

1 Biological, immunological and functional properties of two novel multi-variant
2 chimeric recombinant proteins of CSP antigens for vaccine development against
3 *Plasmodium vivax* infection
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Abstract:

The circumsporozoite protein (CSP) of the *Plasmodium vivax* that carries on of three repeat families (VK210, VK247, and the *P. vivax*-like) is a major target of pre-erythrocyte vaccine. In the present study, computer modelling was employed to select the most appropriate structure of chimeric proteins similar to their native counterpart that included the conserved and different arrangements of the repeat regions of the *P. vivax* CSVK210 and VK247 types. DNA encoding the selected chimeras (named CS127 and CS712) were synthetically constructed based on *E. coli* codons and then cloned and expressed. Mouse monoclonal antibodies (mAbs; anti-Pv-210-CDC and -Pv-247-CDC), recognized the chimeric antigens in ELISA, indicating correct conformation and that the B-cell epitopes are accessible. IgG reactivity in plasma samples from 221 Iranian patients with acute *P. vivax* were also tested by ELISA, and showed that only 49.32% of the examined patients had positive antibody responses to both CS127 and CS712 proteins with no statistically significant ($P = 1.000$, McNemar's test). IgG1 (48%, $OD_{490} = 0.777 \pm 0.420$ for CS127; 48.41%, $OD_{490} = 0.862 \pm 0.423$ for CS712) was the dominant subclass, with no statistically significant difference found for both chimeras ($P > 0.05$; Wilcoxon signed ranks test). The heparan sulphate and HepG2 cells binding assays demonstrated that both proteins bound to immobilized heparan sulphate and hepatocyte cells in a concentration-dependent manner saturable at 80 $\mu\text{g/mL}$. Additionally, mice anti-CS127 and -CS712 antibody recognized the native protein present on the surface of *P. vivax* sporozoite with high intensity, confirming the presence of common epitopes between the recombinant forms and the native proteins. In summary, although both designed constructs had structurally different characteristics at the molecular level, the expression levels were satisfactory, and chimeras had a conformational structure with biological function that signified their potential for use in the development of vivax- based vaccine.

Keywords: *Plasmodium vivax*, vaccine, circumsporozoite protein, chimeric VK210/VK247, conformational structure, biological function

1. Introduction

Plasmodium vivax is the most widespread species of *Plasmodium* outside Africa and is predominant in countries where malaria is in pre-elimination or elimination phase (WHO, 2014). Some strains of *P. vivax* are capable of causing delayed infections, by lasting for several months in the liver stage as hypnozoites, that increase transmission (Robert et al., 2005) and form an obstacle to control program. There are many reports on the severe forms of malaria caused by *P. vivax* infection (Lomar et al., 2005; Rodriguez-Morales et al., 2006; Tjitra et al., 2008; Price et al., 2009; Kochar et al., 2009; Nadkar et al., 2012). Therefore, there is a need for more efforts and investments to develop a vaccine to help control, and eventually eliminate *P. vivax*.

One of the approaches for a malaria vaccine is eliciting the sterile immunity against the pre-erythrocytic stage (Heppner, 2013) by blocking sporozoite and/or hepatocyte invasion and the development of the hepatic stages thereby preventing the blood infection (Hollingdale, 1990; Heppner, 2013). Previous experimental vaccinations with irradiation-attenuated sporozoites induced sterile protection against a sporozoite challenge in animal models and in humans (Clyde, 1975; Hoffman et al., 2002). At present, methods have been developed to produce sufficient

quantities of *P. falciparum* sporozoites for large-scale vaccination with irradiation-attenuated sporozoites (Luke and Hoffman, 2003). However, as *P. vivax* cannot be maintained *in vitro*, a sporozoite-based vaccine is not an practical option, and current efforts have been focused on developing a vivax-based subunit vaccines.

The most abundant protein on the surface of all *Plasmodium* sporozoites is the circumsporozoite protein (CSP). It is involved in the motility from the site of inoculation to the invasion of the hepatocyte (Nussenzweig and Nussenzweig, 1985; Pancake et al., 1992; Mota and Rodriguez, 2004; Kappe et al., 2004; Coppi et al., 2011). This protein is the main candidate of pre-erythrocyte recombinant malaria vaccine (Qari et al., 1993; Gonzalez et al., 2001; Herrera et al., 2007; WHO, 2012), and antibodies against it have been shown to mediate protection against *Plasmodium* sporozoite challenge in preclinical models and humans (Charoenvit et al., 1991; Potocnjak et al., 1980; Tam et al., 1990; Zavala et al., 1987; Kebaier et al., 2009; Kester et al., 2009). Additionally, much evidence has indicated that immune responses targeting the *P. vivax* CSP (PvCSP) are likely play a crucial role in mediating protection against *P. vivax* in clinical and preclinical studies (McCarthy and Clyde, 1977; Charoenvit et al., 1991; Yang et al., 1997).

Three different variant forms of the central repeat region have been detected for PvCSP (VK210, VK247, and the *P. vivax*-like variants) (Rosenberg et al., 1989; Qari et al., 1993; Gopinath et al., 1994), and a previous study has reported no immunological cross-reactivity for VK210 and VK247 variant types (Rosenberg et al., 1989). The lack of cross-reactivity would cause strain-specific immunity (Machado et al., 2003). Thus, a multi-variant chimeric subunit vaccine would be required. A multi-variant chimeric subunit vaccine could be hampered by limited intrinsic immunostimulatory properties due to the modification of the structure of the natural protein, which would diminish the ability of the elicited antibodies to recognise all variants of the native target antigen.

