

Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes

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Abstract

Factors which could influence the success of experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* were investigated in Cameroon. 139 experimental infections with different gametocyte carriers were performed. 86 (62%) gave rise to mosquito infection after dissection of at least 20 mosquitoes. Among succeeding infections, the mean percentage of infected mosquitoes was 18.6% and mean oocyst load per positive midgut was 2.56. Only gametocyte density was identified as a factor which determined the success and the level of mosquito infection. No significant influence was found for sex and age of the gametocyte carrier, body-temperature, presence of asexual erythrocyte stages, rhesus factor, blood group and use of antimalarial drugs (chloroquine and amodiaquine).

Introduction

Mature gametocytes of *Plasmodium falciparum* first appear in the bloodstream about 10 days after the asexual parasites (Smalley, 1976). Successive generations of circulating gametocytes have an estimated half-life of 2.4 days and the overall infectivity to mosquitoes persists for about 3 weeks (Smalley and Sinden, 1977). The density of gametocytes in *falciparum* malaria is variable (Bruce Chwatt, 1980). In *in vivo* and *in vitro* systems, it has been found to be impossible to predict the outcome of an infection of the vector based on the gametocyte density (Muirhead-Thomson, 1957, Ponnudurai et al., 1989). On the other hand Boudin et al. (1989) found a positive relation between gametocyte density and infectiousness for gametocyte densities lower than 450 gametocytes/ μ l and Graves (1980) found that only patients with at least 300 gametocytes/ μ l are likely to produce a high infection in mosquitoes. The hypothesis of specific antibody titer-

correlated immunosuppression of gametocytaemia in *P. falciparum* infections, independent of control of asexual stages, has been postulated (Baird et al., 1991). Different host serum factors are able to influence infectivity of gametocytes. Naotunne et al. (1991) reported that gametocytes of *P. cynomolgi* are killed and lose infectivity in crisis serum due to the presence of the cytokines tumor necrosis factor and gamma interferon. The killing activity is dependent on the presence of additional, yet unidentified serum factors.

Antimalarial drugs too appear to modulate infectivity. Proguanil and pyrimethamine are known to have a sporontocidal effect while primaquine has a gametocidal action, particularly in *P. falciparum* (Bruce Chwatt, 1980). Serum from volunteers on proguanil-chloroquine prophylaxis showed a reduction in transmission (Ponnudurai et al., 1989). However, Chutmongkonkul et al. (1992) found that pyrimethamine-treated gametocytes were more infective than untreated controls. Sera with chloroquine alone did not influence sporogony (Wilkinson et al., 1976; Smalley, 1977; Chutmongkonkul et al., 1992) but chloroquine and other antimalarial drugs can enhance infectivity of gametocytes during an established infection-crisis by suppressing asexual parasites and thus lowering levels of crisis serum factors (reviewed by Sinden, 1991). Experimental infection of mosquitoes with gametocytes of local origin provides a useful model for the estimation of man-mosquito transmission capacity and the analysis of human host factors that influence the infectivity of gametocytes. We studied the infectivity of *P. falciparum* gametocytes to a local strain of *Anopheles gambiae*, the most important vector in Sub-Saharan Africa. Mosquitoes were infected with blood from naturally infected gametocyte carriers by a membrane feeding technique. The influence of gametocyte density and the presence of asexual blood-stages in the infectivity was analyzed as well as the age and sex of the gametocyte carrier, his body temperature, blood group (ABO) and use of chloroquine.

Materials and methods

Mosquitoes. A strain of *A. gambiae sensu stricto*, caught in 1988 in Essos, a quarter in Yaoundé, was adapted to feeding on parafilm membrane feeders (Ponnudurai

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Table 1 Prevalence of malaria related symptoms reported by 248 gametocyte carriers.

