	-	-	-
	TSDILYKDIEENKNTE	185	0.88	+	-	-	-
	SKVTGDSVENINEQTN	269	0.85	+	-	-	-
MSP10 R1 (D29-N188)	KITYDKYNKNKENMNN	10	0.87	+	-	-	-
	NKENMNNEKNDNKDNK	19	0.86	+	-	-	-
	NKDNKDNIYNDNINND	30	0.85	+	-	-	-

Table 2. Details of selected CD4+ T-cell epitopes predicted by NetMHCIIpan 4.0.

Peptide	Epitopes	HLA-II alleles
LSA 3-C (V750-K1433)	VKEIKELESEILEDY	DRB1_0101, DRB1_1201, DRB1_1501
	KKKVRFDIKDKEPKD	DRB1_0301, DRB1_1302, DRB4_0101
	EQNVYVDVDVPAMKD	DRB1_0301, DRB1_0401, DRB1_0405, DRB3_0101
MSA-Tr4 (A805-P1093)	AKNFYNISNENGDNT	DRB1_0401, DRB1_0405, DRB1_0802
	DNTFNNNNNNMDNKK	DRB1_0401, DRB1_0405, DRB3_0202
	LPSLKSIYNNKIKGN	DRB1_1201, DRB1_1302, DRB1_1501
MSP10 R1 (D29-N188)	NENIENIENNENNEN	DRB1_0401, DRB1_0405, DRB4_0101

Table 3. Selected CD8+ T-cell epitopes with MHC supertypes and immunogenicity score.

Peptide	HLA1 epitope	MHC supertype	Immunogenicity score(s)
LSA3-C (V750-K1433)	IISGDAHIK	A3	0.14389
	KVRFDIKDK	A3	0.11244
	RPKLEEVLL	B7, B8	0.13895; 1.1500
	EIKDLEADI	A26	0.8428
	YVDVDVPAM	B39	1.0123
	EEHDITTTL	B39, B44	1.1040; 1.7244
	RSIETSIVI	B58, B62	1.3442; 0.9382
	TEHVEQNVY	B62	0.7923
MSA-Tr4 (A805-P1093)	ERKRYIRKK	B27	0.8928
MSP10-R1 (D29-N188)	ENIENNENV	A26	0.9367

the consistency of the overall structure and limits protein components from joining through effective detachment; hence, it was used to join the ADV with the polypeptide vaccine [92]. The AAY linker improves the vaccine's ability to elicit an immunological response [93]. The need to impinge the development of junctional epitopes and improve epitope presentation necessitates the GPGPG linkers [94]. The KK linker was used to connect linear B-cell epitopes so that their self-regulating immunogenic responses may be preserved [95]. The VC follows the sequence "ADV-CD8+ epitope-CD4+ epitopes-B-cell epitopes- 6X His tag," joined by appropriate linkers to form a 437 amino acid-long peptide as illustrated in Figure 2.

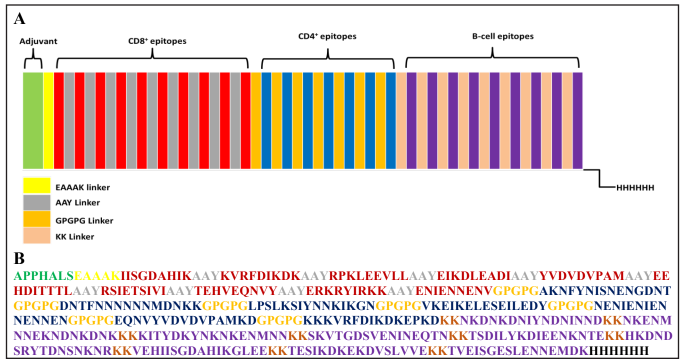

Figure 2. (A) Schematic diagram of polypeptide vaccine. It comprises several segments: an ADV, RS09 (TLR4 agonists), 10 CD8+ epitopes, 7 CD4+ epitopes, 9 B-cell epitopes, and a His tag. These segments are integrated by EAAK, AAY, GPGPG, and kk linkers. (B) Sequence of polypeptide vaccine.

