

Purification Methodology for Viable and Infective *Plasmodium vivax* Gametocytes That Is Compatible with Transmission-Blocking Assays

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Significant progress toward the control of malaria has been achieved, especially regarding *Plasmodium falciparum* infections. However, the unique biology of *Plasmodium vivax* hampers current control strategies. The early appearance of *P. vivax* gametocytes in the peripheral blood and the impossibility of culturing this parasite are major drawbacks. Using blood samples from 40 *P. vivax*-infected patients, we describe here a methodology to purify viable gametocytes and further infect anophelines. This method opens new avenues to validate transmission-blocking strategies.

In a scenario of malaria elimination, strategies based on transmission control, rapid diagnosis, effective vaccines, and specific drugs are vital. Early diagnosis and prompt treatment with effective drugs have led to a considerable decrease in the number of cases of falciparum malaria worldwide (1). However, the control and elimination of *Plasmodium vivax* still constitute a great challenge due to specific features of the organism, including gametocytes on peripheral blood early during infection (2), occurrence of a dormant stage in the liver (hypnozoite), and the emergence of drug-resistant forms (3, 4). The development of transmission-blocking molecules to reduce transmission is crucial for malaria eradication. However, because of the lack of a robust continuous *in vitro* culture system (5), studies on *P. vivax* gametocytes have been hampered. Here, using blood samples from 40 *P. vivax*-infected patients and membrane feeding assays, we describe a unique methodology to purify and concentrate viable gametocytes capable of infecting anophelines. This methodology opens avenues for testing drugs or vaccines against the gametocytes, the nonreplicating sexual stage responsible for parasite transmission to mosquitoes (2).

Patients were recruited at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), a tertiary care center for infectious diseases in Manaus, Amazonas State, Brazil. This study was approved by a Brazilian ethics board (CAAE 0044.0.114.000-11). Up to 9 ml of peripheral blood was collected from infected patients, with parasitemia ranging from 297.5 to 30,600 parasites/ μ l and gametocytemia (mean \pm standard deviation [SD]) of 13.3% \pm 16.7%. White blood cells were removed from the blood using a cellulose column (Sigma), as previously described (6). Afterwards, to separate asexual and sexual parasites from noninfected erythrocytes, we used a Percoll-45% (P45) or Percoll-60% (P60) gradient (7, 8) and/or magnetic purification (MP) using MACS-Columns LD/LS (Miltenyi Biotec) (9). Blood samples were maintained at 37°C during all procedures to avoid gametocyte exflagellation. For this purpose, we used a hot plate inside the tissue culture hood and preheated solutions, and all centrifugations were performed at 37°C. The percentage of gametocytes was determined by counting parasites on thin Giemsa smears before and

after the purification process. Correlations were analyzed using the Spearman test. The normality of the data was evaluated with the Kolmogorov-Smirnov test. Comparisons between groups were analyzed using the Mann-Whitney U test (two groups) or the Kruskal-Wallis test. Statistical analyses were performed using GraphPad Prism version 6.

The P45 treatment led to the recovery of trophozoites, schizonts, and gametocytes. A considerable percentage of parasites at the gametocyte stage was obtained (mean \pm SD, 44.1% \pm 33.88%; Fig. 1A) and a considerable amount of trophozoites as well (50.3% \pm 34.17). Moreover, by performing MP after the P45 treatment, we were not able to improve the gametocyte purity (54.3% \pm 41.65%, $P = 0.70$) or the parasitemia (47.7% \pm 50.54%, $P = 0.46$) (Fig. 1A and B). In contrast, after P60 purification, gametocytes were recovered, with low percentages of other parasite stages (gametocytes, 81.6% \pm 30.50%; Fig. 1A). Despite the high degree of purification, noninfected erythrocytes were also found in the P60 interphase that contained gametocytes (parasitemia, 18.9% \pm 27.34%) (Fig. 1B). The P60 procedure rendered significantly higher percentages of gametocytes than those with the P45 procedure (81.6% versus 44.15%, $P = 0.0049$).

Performing MP after the P60 procedure did not improve the percentage of gametocytes recovered (79.9% \pm 38.64%, $P = 0.54$) or the parasitemia level (34.7% \pm 40.49%, $P = 0.65$).

