Original Article

Optimized Method for Purification of Expressed Plasmodium Vivax Duffy Binding Protein-II (PvDBP-II): Implication for Vivax Malaria Vaccine Development

Vahideh Valizadeh[1], Sedigheh Mirkazemi[1], Behrouz Vaziri[2], Sedigheh Zakeri[1*], Navid D. Djadid[1]

1 Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran.
2 Department of Medical Biotechnology, Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran.

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ABSTRACT

Introduction: The purity and correct folding of a recombinant protein is critical for any structural, biochemical and vaccine design studies. *Plasmodium vivax* Duffy binding protein-II is a leading vaccine candidate for vivax malaria. In the present study, the purification process of recombinant DBP-IX (a variant form of PvDBP-II) was optimized to achieve the highest yield and purity. Moreover, naturally-acquired IgG antibodies to the expressed protein have been evaluated. **Methods:** DBP-IX was cloned and expressed as a his-tagged protein in *E. coli*. The recombinant protein was purified using Ni-NTA agarose and different purification parameters were optimized to achieve the highest yield and purity. The quality of the purified rDBP-IX was assessed by different procedures such as SDS-PAGE gel analysis in both reducing and non-reducing conditions, followed by indirect immunofluorescence antibody test and ELISA using the sera of *P. vivax* infected patients (n= 202). **Results:** DBP-IX was successfully cloned, expressed and optimally purified. Differential mobility of the rDBP-IX on the SDS-PAGE gel in reducing and non-reducing conditions, confirmed the presence of disulphide bonds. In addition, anti-rPvDBP-IX antibody produced in mice recognized the native PvDBP-II, suggesting that epitopes in the recombinant protein were similar to the corresponding native form. Finally by performing ELISA experiments, it was demonstrated that natural *P. vivax* infection produces IgG against rDBP-IX (42.1%) whilecytophilicIgG1 antibody (35.4%) was the predominantly detected IgG subclass. **Conclusion:** The results indicated that the optimally-purified rDBP-IX was of a quality that could be used in vaccine development research and immunological studies of vivax malaria.

KEYWORDS: *Plasmodium vivax*, Duffy binding protein, Recombinant protein, Vaccine

INTRODUCTION

Historically, vaccines have been one of the most effective and simply administered tools for controlling and preventing of infectious diseases such as small pox, rabies, plague, cholera and typhoid [1]. One of the neglected life-threatening diseases is malaria which occurs in nearly 100 countries worldwide and infects at least 300 million people living in tropical regions, annually [2]. Malaria is preventable, treatable and history shows that it can be eliminated. Potential means that could facilitate malaria eradication include highly sensitive diagnostic tests, single-dose treatments and vaccines.

Today, research in the field of malaria vaccine development has accelerated considerably and increased funding and studies are driving the discoveries of new vaccines, especially the recombinant subunit ones [3]. *Plasmodium vivax* has three distinct stages in its life cycle. One of the most important stages at which an individual would start to experience clinical symptoms is the erythrocytic stage. Different antigens from this stage are evaluated as vaccine candidates and one of the most promising is Duffy binding protein [4, 5]. This protein consists of seven regions and a- cysteine rich domain, referred to as region II which has a fundamental rolein adhesion of the parasite to the red blood cells and initiation of the invasion. PvDBP-II is 330 amino acids in length and contains 12 conserved cysteine residues. It is postulated that the interaction of PvDBP-II with the Duffy antigen receptor for chemokines

***Corresponding Author:** Sedighe Zakeri, Ph.D; Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran. No. 69, Pasteur Ave, Tehran, Iran, 1316943551.
Email: zakeris@pasteur.ac.ir, zakerimvrg@gmail.com.
Tel/Fax: (+98) 21 66480749

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(DARC) on the surface of the red blood cells mediates invasion by *P. vivax* merozoites [6, 7]. However, few reports have described the transmission of *P. vivax* in a Duffy-negative population, suggesting that *P. vivax* could have alternative invasion pathways; although these appear to be uncommon [8]. Furthermore, sera obtained from residents of endemic areas can block binding of the erythrocytes to PvDBP-II. Although high titer binding inhibitory antibodies are not acquired following the natural exposure to *P. vivax*, immunization with recombinant PvDBP-II can elicit antibodies that successfully block the interaction of PvDBP with DARC [9-11]. Therefore, a vaccine based on PvDBP-II would be expected to be effective against *P. vivax* isolates.

