Journal of the American Mosquito Control Association, 10(3):419–422, 1994

IMMUNODETECTION OF *PLASMODIUM FALCIPARUM* ZYGOTES AND OOKINETES IN *ANOPHELES* BLOOD MEALS

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ABSTRACT. An indirect fluorescent antibody assay (IFA) using monoclonal antibodies to the 25-kDa *Plasmodium falciparum* ookinete surface antigen was developed to detect and quantify preoocyst stages of *P. falciparum* in mosquito blood meals. This IFA is suitable for the detection and quantitation of zygotes/macrogametes, retorts (immature ookinetes), and ookinetes. Time-course evaluations showed that zygotes represented >52% of the parasites detected between 6 and 24 h. From 24 to 36 h similar densities of all 3 stages were observed. By 42 h, 56% of the detectable parasites were ookinetes. Compared to the hemocytometer the IFA provided a better estimate of stage-specific infection rates and densities. The assay is a useful new tool for evaluating preoocyst stages in laboratory-infected mosquitoes and has the potential for detecting natural infections in vector populations.

INTRODUCTION

Findings that the ookinete is the key transitional stage in *Plasmodium falciparum* sporogony emphasize that ookinete densities are key determinants of sporozoite rates in anopheline mosquitoes (Beier et al. 1992; Vaughan et al. 1992, 1994). Although a technique for examining ookinetes in mosquito blood meals was described 60 years ago (James 1934), few studies have investigated quantitative relationships between the various preoocyst stages. Conventionally, the oocyst has been used as the first indication of sporogonic development in individual mosquitoes. In part this is due to the lack of suitable methods for detecting earlier sporogonic stages. Attempts to isolate ookinetes from mosquitoes have yielded preparations that are considerably contaminated with mosquito tissue and red blood cells (Weiss and Vanderberg 1976).

Recent studies have delineated methods for estimating absolute ookinete densities in individual experimentally infected (Vaughan et al. 1991) and naturally infected (Beier et al. 1992) mosquitoes. However, each method has technical limitations that compromise the accurate detection of low-grade infections. The objective of this study was to develop better methods for detecting and quantifying early stages of *P. falciparum* in mosquitoes.

MATERIALS AND METHODS

Three colonized *Anopheles* species, reared at 28°C and 70% RH, were used in this study. These included *Anopheles albimanus* Wiedemann (Panama 2 strain), *Anopheles freeborni* Aitken (Marysville strain; original F-1 strain isolated in 1944), and *Anopheles gambiae* Giles (G3 strain).

Plasmodium falciparum (NF54 strain) gametocytes were cultured *in vitro* using modifications of the methods of Ponnudurai et al. (1982). Three- to 8-day-old mosquitoes were allowed to feed on gametocyte cultures (diluted 1:8 with a 1:1 mixture of heat inactivated human sera : washed human red cells) contained in water-jacketed membrane feeders at 37°C. Fully engorged mosquitoes were provided with 5% Karo (corn syrup) solution and were held at 27°C and 70% RH.

At selected time intervals, midguts dissected in phosphate buffered saline (PBS) (pH 7.4) were homogenized in 20 µl of 3% acetic acid to lyse the red blood cells. Ten microliters of the suspension were placed in a hemocytometer chamber after which parasites in 4 0.1-µl corners were quantified under phase-contrast microscopy (400×) (i.e., the theoretical limit of resolution for this method is 50 parasites per midgut). For each mosquito, ookinete counts obtained with the hemocytometer were multiplied by 50 (to account for total sample volume) to provide an estimate of absolute ookinete density.

For evaluation by the indirect fluorescent antibody (IFA) method, the residual suspension (after loading the hemocytometer) was diluted with 20 µl Tris-acetic acid, spotted (6 µl/well) on poly L-lysine coated microwell slides, and allowed to air dry. Slides not assayed immediately were wrapped in aluminum foil and held at −70°C. Prior to assay, reagents and samples were allowed to attain room temperature. Then, slides were held in PBS (pH 7.4) for 5 min. Monoclonal antibodies to the *P. falciparum* 25 kDa ookinete surface antigen (Vermeulen et al. 1985) were diluted 1:200 in PBS. A 20-µl aliquot was placed on each well and the slide was incubated in a

