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A cutting-edge immunoinformatics approach for design of multi-epitope oral vaccine against dreadful human malaria

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ABSTRACT

Human malaria is a pathogenic disease mainly caused by *Plasmodium falciparum*, which was responsible for about 405,000 deaths globally in the year 2018. To date, several vaccine candidates have been evaluated for prevention, which failed to produce optimal output at various preclinical/clinical stages. This study is based on designing of polypeptide vaccines (PVs) against human malaria that cover almost all stages of life-cycle of *Plasmodium* and for the same 5 genome derived predicted antigenic proteins (GDPAP) have been used. For the development of a multi-immune inducer, 15 PVs were initially designed using T-cell epitope ensemble, which covered >99% human population as well as linear B-cell epitopes with or without adjuvants. The immune simulation of PVs showed higher levels of T-cell and B-cell activities compared to positive and negative vaccine controls. Furthermore, *in silico* cloning of PVs and codon optimization followed by enhanced expression within *Lactococcus lactis* host system was also explored. Although, the study has sound theoretical and *in silico* findings, the *in vitro*/*in vivo* evaluation seems imperative to warrant the immunogenicity and safety of PVs towards management of *P. falciparum* infection in the future.

1. Introduction

World Health Organization has documented almost 405,000 deaths including 228 million infections globally towards human malaria disease [1]. Five diverse species of *Plasmodium*, i.e., *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, as well as *P. knowlesi* are culprit for the disease outbreak in which *P. falciparum* has stood first for lethality. About 99.7 and 62.8% disease cases were documented merely for *P. falciparum* (Pf) in African as well as South-East Asia realms, respectively, which further supports the above fact [2]. In recent findings, *P. vivax* has also been found capable to develop severe malaria amongst populations living in sub-tropical countries [3]. The only preferred option is cost intensive chemotherapy for human malaria [4,5]. The reason being the fact that currently, none of cutting-edge effective human malaria vaccine is accessible, which can provide protection towards most of the world-wide population together with endemic regions. On the other hand, the exhaustive research from decades has led to development of total 44 malaria vaccine candidates together with 19 subunit, 10 DNA, 10 recombinant vector, 1 recombinant protein as well as 4 live/attenuated vaccine preparations, of which, merely 7 vaccines are revealed for human host (http://www.violinet.org/). Most of these vaccines are either single or multi-antigens derived from various life-cycle stages of the parasites *P. falciparum*, *P. vivax*, *P. yoelii*, *P. berghei and P. chabaudi* [6,7]. For instance, Pf vaccine combination involves multi-antigens namely MSP1, MSP2 and RESA derived from blood-stage [8], while NYVAC-Pf7 includes antigens CS, SSP2, LSA1, MSP1, AMA1, SERA as well as Pfs25 from multi-stage of pathogenic life-cycle [9]. Besides these, *P. falciparum* reticulocyte-binding homologue 5 (PfRH5) was also reported as good antigen for development of malaria vaccine [10,11] that elicits human monoclonal antibody in vaccine trial [12]. Most of the aforesaid vaccines were found to elicit immune responses, but unfortunately, failed to clear phase-III clinical trial owing to rapid waning of vaccine efficacy due to geographical antigenic variation and human leukocyte antigen (HLA) allelic diversity [3,13–15]. Apart from these,

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apoptosis of infected erythrocytes and their inability to express HLA class I molecules on cell surface that assists in avoiding cytotoxic T lymphocytes (CTL) response is also another aspect [16–18]. Thus, there is pressing need towards the development of innovative vaccines using reverse vaccinology together with immunoinformatics that can target majority of the stages of parasite's life-cycle including species level conservation so as to cover the world-wide human population [19].

In last two decades, the reverse vaccinology strategy has been extensively exploited by world-wide research groups for genome-wide screening of vaccine antigens against several pathogens like *Neisseria meningitides* serogroup B, *P. falciparum*, *Leishmania* and so on [20–24]. It has been synergistically progressive with onset of immunoinformatics, which is another cost-effective and quicker strategy towards prediction of B- as well as T-cell epitopes present on antigenic proteins and targeted population coverage analysis [25–29]. In recent years, the aforementioned strategies have been used very frequently in designing of novel vaccines by various researchers against different diseases like Dengue [30], Schistosomiasis [31], Fascioliasis [32], Encephalitis [33], Lassa fever [34], Neonatal meningitis [35] and H7N9 influenza A [36]. Furthermore, Toll-like receptors (TLRs), e.g., TLR-2, TLR-4 and TLR-9 typically present in plasma membrane of host cell recognized as pathogen-associated molecular patterns (PAMPs) that provokes phagocytosis and develop innate immune responses through production of cytokines, interleukins, and antibodies that prohibit the parasite entry in pre-erythrocytic stage of malaria [37–40]. To the best of our knowledge, this is one of the first computational studies for designing of multi-epitope based oral vaccine against human malaria. Overall, this investigation focuses on the designing of 15 innovative polypeptide vaccines (PVs) utilizing predicted B- and/or T-cell epitopes sourced from 5 genome derived predicted antigenic proteins (GDPAP) assembled together with specific linkers and adjuvants towards *P. falciparum* malaria [24].

2. Methodology

The methodological flow chart depicting the strategy for development of innovative PVs is presented in Fig. 1 with following steps: (i) Selection of *P. falciparum* 3D7 protein sequences and homology study, (ii) B-cell epitopes prediction, (iii) Prediction of HLA class I and II restricted T-cell epitope ensemble, (iv) Prediction of IL-10 and IFN-γ inducing T cell epitopes (v) Designing or selection

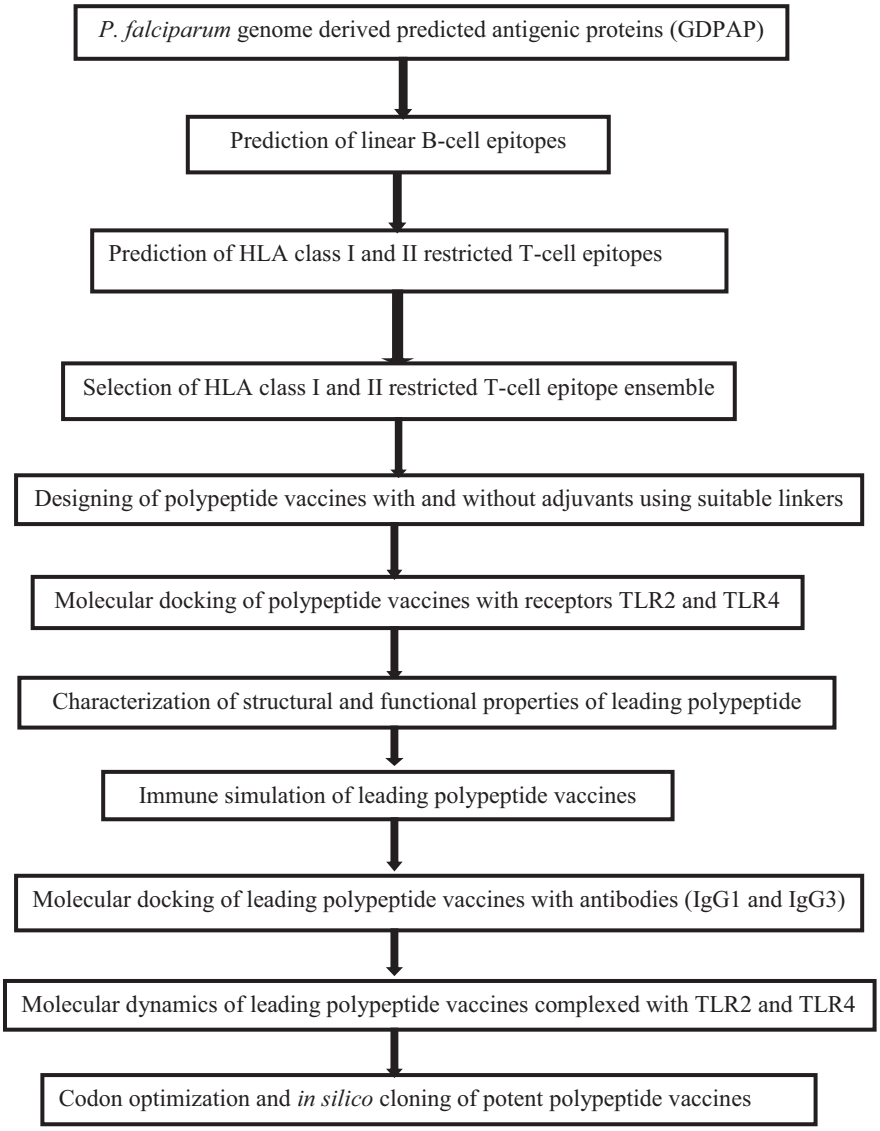


Fig. 1. Strategy of the present work for development of effective malaria polypeptide vaccines.

of test PVs, positive as well as negative polypeptide vaccine controls using chimeric technique, (vi) Tertiary structure prediction and molecular docking of PVs with TLR2 and TLR4 receptors, (vii) Characterization of structural and functional properties viz. secondary structure, physicochemical, adhesion, antigenicity, allergenicity, solubility and biological activity of leading PVs (viii) Immune simulation of leading PVs, (ix) Molecular docking of leading PVs with protective antibodies (IgG1 and IgG3), (x) Molecular dynamics of leading PVs complexed with TLR2 and TLR4 and (xi) *In silico* cloning and expression of potent PVs in *Lactococcus lactis* etc. Further, the accomplishment of aforementioned steps required various bioinformatics tools, which are provided in Table 1.

2.1. Selection of P. falciparum protein sequences

Our previous study revealed five protein sequences of *P. falciparum* 3D7 genome as promising antigenic adhesion proteins [24]. Therefore, in the present study, these malarial adhesion proteins viz. circumsporozoite protein (CSP: PF3D7_0304600), surface protein P113 (P113: PF3D7_1420700), merozoite surface protein 1 (MSP1: PF3D7_0930300), 28 kDa ookinete surface protein (P28: PF3D7_1030900) and 25 kDa ookinete surface antigen precursor (P25: PF3D7_1031000) were considered as platform for designing of new PVs. Further, the BLASTp tool was used to explore homologous sequences amongst human malaria parasites.

2.2. B-cell epitopes prediction

The presence of linear (16-mer) and conformational B-cell epitopes were predicted using BCPREDS and DiscoTope tools, respectively.

2.3. Forecast of T-cell epitopes

The linear B- cell epitope sequences (as forecasted in section 2.2) were used as input for forecast of HLA class I and II restricted T-cell epitopes through IEDB based consensus strategy with threshold criteria of binding affinity (IC_{50}) ≤ 500 nM and percentile rank ≤3, correspondingly.

2.4. Forecast of population coverage and selection of T-cell epitope ensemble

The IEDB based population coverage tool was exploited towards the predicted population coverage (PPC) analysis of forecasted T-cell epitopes with their corresponding HLA binding alleles. Further, HLA class I as well as II epitope ensemble was developed as described previously [24]. Finally, HLA class I and II epitope ensembles were then mapped to forecasted continuous B-cell epitopes.

2.5. Prediction of cytokine responses

The induction of cytokines response predictions, i.e., IL-4, IL-10 and IFN-γ were carried out for epitope ensembles using tools IL-4Pred, IL-10Pred and IFNepitope, correspondingly.

2.6. Designing of multi-epitope PVs

In this study, the new multi-epitope PVs were developed using the linker EAAAK (L1) at N-terminal with or without adjuvant following Ali et al. [30] where Cholera toxin B subunit (A: UniProt accession no. AIE88420.1) and 50S ribosomal L7/L12 (B: UniProt accession no. P9WHE3) were used as adjuvants against TLR-2 (PDB ID: 2Z7X) and TLR-4 (PDB ID: 4G8A), correspondingly. During PVs designing, the epitopes were coupled with linkers by adopting following strategies: HLA class I epitopes with GGGS (L2), HLA class II epitopes with GPGPG (L3), B-cell epitopes with L2 or L3, HLA class I and II epitope with L3, HLA class II epitope and B-cell epitope with L3. Also, the adjuvants were coupled with epitopes using linker

Table 1
Bioinformatics tools used in the present study for designing of polytope vaccines.

