Characterization of Human Malaria Parasites

Ultrastructural Studies on *Plasmodium vivax*

For the first time a detailed ultrastructural study was carried out on *P. vivax.* Fine structural analysis of growth and differentiation of successive stages involved in erythrocytic and sporogonic phases of development of this parasite was done and compared with those of other malaria parasites. In the erythrocytic phase, asynchrony during merozoite formation within the schizont and caveola-vesicle complexes and cytoplasmic clefts observed in all infected erythrocytes (Fig. 3) were important features of *P. vivax* (Nanda 1990). During sporogonic phase, oocysts on a single midgut exhibited differential rate of development. The invasion of sporozoites into the acinal cells of salivary gland resulted in depletion of rhoptries and changes in pellicular membranes (Nanda *et al* 1985). Morphologically two types of sporozoites were observed in salivary gland cells. Apart from providing better understanding of the parasite morphology, this study may provide basis for other investigations like mechanism of drug action, host-parasite interactions, *etc.*

Genetic Diversity Studies

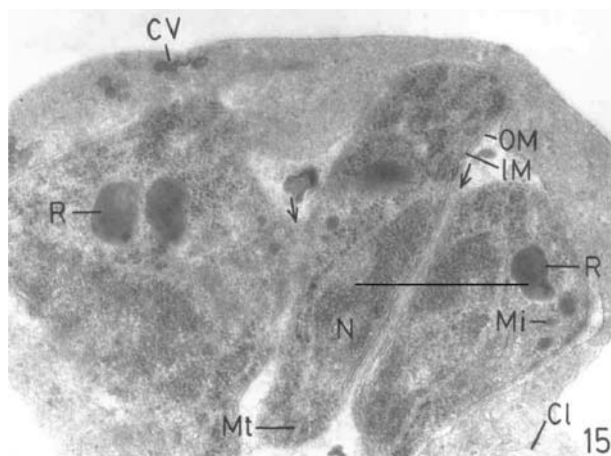Understanding the diversity, extent and distribution of variant forms of human malaria

Fig. 3: **Fully differentiated *P. vivax* merozoites, prior to host cell lysis, showing well-developed pellicular membranes (OM, IM), microtubules (Mt), rhoptries (R), micronemes (Mi), nucleus (N) and surface strands to bridge the adjacent merozoites (arrows). Note the caveola–vesicle complexes (CV) and cytoplasmic clefts (Cl) in the host cell**

parasites (*Plasmodium* species) in different geographic locations as well as the complexity of infections they cause is crucial in developing effective control measures and in malaria surveillance. Therefore, with an objective to understand genetic structure and to estimate the type and the extent of genetic diversity existing among *P. falciparum* and *P. vivax* populations, studies on enzyme and DNA size polymorphism have been carried out in relation to space and time. The sites from where isolates have been characterized are shown in Fig. 4.

Plasmodium vivax

Population Genetic Structure

The polymorphic nature of Indian *P. vivax* isolates was initially established by isoenzyme typing (Glucose phophate isomerase, glutamate dehydro-genase and adenosine deaminase enzyme loci) studies in patient samples from Delhi (Joshi *et al* 1989). Later, longitudinal studies using *P. vivax* isolated from patients in Delhi between 1985 and 1993, together with samples collected from Sundargarh in the hyperendemic Orissa state in 1991, confirmed the extensive diversity of *P. vivax* in India (Joshi *et al* 1997), while simultaneously showing that allelic frequencies did not differ significantly in successive years. Equally important, these studies showed that a similar population structure existed in Delhi and Sundargarh and by extension in different geographical areas in India (because the Delhi samples were presumed to be representative of allelic variants from different regions of the country due to frequent migration). Subsequent studies have used PCR alone or in combination with sequencing and restriction fragment length polymorphisms (RFLPs) to describe the population structure of *P. vivax*. Markers used for the study are surface antigens (MSP-1, MSP-3α, CSP, AMA-1, DBP,), sexual stage antigens (GAM-1, 25S and 28S), 18s SSU RNA-S-type and neutral markers (mini and microsatellites and housekeeping genes).

