



(Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate

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Abstract

The effects of drugs on *Plasmodium falciparum* transmission stages may reduce the spread of parasites in the population and contribute to malaria control. Detailed quantitative studies on (sub)microscopic gametocytaemia have become feasible with the availability of real-time *Pfs25* quantitative Nucleic Acid Sequence-based Amplification (QT-NASBA), which can be used to detect gametocyte densities above 20 gametocytes per millilitre from in vitro cultures. Gametocyte dynamics were investigated in children with uncomplicated *P. falciparum* malaria after treatment with sulphadoxine-pyrimethamine (SP) or a combination of SP and artesunate (SP+AS), in a 28-days drug efficacy study. This study demonstrated that gametocyte prevalence in 873 samples from symptomatic Kenyan children was 2.8 times higher by QT-NASBA compared with microscopy. Microscopy-positive cases showed a significant correlation with QT-NASBA for gametocyte density. At enrolment, gametocyte prevalence was 86% by QT-NASBA compared with 22% by microscopy. Gametocytes were detected in 97% of children in at least one blood sample and in 38% of children in all samples obtained during the 28-days follow-up. Both the risk of gametocyte carriage and gametocyte density were considerably higher after treatment with SP compared with SP+AS. Gametocyte prevalence and density decreased with time in the SP+AS group, but not in the SP-treated children. Our data suggest that the potential of malaria transmission remains high even after treatment with artemisinin combination therapy, although prevalence and density of gametocytes is lower after SP+AS.

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1. Introduction

Clinical symptoms of malaria are caused by cyclical proliferation of asexual *Plasmodium* parasites in the patient's red blood cells. Spread of the disease depends on the presence of mature sexual stage parasites (gametocytes), which do not replicate or cause clinical symptoms, but are essential for transmission from humans to the mosquito vector.

As measures to prevent malaria are not completely efficient, early treatment of symptomatic infections is a major

component of malaria control strategies. Drugs are designed to cure clinical symptoms and therefore mostly target asexual stages; the impact of drug treatment on gametocytes and transmission has long been neglected. Some drugs increase (e.g. chloroquine and sulphadoxine-pyrimethamine) or decrease (e.g. artemisinin derivatives) gametocyte prevalence (Targett et al., 2001; von Seidlein et al., 2001; Sowunmi and Fateye, 2003; Sutherland et al., 2005). Due to increasing resistance to most affordable antimalarial drugs, many countries are forced to revise drug treatment strategies. Generally, artemisinin combination therapies (ACT) are recommended to replace current first line drugs such as chloroquine and sulphadoxine-pyrimethamine (SP). Combination of artemisinin derivatives with other antimalarial drugs

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may protect the other drug from development of resistance. The benefit of better cure rates induced by ACT combined with its effect on transmission due to its alleged gametocytocidal effects (Dutta et al., 1989) may justify increased costs for treatment.

The key factors that trigger sexual stage development are not yet understood. Gametocytes are derived only from a small subset of asexual parasites and only a fraction of patients develop patent gametocytaemia (Shute and Maryon, 1951). However, the apparently low prevalence of gametocytes contrasts with the successful spread of malaria and the difficulties to control malaria transmission (Taylor and Read, 1997). Recently, a number of studies have addressed the effect of antimalarial drug treatment on gametocytes (Sokhna et al., 2001; Osorio et al., 2002; Sutherland et al., 2002; Tjitra et al., 2002; Sowunmi and Fateye, 2003; Suputtamongkol et al., 2003; Hallett et al., 2004). Most of these studies used microscopy for detection and quantification of gametocytes, but it has been shown that patients without microscopically detectable gametocytes can infect mosquitoes (Githeko et al., 1992; Boudin et al., 1993) and submicroscopic gametocytaemia can be common (Babiker et al., 1999; Menegon et al., 2000; Abdel-Wahab et al., 2002; Nassir et al., 2005). Detailed quantitative studies on submicroscopic gametocytes are now possible with the recently developed gametocyte-specific *Pfs25* quantitative Nucleic Acid Sequence-Based Amplification (QT-NASBA) (Schneider et al., 2004). The assay is performed in real-time, and quantifies mature *Plasmodium falciparum* gametocytes in blood samples with a lower detection limit of 20–100 gametocytes per millilitre of blood. The objective of this study was to investigate the effect of SP and of SP plus artesunate (SP + AS) treatment on *P. falciparum* gametocyte dynamics using *Pfs25* real-time QT-NASBA.

