In vivo and in vitro gametocyte production of *Plasmodium falciparum* isolates from Northern Thailand

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Abstract

Understanding why some malaria-infected individuals are infective to mosquitoes while others are not, is of great importance when considering interventions to stop malaria transmission. Whether gametocytes are produced in every individual infected with *Plasmodium falciparum* remains unclear. Using a highly sensitive reverse transcription (RT)-PCR assay, we attempted to detect gametocyte-specific mRNA transcripts in isolates from Thai patients which newly adapted to continuous in vitro culture. We then compared the allelic types of the *pfg377* gene between patient blood and culture-adapted parasites in order to determine whether the same parasite lines were producing gametocytes in vivo and in vitro. Transcripts of *pfg377* were detected in all parasite isolates and in the corresponding cultured isolates, revealing that all patients had gametocytes circulating in their blood at the time of sampling. For isolates in continuous in vitro culture, there was a match between *pfg377* allelic types detected by PCR from genomic DNA (and thus indicative of the dominant allelic type of asexual parasites) and those detected by RT-PCR of mRNA (gametocyte-specific), whereas in freshly isolated patient blood there were some differences between the asexual parasite allelic type and that of the gametocytes in the same infection. Seven isolates contained asexual stage parasites harboring *pfg377* alleles that were not detectable in gametocytes from the same infections, suggesting that some clones were not producing gametocytes at the time of sampling, or that they were below the level of detection.

Keywords: *Plasmodium falciparum,* Gametocyte detection, *pfg377*, Allelic types, malaria

1. Introduction

When malaria gametocytes are taken up by mosquitoes during blood feeding, male and female gametes fuse to form diploid zygotes which then undergo sporogony to form haploid recombinant progeny. Thus, not only are gametocytes responsible for the transmission of the parasite from one host to another, but they also play a role in generating parasite genetic diversity. An understanding of the size and distribution of the gametocyte population is, therefore, of importance when considering malaria epidemiology.

The detection of gametocytes has traditionally relied on the examination of blood smears by microscopy. The recent application of more sensitive molecular detection methods such as real-time nucleic acid sequence based amplification (QT-NASBA) (Schneider et al., 2005), reverse transcription-PCR (RT-PCR) (Maeno et al., 2008) and reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) (Buates et al., 2010) have revealed that the proportion of parasite-infected individuals who harbour gametocytes is much higher than previously thought, and has led to a much clearer picture of the patterns of gametocyte carriage amongst infected individuals (Ouedraogo et al., 2009). The limit of detection of gametocytes by thick-smear microscopy is in the range of five gametocytes per µl, while molecular techniques such QT-NASBA can detect as few as 0.02 gametocytes per µl (Schneider et al., 2007). The application of QT-NASBA to a study of gametocyte carriage rates in uncomplicated malaria cases from Kenya, for example, revealed that 90% of infections harboured gametocytes; when the same infections were assayed by microscopy, only 25% had detectable levels of gametocytaemia (Bousema et al., 2006b). Although it seems unlikely that gametocyte densities below 0.3 per µl would be infectious to mosquitoes, submicroscopic densities (i.e. those below five gametocytes per µl) above this threshold have been shown to result in mosquito infections (Schneider et al., 2007). However, it should be noted that infectivity to mosquitoes does not necessarily correlate with gametocyte density (Nedelman, 1989; Hallett et al., 2006; Gadsby et al., 2009).

The gametocytes of *Plasmodium falciparum* are unique amongst studied malaria parasites in that their maturation time takes up to 8 days, during which time they sequester within the vasculature of the host, thus avoiding clearance by the spleen (Eichner et al., 2001). Their release comes long after the peak of asexual parasites, thereby avoiding exposure to TNF, the production of which is associated

with peak asexual parasitaemia, to which they are particularly sensitive (McKenzie et al., 2007). Following their release from sequestration, the mature and infectious gametocytes have been shown to remain in the circulation for a mean range of 4.6 – 6.5 days (Bousema et al., 2010). Following drug treatment with non-artemisinin combination therapy (ACT) drugs, which should kill all asexual parasites, patients in the same study were estimated to remain gametocytaemic for up to 55 days, however this was reduced to a mean gametocytaemic period of 13.4 days for those treated with ACTs (Bousema et al., 2010).

Previously, when gametocyte detection was performed exclusively by microscopy, it was thought that children below the age of 5 years were the predominant gametocyte-carrying group (Molineaux and Gramiccia, 1980). It was proposed that this situation might be explained by the acquisition of anti-gametocyte immunity with increasing host age. It has been shown that gametocyte prevalence reduces more quickly with age than asexual parasite prevalence, presumably due to the smaller repertoire of gametocyte specific-antigens (Drakeley et al., 2006b). Although recent molecular detection techniques have revealed that there is substantial gametocyte carriage among asymptomatic adult populations (Ouedraogo et al., 2009), the inverse correlation between age and gametocyte prevalence (and, indeed, density) is still apparent. Gametocyte-specific acquired immunity may actually reduce with age, in contrast to that induced against asexual parasites, as a result of infections in adults producing fewer gametocytes; this phenomenon has been proposed to be a deliberate strategy by the parasite in order to limit the acquisition of transmission blocking immunity (Taylor and Read, 1997; Bousema et al., 2006a). Interestingly, recent work has suggested that host genetic factors influence whether asymptomatic infections will produce gametocytes (Lawaly et al., 2010).

