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A Multi-Epitope Vaccine Designed Against Blood-Stage of Malaria: An Immunoinformatic And Structural Approach

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Research Article

Keywords: Malaria, Plasmodiumis, morbidity, physicochemical, bioinformatics

Posted Date: December 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1195734/v1

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Abstract

Malaria is a complex disease caused by genus Plasmodiumis parasites and is the leading cause of morbidity and mortality worldwide. The most severe form of malaria disease is caused by Plasmodium falciparum. A combination of different approaches is needed to control malaria, and on the other hand, resistance to first-line drugs and insecticides makes the need for an effective vaccine more mandatory than ever. Erythrocyte parasites have the most clinical symptoms, so designing the potential vaccine for this stage of infection could be very helpful. In this research, we used various bioinformatics tools to design an effective antibody-inducing multi-epitope vaccine against the blood-stage of malaria infection. For this purpose, we selected the malaria PfGARP protein as the target here. The predicted B and HTL epitopes and flagellin molecule (as an adjuvant) were connected with suitable linkers and the final construct vaccine was designed. The various properties of this construct, including physicochemical properties, 3D structures, molecular docking, molecular simulations, and in silico cloning were then carried out. Based on preliminary findings, our designed fusion construct could be proposed as a novel potential vaccine candidate against Malaria. However, in vitro and in vivo studies are essential for further validation.

Introduction

Malaria is a devastating disease caused by a unicellular protozoan parasite transmitted to humans through the bites of infected mosquitoes [1]. A massive number of people worldwide afflict this disease. The World Health Organization (WHO) has reported that malaria was responsible for about 409,000 deaths globally in the year 2019 [2, 3]. Five parasite species cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. Of these, people who are heavily exposed to the bites of mosquitoes infected with *P. falciparum* cause a more severe form of malaria disease, and they have a higher risk of death and worst of all; *falciparum* has developed resistance to nearly all of the currently available antimalarial drugs [4, 5]. The high level of *falciparum* resistance to chloroquine (CQ) and then to sulfadoxine-pyrimethamine (SP) has created the need for newer and more effective treatments such as vaccines [6]. Genetic and antigenic diversity are major obstacles hindering the development of effective anti-malaria vaccines. For these reasons, many studies have focused on conserved regions or epitopes common between vast ranges of plasmodium strains [7]. *P. falciparum* glutamic acid–rich protein (PfGARP) is an 80 kDa antigen expressed on the surface of infected erythrocytes with *P. falciparum* [8]. Remarkably, the PfGARP gene is absent in other species of human malaria parasites and is expressed in early-to-late-trophozoite-stages [9]. PfGARP localizes to the periphery of infected erythrocytes, and the whole-genome sequence data have indicated that PfGARP is conserved, despite its apparent immunogenicity, and no deletions appear to occur [10]. Studies demonstrated that PfGARP plays a functional role in enhancing the adhesive properties of human erythrocytes that are unique to *P. falciparum*–mediated pathogenesis [11]. Generally, the parasite's adhesive properties, a strategy used to avoid immune surveillance and splenic clearance of infected RBCs, are major factors contributing to virulence [12, 13]. Interestingly, PfGARP has recently been

identified as the target of protective antibodies. In the trophozoite stage, antibodies to PfGARP disrupted mitochondrial membrane integrity and induced programmed cell death of infected RBCs. Then it eventually leads to a reduction in acute malaria. Also, vaccination with PfGARP protects monkeys from being confronted with the malaria parasite [14, 15]. Immunity against malaria parasites is complex and fundamentally stage- and species-dependent [16]. Many attempts have been made to develop a vaccine against malaria, but its complex multistage life cycle exhibits special challenges for vaccine development [17]. RTS, S, or Mosquirix is the world's first malaria vaccine proved to provide partial malaria protection in young children. This vaccination avoids 39% of malaria cases and 30% of severe malaria cases. Despite considerable efforts, there are certain drawbacks. It is less effective, requires four doses, and gives less protection after a few months [18, 19]. Nevertheless, erythrocytic-stage vaccines, because the pathogenesis of malarial disease results from blood-stage infection, will improve the eradication of the disease [20]. According to the abovementioned evidence, the PFGARP antigen reasonably turns infected RBCs into a very promising target for vaccine development, especially in light of the fact that the parasite in the erythrocyte stage is directly exposed to the host humoral immune response. Incorporating some adjuvants in vaccines such as Toll-like receptor (TLR) agonists is promising molecular with high immunogenicity [21]. TLRs are present in macrophages, dendritic cells (DCs), and specific epithelial cells to provoke phagocytosis and develop effective immune responses [22]. Flagellin (fliC) inherently attaches to toll-like receptor 5 (TLR5) as a natural agonist. FliC protein is a highly conserved bacterial protein; it can stimulate the humoral and cellular immune system by producing antibodies, cytokines, and interleukins that prevent the parasite entry in the pre-erythrocytic stage of malaria [23–25]. Therefore, the present study aimed to design a multi-epitope vaccine by utilizing bioinformatics methods based on PfGARP epitopes to elicit a humoral immune response against blood-stage of malaria infection.

