

Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes

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Abstract

Human infectiousness to mosquitoes can be estimated by 2 tests: direct feeding on the skin and membrane feeding on venous blood. To validate the membrane feeding assay, the infectiousness of *Plasmodium falciparum* gametocyte carriers to *Anopheles gambiae* was estimated by these 2 methods in the same individuals in a rural area of Cameroon. Results from 37 experiments showed that direct feeding gave significantly higher infection rates than membrane feeding. We observed an average of 19.4% infected mosquitoes by direct feeding compared with 12.1% by membrane feeding, and a mean oocyst load of 5.63 by direct feeding compared with 2.65 by membrane feeding. However, there was a very good concordance between the 2 tests: 84.3% with the Kappa test on percentages of infected mosquitoes and 98.7% with the interclass correlation coefficient on oocyst loads. In addition, we found a good linear correlation between the 2 methods.

Keywords: malaria, *Plasmodium falciparum*, *Anopheles gambiae*, infectiousness, membrane feeding, direct feeding, Cameroon

Introduction

Estimation of infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes is crucial for comparing and understanding different epidemiological situations of malaria. It is also essential for evaluation of the efficacy of control measures such as future transmission-blocking vaccines (KASLOW, 1993; ROEFFEN *et al.*, 1995).

Determination of gametocyte density in blood smears does not adequately predict the infectiousness of individuals to mosquitoes (JEFFERY & EYLES, 1955; MUIRHEAD-THOMSON, 1957; CARTER & GWADZ, 1980; CARTER & GRAVES, 1988; BOUDIN *et al.*, 1993). Alternatively, infectiousness can be assessed by feeding mosquitoes with patients' blood and recording the rate of infection in mosquitoes. The direct feeding method developed by MUIRHEAD-THOMSON (1957) seems to reflect epidemiological reality (GITHEKO *et al.*, 1992) but is not well perceived by the human population and could be replaced by another technique such as membrane feeding. Infection of mosquitoes through animal membranes has been reported previously by several authors (YOELI, 1938; BISHOP & GILCHRIST, 1946; COLLINS *et al.*, 1964). In 1964, RUTLEDGE *et al.* developed a feeding method using an artificial membrane of cellophane. Since then, this membrane feeding technique has been widely used (GRAVES, 1980; GRAVES *et al.*, 1988; PONNUDURAI *et al.*, 1989; BOUDIN *et al.*, 1993; TCHUINKAM *et al.*, 1993; LENSEN *et al.*, 1996), but without any comparison with the direct feeding method as a tool for assessing infectiousness. Such a comparison is of great importance because of the more common use of this assay as a tool for the study of malaria transmission-blocking activity.

The aim of the present study was to compare mosquito infection rates obtained by membrane and direct feeding on the same individuals in Cameroon. We then evaluated the concordance between the 2 methods to determine the extent to which they could be used as tools for evaluating natural or induced modifications of human-to-mosquito transmission. Finally, we established a linear correlation between results obtained with the 2 methods to allow the calculation of theoretical results for one of the techniques, using observed data obtained by the other, and also a comparison of studies carried out by either method.

Subjects, Materials and Methods

Study area

The study was carried out in the district of Mengang, 100 km east of Yaoundé (Cameroon, 3.9° N, 12.06° E). It is located in a hilly, degraded rain forest area, where annual malaria transmission is around 100 infective bites per human being (J.-Y. Meunier, unpublished data), with a peak at the beginning of the short rainy season (March-April). *P. falciparum* is the dominant parasite species, followed by *P. malariae* and *P. ovale*.

Mosquitoes

A local strain of *Anopheles gambiae*, reared at 25°C with 80% humidity in our insectarium in Yaoundé, was used. This strain was adapted to feeding on a membrane feeder (TCHUINKAM *et al.*, 1993). Mosquitoes were fed daily with sucrose. For experimental feedings, batches of 30 three-day-old females were starved from sucrose 12 h before infection and placed in plastic boxes covered with a net.