Symptoms	fever or chill	headache	listlessness or weariness	stomach pain	body pain	itching	other
Main complaint %	35.5	18.1	15.3	6.9	4.8	2.8	14.6
Present %	89.5	84.3	100.0	56.5	58.9	31.4	-

et al., 1989) and kept in culture under laboratory conditions (Armstrong and Bransby-Williams, 1961). Lights were switched off and on at 2.00 am and 3.00 pm, respectively. Temperature was kept between 26 °C and 28 °C and relative humidity between 70% and 90%. Mosquitoes used for the maintenance of the colony were fed on filtration paper soaked in a 10% sucrose solution. The female mosquitoes were allowed to feed on human blood that was offered through a medium-sized membrane feeder as described by Ponnudurai et al. (1989). Eggs laid on wet filtration paper were collected every morning and placed in separate trays containing filtered pond water, thus restricting the difference in age of successive batches of mosquitoes to 24 hours. Larvae were fed with 150 mg of Tetra Baby Fish Food L[®] per day in each tray containing about 500 larvae at a density of 0.5 larvae/cm³. Eight to ten days later larvae evolved into pupae; they were collected in cups using plastic pipettes and placed in cages. Emergence took place after 25 to 48 hours. A bowl with 500 pupae was put daily in 20×20×20 cm cages to be used for experimental infections at the age of 5 days post emergence. Before the infection experiment, no blood meal was given to the mosquitoes, only sucrose solution which was removed 20 hours before feeding.

Gametocyte carriers. Each morning between 8.00 am and 9.30 am about 30 thick smears were collected from patients with malaria-like complaints at the dispensary at Messa, a central quarter in Yaoundé. The slides were stained with Giemsa and examined at the O.C.E.A.C. laboratory. At 11.00 am patients were informed of the results and gametocyte carriers were asked to cooperate in the experimental study. Those who consented were invited to the O.C.E.A.C. laboratory. Data on the patient history, use of medication, body weight and temperature were collected. All patients were treated with 35 mg/kg amodiaquine over 3 days according to prevailing O.C.E.A.C. procedures (Louis et al., 1992).

Blood collection. Before treatment, intravenous blood was collected into both dry and heparinized vacutainer tubes. The blood from the dry tube was used to determine the blood group and to prepare Giemsa stained thick smears. Gametocyte density was based on a count of the number of gametocytes per 1 000 leucocytes, assuming an average number of 8 000 leucocytes/μl. The heparinized blood was used for experimental infections.

Experimental infections. Experimental feeding took place at 12.00 am, 3 hours before the lights in the insectarium were switched on, a period during which *A. gambiae* has been reported to be most aggressive (Gillies et al., 1968). The tube containing heparinized blood was carefully kept in water at 37 °C to avoid activation of gametocytes. A membrane feeder with a feeding surface area of 1134 mm² (Ponnudurai et al., 1989) was quickly filled with 2 ml of blood using a prewarmed sterile syringe and the mosquitoes were allowed to take blood during 15 minutes. Fed mosquitoes were counted and placed into another cage with permanent access to a 10% sucrose solution. After seven days surviving mosquitoes were dissected. Midguts were stained with 2% mercurochrome and examined for the presence and number of oocysts by normal light microscopy.

Enrolment criteria. *P. falciparum* gametocyte carriers, negative for other plasmodial species and aged at least 4 years, were included in the study if at least 20 mosquitoes could be examined on day 7 after the experimental infection.

Statistical analysis. A simple monofactorial analysis was conducted on the following variables: sex of the gametocyte carrier, body temperature at the time of blood collection for the experimental infection, blood group and rhesus factor and declared use of antimalarial or antipyretic drugs. Differences between proportions were studied by the Chi-square or Fisher's exact test. Differences were considered significant at $P < 0.05$. A multifactorial analysis by logistic regression on the success of infection and by multiple linear regression on the percentage of infected mosquitoes was performed with gametocyte density, presence or density of asexual blood stage parasites, age of the gametocyte carrier as independent variables and with the success of infection or the percentage of infected mosquitoes as dependent variables.

