COMPARISON OF ARTIFICIAL MEMBRANE FEEDING WITH DIRECT SKIN FEEDING TO ESTIMATE THE INFECTIONOUSNESS OF \textit{PLASMODIUM VIVAX} GAMETOCYTE CARRIERS TO MOSQUITOES

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Abstract. The efficacy of a membrane-feeding apparatus as a means of infecting \textit{Anopheles dirus} mosquitoes with \textit{Plasmodium vivax} was compared with direct feeding of mosquitoes on gamocyte carriers. Volunteers participating in the study were symptomatic patients reporting to malaria clinics in western Thailand. Direct mosquito feeds were conducted on 285 \textit{P. vivax}-infected individuals. Four methods of preparing blood for the membrane-feeding apparatus were evaluated. They included 1) replacement of patient plasma with sera from a \textit{P. vivax}-naive donor (n = 276), 2) replacement of patient plasma with plasma from a \textit{P. vivax}-naive donor (n = 83), 3) replacement of patient plasma with that individual’s own plasma (n = 80), and 4) whole blood added directly to the feeder (n = 221). Criteria used to compare the different methods included 1) number of feeds infecting mosquitoes, 2) percent of mosquitoes with oocysts, and 3) mean number of oocysts per positive mosquito. For most parameters, the direct-feeding method was not significantly different from methods that replaced patient plasma with sera from a \textit{P. vivax}-naive donor. However, direct feeding was more effective than use of whole blood or blood that was reconstituted with the patient’s own plasma. These data suggest a possible role of transmission-blocking antibody. The implications towards development of a membrane-feeding assay for the evaluation of candidate transmission-blocking malaria vaccines is discussed.

INTRODUCTION

The successful development and validation of a transmission-blocking malaria vaccine requires a method of estimating the infectiousness of \textit{Plasmodium} gametocyte carriers to mosquitoes.\textsuperscript{1} Determination of gametocyte density in blood smears does not adequately predict ability to infect mosquitoes.\textsuperscript{2–5} Therefore, infectivity is best assessed by allowing susceptible anopheline mosquitoes to feed on gametocyte-containing blood and recording infection rates in the mosquitoes.\textsuperscript{6} Although the direct-feeding method originally developed by Muirhead-Thompson\textsuperscript{4} reflects epidemiologic reality,\textsuperscript{7} membrane feeding is more widely used due to ethical reasons.\textsuperscript{8} A membrane-feeding method using an animal membrane was first used to infect mosquitoes with malaria more than 60 years ago.\textsuperscript{9} Rutledge and others\textsuperscript{10} subsequently developed a method that used an artificial membrane. Since then, the membrane-feeding method has been widely used in malaria studies.\textsuperscript{2,8,11–14} However, few studies have compared the relative efficacy of the membrane-feeding method with that of direct feeding on gametocyteic patients. Yoell,\textsuperscript{9} Dashkova and Rasnitsyn,\textsuperscript{15} Awono-Ambene and others\textsuperscript{16} reported that infection rates by membrane feeding were as high as those obtained by direct feeding on patients, whereas Bonnet and others\textsuperscript{17} reported that infection rates were lower when \textit{Anopheles gambiæ} fed on \textit{Plasmodium falciparum}–infected blood placed in a membrane feeder than when fed directly on the infected host. In spite of the lower infection rates when using the membrane feeder, there was good concordance between the two tests.

