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Polymorphism of K-13, dhfr and dhps Genes in Children with Simple Malaria Infectious in Bangui, Central African Republic

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ABSTRACT

Objective: *To study the currently circulating dhfr and dhps haplotypes and assess the distribution of K-13 gene polymorphism in children under treatment with artemether-lumefantrine (AL) and artesunate-sulfadoxine-pyrimethamine (ASSP) in Bangui.*

Methods: *A single-blind randomized cross-sectional study was conducted from August 10 to November 30, 2024, in 135 children aged 6 months to 14 years who were cared for and followed up for simple malaria access at the pediatric hospital complex in Bangui. Five capillary blood spots were made on Whatman® filter paper for molecular analysis before treatments. The parasitic DNA was extracted using the QIAGEN®kit. Resistance markers dhfr, dhps, and Kelch 13 were analyzed by PCR.*

Results: *The dhfr-N51I-C59R-S108N-dhps-S436A mutants were predominant with a prevalence of 67.44%. For the dhfr gene, the triple mutant N51I-C59R-S108N was detected in 96.21% of cases, the double mutant C59R-S108N and N51I-N108N were detected with a prevalence of 0.76% and 3.03%, respectively. The mutant S436A of the dhps gene was found in 94 isolates, or 69.47%. The K13 gene polymorphism showed 09 mutants synonyms: P413P, C469C, E509E, V510V, E567E, S623S, G690G, P715P, V714V and 04 mutants non-synonyms: I416V, V650F, V520I/V637I.*

Conclusion: *This study shows that dhfr-N51I-C59R-S108N-dhps-S436A mutants are currently circulating in Bangui and that Plasmodia carrying synonymous and non-synonymous mutations have been detected at the paediatric and university complex of Bangui, hence the need to continue monitoring these resistance markers regularly.*

Keywords
Plasmodium falciparum, Resistance, Bangui.

Introduction
In the Central African Republic, since the emergence of cases of resistance to chloroquine (40%), amodiaquine (23%) and sulfadoxine-pyrimethamine (22%), combinations based on artemisinin derivatives (ACT) were introduced as first-line treatment for the management of simple malaria in CAR in 2005 [5, 6]. However, sulfadoxine-pyrimethamine (SP) continues to be used as intermittent preventive treatment (IPT) in pregnant women requiring constant monitoring of parasite resistance levels by monitoring the polymorphism of these genes associated with SP resistance such as point mutations in the dhps S436A gene,

A437G, K540E, A581G and A613T/S and point mutations in the dhfr gene N51I, C59R, S108N and I164L [1,4]. Furthermore, the therapeutic effectiveness of ACTs is strongly threatened by the emergence of artemisinin resistance phenotypes in western Cambodia before spreading to Southeast Asia and then to South China. These resistance markers have been identified in the K-13 gene. Of all these mutations, C580Y was identified as the one most associated with the artemisinin resistance phenotype [3,7]. This could jeopardize efforts to combat this scourge, given the difficulty of finding alternative therapies. It is in this context that we set ourselves the objective: (i) to study the currently circulating haplotypes dhfr and dhps; (ii) to determine the prevalence of single, double, triple, and quatruple mutations present in circulating parasites in Bangui; (iii) to assess the distribution of K 13 gene polymorphism in P. falciparum isolates.

Methodology

This cross-sectional, simple comparative study was conducted from August 10 to November 30, 2024, among children aged 6 months to 14 years treated for simple malaria access and follow-up at the pediatric hospital complex in Bangui.

Inclusion criteria

(i) patient aged 6 months to 14 years suffering from a mono-specific P. falciparum infection with parasitaemia between 700 and 200,000 asexual forms/µL of blood; (ii) axillary temperature greater or equal to 37.2 °C; (iii) possibility of follow-up with easy access to the health center; (iv) informed consent from parents.

Non-inclusion criteria

(i) presence of general signs of danger in children or signs of severe P. falciparum malaria according to WHO definitions; (ii) body weight less than 5 kg; (iii) haemoglobin level > 8g/dl; (iv) mixed or monospecific infestation by another species of Plasmodium; (v) severe malnutrition; (vi) febrile illness due to diseases other than malaria; (vii) history of hypersensitivity to treatments administered.

