Increasing Prevalence of a Novel Triple-Mutant Dihydropteroate Synthase Genotype in *Plasmodium falciparum* in Western Kenya

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The molecular basis of sulfadoxine-pyrimethamine (SP) resistance lies in a combination of single-nucleotide polymorphisms (SNPs) in two genes coding for *Plasmodium falciparum* dihydrofolate reductase (*Pf dhfr*) and *P. falciparum* dihydropteroate synthase (*Pf dhps*), targeted by pyrimethamine and sulfadoxine, respectively. The continued use of SP for intermittent preventive treatment in pregnant women in many African countries, despite SP's discontinuation as a first-line antimalarial treatment option due to high levels of drug resistance, may further increase the prevalence of SP-resistant parasites and/or lead to the selection of new mutations. An antimalarial drug resistance surveillance study was conducted in western Kenya between 2010 and 2013. A total of 203 clinical samples from children with uncomplicated malaria were genotyped for SNPs associated with SP resistance. The prevalence of the triple-mutant *Pf dhfr* C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ genotype and the double-mutant *Pf dhps* S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype was high. Two triple-mutant *Pf dhps* genotypes, S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ and H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃, were found, with the latter thus far being uniquely found in western Kenya. The prevalence of the S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype was low. However, a steady increase in the prevalence of the *Pf dhps* triple-mutant H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype has been observed since its appearance in early 2000. Isolates with these genotypes shared substantial microsatellite haplotypes with the most common double-mutant allele, suggesting that this triple-mutant allele may have evolved locally. Overall, these findings show that the prevalence of the H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ triple mutant may be increasing in this population and could compromise the efficacy of SP for intermittent preventive treatment in pregnant women if it increases the resistance threshold further.

The use of sulfadoxine-pyrimethamine (SP) for the treatment of malaria has been discontinued in many African countries due to high levels of drug resistance, as reviewed in reference 1. However, SP is still used in many countries in Africa for intermittent preventive treatment in pregnant women (IPTp), as there are very few drugs available for safe use in pregnant women (2). One of the factors that allows the selection and maintenance of parasites with genetic backgrounds conferring drug resistance in a population is drug pressure (3). Therefore, the continued use of SP can maintain or even increase the prevalence of SP-resistant parasites (4) in a population. Resistance to SP is conferred by the accumulation of single-nucleotide polymorphisms (SNPs) in two genes that code for enzymes involved in the *P. falciparum* folate metabolism, *P. falciparum* dihydrofolate reductase (*Pf dhfr*) and *P. falciparum* dihydropteroate synthase (*Pf dhps*), which are targeted by pyrimethamine and sulfadoxine, respectively. At least five mutations in *Pf dhfr* are thought to confer resistance to pyrimethamine: C50R, N51I, C59R, S108N, and I164L (mutated amino acids are in bold-face). Similarly, at least five SNPs in *Pf dhps* are reported to be involved in resistance to sulfadoxine, including S436A/F, A437G, K540E, A581G, and A613S/T. In Africa, the most commonly observed genotype conferring pyrimethamine resistance is the *Pf dhfr* triple-mutant genotype C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ (reviewed in references 1 and 5), which was recently found at frequencies of as high as 80% in East Africa (6). The addition of a fourth mutation, I164L, has been shown to lead to a highly resistant phenotype (7, 8), but this I164L mutation is observed only at a low prevalence in western Kenya (6, 9, 10). In *Pf dhps*, two different patterns of resistance have been reported in Africa: the 437G and 540E mutations are commonly reported in East Africa, while in West and Central Africa, the 436A and 437G mutations are common (11–13). As

reviewed in references 1 and 5, the quintuple-mutant genotype (*Pf dhfr* C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ and *Pf dhps* S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃) has been associated with resistance to SP in many parts of Africa.

