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Research Article

Single Nucleotide Polymorphisms of *Pfdhfr* and *Pfdhps* Genes: Implications for Malaria Prophylactic Strategies in Maiduguri, Northeast Nigeria

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Background. The success of Intermittent Preventive Treatment in Pregnancy (IPTp), Intermittent Preventive Treatment in Infancy (IPTi), and Seasonal Malaria Chemoprevention (SMC) depends on sulfadoxine-pyrimethamine (SP) efficacy. *Objective*. The study determined Single Nucleotide Polymorphisms (SNPs) of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) in Maiduguri, Northeast Nigeria. *Materials and Methods*. Giemsa-stained blood smears, capillary blood, and dried blood spot samples were collected from 63 subjects with uncomplicated malaria in Maiduguri between May and October 2018. *Plasmodium species* was determined and parasite density (PD) was estimated using the smears. Genomic DNA (gDNA) of *P. falciparum* was extracted from the dried blood spot samples using QIAamp DNA Mini Kit. The gDNA was subjected to nested PCR followed by restriction fragment length polymorphism (RFLP) to determine SNPs at *Pfdhfr* codons N51I, C59R, and S108N and *Pfdhps* codons S436A/F, A437G, and K540E. *Results*. The subjects' mean age ± standard deviation was 23.6 ± 8.7 (2.0–67.0) years with a geometric mean PD of 8,948 (2,100–13,400) asexual parasites/μl blood. SNPs prevalence at any of the six *Pfdhfr* and *Pfdhps* codons was 85.7% (54/63); the prevalence was higher ($p < 0.05$) in *Pfdhfr* (82.5%; 52/63) than *Pfdhps* (58.7%; 37/63). *Pfdhfr* allele 108N (82.5%; 52/63) was the highest ($p < 0.05$) mutant when compared with alleles 51I (60.3%; 38/63) and 59R (66.7%; 42/63). Triple *Pfdhfr* mutation was observed in 60.3% (38/63) of the isolates and was higher ($p < 0.05$) among female subjects and SP recipients. Prevalence of *Pfdhps* allele 436A (28.6%; 18/63) was similar ($p > 0.05$) to allele 437G (34.9%; 22/63), with double mutation recorded in 4.8% (3/63). K540E mutation was not observed. *Conclusion*. *Pfdhfr* and *Pfdhps* mutations observed in Maiduguri are suggestive of SP resistance level, and this could constitute a setback to malaria prophylactic strategies in the region if unchecked. Thus, there is a need to investigate the clinical efficacy of SP.

1. Introduction

The scourge of malaria persisted in most countries of sub-Saharan Africa [1–4] despite the progressive reduction in global burden [5]. In 2018, the World Health Organization (WHO) reported 228 million cases of malaria worldwide, with the African Region accounting for 213 million cases amounting to 93% of the global burden. With 25% of the global morbidity and 24% of mortality, Nigeria remains the worst hit country in the world [5]. Similarly, malaria is still of public health concern in Northeast Nigeria [4, 6], compounding other health, security, and social challenges in the region. However, the recent WHO World Malaria Report indicated a decline in the global burden when compared with previous years. Specifically, the global incidence rate progressively decreased from 71 cases per 1000 individuals

in 2010 to 57 cases per 1000 individuals in 2018. Within the same period, the incidence rate decreased by 22% in the African Region. *Plasmodium falciparum* is the dominant species of the malaria parasite in Africa, accounting for 99.7% of the estimated cases in 2018 [5].

Malaria disproportionately affects all ages and sexes, with pregnant women and children below five years at the highest risk [7]. In 2018, about 24 and 11 million cases were recorded among children and pregnant women in sub-Saharan Africa, respectively; and 67% of the global mortality occurred in children below five years [5]. Thus, most prophylactic strategies, such as Intermittent Preventive Treatment in Pregnancy (IPTp), Intermittent Preventive Treatment in Infancy (IPTi), and Seasonal Malaria Chemoprevention (SMC), target these vulnerable cohorts. IPTp involves the administration of full doses of sulfadoxine-pyrimethamine (SP) to pregnant women starting early in the second trimester and continues monthly until delivery [8]. IPTi is the administration of full doses of SP to infants (<12 months) in regions with moderate to high malaria transmission at 10 weeks, 14 weeks, and nine months of age [9]. SMC is a complete treatment course with a fixed dose of amodiaquine and SP given monthly to children aged 3–59 months, beginning at the start of the malaria transmission season in highly seasonal transmission areas of sub-Sahara Africa [10]. Previous reports have demonstrated the efficacy of these prophylactic strategies on malaria transmission [11–16].

