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DETECTION OF FALCIPARUM MALARIAL FORMS IN NATURALLY INFECTED ANOPHELINES IN CAMEROON USING A FLUORESCENT ANTI—25-kD MONOCLONAL ANTIBODY

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Abstract. *Anopheles gambiae* s.s. and *An. funestus* were sampled in houses located in a *Plasmodium falciparum*–holoendemic site in southern Cameroon. The midguts of female mosquitoes in half-gravid or gravid stages of blood digestion were incubated with a fluorescent monoclonal antibody directed against the *P. falciparum* zygote/ookinete surface protein Pfs25 and examined using a fluorescent light microscope. Malarial forms were detected in 11.6% of the half-gravid mosquitoes and in 0.0% of the gravid ones ($P = 0.012$). No difference in infections or the occurrence of malarial forms between *An. gambiae* and *An. funestus* was observed. Overall, 127 malarial forms were counted and distributed among round forms, retorts, and ookinetes in 77.2%, 9.5%, and 13.4%, respectively. Round forms include macrogametes, activating microgametocytes, and zygotes. The mean number of malarial forms per infected midgut was 2.16 and the maximum number observed was 13. In four anophelines, round forms, retorts, and ookinetes were simultaneously observed. Sporozoite rates were 5.7% for *An. gambiae* and 3.8% for *An. funestus*. In the human population, the gametocyte index for *P. falciparum* was 38% with a mean density of 1.11 gametocytes per microliter of blood. Differences concerning malarial forms in mosquito midguts were observed between houses (range percentage = 4.7—21.3%; mean range of forms per positive anopheline = 1.1—3.1). In each house, relationships existed between infected vectors and the gametocyte reservoir of their inhabitants. The role in transmission of people with very low gametocytemia, approximately one per microliter, as a reservoir of falciparum malaria in highly endemic areas, is emphasized.

When ingested by the appropriate mosquito, mature gametocytes of *Plasmodium falciparum* in the lumen of the mosquito midgut rapidly escape from the erythrocyte to form gametes, then zygotes. Both macrogametes and zygotes appear spherical, approximately 12 μm in diameter. The zygote protrudes into a retort, considered by some investigators as an intermediate stage between the zygote and the ookinete.[1] The elongated ookinetes (approximately 20 μm in length) reach the epithelium of the midgut, penetrate this layer, and rest on the external surface, where they round up into young oocysts.[2] Recently, Beier and others have presented the first field study on ookinetes in anopheline mosquitoes using a classic Giemsa staining for ookinete detection.[3] In the early stages of parasitic development in the mosquito, namely the female gamete, retort, and ookinete, a 25-kD protein is expressed on the parasite surface. A fluorescent-labeled monoclonal antibody against this neoantigen has been developed and was used in the observation of *P. falciparum* preoocyst stages. Its transmission blocking properties have been evaluated.[4]

The present study examined malarial forms in midguts of anopheline populations from southern Cameroon. The aim was to detect the presence of malarial forms using a fluorescent method and to relate these observations with the anopheline species, the mosquito stages of blood digestion, the presence of sporozoites, and the mean number of gametocytes ingested.

This study is part of a research program on malaria transmission around the major river of southern Cameroon that has been underway since 1989. After preliminary studies along the Sanaga river, the region of Mbebe-Kikot was chosen. *Anopheles gambiae* s.s. was the only species of the *An. gambiae* complex observed in this region.[5] In the Ndonzengue hamlet, the main entomologic parameters were observed during the dry season in 1992, just one year before the survey presented in this report. The biting cycle of *An. gambiae* and *An. funestus* was observed (Figure 1); the median was between 2:00 AM and 3:00 AM for *An. gambiae* and between 3:00 AM and 4:00 AM for *An. funestus*. Transmission was year-round, with about 200 infected bites/human/year. This transmission was due mainly to *An. nili* in all seasons, and to *An. gambiae* in the dry season when breeding sites are provided by the Sanaga river at its lower level;[6] the role of *An. funestus* was less important. The rate of in vivo chloroquine drug resistance was 10—21% at Mbebe-Kikot, mainly at the RI and/or RII levels.[7]

MATERIALS AND METHODS

Mosquitoes were collected weekly from 7:00 AM to 9:00 AM in bedrooms of four houses in the Ndonzengue hamlet by pyrethrum spray catches. Fifteen collections were done on Wednesdays from January 6 to April 14, 1993 during the dry season.

