Prevalence of *Plasmodium falciparum* parasites resistant to sulfadoxine/pyrimethamine in pregnant women in Yaoundé, Cameroon: emergence of highly resistant *pfdhfr/pfdhps* alleles

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Objectives: To determine, 6 years after the adoption of intermittent preventive treatment of pregnant women with sulfadoxine/pyrimethamine (IPTp-SP) in Cameroon, (i) the polymorphism and prevalence of *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) gene mutations associated with sulfadoxine/pyrimethamine resistance and (ii) the consequences of sulfadoxine/pyrimethamine use in the selection of *pfdhfr/pfdhps* alleles.

Methods: *pfdhfr* and *pfdhps* genes from *P. falciparum* isolates collected in Yaoundé (Cameroon) from pregnant women with symptomatic malaria before taking IPTp-SP [SP− group (control) (*n*=51)] or afterwards [SP+ group (*n*=49)] were sequenced.

Results: The *pfdhfr* N51I, C59R, S108N triple mutant had a prevalence close to 100% (96/100) and no mutations at codons 50 and 164 were detected in either of the groups. The most frequent *pfdhps* mutation was A437G with a prevalence of 76.5% (39/51) in the SP− group, which was significantly higher in pregnant women who took sulfadoxine/pyrimethamine [95.9% (47/49)] (*P*=0.012). Our study confirmed the presence of the *pfdhps* K540E mutation in Cameroon, but it remained rare. The prevalence of *pfdhps* A581G and A613S mutations had increased [5.9% (3/51) and 11.8% (6/51) in the control group, respectively] since the last studies in 2005. Surprisingly, the new *pfdhps* I431V mutation was detected, at a prevalence of 9.8% (5/51), and was found to be associated with other *pfdhfr/pfdhps* alleles to form an octuple N51I, C59R, S108N/I431V, S436A, A437G, A581G, A613S mutant.

Conclusions: Significant changes were found in *pfdhps* polymorphism. In particular, we observed several parasites carrying eight mutations in *pfdhfr/pfdhps* genes, which are very susceptible to having a high level of resistance to sulfadoxine/pyrimethamine.

Introduction

Pregnancy-associated malaria (PAM) is a significant public-health problem in sub-Saharan Africa. Each year, 35 million pregnancies are exposed to malaria.[1] PAM is an important cause of maternal and neonatal morbidity, such as severe maternal anaemia, retardation of intrauterine growth, low birth-weight, premature delivery, intra-uterine death and stillbirth, and can lead to maternal or neonatal mortality.[2]

In the mid-1990s, sulfadoxine/pyrimethamine replaced weekly chloroquine prophylaxis as the drug-based strategy to prevent malaria during pregnancy because of the extension of chloroquine resistance. In areas of stable *Plasmodium falciparum* malaria transmission, the WHO recommends intermittent preventive treatment for malaria during pregnancy (IPTp), with at least two doses of sulfadoxine/pyrimethamine that should be taken after quickening (second and third trimesters).[3]

Sulfadoxine/pyrimethamine resistance is conferred by mutations in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthetase (*pfdhps*) genes, which encode enzymes targeting pyrimethamine and sulfadoxine, respectively.[4,5]

Accumulation of *pfdhfr* and *pfdhps* mutations leads to increasing levels of sulfadoxine/pyrimethamine resistance *in vivo*.[6,7] In combination with a *pfdhfr* triple mutant N51I, C59R, S108N allele, the *pfdhps* A437G mutation has been found to be associated with treatment failure in West and Central Africa.[8,9] A quintuple mutant genotype that combines the *pfdhfr* triple mutant with the *pfdhps* double A437G+K540E mutations, which are mainly found in East Africa, is a significant predictor of sulfadoxine/pyrimethamine treatment failure.[10,11]

Recently, Naidoo *et al.*[12] introduced the concept of 'super-resistant' genotypes, which further raises the threshold of drug tolerance in parasites. Two 'super-resistant' genotypes have been well documented. The first corresponds to the additional *pfdhps* A581G mutation in combination with the quintuple *pfdhfr/pfdhps* N51I, C59R, S108N/A437G, K540E mutant. Only found in East African foci,[12,13] the initial indications are that their effect on IPTp with sulfadoxine/pyrimethamine (IPTp-SP) efficacy is highly detrimental.[14] The second genotype, well known in East Asia and South America, has been also identified in East Africa[12] and corresponds to the additional *pfdhfr* I164L mutation to the *pfdhfr* N51I, C59R, S108N allele. This combination confers a very high level of resistance to pyrimethamine and sulfadoxine *in vitro* and *in vivo*,[15,16] especially as it is associated with the *pfdhps* A437G, K540E alleles. A third genotype that combines the *pfdhps* A613S/T mutation with the *pfdhfr/pfdhps* N51I, C59R, S108N/A437G, K540E genotype has also emerged in East Africa[12] and could have this 'super-resistant' phenotype status; however, this assumption is only based on an *in vitro* result.[17]