Increasing interest in the development of transmission-blocking malaria vaccines has resulted in continued refinement of the membrane-feeding method.\textsuperscript{12} Two primary methods have been used to evaluate the ability of patient sera to block transmission of \textit{P. falciparum}. In the first, patient sera is added to cultured gametocytes as described by Lensen and others.\textsuperscript{12} In the second, whole blood is collected from gametocyteic patients and the serum is replaced as described by Mulder and others.\textsuperscript{17} The advantage of the latter method is that transmission reduction is evaluated under more natural conditions. However, large sample sizes are required due to the low mosquito infection rates and the low oocyst numbers normally encountered.\textsuperscript{12,18}

Although Golenda and others\textsuperscript{19} reported a method for the continuous culture of \textit{P. vivax}, this method has not yet produced mature gametocytes capable of infecting mosquitoes. Therefore, studies evaluating vaccines capable of interrupting transmission of \textit{P. vivax} currently must be conducted using gametocytes obtained from naturally infected patients. In this study, we evaluated several methods of preparing gametocyteic blood collected from patients naturally infected with \textit{P. vivax} for membrane feeding. Each of these methods was compared with direct feeding to determine concordance between the methods, thereby allowing us to determine the extent to which the different methods of preparing blood could be used as tools for evaluating human to mosquito malaria transmission.

MATERIALS AND METHODS

Study site and patients. The study was conducted in three provinces in western Thailand. \textit{Plasmodium vivax} and \textit{P. falciparum} are the predominant parasite species in the region, followed by \textit{P. malariae} and \textit{P. ovale}. Individuals who participated in the study were volunteers 15 years of age and older seeking treatment at four local malaria clinics (one clinic in Ratchaburi Province, one clinic in Kanchanaburi Province, and the Mae Sod and Mae Kasa clinics in Tak Province). Thick and thin blood smears were prepared from each individual and stained with 10% Giemsa by malaria clinic staff. Gametocyte and trophozoite densities were determined for all \textit{P. vivax}-positive patients by counting the number of parasites per 500 leukocytes using oil immersion microscopy, and converting raw counts to parasites/microliter by assuming a
count of 7,000 leukocytes/μL. If gametocytes were present, the patient was asked to enroll in the study. After being briefed on the project and completing consent forms, approximately 10 mL of blood was collected by venipuncture and 100 mosquitoes were fed on the arm of the subject (more information on direct and membrane feeding is provided in the Materials and Methods). After mosquito feeding was completed, volunteers were released from the study and received antimalarial treatment from the malaria clinic staff. The complete process (entry into the malaria clinic for initial diagnosis until receiving malaria treatment) took approximately 90 minutes. All human subjects research conducted in these studies was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army.

**Mosquitoes.** *Anopheles dirus* A has been maintained at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, for more than 25 years. Mosquitoes were reared at 26°C and a relative humidity of 70–80% in the insectary in Bangkok. Mosquitoes were provided 10% sucrose and water ad libitum. Groups of 100 mosquitoes were transported from Bangkok to the field sites in pint cartons placed inside a cooler. At the field sites, 6–8-day-old mosquitoes were provided with water only for 12 hours prior to feeding.

**Direct-feeding and membrane-feeding assays.** Infectivity of each selected individual to laboratory-bred mosquitoes was evaluated by both direct- and membrane-feeding assays. For direct feeding, a pint carton containing 100 starved mosquitoes were applied to the inside forearm of the patient. Mosquitoes were allowed to feed for 30 minutes, after which the carton was removed from the patient’s arm and unengorged mosquitoes removed from the carton. Each patient was provided with an anti-histamine cream.

For membrane feeding, lithium-heparinized vacutainer tubes (Franklin Lakes, NJ) were filled with 10 mL of blood drawn by venipuncture from each patient. Blood was treated in one of four different ways (see next section) prior to adding 1 mL final volume to a 5-cm diameter glass feeder closed with a baudruche membrane. To prevent exflagellation of microgametocytes, a constant temperature of 37°C was maintained using a water jacket circulation system. The mosquitoes were allowed to feed for 30 minutes, after which the glass membrane feeder was removed from the top of the carton and all unengorged mosquitoes were removed. After feeding, all mosquitoes were returned to the AFRIMS insectary in Bangkok. Mosquitoes were provided 10% sucrose daily until they were dissected on day 7 after feeding. Midguts were dissected in a drop of mercurochrome in phosphate-buffered saline, and oocysts were counted by microscopic examination.

