Chapter 3

RESULTS
3. RESULTS

3.1 Cloning and characterization of *P. yoelii msp-9* gene

A BlastX analysis of PfMSP-9 nucleotide sequences against all the annotated ORFs in the PlasmoDB database identified the presence of its homologue, on the lower strand of chromosome 1, contig 811, from 5651 to 3612 bases in the *P. yoelii* genome. Primers were designed to amplify the full length gene corresponding to mature PyMSP-9 protein. Amplification was carried out using *P. yoelii* yoelii nigeriensis genomic DNA and the Py5'A forward / Py3'A reverse primer set. A single 2.0 Kb DNA fragment was amplified by PCR (Figure 3.1). The purified PCR product was cloned into pGEM-T vector and the transformants were analyzed by restriction digestion with BamHI-HindIII enzymes. Two positive clones from the restriction analysis were selected and the complete insert was sequenced. The sequence of this insert was found to be identical to the sequence reported in the PlasmoDB database.

![Size (Kb)](image)

Figure 3.1 Ethidium bromide stained 1% agarose gel showing the PCR amplification of Pymsp-9 full-length gene (∼2.0 Kb). The marker positions are indicated on the left.

3.2 Cloning and characterization of *P. berghei msp-9* gene

3.2.1 Screening of *P. berghei* genomic library

To identify the MSP-9 homolog from *P. berghei*, we first screened a *P. berghei* genomic DNA library in lambda zap vector using a *P. falciparum* MSP-9 N-terminal DNA fragment
as a probe (aa 24 to 369). After several rounds of screening, five positive clones were selected for DNA excision and cloning. These clones were analyzed by restriction digestion and southern blot hybridization with DNA probes derived from different regions of *P. falciparum* *msp-9* gene. All the clones released an insert of 2.0 Kb that showed hybridization only with PfMSP-9 (N) probe (aa 24 to 369), but not with PfMSP-9 (Ncys) (short N-terminal, aa 24 to 195) or with PfMSP-9 (M) (aa 370 - 507) probes. Complete sequence of the insert from clone 231(S1) was obtained by primer walking, and BlastX analysis against the Genebank sequences did not show any significant homology of the clone 231(S1) with the known MSP-9 genes. We found that the DNA sequence of clone 231(S1) showed 17% homology with a region in PbMSP-9 (N) probe corresponding to aa 195 and 369. This region is exclusive of N probe and it contains a small stretch of around 50 residues with tandem repeats of the sequence DDED/DEED. Tandem repeats are known to be commonly present in many malaria proteins and most probably, the presence of this repeat sequence in PbMSP-9 (N) probe was the reason for the non-specificity of PfMSP-9 (N) probe to identify the homologue from *P. berghei* MSP-9 genomic DNA library. Figure 3.2 shows the Southern blot hybridization of some of the clones that were screened.

### 3.2.2 Identification of *P. berghei* msp-9 gene by degenerate PCR and RACE

A second approach based on a combination of degenerate PCR and RACE was applied to isolate the *Pbmsp*-9 gene. An analysis of the sequence alignment from the known MSP-9 proteins indicated that the peptide sequences LIEKGKEIGEK, LKDILLRLLYK, RLLIKIKNKMC in the middle region of these proteins were conserved in most of the members of the MSP-9 family, with some differences found in PfMSP-9. It was observed that the sequence RLLIKIKNKMC constituted a part of one PbEST (BF295925), which was found to have homology to all the MSP-9s known at that time. The degenerate PCR primers ENK-1 (forward) and ENK-3 (reverse) corresponding to the conserved sequences LIEKGKEIGE and RLLIKIKNKMC amplified a 0.387 kb segment (termed as middle region) from *P. berghei* genomic DNA.
Figure 3.2  (A) Autoradiogram showing the hybridization of the 2.0 Kb insert in phagemids from positive clones (lanes 3-5 and 7-12). Phagemid DNA was digested with EcoRI restriction enzyme and separated in 1% agarose gel. (B) Schematic representation of PfMSP-9 gene showing the location of PfMSP-9 (Ncys), PfMSP-9 (N) and PfMSP-9 (M) fragments, used as probes in the library screening and southern blot analysis. Location of signal sequence (SS), cysteines (*) and repeat regions (RI, RII) are also marked.

The sequence analysis of this amplicon confirmed that it was indeed a portion of Pbmsp-9 gene having sequence similarity to other members of this family. In order to obtain the rest of the gene, 5' RACE and 3' RACE were performed as described in Materials and Methods (Section 2.2.6, page 56). The 5' RACE reaction yielded a 1.2 kb fragment.
consisting of 406 bp 5' untranslated region (5' UTR) and the 5' end of the gene preceding the middle region. Similarly, 3' RACE yielded a 1.4 kb fragment consisting of the 3' region of the Pb MSP-9 ORF that follows the middle region and 328 bp of 3'UTR (Figure 3.3). Both the RACE products were cloned in TA cloning vector and the insert from three different clones of each reaction were fully sequenced. The sequence analysis confirmed that these amplicons were parts of the whole msp-9 gene. The 5' RACE clones showed the characteristic cluster of four conserved cysteines in the 5' region of the ORF and the 3' RACE clones showed that although the sequence was similar to Pymsp-9 gene, it had some differences, which clearly indicated that it was the related gene Pbmsp-9. The sequence data from all the clones obtained through the different strategies described above were analyzed to obtain the overlaps and the whole Pbmsp-9 gene was assembled which contained an ORF of 1857 bases. Finally, based on the sequence of this ORF, PCR primers were designed to amplify the full length gene from P. berghei genomic DNA and again the sequence of the whole gene was confirmed from this amplicon. The complete nucleotide sequence of PbMSP-9 ORF along with the 5' and 3' UTR regions obtained by RACE was reported in the GenBank TM, EMBL and DDJB databases under the accession number AY302245. Figure 3.4 summarize the strategy to identify P. berghei msp-9 gene.

