Genetic diversity of *Plasmodium vivax* in a hyperendemic area predominated by *Plasmodium falciparum*; A preliminary study

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Abstract. Understanding the genetic diversity, extent and distribution of variant forms of *Plasmodium vivax* parasites is crucial in the development of effective control measures and in Orissa, a hyperendemic state in the eastern part of India, the polymorphic nature of *P. vivax* isolates is largely lacking. The result of the study analyzing two highly polymorphic single copy genes for *P. vivax* circumsporozoite protein (*pvcs*) and *P. vivax* merozoite surface protein 3α (*pvmsp3α*) shows that the parasite population is highly heterogeneous (33 distinct genotype from 35 isolates) in Orissa. However, the observation of the multiplicity of infection value of 1.34 and high frequency distribution of certain genotype with respect to individual marker (the VK247b allele with a frequency of 0.37; VK210e with 0.25 and VK210c with 0.14) suggests that the parasite population are likely to be under selective pressure and may either be due to preferential production of sporozoites carrying these variants in the available anopheline mosquito species of the state or selection of particular genotypes by host immune pressure. Moreover, although *P. vivax* in South-East Asia indicates an overall predominance of VK210 which is thought to be the best adapted variant of *pvcs* repeat type, the almost equal prevalence of both repeat type of *pvcs*; VK210 and VK247 in the present study is unexpected and needs further study for clarification.

INTRODUCTION

Malaria is one of the major public health problems in India and although, *Plasmodium falciparum* has shown an increased incidence during the last few decades, *Plasmodium vivax* continues to be a predominant species contributing around 60% of total cases of malaria in India (Maestre *et al.*, 2004). According to the National Vector Borne Disease Control Programme (NVBDCP) 2009; Orissa, a hyperendemic state in the eastern part of the country contributes 24.5% of total malaria cases, 40.3% of *P. falciparum* cases and 18% of all reported deaths due to malaria, which is the highest in India. The climate of the state is favorable for the perennial transmission of malaria in many parts with a seasonal peak from July to October and all four *Plasmodium* species of human malaria have been reported from the state. Of which, *P. falciparum* is the major cause of malaria (>80%) followed by *P. vivax* (10-15%), *Plasmodium malariae* (up to 3%) in certain foothill areas and *Plasmodium ovale* being restricted to Koraput district of the state (Ranjit, 2006). In a recent study, we have shown the prevalence of *P. malariae* up to 44.6% and *P. vivax* up to 15% during the peak season of malaria transmission while the prevalence of *P. falciparum* remain unchanged by PCR method of diagnosis (Dhangadamajhi *et al.*, 2009).
Genetic diversity study of *Plasmodium* parasite may help to predict the rate of spread of drug resistance mutations or escape from vaccine induced responses and has the practical importance of development and deployment of control strategies (Cui et al., 2003a). Majority of *Plasmodium* genetic diversity studies have been based on genes coding for antigenic determinants such as circumsporozoite surface protein (*csp*) and merozoite surface protein (*msp*) of *P. falciparum*, which is responsible for the most severe disease (Rich & Ayala, 2000). In recent years, although similar genetic diversity studies on *P. vivax* field isolates have been carried out in many geographic regions where *P. vivax* predominates, the study is limited in areas where it is less frequent. Several markers are available for molecular epidemiologic study of *P. vivax* and includes genes for circumsporozoite protein (*pvcs*), merozoite surface protein-1 (*pvmsp1*) merozoite surface protein-3α (*pvmsp-3α*) and 3β (*pvmsp-3β*), apical membrane antigen 1 (*pvama1*) and gametocyte antigen 1 (*pvgam1*) etc (de Souza-Neiras et al., 2007). However, the first 3 markers have been most widely used for population diversity study of *P. vivax* due to their highly polymorphic nature. The *pvcs* gene has a central repeat domain that varies in sequence (VK 210 has a nonapeptide repeat unit Gly-Asp-Arg-Ala-Asp/Ala-Gly-Gln-Pro-Ala and VK247 has Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly) and number of repeat units (Rosenberg et al., 1989; Arnot et al., 1985). The VK210 and VK247 have a worldwide distribution and four subtypes from VK210 and two subtypes from VK 247 can be differentiated by restricted enzyme digestion to show polymorphisms in both the pre- and post-repeat region (Imwong et al., 2005). Similarly, the *pvmsp-3α* gene encodes a merozoite surface protein with an alanine-rich central domain that is predicted to form a coiled-coil tertiary structure and is a possible immunogens and vaccine candidate (Galinski et al., 1999). Use of the *pvmsp-3α* gene as genetic marker has been recently validated (Bruce et al., 1999; Rayner et al., 2002; Cui et al., 2003b; Zakeri et al., 2006). Thus, in the proposed study attempt has been made to study the genetic diversity of *P. vivax* in a region predominated by *P. falciparum* analyzing two highly polymorphic single copy genes the *pvcs* and the *pvmsp-3α*.

