

## EXPERIMENTAL INFECTION OF THE NEOTROPICAL MALARIA VECTOR *ANOPHELES DARLINGI* BY HUMAN PATIENT-DERIVED *PLASMODIUM VIVAX* IN THE PERUVIAN AMAZON

AJAY R. BHARTI, RAUL CHUQUIYAURI, KIMBERLY C. BROUWER, JEFFREY STANCIL, JESSICA LIN,  
ALEJANDRO LLANOS-CUENTAS, AND JOSEPH M. VINETZ\*

University of California San Diego School of Medicine, La Jolla, California; Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; Naval Medical Research Center Detachment, Iquitos, Peru

**Abstract.** Malaria transmission from humans to mosquitoes is modulated by human host immune factors. Understanding mechanisms by which the human host response may impair parasite infectivity for mosquitoes has direct implications for the development of transmission-blocking vaccines. We hypothesized that despite a low transmission intensity of malaria in the Peruvian Amazon region of Iquitos, transmission-blocking immunity against *Plasmodium vivax* might be common, given an unexpectedly high proportion of asymptomatic parasitemic individuals in this region. To test this hypothesis, the ability of symptomatic *P. vivax* malaria patients to experimentally infect wild-caught outbred *Anopheles darlingi* mosquitoes was tested using the indirect membrane feeding technique. Only half (52/102) of *P. vivax* parasitemic patients successfully infected mosquitoes. Transmitters were more likely to have gametocytes (OR 6.35,  $P = 0.003$ ), high parasitemia (OR 3.79,  $P = 0.024$ ), and, in terms of basic clinical parameters, a slower pulse rate (mean  $\pm$  SD:  $82.3 \pm 12.3$  versus  $88.7 \pm 13.5$ ,  $P = 0.016$ ) than non-transmitters. Log<sub>10</sub> gametocytemia and log<sub>10</sub> real-time reverse transcriptase *Pvs25* PCR quantifying gametocytes were significantly and positively correlated with oocyst counts (correlation coefficient 0.505,  $R^2 = 0.26$ ,  $P = 0.001$ ). These experiments are the first to establish a system of determining transmission patterns in experimental infection of outbred natural neotropical malaria vectors in the Amazon region. Patients with *P. vivax* inefficiently infect outbred *An. darlingi* mosquitoes, raising the possibility that some degree of naturally occurring transmission-blocking immunity is present on a population basis in the Peruvian Amazon, an area of low intensity of malaria transmission.

### INTRODUCTION

Malaria transmission begins with uptake of viable, mature gametocytes by female *Anopheles* mosquitoes during ingestion of an infectious blood meal. Patients harboring malaria parasites are not equally infectious to mosquitoes. Naturally occurring transmission-blocking and transmission-enhancing activity (both immune and non-immune mediated) present in serum has been demonstrated both for *Plasmodium falciparum* and *Plasmodium vivax* in endemic regions. Nonetheless, molecular targets of *P. vivax* transmission-blocking immunity have not been delineated in the field setting.<sup>1–3</sup>

Infectivity of *Plasmodium* gametocytes for mosquitoes has been shown to be modulated by antibody,<sup>4</sup> complement,<sup>5</sup> and leukocytes.<sup>6</sup> This combination of factors leads to variability in the ability of gametocytemic individuals to infect mosquitoes. Intuitively, the three major determinants would be parasite infectivity, vector competence, and host factors (genetic, immune, and non-immune). Quantity of gametocytes as determined by light microscopy has not been demonstrated to predict *P. vivax* transmission to mosquitoes.<sup>7</sup>

*P. vivax* is more common than *P. falciparum* as a cause of malaria in many parts of the tropics and subtropics outside of sub-Saharan Africa.<sup>8</sup> Malaria caused by *P. vivax* occurs in many regions of Asia and Latin America but has been understudied and underappreciated as a public health threat there.<sup>8</sup> In Peru, the incidence of malaria has increased dramatically since the early 1990s.<sup>9</sup> *P. vivax* remains the pre-

dominant cause of malaria in the region and is a considerable source of morbidity to people of all ages, especially during pregnancy where it is associated with maternal anemia and low birthweight.<sup>10</sup>

The principal malaria vector in the Amazon basin is *Anopheles darlingi*, an aggressive, anthropophilic mosquito that has invaded the western Amazon over the past 15 years due, at least in part, to deforestation,<sup>11</sup> and has led to epidemic malaria in the region.<sup>12</sup> Despite being outnumbered by another malaria-competent anopheline mosquito, *Anopheles benarrochi* (71% vs. 24%), *An. darlingi* has been found to have a much higher infection rate (1% vs. 0.14%), which, combined with being highly anthropophilic, has led to its being the most important malaria vector in the Amazon basin.<sup>13,14</sup> However, because *An. darlingi* cannot be propagated in colonies, the natural *P. vivax*–*An. darlingi* combination has been difficult to study under experimental settings using infected humans as sources of infectious blood meals to determine the biologic parameters of transmission. To do so requires a logistically challenging system in which *An. darlingi* is caught in the field, induced to lay eggs to obtain uninfected F1 mosquitoes, and infected with blood from a naturally infected human in the laboratory setting. Only in this experimental setting can naturally acquired immunity that closely mimics the field setting be simulated.

