Transmission and Cross-Mating of High-Level Resistance *Plasmodium falciparum* Dihydrofolate Reductase Haplotypes in The Gambia

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Abstract. A high-level pyrimethamine resistance *Plasmodium falciparum* lineage with triple dihydrofolate reductase (*dhfr*) mutations prevails across Africa. However, additional minority lineages were seen. We examined transmission success of mutant *dhfr* haplotypes among 22 children in The Gambia and 60 infected *Anopheles gambiae* mosquitoes fed on their blood. Additional polymorphic genes of the gametocyte-specific protein (*pfg377*) and merozoite surface protein-1 (*MSP-1*) were examined. Similarities were seen between *pfg377* and *MSP-1* alleles in children and mosquitoes and evidence of cross-mating between different parasite genotypes was seen in some infected mosquitoes, reflecting high transmission success of existing clones. With regard to *dhfr*, 16 haplotypes were seen among the children: 2 carried double mutations and 14 carried triple mutations. However, only nine haplotypes, all with triple mutations, were detected among mosquitoes. A single triple-mutant *dhfr* haplotype, similar to that in other countries in Africa, predominated among children (42%) and mosquitoes (60%), supporting the hypothesis of migration of this haplotype across Africa. However, evidence of cross-mating between the above haplotypes signifies the role of local evolution.

INTRODUCTION

Mutations leading to drug-resistant pathogens are adaptive in the face of drug pressure. In environments of continuous drug use, the mutant form is expected to have advantageous selection and transmission success.¹ The high-level pyrimethamine resistance genotype with triple dihydrofolate reductase (*dhfr*) gene mutations (N51I, C59R, S108N) exists at higher frequency in areas where resistance to sulfadoxine-pyrimethamine (SP) is well established.^{2,3} This finding suggests that this genotype is subjected to a relatively higher selection force compared with low-level resistance genotypes, that contain single and double mutations. Direct evidence of *in vivo* selection of mutant genes for *dhfr* and dihydroperoxide synthase (*dhps*) after administration of anti-malarial drugs has been demonstrated.⁴ In addition, it has been shown that growth and gametocyte production of drug-resistant genotypes is enhanced in a dose-dependent manner after drug administration⁵ and they are readily transmitted to mosquitoes.⁶

Analysis of single nucleotide polymorphisms and polymorphic microsatellite loci situated near drug-resistance genes has provided powerful tools to monitor drug-resistant *Plasmodium falciparum* haplotypes that are inherited together.^{7–9} These tools have identified different genetic forms (haplotypes) carrying the same drug-resistance gene and have been used to analyze their origin and spread.⁸ Analysis of *dhfr* and close microsatellites on chromosome 4 showed a distinct haplotype of a triple-mutant *dhfr* genotype occurring at high frequency in different regions, which suggested a single origin of high-level pyrimethamine resistance.¹⁰ However, additional minority haplotypes of this genotype have also been seen in many countries,^{11–15} and similarities between their flanking microsatellites suggest that they resulted by cross-mating and recombination among local parasites.¹⁵

We assessed the transmission success of different resistant *dhfr* haplotypes in The Gambia. At the time of the study, chloroquine (CQ) plus SP was the first-line anti-malarial drug

used for treatment of patients with uncomplicated malaria in this area.⁶ *Plasmodium falciparum* parasites resistant to these drugs were frequently detectable after drug therapy.^{16,17} Previous studies in The Gambia have demonstrated that parasites with a multidrug-resistant genotype had a substantial transmission advantage after CQ/SP treatment.⁶ The present study extends this observation by examining whether diverse lineages of resistant *dhfr* haplotypes (differing in flanking genetic markers) vary in their transmission success to *Anopheles* mosquitoes and if cross-mating occurs between them to generate novel drug-resistant haplotypes.