The *P. vivax* population in India is highly diverse and is highly genetically variable according to studies of isoenzyme markers, antigen genes, DNA based markers (both asexual and sexual stages) as well as neutral markers. The high number of genetically mixed isolates indicate that in India, *P. vivax* is a
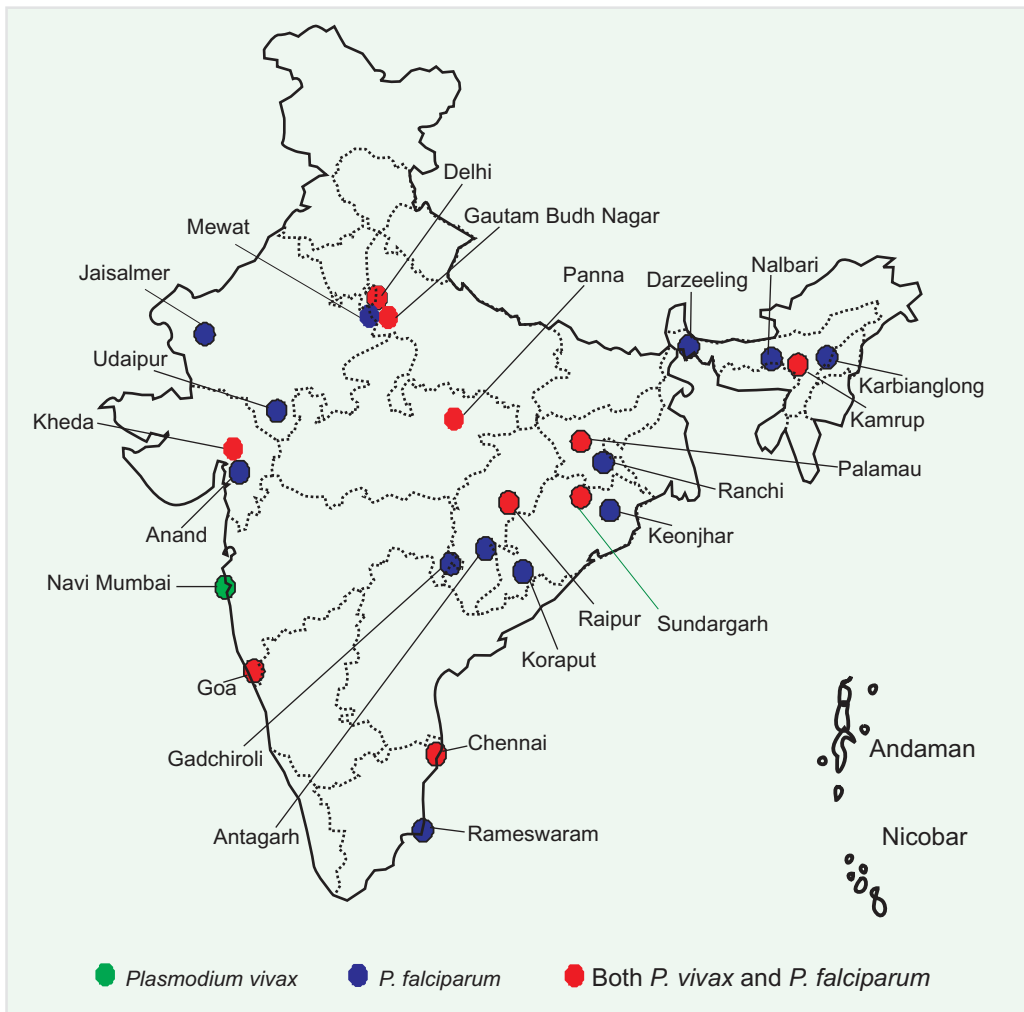
Fig. 4: Map showing study sites from where malaria parasites were collected for genetic diversity studies

randomly mating population, with the high level of genetic diversity likely due to prolonged periods of transmission along with population movement between different regions of the country. Indian isolates have 90 to 100% sequence similarity among themselves and average identity of Indian isolates with the isolates of other regions is in the range of 80 to 99%. Existence of common allelic composition in different parts of the globe and segregation of Indian isolates with the isolates of different regions suggests that Indian isolates have global allelic representation. Little evidence of population sub-structure was observed based on the mean synonymous and non-synonymous sequence diversity. Both synonymous and non-synonymous nucleotide diversity was higher between the populations than within the populations. High degree of allele sharing was observed among five geographical regions (Fig. 5) indicates good level
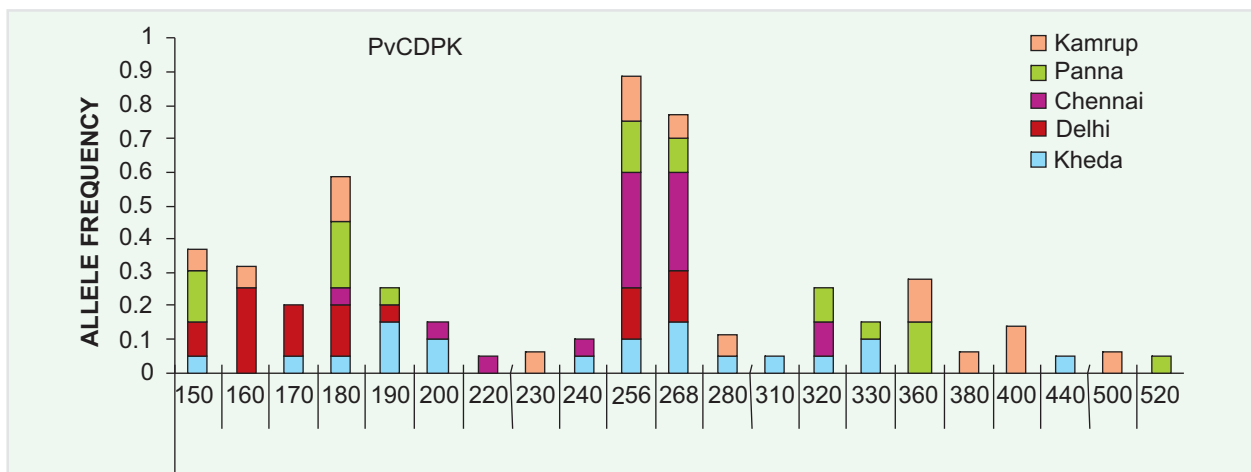