There are marked differences in gametocyte carriage rates in regions of differing transmission intensity. For example, in high transmission areas the inverse correlation between age and gametocytaemia is strong, whereas in low transmission areas, gametocyte carriers are just as likely to be found amongst all age groups (Drakeley et al., 2006b). Furthermore, gametocyte carriage is also affected by the seasonality of malaria transmission, with a larger increase in the proportion of infected individuals carrying gametocytes observed between the non-transmission and transmission seasons in low-transmission areas than in high-transmission areas (Drakeley et al., 2000, 2005).

The degree to which gametocytogenesis is a stochastic process, in which a sub-population of asexual parasites randomly differentiate into sexual stages, or whether it is specifically stimulated by environmental factors such as drug treatment, anaemia or host immune responses (reviewed in Kuehn and Pradel, 2010), is currently poorly understood. Certainly, observational studies linked the appearance of gametocytes to the onset of clinical symptoms (Miller, 1958, reviewed in Drakeley et al., 2006a), and the production of gametocytes may be stimulated by immune stress (Smalley et al., 1981), and factors linked to anaemia (Price et al., 1999; Nacher et al., 2002). The presence of mixed parasite genotype infections may also affect gametocytaemia; mixed species infections with *P. falciparum*, for example, have been shown to be linked with both decreased risk of gametocyte carriage (in the case of *Plasmodium vivax*, Price et al., 1999) and increased risk (in the case of *Plasmodium malariae,* Bousema et al., 2008) compared with single species infections. In *P. falciparum* multi-clone infections, individual genotypes have been shown to produce gametocytes simultaneously, and compared with single infections these gametocytes persist for longer (Nassir et al., 2005; Nwakanma et al., 2008).

The reason(s) why gametocytes are not detected in every *P. falciparum* infection remain unclear. There are two major explanations for this; either there are some situations in which asexual parasites multiply in the blood without the production of gametocytes or our molecular detection techniques are not sensitive enough to detect very low numbers of gametocytes. Of interest is whether all parasites inherently produce gametocytes at low levels throughout infections or whether bursts of gametocytogenesis occur in response to certain environmental stimuli.

It is well-known that *P. falciparum* parasites kept in continuous culture for long periods of time may lose the ability to produce gametocytes and in one specific case this was shown to be caused by a deletion of part of the right arm of chromosome 9 (Day et al., 1993; Gardiner et al., 2005). It is currently unknown, however, whether parasite clones differ in their ability to produce gametocytes from the outset or following adaptation to in vitro culture.

Here, we use a sensitive RT-PCR technique to detect gametocytes in blood samples taken from patients with symptomatic *P. falciparum* malaria. These parasites were subsequently adapted to in vitro culture, and gametocyte production was assessed by the same technique. *Pfg377* is a gametocyte-specific *P. falciparum* gene, in which polymorphisms in the sequence length of region 3 allow the typing of alleles

by PCR (Menegon et al., 2000). We were able to compare the predominant asexual parasite *pfg377* alleles with those of the gametocytes from the same infections, in order to determine which clones were producing gametocytes. We performed further observations on cultured parasites as this allows the exclusion of environmental factors that may trigger gametocytogenesis.

The majority of our isolates consisted of more than one parasite clone. In order to determine which parasites were producing gametocytes, and to compare between gametocyte production in vivo and in vitro, we analysed gametocyte *pfg377* allelic types of both mRNA and genomic DNA (gDNA) extracted from cultured parasites and the corresponding patient blood samples.

2. Materials and methods

2.1. Parasites and in vitro culture conditions

Blood was collected from 44 patients diagnosed with *P. falciparum* malaria by microscopic examination in Mae Sod, Tak province, Thailand from November 1988 to January 1989. The study was approved by the National Research Council of Thailand. Donors or their guardians gave oral consent for the donation of blood. Five milliliters of venous blood was collected in a heparinized tube (200 IU), centrifuged for the removal of plasma and white blood cells, washed with RPMI 1640 medium and frozen at -80˚C as previously described (Meryman and Hornblower, 1972). The frozen blood was thawed and washed in RPMI 1640 medium prior to the establishment of culture. Thawed parasites were re-suspended in 5 ml of complete medium (RPMI 1640 supplemented with 25 mM HEPES, 25 mM sodium bicarbonate, 25 μg/ml gentamicin sulfate and 10% human type AB serum, pH 7.4) (Trager and Jensen, 1976) at 2% hematocrit and cultured in a 60 mm dish at 5% O2, 5% CO2 and N_2 balanced. The period from the commencement of culture to the time when parasitemia reached 0.01%, which corresponds to 1×10^5 parasitized erythrocytes, was designated as the pre-growth period. When parasitemia increased to 0.5%, the culture was scaled up from 5 ml to 20 ml. O-type erythrocytes were added to adjust to a hematocrit of 5% and O-type non-immune serum was used in place of AB serum. When parasitemia reached 1%, corresponding to 1×10^8 parasitized erythrocytes, the

culture was regarded as successfully established. The period during which 1×10^5 parasitized erythrocytes increased at a constant growth rate to 1×10^8 was designated as the "growth period". Fresh erythrocytes were added to cultures every 4 days. Following the successful establishment of culture, lines were frozen in liquid nitrogen. Prior to collection of parasites for DNA and RNA extraction, frozen stabilates were subsequently recovered, thawed and culture was re-established. Fresh erythrocytes were added 1 day after the removal of stabilates from the liquid nitrogen and 4 days later. Four days after this, parasites were harvested. Samples for RT-PCR and PCR were all taken within 1 week of the successful establishment of culture.