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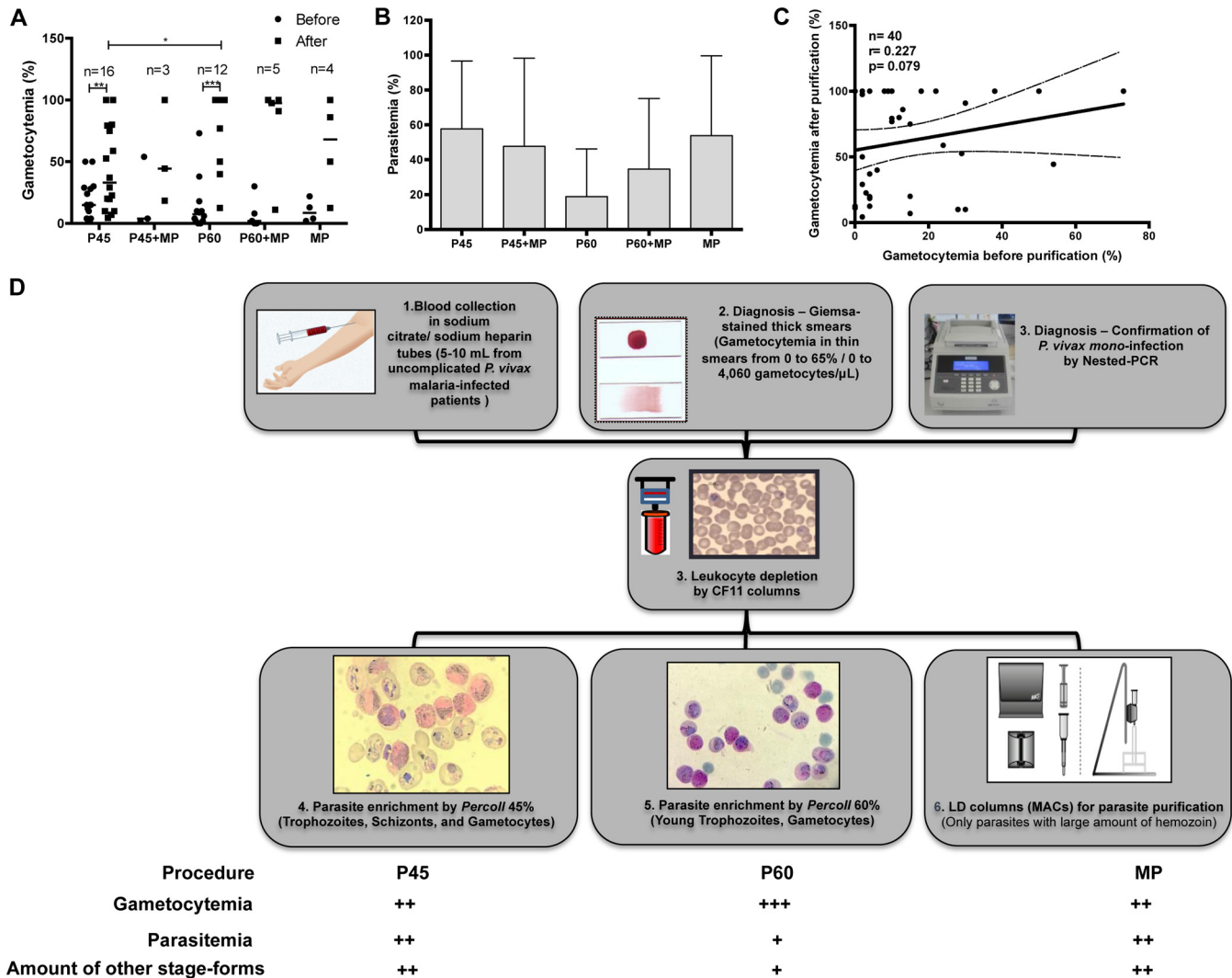


FIG 1 *P. vivax* gametocyte purification. (A) Gametocytemia as gametocytes/total parasites (as a percentage) before and after the indicated purification processes. P45, Percoll 45%; P60, Percoll 60%; MP, magnetic purification. The short horizontal lines represent the analysis between the same procedure before and after purification. The long horizontal line represents the comparison of gametocytemia between P45 and P60 after purification. *, $P < 0.05$; ***, $P < 0.001$. (B) Parasitemia (total parasites/total erythrocyte [as a percentage]) after indicated purification processes. The columns represent the means and the error bars represent the SD. (C). Correlation between gametocytemia before and after purification. The dark solid line represents the linear regression and the dashed lines represent the 95% confidence limit. (D) Step-by-step schematic and comparative representation of the three procedures.

