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Plasmodium vivax merozoite surface protein-3α: a high-resolution marker for genetic diversity studies

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Abstract

Background & objectives: Malaria, an ancient human infectious disease caused by five species of *Plasmodium*, among them *Plasmodium vivax* is the most widespread human malaria species and causes huge morbidity to its host. Identification of genetic marker to resolve higher genetic diversity for an ancient origin organism is a crucial task. We have analyzed genetic diversity of *P. vivax* field isolates using highly polymorphic antigen gene *merozoite surface protein-3alpha* (*msp-3α*) and assessed its suitability as high-resolution genetic marker for population genetic studies.

Methods: 27 *P. vivax* field isolates collected during chloroquine therapeutic efficacy study at Chennai were analyzed for genetic diversity. PCR-RFLP was employed to assess the genetic variations using highly polymorphic antigen gene *msp-3α.*

Results: We observed three distinct PCR alleles at *msp-3α,* and among them allele A showed significantly high frequency (53%, $\chi^2 = 8.22$, $p = 0.001$). PCR-RFLP analysis revealed 14 and 17 distinct RFLP patterns for *Hha1* and *Alu1* enzymes respectively. Further, RFLP analysis revealed that allele A at *msp-3α* is more diverse in the population compared with allele B and C. Combining *Hha1* and *Alu1* RFLP patterns revealed 21 distinct genotypes among 22 isolates reflects higher diversity resolution power of *msp-3α* in the field isolates.

Interpretation & conclusion: P. vivax isolates from Chennai region revealed substantial amount of genetic diversity and comparison of allelic diversity with other antigen genes and microsatellites suggesting that *msp-3α* could be a high-resolution marker for genetic diversity studies among *P. vivax* field isolates.

Key words Genetic marker; malaria; PCR-RFLP; *Plasmodium vivax*; *Pvmsp-3α*

Introduction

The extensive polymorphism exhibited by surface antigens is one of the major factors, why immunity to malaria develops only after repeated infections with the same species[1]. The genes encoding antigens are under strong diversifying selection, i.e. an adaptive mechanism to escape the host immune response[2–5]. Understanding of the mechanisms generating variations in malaria surface antigens is essential for designing immunization strategies to circumvent the emergence of novel polymorphisms[6].

PvMSP-3α, a 148–150 KDa merozoite protein belongs to *Pvmsp-3* gene family. This gene family has structurally related members, namely *Pvmsp-3β*, and *Pvmsp-3γ*[7,8]. *Pvmsp-3α* is characterized by a distinct block of alanine-rich heptad repeats in the central region that are predicted to form an intramolecular coiled-coil, and is polymorphic in nature. A number of substitutions are reported to occur in or around the heptad blocks, although the alanine-residue based framework of the putative coiled-coil is conserved[8,9].

Plasmodium vivax is the most widespread human ma-

laria parasite throughout the globe except sub-Sahara region of Africa where its prevalence is extremely low due to fixation of Duffy negativity trait in African negro population[10]. In India, >50% annual malaria cases are due to *P. vivax*. Understanding towards genetic diversity and population structure of malaria parasites are the crucial steps to make an effective anti-malarial control measure, and for such studies identification of high diversity resolving genetic markers are equally essential. In this paper, genetic diversity of *P. vivax* field isolates from Chennai and assessment of *msp-3α* as high diversity resolving genetic marker are presented.

Material & Methods

Study site: Chennai (Tamil Nadu), a coastal metropolitan city located in the southern region of the country has the problem of urban malaria. Large number of overhead tanks in the city provide good condition for mosquito breeding. Malaria is mainly due to *P. vivax* (>90% of cases) and is being transmitted by *Anopheles stephensi* (Diptera: Culicidae). Malaria transmission is stable in Chennai and occurs throughout the year. Recently, cases of chloroquine resistant *P. falciparum* malaria are also being reported in the Tamil Nadu state[11].

