Parasite Biology

2.1 Molecular Genetics of *Plasmodium falciparum* and *P. vivax*

2.1.1 Sequence diversity of *Plasmodium vivax* MSP-3α in Indian field isolates

A study was undertaken to know the sequence variations existing among the Indian field isolates having three length variants of 1.2, 1.4 and 1.8 kb as revealed from PCR-RFLP. It was observed that Indian isolates have 90 to 100% sequence similarity among themselves and average identity of Indian isolates with isolates of other regions was in the range of 80 to 99% (Fig. 1). Highest level of identity was observed in 1.2 kb variant, while identity observed in 1.8 kb variant was the lowest. Existence of common allelic composition in different parts of the globe and segregation of Indian isolates with isolates of different regions suggests that Indian isolates have global allelic representation (Fig. 2).

2.1.2 Comparison of genetic diversity of *Plasmodium vivax* among Indian, southeast Asian and other regions

Plasmodium vivax apical membrane antigen-1 (*PvAMA-1*) is a potential asexual stage vaccine candidate and highly polymorphic in nature. DNA sequence variation in the *PvAMA-1* gene from 41 *P. vivax* isolates from India were examined and compared with previous reported studies of *PvAMA-1* from Papua New Guinea, Thailand, Sri Lanka, Indonesia, Africa, India, China, Solomon Islands and Philippines. In total, 86 haplotypes, 25 polymorphic sites and 31 mutations were identified. The rate of non-synonymous substitutions was very high compared to the rate of synonymous substitutions indicating that *PvAMA-1* is under positive selection pressure. Twenty out of 83 haplotypes showed sharing among different population and remaining 63 haplotypes were clustered among different populations (Fig. 3).

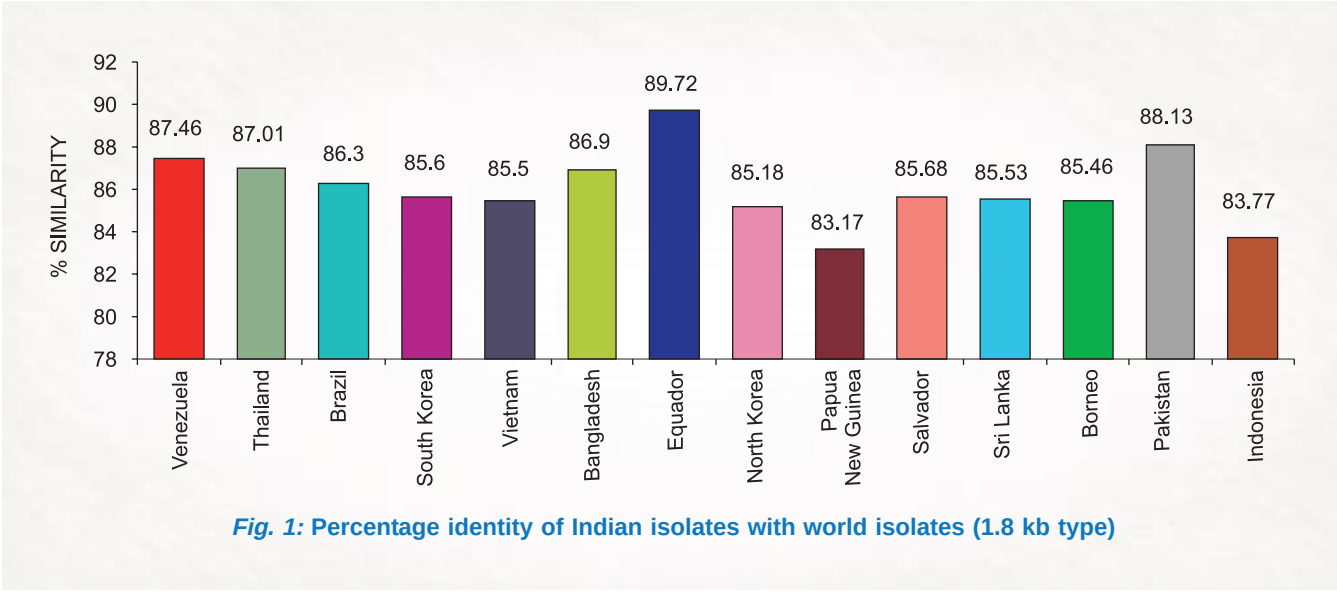

Fig. 1: **Percentage identity of Indian isolates with world isolates (1.8 kb type)**

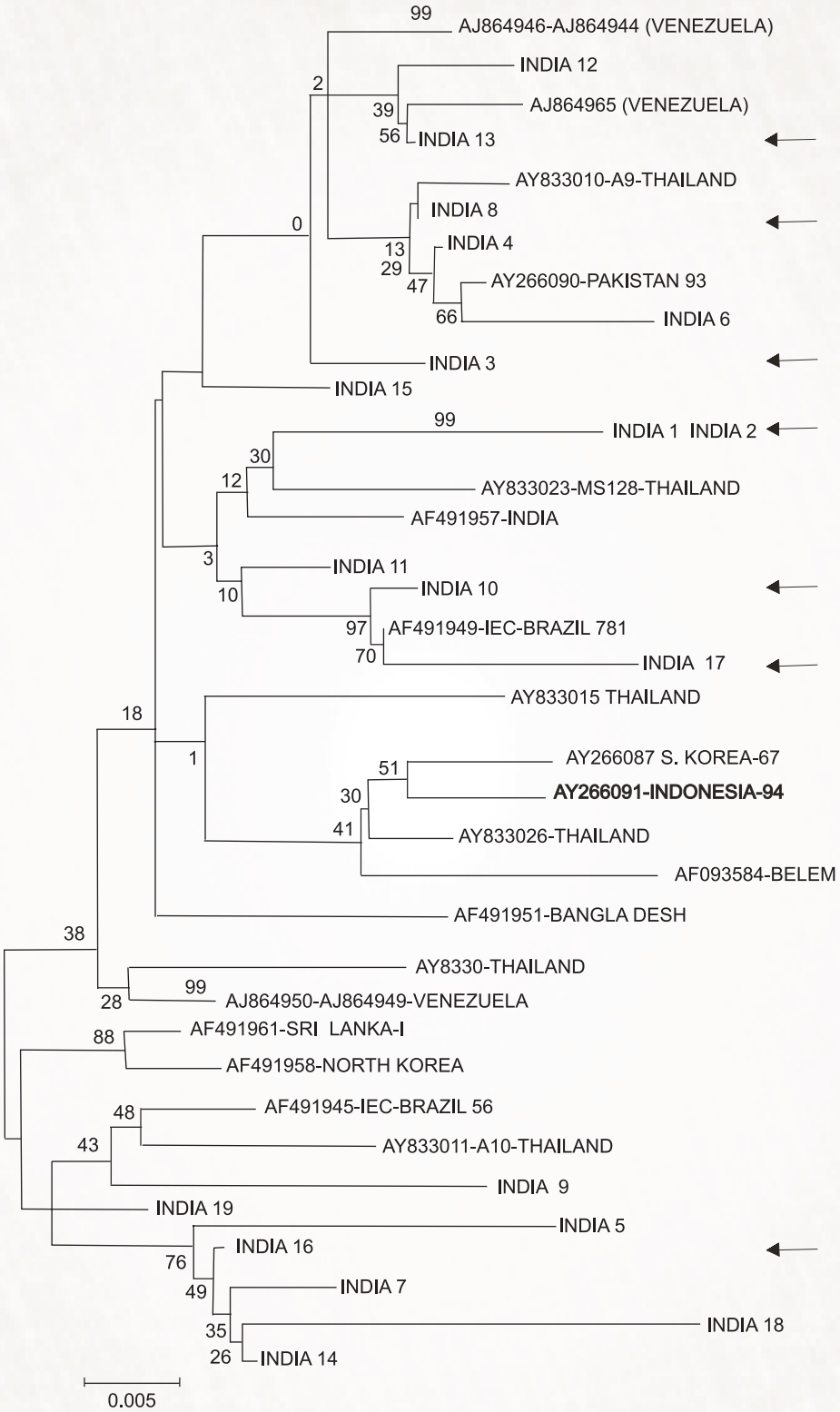

Fig. 2: **Phylogenetic analysis of Indian and other global isolates using MSP-3α sequences**

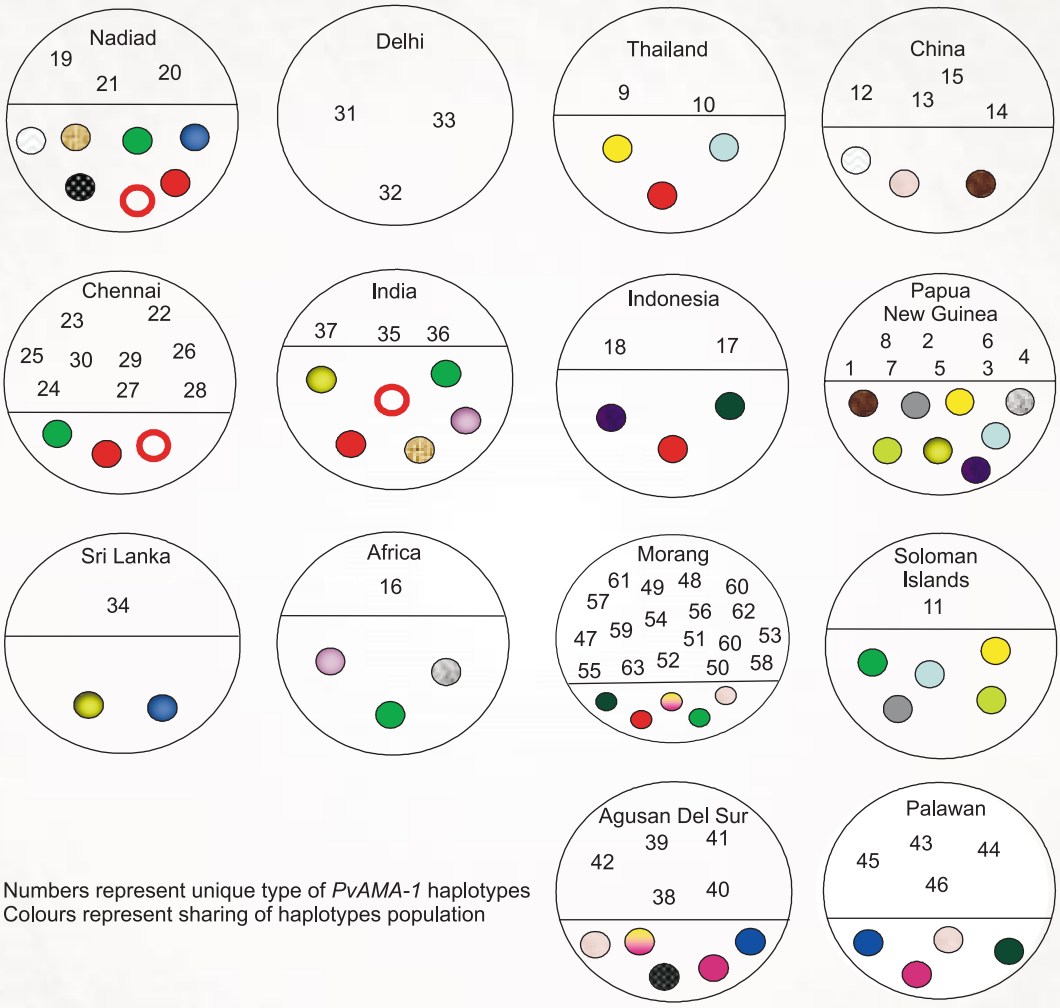

Fig. 3: **Distribution of *PvAMA-1* haplotypes among Indian and other southeast Asian regions**

2.1.3 MSP-3α gene of *Plasmodium vivax*

DNA amplification and sequence analysis of MSP-3α genes of *P. vivax* positive patient isolates from different regions of India like Delhi, Patna, Chennai, and Daltonganj were conducted. PCR/RFLP protocol-based on PvMSP-3α genes has been standardised to demonstrate its utility in analysis of *P. vivax* diversity (Figs. 4 & 5).

