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SUBMICROSCOPIC *PLASMODIUM FALCIPARUM* GAMETOCYTE DENSITIES FREQUENTLY RESULT IN MOSQUITO INFECTION

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Abstract. Submicroscopic *Plasmodium falciparum* gametocytemia (< 5,000 gametocytes/mL) is common and may result in mosquito infection. We assessed the relation between gametocyte density and mosquito infection under experimental and field conditions using real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) for gametocyte quantification. Serial dilutions of NF54 *P. falciparum* gametocytes showed a positive association between gametocyte density and the proportion of infected mosquitoes ($\beta = 6.1$; 95% confidence interval [CI], 2.7–9.6; $P = 0.001$). Successful infection became unlikely below an estimated density of 250–300 gametocytes/mL. In the field, blood samples of 100 naturally infected children showed a positive association between gametocyte density and oocyst counts in mosquitoes ($\beta = 0.38$; 95% CI, 0.14–0.61; $P = 0.002$). The relative contribution to malaria transmission was similar for carriers with submicroscopic and microscopic gametocytemia. Our results show that transmission occurs efficiently at submicroscopic gametocyte densities and that carriers harboring submicroscopic gametocytemia constitute a considerable proportion of the human infectious reservoir.

INTRODUCTION

Transmission of *Plasmodium falciparum* from human to mosquito depends on the presence of infectious sexual stage parasites (gametocytes) that are ingested by mosquitoes taking a blood meal. The likelihood of mosquito infection can be expected to increase proportionally with increasing gametocyte density. However, this relation remains equivocal. A positive relation between gametocyte density and the proportion of infected mosquitoes was detected in experimental^{1–3} and natural^{2,4–6} *P. falciparum* infections, but this relation was weak or non-existing in other studies.^{7–11} Several studies show that mosquitoes can become infected with gametocyte densities below the detection level of standard microscopy.^{3,6,8,9,12–14} Importantly, the recent application of molecular techniques for the detection^{15,16} and quantification^{17,18} of gametocytes indicated that submicroscopic gametocyte densities are common.^{17,19,20} In a previous study, we have shown that submicroscopic gametocyte densities seem sufficient to drive post-treatment malaria transmission.²¹ It is important to quantify this potential reservoir and to determine the relation between submicroscopic gametocyte densities and the infectiousness to mosquitoes to plan and evaluate transmission reducing interventions.²² This can be done in natural infections and under laboratory conditions where the use of cultured, synchronized gametocytes excludes potential confounders such as gametocyte maturity and host immune factors. In this study, we used quantitative nucleic acid sequence-based amplification (QT-NASBA) to determine the relation between *P. falciparum* gametocyte density and the infectiousness to mosquitoes under field and laboratory conditions.

MATERIALS AND METHODS

Infectiousness of cultured gametocytes. *Plasmodium falciparum* NF54 gametocytes were obtained from *in vitro* cul-

ture.²³ Experiments were performed with mature gametocytes. Serial dilutions were made in whole blood to obtain gametocyte dilutions at ~50% hematocrit ranging from 10² to 10⁶ gametocytes/mL. Gametocyte density in serially diluted suspensions was calculated from the number of gametocytes present in the original sample. Of all dilutions, a 50- μ L sample was stored at -70°C for subsequent quantification of gametocytes by real-time *Pfs25* QT-NASBA. All dilutions were fed through a membrane feeding system to 50–100 female *Anopheles stephensi* mosquitoes²⁴ that were 3–5 days old and starved for 1–2 hours before feeding. After feeding, mosquitoes were kept at 26°C and 80% relative humidity on glucose until dissected in 1% mercurochrome for microscopic assessment of oocyst infection on days 7–9.

Infectiousness of naturally infected children. Membrane feedings were conducted on samples from children naturally infected with *P. falciparum* malaria in Mbita, Suba district, western Kenya. Details of the study can be found elsewhere.²¹ The study protocol was approved by the scientific steering committee and ethical review committee of the Kenya Medical Research Institute (SSC No. 791). Children were treated for uncomplicated *P. falciparum* malaria with sulphadoxine-pyrimethamine (SP), SP plus artesunate, SP plus amodiaquine, or artemether-lumefantrine. All children older than 2 years who attended the clinic for their day 14 post-treatment visit in November–December 2004 were invited for membrane feeding experiments, regardless of microscopic gametocytemia. We enrolled 25 children consecutively per treatment arm, giving a total of 100 membrane feeding experiments.