Figure 3.3 Ethidium bromide stained 1% agarose gel showing the PCR amplification of Pbmsp-9 full-length gene (~1.8 Kb), the 3' RACE product (~1.4 Kb) and 5' RACE product (~1.2 Kb).
Figure 3.4  (A) Alignment of middle region of MSP-9 proteins indicating the conserved regions used to design the primers ENK-1/ENK-3 that were used to amplify P. berghei msp-9 middle region. Pv: P. vivax, Pc: P. cynomolgi, Pk: P. knowlesi, Pf: P. falciparum (B) Schematic representation of the products obtained from RACE and the assembly of P. berghei msp-9 ORF.
3.3 Northern blot analysis

In order to confirm the presence of the RNA transcript for Pbmsp-9 and Pymsp-9 genes, northern blot analysis of the RNA obtained from the parasitized mouse erythrocytes was carried out, using the middle regions of both Pymsp-9 and Pbmsp-9 genes as probes. A band of ~2.7 Kb was observed in the RNA preparation from P. yoelii, while in case of P. berghei RNA, the transcript was observed as a 2.5 Kb band, in accordance with the sizes of their respective genes (Figure 3.5). The probes from both the species cross-reacted with the RNA derived from each other. The specificity of these probes was confirmed by southern blot analysis, in which both the probes cross-reacted with the DNA from each other but not with PfMSP-9 gene fragments. These results clearly showed the presence of PbMSP-9 and PyMSP-9 transcripts at the blood stage in the rodent parasites.

![Figure 3.5 Northern blot analysis using total RNA, showing the presence of ~2.7 kb and ~2.5 kb transcripts corresponding to P. yoelii (A) and P. berghei MSP-9 (B).](image)

3.4 Analysis of the primary structure of PyMSP-9 and PbMSP-9 proteins in comparison with the other members of their family

The genes encoding PyMSP-9 and PbMSP-9 produced the ORFs of 678 and 618 amino acids respectively. Both the proteins contain a cluster of four conserved cysteines at the
N-terminus, which is a characteristic of the MSP-9 family. These proteins bear a putative signal peptide containing a hydrophobic core and a predicted signal sequence cleavage site between amino acids Ala (22 aa) and His (23 aa). Careful analysis of the protein sequences revealed that PbMSP-9 lacks the repeats present in the C-terminus in PyMSP-9. However, both the proteins contained a small stretch of YD repeats towards the C-terminus. The salient features of these proteins as well as all other members of the MSP-9 family have been depicted in Figure 3.6. These rodent malaria proteins are hydrophilic and rich in acidic residues with isoelectric points of 4.53 (PyMSP-9) and 4.84 (PbMSP-9) and glutamic acid content of 10.8% (PyMSP-9) and 9.1% (PbMSP-9) respectively. The SOPM program for secondary structure prediction was used for the individual protein sequences. According to the results, PbMSP-9 and PyMSP-9 assume an alpha helical conformation at the N-terminus and a combination of alpha helix and random coil at the C-terminus. Normalized Chou-Fasman and Rober-Garnier algorithms predicted similar structures. Both the proteins had a total of 6 cysteine residues. The SGG motif, presumably associated with chymotrypsin-like activity, was found at position 284 in both the proteins. Alignment of the protein sequences shows that PbMSP-9 and Py MSP-9 share a similarity of 6% and an identity of 73% when the whole sequence including the repeats is considered, but the N-terminal regions appeared more conserved with an identity of 77% and similarity of 8%.

Phylogenetic tree of MSP-9 homologues including the new characterized genes shows that both genes PbMSP-9 and PyMSP-9 are evolutionarily closely related, with a calculated distance of 0.107 between them, and 0.516 with respect to the other members of the family. Using either UPGMA or Neighbor Joining method, or different algorithms to calculate the distances, P. berghei and P. yoelli MSP-9 genes appear together in a separate node. (Figure 3.6 B).

3.5 Expression and purification of recombinant N-terminal cysteine rich region (Ncys) of P. berghei and P. yoelli MSP-9

With the aim of producing antiserum against P. berghei and P. yoelli MSP-9 proteins, the gene segments encoding the N-terminal region containing the four conserved cysteines (termed Ncys) was cloned in the expression vector pQE30. P. berghei MSP-9 (Ncys)
fragment [PbMSP-9 (Ncys)] was amplified with the set of primers ENK-12/ENK-13 (See Table 2.1.1 in Materials and Methods) and P. yoelii MSP-9 (Ncys) [PyMSP-9 (Ncys)] with the primers Py-5/Py-6. Both PCR reactions yielded a band of ~0.45 Kb that was gel purified and cloned in pGEM-T vector. The transformants were analyzed by restriction digestion with BamHI/HindIII and two of the positive clones from each transformation were sequenced. After confirming the identity of the insert and the absence of mutations, the DNA was sub-cloned in the expression vector pQE30 using the same restriction sites. The recombinant clones were selected based on the restriction analysis. The expression of the recombinant proteins was induced in E. coli (M15) cells with 1 mM IPTG for 4h. SDS-PAGE analysis of the cellular fractions showed that both the recombinant proteins with an apparent molecular weight of ~22 kDa, were expressed as inclusion bodies. The authenticity of the proteins was confirmed by western blot analysis with antibodies against the histidine tag.

These proteins were partially purified from the inclusion bodies using Ni²⁺-NTA affinity chromatography. The expression of PbMSP-9 (Ncys) and its purification has been depicted in Figure 3.7. Similar kind of profile was obtained for PyMSP-9 (Ncys). Both protein samples were fractionated in a preparative SDS-PAGE and electroeluted for further use in immunization of either rabbits or mice.

3.6 Localization of native MSP-9 protein in P. yoelii and P. berghei parasites

Using the antiserum raised against both the MSP-9 proteins, indirect immunofluorescence was performed on acetone fixed, schizont-rich pellet of P. yoelii and P. berghei parasites. Both the antibodies reacted strongly with the infected erythrocytes giving a grape-like pattern of rimmed fluorescence around the merozoites contained within the mature schizonts (Figure 3.8), whereas preimmune sera did not show any reactivity. In a Western blot assay, both antibodies strongly and selectively recognized the respective native parasite proteins of ~100 kDa for PyMSP-9 (Figure 3.9, lanes 2 and 3) and ~90 kDa for PbMSP-9 (Figure 3.9, lanes 5 and 6). Preimmune sera did not recognize the specific bands. No cross reaction was detected between anti-PbMSP-9 (Ncys) and anti-PyMSP-9 (Ncys) antibodies either by western blot or by IFA.
Thus, both the assays suggest that PbMSP-9 and PyMSP-9 are expressed in the blood stage parasite and, as expected, these proteins are localized on the surface of the respective merozoites.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. vivax</td>
<td>979</td>
</tr>
<tr>
<td>P. cynomolgi</td>
<td>818</td>
</tr>
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</tr>
<tr>
<td>P. falciparum</td>
<td>743</td>
</tr>
<tr>
<td>P. yoelli</td>
<td>678</td>
</tr>
<tr>
<td>P. berghei</td>
<td>618</td>
</tr>
</tbody>
</table>

