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Detecting malaria sporozoites in live, field-collected mosquitoes

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Abstract

A method is described for identifying malaria-infected mosquitoes, without killing them or hampering their fitness. Individual mosquitoes were induced to salivate on coverslips, and sporozoites, deposited on the glass surface, were visualized by Giemsa staining. Of 21 mosquitoes found to contain sporozoites by salivary gland dissection, 13 had delivered sporozoites on coverslips. A positive correlation was found between the amount of saliva expelled and ejection of sporozoites, indicating that the sensitivity of the method may be increased by improving the probing behaviour of the mosquitoes. The procedure described may be suitable for selecting infected mosquitoes which are able to eject sporozoites during probing. Being applicable to wild *Anopheles* and to large numbers of mosquitoes, the method lends itself for use in field studies on malaria.

Introduction

An easy method for assessing whether a single mosquito harbours *Plasmodium* sporozoites in its salivary glands, and whether it may eject these sporozoites during probing, could be useful for certain studies in malaria research.

Classical methods for identifying malaria sporozoite infection in mosquitoes rely either upon microscopical examination of dissected salivary glands, or upon immunological identification of circumsporozoite (CS) protein in mosquito extracts (COLLINS *et al.*, 1984).

In trypanosomiasis research, an easy and rapid method for identifying trypanosomes in live tsetse flies was published by BURTT in 1946 and is now widely used. The method exploits the behaviour of *Glossina* in responding with salivation when stimulated by a warm surface. In practice, flies are offered a lukewarm glass slide and allowed to deposit saliva for some minutes. The slide is then examined microscopically for the presence of trypanosomes.

Here we describe a modified salivation method, which enables *Plasmodium* sporozoite infections to be detected in live, field-collected *Anopheles*, and propose possible applications in malaria research.

Material and Methods

Anopheles gambiae s.l. mosquitoes were collected in Noungou, a village near Ougadougou (Burkina Faso), in which *P. falciparum* sporozoite rates were about 10% during the study period. The hand-caught females were maintained for 10–12 d in an insectary on 1% sugar, to allow the sporogonic cycle to be completed and a higher percentage of salivary gland-infected specimens to be obtained. For the salivation experiments, mosquitoes—placed individually in paper cups covered with gauze—were stimulated to feed by gently breathing into the cup and by placing the forearm over it. As soon as a female showed exploratory behaviour, searching for a host, a coverslip, warmed by a finger, was held on the gauze. Mosquitoes were allowed to probe and salivate on the glass surface for 5 to 10 min, being restimulated at intervals. Their salivary glands were then dissected and examined microscopically for sporozoites. Coverslips from mosquitoes with sporozoites were fixed with methanol, stained with Giemsa's stain and mounted on slides with Canada balsam. The coverslips were examined for the presence of saliva spots at 100× magnification, and sporozoites were counted at 600× magnification. The amount of expelled saliva was arbitrarily assessed, considering as 1 saliva unit a single saliva deposit or—when saliva had been distributed over an extended area—one-fifth of the microscopical field at 600× magnification.

Results

Screening for *Plasmodium* sporozoite infection was performed on about 200 field-collected *Anopheles* females, by stimulating them individually to salivate on coverslips. At dissection, 21 of these mosquitoes were found to harbour sporozoites in their salivary glands. Of these, 13 had expelled saliva containing sporozoites. The amount of saliva deposited per coverslip varied widely. A positive correlation (Spearman's rank correlation coefficient $r_S=0{\cdot}72$, $P<0{\cdot}001$) was found between the number of sporozoite-positive saliva units per coverslip and the total number of saliva units per coverslip (Figure). Accordingly, sporozoites were found on 9 of the 11 coverslips on which mosquitoes deposited a number of saliva units higher than or equal to the median (median=15, geometric mean=10·25), while only 4 of the 10 coverslips with fewer than 15 saliva units contained sporozoites. On average, about one-fifth of the saliva units on sporozoite-containing coverslips were positive (geometric mean of the ratio positive/total saliva units=0·19, standard deviation [SD]=0·13, range 0·05–0·5).

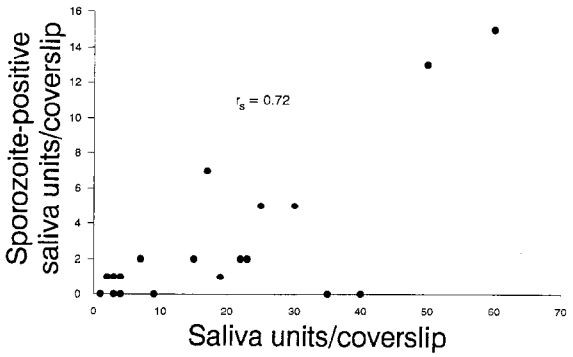

Figure. Correlation between sporozoite-positive saliva units per coverslip and total number of saliva units per coverslip. The value of the Spearman's rank correlation coefficient, $r_S=0{\cdot}72$, is significantly different from 0 ($P<0{\cdot}001$).

As shown in the Table, usually 1 to 3 sporozoites were found in sporozoite-positive saliva units (mode=1; geometric mean=2·3, SD=7·5) with the exception of one coverslip, on which up to 30 sporozoites were noted in one saliva unit. Considering the coverslips as a whole, a geometric mean of 4·4 sporozoites per positive coverslip was recorded (SD=43·3; range=1–166).