Exclusion criteria

(i) patient lost to follow-up; (ii) withdrawal of informed consent; (iii) patient with persistent vomiting; (iv) manifestations of serious adverse events, requiring treatment termination before completion; (v) development of severe malaria within 24 hours of starting treatment.

Two combinations of drugs were used, artemether-lumefantrine (AL) and artesunate-sulfadoxine-pyrimethamine (ASSP) as shown in Tables 1 and 2.

Table 1: Dosage of artemether-lumefantrine (AL) Number of tablets per dose: 20 mg artemether + 120 mg lumefantrine H0, H8, H24, H36, H48 et H60.

weight	Number of tablets in each of the 6 doses
5 à <15 kg	1
15 à <25 kg	2
25 à <35 kg	3
≥35 kg	4

Table 2: Dosage of artesunate-sulfadoxine-pyrimethamine (ASSP) artesunate 50 mg + 500 mg sulfadoxine + 25 mg pyrimethamine.

Age (years)	weight	artésunate (D1-D3)	SP (dose unique D1)
<1	5-8kg	½ cp (25mg) x1/Day	¼ cp
1-5	9-17kg	1cp (50mg) x1/day	½ cp
6-9	18-29kg	2cp (100mg) x1/day	1 cp
10-14	30-39kg	3cp (150mg) x1/day	1,5 cp
≥15 years/adults	≥40kg	4cp (200mg) x1/day	2 cp

cp = comprimé; kg = kilogramme; mg = milligramme.

The size of each study arm (N) was calculated using the formula N=Z2*P (1-P) / E2. The proportion of early failure in the population (P) was 15%, the confidence level (Z) was 95%, and the margin of error (E) was 10% per arm [7]. The minimum number of patients needed was 88 per treatment.

A nurse was taking the axillary weight and temperature. The clinician looked for signs of gravity. A technician performed a thick drop (GE), a thin smear and a hemoglobin assay (Hemocue HB 301 analysis, Kuvettgatan, Sweden). Children with a hemoglobin level above 8 g/dl and a GE between 700 and 200,000 parasites/µl were included after approval by the manager.

A code number was randomly assigned to each patient on Day 0 with a view to making the files anonymous. A coefficient between 0 and 1 was randomly generated on an Excel 2013 file to randomize patients into two different treatment groups. The first dose of treatment was administered at the Health Center by the healthcare staff.

Patients were observed for 30 minutes before leaving the site. In case of vomiting, the same dose was repeated. The following doses were administered at home by those responsible for the child. Adherence to the treatment was checked by the healthcare staff on the third day upon presentation of the rest of the leaflet and a description of the administration of the treatment.

In order to assess the parasitic and thermal clearances, a GE and a temperature check were carried out on days 3, 7, 14, 21, and 28.

The criteria for assessing responses were those defined by the WHO [7]. Responses to treatment are classified as adequate parasitological clinical response (APCR), early therapeutic failure (ETP), late clinical failure (ECT), and late parasitological failure (LPR) as follows: (i) ETP, from Day 0 to Day 3, sign of danger or malaria at Day 1, D2 or D3; fever + increased parasitaemia on D2 or fever + parasitaemia on D3; or parasitaemia on D3 greater than or equal to 25% of the parasitaemia on D0, (ii) FTE, from D7 to D14 or D28, presence of parasites without fever, (iii) ECT, from D4 to D14 or D28, signs of danger or malaria attacks after D3; fever + positive parasitaemia between D4 and D28, (iv) RCPA, absence of detectable parasites between D2 and D28, and absence of fever between D2 and D28.

The microscopic observations were made by two double-read microscopists. Parasite densities were expressed according to

the formula: number of asexual parasites x 8,000/number of white blood cells per µl of blood over 200 leukocyte fields. One out of three slides was subjected to a second reading by a third microscopist accredited by the Pasteur Institut of Bangui for quality control, without any discrepancy being observed.

For molecular analyses, five blood spots were carried out on Whatman filter paper.

Among the 138 samples collected at the University Pediatric Complex of Bangui, 3 (2.17%) were negative and 135 (97.83%) positive during the PCR allowing the detection of the presence of P. falciparum. One hundred thirty-five (135) samples were selected for sequencing, which yielded exploitable results for 133 samples for the K13 gene (98.52%), 132 for the dhfr gene (97.78%), and 131 for the dhps gene (97.04%).