2.1.4 Glutathione S-transferase in both *Plasmodium vivax* and *Plasmodium falciparum*

Glutathione S-transferase (GST), an intracellular antioxidant with complex biological functions and well-established metabolic regulator, when decreased is associated with number of disease states including malaria. GST estimation has been investigated in Indian population infected with malaria. Clinical isolates were analysed to understand the role of GST as biochemical marker in vivax patients as well as to compare GST activity in adults with non-complicated vivax malaria with healthy controls. In continuation to biochemical work on GST, PCR protocol-based on GSTP1, GSTM1 and GSTT1 genes has been standardised.

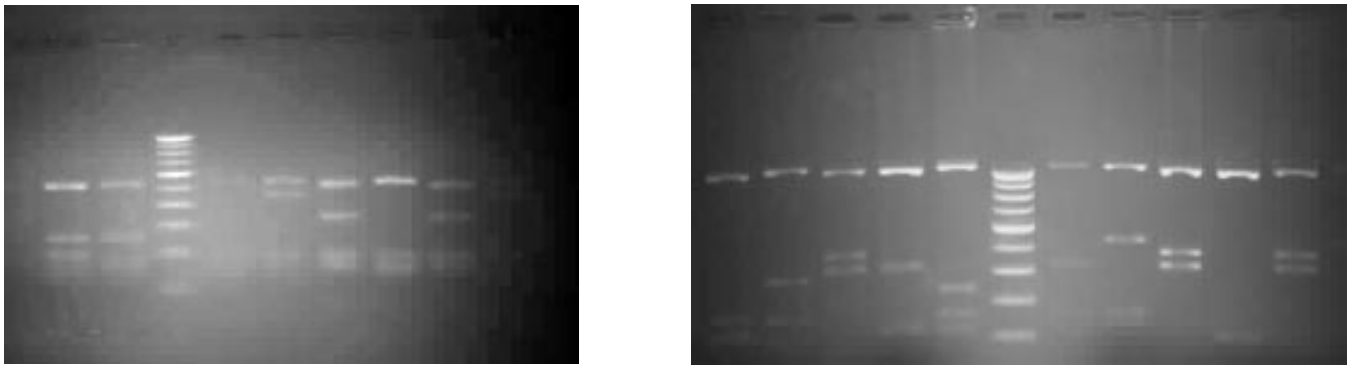

Fig. 4: **Gel electrophoretograms showing size variations of MSP-3α by employing PCR/RFLP among *P. vivax* isolates**

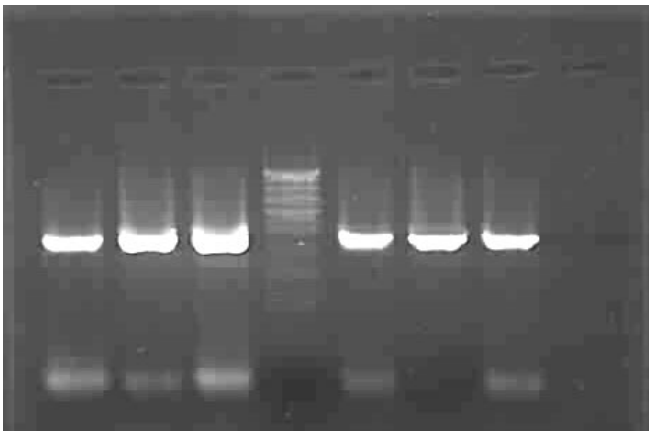

Fig. 5: **Agarose gel electrophoretogram showing 433 bp of GSTP1 allele among *P. vivax* isolates**

2.1.5 Molecular genotyping of clinical resistance in *Plasmodium falciparum*

Samples collected from *P. falciparum* patients on Day 0 and the day of recrudescence during therapeutic efficacy studies of various antimalarials conducted at different parts of the country were analysed for molecular genotyping using highly polymorphic surface protein markers as well as drug resistance related markers. Results revealed high proportion of recrudescent infections with the same genotype that of Day 0 among isolates of Gujarat, Tamil Nadu, Orissa and Assam in case of CQ efficacy studies thus suggesting for resistance. Analysis of mutations in *Pfcrt* and *Pfmdr1* genes revealed prevalence of threonine (T) at codon76 of *Pfcrt* and asparagines (N) at codon86 of *Pfmdr* gene among the isolates of different areas. The study further revealed SVMNT as the predominant haplotype, however, in areas of high malaria endemicity, haplotype diversity observed was much higher (Fig. 6). High prevalence of mutant alleles and haplotypes among the isolates of different

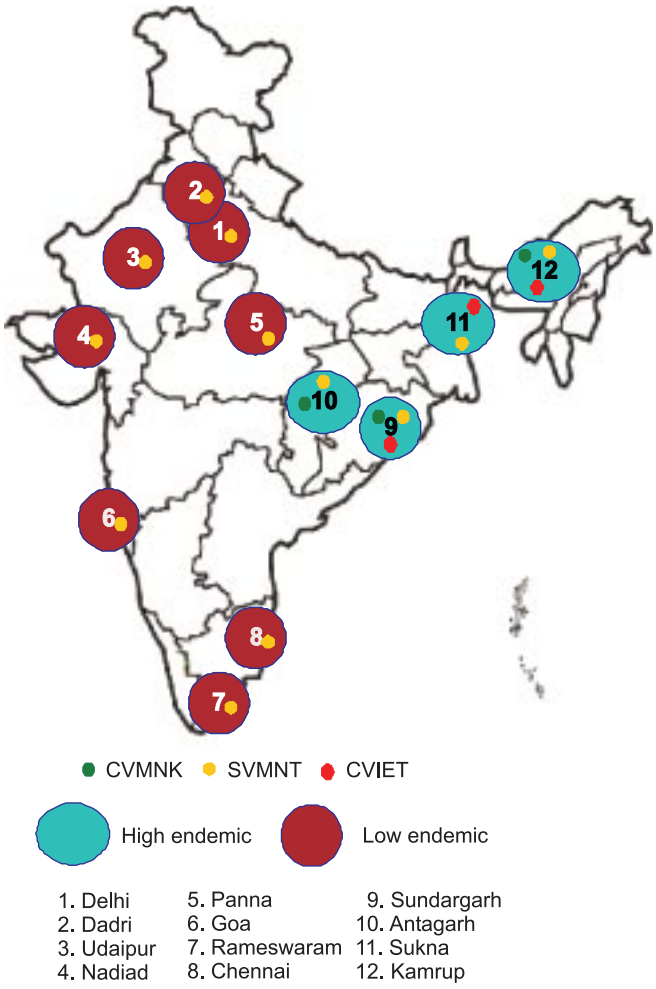

Fig. 6: **Distribution pattern of *Pfcrt* haplotypes in India**

regions definitely reflect presence of CQ resistant strain in the country.

2.1.6 MSP-1 and MSP-2 in genotypes of *Plasmodium falciparum*

The data of *P. falciparum* positive blood samples collected from different villages of Sundargarh, Orissa have been analysed using polymorphic markers and vaccine candidate antigens such as the merozoite surface proteins MSP-1 and MSP-2 after extracting DNA from the samples and performing PCR. Multiplicity of infection and clonality (samples having single and multiple clones) at three different transmission levels—low, moderate and high with respect to different age groups and two ecosystems were calculated (Figs. 7 and 8).

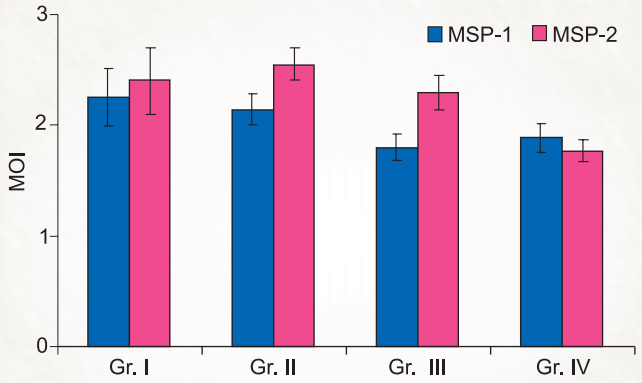

Fig. 7: **MSP-1 and MSP-2 polymorphism in different age groups**

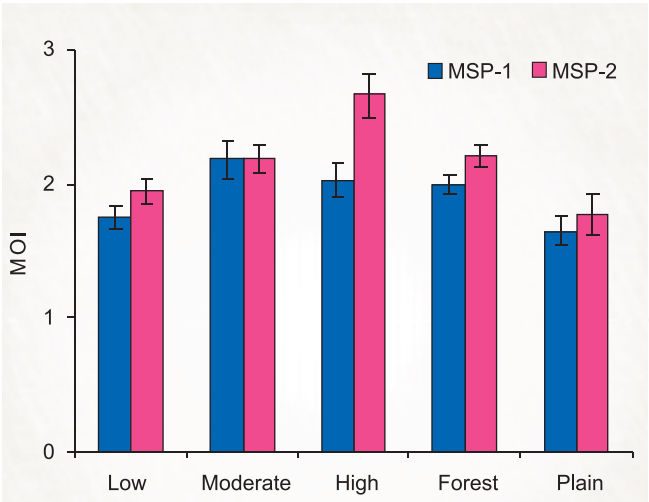

Fig. 8: **MSP-1 and MSP-2 polymorphism in forest and plain areas**

2.1.7 Genetic diversity studies of *Plasmodium falciparum*: SNP analysis in putatively neutral loci

In order to infer haplotype diversity with SNP, it is essential to use field isolates having infection with single parasite type. Therefore, about 400 field isolates collected from nine different study sites were analysed with three highly polymorphic surface protein markers to identify clonality in them. A total of nine PCR assays were carried out for each isolate to identify single clone infection isolates. Fig. 9 shows the distribution of single and multiclone isolates in different study sites and these were used for further analysis by selected sets of primers to

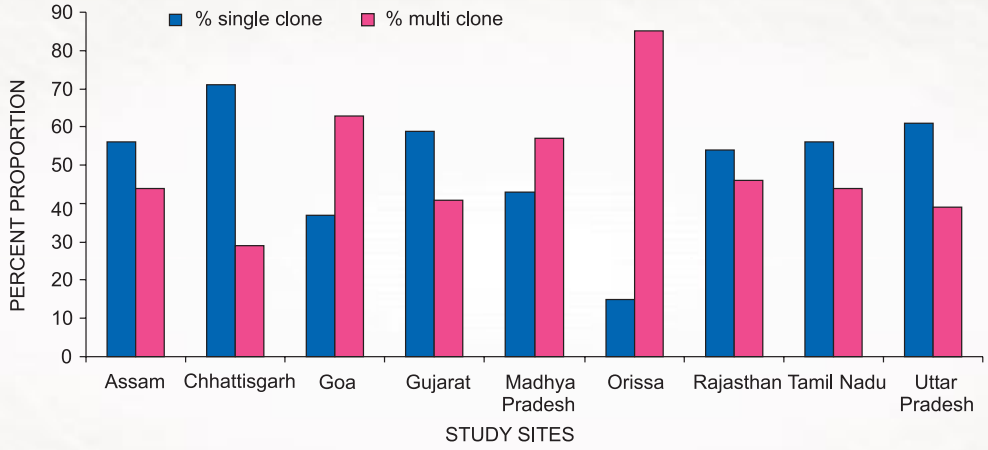

Fig. 9: **Distribution of single and multiclonal isolates in different study sites**

