High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host

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SUMMARY

To investigate the quantitative distribution of *Plasmodium falciparum* gametocytes into the vector bloodmeal, *Anopheles arabiensis* mosquitoes were fed on 3 volunteers naturally infected with gametocytes. The content of each mosquito midgut was smeared on a microscope slide and Giemsa stained. The distribution of gametocytes ingested by mosquitoes followed a negative binomial distribution, with apparently constant overdispersion (parameter $k \pm$ S.E. $= 3{\cdot}105 \pm 0{\cdot}392$) for the 3 series. This aggregation of gametocytes in some midguts probably facilitates the conjunction of gametes and fertilization. This suggests that *P. falciparum* gametocytes in the peripheral blood flow of infected man do not follow an independent, homogeneous pattern but show a significant aggregation.

Key words: *Plasmodium falciparum*, gametocyte, mosquito, aggregated distribution, malaria transmission.

INTRODUCTION

The mature gametocyte of *Plasmodium falciparum* is basically considered as a particle which is free in blood, passively moved around by the blood flow. Its fairly small size, close to the red cell, could suggest its allocation is homogeneous in all the compartment of blood circulation. If it is right, the number of gametocytes taken by a mosquito in its bloodmeal should follow a Poisson distribution with variability only due to sampling fluctuations and to variations in the volume of ingested blood.

Nevertheless it was observed that the oocyst numbers on the individual mosquito midgut are strongly overdispersed, and typically follow a negative binomial distribution, with variable mean depending on the gametocyte carrier, but with constant overdispersion (inverse of parameter k) (Medley *et al.* 1993; Pichon *et al.* 1996). The last authors found a constant value $k \pm$ S.E. $= 0{\cdot}267 \pm 0{\cdot}020$. To explain this observation, one possible hypothesis resides in the number of gametocytes ingested by mosquitoes that would be more variable than expected from a 'random', i.e. Poisson distribution for gametocytes in the blood feeding. To test this hypothesis we have performed the direct numbering of gametocytes in the bloodmeal of a large number of mosquitoes fed on a few volunteers.

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MATERIALS AND METHODS

Three adult men (designated by the letters A, B and C) naturally infected by *Plasmodium falciparum* and carrying gametocytes, were volunteers to let batches of mosquitoes ingest blood through their skin. The carriers were not infected by other plasmodial species and were asymptomatic. Volunteer A had had a simple malaria attack treated with halofantrine 13 days before the experiment; volunteer B, a simple malaria attack treated with chloroquine 8 days before; and volunteer C did not declare any clinical episode or anti-malarial treatment in the previous weeks. The gametocytaemia and the asexual parasite density of these volunteers were measured by means of a classic thick blood film realised at fingertip and Giemsa stained; the number of observed gametocytes (on 1800 microscopic fields for volunteer A who had a low gametocytaemia, and on 200 fields for volunteers B and C) was reported to the mean number of leucocytes per field, based on 8000 leucocytes/μl of blood.

Anopheles arabiensis mosquitoes – a species belonging to the *An. gambiae* complex – were collected at larval stages in Dakar, reared in our insectary at 27–28 °C and 70–85 % humidity, up to adult stage. Three-day-old mosquitoes, which had only been fed on sugar, were starved during 6–8 h. They were divided in batches of 35–40 females in cylindrical paper cups (volume 40 ml, diameter 5·5 cm) covered with a net. For each volunteer the feeding occurred on the same day and during a maximal period of 3 h.

