

## Short Report: Optimized *In Vitro* Production of *Plasmodium vivax* Ookinetes

Colleen M. McClean, Haydee Guerra Alvarado, Victor Neyra, Alejandro Llanos-Cuentas, and Joseph M. Vinetz\*

Division of Infectious Diseases, Department of Medicine, University of California San Diego, La Jolla, California; Asociación Benéfica PRISMA, Lima, Peru; Institute of Tropical Medicine, Universidad Peruana Cayetano Heredia, Lima, Peru

**Abstract.** Previous reports have described obtaining mature *Plasmodium vivax* ookinetes *in vitro* using blood from infected patients using a simplified, field-based protocol. Here, we report protocols that produce improved *P. vivax* ookinete yields and morphological development. Optimal conditions included induction of gametogenesis using 10 mM Tris, 170 mM NaCl, 10 mM glucose, 25 mM NaHCO<sub>3</sub>, and 100 μM xanthurenic acid for 90 minutes at pH 8.0–8.2, followed by culture in RPMI-1640, 50 mg/mL hypoxanthine, 25 mM HEPES, 29 mM NaHCO<sub>3</sub>, 2 mM L-glutamine, and 20% fetal bovine serum at pH 8.4 for 36 hours. Ookinetes were produced in 86% (18/21) of optimized *in vitro* cultures; yields ranged from 6.5 × 10<sup>4</sup> to 2.8 × 10<sup>6</sup>; percent gametocyte conversion ranged from 1.4% to 4.7%. This improved method is suitable for preparation of *P. vivax* ookinetes in quantities sufficient for biochemical, molecular, and cell biological analysis where basic laboratory facilities are in proximity to patients with vivax malaria.

The study of *Plasmodium vivax* biology lags behind that of other *Plasmodium* species.<sup>1,2</sup> Significant differences in details of life cycle, epidemiology, and disease mechanisms of *P. vivax* and other *Plasmodium* species, such as the early appearance of gametocytes compared with *Plasmodium falciparum*, highlight the importance of detailed species-specific investigations.<sup>3</sup>

Relevant to the development of transmission-blocking strategies is an understanding of the sporogonic development of malaria parasites within the mosquito midgut. Once a mosquito ingests gametocytes, parasites transform into diploid zygotes and then into motile ookinetes.<sup>4–6</sup> *Plasmodium* sporogonic development within the mosquito midgut is asynchronous: *Plasmodium vivax* ookinete density in the mosquito midgut peaks at 36 hours and ookinetes persist up to 48 hours.<sup>4,7</sup> The ookinete penetrates the midgut wall and matures as an oocyst, subsequently releasing sporozoites that invade the mosquito salivary glands thereby enabling transmission to the next human on which the mosquito feeds. New strategies to develop and validate transmission-blocking vaccine strategies involve enhancing our detailed knowledge of malaria parasite sporogonic stage biology. Being able to culture the sexual stage forms of human-infecting malaria parasites in quantities sufficient for systems biology analysis (gene expression profiling and proteomic analysis), immunological, cell biological, and biochemical analysis is essential to move the field forward.

Previous reports have described *in vitro* production of *P. vivax* ookinetes. An important study produced *P. vivax* ookinetes *in vitro*, obtaining blood *ex vivo* from patients in Thailand with vivax malaria.<sup>8</sup> Key findings from this study were that of 98 patients (all with patent gametocytemia), only half produced ookinetes (after 45-minute fertilization and 24-hour incubation), with yields ranging from 10 to 248,500 ookinetes from 5 mL blood. Furthermore, these investigators found that two-thirds of patients infected laboratory-reared *Anopheles dirus* mosquitoes, but that gametocyte concentrations did not correlate with oocyst production or *in vitro* ookinete yield. Whether xanthurenic acid was necessary for ookinete formation was not assessed.<sup>8,9</sup> Other brief reports have described

incidental findings of exflagellating *P. vivax* microgametes<sup>10,11</sup> and *P. vivax* ookinete formation produced in peripheral blood left for prolonged periods at room temperature during diagnostic testing.<sup>12</sup>