In areas of high malaria transmission, patients develop clinical immunity as a result of an immune response that controls, but does not eliminate, parasitemia while simultaneously avoiding severe pathology. This phenomenon is well described for *P. falciparum*<sup>15</sup> and more recently for *P. vivax*<sup>16–18</sup> malaria. Interestingly, in the Peruvian Amazon, a site of low malaria transmission intensity, we and others have demonstrated an unexpectedly high prevalence of asymptomatic malaria with one-third of slide-positive and three-fourths of PCR-positive patients being asymptomatic.<sup>19,20</sup> These pa-

\* Address correspondence to Joseph M. Vinetz, University of California San Diego School of Medicine, Division of Infectious Diseases, Department of Medicine, 9500 Gilman Drive, 0741, George Palade Laboratories/Cellular and Molecular Medicine-West Room 125, La Jolla, CA 92093-0741. E-mail: [jvinetz@ucsd.edu](mailto:jvinetz@ucsd.edu)

tients serve as a reservoir of infection, but the transmission pattern in this group is unknown.

This study was conducted to determine the ability of *P. vivax*-infected patients in the Peruvian Amazon to infect *An. darlingi* mosquitoes. We hypothesized that, despite low endemicity, transmission-blocking immunity develops against *P. vivax* in the Peruvian Amazon. Using the natural outbred *An. darlingi*-*P. vivax* pairing, we also determined the relationship between *P. vivax* parasitemia, gametocytemia as determined by light microscopy and quantitative reverse transcriptase PCR, and clinical features and infectivity for *An. darlingi* mosquitoes.

## MATERIALS AND METHODS

**Study site.** Patient enrollment and experimental mosquito infections were carried out in Iquitos, the capital city of the department of Loreto, Peru, over a period of 14 months from May 2004 to June 2005. Enrollment criteria were patients with acute fever and *P. vivax* parasitemia. The region comprises nearly one-fourth of the landmass of Peru and has the ecological characteristics of the Amazon lowlands. Malaria cases occur nearly throughout the year, with seasonal peaks from January to September. *P. vivax* prevalence in this population is 5 times more than that of *P. falciparum* (14% vs. 3%) as has been shown by active surveillance.<sup>19</sup>

**Patient recruitment method and data collection.** This study was approved by the Institutional Review Boards/Ethical Committees of the University of California San Diego, Universidad Peruana Cayetano Heredia, A.B. Prisma, and United States Department of Defense. Permission to carry out the study was provided by the Loreto Director of Health, Iquitos, Peru. Informed consent was obtained from all participants prior to enrollment. All participation was voluntary, and subjects could opt out at any time during the study. All parasitemic patients, regardless of decision to participate, were offered free anti-malarial chemotherapy according to Peruvian Ministry of Health guidelines. Patients were sought from the outpatient department of Hospital Apoyo Iquitos and the health posts at the villages of Varillal and Santo Tomas. No patient had been on anti-malarial therapy on entry to the study. Febrile patients (defined as having an oral temperature > 37.7°C) routinely have blood smears made when presenting for care. Samples were prepared for thick and thin smear microscopy using 5% Giemsa stain, and the slides were examined on site by an experienced Ministry of Health microscopist. This diagnosis was considered standard for inclusion of patients in the study. Patients were excluded if they had severe or complicated malaria as defined by the World Health Organization, serious co-morbid conditions (e.g., HIV/AIDS or malnutrition; none were excluded for these reasons), or were pregnant (2 were excluded). Symptomatic *P. vivax* patients 18 years and older were invited to participate in the study, and informed consent was obtained from those willing. Data regarding age, sex, occupation, place of residence, and history of travel were obtained. Physical examination was done and included general appearance, heart rate, respiratory rate, pallor, icterus, and splenomegaly. Information regarding past history of malaria (type, frequency), date of onset of current symptoms, and use of drugs (anti-malarials and anti-pyretics) was noted.

**Mosquitoes.** *An. darlingi* mosquitoes were caught at night using human bait in the study villages under U.S. Department of Defense-approved protocols, including strict attention to occupational safety, provision of malaria prophylaxis, and surveillance for malaria as the cause of febrile illness. Adult female *An. darlingi* mosquitoes were brought to the Iquitos insectary and fed on chicken blood to induce egg-laying, and F1 mosquitoes were then reared to adults using established methods.<sup>21</sup> Mosquitoes were fed on parasite-containing blood 3–4 days post-emergence.