Recent studies in East Africa have reported the emergence of triple *Pf dhps* mutants involving the addition of an SNP at codon 581 or 613 to the double-mutant A437G/K540E genotype (8, 14–17). These additional *Pf dhps* mutations, A581G or A613S/T, have been reported to be present at a low prevalence in *P. falciparum* isolates collected from western Kenya (6, 10). However, a novel mutation at codon 436 (S436H) was found in western Kenya (6, 10) and, to the best of our knowledge, has not been reported elsewhere. This S436H mutation was first reported in samples from pregnant women collected between 2002 and 2008 (10) and more recently in samples from the general population collected between 2008 and 2012 (6). The acquisition of additional *Pf dhfr* and *Pf dhps* mutations by parasites harboring the quintuple-mutant genotype *Pf dhfr* C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ and *Pf dhps* S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ has led to so-called superresistant parasites

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due to their increased resistance to SP (reviewed by Naidoo and Roper [5]). Therefore, it is important to continuously monitor the prevalence of resistant mutant genotypes in Africa, where SP is still used for prophylaxis in pregnant women, as previously reviewed by Venkatesan et al. (18).

Studies on the dynamics of antimalarial drug resistance in Africa have indicated that pyrimethamine-resistant parasites originated from Southeast Asia and subsequently spread to Africa (19). In addition, a recent study by Mita et al. (20) provided further evidence to suggest that some *Pfdhps* alleles migrated from Southeast Asia to Africa. Nevertheless, multiple local origins of *Pfdhps* single-mutant and *Pfdhps* double-mutant genotypes have also been reported previously (21). However, our understanding of the geographic origin and spread of sulfadoxine-resistant *Pfdhps* genotypes is far from complete, as *Pfdhps* triple-mutant parasites are still evolving in this region. Recently, the A581G mutation, known to confer increased resistance to sulfadoxine, was shown to have a local origination in Malawi and Tanzania (16). Therefore, the appearance of the additional *Pfdhps* S436H mutation in western Kenya warrants some investigation into its origin(s). Did it evolve within the S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ background, which is almost at fixation in this region, or did it arrive with parasites introduced from elsewhere in Africa or Southeast Asia?

We investigated the prevalence of SNPs associated with SP resistance in *P. falciparum* isolates collected from children in western Kenya between 2010 and 2013. In addition, the microsatellite allele heterozygosity in the *Pfdhps* S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ double mutant and H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ triple mutant was investigated to determine whether this gene may have undergone recent selective pressure. In order to determine the genetic relatedness of the *Pfdhps* mutants observed in this study, the haplotypes of eight microsatellite loci flanking the *Pfdhps* locus were determined and compared to the haplotypes of mutant parasites from Cambodia, Ghana, and Thailand.

MATERIALS AND METHODS

Sample collection. The Kenya Medical Research Institute (KEMRI) has initiated a continuous *in vitro* and molecular surveillance study to monitor the evolving patterns of antimalarial resistance in western Kenya. As part of this surveillance, two public hospitals located in the Siaya and Bondo Districts in western Kenya were used to collect blood samples between 2010 and 2013. These hospitals are located in a region of the country where malaria is endemic, with the prevalence of *P. falciparum* infection ranging from 20% to 40% (http://www.pmi.gov/docs/default-source/default-document-library/malaria-operational-plans/fy14/kenya_mop_fy14.pdf?sfvrsn=10). The two hospitals are approximately 60 km from KEMRI in Kisumu, Kenya, where the laboratory studies were conducted. The Bondo District Hospital outpatient department examines up to 50 patients on a single day during peak malaria season. The Siaya District Hospital is the only major hospital serving the Siaya District. The outpatient department examines over 40 patients per day during peak malaria transmission seasons. The aim of the study was to monitor the prevalence of genotypes associated with resistance to different antimalarial drugs. Children between the ages of 6 months and 7 years with a diagnosis of uncomplicated *P. falciparum* malaria and an axillary temperature of $\geq 37.5^{\circ}\text{C}$ or a history of fever in the previous 24 h with no recent treatment (at least in the last 7 days) for malaria were enrolled. This study was approved by the institutional review boards of KEMRI and the Centers for Disease Control and Prevention (CDC). The parents or guardians of all children who met the inclusion criteria were referred by the health facility nurse or clinical officer to study personnel. An informed written

consent to participate in the study was obtained from the parent/guardian of the children who participated in this study.

