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Brief Communication

Genetic polymorphism of merozoite surface protein 1 and antifolate-resistant genes in *Plasmodium falciparum* from Mali and Niger

Mahaman Moustapha Lamine[1,4,*], Rabia Maman[2], Abdoul Aziz Maiga[3], Ibrahim Maman Laminou[4]

[1]*Faculté de Science et Technique, Université André Salifou, Zinder, Niger;* [2]*Molecular Biology Laboratory of Bamako in Mali, Mali;*
[3]*Université de Ouagadougou, Laboratory of Fundamental and Applied Entomology, Ouagadougou Centre, Burkina Faso;*
[4]*Unité de Parasitologie et Entomologie Médicale, Centre de Recherche Médicale et Sanitaire, Niamey, Niger*

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***Correspondence**
(laminemahamanmoustapha@gmail.com)

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Abstract

Since 2015, countries in the Sahel region have implemented large-scale seasonal malaria chemoprevention (SMC). However, the mass use of sulfadoxine-pyrimethamine (SP) plus amodiaquine impacts the genetic diversity of malaria parasites and their sensitivity to antimalarials. This study aimed to describe and compare the genetic diversity and SP resistance of *Plasmodium falciparum* strains in Mali and Niger. We collected 400 blood samples in Mali and Niger from children aged 3–59 months suspected of malaria. Of them, 201 tested positive (Niger, 111, 55.2%; Mali, 90, 44.8%). Polymorphism of merozoite surface protein 1 (*msp1*) genetic marker showed 201 allotypes. The frequency of the RO33 allotype was significantly higher in Niger (63.6%) than in Mali (39.3%). There was no significant difference in the frequency of the K1 and MAD20 allotypes between the 2 countries. The multiplicity of infection was 2 allotypes per patient in Mali and one allotype per patient in Niger. The prevalence of strains with the triple mutants *Pfdhfr*51I/*Pfdhfr*59R/*Pfdhps*436A/F/H and *Pfdhfr*51I/*Pfdhfr*59R/*Pfdhps*437G was 18.1% and 30.2%, respectively, and 7.7% carried the quadruple mutant *Pfdhfr*51I/*Pfdhfr*59R/*Pfdhps*436A/F/H/*Pfdhps*437G. Despite the significant genetic diversity of parasite populations, the level of SP resistance was comparable between Mali and Niger. The frequency of mutations conferring resistance to SP still allows its effective use in intermittent preventive treatment in pregnant women and in SMC.

Keywords: Malaria, genetic diversity, drug resistance, multiplicity of infection, Mali, Niger

Malaria is a parasitic disease caused by *Plasmodium* spp. that is transmitted through the bites of infected mosquitoes. It is a major public health issue in many countries, including Niger and Mali, where it is endemic. In March 2012, the World Health Organization recommended a new strategy for combating malaria: seasonal malaria chemoprevention (SMC) [1,2]. This is intended for countries in the Sahel region with high rates of seasonal malaria transmission, including Mali and Niger. SMC uses antimalarial drugs, such as sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ), which have long been used for malaria treatment worldwide. Following a pilot phase, Niger and Mali are scaling up SMC with the help of funding from donors such as the Global Fund to Fight HIV/AIDS, Tuberculosis, and Malaria, the World Bank, Malaria Consortium, UNITAID, and United Nations International Children's Emergency Fund. This has led to SMC implementation in 61/72 health districts in Niger since 2015, with 4 million children aged 3–59 months receiving SP plus

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Data curation: Maman R, Ibrahim ML
Formal analysis: Maman R, Ibrahim ML
Investigation: Lamine MM, Maman R
Methodology: Lamine MM, Maman R, Ibrahim ML
Validation: Lamine MM, Ibrahim ML
Writing – original draft: Lamine MM, Maiga AA, Ibrahim ML
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ORCID
Mahaman Moustapha Lamine (https://orcid.org/0000-0001-5384-546X)

AQ once a month for the 4 months of the high malaria transmission season [3]. It was estimated that to achieve coverage in the Sahel region in the 6 years from 2015 to 2020, 158 million children would need SMC, requiring 49–72 million SP tablets and 148–217 million AQ tablets [4]. SP is used for intermittent preventive treatment against malaria in pregnant women. A dose of SP is administered to each pregnant woman at each antenatal care visit following the observation of the first fetal movements until delivery [5].