2.2. Molecular analysis

gDNA was extracted both from stabilates of cultured isolates and frozen patient blood by using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). Extraction of RNA and reverse transcription were carried out as previously described (Maeno et al., 2003). Briefly, extracted total RNA was reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan) and the resulting cDNA was PCR-amplified using specific oligonucleotide primers. The first time RNA was subjected to RT-PCR (using 15 samples), all of those samples were also subjected to PCR without reverse transcription in order to check for DNA contamination of RNA. No DNA contamination was detected in any of the 15 samples. RNA was also treated with DNAse prior to RT-PCR. For PCR or RT-PCR analyses of the *pfg377* gene, reaction mixtures (25 μl) comprised 1 μl of cDNA or gDNA as a template, 0.5 mM of each primer, 200 mM dNTP, 0.625 units of Blend Taq DNA polymerase (Toyobo, Osaka, Japan) and 1 X PCR buffer (containing 2 mM MgCl2). Single PCR amplification was carried out under the following conditions: initial denaturation at 94˚C for 2 min, followed by 35 cycles (94˚C, 1 min; 55˚C, 30 s; and 72˚C, 1 min), and a final extension step (72˚C, 5 min). Primer sequences for region 3 of *pfg377* mRNA and gDNA (Menegon et al., 2000) were as previously described, except that only the outer primers described for the nested PCR were used in a single (non-nested) PCR. An ABI 2720 Thermal cycler (ABI, Foster city CA, USA) was used for all PCR reactions. PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Detection and size analyses of PCR products was confirmed with Lane & Spot Analyzer software (Atto, Tokyo, Japan). PCR products of mRNA

and gDNA for *pfg377* were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Tokyo, Japan) according to the manufacturer's instructions, and were then sequenced with a DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Sequencing products were separated with an ABI Prism 310 Genetic Analyzer (PerkinElmer) and the resulting nucleotide sequences were compiled using Vector NTI Advance (Invitrogen, Carlsbad, CA, USA).

2.3. Sensitivity of microscopy, RT-PCR and PCR

The detection limit of the RT-PCR methodology for the identification of *pfg377* mRNA was previously shown to be 0.03 parasites per µl of blood. The detection limit of the PCR for the detection of *pfg377* was determined to be two parasites per µl of blood (data not shown). By microscopy, we were able to detect gametocytes only when they were present at densities of at least four per µl of blood.

2.4. Statistical analyes

The number of days that patients were symptomatic prior to sample collection was analyzed using a Kruskal-Wallis test. The proportion of allele types was analyzed using a Chi-square test with Yates' correction. Statistical calculations were carried out with Epi Info (Centers of Diseases Control and Prevention, Atlanta, GA, USA).
A P value <0.05 was regarded as statistically significant.

3. Results

3.1. Patients and parasite culture

All patients from whom blood samples were obtained were over 14 years of age and presented to the health centre with malaria symptoms. Nine patients were unconscious at admission, 35 were uncomplicated cases (Table 1). The number of days of symptomatic infection was significantly different between severe and mild cases (Kruskal-Wallis value = 6.4455, d.f. = 1, $P = 0.011$). The levels of parasitaemia varied among patients. Gametocytes were detected by microscopy in six samples (MS814, MS817, MS825, MS826, MS828 and MS842) among 37 samples.

Successful in vitro culture was established for all 44 isolates (Table 2). Five isolates (MS801, MS825, MS842, MS843 and MS948) grew immediately after transfer to culture, whilst the remaining 39 isolates underwent a variable period of submicroscopic development at the initiation of culture. The longest such submicroscopic development period was 37 days. Freezing, thawing and culturing of parasites was repeated three to four times for each isolate, during which time parasites were maintained in culture for a total of 4 to 11 weeks from initial thawing of blood samples to the time at which RT-PCR and conventional PCR for the detection of gametocytes were performed.

3.2. Allelic typing of pfg377 in isolates cultured in vitro

Gametocytes were detectable by microscopy in 39 of the 44 cultured isolates (89%). Transcripts of *pfg377* were detected by RT-PCR in all of the 44 culture isolates (Table 2). To confirm that the allelic type of *pfg377* detected in the gDNA of asexual parasites was the same as that expressed by gametocytes, the allelic types of region 3 of *pfg377* were compared between gDNA and complimentary DNA reverse transcribed from mRNA extracted from cultured parasites. A total of five different alleles based on size polymorphisms were detected in the 44 isolates. In all cases there was a perfect match between the allele detected by gDNA typing and the gametocyte transcribed allele as determined by reverse transcription of mRNA. Of the 44 isolates, 40 possessed only one allelic type, whilst the remaining four each possessed two allelic types. As *pfg377* transcripts were detected in all cultured isolates, we concluded that all isolates possessed the ability to produce gametocytes. We refer to the five allelic types of *pfg377* as A, AB, B, C and D, as distinguished by size polymorphism of PCR amplified fragments. Alleles A, B, C and D were the same size as those previously described from Vietnamese malaria patients in 2002 (Maeno et al., 2008), whereas allele AB shared a similar size to an allele previously described from Africa, for which no nucleotide sequence was previously determined. The sizes of RT-PCR products of alleles A, AB, B, C and D, as visualized by agarose gel electrophoresis, were 411, 390, 369, 348 and 306 bp, respectively. On analysis of the deduced amino-acid sequence, seven degenerate amino-acid repeats (D-Q/H-Q/H-D/N-H-H-I) constituting region 3 gave rise to 11, 10, nine, eight and six copies, respectively (Fig. 1).