***Pfdhfr* and *Pfdhps* genotyping analysis.** Genomic DNA was isolated from blood samples collected on Whatman grade 3 filter papers using a QIAamp blood minikit (Qiagen Inc., CA, USA). SNPs at *Pfdhfr* codons 50, 51, 59, 108, and 164 and *Pfdhps* codons 436, 437, 540, 581, and 613 were identified using Sanger sequencing as previously described (22). Sequencing of the nested purified PCR products was performed using a BigDye Terminator (v3.1) cycle sequencing kit on an iCycler thermal cycler (Bio-Rad, Hercules, CA). The DNA from the reaction mixtures was precipitated in 70% ethanol to clean up the dye terminators, rehydrated in 10 μl HiDi formamide, and sequenced using an Applied Biosystems 3130 xl sequencer (Life Technologies, Grand Island, NY). The codons were classified as wild type (having only the wild-type codon present in the sample), mutant (having only the mutant codon present in the sample), or mixed (having both wild-type and mutant codons present in the sample). Mixed infections were excluded when genotype calling was done. However, the presence of mixed infections was reported.

Temporal trend in prevalence of major genotypes conferring SP resistance in western Kenya. In order to determine the temporal trends in the prevalence of the major genotypes conferring SP resistance since SP was introduced in western Kenya, historical published data from the same region (10, 23) were used together with data obtained from the current study. In calculating the prevalence of these genotypes, isolates with mutant codons were analyzed together with isolates with mixed genotypes. The previously published prevalence data reported by the authors were extracted from the tables and figures.

Multilocus genotyping and estimating expected heterozygosity. We genotyped eight microsatellite loci flanking *Pfdhps* on chromosome 8 at -7.5 , -2.9 , -1.5 , -0.13 , 0.03 , 0.5 , 1.4 , and 6.4 kb. The markers with negative values denote loci located 5' (upstream) of *Pfdhps*, while markers with positive values denote loci located 3' (downstream) of the gene. These loci and their respective primers and cycling conditions have been described previously (21, 24, 25) and have been used to investigate signatures of selection around the *Pfdhps* gene. Loci that are under selection tend to show a lower expected heterozygosity (H_e) than loci that are selectively neutral (26). In addition, eight putatively neutral microsatellite loci located on chromosomes 2 and 3 were genotyped in order to exclude the possibility that the samples were infected with multiple strains and to estimate the neutral baseline H_e in the samples analyzed (27). These eight microsatellite loci were as follows: C2M27, C2M29, C2M33, and C2M34 on chromosome 2 and C3M39, C3M40, C3M69, and C3M88 on chromosome 3. The eight neutral microsatellite loci and their respective primers and cycling parameters have been described previously (9, 21, 22, 24).

The sizes of the amplification products were assayed by capillary electrophoresis on an Applied Biosystems 3130 xl sequencer (Life Technologies, Grand Island, NY). The fragments were then scored using GeneMapper software (v3.7; Life Technologies, Grand Island, NY) with default microsatellite settings, where peaks lower than 100 relative fluorescence units (RFU) were defined to be background peaks. The tallest peak was identified to be the predominant allele; peaks whose height was over 33% of the height of the tallest peak were identified to be additional alleles. When more than one allele was identified at any locus, the sample was presumed to be infected with two or more genetically distinct clones. Samples for which amplification of one or more loci was initially unsuccessful were reanalyzed to complete the microsatellite profiles. Only those samples presumed to be infected by a single parasite clone (defined by amplification of only one allele at each of the eight neutral microsatellite loci and each of the eight loci flanking *Pfdhps* on chromosome 8) were used for H_e analysis ($n = 29$). H_e was estimated for each microsatellite locus using the Excel microsatellite tool kit (28). We used the following formulae to calculate H_e : $[n/(n-1)][1 - \sum p_i^2]$ and $[2(n-1)/n^3]\{2(n-2) [\sum (p_i^3 - (\sum p_i^2)^2)]\}$, where n is the number of samples genotyped for the locus and p_i is the frequency of the i th allele.

TABLE 1 Total number of samples with SNPs observed during the study period, 2010 to 2013

Strain	No. of samples with the indicated SNP in ^a :									
	<i>Pfdhfr</i>					<i>Pfdhps</i>				
	C50R	N51I	C59R	S108N	I164L	S436H	A437G	K540E	A581G	A613S/T
Wild type	202	0	7	0	198	170	0	0	200	203
Mutant	0	201	187	201	2	12	203	202	1	0
Mixed	0	1	8	0	2	16	0	1	2	0
Total	202	202	202	201	202	198	203	203	203	203

^a Mutations are in boldface.