Recombinant protein vaccines consist of protein antigens that have been produced in a heterologous expression system (e.g., bacteria, yeast, etc.). Such vaccines are based on the concept that immune responses mounted to an infection are often targeted toward specific localized regions on the surface of protein antigens known as epitopes [12]. Consequently, protein antigens must be produced and purified in a way to have correct secondary and tertiary structures for presenting the right epitopes to human immune system. Since in case of any contamination with the host proteins, the immune responses will be induced against these epitopes, the purification of recombinant proteins is of great importance for the safety and efficacy of the protein vaccines. Therefore, in the current investigation, different purification protocols were evaluated to choose the best method to generate recombinant PvDBP-II with the highest yield, purity and closest conformation to the native protein. Moreover, naturally acquired IgG antibodies and its subclasses to this expressed protein were evaluated as well and it was shown that the protective IgG1 and IgG3 antibodies against malaria were predominant.

MATERIALS and METHODS

Study area and Subjects

The sampling for this study was performed in Chabahar district, Sistan and Baluchistan province, South-East of Iran. The participants were symptomatic uncomplicated *P. vivax*-infected patients attending the Public Health Center in Chabahar. Before blood collection, informed consent was obtained from adults or parents/legal guardians of children who participated in this study. One ml venous blood sample was collected in tubes containing EDTA and the infection in all 202 collected blood samples was confirmed by light microscopic examination of Giemsa-stained blood smears. For final confirmation of the microscopic assay, all blood samples were analyzed for *P. vivax* DNA by nested PCR amplification of a species-specific segment of the 18SrRNAgene of human malaria parasites [13]. This study was verified and approved by the Ethical Review Committee of Research of Pasteur Institute of Iran.

Cloning, expression and purification of recombinant PvDBP-II

DBP-IX (NCBI accession number EU860436.1), one of the variant forms of PvDBP-II reported previously in Iran [14] was cloned and expressed in *E. coli*. DBP-IX is identical to reference strain, Sal-1 (accession number, M61095) which has implication for vaccine research studies. DBP-IX was cloned in pQE30 vector (Qiagen, Hilden, Germany) and expressed in *E. coli* M15 strain as recombinant protein fused to the histidine tag as described previously [15]. Briefly, overnight culture of *E. coli* M15pQE30-DBP-IX was grown in Luria broth (pH~7.2) containing ampicillin (100 µg/ml) and kanamycine (25 µg/mL) at 37°C with shaking (150 rpm) until an optical density (OD) of 0.6-0.7 at 600 nm was reached. The expression of recombinant DBP-IX (rDBP-IX) was induced with 1 mM isopropyl-β-thio galacto pyranoside (IPTG, Sigma-Aldrich, USA). The culture was grown for another 4 h and the cells were harvested by centrifugation and kept at -80°C until use.

