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Genetic diversity of *Plasmodium vivax* Duffy Binding Protein II (PvDBPII) under unstable transmission and low intensity malaria in Sri Lanka

Prasad H. Premaratne [a], B. Ramesh Aravinda [a], Ananias A. Escalante [b], Preethi V. Udagama [a,*]

[a] Department of Zoology, Faculty of Science, University of Colombo, Colombo 03, Sri Lanka
[b] School of Life Sciences, Arizona State University, Tempe, AZ, USA

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ABSTRACT

Elucidating the genetic diversity of the Duffy Binding Protein II (PvDBPII), a leading vaccine candidate for vivax malaria, in different geographical settings is vital. In Sri Lanka malaria transmission is unstable with low intensity. A relatively high level of allelic diversity, with 27 polymorphic nucleotides and 33 (aa) haplotypes was detected among the *PvdbpII* gene in 100 local *Plasmodium vivax* isolates collected from two hypoendemic areas, and from a non endemic area of the country. Mutations, recombination and balancing selection seem to maintain the observed local allelic diversity of *PvdbpII*. Lack of gene flow was evidenced by high *Fst* values between the two endemic study sites. Some of the aa polymorphisms may alter the binding and expression capacity of predicted T cell epitopes in PvDBPII. Of the 8 binding inhibitory linear B cell epitopes, 2 (H2 and M1) in the vicinity of the exact binding region of PvDBPII appeared to be highly conserved in Sri Lankan, Iran and Colombian isolates, while H3, M2, M3 and L3 neutralizing epitopes seem to be polymorphic globally, with H1 and L2 conserved in Colombian, South Korean and Iran isolates. In comparison to the reference Sal-1 strain, among 402 world-wide isolates (302 global and 100 local), 121 aa polymorphisms and 138 haplotypes were recorded of which 3 aa polymorphisms and 21 haplotypes seem to be unique to Sri Lanka. *PvdbpII* phylogeny suggests that local *P. vivax* parasites represent a sample of the global population. The ubiquitous presence of some PvDBPII aa haplotypes among both local and global *P. vivax* isolates may aid future vaccination strategies based on PvDBPII.

1. Introduction

With its global widespread predominance in the tropics, subtropics and temperate regions, and being responsible for 25–40% of the annual cases of malaria worldwide (reviewed by Price et al., 2007), *Plasmodium vivax* is the major cause of malaria outside of Africa, mainly afflicting Asia and the Americas with approximately 2.5 billion people worldwide at risk from this infection (reviewed by Baird, 2009; Guerra et al., 2010). The re-emergence of *P. vivax* in areas where it was considered eradicated, the emergence of drug resistance, and its association with severe and fatal malaria are evidence of its significant public health importance than traditionally considered (reviewed by Price et al., 2007; Tjitra et al., 2008). Given the substantial differences between *P. vivax* and *Plasmodium falciparum* in terms of their biology, pathogenesis, and epidemiology, it cannot be assumed that interventions developed for the control of *P. falciparum* will be similarly successful against *P. vivax*, and highlights the need of developing effective, long-term control strategies to reduce the impact of this disease (reviewed by Mueller et al., 2009). Due to the development of increasing resistance to both insecticides and anti-malarials, these strategies are increasingly becoming insufficient to reduce the global burden of malaria. An important part of any control strategy will be the implementation of a vaccine capable of inducing strain transcending immunity (Ntumngia et al., 2009); such a strategy seems particularly important in *P. vivax*.

Studies in humans and animal models have substantiated that immune responses targeting blood-stage merozoite antigens may hamper the parasite ability of invading the RBC and offer protection against clinical disease (reviewed by Richards and Beeson, 2009). However, extensive antigenic diversity associated with most of the vaccine candidate antigens of the *Plasmodium* merozoite, along with several other factors, hampers the progress of the development of blood stage vaccine(s).

The complex multistep RBC invasion process of *P. vivax* is dependent on the recognition of the Duffy blood group antigen

* Corresponding author at: Department of Zoology, Faculty of Science, University of Colombo, No. 94, Cumaratunga Munidasa Mawatha, Colombo 03, Sri Lanka. Tel.: +94 11 2503399; fax: +94 11 2503148.
E-mail address: dappvr@yahoo.com (P.V. Udagama).

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(DA) receptor on the host RBC surface by the merozoite ligand, the Duffy Binding Protein (DBP), exported to the merozoite surface during invasion (Adams et al., 1992; Chitnis and Miller, 1994). Two key observations, *i.e.* the complete resistance of Duffy negative individuals to *P. vivax* merozoite invasion (Miller et al., 1976), and reduced susceptibility to *P. vivax* of heterozygous carriers of a Duffy-negative allele compared with wild-type homozygotes (Kasehagen et al., 2007), provide strong evidence that complete or partial disruption of the expression of DA reduces invasion ability of the merozoite restricting the blood stage development of *P. vivax*. Although recent observations of transmission of *vivax* malaria in Duffy-negative populations (Ménard et al., 2010) suggest alternative invasion pathway(s) of *P. vivax*, how extended this phenomenon is remains obscure. Thus, *P. vivax* DBP (PvDBP) seems one of the most promising vaccine candidates against this disease.

