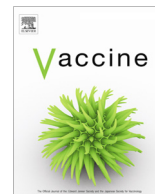




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Development of multi-epitope driven subunit vaccine in secretory and membrane protein of *Plasmodium falciparum* to convey protection against malaria infection

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ABSTRACT

Malaria infection is the severe health concern for a long time. As per the WHO reports, the malarial infection causes huge mortality all around the world and is incomparable with any other infectious diseases. The absence of effective treatment options and increasing drug resistance to the available therapeutics like artemisinin and other derivatives demand an efficient alternative to overcome this death burden. Here, we performed the literature survey and sorted the *Plasmodium falciparum* secretory and membrane proteins to design multi-epitope subunit vaccine using an adjuvant, B-cell- and T-cell epitopes. Every helper T-lymphocyte (HTL) epitope was IFN- γ positive and IL-4 non-inducer. The physicochemical properties, allergenicity, and antigenicity of designed vaccine were analyzed for the safety concern. Homology modeling and refinement were performed to obtain the functional tertiary structure of vaccine protein followed by its molecular docking with the toll-like receptor-4 (TLR-4) immune receptor. Molecular dynamics simulation was performed to check the interaction and stability of the receptor-ligand complex. Lastly, *in silico* cloning was performed to generate the restriction clone of designed vaccine for the futuristic expression in a microbial expression system. This way, we designed the multi-epitope subunit vaccine to serve the people living in the global endemic zone.

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1. Introduction

Malaria is considered as the severe health issue for centuries. It mainly affects the population living in the tropical, subtropical and sub-Saharan Africa region around the globe [1]. As stated by the World Health Organization (WHO) report, half of the world's population is living under the risk of malaria infection and in the year 2015, total 212 million malaria cases and 429,000 deaths were reported, worldwide. Malaria is caused by the intracellular sporozoan parasites belong to the genus *Plasmodium*. Five *Plasmodium* species namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* are known to cause the malaria infection among human being. Among these five *Plasmodium* species, *P. falciparum* causes the most lethal form of malaria with highest complications and mortality rates [2]. Transmission of these parasites to humans is caused by the bite of hitherto infected female *Anopheles* mosquitoes [3]. Malaria infection is associated with the severe illness

and its symptoms appear usually after 10–15 days of infective mosquitos bite. Primary malaria symptoms include fever, headache, body aches, nausea, vomiting, and chill while severe malaria causes multiple organs disorder in adults and anemia and respiratory distress among children [4].

Even after such severe assault by the malaria parasite, the treatment of uncomplicated malaria only relies upon the artemisinin-based combination therapies (ACTs) consisting of two active molecules with a different mechanism of action. Among this two active constituent, one consists of an artemisinin derivative while another consists of antimalarial drugs like mefloquine, amodiaquine, piperaquine, lumefantrine, or sulfadoxine-pyrimethamine. The treatment of severe malaria depends upon the injectable form of artesunate (2.4 mg/kg body weight) for the time duration of minimum 24 h. It should be continued with the ACTs for consecutive 3 days. Both these treatment options have their respective limitations. As per the WHO reports, parasitic resistance has been reported against almost all known antimalarial drugs that were used against 3 major malarial species namely *P. falciparum*, *P. vivax* and *P. malariae* [5]. The resistance against the highly efficacious artemisinin has also been reported from some parts of the

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South-East Asian region [6]. This resistance is aggravated by cross-resistance, a severe situation in which resistance to one drug deliberates resistance to other drugs which have either the same mode of action or belongs to the same chemical family. Additionally, the combination therapy of artemisinin derivative with mefloquine has been reported to cause acute nausea, anorexia, vomiting, and dizziness in the patient with uncomplicated malaria [7].

A malaria vaccine is a special need of society for the public health concern to attain successful malaria elimination. Unfortunately, to date, there is not a single licensed vaccine against malaria infection for the human use. Recent WHO strategies indicate towards the existence of licensed malaria vaccine for human use by 2030s. That vaccine will immunize against both *P. falciparum* and *P. vivax* and will have at least 75% immunization efficacy against malaria infection [8]. The most advanced vaccine nominee reported till date is RTS, S/AS01 but it is in phase III trial in African countries [9]. Apart from this, there are several vaccine candidates that are either in preclinical trial or different phases of a clinical trial but not licensed for the human use [9].

The complicated life cycle of malaria parasite divides the malaria vaccine into three categories namely pre-erythrocytic malaria vaccines, erythrocytic malaria vaccines and transmission-blocking vaccines. All three vaccines have their own importance in immunization, for example, pre-erythrocytic vaccine targets the *Plasmodium* sporozoite getting entry into the liver cell. While erythrocytic vaccine aims to stop the asexual reproduction of malaria parasite within the red blood cells; the last transmission-blocking vaccines target the sexual reproduction stage of the parasite occurs in the mosquito gut. This study was designed to contribute to the path of pre-erythrocytic and blood-stage malaria vaccine candidate design. As out of nine proteins sorted for the vaccine design, only one protein (CS) is pre-erythrocytic in nature and remaining belongs to the blood stage, the designed vaccine construct will primarily protect against the blood stage parasitic infection but also against pre-erythrocyte parasites. Here, we utilized an immunoinformatics approach to design the subunit

vaccine targeting *P. falciparum* membrane and secretory proteins. Subunit vaccine consists of B-cell, cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes along with suitable adjuvant and linkers. Among the physicochemical parameters, thermostability, amphipathic nature, immunogenicity and allergenic nature of vaccine protein was determined. To evaluate the binding affinity of vaccine protein against the TLR-4 immune receptor, the tertiary structure of subunit vaccine was developed followed by molecular docking and molecular dynamics simulation. Lastly, *in silico* cloning was performed to make a restriction clone of optimized codons corresponding vaccine protein into pET28a(+) vector. This study utilizes a combinatorial algorithm to design the subunit vaccine which may have the capability to prevent the pre-erythrocytic and blood-stage malaria parasite. Moreover, this study warrants the experimental validation of subunit vaccine construct to check its immunogenicity and safety behavior.

2. Results and discussion

Malaria is the deadliest parasitic infection covering half of the world's population, but most severely the African region, where highest mortality rate was reported. Increasing drug resistance and the complex life cycle of *P. falciparum* hinders the path of malaria vaccine candidate design. This study was designed to overcome such problems by developing multi-epitope subunit vaccine. Each and every step of this study was designed precisely to obtain an immunogenic and stable subunit vaccine construct which may have the ability to neutralize the futuristic malarial infection (Fig. 1). The different steps of vaccine candidate design and their results are mentioned below ranging from sequence retrieval to *in silico* cloning.

