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Genotyping of *MSP3β* gene in Indian *Plasmodium vivax*

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ABSTRACT

Background & objectives: The search for effective polymorphic markers in *Plasmodium vivax* is highly demanding to understand its transmission in a population. Due to the limited knowledge existing for *P. vivax*, the search for polymorphic markers for population studies is ongoing. The *MSP* gene family of *Plasmodia* has been linked with immune evasion. To study the circulating parasite population *P. vivax* merozoite surface protein 3β (*PvMSP3β*) polymorphic marker was used to investigate the genetic diversity of *P. vivax* in natural infections.

Methods: Polymorphism of *PvMSP3β* gene was determined in 46 *P. vivax* blood samples from six different regions of India by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) with *PstI*.

Results: Two different parasite genotypes, viz. type-A and type-B were detected among 46 samples that were positive for PCR, based on the size of the amplification. RFLP analysis with *PstI* showed 22 allelic groups and 15.2% samples revealed mixed infections on analysis.

Conclusion: PvMSP3β was found to be an effective molecular marker for *P. vivax* as it shows high diversity in India and multiple genotypes easily distinguishable without the need for sequencing.

Key words Genotyping; *MSP3β*; *Plasmodium vivax*; RFLP

INTRODUCTION

Plasmodium vivax causes the most widespread malaria in India in equal frequency as *P. falciparum* and is the reason for high morbidity[1]. It is said to be moving towards severity and is also turning resistant causing urgency in the search for a suitable vaccine for *P. vivax*[2–3]. It requires an understanding of the genetic structure for which identifying effective genetic markers is essential. Several molecular markers like circumsporozoite protein (CSP), merozoite surface protein (MSP) and *var* genes have been studied extensively in *P. falciparum* as large amount of polymorphism exists in making them potential vaccine candidates. As compared to *P. falciparum*, *P. vivax* is much less studied and hence less understood. In *P. vivax*, the polymorphic markers which are studied include CSP, apical membrane antigen-1 (AMA-1), duffy binding protein (DBP), MSP-1 and MSP3α. Not much information about *PvMSP3β* as an epidemiological marker is available in the Indian context.

Merozoites, the invasive stage of *P. vivax* life cycle are coated by layer of MSPs organized into a structurally complex coat. MSP antigen is encoded by several genes such as *PvMSP-1*, *PvMSP3α*, *PvMSP3β*, *PvMSP3γ*, *PvMSP4*, *PvMSP5*[4]. *PvMSP3* contains a central core domain that forms an α-helical secondary structure and coiled-coil tertiary structures with alanine residues. It is reported elsewhere that *PvMSP3β* and *PvMSP3γ* share some structural similarity with *PvMSP3α* but it is designated as a new gene family. The peptide sequences and structures of *PvMSP3α*, *PvMSP3β* and *PvMSP3γ* are similar to *PfMSP3* and MSP of *P. knowlesi*[5]. The *MSP* gene family has been linked with immune evasion and thus the knowledge of the existing polymorphism in the *MSP* gene is essential for understanding the development of malaria parasite on many levels like transmission, epidemiological surveillance and vaccine development. An effective marker requires to be highly polymorphic in order to gauge an understanding of the population evolutionary structure. *PvMSP3α* exhibits high polymorphism because of intragenic recombinations and further maintained by balancing selection whereas polymorphism in *PvMSP3β* sequences from across the globe is seen due to large insertions and deletions in the central alanine-rich region[4, 6–7]. This study genotyped the *PvMSP3β* gene in populations from different regions of India and showed that it is extremely polymorphic across the population.

MATERIAL & METHODS

Sample collection

Plasmodium vivax infected blood samples were collected during the years 2008–11, after obtaining ethical clearance and detailed patient history with informed con-

sent was taken before the collection. The samples were collected from six different regions of India, namely Delhi (D-northern India)—11 samples, Mangalore (M-southern India)—5 samples, Goa (G-western India)—9 samples, Jabalpur (J-central India)—4 samples, Rourkela (R-eastern India)—11 samples, and Chennai (C-southern India)—8 samples. Seasonal transmission of malaria occurs in Delhi and the samples collected from the other regions are endemic for malaria[8–10].

Diagnosis

Preliminary detection and diagnosis of the *Plasmodium* species was done by rapid diagnostic tests (RDTs) (Bioline SD Rapid Test) and microscopic diagnosis was done by thick and thin blood smears. The blood smears were stained with Giemsa and examined at 1000× magnification (100× objective) under the microscope for *P. vivax* species identification. The preliminary diagnosis was followed by the preparation of blood spots on Whatman No. 3 filter paper strips of the positive malaria samples and stored at 4°C.