RESULTS

A total of 203 samples collected during 4 years of continuous surveillance (2010 to 2013) were genotyped for the *Pfdhfr* and *Pfdhps* mutations associated with SP resistance. Table 1 summarizes the different SNPs observed in the samples collected during the 4 years of the study. Of the 202 samples successfully genotyped for the *Pfdhfr* gene, 99.5% harbored the mutant allele 51I, while 1 sample (0.5%) had a mixed infection with both wild-type and mutant parasites at this codon. No mutations were observed at codon 50. One hundred eighty-seven samples (92.6%) had the mutant allele 59R at codon 59, 7 had the wild-type allele at codon 59 (3.5%), and 8 (3.9%) had mixed infections with both wild-type and mutant alleles. All the samples genotyped (100%) harbored the 108N mutation, and 4 samples out of 202 (2%) had the 164L mutation; 2 of the samples with the latter mutation were part of mixed infections with the wild-type allele.

We observed the *Pfdhps* S436H mutation in 28 of the 198 (14.1%) samples successfully genotyped for this codon; over half of these samples (16 out of 28) also harbored the wild-type allele. The 437G mutation was observed in 100% of the samples genotyped. Similarly, 203 out of 203 samples harbored the 540E mutation, except that 1 of these had a mixed infection with both wild-type and mutant alleles. The *Pfdhps* A581G mutation was observed in 3 out of 203 samples (1.5%), 2 of which were mixed infections with the wild-type allele. All the samples genotyped harbored the wild-type A613 allele.

The overall frequency of parasites with the *Pfdhfr* triple-mutant C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ genotype was 91.7%. The *Pfdhfr* double-mutant C₅₀I₅₁C₅₉N₁₀₈I₁₆₄ genotype was present in 2.6% of samples, and 1% of samples had the quadruple-mutant C₅₀I₅₁R₅₉N₁₀₈L₁₆₄ genotype. No wild-type genotypes were de-

tected; however, 4.7% of the isolates had mixed genotypes at codon 51 ($n = 1$), 59 ($n = 7$), and/or 164 ($n = 2$) (Table 2). The frequency of the *Pfdhps* double-mutant S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype was 86.6%. Two different types of *Pfdhps* triple-mutant genotypes were observed: H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃, observed in 5.7% of the samples, and S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃, observed in 0.5% of the samples. The remaining 7.2% of samples had mixed infections harboring both wild-type and mutant alleles (Table 2).

In calculating the prevalence of the observed genotypes, isolates with mutant codons were analyzed together with isolates with mixed genotypes. No differences in the yearly prevalence of the S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ and C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ genotypes were observed over the time period of the study (Table 3). We observed a low prevalence of the *Pfdhfr* double-mutant C₅₀N₅₁R₅₉N₁₀₈I₁₆₄ genotype only in 2013 (1.1%); however, the C₅₀I₅₁C₅₉N₁₀₈I₁₆₄ double mutant was observed in all 4 years. The *Pfdhfr* triple-mutant C₅₀I₅₁C₅₉N₁₀₈L₁₆₄ genotype was observed only in 2013 (2.2%), while the *Pfdhfr* quadruple-mutant C₅₀I₅₁R₅₉N₁₀₈L₁₆₄ genotype was observed in 2010 (2.7%) and again in 2013 (2.2%). We observed an increase in the prevalence of the *Pfdhps* triple-mutant H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype from 2.9% in 2010, to 13.6% in 2011 and 16.3% in 2012. A slight decline to 14.4% was observed in 2013. The *Pfdhps* triple-mutant S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype was observed in 2012 (2.3%) and in 2013 (2.1%).

In order to determine the temporal trends in the prevalence of the major genotypes conferring SP resistance since SP was introduced in western Kenya, we plotted the prevalence of these genotypes, as reported from selected published data collected by others in the same region (10, 23) and data obtained from this study (2010 to 2013), against the year in which the data were collected

TABLE 2 Summary of genotypes identified in samples with complete data

<i>Pfdhfr</i>							<i>Pfdhps</i>						
Total no. of samples	Genotype ^a					% of all samples with the genotype	Total no. of samples	Genotype					% of all samples with the genotype
	C50R	N51I	C59R	S108N	I164L			S436H	A437G	K540E	A581G	A613S/T	
177	C	I	R	N	I	91.7	168	S	G	E	A	A	86.6
6	C	I	C/R	N	I	3.1	11	H	G	E	A	A	5.7
5	C	I	C	N	I	2.6	11	S/H	G	E	A	A	5.7
2	C	I	R	N	L	1	1	S	G	E	A/G	A	0.5
1	C	I	R	N	I/L	0.5	1	S/H	G	E	A/G	A	0.5
1	C	I	C/R	N	I/L	0.5	1	S/H	G	K/E	A	A	0.5
1	C	N/I	R	N	I	0.5	1	S	G	E	G	A	0.5

^a Mutations are in boldface.