2.1. Sequence procurement and functional domain selection

Surface-associated parasitic proteins play a major role in host-parasite crosstalk which is advantageous for the parasite to coun-

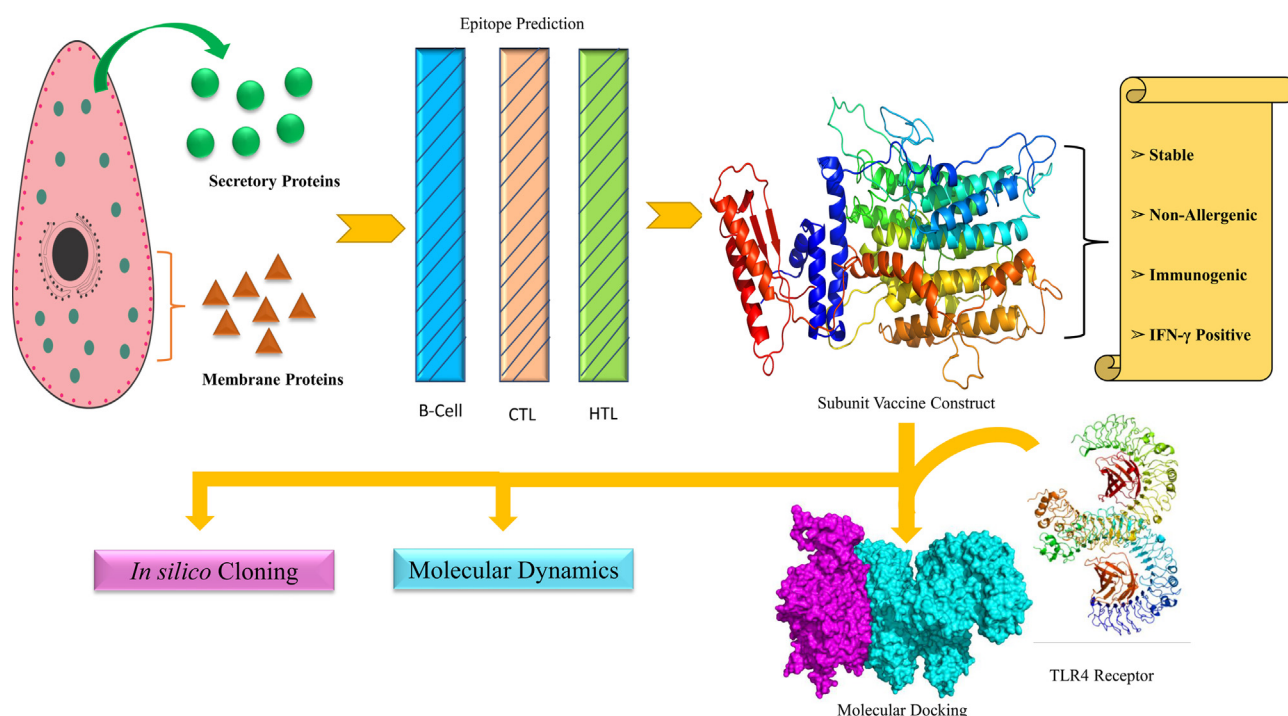


Fig. 1. Schematic representation of multi-epitope subunit vaccine candidate designing using B-cell, CTL and HTL epitopes followed by molecular docking, dynamics simulation and *in silico* cloning.

ter the host immune response. While, the parasitic secretory proteins, after releasing from pathogen into host microenvironment, leads to the symptomatic infectious condition. Three membrane proteins and six secretory proteins of *Plasmodium falciparum* were sorted through a literature survey and their respective FASTA sequence was retrieved from the National Center for Biotechnology Information (NCBI) database. Briefly, four extracellular secretory proteins of *P. falciparum* at asexual blood stage were sorted based on their immunomodulatory roles (Table 1). These secretory proteins are PfB190, PfN462, PfK22, and PfK139. The former two proteins PfB190 and PfN462 consisting of Sel1 repeats while PfK22, and PfK139 consisting of kinase and phosphatase catalytic domain, respectively. All these proteins were found to be exported beyond the infected RBC plasma membrane [10]. *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) helps in the cytoadherence of parasite-infected red blood cells to the host endothelial cells, in this manner hampering the microvasculature and leads to severe malaria. The intracellular conserved domain of PfEMP1 mainly ATS creates that mechanical connection using intracellular protein network [11]. Because of their crucial role in disease severity, ATS-1 and ATS-2 domains were selected as fifth and sixth proteins for the vaccine candidate design. Placental malaria is another form of malaria which targets the pregnant women regardless of previously acquired immunity. This severe pathological condition is mediated by the VAR2CSA, a *Plasmodium* antigen that interacts with the placental chondroitin sulfate-A (CSA). Therefore, VAR2CSA was selected as the seventh protein of this study to design the subunit vaccine, capable of blocking the interaction between pathogen and CSA via specific antibody response [12]. Last two proteins that were included in the vaccine designing are Circumsporozoite (CS) protein and Phospholipid Transfer Protein (PFA0210C). The former appears as a flexible rod-like protein on the surface of *P. falciparum*

sporozoite and helps in hepatocytes invasion. CS protein consists of three regions, to be specific, an N-terminal domain that binds to the heparan sulfate proteoglycans, NANP repeat region, and thrombospondin-like type-I repeat domain at the C-terminal [13]. The latter include PFA0210c, a phospholipid transfer protein with START domain, help in the transfer of phospholipid, fatty acid and ceramide between parasite membrane [14]. Conclusively, aforementioned nine proteins were selected for the subunit vaccine designing because of their crucial role in *Plasmodium* physiology to develop human malaria infection.

2.2. B-cell epitope prediction

Humoral immunity is a part of the adaptive limb of the immune system, which is mediated by antibodies and known to protect from blood-borne infection. Humoral immunity generates antibody mediated specific immune response against invading pathogenic organism, which is necessary for the pathogen clearance [15]. The ABCpred server was used to predict the B-cell epitopes for nine sorted proteins and total 308 epitopes were obtained. Out of them, single highest scoring epitope corresponding to each input protein was selected as final epitope and subjected to the subunit vaccine design. Highest positive prediction score of B-cell epitopes grants that the predicted epitope is actually an epitope (Table 2) [16]. Therefore, total nine B-cell epitopes were used in the vaccine candidate design along with suitable linkers and adjuvant.

2.3. Cytotoxic T-lymphocyte epitope prediction and immunogenicity evaluation

The human immune system consisting of a complex array of cells and molecules which preserve the cellular integrity by the

Table 1
Sorted *Plasmodium falciparum* (3D7) specific secretory and membrane proteins used for the subunit vaccine design.

S. No.	Protein name	Strain	Abbreviation	Accession number & source database	Functional domain sequence range
1	Conserved Plasmodium protein	Plasmodium falciparum 3D7	PfB0190	XP_001349553.1 (NCBI)	Glu1080-Gly1238
2	SEL-1 protein	Plasmodium falciparum 3D7	Pf14_0462	XP_001348636 (NCBI)	Ala382-Ala553
3	Protein kinase	Plasmodium falciparum 3D7	Pf11_0220	XP_001347891 (NCBI)	Lys201-Glu577
4	Protein tyrosine phosphatase	Plasmodium falciparum 3D7	Pf11_0139	XP_001347810 (NCBI)	Lys2-Met218
5	Erythrocyte membrane protein 1 (ATS1 domain)	Plasmodium falciparum 3D7	PfEMP1(ATS1)	CAD51362 (NCBI)	Tyrosine2448-Ile2858
6	Erythrocyte membrane protein 1 (ATS2 domain)	Plasmodium falciparum 3D7	PfEMP1(ATS2)	CAG24990 (NCBI)	Tyrosine2029-Serine2391
7	VAR2CSA	Plasmodium falciparum 3D7	VAR2CSA	N/A	Asparagine1-Aspartic acid640
8	StAR-related lipid transfer protein	Plasmodium falciparum 3D7	Phospholipid Transfer Protein	CAD49002 (NCBI)	Methionine1-Aspartic acid466
9	Circumsporozoite (CS) protein	Plasmodium falciparum 3D7	CS	Q7K740 (UNIPROT)	Phenylalanine20-Serine384

Table 2
B-cell epitope prediction for the input of *Plasmodium falciparum* secretory and membrane protein sequences.

