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DHFR and *DHPS* genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulfadoxine–pyrimethamine treatment efficacy

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***Objectives*: To assess the relationship between the presence of *DHFR* and *DHPS* mutations in *Plasmodium falciparum*, parasite *in vitro* resistance, and *in vivo* efficacy of sulfadoxine–pyrimethamine (SP) treatment.**

***Patients and methods*: Measurement of SP treatment efficacy in malaria-infected children in Gabon was combined with *in vitro* tests of susceptibility to pyrimethamine and cycloguanil, and molecular genotyping at several *DHFR* and *DHPS* loci of parasites isolated before treatment. *DHFR* was studied at codons 108, 51, and 59, whereas *DHPS* gene was typed at positions 436, 437, 540 and 581.**

***Results*: SP treatment was effective in 86% of children by day 28. Seventy-five percent of isolates were *in vitro* resistant to pyrimethamine and 65.5% to cycloguanil. No mutation was detected at codons 540 and 581 of the *DHPS* gene. Most isolates (71.8%) presented with the triple mutant *DHFR* genotype, whereas 64.3% combined at least three *DHFR* and one *DHPS* mutations. The increase in the number of *DHFR* mutations was associated with an increase in *in vitro* resistance to pyrimethamine and cycloguanil; three *DHFR* mutations conferred pyrimethamine and to a lesser extent cycloguanil resistance. Treatment failures only occurred with isolates presenting at least two *DHFR* mutations (S108N and C59R) and one *DHPS* mutation (S436A or A437G), but SP treatment of infections with such parasites gave treatment success in 82.0% of children.**

***Conclusions*: *DHFR* mutations that lead to high-level *in vitro* resistance to pyrimethamine plus 1–2 *DHPS* mutations are not sufficient to induce *in vivo* failure of SP treatment in young children from Gabon.**

Keywords: malaria, drug resistance, antifolates, molecular markers

Introduction

The spread of *Plasmodium falciparum* resistance to cheap drugs is a serious world-wide problem, considering the limited number of drugs available, the lack of vaccine, and the morbidity and mortality impact of malaria. The combinations of proguanil with atovaquone or chlorproguanil plus dapsone, may constitute effective alternative treatments in chloroquine-resistant areas.[1–3] Currently, the most common alternative drug to chloroquine remains the sulfadoxine–pyrimethamine (SP) combination. The extensive use of SP combination has led however to rapid emergence and spread of resistant parasites.[4] Pyrimethamine and proguanil (or cycloguanil, its active metabolite) inhibit the dihydrofolate reductase (*DHFR*) present in *Plasmodium* as a bifunctional enzyme with thymidylate synthase (*DHFR-TS*). The target of sulfadoxine is the dihydropteroate synthase (*DHPS*), also part of a bifunctional enzyme, the 7,8-dihydro-6-

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hydroxymethylpterin pyrophosphokinase-*DHPS* (*PPK-DHPS*). The molecular basis of *P. falciparum* resistance to antifolates consists of point mutations in genes encoding for both *DHFR* and *DHPS*. The understanding of resistance molecular mechanisms is of utmost importance for both designing new drugs and providing molecular markers to monitor drug activity and treatment efficacy.

In vitro resistance to pyrimethamine and cycloguanil has been attributed to the key mutation *DHFR* S108N; additive mutations in *DHFR* N51I and C59R conferring higher levels of resistance.[5–9] Mutations *DHPS* S436A, A437G and K540E were related to *in vitro* resistance to sulfadoxine.[10] Alternative mutations *DHFR* S108T plus A16V or additional mutations *DHFR* I164L, A613S/T, and *DHPS* A581G, are more rare in Africa, but are thought to increase the levels of resistance.[11,12] Relation to treatment efficacy is more controversial, but *DHFR* triple mutant at codons 108, 51 and 59 was mostly associated with SP treatment failure, regardless of *DHPS* genotype.[3,13]

As additional field data are needed for understanding antifolate drug resistance molecular mechanisms, we studied the major mutations present in Central Africa in relation to SP treatment outcome in Gabonese children and measured the *in vitro* susceptibility of isolates to pyrimethamine and cycloguanil.

Materials and methods

Data shown in this article are part of a treatment efficacy study that compared the sulfadoxine–pyrimethamine (SP) combination and amodiaquine treatments, involving 252 children in Gabon.[14] *In vivo* follow-up summarizes the results for the 128 subjects treated with SP, whereas *in vitro* tests, as well as *DHFR* and *DHPS* genotyping, involve the 252 subjects.