We present here an improved methodology for the production of *P. vivax* ookinetes and other sexual stage parasites from field samples in quantities suitable for systems biology, biochemical and cell biological analysis. Photomicrographs of *P. vivax* sexual stage parasites produced *in vitro* that confirm previous work<sup>9</sup> and provide additional morphological exemplars. Elsewhere we have recently reported gene expression profiling of *P. vivax* sporogonic forms.<sup>13</sup>

This study was conducted on 37 *P. vivax* malaria patients reporting to health clinics in Iquitos, Peru. All patients provided written informed consent and the study was approved by the Institutional Review Board/Ethical Committees of the University of California San Diego and Asociación Benéfica PRISMA.

Sources of parasites were adult patients (≥ 18 years of age) with light microscopy-diagnosed, acutely symptomatic patients with *P. vivax* malaria. Mixed infections with *P. falciparum* (as determined by light microscopy) were excluded from the study. For each patient, 10 mL of blood were drawn into heparinized vacutainer tubes and immediately placed in a 37 to 40°C portable incubator for transport from the clinic to the laboratory for processing. Four protocols were attempted before an optimized protocol was selected for ookinete production (Table 1). Within 20 minutes of the initial blood draw samples were pelleted by centrifugation and resuspended in 3 volumes filtration media (FM).<sup>8</sup> To remove white blood cells, resuspended blood cells were allowed to pass through sterile methylcellulose packed into a 20 mL syringe (CF-11, Whatman Ltd., Piscataway, NJ) and allowed to filter by gravity while being maintained in an incubator at 37°C. Cells were then centrifuged (500 g for 5 minutes), resuspended in 3 volumes of gametogenesis solution (GS), and allowed to fertilize by incubation for 45 to 90 minutes at ambient temperature lying sideways in a 50 mL conical tube.<sup>8</sup> Following incubation, parasites were separated by overlaying the suspension on a 47% percoll/RPMI-1640 density gradient followed by 15-minute centrifugation at 500 g.<sup>14</sup> Zygotes were collected from the interface using a 200 μL micropipetter (Gilson, Middletown, WI), twice washed in ookinete medium, pelleted, and resuspended in ookinete medium, and finally incubated for 24 to 48 hours in a 50 mL unvented culture flask, changing the medium every 12 hours.

\* Address correspondence to Joseph M. Vinetz, Division of Infectious Diseases, Department of Medicine, University of California San Diego, 9500 Gilman Drive MC 0741, George Palade Laboratories Room 125, La Jolla, CA 92093-0741. E-mail: jvinetz@ucsd.edu

TABLE 1  
Summary of protocols tested to produce *Plasmodium vivax* ookinetes *in vitro*\*

	Protocol 1	Protocol 2	Protocol 3	Protocol 4
Filtration media	10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.3	10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.3	10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.3	10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.3
Gametogenesis solution	100 $\mu$ M xanthurenic acid in PBS	100 $\mu$ M xanthurenic acid in 25 mM NaHCO <sub>3</sub> /PBS	10 mM Tris, 170 mM NaCl, 10 mM glucose, 25 mM NaHCO <sub>3</sub> , 100 $\mu$ M xanthurenic acid, 10% FBS, pH 8.0–8.2	10 mM Tris, 170 mM NaCl, 10 mM glucose, 25 mM NaHCO <sub>3</sub> , 100 $\mu$ M xanthurenic acid, pH 8.0–8.2
Gametogenesis time	45 min	45 min	90 min	90 min
Percoll separation	Yes	Yes	Yes	No
Ookinete media	RPMI-1640, 50 mg/mL hypoxanthine, 25 mM HEPES, 20% FBS, pH 7.8–8.2	RPMI-1640, 50 mg/mL hypoxanthine, 25 mM HEPES, 20% FBS, pH 7.8–8.2	RPMI-1640, 50 mg/mL hypoxanthine, 25 mM HEPES, 29 mM NaHCO <sub>3</sub> , 2 mM L-glutamine, 20% FBS, pH 8.4	RPMI-1640, 50 mg/mL hypoxanthine, 25 mM HEPES, 29 mM NaHCO <sub>3</sub> , 2 mM L-glutamine, 20% FBS, pH 8.4
% Of cultures producing ookinetes	40%	67%	60%	86%
Mean ookinete yield	$3 \times 10^3$	$1.3 \times 10^5$	$3.7 \times 10^5$	$7.3 \times 10^5$
Mean % gametocyte conversion	0.05%	0.6%	1.6%	3.0%