Serial No.	Protein name	B-cell epitope	Rank	Start position	Score
1	PfB190	KFLKKEELVK	1	67	0.77
2	PfN462	ASKYNNSEAI	1	1	0.78
3	PfK22	NCINVAIKVL	1	40	0.79
4	PfK139	YIKEMKNYNV	1	84	0.79
5	PfEMP1 (ATS1)	SSDAPMNKFT	1	122	0.84
6	PfEMP1 (ATS2)	EWNQLKQDFI	1	143	0.79
7	VAR2CSA	DNEYTKDLEL	1	281	0.81
8	Circumsporozoite Protein	RTKSATLIND	1	142	0.79
9	Phospholipid Transfer Protein	NPNANPNANP	1	204	0.88

Table 3Cytotoxic T-lymphocyte specific epitope prediction for the input of *Plasmodium falciparum* secretory and membrane protein sequences.

S. No.	Protein name	CTL epitopes			A3 supertype	Score-1	Score-2	B7 supertype	Score-1	Score-2
		A2 supertype	Score-1	Score-2						
1	PfB190	AALGEIYYL	1.115	0.248	ALAALGEIY	0.7893	0.235	IVQNVGNVF	0.8954	0.047
2	PfN462	NLAFFYFEL	1.378	0.280	QHAFWFYK	0.7769	0.549	VPINYDKAM	1.3181	–0.105
3	PfK22	ILVQJINVL	0.975	0.170	KSPAIVHVGK	1.0212	0.250	SPIVYHRDL	1.4095	0.168
4	PfK139	GMDPIDAIV	1.158	0.257	IVFIRDRRK	1.0539	0.317	APVLASIVL	1.6802	0.021
5	PfEMP1 (ATS1)	ILDDIEDDI	0.818	0.285	GSAIFLIK	1.207	0.363	IPFGIALAL	1.4091	0.252
6	PfEMP1 (ATS2)	FAAISYFFL	1.101	0.149	LRVINIPKG	1.2209	0.257	RVLNSDVS	0.8324	–0.212
7	VAR2CSA	FLQEWVENF	1.017	0.398	KKYIWTAMK	0.8129	0.315	APWTTYTTY	1.0255	0.201
8	Circumsporozoite Protein	YLNKIQNSL	1.247	–0.301	KQENWYSLK	1.206	0.092	MPNDPNRNV	0.7835	0.043
9	Phospholipid Transfer Protein	KINVNNFIL	0.802	0.204	KIFGIFFNK	1.788	0.407	FPFNECTNL	0.9327	0.107

Score-1: NetCTL score; Score-2: IEDB immunogenicity score.

Table 4Helper T-lymphocyte specific epitope prediction for the input of *Plasmodium falciparum* secretory and membrane protein sequences.

HTL epitope prediction results						IFN- γ inducer		IL4 inducer	
Protein	Allele	HTL epitopes	Method	Percentile rank	Smm_ic50	Result	Score	Prediction	SVM Score
PfB190	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PfN462	DPA1*03:01/ DPB1*04:02	AISLLGYIYLLGLGV	Consensus (comb.lib./ smm/nn)	0.01	18	POSITIVE	1.351	Non IL4 inducer	0.04
PfK22	DRB4*01:01	FNIRINILVQIINVL	Consensus (comb.lib./ smm/nn)	0.1	28	POSITIVE	0.052	Non IL4 inducer	–0.12
PfK139	DRB1*13:02	DYNSDTIINNVIK	Consensus (smm/nn/ sturniolo)	0.1	8	POSITIVE	0.103	Non IL4 inducer	0.16
PfEMP1 (ATS1)	DRB1*11:01	TIPFGIALALGSAIF	Consensus (smm/nn/ sturniolo)	0.14	90	POSITIVE	0.646974	Non IL4 inducer	–0.12
PfEMP1 (ATS2)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
VAR2CSA	DRB1*13:02	SSDTLVVVNVPSPLG	Consensus (smm/nn/ sturniolo)	0.23	21	POSITIVE	0.154906	Non IL4 inducer	–0.32
Circumsporozoite Protein	DPA1*01:03/ DPB1*02:01	FILLSFVFSVVELIF	Consensus (comb.lib./ smm/nn)	0.01	31	POSITIVE	0.055759	Non IL4 inducer	0.16
Phospholipid Transfer Protein	DRB1*08:02	CGNGIQVRIKPGSAN	Consensus (smm/nn/ sturniolo)	0.69	455	POSITIVE	0.026075	Non IL4 inducer	0.15

elimination of foreign particles that found dangerous to the cellular functionality. As a part of cell-mediated immunity, cytotoxic T-lymphocyte recognizes MHC-I driven antigenic peptides and perform the cytotoxic function by perforin pathway. Here, MHC-I specific CTL epitopes were obtained using NetCTL 1.2 server and a variable number of epitopes were obtained for nine input protein sequences [17]. Three MHC-I supertypes namely A2-, A3-, and B7-supertypes were selected with the aim to cover 88.3% of the population, worldwide [3]. Overall 57, 108 and 31 number of CTL epitopes were obtained for nine *Plasmodium* proteins corresponding to the A2-, A3- and B7-supertypes, respectively. NetCTL epitopes output were subjected to IEDB immunogenicity prediction and based on the obtained immunogenicity score, topmost immunogenic CTL epitope was selected to be a part of subunit vaccine (Table 3). Therefore, total 27 CTL epitopes were selected for the vaccine candidate design, corresponding to three MHC class-I supertype.

2.4. Helper T-lymphocyte epitope prediction

Helper T-lymphocytes are the key player of an adaptive limb of the immune system, which regulates almost all adaptive immune responses. They perform diverse functions like activation of B-cell and macrophages to secrete antibodies and microbial destruction, respectively [18]. Also, it activates cytotoxic T-lymphocytes to kill infected target cells. MHC-II specific HTL epitopes were predicted using IEDB web server and a variable number of epitopes were obtained for the input proteins (Table 4). Those HTL epitopes were further sorted based on their lowest percentile rank of <1 to attain

maximum affinity and this way total 871 HTL epitopes were obtained for nine secretory/membrane proteins. All these epitopes were subjected to check their IFN- γ and IL-4 induction capacity.

2.5. Evaluation of HTL epitopes for IFN- γ and IL-4 induction capacity

Antigen presenting cells mediated naïve helper T-lymphocyte activation leads to the T-cell differentiation into either TH1 or TH2 helper cells. Both these effector helper T-cells having different functional aspects based on their cytokine secretion. Upon differentiation into TH1 cells, it releases IFN- γ and TNF- α cytokines lead to the parasitic clearance. While the absence of TH2 mediated IL-4 is necessary to control the chronic parasitemia [19]. Therefore, shorted HTL epitopes were subjected to IFN- γ and IL-4 prediction and found that except for PFB0190 and PfEMP1(ATS1), remaining seven proteins having HTL epitopes with its ability to induce IFN- γ secretion but not the IL-4 inducing ability (Table 4) [3]. This way, only seven HTL epitopes with their lowest percentile rank, lowest SMM IC₅₀ value, IFN- γ positive and IL-4 non-inducer were selected and used for the vaccine candidate design.

2.6. Allelic frequency and population coverage analysis

The allelic frequency of HTL epitopes was determined by the Allele Frequency Net Database and their global alleles distribution was obtained [20]. Most of the alleles have shown their frequency in Sub-Saharan Africa, the major endemic zone of malaria. Other regions include North America, Europe, North Africa, North East Asia, Oceania, South East Asia, Sub-Saharan Africa, and Western

Table 5
Population coverage analysis for the HTL epitope specific alleles.