DNA preparation and PCR analysis

Genomic DNA of positive *P. vivax* samples were isolated from the filter paper blood spots by QIAamp DNA Blood Mini Kit (Qiagen Inc) according to the manufacturer's instructions. To confirm the single *P. vivax* infection in the positive samples these were amplified by nested PCR using genus-specific primers-specific for 18s rRNA as described previously[11].

PvMSP3β marker gene was amplified in all the single *P. vivax* infections from six different regions of India by using the published protocol with some modifications[7]. In the primary PCR, primers F1 (5' GTATTCTTCGC AACACTC 3') and R1 (5' CTTCTGATGTTATTT CCAG 3') were used and for nested PCR; primers F2 (5' CGAGGGGCGAAATTGTAAACC 3') and R2 (5' GCTGCTTCTTTTGCAAAGG 3') were used. The primary PCR contained 1 µM of both forward and reverse primers, 1 unit of Taq DNA polymerase (Bangalore GeNei, 3 U/µl), 1 × buffer with $MgCl_2$, 0.2 mM of dNTP mix (Bangalore GeNei 2.5 mM each) and 1 µl of template DNA in a 25 µl reaction mix. The nested PCR comprised of 1 µM each of nested primers, 1 unit of Taq DNA polymerase, 1 × buffer with $MgCl_2$, 0.2 mM of dNTP mix and 1 µl of primary PCR product as a template in a 25 µl reaction mix. The primary and nested PCR reaction conditions included an initial denaturation of 95°C for 5 min and 35 cycles of 95°C for 30 sec, 56°C for 30 sec, 68°C for 2.5 min and a final extension of 72°C for 8 min. The amplified PCR products were analyzed on 1.5% agarose gel (Fig. 1).

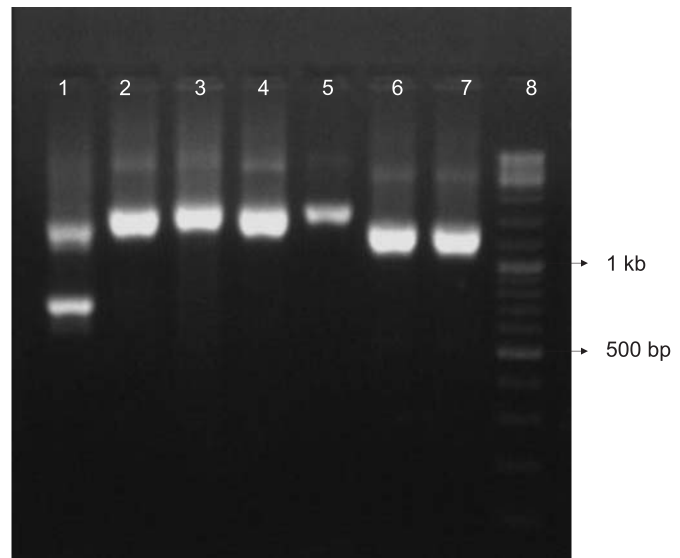

Fig. 1: Gel picture showing the results for *PvMSP3β* gene. Lane 1 shows mixed genotype with two bands, lanes 1–4 and lanes 6–7 show genotype B, lane 5 shows genotype A and lane 8 is a 100 bp DNA marker.

RFLP analysis

RFLP was carried out on all PCR positive samples which showed amplification for *PvMSP3β* gene by *PstI* (10 U/µl, Fermentas). The RFLP reaction mix contained five units of *PstI*. Restriction enzyme and 5 µl of the amplified *MSP3β* gene (PCR product) in 20 µl reaction mix as described by the manufacturer. The digestion mixture was incubated at 37°C for 16 h. The products were visualized on 2.0% agarose gel under UV illumination (Fig. 2).

RESULTS

Microscopy and RDT showed all the 48 samples to be *P. vivax* infections. The analysis of mixed infections by PCR revealed mixed infection in two samples which showed band at both 205 bp (*P. falciparum*) and 120 bp (*P. vivax*) and the remaining 46 samples with single band at 120 bp were only *P. vivax* infections. All the 46 samples were amplified for *PvMSP3β* gene with the primers that have been used previously (Fig. 1)[7]. In our analysis after PCR, we found the samples categorized into two different genotypes—type-A and type-B. Type-A ranged between 1.7 and 2 kb and type-B between 1.4 and 1.5 kb. Type-B corresponds to the reference Belem strain and type-A to the sequences with different insertions in the central alanine-rich domain of the gene. Type-C with a product size of ~0.65 kb has a large deletion of ~780 bp in the central alanine-rich region was not found in any of the *P. vivax* isolates analyzed by us[4].