\* PBS = phosphate buffered saline; FBS = fetal bovine serum.

Following slide preparation and ookinete enumeration by hemacytometer, ookinetes were concentrated and purified on a discontinuous gradient of 80%, 65%, 50%, and 35% Percoll/RPMI-1640.<sup>15,16</sup> Ookinetes were collected from the 35/50% interface using a 200  $\mu$ L micropipetter, washed two times in incomplete culture medium (RPMI-1640, 50 mg/mL hypoxanthine, 29 mM NaHCO<sub>3</sub>) and processed for further study.

The  $\chi^2$  tests and analysis of variance were used to test differences in patient characteristics, parasitemia, and gametocytemia among field samples. Statistical significance was defined at a level of 0.05. Correlation between gametocyte numbers, ookinete yield, and percent gametocyte conversion (the proportion of ookinetes in relation to zygotes + macrogametes [round forms] at the end of the culture incubation period) was tested by Spearman correlation coefficients. Statistical Analysis Software (SAS, version 9.2, Raleigh, NC) was used to perform the analyses.

There was no difference in patient gender ( $P = 0.8$ ), temperature ( $P = 0.3$ ), pulse ( $P = 0.5$ ), number of past clinical cases of vivax malaria ( $P = 0.3$ ), the number of asexual parasites ( $P = 0.4$ ), or the number of gametocytes ( $P = 0.9$ ) observed in the field samples used for the four different culture protocols. The number of *P. vivax* gametocytes observed in the 37 patients ranged from  $1.6 \times 10^6$  to  $7.3 \times 10^7$  gametocytes per 10 mL of blood (mean =  $2.3 \times 10^7$ , median =  $1.8 \times 10^7$ ). Protocol 1 produced ookinetes in 40% (2/5) of cultures with ookinete yields of 840 (percent gametocyte conversion = 0.005%) and 5,120 (percent gametocyte conversion = 0.09%). Protocol 2 produced ookinetes in 67% (4/6) of cultures with ookinete yields ranging from 113 to  $3 \times 10^5$  (mean =  $1.3 \times 10^5$ ) and percent gametocyte conversion ranging from 0.0007% to 1.3% (mean = 0.6%). Protocol 3 produced ookinetes in 60% (3/5) of cultures with ookinete yields ranging from  $9.5 \times 10^4$  to  $9.8 \times 10^5$  (mean =  $3.7 \times 10^5$ ) and percent gametocyte conversion ranging from 1.2% to 2.1% (mean = 1.6%). The best method, Protocol 4, led to ookinete production in 86% (18/21) of cultures with ookinete yields ranging from  $6.5 \times 10^4$  to  $2.8 \times 10^6$  (mean =  $7.3 \times 10^5$ ) and percent gametocyte conversion ranging from 1.4% to 4.7% (mean = 3.0%). The number of gametocytes present in field samples weakly correlated (Spearman  $R^2 = 0.64$ )

with ookinete yield but did not correlate (Spearman  $R^2 = 0.15$ ) with percent gametocyte conversion (Figure 1). Early retort forms were first observed 6 hours after blood was placed in ookinete medium. Maturing retorts were observed 8–24 hours after and mature ookinetes were observed 24–48 hours after initiation of ookinete culture. Ookinetes with the most