Figure 3.6   (A) Schematic representation of the MSP-9 family including the new identified homologs from P. yoelii and P. berghei. The main features of this family of proteins are the presence of four conserved cysteines (*) within the first 72 aa, the SGG/EGG motif located in the middle region of the protein and the tandem repeats (boxes) located mainly in the C-terminal region.
Method: UPGMA; Best Tree; tie breaking = Systematic
Distance: Poisson-correction
Gaps distributed proportionally

Figure 3.6 (B) Phylogenetic tree of MSP-9 family showing the phylogenetic distances for each branch.

Figure 3.7 Expression and purification of recombinant PbMSP-9(Ncys). (A). Coomassie blue stained 10% SDS-PAGE gel showing the protein profile of total cell lysate uninduced (lane 1) and induced (lane 2) of E. coli transformed with the plasmid pQE30/PbMSP-9 (Ncys). Lanes 3-6: Elutions from Ni²⁺-NTA column showing PbMSP-9 (Ncys) protein at ~22 kDa. (B), (C) Western blot analysis of the Ni²⁺-NTA purified protein with the monoclonal antibody against the His tag and with anti-PbMSP-9 (Ncys) antibodies, respectively. Molecular marker positions are indicated on the left.
Figure 3.8  Immunofluorescent staining of the schizonts in the parasitized mouse blood using antibodies raised against PyMSP-9 (Ncys) and PbMSP-9 (Ncys). Cy3 labeled anti-rabbit IgG and anti-mouse IgG were used as secondary antibody. Nuclear staining was carried out by DAPI stain. The same field under visible light shows the parasitized erythrocytes.

Figure 3.9  Western blot of infected erythrocytes and parasite extract from P. yoelii and P. berghei with anti-PyNcys (A) and anti-PbMSP-9 (Ncys) (B). Lane 1: Uninfected mouse erythrocytes; lane 2: P. yoelii infected erythrocytes; lane 3: P. yoelii parasite extract; lane 4: pre-stained marker; lane 5: P. berghei infected erythrocytes; lane 6: P. berghei parasite extract; lane 7: pre-stained marker; lane 8: uninfected mouse erythrocytes.
3.7 Expression and purification of different recombinant constructs from *P. berghei* MSP-9

Different fragments of *Pbmsp-9* gene were amplified using specific primers containing the restriction sites BamHI/HindIII, to allow the cloning in a variety of expression vectors. Figure 3.10 depicts the length and location of the different recombinant constructs of PbMSP-9 protein that were used for this study.

### 3.7.1 Expression of full-length PbMSP-9 protein

The full-length *P. berghei* MSP-9 protein without the signal sequence was amplified with the primers ENK-12/ENK-11 (See Table 2.1.1 in Materials section) using the pGEM-T plasmid containing the sequenced gene as template. A product of 1.8 Kb was obtained as shown in Figure 3.3. This insert was cloned in pGEM-T and after selection of positive clones, the insert was again sub-cloned in the expression vectors pQE30 and pET23a. The expression in small-scale cultures did not show any induced protein in Coomassie staining and no band was detected by western blot with anti-PbMSP-9 or anti-His antibodies. From previous work carried out in our lab and by other groups, it is known that *P. falciparum* and *P. yoelii* MSP-9 full length proteins are very unstable and difficult to express (Kushwaha et al., 2000; Nwagwu et al., 1992). There are no reports of the full-length expression of any other MSP-9 protein.

### 3.7.2 Expression of PbMSP-9 (NM)

This fragment was amplified from *P. berghei* genomic DNA using the set of primers ENK-12/ENK-16 (See table 2.1.1 in Materials section). A PCR product of 1.1 Kb was obtained (Figure 3.11), which was cloned in pGEM-T vector. The clones obtained after the transformation of DH5α cells were checked by restriction digestion with BamHI/HindIII and PCR with the same set of primers. Sequence of the insert from two positive clones did not show any difference compared with the template. Subsequently, the insert was cloned in the expression vectors pQE30, pMALc, pET41a, pET29a, pET23a using BamHI/HindIII sites.
Figure 3.10  Schematic diagram of P. berghei MSP-9 and its different fragments. Amino acid locations of signal sequence (SS), putative proteolytic site (SGG) and the length of the different recombinant fragments are indicated. Positions of cysteine are marked with asterisks (*).
3.7.2.1 Expression of PbMSP-9 (NM) in fusion with Maltose Binding Protein (MBP).

The recombinant plasmid pMAL-c/PbMSP-9 (NM) was transformed into the expression cells TB1. Expression of the protein in this cell culture was induced with 1 mM IPTG for 4h. The expected fusion protein (≈ 83 KDa) was induced in all the clones, but it was detectable only by Western Blot with anti-MBP antibodies and specific PbMSP-9 antibodies [anti-PbMSP-9 (Ncys)]. Additionally, two lower bands were recognized by these antibodies, which indicated that the fusion protein might be getting degraded during the expression in *E. coli* (Figure 3.12). This phenomenon has been observed for similar recombinant fragments of MSP-9 from *P. falciparum* and *P. yoelii* and it has been related with the suspected autocatalytic activity of these proteins.

The fusion protein was detected in the soluble fraction of the cells with less degradation when the culture was induced at an O.D$_{600}$ of 2.0, compared with the induction at O.D$_{600}$ of 0.5. Purification of the soluble protein was carried out by affinity chromatography with amylose resin and elution with maltose protein. SDS-PAGE of the eluted fractions showed that only the lower band at ~56 KDa was purified; which reacted with anti-MBP.
antibody as well as PbMSP-9 specific antibodies. These results indicated that this protein band corresponds to the degradation product of PbMSP-9(NM)-MBP fusion and most probably contains the MBP (~42 KDa) protein fused with a small N-terminal fragment of PbMSP-9 (NM).

