Chapter 5

Introduction

Apicoplast is surrounded by 3 – 4 layered membranes which relate to its secondary endosymbiotic origin. These membranes are known to be permeable to proteins encoded by the parasite nuclear genome. It is now a well established fact that some proteins encoded by parasite nuclear genome are targeted via N – terminal signal bipartite leader sequence (includes a signal peptide and a transit peptide) to the organelle. The bipartite leader sequence helps entry of these external proteins across the initial 2 – 3 membranes of the organelle and then by certain Tic – Toc mechanism, proteins are pushed inside the organelle. The necessity of the bipartite leader sequence mechanism has been proven experimentally where GFP was fused at the N – terminal with a signal peptide, a transit peptide and apicoplast destined Acyl Carrier Protein (ACP). This construct was then transfected into *P. falciparum* where it got concentrated inside apicoplast and gave fluorescence (Waller *et. al.,* 2000). This leader and GFP construct has been used by various workers to localize the presence of nuclear encoded proteins in the apicoplast (Waller *et. al.*, 1998; Yung and Lang-Unnasch, 1999; DeRocher *et. al.*, 2000; Waller *et. al.*, 2000; Foth and McFadden, 2003). These proteins are used in various synthetic (fatty acid, isoprenoid, lipoic acid, etc.) pathways within apicoplast.

Localization of Apicoplast Genome Encoded Proteins

The actual presence of the protein products of major ORFs in the apicoplast genome, except for elongation factor, EF – Tu (Chaubey *et. al.*, 2005), has not been demonstrated as yet. If these products are at all formed, whether they are transported out of apicoplast or not, is not known. Rather they are believed to be used within the organelle. Localization of the apicoplast genome encoded elongation factor, EF – Tu, is proven within the *P. falciparum* apicoplast. Though the protein is synthesized throughout the parasite erythrocytic cycle, it is maximally synthesized at the middle and late trophozoite and at schizont stages (Chaubey *et. al.*, 2005). Several drugs (eg.

Clindamycin, Azithromycin, Thiostrepton and Chloramphenicol) that are believed to be targeted to the apicoplast (Beckers *et. al.*, 1995; Clough *et. al.*, 1997; McConkey *et. al.*, 1997; Camps *et. al.*, 2002) are parasiticidal in action and are known for blocking prokaryotic translation. Among these drugs, Thiostrepton has been shown to have maximum effect on specifically apicoplast EF – Tu translation (Chaubey *et. al.*, 2005).

The SufB protein is believed to be a member of Suf system which is involved in the mobilization of $[Fe - S]^{2+}$ cluster assembly during the electron transfer and redox – non – redox catalysis. The genes encoding other members (SufA, C, D, E, etc.) of this family are present in the *P. falciparum* genome database. All these members of Suf family are known to work as a complex (esp. SufBCD) in bacteria to regulate the $[Fe - S]^{2+}$ cluster. In *P. falciparum* SufC is known to be targeted to the Apicoplast and is thus presumed to interact with SufB (Wilson, 2005). But as yet there are no reports available on the localization, or isolation etc. of SufB protein from *P. falciparum.*

Results and Discussions

Peptides based on the B – cell epitope regions from the protein sequences of EF – Tu and SufB were designed. They were synthesized at CDC, Atlanta, USA and were obtained as a gift. These peptides were used to raise specific monoclonal antibodies in the rodent models which were then used for the localization of proteins from which respective peptide was designed.

Antibody Raising in Swiss – Albino Mice

The peptides were dissolved in 1X PBS based upon their solubility parameters. They were formulated with Freund's Complete (for priming dose) and Incomplete Adjuvants (for booster dose) in a dose of 100 µg (1 µg/ µl) and were injected into Swiss Albino female mice. In total three boosters were given. Blood was collected by tail bleed before priming and after every booster, and sera were separated. Antibody titer was checked using ELISA. A significant titer was obtained after the third booster (Figure 5.1 A, B).