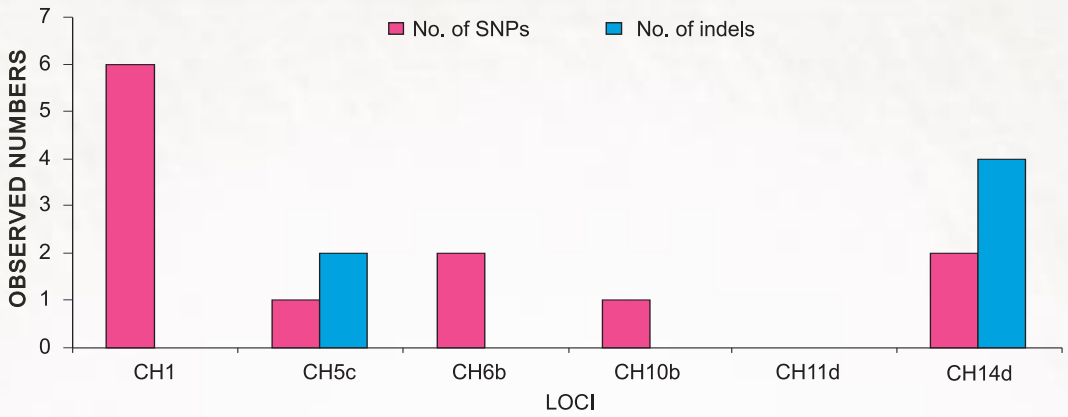

Fig. 10: **Distribution of SNPs and indels (insertion/deletion) observed at neutral loci studied**

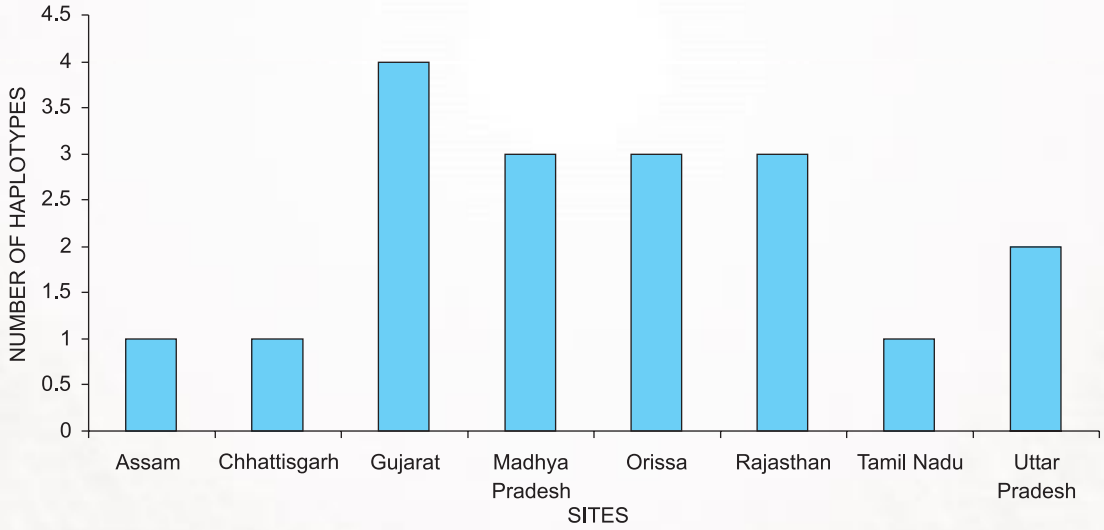

Fig. 11: **Distribution of CH1 haplotypes in different study sites**

amplify intronic regions. PCR amplified products were gel purified and sequenced.

Analysis of sequence data revealed five polymorphic loci (Fig. 10). In addition, a number of indels (insertion/deletions) were also observed and this was in conformity with published data of *P. falciparum.* Analysis of data using population genetic softwares, DnaSP and Arlequine revealed that CH1 locus had six different haplotypes and six polymorphic sites. Isolates of different study sites had shown varied degree of heterozygosity (Fig. 11) but *F* statistics calculated for different populations was non-significant. The phylogenetic tree constructed, based on sequence data, 3D7 and respective *P. reichenowi* sequences using MEGA 3.1 with 1000 boot strap replicates has not shown much divergence between the populations. Analysis of other loci is in progress.

2.1.8 Relatively simple genotype of *Plasmodium falciparum* isolates from northeastern states as determined by Anchored Primer Amplification of DNA (APAD)

Twenty isolates have been studied for genetic

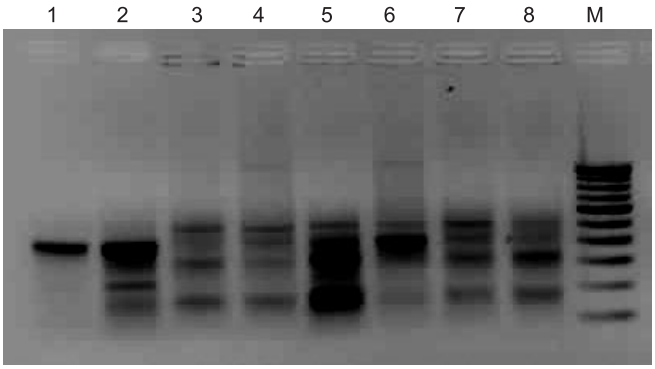

Fig. 12: **Anchored primer amplification of gDNA of *P. falciparum* isolates with primer $(A)_{14}$CATGCC M: Marker, 100 bp DNA ladder; Lane 1:(CQ - 11); 2:(CQ-16); 3:(CQ-19); 4:(CQ-24); 5:(C-4); 6: (C-9); 7:(C-25); 8:(C-26)**

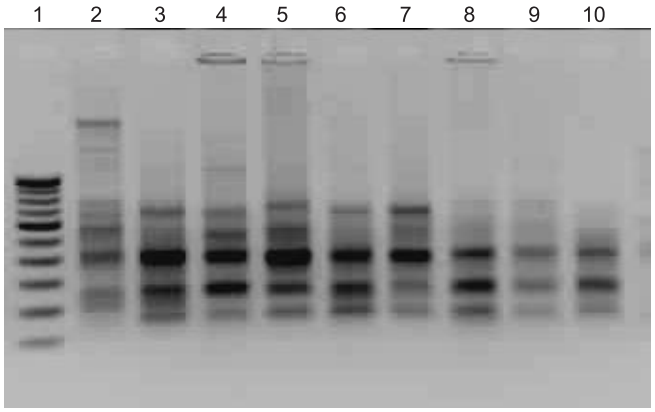

Fig. 13: **Anchored primer amplification O of gDNA of *P. falciparum* isolates with primer $(A)_{14}$GGTTCC M: Marker, 100 bp DNA ladder; Lane 1:(CQ-11); 2: (CQ-16); 3:(CQ-19); 4:(CQ-24); 5:(C-4); 6:(C-9); 7:(C-25); 8:(C-26); 9: (C-29); 10:(C-32)**

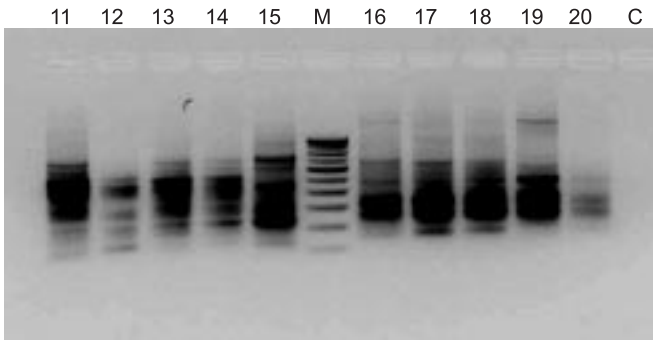

Fig. 14: **Anchored primer amplification of gDNA of *P. falciparum* isolates with primers $(A)_{15}$GTGTA M: Marker,100 bp DNA ladder; Lane 11:(ACT-3); 12:(ACT-4); 13:(ACT-5); 14:(ACT-6); 15:(ACT-7); 16:(ACT-8); 17:(ACT-11); 18:(ACT-12); 19: (ACT-13); 20:(ACT-14); C : Control**

diversity by APAD. Variable multiple bands were obtained, depending on the type and number of nucleotides attached to the 3' end of the oligo-nucleotide primer (Figs.12–14). The appearance of the polymorphic bands also depend upon which anchored primers and DNA templates were used. Thus, by using various combinations of anchored nucleotides, numerous and unique patterns of bands were obtained. By using this method, isolates from the same region could be categorised into groups using the same primer and different primers as well. These results could be used for detecting variations between closely related parasites. Some primers gave multiple and discrete bands with all the isolates and showed difference in the patterns of bands with the same primer (Figs. 12–14). Difference in the patterns of band with the same primer in different isolates revealed the presence of diversity among the isolates. This method can be very useful for identifying polymorphism that can distinguish closely related parasite isolates. This method is very useful for rapid parasite typing because large number of loci in the genome can be checked quickly. The method also provides a potentially powerful tool for developing hundreds of genetic markers from different parasite species.

As proposed in the project, data on all above parameters would be generated in the second study site that has already been selected in Meghalaya state, bordering Bangladesh. Epidemiological data of the selected site, CHC Dalu (Indo-Bangla border) of West Garo Hill district of Meghalaya has shown high *P. falciparum* prevalence with API of 22.4 in 2006 and deaths were also reported.

2.1.9 Molecular analysis of aspartic protease gene in *Plasmodium vivax*

Parasite proteases are essential for parasite survival. Proteases help parasite in host cell invasion, nutrition, growth and the processing of precursor proteins. Proteases are believed to be the promising targets for antimalarial chemotherapy as plasmepsins have been characterised in *P. falciparum*. Aspartic proteases have now been characterised in *P. vivax* (Sharma *et al. J Biochem* 2005; *138:* 71–8). As an extension of our previous work, we have characterised aspartic protease

gene in *P. vivax.* Nucleotide sequences encoding the plasmepsins, PvPM4 and PvPM5 of *P. vivax* were retrieved from the Blast searches of available malaria genome sequences database (The Institute for Genomic Research through the website at http://www.tigr.org) using the nucleotide sequences of *P. falciparum* plasmepsins as query sequences. PCR was performed with ~50 ng of *P. vivax* genomic DNA by using specific oligo-nucleotide primers for each gene.