Anopheles gambiae and *An. funestus* were placed in an isotherm box at 3–7°C and brought by road to the OCEAC laboratory. Samples of the two anopheline species at half-gravid (with the posterior limit of the midgut between abdominal tergits 1 and 2), subgravid (with the midgut not visible at the dorsal side), or gravid blood-feeding stages were selected at each house. Half-gravid mosquitoes had taken their blood meal during the same night, and subgravid or gravid ones had taken theirs during the preceding night.

Mosquito midguts were dissected in saline from noon to 2:00 PM. Midguts were placed in vials with 20 μl of 1%

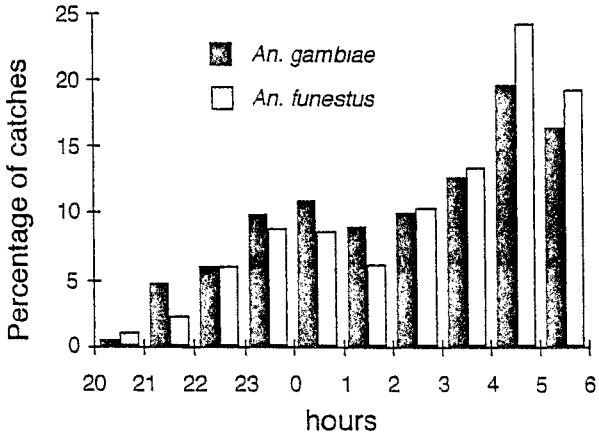

FIGURE 1. Variations in biting rates of *Anopheles gambiae* and *An. funestus* observed with human landing catches in Ndonzengue in southern Cameroon (March–June 1992).

fluorescein isothiocyanate (FITC)–labeled anti–25 kD monoclonal antibody in 0.025% Evans blue. They were homogenized using a pipette. The suspension containing midgut contents, tissue fragments, and FITC was incubated for 30 min at room temperature, then washed with 1 ml of phosphate-buffered saline (PBS, pH 7.2). After the suspension was centrifuged at 5,000 × *g* for 2 min, the pellet was homogenized in 10 μl of PBS. The mixture was mounted between a microslide and cover glass; desiccation was avoided by sealing the periphery of the cover glass with vaseline. The entire blood mixture was examined at 500× (oil immersion lens) with an incident fluorescent light microscope, a procedure that required approximately 15 min per slide.

Mosquitoes processed for preoocyst stage detection were also examined microscopically for the presence of sporozoites in the salivary glands. A sample of half-gravid anophelines not processed for detection of malarial forms was analyzed for the presence of human blood using a dot-enzyme-linked immunosorbent assay in a dipstick, as described by Savage and others;[8] mosquitoes were sampled during five different catches.

Thick blood smears of inhabitants of the four houses where mosquito collecting was conducted were performed on February 10 and on March 24, 1993. The blood thick smears were immediately dried using a microwave oven,[4] which preserves *P. falciparum* gametocyte morphology and avoids confusion with other stages of any *Plasmodium* species such as *P. malariae* trophozoites (unpublished data). Preparations were stained without fixation with a 4% Giemsa solution for 30 min. Examination at 1,000× (oil immersion lens) for asexual stages of *P. falciparum* and for *Plasmodium* other than *P. falciparum* was based on 2,000 leukocytes,

TABLE 1

Blood-feeding status of anophelines collected indoors by pyrethrum spray catches, in Ndonzengue, southern Cameroon (January 1993–April 1993)

Species	Unfed	Freshly fed	Half-gravid	Subgravid and gravid	Total
An. gambiae	10	1	935	54	1,000
An. funestus	2	1	183	73	259

TABLE 2

Repartition of anophelines with Plasmodium falciparum *forms in their midguts, according to their blood-feeding status*

		Half-gravid			Subgravid and gravid	
	No. examined	No. examined	No. positive	%	No. examined	No. positive
An. gambiae	440	421	48	11.4	19	0
An. funestus	144	113	14	12.4	31	0
Total	584	534	62	11.6	50	0

which corresponded to approximately to 0.25 μl of blood. For gametocytes of *P. falciparum*, examination took 45 min, corresponding to approximately 2.0 μl of blood; this was much longer than in routine epidemiologic surveys and served to trace low gametocytemias at the order of 1/μl.

Statistical analysis was performed using the chi-square test, when applicable, and the Fisher's exact test.