Fig. 5: Extent of genetic diversity and allele sharing at minisatellite locus
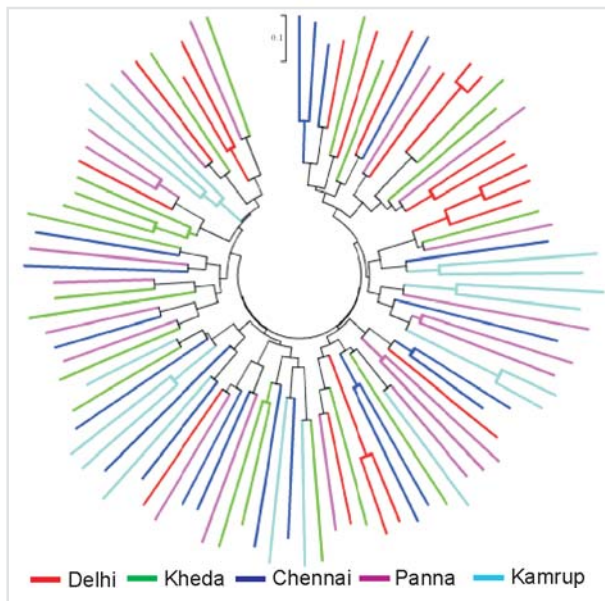
Fig. 6: Neighbor-Joining phylogenetic tree showing genetic relationship among Indian *P. vivax* isolates using ten minisatellites

of gene flow among the different geographical regions of India. Phylogenetic tree reflect that *P. vivax* isolates circulating in different geographical regions clustered together suggesting similar genetic structure among different geographical regions of India (Fig. 6). Observed genetic diversity reflects that Indian *P. vivax* isolates exhibit diversity very close to the Southeast Asian isolates but higher than the South American isolates (Prajapati *et al* 2006; Joshi *et al* 2008).

Evolutionary Parameters and Population History

Asia has been reported as the motherland for *P. vivax* origin (Escalante *et al* 2005, 2006). The expansion of Southeast Asian *P. vivax* population is more likely to led the introduction of this species in India. To understand the history between Southeast Asian and Indian *P. vivax* in real time scale, panels of genome wide neutral markers were explored, namely minisatellites, microsatellites and housekeeping genes. Analysis of microsatellites and minisatellites revealed that Indian isolates displayed extensive genetic diversity and this diversity is very close to the Southeast Asian isolates but is higher than the South American *P. vivax* isolates. Study indicated evidence of long-term population history of *P. vivax* in India suggesting ancient connection between Indian and Southeast Asian *P. vivax*. These findings were further supported by the extensive putatively neutral SNPs observed at ten housekeeping genes. Study indicated very high effective population size suggesting ancient population history of *P. vivax* in India. The coalescence analysis of TMRCA (Time to the Most Recent Common Ancestor) using housekeeping genes neutral SNPs suggest ancient signature of *P. vivax* in India. The TMRCA estimates in our study is overlapped with the earlier

report of TMRCA using mitochondrial genome sequencing from world-wide *P. vivax* isolates suggesting Asia is the centre of *P. vivax* origin (Mu *et al* 2005, Escalante *et al* 2006). The study suggested that observed genetic diversity in respect of genome wide neutral markers reflects ancient population expansion of *P. vivax* in Southeast Asia which led to the introduction of this most prevalent human malaria parasite in India in ancient time scale.

Antigenic Repertoires of Vaccine Candidates

Antigenic diversity in the natural parasite populations is the major obstacle in the development and success of effective antimalarial measures. Antigenic repertoires of human malaria parasites have been widely studied, however, day-by-day new antigenic variants are being reported from different parts of the globe. Local antigenic variations which are hidden in smaller geographical regions are very crucial in the understanding of total antigenic repertoires in a country like India and in turn, planning effective control measures. Antigenic repertoires of *P. vivax* vaccine candidates were investigated in five widely separated geographical regions (Delhi, Panna, Kheda, Chennai and Kamrup) of India to understand the local antigenic repertoires. Sequence analysis of five vaccine candidates from asexual (*Circumsporozoite Protein (CSP), Apical Membrane Antigen-1 (AMA-1)* and *Duffy Binding Protein-II (DBP-II)*) and sexual stages (*Pv25S* and *Pv28S*) revealed differential levels of antigenic repertoires. Asexual stage revealed high antigenic repertoires in comprison to sexual stage. Extensive non-synonymous and synonymous nucleotide substitutions were found and overall number of non-synonymous substitutions exceed over synonymous substitutions suggesting signature of diversifying selection at vaccine candidates. Extensive non-synonymous substitutions found in the vaccine candidates in comprison to the housekeeping genes explained that high antigenic variation among antigenic genes is the adaptive mechanism of parasite to evade the host immune response. Substantial amount of local antigenic repertoires for each candidate in all five regions have been uncovered. The antigenic repertoires of five vaccine candidates for entire regions were very high, however, each region showed a fraction of specific antigenic repertoires. The overall observed SNPs in five vaccine candidates revealed that a good amount of antigenic repertoires are shared among different regions, however, signature of region-specific antigenic-repertoire was also observed (Fig. 7).

