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QUANTITATION OF *PLASMODIUM FALCIPARUM* SPOROZOITES TRANSMITTED IN VITRO BY EXPERIMENTALLY INFECTED *ANOPHELES GAMBIAE* AND *ANOPHELES STEPHENSI*

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Abstract. The frequency and numbers of *Plasmodium falciparum* sporozoites transmitted in vitro and corresponding sporozoite loads were determined for experimentally infected *Anopheles gambiae* and *An. stephensi*. Geometric mean (GM) sporozoite loads in three experiments ranged from 808 to 13, 905 for *An. gambiae* and from 6, 608 to 17, 702 for *An. stephensi*. A total of 44.1% of 68 infected *An. gambiae* and 49.2% of 63 infected *An. stephensi* transmitted sporozoites in vitro. The GM number of sporozoites transmitted was 4.5 for *An. gambiae* and 5.4 for *An. stephensi*. Overall, 86.9% of the mosquitoes transmitted from one to 25 sporozoites, and only 6.6% transmitted over 100 sporozoites (maximum = 369). Sporozoite loads were not a useful predictor of potential sporozoite transmission. Despite higher sporozoite loads, the numbers of sporozoites transmitted in vitro by the experimentally infected mosquitoes were similar to estimates obtained, using the same techniques, for naturally infected *An. gambiae* in western Kenya. The low but highly variable numbers of sporozoites transmitted in vitro by mosquitoes used in malaria vaccine challenge studies appears to be a reasonable simulation of natural sporozoite transmission.

Malaria sporozoite vaccine trials in humans are being conducted by challenging volunteers with the bites of up to five infective mosquitoes.[1–3] The infection status of mosquitoes after feeding is determined by dissection and the examination of salivary glands for the presence of sporozoites. The gland index is used to estimate the number of sporozoites in the salivary glands (sporozoite load) on a log-scale from +1 (≤ 10) to +4 (> 1,000). This provides limited information because it is unclear whether the number of sporozoites transmitted by infective mosquitoes is correlated directly with the intensity of sporozoite loads. Thus, sporozoite vaccine efficacy trials are being conducted without any measurement of potential sporozoite inocula.

The issue has been raised that the failure of sporozoite vaccines to confer complete protection in vaccinated volunteers may be due to an unnaturally large challenge of sporozoites by laboratory-infected mosquitoes.[4,5] The basis for this concern is that sporozoite loads in experimentally infected anophelines appear to be from 10 to 200 times more intense[4] than those reported for naturally infected mosquitoes.[6–9] The biological relevancy of the current sporozoite vaccine challenge model is a difficult question because there is limited information on the numbers of sporozoites transmitted by wild, naturally infected vectors.[10]

This study compares *P. falciparum* sporozoite loads and the sporozoite transmission potential of experimentally infected *An. gambiae* and *An. stephensi*. Methods used previously to estimate sporozoite loads and the number of sporozoites transmitted by naturally infected vectors[9,10] were used to determine how closely infections in laboratory-infected anophelines simulate natural infections.

MATERIALS AND METHODS

Laboratory-reared *An. gambiae* (G-3 strain) and *An. stephensi* (Dutch strain) were infected with *P. falciparum*. Mature gametocytes of *P. falciparum* were obtained in vitro using a modification of a method described previously.[11] The CVD1 clone of the NF54 strain of *P. falciparum* (JR Davis, unpublished data) was used in the first feeding experiment. The NF54 strain, reisolated from a control individual infected during a recent vaccine challenge study, was used in

the second and third feeding experiments. In each feeding, cohorts of 3–5- day-old *An. gambiae* and *An. stephensi* were fed on cultured gametocytes (diluted 1:8 with equal volumes of washed human red cells and heat-inactivated human sera) using a series of glass membrane feeders covered with Baudruche membrane with water jackets heated to 38°C. Mosquitoes were allowed to feed for 15 min. Fed mosquitoes were provided with 5% Karo solution and were maintained in an insectary at 27°C and 70% relative humidity. In each experiment, cohorts of *An. gambiae* and *An. stephensi* were held 15–17 days before individuals were tested for transmission and dissected over a 2–3 day period.