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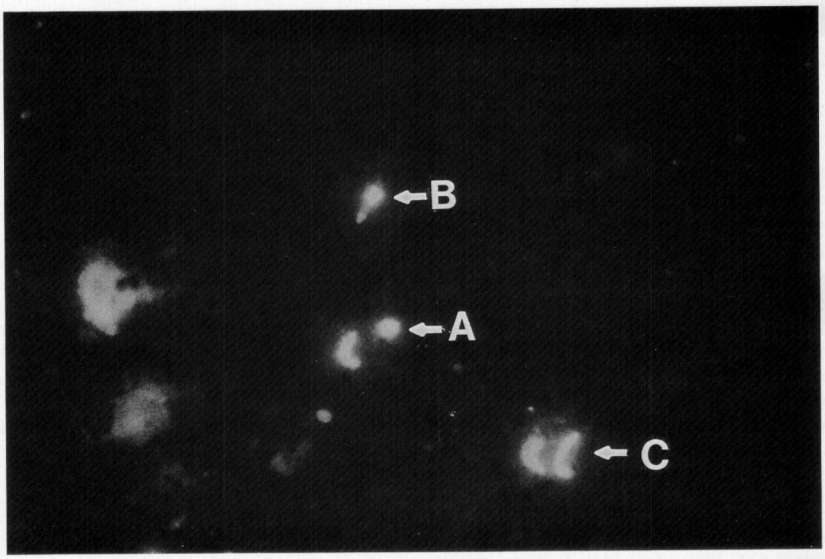

Fig. 1. Immunofluorescence of *Plasmodium falciparum* zygotes/macrogametes (A), retorts (B), and ookinetes (C) isolated from midguts of *Anopheles albimanus* (fluorescent microscopy ×400).

moist chamber at room temperature for 60 min. Following 2 washes in PBS, 20 μl of fluorescein conjugated goat antimouse IgG (Kirkegaard & Perry Inc., Gaithersburg, MD) diluted 1:50 in PBS was added and the slide was held for 60 min after which it was washed 2 times in PBS. After drying, it was examined by fluorescent microscopy. The theoretical limit of resolution for this method is 2 parasites per mosquito. Uninfected bloodfed mosquitoes, cultured gametocytes spotted and dried at 37°C, and parasites that had been allowed to undergo exflagellation served as controls.

A time-course experiment was conducted to examine the developmental kinetics of *P. falciparum* in *An. gambiae*. For each selected time point (6, 12, 18, 24, 30, 36, 42 h postinfection), midguts were dissected from 6 mosquitoes and parasites enumerated by the IFA technique described above.

Prior to conducting the time-course experiment, preliminary experiments were performed to standardize the IFA technique for quantifying ookinetes in the blood meal. Ookinete densities obtained by the IFA were compared with ookinete densities obtained using phase-contrast microscopy for a series of individual infected mosquitoes. During these experiments, Tris-acetic acid (100 mM Tris [hydroxymethyl] aminomethane containing 0.4% [v/v] acetic acid [$C_2H_4O_2$]), pH 7.4, was found to be a better fixative for maintaining parasite integrity than 3% acetic acid. Data obtained by the 2 methods were log transformed and statistically analyzed using SPSS (SPSS Inc., Chicago, IL).

RESULTS

By fluorescent microscopic inspection of infected midgut homogenates it was possible to distinguish 3 sporogonic life stages of the malaria parasite (Fig. 1). Due to their highly intense fluorescence, zygotes and retorts were more readily detectable than ookinetes, which exhibited less intense fluorescence.

The kinetics of parasite development were monitored using the IFA (Fig. 2). Zygotes, retorts and ookinetes were detected initially at 6, 12, and 18 h, respectively. Zygotes were detectable as late as 42 h. Zygotes represented >50% of recoverable parasites from 6 to 24 h. At 30 and 36 h similar densities of all 3 stages were observed. At 42 h, 56% of the detectable parasites were ookinetes. Peak densities of ookinetes at 24 h, a geometric mean of 123 per mosquito, were 7 times lower than peak zygote densities at 6 h, a geometric mean of 907 per mosquito.

Retorts and/or ookinetes were detectable by either method in 84% (91/108) of the mosquitoes examined. There was a significant relationship between ookinete densities obtained using the hemocytometer and those obtained by the IFA for individual mosquitoes ($r = 0.62$, df = 107, $P < 0.0001$). Retort densities obtained by hemocytometer were positively associated with retort densities obtained by IFA ($r = 0.40$, df = 107,

$P < 0.0001$). The correlation coefficient for the association between hemocytometer retorts and IFA retorts ($r = 0.40$) was significantly lower ($t = 11.51$, df = 1, $P < 0.05$) than that for ookinetes ($r = 0.62$).

Table 1 presents a comparison of the test results, using both methods, for a series of 91 infected mosquitoes. The hemocytometer method yielded 1.3 times higher estimates of retort densities than the IFA method ($t = 6.1$, df = 90, $P < 0.001$). The hemocytometer method underestimated both the retort rate and the ookinete rate by *ca.* 30%. Estimates of ookinete densities obtained by the hemocytometer were 3.1 times higher than estimates obtained by the IFA ($t = 2.38$, df = 90, $P < 0.05$).

DISCUSSION

An indirect immunofluorescence assay suitable for detecting and quantifying *P. falciparum* macrogametes/zygotes, immature ookinetes, and mature ookinetes was developed. Ookinete infections in individual mosquitoes can be determined by phase-contrast examination of acetic acid-lysed blood meals loaded in hemocytometers (Vaughan et al. 1991) or by oil immersion examination of Giemsa-stained midgut homogenates (Beier et al. 1992). The former method demands immediate sample evaluation and the latter yields preparations with substantial cellular debris. Neither method is suitable for detecting zygotes. The assay described in this study is easy to perform and can be completed in about 2 h, and fluorescent microscopic examination for each sample requires about 10 min.

