Am. J. Trop. Med. Hyg., 97(3), 2017, pp. 719–725
doi:10.4269/ajtmh.16-1000

Optimizing Direct Membrane and Direct Skin Feeding Assays for *Plasmodium falciparum* Transmission-Blocking Vaccine Trials in Bancoumana, Mali

Mamadou B. Coulibaly,[1]* Erin E. Gabriel,[2] Youssouf Sinaba,[1] Daman Sylla,[1] Adama Sacko,[1] Lakamy Sylla,[1] Boubacar Coulibaly,[1] Jen C. C. Hume,[3] Ibrahima Baber,[1,4] Mahamadoun H. Assadou,[1] Issaka Sagara,[1] Yimin Wu,[3,5] Sara A. Healy,[3] Ogobara Doumbo,[1] Sekou F. Traore,[1] and Patrick E. Duffy[3]

[1]*Malaria Research and Training Center, ICER-MALI-USTTB-NIAID/NIH, Bamako, Mali;* [2]*Biostatistics Research Branch, NIAID/NIH, Bethesda, Maryland;* [3]*Laboratory of Malaria Immunology and Vaccinology, NIAID/NIH, Bethesda, Maryland;* [4]*Abt Associates Inc., Monrovia, Liberia;* [5]*PATH-Malaria Vaccine Initiative, Washington, District of Columbia*

Abstract. Malaria transmission-blocking vaccines (TBV) have been evaluated in field trials in Mali since 2013. However, the assays currently used to measure serum antibody TB activity (TBA) after vaccination are highly variable, in part due to the lack of optimization and standardization for field assays in which mosquitoes feed on gametocytemic blood. Herein, we report a study conducted in Bancoumana village, Mali, where we identify and optimize the parameters that contribute to successful mosquito feeding outcomes in both direct skin feeds (DSFs) and direct membrane feeding assays (DMFA). These parameters include: 1) mosquito age, 2) duration of mosquito starvation prior to feeding, 3) membrane selection for DMFA, 4) anatomical location of DSF feeding (arm, calf, and ankle), and 5) time of day for DSF (dawn or dusk). We found that younger mosquitoes were significantly associated with higher feeding, survival, and infection rates. Longer starvation times were positively, but not significantly, associated with higher infection rates, but were negatively associated with feeding and survival. Membrane type and body location did not affect infection outcome significantly. Although dusk was found to be associated with higher infection rates, this may be confounded by the time from positive blood smear. Based on these findings, we make specific recommendations for optimal feeding parameters in the different assay types to maximize the chance of detecting parasite transmission in a standardized manner.

INTRODUCTION

Malaria remains a major public health issue with an estimated 214 million cases and 438 thousand deaths in 2015, the majority in Sub-Saharan Africa.[1] Current malaria control strategies are based on rapid diagnosis, effective treatment, and vector control. Drugs are available for treating both uncomplicated and complicated malaria, and long-lasting insecticide-treated nets and indoor residual spraying are being used for vector control on a large scale. Although these tools have lowered the malaria burden, malaria continues to claim hundreds of thousands of lives and contributes to the impoverishment of already poor populations.[2] The economic loss due to malaria is estimated to be 12 billion dollars per year for the African continent with a 1.3% decrease in gross domestic product.[3] Malaria control has been hindered by several factors including but not limited to: 1) resistance of both parasite and mosquito to common drugs and insecticides, respectively, and 2) the lack of effective vaccines.

Among many possible malaria vaccine strategies, transmission-blocking vaccines (TBVs) were conceived primarily as tools to interrupt malaria transmission. TBVs are expected to work by inducing antibody responses that inhibit the sexual development of malaria parasites in the midgut of the mosquito, thereby preventing parasite transmission to the next human host.[4] Such a vaccine could play an important role in malaria elimination and/or eradication programs if shown to be efficacious at the community level.

Currently, the activity of TBVs is evaluated by measuring parasite transmission to mosquitoes in membrane and direct feeding assays. These assays allow mosquitoes to ingest parasites together with test antibodies, through artificial membrane feeders or directly on the skin of parasite carriers that received test vaccines, which then enables measurement of parasite development in the mosquitoes. Standard membrane feeding assays (SMFAs) use cultured parasites, whereas direct membrane feeding assays (DMFAs) use freshly collected blood from parasite carriers. DMFA and direct skin feeding (DSF) assays have been used for several decades to measure either the infectivity of gametocyte carriers to mosquitoes or the TB activity (TBA) of treatments. For example, in the 1950s, Muirhead Thompson conducted DSF and identified children as the primary reservoir of gametocytes in west Africa,[5,6] a finding corroborated by Githeko and others in Kenya in 1992.[7] DSFs have proven to be a robust platform for studies of different parasite species and at different sites around the world.[8–10]