Optimizing the purification process of recombinant PvDBP-IX

DBP-IX contains six disulphide bonds and its expression in *E.coli* results in formation of inclusion bodies. Therefore, the first step in purification of rDBP-IX was dissolving the cell pellets in a denaturation buffer. For this purpose, two distinct chaotropic agents were examined for solubilizing the inclusion bodies in lysis buffer and efficacy of each buffer was compared. The first solution containing 6MGITC, 20mM Tris-HCl, 500mM NaCl, 10mM Imidazole (30mM imidazole was also checked) and 1mM phenyl methane sulfonyl fluoride (PMSF) as protease inhibitor (pH~7.9). In the second solution, 8M urea was used instead of GITC while all other reagents were the same. The cells were lysed on ice by sonication (Ultra schallprozessor, Germany) with 12 cycles; each consisted of 20-sec pulses with 40-sec intervals. The bacterial lysate was centrifuged at 12,000 ×g at 4°C for 15 min. The supernatant was incubated with Ni2+nitrilotriacetic acid agarose resin (Ni-NTA Agarose, Qiagen, Germany) at 4°C for 90,120 and 150 min. In order to remove the unbound proteins and non-specifically bound proteins, the resin was packed into a columns and washed with a 10-column volume of wash buffer containing 8M urea, 20mM Tris-HCl, 500mM NaCl, and different concentrations of imidazole (30, 40 and 60mM) and eluted separately in elution buffer (pH~7.9). In addition to reducing the non-specific protein bindings, the concentration of NaCl was increased to 1M in the lysis and wash buffers and the bound protein was eluted with elution buffer containing 8M urea, 20mMTris-HCl, 300mMNaCl and different concentrations of imidazole (150, 250 and 500mM).For correct refolding of the expressed protein and preventing the formation of protein aggregates, the chaotropic agents were removed gradually (6 and 4M urea) and the results were assessed by SDS-PAGE in both reducing and non-reducing conditions. For complete removal of urea, fractions containing rDBP-IX was desalted with Econo-Pac 10DG columns (BioRad, USA) according to the manufacturer's manual and concentrated with a concentrator (Eppendorf, Hamburg, Germany).

Purity analysis and confirmation of recombinant protein

The eluted proteins were analyzed under both reducing [with 1% SDS and 2% 2ME (β –mercapto ethanol)] and non-reducing [with 1% SDS and without2ME] conditions by SDS-PAGE (12%). Western blot assay was carried out by standard protocols using anti-His antibody (Penta His Antibody, Qiagen) as well as *P. vivax*-infected and normal human sera in both reducing and non-reducing conditions to confirm the purified recombinant proteins.

Indirect immune fluorescence antibody test (IFAT)

The similarity of epitopes in the recombinant protein to its corresponding native form was checked by immune fluorescent antibody test (IFAT) using the ability of the anti-DBP-IX sera to recognize the native form of the PvDBP-II antigen on the merozoite surface. Anti-sera against rDBP-IX was produced by immunizing mice with

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purified DBP-IX in combination with complete Freund's adjuvant and the sera used for recognition of native DBP-II on *P. vivax* parasites. Briefly, multi-spot slides of the parasites were prepared from *P. vivax* infected patients, air-dried and then fixed in cold acetone for 10 min. The spots were blocked with 5% BSA diluted in PBS in a wet chamber at room temperature for 30 min. After washing three times with PBS (pH~7.4), 25 µL mouse sera diluted (1:200, 1:100 and 1:50) in PBS were added to the spots and incubated in a wet chamber for 30 min. Slides were washed three times with PBS (pH~7.4) and each well was covered with 25 µL of the fluorescein-conjugated anti-mouse or anti-human (in case of positive control) poly valent IgG (1:40) and then left in a wet chamber for another 30 min. 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 and also the two known human sera infected with *P. vivax* were used as negative and positive controls, respectively.

Measurement of IgG and subclasses antibody responses to recombinant DBP-IX antigen by ELISA