3.3. Allelic type of pfg377 in blood samples

Pfg377 mRNA was detected in all of the 41 patient samples analyzed prior to culture (Table 2). The average number of gDNA *pfg377* allelic types per sample was 1.3 (range 1-3), while for cultured isolates it was 1.1 (range 1-2) (Fig. 2). In 25 out of 41 pre-cultured patient blood samples, the allelic types of *pfg377* determined from gDNA were consistent with those determined by analysis of mRNA (19 single and six double infections). For nine out of 41 of these blood samples, partial overlap between gDNA and mRNA determined *pfg377* allelic type was observed, with at least one allele being identical between them. Therefore, gametocytes of the same allelic types at *pfg377* as those determined from the erythrocytic stages were observed in 83% of the samples.

We assume, therefore, that the asexual stages of the parasite clone that harbored the AB allele were below detectable levels or sequestered in these samples; however, as gDNA of the AB allele carrying clones was detectable in the cultured lines derived from these isolates, sufficient numbers of asexual parasites must have been in circulation in the blood in order to establish infections in culture. Therefore, we conclude that asexual stages of the AB allele carrying clones were present in these isolates, but were below the threshold of detection by our PCR methodology (two parasites per μl). Severe cases have previously been shown to be more likely to have gametocytes (Nacher et al., 2002). We examined whether parasite infection with the same allelic types between gDNA and mRNA were related to severe symptoms. There was no significant difference in disease severity prior to presentation at the health center between the patients with the same allele types between gDNA and mRNA and those with different allele types between gDNA and mRNA ($P = 0.61$).

There were twice as many samples with allele type AB detectable by mRNA than there were by gDNA, a significant difference ($P = 0.03$) (Fig. 1). AB allele types were always detected by mRNA analysis when gDNA samples were positive for this allele. All other allele types showed the opposite trend, not significant, with a higher frequency detectable by gDNA amplification than with mRNA amplification.

3.4. Gametocyte production of individual parasite clones

If we consider only the patients' blood samples, then there were 53 genotypes detected in total. Of these, 40 were also detected by amplification of mRNA.

Therefore, 75% of all parasite clones identified by gDNA PCR had produced detectable levels of gametocytes at the time of sampling.

3.5. Comparison of allele types between blood samples and culture isolates

In 14 cases, there was complete agreement between the gDNA and mRNA allele types derived from freshly isolated samples, and from those same samples following in vitro culture (Table 3). For 17 isolates, the same allele types as determined by mRNA analyses of blood samples were observed in freshly isolated patient blood samples or their corresponding culture adapted lines (Table 4). We assume, therefore, that in these 31 samples (75% of the total number of samples analysed), the same parasites that were producing gametocytes whilst in the circulatory blood system of the patients also produced gametocytes during in vitro culture. For the remaining 10 samples, the allele type of *pfg377* detected by RT-PCR of mRNA differed from the freshly isolated patient blood samples (Table 5) and from the corresponding culture-adapted lines (Table 6).

The proportion of samples exhibiting the AB allelic type of *pfg377* at the asexual stages (as determined by PCR typing of gDNA) significantly increased following in vitro culture ($P = 0.014$), while that of the C type allele significantly decreased ($P = 0.027$).

Of all the genotypes identified here, 36 (out of 68 present in the blood isolated from patients including those that were below detectable levels in patients but subsequently established in culture) (53%) were present both in patients and in the resulting culture lines. Interestingly, 32 genotypes that were present in the patients' blood (47%) were not detected in culture. Fourteen were present in culture but not detected in the blood isolated from patients, and therefore must have been present at a level below the threshold of PCR detection (two parasites per μl).

It is possible that the significant increase in the proportion of parasites expressing the AB allele of *pfg377* may have occurred due to cross-contamination of cultures. Although care was taken to minimize the chances of such cross-contamination, we cannot formally exclude the possibility that cross-contamination of a small number of cultures by parasites bearing the AB allele at *pfg377* occurred.

4. Discussion

To determine whether parasites infecting individual hosts were actively producing gametocytes, we obtained fresh blood samples from malaria patients and assessed the expression of the gametocyte-specific gene *pfg377*. The capacity to produce gametocytes was further assessed in these samples following their adaptation to in vitro culture. Furthermore, we compared the allelic type of the *pfg377* gene in asexual parasites (by PCR of gDNA) with that of gametocytes (as determined by RT-PCR of mRNA), in order to ascertain which parasite clones were producing gametocytes in mixed strain infections.

All isolates produced gametocytes at the time of isolation from patients. However, not all clones within infections were observed to have gametocytes present at the time of sampling. When the genotype if the *pfg377* gene was compared between gDNA and mRNA transcripts, 25% of clones detected by PCR of gDNA had no corresponding mRNA production, suggesting that these clones had not produced detectable levels of gametocytes at the time of sampling. However, as gametocytes take several days to mature to the point at which *pfg377* transcripts are produced, and we tested for gDNA and mRNA at the same time-point, it cannot be stated that these clones were not producing gametocytes at the time of sampling.

As our mRNA detection method is approximately 67 times more sensitive than our gDNA detection technique, we consider it unlikely that the detection of gDNA, but not of mRNA, reflects methodological error. On the other hand, the detection of mRNA, but not gDNA, does not necessarily imply that gametocytes are present and asexual stages are not, but that asexual parasites may be present at levels below our ability to detect those by PCR.