Blood sample collection: Blood samples were collected during *P. vivax* therapeutic efficacy study conducted for chloroquine drug at Chennai Field Unit of National Institute of Malaria Research (NIMR), between 2003 and 2004. This study was approved by the Ethics Committee of the NIMR, New Delhi. All blood spots were collected with the consent of the patients. Microscopically diagnosed *P. vivax*-positive blood was spotted on autoclaved Whatman filter paper strips and dried blood spots were stored at 4°C. Blood smears were stained with JSB stain[12] and examined at a 1000x magnification for identification of *P. vivax* infection.

Genomic DNA extraction: The parasite DNA was extracted from three punches of blood spots (3 mm) on Whatman filter paper using the QIAamp DNA Mini kit (Qiagen, Germany) as per manufacturer's instructions. Genomic DNA was eluted in autoclaved double distilled water and stored at –20°C for further use.

Amplification and restriction fragment length polymorphism (RFLP): PCR amplification and RFLP protocols for *msp-3α* were reported elsewhere[13]. PCR products were visualized on 1.0% agarose gel and samples showing single PCR fragment were only used for RFLP study. Five micro litres of PCR product was digested individually with restriction enzymes (*Hha I* and *Alu I*) at 37°C for 4 h. The restriction products were visualized on 3.0% agarose gel followed by fine genotyping of digested PCR products.

Results

PCR amplification of msp-3α and allele frequency: Analysis of 27 *P. vivax* isolates from Chennai, India revealed three PCR length variants A, B and C, and their sizes were in the range of 1.8–2.0, 1.4–1.5 and 1.1–1.2 kb respectively (Fig. 1). In addition, we have observed a fourth rare allele D with smaller fragment size (0.5 kb) in a single isolate (Fig. 1). This rare allele was previously observed in three monkey-adapted strains of *P. vivax,* with a fragment size in the range of 0.45–0.57 kb[14]. Two samples showed multiple infections with variant A and B. For the calculation of allele frequency, total number of observations for all alleles were considered instead of to-

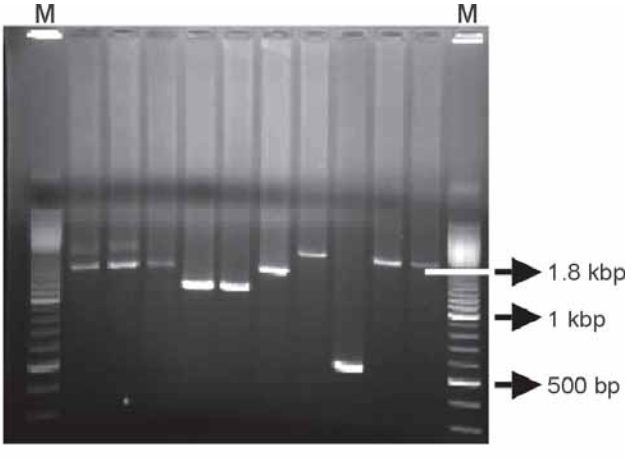

Fig. 1: PCR amplified fragments of *Pvmsp-3α* gene from *P. vivax* field isolates

Table 1. *Pvmsp-3α* RFLP pattern (*Alu 1* and *Hha1* restriction enzymes) and genotyping among *P. vivax* isolates collected from Chennai, India

Sample ID	Fragment size (kb)	*Alu1* pattern (size in bp)					Genotype *Alu1*	Combined genotype	Genotype *Hha1*	*Hha1* pattern (size in bp)				
ch45	1.8	500	450	270	350	200	1	1	1	1000	500	450	275	250
ch8	1.4	500	200	150			2	2	2	1000	250			
ch30	1.2	500	400	250	160	150	3	3	3	1000	200			
ch105	1.2	500	300	190	150		4	4	3	1000	200			
ch104	1.2	500	300	190	150		4	4	3	1000	200			
ch106	1.4	500	200	190	150		5	5	3	1000	200			
ch46	1.8	500	250	190	170	150	6	6	4	1000	320	250		
ch112	1.8	500	420	200	190		7	7	5	1000	250	200		
ch48	1.8	500	350	200	190		8	8	6	1000	400	250		
ch45	1.8	500	320	200			9	9	7	1000	400			
ch82	1.8	500	320	200	190	160	10	10	7	1000	400			
ch33	1.8	500	420	250	190	150	11	11	8	1000	500	250	200	
ch13	1.8	500	200	150			2	12	9	1000	400	250	200	
ch83	1.8	500	320	250	200	190	12	13	9	1000	400	250	200	
ch18	1.8	500	200	150			2	14	10	1000	500	400		
ch12	1.8	500	360	200	190	160	13	15	11	1000	500	420		
ch20	1.8	500	200	150			2	16	11	1000	500	420		
ch81	1.8	500	250	190	150		14	17	11	1000	500	420		
ch31	1.2	500	250	160	150		15	18	11	1000	500	420		
ch84	1.2	500	250	200	160	150	16	19	12	1000	900	400	200	
ch80	1.8	–	–	–	–	–	–	20	13	1000	900	350	250	200
Ch107	1.8	500	400	200	190	150	17	21	14	1000	500			