**Membrane-feeding assays.** A member of the study team accompanied the patients to the insectary, and a physician was present throughout the procedure. Blood samples were obtained in heparinized tubes, maintained at 37°C, and blood smears repeated. Two and a half milliliters of blood was added to a 5-cm diameter hand-blown glass feeder (Perpetual Systems Corp., Rockville, MD) closed with Parafilm (American National Can, Chicago, IL). To prevent exflagellation of microgametocytes, a constant temperature of 37°C was maintained using a water-jacketed circulation system.<sup>22</sup> The mosquitoes (25 per carton) were allowed to feed for 15 min in the dark, after which time the glass membrane feeder was removed from the top of the carton and unengorged mosquitoes were removed. Blood samples from each patient were fed to mosquitoes in 3 separate cartons simultaneously. After feeding, all mosquitoes were kept in the insectary (22–27°C and 70–80% humidity) and provided with 20% sucrose daily until they were dissected on days 7, 8, and 9 after feeding. Midguts were dissected in a drop of phosphate-buffered saline, and oocysts counted by examination under 10× light microscopy. Immediately after blood draw, patients were given anti-malarial drugs (chloroquine and primaquine) per Peruvian Ministry of Health guidelines. A second blood smear was prepared at the time of blood draw to confirm the diagnosis and to quantify parasitemia and gametocytemia. The following system was used: < 1+, at least 1 parasite/100 high-power field (HPF); 1+, 1 parasite/HPF; 2+, 2–20 parasites/HPF; 3+, 21–200 parasites/HPF; 4+, > 200 parasites/HPF.

**Real-time reverse transcriptase PCR.** A real-time reverse transcriptase PCR based on the *Pvs25* gene (GenBank accession no. AB033343) was developed as an alternative method for quantifying *P. vivax* gametocytemia.<sup>23</sup> Primers (forward, 5'-AACGAAGGGCTGGTGCACCTTT-3', 5'-AGCAACCTGCACTTTGGATTTCCG-3') were designed to amplify *Pvs25*. The predicted fragment size of 267 bp was amplified from total genomic DNA obtained from whole blood containing *P. vivax* from a patient. The amplified product was cloned into the pCR 2.1-TOPO plasmid vector. A tenfold dilution of the plasmid DNA starting with 10<sup>10</sup> plasmid copies/μL (as determined by spectrophotometry at OD<sub>280</sub>) was used as a positive control and for making a standard curve. RNA isolation from freshly obtained infected blood from Peru was done using the PAXgene Blood RNA Kit (Qiagen Inc., Valencia, CA). Briefly, 2.5 mL of whole blood was collected in PAXgene Blood RNA Tubes (Becton Dickinson (BD), Franklin Lakes, NJ, catalog no. 76216) and stored at –20°C after being allowed to incubate at room temperature for 2 hours. All samples were processed within 2 weeks of collection using the PAXgene Blood RNA Kit. *TaqMan* probes were designed for the *Pvs25* real-time PCR assays. A 5' 6-FAM reporter dye was selected, with a 3' Black Hole Quencher on the end. Probe concentration was optimized at

a final concentration of 0.1  $\mu\text{M}$  in each reaction, primer final concentration was 0.2  $\mu\text{M}$ , and 10  $\mu\text{L}$  of RNA template from each extraction was used for each reverse transcriptase PCR reaction. The reverse transcriptase PCR cycles were as follows: 50°C  $\times$  15 minutes, 95°C  $\times$  2 minutes, 94°C  $\times$  10 seconds, 55°C  $\times$  45 seconds, plate read, 45 cycles total. For each reverse transcriptase PCR run, RNA samples were run alongside ten-fold dilutions of the plasmid DNA that were performed in triplicate to generate a reliable standard curve. For each RNA sample subjected to reverse transcriptase PCR (using Invitrogen's one-step reverse transcriptase PCR reagents with reverse transcriptase and *Taq* polymerase), a reverse transcriptase minus control was run using the standard PCR reagents with *Taq* polymerase only for contaminating genomic DNA. The real-time probe used was the 5' 6-FAM-AACCCAGACCCAGCACAGGTAACATG-3' Black Hole Quencher.

**Statistical analysis.** For analysis purposes, a person was defined as a "transmitter" if a blood sample from that person had successfully infected at least one mosquito. Univariate analyses, using the  $\chi^2$  test or *t* test to compare means where appropriate, were used to examine which variables were associated with transmission of infection to mosquitoes. A *t* test was also used to compare the mean percentage of mosquitoes infected by parasitemia group, and the Mann-Whitney *U* test was used to compare the number of oocysts per infected midgut with parasitemia group. Relationships between real-time reverse transcriptase PCR quantification of gametocytes, gametocytemia, oocysts per infected midgut, and percentage of mosquitoes infected were compared using simple linear regression. Two-sided *P* values < 0.05 were considered statistically significant. Data were analyzed using the SPSS 12.0 (SPSS, Inc., Chicago, IL) statistical package.

