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Submicroscopic Gametocytes and the Transmission of Antifolate-Resistant *Plasmodium falciparum* in Western Kenya

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**Abstract**

**Background:** Single nucleotide polymorphisms (SNPs) in the *dhfr* and *dhps* genes are associated with sulphadoxine-pyrimethamine (SP) treatment failure and gametocyte carriage. This may result in enhanced transmission of mutant malaria parasites, as previously shown for chloroquine resistant parasites. In the present study, we determine the association between parasite mutations, submicroscopic *P. falciparum* gametocytmemia and malaria transmission to mosquitoes.

**Methodology/Principal Findings:** Samples from children treated with SP alone or in combination with artesunate (AS) or amodiaquine were genotyped for SNPs in the *dhfr* and *dhps* genes. Gametocytemia was determined by microscopy and Pfs25 RNA–based quantitative nucleic acid sequence–based amplification (Pfs25 QT-NASBA). Transmission was determined by membrane-feeding assays. We observed no wild type infections, 66.5% (127/191) of the infections expressed mutations at all three *dhfr* codons prior to treatment. The presence of all three mutations was not related to higher Pfs25 QT-NASBA gametocyte prevalence or density during follow-up, compared to double mutant infections. The proportion of infected mosquitoes or oocyst burden was also not related to the number of mutations. Addition of AS to SP reduced gametocytemia and malaria transmission during follow-up.

**Conclusions/Significance:** In our study population where all infections had at least a double mutation in the *dhfr* gene, additional mutations were not related to increased submicroscopic gametocytemia or enhanced malaria transmission. The absence of wild-type infections is likely to have reduced our power to detect differences. Our data further support the use of ACT to reduce the transmission of drug-resistant malaria parasites.

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**Introduction**

Artemisinin-based combination therapy (ACT) is now widely advocated as first-line antimalarial treatment. Nevertheless, drugs that are now no longer recommended as first-line treatment, such as sulphadoxine-pyrimethamine (SP), will continue to play a role in malaria control. A change in international therapeutic recommendations is not always translated into an immediate, effective policy change at country levels. In Kenya, monotherapy with chloroquine was replaced by SP in 1998 and SP was officially replaced by the ACT Artemether-Lumefantrine (AL) in 2004 [1]. However, health worker training and distribution of AL were not completed until the end of 2006 [1]. Even if official recommendations are followed by government services, monotherapy will remain available in the private sector, as was reported in Nigeria [2]. Furthermore, SP also plays an important role in intermittent preventive treatment to control malaria in infants [3,4] and pregnant women [5] and in some situations the combination of SP with amodiaquine (AQ) may be a cheap and efficacious alternative to ACT [6,7]. The ongoing use of SP, either in treatment of uncomplicated malaria or as method of malaria control by intermittent preventive treatment, underlines the importance of continued studies of SP resistance and the way resistance spreads in populations.

SP resistance is associated with the presence of single nucleotide polymorphisms (SNPs) in the *dhfr* and *dhps* genes [8–10]. This SP resistance starts off with a core SNP at codon 108 (S108N), which is followed by mutations at c51 (N51I) and 59 (C59R) [11] leading to a so-called ‘triple mutation’. Additional mutations in the *dhps* gene (codon 437 and 540) are related with even higher risks for SP treatment failure [12]. Other codons in *dhfr* at c164 and *dhps* at...
c581 and c613) are also associated with high-level SP resistance in Southeast Asia [13,14] and Latin America [9], but until now, these mutations are uncommon on the African continent and have rarely been found in East Africa [15–19].