In the late 1980s and the early 1990s, immunization with a recombinant PvCSP expressed in yeast induced inadequate immunity in non-human primates (Barr et al., 1987) and in humans (Collins et al., 1989; Herrington et al., 1991). Later, multiple antigen constructs were used to develop epitope-based vaccines using the vivax repeat motif, and some levels of protection were observed in *Saimiri* monkeys (Yang et al., 1997; Collins et al., 1997) but due to the absence of a control group, interpretation and final conclusion were not possible. Subsequently, synthetic peptide-based vaccines were considered and tested in non-human primates and humans (Udhayakumar et al., 1998; Herrera et al., 1997; 2005) to improve the immunogenicity of the PvCSP-based vaccine and to overcome the difficulties in production. Recently, a study by Yadava et al., (2007) reported a new PvCSP chimera (named VMP001) that was produced in *Escherichia coli* (*E. coli*), and immunization of mice with this chimera formulated with Montanide ISA-720 showed that it to be immunogenic and to recognize live sporozoites (Yadava et al., 2007; Bell et al., 2009). However, *in vitro* evaluation of antibodies to VK247 and VK210 peptides showed that the response to the VK247 was lower than that to the VK210 peptide, maybe because of a lower VK247 copy numbers of repeats within the vaccine construct (Vanloubbeeck et al., 2013). Furthermore, recent humans phase 1 clinical trial using VMP001/AS01B did not induce sterile protection, and compared to the control group, significant delay in time to parasitaemia was observed in only 59% of vaccinated subjects (Bennett et al., 2016).

In order to obtain an recombinant chimeric antigen comparable in its immunogenicity to its native form, we designed and constructed two novel synthetic chimeric *P. vivax* CSP proteins

based on the two most widespread variant forms (VK210 and VK247). Both synthetic structures consisted of the conserved N-terminal and C-terminal regions of the native protein, flanking a truncated repeat region of the either VK210/VK247 (named CS127) or VK247/VK210 (named CS712). Computer modelling was employed to guide the design of CS127 and CS712 towards physicochemical stability, including RNA stability, epitope mapping, prediction of tertiary structure, and comparison of structure of the both chimeric proteins. Moreover, the predicted 3-dimensional (3D) structure was compared to the natural proteins of similar size to ensure that conformations of both synthetic proteins would be similar to their natural counterparts in terms of internal energy. The amino acid sequences of the new constructs were selected based on these modelling studies. *E. coli* is commonly used for the expression of high-level-heterologous proteins, especially in a commercial setting. Thus, the nucleotide sequences of both *Pvcs127* and *Pvcs712* genes were synthetically constructed based on the *E. coli* codons in order to optimize expression levels. Subsequently, the expression and purification of both chimeric recombinant proteins were carried out, and their biological and functional properties were evaluated using enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence antibody test (IFAT), as well as heparan sulphate and hepatocyte-binding assays.

2. Materials and methods:

2.1. Designing chimeric CSPVK210/VK247 proteins

In this investigation, two synthetic CSP genes were designed based on the complete gene and protein sequences of VK210 (Sal-1, accession no. GU339059) and VK247 (Papua New Guinea [PNG], accession no. M69059) sequences (Fig. 1A and 1B). The first construct (named after CS127 based on the number and the tandem of motif repeats; accession no. KY548403) consisted of 76 conserved N-terminal amino acids (aa: 20-95, Sal-1 sequence, accession no. GU339059), followed by EDGAGNQPG sequence (aa: 96-104; PNG, accession no. M69059) and the first 12 repeats of VK210 (GDRA[D/A]GQPA), followed by the 7 repeats of VK247

(ANGAG[N/D]QPG) (Fig. 1C). Moreover, the post-repeat region of this newly construct was considered to have one copy of 12 inserted amino acid sequences, GGNAANKKAEDA, which is detected in South Korean and Iranian isolates (aa: 267-292, Belem sequence, accession no. EU401923) and conserved C-terminus (aa: 281-350; Sal-1 sequence, accession no. GU339059) (Fig. 1C). For the second construct, (named after CS712 based on the number and the tandem of motif repeats; accession no. KY548404), both N- and C- terminals were the same as the first designed construct. However, the arrangement of the repeats in CS712 was four of ANGAGNQPQ and three of ANGAGDQPQ repeats of VK247 (totally, 7 repeat types of VK247), followed by six of each VK210 (GDRA[A/D]GQPA) (totally, 12 repeat types of VK210) (Fig.1D).

To design both constructs, Sal-1 and PNG reference sequences were retrieved from the GenBank and aligned with ClustalW software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) (Thompson et al., 1994) to define conserved and non-conserved regions. Then the both genes were synthesized based on *E. coli* codon preferences (Sharp and Li, 1987). Two restriction endonuclease recognition sites (*Nde*I for 5' end and *Hind*III for 3' end) were inserted to synthetic constructs as well as to hexa-histidine (6×His)-tag at the C-terminal site to facilitate recombinant protein purification by nickel affinity protein purification. Both constructs were synthesized and delivered as two separate clones in pET26b plasmid by ShineGene Molecular Biotech, Inc. (Shanghai, China). The secondary structure of messenger RNA of the chimeric genes was analyzed by Mfold (version 2.3 energies) online software at <http://www.bioinfo.rpi.edu/applications/mfold> (Zuker, 2003).

2.2. Determination of physico-chemical parameters of the chimeric proteins

The ExPASy Prot-Param (<http://us.expasy.org/tools/protparam.html>) was used to calculate the physico-chemical parameters of the synthetic CS127 and CS712 proteins. The theoretical molecular weight, extinction coefficient, isoelectric point (pI), the total number of positive and negative residues, the half-life of both chimeric proteins, aliphatic index as a positive factor for the increase in the thermostability of globular proteins (Ikai, 1980), grand average hydropathy (GRAVY) (Kyte and Doolittle, 1982), and instability index as a tool for protein stability predication (Guruprasad and Pandit, 1990) were calculated.

2.3. Secondary and tertiary structure prediction

Garnier-Osguthorpe-Robson (GOR) IV secondary structure prediction server (Sen et al., 2005) was used to predict and analyze the secondary structure of chimeric proteins. The 3D structure and confidence score (C-score) of the chimeric proteins were generated by using the I-TASSER online server (Zhang, 2008). The highest C-score represented the proper model (Yang et al., 2015).

2.4. Three dimensional structure validation analyses

Visualization of 3D structure was carried out by PyMOL software version 1.7.2 (DeLano, 2002; Pedretti and Vistoli, 2004). Ramachandran plots were calculated by RAMPAGE server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) (Laskowski et al., 1993). Moreover, the molecular docking approach was used to model the interaction between a small molecule of heparan sulphate binding and both chimeric proteins at the atomic level. The results could characterize the behaviour of ligand in the binding site of target chimeric constructs as well as elucidate fundamental biochemical processes (Morris and Lim-Wilby, 2008). Docking analysis

was performed by Hex Protein Docking (Ghoorah et al., 2013). For docking analysis, the PDB files of both tertiary structures of chimeric proteins predicted by I-TASSER and PDB files of ligand (PDB ID: 1HPN) were used.