Sulfadoxine-pyrimethamine (SP) is an integral component of malaria chemoprevention in infants, children, and pregnant women. Hence, its efficacy is essential to the success of prophylactic strategies [17–19]. Previous studies have reported SP resistance in Nigeria [20–22] and other countries where strategies are deployed [23–26]; thus, the need for periodic monitoring of SP efficacy to ensure early detection of drug resistance. Single nucleotide polymorphisms (SNPs) in *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and *P. falciparum* dihydropteroate synthase (*Pfdhps*) are strong predictors of SP resistance [20, 23, 26, 27]. Pyrimethamine resistance is associated with point mutations at codons N51I, C59R, S108N, and I164L of *Pfdhfr* [28], while sulfadoxine resistance is associated with point mutations at codons S436A/F, A437G, K540E, A581G, and A613S/T of *Pfdhps* [29].

In Maiduguri, Northeast Nigeria, there is intense malaria transmission during the 4–6 months of the rainy season [30] with an annual average prevalence of 22.6%, lowest in April (7.5%) and highest in September (84.2%) [4]. Malaria indicators are poor in the region, only 26% of pregnant women received at least three doses of SP during the last pregnancy and 45.4% of the population used insecticide-treated nets (ITNs) [30]. IPTi and SMC are being considered for routine use in Northeast Nigeria, and a recent study has demonstrated the impact of SMC in Borno State [31]. Despite that SP is widely used, there is a dearth of molecular evidence of its resistance in the region. Thus, the present study determined the SNPs of *Pfdhfr* and *Pfdhps* in *P. falciparum* isolated in Maiduguri, Northeast Nigeria and discussed the implications on malaria prophylactic strategies.

2. Materials and Methods

2.1. Study Area and Study Population. The study was conducted in Maiduguri, Northeast Nigeria, a region with seasonal malaria transmission that peaks in the rainy months of August and September [4]. Maiduguri is the largest city in the region and the capital of Borno State, which shares international borders with republics of Cameroon, Chad, and Niger. It is located on latitude 11°40′N–11°44′N and longitude 13°05′E–13°14′E with an average annual rainfall of 613 mm, an average annual temperature of 25.8°C (11.9–44.0°C), and relative humidity of 15–72% [32]. The weather is categorized into harmattan (October–February), hot (March–May/June), and rainy seasons (June/July–September) [33]. The estimated population is over one million people [34] who are predominantly *Kanuri*. However, other indigenous ethnic groups and non-Nigerians are also present. They mainly engage in farming, livestock keeping, trading, fishing, artisanship, civil service, and nongovernmental organizations.

2.2. Subject Enrolment and Sample Collection. Between May and October 2018, 235 individuals were screened for malaria by light microscopy at the University of Maiduguri Teaching Hospital (UMTH), Muhammed Shuwa Memorial Hospital (MSMH), and Teachers' Village (TV), Maiduguri. Of the 128 individuals with malaria, 106 subjects who met the inclusion criteria and provided informed consent were enrolled. The demographic and medical data were obtained following a comprehensive physical examination. Giemsa-stained thick and thin smears, capillary blood, and dried blood spot samples were prepared for all subjects [35]. Thin and thick smears were used for species identification and parasitaemia quantification, respectively, while capillary samples were used to determine haematocrit values [36]. The dried blood spot samples were labelled, placed in an air-tight zip bag with desiccants, and stored at 4°C until use. The subjects were treated with artemether-lumefantrine (AL) and monitored for 14-day posttreatment. The study protocol was reviewed and approved by the Research Ethics Committee, UMTH.