The presence of mutations in the dhfr and dhps genes may not only predict treatment outcome, but may also be related to the transmission stages of P. falciparum, the gametocytes. The presence of resistant parasites at enrolment was found to be related to microscopically detected gametocytes during follow-up [20,21] or even prior to treatment [21] and to the duration of gametocyte carriage [22]. Mutations in the Pfcrtr and Pfmdr1 genes, encoding CQ resistance, were associated with a higher gametocyte density and subsequent malaria transmission after CQ monotherapy [23,24] or SP/CQ combination treatment [25]. Recently, a study of Méndez and others showed that in Colombia, malaria transmission was enhanced for parasites with low levels of resistance to SP compared to fully sensitive parasites [26]. In addition to a transmission advantage of mutant parasites in the human host through a higher gametocyte prevalence or density [20,23], there may also be a selective advantage in the mosquito midgut, as was suggested for Pfcrtr mutations [23]. These findings are worrying, indicating a very rapid spread of mutant parasites under drug pressure, but need confirmation in areas with different levels of transmission intensity and drug resistance.

Molecular gametocyte detection tools have recently demonstrated that gametocyte carriage can be seriously underestimated by the use of microscopy [27]. Submicroscopic gametocytes are not only common in various populations [27–29], but may also contribute considerably to malaria transmission [27].

The present study was conducted in an area of high resistance to SP with more than 50% treatment failure within one month of follow-up [27]. Samples from a previously published study on submicroscopic gametocytomia following antimalarial treatment [27] were genotyped to determine the relation between parasite mutations, submicroscopic gametocytomia and the transmission of malaria to mosquitoes.

Methods

Ethics statement

The study protocol (SSC No. 791) was approved by the Scientific Steering Committee and Ethical Review Committee of the Kenya Medical Research Institute. Written, informed consent was obtained from a parent or guardian of the participating children. The trial was completed in 2004, before registration became mandatory for clinical trials. The trial was registered after completion under Clinical Trials registration number ISRCTN31291805 (http://www.controlled-trials.com/ISRCTN31291805).

Objectives

We hypothesized that mutant parasites possess a transmission advantage compared to susceptible parasites. To test this hypothesis, our primary objectives were to determine the number of allelic variants before and after treatment and to determine the relation between mutations in the dhfr and dhps genes and: i) gametocyte prevalence and density by microscopy and RNA-based quantitative nucleic acid sequence based amplification (QT-NASBA); ii) mosquito infection rate; iii) oocyst densities in mosquitoes.

Participants

This study was conducted from October to December 2004 (short rainy season) in Mbita, a rural village on the shores of Lake Victoria in Suba District, western Kenya. The main malaria vectors in the area are A. gambiae, A. funestus, and A. arabiensis. Malaria transmission is high and perennial, with parasite prevalences in the human population ranging from 24.4% to 99.0%. Generally, the rainfall pattern is bimodal, with a long rainy season between March and May and a short rainy season between October and December.

The study population and treatment procedures are described elsewhere [27]. Briefly, 528 children (6 months–10 years) with P. falciparum mono-infection with a minimum parasite density of 500/parasites μL, fever or history of fever in the last 48 hours and absence of danger signs were enrolled and randomised to treatment with sulphadoxine-pyrimethamine (SP; Fansidar®, Roche, Switzerland), SP plus amodiaquine (AQ; Camoquine®, Pfizer, Senegal), SP + Artesunate (AS; Arsumax®, Sanofi, France) or Arteether-Lamefaun (AL; CoArtem®, Novartis Pharma, Switzerland). Samples were randomly selected from the study populations of 2003 and 2004 for molecular analysis of gametocyte carriage [27]. In addition, a random selection of 25 children per treatment arm was invited for membrane feeding experiments and molecular analysis of gametocyte carriage, irrespective of gametocyte carriage status [27]. Samples from these two groups were combined and used for parasite genotyping in the current study. Only children who received SP alone or in combination with AQ or AS were included in the current study because we were interested in the transmission potential in relation to mutations in the dhfr and dhps genes (i.e., mutations that are associated with SP treatment failure). In addition, we determined the prevalence of mutations in the Pfcrtr gene that are associated with AQ treatment failure.