DMFAs have been an important tool in situations where DSFs are not possible. Studies that compare DMFA to DSF demonstrate their similarity,[11] but DSFs are considered more accurate, as they represent a scenario closest to the natural setting.[12,13] Although DSF and DMFA have been used for years, the sources of variation are still unclassified and there is no universally standard assay procedure, although a number of laboratories are working towards standardized procedures, particularly for the DMFA. Without standardization of the assay and a better understanding of the sources of variation, the results of these assays can be unreliable endpoints for trials of TBV products. Here, we investigate several potential sources of variation including the starvation time, age of mosquito, the type of membrane (DMFA only), the time of feeding (DSF only), and the anatomical feed position (DSF only) to suggest best practice standards. We examine these parameters for their effect on the rate of mosquito infection, which is the primary endpoint for studies of interventions such as TBV products which seek to reduce the number of infected mosquitoes.

* Address correspondence to Mamadou B. Coulibaly, Faculty of Medicine and Dentistry, Malaria Research and Training Center, University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali. E-mail: doudou@icermali.org

MATERIALS AND METHODS

Study site. This study was performed in Bancoumana village (12°20′N, 80°20′W) located 60 km southwest of Bamako (capital of Mali) where malaria transmission is highly seasonal and intense during the rainy season from July to December. This village contains facilities built by NIAID/NIH for vaccine trials, hosting more than 20 years of collaboration between NIH and the research centers at the University of Sciences, Techniques and Technologies of Bamako. To maximize the number of gametocytemic samples available for these assays, the studies were conducted during the high-transmission season (i.e., August through December from 2013 to 2015).

Human subjects. To maximize the probability of mosquito infection, individuals identified as gametocytemic were used for the experiments. Twenty-five unique gametocyte carriers were invited to participate in DSF assays, and/or give peripheral blood for testing by DMFA. Among the gametocyte carriers the minimum age was 7 years (with approval from parents or guardians), maximum age was 48, with an average age of 24. Thirteen out of 25 carriers (52%) were female.

Mosquito colony and rearing. Mosquitoes used in the feeding assays were *Anopheles coluzzii*. The colony originated from wild-caught mosquitoes that were reared in the laboratory over several generations. The mosquitoes used in these experiments were the 82nd generation. The laboratory rearing environmental conditions were 25 ± 2°C for the temperature and 70 ± 10% for the relative humidity. The light cycle was 12 hours. The larvae were fed with fish food and adults were fed with 10% sugar solution. To maintain the colony, adult female mosquitoes were provided with human blood from the Mali blood bank (screened under safe blood transfusion criteria in Mali and under ethical approval) through membrane feeding using a heated circulating water bath (Model ED, v.2, DIN:12876, Julabo) and Parafilm® (Bemis Company Inc., Neenah, WI) as membrane.

Feeding assays. Adult female mosquitoes were selected and transferred into cups that would hold 30 mosquitoes each with no overcrowding. For direct skin feeds, two cups (60 mosquitoes) were applied to the indicated body location (arm, calf, and ankle) of the volunteer according to the experiments. For membrane feeds, the same number of mosquitoes per cup was used. The number of cups per experiment was set according to the type of comparison (see Supplemental Table 1). DMFA assays were conducted immediately post bleeding using whole blood taken from the individual with no washing or replacement of serum. Feeding assays were conducted at different times of the day (dawn versus dusk), with different age categories (3, 6, and 9 days) of mosquitoes, using different starvation times (8, 14, and 20 hours) and different types of membranes (Baudruche versus Parafilm). When age was not the parameter to optimize, 2–5 days old mosquitoes were used. Feeding assays were conducted in the village of Bancoumana and stored in a field insectary monitored for temperature and humidity. Mosquitoes were left to rest for 24 hours before they were transported in coolers back to the laboratory for dissection for oocyst detection. Mosquitoes remained in the cups that were covered with a wet towel; the whole assembly was then placed in the coolers. In the laboratory, mosquitoes were kept in containment separated from noninfected mosquitoes. After 7–8 days, mosquitoes were dissected in 0.5% mercurochrome under a dissecting scope to extract the midguts. The latter were then observed under an optic microscope (objective 10 and 40) for oocyst detection.