SP analogs, such as cotrimoxazole (trimethoprim-sulfamethoxazole), are also widely used in the Sahel region to manage opportunistic infections in people with HIV/AIDS, and this chemoprophylaxis has been associated with multidrug resistance [6]. Trimethoprim-sulfamethoxazole is a combination of a 2,4 diaminopyrimidine (trimethoprim) and a sulfonamide (sulfamethoxazole) and has the same resistance mechanism as SP, namely, mutations in the *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and the *P. falciparum* dihydropteroate synthase (*Pfdhps*) genes. Strains associated with treatment failure carry a quintuple mutation: the triple *Pfdhfr*N51I/C59R/S108N mutation and the double *Pfhps*A437G/K540E mutation [7-9]. Additionally, *Pfdhfr*I164L and *Pfdhps*K540E mutations confer very high levels of resistance [10]. Thus, the widespread use of SP could lead to genetically diverse strains and the selection of resistant parasites. Against this background, we conducted a comparative study of the polymorphism of the *P. falciparum* (*Pf*) merozoite surface protein-1 (*Pfmsp1*) genetic marker between Mali and Niger and the variability of mutations at codons 59 and 51 of the *Pfdhfr* gene and codons 436 and 437 of the *Pfdhps* gene using high-resolution melting analysis (HRM).

The study was approved by the Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry of Bamako, Mali, and the Ethics Committee of Niger (Deliberation No. 024/2015/CCNE). Informed consent was obtained from the parents or legal guardians of all study participants.

This is a comparative study of the genetic polymorphism of parasitic strains using the *msp1* genetic marker and a descriptive study of molecular resistance to SP through analysis of the *Pfdhfr* and *Pfdhps* genes. The study was conducted in Mali and Niger, which are neighboring countries in West Africa. All study sites were located in the Sahel region, where malaria transmission is seasonal and lasts for 3–4 months. In Mali, the rural communities of Sélingué (Yanfolila Cercle, Sikasso Region) and Missira (Kolokani Cercle) were selected. In Niger, the Aguié Department (Maradi Region) and Ertcharna (Tahoua Region) were included. The samples were collected during the SMC campaigns of 2016 and 2017. All children aged 3–59 months, having a mono-specific *Pf* infection and having received SP and AQ doses during the CPS campaign were included in this study. The inclusion criteria were children aged 3–59 months presenting signs of malaria, in whom infection was confirmed by a rapid diagnostic test (RDT) or a thick smear. Children with severe malaria or comorbidity were excluded. The sample size was 400 children, 200 from Niger and Mali, respectively.

The samples consisted of blood spots from filter papers contained in the RDT cassettes used for biological confirmation in Niger and Wattman grade 3 filter papers in Mali. DNA was extracted from the samples using QIAamp DNA Kits (QIAGEN GmbH, Düsseldorf, Germany). The procedure comprised 4 main steps. First, the samples underwent enzymatic digestion with protease. Next, the DNA was adsorbed on the silica gel membrane of a

collector. Then, the membrane was washed with AW1 and AW2 buffers. Finally, DNA was eluted using AE buffer. The resultant DNA samples were aliquoted, numbered, and stored at −20°C before analysis by PCR or HRM.