## RESULTS

**Patient data.** A total of 102 patients with symptomatic *P. vivax* infection were enrolled in the study (Table 1). The sex ratio was approximately 2:1 with 65 (64%) men and 37 (36%) women. The mean age was 34  $\pm$  12 SD years (range 18–63 years). All had at least one of the following clinical symptoms by history: fever, chills, or headache (except for 1 patient for whom data were missing). Most patients (80%) had all 3 symptoms on presentation and were seen within 7 days from onset of illness. Sixty percent (60/102) of the patients gave history of prior malaria episodes. About two-thirds (69/102) of patients admitted to taking an analgesic, anti-pyretic, or anti-inflammatory medicine (acetaminophen, ibuprofen, naproxen, or aspirin) some time during the course of their illness. At the time of membrane feeding, half (51/102) of the patients were febrile, with a temperature of 37.5°C or more (range 37.7–40.1°C). Pulse rate ranged from 60 to 120 beats per minute with a mean of 85.4  $\pm$  13.2 SD. Pallor was noted in 65% (66/102) of patients, but because the measurements were not standardized, this variable was not included in the analysis.

**Results of light microscopy quantification of *P. vivax* parasitemia.** Diagnoses of all patients reported as having *P. vivax* malaria by Ministry of Health microscopists at all study sites were confirmed independently in our laboratory. The smear results ranged from < 1 + to 3 +, with a majority of them falling in the 2 + group (77/102). Gametocytemia ranged from 0 to 106 gametocytes per 100 HPF with a mean of 20.61.

TABLE 1

Comparison of clinical aspects of patients who transmitted vs. those that did not transmit *P. vivax* infection to mosquitoes\*

	% Patients that infected mosquitoes (n = 52)	% Patients that did not infect mosquitoes (n = 50)	<i>P</i> value
Sex			
% male	64	64	0.96
Age (years)			
< 25	31	35	0.35
25–34	31	18	
$\geq$ 35 +	39	47	
Mean $\pm$ SD	32.6 $\pm$ 10.9	34.8 $\pm$ 13.6	0.37
Past malaria episodes (a)			
Had malaria in past	67	66	0.94
Chills			
Present	96	96	0.97
Headache			
Present	100	98	0.31
Fever			
$\geq$ 37.5°C	44	56	0.24
$\geq$ 40.0°C	6	12	0.31
Fever medications			
Recent history of use	73	72	0.90
Pulse (b)			
Mean $\pm$ SD	<b>82.3 <math>\pm</math> 12.3</b>	<b>88.7 <math>\pm</math> 13.5</b>	<b>0.016</b>
Parasitemia (categories)			
Groups 1–2	<b>8</b>	<b>24</b>	<b>0.024</b>
Groups 3–4	<b>92</b>	<b>76</b>	
Gametocytemia by smear (categories)			
0 vs. any (> 0)	<b>94</b>	<b>72</b>	<b>0.003</b>

\* Boldface indicates statistically significant association.  
(a) *n* = 101 for past malaria episodes; (b) *n* = 98 for pulse.

### Infectivity of *P. vivax* patients for *An. darlingi* mosquitoes.

A total of 4,017 mosquitoes successfully fed on infected blood using the artificial membrane feeding technique, with 2,631 (65%) surviving until the time of midgut dissection. Approximately 50% (52/102) of the patients specimens infected mosquitoes, and 23% (602/2,631) of mosquitoes had at least one reported oocyst. The mean oocyst load per infected midgut was 11.5  $\pm$  25.9 with a range of 1–175.

**Relationship of *P. vivax* parasitemia and gametocytemia to transmission of infection to mosquitoes.** In *P. vivax* transmitters, the frequency of high parasitemia (2 + or 3 +) was higher than in non-transmitters (92% vs. 76%). The odds of *P. vivax* transmission to mosquitoes for those with high (2 + or 3 +) versus low parasitemia (< 1 + or 1 +) were statistically significantly greater (Odds Ratio 3.79, 95% CI: 1.13–12.7, *P* = 0.024). The higher parasitemia groups also had a greater mean percentage of mosquitoes infected (Figure 1a, *P* = 0.001 by *t* test) and a borderline statistically significant greater number of oocysts per infected midgut (Figure 1b, *P* = 0.09 by Mann-Whitney *U* test). After grouping the cases into those with or without gametocytes on smear examination, the odds of *P. vivax* transmission for those with gametocytes versus no gametocytes were significantly higher (OR 6.35, 95% CI: 1.70–23.8, *P* = 0.003).