Study area and population

The study was conducted between January and June 2000 in Bakoumba, a village located in southeast Gabon in the Haut-Ogooué province. This village of 3000 inhabitants is surrounded by the equatorial forest, and belongs to a meso- to hyper-endemic area for *P. falciparum* malaria where parasite transmission is perennial with seasonal variations according to the rains.[15] During this study, the multiplicity of infection (defined as the mean number of parasite genotypes per man) was 4.0, according to *msp-1* and *msp-2* polymorphism (Aubouy *et al.*, unpublished results). Children aged 6 months to 10 years, presenting at the outpatient clinic with non-severe malaria attack were enrolled for a 28 day follow-up according to the WHO protocol.[16] The study was approved by the Centre International de Recherches Médicales de Franceville (CIRMF) ethical committee, and verbal informed consent was obtained from all parents or guardians.

Treatment and follow-up of children

At enrolment, a medical history was taken and a clinical examination was made. A finger-prick blood sample was obtained to measure parasite density, and children were given orally 25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine (Creat, Vernouillet, France) as a single dose on Day 0 under supervision. Treatment was completed with three doses of paracetamol per day (10 mg/kg per day) at Day 0 and Day 1. Children fulfilling the criteria of early or late clinical failure (see below) were given an alternative treatment. Parents were asked to bring their child back on Days 1, 2, 3, 7, 14 and 28, as well as any other day if the child was unwell. Temperature and parasite density were measured at each visit. Following finger-prick puncture, three drops of blood were collected on Whatman 3MM filter paper at Day 0 for *DHFR* and *DHPS* genotyping.

Both clinical and parasitological data were considered to analyse treatment efficacy, according to the revised WHO *in vivo* protocol for areas of intense transmission,[17] but the follow-up was extended to 28 days. This classification differs from the preceding one by the recognition of an additional group (inside the late treatment failures group) of late parasitological failures defined by the presence of parasitaemia on any day after Day 14, without meeting any of the criteria of early treatment failure or late clinical failure.

In vitro drug susceptibility tests

Distilled water and ethanol were, respectively, used to prepare stock solutions and dilutions of cycloguanil (Cy; Astra-Zeneca, Courbevoie, France) and pyrimethamine (Pyr; Sigma Aldrich, Saint Quentin Fallavier, France). The final concentrations ranged from 50 to 40 000 nM for Pyr, and 10 to 20 000 nM for Cy. Twenty microlitres of each concentration were distributed in triplicate, in 96-well tissue culture plates, and dried under a laminar flow hood before conservation at room temperature in dark and dry conditions. The venous blood samples collected at Day 0 were treated within 48 h after sampling. The erythrocytes were washed twice in RPMI 1640 medium, after isolation by centrifugation. The erythrocytes (haematocrit of 1.5% and initial parasitaemia of 0.1–1.0%) were resuspended in RPMI SP 241 medium (Gibco BRL, Paisley, UK) with a low concentration of folic acid and *p*-aminobenzoic acid, containing 10% human non-immune serum (Valbiotech, Paris, France), 25 mM HEPES, 25 mM $NaHCO_3$, and 0.2% [^{3}H]hypoxanthine (specific activity 5 Ci/mmol, Amersham). The *in vitro* drug sensitivity assay was assessed by the isotopic semi-microtest as described.[17]

The 50% inhibitory concentration (IC_{50}) values were calculated, defined as the drug concentration corresponding to 50% of the uptake of [^{3}H]hypoxanthine measured in the drug-free control wells. The calculation was based on linear regression analysis of the logarithm of concentrations plotted against the percentage growth inhibition. Isolates were defined as susceptible to pyrimethamine when IC_{50} values were <100 nM, and resistant when >2000 nM. For cycloguanil, thresholds for susceptibility and resistance were, respectively, defined as <50 nM and >500 nM. Data were expressed as median IC_{50} values and 25th–75th percentiles.