Gametocyte carriers

Individuals who participated in the survey were volunteers and recruited during cross-sectional studies in different villages of the district. For children, parents' consent was asked and neither direct feeding nor venepuncture was made on children aged <5 years. The project was approved by the National Ethical Clearance Committee for Cameroon. For each individual, thick blood smears were prepared and stained with 10% Giemsa in distilled water. Trophozoite and gametocyte densities were determined by microscopy analysis (×100 objective under oil immersion) with a threshold of 6 and 2 parasites per microlitre, respectively. For individuals infected with asexual malaria parasites, amodiaquine treatment was provided (30 mg/kg for 3 days). Experimental infections of mosquitoes were made using individuals with gametocytes. In addition, some experimental infections were performed with apparent non-gametocyte carriers as a control and to define the threshold of infectiousness.

Direct and membrane feeding assays

Infectivity of each selected individual to laboratory-bred mosquitoes was studied by both direct and membrane feeding assays. Mosquito feeding was performed in a dark room of a local field dispensary, during the morning. For direct feeding, boxes containing mosquitoes were applied on the inside thigh of the patient. Mosquitoes were allowed to feed during 15 min. After feeding, an anti-histamine cream was put on the skin. For membrane feeding, lithium heparinized vacutainer tubes

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were prewarmed at 37°C before use and filled with 4 mL of blood quickly drawn by venepuncture. Immediately after bleeding, 2 mL of blood was put into a 5-cm-diameter glass feeder closed with a slightly stretched Parafilm® membrane as described by RUTLEDGE *et al.* (1964). In order to prevent exflagellation of gametocytes, constant temperature (37°C) was maintained by a water-jacket circulation system. Boxes containing mosquitoes were arranged under the feeding apparatus and the blood was presented to the mosquitoes for 15 min.

After infection, mosquitoes were brought back to the insectarium in Yaoundé, transferred to breeding cages and unfed mosquitoes were discarded. Gorged mosquitoes were maintained in the insectarium with a daily sucrose supply until they were dissected on day 7 after feeding. Midguts were dissected in a drop of 3% mercurochrome in phosphate-buffered saline and oocysts were scored by microscope examination.

Statistical analysis

Only experiments with more than 20 surviving mosquitoes at day 7 were included and analysed. Two parameters were chosen: percentage of infected mosquitoes per batch and oocyst load (i.e., the geometric mean oocyst number per midgut in each batch, using only infected midguts). To compare results obtained by the 2 methods of infection, the χ^2 test was applied on percentages of infection and the Wilcoxon test for paired data on oocyst loads. Concordance between the results of membrane and direct feeding was analysed either using the Kappa test (FERMANIAN, 1984a) on qualitative data (percentages of infected mosquitoes) or using the interclass correlation coefficient (FERMANIAN, 1984b) on quantitative data (oocyst loads). To establish a linear correlation between the 2 methods, a simple regression test was applied, on positive values, after log transformation of percentages of infected mosquitoes (considered here as quantitative data) and mean oocyst loads. As additional information, the correlations between gametocyte density and either percentage of infection or oocyst load, after direct and membrane feeding, were evaluated with Spearman's rank correlation test.

Results

Thirty seven individuals were enrolled in the study: 7 apparently without gametocytes and 30 with gametocytaemias ranging from 2 to 856 gametocytes/ μ L of blood. Of the 7 non-gametocyte carriers, 6 gave no infection after both direct and membrane feeding while 1 infected mosquitoes with both tests. Among the feedings on gametocyte carriers, 10 gave negative results with both types of feeding, 17 gave infection with both tests, 2 gave infection only after direct feeding and 1 only after membrane feeding.

The comparison of the results obtained with the 2 methods is presented in the Table. The mean percentage of infected mosquitoes and the mean oocyst load were significantly higher after direct feeding. However, a very good concordance was observed with the Kappa test (84.3% for the percentage of infected mosquitoes) as well as with the interclass correlation coefficient (98.7% for mean oocyst loads) between the results of direct and membrane feeding experiments. This shows that both techniques can be used to evaluate human infectiousness to mosquitoes. In addition, in infectious subjects, there was a significant linear correlation between direct and

membrane feeding, for percentages of infected mosquitoes ($r = 0.84$, $P < 0.0001$) and for oocyst loads ($r = 0.74$, $P < 0.001$). The fit of the linear model was satisfactory for both parameters with $r^2 = 71\%$, and $r^2 = 55\%$ respectively. This result enabled the formulation of 2 linear equations relating the 2 techniques:

$$\log_{10} \text{IMM} = 0.78 \times \log_{10} \text{DIR} - 0.29$$

for percentages of infected mosquitoes and:

$$\log_{10} \text{IMM} = 0.51 \times \log_{10} \text{DIR} + 0.04$$

for oocyst loads, where DIR and IMM represent direct and membrane feeding, respectively (Figure). This linear relationship will permit estimation of results obtained by one technique from those obtained by the other.

