Chapter 2

P. vivax Infected Blood Sample Collection

The samples were collected from different regions of Rajasthan, such as Bikaner, Jaipur, Sikar and Kota, each at least 150 – 350 km apart from the other. All the regions show unstable episodes of *P. vivax* and *P. falciparum* malaria especially after rainy season. About 2 ml to 5 ml of infected blood was sampled in 16% ACD (Acid Citrate Dextrose) solution from malaria patients with their informed consent. For this, proper assistance was taken from trained clinicians. Among the various samples collected, twenty *P. vivax* and three *P. falciparum* infected samples having very good parasitemia (as reported by the microscopic diagnosis) were used for the study, mostly from Bikaner and only one from Kota. Blood samples were collected and stored at –20^{o}C until DNA extraction.

Parasite DNA Extraction from Blood

Stepwise list of reagents used:

1. Lysis Buffer A: 10 mM NaCl, 50 mM Tris, 10 mM EDTA
2. Lysis Buffer B: 10 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% SDS
3. Proteinase K (20 mg/ml stock solution in SMW)
4. Phenol – Tris saturated solution (pH 8.0)
5. Chloroform : Iso – Amyl Alcohol (24:1)
6. Absolute (99.5%) and 70 % Ethanol
7. Sodium acetate solution – 3M (pH 5.2)
8. 1 X TE buffer (pH 8.0)

The *Plasmodium vivax* infected frozen blood samples were thawed at room temperature and subjected to the modified protocol for parasite DNA isolation. The extraction procedure was based on lysis of the cells in a mixture of 10 mM NaCl,

50 mM Tris, 10 mM EDTA in the presence of 1% SDS (Sambrook *et. al.*, 1989). Whole sample was treated with these lysis buffers at 37^0C for 1 hour, with thorough mixing by gentle tapping or inversions. Proteinase K (100 µg/ml) was added and the material was mixed by inversion and incubated for 1 hr at 50^0C. A conventional Phenol: Chloroform: Iso – Amyl alcohol (25:24:1) extraction was performed and then chilled absolute Ethanol in the presence of 0.2 M Na-acetate was added to the obtained aqueous phase. The preparation was stored at -20^0C overnight and the DNA was precipitated by centrifuging the tubes at 8,000 rpm for 25 – 30 minutes. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer (pH 8.0). A diagnostic PCR (Figure 2.1) was done to confirm *P. falciparum* or *P. vivax* infection (Das *et. al.*, 1995; Kochar *et. al.*, 2005). An amplification of 1.4kb would indicate the presence of *P. falciparum* and a band at approximately 500bp would indicate the presence of *P. vivax* DNA.