RESULTS

Most of the anophelines caught indoors were in the half-gravid stage (93.5% of *An. gambiae* and 70.6% of *An. funestus*). The difference was significant (χ^2 = 107.9, degrees of freedom [df] = 1; $P < 0.0001$), corresponding to a higher endophily for *An. funestus* (Table 1). Preoocyst stages were detected in 11.6% of 534 half-gravid anophelines examined. No malarial forms were observed in 50 subgravid and gravid anophelines (χ^2 = 6.49, df = 1; P = 0.012) (Table 2). There were no differences in the frequency of infections between *An. gambiae* and *An. funestus* (for half-gravid alone: χ^2 = 0.08, df = 1; P = 0.77).

Round forms, retorts, and ookinetes were observed at the respective frequencies of 77.2%, 9.5%, and 13.4% (Table 3). One round form was observed with two mobile flagella. Round forms or ookinetes were observed alone, but retorts were always associated with another malarial form (Table 4). No differences in the frequency of distinct forms or in number of these forms were observed between *An. gambiae* and *An. funestus*. The mean number of malarial forms per infected anopheline was 2.16 (Table 5); the maximum observed value was 13 (Figure 2). The presence of human blood in mosquitoes was verified in 100% of the tested mosquitoes (n = 82 for *An. gambiae* and n = 5 for *An. funestus*).

Overall, 28 of 490 *An. gambiae* and six of 157 *An. funestus* showed sporozoites in the salivary glands, yielding sporozoite rates of 5.71% and 3.82%, respectively (χ^2 = 0.85, df = 1; P = 0.35). The ratios of sporozoite to preoocyst forms were 0.49 (5.7:11.6) for half-gravid *An. gambiae* and 0.33 (3.8:11.6) for half-gravid *An. funestus*. There was no

TABLE 3

Anopheline mosquitoes with observed Plasmodium falciparum *in their midguts**

	Round form	Retort	Ookinete	Total
An. gambiae	82 (77)	11 (10)	14 (13)	107 (100)
An. funestus	16 (80)	1 (5)	3 (15)	20 (100)
Total	98 (77.2)	12 (9.5)	17 (13.4)	127 (100.0)

* Values are the no. (%).

TABLE 4

Plasmodium falciparum *forms observed in midguts of half-gravid anophelines*

	No. examined	Round forms	Round forms plus retorts	Re-torts	Retorts plus ookin-etes	Ookin-etes	Round forms plus retorts plus ookin-etes	Total
An. gambiae	421	34	3	0	0	7	4	48
An. funestus	113	10	1	0	0	3	0	14

association between the presence of sporozoites in the salivary glands and the detection of malarial forms in the midgut for either *An. gambiae* or *An. funestus* ($P = 0.51$ and $P = 0.11$, respectively, by Fisher's exact test).

Parasitologic surveys covered all residents of the four houses: 31 residents in February and 35 in March; 30 were present at both surveys. In the three age groups (0—5 years of age, 6—19 years of age, and 20 years of age and older), the respective numbers of individuals in each group were 19, 15, and 32. Results were very constant during the two surveys; thus they were presented together. Plasmodial indices were typical of a highly endemic area (Table 5) for both *P. falciparum* and *P. malariae* (Figure 3). Gametocytes of *P. falciparum* were observed in 38% of the inhabitants with a geometric mean of 1.11/μl of blood (range = 0.5—7). House no. 3 had the three highest values: 1) the proportion of gametocyte carriers of their inhabitants, 2) the percentage of anophelines with malarial forms, and 3) the number of malarial forms per positive anopheline.

DISCUSSION

This is the first study to detect and quantify *P. falciparum* malarial forms in mosquito midguts using an immunofluorescent technique in naturally-infected anopheline vectors. Round forms, retorts, and ookinetes were readily detected and discriminated in midgut preparations from mosquitoes that had fed on humans one night previously.

Observed round forms can be either 1) activated macrogametes, 2) activating microgametocytes, including exflagellation, and/or 3) zygotes. On the other hand, retorts and ookinetes correspond to a single stage.

TABLE 5

Plasmodium falciparum *forms observed in midguts of half-gravid* Anopheles gambiae *and* An. funestus *in different houses and data on* P. falciparum *gametocytemia of inhabitants*

	House				Total or mean
	1	2	3	4	
No. of anophelines	148	104	122	85	459
No. of anophelines parasite-positive	13	8	26	4	51
% anophelines parasite-positive	8.8	7.7	21.3	4.7	11.1
Mean of preoocyst forms/parasite-positive anopheline	1.1	1.1	3.1	1.5	2.16
No. of inhabitants*	6, 9	8, 7	11, 12	6, 7	31, 35
No. of gametocyte carriers†	2.0	3.0	6.0	1.5	12.5
Geometric mean no. of gametocytes/mm³/inhabitant with gametocytes†	1.45	0.95	1.25	0.63	1.11

* The first number was obtained in February 1993 and the second in March 1993.
† Values are the means of two surveys.