Table. Number of sporozoites ejected by individual mosquitoes

No. of sporozoites in each positive saliva unit	per coverslip
1	1
1	1
1,1	2
1,1	2
1,1	2
1,1	2
6	6
1,1,1,1,2	6
1,1,1,2,3	8
1,1,1,1, 2,2,2	10
1,1,1... 3,3,4[a]	29
2,5,6...18,26,30[b]	166

[a]15 saliva units were deposited.
[b]13 saliva units were deposited.

Discussion

In trypanosomiasis research a salivation method, based on stimulation by warmth, is widely used to identify infected flies (BURTT, 1946). This method, slightly modified, proved to be applicable for detecting *Plasmodium* sporozoite infection in live, field-collected *An. gambiae s.l.* Sporozoite-containing saliva deposits were found on about two-thirds (13/21) of the coverslips on which mosquitoes known to be positive by dissection had been probing. Although this proportion of positive coverslips may appear relatively low, taking into account the positive correlation between sporozoite-containing saliva units and the amount of saliva deposited, it could probably be augmented by improving the probing behaviour of the mosquitoes.

Since the 1930s, several attempts have been made to estimate the number of sporozoites a mosquito injects during its bloodmeal (reviewed by ROSENBERG *et al.*, 1990). This topic has enjoyed renewed interest in recent years in connection with research aimed at developing an anti-sporozoite malaria vaccine (DAVIS *et al.*, 1989a, 1989b; RICKMAN *et al.*, 1990; HERRINGTON *et al.*, 1990). Obviously, the salivation method described here, in which a glass surface was used as probing substrate, was not designed to determine the size of parasite inocula. Nevertheless, the results obtained (4·4 sporozoites ejected on average per mosquito, range 1–166) were of the same order of magnitude as estimates of sporozoite inocula derived from different experimental salivation or feeding systems: examining wild *Anopheles*, BEIER *et al.* (1991) reported a value 3·8 sporozoite (range 1–34); studying experimentally infected mosquitoes, ROSENBERG *et al.* (1990) and PONNUDURAI *et al.* (1991) reported values of 15 (range 0–978) and 8 sporozoites (range 0–522), respectively. Interestingly, in the studies referred to, as well as in ours, the upper limit of the range of sporozoites expelled was many times higher than the average value, indicating that single infected bites may deliver a sporozoite load much heavier than that injected by the majority of the mosquito bites.

Our finding that 2 infected mosquitoes, although salivating abundantly (more than twice the median number of saliva units), did not deposit a single sporozoite on the coverslip, was also consistent with previous observations by ROSENBERG *et al.* (1990)—who observed that 9 of the 46 mosquitoes which delivered more than the median amount of saliva did not expel any sporozoites—and PONNUDURAI *et al.* (1991), who, using an experimental design closely matching the natural feeding situation, reported that 9 of 34 mosquitoes failed to inject sporozoites. An explanation for these observations may be found in the architecture of the salivary gland, due to which only sporozoites which have penetrated the distal acinar cells can gain access to the duct lumen (STERLING *et al.*, 1973; PONNUDURAI *et al.*, 1991). Mosquitoes which salivate abundantly but do not expel sporozoites might represent specimens with infections limited to the proximal portions of the salivary glands. Whatever the reason may be, experimental data agree that some sporozoite-harbouring mosquitoes do not eject sporozoites. If it were demonstrated that the inability of infected mosquitoes to eject sporozoites is an inherent property of individuals, the accuracy of entomological inoculation rates, based on the classical sporozoite rate, may have to be questioned.

The results obtained suggest that the method described here may be used for selecting, from a group of malaria-infected mosquitoes, (i) those which are certainly able to eject sporozoites during probing and (ii) those ones which are capable of expelling exceptionally large sporozoite loads. A method with these characteristics might be useful, for example, to select for high or low transmission capacity among laboratory-reared mosquito colonies. An additional feature of the method, which might be useful in field applications, would be the specific identification of the ejected sporozoites. This could be easily done, by processing the slides for immunofluorescence with a species-specific monoclonal antibody (A. Habluetzel and F. Esposito, unpublished data). Recently, a method has been described in which infected *Anopheles* were induced to salivate on a fructose-soaked nitrocellulose membrane and CS protein on the membrane was revealed by a monoclonal antibody (BILLINGSLEY *et al.*, 1991). Since sporozoites shed CS protein during their stay in the salivary glands (POSTHUMA *et al.*, 1989), a positive reaction in this assay is not conclusive as to whether sporozoites or just CS protein had been deposited with the saliva.

Being applicable to field-collected *Anopheles* and to large numbers of mosquitoes, the salivation method described here lends itself for use in field studies. An interesting application might be in vaccine efficacy trials: by preliminarily screening wild *Anopheles* with the salivation method, volunteers could be challenged with a chosen number of vectors ejecting truly natural malaria parasites instead of strains reared in the laboratory.

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Announcement

PRIZES

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1. Two prizes of £200 may be awarded annually in recognition of outstanding projects which increase knowledge of tropical medicine and hygiene in the broadest sense.
2. Candidates shall be nominated by their head of department, supervisor or Dean, with a supporting statement of up to 500 words.
3. The closing date for receipt of project reports is 31 December. The project should have been done or completed in the previous twelve months.
4. A Committee of three shall choose the prize winners.
5. The announcement of the prize winners will be made at the March meeting of the Society.
6. The prizes will be presented by the President of the Society at the Annual General Meeting in June or July.

Please note that the Society cannot provide funds to cover students' elective travel expenses.