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High prevalence of genotypes associated with sulfadoxine/pyrimethamine resistance in the rural area of Fougamou, Gabon

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ABSTRACT

Objectives: Pregnancy-associated malaria (PAM) is a complex form of malaria. To prevent PAM, several African countries have adopted intermittent preventive treatment with sulfadoxine/pyrimethamine (IPT-SP). However, resistance to SP has been reported, associated with mutations in the genes *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps*) and *P. falciparum* dihydrofolate reductase (*Pfdhfr*). The aim of this study was to investigate the prevalence of mutations in *Pfdhfr* and *Pfdhps* in *P. falciparum* isolates from rural areas of Gabon.

Methods: A cross-sectional survey of febrile patients ($n = 202$) who consulted Fougamou Health Center between February–May 2016 was performed. DNA was extracted from patient samples and the *Pfdhfr* and *Pfdhps* genes were genotyped using PCR-RFLP. Statistical analyses were performed.

Results: The malaria prevalence in febrile patients included in the study was 60.4% (122/202). The main parasite species was *P. falciparum* (96.7%; 118/122), followed by *Plasmodium malariae* (3.3%; 4/122). Genotypes on codons 16, 51, 59 and 108 of *Pfdhfr* were highly mutated (>96%). In *Pfdhps*, codons 436, 437, 540 and 613 also expressed high mutation rates. The prevalence of triple mutations of *Pfdhfr* VIRNI and AIRNI was 12.1% and 84.5%, respectively. The prevalence of mutant haplotypes of *Pfdhps* SGEA, SGKA and AGEA was 37.9%, 25.9% and 12.1%, respectively. The prevalence of quadruple mutants IRN-A and IRN-G was 20.0% and 93.1%, respectively, whereas quintuple mutants were found at 57.8% (IRN-GE) and 5.0% (IRN-AE).

Conclusion: Our data show a high prevalence of genotypes associated with SP resistance. Clinical trials to investigate the efficacy of IPT-SP are much needed.

1. Introduction

Malaria remains one of the most prevalent public-health problems. A recent World Health Organization (WHO) report indicated that 219 million cases and 435 000 related deaths were registered globally in 2017 [1]. One of the reasons for the persistence of malaria is the emerging and wide spread of antimalarial drug

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resistance. Drug resistance is defined as the ability of *Plasmodium* parasites to survive after the absorption of antimalarial drugs in amounts above patient-tolerated doses. Several studies have shown that antimalarial drug resistance is associated with certain genotypes, known as drug resistance markers. Following the widespread implementation of chloroquine, mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) were described [2]. Other antimalarial drug resistance markers have also been described [3]. To combat antimalarial drug resistance, the WHO recommends the use of artemisinin-based combinations therapy (ACT) to treat uncomplicated malaria and the use of intermittent preventive treatment with sulfadoxine/pyrimethamine (IPT-SP) for pregnant women.

Sulfadoxine/pyrimethamine (SP) has an antifolate effect derived from the inhibition of two enzymes in the folate synthesis pathway: sulfadoxine acts against dihydropteroate synthase (DHPS) [4,5], whereas pyrimethamine acts against dihydrofolate reductase (DHFR) [6]. *Plasmodium falciparum* undergoes de novo synthesis of folate from GTP precursors, p-aminobenzoic acid and L-glutamine precursors. Pyrimethamine binds far more strongly to the parasite DHFR than to the mammalian enzyme. Inhibition of DHFR and DHPS in parasite folate synthesis pathway leads to decreased levels of tetrahydrofolate, resulting in lowered thymidylate levels that eventually inhibit nuclear division. Following the introduction of pyrimethamine in Africa, resistance quickly emerged in 1954 [7] and therefore its association with sulfadoxine was developed. Unfortunately, resistance against this association also emerged [8].