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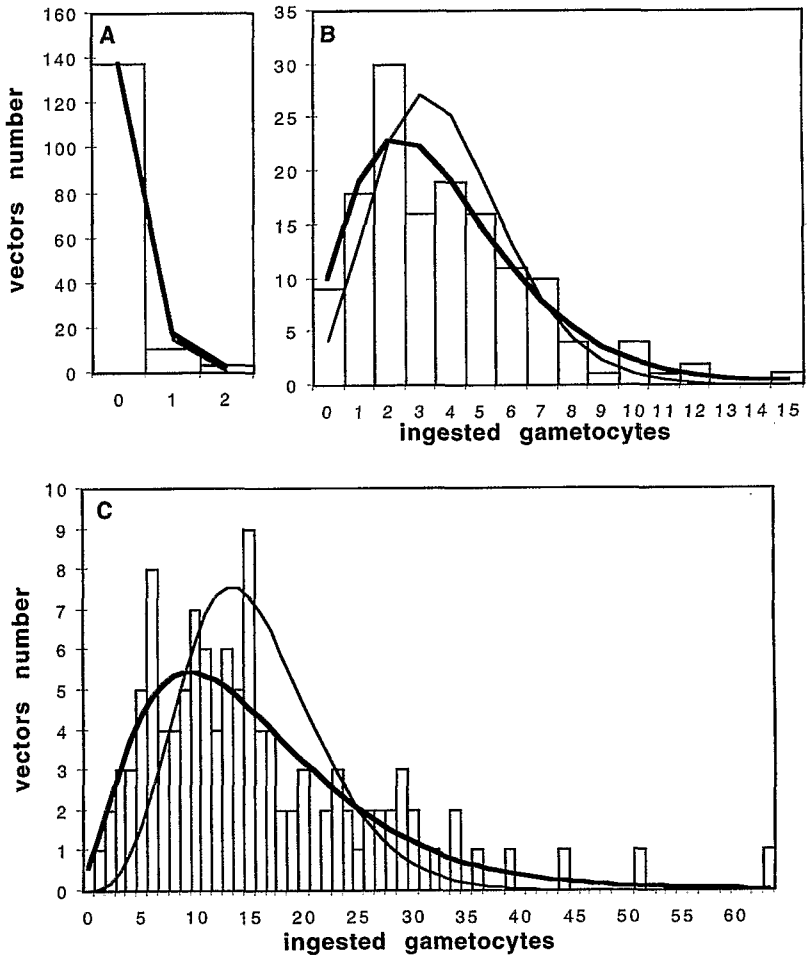

Fig. 1. Distribution of the number of mosquitoes (*Anopheles arabiensis*) as a function of the number of ingested *Plasmodium falciparum* gametocytes. Component parts A, B and C correspond to the 3 volunteers A, B and C. Observed histogram (bars) is compared with negative binomial (thick line) and Poisson (thin line) distributions. For the Poisson distributions in volunteers B and C, notice the deficit shown for frequencies corresponding to low and high intakes, and the excess of frequencies corresponding to medium intakes. For very low mean densities of gametocytes, as in volunteer A, even if overdispersion is found significant, negative binomial and Poisson distributions are difficult to distinguish.

Mosquitoes were fed on volunteers A, B and C, respectively on 26 December 1997, on 26 October 1998, and on 12 October 1998. The cups containing mosquitoes were placed in contact with the skin of volunteers for 15 min, in an insectary under dark conditions. Straight after, mosquitoes were killed using formaldehyde and examined under a binocular microscope. Only the fully fed mosquitoes were dissected using micro-needles for midgut extraction. The midgut and its content were dry-smeared on a microscopic slide. To facilitate the microscopic reading, the smear was spread on to a size of 2–3 mm wide and 10–15 mm long. These slides were dried for 1 day at room temperature, stained with a 6% Giemsa solution for 20 min, washed with water, and dried; in order not to get unstuck, they were continuously maintained flat and did not endure agitation in staining and washing liquids.

The count of gametocytes was done using a microscope and the oil immersion objective 100×. The totality of each blood smear was observed by taking the mean of successive adjacent crossings in its width. The average time of a reading reached 50 min. Thirty slides were read twice with reproducible results, which validate this counting method (results not shown).

The quantitative distribution of gametocytes in the mosquito gut contents was analysed using the methods described by Anscombe (1950), Bliss & Fisher (1953) and Bliss & Owen (1958). These methods are implemented in the freeware ParaDis (Pichon & Mulon, 1998).

RESULTS

In the thick blood film, volunteers A, B and C harboured gametocytaemia of 0·6, 2·9 and 45·7/μl, respectively, and asexual parasite density of 0·0, 11·6 and 5·7/μl. In the bloodmeal smears gametocyte morphology was frequently typical of activating or exflagellating forms.

