## RESEARCH NOTE

# GENETIC DIVERSITY OF *PLASMODIUM VIVAX* MEROZOITE SURFACE PROTEIN-3α (*PVMSP*-3α) GENE IN JHAPA DISTRICT OF NEPAL

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**Abstract.** In Nepal, *Plasmodium vivax* accounts for approximately 80-90% of the malaria cases, but limited studies have been conducted on the genetic diversity of this parasite population. This study was carried out to determine the genetic diversity of *P. vivax* population sampled from subjects living in an endemic area of Jhapa District by analyzing the polymorphic merozoite surface protein-3α (*Pvmsp-3*α) gene by using PCR-restriction fragment length polymorphism. Three distinct genotypes were obtained from 96 samples; type A: 40 (71%), type B: 7 (13%), and type C: 9 (16%) which could be categorized into 13 allelic patterns: A1-A9, B1, B2, C1 and C2. These results indicated a high genetic diversity within the studied *P. vivax* population. As the transmission rate of malaria is low in Nepal, the diversity is most likely due to migration of people between the malaria endemic regions, either within the country or between Nepal and India. Similar prevalence of the three genotypes of *Pvmsp-3*α between the two countries likely supports the latter explanation.

**Keywords:** *Plasmodium vivax*, genetic diversity, merozoite surface protein-3α, Nepal

## INTRODUCTION

Among the four *Plasmodium* species, *Plasmodium vivax* is the leading cause of human malaria in Southeast Asia (SEA) (Hay *et al*, 2004). Around 2.5 billion people are at risk of becoming infected with *P. vivax* resulting in about 132-391 million cases of *vivax* malaria every year (Hay *et al*, 2004; Guerra *et al*, 2006). Although *P. falciparum* is predominant in Africa, *P. vivax* is the major cause of malaria outside Africa, of which 60% cases occur in SEA (Joshi *et al*, 2008). This species rarely causes fatality but has huge social and economic consequences because of its latent stages that can cause a debilitating

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disease that can persist for several months and even years (Carlton *et al*, 2008; Zakeri *et al*, 2010a). Knowledge of the epidemiology of *P. vivax* is limited, which is a major problem in the current efforts to eliminate *vivax* malaria (Marsh, 2010).

Polymorphic *P. vivax* genes such as the circumsporozoite protein gene (*Pvcsp*), merozoite surface protein 1 gene (*Pvmsp-1*) and merozoite surface protein-3α gene (*Pvmsp-3*α) are commonly used in studies of *P. vivax* genetic diversity (Cui *et al*, 2003; Kim *et al*, 2006), where in particular *Pvmsp-3*α is one of the most polymorphic genes studied to date (Zakeri *et al*, 2006; Manamperi *et al*, 2008). It encodes a merozoite surface protein comprising of three characteristic domains: an alanine-rich central domain having a series of heptad repeats predicted to form a coiled tertiary peptide structure, and highly conserved C- and N terminal regions (Galinski *et al*, 1999). The gene is genetically similar to *P. falciparum* merozoite surface protein-3 gene (*Pfmsp-3*) (Bruce *et al*, 1999; Manamperi *et al*, 2008) and Pvmsp3α is considered to be a promising vaccine candidate (Bruce *et al*, 1999). Three major alleles of *Pvmsp-3*α are found worldwide: type A (≈ 1.9 kb), B (≈ 1.4 kb) and C (≈1.1 kb) based on amplified PCR products (Bruce *et al*, 1999). Digestion of these three amplicons by the *Hha*I results in several fragments, which vary from 9-14 depending on the geographical origin, sample size and period of sample collection (Cui *et al*, 2003; Zakeri *et al*, 2006; Cristiano *et al*, 2008; Shahbazi *et al*, 2008; Khatoon *et al*, 2010; Prajapati *et al*, 2010).