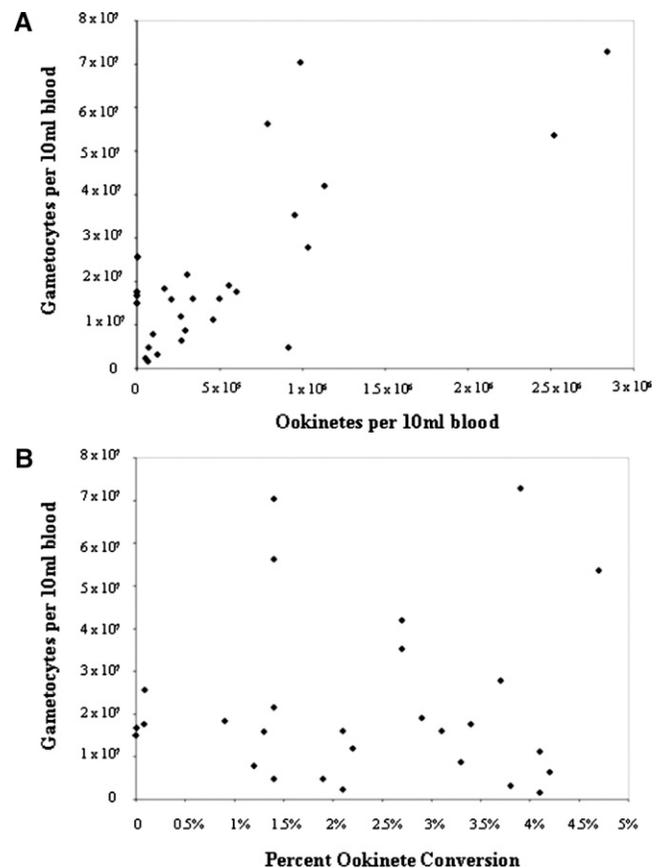


FIGURE 1. Relationship between (A) the number of gametocytes in patient samples and percent gametocyte conversion and (B) the number of gametocytes in patient samples with ookinete yield.

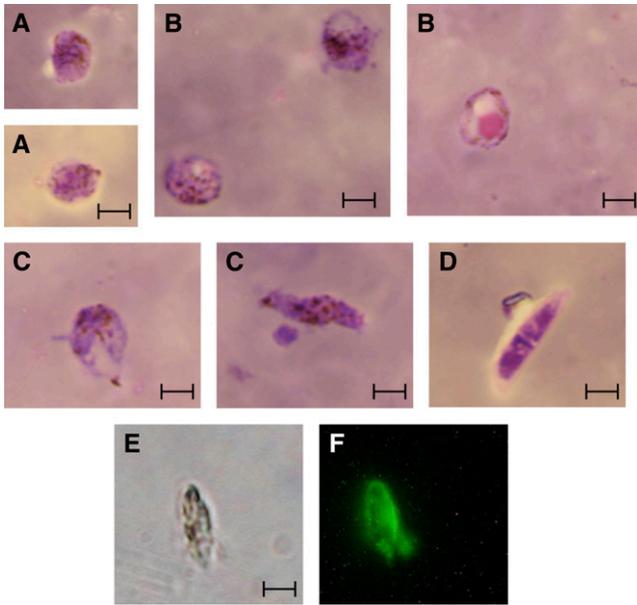


FIGURE 2. Photomicrographs of *in vitro* produced *Plasmodium vivax* sexual stage parasites. Panels: Giemsa-stained thin blood smears: **A**, Zygotes; **B**, Early retort forms; **C**, Maturing retort forms/Early ookinetes; **D**, Mature ookinete; **E**, Bright-field microscope of *P. vivax* ookinete; **F**, Immunofluorescence microscopy of a fixed *P. vivax* ookinete stained with polyclonal rabbit anti-Pvs230, with FITC-labeled goat anti-rabbit antibody used as secondary antibody. Bar 5  $\mu\text{m}$ . This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

characteristic mature morphology were produced after 36 hours of incubation (Figure 2). These *in vitro*-produced ookinetes were used in an indirect immunofluorescence assay to visualize the *P. vivax* sexual stage protein Pvs230, which proteomic analysis of *Plasmodium gallinaceum* ookinetes has shown to be secreted,<sup>17</sup> and which gene expression profiling recently has shown to be up-regulated in both *P. vivax* and *P. falciparum* ookinetes relative to preceding stages.<sup>13,18</sup> We have shown that purified *P. vivax* zygotes and ookinetes have been successfully used to produce sufficient RNA for large scale gene expression profiling.<sup>17</sup>

