Biochemical and immunological characterization of *E. coli* expressed 42 kDa fragment of *Plasmodium vivax* and *P. cynomolgi bastianelli* merozoite surface protein-1

Deep C Kaushal1*, Nuzhat A Kaushal2, Atul Narula1, Niraj Kumar1, S K Puri2,
Shitij Dutta3 and David E Lanar3

Divisions of Microbiology1 and Parasitology2
Central Drug Research Institute, Post Box 173, Lucknow 226001, India
&
3Department of Immunology, Walter Reed Army Institute of Research
Silver Spring, Maryland 20910, USA

Received 14 June 2007; revised 22 October 2007

*Corresponding author

Tel: 522-2612411-18 Ext 4390, 4339
E-mail: deepkaushal@hotmail.com
depkaushal@yahoo.com

*Plasmodium vivax* is one of the most widely distributed human malaria parasites and due to drug-resistant strains, its incidence and prevalence has increased, thus an effective vaccine against the parasites is urgently needed. One of the major constraints in developing *P. vivax* vaccine is the lack of suitable *in vivo* models for testing the protective efficacy of the vaccine. *P. vivax* and *P. cynomolgi bastianelli* are the two closely related malaria parasites and share a similar clinical course of infection in their respective hosts. The merozoite surface protein-1 (MSP-1) of these parasites has found to be protective in a wide range of host-parasite systems. *P. vivax* MSP-1 is synthesized as 200 kDa polypeptide and processed just prior to merozoite release from the erythrocytes into smaller fragments. The C-terminal 42 kDa cleavage product of MSP-1 (MSP-142) is present on the surface of merozoites and a major candidate for blood stage malaria vaccine. In the present study, we have biochemically and immunologically characterized the soluble and refolded 42 kDa fragment of MSP-1 of *P. vivax* (PvMSP-142) and *P. cynomolgi B* (PcMSP-142). SDS-PAGE analysis showed that both soluble and refolded *E. coli* expressed *P. vivax* and *P. cynomolgi B* MSP-142 proteins were homogenous in nature. The soluble and refolded MSP-142 antigens of both parasites showed high reactivity with protective monkey sera and conformation-specific monoclonal antibodies against *P. cynomolgi B* and *P. vivax* MSP-142 antigens. Immunization of BALB/c mice with these antigens resulted in the production of high titres of cross-reactive antibodies primarily against the conformational epitopes of MSP-142 protein. The immune sera from rhesus monkeys, immunized with soluble and refolded MSP-142 antigens of both parasites also showed high titred cross-reactive antibodies against MSP-142 conformational epitopes. These results suggested that the soluble and refolded forms of *E. coli* expressed *P. vivax* MSP-142 antigens were highly immunogenic and thus a viable candidate for vaccine studies.

**Keywords:** *Plasmodium vivax*, *Plasmodium cynomolgi*, Malaria, Vaccine, Merozoite surface protein-1, Merozoite, Rhesus monkey.

Malaria is caused by the protozoan parasite of genus *Plasmodium* and is one of the largest public health problems in the world. About 300-660 million people are affected by malaria worldwide. The disease results in the death of 2-3 million people annually, particularly among African children under the age of five, making development of a malaria vaccine a global health priority.

Most of the work on malaria vaccine development has been done on *P. falciparum* and relatively little efforts have been made towards developing *P. vivax* vaccine. *P. vivax* is infrequently fatal and imposes a serious burden of morbidity in malaria endemic areas outside Africa and according to WHO there may be 15-30 million cases of *P. vivax* annually. Moreover, strains of *P. vivax* resistant to chloroquine have been identified in many areas and are expected to spread, leading to further increase in the worldwide incidence and prevalence of *vivax* malaria. Thus, *P. vivax* continues to impose a major public health burden in endemic areas and necessitates the continued use of chemoprophylaxis. Some of the major constraints for the development of vaccine against *P. vivax* malaria are the relapsing nature of *P. vivax* hepatic stages, lack of *in vitro* culture and suitable *in vivo* monkey model system. The *in vivo* testing of *P. vivax* vaccine candidates requires highly specialized monkey model.
such as Toque and New World monkey species which are available in limited numbers and may not be sufficient to cope with increasing number of *P. vivax* vaccine candidates. It is, therefore, necessary to compare and down-select vaccine candidate antigens in preclinical studies using alternate animal model before going to the clinical trials in humans.