Table 1. Main statistics and results for the 3 experiments with the gametocytes of *Plasmodium falciparum* counted in the bloodmeals of *Anopheles arabiensis*

(k*, Maximum likelihood estimate of k (inverse of the overdispersion). T, Anscombe (1950)'s statistic testing the tail size of the distribution.)

	Volunteer A	Volunteer B	Volunteer C
Sample size: number of mosquitoes	152	142	114
Minimal intake: number of gametocytes	0	0	1
Maximal intake: number of gametocytes	2	15	63
Total number of gametocytes	17	549	1765
% of male gametocytes ± S.E.	23·5 ± 10·3	33·5 ± 2·0	27·6 ± 1·1
Number of mosquitoes without gametocyte	138	9	0
% prevalence in mosquitoes	9·2	93·7	100·0
Arithmetic mean of gametocytes ± S.E.	0·112 ± 0·028	3·866 ± 2·819	15·482 ± 10·51
Williams mean of gametocytes	0·074	3·093	12·606
k* ± S.E.	0·357 ± 0·318	3·788 ± 0·928	2·869 ± 0·441
χ^2 deviation from Poisson (D.F.)	188·65 (151)	289·8 (141)	806·7 (113)
P value (χ^2)	0·020	$< 10^{-4}$	$< 10^{-4}$
χ^2 deviation from neg. binomial (D.F.)	0·0004 (1)	5·247 (9)	9·883 (16)
P value (χ^2)	0·984	0·812	0·872
T ± S.E.	−0·025 ± 0·063	−0·430 ± 6·590	312·6 ± 379·4
P value (T)	0·658	0·526	0·205

D.F., Degrees of freedom.

The count of gametocytes in the mosquito midguts is separately exposed for each volunteer (Fig. 1). The 3 distributions of gametocytes were overdispersed and significantly differed from a Poisson distribution. On the contrary, they were in close agreement with a negative binomial distribution (Table 1). A test for homogeneity was performed with the values of k obtained from the 3 experiments and was not significant ($\chi^2 = 0{\cdot}29$, D.F. = 2, $P = 0{\cdot}87$), allowing the calculation of a common k estimate of $k_c \pm$ S.E. = 3·105 ± 0·392. (In this calculation, the relative weight of the Experiment A is very weak due to its low mean.)

DISCUSSION

This is the first study that highlights heterogeneity in the passage of *P. falciparum* gametocytes from man to mosquito. Is this heterogeneity a consequence of some other heterogeneities? It was possible to dismiss 3 of them, i.e. the blood smear, the mosquito blood volume, and the mosquito pools fed sequentially.

(*1*) *Spatial heterogeneity in thick smears*

A study on the distribution of gametocytes counts in 400 microscope fields was conducted on 10 positive thick smears from different carriers. The mean density per field varied from 0·067 to 4·265. The variance/mean ratios varied from 0·956 to 1·265, with a mean value ± S.D. of 1·046 ± 0·095, not significantly different (Student's $t = 0{\cdot}091$; $P = 0{\cdot}46$) from the random, Poisson expectation 1. The same result was obtained by the ANOVA method of Bliss & Owen (1958): the $1/k$ common value of 0·041 (k = 24·3) was not significantly different from zero (Fisher-Snedecor's $F(1,7) = 4{\cdot}24$; $P = 0{\cdot}077$). In other words, the spatial distribution of gametocytes in each thick smear appeared to be random. But we don't know if the defibrination process used for preparing the thick smear had disaggregated some putative gametocyte clusters.

