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Research

Inter-allelic recombination in the *Plasmodium vivax* merozoite surface protein 1 gene among Indian and Colombian isolates

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Abstract

Background: A major concern in malaria vaccine development is the polymorphism observed among different *Plasmodium* isolates in different geographical areas across the globe. The merozoite surface protein 1 (MSP-1) is a leading vaccine candidate antigen against asexual blood stages of malaria parasite. To date, little is known about the extent of sequence variation in the *Plasmodium vivax* MSP-1 gene (*Pvmsp-1*) among Indian isolates. Since *P. vivax* accounts for >50% of malaria cases in India and in Colombia, it is essential to know the *Pvmsp-1* gene variability in these two countries to sustain it as a vaccine candidate. The extent of polymorphism in *Pvmsp-1* gene among Indian and Colombian isolates is described.

Methods: The sequence variation in the region encompassing the inter-species conserved blocks (ICBs) five and six of *Pvmsp-1* gene was examined. PCR was carried out to amplify the polymorphic region of *Pvmsp-1* and the PCR products from twenty (nine Indian and 11 Colombian) isolates were sequenced and aligned with Belem and Salvador-1 sequences.

Results: Results revealed three distinct types of sequences among these isolates, namely, Salvador-like, Belem-like and a third type sequence which was generated due to interallelic recombination between Salvador-like sequences and Belem-like sequences. Existence of the third type in majority (44%) showed that allelic recombinations play an important role in PvMSP1 diversity in natural parasite population. Micro-heterogeneity was also seen in a few of these isolates due to nucleotide substitutions, insertions as well as deletions.

Conclusions: Intergenic recombination in the *Pvmsp-1* gene was found and suggest that this is the main cause for genetic diversity of the *Pvmsp-1* gene.

Background

There is an intense global effort to develop an effective vaccine in addition to the malaria control measures currently in use. Several vaccine candidate antigens have

been identified against different stages of the two main human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*, and are being developed as a part of a subunit vaccine [1,2]. One of the major concerns in malaria vaccine development is the polymorphic nature of the candidate vaccine antigens. Several *in vitro* and epidemiological studies have demonstrated that natural variations can abrogate immune recognition [3,4]. Thus, studies of sequence and antigenic diversity of malaria vaccine candidate antigens become a subject of considerable importance.

The merozoite surface protein 1 (MSP-1) of *Plasmodium* sp. is a large polypeptide of ~200 kDa and is one of the leading asexual blood stage vaccine candidate antigens [5]. A number of experimental studies with native and recombinant MSP-1, particularly its C-terminal fragments, have demonstrated the vaccine potential of MSP-1 [6,7]. Polymorphism has been reported in the *P. falciparum* MSP-1 (*Pfmsp-1*) gene among different isolates across different geographical areas [3]. Based on sequence variations, the *Pfmsp-1* gene has been divided into a number of blocks that are conserved, semi-conserved and variable in different species and isolates [5]. Broadly, *Pfmsp-1* gene sequences have been classified into two allelic families, Wellcome and PNG-MAD 20 type [5,8]. In addition, intragenic recombinations have been reported among these two parental alleles, resulting in polymorphism among different isolates around the world [9,10].

The gene encoding the merozoite surface protein 1 of *P. vivax* (*Pvmsp-1*) shows many similarities with those from other malaria species. The gene consists of 10 relatively conserved blocks alternating with regions of high diversity [5]. Like the *Pfmsp-1* gene, the *Pvmsp-1* gene has also been shown to be dimorphic; identified as Belem, type 1 [11] and Salvador-1, type 2 [12] forms. In comparison to *Pfmsp-1* polymorphism studies, studies on *Pvmsp-1* polymorphism are limited. Several regions of *Pvmsp-1* have been amplified and sequenced from field isolates collected from Sri Lanka, Colombia, Brazil, Thailand, Korea and China [13-18]. A third allele type (type 3) has been reported among these isolates and it has been suggested that this allele has arisen due to intragenic recombination between the Belem and Salvador alleles [18,19]. Recently, a detailed study of *P. vivax* merozoite surface protein 1 (*Pvmsp-1*) gene of 40 isolates from different geographical areas (Thailand, Brazil, Bangladesh, South Korea, Vanuatu and Japan) revealed mosaic organization of the *Pvmsp-1* gene and heterogeneity in the frequency of allelic recombination among different isolates [16].

