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Genotyping of *Plasmodium vivax* infections in Sri Lanka using *Pvmsp-3α* and *Pvcs* genes as markers: A preliminary report

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Abstract. *Plasmodim vivax* malaria accounts for more than 90% of malaria cases in Sri Lanka. There is limited information on the genetic heterogeneity of *P. vivax* in endemic areas of the country. Here we have assessed the potential of two *P. vivax* genes as genetic markers for their use in genotyping parasites collected from the field. DNA extracted from Geimsa-stained *P. vivax* positive slides were genotyped at two polymorphic loci: the *P. vivax* merozoite surface protein 3-alpha (*Pvmsp*-3α) and circumsporozoite protein (*Pvcs*). Analysis of these two genetic markers revealed 11 distinguishable variant types from the two genetic markers: 4 for *Pvcs* and 7 for *Pvmsp*-3α. The results indicate that the *P. vivax* parasite population is highly diverse in Sri Lanka, despite the low level of transmission.

INTRODUCTION

Vivax malaria is usually a non-lethal infection but its prolonged and recurrent nature can have major deleterious effects on personal well-being, growth and on the economic performance at the individual, family, community and national levels. Currently, it accounts for more that 90% of all malaria infections in Sri Lanka and chloroquine still remains the first line drug for vivax malaria. Rapid genotyping methods have been developed for the study of different aspects of malaria infections including studying parasite population dynamics, epidemiological analyses investigating geographical differentiation of parasites, assessment of drug efficacy, and determining whether an infection is a result of a new infection or a relapse. However, little is known about the genetic diversity of the *Plasmodium vivax* parasite population in Sri Lanka.

Here we present a *P. vivax* Polymerase Chain Reaction (PCR)/Restriction Fragment Length Polymorphism (RFLP) tool that will facilitate the analysis of genetic diversity of *Plasmodium vivax* parasites in Sri Lanka. Two highly polymorphic *P. vivax* single copy genes were used independently for molecular typing in this study: *Pvmsp*-3α gene coding for the merozoite surface protein 3α (Galinski *et al.*, 1999) and *Pvcs* gene coding for the circumsporozoite protein (Rosenberg *et al.*, 1989).

The *Pvmsp*-3α is a member of an *Msp*-3 gene family that is genetically related to *Plasmodium falciparum* merozoite surface protein -3 (*Pfmsp*-3; Oeuvray *et al.*, 1994). Amplification and both size and sequence analysis of a series of *Pvmsp*-3α genes from laboratory strains- Belem (Del Portillo *et al.*, 1991) and Sal-1 (Gibson *et al.*, 1992) and patients isolates of *P. vivax* from diverse geographic regions have indicated a high degree of polymorphism especially in the central domain of the molecule which has been predicted to form a coiled-coil tertiary structure (Bruce *et al.*, 1999; Galinski *et al.*, 1999; Cui *et al.*, 2003; Kim *et al.*, 2006).

The gene coding for the circumsporozoite protein of *P. vivax* comprises a central repetitive domain composed of a 27 bp element that can be repeated a variable time (Rosenberg *et al.*, 1989) that has been shown to be highly polymorphic (Imwong *et al.*, 2005; Kim *et al.*, 2006). Therefore, the genotyping strategy was focused on this repeat region which can generate size polymorphism amenable to detection by electrophoresis.

Using these genetic markers we provide preliminary evidence that the *P. vivax* parasite population is highly polymorphic and that infections with multiple genotypes of *P. vivax* are present in this study area of "Kataragama" in Sri Lanka.

MATERIALS AND METHODS

Study site and transmission kinetics

Malaria transmission in Sri Lanka is unstable, fluctuating seasonally as well as yearly and the population is prone to suffer epidemics at fairly regular intervals (Fonseka & Mendis, 1987). Presently, *P. vivax* infections predominate with a very few *P. falciparum* cases being reported. *P. malariae* was also prevalent till the late 1960s, after which, transmission appears to have been interrupted (AMC, 1992).

The study area "Kataragama" (6° 25' N, 81° 20' E), is situated in the South-Eastern region of Sri Lanka, in the low country coastal plains (60 m above the sea level) of the dry zone. Malaria transmission in this region is seasonally endemic for both *P. vivax* and *P. falciparum* (Mendis *et al.*, 1990), and the majority of the population, mainly rural peasants, is engaged in agricultural pursuits (Gunawardena *et al.*, 1998).

Blood samples

Finger prick blood samples were collected after obtaining informed consent from 12 patients from Kataragama with symptomatic *P. vivax* malaria between February and December 2000. Thick and thin blood smears were prepared for microscopy which were used for extracting parasite DNA. Ethical clearance for this study was obtained from the Ethics Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka.