Distribution of Two Subtypes

P. vivax has been categorized into two distinct lineages, the New and Old World, distinguishable by gene conversion in the SSU rRNA S-type and mutations in an open reading frame (ORF 470) in the apicoplast genome. These two populations of *P. vivax* also differed in their transmission potential. The
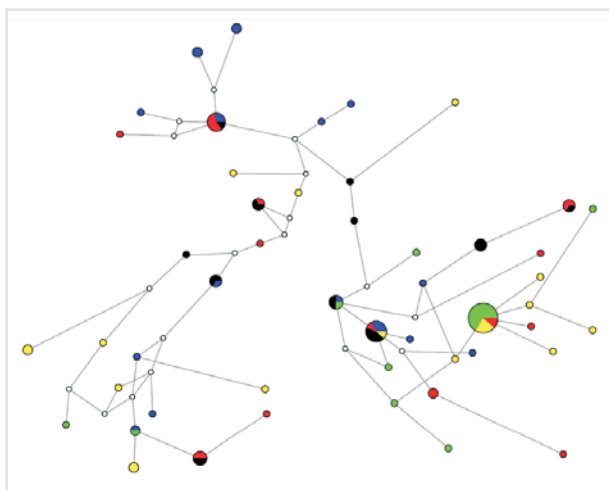
Fig. 7: **Region-specific haplotypes and shared haplotypes of _P. vivax_ as well as phylogenetic relationship between AMA-1 haplotypes. Each circle represents different haplotypes, colour of the circle represents geographical origin of isolates and different colours in a circle represent haplotypes shared between respective regions while colourless circle represents missing haplotypes. Haplotypes are connected with the mutational events, length of connecting line is proportional to the number of mutational events and size of circle is proportional to the frequency of haplotypes**

distribution of two subtypes of _P. vivax_ (Old and New world) based on S type 18s SSU rRNA was studied in field isolates from different locations in India. Comprehensive analysis revealed equal proportions of Old and New world _P. vivax_ subpopulations across India, however, proportions of two subtypes were different according to geographical location (Fig. 8). Analysis of single clone isolates identified using MSP-3α marker has revealed similar pattern of mutations in _dhfr_ and _dhps_ genes, associated with
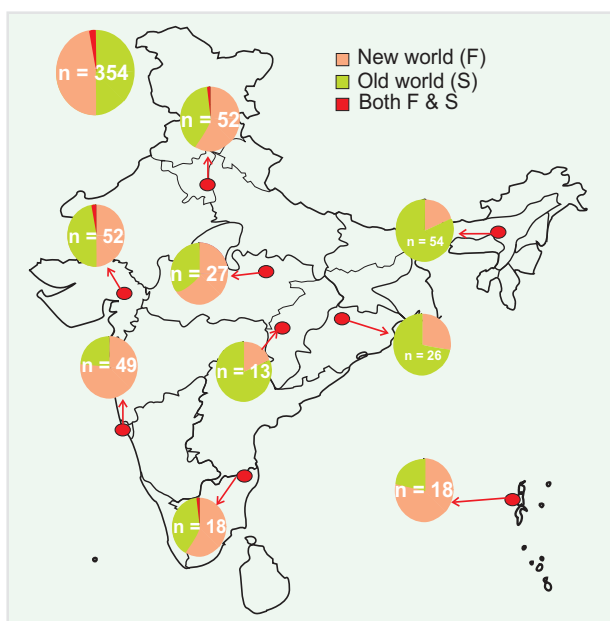
resistance to pyrimethamine and sulphadoxine, respectively.

Drug Resistance Studies

P. vivax has been stated to be intrinsically resistant to pyrimethamine, but recent studies have suggested that acquired resistance is more likely, with mutations in three codons of the gene encoding for dihydrofolate reductase (DHFR): at residue 57 (F to L), 58 (S to R) and 117 (S to N), with double (residues 58 and 117) and triple (57, 58 and 117) mutation genotypes also implicated. The quadruple mutant including mutations affecting residue 61 (T to M) correlates with SP treatment failure _in vivo_. The use of SP to treat chloroquine (CQ) resistant _P. falciparum_ might be creating selection pressure in _P. vivax_ populations due to mixed species infections, therefore, a study has been undertaken to assess mutations in _dhfr_ gene in field isolates. Interestingly, there was a gradual increase in the frequency of mutant genotypes from the north to the south (Fig. 9), with the double mutant genotype more prevalent in areas with sympatricity of _P. vivax_ and _P. falciparum_ infections and with perennial transmission, thus, suggesting the exposure of _P. vivax_ to SP from treatment of CQ resistant _P. falciparum_ (Prajapati _et al_ 2007). However, Indian isolates show limited DHFR polymorphism as compared to isolates from other Southeast Asian countries where SP resistance among field isolates is very high. Based upon these studies, _P. vivax_ appears to remain susceptible to SP treatment in India (Joshi _et al_ 2007).

Plasmodium falciparum

P. falciparum, only species of malaria which causes death has shown an increasing trend in India. Therefore, a study has been taken up to understand

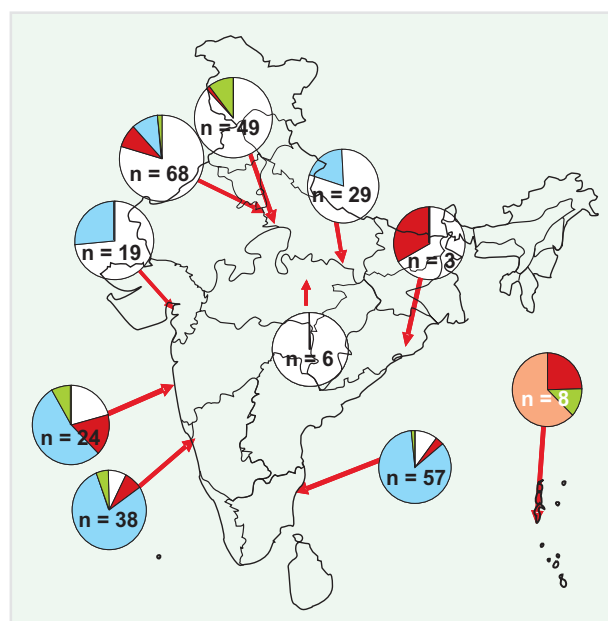Fig. 8: Distribution of two _P. vivax_ SSU rRNA subtypes in India