The genetic characteristics of *P. vivax* circulating in distant endemic regions have not been studied so far. Therefore, the molecular basis underlying phenomena such as resistance of *P. vivax* to chloroquine in America and Asia (20,21) and effective immune responses (which is essential for vaccine design), have not being fully understood. Since PvMSP-1 is a potential vaccine candidate for *P. vivax*, it is important to further analyse the extent of polymorphism from field isolates across the globe. In the present study, the extent of polymorphism in one of the variable regions of *Pvmsp-1* gene among Indian and Colombian isolates was investigated. This study is important as there is no information of *Pvmsp-1* alleles and their diversity among Indian isolates in spite of *P. vivax* being one of the major causes of malaria in India [22].

Subjects and methods

Study area and blood collection

To study the polymorphism in different *P. vivax* isolates, a polymorphic region of the MSP-1 between blocks 5–6 corresponding to base pairs 1984–2653 (Belem strain) was amplified by PCR (Fig. 1). To begin with, venous blood samples were collected from 50 Colombian and 25 Indian symptomatically infected patients after verbal consent. Colombian isolates were from the malaria clinics of the northwest region of Colombia (Turbo). Between 1996–2000, this region recorded highest number of malaria cases with a mean parasite annual index of about 40. *P. vivax* accounts for 60% of the total number of malaria cases (23). Indian isolates were from the north (Aligarh) and northeast (Nagaland) regions of India, which are more than 500 km apart. In Aligarh, *P. vivax* prevalence is high (60%) while in Nagaland, *P. vivax* accounts for 40% of the total number of malaria cases (24). Blood was either collected into EDTA-containing vials or spotted onto Whatman-3 filter sheets.

DNA preparation and PCR amplification

P. vivax genomic DNA was isolated from filter discs using the Chelax extraction procedure [25] and from infected blood by proteinase K digestion, followed by four rounds of phenol-chloroform extraction. PCR was carried out to amplify the polymorphic region of *Pvmsp-1* for 30 cycles of 94°C for one minute 55°C for one minute and 72°C for two minutes and a final primer extension at 72°C for five minutes. The forward primer used was 5'-GGGAATTCTACTTGATGGTCCTC-3' (PV1), and the reverse primer was 5'-GGAATTCTTGTGACATGCGTAAGCG-3' (PV2).

Sequencing, alignment and data analysis

PCR products from twenty (nine Indian and 11 Colombian) isolates were run on a 2% agarose gel and the fragments were purified from gel using a Novagen gel DNA kit. Gel purified fragments were sequenced in both directions using an automated DNA sequencer, ABI Prism 310 (Applied Biosystems). Two independent PCR products were sequenced for each isolate. The deduced amino acid sequences of the PvMSP-1 from twenty of the isolates are

