SHORT COMMUNICATION

Use of Circumsporozoite Protein Enzyme-Linked Immunosorbent Assay Compared with Microscopic Examination of Salivary Glands for Calculation of Malaria Infectivity Rates in Mosquitoes (Diptera: Culicidae) from Cameroon

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ABSTRACT A survey in Cameroon compared the usefulness of the circumsporozoite protein enzyme-linked immunosorbent assay (CSP ELISA) to dissection and microscopic examination of anopheline salivary glands for measuring infectivity rates in anopheline mosquitoes. The salivary glands of 375 females, belonging to four species were examined for sporozoites. After microscopic examination, the glands as well as all the remaining heads and thoraces were tested by ELISA. The sensitivity of ELISA was 100% (18/18), confidence interval (CI) (78.1–100) and the specificity was 99.7% (357/358), CI (98.2–100). The Kappa value, agreement between examination of the glands and salivary gland ELISA, was 0.97. The head–thorax CSP ELISA overestimated the true salivary gland infection rate by 12.0%. The results obtained in Central Africa in a village with perennial transmission highly justified the use of the ELISA for measuring the entomological inoculation rate.

KEY WORDS mosquitoes, *Anopheles*, malaria, enzyme-linked immunosorbent assay, transmission, Cameroon

THE CALCULATION OF the malaria entomological inoculation rate (the number of infective mosquito bites per human being per day) is essential in every study of malaria transmission and depends on an accurate measure of the number of anophelines infected with *Plasmodium* sporozoites. Two methods are generally used for determining infection rates. The first method, which is the "gold standard," is dissection followed by microscopic examination of the salivary glands of the anopheline to observe the presence of sporozoites (WHO 1975). The second is an enzyme-linked immunosorbent assay (ELISA) that detects *Plasmodium* circumsporozoite protein (CSP) from sporozoites in the thoracic salivary glands or mature oocysts on midgut (Burkot et al. 1984, Wirtz et al. 1987).

Both methods present advantages and disadvantages. Dissection allows the direct observation of sporozoites within the glands, and therefore determines the infectiveness of a mosquito. However, dissections must be made quickly after the capture of the mosquitoes, which is not always possible, especially when mosquito densities are high, and technicians must be well-trained to avoid observational errors. This method does not determine which *Plasmodium* species is present. The ELISA permits the testing of all mosquitoes captured, because they can be stored until processed. Because the monoclonal antibodies are species-specific for *P. falciparum*, *P. malariae*, and *P. ovale* (the three species present in Central Africa), the *Plasmodium* species can be identified. However, the ELISA detects not only the sporozoites in salivary glands, but also CSP in other mosquito tissues (Lombardi et al. 1987, Robert et al. 1988), and consequently, more mosquitoes are ELISA positive than dissection positive, even when only head-thoraces are tested.

In a new research program on malaria transmission in villages near Yaounde, Cameroon, in a central African forest area, we decided to use the head–thorax ELISA to evaluate the mosquito infection rate. The objectives of our current research were to compare the sensitivity and the specificity of the ELISA to microscopic examination of salivary glands, and to calculate amount of overestimation by the ELISA.

Materials and Methods

Study Area. Mosquitoes were captured between February and April 1999 in Simbock, a village in a rural forested area located 15 km from Yaounde, the capital city of Cameroon (3° 50′ N, 11° 30′ E).

Mosquito Processing. Mosquitoes were identified to species. Sibling species from the *An. gambiae* complex were identified using polymerase chain reaction (PCR) (Scott et al. 1993). The salivary glands were excised in 0.9% saline solution and examined under a microscope at 630× for the presence of sporozoites.

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Table 1. Number of females of each species positive by each of three techniques

Vector species	N° of salivary glands dissected	Sporozoites	Salivary gland ELISA	Head-thorax ELISA
An. funestus	147	Positive: 13	Positive: 13	Positive: 12 Negative: 1[a]
			Negative: 0	Negative: 0
		Negative: 134	Positive: 0	Positive: 0
			Negative: 134	Negative: 133 (1 not tested)
An. gambiae s.s.	43	Positive: 4	Positive: 4	Positive: 4 (1)
			Negative: 0	Negative: 0
		Negative: 39	Positive: 0	Positive: 0
			Negative: 39	Negative: 39
An. moucheti	150	Positive: 1	Positive: 1	Positive: 1
			Negative: 0	Negative: 0
		Negative: 149	Positive: 1[a]	Positive: 0 Negative: 1
			Negative: 148	Positive: 2[a] Negative: 144 (2 not tested)
An. nili	35	Positive: 0	Positive: 0	Positive: 0
			Negative: 0	Negative: 0
		Negative: 35	Positive: 0	Positive: 0
			Negative: 35	Negative: 35
Total	375	Positive: 18	Positive: 18	Positive: 17 Negative: 1[a]
			Negative: 0	Negative: 0
		Negative: 357	Positive: 1[a]	Positive: 0 Negative: 1[a]
			Negative: 356	Positive: 2[a] Negative: 351 (3 not tested)

All the positive mosquitoes were positive for *P. falciparum*, one *An. gambiae* (1) also was positive for *P. ovale*.
[a] Mosquitoes needing a meticulous interpretation (see text).

Each time sporozoites were observed, the mosquito was recorded as "positive."