As far as the relation between gametocyte density and mosquito infection is concerned, the Spearman's rank coefficients for gametocyte densities and percentages of infected mosquitoes were 0.47 ($P < 0.01$) and 0.51 ($P < 0.01$) for direct and membrane feeding, respectively. Similarly, the rank coefficients for gametocyte densities and oocyst loads were 0.55 ($P < 0.001$) and 0.59 ($P < 0.001$) for direct and membrane feeding, respectively. Thus, the link between gametocyte densities and either percentages of infected mosquitoes or oocyst loads was significant but not sufficient to form any firm conclusions. Indeed, there were exceptions where some medium or heavy gametocytaemias did not lead to infection in mosquitoes whereas some low gametocytaemias (below the detection threshold for example) led to an infection (data not shown).

Discussion

Membrane feeding is one of the available techniques to evaluate malaria transmission and malaria transmission-blocking activity. However, it is crucial to validate this method before using it in the field. One way to validate it is to compare the results obtained by this method with those obtained by direct feeding on individuals (MUIRHEAD-THOMSON, 1957), which is more physiological and therefore reflects the epidemiological reality with more precision.

In 1964 COLLINS *et al.* reported, albeit for only 1 case, higher *P. falciparum* infections in mosquitoes after animal-skin membrane feeding than after a direct feed on human subjects. Since then, several authors have acknowledged that infections in mosquitoes fed through membranes usually equal or exceed infections of mosquitoes fed directly on human skin (GRAVES, 1980; VANDERBERG & GWADZ, 1980). The study presented here is the first comparison of the 2 methods using multiple cases. A detailed analysis showed that direct feeding gave better results in terms of infection than membrane feeding. However, a very good concordance between results obtained by the 2 methods, both for percentages of infected mosquitoes and oocyst loads, was found. In addition, the good correlation obtained between the results of the 2 methods, for both percentages of infected mosquitoes and oocyst loads, enabled formalization of the relationship in terms of linear equations.

In the present work, little emphasis was placed on the highest gametocytaemias. This is explained by the fact that heavy carriers were essentially young children (aged < 2 years) and that neither venepuncture nor direct

Table. Infectivity of 37 individuals with falciparum malaria to *Anopheles gambiae* after direct or membrane feeding infections

	Direct feeding	Membrane feeding	P value
Infected mosquitoes/dissected mosquitoes (%)	199/1025 (19.41%)	131/1082 (12.11%)	<0.0001 ^a
Geometric mean oocyst load	5.63	2.64	<0.01 ^b

^a χ^2 test; ^bWilcoxon test for paired data.

