

# *Plasmodium falciparum* Genetic Diversity and Resistance Genotype Profile in Infected Placental Samples Collected After Delivery in Ouagadougou

Haffsatou Sawadogo<sup>1,2</sup>, Issiaka Soulama<sup>3,4</sup>, Adama Zida<sup>2,5</sup>, Cheikna Zongo<sup>1</sup>, Patindoilba Marcel Sawadogo<sup>2,5</sup>, Kiswendsida Thierry Guiguemde<sup>5,6</sup>, Seni Nikiema<sup>7</sup>, Salimata Emilie Badoum<sup>1,8</sup>, Salam Sawadogo<sup>7</sup>, Aïcha Tou<sup>4</sup>, Salif Sombié<sup>4</sup>, Chanolle Tchekounou<sup>1,9</sup>, Sindié Samuel Serme<sup>1,8</sup>, Rasmata Ouedraogo-Traoré<sup>9</sup>, Tinga Robert Guiguemde<sup>10</sup>, Aly Savadogo<sup>1</sup>

<sup>1</sup>Laboratory of Applied Biochemistry and Immunology (LABIA), Joseph KI - ZERBO University, Ouagadougou, Burkina Faso; <sup>2</sup>Parasitology-Mycology Department, Centre Hospitalier Universitaire Yalgado Ouédraogo (CHU-YO), Ouagadougou, Burkina Faso; <sup>3</sup>Health Science Research Institute (IRSS), Ouagadougou, Burkina Faso; <sup>4</sup>National Malaria Research and Training Center (CNFRP), Ouagadougou, Burkina Faso; <sup>5</sup>Health Sciences Training and Research Unit (UFR/SDS), Joseph KI - ZERBO University, Ouagadougou, Burkina Faso; <sup>6</sup>Centre Hospitalier Universitaire Pédiatrique – Charles de Gaulle (CHU-CDG), Ouagadougou, Burkina Faso; <sup>7</sup>Molecular Biology and Genetics Laboratory (LABIOGENE), Joseph KI - ZERBO University, Ouagadougou, Burkina Faso; <sup>8</sup>Health Action Research Group (GRAS), Ouagadougou, Burkina Faso; <sup>9</sup>International Institute of Science and Technology (Istech), Ouagadougou, Burkina Faso; <sup>10</sup>Parasitology-Mycology Laboratory, National Institute of Health Sciences (INSP), Nazi Boni University, Bobo-Dioulasso, Burkina Faso

Correspondence: Cheikna Zongo, Laboratory of Applied Biochemistry and Immunology (LABIA), Joseph KI - ZERBO University, Burkina Faso 03 BP 7021 Ouagadougou 03, Ouagadougou, Burkina Faso, Tel +226 76579605, Fax +226 25307242, Email Cheikna.zongo@ujkz.bf

**Purpose:** Intermittent preventive treatment with sulfadoxine-pyrimethamine is widely used for the prevention of malaria in pregnant women in Africa. Known resistance cases of sulfadoxine-pyrimethamine during pregnancy need to be follow up to support IPTp implementation in Burkina Faso. However, data on the development and spread of resistance to this molecule are lacking. This study aimed to investigating the genetic diversity of *P. falciparum* and the mutation prevalence in the *dhfr* and *dhps* genes infected from postpartum infected placentas.

**Patients and Methods:** This was a prospective and cross-sectional study conducted between April 2019 and March 2020 in four health districts of Ouagadougou capital city. From the placentas collected after delivery, *P. falciparum* detection and *msp1* and *msp2* polymorphism analysis were performed by nested PCR. The resistance profile was checked after analyzing the mutation point on *dhfr* and *dhps* genes.

**Results:** PCR-positive samples were estimated at 96% for *msp1* and 98% for *msp2*. The polymorphism analysis showed that the RO33 and 3D7 allelic families were the most widespread with 62.5% and 91.83%, respectively. Multiple infections by *msp1* and *msp2* were frequent with 12.50% and 92.92%, respectively. The prevalence of individual *dhfr* mutation point, 51I, 108A, and 59R, was 1.96, 15.68, and 7.84, respectively, and the *dhps* mutation point, 437G, was 3.92. There is no detected mutation at the point 164L and 540E. The triple (51I+108A+59R) in *dhfr* and quadruple (51I+108A+59R+ 437G) mutation were not found.

