

66 kDa Protein Isolated from 24,000 g Fraction of *Plasmodium berghei* Exhibits Humoral Immune Response

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Abstract: The various antigens involved in the induction of humoral immune response provide the opportunity for development of an effective malaria vaccine. In the present study, the immunogenicity and protective efficacy of 24,000 g fraction was evaluated in rodent mouse model. A 66 kDa protein was eluted through immunoabsorption using 24,000g fraction. The eluted protein was characterized by SDS-PAGE and mice immunized exhibited strong humoral immune response analysed by ELISA, IFA, DOT-ELISA and in vitro invasion inhibition assay. About fifty percent inhibition of parasite propagation was observed in vitro. This protein appears to be an important constituent of the parasite for antimalarial studies.

Keywords: *Plasmodium berghei*, malaria, 24,000g fraction, 66 kDa protein.

1. Introduction

The phylum Apicomplexa includes *Plasmodium* the important pathogen, causes malaria in human population. The incidence of malaria cases are very high in the regions affected by poverty. According to WHO, an estimated 3.3 billion people are at risk of being infected with malaria parasite and 1.2 billion are at high risk (WHO, 2014). The greatest obstacle in the development of effective malaria vaccine has been the resurgence of malaria due to drug resistance. Several parasite antigens/proteins have been tested for their role as a vaccine candidate antigen. Most of them are expressed on the parasite's surface or in parasitized erythrocytes. In particular proteins involved in invasion of RBCs (host) are attractive target for vaccine and drug development due to accessibility to host immune system. Despite this for the survival of parasite invasion of erythrocyte is a major step (Ju *et al.*, 2013). Sporozoites and merozoites are two developmental stages of *Plasmodium* which invades hepatocytes and red blood cells (RBCs) respectively (Sharma and Chitnis, 2013). In this study, immunogenicity of 66 kDa protein has been evaluated.

2. Materials and Methods

Maintenance of Parasite: *Plasmodium berghei* (NK-65) was maintained in white Swiss mice, *Mus musculus* (BALB/c) with prior permission of the Institutional Animal Ethics Committee (IAEC) of H.P. University, Shimla vide number IAEC/Bio/4-2011. The asexual erythrocytic stages of parasite were maintained by inoculation of 1×10^5 *P. berghei* - infected erythrocytes from the infected individual to the naive in citrate saline in 2:1 ratio (sodium chloride, 0.85% w/v; sodium citrate, 3.8% w/v) (Banyal *et al.*, 1991). The course of parasitaemia was monitored daily by preparing thin blood smear and cell - free *P. berghei* was isolated according to the method of Banyal and Fitch (1982) using 0.2% (w/v) saponin in 0.01M PBS, pH 7.2 (Kapoor and Banyal, 2011).

Subcellular fractionation: Subcellular fractionation of the homogenized parasite was carried out by the method of Banyal *et al.* (1979).

Protein estimation: Protein of the antigen was estimated spectrophotometrically using bovine serum albumin (BSA) as standard (Banyal and Kumar, 1994).

Immunization of mice: Experimental groups of mice were immunized with P-III (pellet at 24,000 g) fraction. Each mouse was injected intraperitoneally with 100µg of protein and 30µg of saponin on day 0, 14 and 28 whereas placebo control group mice received 30 µg of saponin only in 0.01 M PBS, pH 7.2 on day 0, 14 and 28. A week after last immunization dose, mice from each group were sacrificed to collect immune sera. Sera were stored at -20°C for further use.

Immunoabsorption : The different antigenic proteins from the pellet at 24,000g was isolated by antibody immobilization method of Hudson and Hay (1989).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS – PAGE): The molecular weights of purified antigens were analysed by SDS-PAGE according to method of Sharma and Banyal (2011). 10% resolving and 3% stacking gels were used in mini vertical slab gel electrophoresis apparatus. Molecular weights of the proteins were calculated by the Gel Documentation System. Experimental group of mice were immunized with eluted 66 kDa protein fraction and the collected sera subjected to various serodiagnostic assays.

Enzyme Linked Immunosorbent Assay (ELISA): ELISA was carried out in 96 - well polystyrene microtitre plates according to the method of Banyal and Inselburg (1985).

Indirect Fluorescent Antibody test (IFA): IFA was performed according to Upma *et al.* (1998) using Fluorescein Isothiocyanate (FITC, Genei).

Dot- Enzyme Linked Immunosorbent Assay (DOT-ELISA):

Antibodies were detected by an indirect DOT-ELISA according to Sharma and Banyal(2011).

