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SPOROZOITE LOADS OF NATURALLY INFECTED *ANOPHELES* IN KILIFI DISTRICT, KENYA

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ABSTRACT. The number of salivary gland malaria sporozoites (sporozoite load) was determined by hemacytometer counts for 2,055 field-collected *Anopheles* mosquitoes from Kilifi District, Kenya. Of 48 gland-positive *Anopheles gambiae s.l.*, sporozoite loads ranged from 125 to 79,875, with a geometric mean of 1,743 sporozoites per infected mosquito. About half of the infected mosquitoes had sporozoite loads <1,000. Following hemacytometer examination of salivary gland samples, the same samples were subsequently tested for *Plasmodium falciparum* circumsporozoite (CS) protein by enzyme-linked immunosorbent assay (ELISA). The confirmation by ELISA of CS protein in 89.6% (43/48) of the salivary gland-positive samples compared to only 1.4% (28/2,007) of the dissection-negative mosquitoes indicated that dissection methods with hemacytometer counts of sporozoites were adequate for detecting even low numbers of sporozoites in field-collected mosquitoes. Detection of 17 or fewer sporozoites in blood meals of 7 freshly bloodfed *An. gambiae s.l.* provides a further indication that the actual number of sporozoites transmitted during bloodfeeding may be quite low.

INTRODUCTION

Evaluations of malaria parasite transmission in nature are limited by technical difficulties associated with determining the number of sporozoites in the salivary glands (sporozoite loads) and the number of sporozoites transmitted by mosquitoes during the bloodfeeding process. In Africa, 2 studies have demonstrated that sporozoite loads of naturally infected *Anopheles gambiae s.l.* Giles and *Anopheles funestus* Giles mosquitoes are highly variable, ranging from <10 to >200,000 (Pringle 1966, Beier et al. 1991c). In these 2 studies, the number of sporozoites per mosquito was determined by dissection of the salivary glands and corresponding examination of Giemsa-stained samples of salivary gland material containing sporozoites.

In models of malaria parasite transmission, it is generally assumed that all infected mosquitoes transmit sporozoites during each bloodfeeding event. However, one field-based study (Beier et al. 1991b) and a number of recent studies using experimentally infected mosquitoes indicate that not all infected mosquitoes transmit sporozoites during bloodfeeding and that the average numbers of sporozoites transmitted is typically <20 (Rosenberg et al. 1990; Beier et al. 1991a, 1992b; Ponnudurai et al. 1991). Further, about two-thirds of the transmitted sporozoites are quickly reingested by the mosquito during the bloodfeeding process (Beier et al. 1992a). Thus, the number of ingested sporozoites in the mosquito blood meal directly after feeding provides an indication of the minimum number of sporozoites released by the mosquito during bloodfeeding.

The aim of this study was to examine the sporozoite loads and the natural transmission potential of infected anopheline mosquitoes from Kilifi District, Kenya, an area where there is a high incidence of severe disease due to *Plasmodium falciparum* malaria (Snow et al. 1993, Marsh et al. 1995). The low-level transmission of *P. falciparum* in Kilifi District along the coast of Kenya (Mbogo et al. 1993, 1995) provided an opportunity to obtain information on sporozoite loads directly comparable to that from western Kenya (Beier et al. 1991c), where transmission levels are 20–30 times higher (Beier et al. 1990b).

MATERIALS AND METHODS

Mosquitoes were collected at 9 sites located in Kilifi District along the coast of Kenya. The study area and mosquito sampling methods have been described elsewhere (Mbogo et al. 1995). Briefly, *Anopheles* mosquitoes were sampled by indoor and outdoor all-night human biting catches, day resting indoor (DRI) collections, and by pyrethrum spray catches (PSC) (World Health Organization 1975). Field-collected mosquitoes were transported to a laboratory in Kilifi, where they were identified and processed. The study was conducted from February 1992 to June 1993.

The sporozoite load was determined for each dissected mosquito. First, the salivary glands were dissected in 50 µl of M-199 medium (Gibco Life Technologies Inc., Grand Island, NY) and then ground in glass tissue grinders (Kontes Glass Co., Vineland, NJ) (Beier et al. 1991a). A sample of 10 µl was loaded to a hemacytometer (Hausser Scientific Partnership, Horsham, PA) and sporozoites were counted in each of the 0.1-µl corner squares of the counting chamber using phase-contrast microscopy. The total number of sporozoites in the 4 corners was multiplied by a factor of 125 to provide

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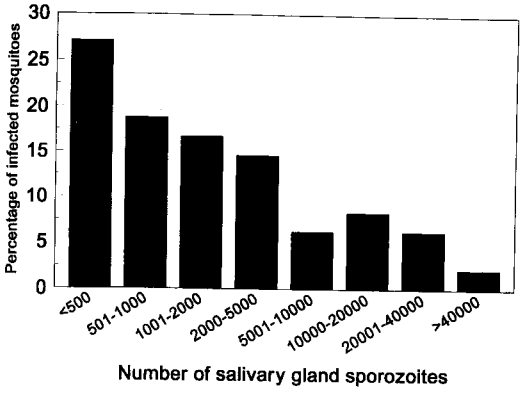

Fig. 1. Frequency distribution of sporozoite loads determined by hemacytometer counts for 48 infected *Anopheles gambiae s.l.* mosquitoes collected at sites in Kilifi District, Kenya.

an estimate of the sporozoite load for each positive mosquito.