TABLE 3 Yearly prevalence of detected genotypes

Gene and genotype ^a	Prevalence (%)			
	2010	2011	2012	2013
<i>Pfdhfr</i>				
CIRNI	91.9	95.2	95.0	87.8
CICNI	5.4	4.8	5.0	6.7
CICNL	0.0	0.0	0.0	2.2
CIRNL	2.7	0.0	0.0	2.2
CNRNI	0.0	0.0	0.0	1.1
<i>Pfdhps</i>				
SGEAA	97.1	86.4	81.4	80.4
HGEAA	2.9	13.6	16.3	14.4
SGEGA	0.0	0.0	2.3	2.1
SGKAA	0.0	0.0	0.0	2.1
HGEGA	0.0	0.0	0.0	1.0
HGKAA	0.0	0.0	0.0	1.0

^a Mutant codons are in boldface.

(Fig. 1). There was a decline in the prevalence of the *Pfdhfr* wild-type genotype from approximately 3% to 0% shortly after the introduction of SP in Kenya in 1999, while the prevalence of the combined double-mutant C₅₀N₅₁R₅₉N₁₀₈I₁₆₄ and C₅₀I₅₁C₅₉

N₁₀₈I₁₆₄ genotypes declined from 69% during the period from 1996 to 2000 to below 10% by 2010 (Fig. 1). This was accompanied by an increase from 26% to above 85% by 2002 of the more resistant *Pfdhfr* triple-mutant C₅₀I₅₁R₅₉N₁₀₈I₁₆₄ genotype. A similar trend was observed for the *Pfdhps* gene. The prevalence of the wild-type *Pfdhps* genotype was observed to go from 56.4% at its highest in samples collected between 1996 and 2000 to 2.3% in samples collected from 2002 through 2008 and to 0% thereafter. A concomitant increase in the prevalence of the *Pfdhps* double-mutant S₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype was observed, increasing in prevalence from 27% in the period from 2000 to over 90% in the period from 2002 to 2008 (Fig. 1). A high prevalence of the *Pfdhps* double-mutant genotype of above 80% persisted through 2013. The prevalence of the *Pfdhps* H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ mutant was reported to be 2.3% in parasites collected between 2002 and 2008, and the prevalence increased to 3.8% in parasites obtained between 2008 and 2009 (10) (Fig. 1). In this study, the H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype was found in 2.96% of samples in 2010, 13.6% in 2011, 16.3% in 2012, and 14.4% in 2013 (Fig. 1). In contrast, the S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype was found in 5.3% of parasites collected in 2008 and 2009 (10); however, we did not observe this genotype in our study in 2010 and 2011 and found it in

FIG 1 Temporal trends over time (including published data from studies conducted by others). The prevalence of the major genotypes conferring SP resistance from selected published data (10, 23), in addition to those obtained from this study (2010 to 2013), was used to determine the temporal trends of these genotypes since the time that SP was first recommended for use as a first-line treatment for uncomplicated malaria in western Kenya. WT, wild type.

FIG 2 The *Pfdhps* gene is under strong selective pressure. The H_e values at microsatellite loci flanking the *Pfdhps* gene in 36 singly infected samples from Kenya are shown. The dashed line crossing the y axis indicates the mean H_e at eight neutral microsatellite loci on chromosomes 2 and 3 (neutral heterozygosity). Error bars indicate standard deviations.

only 2.3% of samples in 2012 and 2.1% of samples in 2013 (Fig. 1).