**Preparation of gametocytemic blood for membrane feeding.** Gametocytemic blood was prepared for membrane feeding in one of four different ways. The first method consisted of placement of 1 mL of the patient’s own whole blood directly into the membrane feeder (Whole-Blood). In the second method, 1 mL of blood from each patient was centrifuged at 500 × g for two minutes. The sera was then removed and replaced with an equal volume of AB serum from a *P. vivax*-naive donor. The packed red blood cells and donor sera were carefully mixed and added to the membrane feeder (Donor-Sera). In the third and fourth methods, patient plasma was removed and the packed red blood cells were reconstituted with either the patient’s own plasma (Donor-Plasma) or with AB plasma from a *P. vivax*-naive donor (Donor-Plasma). One milliliter of each mixture was added to the membrane feeder.

**Statistical analysis.** Three parameters were chosen for analysis: proportion of all feeds that yielded any infected mosquitoes, proportion of infected mosquitoes per positive feed, and oocyst load (mean oocyst number per midgut in each batch, using only infected midguts). We initially evaluated the effect of macrogametocyte, microgametocyte, and asexual stage parasite density on each of these three parameters in mosquitoes fed directly on the patients. A simple regression test was used to determine if there was a linear correlation between parasite load and infection rates.

We subsequently compared the direct-feeding method with the four membrane-feeding methods for each of the three parameters. The chi square test was used to compare the proportion of direct feeds that yielded infected mosquitoes with the proportion of membrane feeds infecting mosquitoes and the proportion of infected mosquitoes per positive feed with each feeding method. The Mann-Whitney U test was used to compare mean numbers of oocysts in the direct-feeding groups with each of the membrane-feeding methods.

Due to the fact that feeds were conducted at four separate clinics and that the interval between drawing blood and conducting membrane feeds varied between clinics, we analyzed effect of the different methods of feeding at each site on oocyst loads. Data were transformed to stabilize variance using the reciprocal transformation (-1/y), then analyzed using analysis of variance. Multiple pairwise comparisons of means were assessed using Tukey’s test for differences between level means with the family error rate set at 0.05.

**RESULTS**

**Patient data.** A total of 285 *P. vivax*-infected individuals were enrolled in the study. There were 226 men, 54 women, and 5 individuals whose sex was not recorded. The mean ± SEM age was 28.3 ± 0.62 years (range = 15–63 years). Mean ± SEM parasite rates in these 285 individuals were as follows: 7,060 ± 301 asexual parasites/μL (range = 336–14,000), 389 ± 36 macrogametocytes/μL (range = 0–5,726), and 169 ± 13 microgametocytes/μL (range = 0–1,456).

**Direct feeds.** Of the 285 patients who had direct feeds conducted on them, 209 (73%) successfully infected mosquitoes and 76 (27%) did not infect mosquitoes (Table 1). Of these patients, five (1.8%) had no macrogametocytes or microgametocytes visible by microscopy, while 17 (6.0%) had macrogametocytes but no microgametocytes. Two of the five patients (40%) with no visible macrogametocytes or microgametocytes infected mosquitoes and seven of the 17 patients (41%) without visible microgametocytes infected mosquitoes. Of the 263 patients with visible macrogametocytes and microgametocytes, 200 (76%) infected mosquitoes. There was no significant correlation between parasite rates (number of asexual parasites, macrogametocytes, or microgametocytes) in the 285 patients and the percentage of mosquitoes becoming infected after direct feeding (Figure 1), and the parasite rates showed no strong correlation with mean observed oocyst numbers per infected mosquito (Figure 2).
Membrane feeds. Results from the paired feeds (direct mosquito feeds on a given patient compared with one or more membrane-feeding methods using blood from the same patient) are presented in Table 1. A total of 221, 276, 80, and 83 direct feeds were compared with the Whole-Blood, Donor-Sera, Own-Plasma and Donor-Plasma membrane-feeding methods, respectively. Significantly fewer Whole-Blood feeds were infectious than were direct feeds, with significantly fewer mosquitoes infected after feeding on whole blood and fewer oocysts produced per mosquito (Table 1). Although there were no significant differences in the percent of direct feeds and Own-Plasma membrane feeds that were infectious, a significantly lower proportion of mosquitoes developed oocysts after the Own-Plasma membrane feeds, with significantly fewer oocysts per positive mosquito. In contrast, replacement of patient sera/plasma with donor sera/plasma (Donor-Sera and Donor-Plasma feeds) resulted in feeds that were not significantly different from or even better than the