An in vitro capillary tube method was used to determine the number of sporozoites transmitted per infected mosquito.[10] After anesthetizing mosquitoes with CO_2 (dry ice) and removing wings and legs, the labium was retracted and stylets were placed into 5 μl of 10% sucrose contained in a drawn capillary tube mounted onto a glass slide. A small piece of wax was placed behind the thorax to secure the mosquito into position. Contact with the sucrose solution stimulated the movement of mouthparts for nearly all mosquitoes tested. After 15 min, mosquitoes were removed and the sucrose solution was expelled on a 5-mm diameter spot on a microslide. This was air-dried and sporozoites were counted at 400× after staining with FITC-labelled 2A10 monoclonal antibody, which recognizes the immunodominant epitope on the circumsporozoite protein of *P. falciparum*.[12]

After mosquitoes were tested for sporozoite transmission, salivary glands were removed by dissection, transferred to a glass grinder, and ground in 35 μl of M-199 solution. After mixing with a pipette, 10 μl were placed in a hemocytometer and sporozoites were counted at 400× phase in the four 0.1 μl corners. Sporozoite loads were determined as the number of sporozoites counted in the hemocytometer times 87.5, to account for the volume examined. An additional 5 μl was spotted on a 5- mm diameter spot on a microslide, air-dried, and stained with FITC-labelled 2A10. Slides were examined for specimens with negative hemocytometer readings to check for positive mosquitoes with sporozoite loads below the sensitivity threshold of the hemocytometer. Sporozoite loads for these specimens were calculated as the number of sporozoites counted in the 5 μl sample times 7, to account for the original volume of gland samples (i.e., 35 μl).

For those mosquitoes transmitting sporozoites, transmission efficiency was calculated as the number of sporozoites transmitted divided by the sporozoite load times 100.

RESULTS

Sporozoite rates ranged from 52.2% to 90.5% for *An. gambiae* and from 84.2% to 91.7% for *An. stephensi* (Table 1). Sporozoite loads were highly variable for both species, ranging from seven to > 100,000. In the first experiment, *An. gambiae* had an eight-fold lower geometric mean (GM) sporozoite load than *An. stephensi*. Differences in sporozoite loads between species decreased as sporozoite loads increased.

Sporozoites were transmitted in vitro into capillary tubes containing sucrose by 44.1% of 68 *An. gambiae* and by 49.2% of 63 *An. stephensi* (Table 2). Overall, *An. gambiae* transmitted a GM of 4.5 sporozoites (range: 1–136) compared to 5.4 (range: 1–369) for *An. stephensi*. There were no significant differences in the number of sporozoites transmitted between *An. gambiae* and *An. stephensi* for the three replicates (Table 2).

Frequency distributions of the number of sporozoites transmitted were similar for *An. gambiae* and *An. stephensi* (Figure 1). Over 80% of both species transmitted from one to 10 sporozoites. Only 6.7% (2/30) of *An. gambiae* and 6.5% (2/31) of *An. stephensi* transmitted over 100 sporozoites.

There were differences between species in the proportion of mosquitoes transmitting sporozoites relative to log classes of sporozoite loads (Table 3). The percent of *An. gambiae* transmitting was not dependent upon the intensity of sporozoite loads ($\chi^2 = 0.55$, df = 2, $P = 0.7587$), but a higher proportion of *An. stephensi* with sporozoite loads > 10^4 transmitted sporozoites than those with < 10^4 sporozoites ($\chi^2 = 5.87$, df = 1, $P = 0.0154$). The GM number of sporozoites transmitted increased for *An. gambiae* from 1.6, when sporozoite loads were < 10^3, to 18.0 when sporozoite loads were > 10^4 ($F = 10.7$, df = 2.27, $P = 0.0004$); this trend was also seen in pairwise comparisons of sporozoite loads and sporozoite transmission ($r = 0.54$, df = 28, $P = 0.002$). In contrast, there was no evidence for a similar relationship for *An. stephensi*, either according to log sporozoite classes ($F = 1.64$, df = 2.28, $P =$

TABLE 1

Plasmodium falciparum *sporozoites detected and counted from salivary glands of experimentally infected* Anopheles gambiae *and* Anopheles stephensi

	An. gambiae				*An. stephensi*				Significance	
									χ^2	2-sided *t*-value
Replicate	No. examined	% positive	Geometric mean	Range	No. examined	% positive	Geometric mean	Range	% positive	Geometric mean
1	67	52.2	808	14–29,575	36	91.7	6,608	7–75,688	0.0001	0.0001
2	21	90.5	1,502	7–101,850	16	87.5	8,446	88–143,500	0.7727	0.0824
3	17	82.4	13,905	88–98,962	19	84.2	17,702	262–94,150	0.8813	0.7220
Total	105	64.8	1,727	7–101,850	71	88.7	8,964	7–143,500	0.0007	0.0001

TABLE 2

Plasmodium falciparum *sporozoites transmitted in vitro during salivation into a capillary tube, containing 5 μl sucrose solution, by experimentally infected* Anopheles gambiae *and* Anopheles stephensi