(*2*) *Heterogeneity due to the volume variation of blood intakes*

Possible variability in the volume of blood intakes interfered poorly with observations for the following reasons. (i) Partly fed mosquitoes were not examined. (ii) Counts of leucocytes were performed on smears of midgut contents and produced homogeneous data (results not shown). (iii) A simulation was carried on the basis of a Gaussian variation of the midgut blood-content, with mean (m_v) ± S.D. of 2·7 ± 0·5 μl (Vaughan, Noden & Beier, 1991): if the distribution for a constant volume is Poisson ($1/k = 0$), the overdispersion resulting from a Gaussian variation in volume of ingested blood is found to be the square of the volume coefficient of variation $1/k = (s_v/m_v)^2$. This simulation gave a value of $1/k = 0{\cdot}0342$ (k = 29·2, much larger than $k = 3$, and undistinguishable from a Poisson distribution). In conclusion, the variation in the ingested blood per midgut was too

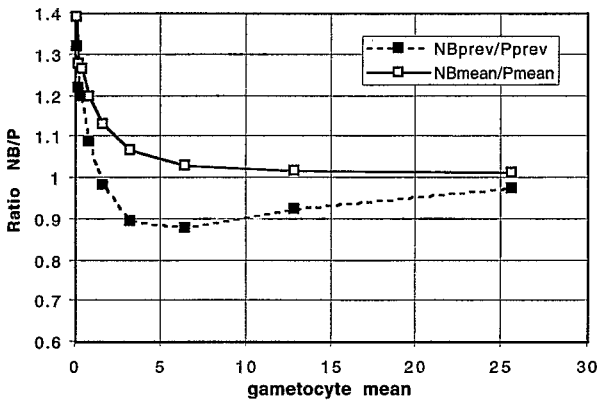

Fig. 2. Advantage of gametocyte binomial distribution ($k = 3$) on Poisson distribution, measured by ratios of potential zygote means (solid line) and of prevalences (dashed line) obtained either through gametocyte negative binomial or Poisson distributions, as a function of mean gametocyte density. Negative binomial is advantageous when the curve is over the 1-ordinate horizontal line.

small to explain the variations in the observed numbers of gametocytes.

(3) *Heterogeneity between mosquito pools*

To check if a part of the variation in gametocyte number per midgut was correlated with pools or time, a study was conducted on the dynamics of the 5 successive mosquito pools infected on each of the volunteers. For each series of 5 pools, 2 statistical methods were employed: (i) simple ANOVA on log $(x+1)$ transformations; this method being suspected to give rise to type I and type II errors (Wilson, Grenfell & Shaw, 1996); (ii) direct estimation by 10000 simulations of negative binomial samples of the same size as the observed ones with $k_c = 3{\cdot}105$. The ANOVA tests showed no significant departure from equality between pools (respectively $P_A = 0{\cdot}39$; $P_B = 0{\cdot}29$; $P_C = 0{\cdot}61$); neither do the direct tests ($P_A = 0{\cdot}108$; $P_B = 0{\cdot}070$; $P_C = 0{\cdot}076$).

Thus, of the 3 invoked possible causes of heterogeneity, none is able to explain the observed overdispersion of gametocyte numbers.

Consequences of heterogeneity

Aggregates of gametocytes were demonstrated in the mosquito midguts. This finding is important and has implications in the following domains.

(1) It increases the zygote yield and, in part, prevalence: 'togetherness' (May, 1977) of many gametocytes and in some mosquitoes increases the probability of conjunction for parasite gametes belonging to different sexes. This is confirmed in a series of 100 simulations on pools of 1000 vectors, each exposed to increasing mean densities (from 0·1 to 25) of gametocytes and following a negative binomial with $k = 3{\cdot}0$ or, for comparison, a Poisson distribution. The gametocyte sex-ratio (male:female) is 3:7 (Robert *et al.* 1996), and the number of efficient flagellae per microgametocyte was supposed to follow a (positive) binomial distribution, with 'canonical' expectance 2·333 (i.e. gametes equality, in accordance with Fisher's (1958) principle) and maximum value 8. The number of potential zygotes was only determined by the numbers of female and male gametes present. For each gametocyte density, the gametocyte load in each vector was randomized either from a negative binomial (with $k = 3$) or from a Poisson distribution, the sex of each gametocyte was randomized according to the fixed sex ratio, for each microgametocyte the number of male gametes was randomized from a positive binomial, allowing to obtain the individual vector content of resulting potential zygotes. After each series of 1000 vectors exposed to the same gametocyte density, the 2 zygote means, namely m_{NB} and m_P (respectively corresponding to the negative binomial and the Poisson distributions) and prevalences, namely p_{NB} and p_P, are calculated for the whole series. For comparison, 2 ratios were calculated, the means ratio $r_m = m_{NB}/m_P$ and the prevalences ratio $r_P = p_{NB}/p_P$. A ratio higher than 1 indicates an advantage in zygote yield or in prevalence when the gametocyte distribution is negative binomial. Negative binomial distribution of gametocytes was always advantageous for zygote mean number; the highest impact was observed at low gametocyte densities, where the ratio is more than 40% over equality of the distributions effect. An analogous situation was found with 32% over equality for zygote prevalence resulting from a negative binomial when the gametocytes density was lower than 2, but over this value, Poisson prevalence was slightly higher than negative binomial. Thus, clumping of gametocytes seems to be advantageous for zygote density without restrictions, and for zygote prevalence when gametocyte density is low.