The correlation coefficient for the association between hemocytometer retorts and IFA retorts ($r = 0.40$) is lower than that for ookinetes ($r = 0.62$). This reflects the fact that under phase-contrast illumination retorts are harder to see than are ookinetes. Relative to the hemocytometer method, the IFA yields a better estimate of stage-specific infection rates and densities. The hemocytometer underestimated both the retort rate and the ookinete rate by *ca.* 30% and overestimated ookinete densities 3-fold. One possible reason for this discrepancy is that whereas one-half of the whole blood meal was assayed by the IFA technique only 1/50 of the blood meal was assayed by the hemocytometer technique.

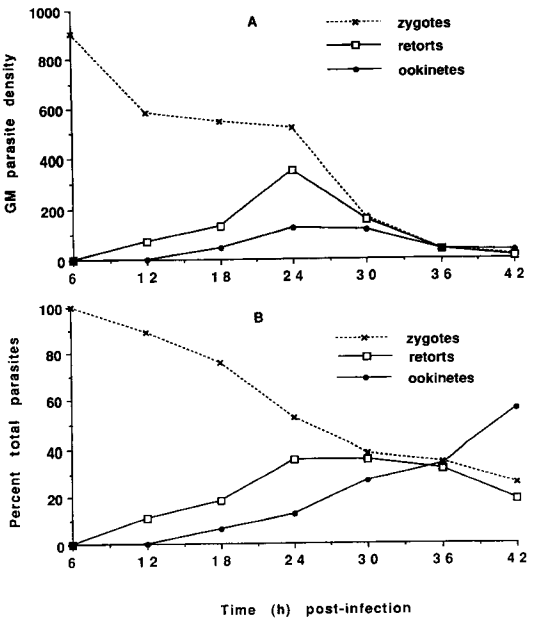

Fig. 2. Developmental kinetics of *Plasmodium falciparum* ookinetes (A) and percentage of zygotes/macrogametes, retorts, and ookinetes (B) at time intervals postinfection for experimentally infected *Anopheles gambiae*. Each time point represents the mean for 6 mosquitoes.

About 4% (4/91) of ookinete-infected mosquitoes were fluorescent negative. This is a negligible proportion but it shows that the assay may fail to detect infections when parasite densities are very low.

The IFA facilitates the monitoring of quantitative relationships between various preoocyst stages. Time-course analysis of the percentage of each parasite stage suggests that substantial proportions of zygotes and retorts fail to develop

Table 1. Comparison of the hemocytometer method and the indirect immunofluorescent antibody assay (IFA) for *Plasmodium falciparum* retort and ookinete detection in a sample of 91 anopheline mosquitoes that were positive by either method.

	Retorts		Ookinetes		Retorts and ookinetes	
	No. positive (%)	Geometric mean	No. positive (%)	Geometric mean	No. positive (%)	Geometric mean
Hemocytometer	57 (62.6)	152.6	49 (53.8)	231.0	65 (71.4)	286.0
IFA	87 (95.6)	120.8	74 (81.3)	73.4	87 (95.6)	192.4

into retorts or ookinetes, respectively. The 7-fold decrease in peak parasite densities between zygotes and ookinetes is within the range observed for macrogametocytes in earlier studies (Vaughan et al. 1992).

The IFA has potential for delayed analysis of field-collected specimens. Although in this study all samples were kept frozen, preliminary studies indicate that after parasites have been spotted on the microwell slides they can be held at room temperature before or after assay for several months without loss of immunoreactivity or fluorescence, respectively.

Immunological methods have been used to detect sporozoites in mosquito tissues but no assay is available for earlier stages. Enzyme-linked immunosorbent assays (ELISAs) are useful tools for detecting sporozoite infections but would be inadequate for detecting earlier sporogonic stages such as ookinetes because: 1) unlike sporozoites, surface proteins of ookinetes do not possess repeat regions (Kaslow et al. 1988), and 2) in nature, these stages occur at low densities (Beier et al. 1992). We have demonstrated the feasibility of using antibody-based IFA methods to detect ookinete infections in individual mosquitoes. Corresponding studies should be conducted to assess early stages of *P. falciparum* in naturally infected vectors.

ACKNOWLEDGMENTS

We thank David C. Kaslow, National Institutes of Health, Bethesda, MD, for donating the anti-*Plasmodium falciparum* 25 kDa ookinete surface protein (Pfs 25) monoclonal antibodies. This research was supported by National Institutes of Health grant AI-29000. G.M.M. Chege was supported by a fellowship from the Kenya Government/United States Agency for International Development *Training For Development* (TFD) program.

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