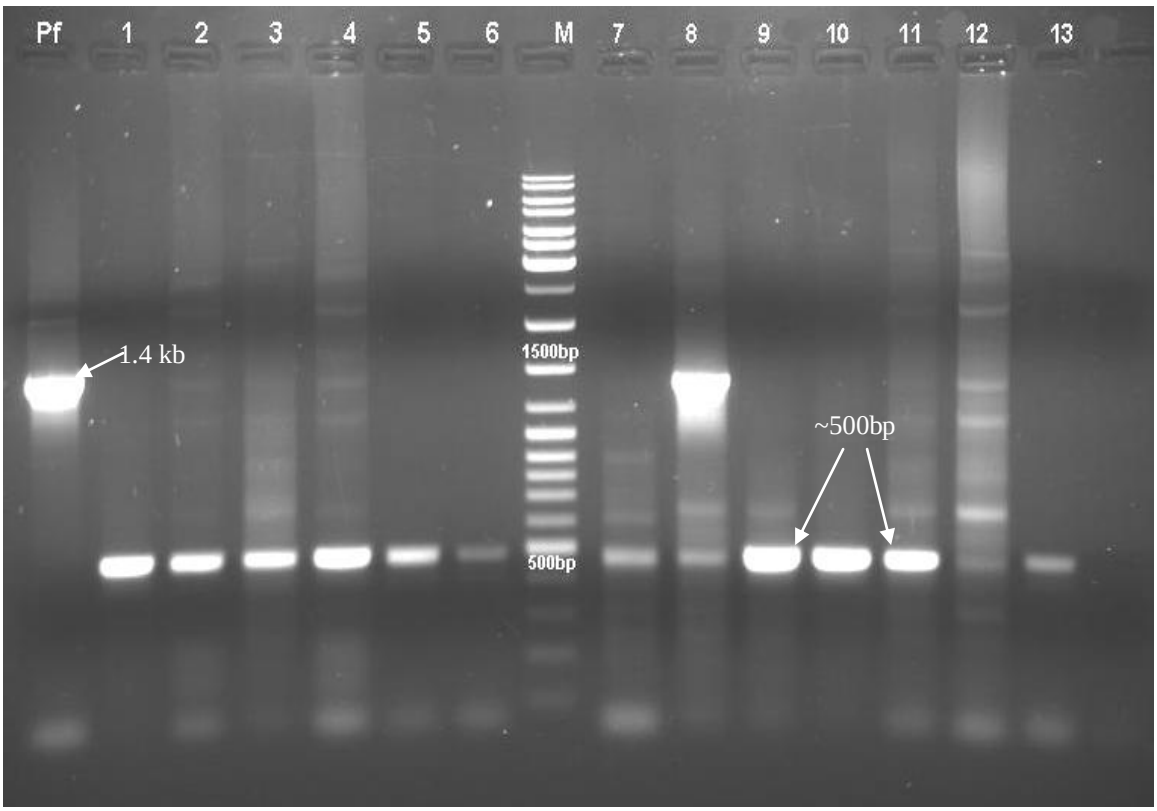

Figure 2.1: Gel image of Diagnostic PCR. Pf= Pure *P. falciparum* DNA; Lanes 1 – 13= *P. vivax* field isolates; M = 1kb DNA Ladder Mix (MBI Fermantas #SM0331)

Apicoplast Genome Sequence and Primer Designing

To amplify the apicoplast genes from *P. vivax*, primers were designed from the apicoplast genome sequence of *P. falciparum*. The 35kb apicoplast genome sequence of *P. falciparum* is established and available on NCBI database. The accession numbers for the two respective regions IR_A and IR_B are X95275 and X95276 respectively. The regions for the genes of interest were marked out from the 15kb (IR_A) and 14kb (IR_B) regions. Primers were designed using Generunner software (Hastings Softwares Inc., USA) from the flanking regions of these genes. A nested PCR approach was also followed for larger genes (greater than 1.2kb in size), for which primers were also designed from the central region of the genes. Proper restriction sites were included at the start of some primers.

The Log T_m value was calculated using the standard formula-

$$\text{Log } T_m = 81.5 + 16.6[\log10(Na^+)] + [41(G+C)/n] - 675/n$$

(n = number of bases)

The presence of any dimers and the loop T_m were also taken into consideration while designing primers. The primers were synthesized commercially. The PCR reactions were carried out in Perkin Elmer 2400 PCR System and Peltier Thermal Cycler (Gradient and Minicycler).

Polymerase Chain Reaction (PCR)

Reagents used:

1) Taq DNA Polymerase (Bangalore Genei)
2) 10 X Taq DNA Polymerase reaction buffer
3) 1M $MgCl_2$
4) 10mM dNTP mix stock solution (Finnzyme)
5) Primers (Commercially synthesized and resuspended in nuclease free sterile water)

Parasite DNA was extracted from the *P. falciparum* and *P. vivax* infected blood samples. After standardization of reactions by *P. falciparum* gene amplification,

various genes from the apicoplast genome of *P. vivax* were amplified using the designed primers. 100 – 250 ng of parasite DNA was taken as template for each amplification reaction, which utilized standard components for a 50 µl-reaction volume. Samples were subjected to 35 cycles of amplification. The initial denaturation at 94°C was kept for 2 – 3 minutes, second denaturation varied with 1 – 1.5 minutes from amplicons to amplicons. Annealing varied from 43 to 52°C for 1.5 - 2.5 minutes and extension at 72°C for 1.5 to 3 minutes. A post – extension step at 72°C was also included for 4 minutes.