To characterize the naturally acquired immune responses towards the target protein anti-PvDBP-II IgG, IgG1 and IgG3 subclasses were determined in the sera of *P. vivax* infected patients by ELISA. Briefly, maxisorp flat-bottomed, 96-well microplates (Grainer, Germany) were coated with 250 ng/well of the rDBP-IX in 0.06 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), microplates were blocked with 200 µl PBS containing 2.5% bovine serum albumin (BSA, pH~7.4) for two hours at room temperature (RT). The plates were then washed and incubated with 100 µl plasma of each patient (n=202) at 1:100 dilution for 90 min at RT. After washing with PBS-T, the plates were incubated with horseradish peroxidase-labeled anti-human IgG (1:30,000; Sigma-Aldrich, USA). The plates were washed again with PBS-T, and the presence of bound IgG was detected using o-phenyle diamine dihydro chloride-H2O2 (Sigma, USA) as substrate and stopped with 2NH2SO4. The absorbance at 492 nm was measured by using a microplate reader (Biotech, USA), and the mean value of each pair of wells was calculated. To detect subclasses of human IgG, an ELISA was performed as described above, but secondary antibodies were added at a dilution of 1:2000 using biotin-conjugated isotype-specific antihuman IgG subclass antibodies (Sigma-Aldrich, USA) at RT for one hour. After washing steps, streptavidin–peroxidase conjugate (Sigma-Aldrich, USA) was added at a dilution of 1:2500 and incubated at RT for 1 h. The enzyme reaction was developed with OPD–H2O2 (Sigma-Aldrich, USA) and stopped with 2NH2SO4. A pool of immune sera (n=5) from adult Iranian malaria patients residingin *P. vivax* endemic region and 35 serum samples from healthy non-exposed Iranians from outside the malaria endemic regions were used as positive and negative controls, respectively. The cutoff value for positive plasma was considered as the mean plus 3 standard deviation (SD) of optical density (OD) readings of the negative samples.

RESULTS

Expression and optimizing purification process of recombinant PvDBP-IX

Transfection of pQE30-DBP-IX into *E. coli* M15 competent bacteria resulted in production of the recombinant protein with the expected sizes of ~47 kDa as revealed by SDS-PAGE

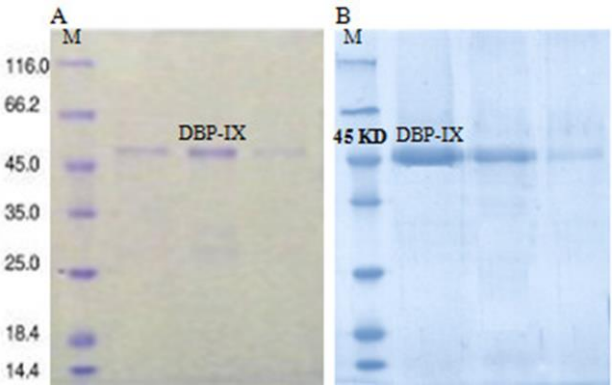


Fig. 1. SDS-PAGE analyses of eluted DBP-IX using different chaotropic reagents in the lysis buffer. (A) lysis buffer containing 6M guanidinium thiocyanate (GITC), (B) lysis buffer containing 8M urea.

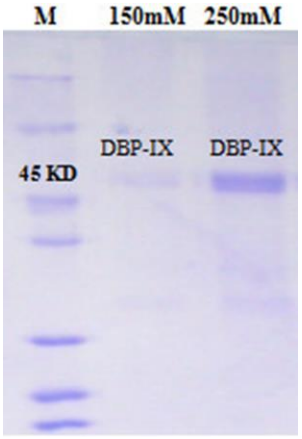


Fig. 2. SDS-PAGE analyses of eluted DBP-IX using different concentration of imidazole in the elution buffer.

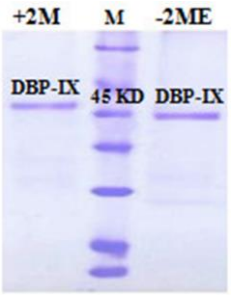


Fig. 3. SDS-PAGE analyses of recombinant DBP-IX purified with the optimized purification protocol under reducing (+2ME) and non-reducing (-2ME) conditions.

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Table. 1. Summary of purification optimization parameters of expressed PvDBP-IX in the prokaryotic system.