PvPM4
(5'ATGGATATAGCAGTGAAAGAACAAGACTACTCAAA-3' and 5'TTAATTCTTTGCGATGGCAAAACCGACACTCTC-3')

PvPM5
(5'-ATGGTCGGAGCGAGCTTGGGGCCCCCCGGT-3' and 5'-CTACGCATCCGCGGGCGCCTTGCCCTCGGAGG-3')

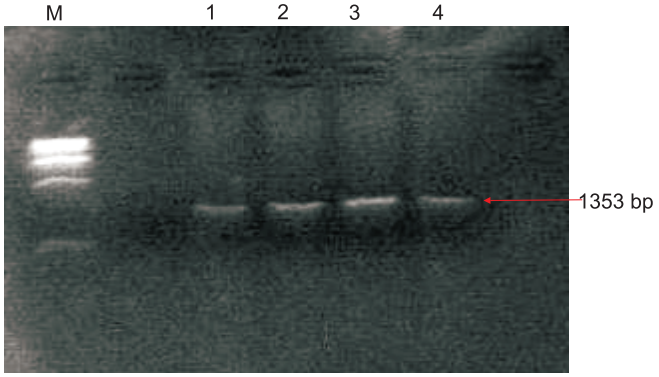

Fig. 15: **PCR amplification of aspartic protease gene using primer PvPM4 in *P. vivax***

P. vivax isolates from Chennai were amplified by PCR using specific primers for PvPM4 and PvPM5 (Data not shown). Each PCR product appeared at the expected sizes of 1353 (Fig. 15) and 1611 bp. Characterisation at the biochemical and molecular level will be helpful for designing new drugs against *P. vivax* and *P. falciparum.*

2.2 Genome Informatics of *Plasmodium falciparum* and *P. vivax*

2.2.1 Bioinformatic study of *Plasmodium falciparum* genome

Distribution and relative position of introns in each gene in all the 14 chromosomes of *P. falciparum* have been studied. The study revealed that introns

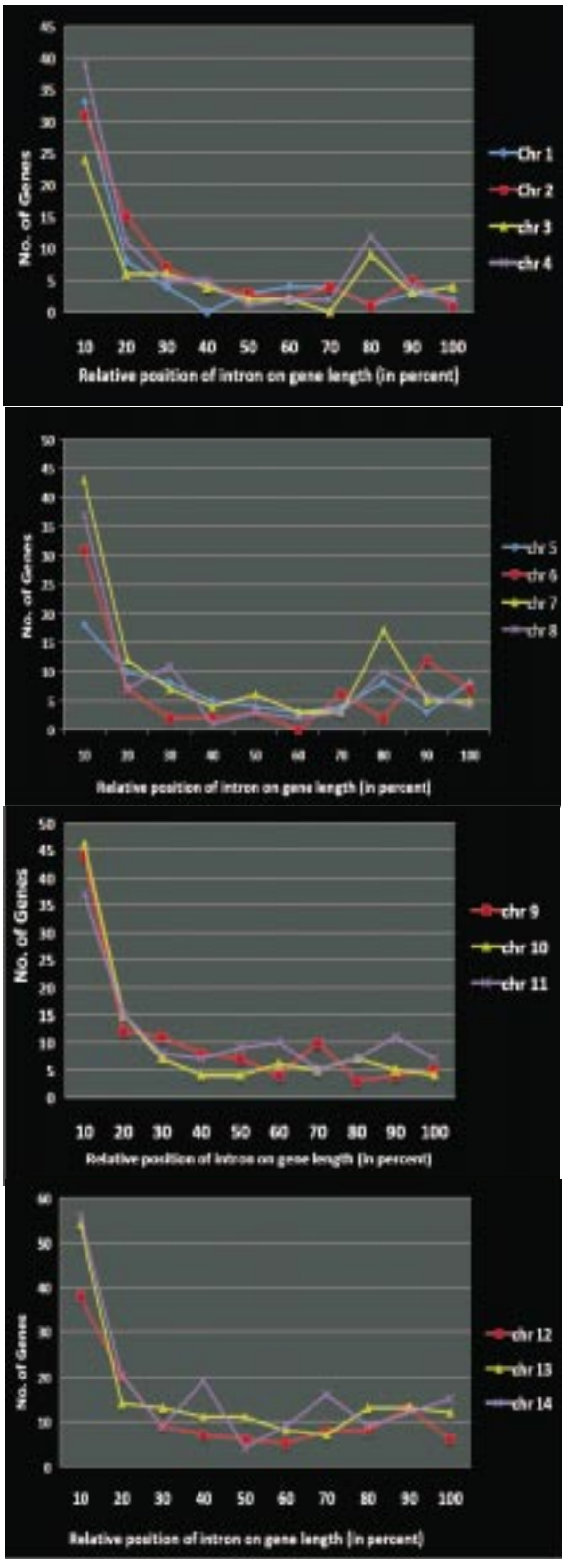

Fig. 16: **Distribution of introns on gene length in *P. falciparum* genes, having one intron per gene**

with length of 100–200 bp were very common in *P. falciparum* genome. Chromosome 13 has maximum number of introns with <400 bp and introns with > 400 bp were mostly present on the chromosomes 10 and 14. Introns in the *P. falciparum* genes were biased towards the 5' end of the genes. Majority of the first introns lies within the first 10% of the total gene length. The results were consistent in all the 14 chromosomes of the *P. falciparum* (Fig. 16). An intron database providing exclusive information about the *P. falciparum* introns with user friendly search and output options was developed. This data base can be assessed from http://www.plasmonimr.org/index_files page1473. htm.

2.2.2 Evolutionary perspective of chloroquine resistant malaria

Drug resistance in *P. falciparum* represents a major health problem in malaria endemic countries like India. *P. falciparum* chloroquine (CQ) resistance transporter (*Pfcrt*) gene has been shown to be primarily responsible in conferring resistance to CQ. In India, CQ resistance in field isolates was first reported from Assam in 1973 and its resurgence has recently been seen in several regions of India. Studies with microsatellite loci in 87 worldwide isolates of *P. falciparum* at the *Pfcrt* gene and flanking DNA sequences have found little variations among CQ resistant isolates but more among CQ sensitive isolates. The observations in India contradict those in global scenario observed for genetic diversity at the *Pfcrt* gene in *P. falciparum*. Indian populations of *P. falciparum* show high genetic variations at the *Pfcrt* gene which do not fit either selective sweep model or the assumptions of origin of drug resistance caused by drug pressure in India. Whereas the global isolates seem to have an almost fixed *Pfcrt* gene following the selective sweep model of evolution by natural selection (Fig. 17a), the *Pfcrt* gene in Indian isolates seems to be in the process of a massive 'genetic reconstruction' (Fig. 17b). Possession of high genetic diversity in Indian isolates is frightening. This would provide a platform where new (beneficial) mutations followed by new associations among different mutations might arise in the population and help a parasite to evade new combination drugs that are now in use in the field. Thus, combining results from recent studies mean that the prospects of testing and using new antimalarial drugs and/or vaccine in India are discouraging. Thus knowledge of the detailed population genetic structure of the parasite in India

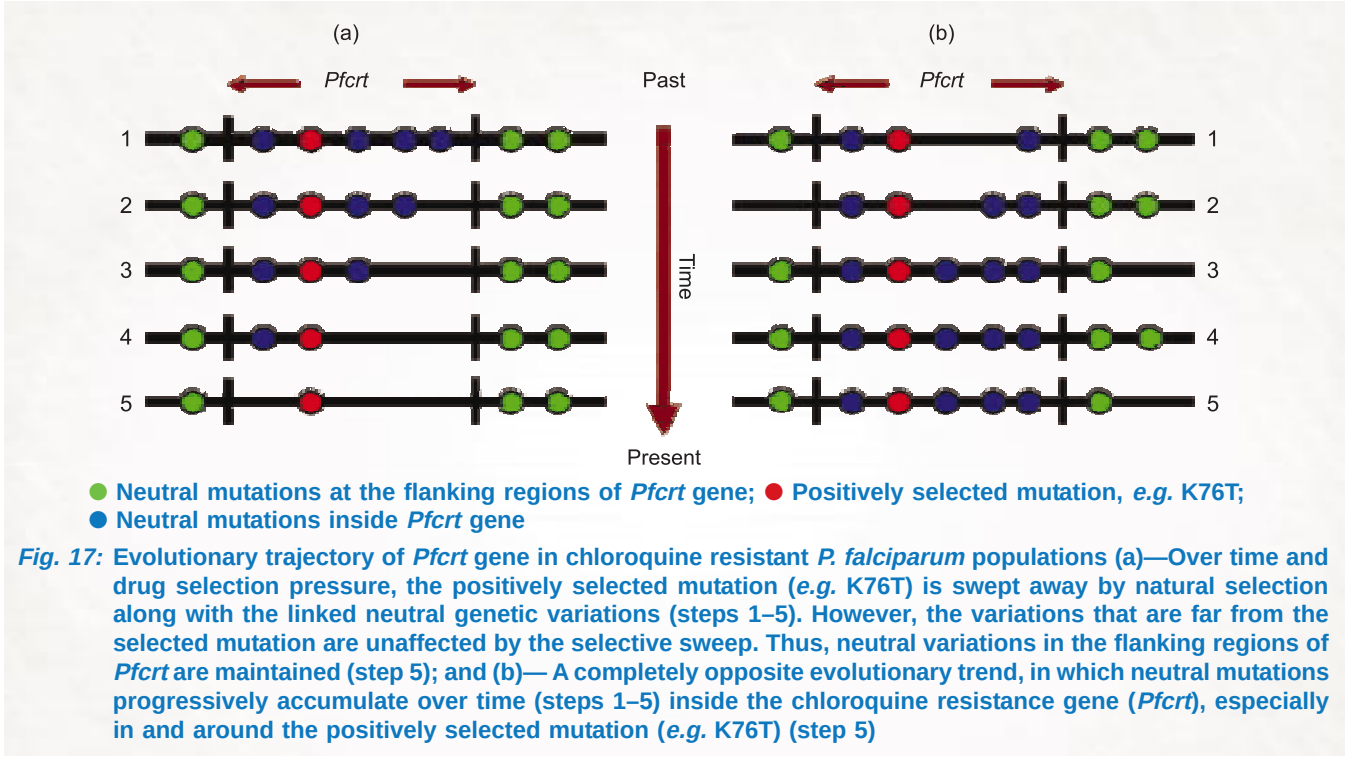

Fig. 17: **Evolutionary trajectory of *Pfcrt* gene in chloroquine resistant *P. falciparum* populations (a)—Over time and drug selection pressure, the positively selected mutation (*e.g.* K76T) is swept away by natural selection along with the linked neutral genetic variations (steps 1–5). However, the variations that are far from the selected mutation are unaffected by the selective sweep. Thus, neutral variations in the flanking regions of *Pfcrt* are maintained (step 5); and (b)— A completely opposite evolutionary trend, in which neutral mutations progressively accumulate over time (steps 1–5) inside the chloroquine resistance gene (*Pfcrt*), especially in and around the positively selected mutation (*e.g.* K76T) (step 5)**

is needed before the field trial of a new drug or vaccine is initiated.