S. no.	Prediction/analysis tools	Function	Accuracy (%)/AUC/R^2	Website
1	AllergenFP	Allergenicity of peptide	88.00%	ddg-pharmfac.net/AllergenFP/
2	ANTIGENpro	Protein antigenicity	76%	http://scratch.proteomics.ics.uci.edu/
3	BCPREDS	Linear B-cell epitopes	0.8	ailab.ist.psu.edu/bcpred/predict.html
4	CamSol	Protein solubility	0.98	http://www-vendruscolo.ch.cam.ac.uk/camsolmethod.html
5	C-ImmSim	Immune simulation	N.A	http://kraken.iac.rm.cnr.it/C-IMMSIM/?page=1
6	ClusPro 2.0	Protein-protein docking	N.A	cluspro.bu.edu/home.php
7	DeepGOPlus	Protein function	0.9	http://deepgoplus.bio2vec.net/deepgo/
8	DiscoTope 2.0	Conformational B-cell epitopes	0.73	www.cbs.dtu.dk/services/DiscoTope/
9	ExPASy-ProtParam	Grand average of hydropathicity	N.A	web.expasy.org/protparam/
10	IEDB-AR	Population coverage analysis of epitopes	N.A	tools.iedb.org/population/
11	IEDB-AR (consensus method)	HLA class I epitope	0.86	tools.iedb.org/mhci/
		HLA class II epitope	0.85	tools.iedb.org/mhcii/
12	IFNepitope	IFN-γ inducing peptides	82.10%	crdd.osdd.net/raghava/ifnepitope/
13	IL-10Pred	Interleukin-10 inducing	72.30%	crdd.osdd.net/raghava/IL-10pred/
14	IL-4Pred	Interleukin-4 inducing peptide	64.76%	webs.iiitd.edu.in/raghava/il4pred/scan.php
15	iMODS	Normal mode analysis	N.A	http://imods.chaconlab.org/
16	JCat	Codon optimization	N.A	http://www.jcat.de/
17	ModRefiner	High-resolution protein structure refinement	N.A	zhanglab.ccmb.med.umich.edu/ModRefiner/
18	PROCHECK	Stereochemical quality of a protein structure	N.A	servicesn.mbi.ucla.edu/PROCHECK/
19	ning of PVs and codon opti	Protein antigenicity prediction	75%	http://imed.med.ucm.es/Tools/antigenic.html
20	Protein-Sol	Protein solubility	0.97	https://protein-sol.manchester.ac.uk/
21	PSIPRED 4.0	Secondary structure	84.20%	bioinf.cs.ucl.ac.uk/psipred/
22	RaptorX	Protein structure modelling	0.89	raptorx.uchicago.edu/
23	Recombinant protein solubility prediction	Protein solubility	88%	http://www.biotech.ou.edu/
24	Secret-AAR	Protein antigenicity	N.A	http://microbiomics.ibt.unam.mx/tools/aar/
25	SOLPro	Protein solubility	74%	http://scratch.proteomics.ics.uci.edu/
26	SPAAN	Adhesin protein	97.4%	http://www.violinet.org/vaxign/
27	VaxiJen 2.0	Protein antigenicity	78.00%	www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html

N.A: not available; AUC: area under ROC curve; R^2: correlation of coefficient.

L1. The linker L1 was also employed to connect adjuvant with HLA class I and B-cell epitope [41–45].

2.7. Tertiary structure prediction and molecular docking of PVs with TLR2 and TLR4 receptors

The forecast of tertiary structures of PVs was performed using RaptorX tool. Further, the refinement as well validation of 3D structure was carried out by tools ModRefiner and PROCHECK, respectively. The molecular docking studies of PVs with molecular complex receptors TLR2-TLR1 (PDB ID: 2Z7X) and TLR4-MD2 (PDB ID: 4G8A) were performed using ClusPro 2.0 tool. The PVs developed without and with TLR2 and TLR 4 specific adjuvants that were docked with receptors TLR2-TLR1 and TLR4-MD2, correspondingly. The ligands *Escherichia coli* heat labile enterotoxin type IIB B-pentamer (C1; PDB ID: 1QB5) and carbohydrate recognition and neck domains of surfactant protein A (C2; PDB ID: 1R13) were used as controls for docking with receptors TLR2 and TLR4, correspondingly [46,47].

2.8. Characterization of structural and functional properties of leading PVs with positive vaccine controls

The self-assembling protein nanoparticles (SAPN) from *P. falciparum* FMP014 (C3) and fusion protein from *Staphylococcus aureus* (C4) were selected as positive vaccine controls as detailed previously in Kaba et al. [48] and Ahmadi et al. [49] for comparative evaluation of several properties of leading PVs., respectively. The physico-chemical properties [Grand Average Hydropathy (GRAVY), molecular weight, isoelectric point (pI) and half-life] were calculated using ExPASy-ProtParam tool. The antigenic properties were predicted with the involvement of VaxiJen2.0, ANTIGENpro, Protein antigenicity prediction by Kolaskar and Tongaonkar and Secret-AAR tools. Further, the recombinant protein solubility was predicted using tools RPSP, Protein-Sol, CamSol and SOLPro. The analysis of secondary structure elements (alpha helix, extended strand and random coil) were performed using PSIPRED tool. Further, tertiary structure analysis was carried using tools ModRefiner and PROCHECK. The biological function and allergenicity were evaluated based on tools DeepGOPlus and AllergenFP, correspondingly.

2.9. Immune simulation of leading PVs

The best docked complex (in terms of lowest docking energy) PVs with receptors TLR2 and TLR4 were chosen for immune simulation study using C-ImmSim tool along with two positive vaccine controls (C3, C4) as mentioned in section 2.8 and one negative vaccine control (C5) so as to compare the simulation results. The C5 was designed using suitable linkers as well as non-binding HLA class I and II epitopes by applying the same strategies as used in PVs. The non-epitopes were screened using the criteria of 14 lowest ranking HLA class I (HLA-A*0201, -B*5301) and 3 lowest ranking HLA class II (HLA-DRB1–0411) as predicted by IEDB based consensus method, correspondingly in a randomly selected highly variable erythrocyte membrane protein 1, (PfEMP1: PF3D7_0617400.1). The C-ImmSim is a simulator of agent-based model, which forecasts the induction of immune response (cellular and humoral response) along with forecast of T-cell epitope as well as B-cell epitope [50]. The default simulation parameters were chosen except HLA allele, number of antigen (10000) and time steps [51]. The host HLA alleles (HLA-A*02:01, HLA-B*53:01 and HLA-DRB1*04:11) were selected based on prevalent alleles associated with human malaria [52–55]. The time steps 1, 42 and 84 were selected following Kaba et al. [48].

2.10. Molecular docking of leading PVs and antibodies IgG1 and IgG3

The molecular docking between antibodies IgG1 (PDB ID: 6B5L) as well as IgG3 (PDB ID: 5BK0) with PVs (PV1A/PV3B) were performed using ClusPro 2.0 tool along with co-crystallized respective control epitopes NPDPNANPNVD (C6, IEDB ID: 756359) and NANPNANPNANPNANPNANP (C7, IEDB ID: 43248) of Pf CSP [56,57].

2.11. Molecular dynamics of leading PVs complexed with TLR2/TLR4

Molecular dynamics of top 2 docked complexes PV1A-TLR2 and PV3B-TLR4 were performed through iMODS server to explain the collective protein motion in the internal coordinates through normal mode analysis (NMA). The NMA in dihedral coordinates naturally mimics the combined functional motions of protein molecules modelled as a set of atoms connected by harmonic springs [58].

2.12. Codon optimization and in silico cloning of leading PVs

The DNA coding sequences of the oral PVs (PV1A and PV3B) were optimized for elevated protein expression using Java Codon Adaptation Tool (JCat) involving following options: i) *Lactococcus lactis* (strain IL1403) as expression host, ii) avoid rho-independent transcription terminators, iii) avoid prokaryotic ribosome binding sites and iv) avoid cleavage sites of restriction enzymes. Further, for *in silico* cloning of PV1A and PV3B cDNA (with stop codon) SnapGene software was used involving insertion at restriction site of *Fsp*I (6006) in plasmid vector pIL1 (Gene bank accession number: HM021326) [59].

3. Results and discussion

According to VIOLIN database (accessed on June 26, 2019), total 16 vaccines available so far for against *P. falciparum* from different life-cycle stages, but they have not succeed to get approval from FDA, USA for world-wide marketing [60]. The RTS,S/AS01 is the only world's first European Medicines Agency (EMA) approved malaria vaccine with partial protection in young children (36.3%) for use to only Sub-Saharan African region along with severe adverse effect (24.2%–28.4%) and incurable adverse effect (1.5%–2.5%) [61,62]. In addition, the efficacy was further declined to almost zero after 4th year and negative in 5th year [63]. The aforementioned facts warrant exhaustive efforts/research towards the development of a more effective PV that can elicit robust immune response globally. The present study is an extension of our previous report [24] that exploits 5 homologous antigens conserved amongst human malaria parasites *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (with minimum 38.62% identity recognized through BLASTp tool) as potential platform for designing of PVs [64].

3.1. Prediction of B- and T-cell epitopes for screening of epitope ensemble

In recent years, epitope based designing of vaccine is a new strategy that has been employed by world-wide researchers towards the development of efficient PVs against numerous diseases such as leishmaniasis, malaria and so on. In this context, the exploitation of computational approaches is not only cost-effective for vaccine development but also diminishes time period and risk of failure in experimental studies [26,27,65,66]. In this study, 82 continuous B-cell epitopes were forecasted from 5 GDPAP using BCPREDS (Supplementary Table S1). These 82 continuous B-cell epitopes were found to possess total 433 T-cell epitopes including 142 HLA class I epitopes and 291 HLA class II epitopes (Supplementary Table S2). These T-cell epitopes

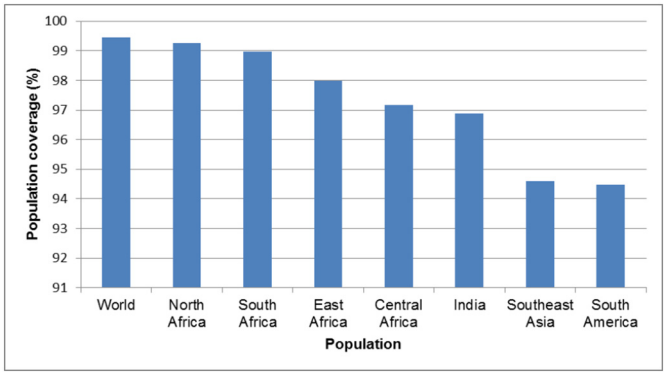


Fig. 2. The malaria endemic population coverage analysis of combined HLA class I and II binding epitope ensemble used in designing of PVs obtained by IEDB analysis tool.

were forecasted from the pool of predicted continuous B-cell epitopes as the antigen presentation to T-cells was supposed to be more efficient if it is recognized by the B-cell. In addition, an antigen-specific B-cell may present multiple T-cell epitopes to the immune system and, thus enhances its ability to be triggered in a specific manner [67–69]. Further, based on the PPC analysis an epitope ensemble of 13 HLA class I epitopes with 98.75% and 3 HLA class II epitopes with 56.85% world coverage were designed using criteria described previously (Table 2) [24]. However, a combined set of 16 HLA class I and II epitope ensemble revealed human population coverage of highest 99.46% and lowest 94.47% for world and South America, respectively (Fig. 2). The aforementioned criteria involved the screening of cross-presented epitopes amongst different set of HLA binding alleles in a selected population with higher PPC and VaxiJen score. The technique of identifying such 'promiscuous' epitopes that cover diverse HLA alleles of affected population are highly desirable as they could enhance the vaccine efficacy [51]. Concerning HLA class I epitope ensemble of *P. falciparum*, epitopes YTLTAGVCV (T1) and YFNDDIKQF (T5) covered 56.56% and 39.26% of world population were also reported in similar study conducted by Pritam et al. [24].

