**Plasmodium falciparum** and **P. berghei**: detection of sporozoites and the circumsporozoite proteins in the saliva of *Anopheles stephensi* mosquitoes

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**Abstract.** Sporozoites and free circumsporozoite (CS) protein were stained immunoenzymatically in 1-min saliva samples collected from *Anopheles stephensi* mosquitoes infected with either *Plasmodium berghei* or *P. falciparum*. The number of sporozoites in 1-min saliva-streak samples significantly increased as the salivary gland index rose from 3+ to 4+. For *P. berghei*-infected mosquitoes from which saliva had been collected before 30 days postfeed, the median sporozoite counts for 3+ and 4+ gland indexes were 4.5 and 116, respectively. For *P. falciparum*-infected mosquitoes, the median counts obtained in two experiments were 4.5 and 14.5 (3+) and 97 and 107 (4+), respectively. The frequency of sporozoite detection in the saliva of mosquitoes containing <100 salivary-gland sporozoites was low (0.1), whereas that in the saliva of mosquitoes with >100 sporozoites was high (0.96). In highly infected 4+ *P. berghei*-infected mosquitoes from which saliva had been collected after 30 days postinfection, both the volume of saliva collected and the median number of sporozoites recovered decreased significantly.

Saliva is the vehicle that transports malarial sporozoites from the mosquito to the host. Immediately after a blood meal, the total protein of the salivary glands is reduced by as much as 35% in *Anopheles stephensi* fed on mice (Poehling 1978), by 30% in *Culex pipiens* Say fed on chicks (Poehling 1978), and by 57% in *Aedes aegypti* (L.) fed on hamsters (Marinotti et al. 1990).

With the advent of vaccine studies and drug trials against the sporozoite stage of human malaria (Egan et al. 1987; Herrington et al. 1987), one concern has been the number of sporozoites delivered by infective mosquitoes on challenge. Estimates from recent in vitro studies indicate that the number of sporozoites ejected into mineral-oil droplets (Rosenberg et al. 1990), sucrose- and blood-filled capillary tubes (Beier et al. 1991a), and mouse skin membrane (Ponnudurai et al. 1991) is relatively low, usually amounting to <25, and does not correlate with the degree of salivary-gland infection (Beier et al. 1991b; Ponnudurai et al. 1991).

Since the number of sporozoites ejected is probably related to the output of saliva (Ponnudurai et al. 1991) and the number of sporozoites in the salivary duct at the time of feeding, we collected saliva from infected and noninfected mosquitoes to determine whether the number of sporozoites in the salivary gland (measured as the gland index) would affect the number of sporozoites ejected and the amount of saliva present in a continuous 1-min flow of saliva. Our primary goal was to observe directly and count the sporozoites in saliva without mimicking the natural process of bloodfeeding. The effect of the interval between bloodfeeding and collection of saliva on the amount and number of sporozoites ejected per minute was also determined. Saliva was collected directly onto microwell slides from legless, wingless female *A. stephensi* mosquitoes and was immunoenzymatically assayed for sporozoites using an alkaline phosphatase-labeled monoclonal antibody to the circumsporozoite (CS) protein of either *P. falciparum* (mAb PF2A10) or *P. berghei* (mAb NBS1).

**Materials and methods**

Female *Anopheles stephensi* mosquitoes aged 3–5 days were infected either with *Plasmodium berghei* (NK 65) by feeding them on anesthetized, infected mice (ICR strain) for 10 min at 18°C or with *P. falciparum* by membrane feeding of mosquitoes (Rutledge et al. 1964) on cultured *P. falciparum* (NF 54) gametocytes suspended in defibrinated human blood and serum. *P. berghei*- and *P. falciparum*-infected mosquitoes were maintained at 18°C and 26°C, respectively, and were fed a 10% sucrose solution before and after the infective meal.

Mosquito saliva was collected directly onto poly-l-lysine (Sigma, St. Louis, Mo.)-treated slides (10 wells, 7 mm, Roboz Surgical Co., Washington, D.C.). For saliva collection mosquitoes were positioned on the well slide after their legs and wings had been removed. The maxillary palps and head were held with one pair of forceps as the fascicle was desheathed using a second pair of
forces. The head was held with forceps such that the fascicle made contact with a glass slide. The fascicle contains the labrum, mandibles, maxillae, and hypopharynx. The labrum comprises the walls of the food canal, whereas the hypopharynx contains the salivary duct (Jones 1978). These structures are readily distinguished when viewed under high-power magnification (× 65) with a dissecting microscope. Direct observation of the ejection of saliva from the hypopharynx was possible at this magnification. If a mosquito did not salivate immediately, the hairs of the labela were tapped to stimulate salivation. Approximately 70% of the mosquitoes responded by salivating on the glass slide.

As the mosquito salivated, it was moved across the slide such that a streak of saliva was deposited. Each microwell contained only a 1-min flow of saliva in which sporozoites were associated with the time of ejection. Saliva samples were then air-dried and fixed in Bouin's solution or cold, absolute ethanol (1 min). In experiments using P. berghei-infected mosquitoes, a second 1-min sample of saliva was collected immediately after the first sample onto a second microwell, washed with 1 μl Medium 199, air-dried, and fixed in Bouin's solution.

The salivary glands from each mosquito that salivated were dissected in Medium 199 and transferred to poly-L-lysine-treated slide wells containing 10 μl medium. Once the tissue had been centrally aligned in the well, excess medium was removed with a fine-tipped pipette (diameter, 0.05 mm). The glands were triturated with a plastic inoculating needle to release sporozoites and were then fixed in Bouin's solution (3 mm) and washed in running tap water (20 min).