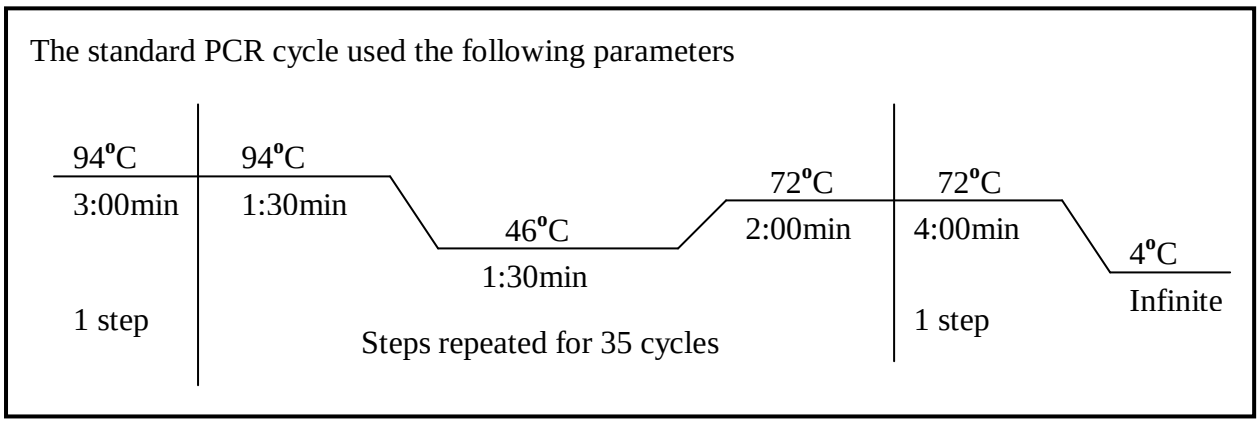

Figure 2.2: Depiction of a standard Polymerase Chain Reaction

A standard PCR reaction (50 µl volume) was set up as follows-

Template DNA	-	100 – 250 ng
10mM dNTP mix (Finnzyme)	-	200 nM
Primers (Both For and Rev)	-	120 – 300 ng each
10X Taq Buffer	-	volume to 1X final concentration
Taq DNA polymerase	-	1 – 3 units
$MgCl_2$	-	1.5 – 8.0 mM
Sterile Millipore Water	-	q. s. to make 50 µl

Plasmid Extraction

The Alkaline lysis method given by Birnboim and Doily (1986) was followed throughout for plasmids (pRSET A, pET21c, etc.) or clone isolation from Gram negative bacterial cultures.

Reagents used:

1. Solution I: 50 mM Glucose, 10 mM EDTA (pH 8.0), 25 mM Tris-Cl (pH 8.0)
2. Solution II: 0.2 N NaOH, 1 % SDS
3. Solution III: 3 M Sodium acetate, Glacial Acetic acid. Adjust pH to 5.2
4. Solution IV: Tris – Saturated Phenol (pH 8.0), Chloroform : Isoamyl alcohol (24:1)
5. Absolute (99.5%) and 70% alcohol
6. RNase A (Sigma)
7. 1 X TE buffer (pH 8.0): 100 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0)

Cells were pelleted down from overnight cultures and re-suspended in ice-cold Solution I. To this freshly prepared Solution II was added and incubated on ice for a couple of minutes with intermittent mixing. Gentle mixing was done by inverting the tubes after adding ice-cold Solution III. The lysed material was then spun at 10,000 rpm for 10 min at 4^{o}C. A conventional Phenol: Chloroform (Solution IV) extraction was then performed and the DNA in the aqueous phase was precipitated by addition of chilled Ethanol in the presence of 0.2 M Na – acetate. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer (Sambrook *et. al.*, 1989).

Preparation of Competent Cells

Reagents used:

1. 0.1 M $CaCl_2$ (Calcium Chloride solution)
2. 60 % Glycerol
3. Luria – Bertani medium broth (LB broth)

The bacterial cells were grown overnight at 37^0C and inoculated into 100ml fresh LB broth in ratio of 1:40. This culture was grown to 0.4 – 0.6 O.D. and the cells were pelleted out. The pellet was re-suspended in 1/10 volume of ice – cold 0.1 M $CaCl_2$ and allowed to stand on ice for 30 minutes. The cells were spun at 8,000 rpm for 6 minutes and re-suspended in 2 ml of ice cold 0.1 M $CaCl_2$. To this Glycerol was added, mixed and the cells were aliquoted and stored at -70^0C till use (Sambrook *et. al*., 1989).

Transformation of *E. coli* with Plasmid/ Ligation Product

Approximately 100 ng of the plasmid DNA was added to the competent cells and incubated on ice for 25 minutes. The microfuge tubes were rapidly transferred to a 42^0C water bath for a heat shock treatment for 90 seconds. These tubes were replaced on ice to which 600 – 900 µl fresh autoclaved LB broth was added in the hood and incubated at 37^0C for 1 hour. This was plated on to LB agar plates with appropriate antibiotics and incubated over night at 37^0C.

Cloning of Apicoplast Genes in Vector Systems

Materials used:

- Restriction enzymes (MBI Fermentas)
- Buffer for restriction digestion: Enzyme specific/ Y^+ Tango (MBI Fermentas)
- Calf Intestinal Alkaline Phosphatase (CIAP) (MBI Fermentas)
- T4 DNA Polymerase (MBI Fermentas)
- T4 Polynucleotide Kinase (MBI Fermentas)
- QIAquick Gel Extraction kit (QIAGEN)
- QIAquick PCR Purification kit (QIAGEN)
- T4 DNA Ligase (New England BioLabs)
- T4 DNA Ligase buffer (New England BioLabs)

The vector was single or double digested with restriction enzymes as per requirement and was purified using QIAquick gel extraction kit. The single digested vector was later treated with CIAP to dephosphorylate the 5' end. The insert, a PCR product, was

purified using PCR purification kit and treated with T4 DNA Polymerase and T4 DNA Polynucleotide Kinase so as to introduce a phosphate group at its 5' end. (Sambrook *et. al.*, 1989). After this, the treated insert and vector were gel eluted and quantified for setting up a ligation.