In Nepal, malaria is endemic and around 22.5 million (80%) of the population are at risk (Epidemiology and Disease Control Division, 2008). The *P. vivax* species predominates and causes 80-90% of total malaria cases, which is one of the highest rates in the SEA region (EDCD/DoHS, 2008; EDCD/DoHS, 2009). Transmission of malaria usually occurs from March to November, with the highest peak in summer (June to August). Information on genetic diversity of *P. vivax* is limited in Nepal and it is necessary to design an effective control program. Therefore, the objective of this study was to examine the genetic diversity of *P. vivax* populations in a limited number of samples collected from subjects living in the malaria endemic Jhapa District of Nepal by studying the *Pvmsp-3*α polymorphism and relate the findings to those of neighboring countries.

## MATERIALS AND METHODS

### Blood samples

A total of 146 blood samples were collected from patients with symptoms of malaria visiting the Amda Hospital, Shrestha Malaria Clinic, Bhadrapur Hospital and Dhulabari Health Center of Jhapa District, Nepal in June to December 2009. Jhapa is a low land area sharing borders with other malaria endemic districts of Nepal (Fig 1). Further, it shares borders with the malaria endemic regions of India namely Assam, Uttar Pradesh and Bihar (EDCD/DoHS, 2008). Laboratory diagnosis was performed by microscopy and positive blood samples were preserved on filter paper (Whatman #3) as dried blood spots.

Informed consents were obtained from patients or legal guardians for children before collecting samples. Ethical clearance was obtained from Nepal Health Research Council, Kathmandu.

### DNA extraction and genotyping of *P. vivax*

DNA from the blood spots were extracted using the chelex method (Wooden *et al*, 1993) and *Plasmodium* species

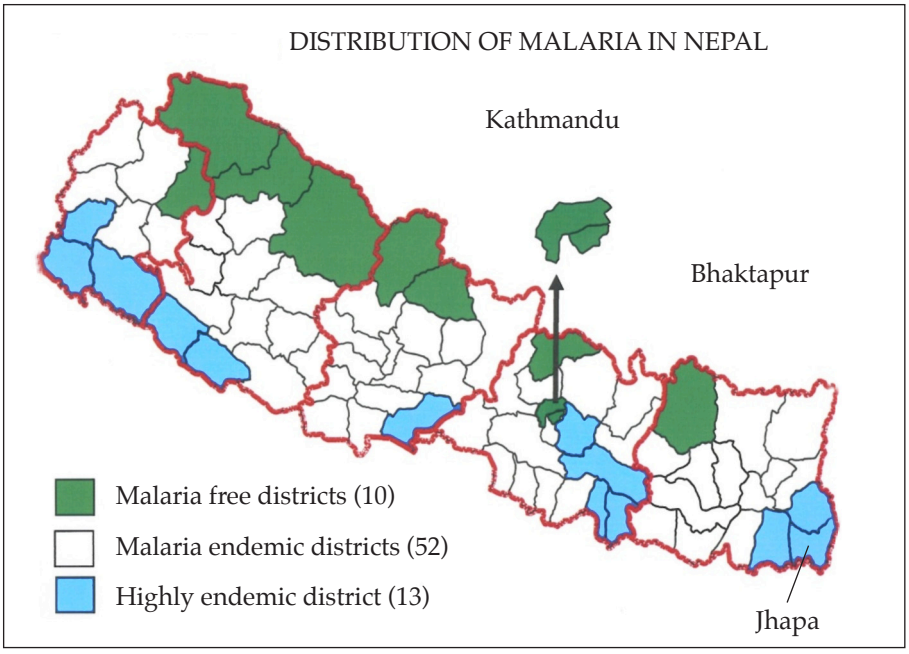


Fig 1–Distribution of malaria in Nepal. High and low malaria endemic districts are categorized on the basis of abundance of two principle vectors: *Anopheles minimus* and *Anopheles fluviatilis*, and malaria transmission rate: where annual malaria transmission rate ≥ 1/1,000 and 0- 1/1,000 population, respectively (EDCD/DoHS, 2008; 2009).

(*P. vivax* and *P. falciparum*) were differentiated by species specific polymerase chain reaction (PCR) (Rupika *et al*, 2010). Allelic diversity of *Pvmsp-3*α gene was determined using the PCR-RFLP (Bruce *et al*, 1999).