The multiplicity of infection (MOI) refers to the number of distinct parasite genotypes present within an infected individual. This can be assessed using PCR amplification of specific genetic markers to identify different parasite strains. We analyzed the genetic polymorphism of *Pf* strains using nested PCR with specific primers for conserved blocks 1 and 3 of the *Pfmsp1* gene. The first round of PCR was performed in a final reaction volume of 25 µl comprising 10 µl distilled H_2O, 5 µl of buffer, 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 1 µl forward primer M1-RO33-F (5′-CTAGAAGCTTTAGAAGATGCAGTATTG-3′), 1 µl reverse primer M1-RO33-R (5′CTTAAATAGTATTCTAATTCAAGTGGATCA-3′), 0.5 µl *Taq* polymerase, and 5 µl of template DNA. The first-round PCR program included 40 cycles of denaturation at 95°C for 1 min, hybridization at 59°C for 2 min, and extension at 72°C for 2 min. The second-round PCR amplified 1 of the 3 allelic families of *msp1* (K1, MAD20 or RO33) in a final reaction volume of 25 µl comprising 10 µl distilled H_2O, 5 µl buffer, 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 0.5 µl *Taq* polymerase, and 5 µl template DNA, and 1 µl each of the following primer pairs: forward primer M1-K1-F (5′-AAATGAAGAAGAAATTACTACAAAAGGTGC-3′) and reverse primer M1-K1-R (5′-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3′), or forward primer M1-MAD20-F (5′-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3′) and reverse primer M1-MAD20-R (5′-ATCTGAAGGATTTGTACGTCTTGAATTACC-3′), or forward primer M1-RO33-F (5′-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3′) and reverse primer M1-RO33-R (5′-CATCTGAAGGATTTGCAGCACCTGGAGATC-3′). The second-round PCR program, used to detect the allelic families, included denaturation at 95°C for 1 min, hybridization at 59°C for 2 min, and extension at 72°C for 2 min. The amplified nucleotide sequences from the nested PCR were electrophoresed in a 1% agarose gel containing ethidium bromide. A UVP program was used to identify polymorphism at different allotypes.

HRM employs a DNA-binding dye that fluoresces when bound to double-stranded DNA and decreases in fluorescence during strand separation to monitor DNA melting behavior upon heating. The resulting fluorescence decrease produces a melting curve, which is a plot of fluorescence intensity versus temperature. The shape of the melting curve provides valuable information about the DNA sequence and genetic variations, such as single nucleotide polymorphisms (SNPs), mutations, and DNA methylation patterns, which are used for genotyping, mutation scanning, and methylation analysis. HRM is a simple, sensitive, and cost-effective technique, making it a valuable tool in molecular biology research and diagnostics [11].

We used HRM to analyze molecular resistance to SP. The PCR program was run in a final reaction volume of 25 µl comprising 10 µl distilled H_2O, 10 µl Light Scanner Master Mix, 2.5 µl primers for the *Pfdhfr* and *Pfdhps* genes, and 2.5 µl of template DNA. The 2.5× Light Scanner Master Mix kit contains buffer, dNTPs, *Taq* polymerase, $MgCl_2$, and primers. A probe is added for the mutation search specification. To detect mutations in the S436/A437 codons of the *Pfdhps* gene, we used forward primer 5′-GAATGTTTGAAATGATAAATGAAG-3′, reverse primer 5′-AGGAAACAGCTATGACGAAATAATTGTAATACAGGTACTACTAAATCTCT-3′ and probe sequence ATCCTCTGGTCCTTTTGTTATACC-

block. To detect mutations in the N51/C59 codons of the *Pfdhfr* gene, we used forward primer 5′-ACATTTAGAGGTCTAGGAAATAAAGGAG-3′, reverse primer 5′-ATATTTACATCTCTTATATTTCAATTTTTCATATTTTGATTCATTCAC-3′, and probe sequence AAATGTAATTCCCTAGATATGAAATATTTTTGTGCAG-block. Strain 3D7 (wild-type, melting temperature: 67.5°C) and strain Dd2 (mutant, melting temperature: 62.8°C) were used as controls. The thermocycler program consisted of denaturation at 94°C for 15 sec; annealing at 63°C for 30 sec for *Pfdhfr*51, *Pfdhfr*59, *Pfdhps*436, and *Pfdhps*437; and extension at 60°C for 1 min. Thereafter, the plate was centrifuged and then subjected to HRM.