In Vitro invasion inhibition assay: The short - term *in vitro* culture of *P. berghei* was carried out as given by Upma *et al.* (1998) using RPMI - 1640 (Gibco) as culture medium.

Culture medium – RPMI - 1640 supplemented with 0.06% HEPES (N – 2 - hydroxyethyl piperazine - N' 2 - ethane sulphonic acid), 5% (w/v) sodium bicarbonate, antibiotics - gentamycin (50µg/ml), penicillin (100µl/ml) and streptomycin (100µg/ml) was used as culture medium. The pH of incomplete medium was adjusted to 7.4 and filtered through 0.22µ millipore syringe filter under sterile conditions. 10% (v/v) inactivated foetal calf serum (FCS)

was added to incomplete medium and the complete medium was prepared.

Invasion inhibition assay - The culture was carried in 12 well culture trays. To each well 1ml of complete medium having 3% haematocrit and 0.3% - 0.8% parasitaemia along with 50µl of normal/immune mice serum was added. The trays were then shaken gently to mix the contents. A little of sample was taken, centrifuged and from resulting pellet portion 0 hour smears were prepared. The culture trays were placed in a candle jar at 37°C in an incubator. After 21hrs of incubation, the trays were removed from incubator and smears from each well were prepared after centrifugation of different samples. Smears were fixed in methanol and stained with Giemsa stain. A differential count of the parasite (ring, trophozoites and schizonts) in smears was done. The percent inhibition of merozoite invasion was calculated as:

$$\% \text{ inhibition} = 100 - \frac{\text{Number of rings in experimental culture}}{\text{Number of rings in control culture}} \times 100$$

3. Results

Immune serum obtained from mice immunized with 24,000g fraction was subjected to immunoabsorption assay. The elution profile of unbound and bound antigens eluted with PBS and glycine-HCl buffer respectively is shown in Fig.1 and Fig.2. Molecular weights of eluted antigens were determined by SDS-PAGE. Antigen/protein having molecular weight of 66 kDa was used to immunize experimental group of mice (Fig. 3). Sera obtained from mice immunized with 24,000g sediment analysed by ELISA gave antimalarial antibody titre of 1:8192, 1:16384, 1:4096, 1:16384, 1:4096, 1:16384 and 1:16384 in mouse number 1 to 7 respectively. The immune sera of mice immunized with 66 kDa protein gave antimalarial antibody titre of 1:4096 in mouse number 1, 4 and 5 while the remaining mice number 2, 3, 6 and 7 showed ELISA titre of 1:8192 (Table. 1). Immune sera of mice immunized with 66 kDa protein gave strong immunofluorescence reaction observed under UV light. While the reference negative or normal serum did not show any

fluorescence (Fig. 4). Presence of antimalarial antibodies was also evaluated by Dot- ELISA which gave positive brown coloured antigen-antibody complex while no colour was seen with malaria reference negative serum (Fig. 5).

The immune sera were also analysed for their antimalarial property by short term *in vitro* invasion inhibition assay. The immune sera obtained from mice immunized with 66 kDa showed significant inhibition of formation of new rings in the culture. The serum of immunized mouse number 1 showed 31.42% invasion inhibition of rings while serum from immunized mouse number 2 showed 42.85% invasion inhibition. Serum from immunized mouse number 3 and 4 showed inhibition 54.28% and 57.14% respectively. Whereas, serum from immunized mouse number 5, 6 and 7 showed invasion inhibition of newly formed rings of 28.57%, 37.14% and 57.14% respectively. These results showed that immune sera collected from immunized mice inhibited merozoite invasion *in vitro*. (Table 2).