Fig. 9: Distribution of DHFR genotypes

the population structure of the field isolates. Isolates from different geographical regions were analyzed for surface protein markers, namely MSP-1 and MSP-2 for length as well as sequence variations and the analysis revealed highly polymorphic nature of Indian *P. falciparum* isolates on the basis length as well as sequence data. Three families of MSP-1 (K1, MAD20 & RO33) are prevalent in all the study sites with a few exceptions. K1 and MAD20 families have shown further allelic polymorphism while RO33 family was observed to be monomorphic with a single allele of 160 bp in all the study areas. In MSP-2, two families FC27 and 3D7 were observed and both the families have shown length variations. Both the families were observed in all the study sites and both were well represented. Fig. 10 shows the proportional prevalence of various families of MSP-1 and MSP-2.

Sequence analysis of K1, MAD20 and RO33 families of MSP-1 and FC27 and 3D7 families of MSP-2 supported the identical allelic composition of isolates in different areas. Further, identical length variants have shown novel deletions in addition to already reported ones. Sequence data had been submitted to genbank. The study suggested that Indian isolates represent a mixture of different alleles reported in different global regions. The study also revealed that Indian isolates present a level of diversity similar to Southeast Asia, Latin America and Papua New Guinea and a high degree of sequence homogeneity with isolates of other global regions. Clustering with isolates of different regions suggests that Indian isolates have global representation. Population sub-structuring was not observed on the basis of geographical location and allele frequency.

Characterization of *P. falciparum* Isolates from Jarawa, an Isolated Tribe of South Andaman

Analysis of *P. falciparum* samples from Jarawa tribe, an isolated tribe of South Andaman, Andaman and Nicobar Islands revealed 90–100% identity of these *P. falciparum* isolates with that of mainland as well as other regions in respect of MSP1 & 2 markers. Sequences were 100% identical to Kuwait isolate with identical deletion.

Molecular Genotyping of Clinical Resistance

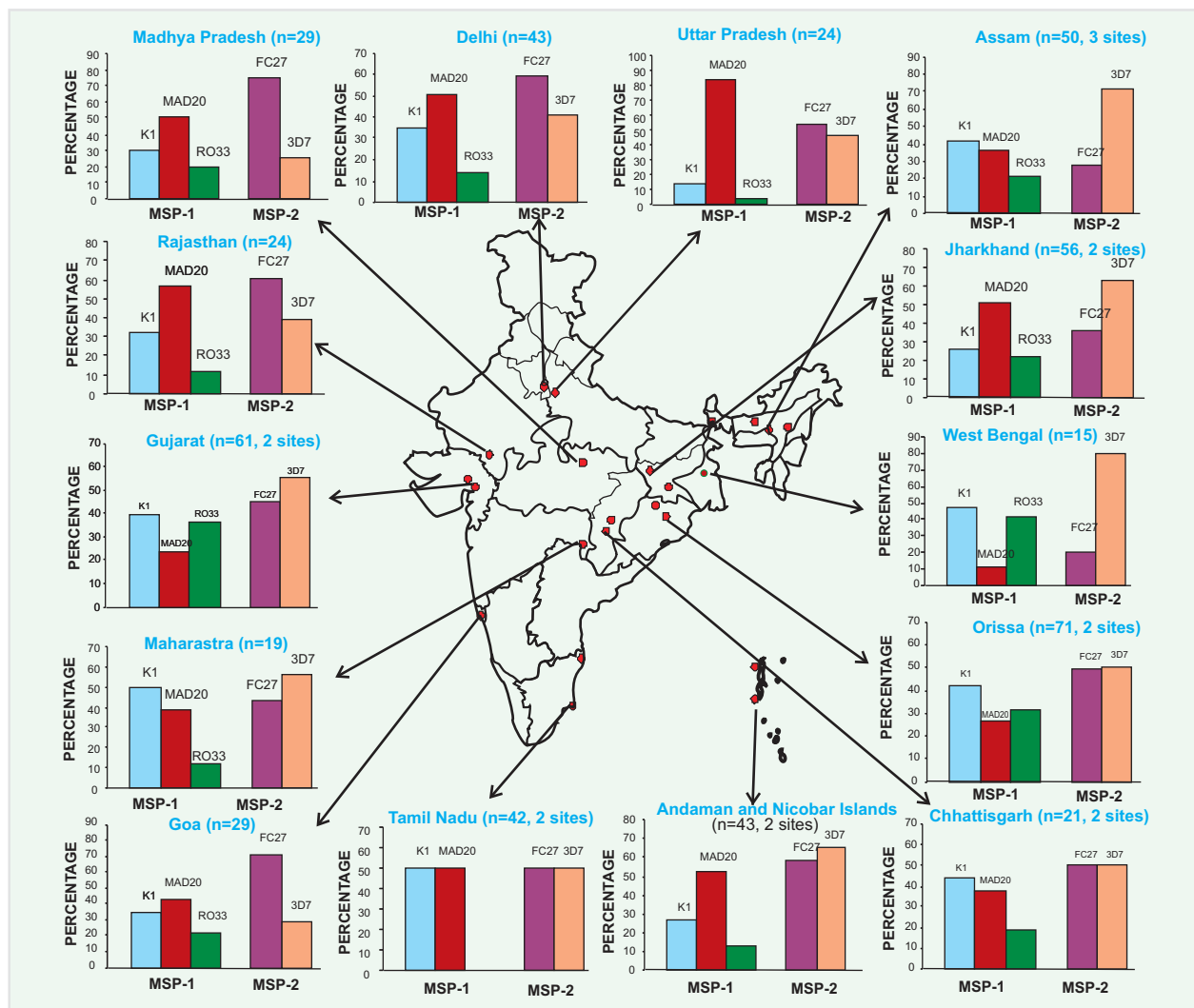Samples collected from *P. falciparum* patients on Day zero and the day of recrudescence during

