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 37^0C 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:

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

3. Results

Immune serum obtained from mice immunized with 24,000g fraction was subjected to immunoadsorption assay. The elution profile of unbound and bound antigens eluted with PBS and glycine-HCI 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).

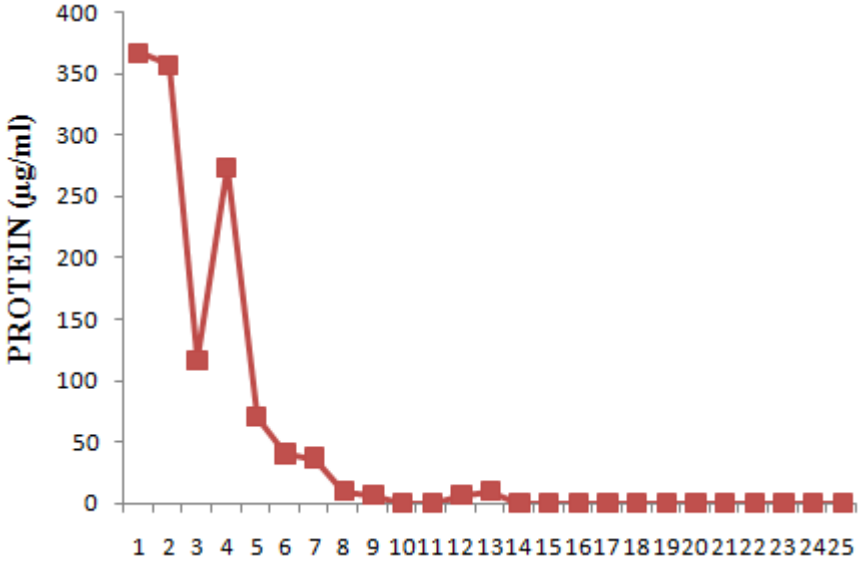

Figure 1: Fractions Eluted with PBS

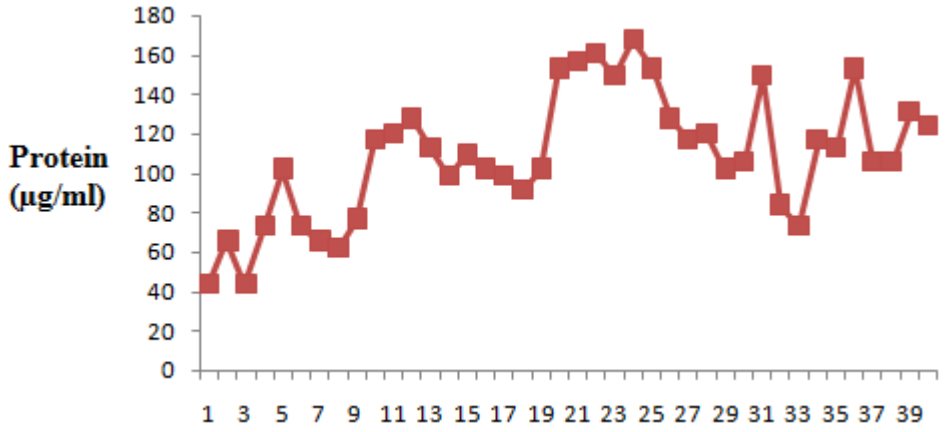

Figure 2: Fractions Eluted With Glycine-Hcl Buffer (µg/ml)

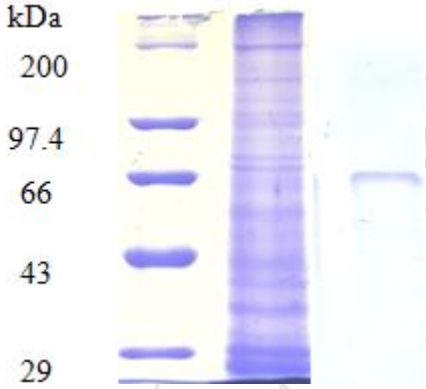

Figure 3: SDS-PAGE of fractions of *P. berghei* stained with Coomassie brilliant blue. Lane 1 = protein standard marker, Lane 2 = 24,000g sediment, Lane 3 = 66 kDa protein

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

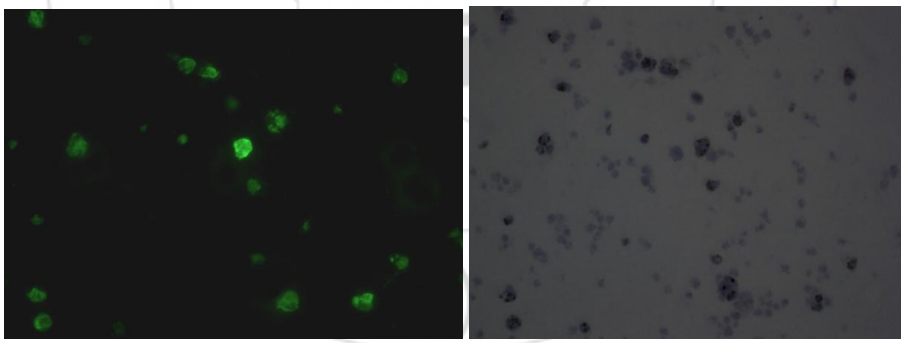

Figure 4: IFA test using sera of mice immunized with 66 kDa protein as seen under (A) UV light and (B) phase contrast (× 1000)

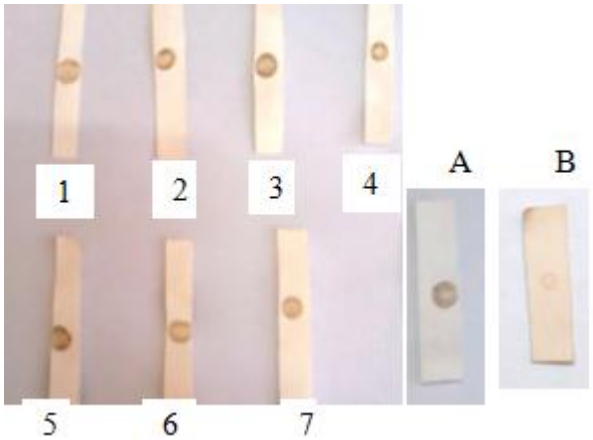

Figure 5: Dot- ELISA of immune sera obtained from mice immunized with 66 kDa protein. (1-7 corresponds to mouse number, A-malaria reference positive, B- malaria reference negative)

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 exists as in the vast array of antigens expressed, in antigen allelic polymorphism and in an antigenic variation (Stanisic *et al*., 20 13). Various antigens have been studied to evaluate their role in protective immune response. In *Plasmodium knowlesi* 66-kDa proteins induces protective immune response in rhesus monkeys. Similar results have also been shown for other 66-kDa AMA1 molecules from the rodent malarias *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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