## RESULTS

### PCR amplification of *Pvmsp-3*α

Species-specific PCR analysis revealed 55% samples positive for *P. vivax* including 11% containing both *P. vivax* and *P. falciparum* (data not shown). The *Pvmsp-3*α allele was successfully amplified for 56 samples and 3 distinct allele size polymorphisms were observed: 40 of type A (71%), 7 of type B (13%), and 9 of type C (16%).

### PCR-RFLP analysis of *Pvmsp-3*α

Restriction digestion of the *Pvmsp-3*α amplicons yielded 13 different fragment sizes (Fig 2). As all samples showed a ~1,000 bp band, only the smaller highly polymorphic fragment sizes, 200-550 bp were used for RFLP analysis. Based on this, 13 different alleles were identified and designated A1-A9, B1, B2, C1 and C2 (Fig 2). Genotype C2 (15%) was the most abundant followed by A1 (13%) and A3 (11%) (Fig 3). Two samples remained undigested while 7 samples (13%) of type A were of mixed genotypes.

## DISCUSSION

Although a significant number of people in Nepal are infected with *P. vivax*,

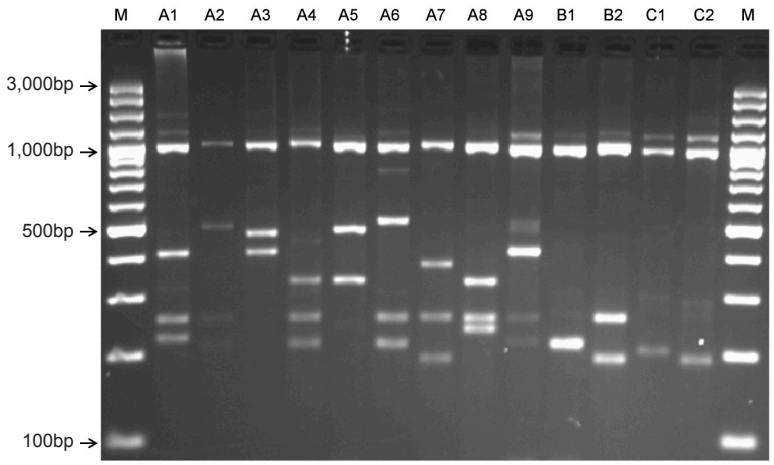


Fig 2–Digestion pattern of *Pvmsp*-3α amplicon using *Hha*I. A1-A9, B1, B2, C1 and C2 are the 13 different alleles resulting from digestion of the *Pvmsp*-3α amplicons with *Hha*I. M is the 100 bp DNA markers.

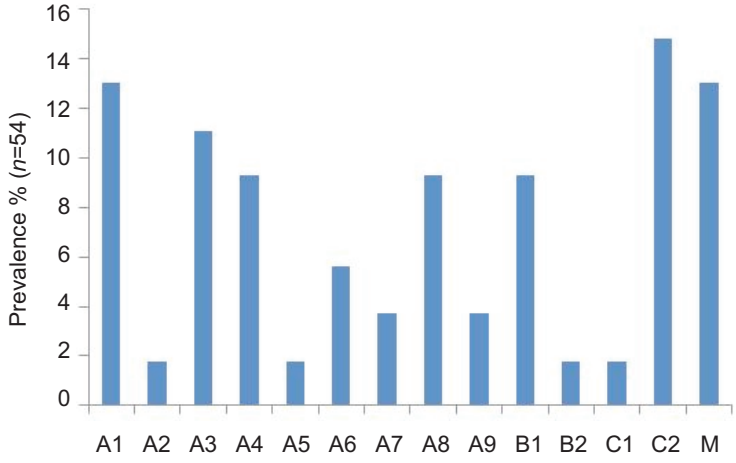


Fig 3–Prevalence of 13 different alleles of *Pvmsp*-3α. A1-A9, B1, B2, C1 and C2 are the 13 different alleles from PCR-RFLP.