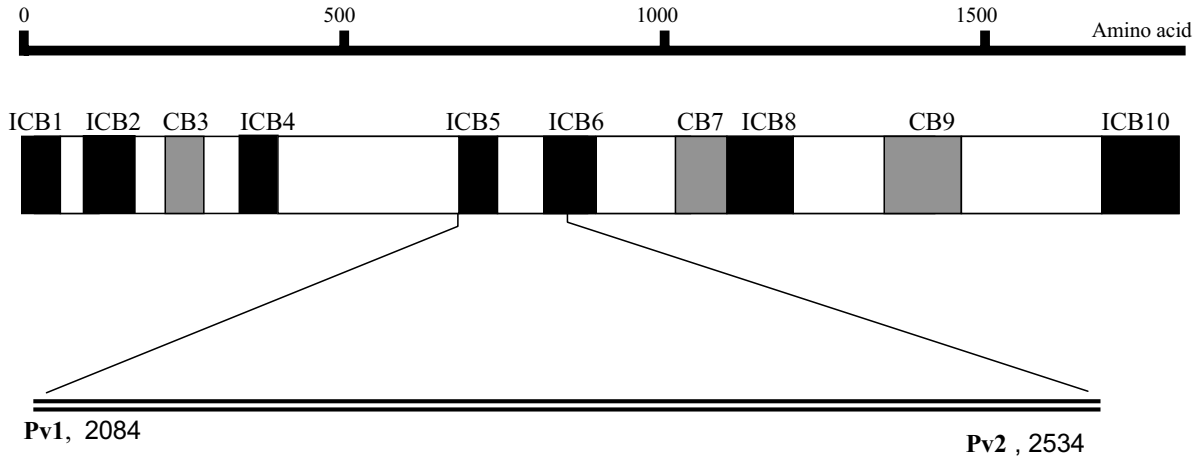

Figure 1
Schematic representation of the *P. vivax msp-1* gene. Boxes representing interspecies conserved blocks (ICBs), conserved blocks (CBs) and variable blocks are filled, hatched and opened respectively. A scale bar for nucleotide number and relative position of primers (Pv1 and Pv2) used for the polymerase chain reaction are indicated at the top and bottom respectively. Sequence and location of the primers used for PCR amplification are indicated at the bottom.

shown in figure 2, aligned with the corresponding Belem and/or Salvador sequences. Based on sequence comparison data, the sequences were classified. These DNA sequences are deposited under GenBank and EMBL Accession Nos. AY 229861 – AY 229867, AJ 582077-79, AJ 582111-20

Results and discussion

PCR amplification

PCR-amplification revealed size variations (~450 bp and ~520 bp) among these isolates. Size variations were seen in both Colombian as well as Indian isolates. Among the Colombian isolates, twenty-nine out of fifty isolates showed amplification and sixteen out of twenty five Indian isolates showed amplification. Agarose gel electrophoresis revealed a predominance of ~520 bp species among Indian isolates.

Classification of isolates

Based on sequence comparison data, we classified these sequences into three basic types: Type 1, Type 2 and Type 3a. This classification was done as previously described by [15] for the Thai isolates. Type 1 had sequences similar to the Belem allele, but with a lesser number of Gln residues in the middle. Isolates CM 12AN, CM 13N, CM 17N and CM 19N showed 16–18 glutamine residues in comparison to Belem allele that had 23 Gln residues. These four Colombian isolates also showed few other variations as a result of nucleotide substitutions: All these isolates showed Val (GTA) at position 742 instead of Asp (GAT) seen in Belem allele. Likewise isolates CM 13N, CM 17N and CM 19N had Asp (GAT) for Gly (GGT) at position 810. Similar substitutions had been reported for Thai isolates TD439A, TD424 and TD 425A. In the present study we did not find Type 1 sequence among Indian isolates. Type 2 sequences, which are similar to Salvador alleles, were found among both Indian and Colombian isolates. These sequences showed slight variations from the Salvador allele. Such variations have been previously reported for the Thai isolates [15]: a 3 nt insertion encoding Gln (CAA) and Pro (CCA) was seen in isolates CM 51, CM 70, IA 14 and IA 6, respectively between Gln 744 and Pro 745 of the Salvador sequence. One such insertion Gln (CAA) has been previously reported for a Thai isolate T128. In addition, four non-synonymous nucleotide substitutions were seen in these Indian as well as Colombian isolates: CM 51 and IN 8 had a change from Val (GTA)→Ala (GCA) at position 750, that was also reported in Thai isolates T117, T128 and TD414. Isolates CM 51, CM 19, CM 70, IN 8, IA 6 and IA 14 had Thr (ACT)→Ile (ATT) at position 796 as seen among Thai isolates TD 207A and T117. There was a change from Ala (GCC)→Val (GTC) at position 813 in isolates CM 19, IA 6 and IA 14 similar to the Thai isolate T 117. Likewise, CM 19, IN 1 and IA 14 had a

