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Markers for population genetic analysis of human Plasmodia species, *P. falciparum* and *P. vivax*

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Present report deals with the genetic diversity existing among the field isolates of *Plasmodium falciparum* and *P. vivax* in India. Isoenzymes and molecular markers were used to analyse field isolates of *P. falciparum* and *P. vivax*. High level of length polymorphism was observed in repeat nucleotide sequences of MSP-1, MSP-2 and GLURP in *P. falciparum* isolates and CSP, GAM-1 and MSP-3α in *P. vivax* isolates. In study populations a high proportion of isolates (up to 60%) were comprised of more than one genetically distinct parasite type—multiclonal. Presence of identical allelic forms of enzyme and DNA variations in different geographical areas and in different years suggest that isolates belong to a single random mating population of *P. vivax* and *P. falciparum*. Observed random combination of alleles in the field isolates suggest the unlinked nature of loci studied. Study supports the feasibility of using molecular markers for the identification of recrudescence in *P. falciparum* from fresh infection.

Key words Genetic markers – India – *P. falciparum* – *P. vivax*

Malaria is a serious public health problem in India and the entire country is endemic for malaria except areas 1800 m above sea level. Two most prevalent human malaria parasite species in the country are *Plasmodium vivax* and *P. falciparum*. *P. vivax* is the predominant species and accounts for about 60–65% of the cases. Recent report has shown an increasing trend in *P. falciparum* incidence including drug resistant strains and also spreading into the earlier *P. falciparum* free areas[1]. Problem of malaria control is becoming difficult day by day because of development of resistance to choloroquine, commonly used antimalarial in parasites, to insecticides in vectors and operational problems. Therefore, to develop suitable and novel control strategies against the parasite, it is important to know the extent of genetic polymorphism existing in the parasite population. Genetic variability of the *Plasmodium* parasites has made them successful against all the eradication efforts made by the human kind. Data generated on the extent of genetic diversity present in *P. vivax* and *P. falciparum* populations from different regions of the country will be important for the development and testing of new drugs and malaria vaccines. 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 gene polymorphism have been carried out in relation to space and time. Fig. 1 shows the sites from where isolates have been characterised.

Polymorphism at glucose phosphate isomerase (GPI), glutamate dehydrogenase (GDH), adenosine deaminase (ADA) and lactate dehydrogenase (LDH) enzyme loci in both *P. vivax* and *P. falciparum* were examined[2–5]. DNA length polymorphism of MSP-1