Figure 3.12  (A) SDS-PAGE showing the expression of MBP-PbMSP-9 (NM) fusion protein after induction with 1 mM IPTG for 4h. Expression of a large fusion protein (~85 kDa) is observed in induced samples along with a lower band at ~56KDa. Lane 1: uninduced cell culture lane 2: Induced cell culture. (B) Western blot analysis of the gel run in parallel, using PbMSP-9 specific antibodies, showing reactivity of a ~86 KDa MBP-PbMSP-9 (NM) fusion protein along with two major degradation products (~70kDa and ~56kDa). Lane 1: uninduced cell culture, lane 2: induced cell culture.

3.7.2.2  Expression of PbMSP-9 (NM) in fusion with GST protein

PbMSP-9 (NM) was cloned in the expression vector pET41a as described in Materials and Methods (Section 2.2.10.7). The E. coli strains BL21 (λDE3) and AD494 were transformed and the cultures from two positive clones were induced for expression of recombinant fusion protein with 1 mM IPTG for 4h. SDS-PAGE from the cell pellet did not show any visible band at the expected size of ~62 kDa (GST ~ 22 kDa). Instead, two bands at ~45 kDa and ~40 kDa were detected by Western blot with anti-PbMSP-9 (Ncys) antibodies only in clones from the transformation of BL21 (λDE3) cells and not in transformation of AD494 cells (Figure 3.13). As in case of MBP fusion expression,
results indicated that PbMSP-9 (NM)/GST fusion protein was getting degraded during the induction.

![Western blot analysis with anti-PbMSP-9 (Ncys) antibodies showing the profile of induction of fusion protein GST-Pb MSP-9 (NM) in two clones of BL21(λDE3) cells. Expected size of the fusion protein was ~ 62 kDa. Lanes 1-2: uninduced cell cultures, lanes 3-4: induced cell cultures.](image)

**Figure 3.13** Western blot analysis with anti-PbMSP-9 (Ncys) antibodies showing the profile of induction of fusion protein GST-Pb MSP-9 (NM) in two clones of BL21(λDE3) cells. Expected size of the fusion protein was ~ 62 kDa. Lanes 1-2: uninduced cell cultures, lanes 3-4: induced cell cultures.

**3.7.2.3 Expression and purification of Pb MSP-9 (NM) with histidine tag**

As discuss in the earlier sections, using pET series of vectors, expression of full-length recombinant protein was not observed in BL21 (λDE3) cells on Coomassie stained SDS-PAGE gel or by Western blot analysis with anti-His or anti-PbMSP-9 antibodies. Therefore, this fragment was sub-cloned in pQE30 vector. Small scale expression studies showed no apparent induction of the protein on Coomassie blue stained gels (Figure 3.14), however, Western blots with anti-His and with anti-PbMSP-9 (Ncys) antibodies detected a band at ~43 kDa [expected size for PbMSP-9 (NM) is 33 kDa] (Figure 3.14). To find out whether the recombinant protein was expressed in soluble or insoluble form, sub-cellular localization studies were carried out after 2h of induction. The sonication of cell pellet from the induced culture, followed by analysis of the proteins from supernatant and pellet by Western blot analysis revealed that the recombinant PbMSP-9 (NM) was
Figure 3.14  A: SDS-PAGE showing the expression of his-tagged PbMSP-9 (NM) in E. coli after induction with 1mM IPTG for different time periods. Lane 1: cell lysate from uninduced culture, lane 2-4: Cell lysate after induction for 2, 4 and 6 hrs respectively No apparent induction of recombinant PbMSP-9 (NM) is observed. B: Western blot of the gel run in parallel using anti-His antibodies. A band at ~43 kDa was detected in lane 2-4, suggesting recombinant protein started to express after 2h of induction.

localized mainly in the insoluble inclusion bodies, with a very little fraction of recombinant protein detected in the cytoplasmic fraction (Figure 3.15). No difference in the levels of expression in cytoplasmic fractions was found when the cultures were induced for 2, 4 and 6h and with different concentrations of IPTG (0.1 to 1.0 mM). Purification of the recombinant protein from soluble fraction was attempted using the cytoplasmic fraction obtained after sonication of cell pellet from 1lt culture, PbMSP-9 (NM) was purified by affinity chromatography on Ni$^{2+}$-NTA column using a gradient of imidazole concentrations (50 – 200 mM). Protein was eluted between 75 and 100 mM imidazole but the yield of the protein was low and the fractions with recombinant protein also contained a number of protein contaminants. The pellet from 6 lt culture was processed in the same way to try to increase the yield of soluble PbMSP-9 (NM), however no significant improvement was observed in the yield of the recombinant protein elutions from Ni$^{2+}$-NTA compared with previous results (Figure 3.16).
Figure 3.15  Western blot analysis using PbMSP-9 specific antibodies showing the localization of recombinant PbMSP9 (NM) in E. coli. Cell pellets from cultures induced with different IPTG concentrations were lysed by sonication, supernatant and pellets were separated on SDS-PAGE and analyzed by western blotting. Lanes 1-4: supernatant from cultures induced with 0.1 mM, 0.2 mM, 0.5 mM and 1 mM IPTG respectively; lanes 5-8: pellets from cultures induced with 0.1 mM, 0.2 mM, 0.5 mM and 1 mM IPTG respectively. Majority of the protein was found to be expressed as inclusion bodies in the pellet fraction.

Figure 3.16  (A) SDS-PAGE showing the Ni²⁺-NTA purification profile of soluble Pb MSP-9 (NM) (band at ~ 45 kDa) Lanes 1-2: elutions with 75 mM imidazole, lanes 3-4: elutions with 100 mM imidazole. (B) Western blot with anti-PbMSP-9 (Ncys) antibodies indicating the presence of soluble PbMSP-9 (NM) at different steps during Ni²⁺-NTA purification. Lane 1: uninduced cells, lane 2: induced cells, lane 3: Pre- Ni²⁺-NTA sample, lane 4: flow through, lanes 5-6: elutions with 75 mM imidazole, lanes 7-8: elutions with 100 mM imidazole.
Because of the low yield and high impurity of purified soluble PbMSP-9 (NM), different methods to purify the recombinant protein from inclusion bodies were tried. The first method was based on the solubilization of the inclusion bodies (IB) in 6 M Gu-HCl buffer; followed by binding of the sample to Ni$^{2+}$-NTA and refolding on column with a gradient of urea (8 M to 0 M) as described in Materials and Methods (Section 2.2.14). The proteins bound to Ni$^{2+}$-NTA were eluted with a gradient of imidazole from 30 mM to 300 mM. SDS-PAGE showed a single band of PbMSP-9 (NM) at 40 kDa that eluted with 75 -100 mM imidazole (Figure 3.17), however, the yield of purified protein was low (~ 0.3 mg/l). Most of the recombinant protein was found to be precipitated in the column during the refolding process and could only be eluted with Gu-HCl at pH 8.0.