2.5. B-cell epitopes prediction

In order to investigate the presence of antigenic sites on chimeric CS127 and CS712 proteins, B-cell epitope prediction analysis for both chimeric structures was performed. The continuous B-cell epitopes (linear B-cell epitope) prediction was carried out using the online BcePred server (<http://www.imtech.res.in/raghava/bcepred/>) (El-Manzalawy et al., 2008) with cut-off of 0.9 (Saha and Raghava, 2004). Both chimeric structures were also subjected to conformational (discontinuous) B-cell epitopes prediction using DiscoTope server 2.0 (<http://www.cbs.dtu.dk/services/DiscoTope>) (Kringelum et al., 2012) and in the present investigation, specific cut-off of 1.9 with 0.95% specificity and 0.17 sensitivity was considered (Kringelum et al., 2012).

2.6. Expression, purification and confirmation of expressed chimeric proteins in *E. coli* host

Upon receipt of two synthetic constructs, both lyophilized constructs were dissolved in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and kept at -20° C until use. Competent *E. coli* DH5 α strain was used as cloning host and transformed by 100 ng/ μ L of each construct. The recombinant plasmids were then purified using Qiagen plasmid purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and were re-sequenced and double-checked with the corresponding Sal-1 and PNG as reference sequences, using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). Both CS127 and CS712 chimeric constructs were expressed in *E. coli* BL21 (DE3) strains as recombinant His-tag fused proteins. Briefly, the overnight *E. coli* culture was grown in Luria broth (pH 7.2) containing kanamycin (50 mg/mL) at 37 °C with shaking (180 rpm) until an optical density (OD) at 600 nm was reached ~1. The expression of CS712 and CS127 recombinant constructs were induced with 0.2 and 0.5 mM isopropylthiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO, USA), respectively. The cultures were grown for another 4 h, and the cells were harvested by centrifugation at 6000 rpm at 4 °C for 15 min and kept at -80 °C until use. Both recombinant proteins were expressed in the form of inclusion bodies, and then the cell pellets were dissolved in denaturation buffer (8 M urea, 20 mM Tris-HCl, 1 M NaCl and 15 mM imidazole, pH 7.9) and incubated on ice for 90 min with gentle shaking. Next the cells were lysed on ice by sonication (Ultraschallprozessor, Deutschland, Germany) with 15 cycles; each cycle consisted of 20-s pulses with 70-s intervals. The bacterial lysate was centrifuged at 6000 rpm at 4 °C for 15 min. The supernatant was incubated with Ni²⁺ nitrilotriacetic acid agarose resin (Ni-NTA Agarose; Qiagen, Hilden, Germany) at 4 °C for 2 h, and the resin was packed into a column and washed with a 10-column volume of wash buffer (6 M urea, 20 mM Tris-HCl, 1 M NaCl, and 30 mM imidazole, pH 7.9). The bound protein was eluted with elution buffer (4 M urea, 20 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole, pH 7.9).

The concentration of the protein was determined using Bradford assay (Bradford, 1976) using a spectrophotometer (Eppendorf, Hamburg, Germany). In addition, the expression level of rCS127 and rCS712 was measured by a densitometer (Bio-Rad, USA). The bovine serum albumin (BSA, Roche, Mannheim, Germany) was used as the standard protein to set up a standard curve from which the unknown protein concentration could be determined. Also, 10

mg/mL stock BSA solution was used to prepare a standard two-fold dilution series (1,000, 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 mg/L).

The purified recombinant proteins were confirmed and analyzed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions (with 1% SDS and 2% mercaptoethanol [2ME]), followed by immune-blotting using standard protocols, with both anti-His antibody (1: 1500; Qiagen, Germany) and the *P. vivax*-infected human sera (1: 100). Moreover, to confirm the recognition of VK210 and VK247 repeat regions in the two newly designed constructs, chimeric recombinant proteins were used as antigens and peroxidase-linked anti-CSP-specific mAbs (anti-Pv-210-CDC, *P. vivax* allelic form VK210 and anti-Pv-247-CDC, *P. vivax* allelic form VK247) were applied using Western blot analysis. These mAbs were obtained from MR4 (the Malaria Research and Reference Reagent Resource Center, MR4, ATCC, Manassas, VA, USA) and used by Dr. G. Snounou (Paris). Each of peroxidase-linked anti-CSP-specific mAbs was incubated at room temperature (RT) for 2 h with gentle shaking in darkness. After washing with PBS-T, the presence of bound mAbs was detected using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA) as a substrate of peroxidase.

2.7. Conformational ELISA

This ELISA was performed according to the instruction provided with the MRA-1028K kit (MRA-1028K, MR4, ATCC® Manassas, VA, USA) with some modifications. Each VK210 and VK247 mAbs (according to the manufacturer's instructions) was coated on Maxisorp flat-bottomed, 96-well microplates (Grainer, Labortechnik, Germany) and incubated at 4 °C overnight. After washing with PBS containing 0.05% Tween-20 (PBS-T), microplates were blocked with 200 µL PBS containing 2.5% BSA (pH 7.4) at RT for 2 h. The plates were then washed with PBS-T and incubated with 1000 ng/mL (50 µL/well) of each recombinant constructs proteins (CS127 or CS712) at RT for 90 min. After washing with PBS-T, the plates were incubated with 50 µL/well peroxidase-linked anti-Pv-210-CDC and -Pv-247-CDC mAbs in darkness for 1 h. The presence of bound mAbs was detected using o-phenylenediamine dihydrochloride-H₂O₂ (OPD, Sigma-Aldrich, St. Louis, MO, USA) as a substrate and stopped with 2N H₂SO₄. The absorbance at 492 nm was read using a microplate reader (BioTek, Winooski, VT, USA).