Graph Pad Prism v6.00 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis which included Chi-square tests and determination of odds ratios (ORs).

Of the 400 patient samples, 201 tested positive (50.3%), including 111 (55.2%) from Niger and 90 (44.8%) in Mali. In Niger, there were 55 (49.5%) malaria-infected patients from Aguié and 56 (50.5%) from Ertcharna. In Mali, there were 54 malaria-infected patients from Sélingué (60.0%) and 36 from Missira (40.0%).

Sample genotyping revealed a total of 201 allotypes (K1: 68, 33.8%; MAD20: 38, 18.9%; and RO33: 95, 47.3%). Table 1 shows the distribution of allotype frequencies by country. The prevalence of the RO33 allotype in Niger (63.6%) showed a statistically significant difference compared with the prevalence of the RO33 allotype in Mali (39.3%) (OR = 0.3696; 95% confidence interval [CI]: 0.2009–0.6791; $P = 0.0015$). However, there were no statistically significant differences between the prevalence of the K1 allotype in Mali (38.5%) versus Niger (24.2%) (OR = 1.958; 95% CI: 1.011–3.793; $P = 0.0566$) or the prevalence of the MAD20 allotype in Mali (22.2%) versus Niger 12.1% (OR = 2.071; 95% CI: 0.8912–4.815; $P = 0.1239$). However, there was a statistically significant difference in the distribution of allotypes by country ($P = 0.0049$).

Table 2 shows the multiplicity of infections by country. In Mali, the MOI was 2 allotypes

Table 1. Distribution of allotypes by country

Country	Allotype			MOI
	K1 *n* (%)	MAD 20 *n* (%)	RO33 *n* (%)	
Mali	52 (38.5)	30 (2.2)	53 (39.3)	2
Niger	16 (24.2)	8 (12.1)	42 (63.6)	1
Total	68 (33.8)	38 (18.9)	95 (47.3)	

The RO33, Mad20, and K1 are alleles of *Plasmodium falciparum merozoite surface protein-1*.

Table 2. Multiplicity of infection by country

Country	MOI			Total *n* (%)
	1 *n* (%)	2 *n* (%)	3 *n* (%)	
Mali	49 (54.4)	37 (41.1)	4 (4.4)	90 (100)
Niger	28 (62.2)	12 (26.7)	5 (11.1)	45 (100)
Total	77 (57)	49 (36)	9 (6.7)	135 (100)

MOI, Multiplicity of Infection.

per patient, compared with one allotype per patient in Niger. Overall, 57.0% of patients had 1 allotype, 36.3% had 2 allotypes, and 6.7% had 3 allotypes. There was a statistically significant difference in the MOI between the 2 countries ($P = 0.0049$). The proportion of patients with 2 *Pf* allotypes was statistically higher in Mali (41.1%) compared with Niger (26.7%) (OR = 2.907; 95% CI: 1.298–6.512; $P = 0.0120$). The proportion of patients with 1 or 3 allotypes was comparable between the 2 countries ($P > 0.05$). However, the MOI in Mali was higher compared to the MOI in Niger.