Description of procedures

Microscopy and Pf625 QT-NASBA. Blood smears were stained with 10% Giemsa for 10 min and then screened for asexual parasites and gametocytes at enrolment and on days 3, 7, 14, and 28. Asexual parasites and gametocytes were counted against 200 and 500 white blood cells (WBCs), respectively, and the counts were converted to parasites per microliter on the assumption of a density of 8000 WBCs/μL. Parasite detection by Pf625 QT-NASBA was done at day 0, 3, 7, 14, and 28 as described elsewhere [30,31], using a NucliSens EasyQ analyser (bioMérieux) as described elsewhere for Pf625 mRNA [30]. Nucleic acids were extracted from 50-μL blood samples as described by Boom and others [32]. The Pf625 QT-NASBA technique is gametocyte specific and has a detection limit of 0.02–0.1 gametocytes/μL. NucliSens Basic kits were used for amplification, in accordance with the manufacturer’s instructions. A standard dilution series of mature, in vitro–cultured NF54 gametocytes [33] was included in each run to determine gametocyte densities in the test samples.

Mosquito membrane feeding and dissection. Membrane-feeding assays were conducted on day 14 after treatment, as described elsewhere [27]. Briefly, 3-mL venous blood samples were obtained and fed to ~150 locally reared [34] 4–5-day-old female A. gambiae sensu stricto mosquitoes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. After 10–15 minutes, fully fed mosquitoes were selected and kept on glucose for 7 days at 27°C–29°C, at which time midguts were dissected in 2% mercuricchrome. Midguts were examined microscopically for oocysts, with a second microscopist confirming their presence if observed. Exactly 30 mosquitoes were dissected per child.

Detection of SNPs in dhfr/dhps and Pfcrtr by PCR and SSOP-ELISA. Samples collected at patient enrolment were screened for SNPs at dhfr c108, c59 and c51, using PCR-SSOP as previously described [25,35] and provided an estimate of baseline allele frequencies at this locus. Samples from individuals participating in membrane feeding experiments were more
extensively studied for SNPs in both the *dhfr* and *dhps* genes. For these samples, SNPs at *dhfr* (at c50/51, c59, c108 and c164), *dhps* (at c436/437, c540, c581 and c613) and *Pfcr* (position 72–76) were determined by SSOP-ELISA of PCR amplified fragments using conditions and reagents as described by Alifrangi and others [36]. For each SNP analyses, parasite samples were categorized into single, mixed but with one SNP in majority or mixed infections with no SNP in majority as described by Alifrangi and others [36]. The mixed samples with one SNP in majority were considered as single mutations. All SSOP-ELISA data were divided into wild or mutant type; samples with a mixed infection (wild-type and mutant) were considered as mutant. Samples with a mutation in c108+c59 or c108+c51 of *dhfr* but no additional mutations were classified as double mutations; samples with mutations in c51+c59+c108 as triple mutations; samples with mutations in c51+c59+c108 of *dhfr* and c437 of *dhps* were considered quadruple mutations; and samples with mutations in c51+c59+c108 of *dhfr* and c437+c540 of *dhps* as quintuple mutations. For confirmation of the rare c164L genotype in *dhfr* the PCR-restriction fragment length polymorphism (RFLP) method described by Duraisingham and others [37] was used.

**Statistical methods and sample size considerations**

The major objective of the current study was to determine post-treatment malaria transmission potential in relation to mutations in the *dhfr* and *dhps* genes. This objective was studied using samples from a previously published drug efficacy trial [27] where the sample size for transmission experiments was based on the comparison of transmission potential after SP monotherapy compared to ACT. In the absence of information on the prevalence of mutations in the *dhfr* and *dhps* genes in the study area or their expected association with submicroscopic gametocytes and malaria transmission, we based sample size considerations for the current study on previously published studies on the relation between mutations in the *Pfcr* gene and malaria transmission. Three previously conducted studies included 11–25 membrane-feeding experiments per treatment arm with an average of 20–30 mosquitoes per experiment [23–25]. We included samples from 75 membrane-feeding experiments in randomly selected individuals after SP (n = 25), SP+AQ (n = 25) or SP+AS treatment (n = 25). Thirty mosquitoes were examined per experiment, giving a total of 2250 mosquitoes [27,30]. Because post-treatment gametocyteemia and malaria transmission was previously shown to be similar for SP and SP+AQ [27], data was grouped as non-ACT (SP mono-therapy and SP+AQ combination therapy) or ACT (SP+AS) for analyses on the relation between mutant genotypes and post-treatment gametocyteemia and malaria transmission. The transmission potential during follow-up was quantified as the area under the curve of *Pfs25* QT-NASBA gametocyte density versus time (AUC). For the cure rate of *Pfs25* QT-NASBA, gametocyteemia was set as 0.001, without a relation with the number of parasite mutations. Gametocyte prevalence at enrolment was 26.3% (50/189) by microscopy and 91.4% (118/129) by *Pfs25* QT-NASBA, and was not associated with the number of *dhfr* mutations. Transmission potential during follow-up was quantified as the area under the curve (AUC) of *Pfs25* QT-NASBA gametocyte density versus time for non-ACT and ACT treated children (table 1). The AUC was significantly lower for ACT compared to non-ACT treated children (p < 0.001). Within the group of non-ACT treated individuals, AUC was positively associated with the presence of asexual parasites on day three after treatment (i.e. a longer parasite clearance time), p = 0.046. There was no relation between AUC and the presence of three mutations in infections for either non-ACT or ACT treated individuals.