**Conclusion:** The results showed that *Plasmodium falciparum* has a high genetic diversity of *msp1* and *msp2*. This suggests that *dhfr* and *dhps* mutant genotypes are potential early warning factors in the increase in the sulfadoxine-pyrimethamine resistance.

**Keywords:** sulfadoxine-pyrimethamine, *Plasmodium falciparum*, *msp1*, *msp2*, *dhfr*, *dhps*, placenta

## Introduction

The number of malaria cases continues to increase worldwide with nearly 247 million cases in 2021, compared to 245 in 2020. Most of this increase was reported in Africa. In 33 countries in the WHO African Region, in 2020, approximately 11.6 million (34%) pregnant women were exposed to malarial infection during pregnancy out of all the 33.8 million

pregnancies recorded in the world.<sup>1</sup> The malarial occurrence during pregnancy could lead to consequences such as worsening of anaemia in pregnant women, prematurity, perinatal mortality, foetal growth retardation, or low birth weight.<sup>2</sup>

To reduce the burden of malaria, the World Health Organization (WHO) recommends chemoprevention in pregnant women by administering sulfadoxine-pyrimethamine (SP) during antenatal visits. This intermittent preventive treatment (IPTp) is widely used in areas with moderate and high malarial transmission. *Plasmodium falciparum* is the most dangerous parasite species due to its strong involvement in the mortality attributed to malaria and its ability to develop drug resistance. Genetically, *P. falciparum* presents a high polymorphism. There exists a great diversity of *Plasmodium* strains that infect people living in malarial-endemic areas.<sup>3</sup> Parasitic diversity and parasitic antigenic variation are the two main factors responsible for the slow acquisition (several years) of protection against malaria. The study of this genetic diversity takes certain factors into account, in particular the transmission intensity and the host's immunity. The *msp1* and *msp2* genes are located on chromosome 9 and chromosome 2, respectively. Variations in the repeat sequences of block 2 (*msp1*) and the polymorphism of the central block (*msp2*) allow the parasitic populations to be subdivided into three allelic families K1, MAD20, and RO33 for *msp1*, and into two allelic families 3D7 and FC273 for *msp2*.<sup>3</sup> The development of malaria vaccine candidates based on the surface proteins MSP1 and MSP2 contributes to further extensively analyze the distinct subpopulations of the parasite during clinical trial.<sup>4</sup> At the same time, the resistance of *P. falciparum* to drugs constitutes a real obstacle to the fight against malaria in endemic countries. Nowadays, the estimation of the level of resistance of *P. falciparum* to antimalarial drugs on a national scale is done using molecular markers. Data obtained by measuring these markers indicate gene mutations for two enzymes (dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*)) in Burkina Faso.<sup>5</sup> These enzymes are thought to be associated with sulfadoxine resistance and pyrimethamine resistance, respectively.<sup>6</sup> However, whether the use of intermittent preventive treatment can greatly affect the spread and distribution of the genotypic resistance profile in pregnant women remains debatable.

This work aimed to study the genetic polymorphism of *P. falciparum* and the prevalence of the mutation in the *dhfr* and *dhps* genes in the placenta after delivery of women living in Ouagadougou, Burkina Faso.

## Materials and Methods

### Study Site and Period

This is a prospective and cross-sectional study conducted between April 2019 and March 2020 in four health districts of the city of Ouagadougou. The health facilities concerned were Noongr-Massom District Hospital, Schiphra District Hospital, Boulmiougou District Hospital, and Paul VI District Hospital as previously described.<sup>7</sup> The climate in the study site is tropical savannah with rainy seasons between June and October.

### Study Population

In 2020, the population of the city of Ouagadougou was approximately 2,684,052 inhabitants. The study population was all pregnant women admitted to the health facilities having given their informed consent to participate in the study ( $n = 630$ ). Those who were infected with HIV were not included in the study. All pregnant women received sulfadoxine-pyrimethamine combination during the antenatal care visits. All doses were administrated at the hospital.

### Sample Collection and Laboratory Analysis

The women's placenta obtained after delivery made it possible to make samples of dried blood (DBS) on Whatman paper and thin smear slides for PCR and microscopy detection, respectively.