Table 3 shows the details of all SNPs identified in the *Pfdhr* and *Pfdhps* genes. The prevalence of the SNP *Pfdhfr*51I was 68.5% for Niger and Mali combined (Niger, 71.8%; Mali, 67%) in Mali. There was no statistically significant difference in the prevalence of mutant *Pfdhfr*51I alleles between Mali and Niger (OR = 0.7993; 95% CI: 0.3494–1.828; $P = 0.6812$). The prevalence of the point mutation *Pfdhps*59R was 63.8% for Niger and Mali combined (Niger, 71.8%; Mali, 60.2%). There was no statistically significant difference in the prevalence of mutant *pfdhfr*59R alleles between Mali and Niger (OR = 0.5949; 95% CI: 0.2626–1.348; $P = 0.2356$). The prevalence of SNP *Pfdhps*436F was 27.5% for Niger and Mali combined (Niger, 37.2%; Mali, 23.5%). There was no statistically significant difference in the distribution of mutations at position 436 of the *Pfdhps* gene between the 2 countries (OR = 0.5207; 95% CI: 0.2227–1.218; $P = 0.1765$). The prevalence of the SNP *pfdhps*437G was 54.2% for Niger and Mali combined (Niger, 48.6%; Mali, 56.5%). There was no statistically significant difference in the distribution of mutant alleles (437G and 437A/G) of the *Pfdhps* gene between Mali and Niger (OR = 1.374; 95% CI: 0.6236–3.026; $P = 0.5458$).

Table 3 shows the details of all multiple mutations identified in the *Pfdhr* and *Pfdhps* genes. The prevalence of the quadruple mutation *Pfdhfr*51I/*Pfdhfr*59R/*Pfdhps*436A/*Pfdhps*437G was 7.7% for Niger and Mali combined (Niger, 9%; Mali, 7.1%), and there was no statistically significant difference between them. The prevalence of the triple mutations *Pfdhfr*51/59/*Pfdhps*436 and *Pfdhfr*51/59/*Pfdhps*437 was 18.1% and 30.2%, respectively, in Niger and Mali combined, and there was no statistically significant difference between them ($P = 0.2369$). The prevalence of double mutants *Pfdhfr*51/59 and *Pfdhps*436/437 was 58.3% and 14.9%, respectively, in Niger and Mali combined, and there was no statistically significant difference between them ($P = 0.2776$).

Table 3. Single and multiple mutations of *Pfdhr* and *Pfdhps*

Mutations	Niger (%)	Mali (%)	Total (%)	*P*-value
SNP				
PfdhfrN51I	71.8	67.0	68.5	0.68
PfdhfrC59R	71.8	60.2	63.8	0.23
PfdhpsS436F	37.2	23.5	27.5	0.17
PfdhpsA437G	48.6	56.5	54.2	0.54
Double mutation				
Pfdhfr (51–59)	71.8	52.3	58.3	0.27
Pfdhps (436–437)	13.9	15.3	14.9	
Triple mutation				
Pfdhfr (51,59) *Pfdhps* (436)	27.3	14.5	18.1	0.23
Pfdhfr (51,59) *Pfdhps* (437)	33.3	28.9	30.2	
Quadruple mutation				
Pfdhfr (51–59) *Pfdhps* (436–437)	9.1	7.1	7.7	-

The prevalence of allelic families of the *msp1* genetic marker and the MOIs were examined by nested PCR, a widely used technique for studying genetic diversity [12,13]. The *msp1* marker is well known for its high rate of polymorphism, and it is recommended by the World Health Organization as one of the major markers for determining genetic diversity [14]. *msp1* is located on chromosome 9 of *Pf* and consists of 17 blocks, of which block 2 is the most variable, enabling the distinction of *Pf* clones. Block 2 includes the K1, MAD20, and RO33 alleles. Our investigation revealed that the prevalence of the *msp1* marker alleles was 34%, 19%, and 47% for K1, MAD20, and RO33, respectively. Although all *msp1* allelic families (K1, MAD20, and RO33) were found in infected children in both Mali and Niger, the prevalence of the RO33 allelic family was the highest. There was a difference in the distribution of *msp1* alleles between Mali and Niger. Epidemiological studies have shown that the genetic diversity of parasite populations can vary from one site to another (e.g., 2 distinct villages in the same country) and according to the state of malaria endemicity (forest zone versus Sahelian zone) [15]. In 2011, 17 different *msp1* alleles and 14 different *msp2* alleles were reported in Niger. The most prevalent allelic family in the population was 3D7 (63%), followed by K1 (43.2%). The rarest allelic families were MAD20 (28.4%) and RO33 (28.4%) [13]. This illustrates the temporal and spatial variability of allelic families of *Pf*.