The presence and apparent increase in the prevalence of the *Pfdhps* $H_{436}G_{437}E_{540}A_{581}A_{613}$ triple mutant, as previously reported by studies conducted in the same study area (6, 10), prompted us to hypothesize that this gene may have undergone recent selection. Therefore, we amplified the microsatellite alleles flanking the *Pfdhps* gene to identify potential signatures of selection. The region flanking the *Pfdhps* gene had very low H_e values in parasites with the $H_{436}G_{437}E_{540}A_{581}A_{613}$ and $S_{436}G_{437}E_{540}A_{581}A_{613}$ genotypes compared to those for neutral microsatellite loci on chromosomes 2 and 3 (Fig. 2), indicating that this gene may have been under some selective pressure immediately prior to or during the time period from 2010 to 2013.

In order to determine the genetic relatedness of the *Pfdhps* mutants observed in this study, the alleles of 8 microsatellite loci flanking the *Pfdhps* locus were amplified, and the resulting haplotypes were compared to the haplotypes of mutant parasites from Cambodia and Thailand and a single sample from Ghana. No comparison to *Pfdhps* wild-type parasites was possible because none of our samples had pure wild-type genotypes. We found that the $H_{436}G_{437}E_{540}A_{581}A_{613}$ isolates had microsatellite haplotypes identical to those of most $S_{436}G_{437}E_{540}A_{581}A_{613}$ mutant isolates from western Kenya (Fig. 3). Additionally, the majority of the double and triple mutants in our study appeared to share an ancestral lineage with parasites from Thailand and Cambodia, given the similarities in their microsatellite haplotypes. The single isolate with the triple-mutant $S_{436}G_{437}E_{540}G_{581}A_{613}$ genotype from our study had a unique haplotype profile which was different from that of the majority of isolates that we compared (Fig. 3).

DISCUSSION

Shortly after the introduction of SP as first-line therapy for malaria in Kenya, a sharp decline in the prevalence of the wild-type *Pfdhfr* and *Pfdhps* genotypes was observed, and this decline was accompanied by an increase in the prevalence of the *Pfdhfr* triple-mutant and *Pfdhps* double-mutant genotypes conferring resistance (9). By early 2000, the prevalence of these genotypes was above 80% (10, 17), and it has remained high, despite the fact that

SP was replaced by artemether-lumefantrine in 2006 as a first-line treatment for uncomplicated malaria (29). SP is still the drug of choice for IPTp. In addition, it is likely that the use of other sulfa drugs by the general population for the treatment of other illnesses, such as respiratory and diarrheal diseases, may be contributing to the sustained selection pressure (30). The emergence of *Pfdhps* triple mutants with the acquisition of the *Pfdhps* A581G or A613S/T mutation and *Pfdhfr* quadruple mutants with the acquisition of the I164L mutation has been described in studies conducted in East Africa (8–10, 16, 17, 31), implying that these genotypes continue to evolve in this region. The presence of the *Pfdhfr* I164L mutants in western Kenya was first reported by McCollum et al. in 2006 (9). Several other studies have shown the presence of this mutation, but it was always present at low frequencies (10, 32). It is surprising that, even about a decade later and despite the continued use of SP for IPTp, our study found only 4 samples out of 202 (2%) harboring this mutation, and 2 of these had mixed infections with the wild-type allele. This slow progression in the evolution of *Pfdhfr* quadruple mutants in this region may be due to the low level of fitness of this genotype in this area of holoendemicity, as has been speculated by others (33). The *Pfdhps* A581G mutation was also found at a low prevalence (1.5%), which is in agreement with the findings of previous studies from western Kenya (6, 10). We did not observe any mutations at codon 613, although previous studies have reported its occurrence at a low prevalence (6, 10, 17). Interestingly, we observed an increase in the prevalence of the *Pfdhps* S436H mutation since it was first described in isolates obtained from pregnant women in western Kenya (10). To date, only parasites collected in western Kenya have been found to harbor this mutation (6, 10).

While the effect of the S436H mutation on SP resistance is not yet known, it is possible that, like other mutations described at this codon (34–36), it may interfere with the efficient binding of sulfadoxine, leading to increased resistance (36). Therefore, it is important to understand the potential impact of this triple-mutant genotype in increasing the resistance threshold for SP treatment and the effectiveness of SP prophylaxis in pregnant women.