Gametocyte production, as determined by RT-PCR of *pfg377* mRNA, was maintained in all isolates following adaptation to in vitro culture. However, we observed that not all of the clones detected in patient blood were able to successfully establish in culture, with 44% of clones present in patient's blood not detected in culture. This could have occurred if these particular clones were only present in low numbers and did not survive the freeze/thawing undertaken at the beginning of culture establishment, or they were simply unable to adapt to in vitro conditions. Alternatively, the gDNA detected in a patient's blood may have originated from gametocytes, rather than from asexual parasites, which may have already been cleared

from the circulation by host immune responses prior to the release of gametocytes from sequestration.

Rodent malaria models have suggested that gametocyte production is positively correlated with multiplicity of infection, with increased gametocyte production being attributed to within-host competition between strains (Taylor et al., 1997). Nassir et al. (2005) showed that the prevalence of *P. falciparum* gametocytes was higher in a multiple-clone infection group compared with a single infection group. We observed gametocytes and detected *pfg377* transcripts in both uncloned isolates during in vitro culture, and in individual parasite clones derived from these isolates cultured under identical conditions, suggesting that multiplicity of infection may not correlate with commitment to gametocytogenesis in *P. falciparum*, at least not during in vitro culture. However, it is important to examine whether gametocyte production quantitatively increases in multiple-clone infections compared with single-clone infections in this system.

We speculate that asexual stages of parasites which carried the AB allele of *pfg377* may more frequently differentiate into gametocytes than those with other alleles, as we detected this allele significantly more frequently by mRNA amplification than we did by gDNA amplification ($P = 0.025$), suggesting the presence of undetectable levels of asexual parasitaemia in these isolates at the time of sampling and a higher copy number of mRNA transcripts of *pfg377* than that of the gene itself. As gametocytes may persist in the blood circulation for up to 3 weeks following release from sequestration (Eichner et al., 2001), it is possible that gametocytes persist after the asexual parasites have been cleared from the blood, either by host immune responses or by the use of antimalarial drugs such as chloroquine which do not have anti-gametocyte properties.

In 31 cases (75%), the allelic type(s) of *pfg377* detected by mRNA RT-PCR in freshly isolated patient blood samples matched that detected by mRNA RT-PCR during in vitro culture. The same parasites were thus able to produce gametocytes both in the blood of patients and in the subsequent in vitro culture. In 10 other cases, the gametocyte *pfg377* allelic types differed from those detected in freshly isolated patient blood and those detected during in vitro culture (Tables 5 and 6). Following in vitro culture adaptation, we observed a decrease in the number of isolates that produced gametocytes with a C allelic type.

In conclusion, we were able to detect gametocytes in all blood samples isolated from patients during acute *P. falciparum* infection by a sensitive RT-PCR detection method. The ability to produce gametocytes was maintained during in vitro culture of the same isolates, although the allelic type of these gametocytes differed from that originally detected in freshly isolated blood for 25% of the cultured lines.

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References

Bousema, J.T., Drakeley, C.J., Sauerwein, R.W., 2006a. Sexual-stage antibody responses to *P. falciparum* in endemic populations. Curr Mol Med 6, 223-229.

Bousema, J.T., Schneider, P., Gouagna, L.C., Drakeley, C.J., Tostmann, A., Houben, R., Githure, J.I., Ord, R., Sutherland, C.J., Omar, S.A., Sauerwein, R.W., 2006b. Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. J Infect Dis 193, 1151-1159.

Bousema, J.T., Drakeley, C.J., Mens, P.F., Arens, T., Houben, R., Omar, S.A., Gouagna, L.C., Schallig, H., Sauerwein, R.W., 2008. Increased *Plasmodium falciparum* gametocyte production in mixed infections with *P. malariae*. Am J Trop Med Hyg 78, 442-448.

Bousema, T., Okell, L., Shekalaghe, S., Griffin, J.T., Omar, S., Sawa, P., Sutherland, C., Sauerwein, R., Ghani, A.C., Drakeley, C., 2010. Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar J 9, 136.

Buates, S., Bantuchai, S., Sattabongkot, J., Han, E.T., Tsuboi, T., Udomsangpetch, R., Sirichaisinthop, J., Tan-ariya, P., 2010. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. Parasitol Int 59, 414-420.

Day, K.P., Karamalis, F., Thompson, J., Barnes, D.A., Peterson, C., Brown, H., Brown, G.V., Kemp, D.J., 1993. Genes necessary for expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on a 0.3-megabase region of chromosome 9. Proc Natl Acad Sci U S A 90, 8292-8296.

Drakeley, C., Sutherland, C., Bousema, J.T., Sauerwein, R.W., Targett, G.A., 2006a. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. Trends Parasitol 22, 424-430.

Drakeley, C.J., Akim, N.I., Sauerwein, R.W., Greenwood, B.M., Targett, G.A., 2000. Estimates of the infectious reservoir of *Plasmodium falciparum* malaria in The Gambia and in Tanzania. Trans R Soc Trop Med Hyg 94, 472-476.

Drakeley, C.J., Carneiro, I., Reyburn, H., Malima, R., Lusingu, J.P., Cox, J., Theander, T.G., Nkya, W.M., Lemnge, M.M., Riley, E.M., 2005. Altitude-dependent and -independent variations in *Plasmodium falciparum* prevalence in northeastern Tanzania. J Infect Dis 191, 1589-1598.

Drakeley, C.J., Bousema, J.T., Akim, N.I., Teelen, K., Roeffen, W., Lensen, A.H., Bolmer, M., Eling, W., Sauerwein, R.W., 2006b. Transmission-reducing immunity is inversely related to age in *Plasmodium falciparum* gametocyte carriers. Parasite Immunol 28, 185-190.