The lack of wild-type *dhfr* loci among this preliminary baseline analysis at three codons suggested that it would not be possible to find associations between parasite genotype and treatment outcomes of interest such as gametocyte carriage and mosquito infectivity using this approach. Therefore, in subsequent analyses we tested for the (rare) substitution of Leucine for isoleucine at codon 164 of *dhfr*, and performed a full analysis of the *dhps* gene at codons 436, 437, 540, 501 and 613. Significant associations with treatment outcome and parasite transmissibility have been shown for mutations at codons 437 [25] and 540 [19].

**Prevalence and post-treatment selection of mutant parasite genotypes in individual included in transmission experiments**

Seventy-five individuals donated blood for random membrane-feeding experiments and subsequent QT-NASBA analyses [27]. Fifty-eight of these individuals contributed to parasite DNA analyses of SNPs in *dhfr* and *dhps* at both day 0 and day 14 (figure 1). In this group, *dhfr* genotyping for day 0 samples was successful in 100% (58/58) for all codons, except for c59 (57/58). For the day 14 samples, approximately half of whom were parasite-negative by microscopy, *dhfr* genotyping was successful in 71% (41/58) for c51 and c59, in 70% (40/58) for c108 and c164.
null
Kenya [45], although a reduction in its prevalence was observed in other countries [46,47]. The Pfcrt gene is also associated with AQ resistance [44,48]. AQ was widely used in the study area in government facilities in the period the study was conducted and continues to be a popular choice for home treatment with shopbought drugs. In the year prior to the current study, we observed that 30.6% (26/85) of children attending the clinic with uncomplicated malaria had metabolites of 4-aminoquinolines in their urine (Bousema, unpublished observations). Although the used dipstick method could not differentiate between AQ and CQ, it is likely that the high frequency of the CVIET haplotype is currently being maintained by the use of AQ, rather than CQ.

Previous studies have found a relation between mutations in the dhfr and dhps genes and treatment outcome, but only when a substantial proportion of the parasite population harboured wild-type alleles [8,21]. This was not the case in our population where we observed no additional risk of treatment failure for infections with $\geq$ three mutations in the dhfr gene compared to double mutations. In line with this, the number of parasite mutations was not related with the proportion of individuals in whom asexual parasites were still detectable on day 3, an indicator of parasite clearance time (PCT).

This is the first report that describes the relationship between submicroscopic gametocytemia and the presence of mutant genotypes. Mutations in the dhfr gene have previously been related to an increased microscopic gametocyte prevalence after SP treatment [20–23]. This observation may be partly explained by a longer PCT for mutant parasites; this prolonged survival time under drug pressure allows parasites to invest in transmission stages [20]. In an earlier study, we have shown that peaks in microscopic gametocyte prevalence after treatment are likely to be the result of an increase in the density of gametocytes that were already present before treatment at submicroscopic concentrations [49]. We therefore hypothesized that post-treatment gametocyte density (measured by QT-NASBA) would be increased for infections with mutations in the dhfr and dhps genes. However, the complete absence of wild-type infections in our study population prevented this comparison being made. When using infections with a double mutation in the dhfr gene as reference group, we did not observe any association between the presence of