DNA extraction and DHFR, DHPS genotyping

Blood collected on Whatman 3MM filter paper before treatment was dried and conserved at room temperature until DNA chelex extraction, as described.[18] The molecular beacons method[19] was used to study the *DHFR* S108N mutation in all isolates with the following primers: 5′ TGTGGATAATGTAAATGATATGCC 3′ (upper) and 5′ CATTTATCCTATTGCTTAAAGGTT 3′ (lower). Point mutations *DHFR* N51I, C59R and *DHPS* S436A, A437G, K540E, A581G were analysed by sequencing in 97 samples from children having been treated with SP. Additionally, one out of five samples (27) corresponding to children treated with amodiaquine were tested for DHPS mutation. Briefly, 4 μL of chelex extracted DNA was amplified in a 50 μL reaction mixture containing 0.3 μM of each primer (*DHFR* 51–59 upper: 5′ CACATTTAGAGGTCTAGGAAATAAAGGA 3′; *DHFR* 51–59 lower: 5′ TCAATTTTTCATATTTTGATTCATTCAC 3′; *DHPS* upper: 5′ TTTGTTGAACCTAAACGTGTCT 3′; *DHPS* lower: 5′ TCTTCGCAAATCCTAATCCAA 3′), 200 μM of dNTPs, buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1 mM $MgCl_2$), and 2.5 U of *Thermus aquaticus* DNA polymerase (AmpliTaq Gold, Perkin Elmer, Courtaboeuf, France). Samples were incubated for 5 min at 94°C for denaturation before cycles (94°C 45 s, 59°C 45 s, 72°C 45 s). After 35 cycles, 5 min at 72°C allowed primer extension. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Courtaboeuf, France), before sequencing with an ABI PRISM Big Dye Terminator Cycle sequencing kit (Perkin Elmer

Cetus), following the manufacturer's instructions (P/N 4303149 revision C, 1998). Fluorescent PCR products were sequenced in an ABI PRISM 3100 Genetic Analyser.

Statistical analysis

The relationship between pyrimethamine and cycloguanil IC_{50} values was assessed by regression analysis. Kruskal–Wallis test and Mann–Whitney *U*-test were used to study the relation between IC_{50} values and genotypes. The relation between *in vivo* or *in vitro* phenotype, with molecular genotypes was studied by χ^2 tests and Spearman correlation tests.

Results

In vivo efficacy of SP

These data were previously reported in detail (Aubouy *et al.*, unpublished results). Briefly, 128 children less than 10 years were enrolled in the *in vivo* study. Mean age (±S.E.) was 3.9 (±0.2) years, and the group was composed of 43% females. Fourteen children were lost before Day 7 of follow-up and subsequently excluded from the analysis. SP treatment gave adequate clinical and parasitological responses (ACPR) in 98 subjects (86.0%), whereas two early therapeutic failures (ETF) occurred (1.8%), and the 14 late therapeutic failures (LTF) were composed of three late clinical failures (2.6%), and 11 late parasitological failures (9.6%). All treatment failures were due to recrudescent parasites, as shown by *msp-1* and *msp-2* analysis.[14] In several cases, appearance of new populations was also observed.

In vitro susceptibility to pyrimethamine and cycloguanil

The 252 isolates obtained from patients at Day 0 were tested for *in vitro* susceptibility to drugs. Sixty (23.8%) isolates gave interpretable results for pyrimethamine and 55 (21.8%) for cycloguanil. These low success rates contrast with the higher rates (63.5 and 62.0%) achieved with chloroquine and monodesethyl-amodiaquine using the same blood samples (data not shown). Seventy-three (29.0%) isolates did not grow in the presence, or absence, of any of the four drugs tested, whereas two (0.8%) additional isolates did not grow in the RPMI SP 241 medium, used for pyrimethamine and cycloguanil activity testing. The growth of 117 (46.4%) and 122 (48.4%) were not inhibited at maximal doses of pyrimethamine and cycloguanil, respectively, or furnished uninterpretable results (no dose/activity response or poor homogeneity of triplicate wells). Consequently, these isolates were excluded from the analysis. Forty-five (75.0%) and 36 (65.5%) isolates were *in vitro* resistant to pyrimethamine and cycloguanil, respectively with IC_{50} median values (25th–75th percentiles) of 7325 (419–12 665) nM and 1614 (63–6826) nM, respectively. Two isolates (3.3%) had an intermediate susceptibility to pyrimethamine with IC_{50} values of 296 and 306 nM, and six (10.9%) to cycloguanil with an IC_{50} median value of 83.5 (67–330) nM. Thirteen isolates (21.7% and 23.6%, respectively) were susceptible to pyrimethamine and cycloguanil with median IC_{50} of 19 (12–49) nM and 6 (4–25) nM, respectively. Pyrimethamine and cycloguanil IC_{50} values were highly correlated (regression analysis, $r = 0.86$, $P < 0.0001$), suggesting *in vitro* cross-resistance to both drugs. In addition, no isolate susceptible to one drug was resistant to the other, although one was susceptible to pyrimethamine (IC_{50} = 77 nM), and intermediate to cycloguanil (IC_{50} = 67 nM).