MATERIALS AND METHODS

Study area. The study was carried out at the Medical Research Council (MRC) field station in Farafenni, The Gambia (13°28'N, 16°34'W), where malaria is seasonal, with transmission occurring mainly from July to November with a peak in September.¹⁸ Entomologic inoculation rates range from less than 1 to more than 30 infected bites/person/year across the country.¹⁹

Patients and drug treatment. During the transmission season (August 2003), we examined *dhfr* haplotypes in children with *P. falciparum* infections and mosquitoes that fed on blood samples taken from these children one week after the start of therapy. This study was undertaken as part of an efficacy trial of three combinations of anti-malarial drugs: amodiaquine (AQ) plus artesunate (AS), AQ plus SP, and chloroquine (CQ) plus SP (Dunyo S and others, MRC Laboratories, Banjul, The Gambia, unpublished data). Briefly, 1,813 children (age range = 6 months to 10 years) with signs of acute malaria infection, including an axillary temperature $\geq 37.5^{\circ}\text{C}$ and a *P. falciparum* parasitemia of 2,000–200,000 parasites/ μL were enrolled after informed consent was obtained from their parents or guardians. The children came from three sites in The Gambia: Brikama (428), Njaba Kunda (682), and Farafenni (703). Patients received either AQ plus AS (717), AQ plus SP (547), or SP plus CQ (549).

On day 7 after treatment, patients in the Farafenni cohort were examined for gametocyte carriage by reading 200 fields

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of stained thick blood films. Approximately 3 mL of blood was collected from gametocyte-positive children, 30 μ L was spotted on a filter paper for DNA isolation, and the rest of the blood was processed for the *Anopheles* mosquitoes infection experiment described below.^{16,20} We report on 22 children treated with CQ plus SP or AQ plus SP who were capable of infecting *Anopheles* mosquitoes that fed on their blood. The study was reviewed and approved by the Medical Research Council Scientific Coordinating Committee and approved by the joint MRC/Gambian Government Ethics Committee.

Mosquito infectivity. At Farafenni, where an insectary facility was available, mosquito infectivity assays were conducted 7 days post-treatment on 100 children who were gametocyte positive according to the protocol described by Targett and others.¹⁶ Briefly, venous blood in citrate phosphate dextrose was centrifuged, and the plasma was removed. Erythrocytes were washed in RPMI 1640 medium and resuspended to a packed cell volume of 33% in pooled AB serum from European donors with no history of malaria. The suspension was then fed to 3–5-day-old laboratory-reared female *Anopheles gambiae* s.s. mosquitoes by an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. Approximately 50 mosquitoes were fed on each blood sample for 30 minutes, after which unfed mosquitoes were removed. Fully engorged mosquitoes were given sugar solution until dissection seven days later. Surviving mosquitoes were dissected and their midguts were examined for oocysts under a dissecting microscope. Infected midguts were transferred into oocyst lysis buffer, incubated for 1 hour at 55°C, and stored at –20°C for DNA isolation and polymerase chain reaction (PCR).²¹

Laboratory analyses. We genotyped *P. falciparum* isolates detected on day 0 blood sample (before treatment) in the 22 patients and in 60 mosquitoes (resulted from feed on blood samples taken on day 7 post-treatment) for *dhfr*, 3 adjacent microsatellites, merozoite surface protein 1 (*MSP-1*), and gametocyte-specific protein (*pfg377*). However, isolates detected on day 7 (post-treatment) blood samples were only examined for *MSP-1* and *pfg377* alleles, because of a shortage of DNA (Figure 1).

Characterization of *dhfr* haplotypes. DNA was extracted from blood of infected children stored on filter paper as described²² and from the midguts of infected mosquitoes using Qiagen Micro DNA Isolation Kit (Qiagen, Valencia, CA).