Venous blood samples of 3 mL were obtained from children whose parent or guardian gave specific consent for the procedure. Samples were fed to ~150 locally reared 4- to 5-day-old female *Anopheles gambiae* s.s. mosquitoes through an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. After 10–15 minutes, fully fed mosquitoes were selected and kept at 27–29°C for 7 days on glucose (10%) until midguts were dissected in 2% mercurochrome. The midguts were examined microscopically for oocysts with a second microscopist confirming their presence if observed. For each experiment, exactly 30 randomly selected mosquitoes were dissected. From each blood sample included

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in the membrane feedings, 50 μ L was used for quantification of gametocytes by real-time *Pfs25* QT-NASBA.

Gametocyte detection by *Pfs25* QT-NASBA. Quantification of gametocytes by real-time *Pfs25* mRNA QT-NASBA was performed as described elsewhere.¹⁷ Briefly, nucleic acids were extracted from the blood samples using the guanidium isothiocyanate/silica procedure.²⁵ QT-NASBA was performed on a NucliSens EasyQ analyser (bioMérieux, Boxtel, The Netherlands) using the NucliSens Basic Kit for amplification according to manufacturer's manual at a KCl concentration of 80 mmol/L in a total reaction volume of 10 μ L per reaction. The number of gametocytes was calculated in relation to a standard gametocyte stage V dilution series, using the time-point of amplification at which the fluorescence detecting target amplicons exceeded the mean fluorescence of three negative controls + 20 SD. A similar protocol was used for the 18S rRNA QT-NASBA¹⁸ to confirm the presence of *P. falciparum* parasites.

Data analyses. Statistical analyses were carried out in SPSS version 12.0 and STATA 8.0. *Pfs25* QT-NASBA gametocyte density was analyzed on a log-scale with negative samples included as zeros. The relation between gametocyte density and mosquito infection rates was fitted to linear, logistic, and exponential functions. The best results were presented based on r^2 . For field samples, the relation between gametocyte density and oocyst counts in individual mosquitoes was determined using negative binomial models. Uninfected mosquitoes were included in these analyses, and a random effect was added to allow for the correlation between observations from the same individual. Regression coefficients β with 95% confidence intervals (CIs) were presented. Proportions were compared using odds ratios (ORs) and the trend in proportions with the Cochran-Armitage test for trend. Normally distributed continuous variables were compared using the Stu-

dent *t* test, and the correlation between continuous normally distributed variables was quantified with the Pearson correlation coefficient.

RESULTS

Infectiousness of cultured gametocytes. The percentage of infected mosquitoes was positively associated with gametocyte density from *in vitro* culture ($\beta = 6.1$; 95% CI, 2.7–9.6) and was described by a linear function ($r^2 = 0.39$; $P = 0.001$; Figure 1). Non-linear functions did not improve the fit. Infected mosquitoes harbored one to four oocysts, and only 14% of the infected mosquitoes had more than one oocyst, making analyses on oocyst counts uninformative. An estimated 7.6% of the feeding mosquitoes was infected at the microscopic detection limit of 5 gametocytes/ μ L. The trendline crossed the y-axis at 250–300 gametocytes/mL, indicating that mosquito infection under the used conditions is unlikely below this threshold. There was a good consensus between the gametocyte density measured in the *Pfs25* QT-NASBA and the estimated density from diluted starting concentrations (Pearson correlation coefficient: 0.88, $P < 0.001$).

Infectiousness of naturally infected children. A total of 100 membrane feedings were successfully carried out in children with a median age of 2.8 years (interquartile range, 2–4 years). At the time of membrane feedings, 25.0% (25/100) of the children harbored microscopically detectable gametocytes, and 75.0% (75/100) were gametocyte positive by *Pfs25* QT-NASBA. A total of 62.0% (62/100) of the children were infectious to mosquitoes, of whom 69.4% (43/62) had no microscopically detectable gametocytes. There was a positive association between gametocyte density and the percentage of infected mosquitoes, described by an exponential function ($r^2 = 0.48$; $P = 0.026$; Figure 2). Infected mosquitoes har-

