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Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan

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Abstract

Malaria in eastern Sudan is characterised by limited seasonal transmission, with the majority of the year remaining transmission-free. Some inhabitants who contract malaria during the transmission season retain long-lasting sub-patent infections, which probably initiate transmission the following year. Here we have monitored *Plasmodium falciparum* infection prevalence and gametocyte production during the dry season, and examined the impact of parasite genetic multiplicity on infection longevity. A cohort of 38 individuals who were infected with *P. falciparum* in November 2001 was monitored monthly by microscopy and PCR until December 2002. Reverse transcriptase polymerase chain reaction of the *pfg*377 gene was used to detect sub-patent gametocytes. In addition, all isolates were examined for *msp*-2 alleles and the mean number of parasite clones per infection was estimated. We found that a large proportion (40%) of the cohort retained gametocytes throughout the dry season. The majority of patients retained asexual infection for at least 7 months. Genetic multiplicity of *P. falciparum* significantly influenced longevity of asexual infection and its gametocyte production. Gametocytes from mixed genotype *P. falciparum* infections persisted three times longer than those from single genotype infections, suggesting that genetic diversity promotes persistence. These findings are discussed in the context of the parasite biology and malaria epidemiology in the study area.

Keywords: *Plasmodium falciparum*; Gametocytes; RT-PCR; Seasonal malaria

1. Introduction

Reproductive success is a central life history determinant of the evolutionary fitness of living organisms. For malaria parasites (*Plasmodium* spp.), the production of mosquito-infective gametocytes is important not only for propagation, but also for generation of new strains—e.g. those capable of evading human immune responses and anti-malarial drug pressure. An understanding of the biology of gametocytogenesis is therefore critical when considering control strategies aimed at limiting the reproductive success of the malaria parasite.

Plasmodium falciparum clones have been found to vary significantly in their capacity to produce gametocytes in culture, and gametocytogenesis has been suggested to be a stable, genetically determined characteristic (Graves et al., 1984). In vertebrates, malaria infection is initiated by mosquito-delivered sporozoites that invade the liver, then differentiate and rapidly multiply as asexual forms in the circulation. Some asexual parasites differentiate into gametocytes, forms that cannot replicate but can be transmitted to mosquitoes. It takes approximately 9–12 days for gametocytes to be produced from asexual forms. Once mature, gametocyte longevity and their infectivity to mosquitoes

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probably vary over time (Hawking et al., 1971; Smalley and Sinden, 1977). Therefore, consistent gametocyte production by individual parasite clones is critical for transmission.

The low density of *P. falciparum* gametocytes in natural infections appears to contradict the apparently high transmission success of this parasite in nature (Taylor and Read, 1997). The recent advent of molecular diagnosis of gametocytes has in part resolved this paradox by demonstrating the occurrence of numerous sub-microscopic gametocyte reservoirs in people living in endemic areas (Menegon et al., 2000; Abdel-Wahab et al., 2002), and has also identified multiple gametocyte genotypes within a single infection (Abdel-Wahab et al., 2002).

In the present study, we have exploited these molecular techniques to examine the longevity of gametocytogenesis of *P. falciparum* during the transmission-free season of eastern Sudan. In this region, malaria transmission is limited to the short rainy season, and pauses over a period of 7–9 months during the dry season (Hamad et al., 2002). Previous studies have indicated that *P. falciparum* appearing in the transmission season gives rise to asymptomatic sub-patent asexual parasitaemia that persists throughout the dry season. Furthermore, genotypes of different clones can survive together in individual infections and fluctuate through this period (Babiker et al., 1998). Limited cross-sectional surveys have suggested that some of these long lasting infections are capable of sustaining gametocyte production during the dry season (Abdel-Wahab et al., 2002). The aim of this study was to elucidate the longevity of individual *P. falciparum* infections, and to examine the impact of clonal multiplicity on longevity and gametocytogenesis among clones that survive the dry season as chronic asymptomatic infections.

2. Materials and methods

2.1. Study area

The study was carried out in Asar village (longitude 35°.30′ E and latitude 13°.30′ N), Gedaref state, eastern Sudan, where malaria transmission is short and distinctly seasonal following the annual rains (July–November), reaching a peak in October. However, by January, the number of malaria cases drops substantially and entomological surveys have shown no evidence of transmission during the long dry season (Hamad et al., 2002). *P. falciparum* is the predominant malaria parasite accounting for more than 90% of all malaria infections. *Anopheles arabiensis* is the main mosquito vector. The entomological inoculation rate is less than one bite per person during the transmission season (Babiker et al., 1997).