Nevertheless, when the MP method was applied alone, we obtained a gametocytemia of $62.13\% \pm 39.20\%$ (Fig. 1A) and a parasitemia of $53.8\% \pm 45.74\%$ (Fig. 1B). This procedure allowed us to enrich for parasites with large amounts of hemozoin, resulting in only the mature asexual stage (>30 h) and gametocytes retained on the magnetic column (9). No association between the yield of gametocytes after purification and the initial gametocytemia of the patients was found (Fig. 1C). Figure 1D shows a step-by-step schematic flow of all purification procedures.

Considering that robust drug tests rely on parasite viability and infectivity, we conducted the artificial membrane feeding assays only after we established the procedures to purify and increase the number of gametocytes. Those assays were performed using an established colony of the *Anopheles aquasalis* mosquito vector, as described elsewhere (10). Successful infec-

tions were determined by the presence of oocysts in the midgut of the mosquitoes.

A. aquasalis, *Anopheles albitalarsis*, and *Anopheles darlingi* are the main vectors in South America and are associated with malaria transmission (11). *A. aquasalis* has successfully been used under laboratory conditions for *P. vivax* infections (10), and an infection protocol with *P. vivax* on *A. darlingi* was recently established (12). For these assays, purified gametocytes were used to prepare blood dilutions at a hematocrit level of 36% using washed uninfected fresh O+ blood and AB serum at a final volume of 1 ml. Next, 100 mosquitoes were fed on these samples and reared accordingly (10). Prior to infection, the viability of the gametocytes was tested by exflagellation assays. Briefly, 10 μ l of purified gametocytes and autologous serum were placed on a microscope slide for 10 min, and exflagellation was visualized with the aid of a light microscope (Fig. 2A).