![Figure 3.17](image)

**Figure 3.17** Refolding on column and purification of recombinant PbMSP9 (NM). SDS-PAGE showing different elution fractions of PbMSP9 (NM) refolded on column and eluted with gradient of imidazole (30 to 300 mM).

A second method was tried in which the inclusion bodies were solubilized in the same way as described in the method for refolding on column, and the solubilized protein was purified on a Ni$^{2+}$-NTA column under denaturing conditions, as described in Materials
and Methods (Section 2.2.12.3). The fractions containing recombinant protein were pooled and refolded by serial dialysis in a step gradient of urea, in presence of reduced and oxidized glutathione. In this protocol, the protein was found to get precipitated when the urea concentration was reduced to 2 M.

Hence, another method to purify and refold PbMSP-9 (NM) was standardized in which the inclusion bodies were solubilized in equilibration buffer containing 6M Gu-HCl at pH 8.0, and the recombinant protein was purified over Ni\textsuperscript{2+}-NTA column under denaturating conditions by eluting with a low pH buffer. The fractions containing the recombinant protein were pooled and the total concentration of protein was adjusted to ~2.0 mg/ml using equilibration buffer. The protein was refolded by rapid dilution method in presence of L-arginine and purified by cation-exchange chromatography on an SP-Sepharose column. By this method it was possible to obtain ~0.5 mg/l of pure and refolded protein (~3.0 mg from 6 l cultures). SDS-PAGE under non-reducing conditions showed a mobility shift in the refolded PbMSP-9 (NM) (Figure 3.18), which indicates the presence of disulfide bonds. The protein obtained by this method was used for the immunological studies of \textit{P. berghei} MSP-9.

![KDa M 1 2 3 4 5 6 7 8](KDa M 1 2 3 4 5 6 7 8)

![KDa M 1 2](KDa M 1 2)

\textit{Figure} 3.18 \textit{(A)} SDS-PAGE showing elution of PbMSP-9 (NM) protein after refolding by rapid dilution and cation exchange chromatography. Lanes 1-8: Elutions from SP sepharose column \textit{(B)} SDS-PAGE showing migration of purified PbMSP-9 (NM) under reducing (lane 1) and non-reducing conditions (lane 2).
To produce sufficient amount of protein for the immunological studies, five liter culture of M15 cells harboring the plasmid pQE30/PbMSP-9 (NM) was grown in a feed-batch bioreactor. Cells were grown for 3h until they reached an OD_{600} of 1.9 and the culture was induced with 1 mM IPTG. After 4h, cells were pelleted and kept at -70°C for further purification of the recombinant protein. From this culture, ~ 90 g of total cell pellet was obtained. This pellet was processed for Ni^{2+}-NTA purification in batches of 20 g each, and from each batch ~ 10 mg of solubilized IB were recovered after purification. Approximately 4.0 mg of recombinant protein elution fractions were used for refolding by rapid dilution in 1 l buffer and from this sample ~ 1.0 mg of pure refolded PbMSP-9 (NM) protein was recovered each time after cation-exchange chromatography.

3.7.3 Expression of recombinant PbMSP-9 (Ncys) with histidine tag.

For functional assays, the recombinant protein was expressed in M15 cells carrying pQE30/PbMSP-9 (Ncys), extracted from inclusion bodies, refolded and purified on Ni^{2+}-NTA column as described earlier in Materials (Section 2.2.14). A major band at 22 kDa was obtained that reacted with anti-PbMSP-9 antibodies. However, it was observed that this protein was unstable when stored at 4°C and it got precipitated after 4 days. The protein was then solubilized and refolded by rapid dilution method as described early for PbMSP-9 (NM). By this method the yield of the purified refolded PbMSP-9 (Ncys) was ~ 1.5 mg/l and the recombinant protein was found to be stable at 4°C (Figure 3.19).

![Figure 3.19 SDS-PAGE gel showing the profile of elutions from anion-exchange chromatography after refolding of Pb MSP-9 (Ncys) by rapid dilution method.](image-url)
3.7.4 Expression of PbMSP-9 (N) fragment

This construct was designed to express a large N-terminal region of PbMSP-9 containing the Ncys region, however it does not include the SGG motif. The primers ENK-12/ENK-15 (Table 2.1.1 in Materials and Methods) were used to amplify this fragment from the pGEM-T plasmid containing the sequenced full-length gene of PbMSP-9. Amplification resulted in a product of 0.9 Kb that was further cloned in pGEM-T as described in Materials and Methods. The insert from a positive clone was digested and cloned in pQE30, pET23a, pET28a and pET29a expression vectors. Expression of the recombinant protein was analyzed in small-scale cultures of *E. coli*. No expression was detected in the clones from transformation with pET23a and pQE30. However, clones from pET28a transformation produced a recombinant protein at ~33 kDa that was detectable only by western blot with anti-PbMSP-9 (Ncys) specific antibodies (Figure 3.20).

![Western blot analysis of pET28a/PbMSP-9 N clones using anti-PbMSP-9 Ncys antibodies, showing a major band at ~33 kDa and lower band at ~19 kDa. Lanes 1-2: Uninduced cell pellets, lanes 3-5: Induced cell pellets](image)

Similarly, expression of a ~33 kDa protein was observed in pET29a/PbMSP-9 (N) clones after induction (Figure 3.21) and this protein was also recognized by anti-PbMSP-9 (Ncys) antibodies in the Western blot analysis. The localization studies indicated that this protein was produced as inclusion bodies. The purification of PbMSP-9 (N) was carried out on Ni²⁺-NTA column under denaturing conditions as it was described for PbMSP-9 (Ncys). SDS-PAGE of the elution fractions showed that the protein did not
bind to the Ni\textsuperscript{2+}-NTA matrix, as none of the elutions contained the 33 kDa protein. On the other hand, the flow through fraction showed the band at 33 kDa in similar concentration to the pre- Ni\textsuperscript{2+}-NTA sample. The 33 kDa obtained after induction reacted with anti-PbMSP-9 (Ncys) antibodies but it did not show any reaction with anti-His antibodies suggesting that the expressed protein might not have a functional His tag. Sequence of the insert from one of the clones used for the expression showed that it contains the insert PbMSP-9 (N) fragment, however a deletion of T was found at position 786 in the gene sequence, which change the frame producing a stop codon, as a consequence, the His-tag at the C-terminal was not present in this protein.