Dihydrofolate reductase mutations. Mutations in the *dhfr* gene were detected by sequencing of amplified PCR products.^{22,23} The amplified fragment encompasses the N51I, C59R, S108N, and I164L mutations associated with pyrimethamine resistance. The PCR products were first purified using ExoSAP-IT® (United States Biochemicals, Cleveland, OH) as described by the manufacturer. Cycle sequencing was carried out using the BigDye Terminator v3.1 system (Applied Biosystems, Foster City, CA) and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems). Sample sequences were compared against the wild-type sequence for automated identification of mutations by using Seqscape (Applied Biosystems) and confirmed by visual inspection of chromatogram peaks for forward and reverse reads at putative mutation sites.

Microsatellite loci. Three single copy microsatellite loci located 5.3, 4.4, and 0.3 kb upstream from *dhfr* were amplified and characterized as described.^{10,11} Alleles of these microsatellites were used to create haplotypes. Microsatellites were amplified in a semi-nested PCR, and products were sized on an ABI 3100 Sequencer using Genotyper software (Applied Biosystems). A control DNA of *P. falciparum* clones (3D7/Dd2) were run in parallel and samples were adjusted for variations in observed allele sizing of this control. We scored multiple alleles per microsatellite locus if a minor peak was more than 30% of the height of the predominant allele detected at each locus.²⁴ Samples with more than one allele at any microsatellites were considered to have multiple *P. falciparum* clones. Microsatellites with comparable intensity were sorted into different haplotypes.

We used microsatellites alleles to construct haplotypes. In instances where two or more alleles per locus are present, the haplotypes were a composite of alleles from two or more parasite clones. To avoid overestimation of possible recombinant haplotypes, we used the predominant alleles detected at each

FIGURE 1. Diagrammatic illustration showing number of samples taken from children on day 0 (before treatment), day 7 (post-treatment) and infected *Anopheles* mosquitoes (resulted from feed on blood samples taken on day 7) successfully analyzed for dihydrofolate reductase (*dhfr*) and three microsatellite loci flanking *dhfr*, *pfg377*, and merozoite surface protein 1 (*MSP-1*). ND = not done (unsuccessful assays).

locus to construct haplotypes. Similarly haplotypes of minority alleles were combined.²⁴

Alleles of the *MSP-1* gene. We examined alleles of polymorphic *MSP-1* in parasites detected in infected children on day 7 post-treatment and in infected mosquitoes. The *MSP-1* alleles were typed by nested PCR using family-specific primers to detect the K1, MAD20, and RO33 allelic families according to the method of Zwetyenga and others.²⁵

Gametocyte-specific protein *pfg377*. We examined the polymorphic single copy gene *pfg377*, which is expressed exclusively in gametocytes of *P. falciparum*. We analyzed genomic DNA (representing all parasite blood stages) from patient's pre-treatment (day 0) and post-treatment (day 7) blood samples and DNA of oocysts obtained from infected mosquitoes as described elsewhere.²⁰

RESULTS

We analyzed *P. falciparum* isolates among 22 infected children treated with either CQ plus SP or AQ plus SP who had microscopically detectable gametocytes on day 7 and among 60 infected *Anopheles* mosquitoes that fed on them. The

TABLE 1
Dihydrofolate reductase (*dhfr*) haplotypes among 11 *Plasmodium falciparum*-infected children before treatment and 24 mosquitoes that fed on blood samples taken on day 7 post-treatment*