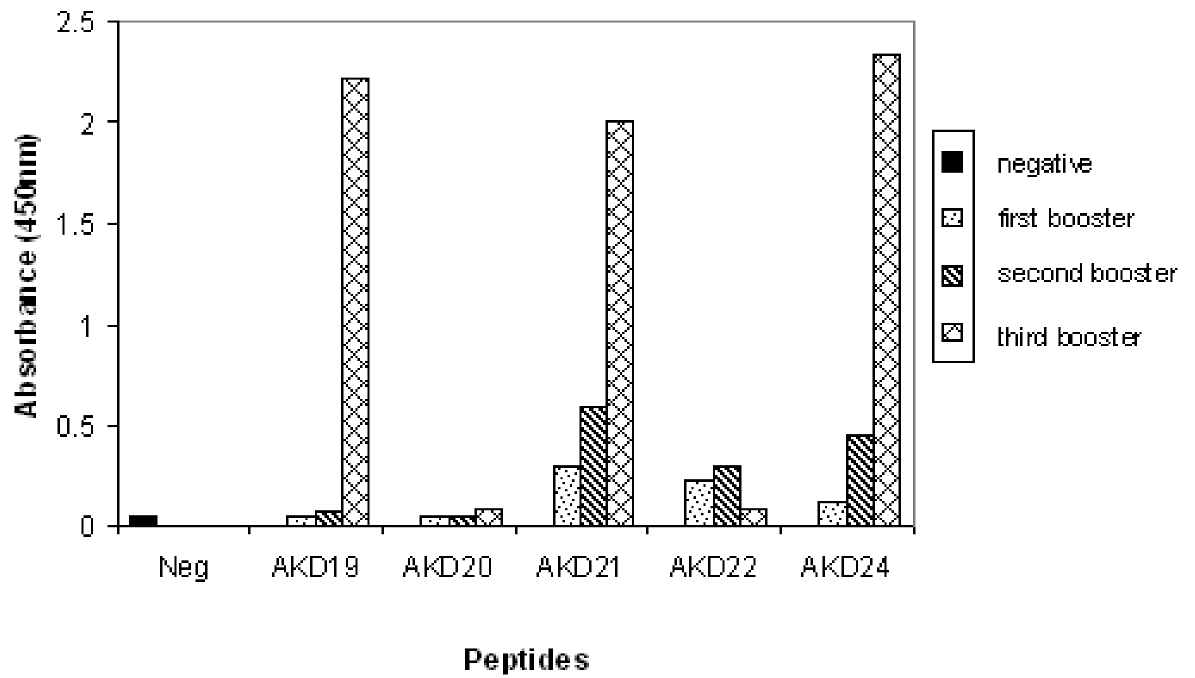


Figure 5.1 A: Antibody profile of TufA peptides in Swiss Albino mice

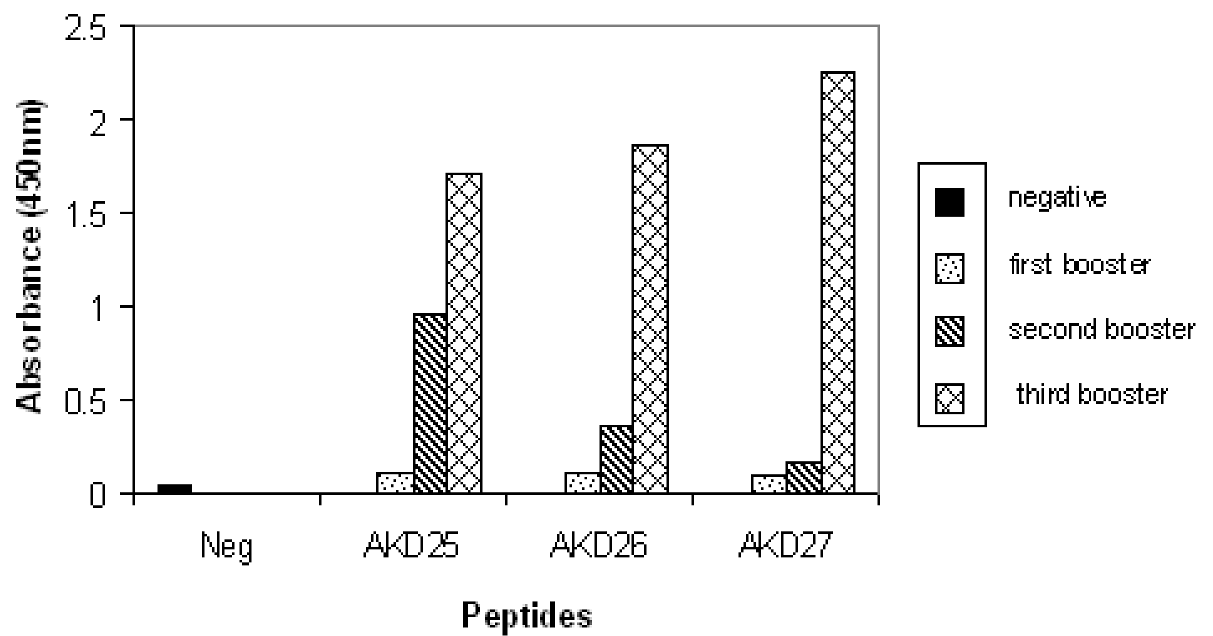


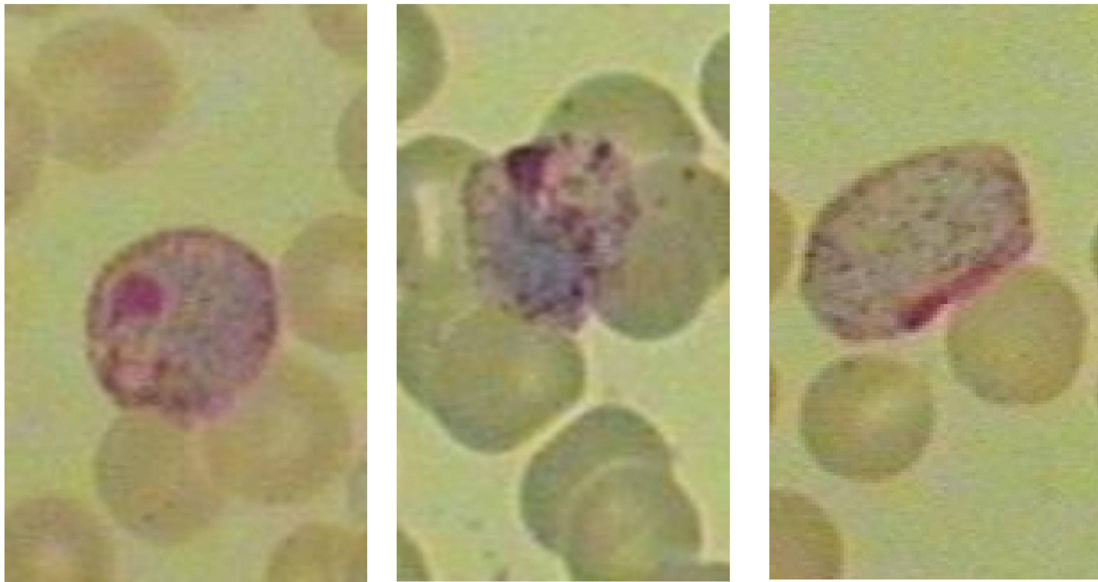
Figure 5.1 B: Antibody profile of SufB peptides in Swiss Albino mice

A peptide designed from the *P. falciparum* EF – Tu sequence was also used as standard. Among the four peptides designed from *P. vivax* EF – Tu, only two (AKD19 and AKD21) gave good antibody response in mice while for other two (AKD20 and AKD22) the response was very poor. In case of SufB peptides, all the three peptides (AKD25, AKD26 and AKD27) gave good antibody response.

Immuno – localization on *P. vivax* field samples

Fresh *P. vivax* thin smear slides, fixed in methanol / methanol: acetone solution, were obtained from the field. The infection was confirmed using Giemsa stained slides (Figure 5.2 A – C) and diagnostic PCR of total blood DNA. For indirect immunofluorescence microscopy, the smeared and unstained slides were first washed with 1X PBS, then treated with raised antibodies, followed by secondary antibodies tagged with fluorescine isothiocynate (FITC). The slides were also treated with a counter stain, nuclear stain Hoechst (no. 33342) and mounted. The slides were viewed with 100X oil – immersion lens in fluorescence microscope (Nikon) and images were captured. The complete protocol was optimized to reduce the background due to stain taken by RBCs.

The images were taken in bright light field to identify the parasite, in green fluorescence to localize the parasite stained with FITC and in blue fluorescence to identify the parasite nucleus stained with nuclear stain Hoechst (no. 33342) in the RBC. The obtained images were identified as trophozoite and schizont stages of *P. vivax* parasite. The best obtained images are shown in Figure 5.3 A – B.



(A) (B) (C)

Figure 5.2 A – C: Trophozoite stages of *P. vivax* as viewed in Giemsa stained slides. Same patient's slides were used in indirect immuno – fluorescence.