In Cameroon, IPTp-SP was adopted in 2004 with significant support from the Ministry of Health as sulfadoxine/pyrimethamine is free of charge for pregnant women attending antenatal clinics from week 16 of gestation. Regardless of prophylaxis, sulfadoxine/pyrimethamine has been used for the treatment of uncomplicated malaria for several years without being recommended as a first-line policy. To date, outside of studies conducted by Tahar and Basco[18] and McCollum *et al.*[19] on samples collected in and before 2005, no work has evaluated changes in the prevalence of whole molecular markers implicated in sulfadoxine/pyrimethamine resistance in Cameroon.

Methods

Study site and design

A prospective study was carried out between April 2010 and March 2011 in the Health Care Center of the CASS (Centre d'Animation Sociale et Sanitaire) in Yaoundé intra-muros (3°52′N, 11°31′E), Cameroon.

The CASS has a maternity centre that oversees nearly 3500 births each year. At this centre, IPTp is routinely administered to pregnant women as three tablets of sulfadoxine (500 mg) and pyrimethamine (25 mg) in a single dose during the consultation in the presence of a nurse, a second dose at least 4 weeks after the first dose, and possibly a third dose if the gestational age permits (i.e. <36 weeks of gestation).

In this study, symptoms suggestive of malaria (fever, shivering, headache, nausea or vomiting) were actively searched for between the first antenatal visit and delivery. If there were symptoms, finger-prick blood samples for thick smears were collected and assessed for the presence of *P. falciparum* malaria. At the same time, capillary blood samples were placed on Whatman 3MM filter paper (Whatman) for DNA conservation. Thick smears were Giemsa stained and then examined microscopically using a 100× oil-immersion objective.
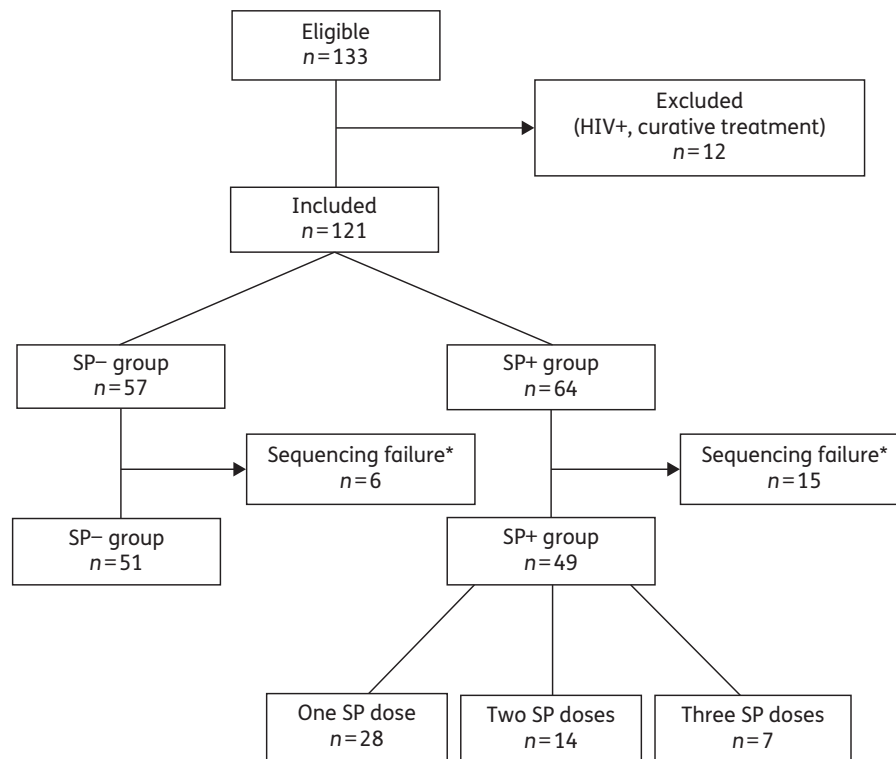

Figure 1. Flow chart of participants through the study. The number of sulfadoxine/pyrimethamine (SP) doses was considered at the time of the blood collection. *The sequencing failure was due to low parasite burden and poor conditions of conservation.