Results

Linear B-cell epitope prediction. Epitopes with 16 amino acid length in (PfGARP) protein were selected for use in the final construct of the vaccine. Five B-cell epitopes that had higher scores are shown in Table 1.

Table1. List of the five B-cell epitopes from PfGARP protein by ABCpred Server.

Rank	Sequence	Start Position	Score
1	HGEENLYEEMVSEINN	162	0.93
2	HETSNDTKDNDKENIS	204	0.91
3	AEEDDDDAEEDDDDAE	596	0.89
4	CEEQHITVESRPLSQP	441	0.89
5	GCGIISSVHETSNDTK	186	0.89

MHC class II peptide-binding prediction (CD4+ T cell epitope). MHC class II peptide binding epitopes with the highest score for human class II alleles were predicted using the IEDB MHC-II server. Then, top predicted epitopes with high scores were only chosen for generating the final vaccine construct (Table 2).

Table 2
List of the top predicted MHC class II peptide from (PfGARP) protein, by IEDB MHC-II server.

Allele	HTL epitopes	Method	Percentile rank	Smm_ic50
2-16 HLA-DRB1*01:03	FSNGLLKNQNILNKS	NetMHCIIpan	20.00	N/A
234-248 HLA-DRB1*01:05	TLDKKERKQKEKEMK	NetMHCIIpan	100.00	N/A
91-105 HLA-DRB1*01:03	SVDKKKDKKEKKHKK	Consensus (comb.lib./smm/nn	100.00	N/A
401-415 HLA-DRB1*01:03	DKGKHKKAKKEKVKK	NetMHCIIpan	82.00	N/A
12-26 HLA-DPA1*01/DPB1*04:01	ILNKSFDSITGRLLN	Consensus (comb.lib./smm)	11.35	1257.00

Design and construction of multi-epitope vaccine candidate sequence. In this step, selected high scored and affinity B-cell epitopes (a total of 5 linear B-cell epitopes) and MHC class II peptide-binding epitopes (a total of 5 epitopes) were joined together using GPGPG and AYY linker to generate the candidate sequence of a vaccine, respectively. Flagellin D0/D1 domains were added upstream of the above sequences as an adjuvant using the EAAAK linker to enhance the immunogenicity of vaccines. As well, six histidine residues codons were placed downstream of the sequence of the multi-epitope construct as a his-tag for the future purifying process. Therefore, the vaccine constructs with 414 amino acid residues were designed (Fig. 1).

Physiochemical parameters and solubility prediction. The final protein's pI and Mw value predictions were 5.01 and 46 kDa, respectively. The half-life was considered to be 30 hours in mammalian reticulocytes (in vitro), 20 hours in yeast (in vivo), and 10 hours in E. coli (in vivo). Aliphatic index and GRAVY were estimated at 65.12 and -1.110, respectively. The instability index (II) was calculated with a 40.11 score. Upon expression, the constructed vaccine protein was soluble in the *E. coli* host with a solubility score of 0.759117.

Antigenicity and allergenicity of the vaccine construct. The ANTIGENpro and VaxiJen 2.0 servers calculated the whole vaccine sequence antigenicity with the adjuvant sequence values of 0.92630 and 0.6976 with a threshold of 0.5 for the parasite model, respectively. The allergenicity of the vaccine was predicted using the AlgPred and AllerTOP v. two servers and resulted in non-allergenic.

Secondary structure prediction. The predicted secondary structure using PSIPRED data was shown 60% alpha-helix, 39% beta-strand, and 0.4% coil in the final protein vaccine (Fig. 2). This secondary structure was used for refining the protein tertiary structure.

3D structure homology modeling and validation. SWISS-MODEL, Phyre2, and I-TASSER are the servers used for 3D structure modeling. In this study, c2ch7A_.11 model was selected from the Phyre2 server (Fig. 3) as the best model according to prior validation analysis. In the chosen model, analysis with PROCHECK's Ramachandran plot shown 96%, 4% of residues are placed in favored regions and allowed, respectively (Fig. 4A) the ProSA z-score and ERRAT were respectively -2.84 and 98.06 (Fig. 4B) and (Fig. 4C).