2.3. Genomic DNA Extraction and Detection of Pfdhfr and Pfdhps SNPs. The parasite genomic DNA (gDNA) was extracted from the dried blood spot samples using QIAamp DNA Mini Kit (QIAGEN, Valencia, California, US) according to the manufacturer's instructions and stored at −20°C until use. *Pfdhfr* mutations at codons N51I, C59R, and S108N were determined by amplification (Supplementary Table 1) of the genes using nested Polymerase Chain Reaction (nPCR) as described by Lau et al. [37] and Duraisingh et al. [38]. This was followed by Restriction Fragment Length Polymorphism (RFLP) with *Tsp509I* (NEB), *XMNI* (NEB), and *AluI* (NEB) targeted at codons N51I, C59R, and S108N, respectively [39]. Amplification of *Pfdhps* by nPCR (Supplementary Table 2) was done as described by Lau et al. [37] and Wang et al. [29] followed by RFLP using *HhaI* (NEB), *AraII* (NEB) and *FokI* (NEB) targeted at codons S436A/F, A437G, and K540E, respectively [40]. The digested products

were resolved by gel electrophoresis and visualized under ultraviolet light.

2.4. Statistical Analysis. The data were analyzed with SPSS version 21.0 for Windows (IBM Corporation, Armonk, NY, USA) and presented as means ± standard deviation, range, and percentages. Categorical variables were compared using Pearson Chi-square or Fisher's exact test, while Student's *t*-test and analysis of variance (ANOVA) were used for continuous variables. Significance difference was inferred at $p < 0.05$.

3. Results

3.1. Baseline Characteristics of the Subjects at Enrolment. The baseline characteristics of the 63 subjects whose samples were analyzed are presented in Table 1. The overall mean age ± standard deviation (range) of the subjects was 23.6 ± 8.7 (2.0–67.0) years with a mean duration of illness of 3.1 ± 1.6 days and anaemia prevalence of 27.0% (17/63). Headache and fever accounted for the highest ($p < 0.05$) proportion of symptoms presented by the subjects at enrolment. The proportion of subjects who consumed SP in the last 12 months (69.8%; 44/63) was higher ($p < 0.05$) than those who did not (30.2%; 19/63). The geometric mean parasite density (range) was 8,948 (2,100–13,400) asexual parasites/μl blood. All subjects responded adequately to AL treatment and the outcomes presented elsewhere.

3.2. SNPs of Pfdhfr and Pfdhps in Maiduguri, Northeast Nigeria. Of the 106 samples collected, gDNA extraction was successful in 63 samples and they were used for subsequent SNPs analysis. The combined prevalence of point mutations at any of the six *Pfdhfr* and *Pfdhps* codons assessed was 85.7% (54/63) and was higher ($p < 0.05$) in *Pfdhfr* (82.5%; 52/63) than *Pfdhps* (58.7%; 37/63). Figure 1 presents the distribution of *Pfdhfr* SNPs at codons N51I, C59R, and S108N. Point mutations were observed in 52 isolates giving a prevalence of 82.5% (52/63); the mutant allele 108N accounted for the highest ($p < 0.05$) proportion of 82.5% (52/63). Prevalence of triple *Pfdhfr* mutant alleles (51I, 59R, and 108N) was 60.3% (38/63) and was higher ($p < 0.05$) than triple wild alleles (N51, C59, and S108) with 17.5% (11/63). The prevalence of triple mutation was similar ($p > 0.05$) among the samples collected from the three study sites (Figure 2) but was higher among females than males ($p < 0.05$) (Figure 3) and among SP recipients (65.9%; 29/44) than nonrecipients (47.4%; 9/19) [$p < 0.05$]. The selected agarose gel images of PCR-RFLP products of the gene are shown in Figure 4.

Table 2 shows the distribution of point mutations at codons S436A, A437G, and K540E of *Pfdhps*. Point mutations were observed in 37 isolates giving a prevalence of 58.7% (37/63); the prevalence of mutants 436A (28.6%; 18/63) and 437G (34.9%; 22/63) were similar ($p > 0.05$). Double mutation of *Pfdhps* at codons S436A and A437G was recorded in 4.8% (3/63) of the samples. No mutation was recorded at codon K540E. In addition, 29 of the 38 isolates with triple *Pfdhfr* mutant alleles of 51I, 59R, and 108N harbour at least one additional *Pfdhps* mutant allele 436A or 437G (33.3%; 21/63) and both mutant alleles (12.7%; 8/63).

Table 1: Baseline characteristics of the subjects whose samples were analyzed.