Eichner, M., Diebner, H.H., Molineaux, L., Collins, W.E., Jeffery, G.M., Dietz, K., 2001. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. Trans R Soc Trop Med Hyg 95, 497-501.

Gadsby, N., Lawrence, R., Carter, R., 2009. A study on pathogenicity and mosquito transmission success in the rodent malaria parasite *Plasmodium chabaudi* adami. Int J Parasitol 39, 347-354.

Gardiner, D.L., Dixon, M.W., Spielmann, T., Skinner-Adams, T.S., Hawthorne, P.L., Ortega, M.R., Kemp, D.J., Trenholme, K.R., 2005. Implication of a *Plasmodium falciparum* gene in the switch between asexual reproduction and gametocytogenesis. Mol Biochem Parasitol 140, 153-160.

Hallett, R.L., Dunyo, S., Ord, R., Jawara, M., Pinder, M., Randall, A., Alloueche, A., Walraven, G., Targett, G.A., Alexander, N., Sutherland, C.J., 2006.

Chloroquine/sulphadoxine-pyrimethamine for gambian children with malaria: transmission to mosquitoes of multidrug-resistant *Plasmodium falciparum*. PLoS Clin Trials 1, e15.

Kuehn, A., Pradel, G., 2010. The coming-out of malaria gametocytes. J Biomed Biotechnol 2010, 976827.

Lawaly, Y.R., Sakuntabhai, A., Marrama, L., Konate, L., Phimpraphi, W., Sokhna, C., Tall, A., Sarr, F.D., Peerapittayamongkol, C., Louicharoen, C., Schneider, B.S., Levescot, A., Talman, A., Casademont, I., Menard, D., Trape, J.F., Rogier, C., Kaewkunwal, J., Sura, T., Nuchprayoon, I., Ariey, F., Baril, L., Singhasivanon, P., Mercereau-Puijalon, O., Paul, R., 2010. Heritability of the human infectious reservoir of malaria parasites. PLoS ONE 5, e11358.

Maeno, Y., Nakazawa, S., Nagashima, S., Sasaki, J., Higo, K.M., Taniguchi, K., 2003. Utility of the dried blood on filter paper as a source of cytokine mRNA for the analysis of immunoreactions in *Plasmodium yoelii infection*. Acta Trop 87, 295-300.

Maeno, Y., Nakazawa, S., Dao le, D., Yamamoto, N., Giang, N.D., Van Hanh, T., Thuan le, K., Taniguchi, K., 2008. A dried blood sample on filter paper is suitable for detecting *Plasmodium falciparum* gametocytes by reverse transcription polymerase chain reaction. Acta Trop 107, 121-127.

McKenzie, F.E., Jeffery, G.M., Collins, W.E., 2007. Gametocytemia and fever in human malaria infections. J Parasitol 93, 627-633.

Menegon, M., Severini, C., Sannella, A., Paglia, M.G., Sangare, D., Abdel-Wahab, A., Abdel-Muhsin, A.A., Babiker, H., Walliker, D., Alano, P., 2000. Genotyping of *Plasmodium falciparum* gametocytes by reverse transcriptase polymerase chain reaction. Mol Biochem Parasitol 111, 153-161.

Meryman, H.T., Hornblower, M., 1972. A method for freezing and washing red blood cells using a high glycerol concentration. Transfusion 12, 145-156.

Miller, M.J., 1958. Observations on the natural history of malaria in the semi-resistant West African. Trans R Soc Trop Med Hyg 52, 152-168.

Molineaux, L., Gramiccia, G., 1980. The Garki Project. Research on the epidemiology and control of malaria in the Sudan savannah of West Africa. World Health Organization, Geneva.

Nacher, M., Singhasivanon, P., Silachamroon, U., Treeprasertsuk, S., Tosukhowong, T., Vannaphan, S., Gay, F., Mazier, D., Looareesuwan, S., 2002. Decreased hemoglobin concentrations, hyperparasitemia, and severe malaria are associated with increased *Plasmodium falciparum* gametocyte carriage. J Parasitol 88, 97-101.

Nassir, E., Abdel-Muhsin, A.M., Suliaman, S., Kenyon, F., Kheir, A., Geha, H., Ferguson, H.M., Walliker, D., Babiker, H.A., 2005. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. Int J Parasitol 35, 49-55.

Nedelman, J., 1989. Gametocytaemia and infectiousness in falciparum malaria: observations and models. Advances in Disease Vector Research 6, 59–89.

Nwakanma, D., Kheir, A., Sowa, M., Dunyo, S., Jawara, M., Pinder, M., Milligan, P., Walliker, D., Babiker, H.A., 2008. High gametocyte complexity and mosquito infectivity of *Plasmodium falciparum* in the Gambia. Int J Parasitol 38, 219-227.

Ouedraogo, A.L., Bousema, T., Schneider, P., de Vlas, S.J., Ilboudo-Sanogo, E., Cuzin-Ouattara, N., Nebie, I., Roeffen, W., Verhave, J.P., Luty, A.J., Sauerwein, R., 2009. Substantial contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. PLoS ONE 4, e8410.

Price, R., Nosten, F., Simpson, J.A., Luxemburger, C., Phaipun, L., ter Kuile, F., van Vugt, M., Chongsuphajaisiddhi, T., White, N.J., 1999. Risk factors for gametocyte carriage in uncomplicated falciparum malaria. Am J Trop Med Hyg 60, 1019-1023.