The microscopy detection was applied according to the WHO reference method for malaria diagnostic. Thick and thin blood films were prepared on clean and grease-free glass microscope slides with the maternal face blood of the placenta and examined by two microscopists after staining with 5% Giemsa solution for 30 minutes as described previously.<sup>7</sup>

The QIAamp kit (QIAGEN) was used for parasite DNA extraction on the DBS according to the manufacturer's instructions. Microscopic examinations were performed on the prepared thin smear slides. The extracted *Plasmodium*

*falciparum* DNA was either used immediately for the amplification reactions or kept at a temperature of  $-20^{\circ}\text{C}$  before being used. The nested polymerase chain reaction (nested PCR) was the DNA amplification technique used based on small subunit ribosomal RNA (ssrRNA) genes.<sup>8</sup> The primer sequences for the first (nested) amplification were: rPLU5 5-CCT GTT GTT GCC TTA AAC TTC-3 (forward) rPLU6 5-TTA AAA TTG TTG CAG TTA AAA CG-3 (reverse). The primer sequences for the second (nested) amplification were used rFAL1 5-TTA ACC TGG TTT GGG AAA ACC AAA TAT ATT-3 (forward) rFAL2 5-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3 (reverse).

Applied Biosystem 2720 Thermal cycler tool was used for amplification. A volume of 20  $\mu\text{L}$  of reaction containing 1  $\mu\text{L}$  of genomic DNA, 2  $\mu\text{L}$  of polymerase chain reaction (PCR) buffer 10X, 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  of each primer, 0.8  $\mu\text{L}$  of 50 mM  $\text{MgCl}_2$ , 1.25  $\mu\text{L}$  of mM of dNTP and 0.1 of 5UI Taq polymerase was used for amplification. The initial denaturation at  $95^{\circ}\text{C}$  was programmed at 5 min, the denaturation at  $94^{\circ}\text{C}$  at 1 min followed by 24 cycles. Each cycle corresponds to a denaturation at  $94^{\circ}\text{C}$  for 1 minute, an annealed primer for 2 min at  $58^{\circ}\text{C}$ , and an extension for 2 minutes at  $72^{\circ}\text{C}$ . The extension was completed by adding an additional 5 minutes of  $72^{\circ}\text{C}$  incubation at the end of the cycle. The 30-cycle condition was set for the second amplification. The *msp1* and *msp2* typing, extracted DNA was amplified by using sequence-specific primers according to a modified protocol. After the primary amplifications, secondary PCR reactions were performed using allelic primers for Mad20, K1, RO33 (for *msp1*), FC27 and 3D7 (for *msp2*).

Then, the amplification of the *dhfr* and *dhps* genes was performed by PCR restriction fragment length polymorphism. PCR- positive samples for *P. falciparum* were analyzed at *dhfr* (codons 51, 59, 108, and 164) and *dhps* (codons 436 and 540). The amplicons (DNA fragment amplified by PCR obtained) were analyzed by electrophoresis on agarose gel or stored at  $+4^{\circ}\text{C}$ .

## Data Collection and Processing

Data on the demographic and clinical information of pregnant women were collected through a structured questionnaire designed for the study. The information covered age, level of education, occupation, marital status, current and previous pregnancies, as well as environmental and living conditions. Information such as the use of intermittent preventive treatment (IPT) and the use of insecticide-treated bed nets (ITN) was also collected.

## Statistical Analysis

R software was used to analyze the data. The chi-square was used to compare the proportions, and the student's test and ANOVA were used for normally distributed continuous data.