Variable	Value
Number enrolled (%)	63 (100.0)
UMTH	20 (31.7)
MSMH	24 (38.1)
TV	19 (30.2)
Age (years)	
Mean ± SD	23.6 ± 8.7
Range	2.0–67.0
Number < 5 (%)	8 (7.6)
Sex	
Female (%)	35 (55.6)
Male (%)	28 (44.4)
Axillary temperature (°C)	
Mean ± SD	38.6 ± 3.7
Range	36.8–40.2
Weight (kg)	
Mean ± SD	49.6 ± 9.4
Range	5.0–74.0
Duration of illness (days)	
Mean ± SD	3.1 ± 1.6
Range	1.0–5.0
Haematocrit (%)	
Mean ± SD	39.1 ± 5.3
Range	25.0–46.0
Number < 30 (%)	17 (27.0)
Symptoms (%)	
Abdominal pain	27 (42.9)
Body ache	21 (33.3)
Fever	33 (52.4)
Headache	42 (66.7)
Joint pain	8 (12.7)
Nausea and vomiting	19 (30.2)
Poor appetite	29 (46.0)
Weakness	11 (17.5)
Parasite density (/μl blood)	
Geometric mean	8,948
Range	2,100–13,400
SP use in the last 12 months (%)	
Yes	44 (69.8)
No	19 (30.2)

MSMH: Muhammed Shuwa Memorial Hospital, SD: Standard Deviation; SP: Sulfadoxine-pyrimethamine, UMTH: University of Maiduguri Teaching Hospital, and TV: Teachers' Village.

4. Discussion

The present study reports the prevalence of *Pfdhfr* SNPs at codons N51I, C59R, and S108N and codons S436A/F, A437G, and K540E of *Pfdhps* and discusses the implications on malaria prophylactic strategies in Maiduguri, Northeast Nigeria. *Pfdhfr* and *Pfdhps* genes have been widely used as biomarkers for monitoring pyrimethamine and sulfadoxine resistance, respectively [23, 24, 26, 27, 36].

The detection of *Pfdhfr* mutations in the present study is an indication of circulating pyrimethamine-resistant *P.*

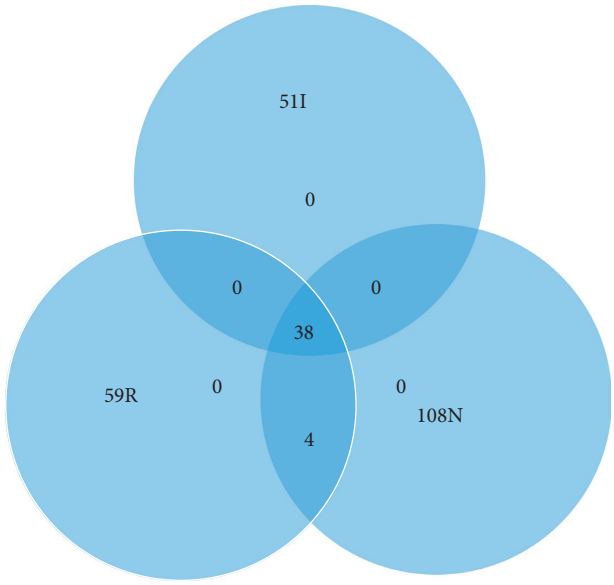

Figure 1: Number of single mutation at *Pfdhfr* codons N51I, C59R, and S108N.

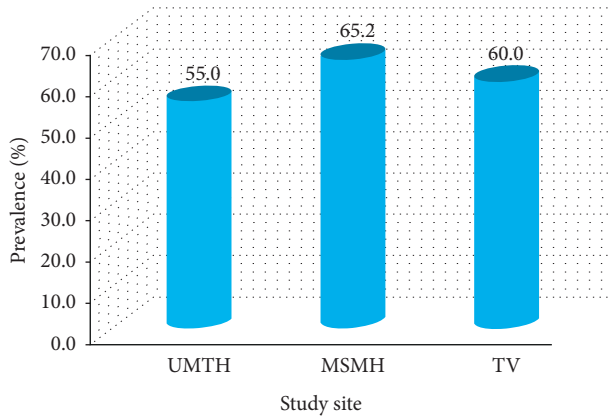

Figure 2: Study site distribution of triple *Pfdhfr* mutant alleles 51I, 59R, and 108N ($p > 0.05$).