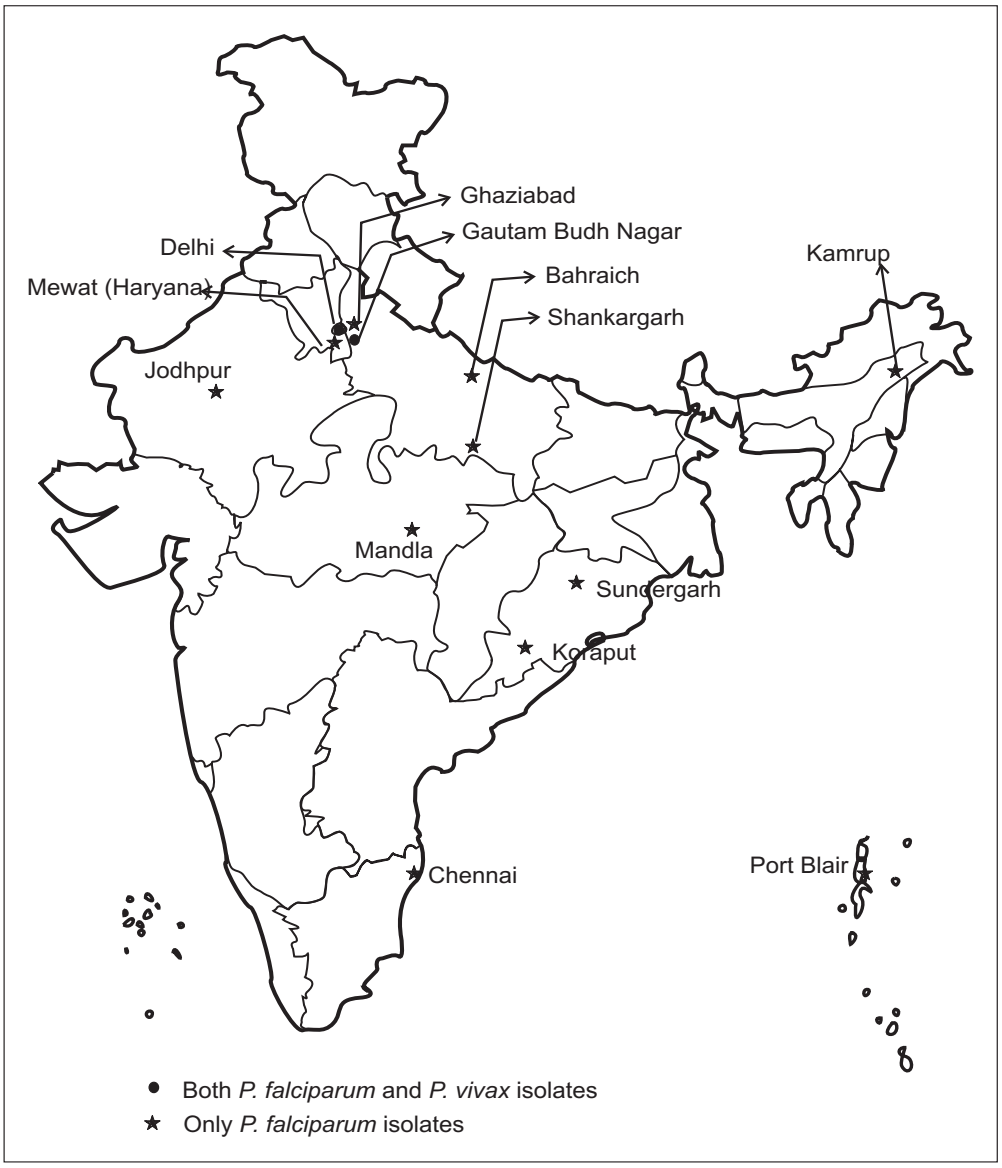

Fig. 1 : Map showing study sites for genetic diversity in malaria parasites

& 2 (merozoite surface proteins 1&2) and GLURP (glutamate rich protein) in *P. falciparum* isolates and CSP (circumsporozoite protein), MSP-3α (merozoite surface protein 3α) and GAM-1 (transmission blocking candidate antigen) genes in *P. vivax* isolates were examined. Table 1 lists the polymorphic markers in Indian *P. falciparum* and *P. vivax* isolates.

Studies carried out on the isoenzymes typing of *P. vivax* and *P. falciparum* isolates collected from patients using GPI, GDH, ADA and LDH systems for the first time showed the random mating nature of *P. vivax* and *P. falciparum* isolates in India[2]. Further studies continued in relation to time and space for both *P. vivax* and *P. falciparum* species showed high degree of genetic diversity and identical electrophoretic forms of different enzyme systems in different geographical areas. Identical allelic forms observed among *P. vivax* isolates studied from Delhi in relation to time are shown in Fig. 2. Study on isoenzymes fur-

Table 1. Markers and polymorphism in human Plasmodia species in India

Markers	*P. vivax*	*P. falciparum*
Enzyme		
GPI	6 alleles	6 alleles
GDH	7 alleles	5 alleles
ADA	5 alleles	3 alleles
LDH	1 allele	1 allele
DNA		
Size variations	GAM-1: 9 alleles	MSP-1 (block II region): 9 alleles in nested
	MSP-3α : Alu 1 : 14 alleles Hha 1 : 11 alleles	24 alleles in 3 families – RO33, MAD 20 & K1
	CSP: 3 alleles	MSP-2 (central region): 8 alleles PCR 23 alleles in 2 families–3D7 & FC27
		GLURP: 11 alleles

ther revealed no difference in the proportions of single and multiclonal isolates during low and high transmission periods of *P. vivax*[3–5] .

Studies on the gene polymorphism of CSP, GAM-1 and MSP-3α in Indian *P. vivax* isolates have revealed more than two variants of CSP differing in nucleotide sequences and nine size variants in GAM-1 in isolates of Delhi. CSP variants were detected by PCR amplification using CSP specific primers[6–7] and hybridising with allele specific DNA probes using digoxigenin labeling systems. GAM-1 gene has been assayed using nested PCR amplification with specific primers[8]. *P. vivax* MSP-3α locus was investigated in Indian field isolates using a combined polymerase chain reaction and restriction fragment length polymorphism (PCR/RFLP) protocol[9]. Isolates collected from symptomatic patients attending malaria clinic of Malaria Research Centre, Delhi showed both size and sequence polymorphism. Size variations of PCR amplified product ranged between 1.2 and 1.8 kb. RFLP pattern with Alu I and Hha I has shown 14 and 11 alleles respectively. Sixteen different genotype combinations of RFLP patterns were observed in a total of 19 isolates analysed (Fig. 3).