Figure 5.3

(A) View 1

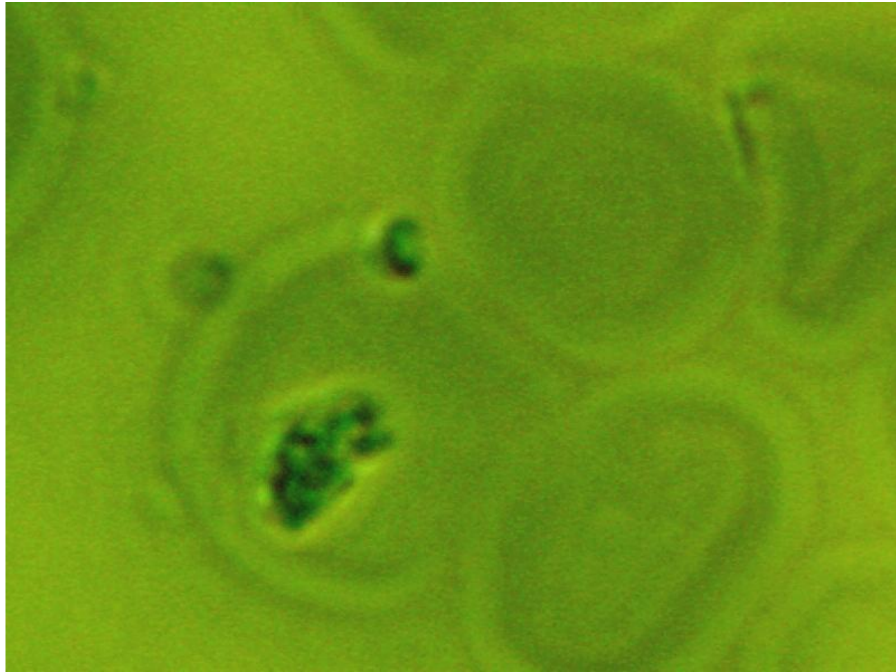


Figure 5.3 A1: *P. vivax* trophozoite in Bright light field

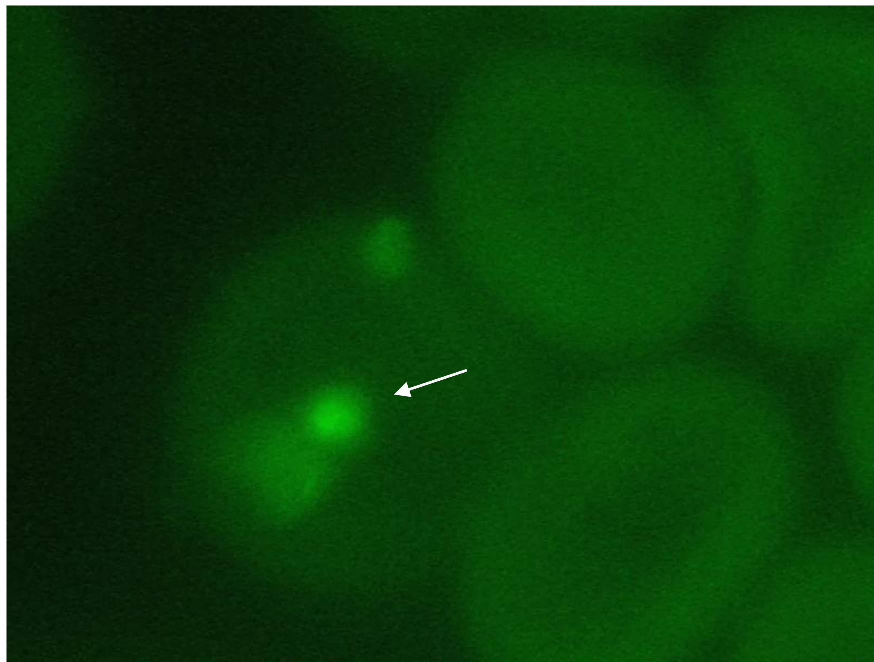


Figure 5.3 A2: *P. vivax* trophozoite stained with FITC

(The arrow indicates the circular structure expected to be apicoplast)

Figure 5.3 A3: *P. vivax* trophozoite stained with Hoechst (No. 33342) nuclear stain