Extraction of parasite DNA from Giemsa-stained slides

Slides were first de-stained with 1% acid alcohol, and, the thin film was scraped using a sterile razor blade into a sterile 1.5 ml eppendorf tube with 50 µl of lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, and 1% Tween 20. This was repeated 2 times with further 50 µl of lysis buffer each time. To this, 10 µl of 20 mg/ml proteinase K was added and incubated for 2 hours at 56 °C, and extracted twice with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1). The aqueous layer was then precipitated with 2.5 volumes of isopropanol at – 20 °C overnight and the resulting pellet was washed with 200 µl of 70% ethanol and dissolved in 20 µl TE, pH 8.0.

PCR amplification

A nested PCR approach was adopted for both *Pvmsp*-3α (Cui *et al.*, 2003) and *Pvcs* (Imwong *et al.*, 2005) genes. Primary PCR amplification of the *Pvmsp*-3α gene was carried out in a reaction volume of 20 µl using 5 µl of DNA extract. One unit of Taq polymerase (Promega, Madison, USA) was used per reaction, with oligonucleotide primers (P1- CAG CAG ACA CCA TTT AAG G, P2- CCG TTT GTT GAT TAG TTG C) at a final concentration of 0.1 µM and each of the deoxynucleotide triphosphates at 150 µM, in reaction buffer containing 2.5 mM $MgCl_2$. The PCR parameters were as follows: an initial denaturation of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, extension at 70 °C for 2.5 min and a final extension step at 70 °C for 5 min.

Nested amplification of the *Pvmsp*-3α gene was carried out in a reaction volume of 20 µl using 1 µl of the primary reaction. One unit of Taq polymerase (Promega, Madison, USA) was used per reaction, with nested oligonucleotide primers (N1-GACCAGTGTGATACCATTAACC, N2-ATACTGGTTCTTCGTCTTCAGG) at a final concentration of 0.1 µM and each of the deoxynucleotide triphosphates at 150 µM, in reaction buffer containing 2.5 mM $MgCl_2$. The PCR parameters were as follows: an initial denaturation of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s, extension at 70 °C for 2.5 min and a final extension step at 70 °C for 5 min.

PCR amplification of the *Pvcs* gene was carried out using VCS-OF (ATGTAGATC TGTCCAAGGCCATAAA) and VCS-OR (TAATTGAATAATGCTAGGACTAACAATATG) outer primers and VCS-NF (GCAGAA CCAAAAAATCCACGTGAAATAAG) and VCS-NR (CCAACGGTAGCTCTAACTTTATC TAGGTAT) nested primers. All amplification reactions were carried out in a total volume of 20 µl and in the presence of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 250 nM of each oligonucleotide primers, 125 µM of each dNTPs, and 0.4 units of Taq polymerase. Primary amplification reactions were initiated with 1 µl of the template genomic DNA, and 1 µl of the primary PCR product was used in all nested amplifications. The PCR parameters were; 95 °C for 5 min for initial denaturation, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 2 min for primary reaction and 62 °C for 2 min for nested reaction, extension at 70 °C for 2 min and a final extension step at 70 °C for 5 min.

For direct analysis of all amplified fragments, 10 µl of the amplified PCR product was mixed with 2 µl of loading buffer and visualized in 0.8% agarose gels containing 0.25 µg/ml of ethidium bromide. One kb ladder (Gibco-BRL, Gaithsburg, MD, USA; 0.1 µg/lane) was used for sizing of the products.

RFLP analysis of nested *Pvmsp*-3α PCR products

RFLP analysis of *Pvmsp*-3α nested PCR products was carried out with *Hha*I restriction enzyme according to Bruce *et al.*, (1999). Four µl of nested PCR product was digested with *Hha*I in 20 µl reaction volumes (5 Units of enzyme per reaction; Promega) in buffer supplied with enzymes at 37 °C for 5 hr. The DNA fragments were visualized under UV illumination after electrophoresis on 1.8% agarose gels containing 0.25 µg/ml of ethidium bromide. Sizing of products was carried out using a DNA marker run adjacently (0.1 µg/lane; Roche Diagnostics, USA). Major alleles were classified based on the differences in restriction banding patterns.

RESULTS

PCR amplification

Of the 12 PCR positive samples, 11 corresponded to samples of primary infection origin (M321, M284, M204, M210, M272, M281, M143, M201, M134, M122, M117, M120) and 1 sample (M294) corresponded to a second infection of the same individual-M134 after 2 months.