The MOI was 2 (135/90) in Mali compared with 1 (66/45) in Niger. This was identical to that of Sasoun Broum in Niger (MOI = 2) in 2011 [13] and similar to that of Ivory Coast (MOI = 1.7) in 2016 [12]. The MOI ranged from 1 to 3 in 2016 in Dangassa, Mali [16]. Our study results showed a high rate of malaria transmission in Sélingué and Missira (Mali). In contrast, there was a decrease in the rate of infection transmission in Niger, supported by a study conducted in Niger in 2011, reporting an average MOI of 2.8 [13].

HRM of DNA was described in 2012. It is a sensitive technique for detecting point mutations as well as for differentiating haplotypes [17]. We used strains 3D7 and Dd2 as controls because 3D7 was sensitive and Dd2 was resistant to SP. Studies on SP resistance were performed in the Maradi region of Niger in 2012, well before CPS implementation. These studies showed a very high prevalence of point mutations *Pfdhfr*51I (68%), *Pfdhfr*59R (61%), *Pfdhfr*108N (69%), *Pfdhps*436A/F/H (65%), and *Pfdhps*437G (83%). Dinzouna-Boutamba et al. recently reported that the prevalence of antifolate resistance-associated polymorphisms was high among parasite isolates in Gabon [18]. In Dakar (Senegal), Wurtz et al. reported the prevalence of isolates with high-level pyrimethamine resistance as 83.5% and resistance to sulfadoxine as 40.2% [19]. Beshir et al. surveyed 13 countries in the Sahel region in 2016 and 2018. They reported that the *dhfr* haplotype (51Ile-59Arg-108Asn) was common at both survey timepoints, but the *dhps* haplotype (431Ile-436Ser-437Gly-540Glu-581Ala-613Ala), crucial for resistance to SP, was rare [20]. These haplotypes are crucial for SP resistance.

Notably, mutations associated with very high levels of resistance, such as *Pfdhfr*I164L and *Pfdhps*K540E were not reported in south central Niger [8]. Similarly, in Mali, a prevalence of 16.1% for the triple mutation *Pfdhfr*51I/*Pfdhfr*59R/*Pfdhfr*108N and 15% for the double mutation *Pfdhps*436A/F/H/*Pfdhps*437G were reported in Dangassa [16]. SP resistance develops easily, and many studies have demonstrated this after SP introduction in 1967, to the extent that some areas, such as the Horn of Africa, have become multiresistant zones. Our findings did not show a significant difference in genetic diversity between the study

areas (Mali and Niger), suggesting that the 2 regions have similar patterns of genetic diversity in *P. falciparum* strains, which may have implications for malaria control and treatment strategies in both areas. The limitation of our study is the absence of mutations at position 108 of *Pfdhfr* and position 540 of *Pfdhps*. Furthermore, the determination of *Pf* resistance to pyrimethamine often requires the study of positions 108 and 164. Additionally, position 540 of the *Pfdhps* gene is well known for its involvement in sulfadoxine resistance. Our study did not include these mutations, which is a limitation.

In conclusion, our study highlights the genetic diversity of *Pf* strains in Mali and Niger, revealing the presence of multiple mutations associated with SP resistance. Our findings underscore the importance of careful monitoring of the status of antimalarial drug resistance and the need for continued surveillance to ensure the success of chemoprevention programs in the Sahel region. Our result also demonstrates the utility of HRM as a rapid and cost-effective tool for detecting mutations associated with antimalarial drug resistance. Thus, HRM could be useful for guiding treatment decisions and informing public health strategies aimed at malaria control and elimination malaria.

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