Sample†	No. oocysts	<i>MSP-1</i> alleles‡	Microsatellites, kb		
			5.3	4.4	0.3
Child 1442-(CQ/SP)		2	196/202	172	84
Mosquito 2	1	2	192/202	174/179	84
Mosquito 3	2	3	202	174	104
Child 1505 (AQ/SP)		1	202	174	104
Mosquito 23	70	1	202	174	104
Mosquito 24	28	1	202	174	84/104
Mosquito 25	7	1	202	174	104
Child 1551(AQ/SP)		2	202	174	100/84/109
Mosquito 26	3	2	202	174	109
Mosquito 27	4	2	216/218	181	100
Mosquito 28	50	2	202	174	104/109
Child 1570 (CQ/SP)		2	202/218	174	104
Mosquito 33	1	1	202	174	104
Child 1621-7 (AQ/SP)		3	202	174	104/107
Mosquito 35	24	3	202	174	107
Child 1678-3 (CQ/SP)		1	202	174	104
Mosquito 36	6	1	202	174	104
Mosquito 37	3	2	202	174	84
Child 1753-4 (CQ/SP)		1	202	174	104
Mosquito 38	36	1	202	174	104
Mosquito 39	53	1	202	174	104
Child 1771-6 (CQ/SP)		1	202	174	104
Mosquito 42	3	2	202	174	104
Mosquito 43	1	1	202	174	84
Child 1811-6 (CQ/SP)		1	202	176	104
Mosquito 47	5	1	202	174	104
Child 1849 (CQ/SP)		3	202	174	104
Mosquito 48	24	3	202	174	104
Mosquito 50	65	1	202	174	104
Mosquito 51	100	1	202	174	104
Mosquito 52	100	3	202	174	104
Mosquito 53	100	3	202	174	104
Mosquito 54	100	2	202	174	104
Child 1950 (AQ/SP)		2	202	174	104
Mosquito 60	1	2	202	174	104

*Drug regimens are shown in parentheses. CQ = chloroquine; SP = sulfadoxine/pyrimethamine; AQ = amodiaquine.

†All samples had the 51I, 59R, 108N *dhfr* genotype, except the sample from child 1442, which had the 51I, 59C, 108N *dhfr* genotype.

‡Number of merozoite surface protein 1 (*MSP-1*) alleles were estimated in blood samples taken on day 7 on which mosquito feeding was carried out.

midguts of infected mosquitoes harbored various numbers of oocysts ranging from 1 to 100 per midgut (Table 1). However, 20 (30%) of 60 mosquitoes were infected with only one oocyst, which enabled us to examine cross-mating between parasites that carry different haplotypes of the *dhfr* gene and other variable alleles of *MSP-1* and *pfg 377* loci.

Merozoite surface protein (*MSP-1*) 1 alleles in children and infected mosquitoes. The *MSP-1* alleles were successfully genotyped in 96% (21 of 22) of day 7 blood samples from children and in 58% (35 of 60) of infected mosquitoes. Fourteen of the children harbored more than one allele of *MSP-1*, and 7 had single clone infections with a mean number of 1.8 clones per child. Similarly, 14 (40%) of 35 mosquitoes typed for *MSP-1* harbored more than one allele and 21 had single clone infections with a mean number of 1.6 clones per infected mosquito.

Three (37%) of eight mosquitoes, in which only one oocyst was detected, harbored more than one *MSP-1* allele (e.g., mosquito 2 and 60; Table 1), which indicated that these oocysts developed from heterozygous zygotes that resulted from cross-mating between *P. falciparum* clones of different genotypes.

Gametocyte-specific protein gene (*Pfg377*) in infected people and mosquitoes. The *Pfg377* alleles were successfully amplified in 73% (16 of 22) of infected children before treatment (day 0) and in 82% (18 of 22) after anti-malarial treatment (day 7), and in 85% (51 of 60) of infected mosquitoes that had fed on the day 7 blood samples. Seven alleles ranging in size between 280 basepairs and 400 basepairs were detected. The most common pre-treatment alleles (340 basepairs and 360 basepairs) were also most prevalent post-treatment and in infected mosquitoes.

The *pfg377* typing detected more multiple clone infections in pretreatment sample 31% (5 of 16) and in mosquitoes 16% (8 of 51) than in day 7 samples, in which only 5% (1 of 18) of isolates had multiple *pfg377* alleles (Figure 1). Of 13 mosquitoes infected with one oocyst, one mosquito harbored more than one *pfg377* allele, which supported the *MSP-1* data and the presence of heterozygous zygotes resulting from cross-mating between *P. falciparum* clones of different genotypes.