2.2.3 *In silico* genetic characterisation of *Plasmodium falciparum* chromosome 7

The fact that malaria still an uncontrolled disease, is reflected by the genetic organisation of the parasite genome. Efforts to curb malaria should begin with proper understanding of the mechanism by which the parasites evade human immune system and evolve resistance to different antimalarial drugs. We have initiated such a study and present herewith the results from the *in silico* understanding of a seventh chromosomal region of the malaria parasite, *P. falciparum* encompassing

Fig. 18: The ~270 kilobase specified region of *Plasmodium falciparum* chromosome 7

the antigenic *var* genes (coding *Pfemp1*) and the drug-resistant gene *Pfcrt* located at a specified region of the chromosome 7 (Fig. 18). We found 60 genes of various functions and lengths (Fig. 19), majority (61.67%) are performing unknown

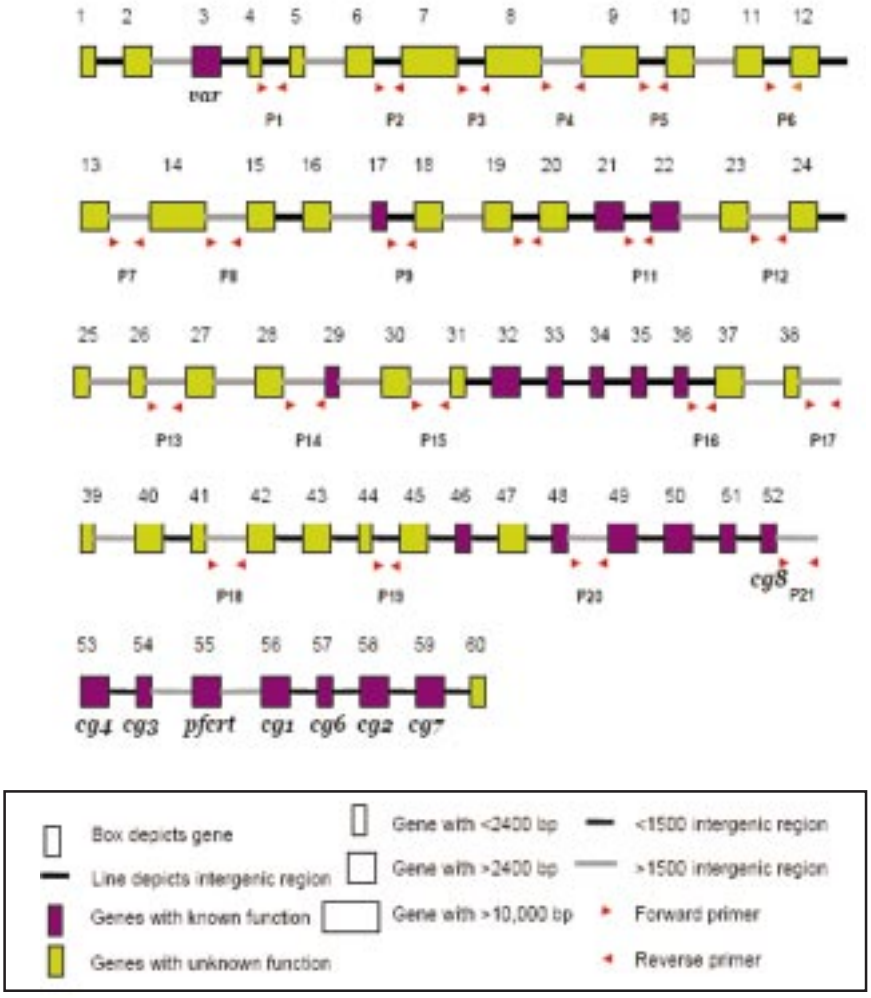

Fig. 19: Fine details of the specified region showing the genes, intergenic regions and locations of the primers designed for amplification of different regions

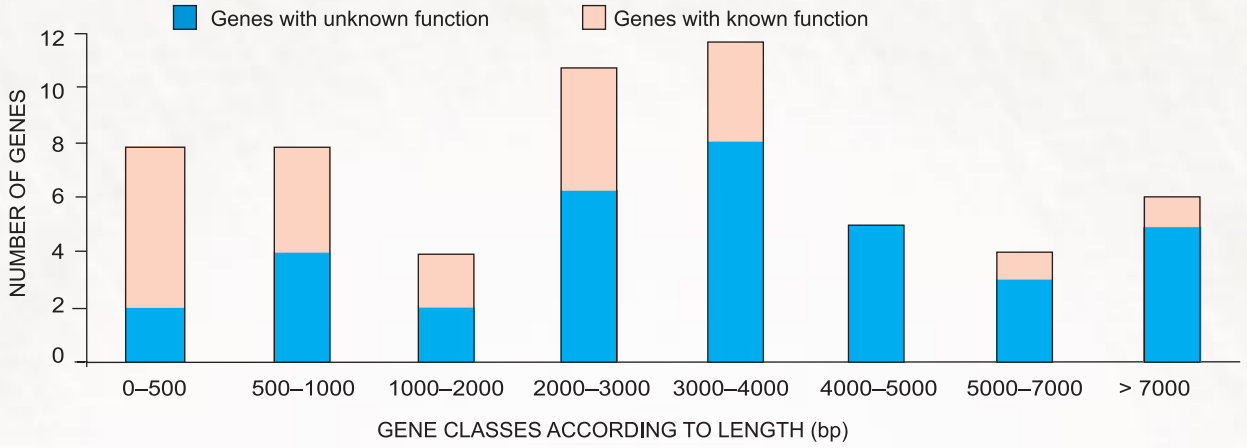

Fig. 20: **Distribution of genes with known and unknown functions in the specified region**

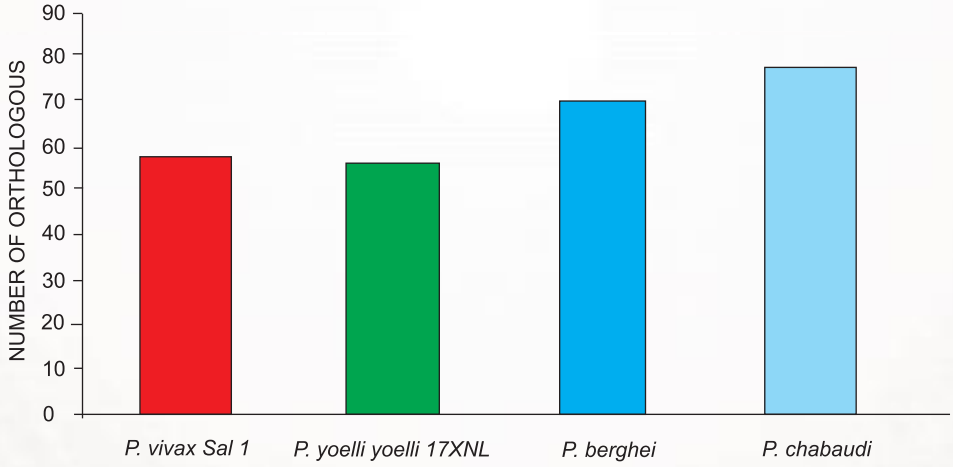

Fig. 21: **Number of genes in the specified region that are orthologous to other species of the genus *Plasmodium***

functions (Fig. 20). Almost all genes have orthologs in other four species of *Plasmodium*, of which *P. chabaudi* seems to be the closest to *P. falciparum* (Fig. 21). However, only two genes were found to have paralogs. Interestingly, the drug-resistant gene, *Pfcrt* was found to be surrounded by seven genes coding for several CG proteins out of which six were reported to be responsible for providing drug resistance to *P. vivax*. The intergenic regions, in this specified region were generally bigger in size, majority (73%) of them were found to be of > 500 nucleotide base pair length. We also designed primers for amplification of 21 non-coding DNA fragments in the whole region for estimating genetic diversity and inferring the evolutionary history of this region of *P. falciparum* genome.

The study, for the first time, provides fine-scale genetic insights into a chromosomal region of the malaria parasite genome of high importance. The study, in addition to characterisation of different genes, their homology pattern, intron-exon compositions and pattern of intergenic regions, also reveal a very interesting phenomenon that the gene conferring chloroquine-resistance to the malaria parasite, *P. falciparum* is flanked by seven CG-protein coding genes otherwise known to be responsible for drug resistance in *P. vivax*. The study would help

furthering malaria research by understanding the pattern of genetic diversity in this specified region and help inferring evolutionary mysteries of both drug resistance and high antigenic variations.

2.2.4 Localisation and characterisation of the antigenic *var* genes in *Plasmodium falciparum* genome

Recent research has shown that around 10% of the 3-Mb *P. falciparum* genome is committed to the expression and generation of diversity of the virulence genes and these genes are randomly distributed throughout the genome. Three families of variant virulence genes have been characterised in *P. falciparum*; the *var* gene encoding *P. falciparum* erythrocyte membrane protein 1 (*Pfemp1*); the repetitive interspersed family (*rif*) of gene; and the sub-telomeric variant open reading frame (*stevor*) gene. The *var* and *rif* gene families are considered to be responsible for the key virulence factors and the functions of *stevor* gene are not yet well understood. Parasite protein *Pfemp1* adherences to certain disease receptors are more commonly associated with certain disease outcomes, such as cerebral and placental malaria. *P. falciparum* infections are persistent, and this chronicity is promoted by antigenic variation at the infected red blood cell surface. Further, expression of *var* genes from a chromosomal domain known for frequent rearrangements has important implication for the mechanism of *var* gene switching and the generation of novel antigenic and adhesive phenotypes.

To understand mechanisms by which the parasite is able to do so, it is important, at the first hand, to localise the position of each of the *var* genes in the genome of *P. falciparum*. Also, by mapping chromosomal locations of *var* genes expressed in *P. falciparum* isolate 3D7, we can identify the *var* gene map within sub-telomeric and centrally placed locations. In the present study, we utilised the *P. falciparum* genome web database to localise each of the *var* genes in all the 14 chromosomes of *P. falciparum* genome. The schematic representation of the results is shown in (Figs. 22 a & b). As shown in the figure, the studies revealed the presence of 104 *var* genes in all the 14 chromosomes. Majority of *var* genes were located at the sub-telomeric regions of the chromosome, the region, generally with high recombination rate. Another small set of *var* genes was found to be arranged in chromosome internal clusters in some of the chromosomes (Figs. 22 a & b). Phylogenetic analysis revealed the ancestry and high evolutionary resemblance among the various *var* genes within the chromosome and also across chromosomes, in the whole genome (Fig. 23).

2.2.5 Genomic characterisation of *vir* multigene family in *Plasmodium vivax*

The estimated global burden due to *P. vivax* is approximately 70 to 80 million cases annually with about 80–90% cases in the middleeast, Asia and western pacific, 10–15% in central and south America, and 10–20% in sub-Saharan Africa. It is now known that the virulence, pathogenicity and successful invasion of the parasites into the host have genetic basis, thus, characterisation of specific genes in the genome should be first step to understand the whole mechanisms. To this respect, the *vir* multigene family is an important virulence determining gene family in *P. vivax.* One of the most striking features of this gene family is the antigenic property and enormous amount of genetic variation that helps the parasite in successfully evading the host immunity and thus, plays a major role in malaria pathogenicity. The almost completed *P. vivax* whole genome sequence available at public domain, provides opportunities to characterise different genes and family of genes. The present study was carried out to characterise the entire *vir* gene family in detail with a genome outlook and establish evolutionary relationships among different gene families. There are altogether 32 genes reported across the entire gene family. Using the PlasmoDB (www.plasmodb.org) web database, data on five (A, B, C, D and E) *vir* gene subfamilies were retrieved. Each of the subfamilies except the subfamily B has about 400–570 different gene sequences. These sequences are nothing but the multiple copies of all 32 genes. Subfamily B has 192 sequences in the database. We divided each subfamily into 32 groups according to the gene similarities and aligned all the genes within each group. We found several conserved domains within