## Ethical Considerations

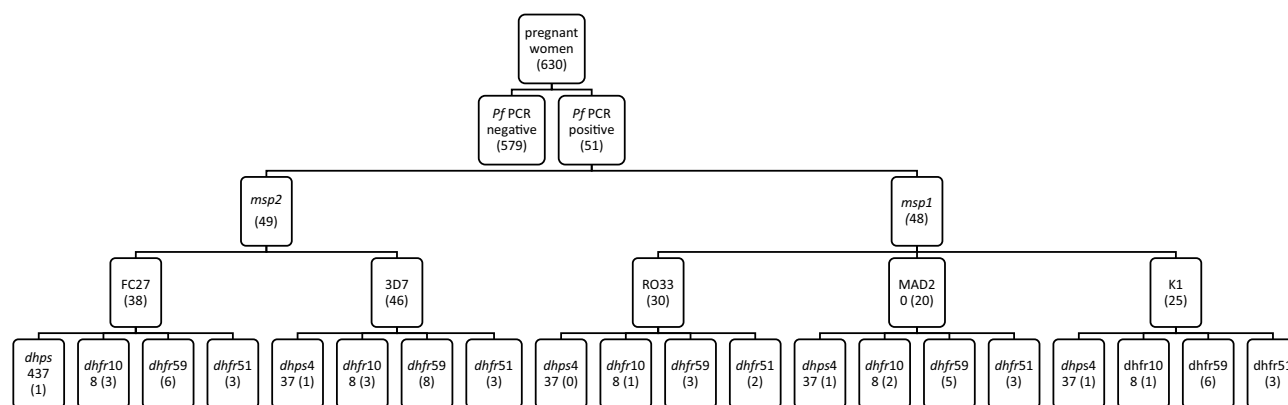
The investigations during this study were carried out following the rules of the Helsinki's Declaration. The protocol of this study was submitted for ethical approval by the National Ethics Committee for Health Research in Burkina Faso (deliberation N° 2019-4-056). The study received the authorization of the chief medical officers of the various health districts, the mayors of the various communes, and the administrative officials of each health structure. Each pregnant woman's informed and signed consent was obtained. This made it possible for them to participate in the study.

## Results

A total of 630 pregnant women participated in the study, and all available samples were systematically considered, including 51 for genotyping. Of the 51 *Plasmodium falciparum*-positive samples by species nested PCR, 48 were positive for *msp1* and 49 were positive for *msp2*. A flowchart was made to explain the results obtained (Figure 1).

## Frequency of *msp1* and *msp2* Genes Family

The allelic distribution of the *msp1* gene (K1, MAD20, and RO33) revealed that there was no statistically significant difference between the frequencies of the allelic families K1 52.08%, RO33 62.50% and MAD20 41.66% ( $P = 0.1418$ ). However, a predominance of RO33 alleles was noted followed by K1 alleles. Allelic analysis of the *msp2* gene (3D7 and FC27) revealed a statistically high frequency of the 3D7 allelic family at 91.83% ( $P = 0.04039$ ) (Table 1).



**Figure 1** Flow diagram showing malaria diagnosis using PCR.

**Abbreviations:** *Pf*, *Plasmodium falciparum*; PCR, polymerase chain reaction; *msp1* et 2, Merozoite Surface Protein 1, 2; *dhfr*, dihydrofolate reductase; *dhps*, dihydropteroate synthetase.

## Frequency of *msp1* and *msp2* Individual Alleles

A total of 42 alleles were found and classified according to their size in base pairs. Eleven different alleles of the *msp1* gene had been obtained with five alleles for K1 (150–200Pb), two alleles for RO33 (150–200Pb), and four for MAD20 (190–400Pb). For the *msp2* gene, 27 alleles were found with 15 alleles for 3D7 (100–1000Pb) and 12 alleles for FC27 (240–700Pb).

Polyclonality of infection was assessed for each gene. It corresponds to the proportion of pregnant women with more than one clone (allele) of *Plasmodium falciparum*. The estimated polyclonality was 12.50% for *msp1* and 95.92% for *msp2*. The study of the polymorphism of the *msp1* gene indicated that the number of parasite clones per person in the study population ranged from 1 to 5 clones and the number of parasite clones per person in the *msp2* gene ranged from 1 to 8 clones. As presented in Figure 2, the monoclonal infection was estimated at 87.50% for *msp1* and 4.8% for *msp2* (Figure 2).

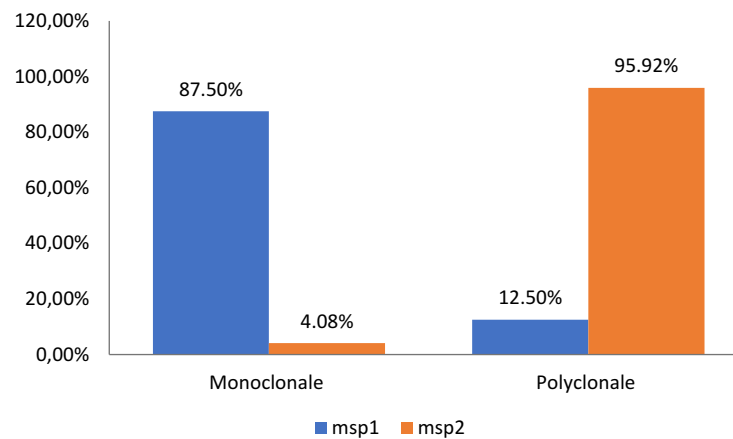
## Prevalence of *dhfr* and *dhps* Gene Mutations