2.8. Human ELISA

ELISA was used to confirm that the B-cell epitopes are preserved in the chimeric recombinant protein and to evaluate the antigenicity of both CS127 and CS712 constructs. To perform this assay, plasma samples were collected from 221 individuals from patients in Chabahar, Sistan and Baluchistan Province in southeastern Iran (2005-2012) where malaria is endemic. The inclusion criteria were patients with uncomplicated malaria symptoms, mono-infection with *P. vivax*, while pregnant women, infants under two years old and malnourished patients were excluded from sampling. In addition, before blood collection, an informed consent was obtained from adults or parents or legal guardians of children who were participant in this survey. The *P. vivax* diagnosis was confirmed by the microscopic examination of blood smears stained with Giemsa, followed by PCR amplification of the 18ssrRNA gene as described previously (Snounou et al., 1993). The collected blood samples were transported cold to the main laboratory at the Pasteur Institute of Iran (Tehran).

ELISA was performed as previously described (Rouhani et al., 2015) with some modifications. Briefly, CS127 and CS712 recombinant proteins (100 ng/well) were diluted in a

coating buffer (in 0.06 M carbonate bicarbonate buffer, pH 9.6), coated in Maxisorp flat-bottomed 96-well ELISA plates (Grainer; Labortechnik, Nürtingen Germany) and kept at 4 °C overnight. After incubation, the plates were washed five times with PBS-T and were blocked with PBS containing 2.5% BSA (Roche, Mannheim, Germany) at RT for 2 h. After the plates were washed again with PBS-T, serum samples were incubated in duplicate wells with 1: 100 diluted plasma in PBS-T containing 0.5% BSA for 90 min. After washing, the plates were incubated with 1: 35,000 dilutions of anti-human IgG antibody conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO, USA) in PBS-T at RT for 60 min. Bound antibodies were visualized after adding the OPD (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. The reaction was stopped with 2N H₂SO₄ after 10 min, and OD was read at 492 nm (OD₄₉₂ nm) using an ELISA microplate reader (BioTek, Winooski, VT, USA). Also, for measurement of the specific IgG subclasses response, the test was performed as described above except for the secondary antibodies that were specific to human IgG1, IgG2, IgG3, and IgG4 antibodies (Sigma-Aldrich, St. Louis, MO, USA) diluted 1: 2500 and incubated at RT for 1 h. After washing, for the detection of antibodies, HRP-conjugated mouse antibody, which was specified to the heavy chains of human IgG1, IgG2, IgG3, or IgG4, was used. Thirty serum samples from healthy non-exposed Iranian from outside malaria-endemic regions were used as negative controls. The cut-off value for positive samples was considered as the mean OD of negative samples plus three standard deviation (SD). Responders were individuals whose plasma samples had an OD upper than cut-off value, and non-responders were those with OD ≤ cut-off value. Samples with discrepancy of greater than 20% between duplicates were re-tested.

2.9. Mouse immunization

The animals in the study were housed and used strictly in accordance with the guidelines set by the National Institutes of Health in 1985. Inbred C57BL/6 female mice (6–8 weeks old) were obtained from Laboratory Animal Science Department, Pasteur Institute of Iran. Mice groups (n = 10) were immunized subcutaneously at the base of tail with 10 µg of the two constructs. In priming and boosting, the antigens were then emulsified in complete Freund's adjuvant (CFA 1:1 ratio, Sigma, St. Louis, MO, USA) and with incomplete Freund's adjuvant (IFA, Sigma), respectively. However, the mice control groups were immunized with PBS alone and PBS in Freund's adjuvant, respectively. The animals were boosted on days 14 and 28 and bled on days 0 (pre-immune), 21, and 35 of immunization. The obtained sera were used in heparan sulfate, HepG2 cells-binding assays, and IFAT.

2.10. Heparan sulphate binding

Heparan sulphate-binding assay was performed as described earlier (Yadava et al., 2007) with some modifications. Briefly, Maxisorp flat-bottomed, 96-well microplates (Grainer, Labortechnik, Germany) were coated with 100 µl/well of 10 µg/mL solution of heparan sulphate (from bovine kidney, Sigma-Aldrich, St. Louis, MO, USA), and the control wells were incubated with 10 µg of BSA/mL. The plates were incubated uncovered at 37 °C overnight and then were washed with a 0.05% Tween-20 in PBS. In the next step, the plates were blocked with 1% BSA solution in PBS at RT for an hour. After washing steps, serial dilutions of CS127 and CS712 antigens were diluted in PBS (starting at 100 µg/mL) and plated at RT for 2 h. After washing with PBS-T, the binding of CS127 and CS712 to heparan sulfate was detected by 100 µl/well either peroxidase-linked anti-Pv-210-CDC and -Pv-247-CDC mAbs (MRA-1028K, MR4, ATCC®, Manassas, VA, USA) or polyclonal antibodies raised in mice against purified CS127,

CS712 in darkness at RT for 2 h. The bound antibodies to CS127 and CS712 were detected by goat anti-mouse IgG-HRP conjugate (1: 25,000; Sigma-Aldrich, St. Louis, MO, USA) at RT for 60 min. The reaction was developed by using OPD (Sigma-Aldrich, St. Louis, MO, USA) as a substrate and stopped with 2N H₂SO₄. The absorbance at 492 nm was read using a microplate reader (BioTek, Winooski, VT, USA).