Conformational (Discontinuous) B-cell epitopes prediction. In the 3D model of the final designed vaccine construct, residues with a value of 0.7 or higher were identified as conformational epitopes (Table 3). Also, discontinuous epitopes predicted in the 3D structure of the final multi-epitope construct are shown (Fig. 5).

Table 3
Predicted conformational epitopes of the final multi-epitope construct by the ElliPro server.

No.	Residues	Number of residues	Score
1	E143, :A144, :K145, :E146, :K147, :D148, :E149, :D150, :T151, :D152, :E153	11	0.934
2	:S8, :L9, :L10, :T11, :Q12, :N13, :N14, :L15, :N16, :K17, :S18, :Q19, :S20, :A21, :L22, :G23, :T24, :A25, :I26, :E27, :R28, :L29, :S30, :S31, :G32,:R34, :I35, :A38, :D41, :E257,:E258, :D260, :D261, :D262, :A263, :E264, :G265, :P266, :G267, :P268, :G269, :C270, :E271, :E272, :Q273, :H274, :I275, :T276, :V277, :E278, :S279	51	0.773
3	:E119, :R122, :G125, :Q126, :K133, :V134, :L135, :A136, :Q137, :D138, :N139, :T140, :E141, :E142, :E154, :A155, :A156, :V157, :E158, :E159, :E160, :D161, :E162, :E163, :K164, :K165, :P166, :K167, :T168, :K169, :V171, :E172, :V175, :W176	34	0.749

Molecular docking of designed vaccine with TLR5. Molecular docking between the final construct model and TLR5 was done using the ClusPro server. The best possible docked complex with the highest binding affinity and total free energy (-1353.6) was selected. The best docking between the final construct model and TLR5 complex is shown in Figure 6. The PyMOL 1.1eval server performs visualization and analysis of the docked complex in the next step.

Molecular Dynamics Simulation. The molecular dynamics of our docked complex were done by CABS-Flex 2.0. The flexibility of the final docked complex has been analyzed at a 1.4°C temperature and 50 cycles' simulation for the 10 ns. The CABS-Flex 2.0 software provides ten alternative models based on parameters such as optimum free energy, structural heterogeneity, and extremely stable configuration. We selected the first model as a stable protein vaccine structure based on the above parameters. The selected docked complex had the highest fluctuations levels in the residues (in chain A) at positions 219

and 617 in values of 4.58 Å and 3.57 Å, respectively (Fig. 7A). The stable protein vaccine structure using Cabs-Flex 2.0 (Fig. 7B).

In silico cloning and codon optimization of vaccine construct. For efficient vaccine protein expression in the E. coli host, nucleotide vaccine sequence was reverse translated and codon-optimized using the GenScript tool. The value of some parameters such as GC content and codon adaptive index (CAI) for the optimized vaccine nucleotide sequence were respectively 46.32% and 0.85. These parameters were considered a good adaptation because they provided a high rate of expression in *E. coli* K12. Finally, using *NcoI* and *XhoI* restriction sites, 1242 nucleotides as the optimized sequence was cloned into the pET28a vector. The 6xHis-tag at the C-terminal of the multi-epitope protein vaccine was placed for the purification process (Fig. 8).

Materials And Methods

Retrieving of PfGARP and flagellin protein sequences. In this research, *P. falciparum* glutamic acid–rich protein (PfGARP) (P13816) (UniProt database at http://www.uniprot.org/.) was selected as parasite antigen and then was evaluated to in silico study for potential B cell and MHC class II peptide binding prediction. A sequence of flagellin (fliC) protein (UniProt ID: Q8IFM5) (an agonist of TLR5) from Salmonella typhimurium was chosen as an adjuvant to enhance the efficacy of the vaccine.

Immuno-informatics analysis

B-cell epitope prediction. B-cell epitopes are potential antigens that are recognized by surface receptors of B-cell lymphocytes. The identification of these epitopes leads to producing a specific humoral response. Therefore, B-cell epitopes play a crucial role in vaccine efficiency [26]. In this step, the ABCpred (http://crdd.osdd.net/raghava/abcpred/) server as a sequence-based tool was used for predicting flexible length linear B-cell epitopes in PfGARP protein. This online web server uses different kernel methods to permit users to select the prediction method.