Reaction for restriction digestion of vector/ insert

Template DNA	=	500 ng – 1 µg
Restriction Enzyme	=	1 – 3 units
Buffer (10X)	=	2µl
Sterile Millipore Water	=	Volume to make 20 µl

Reaction was left at 37^0C for 1 – 2 hours.

Reaction for CIAP treatment of vector

Template DNA	=	10 µl (2-3 µg)
CIAP enzyme (1unit/µl)	=	2 µl
Enzyme Buffer (10X)	=	2 µl
Sterile Millipore water	=	Volume to make 20 µl

Reaction was left at 37^0C for 1 – 2 hours.

Reactions for treatment of insert

Step 1: Treatment with T4 DNA Polymerase for making blunt – ended insert

Template DNA	=	8 µl (1µg)
T4 DNA Polymerase Enzyme (2 unit/µl)	=	2 µl
10X Buffer (Y+/Tango-MBI Fermentas)	=	1.5 µl
dNTP's (10 mM mix)	=	50 nM
Sterile Millipore Water	=	q.s. to 15 µl

Reaction was left at 11^0C for 30 minutes.

Step 2: Treatment with T4 Polynucleotide Kinase for introducing phosphate group at 5' end of the insert

Template DNA (Complete product from step 1)	= 15 µl
T4 Polynucleotide Kinase Enzyme (1 unit/µl)	= 2 µl
10X Buffer (Y+/Tango-MBI Fermentas)	= 0.5 µl
ATP(10 mM)	= 1 µl
Sterile Millipore Water	= q.s. to 20 µl

Reaction was left at 37^0C for 30 minutes.

Ligation

The quantities of both vector and insert were calculated by measuring O. D. at 260 nm. Based on the quantity calculated a reaction mix was prepared as follows –

Vector	=	1 part
Insert	=	3 parts
T4 DNA Ligase Enzyme (NEB)	=	1 unit
Ligase Buffer (10X) (NEB)	=	2 µl
Sterile Millipore Water	=	Volume to make 20 µl

The reaction mixture was left at 16 – 22^0C for 6 – 16 hours depending on the specification mentioned by T4 DNA Ligase enzyme supplier (New England Biolabs/ Bangalore Genei/ MBI Fermentas). Following this, the mixture was transformed into an appropriate host.

Clone Analysis

Reagents used:

- Lysis Buffer: 5 mM EDTA, 10% w/v Sucrose, 0.25% SDS, 100 mM NaOH, 60 mM KCl

The colonies obtained after transformation of ligated product were first analysed using gel lysis method. For this, the obtained colonies were streaked on LB Agar – antibiotic plate. This plate was kept at 37^0C for 15 – 16 hrs to get good growth of colonies. A small part of these colonies was then picked up and suspended into lysis

buffer. The tubes were then kept at 37^0C for 5 minutes, then at 4^0C for 5 minutes and finally at room temperature for 5 minutes. The tubes were centrifuged at 10,000 rpm for 5 minutes and the supernatant was loaded on the gel. If a clone is present in the tested colonies, a shift in the plasmid bands (Figure 2.3) should be seen as compared to others or the standard. This protocol thus differs from the standard plasmid isolation techniques where the plasmid appears as multiple bands. The short – listed colonies were then grown in LB broth and plasmid was isolated using routine alkaline lysis method. The obtained plasmid is further analysed by restriction digestion at the multiple cloning regions. This gives a proper clone carrying only one insert in the vector (Sekar, 1987).