Fig. 10: *Plasmodium falciparum*-distribution of MSP-1 & 2 families among field isolates in India
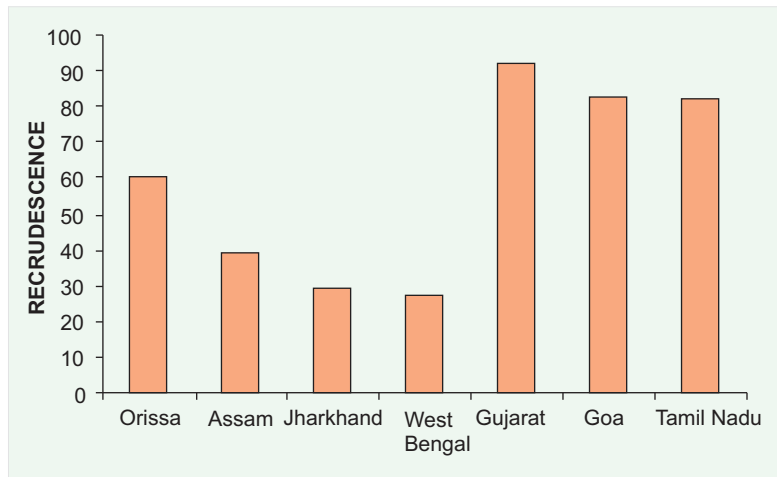
Fig. 11: Percentage of recrudescent infection in *P. falciparum* clinical samples

therapeutic efficacy studies of various antimalarials conducted in different regions of the country were analyzed for molecular genotyping using highly polymorphic surface protein markers. Results revealed high proportion of recrudescent infections with same genotype that of Day zero among field isolates in case of chloroquine (CQ) efficacy studies thus suggesting for resistance (Fig. 11).

Molecular Determination of Chloroquine Resistance in Indian *P. falciparum* Isolates

Widespread use of chloroquine (CQ) for the past decades might have led to chloroquine resistant parasites and emergence of clinical failure of chloroquine treatment. Previous studies on the *Pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) gene, that is responsible for chloroquine
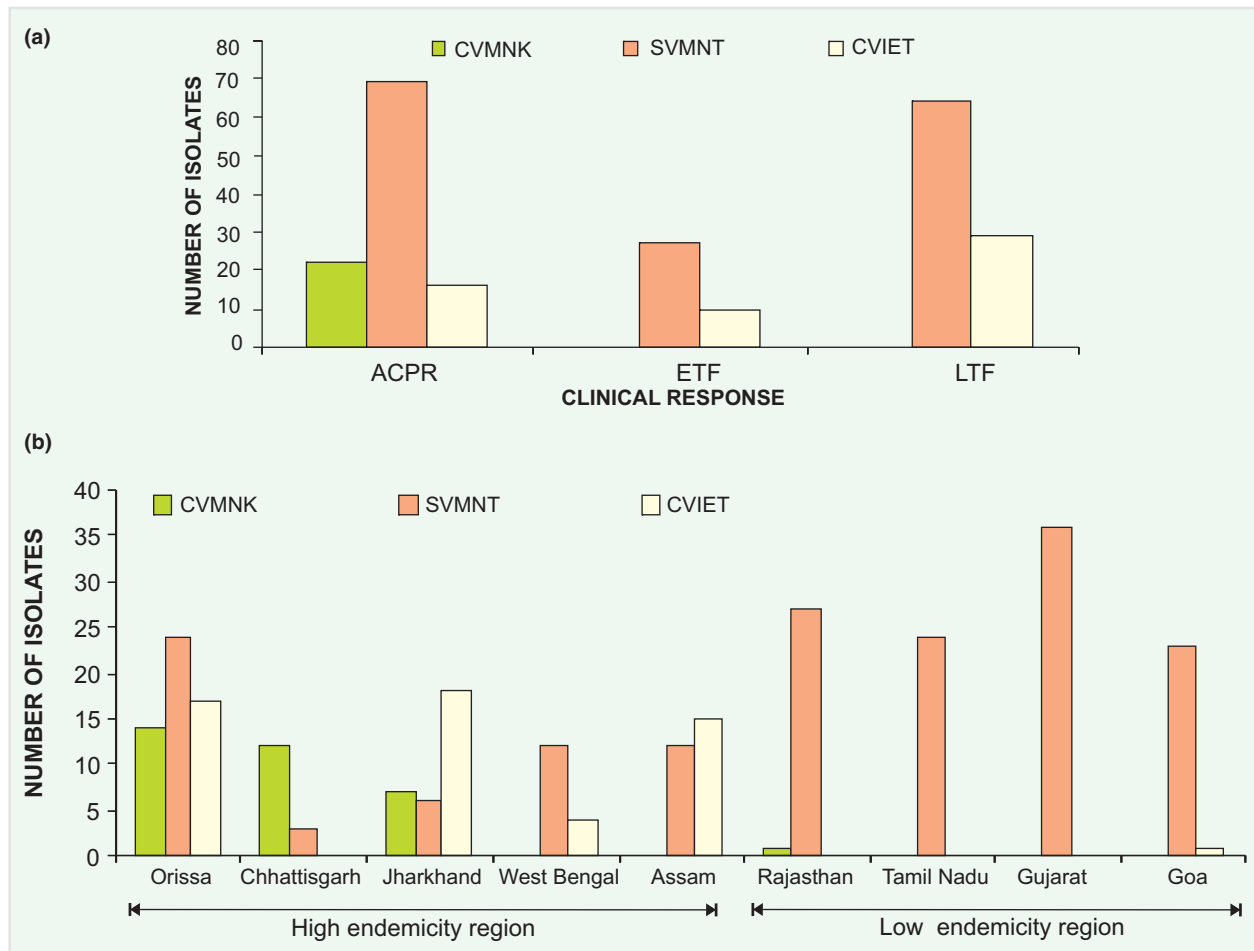