*P. cynomolgi* B, a closely related species to *P. vivax*, infects rhesus macaques in South-east Asia and its transmission in humans is also reported. Two parasites share a similar clinical course of infection, a reticulocyte-specific invasion, presence of Schuffner’s dots on infected erythrocytes, and a dormant liver hypnozoite stage that is responsible for a relapsing blood stage infection. In addition they have similar genomic GC content and rRNA analysis confirming their close taxonomic relatedness. High homology of major vaccine candidates such as the apical membrane antigen-1, circumsporozoite protein, erythrocyte-binding protein and the 42 kDa fragment of merozoite surface protein-1 (MSP-1) have been reported. Earlier, *P. cynomolgi*-rhesus monkey model system has been used to test the efficacy of *P. vivax* recombinant antigens.

The MSP-1 found on the surface of *Plasmodium* merozoites has been a prime vaccine candidate. The 200 kDa precursor molecule of MSP-1 undergoes step-wise proteolytic processing, resulting in 42 kDa protein (MSP-1) on the surface of free merozoites. This 42 kDa intermediate product undergoes secondary processing at the time of invasion, releasing a 33 kDa soluble polypeptide (MSP-1) and leaving behind a glycosyl-phosphatidylinositol-anchored 19 kDa product (MSP-1) on the invading merozoite. Depending on species, MSP-1 contains 10 or 11 cysteine residues that form five disulfide bonds. Immunization with recombinant MSP-1 and MSP-1 raise antibodies that inhibit invasion of merozoites and protect monkeys.

Earlier, we have evaluated the *P. cynomolgi* B-rhesus monkey model system for testing the protective potential of *E. coli* expressed MSP-1 recombinant antigens of *P. vivax* and *P. cynomolgi* and found significant reduction in parasite burden of monkeys immunized with MSP-1 antigens of both parasites. In the present study, we have biochemically and immunologically characterized the soluble and refolded forms of *E. coli* expressed MSP-1 antigens of both parasites using polyclonal and monoclonal antibodies against protective conformational epitopes and have shown that *in vitro* folded MSP-1 antigens of these parasites are correctly folded. In addition we have tested the immunogenicity of soluble and refolded MSP-1 antigens of both parasites in mice using Montanide ISA720 an adjuvant suitable for human use.

**Materials and Methods**

Recombinant MSP-1 proteins and their denaturation, reduction and alkylation

The expression and purification of *P. vivax* MSP-1 and *P. cynomolgi* MSP-1 soluble (PvS and PcS) and refolded (PvR and PcR) proteins were done as described earlier. The recombinant antigens were denatured and reduced by disturbing the disulfide bonds. The soluble and refolded proteins from both parasites were either alkylated or denatured, reduced and alkylated. Briefly, dithiothreitol (DTT, 10 mM) and guanidine hydrochloride (6 M) were added to the recombinant MSP-1 proteins (100 µg/ml) and incubated at 50°C for 1 h. Thereafter, the denatured and reduced proteins were alkylated by adding sodium iodoacetate (100 mM) and further incubated in dark at 37°C for 1 h. The alkylated or denatured, reduced and alkylated proteins were dialyzed overnight at 4°C and kept at -20°C until used.

**SDS-Polyacrylamide gel electrophoresis**

The purity of recombinant MSP-1 antigens was judged by SDS-PAGE using 10% acrylamide gels run under non-reducing conditions following the procedure of Lammeli.

**Immunization of mice**

BALB/c mice (6-8 weeks old) were immunized with MSP-1 soluble and refolded antigens of both parasites. Institutional Animal Ethics Committee of CDRI approved the immunization protocols. The mice were kept on standard diet in animal house facility of our institute and divided into five groups (Group 1 to 5) with each group comprised 10 mice (5 males and 5 females). Briefly, mice in group 1 were immunized with *P. vivax* soluble (PvS), group 2 with *P. vivax* refolded (PvR), group 3 with *P. cynomolgi* soluble (PcS) and group 4 with *P. cynomolgi* refolded (PcR) antigens. Each mouse was immunized subcutaneously with 0.2 ml of antigen formulation containing 20 µg of antigen in 60 µl and 140 µl of Montanide ISA720 adjuvant (Spinc Inc., France). The mice in group 5 were used as controls and received PBS and Montanide ISA720 adjuvant in the same.
ratio as for mice of group 1 to 4. The mice were given three injections at 15 days interval and were bled 1 week after each injection. The sera collected were kept frozen at -20°C.