**MATERIALS AND METHODS**

The present study was a cross-sectional malaria survey carried out in Orissa during the peak season of malaria transmission in the year 2008 as described previously (Dhangadamajhi et al., 2009). Blood samples were collected from volunteered people complaining of malaria and were screened for the presence of *P. vivax* by both light microscopy and PCR diagnosis methods. The study was approved by the Ethics Committee of the Regional Medical Research Centre, Bhubaneswar.

**Genotyping of *pvcs* and *pvmsp-3α***

Only those samples which were found positive for *P. vivax* by PCR diagnosis method were genotyped for the two highly polymorphic single copy genes *pvcs* and *pvmsp-3α*. Genotyping of *pvcs* was done by nested PCR followed by PCR-RFLP method as described by Imwong et al. (2005) and that of *pvmsp-3α* was done following the protocol of Bruce et al. (1999). The DNA fragments obtained for *pvcs* and *pvmsp-3α* gene following PCR were analyzed by 1.8% and 0.8% agarose gel electrophoresis respectively. For PCR-RFLP analysis, the digested products of both the genes were applied to 2% agarose gel. In order to see the cross-species amplification of genes, the primers for both of these genes were also used to amplify the DNA of *P. falciparum* and *P. malariae* monoinfected samples (five samples from each) and no amplifications were observed.

**RESULTS**

A total of 38 *P. vivax* isolates collected during 2008 (as described by
Dhangadamajhi et al., 2009) were genotyped for the two single copy genes pvcs and pvmsp3α. Genotyping of pvcs gene was successful in all the 38 isolates, from which 15 (39.5%) isolates were only VK210 repeat type (with three size variants of approximately a; 680bp, b; 710bp, and c; 740bp), 10 (26.3%) were only VK247 repeat type (with two size variants b and c) and 13 (34.2%) isolates had both repeat types. Thus, 5 different allelic forms of pvcs were detected by simple analysis of repeat type and fragment size. The b variant dominated in 82.1% (23/28) of VK210 repeat type and the c variant 86.9% (20/23) in VK247 type. The pre- and post-repeat analysis by PCR-RFLP method further divided these 5 different pvcs variants into 11 different allelic types (Table 1), 8 for VK210 and 3 for VK 247. Of the 38 samples analyzed, a total of 51 bands were observed with mean multiplicity of infection (MOI) to be 1.34 and the frequency distribution of the pvcs allele showed the highest frequency of 0.25 and 0.14 for VK210e and VK210c of VK210 pvcs type respectively. For VK 247 repeat type, the VK247b allele represented the highest frequency of 0.37 and was the predominant allele of the samples analyzed (Table 1). Genotyping of pvmsp3α was successful in 37 of the isolates (represented by three size variants A; 1100bp B; 1500bp C; 1900bp), from which two isolates identified as mixed genotype infection by pvcs with MOI of 2 for each showed mixed infections by pvmsp3α as well. The frequency of A allele was 72.9% (27/37), B was 13.5% (5/37) and C was 18.9% (7/37). The PCR-RFLP analysis of the samples showed 3 different Alu I patterns and 4 different Hha I patterns (Figure 1). The combinational analysis of the size variants and RFLP patterns differentiated 16 different alleles from 35 isolates (2 samples with mixed infections were excluded since the sum of RFLP fragments’ sizes was less than that of uncut product), all of which were distributed randomly with allele A all h1 had a highest frequency of 0.23. Combined genotype analysis of the two loci pvcs and pvmsp3α revealed that the P. vivax population of the state is genetically highly diverse and a total of 33 distinct genotypes were enumerated from 35 isolates and the most common two marker genotype were VK210e A a1 h1 and VK247b A a1 h1 which occurred at a frequency of 0.086 (3/35) each.

Table 1. Allele frequency of Pvecs variants classed by repeat type, size and presence of pre- and post repeat insertions

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Allele</th>
<th>Size variants</th>
<th>Pre/Post repeat</th>
<th>N (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VK 210a</td>
<td>a</td>
<td>N/Y</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>2</td>
<td>VK 210b</td>
<td>a</td>
<td>Y/N</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>3</td>
<td>VK 210c</td>
<td>b</td>
<td>N/Y</td>
<td>7 (0.14)</td>
</tr>
<tr>
<td>4</td>
<td>VK 210d</td>
<td>b</td>
<td>Y/Y</td>
<td>3 (0.06)</td>
</tr>
<tr>
<td>5</td>
<td>VK 210e</td>
<td>b</td>
<td>Y/N</td>
<td>13 (0.25)</td>
</tr>
<tr>
<td>6</td>
<td>VK 210f</td>
<td>b</td>
<td>N/N</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>7</td>
<td>VK 210g</td>
<td>c</td>
<td>N/Y</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>8</td>
<td>VK 210h</td>
<td>c</td>
<td>Y/N</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>9</td>
<td>VK 247a</td>
<td>b</td>
<td>Y</td>
<td>3 (0.06)</td>
</tr>
<tr>
<td>10</td>
<td>VK 247b</td>
<td>c</td>
<td>Y</td>
<td>19 (0.37)</td>
</tr>
<tr>
<td>11</td>
<td>VK 247c</td>
<td>c</td>
<td>N</td>
<td>1 (0.02)</td>
</tr>
</tbody>
</table>