2. Materials and methods

2.1. Real-time *Pfs25* QT-NASBA and nucleic acid extraction

Nucleic acids were extracted from blood samples using the Guanidiniumisothiocyanate (GuSCN)/silica procedure as described by Boom et al. (1990). Total parasite load was quantified by real-time 18S rRNA QT-NASBA as previously described (Schneider et al., 2005). *Pfs25* mRNA QT-NASBA was performed as described by Schneider et al. (2004) with some adjustments to enable real-time detection. Briefly, real-time QT-NASBA for *Pfs25* mRNA (Genbank accession number AF193769.1) was performed on a NucliSens EasyQ analyser (bioMérieux) using the NucliSens Basic Kit for amplification according to manufacturer's manual at a KCl concentration of 80 mM. Reactions were carried out in a total reaction volume of 10 µl per reaction. Forward primer: 5'-gac tgt aaa taa acc atg tgg aga-3' (nucleotides 204–227); reverse primer: 5'-aat tct aat acg act cac tat agg gag aag gca tt acc gtt acc aca agt ta-3' (T7 promoter sequence, linker and nucleotides 338–359); *Pfs25* molecular beacon: 5'-TexasRed-cga tcg ccc gtt tca tac gct tgt aac gat cg-DABSYL-3' (molecular beacon stem of six paired nucleotides and nucleotides 259–278). The

time point during amplification at which the fluorescence, resulting from detection of target amplicons, exceeded the mean fluorescence of three negative controls + 20 SD was calculated (time to positivity). The use of a standard gametocytes stage V dilution series allowed calculation of the number of gametocytes present in unknown samples (Schneider et al., 2004).

2.2. Detection limit and interassay variability

Blood samples of in vitro parasite culture of *P. falciparum* NF54 (Ponnudurai et al., 1986) were collected as described by Schneider et al. (2004), with the exception that internal controls for quantification were not added to the samples. Ten-fold dilution series of gametocytes ranging from 10⁶ to 10 gametocytes per ml of blood were made, nucleic acids extracted using the GuSCN/silica procedure (Boom et al., 1990) and the number of gametocytes was quantified by real-time *Pfs25* QT-NASBA.

2.3. Field study

A drug efficacy study was performed from October to December 2003 in Mbita, Western Kenya (000 25'S, 340 13'E), according to the WHO protocol (WHO, 2002). Mbita is a rural region on the shores of Lake Victoria experiencing endemic malaria with seasonal variation in transmission intensity. A description of the study site can be found in Bousema et al. (2004) and Gouagna et al. (2004). Study approval was obtained from the ethical and scientific review boards of the Kenya Medical Research Institute (SSC 791).

In total, 245 children aged 6 months to 10 years with uncomplicated clinical *P. falciparum* malaria confirmed by microscopy (500–100,000 parasites/µl) were included in the study after their guardians gave informed consent. Children were randomised to receive either SP (Fansidar, Roche, Switzerland; 125 mg sulphadoxine and 6.25 mg pyrimethamine/5 kg as a single dose) or SP + AS (Arsumax, Sanofi, France; 20 mg/5 kg daily for 3 days) treatment. Details of randomisation and treatment procedures are described elsewhere (Bousema et al., in press). Follow-up was performed by the study clinician on days 1, 2, 3, 7, 14 and 28 post-treatment and at any time the child showed symptoms of disease. At all sampling points, a blood smear for microscopic detection of parasites was made and stained with 10% Giemsa. Asexual parasites were counted against 200 leucocytes, gametocytes against 500 leucocytes and converted to number of parasites per volume assuming 8000 leucocytes/µl blood. Slides were considered negative when no parasites were detected after viewing 100 microscopic fields at a 10×100 magnification. Additional to the standard protocol (microscopy), at all sampling times, a small blood sample on filter paper was collected for genotyping. A blood sample (50 µl) for QT-NASBA, stored in GuSCN lysis buffer until further analysis, was collected on the same days except for day 2. Samples for all methods were obtained from the same finger prick.

2.4. Statistical analysis

Statistical analyses were performed in SPSS version 12.0.1 and STATA 7.0. Mann–Whitney *U*-test was used for comparisons between groups for continuous variables and χ^2 test for discontinuous variables. Results of microscopy and QT-NASBA were compared by Spearman correlation and paired *t*-test.