![Figure 3.21 SDS-PAGE showing the induction of recombinant Pb MSP-9 (N) in E. coli clones harboring pET29a/PbMSP-9 (N) plasmid. Lanes 1,3,5,7: Uninduced cell lysates, lanes 2,4,6,8: Induced cell lysates.](image)

Therefore, using the pET29a clone, a protocol for refolding of the solubilized IB and further purification of PbMSP-9 (N) by anion-exchange was carried out. The inclusion bodies were thoroughly washed with a buffer containing 2 M urea to remove impurities before the solubilization in Gu-HCl buffer. A sample of the solubilized IB was checked by SDS-PAGE and the total protein content was estimated. Approximately 30 mg of the total protein was used for refolding by rapid dilution as it has been described for PbMSP-9 (NM) recombinant fragment. After refolding, the protein was dialyzed and purified by anion-exchange chromatography using Q-sepharose matrix and eluted with a gradient of 0 M to 1 M NaCl (Figure 3.22).
Figure 3.22  SDS-PAGE showing elution of PbMSP-9 (N) protein after refolding by rapid dilution and anion-exchange chromatography. Lane 1: uninduced sample, lane 2: induced sample, lane 3: solubilized inclusion bodies, lanes 4-6: elutions from Q-sepharose column (B) SDS-PAGE showing migration of purified PbMSP-9 (N) under reducing (lane 1) and non-reducing conditions (lane 2).

3.7.5 Expression of PbMSP-9 (C) fragment.

We tried to express the C-terminal region (393 – 618 aa) of PbMSP-9, with the view of using the corresponding recombinant protein in combination with PbMSP-9 (NM) fragment to simulate the expression of the full length protein. A fragment of 226 aa from the C-terminal region of PbMSP-9 (NM) was amplified with the primers ENK-11/ENK-17, using the pGEM-T plasmid of the sequenced full-length gene as template. A product of 0.7 Kb was obtained and this was cloned in pGEM-T vector. The sequence of one of the positive clones was checked and no mutations or deletions were found. The DNA insert digested with BamHI/HindIII was sub-cloned in pQE30 expression vector and the positive clones were screened as described before. Expression of the recombinant protein was not visible on Coomassie stained SDS-PAGE gels or by Western blot analysis using anti-His antibodies.
3.8 Biochemical characterization of different fragments of *P. berghei* MSP-9

3.8.1 Protease activity of PbMSP-9

Based on the initial studies with *P. falciparum* MSP-9 (PfMSP-9), it has been suggested that this protein could be acting as a protease on the surface of the merozoite. It has been shown that both, native and recombinant PfMSP-9 possess chymotrypsin-like activity against synthetic substrates *in vitro* (Kushwaha et al., 2000; Nwagwu et al., 1992).

The protease activity of three recombinant fragments of PbMSP-9 was studied using two fluorogenic substrates for chymotrypsin (NSuc-LLVY-AMC and NSuc-AAPF-AMC). Results indicated that while recombinant PbMSP-9 (Ncys) and PbMSP-9 (N) showed no activity, PbMSP-9 (NM) (23-370 aa) showed hydrolytic activity of NSuc-LLVY-AMC but not NSuc-AAPF-AMC. Activity of PbMSP9 (NM) against NSuc-LLVY-AMC, measured as μmol of free AMC released per min, showed a value of 0.639. Figure 3.23 shows the progress of the reaction over a period of 1 hour. In the same conditions, a control reaction was carried out with chymotrypsin (0.1 μg/ml), which showed an activity of 20.1 μmol AMC/ min.

PbMSP-9 (NM) activity was affected by specific inhibitors of serine proteases. Pre-incubation of PbMSP-9 (NM) with Chymostatin reduced its proteolytic activity by 79%, compared with the positive control without inhibitor. Similarly, PMSF and leupeptin reduced PbMSP-9 (NM) activity by 69% and 66%, respectively (Figure 3.23). Due to the high instability of recombinant PbMSP-9 (NM) fragment, PMSF was always included in the refolding buffer to prevent degradation during this process. It was observed that even after anion-exchange, the protein refolded in the presence of PMSF does not show protease activity and it appears degraded in the SDS-PAGE gel. To obtain active protein it has to be refolded in the absence of PMSF, however under these conditions, the eluted protein degrades faster and the yield is reduced drastically compared with the yield normally obtained when PMSF was included during for refolding. Figure 3.23 shows the comparative activity of the different recombinant fragments and the effect of protease inhibitors on PbMSP-9 (NM) activity.
Figure 3.23  Activity of PbMSP-9 recombinant fragments with the fluorogenic peptide LLVY-AMC. (A) Time-dependent course of the reactions using 20 μM of LLVY-AMC and 3.3 μg/ml of each of the proteins. (B) Comparative activity of the different recombinant proteins against LLVY-AMC measured as μM of AMC released per min. (C) Effect of protease inhibitors on the activity of PbMSP-9 (NM). Protein was diluted in the reaction buffer and incubated for 30 min at RT with each inhibitor before adding the substrate.
3.8.2 Binding of recombinant PbMSP-9 fragments to mouse erythrocytes

To study the binding, mouse erythrocytes previously washed with RPMI were incubated with 20 μg of recombinant protein for 1 h at RT with constant mixing. Bound protein was eluted from the erythrocytes with NaCl solution, the eluted fraction was fractionated on SDS-PAGE and analyzed by Western blot using anti-PbMSP-9 (NM) antibodies. Figure 3.24 shows that bands at ~42kDa, 33kDa and 20kDa are present in the elution of RBCs after binding of PbMSP-9 (NM), PbMSP-9 (N) and PbMSP-9 (Ncys) respectively. We confirmed these results with another method and counted the erythrocytes with bound protein by flow cytometry as described in Materials and Methods (Section 2.2.17.2). Figure 3.25 shows that both the fragments of PbMSP-9 (Ncys) and (NM) bind specifically to the mouse RBCs, producing a shift in the histogram of cells incubated with protein as compared with cells incubated alone, or cells incubated with preimmune sera. These results conclusively show that recombinant PbMSP-9 was correctly folded using rapid dilution method.