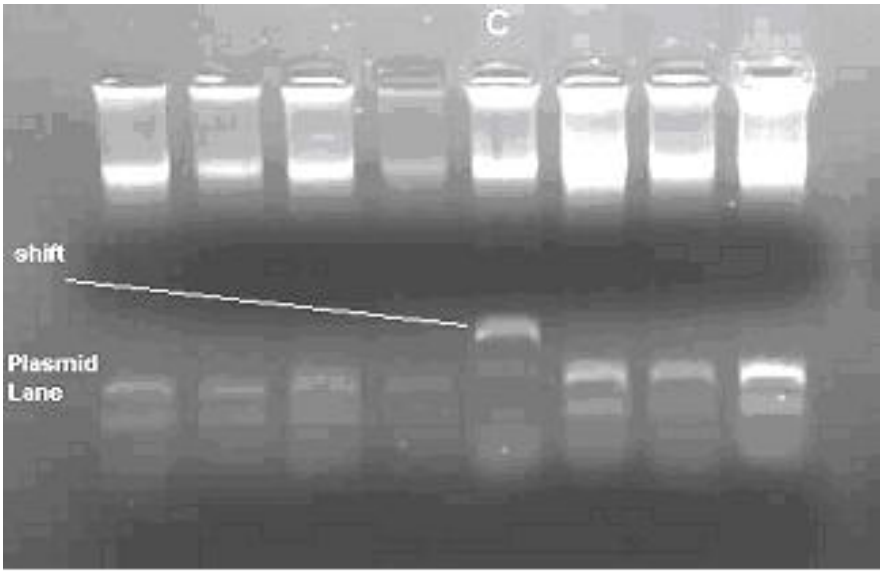

Figure 2.3: Gel Lysis method for checking clones

DNA Sequencing

Sequencing of the clones and amplicons was done at CDFD, Hyderabad and commercially by various service providers. All the completed sequences were submitted to Genbank. Genbank Accession nos. are: **DQ140160** (*tufA*, Isolate1), **DQ533992** (*tufA*, Isolate2), **DQ533993** (*tufA*, Isolate3), **DQ499659** (*sufB*), **DQ503575** (*rpoC*).

Protein Expression

Reagents used:

- 1.5 M Tris solution (pH 8.8)
- 0.5 M Tris solution (pH 6.8)
- Acrylamide solution (Acrylamide 29 parts: Bis – Acrylamide 1 part)
- 10% Ammonium Persulfate solution (Sigma)
- Sodium Dodecyl Sulfate (1% SDS)
- TEMED (Sigma)
- 2X Sample lysis buffer (Bangalore Genei)
- Gel running buffer: Tris Base, Glycine, SDS
- Protein Molecular Weight Marker (Heavy and Light) (Bangalore Genei)
- Prestained Protein Molecular Weight Marker (MBI Fermentas)
- 0.4% Staining solution: Coomassie Brilliant Blue, Methanol
- De- staining solution: Methanol (40 parts), Glacial Acetic acid (7 parts)

The culture of the desired clone was grown overnight at 37^0C with shaking. At 12^{th} hour, 1 part of this primary culture was inoculated in 40 parts of fresh LB broth and kept at 37^0C with shaking. As this secondary culture attains an OD of 0.3 – 0.4 at 600 nm (about 2 – 3 hrs), an aliquot of 1 ml was withdrawn from the culture. IPTG (final concentration 0.5 – 3.0 mM) was added to the culture and again kept in a 37^0C shaker. 1 ml samples were withdrawn from the culture at varying intervals of 1 hr to 8 hrs. Samples were centrifuged at 3,000 rpm for 5 minutes and the obtained cell pellet was resuspended in 2X sample lysis buffer. These were heated at 100^0C for 10 minutes and stored at -20^0C till use. Pre – heated protein samples were loaded on the SDS polyacrylamide gel. The obtained gel was stained with Coomassie Brilliant Blue solution and protein bands were viewed.

Western Blotting

Materials used:

- Tris buffered Saline (pH 7.5): 50 mM Tris, l50 mM NaCl
- Transfer buffer: 48 mMTris, 39 mM Glycine, 20% Methanol
- Blocking buffer: 3% BSA solution
- Washing buffer: 1X TBS, 0.05% Tween 20
- Anti – His Antibodies (QIAGEN)
- Goat anti – mouse IgG ALP conjugate (Bangalore Genei)
- Substrate solution: BCIP/ NBT (Bangalore Genei)
- Nitrocellulose/ Nylon membrane (Schleider & Schulle)
- Western transfer apparatus (BioRad)

A pre – run and unstained SDS polyacrylamide gel was used for the transfer of proteins to the nitrocellulose membrane. The nitrocellulose membrane and filter pads were soaked in the pre-cooled transfer buffer. The assembly was set with the gel at the negative end and the membrane at the positive end. After the transfer, the membrane was washed once with washing buffer and then kept soaked in Blocking buffer for 1 ½ hour. This was followed by washing thrice with Washing buffer and then membrane was left soaked in Anti – His Antibody (1/1000 dilution) for 1 ½ hours. Again the membrane was washed thrice followed by incubation with Goat anti – mouse IgG ALP conjugate antibody (1/2000) for 1 ½ hours. Finally the membrane was washed thrice and incubated for 25 – 30 minutes with substrate solution in dark. The substrate reaction was stopped by adding distilled water. The developed colour was analysed (Sambrook *et. al.,* 1989).