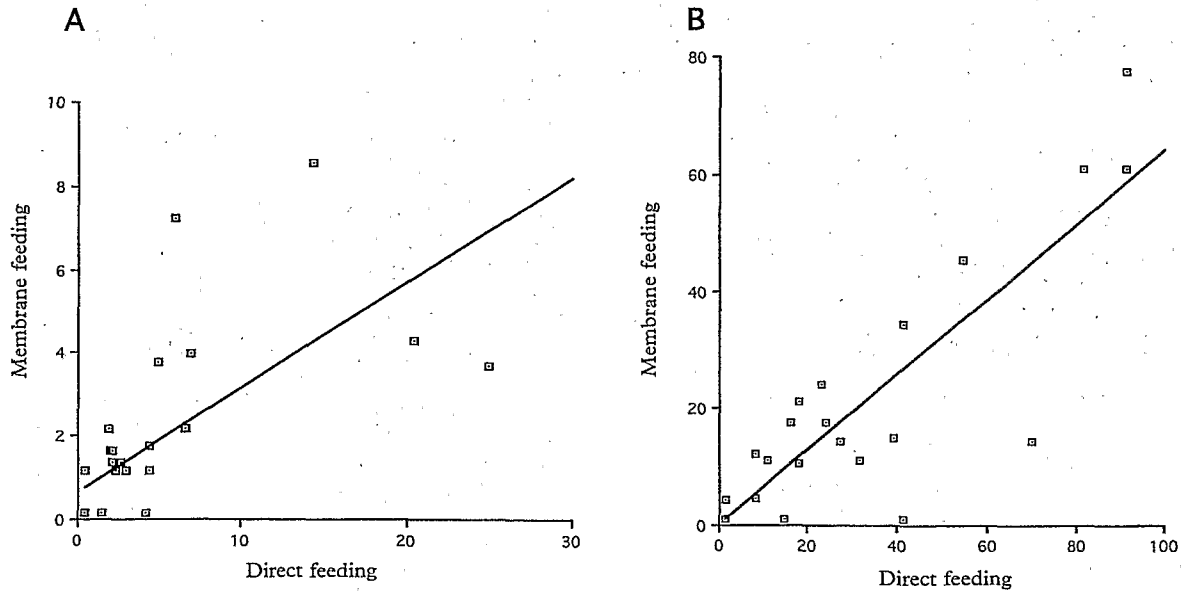


Figure. Graphic representation of a simple regression test between results obtained after direct and membrane feeding experiments with *Anopheles gambiae* and human volunteers with falciparum malaria in Cameroon. (A) Geometric mean oocyst loads, (B) percentages of infected mosquitoes.

feeding could be performed on this age-group. Studies carried out by feeding mosquitoes on individuals of any age exposed to holoendemic malaria indicate that the gametocyte density threshold, for infectiousness to mosquitoes, is remarkably low (CARTER & GWADZ, 1980). In our study, 1 of the 7 apparent non-gametocyte carriers (detection threshold = 2 gametocytes/ μ L) was able to infect mosquitoes by either technique, i. e., direct or membrane feeding. This result suggests that the direct and membrane feeding techniques have similar sensitivities. A weak relationship between gametocyte density and infectiousness of individuals to mosquitoes was observed. Previous studies have produced conflicting results on this subject (JEFFERY & EYLES, 1955; MUIRHEAD-THOMSON, 1957; CONSTANTINESCU & NEGULICI, 1967; GRAVES, 1980; CARTER & GRAVES, 1988; BOUDIN *et al.*, 1993). Like others, we assumed that the prevalence of gametocyte carriers is not a good indication for the infectiousness of a population to mosquitoes.

In conclusion, our results show that the membrane feeding technique seems to simulate adequately the natural feeding process. However, these observations are valid only in the specific epidemiological context of our study. Hence, subject to confirmation of these results in different areas, it may be possible to use the membrane feeding assay to evaluate the infectiousness of a population in the field and to study malaria transmission-blocking activity. Moreover, it might be possible to compare different studies carried out in different epidemiological situations either by direct (MUIRHEAD-THOMSON, 1957; GRAVES *et al.*, 1988; GAMAGE MENDIS *et al.*, 1991; GITHEKO *et al.*, 1992) or by membrane feeding (GRAVES *et al.*, 1988; BOUDIN *et al.*, 1991; BOUDIN *et al.*, 1993) after validation of linear equations in different epidemiological situations. Nevertheless, when using these 2 methods to evaluate transmission, it is essential not to forget that laboratory-adapted insects are used, which could differ from the local mosquito population in their susceptibility to infection.

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Short Report

Infectivity of malaria vector mosquitoes: correlation of positivity between ELISA and PCR–ELISA tests

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Keywords: malaria transmission, anopheline mosquitoes, infectivity, ELISA, PCR–ELISA, Brazil

Studies on the infectivity of malaria vector mosquitoes using the enzyme-linked immunosorbent assay (ELISA) described by WIRTZ *et al.* (1987) have been carried out worldwide for several years. SOMBOON *et al.* (1993) reported false-positive results for the ELISA associated with bovine and swine blood. In order to avoid these false-positive results it is advisable either to use only the anterior part of the mosquito (head and thorax) which, normally, is not contaminated by the ingested animal blood (WIRTZ *et al.*, 1987), or to confirm the ELISA result by another method such as polymerase chain reaction (PCR).

We have carried out the PCR–ELISA to confirm the detection of human malaria parasites in mosquitoes already recorded as positive by ELISA alone. Thirty two such mosquitoes were tested, belonging to different species of the genus *Anopheles* and collected during field trips to different areas of the Amazonia Region (Pará,

Amapá, Rondônia and Roraima States). The same number of mosquitoes known to be negative for human malaria parasites was also tested.