MHC class II peptide-binding epitope (CD4+ T cell epitope) prediction. One of the important purposes of immunology studies and vaccine development is identifying peptide epitopes restricted to MHC class II. The PfGARP protein amino acid sequence was submitted to the IEDB MHCII web server at http://tools.iedb.org/mhcii/ to predict MHC class II peptide binding epitopes. This server uses an artificial neural networks algorithm and classifies strong and weak binders to each HLA (HLADR, HLA-DP, and HLA-DQ) allele based on the affinity of peptides (in nM) and their % rank. In a way, a peptide with a lower percentile rank has a greater affinity. In this study, epitopes with 15-mer length related to HLA-DR supertype alleles were selected.

Multi-epitope vaccine candidate construction. To generate the multi-epitope vaccine construct, the selected candidate epitopes, including 5 linear B-cell epitopes linked to each other using GPGPG and a total 5 high-affinity HTLs epitopes linked together AYY linkers, respectively. As an adjuvant, the fliC sequence was bound in the N-terminal region of the vaccine construct with the EAAAK linker.

Prediction of antigenicity of the designed vaccine construct. Two servers, including Vaxijen 2.0 and ANTIGENpro, were used to study the antigenicity of the above vaccine construct. Vaxijen 2.0 is a free online available server at the address of http://www.jenner.ac.uk/VaxiJen. The basis of this server is the auto- and cross-covariance (ACC) transformation of sequences of target protein into the same amino acid sequence vectors. The VaxiJen v2.0 prediction method is alignment-free with the threshold value of 0.5 based on various protein physicochemical characteristics. Also, ANTIGENpro is the alignment-free, pathogen-independent, and sequence-based predictor available at http://scratch.proteomics.ics.uci.edu. This server applies microarray data of protein antigenicity and shows an antigenicity index. The ANTIGENpro server's accuracy, based on combined dataset and cross-validation experiments, was assessed about 76%.

Allergenicity prediction of designed vaccine construct. The AllerTOP V2.0 and Algpred servers were used to predict the allergenicity ability of the vaccine construct. AllerTOP V2.0 server is available in http://www.ddg-pharmfac.net/AllerTOP and based on various methods such as auto- and cross-covariance transformation, k nearest neighbors (kNN) machine learning, and amino acid E-descriptors for classifying allergens. At 5-fold cross-validation, the accuracy of this server has been presented 85.3%. Algpred server is also available at http://webs.iiitd.edu.in/raghava/algpred and predicts allergenicity based on known epitope similarity in all regions of the target proteins with high accuracy. In this study, among various integrated approaches in Algpred, the hybrid approach with a combined method (MAST + ARPs BLAST + IgE epitope + SVMc) was chosen for allergen prediction.

Structural analysis

Different physicochemical properties and solubility analyses of vaccine construct. Different physicochemical properties of vaccine construct such as theoretical pI, stability profiling, molecular weight, amino acid composition, half-life, instability Index, aliphatic Index, and Average grand hydropathy were evaluated using ProtParam online server at the http://web.expasy.org/protparam/. SOLpro is scratch protein predictor analyzed solubility ability of vaccine construct with corresponding probability (≥ 0.5) and available at http://scratch.proteomics.ics.uci.edu.

Secondary structure prediction of vaccine construct. The vaccine constructs secondary structure was predicted with PSIPRED web free available online server at the http://bioinf.cs.ucl.ac.uk/psipred/. The basis of this server is a highly accurate primary amino acid sequence that uses a stringent cross-validation approach to analyze the efficiency of the method. PSIPRED 3.2 servers can evaluate the obtaining output from PSI-BLAST (Position-Specific Iterated-BLAST) by combining two feed-forward neural networks.

Tertiary structure prediction. Various servers, including SWISS-MODEL, I-TASSER, and Phyre2 were used for 3-dimensional structure modeling. Finally, based on the primary data, Phyre2 tool was chosen for 3D structure modeling of the final vaccine construct. Phyre2 (at http://www.sbg.bio.ic.ac.uk/phyre2) builds 3D models by advanced remote homology detection methods for a user’s protein sequence.

3D structure validation. After predicting 3D models, their performance and accuracy were checked with model validation tools. Some tools such as ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php), ERRAT (http://nihserver.mbi.ucla.edu/ ERRATv2/) and PROCHECK's Ramachandran plot analysis (https://servicesn.mbi.ucla.edu/PROCHECK/) existed for model validation. The ProSA program is frequently utilized for the validation of 3D protein structures. This program was obtained its easy-to-use interface from ProSA-web. ProSA-web calculates the overall quality of the specific input 3D structures and demonstrates them as the z-score of experimentally PDB identified structures in a plot. ERRAT program works based on the statistics of non-bonded interaction between atoms in the described structure and is applied to verify crystallography identified protein structures. PROCHECK's Ramachandran plot program evaluates the protein structure stereochemical quality by analyzing overall structure geometry and residue-by-residue geometry.