Gametocyte prevalence and density after treatment were assessed by use of QT-NASBA in a random subset of 118 children, aged 6 months–5 years, who completed at least 7 days of follow-up (age groups of 1, 2, 3, 4 and 5 years consisted of 32, 23, 20, 21 and 22 children, respectively). This group was representative for the entire study group regarding gender (50% male), mean weight (13.3 kg) and mean age (2.8 years). Gametocyte prevalence at enrolment was not statistically different between the two treatment groups (χ^2 tests, $P=0.770$ for microscopy and $P=0.421$ for QT-NASBA), as well as total parasite and gametocyte densities at enrolment (Mann–Whitney *U*-tests, total parasite density $P=0.345$ and 0.516 ; gametocyte density $P=0.732$ and 0.538 for microscopy and QT-NASBA, respectively). Logistic regression was used to determine the relation of gametocyte prevalence to days after treatment and drug treatment (SP or SP+AS), with adjustment for age in years and outcome of drug treatment (adequate clinical response or late treatment failure (WHO, 2002)). Odds Ratios (OR) were calculated with 95% confidence intervals (CI). For gametocyte density, linear regression was used with inclusion of the same parameters, but only gametocyte-positive samples were used in the analysis. In case of multiple observations per individual, Generalised Estimating equations. (Hanley et al., 2003) models (STATA 7.0) were used to allow for autocorrelation.

Table 1
Interassay variability (SD/mean \times 100%) for real-time *Pfs25* quantitative Nucleic Acid Sequence-based Amplification in 18 independent dilution series from in vitro cultured gametocytes

Log gct/ml	TTP	SD	Interassay variability (%)
6	46.60	1.95	4.2
5	53.48	3.32	6.2
4	64.56	4.26	6.6
3	72.70	5.03	6.9
2	84.06	6.29	7.5
1 ^a	89.85	8.33	9.3

Log gct/ml: $^{10}\log$ of the number of gametocytes per millilitre of blood originally determined by microscopy and diluted. TTP: average time to positivity for *Pfs25* quantitative Nucleic Acid Sequence-based Amplification for each gametocyte concentration.

^a Calculations for eight measurements only, 10 of 18 were below the detection limit and excluded from analysis.

3. Results

3.1. Detection limit and interassay variation of real-time *Pfs25* QT-NASBA

Eighteen independent gametocyte series from in vitro parasite culture were collected and processed over a period of 2 months. Gametocyte densities from 10^2 to 10^6 gametocytes/ml could be detected consistently with 10 gametocytes per millilitre being close to the detection limit of 20–100 gametocytes per millilitre of blood. Interassay variability (SD/mean \times 100%), including variations in culture material and nucleic acid extractions, is below 10% for all measured gametocyte densities and is highest for the lower densities (Table 1). Regression analysis on the $^{10}\log$ number of parasites present in the control samples and the resulting time to positivity showed a consistently significant correlation ($P<0.001$) with a correlation coefficient of on average 0.98 (range 0.92–0.99).

For negative controls, blood samples (50 μ l) were obtained from 20 malaria-naïve blood donors in Nijmegen, the Netherlands and from 10 healthy volunteers at Kenya Medical Research Institute, Nairobi, Kenya with no recent history of malaria. The 30 blood samples were all confirmed negative by *Pfs25* QT-NASBA.

3.2. Field study, comparison microscopy and QT-NASBA for gametocyte quantification

In total, 873 samples obtained during the drug efficacy study in Mbita were quantified for *P. falciparum* gametocytes both by microscopy and by *Pfs25* QT-NASBA. Of these samples, 72% were positive for gametocytes by QT-NASBA, while gametocytes were detected in only 26% of the blood smears by microscopy. The *Pfs25* QT-NASBA confirmed 92% of the microscopically positive samples; 65% of the microscopically gametocyte negative samples was positive in the QT-NASBA with a geometric mean of 6.0×10^2 gametocytes per millilitre (IQR 1.7×10^2 – 2.1×10^3), which is well below the detection limit of microscopy (1.6×10^4 gametocytes/ml). In total, inconsistent results between *Pfs25* QT-NASBA and microscopy were shown in 33/873=3.7% of the samples. Quantification of gametocytes by *Pfs25* QT-NASBA and microscopy showed a statistically significant correlation (Spearman correlation, $\rho=0.452$, $P<0.001$). However, for samples with microscopically detected gametocytes, quantification by QT-NASBA was slightly lower than quantification by gametocyte microscopy (paired *t*-test, $P<0.01$).