(B) View 2

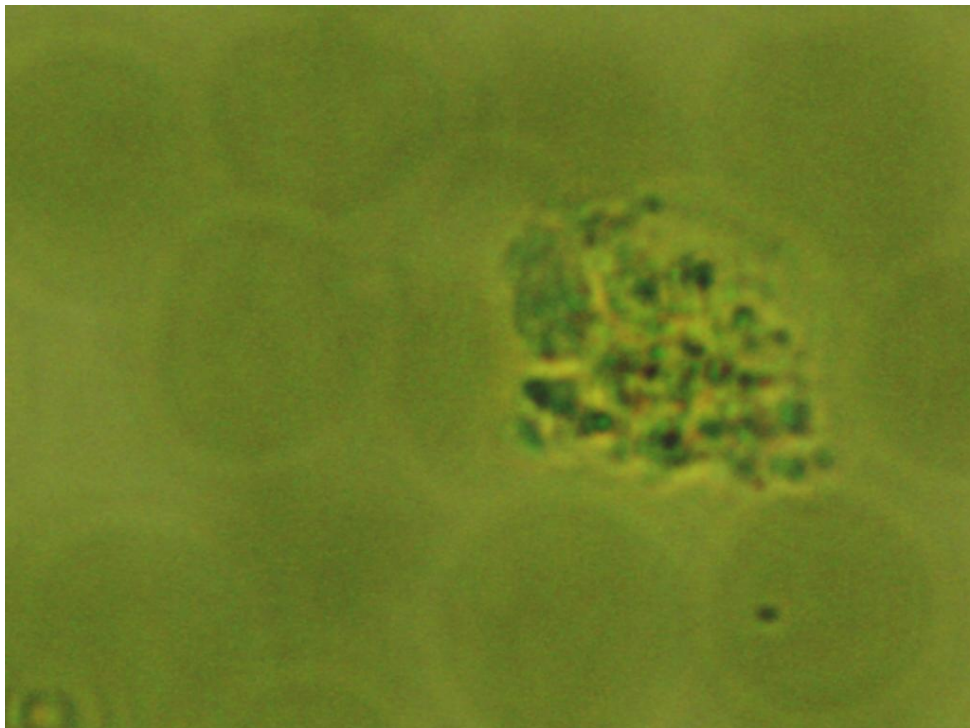


Figure 5.3 B1: A view of *P. vivax* schizont in bright light field

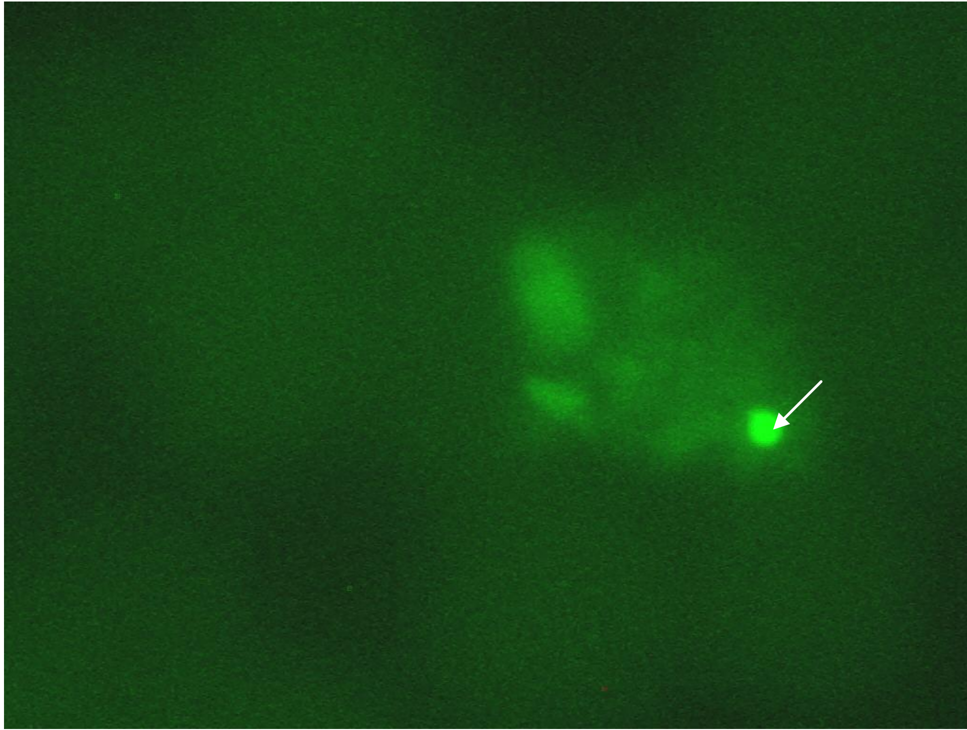


Figure 5.3 B2: *P. vivax* schizont stained with FITC

(The arrow indicates the circular structure expected to be apicoplast)

