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Plasmodium falciparum: the population structure of mature gametocyte cultures has little effect on their innate fertility

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In vitro cultured *Plasmodium falciparum* gametocytes were fed to *Anopheles gambiae* (G3) mosquitoes to identify parasite population characteristics useful for predicting successful mosquito infections. Parameters were collected from an initial study of 90 infections over a two year period and a second study of 55 infections over 12 weeks. Parasite isolate/clone was identified as the most reliable predictor of gametocyte infectiousness. Parameters such as gametocyte age structure (stage IV:V ratio), exflagellation rate and macrogametocyte maturity were not reliable for predicting infectiousness but were useful for monitoring overall culture maturity. Other variables such as gametocyte density, chronological age of the culture at the time of feed, gametocyte sex ratio, asexual parasitemia, and mixing cultures before mosquito feeding were not predictive. Thus, if a reliable parasite isolate or clone is used, there is no need to measure other characteristics of in vitro gametocyte populations because these will not significantly improve one's ability to predict oocyst infection rates.

Key words: *Plasmodium falciparum*; *Anopheles gambiae*; Gametocyte; Malaria

Introduction

The sporogonic cycle of *Plasmodium falciparum* is initiated when mature male and female gametocytes are ingested by anopheline mosquitoes. In the midgut, microgametocytes undergo the process of exflagellation, releasing microgametes in the blood meal where they find and fertilize mature macrogametes. Though this process was first recognized by Grassi in 1898 (reported in Boyd, 1949), many attempts have been made to identify the critical factors determining the innate fertility of *P. falciparum* gametocytes in *Anopheles* mosquitoes (reviewed by Sinden, 1991). Major categories include serum components, vector-related factors, parasite genetic composition, and parasite population structure.

The capability to culture *P. falciparum* gametocytes and to experimentally infect anophelines provides a means to examine how such factors affect the innate fertility of gametocytes. Although serum factors (Naotunne et al., 1993; Jensen, 1979),

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parasite genetic factors (Ponnudurai et al., 1989), and vector-related factors (Vaughan et al., 1994; Pumpuni et al., 1993) have been addressed, the population structure of the parasite has been the most studied (Ponnudurai et al., 1982a,b; Burkot et al., 1984; Ponnudurai et al., 1989). The parasites developing within a culture flask can be considered as a single population and as such, population characteristics such as age, maturity, and sex ratios can be determined for any culture. By determining such characteristics prior to mosquito feeding, it may be possible to determine whether the infectiousness of a gametocyte culture is related to its population structure. Accordingly, asexual parasitemia (Ponnudurai et al., 1982b) affects gametocyte infectiousness whereas female gametocyte and macrogamete density (Ponnudurai et al., 1989), gametocyte sex ratio (Ponnudurai et al., 1982a) and male gametogenesis (Burkot et al., 1984; Ponnudurai et al., 1982a) do not reliably predict infectiousness.

Despite these reports, anopheline infections from static cultivation systems remain frustrating with no apparent means to predict infection rates. Our objective, therefore, was to consolidate parameters used by previous investigators with additional characteristics to determine statistically the feasibility of predicting gametocyte fertility based on the detailed examination of the population structure of *P. falciparum* gametocytes in culture.

2. Materials and methods

Anopheles gambiae (G3) mosquitoes were infected with gametocytes from in vitro *P. falciparum* cultures. Gametocytes were cultured using modifications of the method described by Ponnudurai et al. (1982a). Parasites were maintained as static cultures in 25 ml and 75 ml screw-top culture flasks (Corning; Corning, NY). The media and gas mixture (4% CO_2, 3% O_2 and 93% N_2) was replenished daily to the sealed culture flasks.