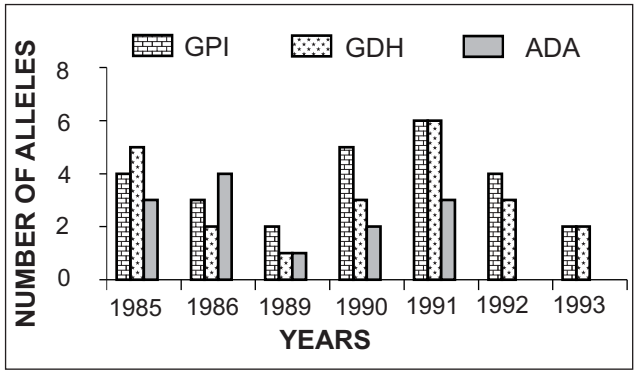

Fig. 2: Allelic polymorphism in *P. vivax* isolates

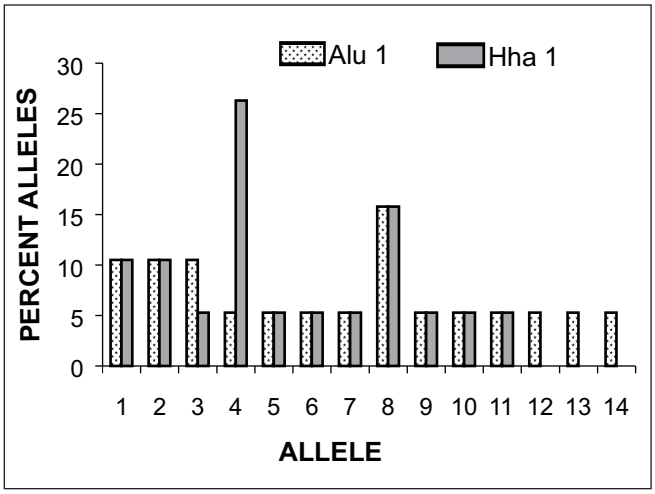

Fig. 3: MSP-3α polymorphism in *P. vivax* isolates

Above observations suggest extensive parasite diversity within *P. vivax* population of India. Polymorphic nature of *P. vivax* isolates has also been observed in drug response and relapse pattern studies[10,11]. In general Indian isolates of *P. vivax* are comprised of a good proportion of multiclonal isolates. Based on isoenzymes markers upto 40% isolates were categorised as multiclonal and proportion has gone up to 60% with DNA size markers.

In *P. falciparum*, studies carried out on length polymorphism MSP-1 (block II) and MSP-2 (central re-