Pregnant women aged >18 years and who had a positive thick blood smear were enrolled as volunteers after they had signed an informed consent form (Figure 1). HIV-positive women and those who had received curative treatment for malaria since the beginning of their pregnancy were excluded.

Women were classified as being within the SP− group (control) if they had not received IPTp-SP since the beginning of their pregnancy. The SP+ group corresponded to women who had received at least a first course of IPTp-SP and had correctly followed prophylaxis as set out by CASS until malaria diagnosis. Following national guidelines, all participants with a positive thick blood smear received a treatment dose of quinine (24 mg/kg for 7 days) after sample collection.

Ethics

All procedures involving human subjects used in this study were approved by the Cameroonian National Ethical Committee (statements no. 230/CNE/SE/2010).

DNA extraction and sequencing

DNA was extracted from filter paper using the chelex-100 (Sigma Aldrich) boiling method.[20] Screening for mutations of *pfdhfr* and *pfdhps* genes was performed as previously described,[18,21] with minor modifications. The Veriti 96-Well Thermocycler (Applied Biosystems) was programmed as follows: denaturation at 94°C for 9 min for the first cycle, and for 60 s for the subsequent 30 cycles; annealing at 50°C (*pfdhfr*) or 52°C (*pfdhps*) for 90 s for the first cycle and 60 s for the subsequent 30 cycles, plus an extension at 72°C for 60 s for 31 cycles.

Sequencing of the *dhfr/dhps* fragments was done on both strands using nested primers with a mixture of 1 μL of amplified product, 2 μL of a Big Dye terminator V1.1 cycle sequencing kit (Life Technologies) and 0.2 μM nested primer, in a final volume of 10 μL, and the following program: 96°C for 60 s for initial denaturation, 96°C for 10 s, 50°C for 5 s and 60°C for 75 s, for 25 cycles. The product was sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical analyses

Data were analysed with R software (version 3.0.2). To compare the frequency of mutations in each group, we used the χ^2 test or Fisher's exact test, as appropriate. A comparison was considered statistically significant if the P value was ≤0.05.

Results

Prevalence of SNPs in pfdhfr and pfdhps genes

A total of 121 isolates were collected between 2010 and 2011, and 100 sequences were successfully obtained for the two genes (Figure 1). The results of the sequencing of *pfdhfr* and *pfdhps* are shown in Table 1 for each SNP. The *pfdhfr* mutations at codons 51, 59 and 108 in the SP− group were predominant, and their prevalence was statistically identical to the SP+ group. No mutations in the *pfdhfr* gene at codons 50 and 164 were detected whatever the group. The most frequent *pfdhps* mutations in the SP− group were S436A and A437G. The prevalence of the A437G mutation was significantly higher in the SP+ group [47/49 (96%)] than in the SP− group [39/51 (76.5%)] (P=0.012). Others, i.e. I431V, K540E, A581G and A613S mutations in the SP− group, had low prevalences and were not significantly distributed between the two groups.

Table 1. Prevalence of SNPs in *pfdhfr* and *pfdhps* genes in both SP− and SP+ groups

Group	No. of samples	*pfdhfr* SNP, n (%)							*pfdhps* SNP, n (%)				
		C50**R**	N51**I**	C59**R**	S108**N**	I164**L**	I431**V**	S436**A**	A437**G**	K540**E**	A581**G**	A613**S**
SP−	51	0 (0)	49 (96.1)	50 (98.0)	50 (98.0)	0 (0)	5 (9.8)	24 (47.1)	39 (76.5)	0 (0)	3 (5.9)	6 (11.8)
SP+	49	0 (0)	49 (100)	48 (98.0)	49 (100)	0 (0)	8 (16.3)	22 (44.9)	47 (95.9)	2 (4.1)	3 (6.1)	5 (10.2)
P		1.000[b]	0.495[b]	1.000[b]	1.000[b]	1.000[b]	0.501[a]	0.987[a]	**0.012**[a]	0.238[b]	1.000[b]	0.944[a]

Mutated alleles are in bold.
Significant P values are in bold.
[a]Calculated by the χ^2 test.
[b]Calculated by Fisher's exact test.