tal number of isolates. Overall, frequency distribution of the four variants A, B, C and D in field isolates was 15/29, 5/29, 8/29 and 1/29 respectively. This observation revealed that variant A is the dominant allele in the isolates. Assuming a similar frequency of the common *msp-3α* alleles in the population, we have estimated expected allele frequency of each allele to be 33%. Comparison of observed verses expected allele frequency revealed biased distribution of *msp-3α* alleles in the Chennai field isolates ($\chi^2 = 9.47$, df = 2, $p = 0.009$) and allele A revealed significantly higher allele frequency (53%, $\chi^2 = 8.22$, $p = 0.001$).

Restriction fragment length polymorphism (RFLP): Among 27 *P. vivax* isolates, 22 were analyzed for RFLP and the RFLP pattern of individual samples for

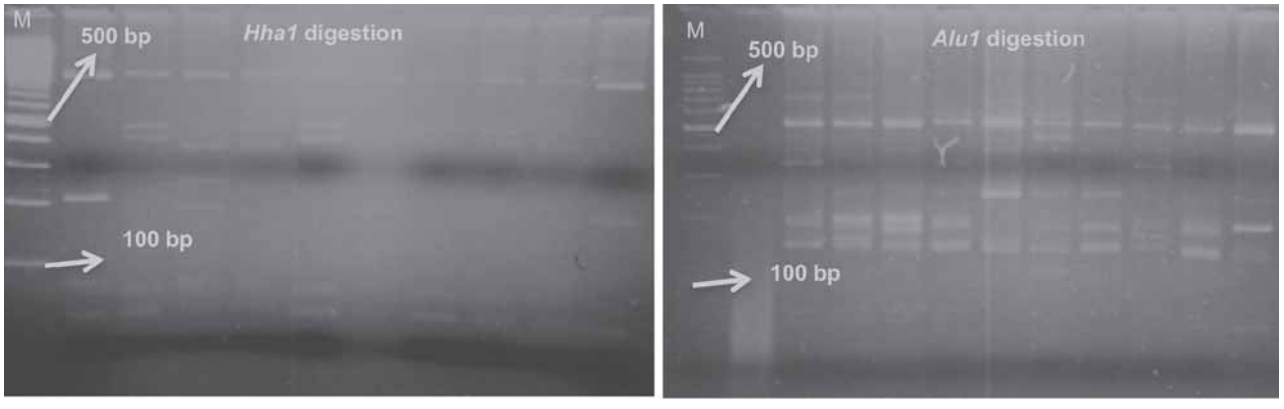

Fig. 2: Restriction fragment length polymorphism of *Pvmsp-3α* using *Hha1* and *Alu1* restriction enzymes.

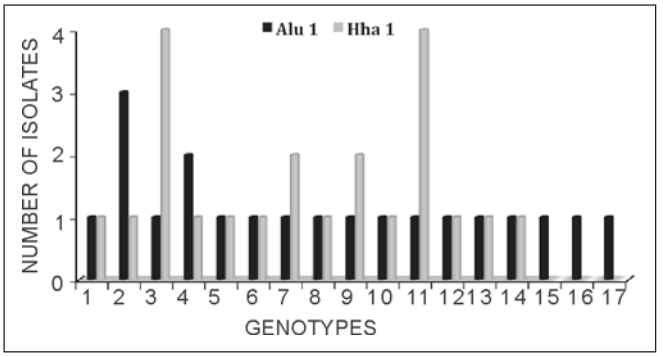

Fig. 3: Distribution of *Alu 1* and *Hha 1* genotypes at *Pvmsp-3α* among *P. vivax* field isolates