Table 1
Volunteers' demographic characteristics

	DSF feeds		
	Mean (SD)	Minimum	Maximum
Age	20 (13)	7	53
Gametocytemia	12 (24)	1	96
Hemoglobin (g/dl)	12 (2)	9	15
Ethnicity	Malinke	Fulani	Other
	16 (94%)	1 (6%)	0 (0%)
		DMFA feeds	
Age	25 (7)	12	36
Gametocytemia	2 (2)	1	5
Hemoglobin	12 (2)	10	14
Ethnicity	Malinke	Bamanan	Other
	8 (89%)	1 (11%)	0 (0%)

STUDY DESIGN AND STATISTICAL METHODS

DMFA: type of membranes, mosquito age, and starvation duration. The DMFA experiments followed a Face-Centered Central Composite Design with extra repetitions in center points of the design; Supplemental Table 1 of the supplement shows the design layout for this experiment. The tests were conducted on samples collected from nine gametocyte carriers. Feeding rate and infection rate were recorded and analyzed to identify the optimal parameters that achieved the highest number of infected mosquitoes. Two membrane systems were tested: Baudruche membrane, which is biological material derived from pig intestine, and Parafilm membrane, which is synthetic and therefore inherently more conducive to standardization. The feeding rate, survival rate, and infection rate were analyzed by two types of regression, with infection rate as the primary outcome. The optimal age, starvation duration, and membrane system were estimated using a response surface regression model; this model was used, as was pre-specified in the statistical analysis plan, to find the values where the highest infection rates were observed. The study was designed for this type of analysis, however inference about which of the characteristics is significantly correlated in the rates is not given by this model. A logistic mixed effects model, conditioning on individual, and feed, was also used to provide inference about the association of each of the characteristics with feeding, survival and infection odds. Three separate models were fit, one for each of three outcomes: feeding, survival, and infection with oocysts. All analyses were performed using *R*[14]: response surface regression used *R* Package rsm,[15] and all mixed logistic regressions were run using the lme4 package.[16] Reported percentages rates are empirical averages with bootstrap 95% confidence intervals.

DSF: anatomical location and timing of feeding. DSF experiments followed a split plot design, where DSFs were performed under multiple different scenarios. These scenarios varied by the anatomical site where DSFs were performed (ankle, leg, or forearm) or the time of feeding (dawn or dusk). Fourteen gametocyte carriers participated in the anatomical location study, in which three cups with 20 mosquitoes each were placed on three different anatomical sites of the same volunteer. The comparison of anatomic location, although intended to be a paired analysis for each of the three-way comparisons, was instead undertaken using a logistic mixed effects regression, conditioning on individual, and feed, to maximize power to detect differences. Unlike the anatomic location experiment, some of the subjects included in the time of feeding experiment received a DSF only at dawn or dusk (eight gametocyte carriers) while six gametocyte carriers received a DSF at both dawn and dusk within the same 24-hour period. Logistic mixed effects regression at the mosquito level, conditioning on the individual and feed, was used to compare feeding, survival, and infection by time of feed. Conditioning on individual and feed in this model makes the results for an average individual and feed; this is also true for the above mixed effects model of DMFA.

Ethical considerations. Approvals were obtained from NIAID-NIH (Protocol No. 11-I-N143) and the FMPOS (N°2011-43) ethics committee. Community permission was obtained from traditional authorities. Written individual informed consent was obtained from each volunteer. For children, approval was obtained from either parents or guardians.

RESULTS

Demographic characteristics of the volunteers. Among the volunteers who were subjects to DSFs, the mean age was 20 years old, the average gametocyte density was 12 per 1,000 white blood cells, and the average hemoglobin rate was 12 g/dL. The majority of the volunteers (94%) were from the Malinke ethnic group. A small proportion was from the Fulani ethnic group (6%). For the volunteers who were subject to DMFAs, the average age was 25 years, the average gametocyte density was 2 per 1,000 white blood cells, and the average hemoglobin level was 12 g/dL. Among these volunteers 89% were from the Malinke ethnic group, whereas 11% were from the Bamanan ethnic group. These demographic data are summarized in Table 1.

DMFA: type of membrane, mosquito age and starvation duration. Table 2 summarizes the results of the mixed effects logistic regression analysis for the two types of membranes, starvation duration, and mosquito age. The infection rates are represented in Figure 1. The feeding, survival, and infection rates were all comparable between the two different membranes (Baudruche versus Parafilm). For Baudruche and Parafilm, the mosquito feeding rates were 87.8% (95% bootstrap CI [81.4, 91.2]) and 86.9% (84.1, 91.4), the survival rates were 70.6% (65.1, 76.3) and 70.7% (67, 74.6), and the mosquito infection rates were 3.5% (0.1, 8.4) and 3.3% (0.5, 8.2), respectively. A response surface regression suggests that Baudruche membrane yielded higher feeding, survival, and infection rates (Table 3), but these differences were not significant when adjusting for starvation duration and mosquito age in the logistic mixed effects model (Table 2).