Total RNA Isolation

Reagents used:

- Diethyl Pyrocarbonate (DEPC) (Sigma)
- Gram negative lysing buffer: 10 mM Tris-Cl (pH8.0), 10 mM NaCl, 1 mM Na-citrate, 1.5% (w/v) SDS

- Protoplasting buffer:15 mM Tris-Cl (pH 8.0), 0.45 M Sucrose, 8 mM EDTA
- Lysozyme solution in DEPC treated water (Ameresco)
- Saturated NaCl solution: NaCl in DEPC treated water
- Chloroform: Iso-Amyl alcohol (24:1)
- MOPS/ Formaldehyde gel running buffer (5X): MOPS, Na – citrate (pH 7.0), formaldehyde.
- RNA gel loading buffer (MBI Fermentas)
- 3% Hydrogen Peroxide solution (Qualigens)

All the reagents were prepared in DEPC treated water and plastic-wares were treated with the same. The culture of the desired clone was grown overnight at 37^0C with shaking. From this a secondary culture was grown as in for protein expression work. For RNA isolation, samples (3 ml) were collected from 0 hr, 1 hr, 3 hrs and 6 hrs IPTG (1 mM) induced cultures. The cells were spun down at 10,000 rpm for 10 minutes and resuspended in equal volume (3 ml) of cold protoplasting buffer. Lysozyme was added to a final concentration of 100 µg/ml and the tubes were incubated on ice for 15 minutes. The tubes were again centrifuged at 7,000 rpm for 5 minutes at 4^0C and the pelleted cells were resuspended in 175 µl of gram negative lysing buffer. 10 µl of DEPC water was added to the tubes, contents were mixed and tubes were incubated at 37^0C for 5 minutes. Tubes were then transferred to ice, saturated NaCl was added and tubes were left on ice for 10 minutes. The tubes were centrifuged for 10 minutes at 10,000rpm. Supernatant was removed in a clean microfuge tube and chilled absolute Ethanol was added to it. Tubes were inverted several times to mix the contents and kept at -20^0C overnight. The tubes were then centrifuged at 8,000 rpm for 20 minutes, pellets were washed with 70% Ethanol and then resuspended in 50 µl DEPC treated water and stored at -70^0C till use (Ausubel *et. al.*, 1999).

cDNA synthesis

Reagents used:

- Sensiscript Reverse Transcriptase kit (QIAGEN)

- Oligo dT (18mer) (Microsynth)
- Bacterial RNA
- PCR reagents (Bangalore Genei)

The Sensiscript RT kit was used as per the manufacturer's instructions, to amplify cDNA from RNA isolated from the clone cultures. Initially the RNA template was kept at 65^0C for 5 mins. along with Oligo dT primer, followed by 1 hour incubation with Reverse transcriptase, dNTPs mix, buffer and water at 37^0C. The product of this reaction was used as template ($1/5^{th}$ of the final reaction volume) for amplification of desired gene using gene specific primers. The amplified DNA product was run on gel to identify the transcription of the desired gene.

Molecular Modeling

Software's used:

- Swiss – Prot database (http://www.expasy.ch/)
- Swiss – Pdb Viewer v3.7b2 (Glaxo Wellcome Experimental Research)
- ViewerLite (Accelerys Inc.)
- Modeller 8v0

Molecular modeling of the desired proteins was tried using freely available softwares based upon the Homology modeling method. The template structures for this were downloaded from Swiss Prot and EMBL database. Modeling was performed following instruction given in help tutorial of the softwares.

Epitope scanning and peptide designing

TufA and ORF470/ SufB protein sequences were scanned for B cell epitopes using the online Bcepred server (IMTECH, Chandigarh, India). Five epitopes from TufA protein (AKD19 – AKD24) and 3 from SufB protein (AKD25 – AKD27) were selected. These peptides were obtained as a gift from CDC, Atlanta. The sequences were as follows:

Table 2.1: Peptides designed using Bcepred

Region	Peptide Name	Sequence
P. vivax TufA	AKD19	LSKKYNYSDIDSAPEEKIRG
	AKD20	KYNFNLNNIHILAGSA
	AKD21	PNKLKVYKSFIAETYILT
	AKD22	YILTKEEGGRHKPFNIGYK
P. falciparum TufA	AKD24	IIQKNKDYELIKSNIWIQ
P. vivax ORF470/SufB	AKD25	YNLNYKYQYKNKINLYLIR
	AKD26	NLSPYFKTNSSDFAQFG
	AKD27	PYIKNYNNTSYVKQEAFV

Peptide formulation using Freund's Adjuvants

Material used:

- Freund's Complete Adjuvant (Bangalore Genei)
- Freund's Incomplete Adjuvant (Bangalore Genei)
- Peptides
- 1X PBS
- Glass syringes
- Tygon tube

Stock solutions of peptides (2 mg/ml) were prepared in 1X PBS (pH 7.4). For the first injection, dose formulation of peptides was prepared using Freund's Complete Adjuvant. The subsequent booster doses were formulated using Freund's Incomplete Adjuvant. These formulations were prepared by mixing the peptide solution and the adjuvant solution in equal ratio using two glass syringes joined together mouth to mouth with the help of a small 2 inch long tygon tube. The two solutions were added to one syringe by removing out its plunger and replacing back the plunger. The plungers of the two syringes were pressed one by one to form a perfect viscous emulsion which was transferred to a clean tube. This emulsion was then injected in the mice.