Study one

The initial study analyzed characteristics of *P. falciparum* gametocytes used for 90 experimental infections over a two year period. The methods used to infect *A. gambiae* with cultures of *P. falciparum* gametocytes were reported in Vaughan et al. (1992) and the morphologic criteria for classifying gametocytes were described by Carter and Miller (1979). *Plasmodium falciparum* NF54 strain, obtained from Walter Reed Army Institute of Research and derived clones, 3D7 (Walliker et al., 1987) and CVD1 (Davis et al., 1992), were fed as individual cultures or sometimes mixed when two or more mature cultures were available. Cultures were fed to mosquitoes after the examination of 9 variables (Table 1). From Giemsa-stained thin smears of culture material, we estimated the age structure of gametocytes (stage IV:V ratio), gametocyte density, and sex ratio. Exflagellation presence and intensity was assessed qualitatively by wet mount. Exflagellation kinetics, also measured by wet mount, were monitored at the time of mosquito infection. The time was recorded when microgametogenesis occurred after the infected blood had been placed into the membrane feeders and the mosquitoes began to feed. The outcome variable was the oocyst infection rate of mosquitoes dissected 8 to 12 days after the feed.

TABLE 1

Variables examined in study one to evaluate the infectiousness of *Plasmodium falciparum* gametocyte cultures to *Anopheles gambiae*[a]

Variable type	Variable	F	df	p
Discrete[c]	Parasite isolate or clone	3.50	5,84	0.006[b]
	Culture age	0.84	6,72	0.52
	Exflagellation intensity	3.40	2,80	0.04[b]
	Exflagellation presence	2.45	1,86	0.12
	Exflagellation kinetics	1.47	10,39	0.19
	Mixed infections	0.10	1,88	0.75
		t	r^2	p
Continuous[d]	Age structure (Stage IV : V)	0.81	0.02	0.42
	Gametocyte density	−1.67	0.07	0.10
	Sex ratio	1.41	0.05	0.17

[a] 90 infections analyzed in data set.
[b] Significance, $p<0.05$.
[c] One-way ANOVA: F=F-statistic; df=degrees of freedom; p=probability.
[d] Multiple linear regression; t=t-statistic; r^2=square of the correlation coefficient.

Study two

Fifty-five infections were conducted over a 12 week period. Each week, a flask containing ca. 25 ml of culture was utilized for four to five daily feeds. Beginning at day 14 and each day thereafter, 5 ml were withdrawn and fed to *A. gambiae*.

Prior to initiating the study, serum lots were screened for inherent inhibitory or stimulatory effects on *P. falciparum* exflagellation. One non-inhibitory serum lot was selected and used throughout the study as a standardized culture diluent for mosquito feeds.

Before each feed, a thin smear and wet mount were taken. A total of 10 variables were evaluated, including two not examined in the first study (Table 2): asexual parasitemia and female gametogenesis. The effect of mixing two or more cultures was not included because the study design focused only on individual gametocyte cultures. Asexual parasitemia was quantified from Giemsa-stained thin smears. Additionally, female gametogenesis was determined from wet mount and interpreted as an indication of what proportion of stage V macrogametocytes within a culture were functionally and morphologically mature. After examining a wet mount for exflagellation, macrogametes were counted under oil immersion using phase-contrast at 1000X. The number of macrogametes was divided by the total number of macrogametes plus stage V macrogametocytes to provide the measurement of macrogamete maturity. The outcome variable for each infection was the oocyst infection rate, determined from 30 mosquitoes dissected 9 to 12 days after infection.

Statistical analysis

Using SPSS (SPSS Inc., Chicago, IL), discrete variables were analyzed using one-way ANOVA. Continuous variables were analyzed in a stepwise manner using multiple linear regression analysis together with standard residual analysis. The outcome variable, oocyst infection rate, was arcsine-transformed from the original

TABLE 2

Variables examined in study two to evaluate the infectiouness of *Plasmodium falciparum* gametocyte cultures to *Anopheles gambiae*[a]

Variable type	Variable	F	df	p
Discrete[c]	Parasite isolate or clone	6.17	2,52	0.004[b]
	Culture age	1.22	7,47	0.31
	Exflagellation intensity	1.40	4,50	0.25
	Exflagellation presence	2.48	1,53	0.12
	Exflagellation kinetics	1.20	8,37	0.33
		t	r^2	p
Continuous[d]	Age structure (Stage IV:V)	4.33	0.26	0.0001[b]
	Gametocyte density	−0.57	0.01	0.57
	Sex ratio	1.15	0.01	0.29
	Female gametogenesis	2.65	0.08	0.01[b]
	Asexual parasitemia	0.74	0.01	0.46