**Immune monkey sera**

Immunization of monkeys with MSP-1$_{42}$ (PvS, PvR, PcS and PcR) was done as described previously$^{21}$. The monkeys were immunized at four weeks intervals and were bled every two weeks for collection of serum. The monkeys in all the five groups were challenged with *P. cynomolgi* B blood stage parasites 4 weeks after the last injection and the number of parasites was counted as described elsewhere$^{21}$. The parasitaemia was expressed as parasites/mm$^3$. After 45 days, the monkeys were cured with chloroquine$^{26}$.

**ELISA**

Enzyme-linked immunosorbent assay (ELISA) was done in 96-well plates essentially as described before$^{27,28}$. Briefly, the wells of microtitre plates were coated with recombinant Pv or PcMSP-1$_{42}$ antigens (40 ng/100 µl/well, diluted in PBS) by incubating the plates at 37°C for 1 h and then overnight at 4°C. The plates were blocked by incubation with 300 µl of 3% non-fat dry milk powder (w/v, prepared in PBS) at 37°C for 2 h. After three washes with PBS-Tween (PBS-T, PBS with 0.05% Tween-20), the plates were incubated with 100 µl of appropriately diluted antibodies (diluted in 1% milk) for 2 h at 37°C, washed again thrice with PBS-T and incubated for 1.5 h with optimally diluted enzyme labeled secondary antibody (horseradish peroxidase-labelled anti-mouse or anti-monkey IgG; Sigma, USA). After washing the plates with PBS-T, the color was developed by adding the substrate solution (1 mg/ml, o-phenylenediamine in citrate-phosphate buffer, pH 5.0, containing 1 µl/ml H$_2$O$_2$). The reaction was stopped after 10 min by adding 5 N H$_2$SO$_4$ and the absorbance was read at 490 nm using Molecular Devices UVmax microplate ELISA reader.

**Isotypic analysis**

The immune mice sera from different groups of mice were tested for IgG, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA antibodies against MSP-1$_{42}$ antigens by ELISA using purified class-specific heavy chain reagents (goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA) and rabbit anti-goat Ig peroxidase conjugate (Sigma, USA).

**Results**

**SDS-PAGE analysis of *P. vivax* MSP-1$_{42}$ and *P. cynomolgi* B MSP-1$_{42}$ antigens**

The SDS-PAGE pattern of recombinant *P. vivax* MSP-1$_{42}$ soluble (PvS), refolded (PvR) and *P. cynomolgi* B MSP-1$_{42}$ soluble (PcS), refolded (PcR) antigens under non-reducing conditions is shown in Fig. 1. All the four recombinant antigens showed a single protein band. The soluble and refolded proteins of both parasites exhibited same mobility on native SDS-PAGE. The SDS-PAGE pattern of soluble and refolded proteins from both parasites before and after alkylation is shown in Fig. 2. The molecular masses of alkylated PvS and PvR of *P. vivax* (Fig. 2a, lanes 2 and 5) are same and showed slight increment as compared to native proteins (Fig 2a lanes 1 and 4). The reduction and alkylation of PvS resulted in increase in size and thus decreased mobility (Fig. 2a, lane 3). The mobility of reduced and alkylated PvR was the same as reduced and alkylated PcS (data not shown). Similar results were obtained with *P. cynomolgi* B MSP-1$_{42}$ PcS and PcR proteins (Fig. 2b).