2.11. Binding of chimeric CS to HepG2 cells

A hepatoma cell line, HepG2 (IBRC C10096; Iranian Biological Resource Center, Tehran, Iran), was maintained in Dulbecco Minimum Essential Medium (DMEM, Biowest, Nuaille, France), Ham's F12 (Biowest, Nuaille, France), 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Biowest, Nuaille, France), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were removed from culture flasks with Trypsin-EDTA (0.25% Trypsin, 1mM EDTA) and pelleted at 3000 × g for 15 min. The cell pellet was resuspended in complete DMEM medium to a concentration of 10⁶ cells/mL. Then, 10⁵ cells/well were plated in 96-well cell culture plates (Orange Scientific, Braine-l'Alleud, Belgium) and allowed to grow overnight. The cells were washed twice with the medium and fixed with 4% para formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at RT for 20 min. Following washings with Tris-buffered saline (TBS; 50 mM Tris, pH 7.4, 130 mM NaCl), the wells were blocked with 1% BSA in TBS at RT for 2 h. After washing step, the cells were incubated with different concentrations of CS127 and CS712 chimeric proteins (starting at 80 µg/mL in TBS) at 37 °C for 1 h. Control wells were incubated with similar dilutions of a recombinant *P. vivax* Duffy binding protein (PvDBP) as control. Unbound proteins were removed by washing with TBS three times for 10 min each, and then the plates were incubated with polyclonal antibodies raised in mice against purified CS127, CS712, or PvDBP (1: 200) at RT for 90 min. The bound antibodies to CS127 and CS712 and PvDBP were detected by goat anti-mouse IgG-HRP conjugate (1: 25,000; Sigma-Aldrich, St. Louis, MO, USA) at RT for 60 min. The binding of CS127, CS712, and PvDBP to HepG2 cells was measured at 490 nm using a micro-plate reader (BioTek, Winooski, VT, USA). Absorbance obtained at 490 nm was plotted against different concentrations of the examined antigens. Wells with only BSA and HepG2 cells served as controls.

2.12. IFAT

IFAT assays were performed in order to test the ability of the anti-PvCSP sera from immunized mice to recognize the native form of the PvCSP antigen on the sporozoite surface and to determine the similarity of epitopes in recombinant forms to corresponding native proteins. *P. vivax* sporozoites were obtained from *Anopheles cracens* that had fed on blood collected from *P. vivax*-infected patients attending malaria clinics at the Shoklo Malaria Research Unit (Mae Sot, Thailand), following a standardized procedure (Andolina *et al.* 2015 Malaria Journal 14:312). Multi-spot slides were coated with 20 µL/well of sporozoites of both *P. vivax* strains VK210 and VK247 (around 500 to 1000) and allowed to dry and then fixed in cold acetone for 10 min. The slides were blocked with 20 µl PBS containing 2.5% BSA (Roche, Basel, Switzerland) in a dark humidified chamber at RT for 30 min. After the slides were washed three times with PBS (pH 7.4), the serial dilution of anti-CS127 and -CS712 mice serum in PBS (1:50-1:102,400) were added to the duplicated wells and incubated in a wet chamber for 60 min. After washing three times with PBS (pH 7.4), each well was covered with 20 µL of the fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (1:40) and Evance blue (1:100)

and then left in a wet chamber for 45 min. Again, after washing three times with PBS, coverslips were placed on each slide and examined under a fluorescence microscope (Nikon E200, Tokyo, Japan) with an oil immersion objective (100×). The serum samples obtained from normal mice were used as negative controls.

2.13. Statistical analysis

A database was generated with SPSS 16.0 for windows (SPSS Inc., USA). As the antibody levels were not normally distributed, non-parametric tests were used. Chi-square comparison of proportions and McNemar's test were applied to assess the differences in the proportions of IgG-positive subjects. Wilcoxon Signed Ranks test was also used to analyze differences between the mean absorbance of chimeric antigens. *P* values < 0.05 were considered statistically significant in all tests.

3. Results

3.1. Chimeric gene optimization

The amino acids of both constructs were back translated to nucleotide sequence based on *E. coli* codon usage and both synthetic chimeras were analyzed for codon bias and GC content. An online database (<http://www.jcat.de/>) for gene optimization was used to predict the expression of the chimeric gene in *E. coli*. The synthetic constructs were further confirmed by the Codon Adaptation Index (CAI), which is a measurement of the relative adaptiveness of the codon usage of a gene compared with the codon usage of highly expressed genes in *E. coli*. The overall GC content, considered as a measure value for transcriptional and translational efficiency, was improved to 63.22% for both constructs in comparison with the reference sequences (Sal-1, accession no. GU339059 for VK210: GC 50% and PNG, accession no. M69059 for VK247: GC 48.4%). Also, instability elements that may have a negative influence on the expression rate were removed.

3.2. Analysis of chimeric proteins properties

3.2.1. Physico-chemical characteristics

Table 1 shows the physico-chemical characteristics of both synthetic constructs in comparison with references sequences (Sal-1 [GU339059] and PNG [M69059]). Both chimeric constructs have 358 amino acids consisting 46 negatively (Asp and Glu) and 37 positively charged residues (Arg and Lys). The molecular weight of both constructs was 35.013 kDa, which reduced in comparison to reference sequences (~37 kDa for both). pI value for each construct was 5.49 that indicated the acidity feature of the designed constructs, which are the same as for the natural structure model of reference sequences of Sal-1 and PNG (5.04 and 5.30, respectively). Based on ExPASy ProtParam, no differences were found between the appraised value of the half-life of both constructs and the reference sequences of Sal-1 and PNG (30 h in mammalian reticulocytes, *in vitro*, more than 20 h in yeast, *in vivo* and near 10 h in *E. coli*, *in vivo*). In accordance with instability index, both constructs of CS127 and CS712 and references sequences were categorized as stable proteins (25.04 and 24.46, respectively).

3.2.2. Secondary and tertiary structure prediction

The secondary structure of full length of CS127 and CS712 is predicted by GOR IV, and the results showed 13.65% and 12.53% alpha helices, 5.85% extended strands and 80.5% and 81.62% random coils, respectively (Fig. 2). Chimeric CS127 and CS712 proteins were successfully modelled using the I-TASSER server. The best tertiary models predicted for both chimeras and references sequences, which have three separate domains, are shown in Fig. 3. The proper models were selected based on the C-score for estimating the quality of the predicted model. C-score is a factor estimating the quality of the suggested model and typically is in the range between -5 and 2, which a higher value signifies the model with a high confidence (Zhang, 2008; Roy et al., 2010; Yang et al., 2015). The estimated C-score, which is used for estimating the quality of predicted models by I-TASSER (ranging from -5 to 3) for the best 3D model for CS127 and CS712, was -0.39 and -0.34, respectively. Additionally, the tertiary models of the complete protein sequences of Sal-1 (C-score: -2.42) and PNG (C-score: -2.10) were predicted as natural sequences (Fig. 3). I-TASSER results from predicting tertiary models for Sal-1 and PNG, as alternative natural sequences, showed an appropriate structure near the predicted structures of both CS127 and CS712 chimeric constructs.