The remaining 40 µl of the salivary gland sample was later tested by *P. falciparum* sporozoite enzyme-linked immunosorbent assay (ELISA) (Wirtz et al. 1987). To the remaining 40 µl of the salivary gland suspension, 50 µl of blocking buffer plus NP40 (grinding solution) and 50 µl of blocking buffer were added (Wirtz et al. 1987). This provided information on the *Plasmodium* species of sporozoites detected and also served as a check to identify mosquitoes where sporozoites could have been missed by microscopic examination. In addition to ELISA tests on the salivary gland suspension (material left after the enumeration of the salivary gland sporozoites), the head–thorax section of the mosquitoes (with salivary glands removed) was also tested to determine concordance with ELISA tests on the salivary gland samples. This was done because salivary glands are sometimes lost during handling due to their small size or break apart during dissections. Before testing, the head–thorax section was homogenized in 50 µl of grinding solution and then 200 µl of blocking buffer was added. The negative control consisted of field-collected male *Anopheles* mosquitoes and a recombinant protein was used as a positive control (Wirtz et al. 1987). The ELISA sample results were evaluated visually (Beier and Koros 1991).

Midguts of *Anopheles* mosquitoes that were bloodfed (those that had visible blood in their guts) and also had sporozoites in the salivary glands were further dissected and examined for ingested sporozoites (Beier et al. 1992a). The mosquito midgut was dissected and rinsed twice in 50 µl phosphate-buffered saline (PBS). The midgut was then suspended in 10 µl PBS and punctured to allow the gut contents to flow out. The contents were mixed well and spotted on slides in preparation for sporozoite detection by indirect fluorescent antibody (IFA) methods (Beier et al. 1992a). After the slides were air-dried, 20 µl of fluorescein isothiocynate-conjugated monoclonal antibody 2A10 was added to the IFA spots. After 30 min in a moist chamber the unreacted material was removed using a pasteur pipette. The slides were rinsed with 25 µl PBS. The slides were blotted dry and examined under a fluorescent microscope to identify the fluorescing sporozoites. The number counted for each mosquito provided an estimate of the minimum number of sporozoites injected during the time of feeding (Beier et al. 1992a).

Table 1. Detection of *Plasmodium falciparum* circumsporozoite protein by enzyme-linked immunosorbent assay (ELISA) in salivary glands and thorax samples of *Anopheles gambiae s.l.* mosquitoes found positive for salivary gland sporozoites by dissection.

Sample tested	Dissection result	*n*	% positive by ELISA
Salivary gland	Positive	48	89.6
	Negative	2,007	1.4
Thorax	Positive	43	83.7
	Negative	1,536	1.4

RESULTS

A total of 2,055 mosquitoes were dissected for sporozoites. These included 2,023 *An. gambiae* Giles *s.l.*, 19 *An. funestus*, 8 *Anopheles nili* Theobald, 3 *Anopheles coustani* Laveran, and 2 *Anopheles squamosus* Theobald. Salivary gland sporozoites were detected only in *An. gambiae s.l.*, with a total of 2.4% infected. Sporozoite loads for the 48 positive *An. gambiae s.l.* ranged from 125 to 79,875 per mosquito with a geometric mean number of 1,743 sporozoites. The frequency distribution of sporozoite loads is shown in Fig. 1. A total of 22 (45.8%) of the 48 mosquitoes had loads of <1,000 sporozoites per mosquito. Only 8 (16.6%) mosquitoes had loads >10,000 sporozoites.

Salivary gland and thorax samples from the dissected mosquitoes were subsequently tested by ELISA for the presence of *P. falciparum* circumsporozoite (CS) protein (Table 1). For the salivary gland samples tested, CS protein was detected by ELISA in 89.6% (43/48) of the dissection-positive samples compared to 1.4% (28/2,007) of the dissection-negative samples. Following the removal of salivary glands by dissection, thorax samples from 1,579 of the mosquitoes were also tested by ELISA. In thorax samples, CS protein was detected in 83.7% (36/43) of the dissection-positive samples and in 1.4% (21/1,536) of the dissection-negative samples.