Note: Y; presence of pre/post repeat region, N; Absence of pre/post repeat region
DISCUSSION

The genetic diversity of *Plasmodium* species depend on complex interplay among themselves, between parasite-host and/or parasite-vector and varies considerably in different geographic regions. Although numerous genetic diversity studies on *P. vivax* field isolates have been carried out in many geographic regions where it predominates, its population structure and distribution is limited in areas where it is less frequent. The present study shows the genetic diversity of *P. vivax* in a region where its frequency distribution is less and mostly predominated by *P. falciparum* and *P. malariae* (Dhangadamajhi et al., 2009).

The result of the study analyzing two highly polymorphic single copy genes *pvcs* and *pvmssp3α* shows that the parasite population is highly heterogenous (33 distinct genotype from 35 isolates). Earlier genetic analysis studies with respect to *pvcs* gene have demonstrated a global distribution of the two variant *pvcs* types; VK210 and VK247 with prevalence of individual genotype that varies geographically (Kain et al., 1992; Cui et al., 2003b; Zakeri et al., 2010 a & b). In general, *P. vivax* in South-East Asia indicates an overall predominance of VK210 (Kho et al., 1999; Kim et al., 2006; Moon et al., 2009). Further, VK210 type is prevalent in all geographical isolates and thought to be the best adapted variant (Machado & Póvoa, 2000) as supported by the observation of significant decrease over time in the proportions of VK247 in a two-year investigation performed in a malaria-hyperendemic area of Papua New Guinea in both the mosquito and human populations (Burkot et al., 1992). Contrast to this, in Mexico, a clear shift from VK210 to VK247 infections had been observed from 1996 to 1998 and the prevalence of *pvcs* phenotype appeared to be dependent on altitudes with VK210 being more prevalent in coastal region, VK247 in region above 170 meters from sea level and both phenotypes in region with intermediate altitudes (Rodriguez et al., 2000). However, the

Figure 1. PCR-RFLP patterns of *P. vivax* isolates based on *pvmssp 3α*. (A) The amplified products were digested with Hha I and the lane labeled with M represents 100bp ladder, L1 represents h1, L2 and L4 represents h4, L3 represents h2 and L5 represents h3 type of banding patterns. (B) The amplified products were digested with AluI and the lane M represents 100bp ladder, L1 and L4 represents a1, L2 and L5 represents a2 and L3 represents a3 type of banding patterns.
result of the present study demonstrated that both VK210 and VK247 are equally prevalent in the state and is in contrast to the study in Kolkata (West Bengal, a neighbor state of Orissa), India where VK210 was the predominant pvcs type (99.3%). While the reasons for geographical variation of pvcs variants in India are unknown, they may reflect (i) differences in vector composition and variability in vector competence for the different genotypes of *P. vivax* as different anopheline mosquitoes were found to be differentially susceptible to infections by VK210 and VK247 variants (Gonzalez-Ceron *et al*., 1999) (ii) transmission intensity and variation in frequency distribution of *Plasmodium* species in these regions, (iii) ethnic differences of human host or (iv) temporal population dynamics of different mosquito vectors which are not equally susceptible to infections by VK210 or VK247 parasite isolates (Rodriguez *et al*., 2000). Moreover, although, the diversity of *P. vivax* parasite could be well differentiated by the combined genotype analysis, the parasite population appeared to be under selective pressure as evident by their high frequency distribution of certain genotype with respect to individual marker. Further, the observation of the multiplicity of infection value of 1.34 suggesting the transmission intensity to be considerably high, the frequency distribution results showed that of the 11 different pvcs allelic types, the VK247b allele was the predominant of all with a frequency of 0.37 followed by VK210e (0.25) and VK210c (0.14) of VK210 pvcs type respectively and may either be due to preferential production of sporozoites carrying these variants in the available mosquito species of the state or selection of particular genotypes by host immune pressure.

In conclusion, the high levels of parasite population diversity and the high frequency observation of certain pvcs genotype belonging to both type of repeat units (VK210 and VK247) suggest that the parasite population are likely to be under selective pressure and may either be due to preferential production of sporozoites carrying these variants or selection of particular genotypes by host immune pressure. Thus, the development of recombinant malaria vaccines which includes the VK210, VK247 and any other variant CS epitopes may be effective in controlling *P. vivax* infections in this part and deserve further study for successful development and deployment of control strategies.

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**REFERENCES**