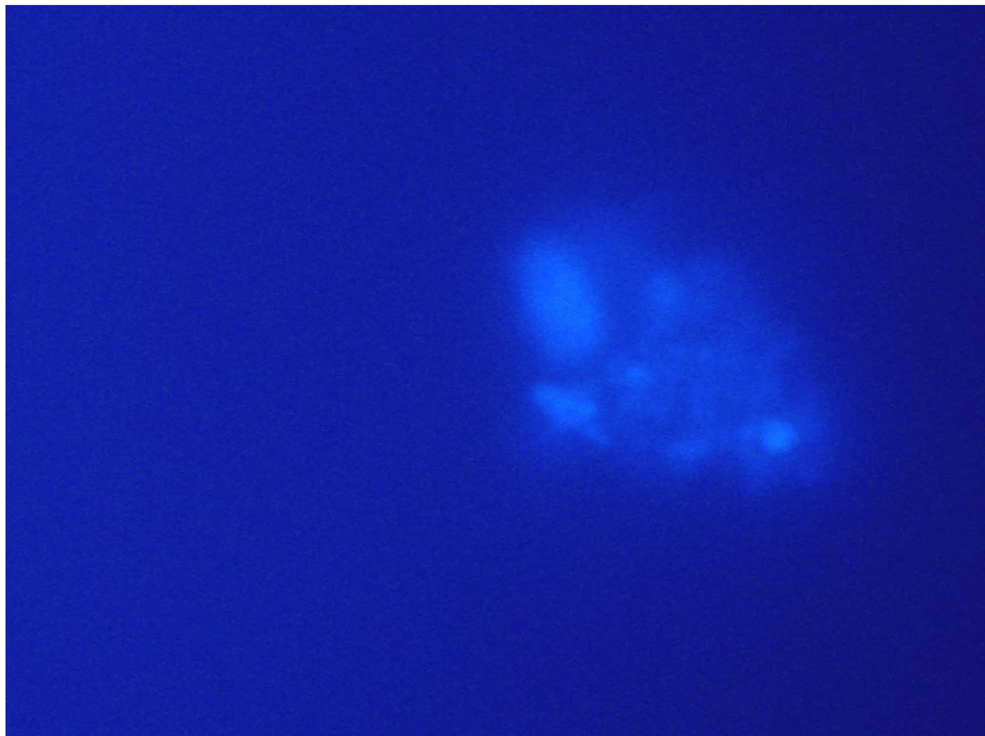


Figure 5.3 B3: *P .vivax* schizont stained with nuclear stain Hoechst (No. 33342)

The negative control slides prepared with the pre immune sera did not show any localization of antibodies on slides. When antibodies raised against TufA peptide were used, the obtained images showed fluorescing green discrete rounded spot within the parasite region. This could very well be indicating the apicoplast in *P. vivax* having maximum of EF – Tu concentration. Adjacent to these discrete rounded spots but within the parasite boundaries green fluorescence was spread out throughout the parasite. This might be indicating the presence of this protein out of the organelle in the parasite. This could mean that the protein may be being released by the organelle in the cytoplasm. But when the sera raised against SufB peptides were used, no florescence could be seen. This may indicate that either the protein may not be available for localization or the conformational sites are not recognized by antibodies raised against designed peptides.

Antibody Profiles in Naturally Infected Individuals, to EF – Tu and ORF470/ SufB Peptides

People naturally exposed to the members of genus *Plasmodium* develop antibodies against various stage specific antigens that are exposed on the surface of the parasite. To investigate whether the proteins from the apicoplast were being exposed to the host immune system or are involved in producing B – cell response in the host, antibody profiles were studied in the naturally infected individuals against the peptides designed from EF – Tu and SufB Apicoplast proteins.

Individual peptide was tested against sera collected from 30 - 35 patients having only *P. vivax* infection. The peptide designed from *P. falciparum* EF – Tu protein was tested against 36 *P. falciparum* positive patients. The peptides which gave high antibody titers in mice showed a feeble or minimal response (Figure 5.4 A – E) against the naturally infected sera even at very low (1:500, 1:1000, 1:2000) dilutions. The results indicated that either these proteins are not accessible to the immune system of the host or these proteins give rise to antibodies which detects the conformational epitopes and not the linear ones as designed in our laboratory. These are the preliminary studies and further studies are required in this regard.

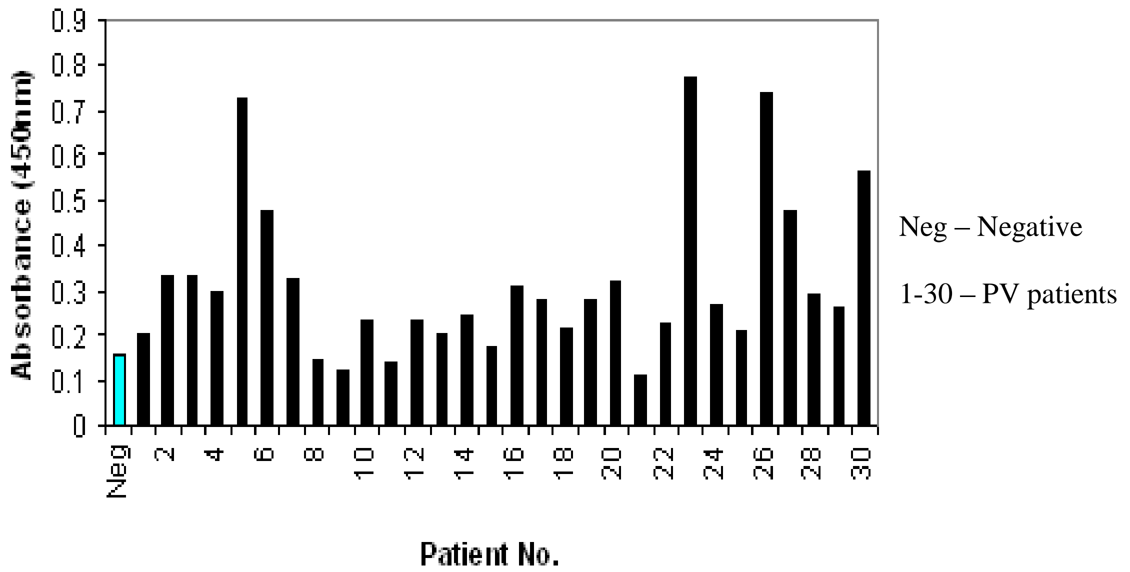


Figure 5.4 A: Antibody titer in *P. vivax* infected human sera (1:1000 dilutions) against TufA peptide AKD19 (Standard Error: ± 0.08)