After examination, the glands (as often as possible all six lobes) from positive and negative mosquitoes were rinsed in 300 μl of blocking buffer before being tested by ELISA for CSP of *P. falciparum*, *P. malariae*, and *P. ovale* as described by Burkot et al. (1984) and modified by Wirtz et al. (1987). *P. vivax* is not present in this region of Africa. This technique is able to detect as few as 100 sporozoites. Monoclonal antibodies were provided by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. Positive controls were *P. falciparum* and *P. malaria* synthetic peptides from CDC; *P. ovale* positive controls were mosquitoes previously positive to *P. ovale* monoclonal antibodies. Mosquitoes were considered positive when the optical density (OD) was higher than the mean plus three standard deviations of negative controls. Negative controls were females from the Organisation de Coordination pour la Lutte Contre les Endemies en Afrique Centrale *An. gambiae* laboratory colony. After dissection, head and thorax remnants were placed individually in 1.5-ml tubes and then crushed in blocking buffer before being processed by the same ELISA protocol as the salivary glands.

Data Analysis. The sporozoite rate, the salivary gland CSP rate, the head-thorax CSP rate, and the 95% exact binomial confidence interval (CI) were calculated (Wayne 1987) as well as sensitivity, specificity and a measure of agreement (Kappa value) (Fleiss 1981). The under- or overestimation of the head-thorax CSP rate, compared with the sporozoite rate, was calculated as (head-thorax CSP rate minus sporozoite rate) divided by sporozoite rate.

Results

Overall, 375 anophelines belonging to four species were dissected (Table 1). All positive mosquitoes were positive for *P. falciparum*, except for one *An. gambiae* s.s., which also was positive for *P. ovale*. The sporozoite indices and the CSP rates for each species with their confidence intervals are presented Table 2.

The sensitivity of the salivary gland CSP ELISA was 100% (18 dissection positive females/(18 salivary glands CSP positive females + 0 salivary glands CSP

Table 2. Infection rates of each species by each of three techniques

Species	Sporozoite rates (CI)	Salivary gland ELISA infection rates (CI)	Head-thorax ELISA infection rates (CI)
An. funestus	8.8% (4.8–14.7)	8.8% (4.8–14.7)	8.2% (4.3–13.9)
An. gambiae s.s.	9.3% (2.6–22.1)	9.3% (2.6–22.1)	9.3% (2.6–22.1)
An. moucheti	0.7% (0.01–3.6)	1.3% (0.16–4.7)	2.0% (0.4–5.8)
An. nili	0	0	0

negative among these 18 dissection positive) = true positive females/(true positive females + false negative females), CI (78.1–100), the specificity was 99.7% (357 true negative females/(357 true negative females + one false positive female), CI (98.2–100). The measure of agreement was excellent (Kappa value = 0.97).

Sporozoites were observed in the salivary glands of 18 mosquitoes, whereas 19 were head–thorax ELISA positive. However, these overall data must be examined in detail (Table 1). One *An. funestus* was positive by dissection, whereas the head–thorax CSP ELISA was negative (OD = 0.018). Salivary gland CSP ELISA clearly confirmed that this mosquito was infected with *P. falciparum*. These data may have indicated that most sporozoites had reached the salivary glands, and then been removed by dissection; those remaining in the thorax were too few to give a significant OD, because the detection threshold of the ELISA was 100 sporozoites (Burkot et al. 1984). This mosquito clearly was a false negative by the head–thorax ELISA technique. One *An. moucheti* was negative by dissection, but positive twice by the salivary gland CSP ELISA (OD = 0.268 for *P. falciparum*), and negative by head–thorax CSP ELISA. It was not possible to say if this mosquito was a false negative both by dissection and by the head–thorax ELISA technique, or a false positive by salivary gland CSP ELISA. The interpretation of two *An. moucheti* positive for head–thorax CSP ELISA, but negative by dissection and salivary gland CSP ELISA, was a more typical result. These mosquitoes probably were infected recently, and sporozoites had not yet reached the salivary glands.

Overall, 18 anophelines out of 375 dissected (4.80%; CI, 2.9–7.5) had sporozoites in their salivary glands, and CSP antigen should have been detected in 20 females (19 plus one false negative) among the 372 tested (5.38%; CI, 3.3–8.2). The head–thorax CSP ELISA overestimation, compared with true salivary gland infection rate, was 12.0% (5.38 versus 4.80%).

Discussion

Our results confirmed that the ELISA provided suitable specificity and sensitivity. Our results also demonstrated that dissections and observation of the salivary glands in the field were well done: only one dissection was a false negative, and none were a false positive.

Few studies really have estimated the specificity and sensitivity of the salivary gland CSP ELISA compared with microscopic examination. In Kenya, Beier et al. (1990) and Adungo et al. (1991) reported an overestimation of 1.45 and 1.10–1.44, respectively, using salivary gland CSP ELISA. More studies have compared microscopic examination to head–thorax CSP ELISA. As expected, the ELISA overestimated the true infection rate, detecting the CSP 2–3 d before the sporozoites had reached the salivary glands (Vaughan et al. 1992). Overestimation rates recorded in the literature varied from 1.1 to 1.9 (Boudin et al. 1988, Robert et al. 1988, Beier et al. 1990, Adungo et al. 1991, Ferreira and Ferreira 1993, Sokhna et al. 1998). In our study, this overall overestimation, which represents the difference between the rates of infected and infective mosquitoes, was 1.12. The high sensitivity and specificity of ELISA justify the use of this technique in our study of malaria transmission in a village of Central Africa.

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