3.3. Gametocyte dynamics after SP and SP+AS treatment

Of the 245 children included in the study comparing treatment efficacy of SP alone versus SP+AS (Bousema et al., in press) a random subset of 118 children was included for QT-NASBA analysis. Gametocyte prevalence at enrolment was 22% as detected by microscopy and 86% by *Pfs25* QT-NASBA. Gametocytes were detected in 97% of all children at

some time point and in 38% at all time points during the 28-days follow-up period by *Pfs25* QT-NASBA.

The SP+AS treated group ($n=55$) showed a significantly lower risk of gametocyte carriage (OR 0.137; 95% CI 0.065, 0.288) and a reduced gametocyte density ($\beta -0.395$; 95% CI $-0.652, -0.139$) compared with the SP treated group ($n=63$) in the *Pfs25* QT-NASBA analysis. Microscopic analysis showed a less pronounced effect of treatment on prevalence (OR 0.322; 95% CI 0.185, 0.561) and no statistically significant effect on gametocyte density. Since SP and SP+AS showed different effects on gametocytes, data were further analysed separately for each treatment group. Gametocyte prevalence by QT-NASBA significantly decreased with time in the SP+AS treated group (OR 0.905; 95% CI 0.877, 0.934) but not in the SP group (Fig. 1). Similar results were obtained with microscopy. However, microscopy data show a peak in gametocyte prevalence at day 7 after SP treatment that was not detected by *Pfs25* QT-NASBA (Fig. 1). Gametocyte density decreases significantly ($\beta -0.032$; 95% CI $-0.048, -0.017$) after treatment with SP+AS but not after treatment with SP, where gametocyte densities peaked at day 7. This is shown in Fig. 2 where the median of gametocyte densities relative to the day 0 density are shown.

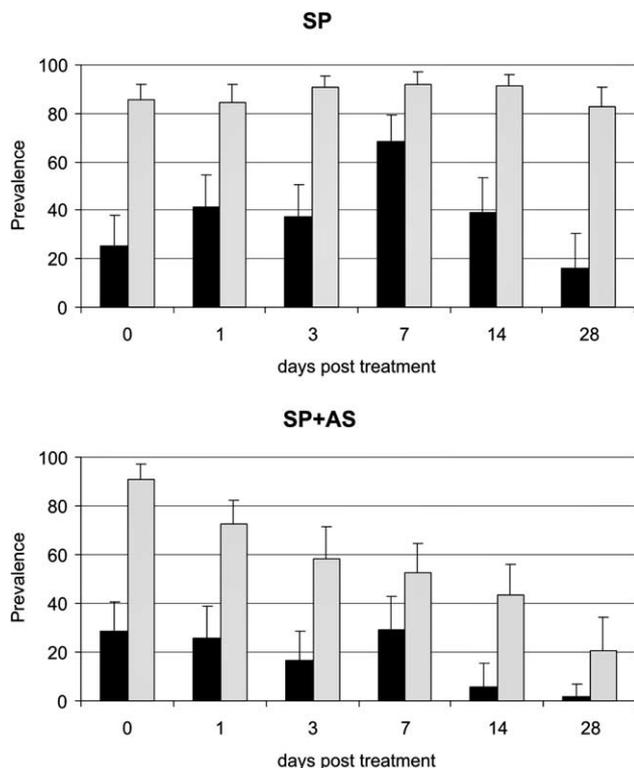


Fig. 1. Gametocyte prevalence from day 0 to 28 after treatment, calculated by microscopy (black bars) and *Pfs25* quantitative Nucleic Acid Sequence-based Amplification (grey bars) for sulphadoxine-pyrimethamine (SP) monotherapy and SP plus artesunate (SP+AS) combination therapy. Error bars indicate the upper limit of the 95% confidence intervals.