3.2. Induction of cytokine responses of epitope ensemble

In case of malaria, adaptive immune system elicits both cellular and humoral immune responses, which are associated with B and T lymphocytes, respectively. However, mainly the CD4+ T lymphocytes (also known as helper T cell (Th), Th1 and Th2) elicit IFN-γ and IL-4, correspondingly) regulate the malaria infection [68,70]. Besides these, TLRs are also involved in the activation of different signalling cascade that ultimately express the genes of pro-inflammatory cytokines like IFN-γ, etc. [71]. The IFN-γ is associated with depletion of liver-stage parasites [72,73]. This is also supported by present study, where the epitopes T2, T7, T8, T10, T11 and T1, T2, T3, T4, T5, T6, T7, T8, T9, T11, T12, T13, T14, T16 were found to induce the IFN-γ and IL-4 responses, correspondingly (Supplementary Table S3). Amongst aforementioned epitopes ensemble, the T14 was recorded as one of the potent candidate to induce IL-10 response that found to suppresses the pathogenic inflammatory responses concerning control of malaria parasite [74].

Table 2
Details of predicted T-cell epitope ensemble including HLA binding alleles along with their source linear B-cell epitope.

S. no.	T-cell epitope number	T-cell epitopes with start and end position	B-cell epitope number (Antigen)	Linear B-cell epitopes with start and end position	Predicted population coverage (%)	HLA binding alleles
HLA class I						
1	T1	100YTLTAGVCV108	B1 (P28)	98TEYTLTAGVCVPNVCR113	56.56	HLA-A*02:06, HLA-A*02:01, HLA-A*68:02, HLA-C*05:01, HLA-C*15:02, HLA-C*12:03, HLA-C*14:02
2	T2	421YPNGIVYPL429	B2 (MSP1)	417PKVPYPNGIVYPLPLT432	53.84	HLA-A*68:02, HLA-B*07:02, HLA-B*18:01, HLA-B*08:01, HLA-B*39:01, HLA-B*35:01, HLA-B*53:01, HLA-C*03:03, HLA-C*14:02, HLA-C*12:03
3	T3	46VLHCEVQCL54	B3 (P113)	45YVLHCEVQCLNGNNEI60	40.93	HLA-A*02:01, HLA-C*14:02
4	T4	90YACKCNLGY98	B4 (P25)	84DGNPVSYACKCNLGYD99	40.73	HLA-A*01:01, HLA-A*29:02, HLA-B*15:01, HLA-B*35:01, HLA-C*12:03
5	T5	1013YFNDDIKQF1021	B5 (MSP1)	1006ILKNNDTYFNDDIKQF1021	39.26	HLA-A*23:01, HLA-A*29:02, HLA-C*14:02, HLA-C*07:02, HLA-C*12:03
6	T6	450LMNPHTKEK458	B6 (MSP1)	447YGDLMNPHTKEKINEK462	38.48	HLA-A*03:01, HLA-A*11:01, HLA-A*30:01, HLA-A*31:01
7	T7	580YRLKENKDY588	B7 (P113)	579YYRLKENKDYDVVSSI594	33.31	HLA-C*07:01, HLA-C*06:02
8	T8	105GVCVPNVCR113	B1(P28)	98TEYTLTAGVCVPNVCR113	25.64	HLA-A*11:01, HLA-A*31:01, HLA-A*68:01
9	T9	1104NVLQNFSVF1112	B8 (MSP1)	1097NSLNNPHNVLQNFSVF1112	23.15	HLA-A*23:01, HLA-B*15:01, HLA-B*15:02, HLA-B*35:01
10	T10	98TEYTLTAGV106	B1(P28)	98TEYTLTAGVCVPNVCR113	19.88	HLA-A*68:02, HLA-B*18:01, HLA-B*40:02, HLA-B*44:02
11	T11	1117KEAEIAETE1125	B9 (MSP1)	1115KKKEAEIAETENTLEN1130	7.81	HLA-B*40:01
12	T12	1310GESEDNDEY1318	B10 (MSP1)	1309FGESEDNDEYLDQVVT1324	6.27	HLA-B*44:03
13	T13	1120EIAETENTL1128	B9 (MSP1)	1115KKKEAEIAETENTLEN1130	5.82	HLA-A*25:01, HLA-A*68:02
HLA class II						
14	T14	1350PLAGVYRSLKKQIEK1364	B11 (MSP1)	1350PLAGVYRSLKKQIEKN1365	41.75	HLA-DRB1*03:08, HLA-DRB1*03:06, HLA-DRB1*03:07, HLA-DRB1*03:09, HLA-DRB1*03:01, HLA-DRB1*03:05, HLA-DRB1*07:03, HLA-DRB1*04:05, HLA-DRB1*08:01, HLA-DRB1*08:17, HLA-DRB1*11:20, HLA-DRB1*08:06, HLA-DRB1*11:01, HLA-DRB1*11:14, HLA-DRB1*08:13, HLA-DRB1*11:07, HLA-DRB1*11:21, HLA-DRB1*11:02, HLA-DRB1*13:21, HLA-DRB1*13:04, HLA-DRB1*13:07, HLA-DRB1*11:28, HLA-DRB1*13:05, HLA-DRB1*13:23, HLA-DRB1*13:01, HLA-DRB1*13:27, HLA-DRB1*13:28, HLA-DRB1*13:22
15	T15	1007LKNNDTYFNDDIKQF1021	B5 (MSP1)	1006ILKNNDTYFNDDIKQF1021	20.03	HLA-DRB1*03:09, HLA-DRB1*03:05, HLA-DRB1*03:01, HLA-DRB1*04:21, HLA-DRB1*04:02, HLA-DRB1*04:10, HLA-DRB1*13:04, HLA-DRB3*01:01
16	T16	125DPANSLTHTCSCNIG139	B12 (P28)	124VDPANSLTHTCSCNIG139	18.25	HLA-DRB1*07:01, HLA-DRB1*07:03

Table 3
Order of linkers, epitopes and adjuvants used in designing of 15 polypeptide vaccines and positive as well as negative vaccine controls.

S. no	Type of polypeptide vaccine	No. of amino acids	Design of polypeptide vaccine/sequence
1	PV1	235	**L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3**
2	PV1A	364	**L1-A-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3** (EAAAKMIKLKFGVFFTVLLSSAYANGTPQNITDLCAEYHNTQIHTLNDKIFSYTESL AGKREMAIITFKNGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMANEAAAKYPNGIVYPLGGGSYFNDDIKQFGGGSLMNPHTKEKGGGSNVLQNFSVFGGGSKEAEIAETEGGGSGESEDNDEYG GGSEIAETENTLGGGSYTLTAGVCVGGGSGVCVPNVCRGGGSTEYTLTAGVGGGSYACKCNLGYGGGSVLHCEVQCLGGGSYRLKENKDYGPGPGPLAGVYRSLKKQIEKGPGPGLKNNDTYFNDDIKQFGPGPGDPANSLTHTCSCNIGGP GPG)
	3	PV1B	**L1-B-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3**
4	PV2	299	**L1**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3**
	5	PV2A	**L1-A-L1**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3**
6	PV2B	434	**L1-B-L1**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3**
7	PV3	529	**L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3**
8	PV3A	658	**L1-A-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3**
9	PV3B	664	**L1-B-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L3**-B2-**L3**-B5-**L3**-B6-**L3**-B8-**L3**-B9-**L3**-B10-**L3**-B9-**L3**-B1-**L3**-B4-**L3**-B3-**L3**-B7-**L3**-B11-**L3**-B5-**L3**-B13-**L3** (**EAAAK**MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAAGAAPAGAAVEAAEEQSEFDVILEAAGDKKIGVIKVVREIVSGLGLKEAKDLVDGAPKPLLEKVAKEAADEAKAKLEAAGATVTVK**E** **AAAK**YPNGIVYPL**GGGS**YFNDDIKQF**GGGS**LMNPHTKEK**GGGS**NVLQNFSVF**GGGS**KEAE IAETE**GGGS**GESEDNDEY**GGGS**EIAETENTL**GGGS**YTLTAGVCV**GGGS**GVCVPNVCR**GGGS**TEYTLTAGV**GGGS**YACKCNLGY**GGGS**VLHCEVQCL**GGGS**YRLKENKDY**GPGPG**PLAGVYRSLKKQIEK**GPGPG**LKNNDTYFNDDIKQF**GPGPG**DPANSLTHTCSCNIG**GPGPG**PKVPYPNGIVYPLPLT**GPGPG**ILKNNDTYFNDDIKQF**GPGPG**YGDLMNPHTKEKINEK**GPGPG**NSLNNPHNVLQNFSVF**GPGPG**KKKEAEIAETENTLEN**GPGPG**FGESEDNDEYLDQVVT**GPGPG**KKKEA EIAETENTLEN**GPGPG**TEYTLTAGVCVPNVCR**GPGPG**DGNPVSYACKCNLGYD**GPGPG**YVLHCEVQCLNGNNEI**GPGPG**YYRLKENKDYDVVSSI**GPGPG**PLAGVYRSLKKQIEKN**GPGPG**ILKNNDTYFNDDIKQF**GPGPG**VDPANSLTHT CSCNIG**GPGPG**)
10	PV4	285	**L1**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11-**L2**-B5-**L2**-B13-**L2**
11	PV4A	414	**L1-A-L1**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11-**L2**-B5-**L2**-B13-**L2**
12	PV4B	420	**L1-B-L1**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11-**L2**-B5-**L2**-B13-**L2**
13	PV5	514	**L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L2**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11 **-L2**-B5-**L2**-B13-**L2**
14	PV5A	643	**L1-A-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L2**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11-**L2**-B5-**L2**-B13-**L2**
15	PV5B	649	**L1-B-L1**-T2-**L2**-T5-**L2**-T6-**L2**-T9-**L2**-T11-**L2**-T12-**L2**-T13-**L2**-T1-**L2**-T4-**L2**-T8-**L2**-T10-**L2**-T4-**L2**-T3-**L2**-T7-**L3**-T14-**L3**-T15-**L3**-T16-**L2**-B2-**L2**-B5-**L2**-B6-**L2**-B8-**L2**-B9-**L2**-B10-**L2**-B9-**L2**-B1-**L2**-B4-**L2**-B3-**L2**-B7-**L2**-B11-**L2**-B5-**L2**-B13-**L2**
16	C3	212	MGHHHHHHDEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKCASVFEDLIDYNKAALSKFKEDGSWQTWNAKWDQWSNDWNAWESDWQAWKDDWAEWRALWMGGRLLLRLE RIRHENRMVLEALEALARFVANLSMRLALMVLSFLRNESRGGSGNA NPNANPNANPNANPNANPNANP
17	C4	468	IRTKGTIAGQYRVYSEEGANKSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQPDFKAAALFMKTRNGSMKAADNFLDPNKASSLLSSGFSPDFATVITMDRKASKQQTN AAAMKKLVPLLLALLLLVAACGTGGKQSSDKSNGKLKVVTTNSILYDMAKNVGGDNVDIHSIVAAADVKPIYLNGEEGNKDKQDPHAWLSLDNGIKYVKTIQQTFIAAAITPGYIWEINTEKQGTPEQMRQAIEFVKKHKLKHLLVETSAAAH TVQAGESLNIIASRYGVSVDQL MAANNLRGYLIMPNQTLAAATPTATTGSNGNASSFNHQNLYTAGQCTWYVFDRRAQAGSPISTYWSDAKYWAGNAANDGYQVNNTPSVGSIMQSTPGPYGHVAYVERVNGDGSILISEMNYTYGPYNMNYRTIPASEVSS
18	C5	248	EAAA KPDNRDKKEGGGGSEKCRGKNKDGGGSPPKRNKRQPGGGSTKAPEKKKEGGGSEEVNGEKDNGGGSEWNKENKNNGGGSTASSEKGKDGGGSGFCRERKKRGGGSPKPPKRNKRGGGSPKRNKRQPKGGGSKCRGKNKDKGGGSPE KQLAGGKGGGSAKKQALGRSGGGSKDCASCKKKGPGP GPAELPKPPKRNKRQPGPGPGAPPKQEEKGGCEPASGPGPGKAPPKQEEKGGCEPAGPGPG

Linkers (**L1, L2** and **L3**), adjuvant (**A** and **B**), T-cell epitopes (T1-T16), and B-cell epitopes (B1-B13).