![Fig. 1—Coomassie blue stained purified *P. vivax* soluble (Lane 1), *P. vivax* refolded (Lane 2), *P. cynomolgi* B soluble (Lane 3) and *P. cynomolgi* B refolded (Lane 4) MSP-1$_{42}$ proteins on non-reducing 10% SDS-polyacrylamide gel](image1)

![Fig. 2—Coomassie blue stained non-reducing 10% SDS-polyacrylamide gel of purified PvMSP-1$_{42}$ (a) and PcMSP-1$_{42}$ (b) proteins [Lane1, soluble; lane 2, soluble alkylated; lane 3, denatured, reduced and alkylated; lane 4, refolded; and lane 5, refolded alkylated]](image2)
The presence of reduction sensitive epitopes was confirmed by reactivity of these antigens with protective monkey sera and conformation-specific monoclonal antibodies against *P. vivax* and *P. cynomolgi* B MSP-1\(_{42}\) antigens. The polyclonal (monkey anti-*P. cynomolgi* B schizont)\(^{25}\) and monoclonal antibodies (MoAb1, 2, 3, and 4) showed same level of reactivity (ELISA OD around 3.00) with soluble and refolded MSP-1\(_{42}\) antigens of both parasites (Table 1).

### Antibody response in immunized mice

The sera obtained from immunized mice were tested in ELISA against MSP-1\(_{42}\) antigens of both parasites to measure the levels of antibodies. The sera from five groups (anti-PvS, anti-PvR, anti-PcS, anti-PcR and control group) collected after each injection, were tested in ELISA against both MSP-1\(_{42}\) antigens. The antibody titre of immune mice sera of different bleeds (1\(^{st}\), 2\(^{nd}\), 3\(^{rd}\)) from four groups are given in Table 2. All the mice sera (Group 1 to 4) showed increase in antibody titres with number of injections.

**Table 1**—Reactivities of soluble, refolded MSP-1\(_{42}\) antigens of *P. vivax* and *P. cynomolgi* B with polyclonal and monoclonal antibodies in ELISA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>ELISA O.D. at 490 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVS</td>
<td>3.134</td>
</tr>
<tr>
<td>PVR</td>
<td>3.092</td>
</tr>
<tr>
<td>PCBS</td>
<td>3.427</td>
</tr>
<tr>
<td>PCRS</td>
<td>3.325</td>
</tr>
<tr>
<td>MoAb1</td>
<td>2.827</td>
</tr>
<tr>
<td>MoAb2</td>
<td>2.979</td>
</tr>
<tr>
<td>MoAb3</td>
<td>3.091</td>
</tr>
<tr>
<td>MoAb4</td>
<td>3.289</td>
</tr>
</tbody>
</table>

**Table 2**—Reciprocal antibody titres of serum pools of different bleeds from four groups of mice with *P. vivax* and *P. cynomolgi* B MSP-1\(_{42}\) antigens

<table>
<thead>
<tr>
<th>Groups</th>
<th>PVS MSP-1(_{42}) Antigen</th>
<th>PCRS MSP-1(_{42}) Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (PVS)</td>
<td>26.927 ± 1.346 (1(^{st}))</td>
<td>219.106 ± 1.592 (1(^{st}))</td>
</tr>
<tr>
<td>Group 2 (PVR)</td>
<td>24.411 ± 1.464 (2(^{nd}))</td>
<td>129.516 ± 1.320 (2(^{nd}))</td>
</tr>
<tr>
<td>Group 3 (PCS)</td>
<td>234.833 ± 1.320 (3(^{rd}))</td>
<td>125.843 ± 1.765 (3(^{rd}))</td>
</tr>
<tr>
<td>Group 4 (PCR)</td>
<td>24.411 ± 1.464 (2(^{nd}))</td>
<td>129.516 ± 1.320 (2(^{nd}))</td>
</tr>
</tbody>
</table>

Higher antibody titres were obtained with the homologous antigens than heterologous. The sera after 3\(^{rd}\) injection showed 6-8 fold and 5-6 fold increase in antibody titres with the homologous and heterologous antigen, respectively. The antibody titres (geometric mean ± 2 SD) observed with sera from groups 1-4 (anti-PvS, anti-PvR, anti-PcS and anti-PcR respectively) with PvS antigen were 207937 ± 1953, 194012 ± 2046, 129516 ± 1708 and 125843 ± 1765. On using PcS antigen, group 1-4 (anti-PvS, anti-PvR, anti-PcS and anti-PcR respectively) sera showed antibody titres of 142120 ± 1760, 136474 ± 1689, 234833 ± 1320 and 219106 ± 1592. The sera from control (Group 5) did not show any reactivity with any of the PvS and PcS antigens (data not shown).

