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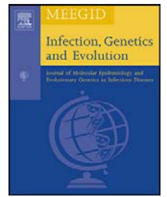

High-throughput molecular diagnosis of circumsporozoite variants VK210 and VK247 detects complex *Plasmodium vivax* infections in malaria endemic populations in Papua New Guinea

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ABSTRACT

Malaria is endemic in lowland and coastal regions of Papua New Guinea (PNG), and is caused by *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. Infection by *P. vivax* is attributed to distinct strains, VK210 and VK247, which differ in the sequence of the circumsporozoite protein (*pvcsp*). Here, based upon sequence polymorphisms in *pvcsp*, we developed a post-PCR ligation detection reaction-fluorescent microsphere assay (LDR-FMA) to distinguish these *P. vivax* strains. This diagnostic assay was designed to detect the presence of both VK210 and VK247 *P. vivax* strains simultaneously in a high-throughput 96-well format. Using this assay, we analyzed human blood samples from the Wosera ($n = 703$) and Mugil ($n = 986$) regions to evaluate the prevalence of these *P. vivax* strains. VK210 and VK247 strains were found in both study sites. In the Wosera, single infections with VK210 strain were observed to be most common (41.7%), followed by mixed-strain (36.8%) and VK247 single-strain infections (21.5%). Similarly, in Mugil, VK210 single-strain infections were most common (51.6%), followed by mixed-strain (34.4%) and VK247 single-strain infections (14%). These results suggest that the distribution of *P. vivax* infections was similar between the two study sites. Interestingly, we observed a non-random distribution of these two *P. vivax* strains, as mixed-strain infections were significantly more prevalent than expected in both study sites (Wosera and Mugil χ^2 *p*-value < 0.001). Additionally, DNA sequence analysis of a subset of *P. vivax* infections showed that no individual *pvcsp* alleles were shared between the two study sites. Overall, our results illustrate that PNG malaria-endemic regions harbor a complex mixture of *P. vivax* strains, and emphasize the importance of malaria control strategies that would be effective against a highly diverse parasite population.

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1. Introduction

Of the four human malaria parasites, *Plasmodium vivax* is the most widespread species currently found in limited parts of Africa, parts of Central and South America as well as the Middle East, and most of Central, Northeast, South, and Southeast Asia, including the Pacific Islands (Carter and Mendis, 2002; Mendis et al., 2001). In 2009, an estimated 2.85 billion people were at risk of *P. vivax* infection, which causes approximately 80 million clinical cases each year (Guerra et al., 2006, 2010; Mendis et al., 2001). *P. vivax* infection begins with the injection of sporozoites into the human host by an infectious mosquito. Sporozoites then migrate to the liver, where they invade hepatocytes and proceed through asexual exo-erythrocytic development. Infected hepatocytes develop either into schizonts, to begin the blood stage development of malaria infection, or into the dormant hypnozoite stage. Hypnozoites can become reactivated and proceed to schizogony many months after initial sporozoite inoculation to cause a relapse infection (Krotoski et al., 1986). As a result, the complexity of *P. vivax* blood stage infections are likely to be high because they may receive contribution from as many as four sources; primary infection, reinfection, recrudescence from surviving blood stage parasites, or relapse caused by an activated hypnozoite.

Consistent with observations from other malaria parasite species, the *P. vivax* sporozoite expresses an abundant surface protein, the circumsporozoite protein (pvcsp) (Arnot et al., 1985; Rosenberg et al., 1989). Earlier studies showed that the central region of *pvcsp* consists of 15–19 repeats, each 27 bp in length (encoding nine amino acids), with polymorphism that leads to classification as two different variants, VK210 and VK247

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(Rosenberg et al., 1989). The VK210 repeat consists of various multiples and combinations of the GDRA[D/A/P]GQPA amino acid motif. The terminal repeat of the VK210 variant has shown to be consistently comprised of the GDRAAGQPA amino acid motif, which is immediately followed by a conserved GNGAGG post-repeat sequence. The VK247 repeat consists of a similar organization of the ANGA[G(N/D)]/[DD]QPG motif. The VK247 terminal repeat is consistently comprised of the ANGAGNQPG motif, which is immediately followed by the conserved ANGAGGQ post-repeat sequence (Rosenberg et al., 1989; Yadava et al., 2007; Zakeri et al., 2006).

Since the sporozoite initiates human infection, the most productive vaccine would block sporozoite invasion of the hepatocyte (Arevalo-Herrera and Herrera, 2001; Bilsborough et al., 1997; Herrera et al., 1997, 2005; Yadava et al., 2007). Currently, several *P. vivax* vaccine candidates are in development, targeting proteins such as CSP, merozoite surface protein, duffy binding protein and thrombospondin-related adhesive protein (Arevalo-Herrera and Herrera, 2001; Castellanos et al., 2007; Herrera et al., 1997; Singh et al., 2002, 2005; WHO, 2005; Yadava et al., 2007). Only two *P. vivax* vaccine candidates have been tested in humans (CSP and Pvs25), with an additional few candidates entering into the pre-clinical developmental stage (Herrera et al., 2005, 2007; Malkin et al., 2005). Using the moderately successful vaccine design based on *Plasmodium falciparum* RTS,S (Alonso et al., 2005), several groups have constructed an anti-CSP vaccine for *P. vivax* (Herrera et al., 1997, 2004; WHO, 2005; Yadava et al., 2007). As *P. vivax* vaccine candidates progress toward advanced clinical trials, monitoring the *pvcsp* variant infection status of post-vaccination populations will become an essential aspect of determining the long-term effectiveness of vaccines targeting both PvCSP variants.