limited information exists regarding the genetic diversity of *P. vivax* parasite populations in the country. Genetic diversity is one of the major factors for their survival by evading host immune mechanisms ( Franks *et al*, 2003; Zakeri *et al*, 2006). Imported malaria due to open borders between Nepal and India as a result of migration of people mainly from India to Nepal is one of the major issues facing malaria control in Nepal (EDCD/DoHS, 2008), and which will most likely introduce new alleles into the indigenous *P. vivax* population resulting in a high genetic diversity within the parasite population (Moon *et al*, 2009; Zakeri *et al*, 2010b). Thus, it is necessary to study the genetic diversity of natural population in order to understand the dynamics of malaria transmission for implementation of effective control strategies and to further develop efficient vaccine (Carlton *et al*, 2008; Zakeri *et al*, 2010a).

*Pvmsp*-3α has been frequently used as a molecular marker for epidemiologic studies of *P. vivax* population globally (Bruce *et al*, 1999; Kim *et al*, 2006; Zakeri *et al*, 2006; Shahbazi *et al*, 2008; Zakeri *et al*, 2010b). A total of 13 different allelic patterns, grouped in the three genotypes (A, B, and C) were observed in *Pvmsp*-3α from 96 samples collected from Jhapa District of Eastern Nepal with type A in the majority of the samples (71.4%, *n*=40). The occurrence of a high number of type A is consistent with observations from India (52-68%) (Kim *et al*, 2006; Prajapati *et al*, 2010).

Likewise, it was also observed 59.3% in Venezuela (Ord *et al*, 2005), 70.5% in Papua New Guinea (Bruce *et al*, 1999), 78% in Iran (Shahbazi *et al*, 2008), 70.8% in Afghanistan (Zakeri *et al*, 2010b), 85.1% in French Guiana (Veron *et al*, 2009 ) and 96.5% in Columbia (Cristiano *et al*, 2008). Furthermore, the occurrence of 13 different alleles in this study indicates a high genetic diversity within the studied *P. vivax* population in Jhapa, which is similar to a study conducted in India, where 14 different alleles were observed (Prajapati *et al*, 2010). A high genetic diversity in parasite population might be a result of a high transmission rate or/and a result of high migration of people between endemic areas (Moon *et al*, 2009; Zakeri *et al*, 2010b). Transmission rate of malaria is low in Asia (Laufer *et al*, 2006), as well in Nepal (Banjara *et al*, 2010), which probably indicates that the migration of people between the malaria endemic regions, either within the country or between Nepal and India, might be the possible cause of the high genetic diversity. Additionally, the presence of similar prevalence of type A (52-68%), type B (12-17%) and type C (20-28%) in India (Kim *et al*, 2006; Prajapati *et al*, 2010) further supports this notion.

A small number (7 samples, 13%) showing mixed genotype infections was comparable with results observed in India (10.6%) (Prajapati *et al*, 2010), French Guiana (13.8%) (Veron *et al*, 2009) and Thailand (19.3%) (Cui *et al*, 2003) but higher than in Pakistan (5-6%) (Khatoon *et al*, 2010; Zakeri *et al*, 2010a) and Iran (2%) (Zakeri *et al*, 2010a). Mixed genotypes can occur due to high transmission rate of parasites and also to migration of people (Khatoon *et al*, 2010). This further indicates the possibility of migration of people to Jhapa. A study conducted in Eastern Nepal in 2007 showed 31.7% of patients had a history of recent visits to known malaria endemic areas of either Nepal or India (Parajuli and Ghimire, 2010). Another study in 2008 indicates 37% of malaria patients with recent travel history to India (Banjara *et al*, 2010). Together, this supports the migration of people as the most likely cause of high genetic diversity of *P. vivax* population in Jhapa.

In conclusion, a high genetic diversity was observed in *P. vivax* population of Jhapa using *Pvmsp-3*α as marker gene, despite the low malaria transmission rate. The results from this study can be used as baseline data for future malaria research in Nepal. However, other studies are needed with a larger sample size collected from different malaria endemic areas in Nepal including a questionnaire with information of history of migration of people in order to determine the nationwide genetic diversity of *P. vivax*.

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