Discontinuous B-cell epitope prediction in final MEV construct. Conformational epitopes play a major role in the antigen-antibody response [27]. The ElliPro server at the address http://tools.iedb.org/ellipro/ was employed on the validated 3D structure to predict conformational B-cell epitopes. ElliPro, a new web tool, predicts antibody epitopes according to protein structure geometrical properties and assigns a score as a Protrusion Index (PI) value averaged to each output epitope over epitope residues. In the ElliPro method, the shape of the 3D protein structure is estimated by several ellipsoids. The ellipsoids with PI = 0.9 mean 90% and 10% of the protein residues are inside and outside the ellipsoid, respectively.

Molecular docking of designed vaccine with TLR-5 receptor. In this step, ClusPro 2.0 as a web-based server at the address https://cluspro.org was used to evaluate interaction patterns between the final vaccine construct and TLR5 receptor (PDB ID: 3J0A). This server performs the direct docking in 3 computational steps: i) rigid-body docking, ii) clustering of the structures based on the 1000 lowest RMSD value, iii) energy minimization and steric clashes removal.

Molecular Dynamics (MD) Simulations. To comprehend the biological functions of protein structures, the recognition of flexible regions of protein structures is crucial. As an efficient alternative approach to conventional all-atom molecular dynamics (MD), CABS-flex has been developed for predicting protein structure fluctuations from a single protein model. Therefore, molecular dynamics simulations were done using the CABS Flex 2.0 server in this work. The protein PDB code or the protein structure PDB format is only input data in this web server. The study applied the chosen docked TLR5-vaccine construct complex as the input data for a quick flexibility simulation. Besides protein flexibility, the contact map and root-mean-square fluctuations (RMSFs) of the atoms in the protein complex is measured in this server. All amino acid residues RMSF simulation in input protein is calculated in a nanosecond time by the CABS-flex server. The highest and lowest RMSF values indicate more flexibility and the limited motion of the system during the simulation process, respectively.

In silico cloning and codon optimization of MEV construct. To express the vaccine construct in an appropriate expression vector, the GenScript Rare Codon Analysis in the address https://www.genscript.com/tools/rare-codon-analysis was applied for the study of the vaccine sequence

codon optimization and reverse translation. The *E. coli* was selected as a host to express the vaccine sequence in the study. Two output indexes for high-level protein expression, including the percentage GC content and the codon adaptation index (CAI), were analyzed. The optimal CAI score is at 1.0, although the score > 0.8 is also good. The optimal GC content can vary between 30–70%. These values usually are desired effects on transcriptional and translational efficiencies. Also, for cloning of final vaccine construct in pET-28a (+) vector, two Identification and cutting sites for *NcoI* and *XhoI* restriction site were added in N and C-terminals of cloned sequence, respectively. Then the cloning of the final vaccine constructs in the pET-28a (+) vector was performed in the SnapGene tool.

Discussion

P. falciparum, the most lethal form of malaria, is the leading cause of infection-related death. Malaria mortality is a global health and economic concern, despite effective treatments. This is related to an increase in P. falciparum's resistance to first-line antimalarial medicines [28]. As a result, a successful vaccination would be a helpful tool for controlling or even eradicating malaria. The sporozoite stage targets two of the most promising malaria vaccine candidates, although sporozoites are only exposed to the host's immune system for a few minutes. Each cycle in the RBC lasts 2-3 days, but the trophozoite remains undetectable since it is concealed within the cell. The trophozoite exports antigens to the RBC membrane's outer surface and these antigens could be identified [29]. Plasmodium conducts numerous cycles of RBC infection, giving infected people time to develop antibodies that identify these proteins. They are temporarily exposed to the host immune response at this phase, making them accessible to antibodies that target their antigens on the surface [30, 31]. Because the parasite is directly exposed to the host humoral immune response during the erythrocytic stage, making it an appealing vaccine target. The PfGARP gene is only produced during the early stages of the parasite life cycle, and immunolocalization experiments revealed that PfGARP is found on the outer surface of trophozoite-infected RBC. In the absence of immune effector cells or complements, Anti-PfGARP inhibits parasite growth by blocking and destroying trophozoite-infected RBCs [14]. Vaccine development and production are often lengthy, challenging, and costly processes. Researchers can benefit from *in silico* methodologies for the rational design of vaccines, particularly for pathogens, because of advancements in molecular immunology and the identification of immune-dominant epitopes, and progress in bioinformatics and immune informatics approaches [32]. Accordingly, in the present study, we use bioinformatics methods to design a potential candidate epitope-based vaccine against malaria based on the PfGARP protein, one of the major antigenic proteins of blood-stage malaria [8]. For this purpose, we first selected PfGARP as the target antigenic protein for further analysis. T helper cells, which are necessary for almost all adaptive immune responses, are the principal mediators of cell-mediated immunity. They aid in activating B cells, which release antibodies and macrophages, and cytotoxic T lymphocytes, which kill infected target cells [33]. Additionally, in the following section, we predicted probable B-cell and T helper cell epitopes from the PfGARP protein in order to develop a multi-epitope vaccine (MEV) capable of inducing a humoral response. Then, to generate the MEV, the predicted epitopes (B-cell and HTL epitopes) were fused using suitable linkers (AAY and GPGPG linkers) as