Fig.12: Distribution of Pfcrt haplotypes among field isolates. (a) Prevalence of haplotypes in chloroquine treated malaria cases. ACPR (adequate clinical and parasitological response), ETF (early treatment failure) and LTF (late treatment failure); (b) Prevalence of haplotypes in high and low malaria endemic area

resistance, revealed a heterogeneous situation of chloroquine resistance in Indian *P. falciparum* isolates. These studies analyzed the randomly collected samples and were not supported with clinical assessment of chloroquine efficacy, however, their results indicate *Pfcrt* gene as an attractive target for studying the epidemiological and molecular aspects of drug resistance. About 200 malaria patients enrolled for the chloroquine (CQ) therapeutic efficacy studies and 68 uncomplicated malaria patients from different geographical regions were assessed for genetic basis of chloroquine resistance using monitoring molecular markers. The status of point mutation responsible for CQ resistance was assessed by PCR amplification of *Pfcrt* gene. Three types of amino acid haplotype encoding 72aa-76aa (amino acid) of PFCRT protein, namely SVMNT, CVIET and CVMNK were observed. Results showed the prevalence of SVMNT haplotype in both clinically sensitive (70%) and in clinically resistant (74%) isolates. The wild type CVMNK was found only in clinically sensitive cases (20%) (Fig. 12a). Prevalence of SVMNT haplotype is observed in all geographical regions irrespective of malaria endemicity. Proportion of SVMNT were nearly cent percent in low endemicity areas while in high endemicity area all the three haplotypes were observed (Fig. 12b).

Studies on Genetic Variations in T-helper cell Epitopic Regions (Th2R and Th3R) of Circumsporozoite Protein (CSP) of *Plasmodium falciparum* Isolates from India

The role of T cells in malaria immunity has been appreciated for a long time. CSP has two T helper cell epitopes flanking the highly conserved region RII and spanning amino acid residue 326 to 343 (Th2R) and 361 to 380 (Th3R). However, these regions show polymorphism. Studies were performed to find out whether the genetic variations are regionally unbiased, polymorphism is restricted and can be categorized into groups because the T cell domains

could be included in a polyvalent sporozoite vaccine and such a strategy might largely depend on the extent of polymorphism in these epitopes. Study revealed that majority of the Indian isolates are regionally unbiased and could be categorized into six groups and sequences of the two groups showed similarity with the sequences of *P. falciparum* isolates from other geographical regions of the world, although some of the isolates showed wide sequence variations and could not be categorized into any group. However, analysis of isolates from different transmission seasons revealed low diversity during pre- and post-transmission than peak transmission season (Table 10). Therefore, the prototype variants from each group could be included in a subunit polyvalent vaccine against sporozoites.

Study of immunogenicity of T-helper Cell Epitopic Region of *Plasmodium falciparum* CSP as a Recombinant Fusion Protein

The development of an effective antimalarial drug or vaccine has occupied a large part of antimalarial research. A prime and reported target for vaccine development is the sporozoite stage of the parasite life cycle. The T-helper cell sequence corresponding to circumsporozoite protein is termed as a universal epitope as it is examined by different vaccine trials to be highly immunogenic. T-cell epitopic region, residues 326–345 of the *P. falciparum* circumsporozoite (CS) was undertaken to study its immunogenicity. Since the peptide units are small and are not much stable in biological system, it was cloned and expressed as recombinant fusion protein fused to DHFR protein of *E. coli* vector pQE40. The recombinant cells were transformed and cloned into *E. coli* pREP-4 vector, under appropriate antibiotic selection. The recombinant fusion protein was expressed by IPTG induction. This expressed protein was purified and its immunogenic properties were studied by cell proliferation assay, using the peripheral blood mononuclear cells (PBMCs) isolated