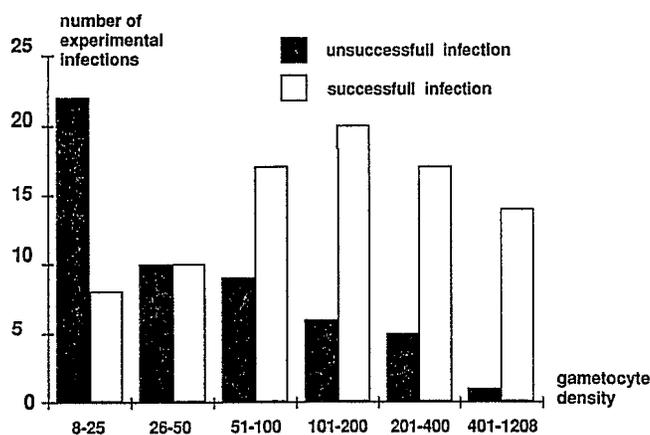
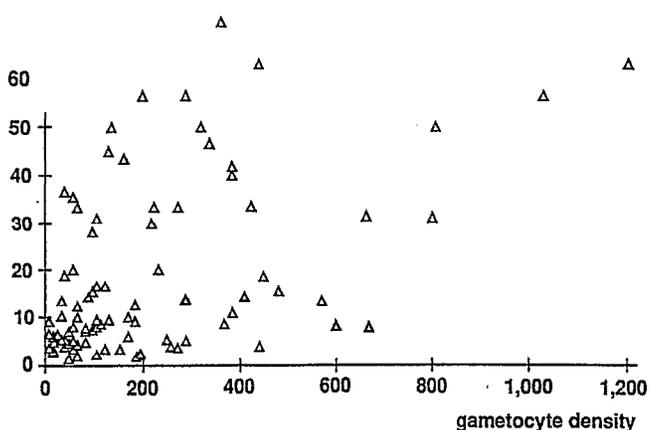
Results

Mosquitoes. The hatching percentage of the matured eggs was 42% and the daily average larval mortality 1.9%. The pupae production of the colony was more than 5 000 per day, which guaranteed the maintenance of the colony and met the needs for the experimental infections. From every 500 mosquitoes, an average of 52 died before the day of the infection experiment. A mean of 82 females became engorged during a feeding.

Gametocyte carriers. Parasitological examination of 10 781 people from October 1990 to January 1993 showed a plasmodial index of $37.1 \pm 7.8\%$ with low seasonal variation. Species division was of: 90.5% *P. falciparum*, 13.0% *P. malariae* and 2.0% *P. ovale*. 5.5% of these infections were mixed and in all but 4 consisted of *P. falciparum* with one of the other species. The gametocyte index of *P. falciparum* was $5.4 \pm 1.8\%$ without seasonal variation. This stability enabled us to carry out experimental infections throughout the year. Almost all of the gametocyte carriers agreed to cooperate in the study. Male and female gametocyte carriers were not equally distributed: 58.4% of the patients who came to the dispensary and 64.0% among the gametocyte carriers were male. Clinical data indicate that malaria related complaints (fever, chill, headache, weariness) within 2 weeks before presentation at the dispensary were common in almost all gametocyte carriers (Table 1). Mean age of the gametocyte carriers was 19.5 years (range 4–60). Trophozoites were found in 68% of the patients carrying gametocytes. They had a mean gametocyte density of 163 gametocytes/μl. Table 2 shows the mean parasite density by age. Age and asexual parasite densities were significantly correlated ($r = 0.143$, $df = 138$, $p = 0.047$), as were

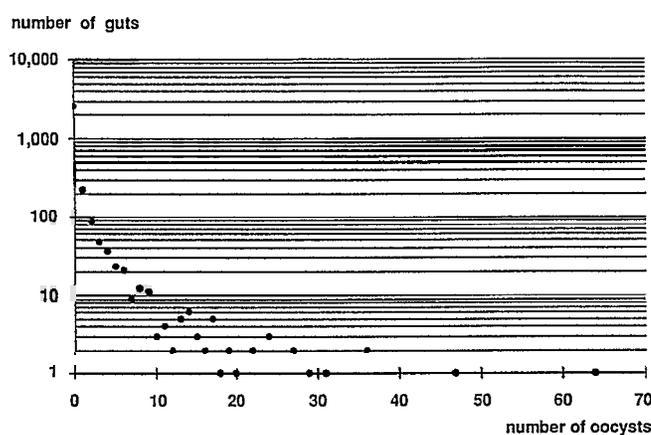
Table 2 Prevalence, parasite density per μl of blood and percentage of infected mosquitoes in different age groups of gametocyte carriers.

Age group	5-10	11-15	16-25	>25	mean
Asexual parasite prevalence %	69	85	63	59	68
Mean asexual parasite density	6283 \pm 4174	6001 \pm 3336	5141 \pm 2417	3479 \pm 2043	5212 \pm 1478
Mean gametocyte density	136.9 \pm 51.2	217.3 \pm 90.8	168.3 \pm 57.1	114.2 \pm 59.4	162.8 \pm 34.1
Mean % of infected mosquitoes	12.8 \pm 7.2	10.6 \pm 5.2	14.5 \pm 5.4	6.1 \pm 3.2	11.5 \pm 2.8

**Fig. 1** Relationship between gametocyte density and the outcome of 139 experimental infections of *A. gambiae* with *P. falciparum*.**% infected mosquito****Fig. 3** Relationship between the percentage of infected *A. gambiae* and the gametocyte density in 86 experimental infections with *P. falciparum*.

age and gametocyte densities ($r = 0.141$, $df = 138$, $p = 0.049$), and gametocyte densities and asexual parasite densities ($r = 0.247$, $df = 138$, $p = 0.002$).