**Table 1**

Comparison of membrane feeding with direct feeding on a patient as a method of infecting *Anopheles dirus* mosquitoes with *Plasmodium vivax*.

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>% of feeds infecting mosquitoes (Positive feeds/total feeds)‡</th>
<th>% of mosquitoes fed on all infectious patients that developed oocysts (Positive mosquitoes/total mosquitoes)¶</th>
<th>Mean (geometric) number of oocysts per positive mosquito (SEM)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct feeds</td>
<td>72.9 (161/221)</td>
<td>56.9 (570/1,001)</td>
<td>13.4 (1.1)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>57.9 (128/221)</td>
<td>50.3 (263/522)</td>
<td>8.8 (1.1)</td>
</tr>
<tr>
<td>Direct feeds</td>
<td>73.2 (202/276)</td>
<td>57.1 (720/1,262)</td>
<td>12.8 (1.1)</td>
</tr>
<tr>
<td>Donor sera</td>
<td>70.7 (195/276)</td>
<td>54.2 (619/1,143)</td>
<td>12.8 (1.1)</td>
</tr>
<tr>
<td>Direct feeds</td>
<td>68.8 (55/80)</td>
<td>65.7 (207/315)</td>
<td>13.7 (1.1)</td>
</tr>
<tr>
<td>Own plasma</td>
<td>67.5 (54/80)</td>
<td>53.1 (169/318)</td>
<td>9.2 (1.1)</td>
</tr>
<tr>
<td>Direct feeds</td>
<td>68.7 (57/83)</td>
<td>59.5 (203/341)</td>
<td>10.9 (1.1)</td>
</tr>
<tr>
<td>Donor plasma</td>
<td>57.8 (48/83)</td>
<td>52.6 (153/291)</td>
<td>17.4 (1.1)</td>
</tr>
<tr>
<td>Total direct feeds</td>
<td>73.3 (209/285)</td>
<td>56.9 (747/1,312)</td>
<td>30.9 (1.1)</td>
</tr>
</tbody>
</table>

* Four methods of preparing blood for membrane feeding were compared with direct feeding. Patients were enrolled from four separate malaria clinics and data were pooled.
† Direct feeds—mosquitoes fed on the arm of a patient; whole blood—whole blood from a patient added directly to the membrane-feeding apparatus; donor sera-serum removed from patient blood and packed cells reconstituted with AB serum from a *P. vivax*-naive donor; own plasma-plasma removed from patient blood and packed cells reconstituted with the patient’s own plasma; donor plasma-plasma removed from patient blood and packed cells reconstituted with AB plasma from a *P. vivax*-naive donor.
‡ Membrane-feeding values followed by a ¶ symbol are significantly different (*P* < 0.05, by chi-square test) from corresponding direct-feeding values.
§ Membrane-feeding values followed by a # symbol are significantly different (*P* < 0.05, by Mann-Whitney U test) from direct-feeding values.

![Effect of Parasitemia on Mosquito Infection Rates](image1)

![Effect of Parasitemia on Mosquito Oocysts Rates](image2)

**Figure 1.** Relationship between the number of *Plasmodium vivax* macrogametocytes, microgametocytes, and asexual stage parasites per microliter of blood and the percentage of *Anopheles dirus* mosquitoes becoming infected after feeding directly on 285 patients.