### Reactivity of immune mouse serum pools with soluble, refolded and denatured antigens

The reactivities of serum pools from different groups (Group 1–4) of mice were tested with soluble, refolded and denatured MSP-1\(_{42}\) antigens of both parasites in ELISA. Serum pools (anti-PvS, anti-PvR, anti-PcS, anti-PcR and control group) collected after each injection, were tested in ELISA against both MSP-1\(_{42}\) antigens. Table 2 gives the antibody titres of serum pools from different bleeds (1\(^{st}\), 2\(^{nd}\), 3\(^{rd}\)) from four groups of mice.
anti-PcS, anti-PcR) from different groups of immunized mice showed significantly high reactivity with both soluble and refolded (antibody titres 48,000-128,000 and 32,000-128,000 respectively) MSP-142 antigens of both parasites, while very little reactivity (<20%) was observed with denatured antigens of these parasites (Fig. 3).

**Isotype analysis of immune mice sera**

The reciprocal antibody titres for each group of mice with PvS and PcS antigens are shown in Fig. 4a and b. All the four groups (PvS, PvR, PcS, PcR) showed high antibody titres for the total IgG and among isotypes of antibodies, IgG1 titre being the highest, followed by IgG2b, IgG2a and IgM. No significant IgG3 and IgA reactivity was observed with both antigens.

**Antibody responses and parasitaemia in immunized monkeys**

The serum pools from five groups of monkeys immunized with PvS, PvR, PcS, PcR (Groups 1 to 4 respectively) and Montanide ISA720 adjuvant (Group 5) were tested against PvS and PcS antigens in ELISA and results are given in Table 3. Almost same antibody titres were observed for group 1 and 2 (anti-PcS and anti-PcR) monkey sera with both PvS and PvR antigens. The group 3 and 4 (anti-PcS and anti-PcR) monkey sera showed high antibody titres with PcS antigen. The titration curve of serum pools from different groups of monkeys (Groups 1 to 4) with PvS and PcS antigens are shown in Fig. 5. Comparable titration curves were obtained with serum pools from all the four groups with both PvS and PcS antigens. The serum pool from group 5 did not show any reactivity (data not shown).

---

**Fig. 4—Antibody titres of different isotypes in immune mice sera pools of different groups with *P. vivax* MSP-142 and *P. cynomolgi* B MSP-142 antigens in ELISA**

**Table 3—ELISA titres and course of infection in different groups of rhesus monkeys after challenge with *P. cynomolgi* B parasites**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ELISA Titre × 1000</th>
<th>Primary peak</th>
<th>Parasiatemia</th>
<th>Daily Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>31309.6 ± 21775.59</td>
<td>16034.6 ± 11440.1</td>
<td>5248.8 ± 3334.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12702-56100)</td>
<td>(5785-34532)</td>
<td>(2381-9366)</td>
</tr>
<tr>
<td>Group 1</td>
<td>PvS</td>
<td>111.431 ± 1.859</td>
<td>97.006 ± 2.137</td>
<td>9584.2 ± 2116.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12702-56100)</td>
<td>(15132-49824)</td>
<td>(6792-1252)</td>
</tr>
<tr>
<td></td>
<td>PcS</td>
<td>114.035 ± 1.761</td>
<td>101.593 ± 2.046</td>
<td>10068.0 ± 3001.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(31239-61040)</td>
<td>(12729-50799)</td>
<td>(4632-12719)</td>
</tr>
<tr>
<td>Group 2</td>
<td>PvS</td>
<td>114.035 ± 1.761</td>
<td>101.593 ± 2.046</td>
<td>10068.0 ± 3001.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(31239-61040)</td>
<td>(12729-50799)</td>
<td>(4632-12719)</td>
</tr>
<tr>
<td></td>
<td>PcS</td>
<td>86.272 ± 1.988</td>
<td>143.675 ± 2.839</td>
<td>7659.3 ± 3503.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20025-65410)</td>
<td>(3913-41185)</td>
<td>(3640-11400)</td>
</tr>
<tr>
<td>Group 3</td>
<td>PvS</td>
<td>86.272 ± 1.988</td>
<td>143.675 ± 2.839</td>
<td>7659.3 ± 3503.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20025-65410)</td>
<td>(3913-41185)</td>
<td>(3640-11400)</td>
</tr>
<tr>
<td></td>
<td>PcS</td>
<td>80.635 ± 2.046</td>
<td>114.035 ± 1.761</td>
<td>74779.8 ± 33688.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15288-55472)</td>
<td>(42284-118125)</td>
<td>(14198-31476)</td>
</tr>
<tr>
<td>Group 4</td>
<td>PcS</td>
<td>80.635 ± 2.046</td>
<td>114.035 ± 1.761</td>
<td>74779.8 ± 33688.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15288-55472)</td>
<td>(42284-118125)</td>
<td>(14198-31476)</td>
</tr>
<tr>
<td>Group 5</td>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The reactivities of PvS, PvR, PvD, PcS, PcR and PcD MSP-142 antigens were tested with immune monkey serum pools (taken 1 week before challenge) from four groups of immunized monkeys (Groups 1-4) in ELISA and the antibody titres are shown in Fig. 6. The denaturation of MSP-142 antigens resulted in significant reduction of ELISA reactivity with immune monkey serum pools. The reciprocal antibody titres of 2000-4000 were obtained with denatured MSP-142 antigens of both parasites. High antibody titres (64,000-128,000) were observed with soluble and refolded antigens (PvS, PvR, PcS and PcR). These results suggested that most of the antibodies in these immune sera (produced by immunization with recombinant MSP-142 antigen) may be directed against the disulphide bonded conformational epitopes of MSP-142 antigens of both parasites.