Optimized parameters	Chaotropic agent	NaCl	Imidazole	Final optimized condition
lysis buffer	8 M urea 6 M GITC[1]	500 mM 1M	10 mM 30 mM	8 M urea, 1 mM DTT, 1 M NaCl, 30 mM Imidazole
Wash buffer	8 M 6 M	500 mM 1 M	40 mM 50 mM 60 mM	6 M urea, 1 M NaCl, 60 mM Imidazole
Elution Buffer	8 M 6 M 4 M	300 mM	150 mM 250 mM 500 mM	4 M urea, 300 mM NaCl, 250 mM Imidazole

* The optimum incubation time with Ni-NTA resin was 120 min
1GITC: Guanidinium thiocyanate

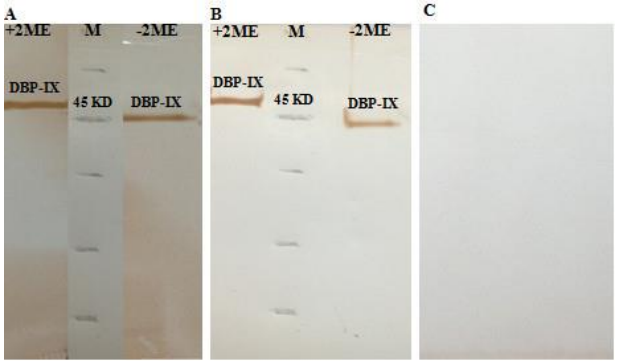


Fig. 4. Confirmation of purified DBP-IX protein by Western blot analyses under both reducing (+2ME) and non-reducing (-2ME) conditions: (A) using *P. vivax*-infected human sera; (B) Anti-His antibody; (C) Normal human sera.

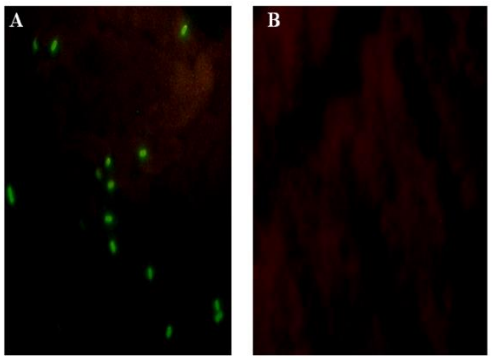


Fig. 5. IFAT results demonstrating the recognition of native PvDBP-II on the surface of *P. vivax* merozites using anti-rPvDBP-IX anti mouse antibody (A) and no detection by normal mouse sera (B).

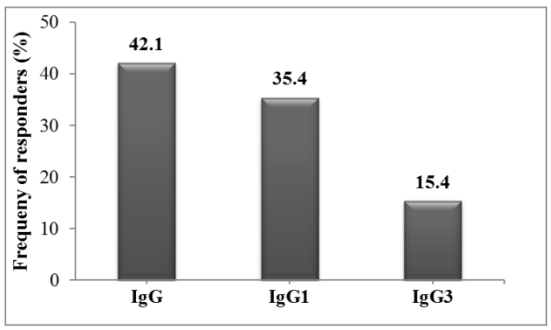


Fig. 6. The prevalence of IgG and its subclasses (IgG1 and IgG3) responses to the DBP-IX antigen among patients sera (n=202) with *P. vivax* infection from south eastern regions of Iran. Cut-off values were 0.34, 0.26 and 0.24 for IgG, IgG1 and IgG3, respectively.

the expected sizes of ~47 kDa as revealed by SDS-PAGE analysis. Over expression of heterologous proteins in *E. coli* resulted in formation of protein aggregates and for their solubilization, both 6M GITC and 8M urea were used and the results were compared. In contrast to 6M GITC, the yield of the purified protein on the SDS-PAGE was higher when 8M urea was used. However, by using the lysis buffer containing urea (8M urea, 20 mM Tris-HCl, 500 mM NaCl, 10 mM Imidazole), a few non-specific protein bands were observed on the SDS-PAGE gel (Fig. 1).