Ingested sporozoites were detected by IFA in blood meals from 5 of 7 gland-positive mosquitoes examined. For the 5 positive mosquitoes, the numbers of sporozoites in the midgut were 3, 5, 7, 13, and 17. The number of ingested sporozoites in-

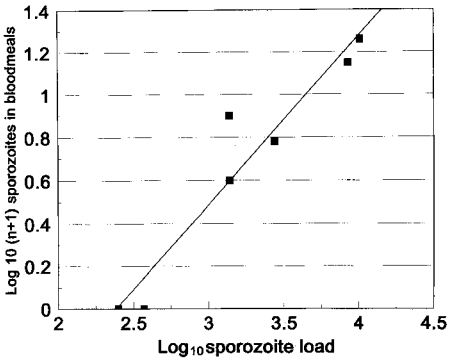

Fig. 2. Relationship between the numbers of sporozoites detected in blood meals of freshly bloodfed mosquitoes and sporozoite loads determined for field-collected *Anopheles gambiae s.l.* from Kilifi District, Kenya.

creased linearly as a function of the number of sporozoites in the salivary glands (Spearman's rho, $r = 0.927$, df = 5, $P = 0.003$) (Fig. 2).

DISCUSSION

Most naturally infected *Anopheles* mosquitoes in Africa have relatively low numbers of sporozoites in their salivary glands. Our findings that about half of the naturally infected *An. gambiae s.l.* from Kilifi District, Kenya, have sporozoite loads <1,000 are similar to results reported previously by Beier et al. (1991c) from western Kenya. Earlier reports of geometric mean sporozoite loads >6,000 by Pringle (1966) in Tanzania could have been overestimated due to methodologic problems associated with detection of low-intensity infections.

The use of direct hemacytometer counts of sporozoites from salivary glands of field-collected mosquitoes provides a level of accuracy not attainable by other methods used to determine sporozoite loads (Pringle 1966, Beier et al. 1991c). Of the 48 mosquitoes positive for sporozoites by dissection, 43 (89.6%) were also positive by ELISA. The 5 ELISA-negative (but dissection-positive) mosquitoes had sporozoite loads of 125, 250, 375, 500, and 6,125. Because these same samples also tested negative for *Plasmodium malariae* CS protein, the identity of the *Plasmodium* species remains uncertain. Of the 2,007 mosquitoes that were sporozoite-negative by dissection, 1.4% tested positive by ELISA. In a similar study in western Kenya by Beier et al. (1990a), 5.2% of salivary gland sporozoite-negative mosquitoes were positive by ELISA and 45.4% of ELISA-positive mosquitoes did not have sporozoites in the salivary glands. Thus, testing of salivary gland and thorax samples by sporozoite ELISA provided a reasonable means for monitoring the accuracy of sporozoite detection methods.

Findings of 17 or fewer sporozoites in the midguts of recently fed *An. gambiae* from Kilifi District indicate that naturally infected mosquitoes from this area likely transmit few sporozoites. This conclusion is based on laboratory evidence that only about one-third of the sporozoites ejected during bloodfeeding remain in the host; the other two-thirds are ingested and can be found in the midgut of the fully engorged female mosquitoes (Beier et al. 1992a). As in the laboratory studies with 2 species of *P. falciparum*-infected mosquitoes, the numbers of sporozoites in the midguts increased as a linear function of sporozoite loads. Thus, the presence of only a few sporozoites in the midguts of the naturally infected mosquitoes from Kilifi District is consistent with predictions from a series of laboratory studies that most infected mosquitoes transmit few sporozoites, generally <20, during the bloodfeeding process (Rosenberg et al. 1990; Beier et al. 1991a, 1992b; Ponnudurai et al. 1991).

Low population densities of anopheline mosquitoes and correspondingly low intensities of *P. falciparum* transmission in Kilifi District (Mbogo et al. 1993, 1995) precluded a detailed analysis of temporal and spatial variation in sporozoite loads of naturally infected mosquitoes. No apparent differences in sporozoite loads occurred over time. In 2 sites where 15 or more sporozoite-positive mosquitoes were detected, no significant differences were found in sporozoite loads. Thus, similar to previous results in western Kenya (Beier et al. 1991c), there is still no convincing evidence that sporozoite loads vary in time and space.

In conclusion, there are 2 important findings relative to malaria parasite transmission. First, under conditions of low-intensity transmission in Kilifi District, sporozoite loads of *An. gambiae s.l.* are remarkably similar to those associated with much higher intensities of natural transmission in western Kenya (Beier et al. 1990b, 1991c). Thus, sporozoite loads may not vary significantly as a function of transmission intensity. Second, it remains difficult to assess whether the inoculation dose of sporozoites influences the outcome of malaria infections (Greenwood et al. 1991, Lines and Armstrong 1992, Marsh 1992). Our studies provide further indications that not all naturally infected mosquitoes transmit sporozoites and that the actual numbers of sporozoites transmitted per bloodfeeding event are likely to be quite low.

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