Serial No.	HTL-epitopes	Alleles	Population coverage
1	AISLLGYIYLLGLGV	DPA1*03:01/ DPB1*04:02	Sub-Saharan Africa Europe North America South and Central America
2	FNIRINILVQIINVL	DRB4*01:01	North America
3	DYNSDTIINNVIKY	DRB1*13:02	North Africa Sub-Saharan Africa Western Asia
4	TIPFGIALALGSIAF	DRB1*11:01	Europe Oceania Western Asia
5	SSDTLVVVNPSPPLG	DRB1*13:02	North Africa Sub-Saharan Africa Western Asia
6	FILLSFVFSVVELIF	DPA1*01:03/ DPB1*02:01	Europe North America North East Asia Oceania South and Central America South Asia South East Asia Sub-Saharan Africa Western Asia
7	CGNGIQVRIKPGSAN	DRB1*08:02	Europe North Africa North America North East Asia Oceania South East Asia Sub-Saharan Africa Western Asia North America South and Central America

Asia (Table 5). Aforementioned allelic frequency data confirms the worldwide distribution of selected HTL epitopes, therefore subjected to the subunit vaccine candidate design.

2.7. Multi-epitope subunit vaccine candidate design

Multi-epitope subunit vaccine candidate was designed by combining adjuvant, B-cell epitopes, and CTL- and HTL epitopes as reported elsewhere [3,21]. An adjuvant is an immunogenic component that may potentiate the vaccine protein-mediated specific immune response by inducing the B-cell mediated antibody secretion and long-lasting immunity. As reported earlier, a TLR-4 agonist namely 50S ribosomal protein L7/L12 (130 amino acid) [22] was retrieved from UniProt database and used as an adjuvant. For the effective separation of each and every epitope within the human body, EAAAK, KK, AAY and GPGPG linkers were used. EAAAK linker was designed to effectively separate the domains of bifunctional proteins [23], therefore, utilized to join the adjuvant and B-cell epitopes. The bi-lysine (KK) linker was used between B-cell epitopes to preserve their independent immunogenic activities. Cathepsin-B targets the KK sequence of antigen before MHC-II mediated antigen presentation to the Th₀ cell, in the context of antigen processing [24]. AAY and GPGPG linkers were used to increase the recognition of subunit vaccine. This way, total one EAAAK linker, eight KK linkers, twenty-seven AAY linkers and seven GPGPG linkers were added to design the subunit vaccine. The final subunit vaccine consists of 704 amino acid residues having all its components.

2.8. Physico-chemical characterization of a subunit vaccine

The designed subunit vaccine must be stable and immunogenic in nature, therefore, the physicochemical properties of subunit vac-

cine were determined and found that the vaccine protein having a molecular weight of 76.5 kDa, and theoretical PI of 8.97. These two properties reflect the high immunogenic and basic nature of vaccine protein, respectively [21]. Its amino acid composition has shown the presence of 61 negatively charged and 73 positively charged residues. The instability index of vaccine protein was computed to be 26.48, classifying the protein as stable in nature. The aliphatic index represents the volume occupied by the aliphatic side chains and it's obtained value of 100.51 defines the thermostable nature of vaccine protein construct. The GRAVY represents the amphipathic nature of proteins where negative and positive value denoted the hydrophilic and hydrophobic nature of the amino acids side chain, respectively. The subunit vaccine has GRAVY value of 0.175, showing hydrophobic nature which necessitates the use of micelles for better interaction of vaccine protein within the polar environment of the body [3]. Overall, physicochemical evaluation grants the stable, immunogenic, thermostable, and basic nature of vaccine protein.

2.9. Antigenicity and allergenicity prediction

A subunit vaccine must be immunogenic and devoid of allergenicity. The vaccine protein was found to be probable antigenic with the probability score of 0.5247 [21]. While, on the allergenicity parameter, non-allergenic nature of vaccine protein was obtained. Both the characteristic output represents the safe and immunogenic behavior of the vaccine protein construct.

2.10. Tertiary structure development

For the functional characterization of subunit vaccine protein, its tertiary structure was developed by using RaptorX server. The server output has shown that 100% residues were modeled into three-dimensional shape with three functional domains. The best template used for the alignment and model generation was B-chain of the membrane domain of respiratory complex-I from *E. coli* (PDB ID-3RKO). The p-value and uGDT value were obtained as 1.19e-04 and 148, respectively. Smaller the p-value score, higher will be the model quality, thus, predicted model reflects good quality [25]. uGDT represents the un-normalized global distance test score and measures the absolute model quality. The uGDT score of >50 represents the good model quality (Fig. 2a). As a part of secondary structure component, the modeled structure consists of 57% helix, 4% beta sheet and 37% coils [20]. While the solvent accessibility result shows that 28% residues are exposed, while, 37% and 34% residues are medium and buried, respectively. The 3D model represents that all the B-cell epitopes occupied the surface position and will be available for the B-cell recognition (Supplementary Figure-1).

2.11. Tertiary structure refinement and validation

The Ramachandran plot assessment using RAMPAGE represents that higher the number of residue in the favored region, greater will be the model quality. When RaptorX output model was subjected to the RAMPAGE only 87% residues were present in the favored region [26]. Therefore, the vaccine 3D model was subjected to the GalaxyRefine server and surprisingly the number of residues in the favored region reached up to 90.9% while only 6.7% and 2.4% residues were present in allowed and outlier region, respectively. The refined model was subjected to the binding energy analysis (Fig. 2b).

2.12. Molecular docking and binding energy calculation

Binding energy between subunit vaccine and TLR-4 immune receptor (PDB ID: 4G8A) was analyzed by using ClusPro server

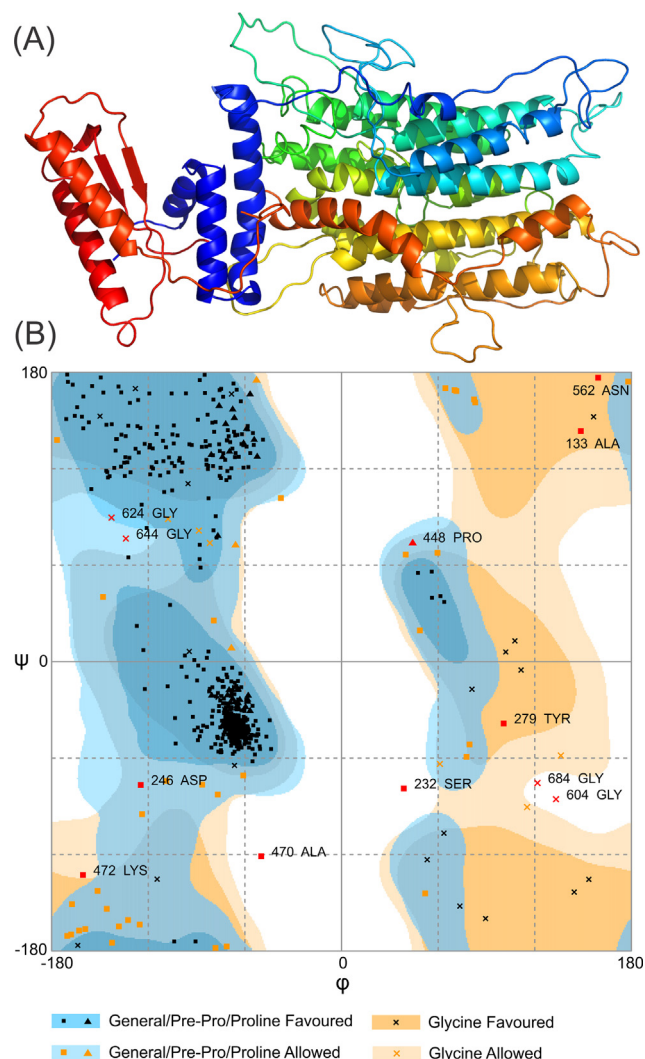


Fig. 2. Tertiary structure model development for the subunit vaccine construct and its validation (A) Multi-colored ribbon and coil structure represents the helix, sheets and coiled secondary structure component of the 3D model obtained for the vaccine protein, while (B) represents the Ramachandran plot shows the presence of amino acid residues in favored, allowed and outlier region.

and total 30 clusters were obtained [27]. All these clusters have their respective weightage score. The lowest energy weightage score was obtained for the cluster-6. Cluster-6 having 21 members within 9 Å C-alpha RMSD radius and its center and the lowest energy weightage score was −1114.2 and −1138.5, respectively. This way, cluster-6 was selected as the best-docked complex with lowest weightage score (Fig. 3).