[a] 55 infections analyzed in data set.
[b] Significance, $p<0.05$.
[c] One-way ANOVA: F=F-statistic; df=degrees of freedom; p=probability.
[d] Multiple linear regression; t=t-statistic; r^2=square of the correlation coefficient.

percentage data (Rohlf and Sokal, 1969) to comply with the assumptions necessary for regression analysis (Draper and Smith, 1981).

Results

Study one

The frequency distribution of oocyst infection rates is shown in Fig. 1a. Over 70% of the culture feeds produced oocyst infections in *A. gambiae*, but infection rates were highly variable. Parasite isolate/clone was identified as the most significant variable for predicting successful infections (Table 1). Exflagellation intensity was also a significant variable.

Study two

In general, the population structure of the gametocytes in culture matured with increasing chronological age. Specifically, the stage IV:V ratio decreased, asexual parasitemia waned and female gametogenesis became more prevalent. High variability was observed again with the frequency distribution of oocyst infection rates (Fig. 1b). Analysis of the 5 discrete and 5 continuous variables identified parasite isolate/clone, gametocyte age structure and female gametogenesis to be significant (Table 2). Residual analysis of gametocyte age structure and female gametogenesis revealed that the assumptions of the regression model were not constant at higher infection rates (i.e., >60%) and created a linear instead of a random pattern. This linear trend could not be corrected by any appropriate transformation.