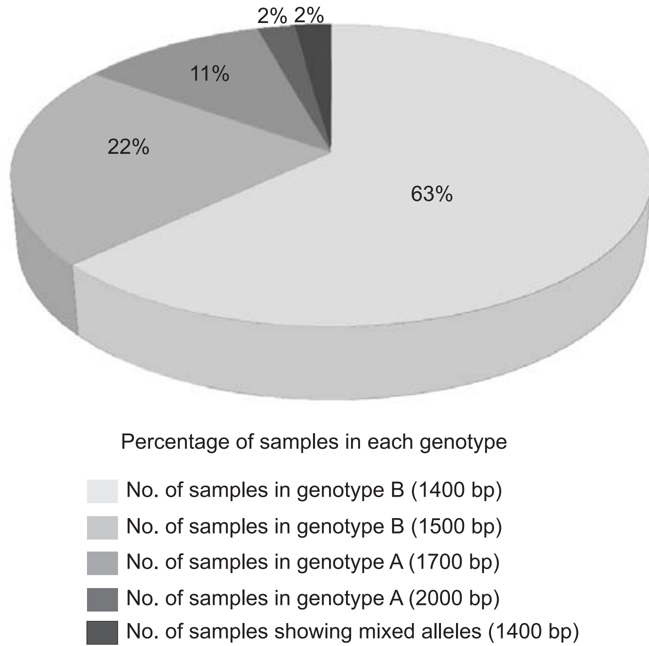

Fig. 2: Pie chart showing two different genotypes for *PvMSP3β* found in the samples by PCR assay. Type-B was in 85% samples, type-A in 13% samples and mixed infection was found to be in 2% samples by this PCR.

On PCR analysis, type B was found to be more prevalent in the population as 85% samples were type-B genotype and the remaining 13% isolates were type-A. Only 2% isolates from Delhi showed mixed genotype by PCR (Fig. 2). The restriction analysis by *PstI* of the positive PCR samples further subcategorized into two genotypes (Fig. 3). The restriction fragments of *PstI* showed 22 different banding patterns and the fragments ranged from 150 to 1500 bp. RFLP analysis showed 17 different alleles in type B genotype (B1–B17) and 5 alleles in type A genotype (A1–A5) (Table 1). Alleles B5, B7, B9 and B10

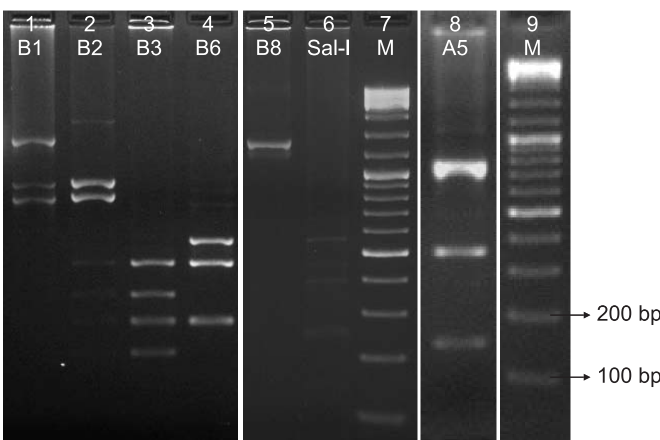

Fig. 3: Gel picture showing restriction digestion by *PstI* with the different alleles. Lane 6 shows the restriction fragments of *P. vivax* Sal-I reference. Lanes 7 and 9 are 100 bp DNA markers.