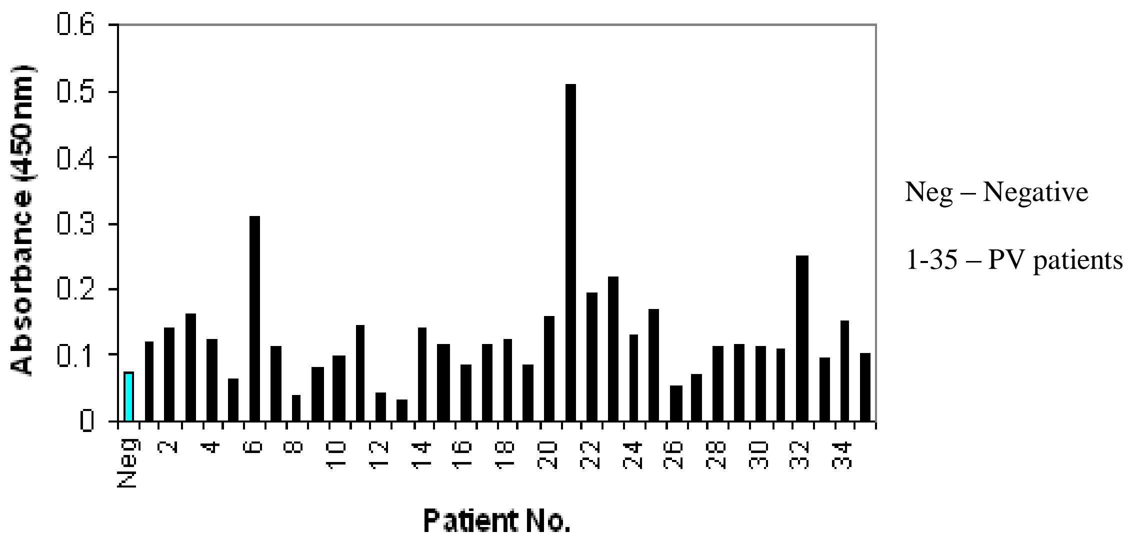


Figure 5.4 B: Antibody titer in *P. vivax* infected human sera (1:1000 dilutions) against TufA peptide AKD21 (Standard Error: ± 0.08)

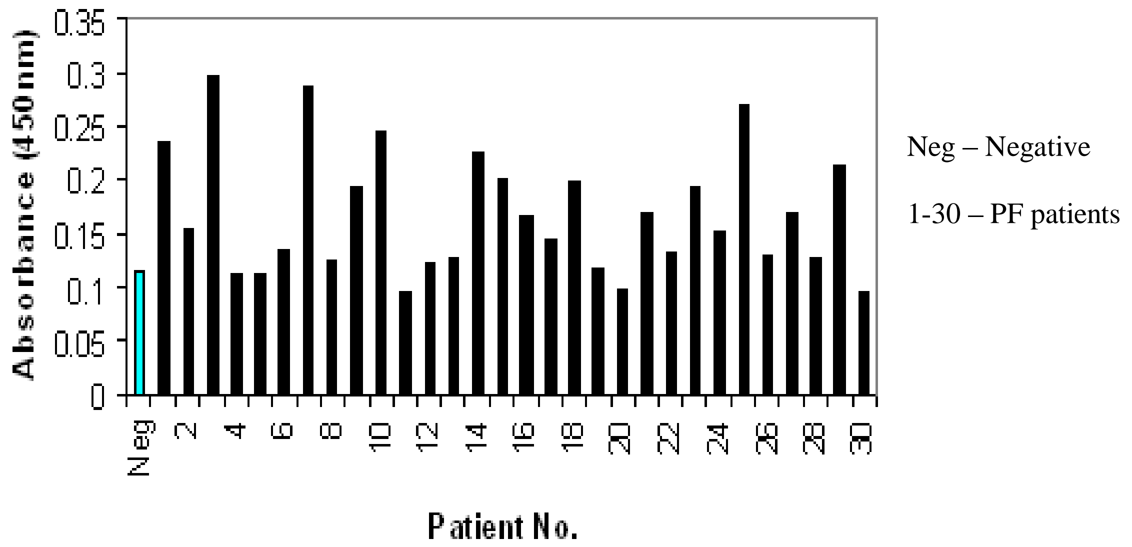


Figure 5.4 C: Antibody titer in *P. falciparum* infected human sera (1:1000 dilutions) against TufA peptide AKD24 (Standard Error: ± 0.08)