2.13. Molecular dynamics simulation

The stability of the protein-protein cluster was determined by the molecular dynamics simulation study. GROMACS, a molecular dynamics package [20,28,29], was utilized to define the microscopic interaction between the protein-protein complex i.e., vaccine protein and TLR-4 receptor. Since TLR-4 is localized on the cell surface of immune cells; the pH of the system was set to 7.4 to mimic the neutral condition of the human body. The RMSD plot obtained for the backbone atom of docked complex reveals that it reaches up to equilibrium within 6–8 ns with the RMSD value of 0.55 nM and remains stable for rest of the time period. RMSF plot for the side chain of docked complex reveals that the complex is stable with

the RMSF value of ~0.3 nM (Fig. 4). All these calculations were performed at the ~300 K temperature and 1 bar pressure.

2.14. In silico cloning into a microbial expression vector

The end goal of this study was to clone the designed vaccine into an expression vector, so that, anyone can easily express it. But, before cloning the subunit vaccine protein was codon optimized and subjected to NEBcutter and found that none of the commercially available restriction sites are present in it [3]. Next, to complement the translation direction of pET28a(+) expression vector, the optimized codon sequence was reversed, and *Xho*I and *Bam*HI restriction sites were added to the N- and C-terminal of the optimized sequence. Lastly, SnapGene tool was used to make a restriction clone of vaccine protein into pET28a(+) vector. The restriction clone having a length of 7447 base pairs, among this the desired gene constitutes a length of 2112 base pairs (Fig. 5).

3. Conclusion

Malaria is the severe health issue for centuries which affects the people living in the tropical, subtropical and sub-Saharan Africa regions, worldwide. As per the recent WHO report, half of the world's population is under the risk of malaria. Lack of effective vaccine candidate and increasing resistance to Artemisinin-based combination therapy needs an effort to find a novel vaccine candidate to tackle this situation. This study, driven a combinatorial approach to devise multi-epitope subunit vaccine construct against *P. falciparum* mediated malaria infection. The designed vaccine consists of B-cell, CTL and HTL epitopes and also having suitable adjuvant and linkers. Further, physicochemical characterization, 3D modeling, Molecular docking and molecular dynamics simulation study was performed for the validation purposes. This study also employed *in silico* cloning to ensure future vaccine expression using microbial expression system. In future, the experimental evaluation of the designed subunit vaccine in an animal model may result in a novel and immunogenic vaccine tackling pre-erythrocytic and blood-stage malaria infection.

4. Methodology

4.1. Sequence retrieval and functional domain shorting

In order to find the different constituents of a subunit vaccine, an in-depth literature survey was performed to obtain the parasitic proteins having a major role in disease severity. This way, total nine secretory and membranous proteins of *Plasmodium falciparum* (3D7) were obtained. All these proteins having a different functional characteristic, therefore, following sequence retrieval, only the functional domain of sorted proteins was selected and subjected to the vaccine designing. The region belonging to the functional domain of sorted proteins, their accession number and source database is reported in Table 1. This way we obtained only nine secretory and membranous proteins and subjected to the subunit vaccine candidate design.

4.2. B-Cell epitope prediction

B-cell epitopes play an essential role in humoral immunity so as to design the multi-epitope subunit vaccine, nine secretory and membranous protein of *Plasmodium falciparum* (3D7) were subjected to the ABCpred (<http://crdd.osdd.net/raghava/abcpred/>) online server [30] to predict 10 amino acids long B-cell epitopes. This server retrieves B-cell epitopes from B-cell epitope database (BCIPEP), which consists of different epitope sets mainly

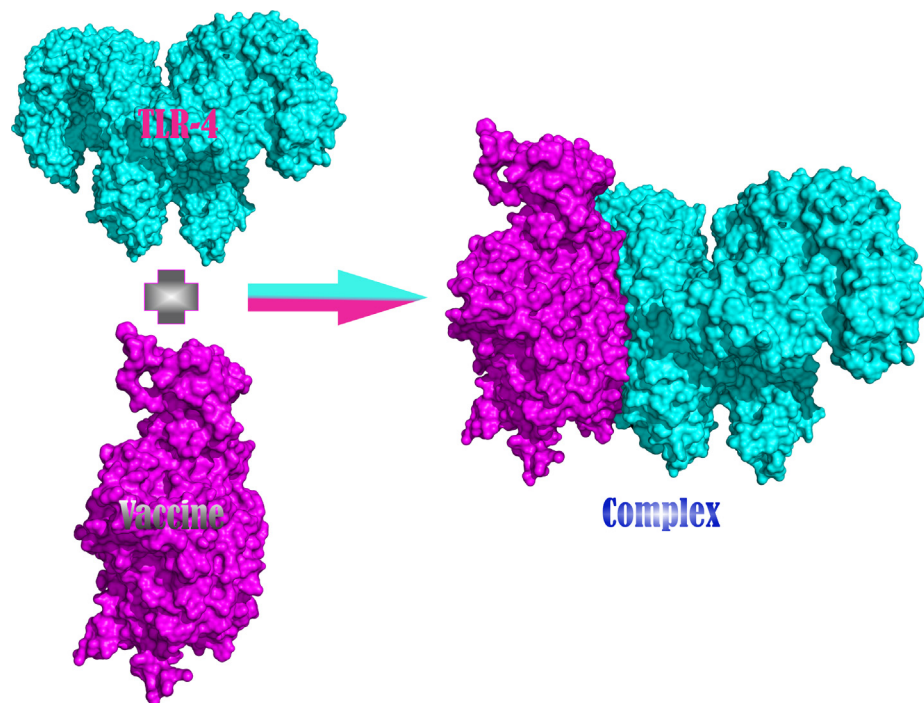


Fig. 3. Molecular docking output of vaccine protein (Magenta) and TLR-4 receptor (Cyan) obtained from ClusPro server represents the formation of stable complex (Magenta-cyan complex). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

continuous epitopes, immunodominant epitopes, and immunogenic epitopes. ABCpred utilizes artificial neural network (ANN) approach for the prediction of B-cell epitope. ANN is a collective approach to a mathematical representation which imitates some of the activities of the biological nervous system by its observations. ANN imitates the neuronal networks by its large number of processing elements that are interconnected as neurons.

4.3. Cytotoxic T-lymphocytes epitope prediction

Cytotoxic T-lymphocytes (CTL) epitope prediction is an essential step prior to design a multi-epitope subunit vaccine. CTL epitopes for nine secretory and membrane proteins were predicted by using NetCTL 1.2 online server (<http://www.cbs.dtu.dk/services/NetCTL/>) [31]. This server predicts the binding ability of the peptide epitope to MHC class-I molecule along with proteasomal cleavage at the C-terminal end and efficiency of TAP transporter. CTL epitope prediction by this server was restricted to only 12 MHC class I supertypes. While, we choose only three allelic supertypes (A2, A3 & B7) having 88.3% population coverage, worldwide. An artificial neural network system was used to predict MHC class I peptide binding, while prediction of proteasomal cleavage was performed by NetChop version at the C-terminal end of the CTL epitope. The weight matrix approach was used to predict the TAP transporter efficiency. These abovementioned three predictions were integrated together to achieve sensitivity and specificity values from different thresholds of the integrated scores. While performing CTL epitope prediction, epitopes having a score of 0.75 and above were selected. The selected epitopes were further examined for their immunogenic nature by using IEDB server (<http://tools.iedb.org/immunogenicity/>) [32], studies show that more immunogenic peptides act as a better T-cell epitope.