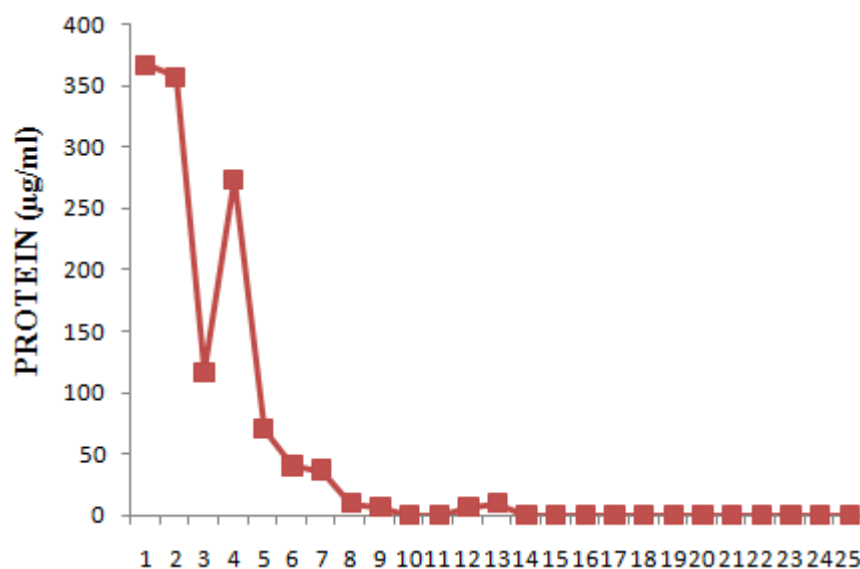


Figure 1: Fractions Eluted with PBS

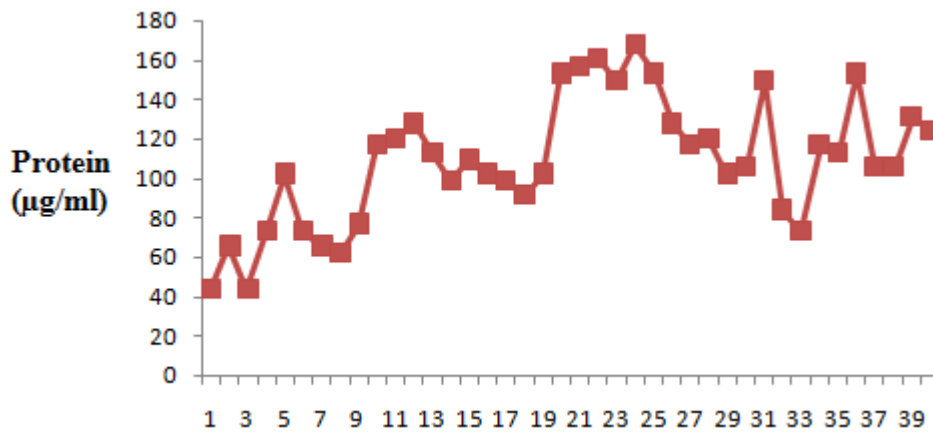


Figure 2: Fractions Eluted With Glycine-Hcl Buffer (µg/ml)

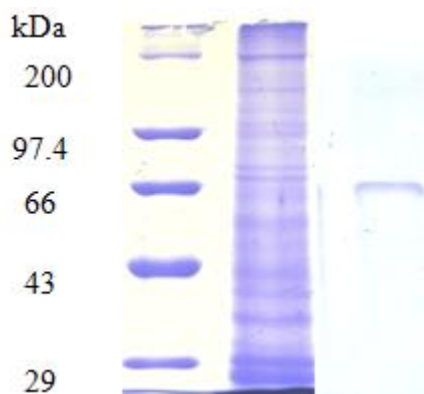


Figure 3: SDS-PAGE of fractions of *P. berghei* stained with Coomassie brilliant blue. Lane 1 = protein standard marker, Lane 2 = 24,000g sediment, Lane 3 = 66 kDa protein

Table 1: Table showing levels of antiparasite antibodies in sera of mice immunized with 24,000g sediment and with 66 kDa protein.

Mouse Number	Elisa Titre Of Mice Immunized With 24,000g Fraction	Elisa Titre Of Mice Immunized With 66 kDa Protein
M1	1 : 8192	1 : 4096
M2	1 : 16384	1 : 8192
M3	1 : 4096	1 : 8192
M4	1 : 16384	1 : 4096
M5	1 : 4096	1 : 4096
M6	1:16384	1: 8192
M7	1:16384	1: 8192

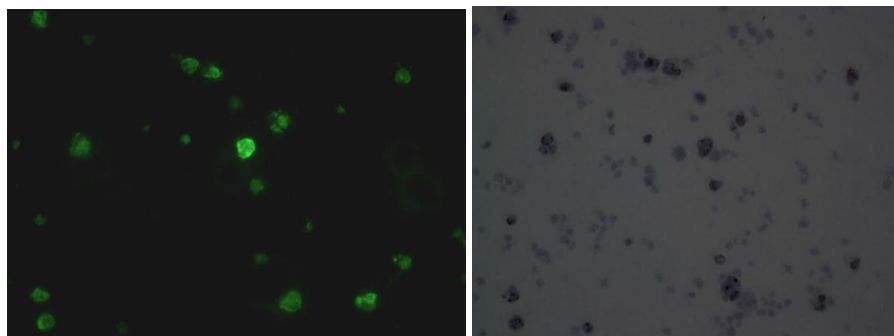


Figure 4: IFA test using sera of mice immunized with 66 kDa protein as seen under (A) UV light and (B) phase contrast ($\times 1000$)

number, A-malaria reference positive, B- malaria reference negative)