Table 2 represents the proportion of different *dhfr* and *dhps* points of mutation. The prevalence of individual *dhfr* mutation, 51I, 108A, and 59R, was 1.96%, 15.68%, and 7.84%, respectively, and that of *dhps*, 437G, was 3.92. There was no mutant parasite with 164L or 540E point of mutation (Table 2).

Out of the samples that showed mutations, 25.49% (13/51) contained at least one *dhfr* gene mutation. The *dhfr*'s gene mutation at codon 59 was the most represented one with a proportion of 15.68% followed by *dhfr* 108 mutation (7.84%).

**Table 1** Prevalence of Allelic Families of *msp1* and *msp2* Genes

Gene	Allele	Prevalence	P value
<i>msp1</i>	K1	52.08%	0.1418
	RO33	62.50%	
	MAD20	41.66%	
	K1+RO33	20.83%	
	K1+MAD20	22.91%	
	RO33+MAD20	27.08%	
	K1+RO33+MAD20	8.33%	
<i>msp2</i>	3D7	91.83%	0.04039
	FC27	83.67%	
	3D7+FC27	77.55%	



**Figure 2** Polyclonal and monoclonal infection.

The double mutation *dhfr* (51 + 59) and (59+108) were found with a proportion of 3.92% and 1.96%, respectively (Table 3). A level of the *dhps* gene, only the mutation at point 437 was found with a proportion of 3.92%. We did not find any mutation of *dhfr* I164L and *dhps* K540E mutation.

## Discussion

The genetic variation of *P. falciparum* strains and their complexity in pregnant women using intermittent preventive treatment with sulfadoxine-pyrimethamine were described in our study. Our study showed that the frequency of successfully analyzed samples was higher with *msp2* than *msp1*. All polymorphic profiles of *msp1* (K1, MAD20, and RO33) and *msp2* (3D7 and FC27) genes were encountered in our study. The RO33 and 3D7 allelic families were the most dominant one followed by the FC27 allelic family, K1 and MAD20. The dominance

**Table 2** Proportion of the Different *dhfr* and *dhps* Points of Mutations

Gene	Mutant	Savage	Mixed
<i>Dhfr51</i>	1 (1.96%)	48 (94.11%)	2 (3.92%)
<i>Dhfr59</i>	8 (15.68%)	43 (84.31%)	0 (0%)
<i>Dhfr108</i>	4 (7.84%)	47 (92.15%)	0 (0%)
<i>Dhfr164</i>	0 (0%)	51 (100%)	0 (0%)
<i>Dhps437</i>	2 (3.92%)	49 (96.07%)	0 (0%)
<i>Dhps540</i>	0 (%)	51 (100%)	0 (0%)

**Table 3** Prevalence of Different Mutations

Mutation	Effective	Percentage
<i>dhfr</i> 51	1	7.69%
<i>dhfr</i> 59	8	61.54%
<i>dhfr</i> 108	4	30.77%
<i>dhfr</i> 164	0	0%
<i>dhps</i> 437	2	15.38%
<i>dhps</i> 540	0	0%
Double mutation <i>dhfr</i> (51+59)	2	15.38%
Double mutation <i>dhfr</i> (51+108)	0	0%
Double mutation <i>dhfr</i> (59+108)	1	7.69%