Dihydrofolate reductase (*dhfr*) haplotypes among infected children. Two *dhfr* alleles were detected among 20 *P. falciparum* isolates obtained from the 22 children. One child had the double-mutant allele (51I, 59C, 108N), and the remaining 21 children had the triple-mutant allele (51I, 59R, 108N).

Three polymorphic microsatellite loci located 5.3 kb, 4.4 kb, and 0.3 kb upstream from the *dhfr* gene and examined among the above *P. falciparum* isolates had eight (192–218 basepairs), five (172–181 basepairs), and six (84–109 basepairs) alleles, respectively. Microsatellite alleles showed remarkable diversity among *P. falciparum* parasites in infected children. The two *dhfr* genotypes detected among the infected children were then sorted into 16 distinct *dhfr* haplotypes based on alleles of flanking microsatellites. Two haplotypes had the double-mutant allele (51I, 59C, 108N) and the remaining 14 haplotypes had the triple-mutant allele (51I, 59R, 108N) (Figure 2).

Eight (40%) of the 20 children had *P. falciparum* infections containing more than one allele in at least one of the examined microsatellite loci. We used either predominant or minority alleles at each locus to construct haplotypes. Using this conservative estimate, we identified 33 clones among the 20 infected children. All *dhfr* haplotypes identified among the 33 clones occurred at a limited frequency (3–6%), except for one dominant haplotype present in 14 (42%) of the 33 examined clones (Figure 2).

FIGURE 2. Dihydrofolate reductase (*dhfr*) haplotypes detected in *Plasmodium falciparum* clones in infected children (A) and mosquitoes that fed on them on day 7 after drug treatment (B). Black bars show haplotypes with double-mutant allele (51I, 59C, 108N) and white bars show haplotypes with the triple-mutant allele (51I, 59R, 108N).

Dihydrofolate reductase haplotypes in infected mosquitoes.

We successfully characterized 24 of the 60 infected mosquitoes at the *dhfr* locus. Similar to infected children, 4 (17%) of the 24 mosquitoes harbored more than one allele of the examined microsatellites, which was indicative of presence of more than one *P. falciparum* genotype per infected mosquito. We identified 30 *P. falciparum* clones based on possible combinations of either dominant or minority microsatellites alleles.

The combined *dhfr* alleles and microsatellites showed nine distinct haplotypes among the 30 clones in the infected mosquitoes (Figure 2). All of these haplotypes carried the triple-mutant *dhfr* genotype (51I, 59R, 108N). The predominant haplotype in infected children was also the most frequent among infected mosquitoes ($P = 0.21$) (Figure 2). However, none of the two haplotypes with the double-mutant *dhfr* allele (51I, 59C, 108N) were seen in infected mosquitoes. Likewise, five haplotypes, with triple mutations, seen among infected mosquitoes were not detected in infected children before the start of treatment (Figure 2).

Transmission of *dhfr* haplotypes within a single infection.

To illustrate dynamics of transmission within individual infections, Table 1 shows *dhfr* haplotypes detected on day 0 (before treatment) among 11 children and mosquitoes that fed on blood samples taken on day 7 (post treatment) and *MSP-I* alleles detected on day 7 and in infected mosquitoes. Most multiple clone infections based on microsatellite haplotypes on day 0 (prior to treatment) had multiple *MSP-I* alleles on day 7 (post-treatment) (Table 1; child 1442, child 1551, and child 1570).

Mosquitoes that fed on blood samples containing multiple *dhfr* haplotypes acquired multiple *dhfr* haplotypes and *MSP-I* alleles (Table 1; e.g. mosquitoes 2, 27, and 28). Some of these mosquitoes were infected with one oocyst (e.g., mosquito 2), which indicated that cross-mating had occurred between *P. falciparum* clones with different *dhfr* haplotypes (Table 1).