The parasitic DNA was extracted by QIAGEN (QIAamp DNA Blood Minikit) [2]. Real-time PCR using SYBR and primers specific for the cytochrome B gene of P. falciparum was performed to confirm the infecting plasmodial species at day 0. The reagents marketed in the SYBR Green Master Mix I kit (Roche Diagnostics) include two mixtures 1a and 1b containing the fluorescent intercalator SYBR green, dNTP, Taq polymerase and MgCl2. The reactions take place in the Light cycler and under a final volume of 20 µl, including 5 µl of extracted DNA to be amplified. A couple of primers specific to P. falciparum: PanPalu 5'-AGTGTGTATCAATCGAGTTTC-3' and PfaIR 5'-AGTTCCCCTAGAATAGTTACA-3' was used. Positive samples were selected for the analysis of artemisinin (k13), pyrimethamine (dhfr) and sulfadoxine (dhps) resistance markers with specific primers presented in Table III.

The regions that encoded these genes were amplified and sequenced by amplicon sequencing at the Institut Pasteur de Madagascar.

The PCR amplification program with real-time sybr green is recorded in Table IV.

Means are given with 95% confidence intervals and extreme values of the distribution.

The statistical tests used to compare means and proportions used the threshold of $p<0.05$.

Results

A total of 135 patients were enrolled, including 78 for AL and 57 for ASSP. Three (3) were lost to view (PDV) with AL and 2 withdrawals with ASSP. Patients followed up to day 28 were 75 for AL and 55 for ASSP (Figure 1).

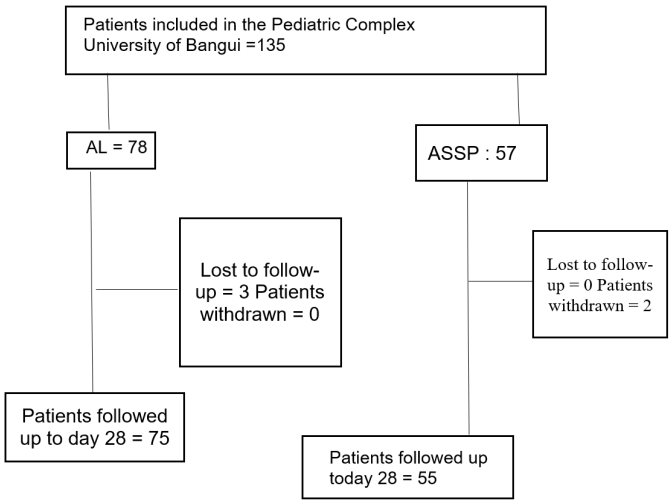

Figure 1: Distribution of patients at site and by treatment groups.

The mean age was 5.69±0.79 years with extremes (1 to 14 years) for AL and 5±3.01 years with extremes (1 to 13 years) for ASSP without significant difference between the two groups ($p = 0.61$). The most represented age groups were children aged 3-4 years. The sex ratio was 1.28. The average temperature was 38.71±0.66°C for AL with extremes (36°C to 40.60°C) and 38.84±1.15°C for ASSP with extremes (36°C to 41°C). The

Table 3: Sequences of specific primers for the resistance genes studied.

	Gène *K13* (5'----3')	Gène *dhfr* (5'---3')	Gène *dhps* (5'---3')
F	GCCAAGCTGCCATTCATTTG	TTTATATTTTCTCCTTTTTA	CCATTCCTCATGTGTATACAACAC
R	GCCTTGTTGAAAGAAGCAGA	CATTTTATTATTCGTTTTCT	CTTGGTCTATTTTTGTTAAAACATCC

F : amorces sens; R: amorces anti sens.

Table 4: Real-time PCR program for amplification of the cytochrome B gene of *Plasmodium falciparum*.

	Steps	Times and Temperatures
	Initial denaturation	8 minutes à 95 °C
Repeated amplification cycles 40 times	Denaturation	10 secondes à 95 °C
	Hybridization of primers	10 secondes à 58 °C
	Elongation	30 secondes à 72 °C
	Denaturation	2 secondes à 95 °C
Merge Curves	Hybridization	20 secondes à 50 °C
	Progressive denaturation	0,2°C/seconde jusqu'à 95 °C
	Cooling	30 secondes à 95 °C

Means are given with 95% confidence intervals and extreme values of the distribution.
The statistical tests used to compare means and proportions used the threshold of $p<0.05$.