Three starvation durations (8, 14, and 20 hours) were assessed for their effects. For 8, 14, and 20 hours, the feeding rates were 90.3% (87.4, 92.4), 88.9% (84.7, 92.5), and 81.2% (73, 88.4), the survival rates were 72.1% (67.4,77.5), 71.9% (66.3, 77.3), and 66.7% (61.7, 72.4), and the infection rates were 2.9% (0, 6.8), 3.3% (0.2, 8.3), and 3.9% (0.5, 10), respectively (Table 2). The response surface regression suggests that starvation of ~11 hours is optimal for feeding and survival, while starvation duration of 15.5 hours is optimal for infection rate (Table 3). Although odds of infection did not significantly differ between starvation durations, there were significantly lower odds of survival and feeding probability at 20 hours compared with 8 hours ($P < 0.05$ for both, Table 2) based on the mixed effects logistic regression.

Three groups of mosquitoes (3, 6, and 9 days) were assessed for effect of age on mosquito feeding, survival, and infection rates. For 3-, 6-, and 9-day old mosquitoes, the feeding rates were 95.2% (92.4, 97.4), 88.0% (84.7, 91.3), and 78.1% (67.9, 86.3), the survival rates were 83.5% (80.8, 86.2), 72.2% (65.9, 77.8), and 54.2% (46.2, 62.3), and infection rates were 4.8% (0.5, 11.4), 3.3% (0.4, 7.9), and 2.0% (0, 5.7), respectively. The response surface regression suggests that mosquito ages of 1 day or less are optimal for feeding and survival, while an age of 5.4 is optimal for infection rates (Table 3). As mosquitoes aged less than 3 days do not feed well and were not included in this study, the suggestion that mosquitoes aged less than 3 days are optimal is an artifact of the model fit to continuous age. Therefore, combining the suggested optimal ages for feeding, survival, and infection, the youngest age at which mosquitoes are able to feed is our

Table 2

Mosquito feeding, survival, and infection results by type of membrane, mosquito starvation duration, and age at time of direct membrane feeding assays (DMFA)

Feeding time	Total number of mosquitoes in cups	Empirical average feeding rate %*	Total number fed	Adjusted mixed model feeding *P* value	Empirical average survival rate %*	Number surviving of those fed	Adjusted mixed model survival *P* value	Empirical average infection rate %*	Number infected of those surviving	Adjusted mixed model infection *P* value
Baudruche	3,570	87.82	3,109	Ref	70.57	2,185	Ref	3.46	81	Ref
Parafilm	3,600	86.89	3,128	0.36	70.68	2,230	0.74	3.27	79	0.93
Starvation 8 hours	1,800	90.28	1,625	Ref	72.1	1,162	Ref	2.92	40	Ref
Starvation 14 hours	3,600	88.91	3,174	0.48	71.86	2,277	0.22	3.31	77	0.62
Starvation 20 hours	1,770	81.24	1,438	< 0.001	66.7	976	0.02	3.92	43	0.21
Age 3 days	1,800	95.22	1,714	Ref	83.47	1,433	Ref	4.78	68	Ref
Age 6 days	3,600	87.96	3,140	< 0.001	72.19	2,258	< 0.001	3.31	77	0.03
Age 9 days	1,770	78.14	1,383	< 0.001	54.18	724	< 0.001	1.97	15	<0.001

Ref = reference group for calculation of odd ratios *P* values.

* Empirical averages are over DMFA rates rather than % of totals. For example, the empirical average infection rate % for Age 3 is calculated by taking the infection rate for each DMFA and then taking the average, it therefore is not equal to 100* (68/1,433).

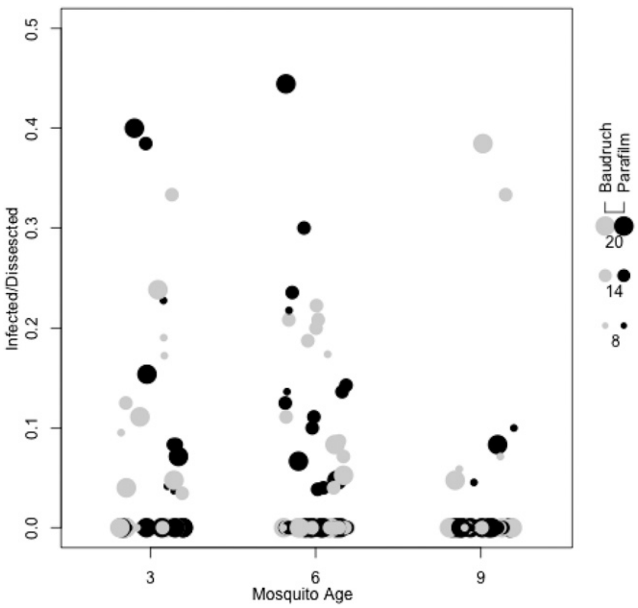

FIGURE 1. Infection rate of mosquitoes by direct membrane feeding assays (DMFA)

suggested optimum. This is also inferred from the logistic mixed effects model, which uses categorical age groups and gives evidence that mosquitoes aged 3 days have significantly higher feeding, survival, and infection odds (Table 2).