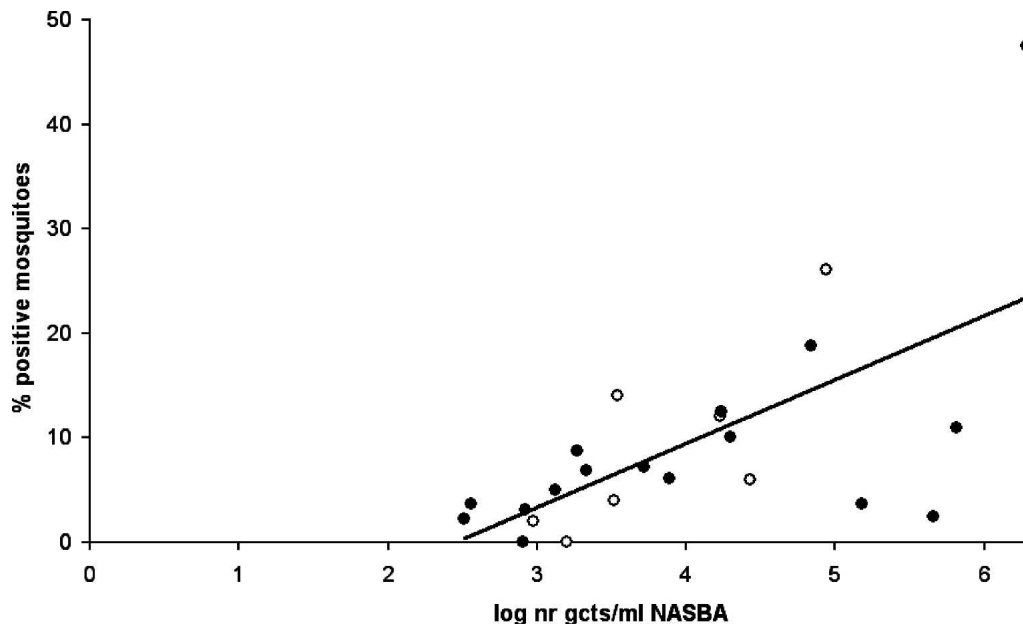


FIGURE 1. Mosquito infection rates against log gametocyte density for feeding experiments on serial dilutions of *in vitro* cultured *P. falciparum* gametocytes. Results are shown from three membrane feeding experiments with serial dilutions of non-synchronized (○) and synchronized (●) gametocytes. Gametocyte density was determined by *Pfs25* QT-NASBA; mosquito infection rates were determined by examination of 50–100 dissected mosquitoes per sample. The curve represents the linear relation between log gametocyte density and mosquito infection rates ($y = 6.13x - 15.06$; $r^2 = 0.39$, $P = 0.001$).