2.2. Patients and blood samples

Inhabitants with malaria symptoms attending the clinic in Asar during the transmission season (November 2001) were examined microscopically for *P. falciparum* infections. A cohort of 121 patients, including adults and children, with microscopically diagnosed *P. falciparum* malaria (asexual parasitaemia) was recruited and followed monthly until the end of December 2002. All patients were treated with the standard curative dose of chloroquine (25 mg/kg). Here we report on 38 patients from whom complete parasitological and molecular data (PCR for identification of sub-patent *P. falciparum* infection, typing of three polymorphic genes and detection of sub-patent gametocytes using RT-PCR) were collected.

Before treatment and on subsequent visits, approximately 2 ml of venous blood was collected from each participant. These blood samples were processed and stored for RNA isolation (Babiker et al., 1999). At the same time, a few drops of blood were spotted onto filter paper and stored for genomic DNA isolation (Plowe et al., 1995). Thick and thin blood smears were stained with Giemsa's stain and examined microscopically for *P. falciparum* asexual and/or gametocyte infection. Parasites were counted per 300 leukocytes, assuming 6000 leukocytes/μl of blood for inhabitants of this area (Bayoumi et al., 1989).

The blood samples were collected with the informed consent of all patients involved. The project was given ethical clearance by the Ethical Committee of the Ministry of Health, Sudan.

2.3. Detection of sub-patent gametocytes

PCR was used to detect the presence of sub-patent *P. faciparum* infection in the blood samples. DNA was first prepared as described elsewhere (Plowe et al., 1995) and the *pfg*377 gene was amplified as described earlier (Menegon et al., 2000). Reverse transcription and subsequent amplification of cDNA (RT-PCR) was used to examine the presence of RNA of the gametocyte specific gene *pfg*377. Since *pfg*377 is expressed only in *P. falciparum* gametocytes (Alano et al., 1995), detection of RT-PCR products of this gene indicate the presence of gametocytes in examined parasite samples, while absence of *pfg*377 mRNA indicates that the infection consists only of asexual forms. Total RNA was first isolated using a high pure RNA isolation kit (Roche, UK). RT-PCR of *pfg*377 was performed in a two step reaction using GenAmp RNA PCR core kit (Roche, UK). The conditions and primers used were as previously described (Menegon et al., 2000).

2.4. Characterisation of pfg377 and msp-2 alleles

The *pfg*377 gene contains four regions with repetitive sequences, the most polymorphic being region three which encodes seven degenerate amino-acid repeats; alleles of this gene thus vary by multiples of 21 base-pairs (Alano et al., 1995). Precise sizing of *pfg*377 alleles was carried out following conventional PCR (genomic DNA, all parasite stages) and RT-PCR (RNA, gametocytes) using

a fluorescent-labelled primer. The PCR products were run on an ABI automated sequencer and the polyacrylamide gel images then analysed with Genescan and Genotyper software (Applied Biosystems) as described by Abdel-Wahab et al. (2002).

Alleles of the highly polymorphic merozoite surface protein-2 (*msp*-2) were analysed, in genomic DNA only, using sequence-specific primers. *msp*-2 primers specific to the known two allelic types IC1 and FC27 were used as described by Zwetyenga et al. (1998), and alleles were identified based on their sequence types.

2.5. Statistical analysis

The overall purpose of the statistical analysis was to examine the relationship between *P. falciparum* infection, genetic diversity and the prevalence and longevity of asexual and gametocyte forms. Chi-squared tests were used to evaluate whether the frequency of gametocyte production in mixed infections was higher than in those designated as single infections. We conducted survival analysis to evaluate the relationship between gametocyte longevity (defined as number of months detectable) and multiplicity of infection (scored as one for single allele infection, and two for multiple infections that had more than one allele). Each patient was assigned a longevity value for the number of months over which they harboured detectable parasites (by microscopy or molecular analysis). Separate values of longevity were computed for asexual and gametocyte forms. Cox regression analysis was then used to investigate whether the longevity of asexual and gametocyte forms was related to the genetic diversity of infection (SPSS version 12, www.SPSS.com). Here we designated multiplicity as the number of genotypes detected throughout the entire study period (patients who exhibited only one genotype were designated as having a single infection, those that exhibited more than one throughout the study period were designated as having mixtures). In determining longevity, patients were assumed to have persistent infection if parasites were detected in consecutive months, or disappeared but reappeared again during the transmission-free season (January–August). Patients were considered to have cleared their infections if parasites disappeared and remained undetected for the remainder of the study period.