We found that the $H_{436}G_{437}E_{540}A_{581}A_{613}$ genotype had microsatellite haplotypes identical to those in most of the double-mutant $S_{436}G_{437}E_{540}A_{581}A_{613}$ genotype from western Kenya. Given this similarity and the fact that the $H_{436}G_{437}E_{540}A_{581}A_{613}$ genotype has not been reported outside Kenya, we speculate that this novel triple-mutant genotype could have evolved locally on the double-mutant $S_{436}G_{437}E_{540}A_{581}A_{613}$ genotype background. In our study, this double-mutant $S_{436}G_{437}E_{540}A_{581}A_{613}$ genotype from Kenya was also ancestrally related to the double-mutant $S_{436}G_{437}E_{540}A_{581}A_{613}$ genotype and triple-mutant $S_{436}G_{437}E_{540}G_{581}A_{613}$ genotype from Thailand and Cambodia, demonstrating that it likely originated from Southeast Asia, as previously suggested by Mita et al. (20). However, the $H_{436}G_{437}E_{540}A_{581}A_{613}$ genotype appears to be an indigenous type that evolved in Kenya. This study is not the first to show the local appearance of novel *Pfdhps* genotypes in East Africa; recently, Taylor et al. demonstrated that parasites in Malawi with the *Pfdhps* triple-mutant $S_{436}G_{437}E_{540}G_{581}A_{613}$ genotype originated and spread locally and are distinct from parasites with the same genotype found in northern Tanzania (16). However, further surveillance will be necessary to monitor if, indeed, the $H_{436}G_{437}E_{540}A_{581}A_{613}$ genotype is on the rise and, if so, to track its evolution in this setting.

With the exception of the report of Spalding et al. (17), the triple-mutant $S_{436}G_{437}E_{540}G_{581}A_{613}$ genotype has been reported

Allele	-7.5	-2.9	-1.5	-0.13	0.03	0.5	1.4	6.4
KEN_SGEAA	152	190	172	134	138	143	252	286
KEN_SGEAA	152	190	172	134	138	143	252	295
KEN_SGEAA	166	190	172	134	138	143	252	286
KEN_SGEAA	152	190	172	134	138	145	256	286
KEN_HGEAA	152	190	172	134	138	145	252	286
KEN_HGEAA	152	190	172	134	138	143	252	286
KEN_SGEAA	152	190	172	134	140	143	250	286
KEN_HGEAA	152	190	172	134	140	143	250	286
KEN_SGEAA	152	190	172	134	140	143	252	286
KEN_SGEAA	152	190	172	134	140	143	254	286
KEN_SGEAA	152	190	172	134	140	143	276	286
KEN_SGEAA	166	190	172	134	140	143	252	286
KEN_SGEAA	168	190	172	134	140	143	252	286
KEN_HGEAA	152	190	172	134	140	143	252	286
KEN_SGEAA	152	190	172	134	140	145	254	286
KEN_HGEAA	152	190	172	134	140	145	252	286
KEN_SGEAA	152	190	172	129	142	143	252	286
KEN_SGEAA	152	190	172	134	142	143	252	293
KEN_SGEAA	152	190	172	134	142	145	254	286
KEN_SGEGA	174	192	187	132	123	139	264	291
THA_SGEGA	164	190	174	134	142	145	252	286
THA_SGEGA	168	190	172	134	142	145	252	286
THA_SGEGA	172	190	166	134	142	145	252	286
THA_SGEAA	154	192	172	134	142	145	252	286
THA_SGEAA	194	192	172	134	142	145	252	286
THA_SGEGA	154	NA	172	134	142	145	252	286
THA_SGEGA	154	192	172	134	142	145	252	286
THA_SGEGA	154	192	172	134	144	145	252	286
THA_SGEGA	154	192	172	134	167	145	NA	286
THA_SGEGA	154	192	174	134	142	145	252	286
THA_SGEGA	154	192	176	NA	142	145	227	NA
THA_SGEGA	154	192	172	134	142	145	254	286
THA_SGEGA	154	192	174	134	142	145	227	286
THA_SGEGA	154	192	169	134	142	145	252	286
THA_SGEGA	154	192	172	134	NA	145	NA	NA
THA_SGEGA	154	192	172	134	128	145	252	299
THA_SGEGA	154	192	172	134	132	145	252	295
THA_SGEGA	154	192	172	134	138	145	NA	286
THA_SGEGA	154	192	172	134	142	145	NA	286
THA_SGEGA	154	192	172	134	142	145	250	299
THA_SGEGA	168	192	172	134	142	145	250	286
THA_SGEGA	168	192	172	134	142	145	252	286
THA_SGEGA	168	192	172	134	142	145	252	299
THA_SGEGA	168	192	172	134	142	145	254	286
THA_SGEGA	168	NA	172	134	142	145	250	286
THA_SGEGA	168	NA	172	134	142	145	252	286
THA_SGEGA	168	183	172	134	126	145	250	299
THA_SGEGA	168	183	172	134	126	145	252	286
THA_SGEGA	168	183	172	134	142	145	252	286
THA_SGEGA	168	183	172	134	142	145	272	286
THA_SGEGA	168	183	172	134	146	145	252	286
THA_SGEGA	168	183	174	134	142	145	252	286
THA_SGEGA	170	183	172	134	126	145	250	299
THA_SGEGA	176	187	172	134	142	145	250	NA
THA_SGEGA	176	192	172	134	142	145	227	286
THA_SGEGA	176	192	172	134	142	145	252	NA
THA_SGEGA	178	NA	172	134	142	145	252	286
CAM_SGEAA	168	190	172	134	126	145	250	299
CAM_SGEAA	168	190	172	134	126	150	248	295
CAM_SGEAA	174	190	172	134	126	150	248	295
CAM_SGEAA	176	190	172	134	126	150	248	295
CAM_SGEAA	194	190	172	134	126	150	248	286
CAM_SGEAA	194	190	172	134	126	150	248	295
CAM_SGEAA	194	190	172	134	126	150	248	295
CAM_SGEAA	194	190	172	134	132	150	248	295
CAM_SGEAA	154	192	172	134	142	145	272	286
CAM_SGEGA	154	192	172	134	142	145	252	286
CAM_SGEGA	168	192	172	134	142	145	252	286
GHA_SGEAA	154	192	176	134	142	145	252	284