Mice Strains and Formulations

Female Swiss Albino (Out bred) mice of 7 to 8 weeks of age, bred under specific-pathogen free conditions and supplied by Animal House Facility, BITS, Pilani, India, were used in groups of 5 for each of the peptides. All the peptides were formulated with Freunds Adjuvants. The primary doses were formulated in Complete Freunds Adjuvant. Each mouse was administered with 100 µg of peptide intra-peritoneally (Harlow *et. al.*, 1988). Subsequently three boosters were given every 21st day (after priming) with Incomplete Freunds Adjuvant. Animals were bled the 9th day after every booster and sera were assayed for antibodies by ELISA.

Immunizations – *Intra – peritoneal injections*

The mouse was held by the left hand by pinching off the skin below the base of the neck using the thumb and index finger, stretching the tail with little finger. The mouse was then inverted to expose the abdominal region. The skin on the abdomen was swabbed with alcohol. Using a 23-gauge needle, 100 µl of the antigen formulation was injected in the peritoneal cavity.

Serum preparation from mouse blood

The mouse was placed in a mouse restrainer and heated with an infrared lamp for a few minutes. The heat increases the flow of blood to the tail. A small portion of the tail, about 1.5 – 2 inches from the body was swabbed with alcohol. Using a sterile scalpel, a nick was made on the underside of the tail across one of the lower well prominent veins. The tail was gently stroked to collect the blood in a 1.5 ml centrifuge tube kept below it. After blood collection, the blood was allowed to clot for about an hour at 37^{0}C. The tube was flicked several times to dislodge the blood clot. This clot was then kept at 4^{0}C for overnight to allow it to contract. The serum was then separated from the clot by centrifugation at 10,000 rpm for 10 min at 4^{0}C. The serum was stored at –20^{0}C till the analysis (Harlow *et. al.*, 1988).

ELISA (Enzyme Linked Immunosorbant Assay) – *For analyzing mice sera*

Materials used:

- 96 well Microtitre plates (Nunc, Denmark)
- Peptides
- 1 X PBS (Phosphate Buffered Saline)
- Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6)
- Wash buffer: 1 X PBS – Tween 20
- Blocking buffer: 5 % casein in 1 X PBS
- Conjugate: Goat anti-mouse IgG peroxidase (Bangalore Genei)
- Substrate: TMB/H_2O_2 (Bangalore Genei)
- Stop solution: 2N H_2SO_4

Mouse sera were assayed at regular intervals for antibodies (total IgG) by enzyme-linked immunosorbent assay (ELISA) (Harlow *et. al*., 1988). Micro titer plates were coated with the appropriate antigen (150 ng of peptide per well, diluted in 100 µl of 1X coating buffer) and incubated at 4^oC overnight. Wells were washed thrice with 1X PBS -Tween 20 (0.2 %), blocked with the blocking buffer and incubated at 37^0C for 2 hrs. After washing with the wash buffer three times, serum diluted (1/50, 1/100, 1/200) in blocking buffer was added (100 µl/well) and incubated at 37^0C for 2 hrs. After washing thrice with wash buffer, 100 µl of secondary antibody diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^0C for 2 hrs. Again after washing, 100 µl of substrate solution diluted in distilled water (as per manufacturer instructions) was added in dark and left at room temperature for 15 to 30 min, for the color to develop. 100 µl of stop solution was added and the absorbance was read at 450 nm using ELISA Reader (STATFAX 2100).

Study area, Human subjects, and sera collection

The study region chosen for sera collection was Bikaner, Rajasthan, which lies in the western part of India. About 1 – 3 ml of *P. vivax* infected blood sample was collected randomly at different stages from different areas in this region, where seasonal

episode of *P. vivax* malaria was present during the years 2002 – 2005. Blood samples were collected from patients in the age group of 20 to 40 years, with informed consent.

Serum separation from the *P. vivax* infected blood samples

After blood collection, the blood was left at 4^0C for over night and the cells were allowed to settle. The serum was then separated from the clot by centrifugation at 10,000 rpm for 10 min at 4^0C. The serum was stored at -20^0C till the analysis.