Single nucleotide polymorphisms (SNPs) in the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) are associated with pyrimethamine resistance. The S108N mutation is the main mutation conferring in vitro pyrimethamine resistance [9] and addition of the mutations N51I and C59R increases the level of resistance from 2 to 16 times [5,10]. Parasites with triple mutations (N51I+C59R+S108N and C59R+S108N+I164R) show higher resistance than those with two mutations, while parasites with quadruple mutations show the highest resistance level [11,12]. Regarding sulfadoxine, mutations in the *P. falciparum* dihydropteroate synthase (*Pfdhps*) gene are associated with resistance. These mutations induce a decrease in affinity between sulfadoxine and its target [13]. The A437G mutation increases the IC_{50} by 5. Addition of other mutations in *Pfdhps* leads to a further increase in the resistance level. Therefore, the triple mutations S436A+437G+K540E and S436A+A437G+A613S induce an increase in the IC_{50} by 9.8 and 24, respectively [14]. The risk of treatment failure with SP is increased by the association of mutations N51I+C59R+S108N in *Pfdhfr* and double mutants G437E540 or G437S581S in *Pfdhps* [15].

In Gabon, antimalarial drug resistance is generally described [16,17]. SP resistance was described as soon as 1988 [18]. In 1992, 30% of isolates from Lambaréné were resistant to SP [19]. Consequently, failure rates of SP treatment were reported from 9.1–15% in the country [20–22]. Molecular investigations showed that SP resistance markers circulated at high levels. In Bakoumba, 71.8% of isolates expressed the IRN triple mutations, whereas 57.3% were mutated in codon 437 of *Pfdhps* [23]. According to WHO recommendations, IPT-SP was implemented. Therefore, several data have reported a decrease in pregnancy-associated malaria in Gabon, mainly in urban areas [24,25]. Consequently, the prevalence of double mutants (51I+59R, 51I+108N and 59R+108N) increased significantly around 100% [26]. These results are mainly from urban areas, despite the fact that rural areas show specific malaria challenges. Little is known about malaria in rural areas even though we previously showed that the malaria prevalence is higher in rural areas than urban areas [27–29]. Drug resistance in rural areas is poorly investigated. In Fougamou, a rural area in the centre of Gabon, antenatal care is effective and several women have received IPT-SP [30]. Therefore, the aim of this study was to investigate the malaria prevalence and the level of SP resistance markers (*Pfdhfr* 16, 51, 59, 108 and 164 and *Pfdhps* 436, 437, 540 and 613) in the rural area of Fougamou.

2. Methods

2.1. Study area and population

The study was conducted in the Gabonese rural town of Fougamou located in the centre-west region of Gabon in Ngounié Province. Fougamou is the chief town of the Tsamba-Magotsi Department and therefore its hospital receives patients from villages throughout the department (Fig. 1). The study population comprised febrile patients consulting Fougamou Health Center between 2 February 2016 and 31 May 2016 with the following criteria: (i) patients with an axillary temperature ≥37.5°C at the time of consultation or who had a history of fever in the 48 h preceding the consultation; and (ii) patients who gave informed consent, or the children of parents or guardians who gave their informed consent.

2.2. Malaria diagnosis

Plasmodium falciparum malaria was diagnosed using the malaria rapid diagnostic test OptiMal-IT®, and the parasite load was estimated using the microscopic Lambaréné method [31]. Briefly, 10 μL of blood is evenly distributed on a 10 mm × 18 mm area of a microscope slide. Each high-power field (HPF) on this thick smear is 1/500th of a microlitre (on a standard microscope at 1000 × magnification) and a count is made per 10 HPFs. Parasitaemia per microlitre is calculated by a pre-calibrated appropriate multiplication factor (500).

2.3. DNA extraction

DNA extraction was done from archived whole blood using an E.Z.N.A® Blood DNA Kit (Omega Bio-tech, Norcross, GA, USA) according to the manufacturer's protocol as previously described [32]. Briefly, 250 μL of infected blood was added to 25 μL of OB protease and 250 μL of Buffer BL and was incubated for 10 min at 65°C. DNA was then precipitated by adding 260 μL of ethanol and mixing for 20 s at a maximum speed of 13 000 rpm. DNA was washed with 500 μL of Buffer HB added to the mini-columns and was then centrifuged for 1 min at 10 000 rpm. DNA was eluted with 100 mL of sterile water pre-heated to 70°C. DNA aliquots were kept at 20°C until use.

2.4. Genotyping

Codons 16, 51, 59, 108 and 164 of *Pfdhfr* and codons 436, 437, 540 and 613 of *Pfdhps* were genotyped using PCR–restriction fragment length polymorphism (PCR-RFLP) as previously described [33]. Specific primers and enzymes are summarised in Table 1.