DSF: anatomic location. Three body locations (arm, calf, and ankle) were assessed for their effect on mosquito feeding, survival, and infection rates. For arm, calf, and ankle, the feeding rates were 89.2% (81.8, 94.9), 88.2% (82.2, 93.1), and 81.3% (72.6, 89.1), the survival rates were 70.4% (61.9, 79.4), 61.5% (55.1, 67.9), and 72.5% (63.6, 81), and infection rates were 12.2% (0, 25.5), 10.6% (1.7, 23.4), and 7.0% (0.3, 16.3), respectively (Table 4). There was not sufficient evidence to support a claim that any specific body location had superior odds for transmitting infection based on the mixed effects logistics regressions, although the data suggest that a mosquito feeding on the calf has lower survival odds compared with one feeding on the ankle (P = 0.046). There was also borderline evidence that both arm and calf are superior to ankle for feeding rates (P = 0.08 and 0.058, respectively). The highest infection rate was observed among mosquitoes that fed on the arm, but this was not significantly different from the calf or ankle (Table 4, also see Figure 2A).

DSF: time of feeding. Two feeding time points (dusk and dawn) were assessed for their effect on mosquito feeding, survival, and infection rates. For dusk and dawn, the feeding rates were 91.1% (85.5, 95.5) and 86.5% (82, 91), the survival rates were 76.0% (70, 81.6) and 61.2% (54.1, 67.9), and the infection rates were 10.0% (0.2, 23.3) and 3.9% (0, 11.3), respectively (Table 5). Although there was no significant difference in odds of feeding between the two feeding times, there was evidence of higher odds of survival (P = 0.004) and infection (P = 0.008) at dusk compared with dawn (see Figure 2B for infection rates). Of note, the dusk feeding assays were usually performed on candidates selected on the basis of blood smears prepared that same day, whereas the dawn feeding assays were usually performed a day or two after the day of blood smear preparation for logistical reasons. An adjusted regression model including age, gender, ethnicity, and village of participants was also run for these outcomes; inference did not differ from the unadjusted model outlined above.

TABLE 3
Estimated optimal parameters for direct membrane feeding assays from response surface regression

Outcome	Membrane	Starvation (hours)	Age (days)
Infection	Baudruche	15.5	5.4
Survival	Baudruche	11.41	1.02
Feeding	Baudruche	11.39	0

DISCUSSION

DMFAs and DSFs are important tools for studying the transmission of parasites from host to host and for evaluating antimalarial drugs as well as malaria TBV candidates. Although SMFAs represent the primary platform for measuring the transmission reduction activities of potential molecules or compounds in laboratory conditions,[17,18] DMFAs and DSFs are performed in the field and are therefore closer to natural transmission conditions. One important goal for use of these tools is the evaluation of drugs and vaccines that might be implemented in the future as part of a malaria eradication package. However, past studies have shown that transmission from human hosts to mosquitoes using feeding experiments (both direct and via membrane) is subject to extreme variation,[19–21] impairing our ability to draw inferences or compare results from different studies.

For DMFA, our suggested optimal parameters for infection are the use of Baudruche membrane, starvation times between 12 and 15 hours, and a mosquito age of 3 days. Although only 3 days of age is supported by statistical analysis for infection, feeding and survival, Baudruche membranes resulted in the highest observed infection and feeding probabilities and very similar survival rates. Although starvation rates of 20 hours resulted in the highest observed infection rates, this length of starvation also significantly reduced feeding and survival; we therefore suggest an optimal range of 12–15 hours. Starvation intervals such as 12–24 hours have often been used,[22] but a more recent protocol indicated a minimum duration of 5 hours before feeding.[21] Although we suggest longer starvation is optimal for infection, too long a duration may impact mosquito health and survival.

The natural membrane made from pig intestine (Baudruche) tended to yield slightly higher feeding, survival, and infection rates, though these differences were not statistically significant when adjusted for other covariates. Improved outcomes may in part be explained by the Baudruche being a natural product, as previous evidence has shown that natural membranes are conducive to better feeding performance.[22] However, artificial membranes, such as Parafilm, have been used more extensively[23–25] and are generally easier to use, more consistent, more cost-effective,[26] and more accessible in certain field conditions. Because any differences between the natural and synthetic membranes appeared modest and were not significant, the convenient and consistency of Parafilm argues in favor of its use.