**Results of real-time reverse transcriptase PCR.** Real-time PCR data were available for 37 patients; linear regression showed a significant and positive correlation between log<sub>10</sub> gametocytemia and log<sub>10</sub> real-time reverse transcriptase PCR results (*R*<sup>2</sup> = 0.26, *P* = 0.001; Figure 2); Log<sub>10</sub> gametocytemia and the percentage of mosquitoes infected (*R*<sup>2</sup> = 0.23, *P* < 0.001; Figure 3); and log<sub>10</sub> gametocytemia and log<sub>10</sub>

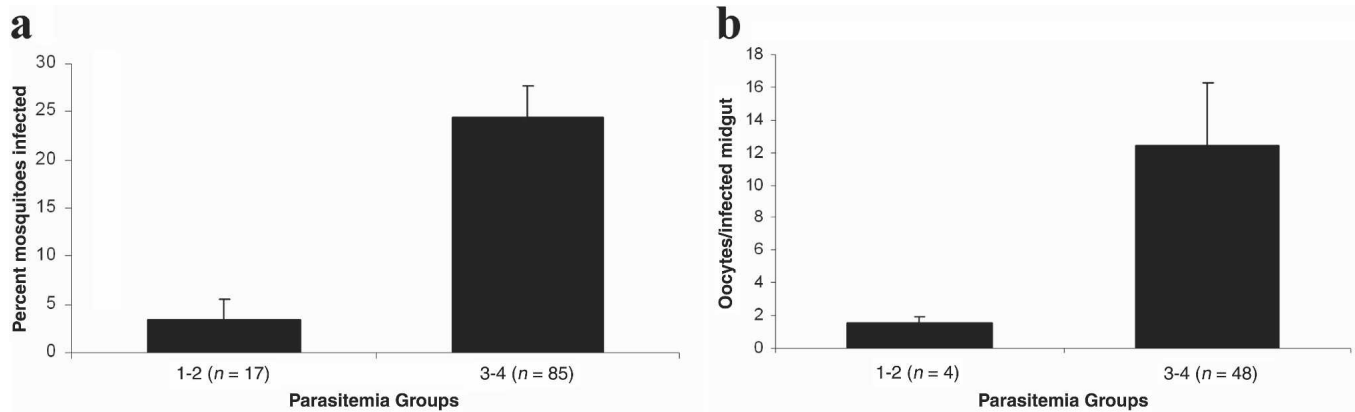


FIGURE 1. Relationship between *P. vivax* parasitemia and (a) percent of *An. darlingi* mosquitoes infected ( $P = 0.001$  by  $t$  test) or (b) oocyst density in infected midguts ( $P = 0.09$  by Mann-Whitney  $U$  test). Groups < 1+ and 1+ vs. 2+ and 3+ include patients with < 2 and  $\geq 2$  parasites per HPF, respectively, on peripheral blood smears. Error bars indicate standard error of mean.

oocysts/midgut ( $R^2 = 0.26$ ,  $P = 0.001$ ; Figure 4). The correlation between  $\log_{10}$  real-time reverse transcriptase PCR and  $\log_{10}$  oocysts/midgut was not statistically significant, but that between  $\log_{10}$  real-time reverse transcriptase PCR and percentage of mosquitoes infected was statistically significant ( $R^2 = 0.21$ ,  $P = 0.004$ ; Figure 5). Univariate analysis of pulse using Student's  $t$  test to compare means shows that transmitters had a lower mean pulse than non-transmitters (mean  $\pm$  standard deviation:  $82.3 \pm 12.3$  vs.  $88.7 \pm 13.5$ , respectively;  $P = 0.016$ ). There was no clear relationship between fever ( $\geq 37.5^\circ\text{C}$ ) at time of membrane feed and transmission (44% vs. 56% for transmitters versus non-transmitters, respectively).

## DISCUSSION

Four major findings emerged from this study. First, we successfully infected an outbred group of *An. darlingi* mosqui-

toes with *P. vivax* obtained *ex vivo* from malaria patients. Second, there was incomplete and inefficient transmission of infection from patients (half of *P. vivax* parasitemic patients) to outbred *An. darlingi* mosquitoes in an area with low malaria endemicity. Third, *P. vivax* transmitters were more likely to have gametocytes on smear, high parasitemia, and a lower mean pulse than non-transmitters. Fourth, there was a correlation between smear and real-time reverse transcriptase PCR gametocytemia.

Our findings are in agreement with other published data where symptomatic *P. vivax* patients were studied. In studies where mosquitoes were allowed to feed directly on symptomatic Thai *P. vivax* patients, there was successful transmission of infection from 63% to 73% cases with a mean oocyst load of 9–13.4 and a mean frequency of infection of 43–57% of mosquitoes fed.<sup>7,24</sup> Similarly, membrane feeds using blood from *P. vivax*-infected symptomatic Thai patients resulted in