Naturally acquired antibodies to PvDBP are prevalent in residents of areas where malaria is endemic (Fraser et al., 1997; Xainli et al., 2003; Tran et al., 2005; Cerávolo et al., 2008; Souza-Silva et al., 2010). Few studies demonstrate the presence of naturally acquired binding inhibitory antibodies directed against the PvDBP (Grimberg et al., 2007; Cerávolo et al., 2008; King et al., 2008; Souza-Silva et al., 2010) which contained both strain specific and strain transcending components (Cole-Tobian et al., 2009). Some such observations were confirmed in a recent study from Sri Lanka (P.V. Udagama-Randeniya, unpublished data). The fact that PvDBP specific binding inhibitory antibodies confer protection against blood stage *in vivo*, provide ample evidence that protective immune response to *P. vivax* is at least partially directed against PvDBP, and reiterate the importance of developing a vaccine based on this antigen (King et al., 2008).

The critical binding motif (CBM) was mapped to a central 170 aa stretch within the 330-aa residue binding motif (with 12 cysteines) located in region II of the DPB (PvDBPII). Six residues that lie between cysteines 4 and 6 come together in three dimensional space to form the exact DA recognition site of PvDBPII during RBC invasion (Mayor et al., 2005; Singh et al., 2006). Though the cysteines and the exact DA binding residues are conserved, extensive polymorphisms are associated with many other residues of the CBM (reviewed by Chitnis and Sharma, 2008). Many of these polymorphic residues, (i) were non-synonymous and organized into clusters of two contiguous stretches that lie opposite to the DA recognition site of PvDBPII (Singh et al., 2006), and (ii) can collectively alter the antigenic character of the molecule which can significantly change the sensitivity to inhibitory antibodies directed against PvDBPII (VanBuskirk et al., 2004). The pattern of polymorphism associated with the CBM imply the existence of selection pressure, suggesting that allelic variation functions as a mechanism for immune evasion (Cole-Tobian and King, 2003; Chootong et al., 2010). Such newly selected mutants will spread affecting the population structure, and may provide insight to the evolution and selection of parasite populations over time (Rich et al., 1997; Gosi et al., 2008). Many similar PvDBP alleles are widely distributed among different geographical areas worldwide (reviewed by Chitnis and Sharma, 2008). Although DBP represents an ideal vaccine target, the allelic variation and the associated strain specific immunity represent challenges for development of a broadly effective vaccine.

Given the complex geographic structure of *P. vivax* that may affect the observed genetic diversity of putative vaccine antigens (Cornejo and Escalante, 2006), the characterization of *PvdbpII* in different geographic regions will be particularly important in vaccine development and deployment. Such vital information was neither available from the Indian sub continent, nor from Sri Lanka where *P. vivax* is responsible for 65–80% of the total reported annual malaria incidence. Although, malaria conditions are described to be unstable transmission with low intensity (Mendis et al., 1990), the geographic isolation of Sri Lanka may impose unique selection constraints on the local parasite population. We thus analysed the nature of the genetic polymorphism, including that of the predicted linear B and T cell epitopes, of the *PvdbpII* gene in Sri Lanka by examining 100 local *P. vivax* clinical isolates, and the forces driving the maintenance of this genetic diversity. Also, the associations among local and global PvDBPII sequences were investigated by comparison of their phylogeny.

2. Materials and methods

2.1. Study sites and sample collection

This study protocol received approval from the Ethical Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka (EC/04/103). Blood samples were collected between December 1998 and March 2000 (Wickramarachchci et al., 2010), with informed consent, from adults (age >15 years) with microscopically confirmed *P. vivax* infections, prior to anti malarial treatment. The patients were selected from two *P. vivax* endemic areas, Anuradhapura (8°22′N, 80°20′E; $N = 42$) and Kataragama (6°25′N, 81°20′E; $N = 73$), situated 250 km apart, and from residents of the non-endemic malaria area, Colombo (7°55′N, 79°50′E; $N = 52$), where the patients were acquired the disease only after visiting to the areas with natural malaria transmission in the island (Fig. 1). During sample collection, the annual parasite incidence due to *P. vivax* was 20–40 and 80–160 per 1000 individuals from Anuradhapura and Kataragama, respectively (Briët et al., 2003). Though the samples from Kataragama were collected from patients living in 7 contiguous villages comprising an area of only

Fig. 1. Map of Sri Lanka indicating the locations of the three study sites.