4.4. Helper T-lymphocytes epitopes prediction

Helper T-lymphocyte (HTL) epitopes prediction was performed by utilizing IEDB MHC-II server (<http://tools.iedb.org/mhcii/>) [33].

The HTL epitopes of 15mer length were predicted for all the shortlisted *Plasmodium* proteins. Prediction of HLA epitopes was performed for MHC class II reference alleles set covering 99.9% of the world's population (Supplementary Table-1). Predicted epitopes were sorted based on their percentile rank and IC_{50} values. By comparing IC_{50} values of the different predicted epitope with the random set of epitopes on SWISS-PROT database, a percentile rank is generated, which is inversely proportional to the affinity of the epitope. Epitopes showing highest affinity will have IC_{50} value < 50 nm, while epitopes with intermediate and lowest affinity will have IC_{50} values < 500 nm and < 5000 nm, respectively. Conclusively, lower the IC_{50} value higher will be the affinity of HTL epitope to MHC-II molecule [27].

4.5. IFN- γ inducing epitope prediction

IFN- γ (interferon gamma) belongs to the type-II interferon and plays a major role in adaptive and innate immunity. It basically activates the cascade of macrophages and natural killer cells during various protozoan, viral and bacterial infections [34]. As activated helper T-cells plays an important role in the secretion of IFN- γ [35], HTL epitopes having a percentile rank of <1, were used as an input sequence for the prediction of IFN- γ epitopes using IFNepitope server (<http://crdd.osdd.net/raghava/ifnepitope/>) [36]. This server utilizes two datasets which consist a total of 8188 IFN- γ inducing and 8888 IFN- γ non-inducing epitopes. The server uses motif and SVM (support vector machine) based hybrid approach for the prediction of IFN- γ epitope by constructing overlapping sequences [36].

4.6. IL-4 prediction for the HTL epitopes

IL-4 is known to be produced by the TH2 cell subtype of activated helper T-lymphocytes, having a role in exacerbating parasitemia during early infection stage. Higher expression of IL-4 also affects the TH1 cell subtype functioning by suppressing IFN- γ production [37]. Therefore, the HTL epitope must not be able to induce

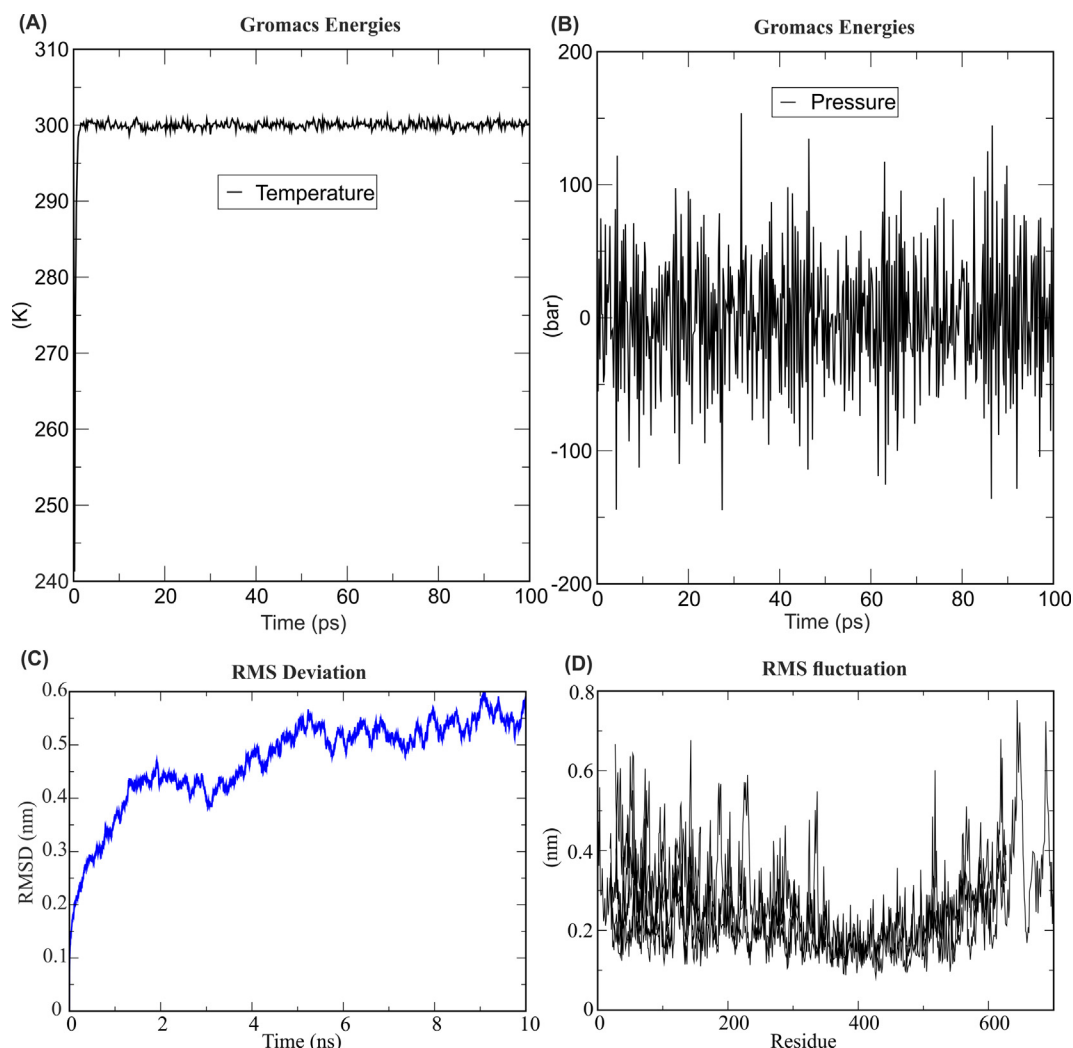


Fig. 4. Molecular dynamics simulation of protein-ligand complex (A-B) represents the temperature and pressure fluctuation curve during NVT and NPT ensemble (C-D) shows the RMS deviation for the amino acid backbone of the receptor-ligand complex and RMS fluctuation of amino acids side chain of the same.

the IL-4 secretion. All those HTL epitopes having a percentile rank of <1.0, were evaluated for their IL-4 induction ability using IL4Pred server (<http://crdd.osdd.net/raghava/il4pred/>) [38]. Only, IL-4 non-inducer HTL epitopes were selected to be a part of a sub-unit vaccine.

4.7. Allelic frequency and population coverage analysis

A subunit vaccine must be broadly applicable means it must have a broader population coverage in the endemic regions, world-wide. The population coverage of HTL epitopes were determined by using “The Allele Frequency Net Database” (<http://www.allele-frequencies.net/default.asp>) [39]. All the HTL epitopes having major population coverage in Sub-saharan Africa region were selected to be a part of vaccine protein.