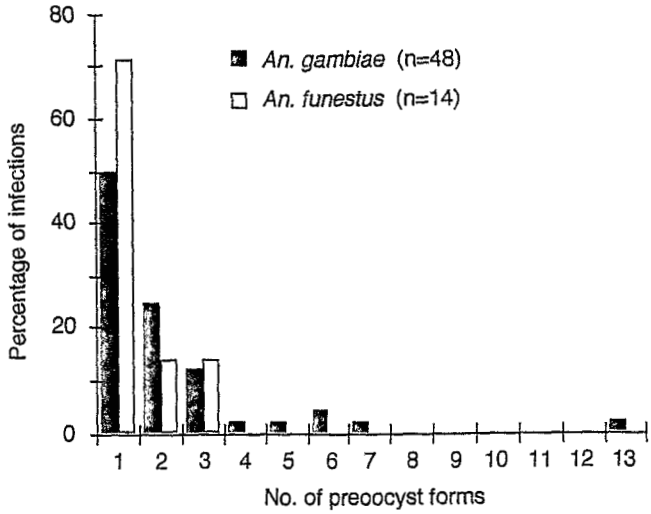

FIGURE 2. Frequency distributions of *Plasmodium falciparum* forms detected with an immunofluorescent method in the midguts of *Anopheles gambiae* and *An. funestus* inside houses in Ndonzengue in southern Cameroon.

The prevalence of malarial forms in anopheline midguts in an area holoendemic for *P. falciparum* indicated that 11.6% of human blood meals contained *P. falciparum* gametocytes that had undergone development in anophelines. Retorts and ookinetes were detected in 3.4% (18 of 534) of half-gravid anophelines and the density of forms per mosquito midgut was 2.16. These results are similar to those of Beier and others,[3] who observed 4.4% of the anophelines with ookinetes and 2.2 ookinetes per mosquito. However, our results and those of Beier and others are difficult to compare because the success of transformation from round forms to ookinetes is unknown. Nevertheless, such low densities of malarial forms associated with such consequential sporozoite rates suggest in the field a high efficiency of the falciparum parasite development within the mosquito. This observation contrasts greatly with experimental infections using cultured *P. falciparum* gametocytes, in which such low

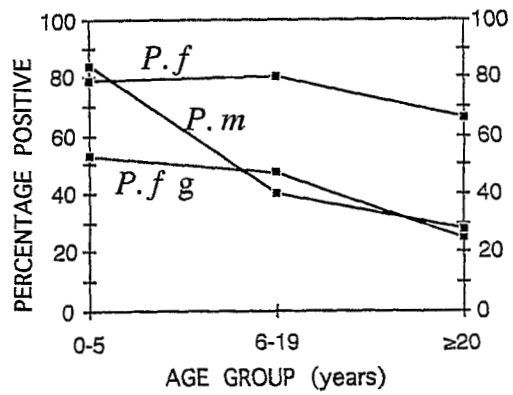

FIGURE 3. Percentage of thick blood smears (n = 66) in each age group positive for *Plasmodium falciparum* (*P. f*), *P. malariae* (*P. m*), and gametocytes of *P. falciparum* (*P. f* g).

densities of ookinetes would not be expected to yield sporozoite infections.[10]

Assuming that normal development of malarial forms was stopped after the catches of mosquitoes, our sample of half-gravid anophelines ranged from 2-hr to 12-hr postfeeding. That is to say, the first ookinetes appear between 12 and 15 hr. No forms were observed in mosquito midguts more than 26-hr postfeeding, at a subgravid or gravid stage. On this point, further investigations must be undertaken: our results differ from those of Beier and others, who observed ookinetes 20—40-hr postfeeding with highest densities at 31 hr. The absence of retorts observed alone is probably due to their short life span; the kinetics of the transformation from round forms to ookinetes needs to be studied further.

The technique described in this study is very useful; the only sophisticated apparatus required is a fluorescent light microscope. Reducing the microscopic examination time for each preparation (15 min in our procedure) would permit an increase number of mosquitoes to be examined.

We attempted to observe the relationships between anopheline infection and gametocyte carriers by house. The results confirm the importance of low gametocytemias of approximately one gametocyte/μl as a natural reservoir of malaria infection.[11] It must be emphasized that such low gametocytemias are normally undetected in routine examinations of thick blood films.

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