Table 10. Categorization of Th2R and Th3R sequences into groups

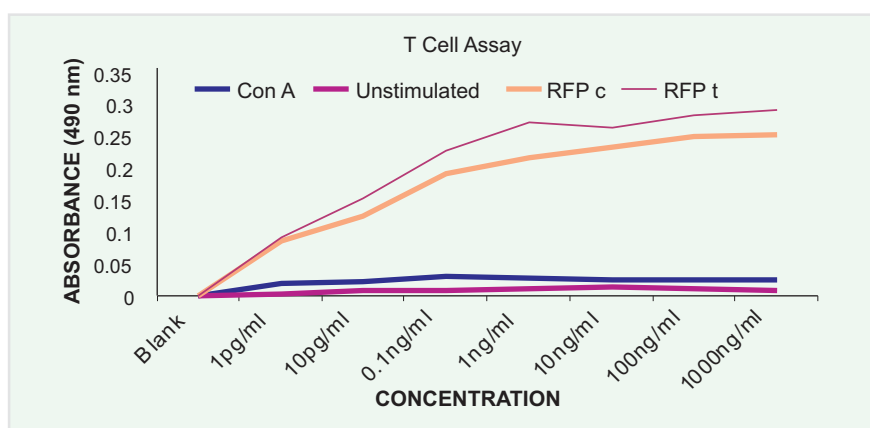| Group	T-helper cell region of circumsporozoite protein	Pre-transmission period (%)	Peak transmission period (%)	Post-transmission period (%)
Group I	Th2R(QQKKKNTTQ)	26	16	24
	Th3R(DQQD)	23	15	24
Group II	Th2R(QQKKKEKNIIQL)	25	20	26
	Th3R(DQRDEAD)	26	21	25
Group III	Th2R(QHIEEYNTQNL)	24	17	25
	Th3R(QEENTQE)	27	23	27
Group IV	Th2R(QHKKRTL)	24	15	25
	Th3R(DQRQAD)	24	16	24
Group V	Th2R(QEENTQ)	–	16	–
	Th3R(A)	–	11	–
Group VI	Th2R(QKRQ)	–	17	–
	Th3R(DAD)	–	14	–

Fig. 13: Immunogenicity of recombinant fusion protein by cell proliferation assay

from the *P. falciparum* infected patients. The PBMCs from healthy individuals (who did not have any clinical history of malaria) and Con A served as negative and positive controls respectively in the cell proliferation studies. The immunogenicity study by T-cell proliferation study revealed that the recombinant fusion protein was more immunogenic than the corresponding peptide unit (Fig. 13). Therefore, to be effective in protection against malaria, T-cell epitopes could be included into a prototype subunit vaccine capable of eliciting protective immune response in genetically diverse population.

Molecular Analysis of Invasion of Indian *Plasmodium falciparum* Field Isolates and Cytoadherent Properties of Infected Erythrocytes

The invasion of erythrocytes by *Plasmodium* merozoites is mediated by specific molecular interactions between host receptors and parasite ligands. Most laboratory strains of *P. falciparum* use sialic acid residues on glycophorin A as receptors for erythrocyte invasion. A 175 kD *P. falciparum* protein known as EBA-175 (for erythrocyte binding antigen-175kD), mediates binding to sialic acid residues of glycophorin A during invasion. Some *P. falciparum* laboratory strains are known to possess alternative invasion pathways and can invade neuraminidase-treated RBCs. It is not known how commonly such alternative pathways are used by *P. falciparum* field isolates. We have studied the invasion phenotypes of *P. falciparum* field isolates collected from different regions of India (Okoyeh *et al* 1999). Out of 15 *P. falciparum* isolates tested, 5 showed invasion and

multiplication in both neuraminidase and trypsin-treated erythrocytes, 3 in neuraminidase-treated but not in trypsin-treated erythrocytes and 4 in trypsin-treated but not in neuraminidase-treated erythrocytes. These studies indicate that *P. falciparum* field isolates commonly use alternative invasion pathways that do not depend on sialic acid residues of glycophorin A.

Cytoadherence refers to the ability of erythrocytes infected with blood stage parasites, trophozoites and schizonts, to adhere to the vascular endothelium in the human host and bind to uninfected erythrocytes to form rosettes. Cytoadherence of *P. falciparum*-infected erythrocytes in brain capillaries have been implicated in cerebral malaria and sequestration in the placenta results in complications in pregnancy. The endothelial receptors used for cytoadherence include ICAM-I, CD36, VCAM, E-selectin, CD31 and chondroitan sulfate A (CSA). Cytoadherent phenotypes of Indian *P. falciparum* field isolates collected from different regions of India have been studied. In a preliminary study, out of 13 isolates screened, 9 showed binding property and 4 did not. JDP-2 (*P. falciparum* isolates collected from tribal areas of Jagdalpur (Chhattisgarh) showed high binding with CD36 and JDP-8 showed high binding with ICAM-I and also forms rosettes. Another *P. falciparum* isolate RAJ-86 collected during Rajasthan epidemic in 1994, showed binding with CD36 and CSA (Chitnis *et al* 1998). Information is important for the development of novel strategies that block cytoadherence to receptors such as ICAM-I and prevent or reverse pathological outcomes such as cerebral malaria.

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