Secondary and tertiary structure prediction, evaluation, and validation

The VCs comprise 26% α-helix, 7% β-strand, and 66% coil, as predicted by Psipred, and RaptorX in plots representing the structural moieties (Fig. 3). Also, 76% was forecasted to be exterior, 8% mildly exterior, and 14% to be buried in relation to the availability of amino acid residues. The α-helix and β-turn have internal localization within the protein and are less likely to act as epitopes; instead, they maintain the structure of the protein due to the high chemical bond energy they contain. Random coils are exposed on the protein surface, have fewer rigid regions of the protein, and may be probable epitopes [96]. Over half the overall structure of the construct comprises random coils, hinting at the greater potential of the protein to trigger immune responses. The 3D structure was predicted by the Robetta server, of which five models were generated, and model 1 was selected as it had higher comparative model quality following subjection to the Saves v.6.0 server for the ERRAT and Ramachandran plot analysis. GalaxyRefine server was used to refine and improve the quality of the selected model (Fig. 4A). Structural validation involves the identification of probable faults in the forecast 3D structure [97]. The model had a *Z*-score of -7.37, predicted by ProSA-web (Fig. 4B). Also, to determine the nonbonded atom-atom interactions, the ERRAT server was utilized [57], which revealed an overall quality factor of 94.72, suggesting a high quality. The Ramachandran plot enables the visualization of energetically favorable and unfavorable dihedral angles psi (ψ) and phi (ϕ) of amino acids, which delineates the stereochemical quality of the 3D structure [98]. The Ramachandran plot analysis revealed that 93.8% of the initial model was in the favored regions, 5.2% in the allowed regions, and 0.8% in the disallowed regions. However, the refinement process improved the protein quality to 95.3% favoured, 3.4% allowed, and 0.5% disallowed regions, representing a very good model (Fig. 4C and D).

Conformational B-cell epitopes

B-cell epitopes usually form discontinuous epitopes resulting from the spatial proximity of a bunch of amino acid residues on the tertiary structure of the antigen [99]. Ellipro forecasted 4 epitopes with a maximum score of 0.758 and a minimum score of 0.512, and the number of epitope residues ranged from 3 to 81 (Table S3). Figure 5 reflects the structure of the epitopes in relation to the VC.