Table 1. Genotyping *P. vivax MSP3β* gene showing the genotypes and their alleles

S.No.	Genotype	Allele	*PstI* restriction fragments	No. of samples* showing the allele
1.	B-1400	B1**	1400+900+800	1
2.		B2	900+800	2
3.		B3	450+350+280+200	3
4.		B4**	1000+700+400+300+250	1
5.		B5	800+600+300	4
6.		B6	550+450+280	1
7.		B7	800+600	4
8.		B8	1400	3
9.		B9	700+500+300	4
10.		B10	700+350+300+200	5
11.		B11	1500	1
12.		B12**	800+600+400+300	1
13.	B-1500	B13**	1000+900	3
14.		B14**	1200+900	1
15.		B15	700+350+150	2
16.		B16	800+600+300	3
17.		B17	900+800	1
18.	A-1700	A1	700+500+300	1
19.		A2	700+350+150	1
20.		A3	850+800	2
21.		A4	800+350+300	1
22.	A-2000	A5	800+350+150	1

Type-A (A1–A5); Type-B (B1–B17); * Total number (n=46); **Mixed alleles after RFLP analysis.

were the most predominant alleles accounting for 36.9% of all the isolates (Fig. 4). The RFLP analysis further revealed the presence of five mixed alleles in type-B genotype but no such feature was observed in type-A.

Samples were categorized as mixed infections when PCR resulted in more than one band of different sizes or when the sum of all the restriction fragments of a PCR product was more than the size of the uncut PCR band[7]. The mixed infections in the total population were found to be 15.2% by RFLP and only 2% by PCR analysis alone,

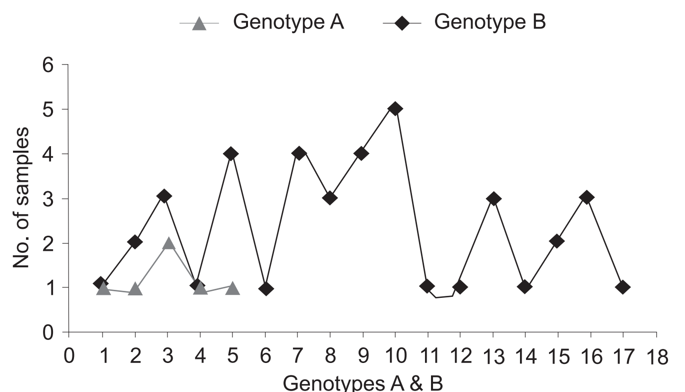

Fig. 4: Frequency of *PvMSP3β* alleles of genotypes A and B (n=46).

hence, indicating higher sensitivity and easy detection of mixed genotype infections by RFLP, as the RFLP analysis detected more number of mixed infections in comparison with the PCR method.

DISCUSSION

We genotyped *PvMSP3β* gene in 46 samples from six different malarious regions of India. In the present study we categorized the isolates majorly into two types, viz. type-A and type-B. The size of the PCR products ranged from 1.4–2 kb encompassing both the types and type-B (1.4–1.5 kb) was found to be more prevalent than type-A (1.7–2 kb) occurring in 40 isolates on PCR- RFLP analysis. The minor type-C with a size range of 0.65 kb reported earlier was not seen in this study[7].

There have been studies in *P. vivax* for polymorphic genes as *PvMSP3α*, *PvCSP*, *PvMSP* but no data are available in Indian context for *PvMSP3β* gene[12]. This study was undertaken with the objective of analyzing the existing diversity in *PvMSP3β* gene for its utility as an epidemiological marker. It has been reported earlier that the *PvMSP3β* gene has shown three major allele types in Asian origin parasite strains[7]. We also observed two allele types in our population but type-C was not found in our isolates. The areas from where the samples were collected are mostly endemic and the multiplicity of the infection is seen randomly distributed in the present study. Only 2% of the total samples were found to be mixed infections by PCR analysis whereas the PCR products were subjected to RFLP, and 15.2% of the total samples were mixed infections. This study thus suggests that this detection method of PCR along with RFLP using *PvMSP3β* gene as a marker is suitable for genotyping *P. vivax* isolates in various epidemiological areas. There have been few studies on *PvMSP3α* gene in India to assess its suitability as a genetic marker for population studies[13]. But no study in the Indian context of *PvMSP3β* has been conducted to evaluate its suitability as a polymorphic marker for genetic studies. With this objective this study was undertaken and the detection of mixed infections in different isolates from different epidemiological regions is indicative of its variability as a genetic marker.

The genotypic pattern of *P. vivax* in this study was observed to be similar among different endemic areas indicative of random distribution of the parasite within the country. The patterns of *P. vivax* identified in other studies are similar to the present study suggesting overall distribution of the parasite[14]. The genetic pattern on comparison with the similar study done in Pakistan shows type-B to be more prevalent in India unlike Pakistan and type-C not found in either of the two countries indicative of the probable absence of the type-C[15]. The type-B genotype found in abundance is in tandem with the other study in western Thailand where type-B was more prevalent[14]. On comparison with *PvMSP3α*, *PvMSP3β* gene displays more polymorphism as we could observe 22 alleles in this study, therefore, *PvMSP3β* gene could be a better epidemiological marker for *P. vivax* in Indian population. As no prior study has taken place on *PvMSP3β*, this study could serve as a basis of further analysis of this polymorphic gene with a bigger sample size to gain a clearer picture on the Indian scenario. RFLP analysis is a very effective and a rapid means of genotyping a particular gene in a population as polymorphisms in different isolates can be detected without resorting to the tedious and lengthy process of sequencing in the population.