Nested amplification of *Pvmsp*-3α gene

Based on the size of the PCR products, two major types were identified by nested amplification of the *Pvmsp*-3α gene. The product sizes corresponded to approximately 1.9 kb – type I (Figure 1(A); lanes 2-11) and 1.1 kb – type II (Figure 1(A); lanes 12-13) in size. Nested amplification products generated from the *P. vivax* Belem laboratory strain was of approximately 1.9 kb in size (Figure 1(A); lane 1) as expected from known sequences. Type I, which corresponded to the expected size of the published sequence of the Belem strain, was the most predominant (10/12).

Pvmsp-3α RFLP Product Analysis

All PCR-RFLP products showed a major size polymorphism (Figure 1 (B)).The sizes of the RFLP fragments of the Belem control DNA was as expected from their sequence characterization. The RFLP pattern of all 12 isolates showed size conservation of the largest fragment (Figure 1 (B); approximately 1000 bp), while smaller fragments showed variation in size. Although the ~ 1kb band of all samples was slightly polymorphic, it was not included for distinguishing different *Pvmsp*-3α alleles because the size difference could not be easily resolved with our gel electrophoresis conditions. Smaller bands ranging from ~ 200 bp to ~500 bp were used for RFLP analysis, from which 7 different RFLP patterns were identified (Figure 1(B); Table 1). Although we did not detect any samples which resembled the Belem prototype based on the RFLP patterns generated, sample M210 corresponding to type Ib appears to resemble the predicted RFLP pattern of Sal-1 prototype.

However, in the samples of M281, M134 and M143, the sum of the RFLP fragment sizes was significantly greater than the size of the uncut PCR product (Figure 1(A); lanes 6, 7 and 9), indicating the presence of more than 1 *Pvmsp-3*α allele. Therefore, these 3 samples showed evidence of infections with mixed genotypes. This is of particular importance and interest in samples M134 and M294, as the latter sample is from the same patient after 2 months of the primary infection. Parasites of M134 infection harbour two genotypes (Ia and Id) at the *Pvmsp*-3α locus and parasites corresponding to infection M294 has the variant type Id at this locus. Therefore, it could be that the secondary infection (M294) is a relapse of one of the strains (Id) in the primary infection. From the 9 samples in which single infections were detected (Figure 1(B); lanes 2-5 and 8, 10-13), 6 different *Hha*I RFLP-patterns were detected.

The observed PCR-RFLP patterns of the *Pvmsp-3*α gene further demonstrate that the *P. vivax* parasites in Sri Lanka are highly diverse. The polymorphic nature of *Pvmsp-3*α makes it an ideal marker for distinguishing different infections in epidemiologic studies.

Nested amplification of *Pvcs* gene

After nested PCR, bands of ~ 600-750 bp were obtained which generated 4 product variants based on the size of the fragment (Figure 1 (C)). The most common allele type was "4" (5/12; Figure 1 (C)).

DISCUSSION

We have presented here the first molecular analysis of *Pvmsp-3*α and *Pvcs* genetic markers in *P. vivax* infections in Sri Lanka to determine the genetic heterogeneity of the parasite population.

The gene coding for the circumsporozoite protein of *P. vivax* comprises a central repetitive domain composed of a 27 bp element that can be repeated a variable time. Therefore, the genotyping strategy was focused on this repeat region which can generate size polymorphism amenable to detection by electrophoresis. Four allelic types distinguishable by size were observed in the Sri Lankan isolates analyzed (Figure 1 (C)).

Nested PCR amplification of the *Pvmsp-3*α locus generated 2 different amplified products based on size, which corresponded to ~1.9 and 1.1 kb, indicating size differences in the major central ± helical repeat region of the MSP3-α molecule (Galinski *et al.*, 1999). These two size variations have been previously detected in samples from Kolkata, India (Kim *et al.*, 2006). In order to increase the genotyping resolution of *Pvmsp*-3α as a marker, an additional RFLP step was used, and based on this, 7 different variant types were identified. By combining the two genetic markers, it was possible to show that 11 out of 15 parasites were present with different, distinct genotypes (Table 1).