In initial studies, saliva was collected from P. berghei-infected mosquitoes at 19–24 days postfeed (n=66) and again from the same cohort 7 days later at 31–34 days postfeed (n=42) to determine whether the postfeed interval would influence the number of sporozoites recovered from saliva. Saliva was also collected from P. berghei-infected mosquitoes both as a saliva streak and as a washed saliva sample to determine whether sporozoite counts from washed samples would prove to be more accurate. In other studies, saliva was collected from P. falciparum-infected mosquitoes at 19–28 days after the blood meal (n=27 and 38, 2 experiments) to determine whether the amount of saliva and number of sporozoites ejected would correlate with the number of sporozoites in the salivary glands (measured as the gland index).

Slides containing saliva samples and dissected salivary glands were placed in a moist chamber at 24°C–26°C, and 30 μl blocking buffer [BB; Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, Mo.) containing 1.0% bovine serum albumin (BSA), 0.5% casein, 0.01% thimersol, and 0.002% phenol red] was added with a digital multichannel pipette (Flow Laboratories, McLean, Va.). After 10 min, the BB was removed and 30 μl of an alkaline phosphatase-labeled monoclonal antibody to the CS protein of P. berghei (mAb NBSI) or P. falciparum (mAb PF2A10) was added for 30 min at a concentration of 0.15 μg/well (5 μg/ml). Slides were then washed (3 × 5 min) in PBS and assayed for alkaline phosphatase (HistoMark Red; Kirkegaard and Perry Laboratories, Gaithersburg, Md.) using 50 μl of the buffered substrate/dye mixture from the kit. After 10 min, the reaction was stopped by washing slides for 3 min in running tap water. Slides were mounted in Crystal/Mount (Biomedca Corp., Foster City, Calif.). All immunostains were observed using bright-field microscopy.

For each mosquito, three variables were measured: (1) the number of sporozoites in a 1-min saliva streak; (2) the number of high-power (×1000) fields traversed during counting of the sporozoites, which gave an approximation of saliva output; and (3) the number of sporozoites in the triturated salivary gland, which gave the gland index. Gland indices measure the level of salivary-gland infections. Five classes are used: 0, no sporozoites; 1+, 1–10 sporozoites; 2+, 11–100 sporozoites; 3+, 101–1000 sporozoites; and 4+, >1000 sporozoites (Vanderberg and Gwadz 1980). For each mosquito infected with P. berghei, the number of sporozoites in each washed 1-min saliva sample was also counted.

The mean and median numbers of sporozoites in a 1-min saliva streak or a washed saliva sample were determined for each gland.

To determine whether we could accurately count the sporozoites in saliva-streak samples and whether the nonreactivity of some sporozoites to anti-CS protein monoclonal antibody would be related to their occurrence in fixed saliva samples, we compared unwashed, fixed saliva-streak samples with washed, fixed saliva index. Similarly, the mean and median numbers of fields traversed during counting of the sporozoites (as a measure of saliva output) were also calculated for each gland.

Two group comparisons were made using appropriate nonparametric statistical methods. The Wilcoxon signed-rank test was used to compare differences in sporozoite counts between unwashed and washed saliva samples from P. berghei-infected mosquitoes. A Mann-Whitney test was used to compare the difference in both sporozoite recovery and field counts between P. falciparum-infected mosquitoes for which gland indices of 3+ and 4+ had been determined.

Analysis of variance (ANOVA) was used to test the effect of each gland index and the sampling period, i.e., the number of days after the blood meal, on the recovery of sporozoites from 1-min saliva-streak samples obtained from P. berghei-infected mosquitoes. Data were normalized using the common logarithm transformation.

Results

Direct viewing of the salivation process under high-power magnification of the dissecting microscope demonstrated that the secretion collected was released from the hypopharynx (the external opening of the salivary duct) and not from the labrum (food canal). Often, the mandibles and maxillae moved back and forth during the ejection of saliva, but saliva was also ejected without mouthpart movements. In other mosquitoes, movement of the mandibles and maxillae occurred without the ejection of saliva from the hypopharynx. The viscosity of the saliva varied greatly among mosquitoes. In some cases it was a syrup-like solution, and in others it was waxy. Regardless of the consistency, all saliva samples were highly soluble in water. Fixation in Bouin's solution was necessary to prevent washoff of the saliva during immunostaining.

The immunoenzymatic assay using alkaline phosphatase conjugated to a monoclonal antibody directed against the CS protein of the sporozoite produced a pink-to-red color at sites of enzyme activity. Immunostained saliva from infected mosquitoes was often tinted pink (Fig. 1), which made it easier to locate on microwell slides than the colorless saliva from noninfected mosquitoes (Fig. 2). Although the pink/red color, which was indicative of the CS protein, was localized on the surface of the sporozoite, it was also found unassociated with the surface of the sporozoite (Fig. 3). The free CS protein in saliva samples from heavily infected mosquitoes was often solubilized by ethanol fixation such that a halo of pink surrounded the saliva streak. Bouin's solution reduced this effect but did not eliminate it. Some sporozoites were not immunoreactive for CS protein (Fig. 4). Counting of sporozoites in heavily infected saliva-streak samples was often difficult because of the amount of free CS protein and the nonreactivity of some sporozoites.