3. Results

3.1. P. falciparum infection and gametocytes among the cohort

At the start of the study in November 2001 (transmission season), all 121 patients recruited to the study harboured microscopically detectable *P. falciparum* asexual infections. Following chloroquine treatment, both asexual parasite and gametocyte densities dropped dramatically and by the beginning of March 2002, none of the patients harboured microscopically visible asexual forms or gametocytes (Fig. 1(A)).

Polymerase chain reaction (PCR) and RT-PCR were carried out on 494 blood samples collected from 38 patients, over a period of 13 months. The microscopically positive parasite rate among these patients decreased to 28.9, 18.4 and 10.5% in December 2001, January 2002 and February 2002, respectively. In contrast, the parasite rate as estimated by PCR for the same period was 71.1, 63.2 and 45.9%, respectively. No parasites were detected by microscopy between March and September 2002 (the dry season) in this cohort, however, a large proportion of the cohort maintained *P. falciparum* parasites detectable by PCR through the dry season until the start of the next transmission season in October 2002 (Fig. 1(B)).

With regard to transmission potential, 82% of the 38 infected individuals examined produced gametocytes at some point during the study. In the transmission season (November 2001), 64.9% of the cohort carried sub-patent *P. falciparum* gametocytes, a proportion that was almost five times higher than what would have been estimated by microscopy alone (13.2%). In subsequent surveys in December 2001 and January 2002, the gametocyte microscopy rate was 3 and 8%, respectively, in comparison

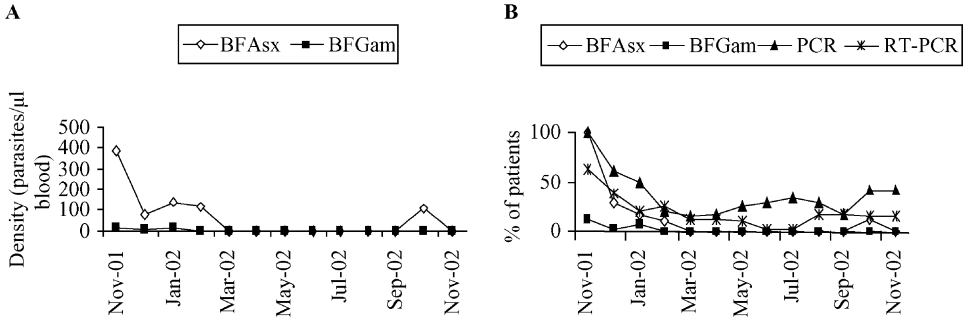

Fig. 1. *P. falciparum* asexual form (Asx) and gametocytes (Gam) during the study (November 2001–December 2002) (A) mean parasite density revealed by microscopy (BF) among 121 patients who completed the study, and (B) parasite prevalence among 38 patients investigated by microscopy (BF), PCR and reverse transcriptase PCR (RT-PCR).

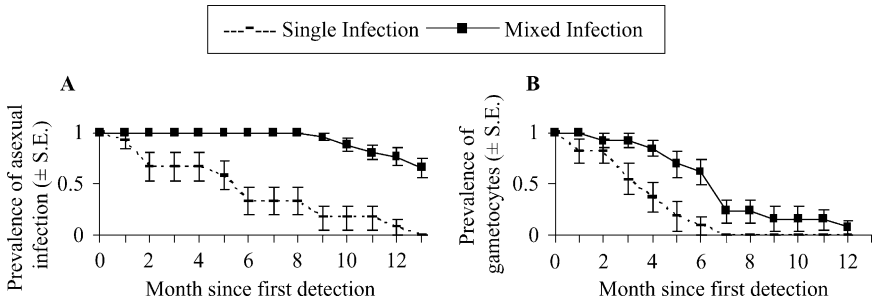

Fig. 2. Longevity of *P. falciparum* infections (A) and gametocyte carriage (B) among patients with single and multiple infections in Asar village between November 2001 to December 2002.

to 39 and 21% as detected by RT-PCR. During the dry season (February 2002–July 2002), no gametocytes were detected by microscopy. However, sub-patent gametocytes persisted throughout this period, although with progressive reduction (Fig. 1(B)). In total, 42% of those recruited maintained sub-patent gametocytes throughout the dry season until the next transmission season (Fig. 1(B)).