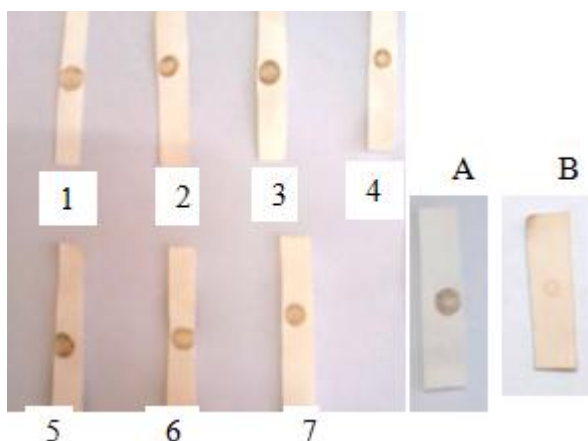


Figure 5: Dot- ELISA of immune sera obtained from mice immunized with 66 kDa protein. (1-7 corresponds to mouse

Table 2: Merozoite invasion-inhibition *in vitro* in the presence of sera of infected mice

Sera	Parasitaemia	Number of Parasitized Cells/5000 RBCs			% Invasion inhibition
		R	T	S	
0 Hour					
	0.5	6	8	11	
AFTER 21 HOURS					
Control	1.02	35	7	9	-
M-1	0.62	24	2	5	31.42%
M-2	0.44	20	1	1	42.85%
M-3	0.36	16	2	0	54.28%
M-4	0.42	15	4	2	57.14%
M-5	0.56	25	3	0	28.57%

M-6	0.44	22	0	0	37.14%
M-7	0.38	15	3	1	57.14%

4. Discussion

Diversity in antigens of *Plasmodium* is a major adaptation enabling parasite to avoid immune responses. Diversity can exist as in the vast array of antigens expressed, in antigen allelic polymorphism and in an antigenic variation (Stanisic *et al.*, 2013). Various antigens have been studied to evaluate their role in protective immune response. In *Plasmodium knowlesi* 66-kDa proteins induce protective immune response in rhesus monkeys. Similar results have also been shown for other 66-kDa AMA1 molecules from the rodent malaria *Plasmodium chabaudi* and *Plasmodium yoelii* (Kocken *et al.*, 2000). 66kDa antigen isolated from 24,000g fraction found proteolytic in nature and also gave protection against parasite by inducing endogenous IL-1 response (Bagai and Pawar, 2013). Two blood stage antigens 43 kDa and 66 kDa induce immune response in *Plasmodium berghei* malaria (Pirta and Banyal, 2014).

In the present study humoral immune response exhibited by 66 kDa protein isolated from 24,000 g fraction of *Plasmodium berghei* has been evaluated. The mice immunized with sedimented fraction at 24,000g fraction exhibited strong humoral immune response. Previous studies (Upma *et al.*, 1998, Pirta and Banyal, 2012) were shown that mice immunized with 24,000g fraction gave protection against parasite infection. Serodiagnostic assays show the inhibition of antibodies to parasite. Therefore, different antigens from this fraction were isolated by antibody immobilization method. The eluted fraction which showed 66 kDa protein band had been selected for immunization of mice. The immune sera obtained from immunized mice were subjected to ELISA, IFA, Dot-ELISA and an *in vitro* invasion inhibition assay. All the mice immunized with 66 kDa protein exhibited high titre of antibodies range from 1:4096 to 1:8192, as determined by ELISA. The antibody titres range from 1 : 4096 to 1 : 16384 in sera immunized with 24,000g fraction. This shows that the 66 kDa protein/antigen present during inoculation activated the immune system of mice and triggered humoral immune response. The specificity of antibody produced by 66 kDa protein isolated from 24,000g fraction was also analysed by Indirect Fluorescent (IFA) test. The specific antigen – antibody reaction was seen as through UV and phase microscopy.

66kDa protein exhibited humoral response in mice. This antigen necessitated to be investigated further so as to know its antimalarial role against malaria. Despite this there are various classes of parasite proteins/antigens presently under investigation for developing effective malaria vaccine. Studies on these proteins may lead to new novel drug targets.

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