3.2.3. Model stability evaluation

The Ramachandran plot was used to predict the structure validation of both chimeras for their reliability and structural quality (Hollingsworth and Karplus, 2010). Based on the results of RAMPAGE server (Fig. 4), for CS127, 237 residues (66.8%) are located in the favoured region, 79 (22.3%) residues in the allowed region, and 39 (11.0%) residues in the outlier region. Regarding CS712, 258 (72.5%) residues are estimated in the favoured region, 74 (20.8%) residues in the allowed region, and 24 (6.7%) residues in the outlier region. Based on this design, 89% of residues of CS127 and 93.3% of residues of CS712 were on acceptable regions. Moreover, based on docking results of PNG, Sal I and both CS127 and CS712 chimeric structures, N- and C- terminals of all analyzed proteins play a main role in the interactions of sporozoite with its ligand on the hepatocyte cells. Table 2 shows the free energy of secondary structures of RNA at the 5' terminus of both chimeric genes. In comparison with reference sequences, both constructs had lower free energy in 5'-terminus (-5.40 for Sal-1 and PNG).

3.2.4. Prediction of B-cell epitopes

To verify the potentiality of the CS127 and CS712 chimeras as a *P. vivax* vaccine candidate, the B-cell epitope prediction analysis was used to identify the presence of antigenic sites on these chimeras, as well as Sal-1 (accession no. GU339059) and PNG (accession no. M69059), as references. The linear epitope prediction analysis predicted several similar B-cell epitopes with high affinity (cut-off > 0.9) at N- and C-terminals of both chimeras, as well as at the reference structures. In case of discontinuous B-cell epitopes, using the DiscoTope server with the cut off score > 1.9, new epitopes in N- but not C-terminals of both CS127 and CS712 were predicted in comparison with Sal-1 and PNG reference strains. However, in case of the repeat region, as we used different arrangements of repeat types of VK210 and VK247, linear and new discontinuous B-cell epitopes were predicted for both structures. The surfaced epitopes of both chimeric proteins are shown in Fig. 5.

3.3. Recombinant CS712 and CS127 expression, purification, and characterization

Both CS127 and CS712 constructs were successfully cloned and expressed in *E. coli* strains, and the optimum condition for expression was obtained in LB broth when 0.5 (CS127) and 0.2 (CS712) mM IPTG were added to the culture ($OD_{600nm} = 0.8-1$). The expression levels of rCS127 and rCS712 were 16.9 mg/L and 194.18 mg/L, respectively. The expressed and purified proteins were analyzed by SDS-PAGE (Fig. 6A), and the result showed a single band with a molecular weight of approximately 55 kDa. In Western blot analysis, using anti-His mAb (Fig. 6B) and *P. vivax*-infected human sera (Figs. 6C and 6D), both expressed chimeric proteins were recognized and the purity and fidelity of both proteins were confirmed. Furthermore, the mAbs (peroxidase-linked anti-Pv-210-CDC and -Pv-247-CDC) (Fig. 6E) were used to specifically recognize the dominant repeat units on VK210 and VK247 sporozoites, respectively. None of the unrelated recombinant proteins (PvAMA and PvTRAP) were recognized by these mAbs (Fig. 6F).

3.4. ELISA

The results of conformational ELISA (Table 3) showed that mouse mAbs -(anti-Pv-210-CDC and -Pv-247-CDC) are able to recognize the expressed chimeric CS127 and CS712 antigens. To determine the presence of natural infection-induced immune responses for recognition of the expressed chimeric CS127 and CS712, IgG reactivity in plasma samples from 221 patients (aged 2 to 61 years; mean = 21.6 ± 13.09 years), from Sistan and Baluchistan Province in Iran, who were diagnosed with acute *P. vivax* infections, were tested by ELISA. The result showed that only 49.32% (109/221) of the examined patients had positive IgG antibody responses to both CS127- and CS712- expressed proteins (Table 4, Fig. 7). Furthermore, difference in the prevalence of anti-PvCSP IgG responses in the studied patients was not statistically significant to the two expressed CS127 and CS712 antigens ($P = 1.000$, McNemar's test; Table 4, Fig. 7). Analysis of the antibody responses to CS127 and CS712 showed high (3.1% and 2.2%), medium (21.7% and 16.74%), and low (24.43% and 30.31%) responses ($OD \geq 2$, high-positive responses; $1 \leq OD < 2$, medium-positive responses; cut-off $< OD < 1$, low-positive responses; $OD < \text{cut-off}$, negative-responses, data not shown). None of the sera from healthy individuals (control group) contained IgG antibody to both expressed antigens, which confirms the specificity of the present results. Regarding the analysis of the subclasses among the samples positive for anti-CS127 and -CS712 total IgG ($n = 109$), IgG1 (47.96%, $OD_{490} = 0.777 \pm 0.420$ for CS127; 48.41%, $OD_{490} = 0.862 \pm 0.423$ for CS712) was the dominant subclass, whereas the second frequent subclasses was IgG3 (47.96%, $OD_{490} = 1.137 \pm 0.653$ for CS127; 47.51%, $OD_{490} = 1.390 \pm 0.721$ for CS712) in which both were not statistically different ($P > 0.05$; Wilcoxon Signed-Ranks test) (Table 4, Fig. 7). Regarding IgG2 and IgG4, the frequency distribution of individuals was zero for both CS127 and CS712 chimeric antigens. Furthermore, heterogeneity in IgG1 and IgG3 isotype responses of the individuals was not statistically different in the two chimeric antigens ($P = 0.952$, χ^2 test; Table 4).

3.5. Biological characterization of CS127 and CS712 by heparan sulphate and HepG2 binding

In order to characterize the interaction between chimeric CS127 and CS712 and heparan sulphate, binding assay was carried out over a range of concentrations. The results demonstrated that both expressed proteins could bind to immobilized heparan sulphate in a concentration-dependent manner and are saturable at 80 $\mu\text{g/mL}$ concentrations of both chimeric proteins (Fig. 8). Moreover, both expressed chimeric proteins, CS127 and CS712 were tested for binding to

hepatoma cell line HepG2 (IBRC C10096), and the results showed binding to HepG2 cells in a dose-dependent manner (Fig. 9).