3.3. Design of PVs for malaria

Linear B-cell epitopes is linked to antibody generation, where identification of such epitopes using traditional approaches is not only costly but also time consuming with involvement of difficult processes [75]. In order to overcome aforementioned issues, the present study involved the prediction of T-cell epitopes using linear B-cell epitopes as input instead of whole antigen so as to minimize not only the size of PV but also elicit both cellular (T-cell epitope) as well as humoral (B-cell epitope) immune responses. Further, the non toxic nature of adjuvants A and B also helps in production of several cytokines (e.g., INF-γ, TNF-α, IL-2, IL-4, IL-6, IL-12) through induction of dendritic cell, B-cell, macrophage and T-cell, which ultimately boost the concentration of the antibodies reported in several studies linked to various disease causing agents including human rotavirus, HIV, *Helicobacter pylori*, Influenza virus [76–79]. Therefore, 15 PVs were designed through epitope ensemble of T-cell epitopes and/or linear B-cell epitopes having epitope ensemble with different linkers as well as adjuvants, which are responsible for the activation of TLR2 and TLR4 receptors pertaining to malaria. Initially, five non-adjuvant PVs (PV1-PV5) were designed followed by incorporation of TLR2 and TLR4 binding specific adjuvants that resulted into respective design of 10 adjuvant PVs, i.e., PV1A-PV5A and PV1B-PV5B (Table 3). Further, EAAAK linker was incorporated at N-terminal of PVs as it is stiff and prevents the assembly of adjuvant with other vaccine domain [80,81]. Although, the adjuvants are found to enhance the immunogenicity of vaccines but they may cause toxicity/adverse reaction. Therefore, we have designed 5 PVs without adjuvants, where the designing of PV1 having only T-cell epitopes (HLA class I and II) and they were joined together by using linker L_2 and L_3. Likewise, in PV2, we have exploited merely linear B-cell epitopes attached together with linker L_3. Similarly in PV3, both T- and B- cell epitopes were joined with linkers L_2 and L_3 while, in PV4, we have exploited merely linear B- cell epitopes attached together with linker L_2. Amongst these two linkers, L_3 is a universal linker, which can enhance the proteasome processing along with immunogenicity, while L_2 is a flexible linker that can stimulate better immune response [42,77,82]. As exemplary vaccine is found to induce multi-immune response (B- and T-cell immune response), therefore in the designing of further PVs both the T- and B- cell epitopes were used so as to elicit humoral/cellular response [83]. The PV3 and PV5 were differing from each other with respect to linkers L_3 and L_2, respectively used for joining continuous B-cell epitopes. However, in case of designing a negative polypeptide vaccine control, linkers L2 and L3 were employed to connect non-HLA class I and II T-cell epitopes (Table 3). Fig. 3 (a, b) depicts the exemplar design of PV1 and PV3 with adjuvants A and B i.e., PV1A and PV3B. The advantage of using linkers and adjuvants used in the present study for designing of multi-epitope malaria PVs have been also revealed by several contemporary researchers against other diseases [36,84,85] to enhance the antigen processing and presentation ability as well as immunogenicity. Also, the cost effective Cholera toxin B subunit adjuvant is cytokines inducer (Th1 and Th2 response), which increases the antibody titration [86]. Thus, the use of both T-cell and B-cell epitopes together with linkers and adjuvants can increase the potential of PVs towards induction of multi immune responses.

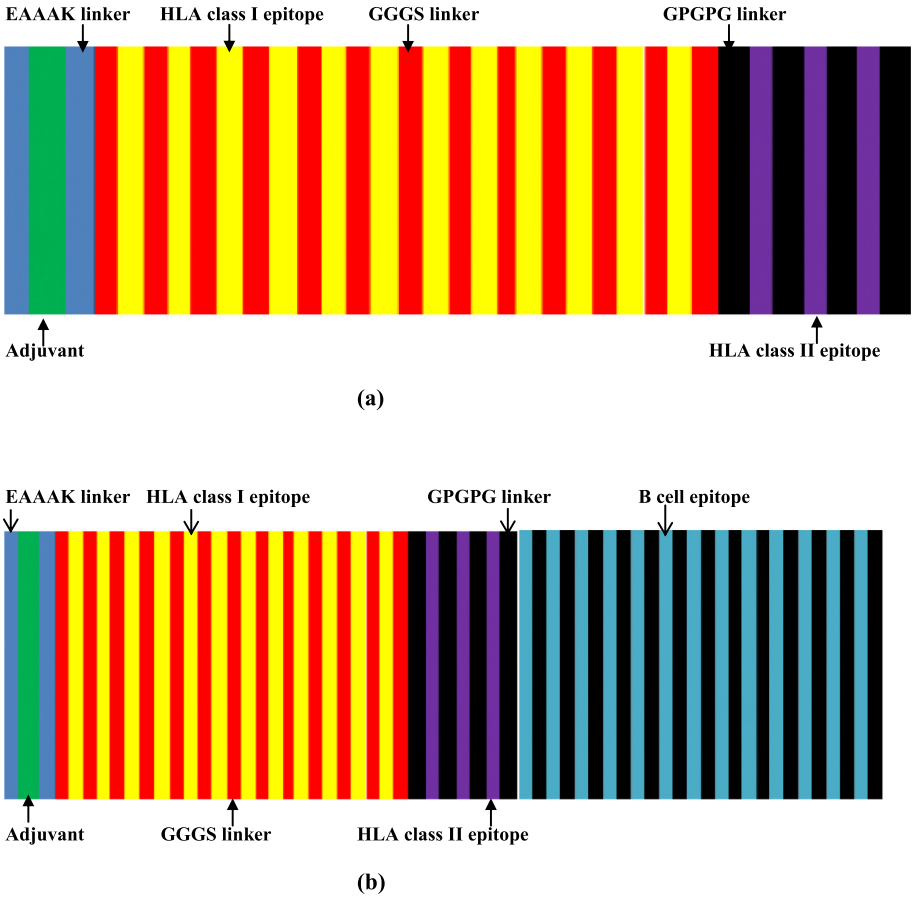


Fig. 3. Schematic diagram of polypeptide vaccines PV1A (a) and PV3B (b) in which adjuvant, T-cell epitopes (HLA class I and II), B-cell epitopes and linkers are shown in different colours.

Table 4
Details of molecular docking energies of polypeptide vaccines with their respective model number.

S. no.	Name of polypeptide vaccine/control	ClusPro 2.0 docking energy (Kcal/mol)	Model number
TLR2 receptor			
1	C1	−685.9	M1
2	PV1	−1153.1	M2
3	PV1A	−1275.5	M3
4	PV2	−1117.1	M4
5	PV2A	−1214.2	M5
6	PV3	N.A	N.A
7	PV3A	−1081.3	M6
8	PV4	−1180.9	M7
9	PV4A	−1115.7	M8
10	PV5	−1047.4	M9
11	PV5A	N.A	N.A
TLR4 receptor			
12	C2	−794.9	M10
13	PV1	−1070.7	M11
14	PV1B	−1111.1	M12
15	PV2	−1117.9	M13
16	PV2B	−1139.7	M14
17	PV3	N.A	N.A
18	PV3B	−1269.2	M15
19	PV4	−1166.7	M16
20	PV4B	N.A	N.A
21	PV5	−1076.5	M17
22	PV5B	−835.8	M18

N.A-not available.

3.4. Molecular docking of PVs with receptors TLR2 and TLR4

The TLRs, especially the surface one, viz. TLR2 as well as TLR4 are available not only on the immune cells, but also on epithelial cells and fibroblasts that recognizes PAMPs and bridge the innate as well as adaptive immunity of the host by regulating the balance between Th1 and Th2 type of responses [87–90]. For example, the merozoites stage of *P. falciparum* releases glycosylphosphatidylinositol (GPI) anchored surface antigens, which act as ligands recognized by both TLR1-TLR2 heterodimers and TLR4 homodimers of host immune cells. Such events indeed results in decreasing the parasitic load from host by triggering the production of various pro-and anti-inflammatory cytokines as well as antibody isotype switching [38–40,91,92,]. Thus, for enhanced protection, selection of respective TLR2 and 4 mucosal protein adjuvant A (CTB) and B (50s ribosomal L7/L12) in designed PVs could be the good choice against *P. falciparum* [77,78,86,93]. Even combining two distinct TLR agonists into an adjuvanted subunit vaccine have showed synergetic protective efficacy [94,95]. Altogether, these facts led to the hypothesis of using both TLR2 and 4 receptors agonists A and B, respectively in the designed PVs and subsequently docking experiment was performed to reveal the possible association amongst PVs and TLR [96,97]. For molecular docking, the tertiary structures of 15 PVs were predicted that revealed >80% of amino acids in favoured regions. Overall 22 docking studies were carried out using ClusPro2.0 tool including control C1 and C2 against receptors TLR2 and TLR4, respectively (Table 4). This resulted into total 18 docked models, i.e., M1 to M18 including 16 PVs and 2 controls. It is quite interesting to note that the PVs designed without adjuvants were also able to interact (dock) with TLR2 and TLR4 (having good energy scores) over control except PV3. Therefore, they might be capable to elicit innate immunity [98–100], which are in well agreement with earlier studies regarding the rapid production of IFN-γ [101,102]. Amongst 15 designed PVs, PV3, PV5A and PV4B were not able to dock by ClusPro tool with their respective receptors. So, a total 12

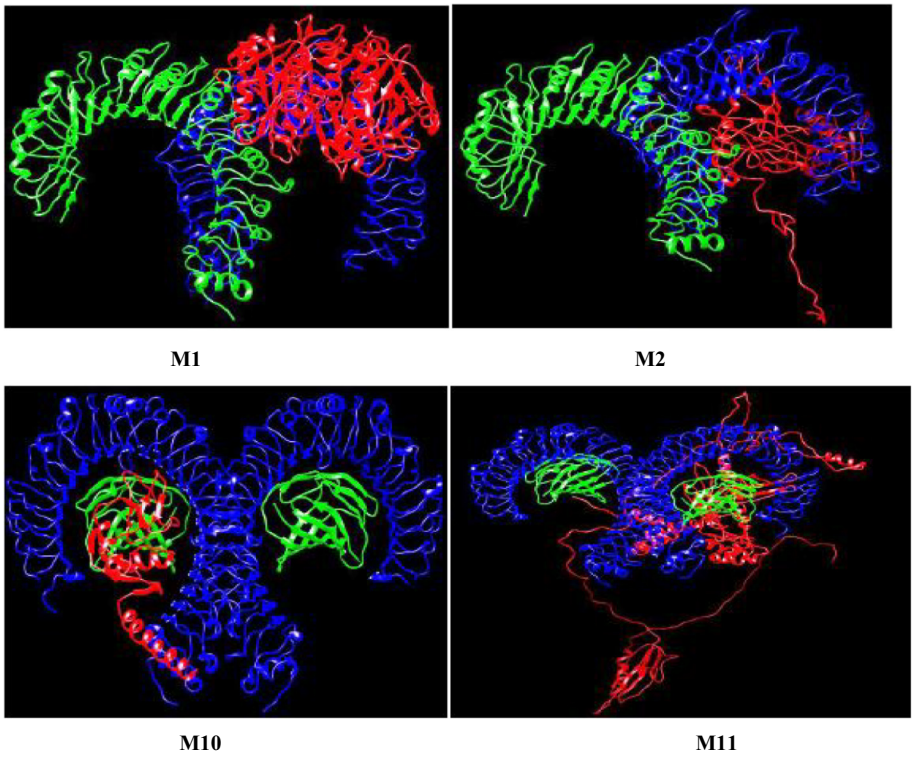
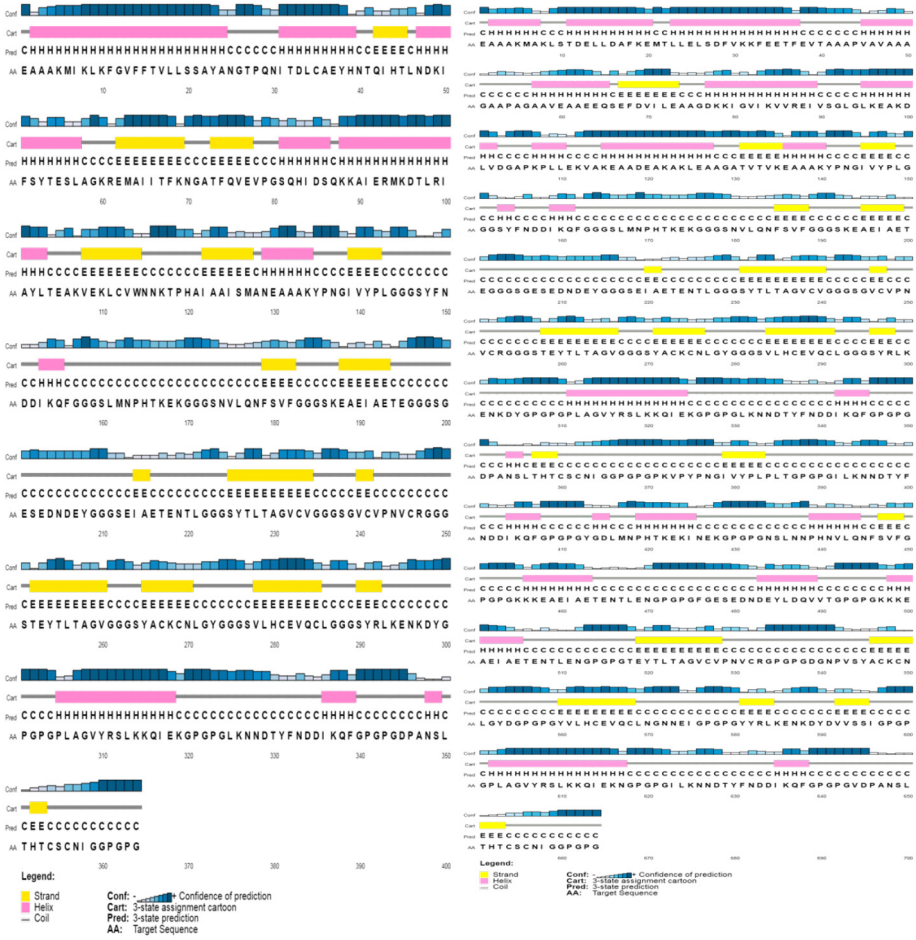