The parasitaemia data on immunized monkeys (challenged with *P. cynomolgi* B blood stage parasites) in all the five groups are given in Table 3. The parasitaemia of different groups was compared in terms of primary and secondary peaks parasitaemia, and average daily parasitaemia. All the four vaccinated groups showed significantly lower parasite burden (*p*<0.05) as compared to the control group. No significant difference was observed between the groups immunized with the soluble and refolded proteins (*p*>0.05).

**Discussion**

The MSP-1 expressed on the surface of invasive merozoites of *Plasmodium* is one of the important targets for development of an effective malaria vaccine. Thus correct folding, purity and yield of recombinant antigen are important criteria for developing MSP-1 malaria vaccine. The MSP-142 fragments of MSP-1 are well characterized in *P. falciparum*31-34, *P. cynomolgi*7 and *P. yoelii*35 and are shown to be the targets of protective immunity. Different expression systems have been evaluated for development of recombinant MSP-1 malaria vaccine. These include production of MSP-1 in *Saccharomyces cerevisiae*36, *Pichia pastoris*37, baculovirus-infected insect cells34 and milk of transgenic mice38. The *E. coli* protein expression system, the first commercialized system for recombinant protein is cost-effective and very efficient for non-glycosylated proteins, such as MSP-142. In our earlier study, we have evaluated protective potential of *P. vivax* and *P. cynomolgi* B MSP-142 recombinant antigens in *P. cynomolgi* B- rhesus monkey model system.21 In the present study, we have done the biochemical and immunological characterization of
MSP-1 patient sera very little reactivity with denatured antigens soluble and refolded antigens of both parasites and different groups of immunized mice with both were directed against the conformational epitopes. Suggested that the antibodies in immune mice sera polyclonal antibodies Kaushal, unpublished observation) and protective conformation-specific monoclonals (Kaushal and refolded and soluble proteins of both parasites with further evidenced by the same level of reactivity of these proteins were correctly folded. This was compared to native proteins thereby suggesting that the formation of disulfide bond. In order to test whether refolded protein also has one cysteine free, both soluble and refolded proteins from both the parasites were alkylated and analyzed on SDS-PAGE under non-reducing conditions. The alkylated \textit{P. vivax} (PvS, PvR) and \textit{P. cynomolgi} B (PcS, PcR) MSP-1\textsubscript{42} proteins revealed same molecular mass and showed slight increment in the molecular mass as compared to native proteins thereby suggesting that these proteins were correctly folded. This was further evidenced by the same level of reactivity of refolded and soluble proteins of both parasites with conformation-specific monoclonals (Kaushal and Kaushal, unpublished observation) and protective polyclonal antibodies\textsuperscript{29}. The reduction and alkylation of PvS, PvR, PcS and PcR resulted in significant increase in molecular mass and thus decrease in mobility. These results suggest that reduction may result in breakage of disulfide bonds and make the sites available for alkylation.