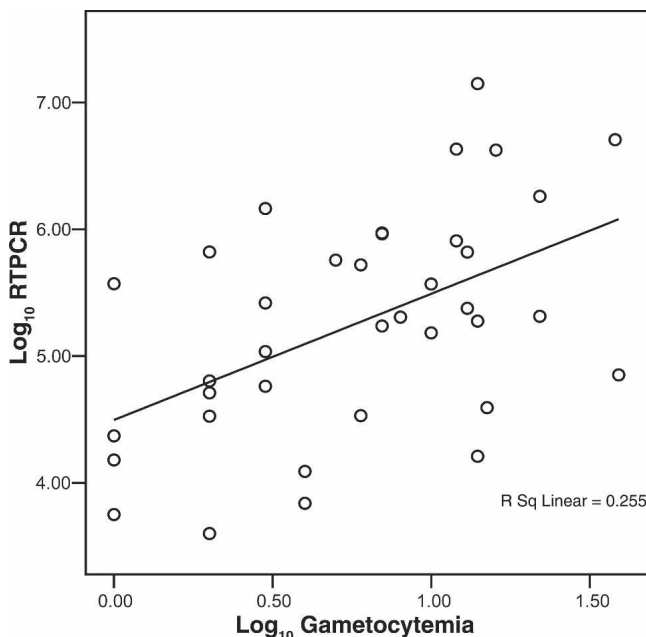


FIGURE 2. Association of  $\log_{10}$  *P. vivax* gametocytemia (excluding null values) and  $\log_{10}$  real-time reverse transcriptase PCR quantification of *Pvs25* mRNA;  $R^2 = 0.26$ ,  $P = 0.001$ ,  $N = 37$ .

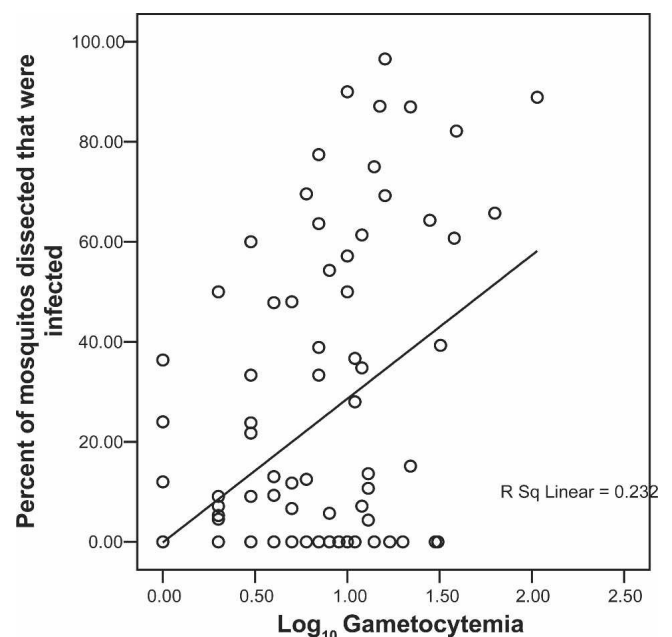


FIGURE 3. Association of  $\log_{10}$  gametocytemia and percent of *An. darlingi* mosquitoes infected by membrane feeding assay with *ex vivo*, human-derived *P. vivax*;  $R^2 = 0.23$ ,  $P < 0.001$ ,  $N = 98$ .



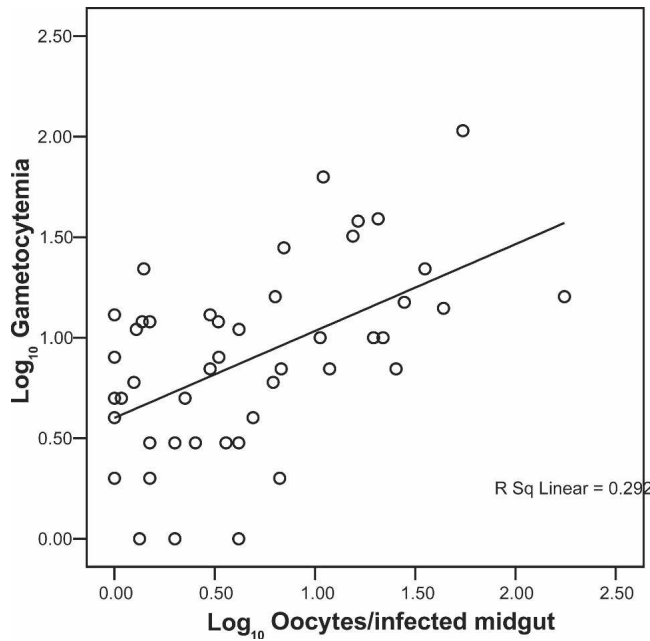


FIGURE 4. Association of  $\log_{10}$  gametocytemia and  $\log_{10}$  oocysts per midgut of *An. darlingi* infected by membrane feeding assay with ex-vivo, human-derived *P. vivax*;  $R^2 = 0.29$ ,  $P < 0.001$ ,  $N = 50$ .