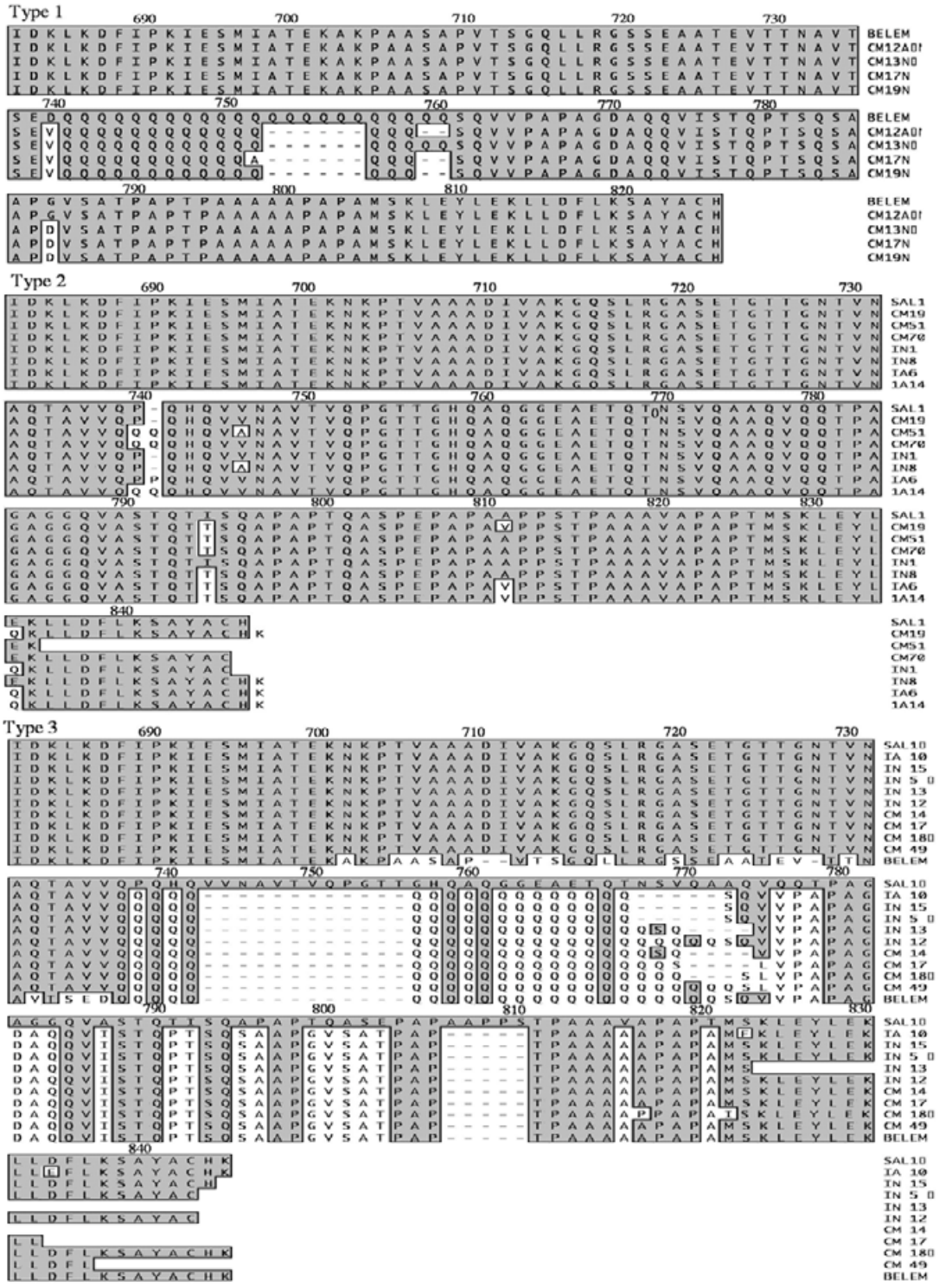

Figure 2
Amino acid sequence alignment of *PvMSP-1* polymorphic region between blocks V-VI of twenty field isolates from India and Colombia with that of Salvador-1 (Sal-1) and Belem isolates. Dots and Dashes represent identical residues and deletions respectively.