Table 5: Sociodemographic and biological characteristics of the study population at inclusion.

Characteristics		AL N = 78 (%)	ASSP N = 57 (%)	Total N=135	P
Sex (sex-ratio 1.28)	Masculin	40 (52.63)	36 (47.37)	76 (56.30)	**0.11**
	Féminin	38 (64.41)	21 (35.59)	59 (43.70)	
Age (years)	mean	3.76±1.58	4.37±0.58		
	Extreme	6 month - 13 years	6 month – 14 years		
Parasitémie /µl de sang	mean	4499.85±6700	4372.07±4300	4445. 90	**0.72**
	Extreme	745 - 12348	1010 - 12400		
Hémoglobine (g/dl)	mean	10.78±0.47	10.67±0.58	10.95	**0.69**
Température (°C)	mean	36.33±0.66	36.50±1.15	37.74	**0.51**
	Extreme	40,60±1.16	41±0.67		

mean parasitaemia was 4499,85±6200 parasites/ml for AL with extremes (740 to 12348 parasites/ml) and 4372,07±6500 parasites/ml for ASSP with extremes (1010 to 12400 parasites/ml) without significant difference between the two groups (P=0.72). The mean haemoglobin concentration was 10.67±0.58g/dl for AL and 10.78±0.47 g/dl for ASSP, Table 5.

On Day 3, 100% or 78 patients had negative parasitaemia for AL and 100% or 57 patients had negative parasitaemia for ASSP. The situation remained stable at days 7, 14, 21, and 28. The same was true for the improvement of fever and anemia.

On day 28, the total number of patients per protocol was 130. The lost to follow-up were 3 for AL and 2 withdrawals of consent for ASSP. We observed no cases of ETP, ECT, or EPT. Furthermore, we observed 130 cases of cumulative RCPA, i.e. 100%, with no significant difference between the two groups (p > 0.05) (Table 4).

Among the 135 samples analyzed, 98.52% (133/135) yielded exploitable results for the sequencing of K13 genes, 97.78% (132/135) for the dhfr gene, and 97.04% (131/135) for the dhps gene.

For the K13 gene, the wild-type allele was detected in 120 samples, or 90.23%. Nine (9) synonymous mutations were detected, namely: P413P, C469C, E509E, V510V, E567E, S623S, G690G, P715P with a prevalence of 0.75 except for V714V which was 1.50%. Four (4) non-synonymous mutations were detected, namely: I416V (0.75%), V650I (0.75%) and V520I associated with V637I in a proportion of (0.75%). No mutations considered to be associated with decreased artemisinin efficacy were observed during this study (Figure 2).

The analysis of the dhfr gene showed that all samples analyzed contained mutations. The triple mutant N51I-C59R-S108N was detected in 96.21% of cases, and the double mutant C59R-S108N and N51I-N108N were detected 1 and 4 times respectively (0.76% and 3.03%) (Figure 3).

The dhps gene had wild-type alleles in 39 isolates, or 29.77% of cases. The mutant S436A was found in 94 isolates, or 69.47% of cases, and the mutant alleles I431V-S436A-A581G-A613S in 1 sample (Figure 4).

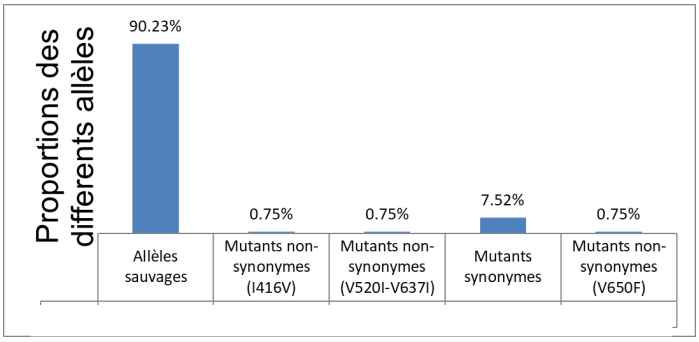

Figure 2: Proportions of wild and mutant alleles of the Kelch13 propeller gene.