specialized spacer sequences. The AAY linkers play a role in increasing epitope presentation and removing junctional epitopes. In the about of GPGPG linkers, these types of linkers can cause stimulate T-helper responses and conformational dependent immunogenicity of helper and antibody epitopes [34–36]. In this study, to compensate for these vaccines' low immunogenicity, the D0/D1 domains of the flagellin protein from S. Typhimurium bacteria were utilized as an adjuvant to boost the effectiveness of immune system stimulation [37]. Also, The EAAAK linker was employed to connect the D0/D1 domains at the N-terminal region of the multi-epitope sequence. For effectively separating the adjuvant interference with protein segments, the EAAAK linker helps to reduce interruption and increases the degree of expression and bioactivity of the target fusion protein. Finally, a candidate vaccine with a length of 414 amino acids, including some linear B-cell and HTL epitopes fused to the adjuvant sequence, was constructed. MEV was predicted to be antigenic with the probability of antigenicity 0.926395 and non-allergenic. This means that our MEV potentially produces a strong immune response without an allergic reaction, making it a potent vaccine. The structure's physicochemical properties were examined: The MEV construct has a molecular weight of 46 kDa. MEV has a theoretical pI value of 5.03, indicating that it is acidic. An extinction coefficient index for a chemical can be explained as the amount of light absorbed at a specific wavelength. The build has an extinction coefficient of 35995M-1 cm-1. The candidate vaccine protein had an instability index (II) value of 40.11, indicating that our protein construct is relatively a stable protein (II >40 implies instability). The aliphatic index measures how much space aliphatic amino acids (alanine, valine, isoleucine, and leucine) take up in the side chains of proteins. It could be viewed as a beneficial factor in increasing globular protein thermostability. The construct had an aliphatic index of 65.12, indicating that it was a thermostable protein. The GRAVY (Grand Average of Hydropathy) value for a peptide or protein is calculated by adding the sum of the hydropathy values of all amino acids by the number of residues in the sequence. Positive and negative values, respectively, represent the hydrophobic and hydrophilic qualities of a substance. The GRAVY value of our suggested construct was -1.110, indicating that it is a hydrophilic protein [38]. The half-life is a forecast of how long it takes for half of the protein in a cell to vanish after production. ProtParam tool predicted the half-life of our construct in the following; 30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo) and >10 hours (Escherichia coli, in vivo). For many biochemical and functional evaluations of recombinant proteins, the solubility overexpression in the *E. coli* host is one of the requirements that help in the efficient purification process in later stages [39]. Here, the solubility upon overexpression of our multi-epitope protein construct predicted (by SOLpro server) with probability 0.759117, which indicates the overexpression of our multi-epitope protein in *E. coli*, insoluble form.

The PSIPRED method was used to analyze the secondary structure of our protein construct, which revealed that it mainly was alpha-helical (60 percent), 39 percent coils, and 0.4 percent of the amino acids in strand formation. Various servers got the vaccine constructs tertiary structure (SWISS-MODEL, phyre2, and I-TASSER). ProSA-web, RAMPAGE, and ERRAT servers were used in the validation process to identify potential faults and improve the quality of the projected 3D model. Based on validation data, the selected model had a high validation score and did not require further refinement. According to the Ramachandran plot, most residues are favored (96.0%) and 4.0% in permitted regions. This proposal used the Cluspro

server to test the immunological interaction between the designed MEV construct and the TLR5 receptor. Cluspro presented dozens of docked models scored based on protein surface hydrophobicity, geometry, and electrostatic complementarity. Between the hydrophobicity models, we choose the best possible docked model. As a result, the best-docked complex was selected as the docked structure with the lowest energy score (-1353.6).