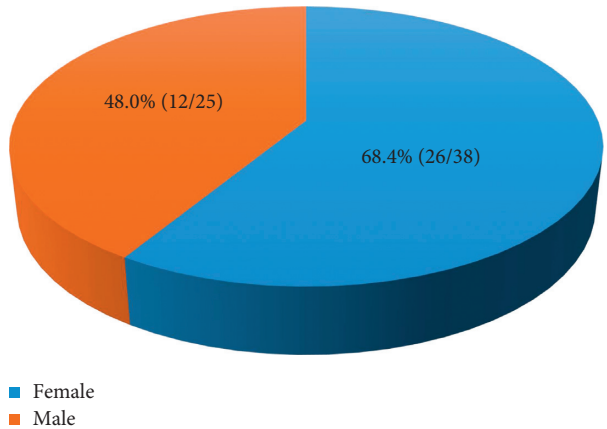

Figure 3: Sex distribution of triple *Pfdhfr* mutant alleles 51I, 59R, and 108N ($p < 0.05$).

falciparum strains in the region. This finding is in accordance with studies that have previously established pyrimethamine resistance in Nigeria [20–22] and other African countries [17, 24, 25]. Among the codons of *Pfdhfr* evaluated, codon S108N accounted for the highest mutation, with all 52 mutant strains carrying the mutant allele 108N. This is similar to previous reports of a preponderance of the mutant allele 108N in populations where SP is deployed [21, 37]. Although the mutant allele 108N is said to confer low pyrimethamine resistance [21, 23], the presence of mutant alleles 51I and/or 59R could have reinforced the level of pyrimethamine resistance in the region. High pyrimethamine resistance is associated with triple *Pfdhfr* mutants (51I, 59R, and 108N) in West Africa [28, 40]. The high proportion (60.3%) of triple mutant alleles observed in the present study points to the extent of pyrimethamine resistance in Maiduguri. The present value is significantly lower than 81.3% reported in Damboa, another town in the region [22]. Oguike et al. [22] studied pregnant women who might have been exposed to pyrimethamine pressure from IPTp, resulting in a higher resistance level. Similarly, the present value is lower than the values (92.9–98.7%) reported from Southern Nigeria [15, 22]. This disparity could be largely attributed to malaria transmission intensity and higher IPTp coverage in Southern Nigeria [30]. Furthermore, the significantly higher prevalence of triple *Pfdhfr* mutations in *P. falciparum* isolated from female subjects and SP recipients is clearly suggestive of the influence of routine IPTp. The majority of the female subjects who participated in the study admitted previous consumption of SP within the last 12 months prior to the study.

Pyrimethamine is mainly coformulated with sulfadoxine and is coadministered for malaria prevention in pregnant women and children [8–10]. Thus, the present study also examined point mutations in *Pfdhps*, a gene associated with sulfadoxine resistance. Two mutant alleles 436A/F and 437G were observed among the parasite population, and the prevalence is similar to the report from other parts of the region [22]. It is worthy to note that the lack of K540E mutation in the present study is similar to previous studies that reported the rare mutant allele 540E in West Africa [15, 21, 22]. However, this does not undermine the possibility of sulfadoxine resistance among the population since the majority of the isolates with the mutant alleles 436A and 437G also have triple *Pfdhfr* mutant alleles indicating a high level of SP resistance.

Sulfadoxine and pyrimethamine are essential components of the WHO prescribed malaria prophylactic strategies such as IPTp, IPTi, and SMC [8–10]. Thus, the success of these strategies primarily depends on the clinical efficacy of both drugs. The present study revealed the presence of sulfadoxine- and pyrimethamine-resistant *P. falciparum* strains in Maiduguri as implied by the detection of *Pfdhfr* and *Pfdhps* mutant alleles. IPTp is routinely used in Nigeria to prevent malaria in pregnancy [30]. The poor coverage of this strategy in Northeast Nigeria [30] is often implicated as the cause of persistent malaria in pregnancy in the region. Besides, the current evidence of SP resistance in the region provides an additional explanation as to why malaria