4.8. Multi-epitope subunit vaccine candidate design

Subunit vaccine was designed by the linking of adjuvant, B-cell epitope, CTL epitope and HTL epitope along with the suitable linkers. TLRs have shown their important role to mediate the innate immune response against parasitic pathogens including malaria parasite. Among human TLRs, TLR-4 has shown their ability to detect the malaria parasite and induce the anti-parasitic immune

response. Recent studies have shown the role of TLR-4 agonist as a malarial vaccine adjuvant to improve the vaccine efficacy [40]. Therefore, TLR-4 agonist namely 50S ribosomal protein L7/L12 (130 amino acid) was selected as an adjuvant and used for the vaccine designing. This adjuvant will help in the interaction of vaccine protein to the TLR-4 receptor in the experimental system. Adjuvant and B-cell epitopes were joined together by EAAAK linker [23], Intra B-cell epitopes were linked by KK linker [41] while AAY and GPGPG linkers [3] were used to link the intra CTL and HTL epitopes, respectively. This way multi-epitope subunit vaccine construct was designed. Here, we used top one B-cell epitope and most immunogenic CTL epitopes corresponding to each super-type. While, only those HTL epitopes having lowest percentile rank, high to an intermediated binding affinity for the T-cell receptor, IFN- γ positive, IL-4 non-inducer and having wide allelic distribution were used for the vaccine candidate design.

4.9. Prediction of physicochemical properties

The physicochemical properties of designed vaccine was investigated by using ProtParam tool of ExPASy database server (<https://web.expasy.org/protparam/>) [42]. ProtParam utilizes amino acid sequence and pKa values of amino acids present in protein for the analysis of various physicochemical parameters. Prediction of

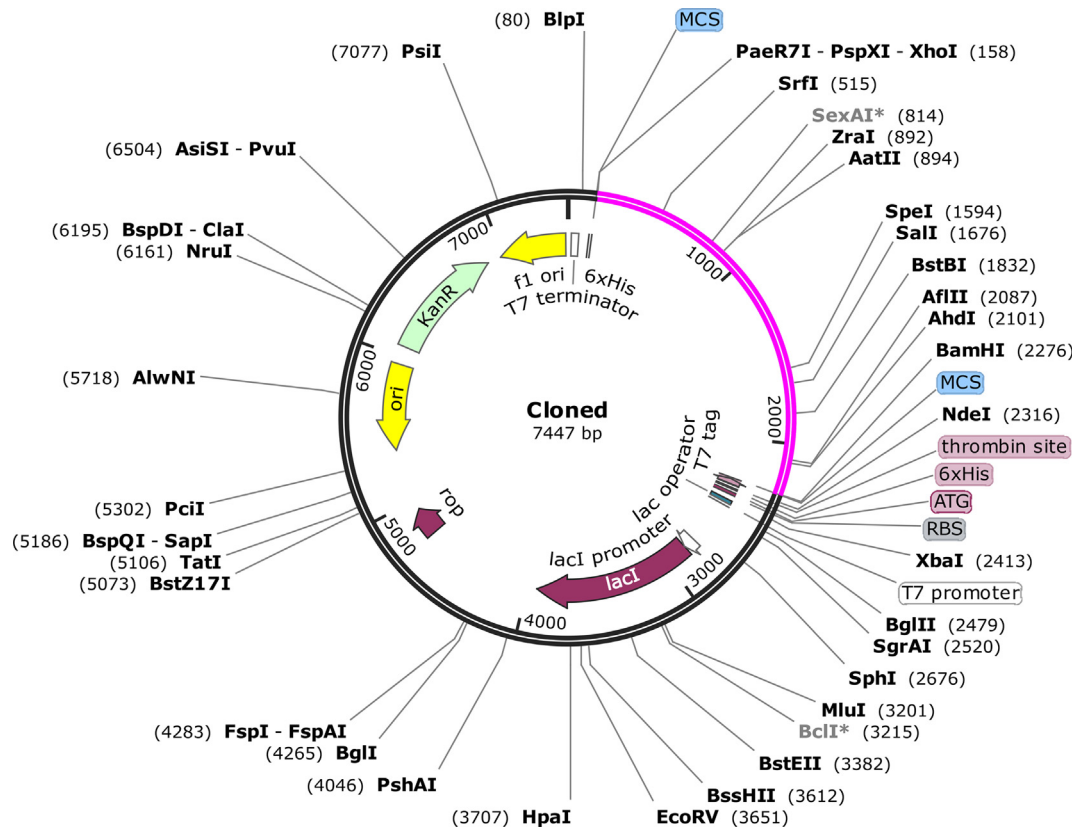


Fig. 5. *In silico* cloning map showing the insert of vaccine protein specific optimized codons (magenta) into the pET28a(+) expression vector. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in vivo half-life of the vaccine protein employed “N-end rule”, which states that *in vivo* half-life of the protein is related to N-terminal amino acid residue [43]. *In vitro* stability of vaccine protein was identified as instability index score. The instability score value of less than 40 reflects the stable nature of input protein or vice versa. Aliphatic index increases the thermostability of proteins, it is determined by the total relative volume of aliphatic side chains [44]. GRAVY was calculated by the total hydrophathy of all amino acids divided by the total number of amino acids present in the protein [45].

4.10. Antigenicity and allergenicity prediction

Vexigen V2.0 (<http://www.ddg-pharmfac.net/vaxijen/Vaxijen/Vaxijen.html>) [20] server was used for the evaluation of probable antigenicity of final vaccine construct; antigenicity prediction was based on an alignment-independent algorithm. It depicts that antigenicity of protein solely based on the physicochemical properties of input vaccine sequence. The accuracy of this server ranges from 70% to 89% which varies from organism to organism [46].

AllerTop (<http://www.ddg-pharmfac.net/AllerTOP/>) [3] server was used to predict the allergenic nature of final vaccine construct. Auto cross-covariance (ACC) transformation of protein sequence into equal length vector is the basis of allergen prediction. It is also an alignment-independent approach and utilizes chemical nature of amino acids for allergen recognition [47].

4.11. Tertiary structure prediction

The tertiary structure of multi-epitope vaccine subunit vaccine was predicted by using RaptorX online server (<http://raptorx.uchicago.edu/StructurePrediction/predict/>) [25]. It can predict tertiary structure of given protein sequence without any close homology

to structure in protein data bank (PDB). Tertiary structure prediction by RaptorX is multiple template-based approaches, basically improves modeling accuracy and quality of generated structure in comparison to the single template-based model prediction [48,49]. Job for the tertiary structure prediction was submitted in the form of amino acids sequence which was further analyzed by the server in the form of different small templates and finally predicts top modeled structures.

4.12. Tertiary structure refinement

Tertiary structure prediction was strictly relying on the template-based approach. To improve the quality of template-based protein model prediction, vaccine protein tertiary structure was refined by using the freely accessible online tool GalaxyRefine (<http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE>) [50]. It performs repackaging of the protein side chains and replaces them with their most probable rotamers, but, if any steric hindrance reported during replacement of rotamer then next probable rotamer was attached. It enhances the structural and functional stability of protein as refinement was initiated in protein core and extending towards the external surface. This way refinement improves both local and global structural quality of protein. GalaxyRefine uses two structural perturbation models. In model-1, structural perturbation was applied strictly to side chains only, but in model-2 structural perturbation was applied more aggressively to secondary structural elements. Tri-axial loop closure method was used to prevent breakage in the modeled structure during perturbation.

4.13. Tertiary structure validation

The tertiary structure of vaccine protein was validated by generating Ramachandran plot using RAMPAGE online

server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) [26]. Ramachandran plot gives us an idea about energetically favored, allowed and disallowed torsional angles; ψ (psi) against ϕ (phi) of amino acid residues on the basis of Van der Waal radius of their side chains.