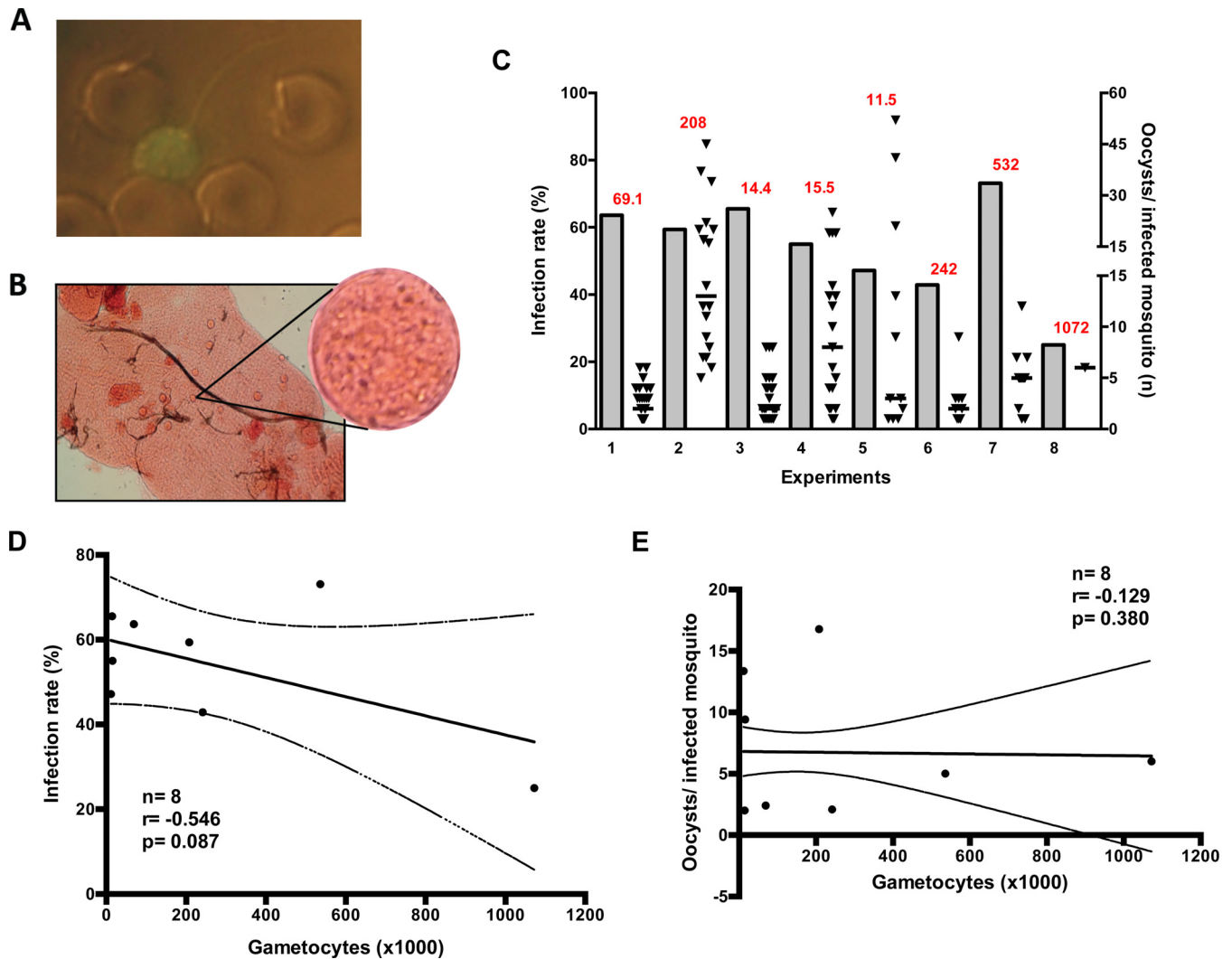


FIG 2 Purified *P. vivax* gametocytes are viable and infective. (A) *P. vivax* gametocyte exflagellation was observed in all purified samples tested. (B) *P. vivax* oocysts in *A. aquasalis* midgut stained with Mercurochrome (Sanvall). (C) Schematic summary of the 8 membrane feeding assays (MFAs) performed for the P45 purification process. An average of 28.6 ± 14.08 mosquitoes were dissected per assay. The infectivity rates are represented as bars. Oocyst numbers in infected mosquitoes are represented in the scatter plot. The number in red above the bars represents the number of gametocytes $\times 1,000$ fed to 100 mosquitoes in each experiment. (D) Correlation between the numbers of gametocytes fed to mosquitoes and the infection rate. (E) Correlation between the numbers of gametocytes fed to mosquitoes and the number of oocysts. The dark solid lines represent the linear regression and the dashed lines represent the 95% confidence limit.

The capacity of *P. vivax* purified gametocytes to infect mosquitoes was evaluated in P45 purification and confirmed by the visualization of oocysts in the midgut (Fig. 2B). As shown in Fig. 2C, the infectivity rate of mosquitoes fed with dilutions prepared with P45 purified gametocytes ranged from 25.0% to 73.1% (mean \pm SD, $54.0\% \pm 15.2\%$). The median number of oocysts produced in mosquitoes fed with *P. vivax* P45 purified gametocytes was 4.0 (interquartile range [IQR], 2.0 to 8.0 oocysts). Moreover, we found no correlation between the number of gametocytes given to mosquitoes and the infection rate (Fig. 2D) or the number of oocysts in infected mosquitoes (Fig. 2E). These data, a representation of the results from 8 independent experiments (Fig. 2A to C), demonstrate that the purified gametocytes were viable (exflagellation) and infective (oocyst production in the midgut of the mosquitoes).

Based on the different gametocyte purification method out-

comes, the choice of procedure will depend on the goal to be achieved. The P60 method will be more appropriate for obtaining gametocytes in the absence of asexual parasite stages. To achieve high concentrations of parasites, the use of P45 or MP alone will be the best choice, because the combination of these methodologies (P45 plus MP or P60 plus MP) did not improve the gametocytemia or parasitemia levels. Collectively, these data validate a methodology to purify viable and infective *P. vivax* gametocytes, assisting in the discovery of new antimalarial drug leads and vaccine candidates.

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