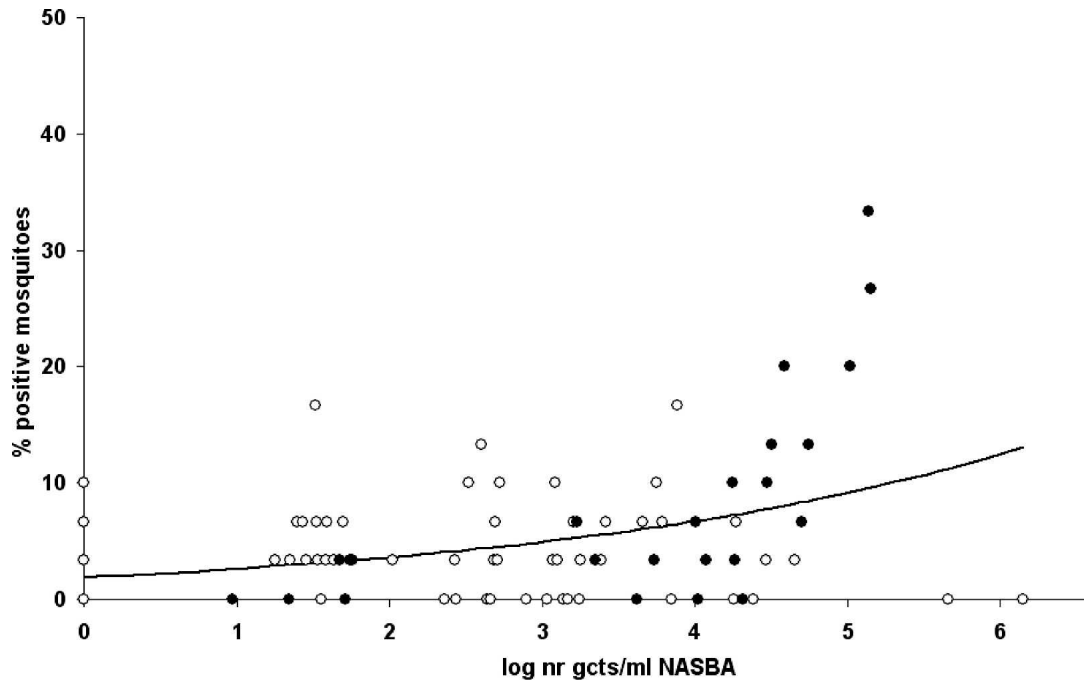


FIGURE 2. Mosquito infection rates against log gametocyte density for feeding experiments on venous blood samples of naturally infected Kenyan children. Results are shown for individual venous blood samples; microscopically confirmed gametocyte carriers are indicated with filled circles. Gametocyte density was determined by *Pfs25* QT-NASBA; mosquito infection rates were determined by examination of 30 dissected mosquitoes per sample. The curve represents the exponential relation between log gametocyte density and mosquito infection rates ($y = 1.93e^{0.31x}$; $r^2 = 0.48$, $P = 0.026$).

bored 1–13 oocysts, and 26% of the infected mosquitoes had > 1 oocyst. We observed a positive association between gametocyte density and oocyst counts in mosquitoes ($\beta = 0.38$; 95% CI, 0.14–0.61; $P = 0.002$) and an independent borderline significant positive association between age and oocyst counts ($\beta = 0.11$; 95% CI, –0.01 to 0.23; $P = 0.075$). Eight children were infectious to mosquitoes with no gametocytes detected in their samples by *Pfs25* QT-NASBA. *P. falciparum* 18S ribosomal RNA was detected in all these samples (eight of eight), indicating that RNA extraction had been successful and *P. falciparum* parasites were present.

Infectiousness of children with submicroscopic gametocytemia. Successful mosquito infection was observed in children with microscopic and submicroscopic gametocyte densities (Table 1). Compared with children with microscopically detectable gametocytes, the proportion of infectious children

was lower in children with submicroscopic gametocytemia and lowest in children who were negative in both microscopy and *Pfs25* QT-NASBA (Cochran-Armitage test for trend, $P = 0.001$). The same trend was observed for the proportion of infected mosquitoes (Cochran-Armitage test for trend, $P < 0.001$). Importantly, 70.0% (35/50) of the children with submicroscopic gametocytemia were capable of infecting at least one mosquito. Of those children who were gametocyte negative in both microscopy and *Pfs25* QT-NASBA, 32.0% (8/25) were infectious to mosquitoes. In general, the association between gametocyte prevalence and the proportion of infectious children in the random feeds was weak for microscopy (OR = 2.36; 95% CI, 0.85–6.57) and much stronger for the *Pfs25* QT-NASBA (OR = 5.46; 95% CI, 2.05–14.56). The contribution to malaria transmission of children with microscopically confirmed gametocytes and children with submi-

TABLE 1
Gametocyte carriage detected by microscopy and *Pfs25* QT-NASBA and infection of mosquitoes after membrane feeding

	Gametocyte carriage			<i>P</i>
	–	–	+	
Microscopy	–	–	+	
<i>Pfs25</i> QT-NASBA	–	+	+	
Number of samples	25	50	25	
Density, GM (IAR)*	–	592.5 (42.0–2,486.5)	4,058.1 (301.0–34,798.3)	0.007†
Infectious children, % (N/N)	32.0 (8/25)	70.0 (35/50)	76.0 (19/25)	0.001‡
Infected mosquitoes, % (N/N)	1.7 (13/750)	4.1 (62/1,500)	8.3 (62/750)	< 0.001‡
Contribution to transmission, %§	9.5	45.3	45.3	

* Geometric mean density of gametocytes per mL of blood in the *Pfs25* QT-NASBA for positives only.

† *P* no value for the Student *t* test on log-transformed gametocyte density.

‡ *P* value for the Cochran-Armitage test for trends.

§ The contribution of each group to transmission was estimated by dividing the number of mosquitoes infected by this group by the total number of mosquitoes infected by all 100 children. Equal mosquito exposure of all groups is assumed.