Fig. 4. Docking model of controls (C1, C2) and polypeptide vaccines. The models M1 (TLR2-TLR1-C1) and M10 (TLR4-MD2-C2) are controls while M2 (TLR1-TLR2-PV1A) and M11 (TLR4-MD2-PV3B) are polypeptide vaccines. In case of models M1 and M2, the TLR1, TLR2 and ligands (C1 and PV1A) are shown in green, blue and red colour, respectively whereas in models M10 and M11, TLR4, MD and ligands (C2 and PV3B) are shown in blue, green and red colour, respectively.

potential PVs with 16 docked models were obtained for TLR2-TLR1 (M2-M9) and TLR4-MD2 (M11-M18). The docking energy of control models M1 (−685.9 Kcal/mol) and M10 (−794.9 Kcal/mol) for complexes TLR2-TLR1-C1 and TLR4-MD2-C2 were found higher over designed potential PVs, which indicates that all the docked PVs have formed stronger immunological complexes over control ligands. Amongst the designed PVs without adjuvants (PV1, PV2, PV4 and PV5), PV4 showed the lowest docking energies −1180.9 Kcal/mol and −1166.7 Kcal/mol with respect to TLR2 and TLR4 receptors, correspondingly. These clearly indicated that the PVs without adjuvants have interacting domain to induce innate immune system. This is in agreement with the recent study where human TLR4-derived self-assembling peptide nanoparticles have been used as non toxic vaccine adjuvant with filarial antigenic protein to induce the immunological responses in mice [103]. Besides these, the linker L2 has been utilized in the designing of PV1A, PV3B and PV4, which can provide better flexibility during interaction as compare to L1 and L3. Amongst the two adjuvants used in designing of PVs, average docking score of PVs (PV1, PV2 and PV3) involving cholera toxin B subunit was lower (−1190.3 Kcal/mol) compare to PVs (PV1, PV2 and PV3) involving 50S ribosomal L7/L12 (−1173.3 Kcal/mol) (Table 4). However, based on overall docking score, PV1A (−1275.5) and PV3B (−1269.2) against receptors TLR2 and TLR4, respectively were selected as leading PVs for further structural and functional analysis (Fig. 4).

3.5. Comparative evaluation of structural and functional properties of leading PVs with positive as well as negative vaccine controls

The negative GRAVY values of both PVs PV1A (−0.377) and PV3B (−0.479) were pointing towards their hydrophilic nature (that exposed on outer surface) and, therefore may elicit elevated humoral immune response [93]. Generally, *in vitro* protein stability is determined by instability index <40. Considering this, the present study depicted PV1A and PV3B as stable proteins with their corresponding instability index values of 36.35 and 26.22. However, *in vivo* half-life of PV1A and PV3B showed >10 h and, therefore reflecting the stabilities of these two PVs, which might enhance the durability as well as strength of immune response [104,105]. The leading PVs, i.e., PV1A and PV3B were predicted as probable antigens in this study using several antigenicity forecasting tools viz. VaxiJen, ANTIGENpro, protein antigenicity prediction and Secret-AAR including SPAN at default threshold values. Nevertheless, non-allergenicity of PV1A and PV3B were forecasted by AllergenFP tool at threshold value >0.8. Also, the secondary structure analysis (SSA) of a protein is beneficial for understanding its folding, stability as well as function [106–110]. In this context, the present study revealed alpha helices of 31.31 and 25.75%, β-strands of 9.89 and 16.71% and coils of 58.79 and 57.53% for PV1A and PV3B, respectively (Fig. 5). The predicted tertiary structures of PV1A and PV3B were refined by ModRefiner tool in which the Ramachandran plot exhibited respective favoured regions of 92.3 and



(a) (b)

Fig. 5. Predicted secondary structural elements (H: helix, E:beta strand, C: coil) of PV1A (a) and PV3B (b) by PSIPRED. The bar chart represents the percentage of confidence.

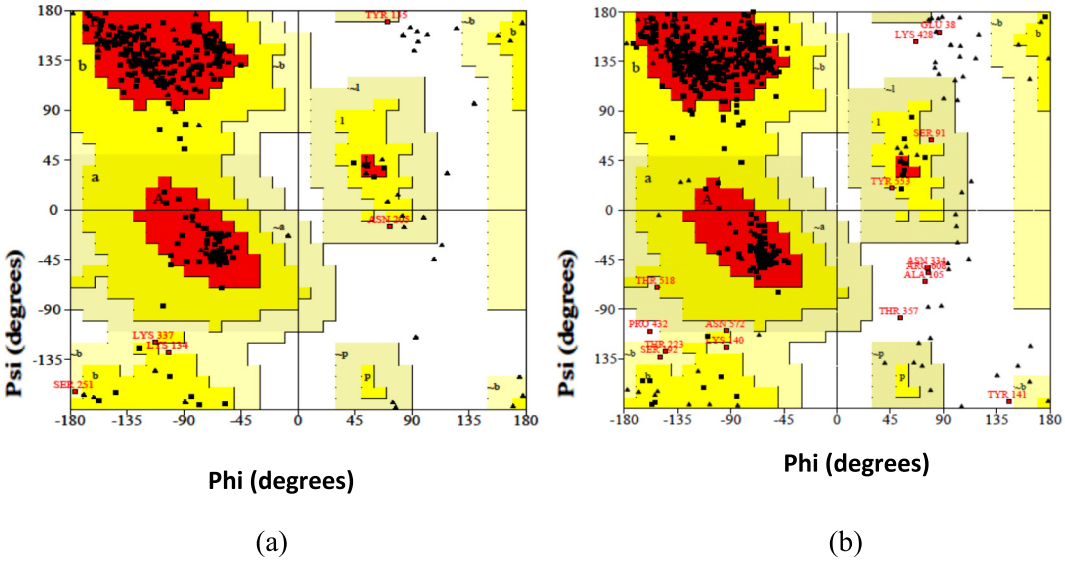


Fig. 6. Evaluation of three dimensional models of PV1A (a) and PV3B (b) using Ramachandran plot. The glycine amino acids are represented by black triangles while other amino acids of polypeptide vaccines are displayed in black squares.

91.4% as well as allowed regions of 6 and 5.7%. These values indicated high quality and stability of refined protein structure model based on Ramachandran plot as described previously [111] (Fig. 6). Further, PV1A and PV3B were forecasted to possess respective 8 and 18 linear as well as 104 and 315 discontinuous B cell epitopes at default thresholds (Supplementary Table S4). These leading PVs were also predicted to be involved in multi-organism process as well as cell adhesion and immune system process, respectively, as predicted by DeepGOPlus tool, which is based on deep convolutional neural network model and Gene Ontology (GO) scheme. The overall structural and functional analysis of leading PVs showed comparatively similar properties over positive vaccine controls C3 and C4 (Table 5). Thus, the leading polypeptide vaccines PV1A and PV3B have the capability to induce both humoral as well as cellular immune responses. However, the orally administered polypeptide vaccines suffer from the poor stability, insolubility, weak bioavailability and low immunogenicity due to acidic environment of the upper GI-tract and inefficient delivery to the mucosa-associated lymphoid tissue. Therefore, genetically engineered *L. lactis* expression host can be used for production and delivery of vaccine antigens due to several advantageous properties viz. easy and safe production as well as storage, survival in gastric environment and self-adjuvanticity [112,113].

3.6. Immune simulation of leading PVs

In the course of human malaria infection, pro-inflammatory (TNF-α, IFN-γ and IL-12) and anti-inflammatory (IL-4 and IL-10) cytokines were produced by Th1 and Th2 cells, respectively [114]. In addition, cytotoxic T lymphocyte, natural killer cells and macrophages were activated by elicitation of IL-4, which helps to control pathogen effect [115,116]. Even, the most successful vaccine candidate of malaria, RTS,S was reported to elicit IFN-γ, IL-2, IgG titers, and activation of CD4+ T cell responses [72,117]. In this background, the present study involved the immune simulations of PV1A and PV3B using C-ImmSim tool along

Table 5
Comparative evaluation of structural and functional properties of positive vaccine controls (C3, C4) and leading polypeptide vaccines (PV1A and PV3B).

Properties	Parameter/tools	Value/Score/Probability			
		C3	C4	PV1A	PV3B
Physicochemical	Molecular weight	2.44 kDa	5.05 kDa	3.79 kDa	6.80 kDa
	Isoelectric point (pI)	6.24	8.67	5.72	4.75
	Instability index (II)	28	22.78	36.35	26.22
	GRAVY	−0.88	−0.32	−0.38	−0.48
Antigenicity	VaxiJen	0.65	0.67	0.56	0.46
	ANTIGENpro	0.67	0.94	0.94	0.90
	Protein antigenicity prediction	0.99	1.02	1.01	1.01
	Secret-AAR	42.6	27.59	33.18	31.67
Adhesion	SPAAN	0.32	0.82	0.76	0.45
Recombinant protein solubility	RPSP	0.1	100	0.0	99.9
	Protein-Sol	0.53	0.28	0.48	0.75
	CamSol	2.00	0.34	0.74	1.56
	SOLPro	In soluble (0.54)	In soluble (0.78)	Soluble (0.87)	Soluble (0.98)
Secondary structure stability	alpha helix	9.9%	20.29%	31.31%	25.75%
	β-strands	25.94%	31.83%	9.89%	16.71%
	coils	64.15%	47.86%	58.79%	57.53%
Protein function	DeepGOPlus	Killing of cells of other organism and regulation of cell processes	Molecular and biological process	Multi-organism process	Immune system process and cell adhesion

Table 6
Details of immune simulation results of positive controls (C3 and C4) and leading PVs (PV1A, PV3B).