2.5. Statistical analysis

Statistical analysis was performed using Epi Info™ v.3.3.2 (CDC, Atlanta, GA, USA). The χ^2 test was used to compare categorical variables among groups. The non-parametric Mann–Whitney *U*-test, Pearson's test and Fisher's exact test were used for group comparisons, as appropriate. A *P*-value of <0.05 was considered to indicate statistical significance.

Fig. 1. Map of Gabon, showing the location of Fougamou (from the Division géographique of the Direction des archives, Ministère des affaires etrangères du Gabon).

Table 1
Sequences of primer sets and restriction enzymes used to characterise gene polymorphisms

Gene	PCR	Primer name	Primer sequence (5′→3′)	*T* (°C)	Size of PCR product (bp)	Mutation	Restriction enzyme	Genotype	Fragment size (bp)
Pfdhfr	PCR	M1	TTTATGATGGAACAAGTCTGC	45	648				
	I	M5	AGTATATACATCGCTAACAGA						
	PCR			45	522	A16V	*Nla*III	16V	376+146
	II	M3	TTTATGATGGAACAAGTCTGCGACGTT			N51I	*Tsp*509I	51I	218+120
		F/	AAATTCTTGATAAACAACGGAACCTttTA			S108N	*Alu*I	108N	522
						I164L	*Dra*I	164L	245+143+107
		F	GAAATGTAATTCCCTAGATATGgAATATT	45	325	C59R	*Xmn*I	59R	162+163
		M4	TTAATTTCCCAAGTAAAACTATTAGAgCTTC						
Pfdhps	PCR	R2	AACCTAAACGTGCTGTTCAA	45	710				
	I	R/	AATTGTGTGATTTGTCCACAA						
	PCR	K	TGCTAGTGTTATAGATATAGGatGAGcATC	45	437	A437G	*Ava*II	437G	404
	II	K/	CTATAACGAGGTATTgCATTTAATgCAAGAA			K540E	*Fok*I	540E	320+85
						S436A	*Mn*lI	436A	317+121
		L	ATAGGATACTATTTGATATTGGAccAGGATTcG	45	160	A613S	*Mwo*I	613S	161
		L/	TATTACAACAATTTGATCATTCgcGCAAccGG						

3. Results

3.1. Clinical and biological characteristics of patients

A total of 202 febrile patients were included in the study, with a mean age of 167.75 ± 138.83 months. The sex ratio (M/F) was 1.1 (106 males and 96 females). Children under 6 years old were the most prevalent (46.5%; $n = 94$). The malaria prevalence was 60.4% ($n = 122$). *Plasmodium falciparum* was the most commonly found malaria parasite (96.7%; $n = 118$), followed by *Plasmodium malariae* (3.3%; $n = 4$). The mean parasite density was 52 375 parasites/μL (16–347 300 parasites/μL). Children under 7 years old represented the most infected group (65.6%; $n = 80$). Haematological parameters were compared between malaria patients and uninfected patients. The data showed that the red blood cell count, haemoglobin and platelets were significantly lower in patients with malaria compared with uninfected patients (Table 2).

3.2. Single nucleotide polymorphisms (SNPs) in Pfdhfr and Pfdhps

SNPs in *Pfdhfr* and *Pfdhps* were investigated from 116 malaria isolates using PCR-RFLP. For *Pfdhfr*, we analysed codons 16, 51, 59, 108 and 164. The frequencies of genotype I51, N108 and I164 were 100% (116/116), whereas the frequency of A16 was 91.4% (106/116), V16 was 8.6% (10/116) and R59 was 96.6% (112/116) (Table 3).

Table 2
Comparison of haematological parameters between *Plasmodium falciparum*-infected and uninfected patients

Parameter	Infected patients	Uninfected patients	*P*-value
White blood cells ($\times 10^3/\mu$L)	8.13 ± 4.22	8.25 ± 5.49	0.85
Red blood cells ($\times 10^6/\mu$L)	4.03 ± 0.78	4.46 ± 0.67	0.03
Haemoglobin (g/dL)	9.61 ± 1.71	11.18 ± 1.96	0.0001
Platelets ($\times 10^3/\mu$L)	121.76 ± 73.32	190.99 ± 125.53	0.002

NOTE: Data are mean ± standard deviation.