![Figure 1. Prevalence of mutant genotypes for dhfr and dhps genes prior to treatment and on day 14 after non-ACT or ACT treatment in samples included in the membrane-feeding assays. Non-ACT = sulphadoxine-pyrimethamine (SP) alone or in combination with amodiaquine; ACT = SP in combination with artemether; $\geq$ three mutations = mutations in c51+c59+c108 and possible additional mutations The number of observations on day 0: all mutations (n = 57–58) (see text); on day 14 after non-ACT treatment: c51, c59, c108, c164 (n = 33); c436, c437 (n = 23); c540, c581, c613 (n = 25); $\geq$ three mutations (n = 33); on day 14 after ACT treatment: c51, c59 (n = 8); c108, c164 (n = 7); c436, c437 (n = 4); c540, c581, c613 (n = 5); $\geq$ three mutations (n = 7). doi:10.1371/journal.pone.0004364.g001]
additional mutations in the \(dhfr\) or \(dhps\) genes and the prevalence or density of submicroscopic gametocytes. The transmission potential, quantified as the area under the curve of \(Pf25\) QT-NASBA gametocyte density versus time, was not associated with additional mutations compared to the reference group. In line with this, we also observed no increased malaria transmission measured by membrane feeding experiments on day 14 after treatment in relation to additional \(dhfr/dhps\) mutations. This contrasts with studies from the Gambia where a substantial proportion of parasites carried wild-type \(Pfcr\) alleles. Here, increased transmission of \(CQ\) resistant parasite strains was observed after examination of a similar number of transmission experiments and (infected) mosquitoes [22–25]. A recent study from Colombia also observed that infections with two \(dhfr\) mutations (in \(c108\) and \(c51\)) had a 10-fold higher probability of infecting mosquitoes than infection with wild-type parasites [26].

**Limitations**

In our study area of moderate transmission intensity, many of the study participants will have harboured multiple clone infections. We have presented estimates of mutation prevalence, and described infections as harbouring two, three or more mutations. However, in mixed infections individual mutations may be present on different parasite strains, and so it is not always possible to confidently infer which haplotypes are present.

None of the infections in our population consisted of pure wild-type parasites. This is likely to have obscured any relation between parasite resistance and malaria transmission. Our initial power calculations were based on assumptions derived from studies where wild-type samples were used as comparator group [23–25]. Using infections with a double mutation in the \(dhfr\) gene as comparator arm will have reduced our power to detect statistically significant associations, but allowed us to study the influence of additional mutations on transmission potential in an area where no infections contained fewer than two mutations in the \(dhfr\) gene.

Barnes and colleagues recently showed a gradual increase in transmission potential with increasing number of mutations in the \(dhfr\) and \(dhps\) genes [22]. Despite the relatively small number of individuals with the quintuple mutation in that study (\(n = 17\)), the study detected a statistically significant 2.6-fold increased AUC compared to individuals with fewer mutations (\(n = 76\)) [22]. We did not observe a similar relation in our population.

**Overall evidence**

Based on the available literature, it seems plausible that newly-arising resistance mutations result in a transmission advantage compared to wild-type parasites under drug pressure. However, in our study population, where the majority of infections harbour many resistance-associated mutations in the \(dhfr\) and \(dhps\) loci, we found no evidence that parasites with three or more mutations in the \(dhfr\) or \(dhps\) genes have an additional transmission advantage compared to double mutations. Our data also indicate that even at very high levels of \(SP\) resistance, the addition of \(AS\) to \(SP\) monotherapy can have a beneficial effect on malaria transmission.

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**Author Contributions**

Conceived and designed the experiments: CJS SO LG SR TB. Performed the experiments: MO MA PS CH LG TB. Analyzed the data: MO MA CJS TB. Contributed reagents/materials/analysis tools: PS. Wrote the paper: MO MA CJS SO SR TB.