Types of immune response	C3	C4	PV1A	PV3B
Antigen count (I^{st} dose)	Decreases to zero count after 5th day of injection	Decreases to zero count after 5th day of injection	Decreases to zero count after 5th day of injection	Decreases to zero count after 5th day of injection
Antigen count (II^{nd} and III^{rd} dose)	Decreases to zero after 2nd day of injection	Decreases to zero after 2nd day of injection	Decreases to zero after 2nd day of injection	Decreases to zero after 2nd day of injection
Antibody titers (IgG + IgM and IgG1 + IgG2)	Elicited high level of antibody titers	Elicited high level of antibody titers	Elicited high level of antibody titers	Elicited high level of antibody titers
Total B cell population per state at end of III^{rd} dose (cells per mm^3)	~ 3000	~ 2700	~ 2800	~ 2700
Active B cell population at end of III^{rd} dose (cells per mm^3)	~ 2900	~ 2700	~ 2700	~ 2500
Plasma B lymphocytes at end of III^{rd} dose (IgG1)	~ 550	~ 550	~ 550	~ 500
IFN-γ (ng/ml)	~ 7.2×10^5	~ 7.4×10^5	~ 6.9×10^5	~ 6×10^5
TGF-β (ng/ml)	~ 9.2×10^5	~ 6.5×105	~ 8.9×10^5	~ 1.1×10^6
IL-2 (ng/ml)	~ 2×10^6	~ 2.1×10^6	~ 1.8×10^6	~ 1.5×10^6
IL-10 (ng/ml)	~ 9×10^4	~ 9×10^4	~ 9×10^4	~ 9×10^4
IL-12 (ng/ml)	~ 9×10^4	~ 11×10^4	~ 9×10^4	~ 8×10^4
Memory T-helper lymphocytes count (cells per mm^3)	~ 7000	~ 7100	~ 6300	~ 5200
Active T-cytotoxic lymphocytes population per state (cells per mm^3)	~ 900	~ 1100	~ 1100	~ 1100
Active macrophages (cells per mm^3)	~ 90	~ 90	~ 80	~ 80
Macrophages presenting (cells per mm^3)	~ 110	~ 145	~ 100	~ 90

with the positive vaccine controls (C3, C4) (Table 6). The C3 is a self-assembling polypeptide nanoparticle (SAPN) based *P. falciparum* malaria vaccine candidate that elicit IFN-γ, TNF-α, IL-4, IL-10 and IgG antibody titers in mice [48,118]. The C4 is a novel fusion protein of *Staphylococcus aureus* that indicated a high titer of specific antibodies (IgG1 and IgG2a) responses and decrease the viable cell counts through elicitation of mixture of Th1, Th2, and Th17 immune responses. The simulation results were displayed no alteration in antigen level as well as immunogenic responses except generation of IFN-γ against negative control (C5). While the positive vaccine controls as well as PV1A/PV3B showed drastic decrease in antigen counts that ultimately reached to zero after 5th day of injection (Supplementary Fig. 1). Besides these, they were also involved in the elicitation of B lymphocytes, cytotoxic T lymphocytes, helper T lymphocytes and macrophages responses that lead to generation of cytokines (IFN-γ, TGF-β, IL-2, IL-10 and IL-12) as well as antibody titration (IgG + IgM and IgG1 + IgG2).

The generation of high level of IgM under study pointing towards better primary immune response as well as decrease in antigen level with enhancement in B cell population with antibodies (IgM, IgG1 + IgG2 and IgG + IgM), which further reflecting good secondary and tertiary immune responses. These results agree well with the earlier finding of Shey et al. [66]. Utilizing similar *in silico* approach, the leading PVs designed and characterized in the present study was compared with the wet lab experimental data of Kaba et al. [48] and Ahmadi et al. [49] (Table 6). The predicted result of immune simulation indicated the elicitation of macrophages, B and T lymphocytes for the production of cytokines (IFN-γ, TGF-β, IL-2, IL-10 and IL-12) as well as antibodies (IgG + IgM and IgG1 + IgG2) against proposed top two PVs, which seems to be similar observations obtained by aforementioned research group in mice. Fig. 7 summarizes the comparative account on immune simulations of C3, C4 with one of leading PVs (PV1A) having higher potential to induce protective immune responses that might be owing to use of Cholera toxin B subunit adjuvant. Therefore, designing strategy used in PV1A/PV3B could be highly effective in stimulation immune responses. Moreover, validity of immunoinformatics tools for prediction of epitopes, protective immune response analysis, constructing chimeric multi-epitope vaccine, assessment of vaccine safety as well as efficacy and immunization modelling have been exercised in the last five years with >500 literatures in the PMC database that assisted in the preclinical and clinical studies of several vaccine project including Hepatitis B Virus, Dengue, *Schistosoma haematobium*, *Treponema pallidum*, *S. aureus*, *Trypanosoma cruzi*, *Helicobacter pylori*, Middle East Respiratory Syndrome Coronavirus, Zika virus [26,45,119,120]. Therefore, the use of bioinformatics tools for prediction of antigenicity, epitopes and molecular interaction are convenient and adequate approach in vaccine design and development [47,84,121].

3.7. Molecular docking of leading PVs with antibodies IgG1 and IgG3

When an antigen interacts with antibody it induces the humoral immune response and helps in clearance of pathogen. The IgG antibodies (named in order of decreasing abundance IgG1, IgG2, IgG3, and IgG4) are one of the most abundant pathogens neutralizing molecules found in human serum. These antibodies share >90% amino acid sequence identity but each subclass has exclusive effector properties including half-life, epitope binding, immunological complex formation, complement activation, triggering of effector cells and placental transport. Moreover, the IgG profile of a given individual is determined by their inherited allotypes that can potentially influence the clinical manifestation of the immune response [122]. However, broadly neutralizing antibodies (bNAbs) have been found in a rare population of patients that control the infection [123–125]. These bNAbs tend to target different conserved antigenic regions exposed on the outer surfaces of a pathogen across the circulating strains. Here, in the present study, a protein-protein global docking method (ClusPro server) was used to reveal the shape complementarity between PVs (as ligands) and the interacting domains of antibodies IgG1 and IgG3 (as the receptors) to eliminate the need of a long term exposure of malaria patients to selected antigen mimetics PV1A and PV3B involving the epitopes (B1, B4 and B5) of *P. falciparum* strains. These antibodies could be considered as bNAbs if they found with a well detectable neutralization activity in wet lab experimental studies [126–130]. Furthermore, the respective source proteins P28, P25 and MSP1of epitopes B1, B4 and B5 have been characterized as leading vaccine candidates [130,131]. Also, the antibodies IgG1 and IgG3 have been found associated with human malaria protection [132,133]. Thus, a structure based vaccinology approach could be exploited to predict the probability of potent PVs that might be able to block infection even more effectively

[134]. These data lead to provoke the molecular interaction studies of leading PVs (PV1A as well as PV3B) along with co-crystallized control epitopes towards antibodies IgG1 and IgG3 (Table 7 and Fig. 8 B1- B6). For IgG1 and IgG3 antibody the obtained lowest score for molecular docking of IgG1 and IgG3 control was −449.4 and − 630.9, correspondingly. The obtained ClusPro docking energies and PatchDock scores of PV1A and PV3B against both antibody receptors IgG1 and IgG3 were found lower as compared to their respective controls C6 and C7 (Table 7). Besides these, PV1A showed hydrogen bond interaction through amino acids Leu-9, Val-13, Phe-15 and Lys-326, Asp-329 of B5 epitope with antibody receptors IgG1 and IgG3, correspondingly. Moreover, PV3B exhibited similar interaction with IgG1 and IgG3 through Gly-255, Gly-280 and Gly-525 of B1, Leu-551 of B4, respectively [135].

3.8. Molecular dynamics of the PV1A/PV3B -TLR2/TLR4 complexes

Molecular dynamics study is crucially for evaluating the stability of the protein-protein complex, which can be determined by comparing

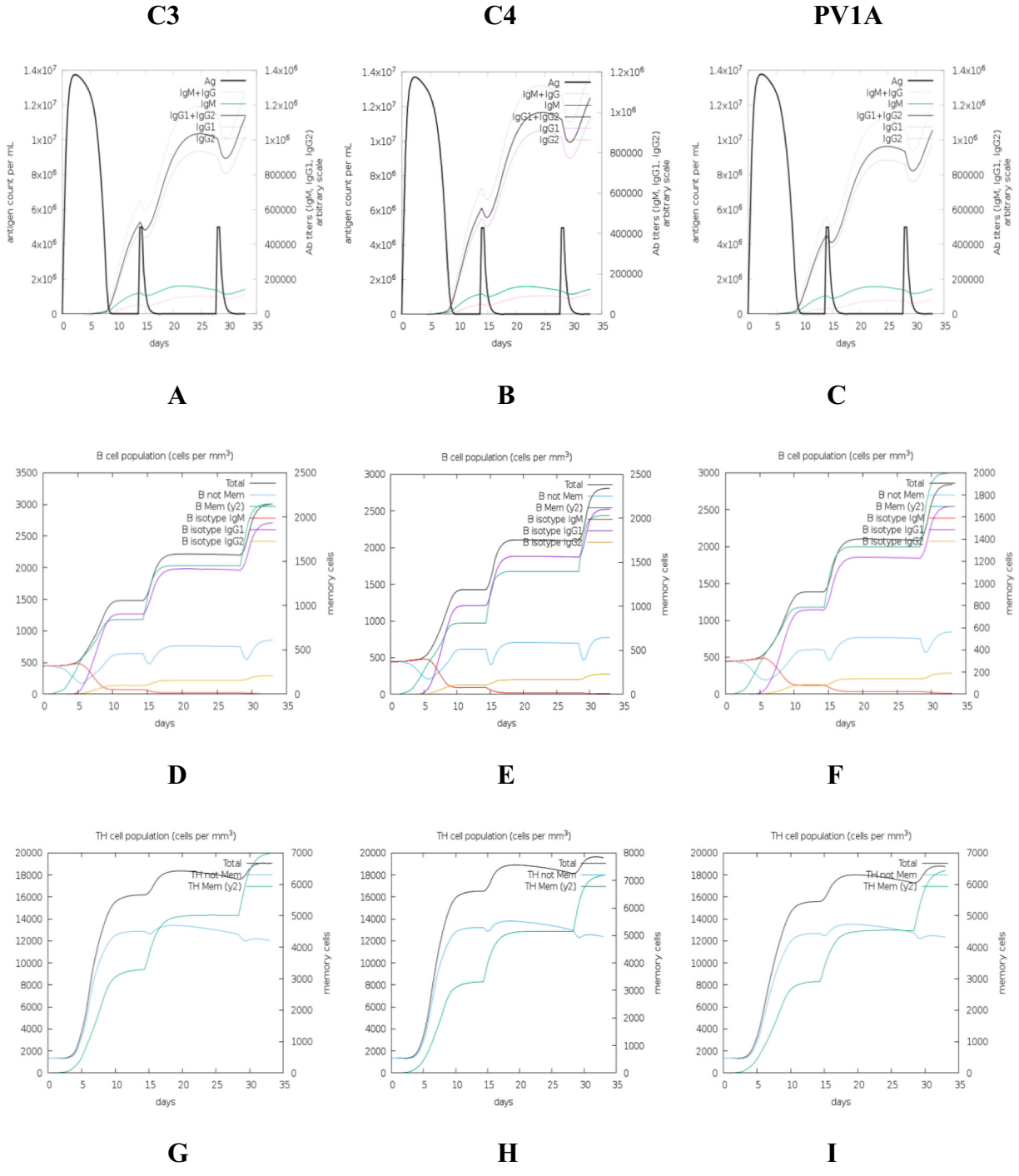


Fig. 7. Immune simulation results of positive vaccine controls C3 (A, D, G, J, M) and C4 (B, E, H, K, N) along with test polypeptide vaccine PV1A (C, F, I, L, O).