Experimental infections. Mosquitoes were fed on blood from 171 individual gametocyte carriers. Based on the enrolment criteria, 139 experiments were

**Fig. 2** Distribution of oocysts observed on mosquito midguts in 86 successful experimental infections of *A. gambiae* with *P. falciparum*.

selected. They were done between October 1990 and January 1993. A total of 5149 mosquitoes were dissected. The mean number of mosquitoes dissected was 37 (range 20-88). An average of 38% of the engorged mosquitoes died before the seventh day after the feeding and were not dissected. In the 139 experiments the overall mean percentage of infected mosquitoes was 11.5% and the mean oocyst load per midgut was 1.59 (Table 3a). Gametocytes from 53 carriers (38%) were not infective to mosquitoes (range of gametocyte density 8-552/ μl). Gametocytes from 86 carriers (62%) were infective to mosquitoes. In those 86 infections the overall mean percentage of infected mosquitoes was 18.6% (Fig. 1) and the mean oocyst load per midgut was 2.56 (Fig. 2 and Table 3b). The highest infection rate was obtained after feeding on a 24 year old man: 72% of the mosquitoes were infected with a mean oocyst load per gut of 4.14.

Factor influencing the result of experimental infection. The correlation coefficient between gametocyte density and percentage of infected mosquitoes was 0.31 (Fig. 3) and it was 0.66 between mean oocyst load and gametocyte density ($p < 0.001$ in both cases). Stepwise logistic regression analysis showed that the success of infectivity to mosquitoes depended only on gametocyte density ($\beta = 0.0073$, improvement $\chi^2 = 24.29$, $p < 0.0001$). Multilinear regression performed on the percentage of infected mosquitoes showed similar results ($B = 0.045$,

Table 3a Relationship between gametocyte density and infectivity in 139 experimental infections of *A. gambiae* with *P. falciparum*.

gametocyte n	≤25 30	26-50 20	51-100 26	101-200 26	201-400 22	>400 15	total or mean 139
mean % infected mosquitoes	1.40±0.92	5.37±3.99	8.40±4.00	13.28±6.55	21.61±9.20	27.38±11.03	11.51±2.79
mean oocyst load per positive gut	0.33±0.20	0.66±0.33	1.08±0.36	1.82±0.75	2.33±0.91	4.72±2.25	1.59±0.39

Table 3b Relationship between gametocyte density and infectivity in 86 transmission experiments resulting in at least one mosquito becoming infected.

gametocyte n	≤25 8	26-50 10	51-100 17	101-200 20	201-400 17	>400 14	total or mean 86
mean % infected mosquitoes	5.24±1.48	10.73±6.53	12.85±4.98	17.26±7.71	27.97±10.05	29.34±11.11	18.60±3.80
mean oocyst load per positive gut	1.24±0.19	1.32±0.31	1.65±0.29	2.37±0.83	3.01±0.95	5.06±2.31	2.56±0.53

Table 4 Characteristics of 139 gametocyte carriers related to the success of experimental infections.

Experimental infection	sex		blood group					rhesus factor			total
	male	female	A	B	O	AB	not done	+	-	not done	
+	57	29	21	12	48	3	2	81	2	3	86
-	32	21	16	13	20	4	0	49	4	0	53
Total	89	50	37	25	68	7	2	130	6	3	139

T test = 7.83, $p < 0.0001$). None of the remaining variables was significant.