Although there were no significant differences observed in the infection probabilities in the DSFs performed on the arm,

Table 4
Mosquito feeding, survival, and infection results by anatomic location of direct skin feed (DSF)

Anatomical location	Total number of mosquitoes in cups	Empirical average feeding rate %*	Total number fed	Mixed model feeding *P* value	Empirical average survival rate %*	Number surviving of those fed	Mixed model survival *P* value	Empirical average infection rate %*	Number infected of those surviving	Mixed model infection *P* value
Arm	340	89.17	299	0.08	70.44	214	0.72	12.22	29	0.61
Calf	670	88.21	590	0.058	61.52	367	0.046	10.60	25	0.63
Ankle	610	81.32	478	Ref	72.52	349	Ref	7.00	29	Ref

Ref = reference group for calculation of odd ratios *P* values.
* Empirical averages are over DSF rates rather than % of totals. For example, the empirical average infection rate % for arm is calculated by taking the infection rate for each DSF and then taking the average, it therefore is not equal to 100* (29/214).

ankle, and leg, the arm tended to yield higher infection rates. Although there were borderline significantly higher feeding odds for the calf, there were also significantly lower survival odds compared with the ankle, and the empirical feeding and infection rates in the arm were the highest of any location. The lower number of mosquitoes fed on the arm likely reduced our power to detect a statistically significant difference based on the mixed effect logistic regression.

A relationship between higher feeding and infection rates at different anatomic locations might have biological explanations, much as the periodicity of microfilariae appears to coincide with the blood-feeding times of their mosquito vectors to maximize transmission.[27,28] In Uganda and South Africa, wild Anopheline mosquitoes demonstrated a propensity for feeding close to ground level, which corresponded to lower extremities when individuals are seated or standing, and a more random distribution for those lying down.[29] In our study, mosquitoes were not given the choice to feed on different sites, only the choice to feed or not on the single site offered to them, and we found that feeding rates were similar on arms and calves, which is consistent with earlier findings with wild mosquitoes.

Although the infection rate was significantly higher at dusk than dawn, this could be explained by the fact that the feeds at dusk were often conducted on the same day the gametocytes were detected in the volunteers. In contrast, dawn feeds were often conducted the following day or sometimes two days after gametocyte detection. Although we attempted to account for this confounding by including an indicator in the model for day of gametocyte detection, the co-linearity with feed time made the model unstable (results not shown). Repeating the feeds by inverting the system might provide a more precise answer to this question, and the circadian periodicity of human *Plasmodium* remains largely unknown and therefore of interest. Magesa and others in 2000 were able to show that *P. falciparum* gametocytes were semidiurnal periodic,[30] but they concluded that the biological ramification of this was unknown. This semidiurnal periodic characteristic may be explained by the increased survival probability for mosquitoes that feed at dusk.

We suggest best practices based on our findings: for DMFA, use 3-day old mosquitoes starved for 12–15 hours and Parafilm membrane; for DSF, follow parameters for DMFA mosquitoes and conduct assays on the arms preferentially over calf or ankle. However, the scope of this study is limited to only a small number of feeding parameters in a single mosquito species and further investigation is needed to truly optimize the assays on a global scale. Differences between mosquito species in terms of feeding preference—location and time of day—will clearly affect results in different populations. Similarly, we acknowledge that natural transmission blocking activity and gametocytemia levels vary across populations affecting assay outcome. However, it is clear that optimization of evaluation tools will maximize the likelihood for reliably detecting TBV activity in endemic field trials.

CONCLUSIONS

Although our results suggest that Baudruche yields slightly higher infection rates than Parafilm, we feel that using an

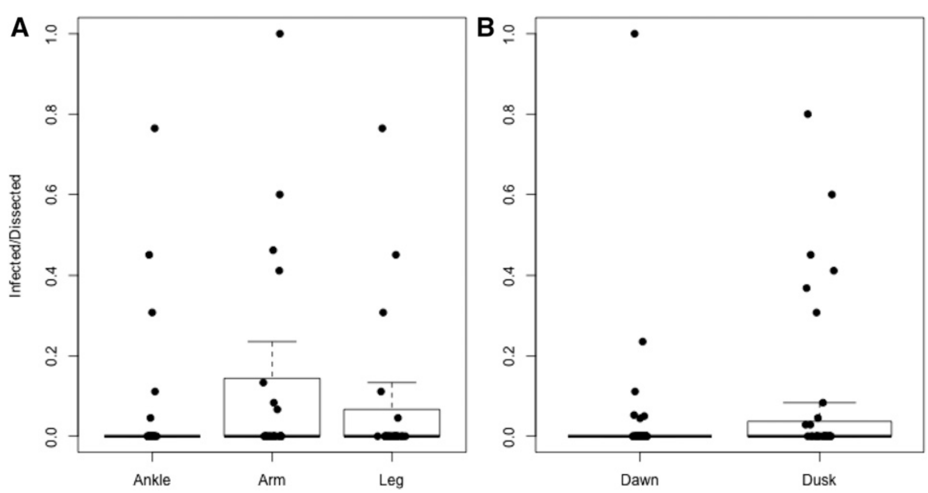

Figure 2. Infection rate of mosquitoes in direct skin feeding assays (DSF) by (**A**) anatomical location, (**B**) time of day