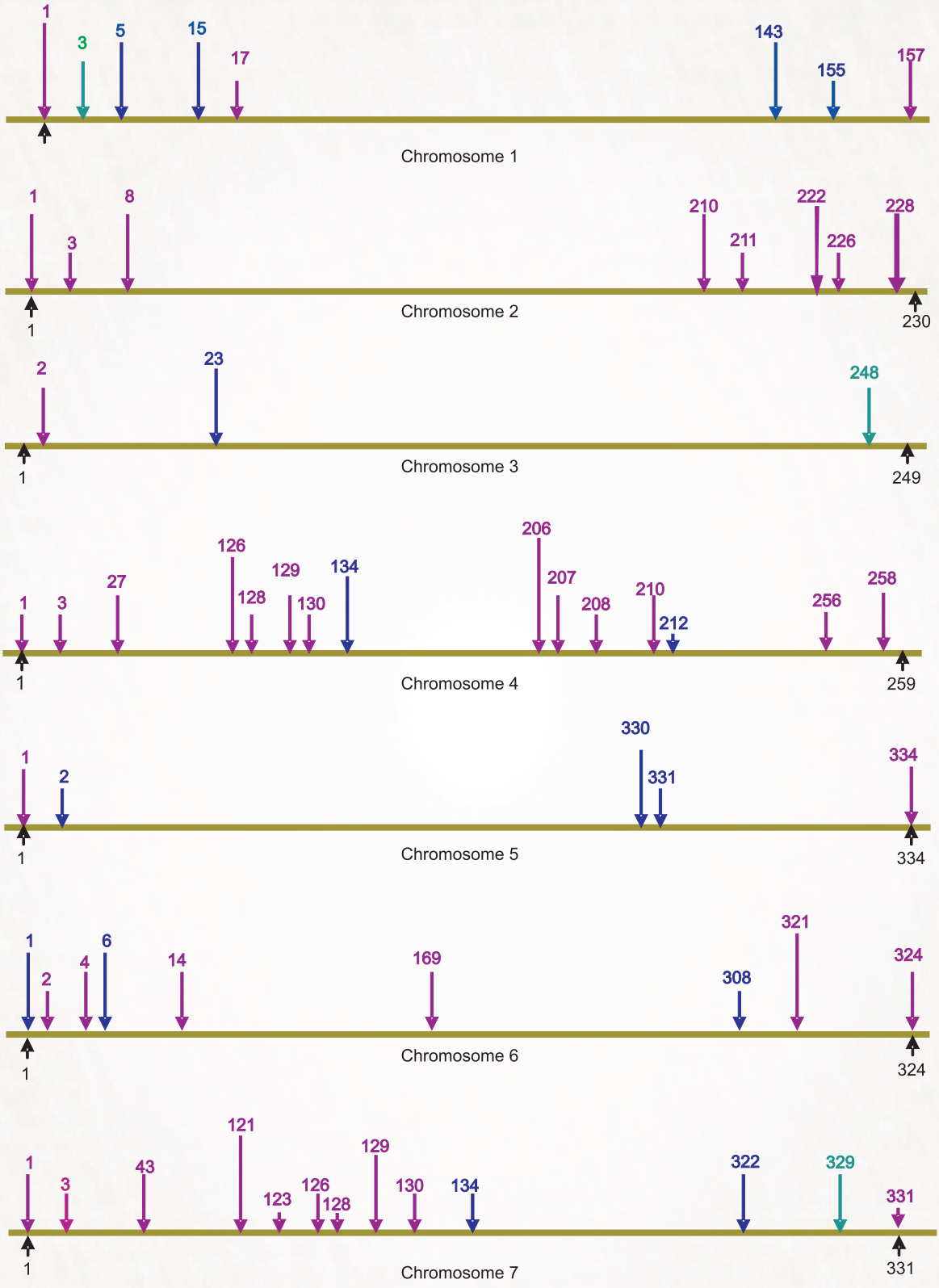

Fig. 22a: **Schematic map of *P. falciparum* chromosomes showing the location of *var* genes (chromosomes 1–7)**

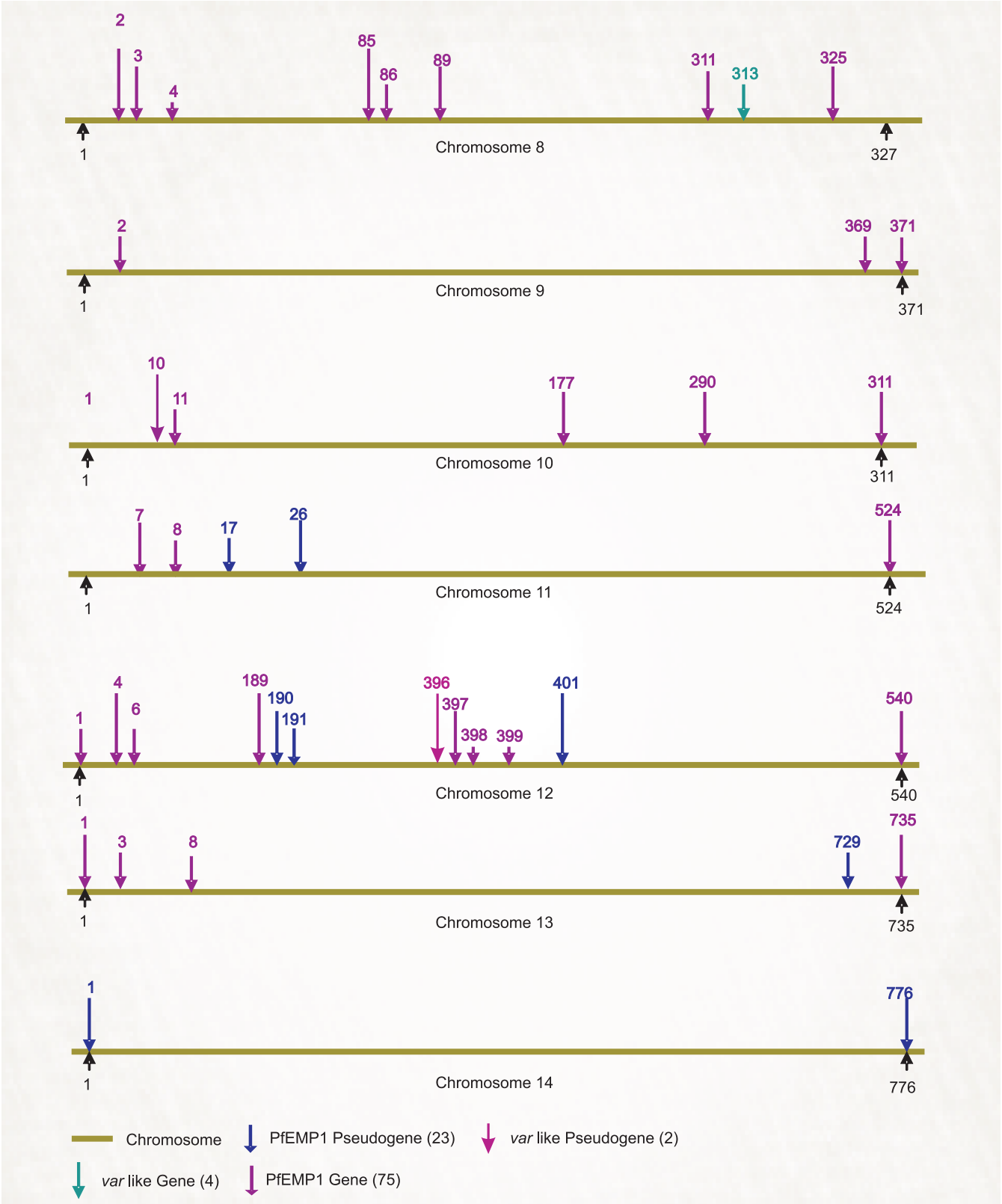

Fig. 22b: **Schematic map of *P. falciparum* chromosomes showing the location of *var* genes (chromosomes 8–14)**

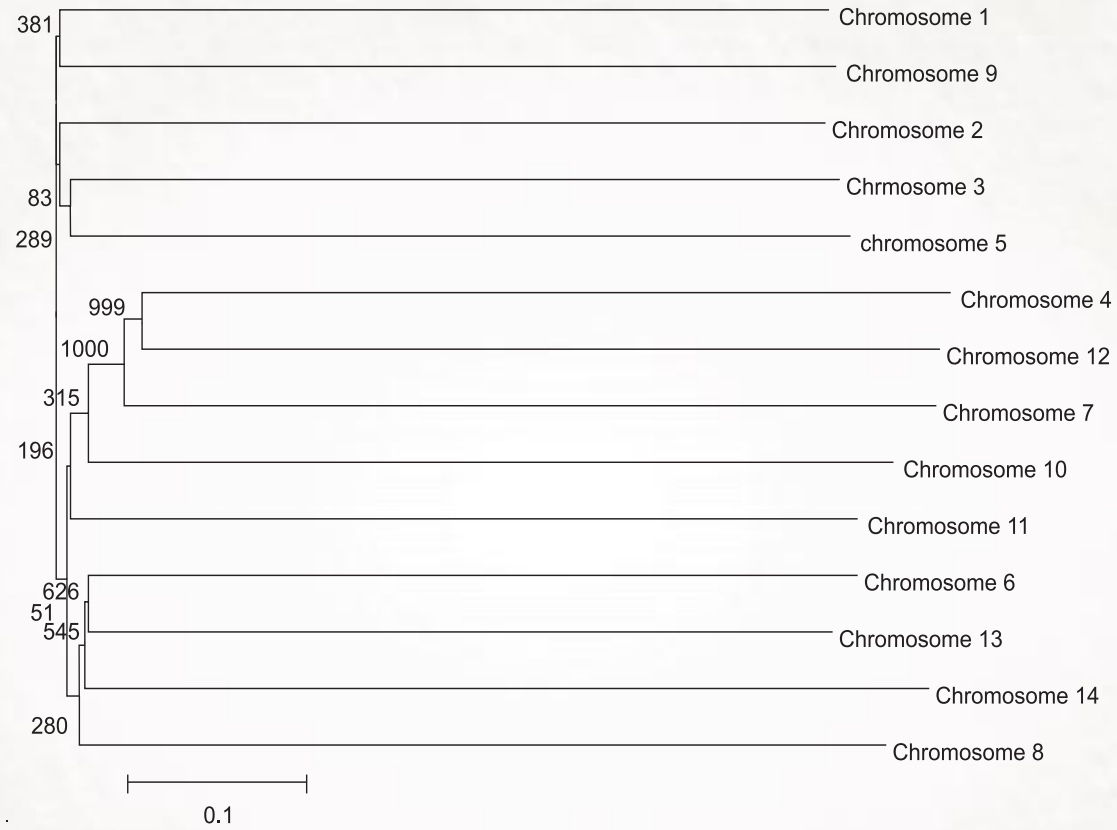

Fig. 23: **Phylogenetic relationships among the *var* genes across the *P. falciparum* genome. Consensus sequences were generated for each chromosome using the computer program "Bioedit". The numbers show the strength of the internal node through generation of bootstrapped trees**

each group, in certain cases, this extends even up to 80%. Fig. 24 shows the contribution of each gene subfamily into each of the 32 divided groups. It was evident from the figure that some of the genes show high number of copies and few show very less number in different groups.

We have conducted phylogenetic studies by constructing neighbour-joining trees among all the five subfamilies of the *vir* gene family and the results are shown in Fig. 25. The analysis showed a close relationship between the subfamily A and B (Fig. 25). Since, genes in the *vir* gene family were responsible for the antigenic properties of *P. vivax* which also help the parasite in virulence and pathogenicity the evolutionary bioinformatics studies presented here would be helpful in furthering research in this area.

2.3 Parasite characterisation and Immunology

2.3.1 Characterisation of the *Plasmodium falciparum* strains prevalent in northeastern states

As per study protocol, malaria prevalence studies were started in Dhubri and Nalbari districts to recruit patients for therapeutic efficacy studies.