The next step for optimizing the recombinant protein purification process was removing the non-specific proteins. Different procedures were examined and the first modified parameter was changing NaCl concentration which was increased from 500mM to 1M in the lysis and wash buffers. Besides, imidazole concentration in both solutions was optimized and was increased to 30 mM. The concentrations of 40, 50 and 60 mM imidazole in the wash buffer were evaluated and after analyzing the purified protein with SDS-PAGE, it was shown that using 60 mM imidazole in the wash buffer yielded the highest purity. Incubation time of the supernatant with Ni-NTA resin can also influence the binding rate of the proteins and among the incubation times examined (90, 120 and 150 min), the most effective time for higher yield and reduced background was 120 min.

Furthermore, the concentration of imidazole in the elution buffer was also optimized and our results showed that the yield of the purified protein was markedly higher when 250 mM imidazole was used (Fig. 2), but the difference between 250 and 500 mM imidazole was not significant.

Therefore, 250 mM imidazole was chosen for the optimized purification protocol. Additionally, in order to prevent aggregate formation, the concentration of the chaoteropic agent was decreased and the highest yield was obtained by 6 and 4 M urea in the wash and elution buffers respectively (Table 1).

Comparison of the mobility shift of the protein in SDS-PAGE under reducing and non-reducing conditions was used as an indicator of disulphide bond formation in rDBP-IX. The estimated size of the purified rDBP-IX was ~45 kDa in the absence of 2ME which was noticeably smaller than the denatured DBP-IX (~47 kDa). The purified DBP-IX protein was confirmed by Western blot analysis in both reducing and non-reducing conditions (Fig. 4).

Indirect immunofluorescence antibody test (IFAT)

The ability of the sera from mice immunized with the recombinant protein to detect the native DBP-II on the *P. vivax* merozoites was confirmed by IFAT. Anti-rDBP-IX antibody

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produced in mice recognized the native protein present on the surface of *P. vivax* merozoite with high intensity (Fig. 5A). Moreover, the control sera did not react with the native protein on *P. vivax* parasite (Fig. 5B), confirming that there are common epitopes presented in the recombinant and the corresponding native form of the protein.

Measurement of IgG and IgG subclasses antibody responses to recombinant rDBP-IX antigen by ELISA

Naturally-acquired anti-DBP-II IgG responses to the purified target protein were examined in the sera from 202 patients and the results showed that natural *P. vivax* infection produces IgG against rDBP-IX in 42.1% (cut off=0.34) of patients. Moreover, analysis of the antibody responses to DBP-IX showed high (5%), medium (19.3%) and low (17.8%) positive as well as negative (57.9%) responses and the mean OD±SD values were1.3±0.26, 0.68±0.13, 0.44±0.02 and 0.27±0.06 in each group, respectively. None of the sera from healthy individuals (control group) contained IgG antibodies to rDBP-IX which confirms the specificity of the present results. Furthermore, sera that were positive for total anti–PvDBPIgG were characterized for IgG subclass responses to DBP-IX antigen. In individuals who were infected with *P. vivax*, the IgG1 (35.4%, mean OD490=0.77) was the predominant subclass whereas IgG3 (15.4%, mean OD490=0.55) was the second most prevalent subclass (Fig.6).

DISCUSSION

Vaccines have been the most useful medical tools to combat infectious diseases during the last century. However, there is no licensed vaccine against any human parasitic disease such as malaria which causes massive human suffering. However, a malaria vaccine is essential if the goal of malaria eradication is to be realized [16]. In recent years, attempts to develop a useful vaccine for malaria and especially vivax malaria have been increasing significantly and scientists are using diverse technologies to isolate and deliver specific antigens for this purpose instead of attempting to produce a live attenuated vaccine [17]. Meanwhile, the purity and correct folding of a recombinant protein are essential factors which should be considered for structural, biochemical and developmental studies of the vaccines.