–, gametocyte negative; +, gametocyte positive; GM, geometric mean; IQR, interquartile range.

croscopic gametocytemia was estimated by determining the proportion of the total number of infected mosquitoes that they infected. Although individual submicroscopic gametocyte carriers infected fewer mosquitoes, submicroscopic gametocytemia was more common than microscopic gametocytemia, resulting in an estimated contribution to transmission of 45.3% for both groups (Table 1).

DISCUSSION

In this study, we showed that submicroscopic gametocyte densities can efficiently infect mosquitoes under both laboratory and field conditions. Although it has previously been suggested that submicroscopic gametocyte densities can infect mosquitoes,^{3,6,8,9,12–14} this is the first study that actually quantifies submicroscopic gametocytemia in relation to mosquito infection. We found that the contribution of submicroscopic gametocytemia to malaria transmission can be similar to that of microscopically confirmed gametocyte carriers.

Data from cultured gametocytes showed a clear reduction in the proportion of infected mosquitoes with decreasing gametocyte density. Such a relation has been observed before^{24,26} but could not be extended to submicroscopic densities until recently. An estimated 7.6% of mosquitoes became infected after feeding on a sample with a gametocyte concentration at the threshold of microscopical detection. Successful mosquito infection becomes unlikely below an estimated density of 250–300 gametocytes/mL.

Under field conditions, submicroscopic gametocyte densities can also infect mosquitoes, although the association between gametocyte density and mosquito infection rates was less straightforward than in the laboratory experiments. A direct comparison between the experiments in the laboratory and the field can not be made for a number of reasons. There may be differences in gametocyte fitness or maturity between cultured NF54 gametocytes and natural infections, as well as differences in compatibility of parasite isolates and vector species.^{27,28} Natural infections also regularly contain multiple parasite genotypes, opposed to a single infecting genotype in the *in vitro* culture. This could affect gametocyte development through in-host competition between parasite genotypes.^{29,30} Sexual stage-specific immune responses may also have influenced transmission efficiency in the field experiments.³¹

In the field experiments, we frequently observed mosquito infection at very low gametocyte densities. Successful mosquito infection by eight children with negative Pfs25 QT-NASBA results suggests that infection can even occur below the detection level of the *Pfs25* QT-NASBA, which is 20–100 gametocytes/mL.¹⁸ Unlike for the laboratory experiments, we could not estimate a minimum gametocyte density for mosquito infection in the field. Gametocytes below levels of 100/mL may not be detected by *Pfs25* QT-NASBA, and this may explain transmission without detection of *Pfs25* mRNA. Findings from laboratory and field experiments indicate that the threshold gametocyte density necessary to infect mosquitoes is below the theoretical threshold of two gametocytes per mosquito blood meal (2 μ L). Because the uptake of gametocytes by mosquitoes follows a Poisson or negative binomial distribution,³² some successful infections can occur when feeding on samples with lower gametocyte densities. Several biologic mechanisms could facilitate successful mosquito in-

fections at low gametocyte densities. Motility or aggregation of gametocytes may occur, favoring the encounter of males and females.³² This phenomenon could have influenced transmission efficiency in our experiments. Gametocytes may also preferentially accumulate in the subdermal capillaries.^{33,34} Although this could increase the transmission success of individuals with low peripheral gametocyte density under natural conditions, it will not have influenced our findings because we used the same venous blood samples for feeding experiments and gametocyte quantification.

Both laboratory and field experiments clearly show the efficient transmission of low-density gametocytemia, despite *Anopheles* immune responses that can substantially reduce transmission success.³⁵ We observed a 2-fold lower proportion of infected mosquitoes for children harboring submicroscopic gametocyte densities (4.1%) compared with microscopic gametocyte densities (8.3%). This lower proportion of infected mosquitoes was counterbalanced by the higher prevalence of submicroscopic gametocytemia in our population, resulting in a similar contribution to malaria transmission of carriers with microscopic and submicroscopic gametocytemia at the time we conducted our experiments. The duration of gametocyte carriage is obviously a crucial factor to determine the overall contribution of submicroscopic gametocytemia to malaria transmission, and this requires further research. Further studies should also determine the prevalence and infectiousness of submicroscopic gametocytemia in other populations and the possible relations with seasonality and transmission intensity.

Despite our selected population of children after antimalarial treatment, we feel that our findings are of public health importance. Interventions that aim to interrupt transmission will have to reduce gametocytemia to densities well below the microscopic detection limit and should also target carriers with submicroscopic gametocytes.

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