**Figure 2.** Relationship between the number of *Plasmodium vivax* macrogametocytes, microgametocytes, and asexual stage parasites per microliter of blood and the mean number of oocysts per positive *Anopheles dirus* mosquito after feeding directly on 285 patients.
Effect of clinic location on infection rates. Although direct mosquito feeds and blood draws were conducted at four separate clinics, membrane feeds were only conducted at two of the malaria clinics (Kanchanaburi and Mae Sod) and at the AFRIMS laboratory in Bangkok. Blood drawn from patients reporting to the Kanchanaburi and Mae Sod clinics was placed in the membrane feeders within 30 minutes of being collected. In contrast, blood drawn from patients reporting to the Mae Kasa and Ratchaburi clinics had to be transported to the Mae Sod clinic and AFRIMS, respectively, prior to being placed in the membrane feeders. Blood collected from the Mae Kasa clinic was normally added to the membrane feeders 30 minutes to four hours after being drawn, while blood collected at the Ratchaburi clinic was placed in the membrane feeders from four to eight hours after being drawn. The effect of the clinic at which blood was collected on mosquito infection rates is presented in Tables 2 and 3.

Between 67% and 78% of the direct feeds conducted at all four clinics infected mosquitoes, and 48–67% of the mosquitoes contained at least one oocyst (Table 2). At the Kanchanaburi and Mae Kasa clinics, significantly fewer cases infected mosquitoes by the Whole-Blood membrane-feeding method than when using the direct-feeding method. In contrast, no effect was seen at the Mae Sod and Ratchaburi clinics. Similar results were observed with the proportion of mosquitoes that developed oocysts after being fed using the Whole-Blood method compared with direct feeds. At the Mae Sod and Mae Kasa clinics, Whole-Blood feeds yielded significantly fewer positive mosquitoes than did direct feeds, while at the Kanchanaburi and Ratchaburi clinics, there was no significant difference. At all four clinics, the Whole-Blood membrane-feeding method resulted in significantly fewer oocysts per mosquito than did direct feeds (Table 2).

In contrast to the whole-blood membrane-feeding method (which was significantly worse than the direct-feeding method for most of the criteria examined), the Donor-Sera membrane-feeding method was not significantly different than the direct-feeding method for most of the criteria examined. A significantly lower proportion of mosquitoes were infected using the Donor-Sera method at the Kanchanaburi and Ratchaburi clinics compared with direct feeds, and the Donor-Sera method at the Ratchaburi clinic resulted in mosquitoes with significantly fewer oocysts than did the direct-feeding method (Table 2). Overall (data from all four clinics pooled), the Donor-Sera method was not significantly different from the direct-feeding method whereas the Whole-Blood method was significantly different. A summary of the statistical analysis of the effect of Whole-Blood and Donor-Sera membrane-feeding methods compared with the direct-feeding method on the mean number of oocysts produced per mosquito at the four clinics is presented in Table 3. This analysis clearly demonstrates that the Whole-Blood feeding method was statistically worse than the direct-feeding method at all four malaria clinics, whereas the Donor-Sera method was not statistically different from the direct-feeding method.

**DISCUSSION**

In this report, we present the first comprehensive comparison of the membrane-feeding method with direct feeding as a means of infecting anopheline mosquitoes with *P. vivax*. In contrast to previous studies with *P. falciparum* that used relatively few infected volunteers as a source of gametocytemic blood, we report results acquired using a total of 285 *P. vivax*-infected volunteers. Almost all Thai patients 15 years of age and older who were infected with *P. vivax* were willing to allow us to feed up to 200 mosquitoes directly on their arms. In more than 20 years of conducting direct mosquito feeds, we have rarely encountered any reluctance regarding this procedure (Sattabongkot J, unpublished data). In Thailand, the main advantage of using the membrane-feeding system therefore results from the ability to manipulate the blood rather than any difficulty in recruiting volunteers for direct mosquito feeds.