TABLE 5
Mosquito feeding, survival, and infection results by time of day of direct skin feed (DSF)

Feeding time	Total number of mosquitoes in cups	Empirical average feeding rate %*	Total number fed	Mixed model feeding *P* value	Empirical average survival rate %*	Number surviving of those fed	Mixed model survival *P* value	Empirical average infection rate %*	Number infected of those surviving	Mixed model infection *P* value
Dusk	900	91.12	810	0.22	76	593	0.004	10.01	12	0.008
Dawn	1,140	86.45	996	Ref	61.15	634	Ref	3.93	46	Ref

Ref = reference group for calculation of odd ratios *P* values.
* Empirical averages are over DSF rates rather than % of totals. For example, the empirical average infection rate % for dusk is calculated by taking the infection rate for each DSF and then taking the average, it therefore is not equal to 100* (12/593).

artificial membrane allows for greater experiment standardization and believe this should be prioritized over "optimizing" for infection rate. The duration of the starvation before blood feeding was estimated to be optimal between 11 and 15.5 hours for feeding survival and infection, but starvation of 20 hours was associated with lower fitness of the mosquitoes. There was no statistical evidence of a superior starvation time for infection, but there was for feeding and survival; therefore, we suggest that a starvation time between 11 and 15 hours is optimal and that longer starvation times should be avoided. We also provide evidence that younger (3-day old) mosquitoes achieve higher feeding, survival, and infection rates.

A higher, though not significant, infection probability was observed in feeds on the arms. Arms were not significantly better than the calf, and the calf had significantly higher feeding, but lower survival than ankle. In the absence of other constraints, both calves and arms may be better anatomical locations for DSF.

The significantly higher infection rate observed at dusk needs to be further investigated by repeating the experiments with more feeds at dawn preceding those at dusk. The increased survival rate, however, may be reason enough to prefer dusk.

Received December 19, 2016. Accepted for publication April 17, 2017.

Published online June 26, 2017.

Note: Supplemental table appears at www.ajtmh.org.

Acknowledgments: We would like to thank Richard Sakai, Chata Doumbia, Abdrahamane Fofana, Amadou Guindo, Moussa Diallo, Brehima Diallo, Fatoumata Sissoko, and Souleymane Karambe for logistical and technical support, Jeff Skinner for helping with study design, and J. Patrick Gorres for editing the manuscript.

Financial support: This study was sponsored by the NIAID/NIH intramural research program, and by the PATH Malaria Vaccine Initiative.

Disclosures: The authors would like to disclose that there are no conflicts of interest regarding the publication of this paper.

Authors' addresses: Mamadou B. Coulibaly, Youssouf Sinaba, Daman Sylla, Adama Sacko, Lakamy Sylla, Boubacar Coulibaly, Ibrahima Baber, Mahamadoun H. Assadou, Issaka Sagara, Ogobara Doumbo, and Sekou F. Traore, University of Sciences, Techniques and Technology of Bamako, Malaria Research and Training Center, Bamako, ML, E-mails: doudou@icermali.org, ysinaba@icermali.org, dsylla@icermali.org, asacko@icermali.org, lsylla@icermali.org, bcoulibaly@icermali.org, baber@mrtcbko.org, mmaiga@icermali.org, isagara@icermali.org, okd@icermali.org, and cheick@icermali.org. Erin E. Gabriel, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Biostatistics Research Branch, Rockville, MD, E-mail: erin.gabriel@nih.gov. Jen C. C. Hume, Sara A. Healy, and Patrick E. Duffy, National Institutes of Health, Laboratory of Malaria Immunology and Vaccinology, Rockville, MD, E-mails: jennifer.hume@nih.gov, sara.healy@nih.gov, and patrick.duffy@nih.gov. Yimin Wu, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Laboratory of Malaria Immunology and Vaccinology, Rockville, MD and PATH, Malaria Vaccine Initiative, Washington, DC, E-mail: ywu@path.org.