Glu (GAG)→Gln (CAG) substitution at position 839, which had been reported in isolates from other geographical origins [16].

Type 3a was the third type of sequence seen among these isolates (Fig. 3). This type of sequence is characterized by a combination of a Salvador-like sequence at the 5' half of this region and a Belem-like sequence at the 3' end. Nine of the twenty isolates (~44%) sequenced showed this type of recombination. Similar types of recombination have been reported earlier for Thai, South Korean, Chinese, Sri Lankan as well as in Colombian isolates (16). The number of Gln repeats varied between 15 to 18 among these isolates. These sequences were conserved in regions 5' to Gln repeats. However, two out of nine isolates showed amino acid changes at two positions in the 3' end (Fig 2). The presence of a type 3a sequence in more than 40% of the isolates in the present study indicates that inter-allelic recombination is one of the main causes for *Pvmsp-1* gene diversity among *P. vivax* Indian and Colombian populations.

The main goal of the present study was to determine and compare the extent of polymorphism in *Pvmsp-1* in two different geographical regions of the world (India and Colombia). This is important, as data regarding the sequence variations among *P. vivax* Indian field isolates is scanty. A region was selected between the two interspecies conserved blocks 5 and 6 of *Pvmsp-1* for this study. Though many recombination sites have been identified within and between the variable blocks of the *Pvmsp-1* gene, this region was chosen as it has emerged as an important genetic marker for *P. vivax* polymorphism to detect mixed infection as well as recombination events between the two parental Salvador and Belem alleles [19]. Results of the present study showed that in both Indian and Colombian isolates, a similar degree of polymorphism for the *Pvmsp-1* gene exists and inter-allelic recombination is quite common among these isolates. It is noteworthy that most of the amino acid changes among Indian and Colombian isolates match with the changes seen in other isolates from other geographical areas in particular, the Thai isolates. Though Belem-like sequence was not observed among any Indian isolates in the present study, it may be due to limited sample size. The presence of the Type 3a sequence among Indian isolates clearly shows that Belem-like isolates exist among Indian population. There are two schools of thought with regards to *Pvmsp-1* polymorphism. Some authors have stated that intragenic recombination is a rare event in *P. vivax* and that is not the main cause for antigenic polymorphism [26,27]. Other groups argue that recombination is the main source of antigenic diversity in PvMSP-1 [17,13,15,16]. Another feature of the recombination events in *P. vivax* isolates in the present study was that the recombination took place in a polymorphic segment of the gene, the polyQ region. This is very much similar to that reported for other isolates in Asia [16,18]. Thus, the recombination events in *Pvmsp-1* differ from *Pfmsp-1* where recombination between *MSP-1* alleles occurs in the conserved segments surrounding the polymorphic regions whereas in *Pvmsp-1*, the recombination occurs at the polymorphic regions [8].

Conclusion

The existence of a large number of Type 3a sequences among Indian and Colombian isolates generated by the fusion of Belem and Salvador sequences suggests that recombination is the main mechanism in Pvmsp-1 allelic diversity generation among the isolates around the globe. It is believed that long lasting and recurrent parasitemia often associated with relapse, a characteristic of *P. vivax* is responsible for this high rate of interallelic recombination. As allelic polymorphism is one of the greatest hurdles in mounting a protective immune response against the genetically diverse *P. vivax*, further experiments are required to determine the impact of recombination events in the immunogenicity of PvMSP-1 antigen in natural populations.

Authors' contributions

MA was involved in amplification of the samples. SS was involved in amplification, sequencing of the samples and preparation of the manuscript. Both MA and SS contributed equally. GA participated in sample collection in India. AM was involved in sample collection and sequence alignment. EM, CM and BS were involved in sample collection from Colombia. VSC, the group leader participated in the design and co-ordination of the study. PM supervised the overall work.

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