PvDBP-II is the most important blood stage vaccine candidate antigen for vivax malaria. It has been studied extensively by many researchers using sera from the infected patients [18, 19] and laboratory animals which had been immunized by recombinant forms of the target protein [10]. In all these studies, recombinant forms of PvDBP-II have been produced and purified with different protocols using affinity tags. In a number of studies, rPvDBP-II was expressed as a soluble glutathione S-transferase (GST) fusion protein [8, 18, 20, 21]. Although such GST-tagged proteins were produced in soluble forms, their GST component had to be removed from DBP-II, because of its possible interference with the co-purified recombinant protein in the immunological assays. The cleavage of GST from DBP-II by thrombin should be followed by its removal from the mixture which would make the purification procedure cumbersome and time consuming. In the present study, rDBP-IX was expressed as an N-terminal his-tagged fusion protein and the purification process was optimized to obtain a higher yield of the purified protein.

Furthermore, in the previous studies in which rPvDBP-II were expressed as a his-tagged protein, the inclusion bodies were dissolved using 6 M GITC in the solubilizing buffer [22-24, 26]. However, in the present investigation, by comparing 6MGITCand 8M urea as chaotropic agents, we concluded that 8M urea was more effective in dissolving the inclusion bodies and the obtained yield of the recombinant protein was much higher (i.e. 67% increase), although some non-specific protein bands were observed with this method. In our optimization procedures to remove non-specific proteins, different concentration of imidazole and NaCl in the lysis and wash buffers were also compared.

In general, the lack of in vitro continuous cultures for *P. vivax* represents a major obstacle in developing a functional malaria vaccine. Therefore, vivax malaria vaccine researches widely focus on recombinant protein technology. As one of the most critical features of a recombinant protein to be used in vaccine researches is the proper conformation to present correct epitopes. Here by stepwise reduction of urea concentration, we tried to purify rDBP-IX in a way that could resemble its native form. The proper folding of purified DBP-IX as a vaccine candidate antigen was confirmed by different assays, namely SDS-PAGE analyses in both reducing and non-reducing conditions, IFAT and ELISA. The different sizes of rDBP-IX in reducing and non-reducing conditions and the recognition of native protein expressed on the surface of *P. vivax* merozoites by antibodies produced in mice against rDBP-IX, were indicators of native-like conformation of the purified target protein, similar to those reported previously [24-26]. Finally, the recognition of rDBP-IX by P.vivax patients' sera was further evidence of its correct representation of the epitopes of the target protein.

The ELISA results showed that 42.1% of the patients were positive responder for rDBP-IX which was higher than a former study in Iran (32%) [27], however this result was comparable with those from unstable malaria transmission regions such as Colombia (40%) [28] and Brazilian Amazon region (50%) [19]. According to previous studies, the dominance of cytophilicIgG1 and IgG3 subclasses has been reported in response to malaria infection [27, 29]. The suggested mechanism by which these subclasses are protective includes their binding to the Fc receptors on the monocytes, leading to antibody dependent cell mediated inhibition of the parasite replication. Our results confirmed this hypothesis as the two most predominant IgG subclasses detected by rDBP-IX in sera of *P. vivax*-infected patientswereIgG1, followed by IgG3.

In summary, in the present investigation, different purification parameters were optimized to achieve the highest purity and yield of a properly folded rDBP-IX. Our aim was to optimize the purification of this recombinant protein by examining different parameters which could lead to the higher quality of the target protein. Based on our IFAT and ELISA results, we envisage this recombinant protein is suitable for use in vaccine developing research and immunological studies for vivax malaria.

AUTHOR'S CONTRIBUTION

VV (Ph.D. student) contributed in the laboratory work, analysis of the data and drafted the paper. SM helped with the experimental assays and BV gave critical suggestions for optimizing the protein purification procedure. SZ designed the work, supervised the study and critically revised the manuscript NDD helped with data analysis and critical reading of the manuscript.

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CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

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