In Papua New Guinea (PNG) *P. vivax* causes significant infection and disease. Our studies in northern coastal regions of East Sepik and Madang Provinces over the past ten years have shown that prevalence of malaria has varied with significant reduction in all four species in recent years (Kasehagen et al., 2006; Mehlotra et al., 2000, 2002; Mueller et al., 2009). During this time, minimum PCR-based prevalence of infection by the four human malaria parasite species have been reported as: *P. falciparum*, 30%; *P. vivax*, 25%; *Plasmodium malariae*, 12%; *Plasmodium ovale*, 4%. Minimum infection prevalence determined by less sensitive blood smear light microscopy has always been significantly lower: *P. falciparum*, 20%; *P. vivax*, 9%; *P. malariae*, 1%; *P. ovale*, not detected. Monitoring population level diversity of *P. vivax* will require molecular diagnostic tools and sequence analysis (Cole-Tobian and King, 2003; Cole-Tobian et al., 2002). Here, we analyzed several *pvcsp* representatives in PNG in order to develop a new high-throughput molecular method for assessing prevalence and diversity of VK210 and VK247 variants. Our results provide new insight into *P. vivax* strain diversity in two malaria endemic regions of northern PNG.

2. Materials and methods

2.1. Study areas, populations and blood sample collection

Cross-sectional studies were conducted in two regions of PNG. Twenty-nine villages in the Wosera region located in East Sepik Province and three villages in Mugil located in Madang Province were surveyed as part of collaborative research studies between Case Western Reserve University and PNG Institute of Medical Research (PNGIMR). These two regions of PNG represent distinctly different populations, which are separated by 350 km of rugged terrain. Migration in between locations is not possible by road. Genton et al. (1995) and Mehlotra et al. (2002) have further discussed details about the Wosera and Mugil study sites, respectively.

A total of 703 blood samples were collected from study participants in the Wosera region between August 2001 and June 2003. This sample set, randomly selected from 29 villages (Kasehagen et al., 2006), represented 7.9% of the population (total population (n = 13,000)) and included individuals <1–87 years old. A total of 986 blood samples were collected from three villages in the Mugil study site between June and July 1999. This sample set represented 79.4% of the population (total population n = 1242) and included individuals <1–85 years old (Cole-Tobian et al., 2002). Demographic information and finger prick blood samples were collected from each study participant. Blood samples were used for thick and thin smears and enable human host and parasite DNA extraction for further diagnostic analysis. Informed consent was obtained from all adult participants and from the parents/legal guardians of minor participants. All protocols were approved by Human Investigations Institutional Review Boards of Case Western Reserve University, University Hospitals Case Medical Center and the Papua New Guinea Medical Research Advisory Committee.

2.2. DNA template preparation

DNA was extracted from whole blood (200 μL) using the QIAamp 96 DNA Blood Kit (QIAGEN, Valencia, CA).

2.3. Molecular diagnosis of Plasmodium species infection

We have previously described strategies and methods to perform PCR and post-PCR ligation detection reaction-fluorescent microsphere assay (LDR-FMA) diagnosis of *Plasmodium* species (McNamara et al., 2006).

2.4. Molecular diagnosis of P. vivax CSP variants

For *P. vivax* strain analysis, nested PCR was preformed to amplify the *pvcsp* region of the *P. vivax* genome. Previously published sequences for VK210 [GenBank accession no. M11926 (Arnot et al., 1985)] and VK247 [GenBank accession no. M28745 (Rosenberg et al., 1989)] were used to design PCR primers to amplify region I, pre-repeat, central repeated region, and post-repeat conserved region of *pvcsp*, consisting of a fragment 600–750 bp in length (Imwong et al., 2005; Zakeri et al., 2006).

PCR amplification reactions (25 μl) were performed in buffer containing 3 pmol of appropriate upstream and downstream primers, 67 mM Tris–HCl, pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 100 μM dATP, dGTP, dCTP, and dTTP, and 2.5 units of thermostable DNA polymerase using the MJ Research PTC-225 thermocycler (Watertown, MA). The nest-1 primers were Up 5′-ATGTAGATCTGTCCAAGGCCATAAA-3′ and Dn 5′-TAATTGAATAATGCTAGGACTAACAATATG-3′; the thermocycling conditions were: 95 °C 2 min (1×), 95 °C 30 s, 64 °C 30 s, 72 °C 1 min 30 s (30×), 72 °C 4 min (1×). The nest-2 primers were Up 5′-GCAGAACCAAAAAATCCACGTGAAAATAAG-3′ and Dn 5′-CCAACGG-TAGCTCTAACTTTATCTAGGTAT-3′; the thermocycling conditions were: 95 °C 2 min (1×), 95 °C 30 s, 64 °C 30 s, 72 °C 1 min 30 s (30×), 72 °C 4 min (1×). To evaluate overall amplification efficiency, PCR products were separated by electrophoresis on 2% agarose gels (1× TBE), stained with SYBR® Gold (Molecular Probes, Eugene, OR), and visualized on a Storm 860 using ImageQuant, 5.2 software (Molecular Dynamics, Sunnyvale, CA) (Fig. 1).

Following nest-2 PCR amplification, products were subjected to a multiplex ligation detection reaction (LDR) where variant-specific upstream primers ligate to conserved sequence primers when appropriate target sequences are available (Table 1). Previous publications in addition to our DNA sequence results have reported (regardless of the number of repeats within the VK210 and VK247 alleles) that the terminal repeat segment of

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