4.14. Molecular docking of the vaccine candidate

Molecular docking is a computational approach applied for the prediction of receptor and ligand interaction at their stable conformation. The interaction between ligand and receptor depends on their binding affinity, higher the binding affinity more will be the stable interaction between ligand and receptor molecule. According to past studies on *Plasmodium* spp. one can analyze the involvement of TLR-4 in proinflammatory responses during malaria infection [51]. Thus, for molecular docking study TLR-4 (PDB ID: 4G8A) was treated as receptor molecule whereas refined tertiary model of the vaccine protein was used as ligand molecule. PDB file of both receptor and ligand was submitted in an online server ClusPro 2.0 (<https://cluspro.bu.edu/login.php>) [52] for protein-protein docking study to generate docked complexes with good desolvation free energy and electrostatic interaction.

4.15. Molecular dynamics simulation

Molecular dynamics (MD) simulation is a commonly accepted approach used for the structural and functional study of macromolecules at the microscopic level. Here we used GROningen MACHINE for Chemical Simulations (GROMACS v5.1.5) tool to perform a molecular dynamics simulation of receptor-ligand docked complex. Particle mesh Ewald summation method and GROMOS96 43A1 force field were used to perform MD simulation study. Bonded and non-bonded characterization of protein topology was analyzed by using Pdb2gm. Also, ligand topology was generated by using the PRODRG2 server. Sodium and chloride ions were added to perform system neutralization. Steepest descent algorithm approach was used to perform energy minimization step before simulation study to make assurance of no steric hindrance and presence of appropriate geometry of the system. NPT and NVT ensemble a two-step process, which is based on leapfrog algorithm, was used for system equilibration study. While performing system equilibration pressure and temperature of the system was raised to 1 bar and 300 K, respectively. Finally, molecular dynamics simulation was attained for a 10 ns time duration which ultimately determines RMSD and RMSF for protein backbone and side chains, respectively [53].

4.16. Codon optimization and in silico cloning

Codon optimization index is a newly accepted approach which ensures the relationship between codon usage and gene expression. Java Codon Adaptation Tool (JCAT) (<http://www.jcat.de/>) was used for codon optimization study. Here, the constructed vaccine sequence was used as input sequence followed by the selection of *Escherichia coli* K12 strain as host organism to ensure high expression rate. Further, as an additional option, avoid rho-independent transcription terminators, avoid prokaryotic ribosome binding sites and avoid cleavage sites of restriction enzymes were selected. JCAT gives output result in the form of cDNA sequence which was further analyzed in NEBcutter (<http://nc2.neb.com/NEBcutter2/>) for the presence of commercially available restriction enzyme cleavage sites. Finally, optimized vaccine sequence was reversed and at the N- and C-terminal site *Xho*I and *Bam*H1 restriction sites were added, respectively. *In silico* cloning was performed by using SnapGene restriction cloning tool and

the optimized codon sequence was inserted into pET28a(+) plasmid vector [17].

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Author contribution

Protocol designed by RKP, TKB, VKP.
Methodology performed by RKP, MA, RO, VKP.
The manuscript was written by RKP, MA, VKP.

Competing financial interests

The Authors have declared no competing interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vaccine.2018.05.082>.

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Development of multi-epitope driven subunit vaccine in secretory and membrane protein of *Plasmodium falciparum* to convey protection against malaria infection

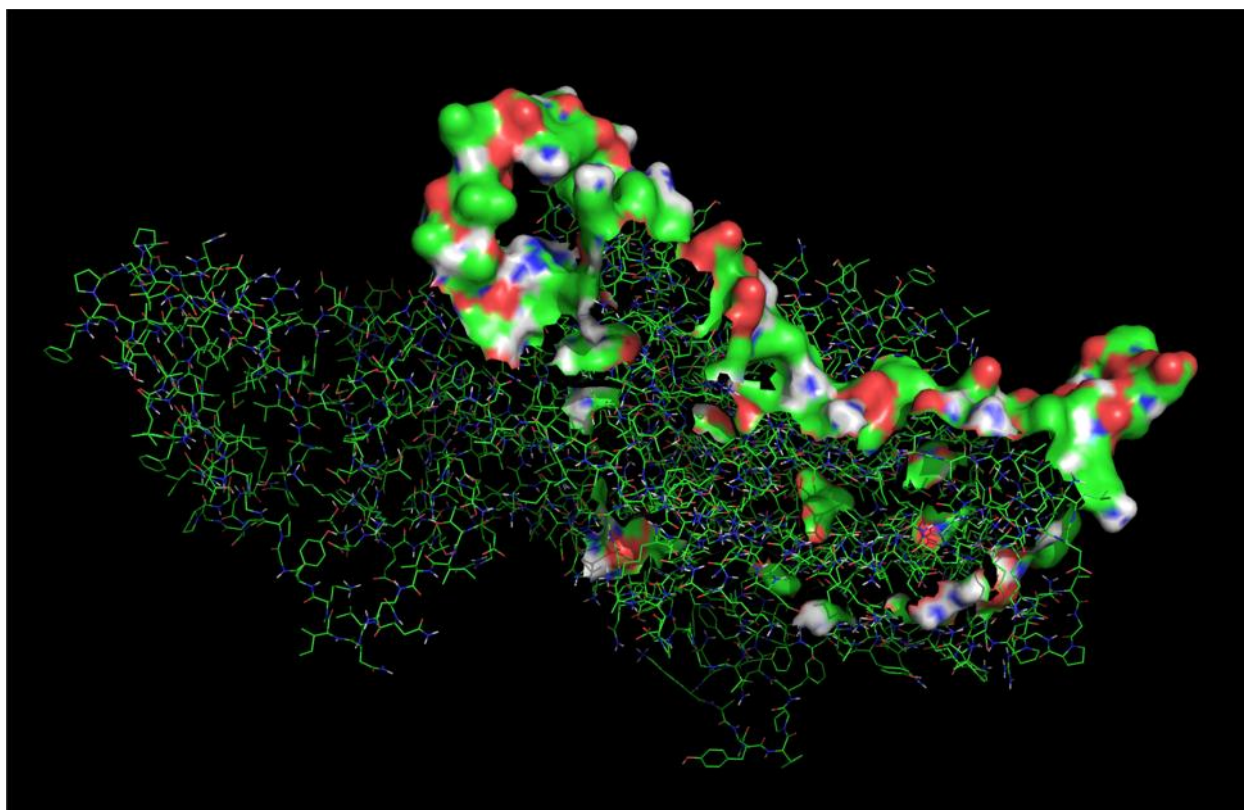
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Supplementary Table 1: List of reference alleles set covering 99.9% of the worldwide population

Serial No	Reference allele set
1	HLA-DRB1*01:01
2	HLA-DRB1*03:01
3	HLA-DRB1*04:01
4	HLA-DRB1*04:05
5	HLA-DRB1*07:01
6	HLA-DRB1*08:02
7	HLA-DRB1*09:01
8	HLA-DRB1*11:01
9	HLA-DRB1*12:01
10	HLA-DRB1*13:02
11	HLA-DRB1*15:01
12	HLA-DRB3*01:01
13	HLA-DRB3*02:02
14	HLA-DRB4*01:01
15	HLA-DRB5*01:01
16	HLA-DQA1*05:01/DQB1*02:01
17	HLA-DQA1*05:01/DQB1*03:01
18	HLA-DQA1*03:01/DQB1*03:02
19	HLA-DQA1*04:01/DQB1*04:02
20	HLA-DQA1*01:01/DQB1*05:01
21	HLA-DQA1*01:02/DQB1*06:02
22	HLA-DPA1*02:01/DPB1*01:01
23	HLA-DPA1*01:03/DPB1*02:01
24	HLA-DPA1*01/DPB1*04:01
25	HLA-DPA1*03:01/DPB1*04:02
26	HLA-DPA1*02:01/DPB1*05:01
27	HLA-DPA1*02:01/DPB1*14:01



Supplementary Figure 1: B-cell epitope localization to the subunit vaccine protein