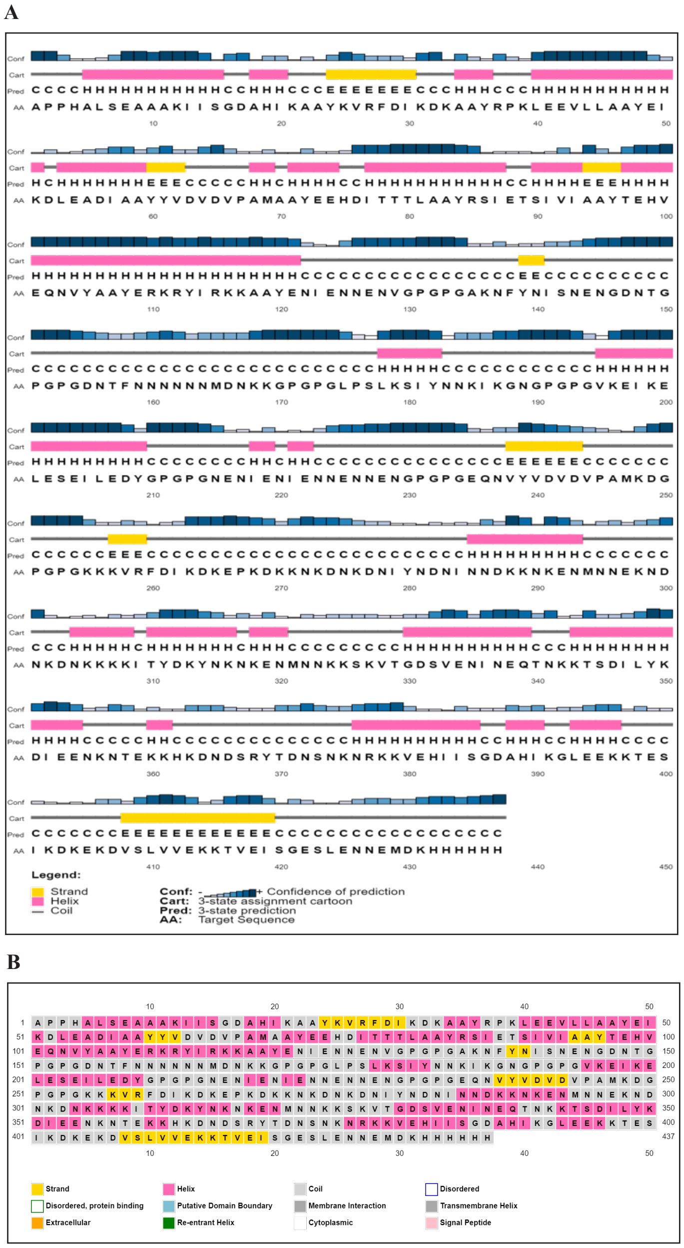

Figure 3. Schematic diagram of the secondary structure of VC. (A) Predicted secondary structure (H: α-helix, E: β-strand, C: coil) by PSIPRED. The bar chart represents the percentage of confidence. (B) RaptorX generated the chimeric protein's secondary structure, and the results show a structure made up of 26% α-helix, 7% β-strand and 66% coil.

Structural and functional characterization of VC

The physicochemical properties prediction revealed that the protein has a molecular weight and theoretical pI of 49.2 kDa and 5.99, respectively. The half-life was predicted to be 4.4 hours (mammalian reticulocytes, *in vitro*), >20 hours (yeast, *in vivo*), and >10 hours (*Escherichia coli*, *in vivo*). The instability index and GRAVY were forecasted to be 39.36 and −1.294, respectively. The score from the instability index suggested that the protein is stable. The GRAVY score represented that the protein is hydrophilic, which is a desired quality for a vaccine as it indicates the ability to trigger an elevated humoral immune response [100]. The aliphatic index was predicted to be 61.40, reflecting thermostability (Table S2).

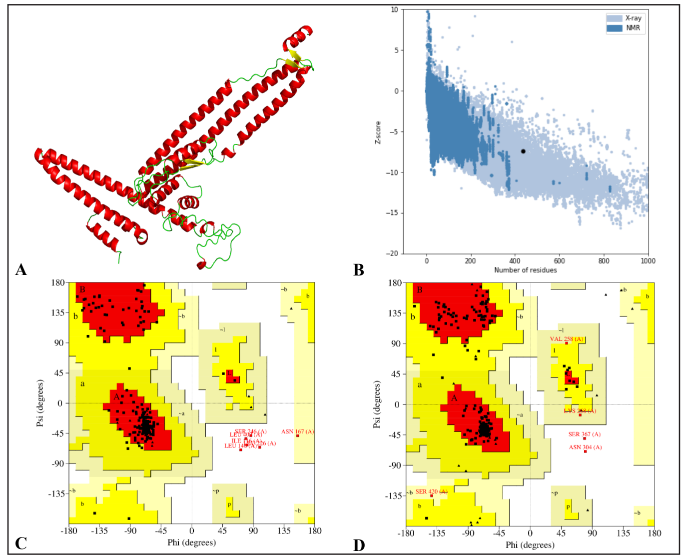

Figure 4. (A) 3D structure cartoon representation (Red, green, and yellow represent α-helix, coil, and β-sheet). (B) ProSA map of the sub-unit vaccine with *Z*-score of −7.37. (C) Ramachandran plot before protein refinement with 93.8% in the favored region, 5.2% allowed region, 0.8% in the disallowed region. (D) Ramachandran plot after protein refinement with 95.3% favored region, 3.4% allowed region, and 0.5% disallowed region.