In this research, CABS-Flex 2.0 software was used for MD simulation. CABS-Flex presents the stable arrangement of the TLR5-designed vaccine complex. The individual amino acid residue's root means square fluctuation (RMSF) values (using CABS Flex 2.0) were described. The highest and lowest RMSF values show that our complicated structure fluctuates more and less during the simulation process, respectively. The MEV's structure fluctuates, indicating its considerable flexibility and validating it as a suitable vaccine structure.

To achieve high-level production and translation efficacy of our multi-epitope protein in *E. coli*, we used a codon optimization technique (strain K12). The CAI value (0.85) and GC content (46.32%) data were obtained in this study, indicating that the protein vaccine may be expressed more strongly in the E. coli K-12 system [40, 41]. Finally, the MEV sequence was included in the pET-28a vector, which efficiently and effectively encoded the MEV protein in E. coli cells. We urge that validation experiments containing in vitro and in vivo studies be undertaken in the future to develop our candidate vaccine against malaria, based on the findings of this study.

Conclusion

In the present work, to design a protective antibody-inducing multi-epitope vaccine against the blood-stage of *P. falciparum* based on B cell and T- helper cell epitopes of PFGARP antigen of *P. falciparum.* Flagellin (fliC) protein, which acts as adjuvants, was also incorporated into the vaccine construct to enhance the vaccine's immunogenicity and induce, enhance, and deviate/direct the best form of the humoral immune response against P. *falciparum* trophozoite surface antigens. One of the advantages of the multi-epitope vaccine method is that it can elicit both humoral and cellular immune responses, and antibodies are required for the ultimate clearance of malaria parasites from the host's blood. The flexibility of the recombinant vaccination method could be helpful in the initial selection of promising candidates from thousands of candidates and subsequently improve their design and formulations. Therefore, the designed vaccine can induce long-term protective humoral immunity against the blood stage of *P. falciparum*. Nevertheless, to confirm the functionality of this multi-epitope vaccine, in vivo and in vitro immunological experiments are required.

Declarations

Author contributions

AA. and P.V.: conceptualization, methodology, software, writing- original draft preparation.

AA. S.J and GAS.: reviewing, editing, validation. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

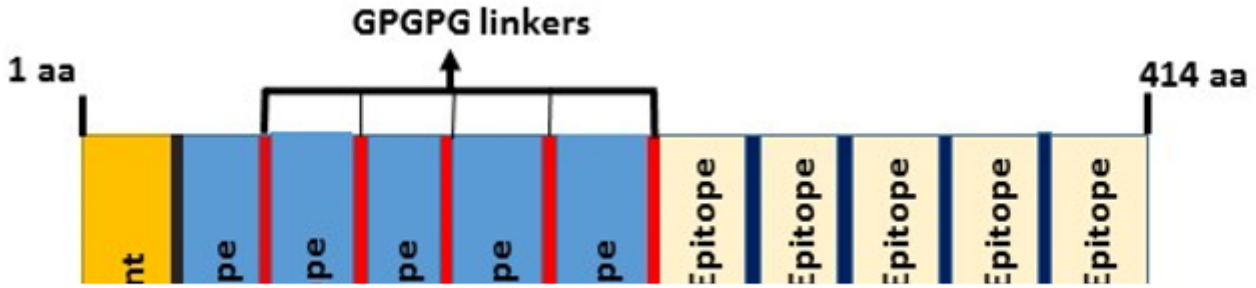

Figure 1

The schematic map of the final vaccine construct. In this construct, the adjuvant sequence was placed at the N-terminal and joined a multi-epitope sequence with the EAAAK linker (Black). GPGPG (Red) and AYY linkers (Blue) were used to bond B and HTL epitopes. 6x-His-tag was placed at the C-terminal of the multi-epitope sequence to identify and purification aims.

Figure 2

Graphical demonstration of vaccine secondary structure with 60% alpha-helices, 39% beta strands, and 0.4% coils.