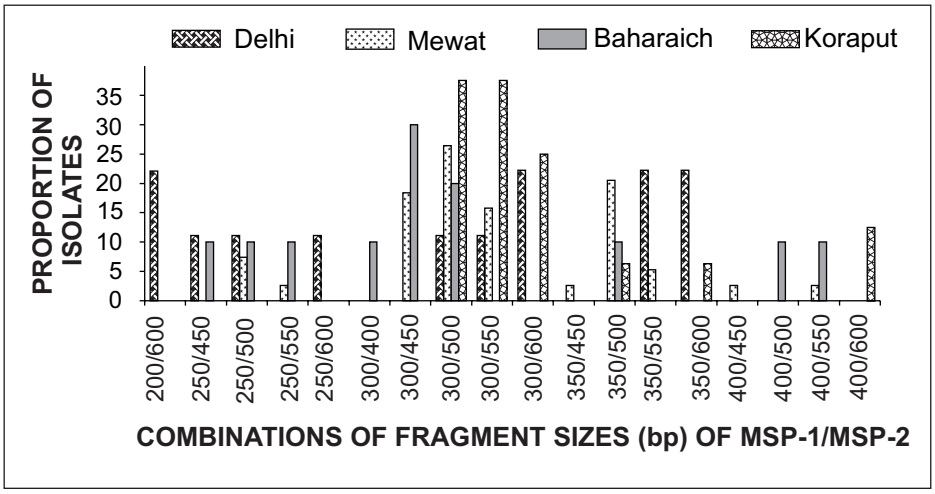

Fig. 4: Genotypes observed in *P. falciparum* isolates

gion) by simple PCR assay[12] have shown 18 different genotypes (Fig. 4) among *P. falciparum* isolates studies from Haryana, Delhi, Orissa and Uttar Pradesh. Analysis of isolates from different parts of the country using family specific assay revealed presence of all the three families of MSP-1 namely K1, MAD20 and RO33 and both of MSP-2—3D7 and FC27 with prevalence of K1 and MAD20 families. Proportional distribution of families of MSP-1 and MSP-2 is given in Fig. 5. About 10% of *P. falciparum* isolates were categorised as multiclonal based on isoenzymes profile and simple PCR assay. However, nested PCR assay[13] and family specific protocol[14] increased the sensitivity of characterisation of isolates and high degree of polymorphism was observed. Proportion of multiclonal isolates among study samples was observed to be about 60%. High degree of genetic diversity among Indian *P. falciparum* isolates has also been reported earlier[15,16].

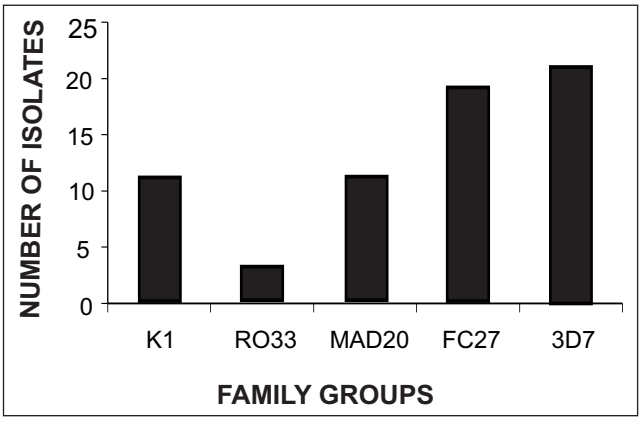

Fig. 5: Distribution of family groups of MSP-1 & 2 of *P. falciparum* isolates

In *P. falciparum* infection treatment failure causes recrudescence, which may increase the proportion of gametocyte carriers in the population, and consequently increase in the transmission risk. Thus it is of utmost importance for the control programme to accurately identify the treatment failure (recrudescence) infection from fresh infections. Therefore, above markers were assessed for the identification of fresh infection from recrudescence in *P. falciparum* positive cases. Paired samples collected from patients with repeat episode of malaria on Day 0 and day of reinfection were analysed using nested PCR assays of MSP-1 and MSP-2.

Isolates collected from three patients on Day 0 and Day 14 showed different genotypes of MSP-1/MSP-2 suggesting new infection. However, in another two patients, isolates collected on Day 0 and Day 14 showed same genotype of both MSP-1 and MSP-2. Fig. 6 shows the genotypes of paired samples. To get more conclusive results, analysis of more samples using more markers is proposed.

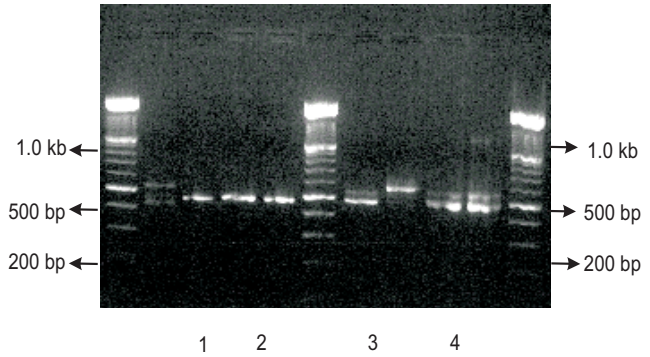

Fig. 6: Gel electrophoretogram showing genotyping of paired samples : Pairs 1 & 3 with different size variants and pairs 2 & 4 are with same size variants of MSP-1

In conclusion, it is summarised that Indian isolates of *P. vivax* and *P. falciparum* exhibit high level of genetic diversity in respect to GPI, GDH, ADA and LDH enzyme systems. High level of length polymorphism was also observed in repeat nucleotide sequences of MSP-1, MSP-2 and GLURP in *P. falciparum* isolates and CSP, GAM-1 and MSP-3α in *P. vivax* isolates. In general a high proportion of isolates (upto 60%) were comprised of more than one genetically distinct parasite type—multiclonal. Presence of identical allelic forms of enzyme and DNA variations in different geographical areas and in different years suggest that isolates belong to a single random mating population of *P. vivax* and *P. falciparum*. Observed random combination of alleles in the field isolates support the unlinked nature of loci studied. Study support the feasibility of using molecular markers for the identification of recrudescence in *P. falciparum* from fresh infection.

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