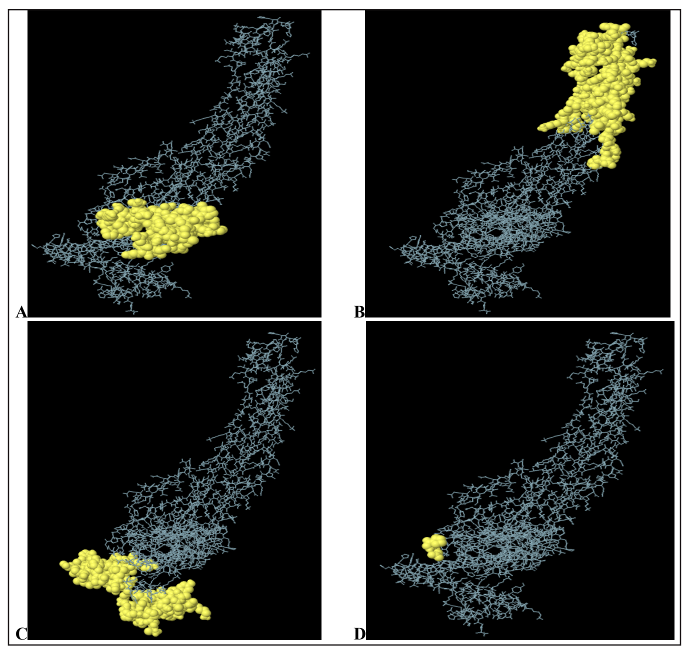

Figure 5. 3-D representation of the four conformational B-cell epitopes. (A–D) A yellow surface represents the epitopes.

Molecular docking of vaccine construct with toll-like receptors

The contact between ligand and receptor molecules is embroiled in molecular docking to generate a stable ligand-receptor product [101]. The interaction between antibodies

Table 4. Molecular docking score between VC and TLR-2 and -4.

	Cluster	Members	Representative	Weighted score (kcal/mol)
VC-TLR-2	0	81	Centre	−791.7
			lowest energy	−945.1
VC-TLR-4	0	72	Centre	−796.1
			lowest energy	−919.8

and their targeted antigens is critical to the humoral immune response to aid pathogen elimination. The VC-TLR2 complex has a total of 81 members in its cluster, while VC-TLR4 has 72 members in its cluster. The lowest energy score and binding affinity of −945.1 and −919.8 kcal/mol were obtained for the VC-TLR2 and VC-TLR4 complexes, respectively (Table 4). These complexes were stable as shown in Figure S1.

Immune simulation of vaccine construct

The immune simulation was performed to determine the natural immune response to the VC. The immune simulator C-ImmSim tool was employed to simulate the natural responses formed by the immune system. Figure 6 indicated that the antigenic recognition and desired corresponding immune responses would manifest. There was a marked increase in IgM and IgG production and the expansion of Helper T-cells due to memory development following primary immunization. There was also a depletion in antigen concentration, reflecting a rise in memory B-cell production (Fig. 6A–D). Figure 6F shows an elevation in the concentration of cytotoxic T-cells following vaccine administration. Similarly, there is an increase in the proliferation of cytokines, including IFN-γ, TGF- β, IL-10, and IL-12 (Fig. 6I), indicating that the vaccine is capable of provoking the immune response to act against malaria.

MD simulation of vaccine construct-TLR complexes

MD simulation is useful for analyzing the stability of protein-ligand complex by incorporating Newton's laws of motion [102]. It was done by enumerating protein dynamics to their normal modes using the iMODS server [103]. NMA is commonly used to evaluate the collective functional motions of docked protein-protein complexes [66]. The 3D interaction between VC-TLR2 and VC-TLR4 complexes is presented, with the arrows representing the direction of amino acids (Fig. 7A and B). The peaks on the deformability graph delineate deformability, as higher peaks depict higher deformability. From the result, the deformability plots report the stability of the complexes with individual amino acid residues having a lesser likelihood of deforming (Fig. 7C and D). The B-factor graph comparatively evaluates the NMA and the protein data bank field of the docked complexes (Fig. 7E and F). The eigenvalue corresponds with motion stiffness; a lower value is congruent with higher deformability and vice versa. The predicted high values of 9.12572e^{-07} and 1.117255e^{-06} for VC-TLR2 and VC-TLR4, respectively, reflect the less deformability and high stability of both complexes (Fig. 7G and H). The variance corresponding to each normal mode is inversely proportional to the eigenvalue