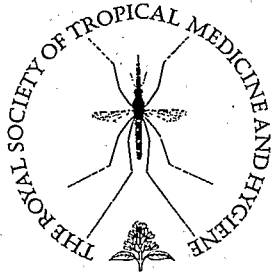
The source of *Plasmodium* DNA was the same material used for the ELISA test [trituration mosquitoes—head and thorax ground in a blocking buffer containing 0.05% nonidet P-40 (WIRTZ *et al.*, 1987)], either 20 µL spotted on a glass fibre membrane (GFM) prepared for PCR, using 1/8 of the spot as DNA source directly into the PCR mixture as described by WARHURST *et al.* (1991), or extracted as follows: 40 µL of ELISA solution was centrifuged for 10 min at 22 500 g, the pellet was lysed by adding 25 µL of lysis buffer and incubating at 65°C for 30 min, afterwards adding potassium acetate and placing on ice for at least 60 min. The isolated DNA (obtained by ethanol precipitation) was dissolved in 15 µL of TE-buffer containing ribonuclease (WILSON *et al.*, 1998). The DNA was amplified as described by MACHADO *et al.* (1998) using primer sequence, concentrations and reaction conditions indicated by OLIVEIRA *et al.* (1995). For the identification of the human malaria parasites we used the liquid-phase, non-isotopic hybridization ELISA technique, following the protocol of OLIVEIRA *et al.* (1995). For negative controls we used distilled water, male anopheline and culicine mosquitoes, and human DNA. Positive controls included strain K1 of *P. falciparum*, and mosquitoes experimentally infected with *P. falciparum* and *P. vivax*.

Our PCR results confirmed the ELISA test results for all positive and all negative mosquitoes, and in 5 (15.6%) of the positive mosquitoes (3 *An. albivittatus* and 2 *An. darlingi*) the PCR–ELISA technique detected other species of human *Plasmodium* that were not found by the ELISA test alone (Table). The DNA source was obtained only by DNA extraction, which means that the GFM technique is not applicable for this type of material. These results indicate that the PCR–ELISA is more sensitive than a simple ELISA test which, however, still remains a very good and useful tool for testing mosquito infectivity.

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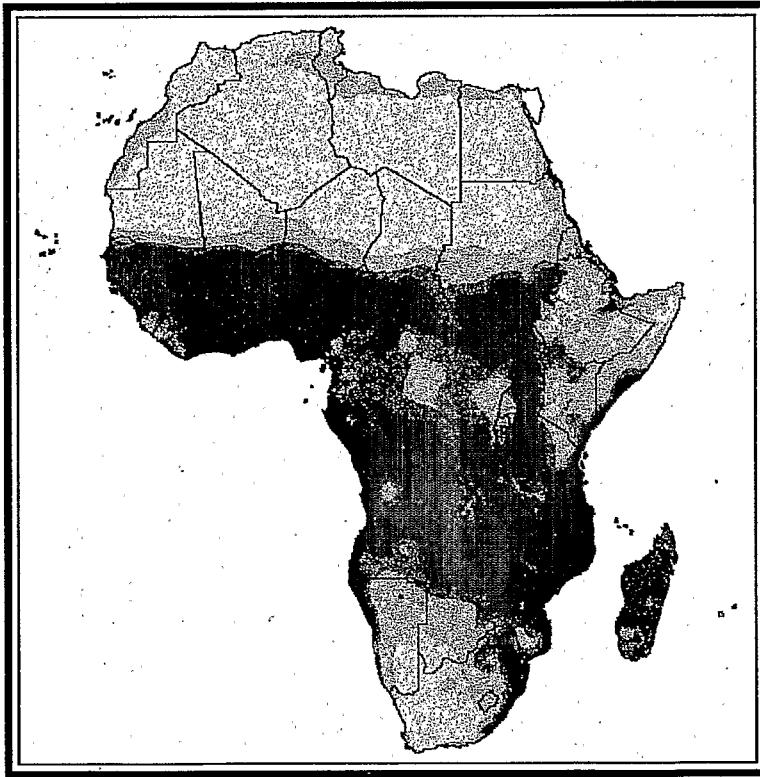
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