3.6. Recognition of native CSP on *P. vivax* sporozoite by mice polyclonal antibodies to the chimeric CS127 and CS712

Anti-CS127 and -CS712 antibodies produced in mice recognized the native protein present on the surface of *P. vivax* sporozoite with high intensity, as indicated by the worm-like fluorescence pattern (Fig. 10A-D). In addition, none of the control mice sera recognized the native protein on *P. vivax* parasite (Fig. 10E), confirming that there are common epitopes in recombinant forms with corresponding native proteins.

4. Discussion

In order to have a highly effective PvCSP-based vaccine, a combination of key epitopes from different variants of the antigen would be required to induce both proper and protective humoral and cellular immune responses, as well as to overcome the antigenic variability due to infection with heterologous parasite strains. For PvCSP, a broad range of sequence diversity was detected in the repeat region of both VK210 and VK247 isolates of the parasites (Mann et al., 1994; Zakeri et al., 2006; Shabani et al., 2016). However, two conserved regions were identified in the molecule's region I (a five-amino-acid sequence immediately preceding the repeat region in N-terminal) and region II (located within the C-terminal region) that are involved in binding to heparan sulphate (Ying et al., 1997). The immune responses against these two conserved regions could block a receptor-ligand interaction between the sporozoite and the hepatocyte and prevent the establishment of infection (Rathore et al., 2002; 2005). In addition, several B- and T-cell epitopes have been identified on the N- and C-terminal regions of the PvCSP (Arévalo-Herrera and Herrera, 2001; Arévalo-Herrera, et al., 2002), and hence, the inclusion of these regions in a vaccine might facilitate the induction of the similar and adequate immune responses as occurs in the natural infection with *P. vivax* (Franke, et al., 1992; Billsborough, et al., 1997; Arévalo-Herrera et al., 1998).

Recently, efforts to develop vaccines based on PvCSP have been made, and the most advanced is VMP001, a chimeric structure of PvCSP (Yadava et al., 2007). In immunization experiments, VMP001-containing vaccines (formulated with different types of adjuvant or as nanoparticles) have been shown to induce high levels of humoral immune responses in mice and rhesus monkeys, but not acceptable cellular response (Bell et al., 2009; Lumsden et al., 2011; 2012; Moon et al., 2012a; 2012b). Moreover, many other attempts have been carried out during last decade to develop a potential chimera as a vaccine such as HBcAgPvCSP and rPvCSP-ME (Almeida et al., 2014), PvCS-NRC (Cespedes et al., 2013), PvNR1R2 (Cespedes et al., 2014), and PvRMC-CSP (Cabrera-Mora et al., 2015) but none of these was able elicit cell-mediated immune response or protection. In the present investigation, our strategy in the development of a multi-variant *P. vivax* vaccine was to design conceptually novel constructs that contain entire N- and C-terminal regions of both VK210 and VK247 backbones with a tandem arrangement of distinct nonapeptides repeats: GDRA(D/A)GQPA in the VK210 CSP and ANGA(G/D)(N/D)QPG in the VK247 CSP. Moreover, by mimicking the natural PvCSP, the least number of both VK210- (12 repeats) and VK247- (7 repeats) sequenced types reported so far from global strains of *P. vivax* were considered, and both these newly designed synthetic genes were expressed in an *E. coli* expression system. In comparison with the VMP001 (Yadava et al., 2007), almost the N- and C-terminal parts of both (the present chimeras and VMP001) are

identical; however, the repeat sequences from VK210 and VK247 parasite subtypes are different. In VMP001, at least one copy of each of the seven unique repeats was found in the wild-type Korean VK210 isolate, and a single copy of the VK247 repeat was included. Nonetheless, our new strategy to increase the number of repeat motifs in the PvCSP-based vaccine construct might increase the epitope density to enhance simultaneously anti-repeat-specific responses to parasite strains carrying either VK210 or VK247 s.

As far as the epitope-specific antibody responses are concerned, a tandem arrangement of the epitopes (particularly the repeats of two VK210 and VK247 variants) in the designed construct might be considered. The reason is that it might cause a failure to elicit proper epitope-specific immune responses due to missing conformational of target epitopes and maybe interfere with antigen processing and presentation. Therefore, in our strategy, two different arrangements of the sequences of VK210 and VK247 (CS127 and CS712) repeat regions flanking with N- and C-terminals were considered in an order to ensure the accessibility of B-cell epitopes for the induction of adequate immune responses. These constructs were codon-optimized for optimal recombinant protein production in a heterologous host. Both CS127 and CS712 were expressed in *E. coli* host with a significant difference between expressions levels, possibly due to the role of messenger RNA secondary structure of each construct (Gaspar et al., 2013). This discrepancy could also explained by the regulation in gene expression that is significantly dependent on forming stable structures by nucleotide pairing in 5'-end (Gaspar et al., 2013). The mRNA prediction also showed that the mRNA of both constructs were stable enough for efficient translation in the *E. coli* host. The 5' untranslated regions (5'-UTRs) of mRNAs play major roles in increasing the translation rates and half-lives of transcripts. In our investigation, the first nucleotides at 5'-UTR of both CS712 and CS127 mRNA secondary structures did not show a pseudo-knot and/or hairpin formation. The folding free energies of 5'-UTRs were higher than other mRNA regions, indicating that region could be available for cell translation machinery for a longer time. It seems that the better expression level in the case of CS712 could be explained with the difference in the energy in these regions (-9.8 for CS712 in comparison to -6.9 for CS127). Furthermore, the higher yield of CS712 signifies that this construct can provide tremendous flexibility in terms of scaling-up and cost in large-scale vaccine production by fomenter.