![Western blot using specific anti-MSP-9 (Ncys) antibodies showing the elution of recombinant proteins after binding to mouse erythrocytes. Recombinant proteins (20 μg) were incubated with 100 μl of packed mouse erythrocytes. Bound proteins were eluted with 300 mM NaCl, separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Lane 1: Elution from reaction with PbMSP-9 (Ncys), lane 2: elution from reaction with PbMSP-9 (N), lane 3: elution from reaction with PbMSP-9 (NM), lane 4: elution from mouse erythrocytes, lanes 5-7: respective recombinant proteins used in the binding reactions.](image-url)
Figure 3.25 Histograms showing binding of PbMSP-9 (Ncys) and PbMSP-9 (NM) fragments to mouse erythrocytes as determined by FACS. PvRll and DNA Helicase were used as positive and negative controls respectively. Shaded histograms represent the overlapped background of unstained cells and cells incubated with preimmune sera.
3.9 Immunogenicity of *P. berghei* MSP-9 (NM) in mice

3.9.1 Specific antibody response

Two strains of mice were used to assess the immunogenicity of *P. berghei* MSP-9: BALB/c and C57BL/6. A total of 12 mice per group were immunized with recombinant PbMSP-9 (NM) protein (20 µg/mice) using Freund's complete/incomplete adjuvant (CFA) or Alum as adjuvants. The schedule of immunization and bleedings is shown in Table 2.2.2 (Materials section). The mice were followed for 3 months and the blood samples were collected every two weeks to check for the antibody production.

Results from ELISA show that PbMSP-9 (NM) recombinant protein was immunogenic in both strains of mice with significant increase in the antibody titers even after the first boost at day 21. A second boost at day 56 had significant effect in the increase of antibody titer in BALB/c mice, reaching the peak at day 65 with an end-point titer $64 \times 10^4$ for CFA group and $32 \times 10^4$ for Alum group. In C57BL/6 mice, the second boost did not have any significant effect in increasing the titer of antibodies. The peak of antibody production was observed at day 36 with a titer of $16 \times 10^4$ for CFA group and $32 \times 10^4$ for Alum group. Preimmune sera (Day 0) showed no reactivity with the recombinant protein by ELISA using a dilution 1:1000. Similarly, serum samples of naïve animals and those immunized with PBS did not contain detectable antibodies against the recombinant PbMSP-9 (NM) (OD490nm $<0.07$ at 1:1000 dilution).

Figure 3.26 shows the antibody response after the immunization with PbMSP-9 (NM) in BALB/c and C57BL/6 mice and the comparison of the antibody titers. Comparison of the titers of anti-PbMSP-9 (NM) antibodies among the immunized groups, at day 65 after primary immunization did not showed statistically significant difference, (paired t test p values $<0.005$ in all the cases). The highest antibody titers were observed in the group of BALB/c mice immunized with CFA formulation, as compare to all the other three groups. The antibody titers in this group remained stable for longer time as compared with the titers induced by Alum in BALB/c mice, in which the levels started to decrease 84 days post immunization.
The proportion of different IgG isotypes was studied at two points during the immunization using recombinant PbMSP-9 (NM) as a capture antigen and affinity-purified anti-mouse IgG1, IgG2a, IgG2b or IgG3 as secondary antibodies. It was observed that generally, PbMSP-9 (NM) mainly induced IgG1, followed by IgG2b, IgG2a and IgG3 in C57BL/6 mice, however the proportion of IgG2a was higher than IgG2b in BALB/c group (Figure 3.27). No statistically significant difference was found when the levels of each IgG isotypes were compared among the groups using paired t test (P value > 1.0 in all the cases).

The sera from mice immunized with PbMSP-9 (NM) were analyzed for their reactivity against the different recombinant fragments of \textit{P. berghei} MSP-9. As shown in Figure 3.28, results of ELISA indicate that PbMSP-9 (N) region showed 90 – 100% reactivity with immunized mice sera from all the groups as compared to the reactivity with PbMSP-9 (NM), whereas PbMSP-9 (Ncys) showed 65 – 70% reactivity. A homologous fragment of \textit{P. yoelii} MSP-9 [PyMSP-9 (NM)] also showed reactivity with these sera, however, the cross reaction showed variation among the different groups from 25% in C57BL/6 - CFA group to 72% in BALB/c - Alum group.

**3.9.2 Cytokine levels secreted by T-cells**

In order to compare the type of cellular immune response induced by PbMSP-9 (NM) immunization, two mice from each group were immunized according to the schedule mentioned before. Two weeks after first boost, mice were sacrificed and the splenocytes were isolated for \textit{in vitro} stimulation with increasing concentrations of PbMSP-9 (NM) antigen, to test the secretion of different cytokines. Results from ELISA showed that there was no production of IL-2, IL-4 and IL-5 at detectable levels for any of the studied groups. On the other hand, maximum levels of INF-\(\gamma\) were observed in BALB/c mice immunized with Alum/PbMSP-9 (NM). In contrast, there was no INF-\(\gamma\) production in C57BL/6 mice immunized with CFA/PbMSP-9 (NM). In case of IL-10, CFA immunization induced comparable levels in both strains of mice, higher than the observed levels in mice immunized with Alum (Figure 3.29). Paired t test for comparison of the levels of IFN\(\gamma\) and IL-10 among the groups, shows statistically significant differences in the levels of these cytokines among all the groups, with p values < 0.001.
in all the comparisons. Comparing the cellular immune response between the strains of mice, it can be concluded that PbMSP-9 (NM) induced a stronger response in BALB/c mice compared with C57BL/6.