individual enzymes is listed in Table 1 and Fig. 2. In RFLP, 14 distinct *Hha1* and 17 *Alu1* RFLP patterns were observed among 22 isolates. Distribution of individual RFLP genotypes is shown in Fig. 3. Further, combination of *Hha1* and *Alu1* patterns revealed a total of 21 distinct genotypes. RFLP pattern of *P. vivax* isolates showed *Alu1* is a better enzyme to uncover most of the variations compared with *Hha1*. RFLP analysis is also able to reveal differential level of diversity among *msp-3α* alleles and variant A is more diverse in field isolates compared to variants B and C (Table 1).

Discussion

This study presents genetic diversity among *P. vivax* isolates collected from Chennai using a highly polymorphic genetic marker *Pvmsp-3α*. Present study revealed high degree of genetic diversity among Indian *P. vivax* isolates and *msp-3α* is highly polymorphic in the population. This observation is in agreement with the earlier studies, which showed high degree of genetic variation at *msp-3α* in the field isolates of Delhi[15] and Kolkata[16]. Transmission of *P. vivax* in Delhi (seasonal), Chennai (perennial), and Kolkata (low) is varied, however, genetic diversity level at *msp-3α* is in similar magnitude suggesting *msp-3α* could be a potential genetic marker. The observed enormous genetic diversity at *msp-3α* is supported by earlier studies suggesting *P. vivax* isolates are highly diverse using various category of phenotypic and genetic markers such as infection phenotypes, relapse pattern and clinical profiles, isoenzyme markers, antigen genes, and drug resistant gene (reviewed by Joshi *et al*[17]). Substantial amount of genetic polymorphism at *msp-3α* have also been reported from Thailand[18], Papua New Guinea[13], Korea[19], Iran[20] and South America[14], thus, supporting high degree of genetic polymorphism observed in the present study. These observations suggest that high genetic diversity is a rule of *P. vivax* isolates rather than exception. The factors influencing higher genetic diversity at *msp-3α* could be: (i) inter-allelic recombination[14]; and (ii) presence of polymorphism prone region such as alanine rich heptad repeats.

Comparison of *msp-3α* alleles from Chennai field isolates with Delhi and Kolkata *P. vivax* isolates revealed similar distribution pattern, i.e. higher frequency of allele A, however, it is variable in nature[15,16]. The RFLP pattern of Delhi isolates revealed higher number of unique genotype like Chennai isolates. The numbers of unique genotypes were less in Kolkata[16] isolates compared to Delhi[15] and Chennai isolates. The difference in RFLP pattern could be due to different transmission patterns and sample collection time. All the isolates from Delhi as well as from Chennai were collected at a single time point (up to a month) whereas isolates from Kolkata were collected at several time points (up to a year). This probably increases the probability of getting higher number of common RFLP pattern.

Power of a genetic marker in terms of its ability to uncover the existing genetic diversity of an organism in a population is of great concern especially for selecting a marker for population genetic study. Comparison of genetic diversity at antigen genes in *P. vivax* field isolates employing simple PCR or PCR-RFLP methods for three antigen genes revealed 2–5 alleles at *Pvgam-1*[21–23], 11–23 at *Pvcsp*[16,24] and 36 at *Pvmsp-1*[16] using substantial number of field isolates (>100). Similarly, studies on microsatellites in *P. vivax* field isolates revealed 5–18 alleles per locus[25–27]. Comparison of allelic diversity of the above markers with the *msp-3α* revealed a higher resolution power of *msp-3α* (21 distinct genotypes among 22 field isolates) using simple PCR-RFLP method. This suggests that *msp-3α* could be a very useful genetic marker for the studies on: (i) molecular epidemiology,

(ii) relapse infection, and (iii) differentiating recrudescence from re-infection during drug efficacy study.

The present study concluded that Indian *P. vivax* isolates displayed high diversity even in varied malaria endemicity using *msp-3α*. A high resolution power of *msp-3α* suggests for its utility as a genetic marker for molecular epidemiological and relapse/recrudescence studies.

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