Higher antibody titres were obtained in sera from four groups of mice (anti-PvS, anti-PvR, anti-PcS, and anti-PcR) with soluble and refolded MSP-1\textsubscript{42} antigens of both parasites suggesting that these antigens are highly immunogenic in nature. Isotype analysis revealed a predominance of IgG1 antibody in immune mice sera. Earlier similar observations for predominance of IgG1 responses were reported in mice immunized with \textit{P. yoelii} MSP-1\textsubscript{19} and \textit{P. vivax} MSP-1\textsubscript{19} as well as in \textit{P. vivax}-infected patient sera\textsuperscript{40}. High reactivity of serum pools from different groups of immunized mice with both soluble and refolded antigens of both parasites and very little reactivity with denatured antigens suggested that the antibodies in immune mice sera were directed against the conformational epitopes.

The protective response to MSP-1 is shown to be antibody-mediated in \textit{in vitro} invasion inhibition assay and passive transfer experiments\textsuperscript{41}. Monoclonal and polyclonal antibodies against several antigens of \textit{P. cynomolgi} and \textit{P. vivax} are found to be cross-reactive\textsuperscript{42,43} and \textit{P. cynomolgi} sporozoites protect humans against a \textit{P. vivax} challenge\textsuperscript{44}. In our earlier study\textsuperscript{21}, because of close phylogenetic relationship between \textit{P. cynomolgi} and \textit{P. vivax}, we used \textit{P. cynomolgi} B rhesus monkey model to compare the immunogenicity and efficacy of soluble and refolded \textit{P. vivax} MSP-1\textsubscript{42} products. We found that the soluble and refolded PvMSP-1\textsubscript{42} products showed similar immunogenicity and efficacy. Immunized monkeys in both the homologous and heterologous challenge groups showed similar reduction in parasite burden as compared to the adjuvant control groups\textsuperscript{21}.

The Montanide ISA720 adjuvant has been used in several human vaccine trials\textsuperscript{45} and in combination with PvMSP-1\textsubscript{42} induces both B and T cell responses in mice\textsuperscript{40,46}. In the present study, immunization of mice with \textit{P. vivax} and \textit{P. cynomolgi} B MSP-1\textsubscript{42} recombinant antigens adjuvanted with Montanide ISA720 induced high level of MSP-1 specific antibodies. In our earlier study, vaccination of rhesus monkeys with these antigens in combination with Montanide ISA720 induced a partially protective immune response in primates using an adjuvant acceptable for human use\textsuperscript{21}. Similarly, partial protection has been reported in vaccine trials using PvMSP-1\textsubscript{19} adjuvanted with a non-ionic block copolymer in the splenectomized \textit{Saimiri} monkey model against \textit{P. vivax} challenge\textsuperscript{8,47}. Baculovirus produced MSP-1\textsubscript{42} and MSP-1\textsubscript{19} fragments of \textit{P. cynomolgi} induce a high degree of sterile protection; however, Freund’s complete adjuvant (not suitable for human use) was employed\textsuperscript{7}.

Conclusion

In the present study, we have biochemically and immunologically characterized the soluble and refolded \textit{P. vivax} and \textit{P. cynomolgi} B MSP-1\textsubscript{42} antigens expressed in \textit{E. coli}. SDS-PAGE analysis shows that both soluble and refolded \textit{E. coli} expressed MSP-1\textsubscript{42} antigens of both parasites are homogenous in nature. The reactivities of MSP-1\textsubscript{42} refolded antigens with antibodies against conformational epitopes suggest that these proteins are correctly refolded. Immunization of mice with MSP-1\textsubscript{42} antigens of both
parasites induced high titres of antibodies primarily against the conformational epitopes. These findings suggest that E. coli expressed soluble and in vitro refolded P. vivax MSP-1α antigens are immunogenic in nature and can be used for vaccine studies.

Acknowledgements
This study was funded by grants no. 990352 and no. 990339 from the WHO to DCK and DEL. We would like to thank Dr. C M Gupta, Director, CDRI for his support for this work. One of the authors (NK) is grateful to the Council of Scientific and Industrial Research for Junior Research fellowship (CDRI Communication no.7262).

References