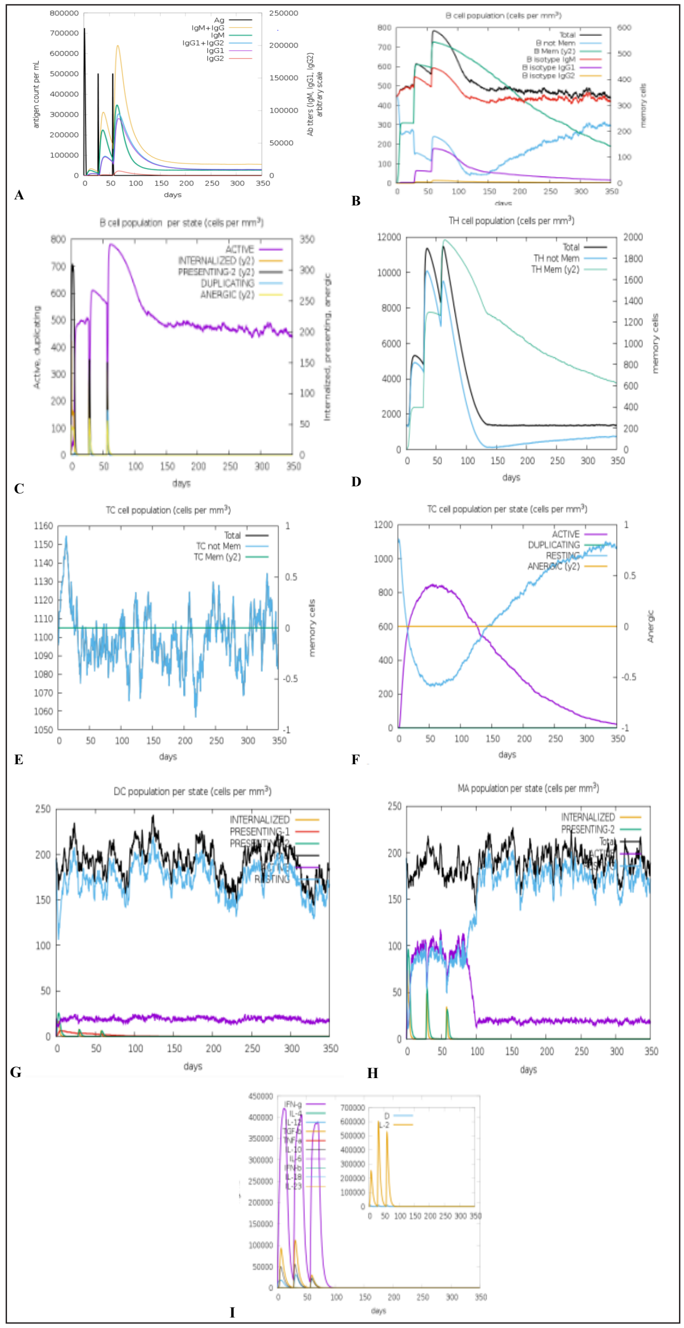

Figure 6. Characterisation of the immune profile of the construct. (A) The antibody production denotes a rise in immune response following the vaccine shot. Antibody subtypes (IgM, IgG1, and IgG2) are depicted as colored peaks. (B) The active B-cell population is observed with the vaccine shots. (C) B-lymphocyte population per entity-state (D) CD4+ T-helper lymphocytes count sub-divided per entity-state (E) The generation of cytotoxic-T cells. (F) CD8+ T-cytotoxic lymphocytes count per entity-state (G) Dendritic cells for MHC class I and II. Shows the total number of active, resting, internalized, and presenting antigen (H) Macrophages. Total count, internalized, presenting on MHC class-II, active and resting macrophages (I) Concentration of cytokines and interleukins.