a mean oocyst load of 8.8 with 50.3% mosquitoes infected.<sup>24</sup> One important difference in these studies was that the mosquitoes used were *Anopheles dirus*; these have been raised in colonies for over 25 years and are genetically inbred. Our study confirms a previous, smaller study in which wild-caught *An. darlingi* mosquitoes were infected in the Brazilian Amazon.<sup>25</sup> We, too, demonstrate that experimental infection of a non-colonizable *Anopheles* spp. mosquito is feasible. The

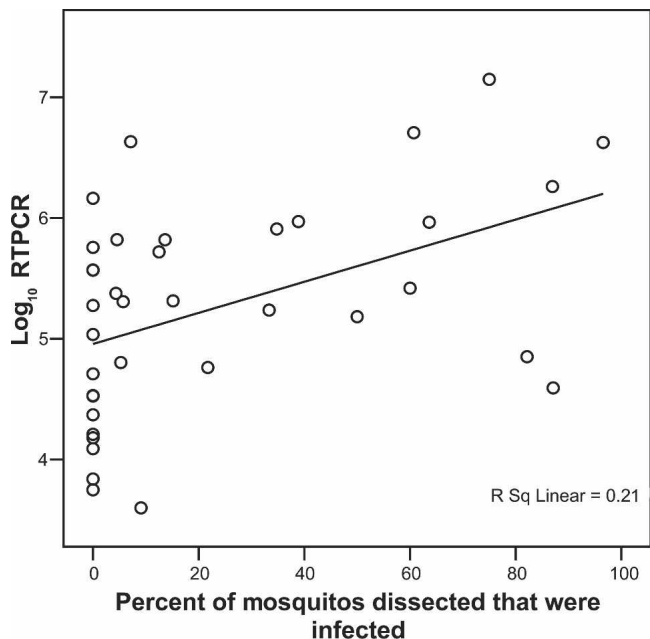


FIGURE 5. Association of  $\log_{10}$  real-time PCR-determined *Pvs25* copy number and proportion of *An. darlingi* that were successfully infected with at least one oocyst;  $R^2 = 0.21$ ,  $P = 0.004$ .

present study, however, provides a basis for applying such a system to assess mechanisms of transmission-blocking immunity in the endemic field setting.

Naturally occurring transmission-blocking immunity has been demonstrated both for *P. falciparum* and *P. vivax* but is incompletely present in human populations in endemic regions.<sup>1-3</sup> The presence of symptomatic or asymptomatic malaria can be used as an indicator of host immunity. Asymptomatic patients, through repeated infections, are more likely to have transmission-blocking antibodies like those against *Pvs25* and *Pvs28* that have been shown to reduce infectivity to mosquitoes.<sup>26</sup> Indeed, asymptomatic *P. vivax*-infected individuals are less efficient transmitters, with only 13.5% patients infecting 0.45% of the mosquitoes with an average of 1.44 oocysts ( $\pm 0.18$ ).<sup>27</sup> Similar results were reported by Alves et al., where the infection rate in mosquitoes was 1.2% for asymptomatic carriers versus 22% for symptomatic patients.<sup>25</sup> In symptomatic patients, other serum factors may modify the ability of *P. vivax* gametocytes to infect mosquitoes, as seen by enhanced mosquito infection rates after replacement of patient plasma with *P. vivax*-naïve sera or plasma.<sup>24</sup> Future studies will compare mosquito infectivity of asymptomatic patients to that of symptomatic patients in association with determination of transmission-blocking antibodies and pro-inflammatory mediators.

It is possible that different genetic types of *P. vivax* could have contributed to variability in transmission. *P. vivax* has been classified into two types, VK210 and VK247, based on the differences in the *Csp* gene that encodes for the circumsporozoite protein, the major sporozoite surface protein. Both of these strains are equally prevalent in Peru, as shown by an equal number of pools of *An. darlingi* being positive for VK210 and VK247.<sup>14</sup> It will be of interest to investigate possible relationships of VK or other genetic types of parasite to transmission.

The presence or absence of gametocytes and their numbers might be considered a good predictor of mosquito infection by malaria parasites. Our study shows that the presence of gametocytes was higher in *P. vivax* transmitters than non-transmitters (94% vs. 72%), and there was a strong correlation between gametocytemia and oocysts/midgut. This observation stands in contrast to an earlier study from Thailand that showed little relation between gametocyte density and either oocyst number or frequency of mosquito infection in *P. vivax* malaria.<sup>7</sup> Unlike *P. falciparum*, the pyrogenic density of *P. vivax* is significantly lower and patients present earlier for medical care. Therefore, it may be possible that the peak gametocyte levels have not yet been achieved and there may be a relationship between the duration of symptoms and gametocytemia. Patients presenting later in the course of the disease may be contributing more to the continued transmission of *P. vivax*.