However, the zygote overdispersion index k_z tends towards infinity when the gametocyte distribution is Poisson, and is slightly lower than 3·0 in the negative binomial situation. The gametocyte heterogeneity and the mating process thus maintain or increase overdispersion of later stages, but not enough to explain the observed overdispersion of oocysts ($k_o = 0{\cdot}3$) (Billingsley *et al.* 1993; Medley *et al.* 1993; Pichon *et al.* 1996).

(2) It partly explains the fact that very exceptionally 100% of mosquitoes become infected after 1 experimental infective bloodmeal on gametocyte carrier, some of them had been ingesting zero, 1 or several gametocytes of the same sex. In particular, it can be found that the probability of getting less than 2 gametocytes (zygote impossibility) is greater for a negative binomial than for a Poisson: the probability 1/10000 is reached respectively for a mean $m = 100$ gametocytes, and $m = 16$.

(3) It suggests that *P. falciparum* gametocytes

do not follow a homogeneous pattern in the peripheral circulation of infected man. Do clusters of mature gametocytes in the peripheral blood exist? If yes, is a cytoadherence process, well known in *P. falciparum*, implicated? If yes, does the parameter k constitute an indirect measure of the global cytoadherence for an infected individual? At this time these questions remain open.

Gametocytaemia and number of gametocytes per midgut

In our study, the ratio between the mean number of ingested gametocytes and the gametocytaemia estimated on a thick smear realized at fingertip would indicate that means of 0·18, 1·33 and 0·34 μl of blood were ingested by mosquito from volunteers A, B and C, respectively. However, *An. arabiensis* takes approximately $2{\cdot}7 \pm 0{\cdot}5$ μl of blood at each feeding (Vaughan *et al.* 1991). It suggests that the gametocyte density of *P. falciparum* is lower in the blood compartment accessible for mosquitoes than in the compartment sampled at fingertip for the estimation of gametocytaemia. In other studies, for *P. yoelii* and *P. inui*, important differences had been also noticed in the density and relative age pattern of gametocytes in the bloodmeal of recently fed mosquitoes as compared to blood drawn from the peripheral circulation (Landau *et al.* 1979; Dei-Cas *et al.* 1980).

Negative binomial distribution

Distributions following a negative binomial pattern are frequent in parasitology. Among macroparasites, this aggregative process had been often observed and analysed. In natural populations, for example in human foci of lymphatic filariasis, the distribution should be zero-truncated, (Pichon *et al* 1980*a*; Grenfell *et al.* 1990). The combinations of Poisson distributions with different means was proposed to explain the overdispersion among hosts (Jaenike, 1994). The intake of *Wuchereria bancrofti* microfilariae by the mosquitoes follows a geometric distribution, a special case ($k = 1$) of the negative binomial (which can be explained by the constitution of 'waiting queues' in the capillaries (Pichon *et al.* 1980*b*)). It is possible that our observations suggesting a high variability in the flow of gametocytes in the blood vessels and capillaries in one host are also under control of mechanical constraints.

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