3.2. Multiplicity of *P. falciparum* infection

At the start of the study (transmission season, November 2001), 18 (47%) out of the 38 patients examined had multiple-clone infections. At this time, the estimated minimum mean number of *P. falciparum* clones per patient among the cohort was 1.9. However, parasite multiplicity fell during the dry season so that only single infections were detected by April. After this time multiplicity increased slightly, with mixtures constituting 10–30% of all infections detected during the remainder of the study period. In contrast to the mean multiplicity estimated during the transmission season (1.9 clones/individual), the mean minimum number of clones dropped to 1.3 during the dry season (February–August 2002), and did not increase significantly in the peak of the next transmission season (October/November 2002).

With regard to gametocytes, 11 out of 25 patients were found to harbour a single gametocyte genotype, and 14 had multiple clones detectable by the presence of more than one RNA form of *pfg*377.

3.3. Longevity of *P. falciparum* infection and gametocytes

3.3.1. Asexual forms

Longevity of *P. falciparum* infection varied markedly between individuals, with the majority, 25 out of 38, retaining chronic sub-patent infection until the next transmission season. All of the 13 patients who cleared their infection did so during the first 3 months after treatment.

Twelve of the 38 individuals in the cohort were designated as having single clone infections, and 26 had mixed clone infections. The relationship between parasite multiplicity and survival of asexual infection was highly significant ($\chi^2_1 = 31.37$, $P < 0.01$). Overall, the parasite clearance rate in the single clonal infection group was nine times faster than in the multiple-clone group (Fig. 2(A)). Whereas 50% of single-clone infection had cleared within 5 months, 65% of multiple infections remained until the end of the monitoring period (13 months).

3.3.2. Gametocyte forms

Ten (40%) of the 25 patients who harboured gametocytes during the study period retained them as a sub-patent infection for up to 7 months until the start of the next transmission season.

The prevalence of gametocytes was higher in the multiple-clone infection group compared to the single-clone infection group. Seven (58%) out of 12 patients with single clone infection produced gametocytes compared to 22 (85%) out of 26 ($\chi^2 = 3.14$, $P = 0.08$) with multiple clones.

The longevity of gametocytes was examined only amongst the cohort of 25 patients who exhibited gametocytes during the course of the study, with 11 and 14 having genotypes representative of single and multiple genotypes, respectively, detectable by the presence of more than one RNA form of the *pfg*377 gene.

Patients with mixed gametocyte genotypes sustained their gametocytes for a significantly longer period of time than those with a single genotype. ($\chi^2_1 = 7.66, P < 0.01$, Fig. 2(B)). *P. falciparum* gametocyte infections that were genetically homogeneous were cleared three times faster than those consisting of multiple clones (odds ratio 3.2, 95%, confidence interval 1.4–8.4). The median survival of infection consisting of multiple gametocyte genotypes was almost two times greater than those having only a single gametocyte genotype (Fig. 2(B)).

Contrasting between parasite forms but pooling across multiplicity levels, gametocytes were cleared at a much faster rate than asexual infections. Overall, the mortality of gametocyte infections was five times higher than that of asexual infections ($\chi^2_1 = 33.39$, $P < 0.01$).

4. Discussion

We have exploited the unique malaria epidemiology setting of eastern Sudan to examine *P. falciparum* gametocytogenesis and longevity during the lengthy dry and transmission-free season (Hamad et al., 2002). We have investigated within-host survival and transmission potential (gametocytogenesis) of individual *P. falciparum* clones during this period, and whether they are influenced by clonal multiplicity. The longevity of *P. falciparum* infection and gametocyte carriage varied markedly between individuals. A large proportion of patients maintained sub-patent *P. falciparum* parasites, which sustained gametocyte production throughout the dry season into the next transmission season. The average length of *P. falciparum* gametocyte carriage was significantly shorter than that of asexual forms.