Figure 3.26  Antibody response in BALB/c mice (A) and C57BL/6 mice (B) immunized with PbMSP9 (NM) formulated in CFA or Alum. Antibody titers were calculated from the readings of ELISA where PbMSP-9 (NM) recombinant protein was used as the capture antigen, the highest dilution sera showing OD ≥ the reading of the preimmune sera + 2 SD was considered as the end point titer. A parallel set of these immunized mice from each group (n=5) were challenged with P. berghei NK65 infected erythrocytes at day 70 after primary immunization.
Figure 3.27  Levels of PbMSP9 (NM) specific IgG isotypes in sera of BALB/c and C57BL/6 immunized mice. Pooled sera of mice (n = 5) collected on day 36 post immunization was tested by ELISA in triplicate at dilution of 1:500. Results are depicted as mean O.D$_{490}$ nm of triplicates. Error bars indicate the standard deviations.

Figure 3.28  Reactivity of sera from PbMSP-9 (NM) immunized mice with recombinant proteins corresponding to different N-terminal fragments of PbMSP-9 (NM). Sera from day 65 after immunization was pooled for each group (n=5) and diluted 1:10,000, their reactivity was analyzed by ELISA using PbMSP-9 (NM) [Pb NM], PbMSP-9 (N) [Pb N], PbMSP-9 (Ncys) [Pb Ncys] and P. yoelii MSP-9 (NM) [Py NM] as capture antigen, plates were coated with 20 ng per well of each of the recombinant proteins.
Figure 3.29  (A) Cytokine levels (pg/ml) in BALB/c and C57BL/6 mice immunized with PbMSP-9 (NM) formulated with CFA or Alum. Splenocytes were in vitro stimulated with recombinant PbMSP-9 (NM) and culture supernatants were analyzed by ELISA for IL-2 and IL-4 levels after 48h of culture and for IL-4, IL-5, and INFγ after 72h of culture. (B) Levels of cytokines (pg/ml) detected in the respective cultures of splenocytes from mice immunized with adjuvant alone.

3.10 Protective efficacy of the immune response elicited by PbMSP-9 (NM) in BALB/c mice

In order to test if the immune responses induced by PbMSP-9 (NM) were protective, the groups of 5 immunized mice were challenged intravenously with *P. berghei* NK65 asexual blood-stage parasites, at day 80 after primary immunization, as described in Materials and Methods (Section 2.2.21). Protection was determined by comparison of the pre-patent period, the parasitemia and the survival rate in experimental and control groups. Naïve mice and control groups immunized with CFA-PBS or Alum-PBS developed patent infection between day 5 and 7 after challenge and reached the peak parasitemia by days 17 to 20 (63.5% ± 4.2 for CFA-PBS and 44.2% ± 11.4 for Alum-PBS). The experimental mice from the CFA group showed patent infection by day 7, except for two of the animals which showed parasitemia after day 9 and 11 respectively.
In this group, the peak of parasitemia was observed between day 17 and 20, with an average of $37.8\% \pm 17.5\%$). In contrast, none of the mice immunized with Alum/PbMSP-9 (NM) developed patent infection at any point during the 30 days period of observation and all of them survived (Figure 3.31). All mice in the control groups as well as in the CFA experimental group died by day 20. Figure 3.30 shows the survival proportions for the different groups.

**Figure 3.30**  Survival curves for mice immunized with PbMSP-9 (NM) and challenge with *P. berghei* NK65. Mice deaths were recorded daily for 30 days after the challenge.
In order to evaluate the duration of the protection observed in the BALB/c mice immunized with Alum formulation and the effect of the reduction in anti-PbMSP-9 (NM)
antibodies, we decided to repeat the challenge after two months for the BALB/c mice that survived the first challenge, along with another set of mice that were immunized at the same time but were kept to study the antibody production. At the time of the second challenge, mice were at day 138 after primary immunization and the antibody titers were $16 \times 10^4$ for CFA group and $4 \times 10^4$ for Alum group. Control mice from CFA and Alum group developed patent infection by day 5 after challenge and all of them died between day 15 and 21, with peak parasitemias higher than 40% in most of the cases. There was no significant difference in the development of the infection in the experimental groups as compared with the controls. Mice from Alum group that showed protection in the previous experiment also behaved similar to the controls and died between day 16 and 22. Figure 3.32 shows the progress of parasitemia in these groups of mice.

From the challenge experiments in BALB/c mice it can be concluded that immunization with PbMSP-9 (NM) induced complete protection when it was administered with Alum as adjuvant, but this effect was lost by day 138 after immunization.

3.11 Protective efficacy of the immune response elicited by PbMSP-9 (NM) in C57BL/6 mice

The C57BL/6 mice were challenged by day 80 after primary immunization with *P. berghei* NK65 by intraperitoneal inoculation. In general, the infection in individual mice from each group behaved more heterogeneous than in BALB/c mice. Patent parasitemia was observed in control groups by day 5 after challenge, with a peak parasitemia between day 20 and 26 of 46.8% for CFA-PBS group, except for one mice that reached the peak parasitemia by day 12 (25.2%). In contrast, CFA experimental group reached the peak parasitemia relatively early (day 8 to 15), with values less than 10% in 3 of the mice (Figure 3.33). In Alum controls all the mice developed patent parasitemia by day 5, but the peak was observed at day 8 (one mice, 5.6%), day 12 (one mice, 27.2%) and day 23 (two mice, 58.4 and 63%).
Figure 3.32  Time course of P. berghei parasitemia in BALB/c mice immunized with PbMSP-9 (NM) (experimental) or with adjuvant alone (control) after challenge on day 138 with 5x10^6 parasitized erythrocytes by intravenous inoculation. Each line represent a individual mouse of the group.
Compared with this, the Alum experimental group behaved similar to CFA experimental group with peak parasitemias less than 15% observed between day 8 and 15. None of the mice in the control or experimental groups survived more than 22 days after challenge. Figure 3.34 shows the survival curves for the different groups.

Figure 3.33 Time course of P. berghei parasitemia in C57BL/6 mice immunized with PbMSP-9 (NM) (experimental) or with adjuvant alone (control) after i.p. with $1 \times 10^8$ parasitized erythrocytes. Each line represent an individual mouse of the group.
Figure 3.34 Survival curves for C57BL/6 mice immunized with PbMSP-9 (NM) (experimental) or adjuvant alone (control) after challenge with P. berghei NK65. Mice deaths were recorded daily for 30 days after the challenge.