	An. gambiae				*An. stephensi*				Significance	
									χ^2	2-sided *t*-value
Replicate	No. tested	% transmitting	Geometric mean	Range	No. tested	% transmitting	Geometric mean	Range	% transmitting	Geometric mean
1	35	45.7	3.3	1–48	33	48.5	2.9	1–20	0.8191	0.7530
2	19	52.6	6.7	1–132	14	50.0	34.3	3–369	0.8812	0.0806
3	14	28.6	5.4	1–136	16	50.0	2.8	1–20	0.4112	0.5457
Total	68	44.1	4.5	1–136	63	49.2	5.4	1–369	0.6832	0.6637

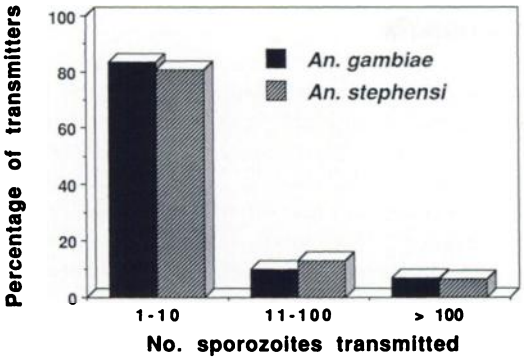

FIGURE 1. Frequency distribution of the number of *P. falciparum* sporozoites transmitted in vitro by experimentally infected *An. gambiae* and *An. stephensi.*

0.2118) or in pairwise comparisons (r = 0.04, df = 29, $P = 0.8508$).

Transmission efficiency averaged 0.78% for *An. gambiae* and 0.38% for *An. stephensi* (Table 3). There was a significant decrease in transmission efficiency with increasing sporozoite loads for *An. gambiae* (r = −0.59, df = 28, $P = 0.0006$) but not for *An. stephensi* (r = −0.26, df = 29, $P = 0.1516$).

DISCUSSION

The degree to which *P. falciparum* infections in experimentally infected anophelines simulate natural infections is difficult to determine because there is limited information on sporozoite loads and the sporozoite transmission potential of naturally infected vectors. There is no information on the natural sporozoite transmission potential of *An. stephensi,* the species used commonly in malaria vaccine challenge studies. In this study, experimental infections of *P. falciparum* in *An. stephensi* were compared with those in *An. gambiae,* the only colonized malaria vector where sporozoite loads and sporozoite transmission have been studied in nature.

There were only slight differences between species in both sporozoite rates and sporozoite loads when infection intensities were highest. Overall, the numbers of sporozoites in the salivary glands of experimentally infected *An. gambiae* and *An. stephensi* (Table 1) were similar to those reported for *An. gambiae* in Tanzania (GM = 6, 380, range: 130–245, 760)[6] and in Kenya (GM = 962, range: 2–117, 544).[9] The highest geometric mean sporozoite load for *An. gambiae* in the third experiment (GM = 13, 905) was only about twice as high as estimates for *An. gambiae* from Tanzania. These differences are minimal because sporozoite loads decrease over time in the vector.[13] Estimates for cross sections of natural populations should be lower than estimates from laboratory-infected mosquitoes sampled a few days after the release of sporozoites from oocysts, a time of maximal sporozoite loads.

Our estimates of geometric mean sporozoite loads for experimentally infected *An. stephensi* were 12–33 times lower than estimates of approximately 220,000,[4] which were determined using immunoassays on whole mosquitoes. Estimates of the number of sporozoites determined by immunoassays on whole mosquitoes, head/thorax parts, or isolated salivary glands are confounded by the detection of soluble circumsporozoite protein.[14, 15] Microscopic techniques for determining sporozoite loads for individual mosquitoes, first described in 1945 by Shute,[16] provide a basis for assessing variability and for determining geometric mean sporozoite loads. The simple techniques used in this study offer advantages over counts from pools of salivary glands,[17] and the gland index, which is subjective[15] and cannot adequately differentiate infections in mosquitoes with sporozoite loads over 1,000.

There were no differences between species in

TABLE 3

Plasmodium falciparum *sporozoite transmission in vitro in relation to sporozoite loads of experimentally infected* Anopheles gambiae *and* Anopheles stephensi

	An. gambiae				*An. stephensi*			
Sporozoite load	No. tested	% transmitting	Geometric mean	Mean transmission efficiency	No. tested	% transmitting	Geometric mean	Mean transmission efficiency
$<10^3$	28	39.3	1.6	1.78	8	37.5	1.9	0.44
10^3–10^4	20	50.0	3.1	0.18	20	30.3	13.3	1.56
$>10^4$	20	45.0	18.0	0.20	35	62.9	4.7	0.04

the geometric mean number of sporozoites transmitted despite variation in sporozoite loads. The low number of sporozoites transmitted in vitro by the experimentally infected *An. gambiae* and *An. stephensi* was nearly identical to estimates for naturally infected *An. gambiae* from Kenya. In the Kenyan study, over 98% of 226 infective *An. gambiae* transmitted less than 25 sporozoites.[10] The small proportion of experimentally infected mosquitoes transmitting > 100 sporozoites was similar to observations on naturally infected *An. gambiae*.[10] Interestingly, transmitted sporozoites were observed frequently on microslides to be clumped together in a viscous-like substance. The significance of this observation is unknown.