(Fig. 7I and J) [104]. The covariance matrix reflects if the pairs of residues' motions were correlated (red), uncorrelated (white), or anti-correlated (blue) (Fig. 7K and L). The elastic

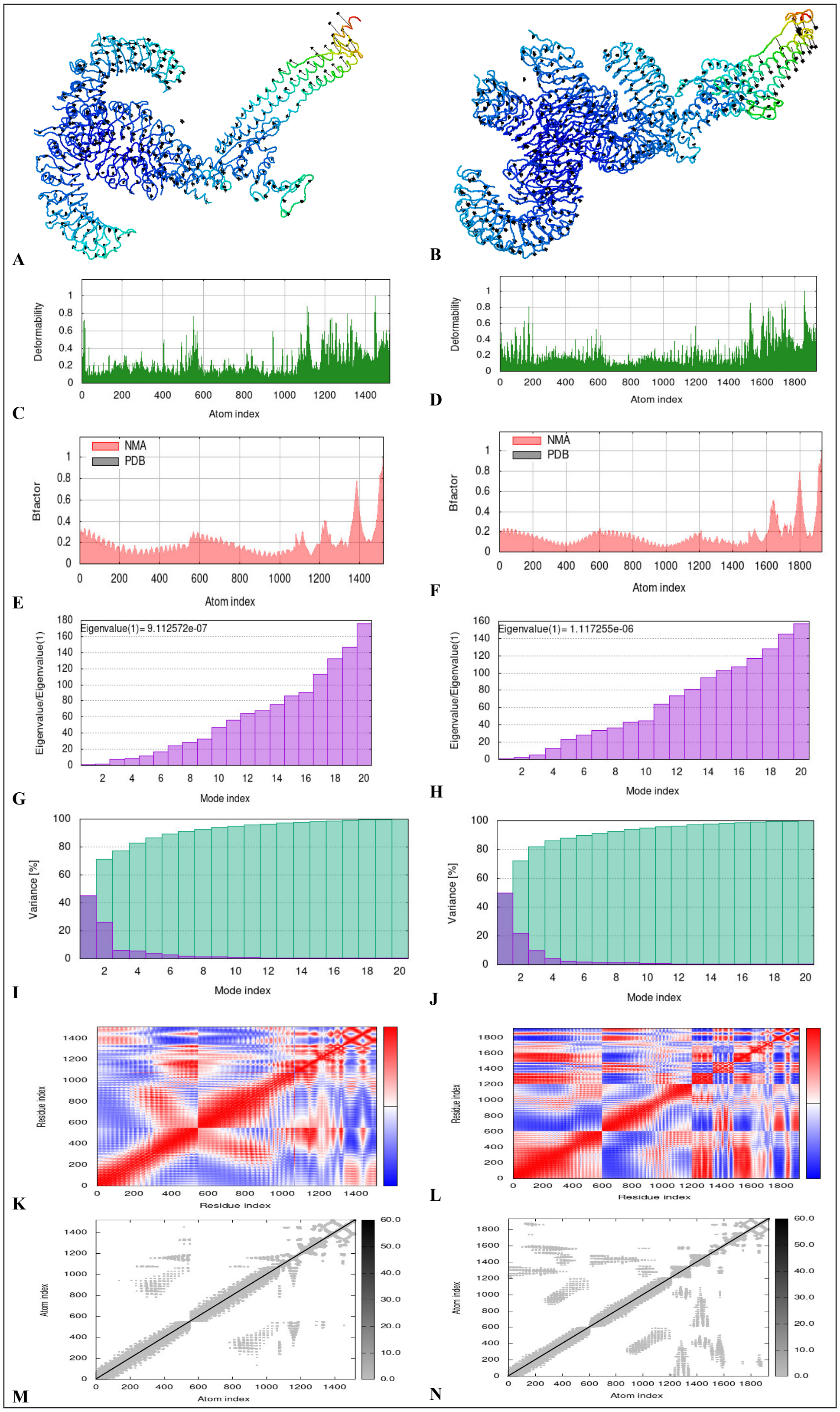

Figure 7. (A, B) NMA mobility, (C, D) deformability, (E, F) B factor, (G, H) Eigenvalue, (I, J) variance map, (K, L) Covariance map and (M, N) Elastic network graph of docked complexes VC-TLR2 and VC-TLR4, respectively. For the covariance map, red: correlated, white: uncorrelated and blue: anti-correlated motions, while for the elastic network graph of docked complexes, darker grey regions correlate with stiffer regions. VC: Vaccine construct.

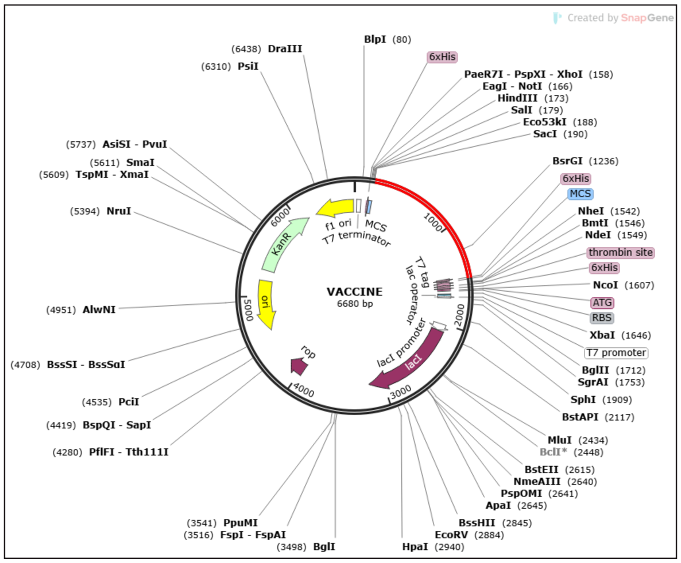

Figure 8. *In silico* cloning of the leading VCs. The red fragment designates the inserted vaccine and the pET-28a(+) vector is presented in a black circle.

network graph reflects atom pairs joined by springs, which are correspondingly colored by the degree of stiffness. The lighter grey area corresponds to less rigid springs and vice versa (Fig. 7M and N).

Codon optimization and *in silico* cloning of VC

Successful cloning and expression of the designed VC are critical for developing a vaccine since a poorly optimized codon could translate to a minimal expression rate in the host. The improved DNAs were 1,311 nucleotides long. A good guanine-cytosine (GC) value ranges between 30 and 70, while the Codon adaptation index (CAI) above 0.5 is good. Our chimeric proteins have good GC and CAI values of 42.94 and 0.99, hinting at a high likelihood of good expression in the prokaryotic host. Finally, the optimized sequence (highlighted in red) was incorporated into the *E. coli* pET-28a(+) vector for optimal gene expression (Fig. 8).

CONCLUSION

A multi-immune inducer that targets several antigens or various points of the parasite's entire life cycle may effectively engender robust clinical protection against malaria. To the best of our knowledge, this is the first study to use short regions rather than full-length proteins of experimentally validated vaccine candidates to contrive an antibody-inducing multi-epitope subunit vaccine targeting the blood stage of *falciparum* malaria. This is important because regions of vaccine candidates that have been experimentally validated to be most immunogenic will be most attractive for mapping immunodominant epitopes capable of being integrated to form a potent subunit vaccine. The VC demonstrated the ability to evoke combined innate and adaptive immune responses via interaction with the TLR-2 and -4, triggering cellular and humoral immunity in addition to forming a stable VC-receptor complex. The limitation of this study is that it is completely performed *in silico*. Therefore, an experimental study is necessary to determine the full potential of the proposed vaccine as a possible intervention in neutralizing malaria.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took p art in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/ guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the journal's website:
Link Here [https://japsonline.com/admin/php/uploadss/4324_pdf.pdf].