Schneider, P., Wolters, L., Schoone, G., Schallig, H., Sillekens, P., Hermsen, R., Sauerwein, R., 2005. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J Clin Microbiol 43, 402-405.

Schneider, P., Bousema, J.T., Gouagna, L.C., Otieno, S., van de Vegte-Bolmer, M., Omar, S.A., Sauerwein, R.W., 2007. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. Am J Trop Med Hyg 76, 470-474.

Smalley, M.E., Brown, J., Bassett, N.M., 1981. The rate of production of *Plasmodium falciparum* gametocytes during natural infections. Trans R Soc Trop Med Hyg 75, 318-319.

Taylor, L.H., Read, A.F., 1997. Why so few transmission stages? Reproductive restraint by malaria parasites. Parasitol Today 13, 135-140.

Taylor, L.H., Walliker, D., Read, A.F., 1997. Mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections. Parasitology 115, 121-132.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673-675.

Figure legend

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3D7      1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHIDHHNHHIDHHNHHIDHQDHH 60
A        1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHIDHHNHHIDHHNHHIDHQDHH 60
AB       1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHI-------DHHNHHIDHQDHH 53
B        1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHI-------------DHQDHH 46
C        1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHI-------------DHQDHH 46
D        1:IFHHRNITPYHVNHHNQHMDQHDHHIDHHNHHIDHHNHHI------------------- 40

3D7     61:IDHHNHHIDHHNHHIDHHDHHIDHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 120
A       61:IDHHNHHIDHHNHHIDHHDHHIDHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 120
AB      54:IDHHNHHIDHHNHHIDHHDHHIDHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 113
B       47:IDHHNHHIDHHNHHIDHHDHHIDHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 106
C       47:IDHHNHHIDHHNHHI-------DHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 99
D       41:---------------DHHDHHIDHHDHHIDHHNHHIDHKSNNQFLQKHQNYVRGHSSFIT 85

3D7    121:ISEGEENHDNRELRKKIE                                            138
A      121:ISEGEENHDNRELRKKIE                                            138
AB     114:ISEGEENHDNRELRKKIE                                            131
B      107:ISEGEENHDNRELRKKIE                                            124
C      100:ISEGEENHDNRELRKKIE                                            117
D       86:ISEGEENHDNRELRKKIE                                            103
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Fig. 1 Deduced amino-acid sequences of region 3 of pfg377, a gene specifically expressed by gametocytes of *Plasmodium falciparum.*