ELISA – *For analyzing human sera*

Materials used:

- 96 well Microtitre plates (Nunc, Denmark)
- Peptides
- 1 X PBS (Phosphate Buffered Saline)
- Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6)
- Wash buffer: 1X PBS – Tween 20
- Blocking buffer: 5 % casein in 1X PBS
- Conjugate: Goat anti-human IgG peroxidase (Bangalore Genei)
- Substrate: TMB/H_2O_2 (Bangalore Genei)
- Stop solution: 2N H_2SO_4

Serum samples from 25 – 35 patients were analyzed by enzyme-linked immunosorbent assay (ELISA) (Harlow *et. al.*, 1988) for total IgG titres against TufA and ORF470/ SufB peptides. Micro titer plates were coated with the appropriate antigen (150 ng of peptide per well, diluted in 100 µl of 1X coating buffer) and incubated at 4^0C overnight. Wells were washed thrice with 1X PBS – Tween 20 (0.2 %), blocked with the blocking buffer and incubated at 37^0C for 2 hrs. After washing with the wash buffer three times, human serum diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^0C for 2 hrs. After washing thrice with wash buffer, 100 µl of secondary antibody diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^0C for 2 hrs. Again after washing, 100 µl of

substrate solution was added in dark and left at room temperature for 15 to 30 mins., for the color to develop. 100 µl of stop solution was added and the absorbance was read at 450 nm using ELISA Reader (STATFAX 2100).

Immuno – Fluorescence Microscopy

Materials used:

- Slides with *P. vivax* infected blood smears
- Goat anti – mouse IgG FITC conjugate (Bangalore Genei)
- Mouse sera containing antibodies for specific peptides
- Fluorescence Microscope (Nikon)
- 1X PBS
- 3% BSA in 1X PBS
- Stains : Hoechst (No. 33342)
- Mountant: PPD (p – Phenylene Diamine) in 90% glycerol

Thin smear slides were prepared from *P. vivax* infected patients blood, air – dried and immediately fixed with cold methanol. After fixation the slides were washed once with PBS and air dried. Permeabilisation was done with 0.05% saponin or 0.1% Triton X 100 for 30 minutes at room temperature. Slides were washed with 1X PBS thrice and blocked in 3% BSA (prepared in 1X PBS). Slides were left for incubation for 4-6 hours at room temperature. Primary antibody prepared in blocking buffer in the ratio 1:50 and 1:100 was added and the slides were incubated at room temperature for 2 – 4 hours. After washing with 1X PBS thrice, secondary antibody was added in dark (Goat anti – mouse IgG FITC conjugate made in blocking buffer, 1:2000 dilution) and incubated for 1-2 hours at room temperature in dark. After washing thrice, Hoechst (no. 33342) stain was added (1:1000 dilution in PBS) for 10 mins. for nuclear staining. Washing was done with 1X PBS for 6 times. Slides were air dried and mounted in 0.05% PPD prepared freshly in 90% glycerol. The slides were then viewed using 100X oil – immersion lens in fluorescent light.

Phylogenetic Analysis

Phylogenetic analysis of obtained gene sequences was performed against the sequences present in database using software MEGA 3.1 (Kumar *et. al.*, 2004). The software was downloaded from its web site, installed and used following developers instructions.

Materials used for various reactions

Vectors and Bacterial strains used

- pRSET A (Invitrogen USA)
- pET 21c (Invitrogen USA)
- *E.coli* DH5α (IMTECH, Chandigarh)
- *E. coli* BL21DE3 (Invitrogen USA)
- *E. coli* BL21 pLysS (Invitrogen USA)

Various kits used for this study

- Qiagen Gel Extraction kit (QIAGEN USA)
- Qiagen PCR Purification (QIAGEN USA)
- Qiagen Anti – His Antibody kit (QIAGEN USA)
- Qiagen Sensiscript Reverse Transcriptase kit (QIAGEN USA)

Medium for bacterial culture

- Luria Bertani medium (HiMedia)
- Plating agar – bactoagar (1.5 – 2.0%)
- Antibiotics used : Chloramphenicol (Hi Media), Ampicillin Na salt (Ameresco)

Composition of various buffers and solutions used in the study

- **TE buffer pH (8.0):**

  10 mM Tris-Cl (pH 8.0)

1 mM EDTA (pH 8.0)

- **Tris – Borate buffer (10 X):** (1000 ml)

  Tris base 108 gm
  Boric acid 55.0 gm
  0.5 M EDTA (pH 8.0) 40 ml

- **Tris – Acetate buffer (50X):** (1000 ml)

  Tris base 242 gm
  Glacial Acetic Acid 57.1 ml
  0.5 M EDTA (pH 8.0) 100 ml

- **Tris Glycine 5X buffer:** (1000 ml)

  Tris base 15.1 gm
  Glycine (electrophoresis grade) (pH 8.3) 94 gm
  SDS (10%) 50 ml

- **Phosphate Buffered Saline pH 7.4 (1X):**

  137 mM NaCl
  2.7 mM KCl
  4.3 mM Na_2HPO_4
  1.4 mM KH_2PO_4

- **Coating buffer For ELISA (10X):** (100ml)

  Na_2CO_3 1.59 gm
  $NaHCO_3$ 2.93 gm

- **Buffers for restriction endonucleases, T4 DNA Ligase and Taq DNA Polymerase**

  The various buffers used were provided by the manufacturers for these enzymes and used as per the instructions provided.