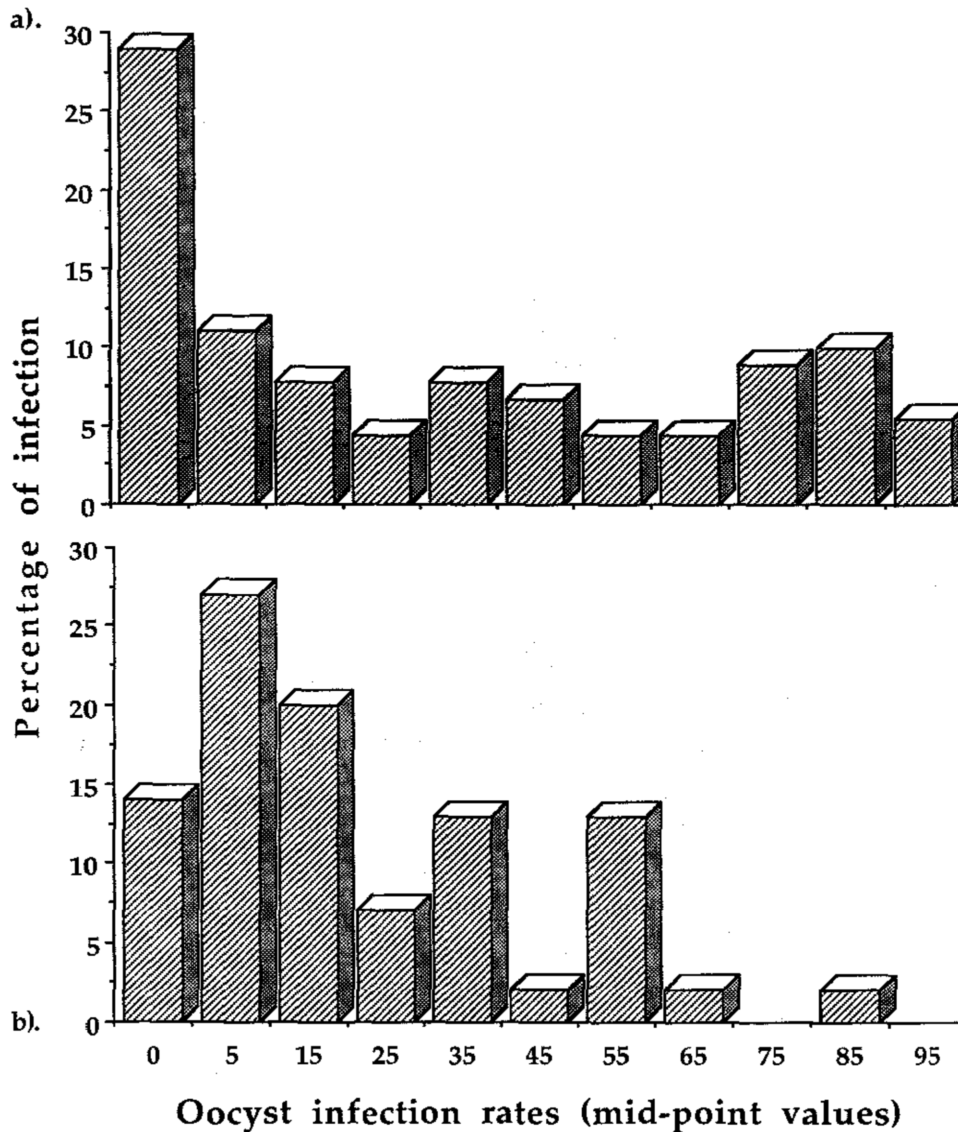

Fig. 1. Frequency distributions of *Plasmodium falciparum* oocyst infection rates in *Anopheles gambiae*: (a) 90 infections over a 2-year period (Study one) and (b) 55 infections during a 12-week period (Study two).

Discussion

No measurable parameter of *P. falciparum* gametocyte population structure reliably and consistently predicted a gametocyte culture's infectiousness to *A. gambiae*. Although exflagellation intensity was notable, there were seven successful infections in which exflagellation was not detected. Thus, exflagellation was not useful for predicting infection. Obviously, exflagellation must precede fertilization, but in these instances, microgametogenesis occurred below the threshold of our detection. Also,

residual analysis identified the unreliability of gametocyte age structure and female gametogenesis even though they were significant in the regression analysis. The consistent shifts in population structure may account for some of the variability observed. Although unreliable for predicting infection outcome, these variables are useful for monitoring gametocyte maturity (Burkot et al., 1984).

The key determinant of gametocyte infectiousness was the parasite isolate or clone used to infect *A. gambiae*. Although several NF54 isolates and derived clones were used during this study, only a few produced consistent infections. However, the infectiousness of these 'reliable' parasite isolates varied from one to three months. This trend was recently improved when a NF54 isolate was obtained from the Institute of Medical Parasitology in Nijmegen, The Netherlands. This isolate consistently produced oocyst infection rates above 80% in *A. gambiae* for 5 months before its ability to produce gametocytes waned. Ponnudurai et al. (1989) also found that a 'reliable' parasite is essential for successful infections in mosquitoes using an automated cultivation system. The present study, however, demonstrated the adequacy of static systems for producing infectious gametocytes using a 'reliable' parasite without the extra cost and space inefficiency involved with automated systems.

The high degree of significance for parasite isolate indicates that genetic differences among parasite isolates and homologous clones account for most of the variability seen in the infectiousness of gametocytes in culture. However, the 'phenotypes of infectiousness' cannot be detected by measuring parasite population parameters. Evidence for this was found as neither gametocyte sex ratios nor the mixing of two clones were significant predictors of infectiousness despite the recent attention directed toward gametocyte sex ratios (Read et al., 1992) and the role of cross-fertilization (Ranford-Cartwright et al., 1993) in malaria parasite populations.

In conclusion, this study highlights that the innate infectiousness of cultured *P. falciparum* gametocytes to *A. gambiae* cannot be determined by measuring specific parameters on a Giemsa-stained smear and a wet mount. The key to infectiousness lies in the parasite isolate or clone used which can only be identified by experimentation. A 'reliable' parasite negates the need to examine additional population variables because such efforts will not significantly improve one's ability to predict infection rates in *A. gambiae*.

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