The frequency of gland-positive mosquitoes transmitting sporozoites into the sucrose solution, 44.1% for *An. gambiae* and 49.2% for *An. stephensi*, was lower than the 85.1% reported for naturally infected *An. gambiae* in Kenya.[10] The frequency of transmission for mosquitoes tested by in vitro systems and during normal blood feeding is probably related to the output of saliva and to the number of sporozoites in the salivary duct at the time of feeding.[10] It is difficult to explain lower rates of transmission for laboratory-reared compared to wild mosquitoes because almost nothing is known about factors affecting transmission potential.

The classical use of the term "infective" for mosquitoes with sporozoites in the salivary glands is probably misleading, because not all gland-positive mosquitoes transmit sporozoites during each blood meal. This is supported indirectly by observations that only 1–20% of the bites from gland-positive mosquitoes produce infections in residents of endemic areas.[18] Recently, Rickman and others [19] observed that feeding two infective mosquitoes on control volunteers in vaccine challenge studies did not routinely cause infections, and concluded that it is reasonable to continue using five gland-positive mosquitoes [20] to reliably transmit *P. falciparum*. Our findings support this conclusion; if only 50% of infective mosquitoes release sporozoites during feeding, then each volunteer in a five-bite challenge would have a 96.9% (0.5^5) chance of receiving at least some sporozoites.

The number of sporozoites in the salivary glands was not a useful predictor of either the probability of transmission or the number of sporozoites transmitted. Some mosquitoes with less than 100 sporozoites transmitted, while some with over 100,000 did not. For both *An. gambiae* and *An. stephensi*, the number of sporozoites transmitted represented less than 1% of the sporozoites in the salivary glands. Linear regression of sporozoite loads on the number of sporozoites transmitted explained only 29% of the variation in sporozoite transmission for *An. gambiae*. For *An. stephensi*, there was no relation between sporozoite loads and the number of sporozoites transmitted. From a practical view, the retrospective determination of the number of sporozoites in the salivary glands may not provide a useful indication of the number of sporozoites transmitted either by naturally infected mosquitoes[10] or by those used in vaccine challenge studies.[19]

Estimates of the numbers of sporozoites transmitted by infective mosquitoes are subject to the limitations of in vitro techniques, which may not adequately stimulate normal probing and blood feeding behavior.[21] Attempting to measure the number of sporozoites released during salivation is probably more realistic than attempting to recover sporozoites from human tissue at the site of feeding.[22] Despite the limitations of the methods used in this study, the numbers of sporozoites transmitted by the experimentally infected *An. gambiae* and *An. stephensi* were similar to results from four other laboratory studies, which employed different techniques. Rosenberg and others [23] showed that *P. falciparum*-infected *An. stephensi* ejected a median of 15 sporozoites (range: 0–978) into mineral oil. Counts of exoerythrocytic schizonts for *P. berghei* indicate that only about 1% of sporozoites in the salivary glands are injected during blood feeding by *An. stephensi*.[24] An analysis using mathematical modelling indicated that observed pre-patent intervals of volunteers challenged by *P. falciparum*-infected *An. stephensi* were due to the inoculation of about 24 sporozoites per mosquito.[25] Recently, Ponnudurai and others [26] found that 73.5% (n = 34) of *P. falciparum*-infected *An. stephensi* with a median of 14, 500 gland sporozoites inoculated a median of 22 sporozoites (range 1–522). Their test system involved feeding mosquitoes through a mouse skin membrane on 200 μl of continuously-stirred blood. This approach minimized interference with the active process of probing and controlled for the possibility that ejected sporozoites could be ingested during the feeding process, even though the in-

gestion of salivary secretions containing sporozoites is a normal phenomenon associated with blood feeding.[27] Thus, all studies to date indicate that most infective mosquitoes transmit few sporozoites and that only a very small proportion transmit hundreds of sporozoites.

In conclusion, the sporozoite transmission potential of *An. gambiae* and *An. stephensi* experimentally infected with *P. falciparum* appears to be similar to that of naturally infected vectors. The actual number of sporozoites transmitted was generally low for both species, regardless of differences in sporozoite loads. Current methods for infecting mosquitoes with *P. falciparum* and challenging volunteers in malaria vaccine studies appear adequate, given the limited information available for naturally infected vectors.

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