In a previous study conducted in the same site "Kataragama" in Sri Lanka using another highly diverse genetic marker, the merozoite surface protein-1 (*Msp*-1) revealed that the *P. vivax* parasite population in this region is highly diverse and also the presence of infections with multiple genotypes (Manamperi, 2002). In this study, sequence analysis of 19 *P. vivax* isolates in

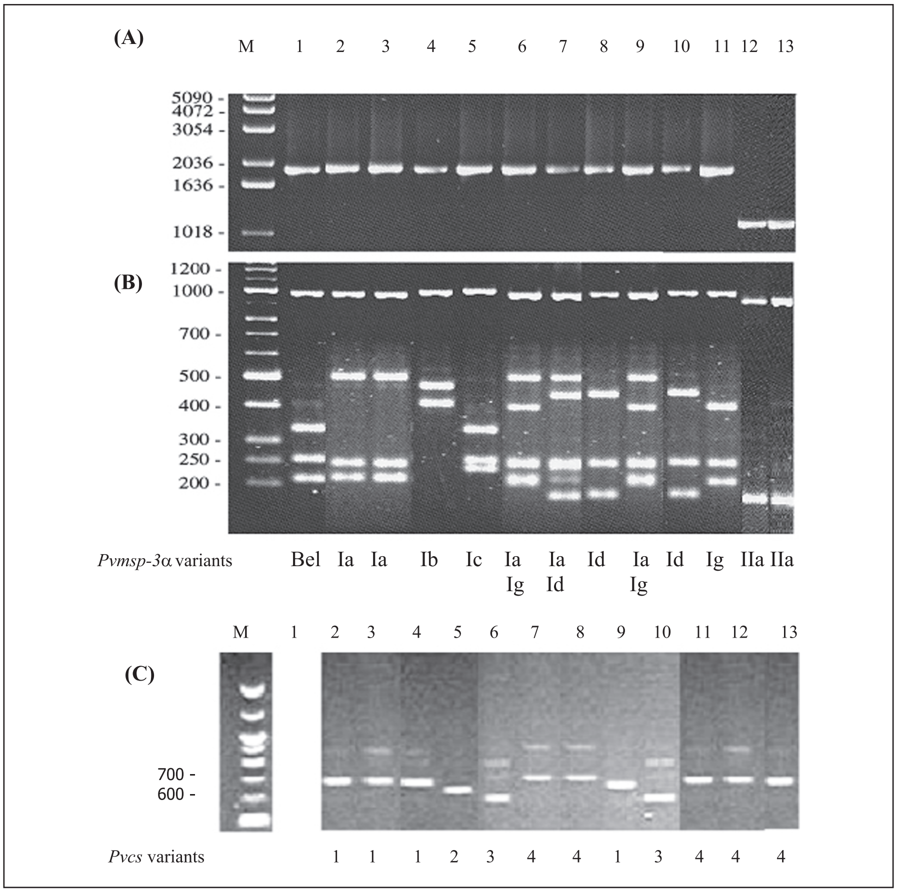

Figure 1: (A) Undigested nested PCR products of *Pvmsp-3α* gene; "M" and "1" denote the marker and Belem control DNA respectively; lanes 2-13 contain amplified DNA from patient's samples M272, M321, M210, M284, M281, M134, M294, M143, M201, M204, M122, and M117. **(B)** Nested PCR products digested with *Hha*I; Predicted RFLP patterns from standard laboratory strains are (Bruce *et al.*, 1999): Belem allele with *Hha*I: 963 bp, 330 bp, 254 bp, 211 bp, 75 bp, and 63 bp; Sal-1 allele with *Hha*I: 975 bp, 465 bp, 405 bp, and 63 bp. **(C)** Nested PCR products of *Pvcs* gene.

Table 1: Genotype analysis with *Pvmsp*-3α and *Pvcs* genes

Sample	*Pvmsp*-3α allele (7 variable types)	*Pvcs* allele (4 variable types)	Total no. of variable types (with 2 markers)(11)
M272	Ia	1	Ia 1
M321	Ia	1	Ia 1
M210	Ib	1	Ib 1
M284	Ic	2	Ic 2
M281	Ia + Ig	3	Ia 3 + Ig 3
M134	Ia + Id	4	Ia 4 + Id 4
M294	Id	4	Id 4
M143	Ia + Ig	1	Ia 1 + Ig 1
M201	Id	3	Id 3
M204	Ig	4	Ig 4
M122	IIa	4	IIa 4
M117	IIa	4	IIa 4

the 3' region of the *Msp*-1 gene (1200 bp fragment) revealed 10 different genotypes. Although DNA sequencing of different isolates would have provided finer details of genetic heterogeneity, the alternative use of the PCR/RFLP protocol to detect sequence diversity is more appropriate in which large numbers of samples could be analyzed. In the present study, detection of multiple genotype infections in 3 of 12 samples was possible from size determination of products in RFLP analysis. It could be concluded that these two genes: *Pvmsp*-3α and *Pvcs*, in combination could be considered suitable genetic markers for the analysis of *P. vivax* population in Sri Lanka. The methodology combining the two genetic markers described here could be useful in the assessment of drug efficacy, differentiating primary infections with relapse infections and elucidating information on the epidemiology of this parasite species.

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