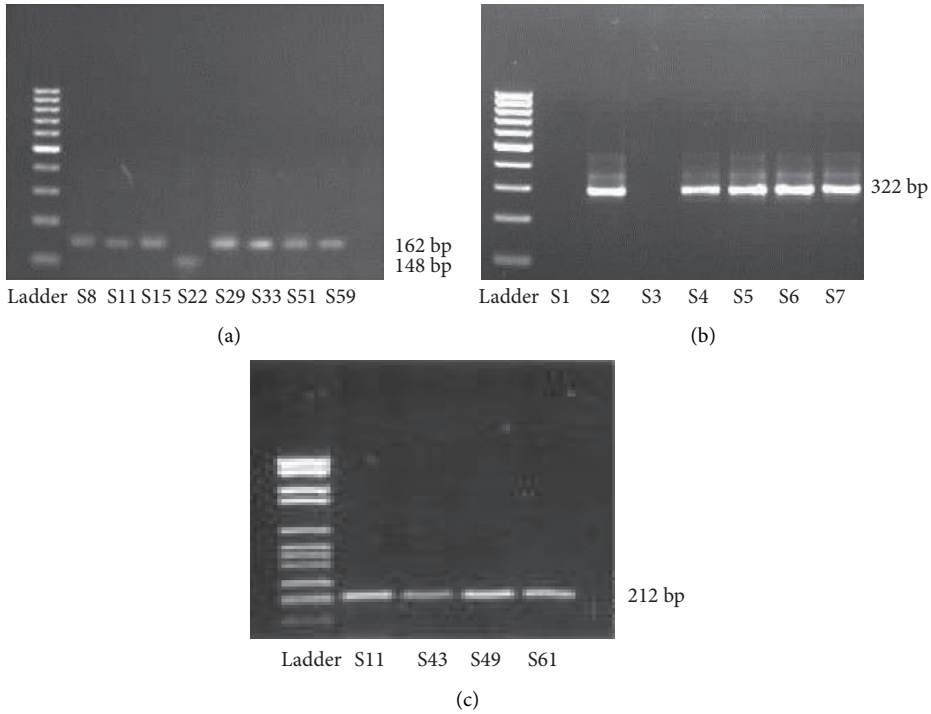

Figure 4: Selected agarose gel images of PCR-RFLP products of *Pfdhfr* wild and mutant alleles: (a) codon C59R with 162 bp indicating mutant allele; (b) codon S108N with 322 bp indicating wild allele; (c) codon N51I with 212 bp indicating mutant allele. S: sample. Lad: ladder.

Table 2: Single nucleotide polymorphisms of *Pfdhps* codons S436A, A437G, and K540E.

Codon	Allele	Prevalence (%)
S436A	S436 (wild)	45 (71.4)
	436A (mutant)	14 (22.2)
	S436/436A (mixed)	4 (6.4)
A437G	A437 (wild)	41 (65.1)
	437G (mutant)	17 (27.0)
	A437/437G (mixed)	5 (7.9)
K540E	K540 (wild)	63 (100.0)
	540E (mutant)	0 (0.0)
	K540/540E (mixed)	0 (0.0)
S436A/A437G	436A/437G (mutant)	3 (4.8)

transmission continues among pregnant women despite the use of IPTp. Thus, efforts should be directed towards improving IPTp coverage and other preventive measures (e.g., ITNs). IPTi is yet to be implemented in Maiduguri. Hence, it is ideal to ascertain SP clinical efficacy among infants prior to the intervention. Ambe et al. [31] recently demonstrated that SMC suppressed the burden of malaria and malaria indicators in Borno State, Nigeria. Notwithstanding, few of the children who received SMC tested positive for malaria within the study period indicating potential resistance among other possibilities. The SMC impact could be partly attributed to the component drug (amodiaquine) which remains very efficacious in Northeast Nigeria [41]. This is in agreement with previous studies that reported SMC efficacy in areas with high SP resistance and low amodiaquine resistance [42].

5. Conclusion

The detection of *Pfdhfr* and *Pfdhps* mutant alleles in Maiduguri is an indication of compromised SP clinical efficacy in Northeast Nigeria and could adversely affect malaria prophylactic strategies if unchecked. However, the implications can be minimized by improved IPTp coverage, systematic IPTi deployment, sustained amodiaquine efficacy, and the use of other malaria preventive measures such as ITNs.

Data Availability

The data are available with STB and can be accessed by readers following a convincing request clearly stating while access to the data is required.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Supplementary Materials

Supplementary Table 1: Primers, master mix, and cycling conditions for primary and secondary amplification of *Pfdhfr*. Supplementary Table 2: Primers, master mix, and cycling conditions for primary and secondary amplification of *Pfdhps*. (*Supplementary Materials*)

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