Generally, similar *dhfr* haplotypes were detected in children and in mosquitoes that fed on their blood samples. Nevertheless, in some cases, clear differences between haplotypes

in infected children and mosquitoes were seen (Table 1). For example, in patient 1442, all haplotypes detected before treatment carried the double mutations (51I, 59C, 108N). However, *dhfr* haplotypes detected in mosquitoes that fed on post-treatment blood sample all carried the triple-mutant allele (51I, 59R, 108N) (Table 1). In child 1551-0, three distinct alleles at the 0.3-kb locus were detected, which indicated the presence of at least three haplotypes before treatment. However, two infected mosquitoes (27 and 28) that fed on blood taken from this patient had different alleles that were not seen before treatment (Table 1).

DISCUSSION

We characterized drug-resistant *dhfr* haplotypes among *P. falciparum*-infected children in Farafenni in The Gambia and examined their infectivity to *Anopheles* mosquitoes. Our study attempted to examine whether the dominant triple-mutant *dhfr* haplotype in Africa, which originated in Asia, has a relative intrinsic higher transmission capacity compared with others and if this haplotype is transmitted as an independent lineage or recombines with others. Twenty haplotypes carrying triple *dhfr* mutations were detected among infected children. However, a one haplotype existed as a predominant lineage. This haplotype was also the most frequently observed in the mosquitoes after drug treatment of the children. In a limited number of examined mosquitoes, cross-mating between *P. falciparum* carrying different triple-mutant *dhfr* haplotypes was seen, which indicated recombination between haplotypes that carry triple mutations.

The major haplotype seen in The Gambia shares microsatellites with a wide spreading haplotype present in other countries in Africa where similar analysis was carried out, which suggests a common source of this haplotype.^{10-12,26} This finding has been confirmed by simultaneous analysis of *P. falciparum* isolates in 11 countries in Africa, which found that 85% of isolates carrying the triple-mutant *dhfr* shared the same flanking microsatellite alleles.¹³ These findings support the hypothesis that gene flow and migration is a primary source of spread of pyrimethamine resistance in Africa.²⁶ Nonetheless, as we have seen in The Gambia, multiple triple-mutant *dhfr* haplotypes exist at limited frequency in many countries.^{11-13,15} Some of these triple-mutant haplotypes have markers that characterize the wild type and low-level pyrimethamine-resistant parasites. For example, haplotypes 84, 174, and 202 (Figure 2), which exist at a high frequency, carry a microsatellite (84 at 0.3 kb) that have been seen mostly in wild-type parasites.^{11,27} In addition, this allele has also been seen in two different haplotypes (84, 172, 202 and 84, 172, 196) (Figure 2) with double mutations.

As demonstrated in our study, cross-mating between *P. falciparum* clones with different triple-mutant *dhfr* haplotypes can result in generation of multiple *dhfr* lineages with this allele. In the present study, the major resistant haplotype shared some flanking microsatellites with other minor haplotypes with triple *dhfr* mutations. This finding suggests that these haplotypes have been generated by recombination between existing clones with triple-mutant *dhfr* genotype, which demonstrates the role of local evolution of pyrimethamine resistance.^{8,10,15} In addition, two *dhfr* haplotypes with double mutations (Figure 2 and Table 1) also shared similar flanking microsatellites with other haplotypes harboring triple mutations. Therefore,

although migration can be a major sources of spread of a high-level resistant *dhfr* haplotype,²⁶ cross-mating and recombination can generate novel haplotypes in areas with recent history of resistance to the drug of concern.^{8-10,15} However, the most successful *dhfr* haplotype might have higher intrinsic transmission success over others, and therefore can readily sweep across a sensitive parasite population driven by drug pressure.^{9,10}

Two major limitations hampered our attempt to acquire a large enough sample size for detailed statistical analysis. First, we were unable to type *dhfr* haplotypes in most of the blood samples collected on day 7, on which *Anopheles* mosquitoes were fed. This could have resulted if the *dhfr* primers were less sensitive than those for other genes and would have been more pronounced in post-treatment samples, in which asexual parasites had been cleared or reduced below microscopic detection after drug treatment. Second, only a limited number of infected mosquitoes were successfully typed for *dhfr* haplotypes probably because of the limited amount of parasite DNA that would have been isolated especially from mosquitoes harboring only one oocyst. Nonetheless, by comparing *dhfr* haplotypes seen before treatment and those transmitted to mosquitoes, two main observations can be made. First, only 9 (64%) of 14 with triple mutations seen on day 0 were transmitted to mosquitoes, assuming that all of them were capable of surviving drug treatment. Second, the dominant haplotype (202, 174, 104) seen in infected children was also highly dominant in infected mosquitoes.