Table 3
Frequency of mutated single genotypes of *Pfdhfr* and *Pfdhps*

Gene	Codon	Genotype mutated	Frequency [% (*n*/*N*)]
Pfdhfr		16V	8.6% (10/116)
	51	51I	100% (116/116)
	59	59R	96.6% (112/116)
	108	108N	100% (116/116)
	164	164L	0% (0/116)
Pfdhps	436	436A	15.5% (18/116)
		436A/S	6.9% (8/116)
	437	437G	100% (116/116)
	540	540E	53.4% (62/116)
		540E/K	8.6% (10/116)
		613S	1.7% (2/116)

Table 4
Prevalence of haplotypes of *Pfdhfr* and *Pfdhps*

Gene/haplotype	Prevalence [% (*n*/*N*)]
Pfdhfr	
$V_{16}I_{51}R_{59}N_{108}I_{164}$	12.1% (14/116)
$A_{16}I_{51}R_{59}N_{108}I_{164}$	84.5% (98/116)
$V_{16}N_{51}C_{59}S_{108}I_{16}$	3.4% (4/116)
Pfdhps	
$S_{436}G_{437}E_{540}A_{613}$	37.9% (44/116)
$S_{436}G_{437}K_{540}A_{613}$	25.9% (30/116)
$A_{436}G_{437}E_{540}A_{613}$	12.1% (14/116)
$S_{436}G_{437}E_{540}A_{613}/S_{436}G_{437}K_{540}A_{613}$	12.1% (14/116)
$A_{436}G_{437}E_{540}A_{613}/A_{436}G_{437}K_{540}A_{613}$	12.1% (14/116)
Associated haplotypes	
Quadruples	
$I_{51}R_{59}N_{108}$-A_{436}	20.0% (20/100)
$I_{51}R_{59}N_{108}$-G_{437}	93.1% (108/116)
Quintuples	
$I_{51}R_{59}N_{108}$-$A_{436}E_{540}$	5.0% (5/100)
$I_{51}R_{59}N_{108}$-$G_{437}E_{540}$	57.8% (67/116)

Regarding the *Pfdhps* gene, the frequencies of the A436 and S436 genotypes were 15.5% (18/116) and 77.6% (90/116), respectively, while the frequency of the A/S436 mixed genotype was 6.9% (8/116). The frequency of genotype G437 was 100% (116/116), while that of genotype E540 was 53.4% (62/116), genotype K540 was 37.9% (44/116) and the E/K540 mixed genotype was 8.6% (10/116). The A613 and S613 genotypes had frequencies of 98.3% (114/116) and 1.7% (2/116), respectively (Table 3).

3.3. *Pfdhfr and Pfdhps haplotypes*

An analysis of the haplotypes of the two genes (*Pfdhfr* and *Pfdhps*) was then performed and the results are presented in Table 4. The *Pfdhfr* gene highlighted two triple mutants on haplotypes $V_{16}I_{51}R_{59}N_{108}I_{164}$ and $A_{16}I_{51}R_{59}N_{108}I_{164}$ with a prevalence of 12.1% and 84.5%, respectively. Only four isolates harbouring the wild-type NCSI haplotype were found. The analysis of associated haplotypes showed that the rate of the triple mutant $A_{436}G_{437}E_{540}A_{613}$ of *Pfdhps* was 12.1% and the double mutant $S_{436}G_{437}E_{540}A_{613}$ was 37.9% (Table 4). Haplotypes associated with *Pfdhfr* (51, 59 and 108) and *Pfdhps* (436, 437 and 540) were also investigated. The quadruple mutant $I_{51}R_{59}N_{108}$-G_{437} was found in 93.1% (108/116), whereas $I_{51}R_{59}N_{108}$-A_{436} had a prevalence of 20.0% (20/100). The quintuple mutant $I_{51}R_{59}N_{108}$-$G_{437}E_{540}$ was also high at 57.8% (67/116), whereas $I_{51}R_{59}N_{108}$-$A_{436}E_{540}$ was lowest at 5% (5/100).

4. Discussion

Drug resistance remains the main obstacle to eradicating malaria. In this study, we investigated the effect of large-scale implementation of IPT-SP on molecular drug resistance in the rural area of Fougamou, a town located in the centre of Gabon. The study was performed on all febrile patients. Indeed, IPT-SP is given to pregnant women because when resistant parasites are not eliminated by treatment they may be transmitted through another mosquito bite. When the infected mosquito transmits the parasite, other people may be infected with SP-resistant parasites. Therefore, the SP pressure in women impacts the entire population.