Gametocytemia as determined by real-time reverse transcriptase PCR correlated well with smear results. However, microscopic smear examination is quite variable and dependent on operator experience, so one might presume that real-time reverse transcriptase PCR might be more precise in measuring gametocytemia. Nonetheless, there was no correlation between molecular quantification of gametocytes and transmission of infection to mosquitoes, as determined by number of oocysts/midgut. Possible explanations for this might be (1) maturity of gametocytes (i.e., *P. vivax* gametocyte maturity

might be heterogeneous, which would give a variable number of *Pvs25* mRNAs per cell), (2) gametocyte sex ratio that might vary between strains, and (3) host response mechanisms that could alter gametocyte infectivity, such as anti-gametocyte antibodies, anemia, cytokines, nitric oxide, and chitotriosidase levels. Pro-inflammatory cytokines (TNF- $\alpha$ , IL-1, and IL-6) and nitric oxide might influence the transmission of infection to mosquitoes by affecting gametocyte levels or their patency. At physiologic concentrations, recombinant TNF- $\alpha$  is anti-parasitic, synergizing with IFN- $\gamma$  to induce production of nitric oxide and other toxic radicals.<sup>28</sup> In the mouse model, C57BL/6 IFN- $\gamma^{-/-}$  mice infected with *P. chabaudi* AS produce lower amounts of other pro-inflammatory mediators, including TNF- $\alpha$  and NO, with significantly higher parasitemia and mortality in comparison with wild-type controls.<sup>29</sup> Nitric oxide has been shown to be cytostatic as well as cytotoxic to *P. falciparum*, *P. berghei*, and *P. chabaudi in vitro*.<sup>30</sup> The infectivity of gametocytes, as shown by direct feeding of *Anopheles stephensi* mosquitoes on mice infected with *P. vinckei petteri*, has also been experimentally observed to be reduced during periods of schizogony that was reversed by injecting the L-arginine analogue, *N*<sup>ω</sup>-nitro-L-arginine (NwNLA), showing that the altered infectivity was due to reactive nitrogen intermediates (RNI).<sup>31</sup> Although our study did not measure cytokine or NO levels, transmitters had lower temperature (although not statistically significant) and had significantly lower pulse rates, possible indicators of lower inflammatory mediators (IL-1, TNF- $\alpha$ ), resulting in less damage to gametocytes with resulting higher level of transmission. Further studies are required to confirm this observation and determine the mechanistic relationship of pro-inflammatory mediators and parasite transmission.

The membrane feeding method to test transmissibility of parasites from humans to mosquitoes has been widely used with blood collected from both *P. falciparum* and *P. vivax* infected patients.<sup>32–37</sup> When compared with direct feeds, membrane feeds have been found in Thailand to result in significantly fewer membrane feeds being infectious (53% vs. 73%). In one study, fewer mosquitoes were infected (50% vs. 57%) and fewer oocysts were produced per mosquito (8.8 vs. 13.4).<sup>24</sup> Possible explanations for this difference are the artificial environment for mosquito feeding, effect of temperature change on gametocyte viability, the effect of handling blood on infectivity, and the use of colonized (used in Thailand) versus outbred mosquitoes (used in the present study). In situations where both experimental and control groups have used the membrane feeding method, this difference in transmission did not affect the final results. We used the membrane feeding method because it is preferred by volunteers and by international institutional review boards, and it has the additional advantage of allowing for experimental manipulation of patient blood. Importantly, we have shown here that *P. vivax* infection can be successfully transmitted to an outbred group of *An. darlingi* mosquitoes. Most important, even in an area of low malaria transmission intensity, there appears to be a high level of transmission-blocking activity.

Transmission-blocking vaccines (TBV) work by inducing antibodies against the sexual stages (gamete, zygote, and ookinete) of the parasite that, when ingested along with a blood meal, block parasite development in the mosquito. Unlike vaccines against pre-erythrocytic and erythrocytic stages, TBVs will not prevent disease but are designed to reduce

malaria transmission. *Pvs25*, expressed on the ookinete surface, is one such vaccine candidate that is undergoing clinical trial.<sup>38</sup> A major emphasis in ongoing studies of transmission-blocking vaccines is to identify a developmentally regulated protein that induces transmission-blocking antibodies boosted by natural infection.

Measuring human infectivity and elucidating factors affecting it is the first step toward identifying potential transmission-blocking interventions that will complement currently available means. Knowing baseline transmission patterns of human-derived parasite infectivity for natural vectors in the field setting will provide the basis for determining the efficacy of transmission-blocking vaccines.

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Authors' addresses: Ajay R. Bharti, Kimberly C. Brouwer, Jessica Lin, and Joseph M. Vinetz, University of California San Diego School of Medicine, La Jolla, California, E-mails: abharti@ucsd.edu, kbrouwer@ucsd.edu, jesstlin@gmail.com, and jvinetz@ucsd.edu. Raul Chuquiayari and Alejandro Llanos-Cuentas, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru, E-mails: raulharo@yahoo.com and allanos@upch.edu.pe. Jeffrey Stancil, Naval Medical Research Center Detachment, Iquitos, Peru, E-mail: stancil@nmrcd.med.navy.mil.

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