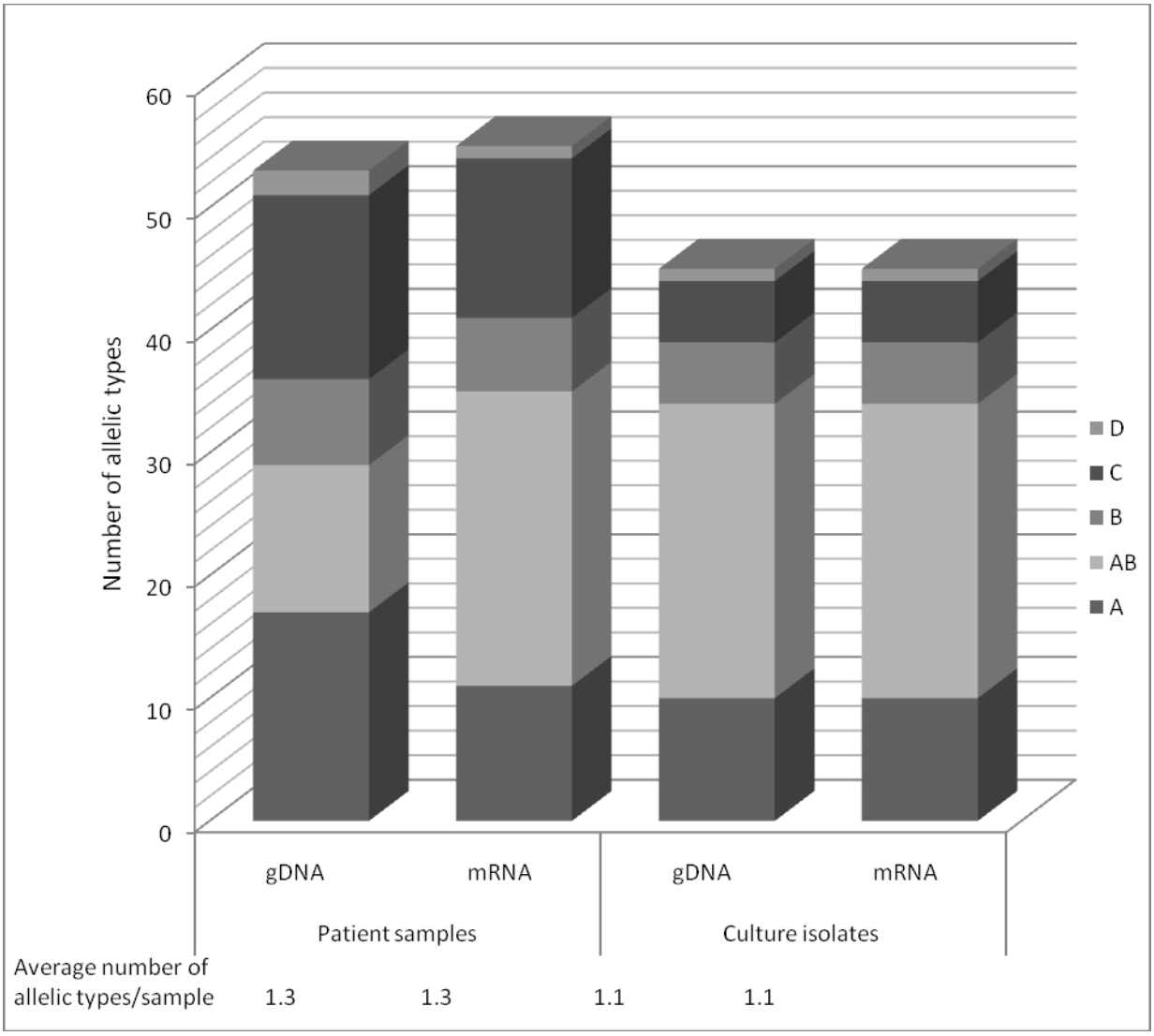

Fig. 2 Appearance of *Plasmodium falciparum* pfg377 allelic types. Detected allelic types (A-D, AB) of pfg377 in genomic DNA (gDNA) and mRNA of every blood samples and corresponding cultured isolate were counted and tallied.

Table 1. Characteristics of patients in the study.

Variables	
Age, average (range)	26.1 ± 9.4 (14-55)
Sex, % male (n/N)	81.8 (36/44)
Symptomatic malaria, % (n/N)	100 (44/44)
Severe cases, % (n/N)	20.1 (9/44)
Level of parasitemia, average (range)	0.61±0.81 (0.01-2.70)
Microscopic gametocyte prevalence, % (n/N)	16.2 (6/37)
Gametocyte count, average/µl (range)	3650 (4-127)
Days with symptoms, average (range)	3.2 ± 1.5 (1-8)
Gametocyte detection by RT-PCR, % (n/N)	100 (41/41)

N, total number; RT-PCR, reverse transcription-PCR

Table 2. Culture duration of *Plasmodium falciparum* isolates and gametocyte detection in culture.

Establishing (weeks)	Isolate (number)	Growth period[a] mean ± S.D. (days)	Total culture[b] mean ± S.D. (weeks)	Gametocyte prevalence (*n*/N)[c]
1	5	6.2 ± 1.3	7.0 ± 3.7 (4-11)	5/5
2	21	9.3 ± 1.1	6.0 ± 1.0 (5-7)	17/21
3	6	11.1 ± 2.9	6.7 ± 1.0 (6-8)	5/6
4	7	8.6 ± 1.5	7.6 ± 1.0 (7-9)	7/7
5	3	8.7 ± 2.1	8	3/3
6	2	11.0 ± 1.4	11	2/2
Total	44			39/44

N, total number.

[a] The period during which 1×10^5 parasitised erythrocytes increased at a constant growth rate to 1×10^8 in the first culture.

[b] Total number of weeks in which parasites were maintained in culture before molecular detection of gametocytes.

[c] Gametocytes were observed in Giemsa-stained thin smears from the culture prior to molecular analysis.

Table 3. *Plasmodium falciparum* isolates, of which allelic types of *pfg377* mRNA in blood samples completely matched those of genomic DNA (gDNA) of blood samples and cultured isolates.

Identification number	Blood samples		Cultured isolates[a]
	gDNA	mRNA	
MS805	B	B	B
MS812	AB	AB	AB
MS815	AB	AB	AB
MS822	AB	AB	AB
MS825	B	B	B
MS828	AB	AB	AB
MS830	AB	AB	AB
MS837	A	A	A
MS838	AB	AB	AB
MS842	C	C	C
MS843	A	A	A
MS946	AB	AB	AB
MS947	A	A	A
MS948	A	A	A

[a] In cultured isolates, gDNA and mRNA results were perfectly matched.

Table 4. *Plasmodium falciparum* isolates, of which allelic types of *pfg377* mRNR in blood samples matched either those of genomic DNA (gDNA) of blood samples or those of cultured isolates.

Identification number	Blood samples		Cultured isolates[a]
	gDNA	mRNA	
MS803	A, C	AB	AB
MS807	AB, C	AB, C	AB, B
MS808	C	AB, C	C
MS811	A	AB	AB
MS813	A, C, D	AB	AB
MS814	A, C	AB, C	A, C
MS816	A	AB	AB
MS818	A	A, C	A
MS819	A, C	A	A
MS820	B	AB, B	AB, B
MS824	AB	AB, C, D	AB
MS831	A, C	A, C	A
MS833	AB, C	AB	AB
MS834	AB, C	AB, C	AB
MS835	AB, C	AB, C	AB
MA836	C	AB, C	AB
MS844	C	AB, C	C

[a] In cultured isolates, gDNA and mRNA results were perfectly matched.

Table 5. *Plasmodium falciparum* isolates, of which allelic types of *pfg377* mRNR in blood samples differed from those of genomic DNA (gDNA) of blood samples and cultured isolates.

Identification number	Blood samples		Cultured isolates[a]
	gDNA	mRNA	
MS804	A	AB	A
MS806	D	AB	D
MS841	B	AB	B

[a] In cultured isolates, gDNA and mRNA results were perfectly matched.

Table 6. *Plasmodium falciparum* isolates, of which allelic types of *pfg377* mRNR in blood samples matched completely those of genomic DNA (gDNA) of blood samples, but differed from those of cultured isolates.

Identification number	Blood samples		Cultured isolatesa
	gDNA	mRNA	
MS809	A	A	AB
MS810	B, C	B, C	AB
MS817	B	B	AB
MS826	B, C	B, C	A
MS827	A	A	AB
MS829	A	A	AB
MS840	A	A	AB

[a] In cultured isolates, gDNA and mRNA results were perfectly matched.