The best *in vitro* ookinete culture protocol presented here allowed for the reliable production of large quantities of high quality *P. vivax* ookinetes suitable for further studies. By properly solubilizing xanthurenic acid in  $\text{NaHCO}_3$  during gametogenesis a 43-fold increase in ookinete yield over a previously published protocol was obtained.<sup>8</sup> An additional 3-fold increase was obtained using a gametogenesis solution modified from Peiris and others.<sup>19</sup> The final 2-fold increase in ookinete yield was achieved by further modifying the gametogenesis solution by removing fetal bovine serum and by not carrying out Percoll gradient purification before ookinete culture; such handling of the parasites seems to impair the efficiency of ookinete development.<sup>20</sup> The final protocol achieved a 243-fold increase in ookinete yield, a 2-fold increase in the percentage of cultures that produced ookinetes, and a 60-fold increase in the percent gametocyte conversion over previously published protocols.

There were three important limitations of this study. First, *in vitro* ookinete formation was not compared with natural ookinete formation within the mosquito gut of experimentally infected mosquitoes. In the previous study from Thailand, there was only a loose, non-statistically significant association

between mosquito midgut and *in vitro* ookinete formation.<sup>9</sup> Second, parasitized blood was only obtained from patients with symptomatic acute vivax malaria accompanied by low parasitemia ( $\sim 0.1\%$ ) as convenience samples. Obtaining blood from patients with higher parasitemia would likely improve ookinete yields. Third, mature *P. vivax* ookinetes were not assessed for motility or mosquito infectivity. Future work will address these limitations.

Although this improved protocol will allow for advances in the study of *P. vivax* sexual stages, the production of sexual stage parasites will remain difficult until reproducible methods to continuously propagate *P. vivax in vitro* are developed, including the production of functional gametocytes.<sup>21,22</sup> Obtaining parasites *ex vivo* from humans introduces unavoidable variability into laboratory studies of *P. vivax*, which may be caused by host factors or differences in the strains of parasites used in a single study. The yield of *P. vivax* ookinete remains far less than from the rodent parasites *Plasmodium berghei* and *Plasmodium yoelii*, but nonetheless enables investigators to obtain sufficient material to directly study the ookinetes of this human malaria parasite without necessitating the need to analogize with animal models. The development of an *in vitro* culture method allowing for the enrichment of *P. vivax* gametocytes would greatly assist in the high volume production of sexual stage parasites.

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Authors' addresses: Colleen M. McClean, Division of Infectious Diseases, Department of Medicine, University of California San Diego, La Jolla, CA; current address: PCV, Peace Corps/TOGO, Lomé, Togo, E-mail: [cmmcclean@gmail.com](mailto:cmmcclean@gmail.com). Haydee Guerra Alvarado, Asociación Benéfica PRISMA, Lima, Peru, E-mail: [haydeeprisma@gmail.com](mailto:haydeeprisma@gmail.com). Victor Neyra and Alejandro Llanos-Cuentas, Universidad Peruana Cayetano Heredia, Instituto de Medicina Tropical Alexander von Humboldt, Lima, Perú, E-mails: [victor.neyra@upch.pe](mailto:victor.neyra@upch.pe) and [elmer.llanos@upch.pe](mailto:elmer.llanos@upch.pe). Joseph M. Vinetz, Division of Infectious Diseases, Department of Medicine, University of California San Diego, La Jolla, CA, E-mail: [jvinez@ucsd.edu](mailto:jvinez@ucsd.edu).

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