Factors not influencing the result of experimental infection. The average age of the gametocyte carrier did not differ significantly in the groups with infections (18.4 years) and without infections (21.4 years), as was asexual parasitaemia. None of these variables appeared to be significant in the stepwise logistic regression ($p = 0.41$ and $p = 0.29$, respectively). The outcome of the experimental infections was not influenced by the sex of the gametocyte carriers ($\chi^2_1 = 0.50$, $p = 0.48$), the A B O blood group ($\chi^2_2 = 4.65$, $p = 0.10$), or the rhesus factor of the gametocyte donor (Fisher's exact test, bilateral probability $p = 0.21$), (Table 4). The axillary body temperature at the moment of the withdrawal of blood had no effect on infection success: the average body temperature was 37.15 °C for the positive infections and 37.31 °C for the negative ones. There were 23 cases of fever (temperature ≥ 38 °C) among which 13 gave positive infections and 10 did not. The use of drugs such as chloroquine (24 cases), amodiaquine (17 cases) and/or aspirin (28 cases) during the days before the withdrawal of blood had no effect on the success of the infection.

Discussion

In this study the infectivity of gametocytes has been merely defined as the capability to infect mosquitoes and was not based on oocyst density. The number of oocysts developing in individual mosquitoes

has a high degree of variability. It is not an appropriate criterion for infectiousness since a mosquito bearing one oocyst on its midgut is capable of transmitting the disease (Ponnudurai et al., 1991). Experimental infections of mosquitoes with blood from gametocyte carriers showed that 62% of the carriers were infective. This value is comparable with the data reviewed by Vanderberg and Gwatz (1980) and with the 45%, observed in the Madang area, Papua New Guinea, by Graves et al. (1988). In not one feeding experiment, did one hundred percent of the blood fed mosquitoes become oocyst positive. This might be related to the low number of gametocytes that actually was ingested by the mosquito, to the fact that partially fed mosquitoes were not discarded and to entomological factors such as difference in the speed of digestion of the bloodmeal by individual mosquitoes (Ponnudurai et al., 1989).

The question remains as to why 38% of the attempted feedings on gametocyte carriers did not lead to any infected mosquito. The results of the multiple regression analysis pointed out that gametocyte density is one of those factors. Boudin et al. (1989) found a positive relation between gametocyte density and percentage of sporozoite containing mosquitoes for gametocyte densities up to 450 gametocytes/ μ l. Our results confirm this observation; however, no limit was found. Similar results were recently found with *P. vivax* (Gamage-Mendis et al., 1993). Boyd (1949) already described other factors than gametocyte density, that co-determine infectiousness of the parasite donor, i.e. an elusive characteristic desig-

nated as "quality", maturity of the gametocytes and the proportion of the sexes present. Determination of sex ratios and densities of female gametocytes in thick smears was attempted but turned out to be unsatisfactory in this study: a high prevalence of indistinguishable (activated?) gametocytes was found, especially in slides from gametocyte carriers that gave high infection percentages.

In contrast to our observations Ponnudurai et al. (1989) found no relation between the number of gametocytes and the number of oocysts produced in their laboratory experiments. Apparently their *in vitro* system differs from the field situation. Particularly the number of gametocytes used in the infective blood meal in this system is much higher than those occurring naturally in our population of gametocyte carriers. Although the gametocyte density showed a highly positive correlation with infection success, the density of asexual blood stage parasites appeared of no importance in our analysis. On the contrary, Rutledge et al. (1969) found that high parasitaemias in some individuals, mostly children with more than 10^3 parasites per mm^3 of blood, are often associated with reduced infectivity of gametocytes. Chloroquine intake did not influence the infectivity of gametocytes. Acting only against asexual parasites and gametocytes up to and including stage III, chloroquine is not capable of interfering with transmission of *P. falciparum* once gametocytes have appeared in the bloodstream. This observation is consistent with previous publications (Rosario et al., 1988; Wilkinson, 1976; Smalley, 1977).

The results of the patient's histories show that the majority of the population of gametocyte carriers in this endemic area presenting at the dispensary likely experienced a recent malaria attack. However, the concomitantly lower density of asexual parasites and gametocytes in the age group of older people indicates some degree of premunition in these city dwellers. In a population recently exposed to epidemic falciparum malaria, no relation between age and gametocyte density was found (Gamage-Mendis et al., 1991). Baird et al. (1991), comparing native and transmigrant populations in an epidemic situation suggested the possibility of a specific antibody-mediated suppression of sexual stages. Recently, a study in a population living in an area of holoendemic malaria described that gametocyte density was constant throughout all age groups, while infection levels decreased with age (Githeko et al., 1992). Based on these observations interesting suggestions were brought forward with regard to the reservoir of *P. falciparum* malaria. However, our study does not permit discussion about the reservoir because our gametocyte carriers sample was not representative of the general population of gametocyte carriers.

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