Natural *P. falciparum* infections often consist of multiple parasite genotypes coexisting in the same host (e.g. Conway et al., 1991; Babiker and Walliker, 1997; Smith et al., 1999). This is primarily due to the continuous burden of transmission in endemic countries, which puts people at high risk not only of infection, but also of cumulative super-infection (Gu et al., 2003). The impact of such genetic multiplicity on the biology and epidemiology of *P. falciparum* is not clearly understood. Previous work has demonstrated evidence of within-host interaction between co-existing parasite clones of the rodent malaria parasite *P. chabaudi* and *P. falciparum*, leading to an increase in parasite transmission—presumably due to competitive stress (Taylor et al., 1997; Smith et al., 1999). However, there is only limited information on the longevity of individual *P. falciparum* clones and their ability to produce gametocytes in nature. Here, we have demonstrated that clonal multiplicity significantly enhances both the longevity of *P. falciparum* infection and their ability to produce gametocytes. A large proportion of *P. falciparum* clones identified at the start of the dry season in January 2002 persisted until the end of the dry season September 2002. No novel parasite genotypes were seen among the examined cohort during the dry season, showing that our analysis was not confounded by re-infection during the period of the study; this is consistent with our previous surveys that found no evidence of transmission throughout the dry season (Hamad et al., 2002).

All patients were treated with chloroquine at the start of the study as they all had clinical malaria. Antimalarial drug usage has been shown to induce gametocytogenesis in surviving malaria parasites (Buckling et al., 1997), although, there was no obvious association between drug resistant phenotype and gametocytogenesis in some laboratory clones of *P. falciparum* (Buckling et al., 1999). In our current work, the examined patients remained asymptomatic and did not take extra anti-malarial drugs during the long dry season.

Survival and longevity of *P. falciparum* among immune and semi-immune inhabitants of the tropics is still poorly understood. The use of PCR has demonstrated that a large proportion of *P. falciparum* infections exist asymptomatically at sub-patent levels (e.g Babiker et al., 1998; Bottius et al., 1996). It has previously been suggested that infections of long duration have a strong tendency to produce gametocytes (Smalley et al., 1981). In the present study, we have shown that longevity of both asexual forms and gametocytes of *P. falciparum* were significantly enhanced by clonal multiplicity. The probability of clearance of a single-clone infection was nine and three times that of multiple-clone infection for asexual forms and gametocytes, respectively. This is consistent with previous findings from Tanzania where *P. falciparum* multiplicity was found to be associated with chronic infection (Smith et al., 1999). Work on the rodent malaria model *P. chabaudi* has also suggested that multiple clone infections tend to persist longer as a chronic infection than a single clone infection (De Roode et al., 2003). It has been suggested that multiple clone infections can elicit greater antigenic variation than single clone infections, and can thus overcome rising immune responses more effectively (e.g. Biggs et al., 1991). However, we did not see sequential peaks in parasite density during the dry season and the parasitaemia has remained constantly low, at sub-patent levels. This suggests that a density dependent mechanism, which is not strain-specific (Bruce et al., 2000), may limit parasite multiplication, while antigenic variation may sustain parasite survival. Such a state of premunition is evident among patients who maintained sub-patent infections, during the dry season into the next transmission season, who did not encounter fresh infection, develop high parasitaemia, or show an increased number of clones.

For *P. falciparum*, gametocyte production from asexual forms takes approximately 9–12 days. Once mature, it is thought that gametocyte longevity and mosquito infectivity potential are limited (Hawking et al., 1971). The average gametocyte circulation time has recently been estimated as 6.4 days (Eichner et al., 2001), which is more than twice the previous estimated half-life of 2.4 days (Smalley and Sinden, 1977). Factors that can enhance and lead to sustained gametocyte production in long-lasting infection can therefore have significant epidemiological impact. Ecological genetic theory suggests that within host competition, aimed at optimising the balance between parasite asexual growth and sustainable transmission, can instigate gametocyte production and parasite transmission (Read and Taylor, 2001). Some studies on the rodent malaria model *P. chabaudi* have examined the relationship between parasite genetic complexity and transmission potential. Gametocyte and mosquito infectivity were found to be enhanced in multiple-clone infections compared with single clone infections (Taylor et al.,1997), but the mechanism for this increase was not determined. However, another recent study on *P. chabaudi* has demonstrated lower transmission potential in multiple clone infections

compared to single-clone infections (De Roode et al., 2003). The different results in each study have been attributed to variation in genetic backgrounds of the clones used (De Roode et al., 2003). The relationship between parasite diversity and transmission is critical for understanding within host dynamics and its epidemiological and evolutionary consequences. Detailed studies of within-host interactions between malaria parasites can be addressed adequately once quantitative assays for gametocyte stages are developed.

