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Infectivity of symptomatic and asymptomatic *Plasmodium vivax* infections to a Southeast Asian vector, *Anopheles dirus*

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ABSTRACT

Plasmodium vivax is now the predominant species causing malarial infection and disease in most non-African areas, but little is known about its transmission efficiency from human to mosquitoes. Because the majority of *Plasmodium* infections in endemic areas are low density and asymptomatic, it is important to evaluate how well these infections transmit. Using membrane feeding apparatus, *Anopheles dirus* were fed with blood samples from 94 individuals who had natural *P. vivax* infections with parasitemias spanning four orders of magnitude. We found that the mosquito infection rate was positively correlated with blood parasitemia and that infection began to rise when parasitemia was >10 parasites/μl. Below this threshold, mosquito infection is rare and associated with very few oocysts. These findings provide useful information for assessing the human reservoir of transmission and for establishing diagnostic sensitivity required to identify individuals who are most infective to mosquitoes.

1. Introduction

Over the past decade, malaria incidence has steadily declined in various parts of the worlds. In many places where *Plasmodium falciparum* and *Plasmodium vivax* coexist, including South America, Southeast Asia and the Western Pacific region, the latter has now become the predominant species (Oliveira-Ferreira et al., 2010; Rodriguez et al., 2011; Imwong et al., 2015; Waltmann et al., 2015). The resilience of *P. vivax* relative to *P. falciparum* against malaria controls can be attributed, at least partially, to the parasite's ability to remain dormant as hypnozoites in the host's liver (Krotoski et al., 1982; White et al., 2014; Robinson et al., 2015) and its greater transmission efficiency (Boyd, 1937; Pethleart et al., 2004). *Plasmodium vivax* thus poses a great challenge for malaria eradication. Due to the lack of an in vitro culture system that produces infectious gametocytes, information about *P. vivax* transmission efficiency is limited, and has mostly relied on direct or membrane feeding experiments using blood from human malaria infections (Sattabongkot et al., 1991, 2003; Gamage-Mendis et al., 1993; Zollner et al., 2006; Rios-Velasquez et al., 2013; Vallejo et al., 2016).

Several studies have reported that the majority of *P. vivax* infections in the endemic areas of Asia, South America, and Oceania are asymptomatic (Harris et al., 2010; Baum et al., 2015; Imwong et al., 2015; Waltmann et al., 2015; Vasquez-Jimenez et al., 2016) and submicroscopic (Cheng et al., 2015), even in areas where malaria transmission intensity has declined. Previous studies have also shown that blood from both *P. vivax*-infected patients and asymptomatic carriers can infect *Anopheles dirus*, a Southeast Asian vector (Sattabongkot et al., 1991, 2003; Coleman et al., 2004; Pethleart et al., 2004), but the relationship between *P. vivax* parasitemia and the mosquito infection rate is often described as weak if not absent (Graves et al., 1988; Gamage-Mendis et al., 1991; Bharti

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et al., 2006; Coleman et al., 2004). At present, the relative contributions to transmission of asymptomatic and symptomatic *P. vivax*-infected populations remain unclear. Such information is important for improving the current disease control and elimination programs. If asymptomatic carriers are contributing substantially to transmission, then malaria interventions will need to also target these carriers to be effective.

To determine how well *P. vivax* transmits from humans to mosquitoes and to assess the contribution of asymptomatic carriers to transmission, we performed membrane feeding experiments on *An. dirus* using blood samples from both *P. vivax* malaria patients and asymptomatic carriers. These samples covered a broad range of parasitemias, from submicroscopic to 10,000 parasites/μl.

2. Materials and methods

2.1. Study sites

The study was conducted in Tha Song Yang District of Tak Province and Sai Yok District of Kanchanaburi Province in western Thailand between 2014 and 2015. Both areas were mountainous and populations were composed mainly of Thai and Karen ethnicities. The main occupation of the study participants was farming. Malaria in the study areas was seasonal, with the major peak season lasting from May to August, and a secondary peak in November to December. The prevalence of *P. vivax* and *P. falciparum* in Tha Song Yang in 2011–2012 was approximately 10% and 3.7% by PCR (Parker et al., 2015). Four anopheline species were recently found to be infected by *Plasmodium* in this area, including *Anopheles maculatus*, *Anopheles minimus*, *Anopheles annularis* and *Anopheles barbirostris* (Sriwichai et al., 2016). The prevalence of *P. vivax* and *P. falciparum* in our study site in Sai Yok was 3.8% and 1.4% by PCR in 2012 (Nguitragool et al., unpublished data). No information is currently available about the vectors in this area.