Table 2. Prevalence of combined *pfdhfr* (codons 50, 51, 59, 108, 164) and *pfdhps* (codons 431, 436, 437, 540, 581, 613) alleles in both SP− and SP+ groups

Allele			Group		
pfdhfr	*pfdhps*	number of mutations	SP−, n (%)	SP+, n (%)	P
CNCSI	IaAKAA	1	1 (2%)	0 (0%)	1.000[b]
CNrnI	ISgKAA	3	1 (2%)	0 (0%)	1.000[b]
CNrnI	IaAKAA	3	1 (2%)	0 (0%)	1.000[b]
CiCnI	ISgKAA	3	0 (0%)	1 (2%)	0.490[b]
CirnI	IaAKAA	4	8 (15.7%)	1 (2%)	**0.031**[b]
CirnI	ISgKAA	4	24 (47.1%)	23 (46.9%)	0.851[a]
CirnI	vaAKAA	5	1 (2%)	0 (0%)	1.000[b]
CirnI	IagKAA	5	7 (13.7%)	11 (22.4%)	0.382[a]
CirnI	ISgeAA	5	0 (0%)	2 (4.1%)	0.238[b]
CirnI	ISgKAs	5	1 (2%)	0 (0%)	1.000[b]
CirnI	vagKAA	6	2 (3.9%)	6 (12.2%)	0.156[b]
CirnI	IagKAs	6	1 (2%)	2 (4.1%)	0.614[b]
CirnI	IaAKgs	6	1 (2%)	0 (0%)	1.000[b]
CirnI	ISgKgs	6	1 (2%)	1 (2%)	1.000[b]
CirnI	vagKAs	7	1 (2%)	0 (0%)	1.000[b]
CirnI	vagKgs	8	1 (2%)	2 (4.1%)	0.614[b]
Total			51	49	

WT alleles are in upper case and mutated alleles are in lower case.
Significant P values are in bold.
[a]Calculated by the χ^2 test.
[b]Calculated by Fisher's exact test.

Prevalence of combined *pfdhfr* and *pfdhps* alleles

There was broad polymorphism of *pfdhfr*/*pfdhps* genes, i.e. in the 100 samples there were 16 different allele combinations (Table 2). Considering only the *pfdhfr* gene, the triple N51I, C59R, S108N (CirnI) mutant was the most prevalent combination, with 94.1% (48/51) and 98% (48/49) in the SP− and SP+ groups, respectively. The *pfdhps* alleles were more heterogeneous, but the two IaAKAA and ISgKAA combinations represented 60% (60/100) of the alleles in both groups. Regarding the *pfdhfr* and *pfdhps* genes, the quadruple mutant N51I, C59R, S108N/A437G (CirnI/ISgKAA) allele was the most common mutant. The only statistically significant difference observed between the two groups concerned the mutated N51I, C59R, S108N/S436A (CirnI/IaAKAA) allele, which was significantly less frequent in the SP+ group compared with the SP− group (P=0.031).

The *pfdhfr*/*pfdhps* quintuple N51I, C59R, S108N/A437G, K540E (CirnI/ISgeAA) mutant, associated with a high level of resistance to sulfadoxine/pyrimethamine, was only observed in the SP+ samples. No genotype classified as super-resistant, associating the quintuple mutant with the *pfdhfr* I164L, *pfdhps* A581G or *pfdhps* A613S/T mutated codon, was identified.

Discussion

This study of *pfdhfr*/*pfdhps* resistance alleles circulating in Yaoundé (Cameroon) at 6 years after IPTp-SP adoption for

pregnant women showed that the *pfdhfr* CirnI mutant allele was the most prevalent. Its prevalence rose strongly between 1993 and 2006 in sub-Saharan Africa,[22] and it was already high (100%) in Yaoundé in 2005.[23]

The *pfdhfr* I164L SNP is still absent in Cameroon, as previously reported.[18,19,23] This mutation is mainly found in East Africa, Madagascar and Comoros,[12] but was also reported in the Central African Republic.[24]

The *pfdhps* A437G SNP is very common across Africa and its prevalence in Cameroon was 69% in 2005.[23] This mutation is involved in resistance to sulfadoxine in endemic areas and A437G selection by sulfadoxine/pyrimethamine has been previously described during intermittent preventive treatment in infants.[25,26] The current study showed an increase in A437G prevalence and the mutation was significantly more frequent in women who had received sulfadoxine/pyrimethamine treatment.