FIG 3 Genetic relationships among parasites with *Pfdhps* double and triple mutations collected in Kenya, Cambodia, Thailand, and Ghana. The eight-locus microsatellite haplotype profiles (the three-digit numbers in the boxes) flanking the *Pfdhps* gene of parasites obtained from Kenya (KEN), Thailand (THA), Cambodia (CAM), and Ghana (GHA) were determined. Three genotypes are represented: SGEGA (black), HGEAA (green), and SGEAA (red). Identical colors (yellow, pink, orange, green, blue, and gray) represent proposed common lineages. NA, not amplified (therefore, the allele size was not determined).

only at a low prevalence in western Kenya in recent years (6, 10). In our study, only one sample had this genotype. Therefore, it appears that the triple-mutant H₄₃₆G₄₃₇E₅₄₀A₅₈₀A₆₁₃ genotype is more prevalent in western Kenya and that its prevalence is increasing faster than that of the triple-mutant S₄₃₆G₄₃₇E₅₄₀G₅₈₀A₆₁₃ genotype. This raises the question as to whether the novel *Pfdhps* triple-mutant H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype is biologically fitter than the *Pfdhps* triple-mutant S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype. However, this hypothesis needs further validation. Interestingly, the single isolate with the triple-mutant S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype observed in our study had a unique microsatellite haplotype profile compared to the profiles of the other isolates from Kenya as well as those from Thailand, Cambodia, and Ghana. The

possibility of local migration from neighboring countries, such as Tanzania, cannot be ruled out, given that the *Pfdhps* triple-mutant S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ genotype has been reported in this region (8, 15–17, 37). However, further studies using more samples are needed to definitively answer this question. Several studies from East Africa have reported on the decreased efficacy of SP due to the high prevalence of genotypes conferring SP resistance in this region (8, 14, 15, 37, 38). The use of SP in IPTp was associated with increased placental inflammation and appeared to select for parasites with the *Pfdhps* 581G allele, which confers high levels of resistance (37). These studies have raised some concerns about the continued use of SP in IPTp in areas where *Pfdhfr* triple-mutant genotypes are near fixation. The WHO

recommends the discontinuation of SP in IPTp when the prevalence of the *Pfdhps* A581G mutation is greater than 10% (39). In addition, a trend toward an increase in the level of the novel H₄₃₆G₄₃₇E₅₄₀A₅₈₁A₆₁₃ genotype, whose role in SP resistance remains to be elucidated, shows that the *Pfdhps* gene continues to evolve. These findings warrant continued vigilance to determine if SP is going to remain efficacious for IPTp in the future in this region.

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