The membrane-feeding method has been widely used as a means of infecting mosquitoes with blood collected from both *P. falciparum* and *P. vivax* -infected volunteers. Although the direct-feeding method first developed by Muirhead-Thomson more accurately reflects epidemiologic reality, volunteers frequently prefer to provide blood by venipuncture rather than allowing mosquitoes to feed directly on their skin. In addition, in contrast to the direct-feeding method, the membrane-feeding method allows for the controlled manipulation of the patient blood. Due to increasing interest in the development of transmission-blocking malaria vaccines, it is crucial that the membrane-feeding method be validated before being used in the field. In spite of the numerous advantages of the membrane-feeding method
that have led to its widespread use, few studies have conducted controlled comparisons of this method with direct feeding on mosquitoes. Although Gamage-Mendis and others\textsuperscript{28} conducted both direct feeds and membrane feeds on \textit{P. vivax}-infected patients in Sri Lanka, no attempt was made to compare the relative efficacy of the two methods. Collins and others\textsuperscript{20} reported, albeit for only a single case, higher \textit{P. falciparum} infections in mosquitoes following membrane feeding than after a direct feed on human subjects. Toure and others\textsuperscript{21} compared membrane feeding with direct feeding with 11 \textit{P. falciparum}-infected patients and found no obvious correlation in either intensity of infectivity or percent of mosquitoes infected between the two methods. Awo-no-Ambene and others\textsuperscript{16} and Bonnet and others\textsuperscript{6} recently compared membrane and direct feeding as a means of infecting anopheles mosquitoes with \textit{P. falciparum}; however, similar studies have not been conducted with \textit{P. vivax}.

Results from this study confirm previous findings\textsuperscript{5} that \textit{P. vivax} gametocyte density is not a good indicator of infectivity to mosquitoes (Figures 1 and 2). Many patients with high numbers of macrogametocytes and/or microgametocytes produced relatively few oocysts, while a number of patients with few gametocytes successfully infected mosquitoes.

Our data also clearly demonstrates that placement of patient whole blood directly into the membrane-feeding system is a less effective method of infecting mosquitoes than direct feeding on a patient (Tables 1 and 2, Figure 3). Almost all criteria that we assessed were significantly lower following placement of whole blood in the membrane feeder than when using the direct-feeding method. Although we are not certain why the Whole-Blood membrane-feeding method was less efficient than the direct-feeding method, there are a number of possible explanations that we did not evaluate. These possible explanations include the artificial environment that the feeding mosquito is faced with (probing through a baudiene membrane into a pool of blood versus probing through human skin in an effort to locate a capillary), changes in blood temperature that may affect gametocyte viability, and effect of handling the blood on infectivity, among others.

Results from this study also suggest that serum factors play a key role in the ability of \textit{P. vivax} gametocytes to infect mosquitoes. Although the use of the Whole-Blood membrane-feeding method was universally worse than direct feeding as a means of infecting mosquitoes, replacement of patient plasma with \textit{P. vivax}-naive sera or plasma enhanced mosquito infection rates in a number of instances (Tables 1 and 2, Fig-
uring 3). Although we did not specifically identify host factors that were responsible for reductions in mosquito infectivity, it seems likely that transmission-blocking antibody 1,3,12 may have played a key role. All patients evaluated in this study were symptomatic adults reporting to local malaria clinics for treatment. The fact that the patients were symptomatic suggests that transmission-blocking antibody levels may have been relatively low. We are currently using procedures similar to those described in this study to evaluate patient infectivity to mosquitoes in a highly endemic village in western Thailand.33 More than 95% of P. vivax malaria patients in this village are asymptomatic, suggesting that transmission-blocking antibody levels may be relatively high. We hope to quantify transmission-blocking antibody rates in each patient and then compare mosquito infection rates after feeding the mosquitoes on blood in which the patients packed blood cells have been reconstituted with plasma from a P. vivax-naïve donor and with the patient’s original plasma. We hope that results from this additional study will provide additional data on the possible role of transmission-blocking antibody on the infectivity of P. vivax gametocytes.

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