In view of the high frequency of triple *dhfr* mutants in the study site, differences between haplotypes seen on day 0 and in infected mosquitoes cannot solely be attributed to drug selection. Apart from possible preferential transmission of the common haplotype, other explanations as to why some haplotypes seen on day 0 did not transmit to mosquitoes fed on blood samples taken on day 7 may be that gametocytes of some haplotypes detected on day 0 may have been sequestered in the spleen and so were not accessible to mosquitoes, or that some minority resistant haplotypes at a lower density were removed during PCR by the more abundant clones detected on day 0. However, after SP treatment, the minority highly resistant haplotypes could have been selected and increased in density and successfully transmitted to mosquitoes. For example, in patient 1442 (Table 1), all *dhfr* haplotypes detected before treatment harbored double mutations (51I, 108N). However, infected mosquitoes that fed on day 7 blood (post-treatment) carried triple-mutant (51I, 59R, 108N) haplotypes. These haplotypes contained some microsatellites that were distinct from those flanking the double-mutant allele seen prior to drug treatment. Thus, *in vivo* selection is not a likely scenario. In a multiple infection of sensitive and resistant parasite, when a drug sensitive clone is cleared by an anti-malarial drug, the resistant clone can expand the density of its asexual forms and gametocytes,¹³ which potentially enhances the spread of the drug resistant clones.

In addition to impact of drug pressure on the dynamics of different clones within an infection, host genotype and parasite genotype interactions are important determinants of transmission success.²⁸ Because we used a colony of *Anopheles* mosquitoes, the host variability effect would have been minimized. However, the influence of the parasite genetics cannot be ruled out because different *P. falciparum* parasite genotypes can vary in their ability to produce gametocytes and transmit

them to mosquitoes.^{29–31} The minority parasite genotypes within an infection can therefore compete efficiently with the majority parasites for mosquito transmission.²⁰

Detection of two *dhfr* haplotypes in a mosquito infected with one oocyst demonstrates the ability of drug-resistant *P. falciparum* clones to cross-mate to produce new lineages. This is the first study to demonstrate cross-mating between different drug-resistant haplotypes in the field. However, the fate of the progeny of this cross-mating is not clear because not all midgut oocysts mature to infectious sporozoites. The ideal sample in which to test for transmission success of recombinant drug-resistant haplotypes resulting from cross-mating should be sporozoites isolated from the salivary gland of infected mosquitoes. However, such an approach would fail to differentiate between haplotypes generated by recombination and multiple oocysts of independent mating events simultaneously acquired by the same mosquito. The extent of cross-mating between different *P. falciparum* clones in the field varies greatly between different areas depending on the intensity of transmission and rate of multiplicity of infection.^{32–35} In addition, the impact of recombination on generating novel lineages of drug resistance can be offset by the level of drug pressure.¹ This finding may explain the variation in the extent of diversity hitchhiking around triple-mutant *dhfr* genotypes in different countries, such that although one haplotype can be seen in areas with high drug pressure,²⁶ multiple lineages are detected in areas with lesser drug pressure and a relatively recent history of resistance to the anti-malarial drug in question.^{8,9}

In view of the widespread use of artemisin-based combination therapy and SP as treatment for patients with malaria, further studies should examine whether certain triple-mutant *dhfr* haplotypes vary in their resistance to pyrimethamine and ability to transmit to mosquitoes. Such information may provide a better predictive index of failure of SP treatment and spread of resistance.

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