CONCLUSION

PvMSP3β gene was characterized in 46 samples across India. Two genotypes A and B were found, each having 5 and 17 alleles respectively. Type-B genotype was found to have seven isolates with mixed infections after RFLP analysis with *PstI*. Due to the lack of *PvMSP3β* studies in India to establish its role as an effective polymorphic marker, this study was done where high variability of *PvMSP3β* gene and the percentage of mixed infections proved its aptness as a genetic marker.

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REFERENCES

1. Singh V, Mishra N, Awasthi G, Dash AP, Das A. Why is it important to study malaria epidemiology in India? *Trends Parasitol* 2009; *25:* 452–7.
2. Carvalho BO, Lopes SC, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, *et al.* On the cytoadhesion of *Plasmodium vivax*-infected erythrocytes. *J Infect Dis* 2010; *202:* 638–47.
3. Price RN, Douglas NM, Anstey NM. New developments in *Plasmodium vivax* malaria: Severe disease and the rise of chloroquine resistance. *Curr Opin Infect Dis* 2009; *22:* 430–5.
4. Rayner JC, Huber CS, Feldman D, Ingravallo P, Galinski MR, Barnwell JW. *Plasmodium vivax* merozoite surface protein PvMSP-3beta is radically polymorphic through mutation and large insertions and deletions. *Infect Genet Evol* 2004; *4:* 309–19.
5. Galinski MR, Ingravallo P, Corredor-Medina C, Al-Khedery

B, Povoa M, Barnwell JW. *Plasmodium vivax* merozoite surface proteins-3beta and -3gamma share structural similarities with *P. vivax* merozoite surface protein-3alpha and define a new gene family. *Mol Biochem Parasitol* 2001; *115:* 41–53.

6. Rayner JC, Corredor V, Feldman D, Ingravallo P, Iderabdullah F, Galinski MR, *et al*. Extensive polymorphism in the *Plasmodium vivax* merozoite surface coat protein MSP-3alpha is limited to specific domains. *Parasitology* 2002; *125:* 393–405.
7. Yang Z, Miao J, Huang Y, Li X, Putaporntip C, Jongwutiwes S, *et al.* Genetic structures of geographically distinct *Plasmodium vivax* populations assessed by PCR/RFLP analysis of the merozoite surface protein 3beta gene. *Acta Trop* 2006; *100:* 205–12.
8. National Institute of Malaria Research. Available from: *http://www.mrcindia.org/* (Accessed on January 25, 2013).
9. *Malaria Site (India).* Available from: *http://www.malariasite.com/*.
10. Health and Family Welfare Department (Government of Tamil Nadu). Available from: *http://www.tnhealth.org/dphdbmal.htm* (Accessed on January 25, 2013).
11. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ. PCR as a confirmatory technique for laboratory diagnosis of malaria. *J Clin Microbiol* 2006; *44:* 1087–9.
12. Kim JR, Imwong M, Nandy A, Chotivanich K, Nontprasert A, Tonomsing N, *et al.* Genetic diversity of *Plasmodium vivax* in Kolkata, India. *Malar J* 2006; *5:* 71.
13. Prajapati SK, Joshi H, Valecha N. *Plasmodium vivax* merozoite surface protein-3alpha: A high-resolution marker for genetic diversity studies. *J Vector Borne Dis* 2010; *47:* 85–90.
14. Rungsihirunrat K, Chaijaroenkul W, Siripoon N, Seugorn A, Na-Bangchang K. Genotyping of polymorphic marker (*MSP3α* and *MSP3β*) genes of *Plasmodium vivax* field isolates from malaria endemic of Thailand. *Trop Med Int Health* 2011; *16:* 794–801.
15. Khatoon L, Baliraine FN, Bonizzoni M, Malik SA, Yan G. Genetic structure of *Plasmodium vivax* and *Plasmodium falciparum* in the Bannu district of Pakistan. *Malar J* 2010; *9:* 112.

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