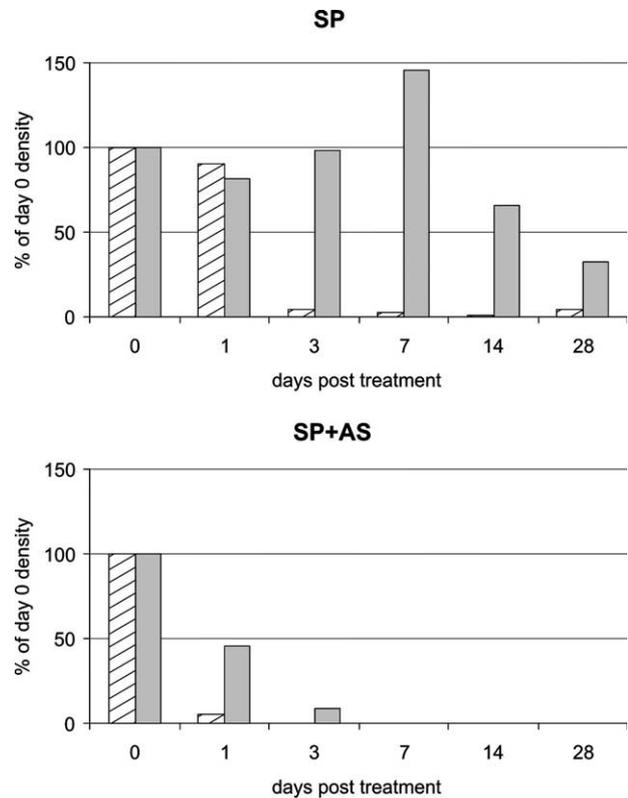


Fig. 2. Median quantitative Nucleic Acid Sequence-based Amplification total parasite (hatched bars) and gametocyte (grey bars) densities relative to day 0 for sulphadoxine-pyrimethamine (SP) monotherapy and a combination of SP and artesunate (SP+AS) combination therapy.

4. Discussion

The real-time *Pfs25* QT-NASBA is more sensitive for quantification of *P. falciparum* gametocytes compared with standard microscopy (detection limits of 20–100 and 16,000 gametocytes per millilitre of blood, respectively). Inconsistent results between gametocyte detection by microscopy or *Pfs25* QT-NASBA, including both false positive and false negative results, occurred in 3.7% of all samples. Similar low percentages of inconsistent results were found during a cross-sectional study in Burkina Faso (Ouedraogo et al., unpublished data). The observation that QT-NASBA results in lower gametocyte densities compared to microscopy may be explained by an overestimation of gametocyte densities by microscopy. Counting of gametocytes against 500 leucocytes was started only after the first gametocyte has been detected, which introduces a positive bias in quantification.

Over 50% of the microscopically gametocyte-positive *in vivo* samples from the study in Kenya contained only 1-gametocyte/500 leucocytes, a density just above the detection limit of microscopy. The increased sensitivity of gametocyte detection by *Pfs25* QT-NASBA revealed high prevalence of submicroscopic levels of gametocytes at enrolment and during the 28-days follow-up period. This confirms previous studies showing submicroscopic gametocytaemia in endemic samples (Muirhead-Thomson, 1954; Boudin et al., 1993; Abdel-Wahab et al., 2002; Coleman et al., 2004;

Nassir et al., 2005). Almost all children harbour gametocytes at some time point during the study and 38% show persistent gametocytaemia during follow-up. This suggests that the majority of children with uncomplicated symptomatic *P. falciparum* infection contribute to the potential infectious reservoir both before and after treatment with antimalarial drugs.

Our data further show that the gametocyte reservoir in symptomatic children may be much larger than previously indicated by microscopic analyses. Many gametocyte carriers carry very low densities of gametocytes (geometric mean 600/ml) in the circulation. Data suggest that gametocyte densities below the microscopy detection limit can infect *Anopheles stephensi* mosquitoes in membrane feeding experiments (P. Schneider, unpublished data). Therefore, low gametocyte densities in vivo may infect mosquitoes, especially when taking into account that *P. falciparum* gametocytes in the peripheral blood may not be homogeneously distributed (Pichon et al., 2000; Gaillard et al., 2003).

Both gametocyte carriage and higher gametocyte densities are considerably more prevalent in the SP group compared to SP + AS. A post-treatment reduction of gametocyte prevalence and density over time was present in the SP + AS but not in the SP treatment group. The peak gametocyte prevalence at day 7 after SP treatment observed by microscopy confirms data from previous studies (Akim et al., 2000; Sokhna et al., 2001; Targett et al., 2001) but was not confirmed by QT-NASBA. In contrast, QT-NASBA showed a peak in gametocyte density in the SP group at day 7. This difference may be explained as higher gametocyte densities being more likely to be detected by microscopic analysis. The peak of gametocyte density at day 7 after SP treatment cannot be the result of de novo induction under drug pressure, as gametocyte development takes longer than 7 days. This peak may reflect the slower clearance of immature gametocytes after SP treatment compared to SP + AS treatment. These results show that data on gametocyte prevalence are highly dependent on the sensitivity of the detection method and microscopy is inferior for this purpose. In conclusion, the *Pfs25* QT-NASBA is the preferred technique to detect and quantify *P. falciparum* gametocytes. The use of *Pfs25* QT-NASBA in transmission studies provides more detailed data and makes studies, especially those with small sample sizes, more efficient.

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