The *pfdhps* S436A SNP occurred at the same frequency within the SP+ and SP− groups, which suggests that the mutation is not selected by sulfadoxine/pyrimethamine. Moreover, the S436A mutation may maintain a high level of susceptibility to sulfadoxine/pyrimethamine as the *pfdhps* IaAKAA combination (only mutated on S436A) was much less frequent in the SP+ group.

The *pfdhps* K540E SNP is always included in the quintuple mutant, which combines the *pfdhps* A437G SNP and the *pfdhfr* triple CirnI mutant. This mutation is common in East Africa, where its prevalence increased after 2004, reaching 100%.[12] By contrast, the *pfdhps* K540E has a low prevalence in West and Central Africa. The mutation prevalence reported in the countries neighbouring Cameroon was 6.25% in Gabon in 2007,[27] 0.8% in Congo in 2004,[27] 5.2% in the Central African Republic in 2004,[24] 11% in Sao Tome and the Principe islands in 2004[28] and 24% in Nigeria in 2004.[29] In Cameroon, the *pfdhps* K540E mutation was previously found between 2004 and 2006 at a low prevalence (0.3%) in samples collected in Mutengene.[30] In the present study, we observed only two mutant isolates (2%), in the SP+ group, which confirms the circulation of this SNP in the country, but still with a low prevalence.

The *pfdhps* A581G and A613S/T mutations have been detected at a low prevalence in West and East Africa, but a rapid emergence of these SNPs has been described in some areas of Kenya and Uganda.[31,32] Apart from Nigeria and Cameroon, these mutations have not been found in Central Africa.[33] In Yaoundé, the prevalence of *pfdhps* A581G and A613S mutations was low (<3%) between 1999 and 2005.[18,19,34] Subsequently, our work shows an increase in the prevalence of the two *pfdhps* A581G and A613S mutations, reaching 5.9% (3/51) and 11.8% (6/51), respectively, with a similar proportion in the SP+ group (Table 1). It is worth noting that six isolates (6%) in this study had both the A613S and A581G mutations. There are several arguments for considering this allele as super resistant: (i) Triglia *et al.*[17] have shown, *in vitro*, that the *pfdhps* SgKgA or fgKAs allele had a 5.3- or a 24-fold higher IC_{50} for sulfadoxine than the WT *pfdhps* SAKAA allele, respectively; and (ii) in the field, close alleles (*pfdhps* agKgt) with a threonine instead of a serine at codon 613, only described in India (West Bengal), have all shown very high levels of *in vitro* resistance to sulfadoxine, with an IC_{50} >3000 nM for seven of the eight isolates.[35]

In our study, the *pfdhps* I431V mutation was detected in both groups. The *pfdhps* I431V SNP has only been described so far by Sutherland *et al.*[33] in Nigeria between 2006 and 2007, in multiple

association. Here, this SNP was associated with the double *pfdhps* S436A/A437G mutation in 12 of the 13 isolates and with mutations at positions 581 and 613 in 3 of the 13 isolates. In these three isolates, these SNPs formed an octuple N51I, C59R, S108N/I431V, S436A, A437G, A581G, A613S (CirnI/vagKgs) mutant, which was never found together with the *pfdhps* K540E substitution. Nonetheless, the presence of octuple mutants reinforces the idea of the presence of genotypes with a high level of resistance to sulfadoxine/pyrimethamine in Central Africa for which the efficacy of IPTp-SP may be limited.

Finally, the present study provides an update on the prevalence of mutations conferring sulfadoxine/pyrimethamine resistance in Cameroon. Despite the small sample size and single-centre study, our findings indicate changes in SNP prevalence over time and the emergence of new mutants in Cameroon calls for continued efforts to prevent the spread of highly resistant parasites. In particular, the presence of the *pfdhps* K540E mutation, found so far in isolates that originated from eastern Africa,[22] raises questions about the significance of its associated high level of resistance to sulfadoxine/pyrimethamine.[27,36] Our results also suggest that *P. falciparum* with a genetic background in West, Central or Southwest Africa, corresponding to the agK (S436A, A437G, K540) allele,[27] could acquire a high level of resistance to sulfadoxine/pyrimethamine. The exact role of the new octuple *pfdhfr/pfdhps* N51I, C59R, S108N/I431V, S436A, A437G, A581G, A613S (CirnI/vagKgs) mutant allele in the resistance to sulfadoxine/pyrimethamine needs to be specified. Further work is required to evaluate the *in vitro* and *in vivo* susceptibility of this parasite genotype to a variety of antifolate drugs.

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