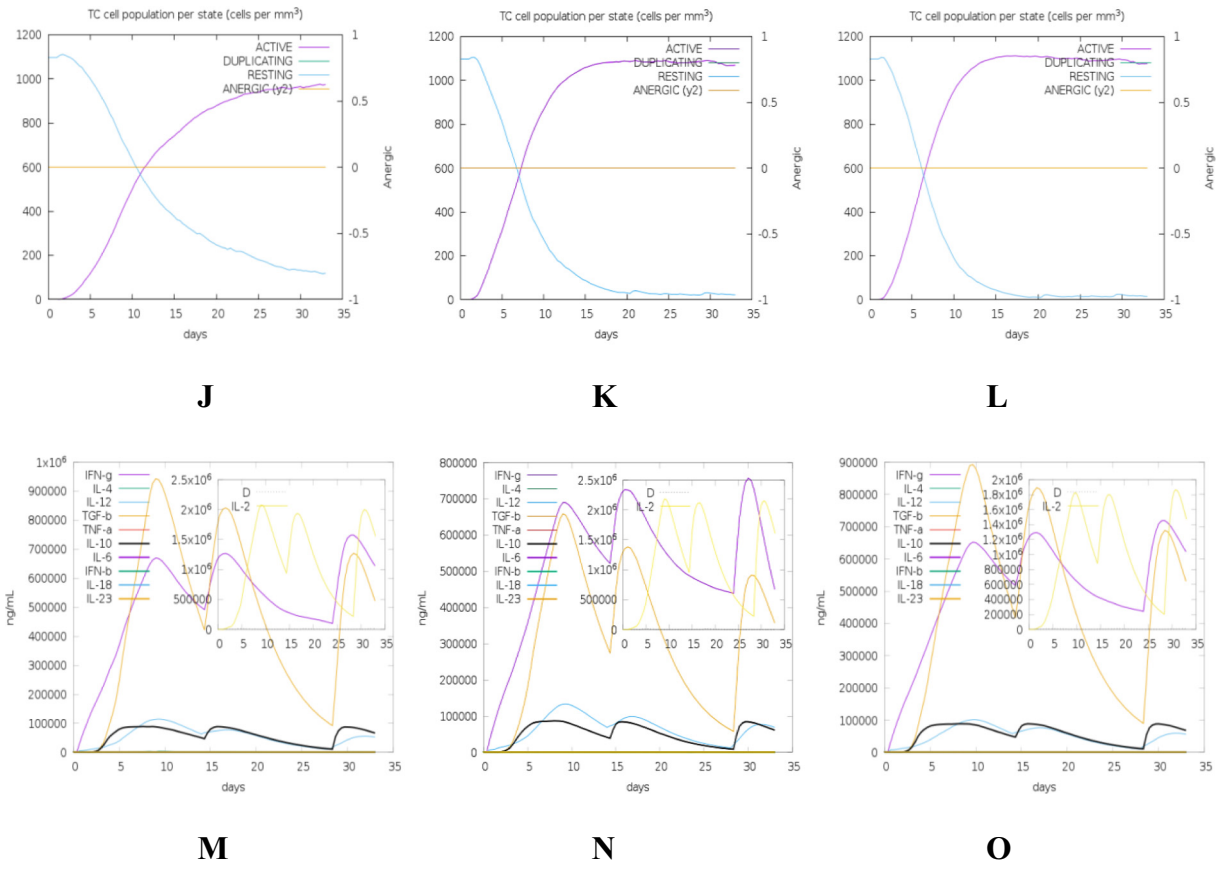


Fig. 7 (*continued*).

necessary protein dynamics to their normal modes [136]. The NMA allowed the demonstration of docked protein-protein complex mobility and stabilization. Fig. 9(a, b) showed the 3D interaction model of respective polypeptide vaccines PV1A and PV3B complexed with TLR2 and TLR4. The direction of each amino acid residue was given by arrows and the length of the arrow corresponded to the degree of mobility. It also provided the profiles of deformability (c, d), mobility (e, f), eigenvalue (g, h), variance map (i, j), covariance matrix (k, l) and elastic network (m, n). The value of NMA-B-factors (mobility) indicated the relative amplitude of the atomic displacements around the equilibrium confirmation. While the deformability calculated the gradient of the atomic displacements summed over all modes at every atomic position. High values are expected in flexible regions such as hinges or linkers between domains, whereas low values usually correspond to rigid parts. The obtained higher and lower values of maximum mobility and deformability for PV3B (2.038E+02, 1.088E−06) indicated towards more flexible regions compare to PV1A (3.443E+01, 4.740E−06). The eigenvalue associated to each normal mode represented the motion stiffness. Lower the eigenvalue, easier the deformation i.e., lower energy is required to deform the complex structure. The respective eigenvalues for PV1A and PV3B complexed with TLR2 and TLR4 were found 1.064E−06 and 7.498E−09 that indicated the greater stability of complex PV1A-TLR2. The individual and cumulative variances associated to each normal mode were inversely related to the eigenvalue. The covariance matrix indicated the coupling between pairs of residues, i.e. whether they experience correlated (red), uncorrelated (white) or anti-correlated (blue) motions whereas elastic network graph characterizes pairs of atoms connected by springs and each dot in the graph represented one spring between the corresponding pair of atoms [137].

Table 7
Molecular docking details of ClusPro docking energy and PatchDock score of PV1A and PV3B as well as controls C6 and C7 towards antibodies IgG1 and IgG3.

Model number	Receptor (antibody)	Ligand (PV/control)	ClusPro 2.0 docking energy (Kcal/mol)	PatchDock Score
B1	IgG1	C6	−449.4	6482
B2		PV1A	−918.8	18,294
B3		PV3B	−929.0	18,512
B4	IgG3	C7	−630.9	7834
B5		PV1A	−1058.5	22,930
B6		PV3B	−1025.1	19,814

3.9. Codon optimization, in silico cloning and expression of PV1A and PV3B

The sequence length of obtained cDNA for PV1A and PV3B were 1092 bp and 1992 bp, correspondingly. The Codon Adaptation Index (CAI) values for PV1A and PV3B were 0.9857 and 0.9584, respectively. For reliable optimization of codon, CAI value should lie between 0.9 and 1.0 [138]. However, the GC content of improved DNA sequence of PV1A and PV3B were found 42.12% and 43.12%, which are lying in the optimal range (30% to 70%) that could be easily expressed in any suitable expression host [139]. Although, *P. falciparum* antigens could be expressed in *E. coli* but require the codon harmonization (reduction of amino acid misincorporation) to improve the immunogenicity [140]. In the present study, the solubilization probability of recombinant proteins (PV1A and PV3B) to be expressed in *E. coli* revealed by bioinformatics tools RPSP, Protein-Sol, CamSol and SOLPro was lower compare to positive vaccine controls (C3, C4) that indicated to look for

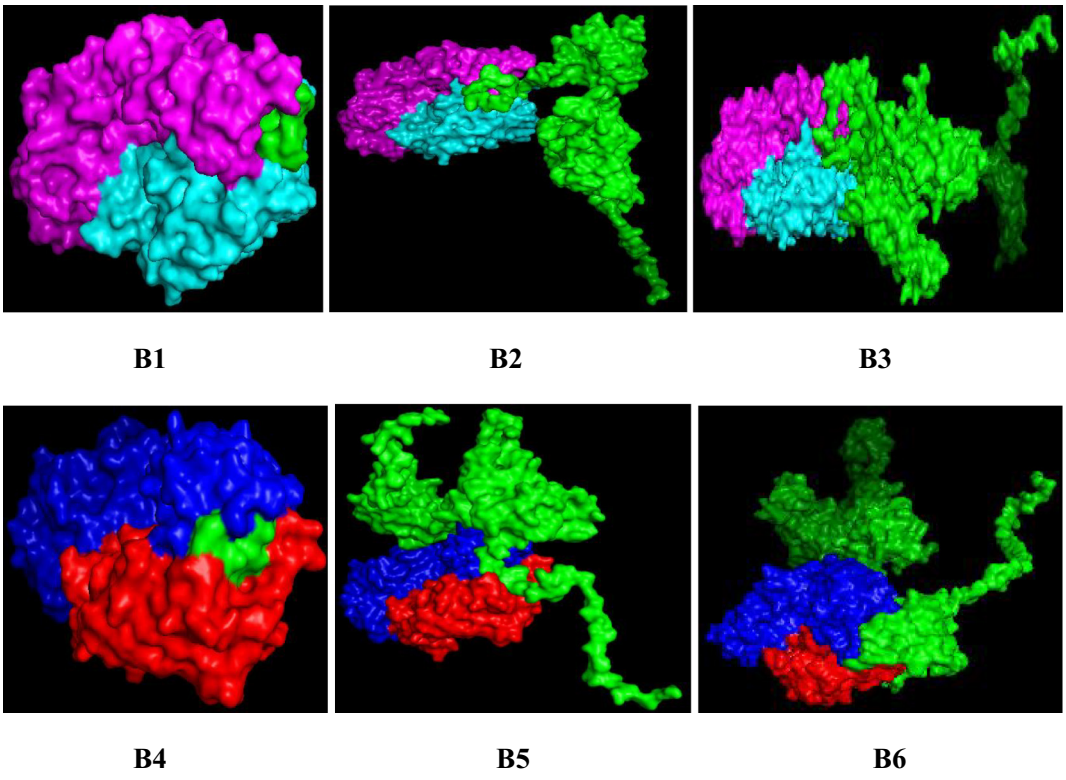


Fig. 8. Visualization of docking models of C6 (B1), PV1A (B2), PV3B (B3) against antibody IgG1 and C7 (B4), PV1A (B5), PV3B (B6) against antibody IgG3. The respective colours of heavy and light chains of IgG1 and IgG3 are shown in cyan and red as well as magenta and blue.

alternative expression host (Table 5). Additionally, *L. lactis* was used as expression host alternative to *E. coli* due to following advantageous properties i) generally recognized as safe (GRAS) microorganism ii) lack of outer membrane (iii) insignificant extracellular proteolysis activity (iv) free of endotoxins (v) no lipo-polysaccharide contamination (vii) accommodates cysteine-rich proteins (vii) accessibility of both inducible and constitutive genetic control systems (viii) able to express prone-to-aggregate and/or difficult-to-purify proteins (ix) presentation to the host immune system in the context of micro-particles to avoids the immunotolerance, which is normally provoked by oral delivery of soluble antigens (x) exhibits similar codon bias to *P. falciparum,* which makes it efficient protein expression and secretion system to outer surface that could easily interact with host immune system [113,141–143]. In recent years, several wet lab studies have confirmed the utilization of *L. lactis* as an expression host to produce properly folded, pure and stable chimeric and/or single antigenic proteins of many pathogens that elicited high levels of functional antibodies/cytokines including *P. falciparum* [144–148], *Mycobacterium bovis* [149], *Mycobacterium tuberculosis* [150], *Helicobacter pylori* [151], Polish avian H5H1 influenza [152], cancer [153] and *Staphylococcus aureus* [154]. Moreover, *L. lactis*-mediated delivery of DNA vaccines also lead to the expression of post-translationally modified antigens by host cells resulting in presentation of conformationally restricted epitopes to the immune system for induction of both cellular and humoral immune responses [112].

Also, with the aforementioned properties, the last two decades witnesses the use of genetically engineered *L. lactis* system as effective oral based vaccine vehicles for delivering antigens of viruses, bacteria and parasites to elicit both systemic and mucosal immunity [155–158]. Finally, the size of PV1A and PV3B recombinant DNA (obtained after insertion of cDNA into pIL1 expression vector) was observed as 7477 bp and 8377 bp, respectively which lies inside the ORF and could be translated into respective protein sequences with four additional amino acids (MCKC) at the N-terminus (Fig. 10). Therefore, an ideal multi-epitope polypeptide vaccine should compose of a series of epitopes and/ or adjuvants that can elicit simultaneous and strong innate and adaptive (humoral and cellular) immune responses involving T- and B-cells responses against a targeted pathogen of malaria. In contrast to traditional killed/live attenuated or single-epitope vaccines, multi-epitope vaccines have distinctive properties such as involvement of numerous HLA-restricted epitopes derived from different antigens of various *Plasmodium* species/strains that can be recognized by various T-cells, bringing of additional components with adjuvant capability to enhance the immunogenicity as well as long-lasting immunity and reduction of unnecessary parts that can trigger the pathogenicity/adverse effects. Well-designed multi-epitope vaccines with such advantages should become powerful prophylactic and therapeutic agents against malaria infections. However, the present problems in the field of multi-epitope vaccine design include the selection of appropriate candidate antigens and systematic arrangement of their immunodominant epitopes for effective oral delivery through virus-like particles and SAPN. The present study successfully utilized the immunoinformatics tools for prediction of suitable epitope ensemble of target proteins for designing a multi-epitope malaria oral vaccine.