We have used the highly polymorphic gene *msp*-2 (Felger et al., 1999) as a marker to distinguish different parasite clones in this study. It is possible that some of the infections identified as single clones may have different alleles of other unlinked polymorphic genes and therefore be multiclonal. However, *msp*-2 was shown to have a high resolution for distinguishing individual *P. falciparum* clones compared to other polymorphic genes (Farnert et al., 2001). In view of the lower parasite diversity in eastern Sudan compared to areas of high malaria transmission (Babiker et al., 1997) we consider that *msp*-2 was an adequate marker for detection of clonal multiplicity.

We have demonstrated a clear association between clone multiplicity and longevity of *P. falciparum* infection and its ability to produce gametocytes. These findings have several implications for malaria epidemiology. First, multiple-clone infections, which are often found in nature, are more likely to survive and transmit to mosquitoes than single-clone infections. While we have not examined the infectivity of gametocytes that persist at sub-patent level during the dry season, it has been established that, gametocytes that exist at levels below that diagnosed by microscopy can be infectious to mosquitoes (Muirhead-Thompson, 1954). This in turn leads to a high probability of crossing and recombination between clones (Hill and Babiker, 1995), and subsequent generation of novel parasite strains. An extensive degree of genetic diversity is seen in most natural parasite populations, even in areas of low and seasonal malaria transmission such as eastern Sudan. Such a high rate of sexual reproduction is advantageous for parasite evolution in the face of drug pressure (Mackinnon and Hastings, 1998). Second, in areas of seasonal malaria transmission such as eastern Sudan, the large proportion of multiple-clone infections that persist during the dry season are the most likely source of continual transmission in subsequent years. Anti-malarial drugs that have a wide spectrum of activity, such as combination therapy, would have a substantial probability of reducing parasite multiplicity and destabilising transmission in this area. Parasite strains that give rise to secondary infections at the start of the transmission season are therefore likely to be novel and diverse, and capable of precipitating malaria outbreaks among the semi-immune inhabitants of these areas. These novel strains are likely to be different from pre-existing parasites among the asymptomatic carriers and thus will enhance parasite multiplicity. Analysis of clinical malaria cases that arise at the start of the transmission in an area of high transmission in Ghana demonstrated that multiplicity is likely to be associated with malaria morbidity (Ofosu-Okyere et al., 2001). Several field studies have pointed out that clonal multiplicity is associated with greater morbidity and disease severity (e.g. Robert et al., 1996).

Thus, within-host parasite diversity can be advantageous to persistence and evolution of the malaria parasite. The biological and epidemiological mechanism that drives parasite genetic diversity in nature is as yet uncertain. For example, it is not clear whether genetic variation is exclusively due to the high rate of super-infection in the human host, or possibly favourable processes in the mosquito vector (Ferguson and Read, 2002). Super-infection can happen often in nature due to the cluster pattern of mosquito distribution and infection risk (Smith et al., 1995). Therefore, even in areas of low and seasonal transmission such as eastern Sudan, inhabitants with previous infections can be exposed to infective bites in the next transmission season. In the present study we have observed a two-fold increase in parasite multiplicity in the transmission season among members of the cohort who maintained their infection throughout the dry season. In this village, the entomological inoculation rate is very low, and it is unlikely that an inhabitant would encounter more than one infective bite per transmission season (Babiker et al., 1997).

Within host diversity of *P. falciparum* is an important factor that contributes to both malaria epidemiology and parasite biology. The observed aggregated distribution of malaria parasites in nature is presumably driven by vector behaviour. This heterogeneity is advantageous to the evolution of the parasite. Control measures aimed at reducing within host parasite diversity should therefore make an important contribution to prevention of transmission of *P. falciparum*.

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