of RO33 is reported by similar studies conducted in Burkina Faso by Nèbié in 2002,<sup>9</sup> and by Hamid in Sudan in 2013.<sup>10</sup> In contrast, other studies conducted in Burkina Faso by Soulama in 2009 and Somé in 2018,<sup>4,11</sup> and Issoufou in 2001 in Bénin<sup>12</sup> found a predominance of the K1 allelic family. As regards the dominance of the 3D7 allele of *msp-2*, there was a consensus with previous studies in Burkina<sup>4</sup> in 2018, in Côte d'Ivoire, and in Gabon in 2016.<sup>13</sup> On the other hand, they are different from previous studies in Africa. Indeed, the Soulama's studies in 2009<sup>9</sup> and Badoum<sup>14</sup> in 2019 in Burkina Faso and Hounto's in Benin in 2013<sup>15</sup> reported that the K1 and FC27 allelic families were the most predominant one for *msp-1* for *msp-2* respectively. The predominance of RO33 and 3D7 alleles in our study could be explained by the effect of IPT/SP in favour of an increase in the "biological fitness" of these parasitic clones. In addition, IPT/SP seems to have an effect on allelic variability leading to a predominance of RO33 and 3D7. Finally, it could be explained by the fact that our study population consisted of asymptomatic individuals. Indeed, previous studies have already shown the association of the RO33 and 3D7 alleles in the case of asymptomatic malaria.<sup>16</sup> For mixed infections, RO33+MAD20 has the highest frequency followed by K1+RO33. The two genes *msp1* and *msp2* are highly polymorphic, as there are 11 alleles of the *msp1* gene and 27 alleles of the *msp2* gene. Similar results are observed in Senegal<sup>17</sup> and Benin where 23 *msp1* and 12 *msp2* alleles are reported.<sup>18</sup>

As regards mutation associated with resistance to SP, only *dhfr59* mutation prevalence was high (61.54%). The high prevalence of *dhfr59* mutation was found at 99.9% in Mali<sup>19</sup> in 2014. Thus, the study states that this mutation is no longer relevant for monitoring sulfadoxine-pyrimethamine resistance. The other *dhfr* (51, 108, 164) and *dhps* (437,540) mutations were poorly represented, negligible, or absent in our study. *dhps* 437 mutation was 15.38%, whereas a study conducted in Mali by Coulibaly found 21.4%. In Cameroon, Chauvin published a high prevalence of the *dhps* 437 gene mutation (76.5%).<sup>20</sup>

In this study, we did not record *dhfr* 164L and *dhps* 540E mutations, suggesting that they are still absent in Burkina Faso. This is consistent with previous reports in the country. This result is similar to data reported in other studies in Burkina Faso in pregnant women<sup>21,22</sup> on the one hand and in children and in the general population on the other hand.<sup>2,23</sup>

Our samples did not carry any triple and quadruple mutations. A study by Nikiema in 2022<sup>24</sup> shows little to no prevalence of triple and quadruple mutation. The quintuple mutation is strongly associated with the clinical failure of SP treatment.<sup>25</sup>

In Mali, Coulibaly also reported lower rates of triple mutation (4.7%).<sup>26</sup> In Nigeria, Iwalokun reported a higher prevalence of triple mutation of 35%.<sup>27</sup> In Central Africa in Gabon, Bouyou-Akotet in a survey reported triple mutation and quadruple mutation at high proportions of 80% and 53%, respectively.<sup>28</sup> In Cameroon, Chauvin published a high prevalence of the triple mutation at 96%.<sup>20</sup>

This different reported prevalence is higher than those found in our study area and could be related to drug pressure being higher in these different areas of Africa than in Burkina Faso.<sup>29–31</sup> The difference could be related to the higher level of endemicity in these regions than in our study area. Indeed, according to some authors, it is likely that in hyper-endemic areas, resistance spread more rapidly in the population. This is especially true for resistance induced by mutations in a single gene for a given molecule. Even with a multiplicity of clones, there is competition between strains, which will facilitate the recolonization of the organism by mutant strains after the elimination of susceptible strains by chemoprevention.

## Conclusion

This study using *msp1* and *msp2* genes in the population of low parasitemia strains from placentas obtained after delivery confirmed a high polymorphism of these two genes with statistically high frequency of the 3D7 allelic family. The study also showed a high polyclonality using *msp2* gene. Also, it is to be noted that there is a contribution to the reduction of the prevalence of *Plasmodium falciparum* infection by sulfadoxine-pyrimethamine.

Mutations in the *Pf dhfr* and *Pf dhps* genes associated with SP resistance were relatively common among pregnant women in Ouagadougou. Nevertheless, the absence of *dhfr* triple mutation shows the efficacy of intermittent preventive treatment with SP, and molecular markers linked to SP resistance should continue to be monitored.



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## Author Contributions

All the authors made a significant contribution to the work reported, whether it was in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no competing interests in this work.

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