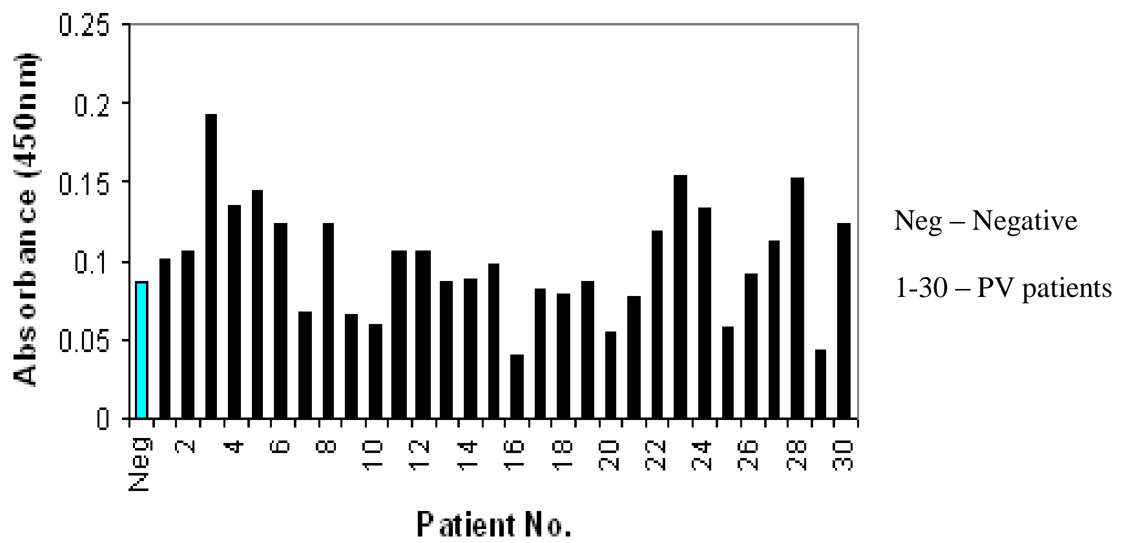


Figure 5.4 D: Antibody titer in *P. vivax* infected human sera (1:1000 dilutions) against SufB peptide AKD25 (Standard Error: ± 0.08)

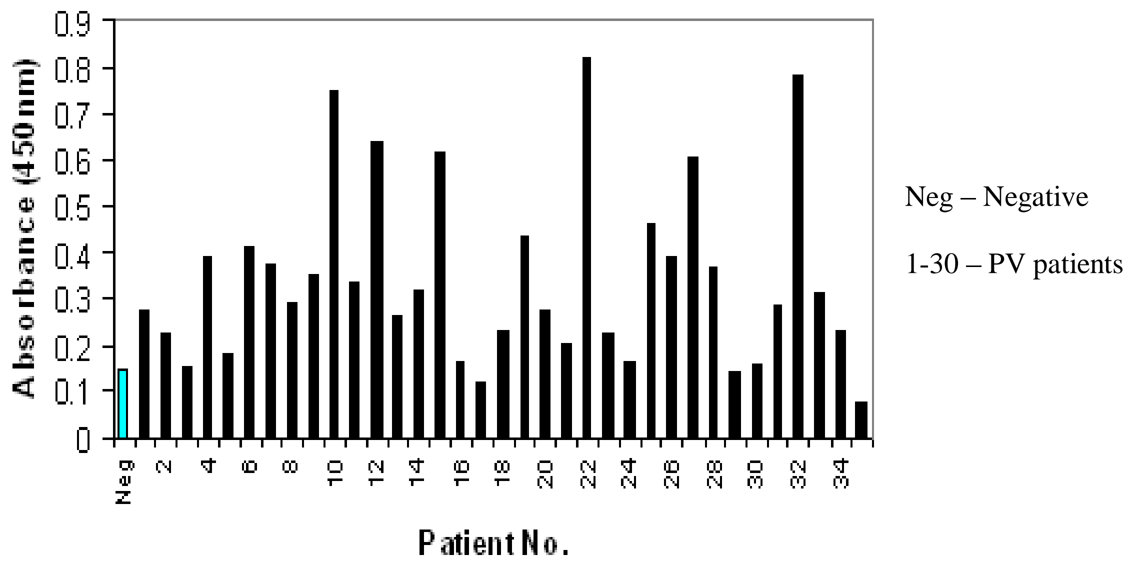


Figure 5.4 E: Antibody titer in *P. vivax* infected human sera (1:1000 dilutions) against SufB peptide AKD26 (Standard Error: ± 0.08)