First, we confirmed the high prevalence of malaria in febrile patients in Fougamou, as previously reported 2 years before this study in 2014 [29]. The data suggest that the burden of malaria in rural areas remains stable but the high prevalence can be considered as a reservoir of *Plasmodium* that may maintain resistant strains of malaria in the country. We also confirmed that children under 6 years old are the most susceptible to malaria in Fougamou. A decrease in red blood cells and platelets induced by malaria was also confirmed.

Molecular analysis of the samples allowed us to genotype codons 16, 51, 59, 108 and 164 of *Pfdhfr* and codons 436, 437, 540 and 613 of *Pfdhps* associated with SP resistance (for review see [34]). Regarding the *Pfdhfr* gene, A16 was the most prevalent genotype. That is consistent with data from India were this genotype was 100%. This wild-type is mainly conserved, the mutant N51I and S108N genotypes had the highest prevalence (100%) similarly to those reported in Yemen [35]. The mutant genotype R_{59} had a prevalence of 96.6%. The higher prevalence of N51I and S108N mutant genotypes confirm the drug pressure. These data could be the result of parasite selection since the deployment of IPT-SP, as reported recently in Oyem [26]. In Franceville, Lastourville and Koulamoutou, we also reported a higher level of the IRN haplotype [36] suggesting a similar pressure of SP over all the country. This pressure could indicate the same implementation of SP in the country or the spread and circulation of parasites over the country from areas with drug resistance. Such a selection has been reported in other countries such as Kenya [37,38]. The data show that (51-59-108-164) IRNI is the main haplotype. This haplotype dramatically decreases the efficacy of pyrimethamine [39] and is maintained by pyrimethamine pressure [40]. This prevalence is similar to that reported in Congo and Ghana [41]. On the other hand, in the Ivory Coast this haplotype accounted for only 26% [42]. In Swaziland, a decrease in the prevalence of this haplotype was observed between 1999 and 2007 [43]. This could be explained by the lack of use of SP, which is a second-line treatment for chloroquine-resistant parasites. Regarding the *Pfdhps* resistance marker, the predominant mutation associated with resistance to sulfadoxine (437G) had a prevalence of 100%. Double mu-

tants also had a very high prevalence. These data are also similar to those obtained in Oyem where administration of IPT-SP has led to an increase in the prevalence of *Pfdhps* genotypes associated with sulfadoxine resistance [26]. Recently, we confirmed this data in Franceville, Koulamoutou and Lastourville (urban, semi-urban and rural areas of Gabon, respectively) [36]. In other African countries, similar data have been reported [44], which also testifies to the strong pressure of sulfadoxine. However, in Ghana, it has been reported that the prevalence of the 437G-540E double mutant remained low between 2003 and 2010, despite the large use of SP in IPT [45]. Although a study carried out in 2005 reported a rate of therapeutic failure of 11.6% in Oyem [46], the very high frequency of *Pfdhfr* and *Pfdhps* gene mutations in Fougamou is an alarm signal regarding a possible decrease in the effectiveness of SP. Indeed, quadruple $I_{51}R_{59}N_{108}$-G_{437} and quintuple $I_{51}R_{59}N_{108}$-$G_{437}E_{540}$ mutants were found to be dramatically high. These mutants are considered as markers for SP prophylactic failure [47,48] and call for an investigation of the efficacy of IPT-SP.

5. Conclusion

It was important to investigate the level of drug resistance several years after the large-scale implementation of IPT-SP in pregnancy in Gabon. The data show a high prevalence of malaria in the rural area of Fougamou. *Plasmodium falciparum* infection induced a decrease in red blood cell and platelets levels. We found a high level of markers associated with SP resistance induced by the pressure of this drug association largely used in IPT. The high level of the quintuple mutant IRNGE/IRNAE is an alarming signal. Clinical trials to evaluate the efficacy of IPT-SP in pregnancy are very much needed.

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Competing interests

None declared.

Ethical approval

This study was approved by the Gabonese National Ethics Committee and registered under PROT 0020/2015/SG/CNE. It was performed in accordance with the principles of the committee. To ensure their voluntary participation, informed consent was obtained from all of the participants or their parents or guardians.

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