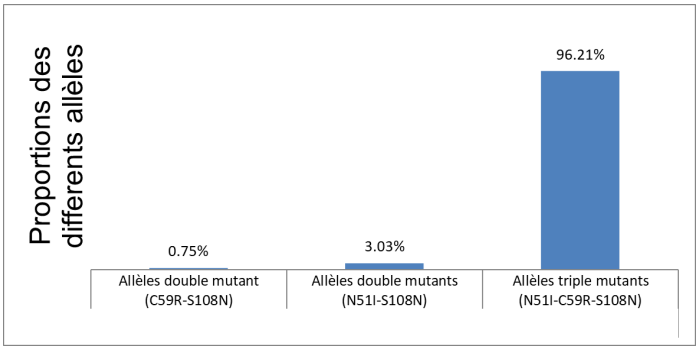

Figure 3: Proportions of wild and mutant alleles of the *dhfr* gene resistant to pyrimethamine.

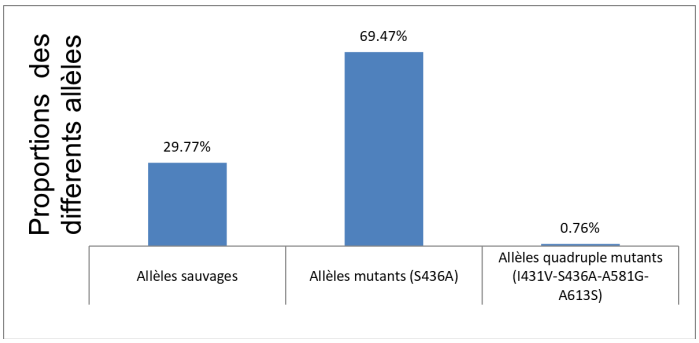

Figure 4: Proportion of wild and mutant alleles of the *dhps* gene resistant to sulfadoxin.

Discussion

The assessment of the prevalence of mutants of the dhfr and dhps genes currently circulating in Bangui after discontinuing the use of the sulfadoxine-pyrimethamine (SP) combination for curative treatment and its use for intermittent preventive treatment (TPi) in pregnant women showed for the dhfr gene the presence of triple mutant N51I-C59R-S108N in 96.21% of cases, double mutant C59R-S108N and N51I-N108N with a prevalence of 0.76% and 3.03%, respectively. For the dhps gene, the mutant S436A was present in 69.47% of cases. These expected results reflect that, despite the lack of use of SP in curative therapy for mutants at the level of different codons 5, 59, and 108 of the dhfr gene and at the level of codon 436 of the dhps gene, it continues to circulate in Bangui as well as in other countries in Africa [4,15] hence the need for regular monitoring of these genes. However, the absence of mutation on codon 540 supports the efficacy of sulfadoxine in TPi.

Moreover, the distribution of the K13 gene showed nine (9) synonymous mutations with a prevalence of 0.75 except for V714V which was 1.50% and four (4) non-synonymous mutations with a proportion of 0.75%. These mutations are not among the validated resistance markers or resistance-associated candidates reported by WHO [7]. These mutations reflect the genetic diversity of the K13 gene and have been reported in other work elsewhere [6, 8, 13, 14]. However, the data collected concern a single locality with different epidemiological profiles, which makes it necessary to extend this work to other regions of the country in order to obtain conclusive results.

Conclusion

Mutations in codons 51, 59 and 108 of the dhfr gene and in codon 436 of the dhps gene are circulating in Bangui, CAR in 2024. Similarly, the analysis of the K13 gene in children at the pediatric and university hospital complex in Bangui has mainly highlighted wild-type alleles. The synonymous and non-synonymous mutations observed reflect the genetic diversity of the K13 gene and are not associated with resistance to artemisinin derivatives. It would be wise to continue to monitor these genes regularly in order to prevent the emergence of resistance.

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Ethical aspect

The study received the opinion of the Ethics and Scientific Committee of the Faculty of Health Sciences of the University of Bangui, N°_50/UB/FACSS/IPB/CES/022, November 2022, and that of the WHO AFRO in Switzerland dated December 2023.

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