District Dhubri

As per discussions with state/district health officials, Chappar and Gazarikandi PHCs were screened for prevalence of *P. falciparum* malaria. During 12–24 July 2006, 411 subjects of Chappar Tea Estate were screened for blood smears and only 8 (1.9%) were positive for malaria. Of these, 6 (75%) were

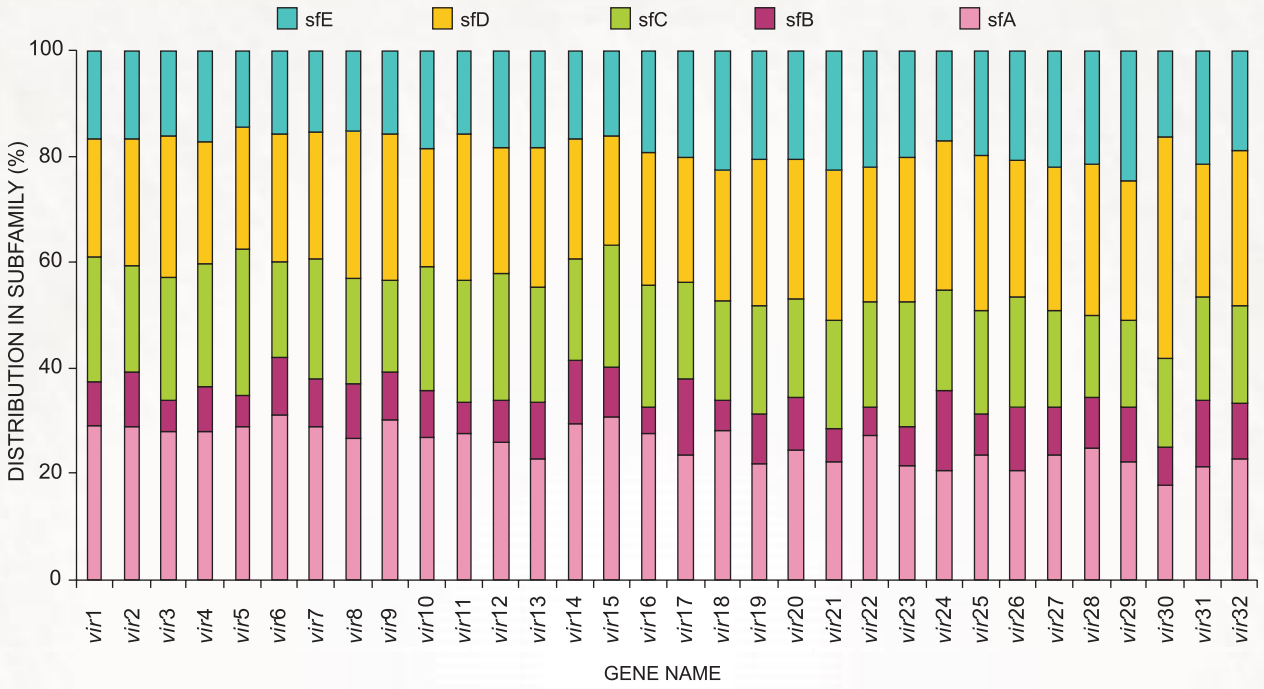

Fig. 24: **Distribution of different *vir* gene subfamilies across 32 different groups**

Fig. 25: **Phylogram showing the relationship among different subfamilies of *vir* gene. The numbers indicate the strength of the internal nodes, established through generation of bootstrapped trees. A high number (1000) separating the subfamily A and B from C, D and E are highly statistically significant**

P. falciparum, and the remaining were *P. vivax* cases. Due to low prevalence of malaria at the given time, the team was shifted to Gazarikandi PHC located along Indo-Bhutan border in consultation with the District Malaria Officer.

Active fever surveys were conducted in villages of Sadullabari, Kakrapara and Mankachar for detection of malaria cases. Of 456 blood-smears examined, 8 (1.7%) were positive for malaria. Most were *P. falciparum* (88%) cases. Among a total of 14 cases that were positive for *P. falciparum*, only one subject met the inclusion criteria. The site has to be abandoned due to low malaria prevalence for the period of study and work started in District Nalbari.

Therapeutic efficacy of drugs used by the State Health Authorities was ascertained for treatment of uncomplicated *P. falciparum* malaria in endemic villages located along Indo-Bhutan border in Tamulpur PHC of Nalbari district of

Fig. 26: **Map of the Northeastern region of India showing Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland and Tripura states and the international borders with neighbouring countries. The location of Tamulpur (Nalbari distrcit), Gazarikandi (Dhubri district), and the proposed site in Tura, Dula CHC (Meghalaya) are indicated**

Assam (Fig. 26). For enrolment of study subjects, in addition to active surveillance in high-risk villages, a clinic was established in Menoka Tea Estate, Kumarikata. During the study period from August–October 2006, all those reporting fever were screened for malaria parasites in their peripheral blood smear (Table 1).

Chloroquine efficacy

Based on inclusion criteria, 33 subjects were selected for treatment with standard regimen of chloroquine (CQ), out of these, 30 completed the 28-day follow-up investigations, 8 (27%), 14 (46%) and 8 (27%) subjects were observed to be ETF, LCF and ACPR respectively.

TABLE 1

The prevalence of malaria among the febrile villagers in Kumarikata, Tamulpur PHC, Nalbari district, Assam*

Study village	No. of blood-smears examined	No. positive for malaria	No. positive for *P. falciparum*
Angarkata	220	95 (43)	47 (49)
Pani Tanki	36	10 (28)	7 (70)
Vinoypur	14	2 (14)	2 (100)
Palasbari	22	7 (32)	1 (14)
Menoka Tea Estate	626	168 (27)	103 (61)
Total	918	282 (31)	160 (57)

*Study period: August–October 2006; Figures in parentheses indicate percentage.

ACT (SP+Artesunate) efficacy

Using this combination therapy, 53 subjects that met the inclusion criteria were included. Of these, 47 (89%) were treatment successes (ACPR) and 6 (11%) were LCF owing to recrudescence on Day 21 (4 cases) and Day 28 (2 cases). All drug failure cases were referred for curative therapy under hospital care. Table 2 shows the baseline characteristics of patients in Tamulpur PHC of Nalbari district. Table 3 presents summary of treatment response among the patients in Tamulpur PHC of District Nalbari.

TABLE 2

Baseline characteristics of patients in Tamulpur, District Nalbari, Assam

Drug: Chloroquine	Dose 25 mg/kg over 3 days
No. of cases	30
Male/Female	18/12
Age (Range)	6–45 yr
Parasitaemia (Range)	1280–63, 520 µl
Drug: AS + SP	AS: Dose 4 mg/kg/day × 3 SP: 25mg/kg single dose
No. of cases	53
Male/Female	30/23
Age (Range)	2–52 yr
Parasitaemia (Range)	1040–99, 280 µl

TABLE 3

Summary of classification of treatment response in Tamulpur, District Nalbari, Assam

	Chloroquine		SP + ACT	
	No.	Prevalence	No.	Prevalence
ETF	9	0.31	0	0
LCF	12	0.414	6	0.113
LPF	0	0	0	0
ACPR	8	0.276	47	0.887
Total analysis	29	–	53	–
Withdrawals	0	–	0	–
Lost to follow-up	1	0.033	0	0
Total	30	–	53	–

Genotyping studies

Genotyping of recrudescence infection observed in CQ and ACT drug therapy was carried out using three surface protein markers, MSP-1, MSP-2 and GLURP. Nested PCR assays were carried out. Results revealed same genotype in three out of five paired samples (zero day and on the day of recrudescence) tested for LTF of chloroquine efficacy, suggesting of recrudescence. Similarly, in ACT efficacy trial, four LTF cases tested showed different genotypes thus suggesting for new infection.

Drug-resistant markers

Samples of clinical isolates collected from individual *P. falciparum* malaria patients participated in the therapeutic efficacy study were analysed by PCR for mutations in *Pfcrt* and *DHFR* genes. In *Pfcrt* analysis, 13.34% demonstrated wild type K76, 33.33% showed mutant type T76, while 53.33% had mixture of both K76 and T76.

Point mutations in *DHFR* codons 16, 51, 59, 108 and 164 have also been analysed. In the study group, 80% demonstrated wild type A16, 3.33% showed mutant type V16 and 16.67% had mixture of both A16 and V16. At codon 51, 70% demonstrated wild type N51, 6.67% showed mutant type I51 and 23.33% showed mixture of both N51 and I51. Codon 59 demonstrated 46.67% wild type C59, 30% showed mutant type R59, while 23.33% had mixture of both C59 and R59. Number of wild type variants in codon 108 are 16.67% (S108); mutant type of N108 found in 60% and 23.33% showed mixed type S108N. Mutant type Thr108 has not been detected among these isolates. Codon 164 demonstrated 76.67% wild type I164, 10% showed mutant type L164, while 13.33% showed mixture of both I164 and L164.

Blood samples collected before and at the time of recurrence of asexual parasitaemia were also analysed. Ten patients who did not respond to the drugs showed selection of resistant subpopulation. All of them had mutant type T76 in case of CQ. In DHFR, presence of double (1), triple (5) and quadruple (4) mutant types have been observed as a result of treatment failure by selecting resistant parasite population (Table 4).

TABLE 4

Variants of *Pfcrt* and DHFR present in *P. falciparum* in samples collected before (D_0) and after treatment (R)

Pairs	Code No.	Sample on D_0 / R	*Pfcrt* K76T	DHFR				
				A16*V*	N51*I*	C59*R*	S108*N/T*	I164*L*
1	CQ-2	D_0	K+*T*	A+*V*	N	C+*R*	S+*N*	I
	ACT-5	R-D_{21}	T	*V*	N	*R*	*N*	I
2	C-1	D_0	*T*	A	*I*	R	N	I
	C-1	R-D_{14}	*T*	A	*I*	*R*	*N*	I
3	C-3	D_0	K+*T*	A	N	C+*R*	S+*N*	*L*
	C-3	R-D_7	*T*	A+*V*	N	C+*R*	*N*	I
4	C-9	D_0	K+*T*	A+*V*	N	C+*R*	S+*N*	I+L
	ACT-11	R-D_{21}	*T*	*V*	N	C+*R*	*N*	I+*L*
5	C-27	D_0	K+*T*	*V*	N	C+*R*	S+*N*	I
	ACT-27	R-D_3	*T*	A	N	*R*	*N*	I+*L*
6	C-33	D_0	K+*T*	A+*V*	N	C+*R*	*N*	I+*L*
	ACT-33	R-D_{21}	*T*	A+*V*	N	C+*R*	S+*N*	I+*L*
7	ACT-18	D_0	*T*	A	*I*	*R*	*N*	I+*L*
	ACT-18	R-D_{21}	*T*	A	N+*I*	*R*	*N*	*L*
8	ACT-20	D_0	*T*	*V*	N	*R*	*N*	I
	ACT-20	R-D_{21}	*T*	*V*	N	*R*	*N*	*L*
9	ACT-23	D_0	*T*	A	*I*	*R*	*N*	*L*
	ACT-23	R-D_{23}	*T*	A	N	*R*	*N*	I
10	ACT-28	D_0	*T*	A	N	*R*	*N*	I
	ACT-28	R-D_{21}	*T*	A	*I*	*R*	*N*	I

Mutant genotypes are indicated in italics.