2.2. Enrolment for membrane feeding experiments

Enrolment was limited to local residents ⩾13 years old. Participants were enroled all year round either at local malaria clinics where *P. vivax* was first identified by light microscopy, or through mass blood surveys of the general village population by genus-specific loop-mediated isothermal amplification (LAMP) (Han et al., 2007) or quantitative reverse transcription (qRT)-PCR (Wampfler et al., 2013). During the enrolment process, the body temperature of each participant was recorded using an infrared thermometer. A history of recent malaria infection was obtained through interview. Symptomatic individuals were classified as those who felt sick with malaria-like symptoms (fever, headache or chill) or had a temperature >37.5 °C at the time of the blood survey. Asymptomatic carriers were *Plasmodium*-positive individuals whose temperature at the time of the blood survey was <37.5 °C and reported an absence of fever during the preceding 2 weeks. Informed consent or assent was obtained from each participant as well as his/her legal guardian if the participant was <18 years old. This study was approved by the Ethics Committees of Mahidol University, Thailand and Pennsylvania State University, USA.

In total, 222 individuals were included in the final data analysis. Individuals who were evidently infected with *P. falciparum* were excluded. The 222 participants comprised 93 individuals who were infected with *P. vivax* (70 symptomatic, 23 asymptomatic), 22 individuals who were infected with *Plasmodium* parasites of unknown species, and 107 individuals whose blood was virtually free of *Plasmodium* by genus-specific LAMP and qRT-PCR (see Sections 2.5 and 2.6).

2.3. Blood collection

Five millilitres of venous blood were collected from each participant in a heparinised tube. From each whole blood sample, 200 μl of blood were collected for parasite species identification by species-specific LAMP assays (Han et al., 2007). A second portion of 200 μl was mixed with 1 ml of RNAprotect Cell Reagent (Qiagen, Germany) and stored at −80 °C for RNA extraction and purification. Thick and thin blood smears were prepared in duplicate using 1 μl of whole blood for each spot for microscopic examination. The rest of the sample was kept at 37 °C and used in the membrane feeding experiment within 4 h of collection.

2.4. Microscopic examination of blood smears

Thick and thin smears were prepared from whole blood. The thin smear was fixed with methanol, while the thick smear was left unfixed, before being stained with 10% Giemsa solution for 10 min and examined for *Plasmodium* spp. and developmental stages. Parasite and gametocyte densities were determined by counting the entire 1 μl of blood spots.

2.5. LAMP assays

Genus-specific as well as species-specific (*P. falciparum* or *P. vivax*) LAMP assays (Han et al., 2007) were performed to detect *Plasmodium* parasite infection in blood samples from the cross-sectional surveys. To perform these LAMP assays, 150 μl of distiled water was added to 50 μl of blood and boiled for 5 min. The sample was then centrifuged for 3 min at 13,000*g*. A total volume of 5.5 μl of the supernatant containing genomic DNA was used as the template in a 25 μl LAMP reaction, using the Loopamp DNA amplification kit (Eiken Chemical Co, Japan) and an appropriate primer set as previously described (Han et al., 2007; Sattabongkot et al., 2014). Only samples that were free of *P. falciparum* were included in the final data analysis. The limits of the LAMP assays for *P. falciparum* and *P. vivax* were 100 copies/reaction (Han et al., 2007).

2.6. qRT-PCR analyses

RNA was extracted from 100 μl of whole blood in 500 μl of RNAprotect Cell Reagent and eluted with 50 μl of elution buffer, following the instruction of the RNeasy Plus 96 kit (Qiagen). A genus-specific quantitative PCR (qPCR) assay, QMAL (Wampfler et al., 2013), using 4 μl of purified RNA as template, was performed on all RNA samples to ensure there was no contamination of genomic DNA.

For blood samples from cross-sectional surveys, 4 μl of purified RNA were subjected to genus-specific, as well as *P. vivax*-specific, qRT-PCR assays targeting the 18S rRNA transcripts. The primer and probe sequences for this assay have been described previously (Rosanas-Urgell et al., 2010).

To detect *P. vivax* gametocytes, 4 μl of purified RNA were used as the template in qRT-PCRs to amplify the *pvs25* transcripts (Wampfler et al., 2013). Copy numbers were determined from an in-plate standard curve prepared by 10-fold serial dilution of a plasmid harbouring the target sequence. Values are reported as copies per μl of equivalent blood volume. To ensure that oocysts in mosquitoes were not due to *P. falciparum* co-infection, qRT-PCRs for *pfs25* transcripts were also performed on all samples (Wampfler et al., 2013). Samples positive for *pfs25* transcripts were excluded from the final analysis. The detection limits of qRT-PCR for *pvs25* and *pfs25* were 12 copies/reaction (Nguitragool et al., unpublished data).

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