The analysis of physico-chemical parameters of CS127 and CS712 chimeric genes by ProtParam software; showed the acidic nature of both chimeric proteins (pI value = 5.49), the same as natural CSP. High extinction coefficient was predicted for both chimeric proteins at 280 nm, which was confirmed by the high yield of protein expression. ExPASy ProtParam classifies both recombinant CS127 and CS712 chimeric structures as stable on the basis of instability index, which is necessary for chimeric recombinant structures. One of the most significant obstacles in designing a recombinant chimeric protein is to have functional properties that are highly dependent on the 3D structure of the designed sequences. This concern is usually solved by the analysis of the predicted 3D structures of the designed protein. Ab-initio I-TASSER software, a reliable analysis tool, predicts precisely the CS127 and CS712 proteins folds and creates a good high-resolution model for these chimeric proteins. However, the theoretical predicted 3D structures of both constructs are slightly different, which is in the consequence of different positions of VK210 and VK247 repeats in both constructs. Despite these discrepancies, they showed similar biological activity using heparan sulphate and hepatoma cell line HepG2-binding assay. This finding suggests that, the arrangement of repeat types can change the 3D structure of proteins though; no significant biological activity was identified. All of these

findings could help us to understand and predict the activity of a synthetic biological molecule, especially for vaccine development.

It is also not known whether synthetically created multi-epitope vaccine constructs, which contain B and/or T cell epitopes, can be processed properly to generate specific immune responses. Therefore, a crucial step in the development of CSP-based vaccine is identifying B-cell epitopes, which plays a central role in development of neutralizing antibodies. Both continuous and non-continuous B-cell epitopes were identified on both novel chimeric CS127 and CS712 proteins. Based on BcePred server analysis, it seems that the continuous and conformational B-cell epitopes are located on the surface of both chimeric proteins and are flexible to interact easily with antibodies.

In silico analysis indicated that the two selected chimeric constructs were codon-optimized to *E. coli* codons. This codon-optimized gene was cloned into an *E. coli* expression plasmid, and the recombinant construct of CS712, but not CS127, produced the high levels of protein in the BL21(DE3) strain of *E. coli* to yield 194.18 and 16.9 mg/L, respectively. Both expressed CS127 and CS712 were recognized in Western blot analysis and ELISA using anti-Pv-210-CDC (specifically recognize repeat motifs on VK210 type) and Pv-247-CDC (recognize repeat motifs on VK247 type). Recognition of both recombinant proteins by the mAbs suggests that the B-cell epitopes are properly expressed and are accessible on the protein's surface for the antibodies, whereas the conformational ELISA test could prove the accuracy of conformational epitopes formation. Moreover, anti-CS127 and -CS712 sera of immunized mice recognized the native form of the PvCSP antigen on sporozoite surface by using IFAT, and this finding indicates the similarity of epitopes in recombinant forms to corresponding native protein of both sporozoites types.

Additionally, in order to confirm that the B cell epitopes are preserved in the two recombinant chimeric proteins and also to evaluate the antigenicity of CS127 and CS712, ELISA was performed using plasma samples collected in a malaria hypoendemic region of Iran from 221 patients who were naturally exposed to malaria and diagnosed with acute *P. vivax* infections by both microscopy and PCR assays. Almost 49% of the patients from these hypoendemic malaria settings had anti-CSP antibodies demonstrating that both expressed chimeric proteins are recognized after natural *P. vivax* infection. Interestingly, this result suggested that the naturally acquired antibodies were able to recognize the modifications introduced in both chimeric proteins and confirms that the antigenic domains of target protein are conserved in designed constructs (Ferreira et al., 2014). The present seroprevalence of anti-CS127 and -CS712 antibodies in individuals living in a low malaria transmission of Iran (49%) was similar to other reported results from areas with a low incidence of malaria in western Amazon region (45.3%), Colombia (24 to 58%), and Brazil (24 to 34.2%) (Oliveira-Ferreira et al., 2004; Cespedes et al., 2014; Cabrera-Mora et al., 2015). Moreover, although there is not enough information about the IgG subclasses associated to PvCSP chimeric protein, studies on IgG1 and IgG3 analysis against CSP of *P. falciparum* in adults and children in low malaria-endemic areas, which showed dominant cytophilic antibodies to pre-erythrocytic stage, indicated a similar result to the present finding (John et al., 2005; 2008; Noland et al., 2015).

The heparan sulphate and hepatoma cell line HepG2-binding assays were performed to verify that the modification on the CS127 and CS712 structures did not affect the functionality of the CSP-conserved domains. This is an important concern, since the binding of CSP to heparan sulphate proteoglycans on the surfaces of hepatocytes is the main event in the rapid and specific localization of sporozoites to the liver cells after the bite of an infectious anopheline (Pinzon-

Ortiz et al., 2001; Sinnis and Coppi, 2007; Pradel et al., 2002). Our results indicate that both chimeric proteins are bound to heparan sulphate and HepG2 cells in a dose-dependent manner, suggesting the availability and the conservation of region I (a cell-adhesive motif exposed by the proteolytic cleavage of CSP after the sporozoite-hepatocyte interaction [Ejigiri and Sinnis, 2009; Coppi et al., 2011]) in the N-terminal and region II-plus in the C-terminal regions of both chimeric molecules. Therefore, this result demonstrates that both novel expressed chimeric recombinant PvCS proteins are biologically functional and maintain the key role of binding to hepatic cells.

5. Conclusions

In summary two novel chimeric recombinant PvCSPs proteins, which included the conserved and different arrangements of repeat regions of the *P. vivax* CS VK210 and VK247 types, were generated. Although both designed constructs had structurally different characteristics at the molecular level, the expression level and the degree of purity were satisfactory, and both chimeric proteins had a conformational structure that allowed recognition by two strain-specific mAbs. Moreover, as both CS127 and CS712 chimeric proteins were recognized by sera from the individuals naturally infected by *P. vivax* and also bound to both heparin sulphate and HepG2 cells, it could be concluded that both have the potential to be a strong candidate for a anti-vivax vaccine designed to elicit protective anti-sporozoite antibodies in humans. Although this needs further study, the present investigation allowed the side-by-side comparison of these two malaria vaccine constructs that will require further pre-clinical evaluation to confirm the immunogenic ability of the new construct in combination with different adjuvant formulae. Ultimately, both chimeric recombinant proteins have the biological and functional properties required for the development of a vaccine against *P. vivax*, an important tool toward the goal of global malaria control and elimination/eradication strategies.

Conflict of Interest

Authors declare that we have no conflict of interest.

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