4. Conclusion

Surprisingly, so far no licensed malaria vaccine is available in the market to protect world-wide human populations regardless of decades of research. One of the major bottlenecks of malaria vaccine development is immune escape mechanism of pathogen through antigenic variation and/or HLA diversity. The designed PVs (PV1A and PV3B) under present study may overcome the aforementioned issues

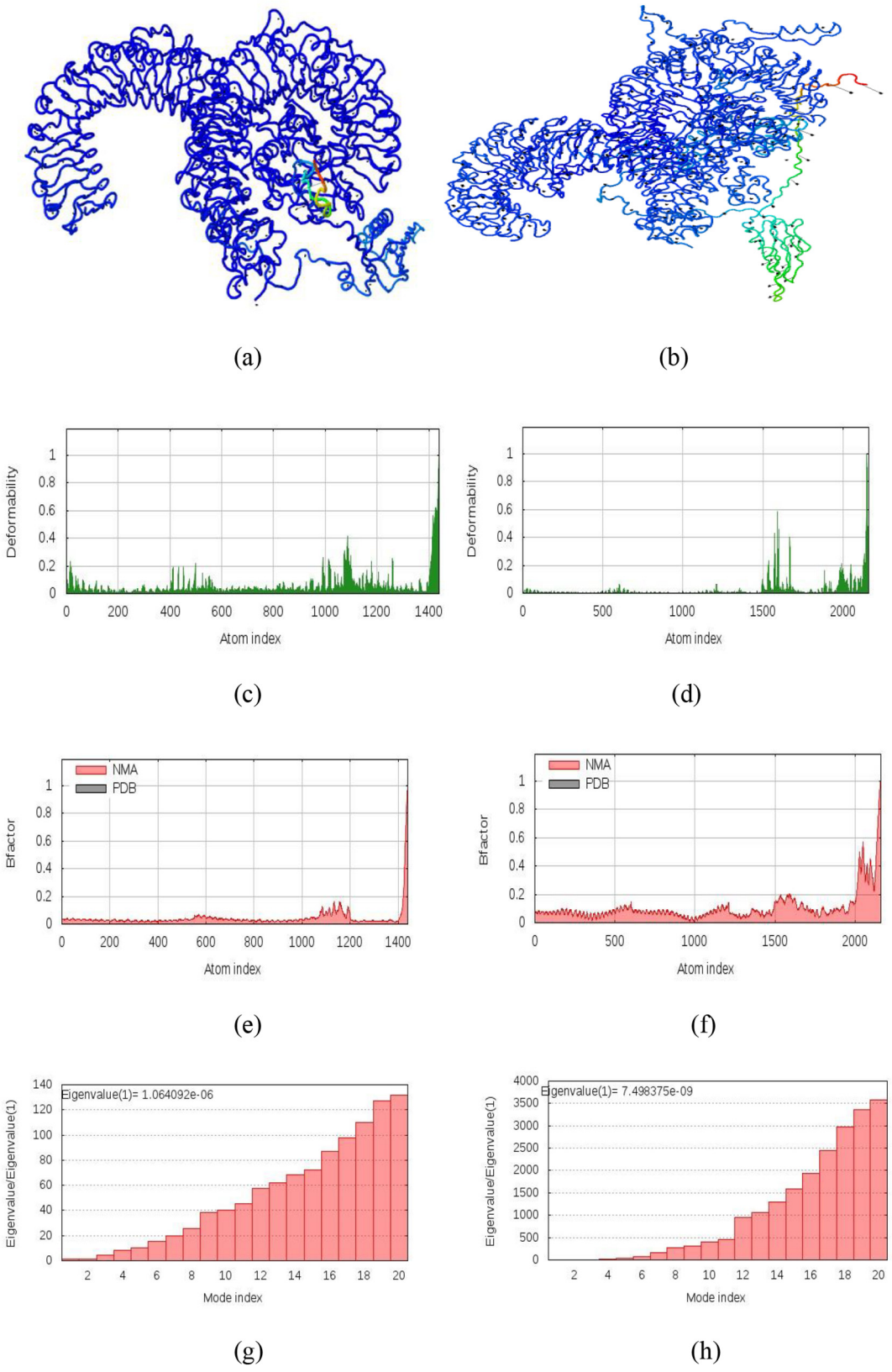


Fig. 9. Molecular dynamics simulation of respective polypeptide vaccines (PV1A and PV3B) complexed with TLR2 and TLR4 (a, b), deformability (c, d), eigenvalue (e, f), variance map (g, h), correlation matrix (i, j) and elastic network model (k, l). Coloured bars showed the individual (red) and cumulative (green) variances in the correlation matrix. In the elastic network graph, dots are coloured according to their stiffness, the darker greys indicate stiffer springs and vice versa (m, n).

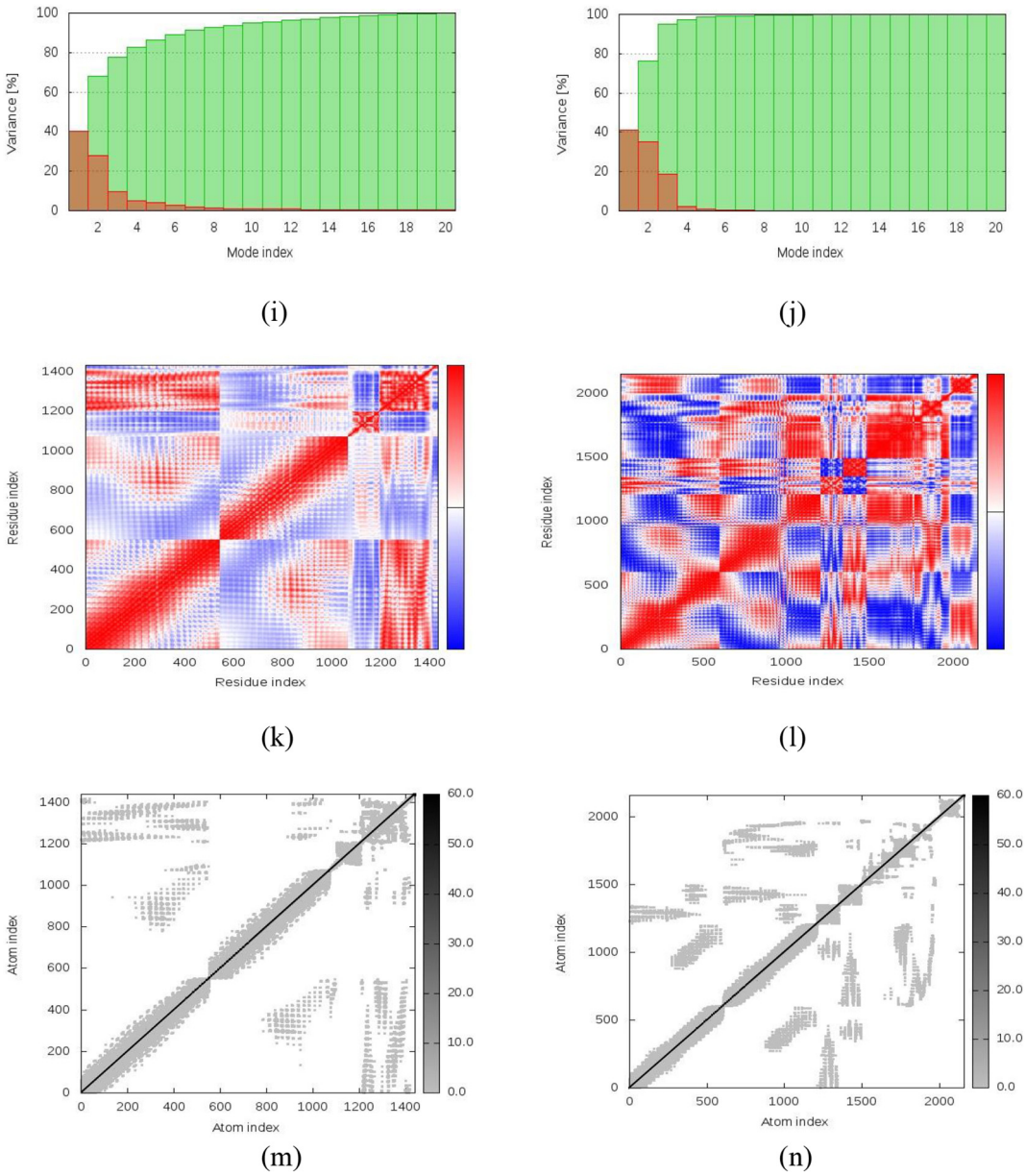


Fig. 9 (*continued*).

as they possess both B- and T-cell epitopes derived from 5 antigenic proteins that involve multi -stages of pathogen life-cycle with world-wide human population coverage (99.46%). Moreover, these PVs have the higher potential to elicit both innate (TLR2 and TLR4) and adaptive (cellular and humoral) immune responses. However, this warrants further experimental validation so as to evaluate their efficacy in the preclinical studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.04.191.

Author statement

Manisha Pritam: Performed the experiments and analyzed the results.

Garima Singh: Involved in analyzing the results.

Suchit Swaroop: Involved in study design.

Akhilesh Kumar Singh: Involved in designing of study and revision of the manuscript.

Brijesh Pandey: Contributed substantially in review and editing of revised manuscript.

Satarudra Prakash Singh: Involved in designing of study, analyzing results and finalized the manuscript.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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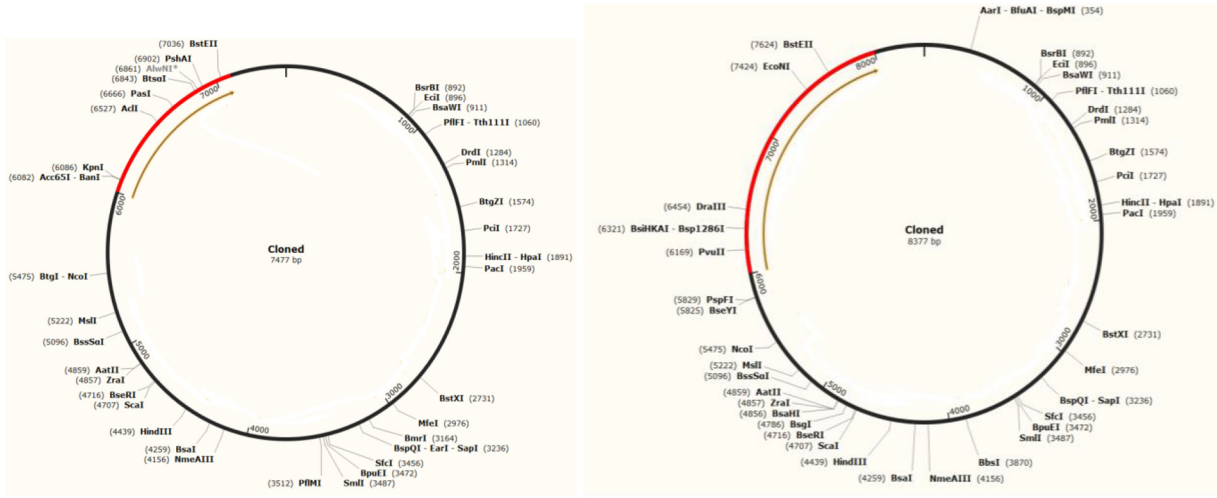

Fig. 10. The cloning map of leading polypeptide vaccine PV1A (a) and PV3B (b) into pIL1 expression vector. The plasmid DNA, inserted cDNA and ORF are shown in black, red and orange colour, respectively.

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