REFERENCES

1. WHO, 2015. *World Malaria Report.* Geneva, Switzerland: World Health Organization.
2. WHO, 2014. *World Malaria Report*. Geneva, Switzerland: World Health Organization.
3. Gallup JL, Sachs JD, 2001. The economic burden of malaria. *Am J Trop Med Hyg 64:* 85–96.
4. Duffy PE, Kaslow DC, 1997. A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines. *Infect Immun 65:* 1109–1113.
5. Muirhead-Thomson RC, 1954. Factors determining the true reservoir of infection of *Plasmodium falciparum* and *Wuchereria bancrofti* in a West African village. *Trans R Soc Trop Med Hyg 48:* 208–225.
6. Muirhead-Thomson RC, 1957. The malarial infectivity of an African village population to mosquitoes (*Anopheles gambiae*); a random xenodiagnostic survey. *Am J Trop Med Hyg 6:* 971–979.
7. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, Collins FH, 1992. The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Trans R Soc Trop Med Hyg 86:* 355–358.
8. Toure YT, Doumbo O, Toure A, Bagayoko M, Diallo M, Dolo A, Vernick KD, Keister DB, Muratova O, Kaslow DC, 1998. Gametocyte infectivity by direct mosquito feeds in an area of seasonal malaria transmission: implications for Bancoumana, Mali as a transmission-blocking vaccine site. *Am J Trop Med Hyg 59:* 481–486.
9. Gamage-Mendis AC, Rajakaruna J, Carter R, Mendis KN, 1991. Infectious reservoir of *Plasmodium vivax* and *Plasmodium falciparum* malaria in an endemic region of Sri Lanka. *Am J Trop Med Hyg 45:* 479–487.
10. Graves PM, Burkot TR, Carter R, Cattani JA, Lagog M, Parker J, Brabin BJ, Gibson FD, Bradley DJ, Alpers MP, 1988. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. *Parasitology 96:* 251–263.
11. Awono-Ambene HP, Diawara L, Robert V, 2001. Comparison of direct and membrane feeding methods to infect *Anopheles arabiensis* with *Plasmodium falciparum*. *Am J Trop Med Hyg 64:* 32–34.
12. Bonnet S, Gouagna LC, Paul RE, Safeukui I, Meunier JY, Boudin C, 2003. Estimation of malaria transmission from humans to mosquitoes in two neighbouring villages in south Cameroon: evaluation and comparison of several indices. *Trans R Soc Trop Med Hyg 97:* 53–59.
13. Diallo M, et al., 2008. Evaluation and optimization of membrane feeding compared to direct feeding as an assay for infectivity. *Malar J 7:* 248.
14. Team RC, 2015. *A Language and Environment for Statistical Computing*. Available at: https://www.R-project.org/.
15. Russell V, 2009. Response-surface methods in R, using RSM. *J Stat Softw:* 1–17.
16. Bates D, Maechler M, Bolker B, Walker S, 2015. Fitting linear mixed-effects models using lme4. *J Stat Softw:* 1–48.
17. van der Kolk M, de Vlas SJ, Sauerwein RW, 2006. Reduction and enhancement of *Plasmodium falciparum* transmission by endemic human sera. *Int J Parasitol 36:* 1091–1095.
18. van der Kolk M, De Vlas SJ, Saul A, van de Vegte-Bolmer M, Eling WM, Sauerwein RW, 2005. Evaluation of the standard membrane feeding assay (SMFA) for the determination of malaria

transmission-reducing activity using empirical data. *Parasitology 130:* 13–22.
19. Bousema T, Churcher TS, Morlais I, Dinglasan RR, 2013. Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? *Trends Parasitol 29:* 53–59.
20. Bousema T, et al., 2012. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS One 7:* e42821.
21. Ouedraogo A, et al., 2013. A protocol for membrane feeding assays to determine the infectiousness of *P. falciparum* naturally infected individuals to *Anopheles gambiae*. *MWJ 4:* 16.
22. Novak MG, Berry WJ, Rowley WA, 1991. Comparison of four membranes for artificially bloodfeeding mosquitoes. *J Am Mosq Control Assoc 7:* 327–329.
23. Harada M, Matsuoka H, Suguri S, 1996. A convenient mosquito membrane feeding method. *Medical and Entomological Zoology* 103–105.
24. Mourya DT, Gokhale MD, Barde PV, Padbidri VS, 2000. A simple artificial membrane-feeding method for mosquitoes. *Trans R Soc Trop Med Hyg 94:* 460.
25. Tseng M, 2003. A simple parafilm M-based method for blood-feeding *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol 40:* 588–589.
26. Montes C, Cuadrillero C, Vilella D, 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera: Cimicidae) using an artificial feeding technique. *J Med Entomol 39:* 675–679.
27. Hawking F, 1967. The 24-hour periodicity of microfilariae: Biological mechanisms responsible for its production and control. *Proc R Soc Lond B Biol Sci 169:* 59–76.
28. Khan AM, Dutta P, Das S, Pathak AK, Sarmah P, Hussain ME, Mahanta J, 2015. Microfilarial periodicity of *Wuchereria bancrofti* in Assam, northeast India. *J Vector Borne Dis 52:* 208–212.
29. Braack L, Hunt R, Koekemoer L, Gericke A, Munhenga G, Haddow A, Becker P, Okia M, Kimera I, Coetzee M, 2015. Biting behavior of African malaria vectors: where do the main vector species bite on the human body? *Parasite Vectors 8:* 76.
30. Magesa SM, Mdira YK, Akida JA, Bygbjerg IC, Jakobsen PH, 2000. Observations on the periodicity of *Plasmodium falciparum* gametocytes in natural human infections. *Acta Trop 76:* 239–246.