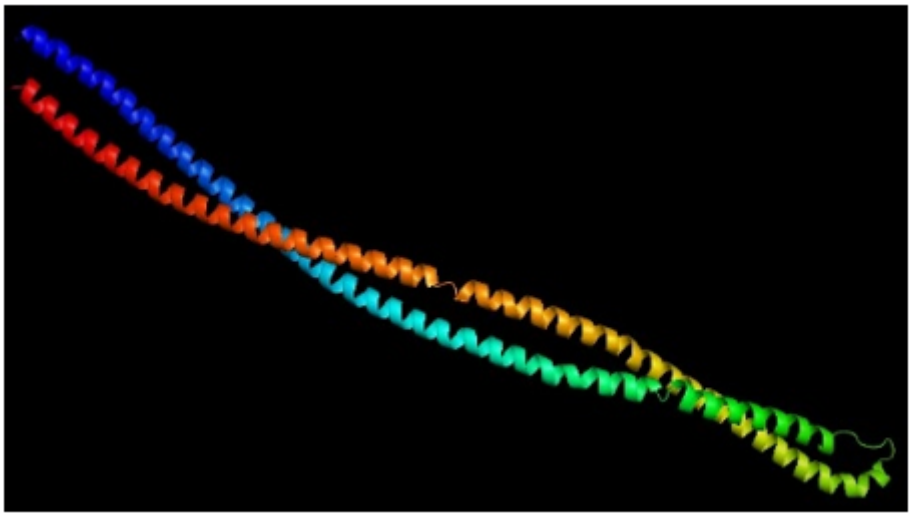

Figure 3

The 3D model of final multi-epitope vaccine.

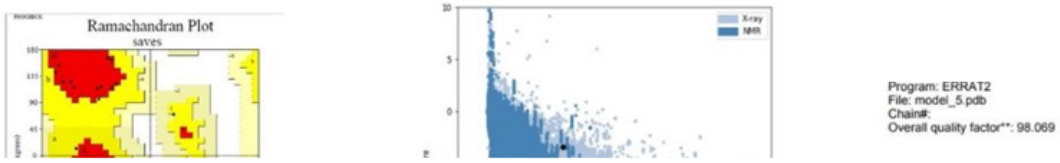

Figure 4

(A) PROCHECK's Ramachandran plot illustrates that the residues are placed in the allowed (96%) and favored (4%) regions. (B) ProSA Z-score plot shows a -2.84 score, in the range of conformation of the native protein. (C) ERRAT plot in a score of 98.06.

Figure 5

3D demonstration of the predicted conformational B-cell epitopes in the final multi-epitope construct.

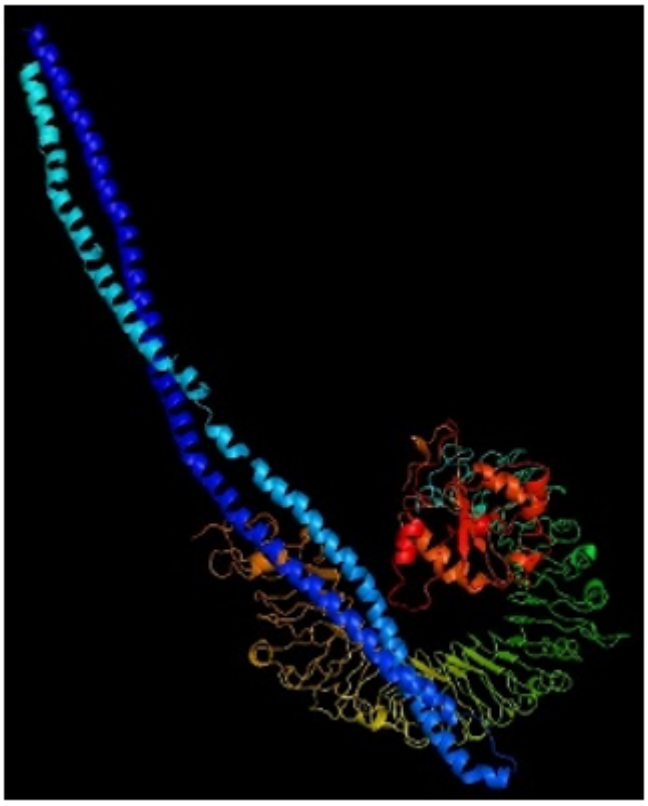

Figure 6

The docked model between the TLR5 molecule and final vaccine construct using the ClusPro server.

Figure 7

(A) The Root Mean Square Fluctuation (RMSF) plot of the protein vaccine structure demonstrates the fluctuations of MEV residues during the fast simulations. In the residues at positions 219 and 617, high degree fluctuation identified in 4.58 Å and 3.57 Å. (B) The identified stable protein vaccine structure with fast flexibility simulations using the CABS-Flex server.

Figure 8

In silico cloning of multi-epitopes vaccine sequence into pET28a (+) expression vector using SnapGene software free-trial (https: //www.snapg ene.com/free-trial /), the red colored semicircle and gray colored semicircle respectively illustrate multi-epitopes vaccine sequence and pET28a (+) backbone.