2.3.2 Naturally acquired humoral immune responses to defined *Plasmodium falciparum* erythrocytic stage antigens in a population of eastern India

In malaria endemic areas, acquired protective immunity to *Plasmodium* is achieved in adults as a cumulative effect after repeated exposure. The humoral response to various antigens of parasite is thought to be one of the important mechanisms mediating this immune state. Human sera of various age groups living in malaria endemic regions, naturally exposed to seasonal or perennial transmission, have been used as probes in defining *P. falciparum* antigens that may be involved in the induction of immunity. Several antigens of *P. falciparum* erythrocytic merozoite stage were under investigation for their inclusion in a subunit vaccine; among them merozoite surface proteins 1 and 2 (MSP-1 and MSP-2), the apical membrane antigen-1 (AMA-1), rhoptry associated protein-1 (RAP-1) and erythrocyte binding antigen-175 (EBA-175) showed high immunogenicity eliciting partial or total protection in monkey malaria. By conducting epidemiologic studies, sera of the inhabitants living in malaria endemic areas showed marked immunoglobulin-G reactivities to these antigens.

Individuals of two subpopulations of town and forest areas were studied for their parasitologic and immunologic profiles (Fig. 27). Sera of three different age-matched groups were screened by ELISA to measure antigen-specific IgG for characterising antibody responses to the B-cell epitopic sequences of *P. falciparum* MSP-1, AMA-1, RAP-1, EBA-175

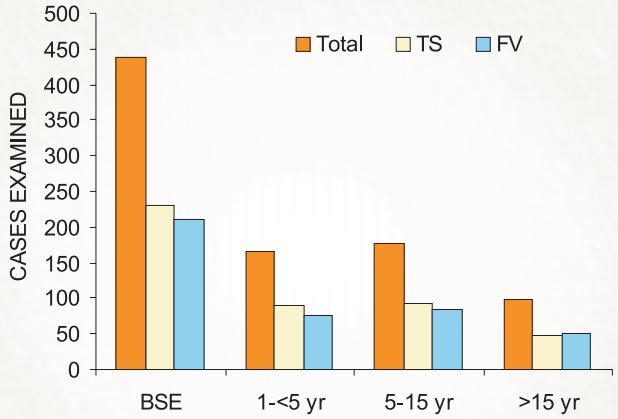

Fig. 27: **Screening of fever cases of different age groups**

and *P. falciparum* infected erythrocyte lysate (PfC) (Fig. 28). In addition, the sera with high antibody titres were also assessed in *P. falciparum* culture in the presence of peripheral blood monocytes by antibody dependent cellular inhibition (ADCI) assay to determine the cytophilicity of circulating antibodies. The results demonstrated that humoral responses to the erythrocytic stage antigens were acquired in an age-dependent manner in these two populations during natural course of infection, and those sera containing antigen-specific antibodies mediated in parasite phagocytosis.

2.3.3 Differential recognition of antigens expressed on the surface of *Plasmodium falciparum*-infected erythrocytes by sera collected from villages of northern India

The natural isolates of *P. falciparum* mainly consist of heterogeneous population of parasites. The antigenic diversity found to be common among various *P. falciparum* strains. The antigens expressed on the surface of *P. falciparum*-infected erythrocytes recognised differentially by the sera of children and adults even from the same location.

P. falciparum-infected blood from a group of young children was collected before giving antimalarial treatment. Blood was collected from the same subjects during convalescent period. Adults of the same area with a past history of repeated malarial infections were also included as immune subjects. Sera from adults of the same area were also tested for antigenic diversity associated with the *P. falciparum* parasites isolated from children. The test parameters included were *in vitro* parasite growth inhibition, erythrocytic stage-specific antibodies, agglutination of parasites with various sera, direct antiglobulin test, *in vitro* rosette formation and identification of proteins on western blot with sera of children and adults.

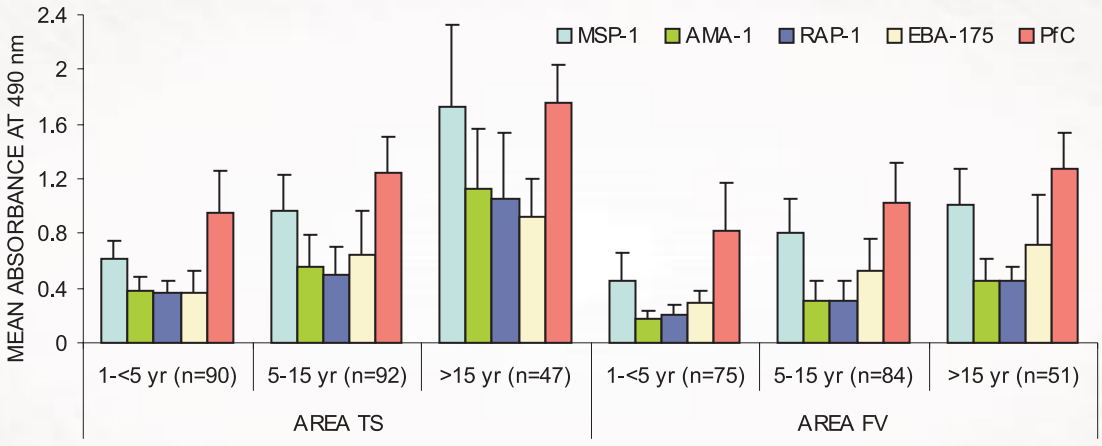

Fig. 28: **Antibody profile of subpopulations of town and forest areas**

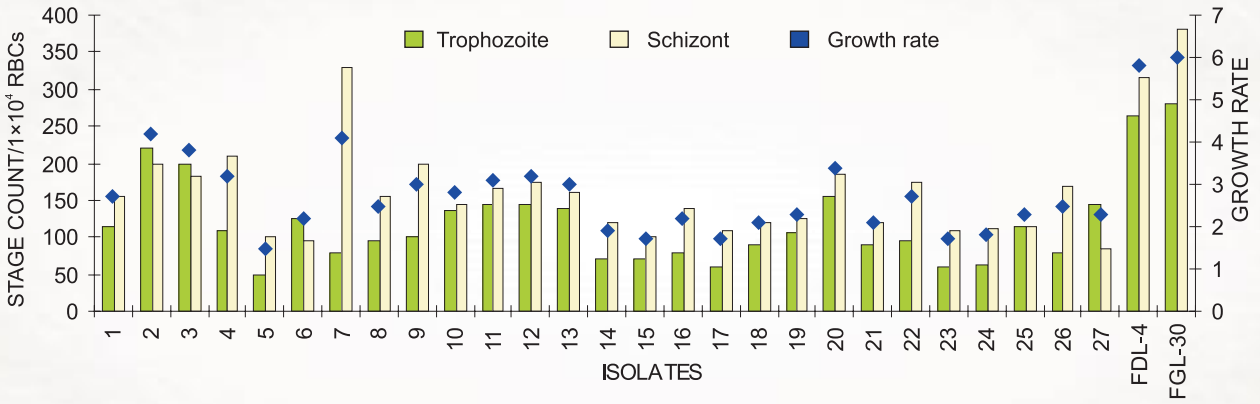

***Fig. 29: P. falciparum* stages and growth rate in culture after 96 hours**

Sera positive for circulating antibodies showed *in vitro* growth inhibitory activity in *P. falciparum* and there was a trend in higher activity with increased age. Rosette formation was observed in some of the isolates and they got disrupted in the presence of autologous sera of children during convalescent stage. Growth inhibitory activity was noticed as a result of agglutination of parasites with autologous convalescent sera. Some of the children developed anaemia at the time of recovery from malaria. The erythrocytes showed agglutination in direct Coombs test. On western blot, sera of these children recognised less number of proteins in comparison to adult immune sera (Fig. 29).

From this study, it was observed that children during acute phase of infection were lacking humoral immunity to combat the infection, but there was a progressive accumulation of antigen-specific antibodies during convalescent period. Variability in response to different isolates and the differential characteristics showed serological diversity in *P. falciparum* within small area experienced with seasonal transmission of malaria.

2.3.4 Immunochromatographic test for determining antimalarial antibody in patients' blood

This study was undertaken to develop an immuno-chromatographic test by dot-immuno binding assay and to compare the results with Plate- ELISA. Blood samples were collected from 92 subjects of age ranging from 5–20 yr, who belong to Nanoo village of Loni PHC, Ghaziabad district after obtaining informed consent. Almost all of them had previous history of malaria. Indirect ELISA was done to estimate antimalarial antibodies in sera samples. Of the 92 sera, 34 had high, 51 had moderate and 7 had low levels of *P. falciparum* erythrocyte stage antigen-specific antibodies. Sera of the children demonstrated low or moderate and sera of adults demonstrated moderate or high antibody profiles. The ELISA results of 92 individuals were compared with that of healthy individuals (negative sera) and malaria immune subjects (positive sera). The results of Dot-blot assay on nitrocellulose membrane were comparable with Plate-ELISA results. Sera containing low titre showed very faint appearance of coloured dots, whereas reactions of moderate and high titre sera showed prominent coloured dots of various intensities when blots were read freshly after completion of reaction.

High level of antibodies determined both by Plate-ELISA and Microdot ELISA indicate past experience of malaria in these individuals. The area where the study subjects reside comes under the influence of seasonal malaria transmission. Since the results of two assays were comparable, they could be equally useful for estimation of antibodies in

patient's serum. The merits of plate or conventional ELISA remain in the measurement of the reactions outcome by an ELISA reader, whereas dot-blot assay results are only readable visually. Therefore, in this case the test outcome is subjective to decide the highest or high or moderate reactions. But antibody positive serum sample always gives positive reaction as a coloured dot, which is easily readable. Moreover, the requirement of antigens, sera, conjugate and other reagents are minimal. The total time requirement for completion of dot-immuno binding assay is shorter than conventional Plate-ELISA. The assay was found to be easy to perform and rapid for producing results.

2.3.5 Source of mosquito blood meal and determination of antimalarial antibodies in ingested blood meal

A total of 576 fed mosquitoes were caught from different areas of Delhi and Ghaziabad. Elution of blood meal was done in 100 µl PBS in 96-well microtitre plates. The eluates were tested by Microdot ELISA to determine the source of blood meal. Of the 576 eluates, 369 were found positive for human blood, whereas 207 blood eluates were cattle positive. Overall human blood meal positivity was 64.1% in these batches of samples. In the study, only 60 mosquitoes (10.4%) were anophelines out of 576, rest were non-vector species. Indirect ELISA tool using *P. falciparum* erythrocytic antigen was applied in testing the eluates positive for human blood meal for antimalarial antibodies. Seropositivity was determined for 325 samples. In some areas almost all the eluates were detected antimalarial antibody negative, but in some pockets samples were found positive for antibody. Overall seropositivity was detected in 78 blood eluates (24%) out of 325.

2.3.6 Allelic variation and immunogenicity of synthetic peptides of T-helper cell epitopic regions of circumsporozoite protein of *Plasmodium falciparum* isolates from India: relevance for vaccine development

T-helper cell epitopes (Th-2R and Th-3R) of circumsporozoite protein of *P. falciparum* show variation which is a serious impediment for development of T-cell epitope-based vaccine against sporozoite. However, if the variants are restricted and could be categorised into groups then the prototype variants from the group could be included into a subunit polyvalent vaccine against sporozoites. We studied altogether 283 isolates of *P. falciparum* from different geographical regions of India. The variants were found to be restricted, could be categorised into groups. The variants were also found to be regionally unbiased in the sense that similar types of variants were found in different geographical regions of India. Studies on the immunogenicity of synthetic peptides of different allelic variants of T-helper cell epitopes found in these studies revealed that combinations of peptides were more immunogenic than individual peptides or peptides of Th-2R and Th-3R from a single group. Therefore, prototype variants from the groups could be used in a subunit polyvalent vaccine against sporozoites.

❑