Table 1. Distribution (%) of *DHFR* and *DHPS* genotypes in 246 blood samples from Gabonese children presenting with a *P. falciparum* malaria attack, 2000

	n	Wild	Mutant	Mixed
DHFR 108	246	43 (17.5)	155 (63.0)	48 (19.5)
DHFR 51	90	12 (13.3)	75 (83.3)	3 (3.3)
DHFR 59	90	24 (26.7)	61 (67.8)	5 (5.6)
DHPS 436	110	72 (65.5)	31 (28.2)	7 (6.4)
DHPS 437	110	40 (36.4)	63 (57.3)	7 (6.4)
DHPS 540	55	55 (100)	0	0

DHFR and DHPS genotypes

The molecular beacons technique was used to study the mutation S108N in 252 isolates with an efficiency of 79.8%. Ninety-seven samples were from children having been treated with SP for the study by sequencing of N51I and C59R *DHFR* mutations, and of *DHPS* mutations. Twenty-seven isolates were sequenced in addition at *DHPS* 436 and 437 loci. Fifty-five isolates were sequenced for mutations at positions 540 and 581, and were all wild-type. As this result was consistent with previous data from the same area,[20] the remaining isolates were not analysed. The overall efficiency rate of sequencing was 92.4%. Consequently, results are available for 246 *DHFR* 108 genotypes, 90 *DHFR* 51 and 59 genotypes, 110 *DHPS* 436 and 437 genotypes, and 55 *DHPS* 540 and 581 genotypes.

As presented in Table 1, the frequency of samples composed of *DHFR* mutant isolates at codons 108, 51 and 59 were, respectively, 63.0%, 83.3% and 67.8%. In addition, the prevalence rates of mixed genotypes with regard to the *DHFR* loci, containing both wild and mutant parasites, were 19.5%, 3.3% and 5.6%, respectively. The higher proportion of mixed isolates at codon 108 is probably the consequence of the higher ability of molecular beacons than sequencing to detect minor genotypes. Among the 85 samples typed for the three *DHFR* loci, 74 contained unmixed (as regards to these loci) parasite populations, and most of these (68.9%) harboured all three *DHFR* mutations, whereas 16.2% harboured two mutations, and 5.4% a single mutation. Among the mixed genotypes, one resulted in two mutations at codons 108 (mixed) and codon 51 (unmixed). The 10 remaining mixed genotypes with regard to the *DHFR* loci resulted in three mutations, with mix at codon 108 (five cases), codon 59 (two cases), or at both codons 51 and 59 (three cases), increasing the rate of samples containing isolates presenting with three *DHFR* mutations to 71.8%. No other polymorphism was detected in the $DHFR_{39–59}$ sequenced region. At the *DHPS* gene, mutations K540E and A581G were not detected, whereas 28.2% and 57.3% of samples presented the mutations S436A and A437G, respectively (Table 1). Both wild and mutant parasites were detected at a frequency of 6.4% for each of these two *DHPS* point mutations. Among the 110 samples typed for both 436 and 437 *DHPS* loci, 103 contained unmixed parasite populations (as regards to these loci). In the wide majority (87.1%), unmixed isolates presented a single *DHPS* mutation, A437G being the most frequent (Table 1). Two unmixed isolates (2.0%) presented with both S436A and A437G mutations. Two mixed samples at the *DHPS* loci resulted in one mutation at either codon 436 or 437. In two more samples, mixed genotypes at either codon 436 or 437 resulted in two mutations. Both mixed genotypes at codons 436 and 437 were

Table 2. *DHFR* and *DHPS* genotypes in relation to *in vitro* susceptibility to pyrimethamine and cycloguanil, in *P. falciparum* isolates from Gabonese children

	DHFR genotypes			*DHPS* genotypes		*In vitro* susceptibility to pyrimethamine ($n = 49^a$)			*In vitro* susceptibility to cycloguanil ($n = 41^a$)		
n^b	108	51	59	436	437	S	I	R	S	I	R
3	S	–	–	–	–	1	1	0	1	1	1
1	S	–	–	S	**G**	1	0	0	0	0	0
1	**S**	N	C	S	A	1	0	0	0	1	0
2	**S**	N	C	**A**	A	2	0	0	2	0	0
2	**S**	N	C	S	**G**	2	0	0	2	0	0
2	**S**	**I**	C	A	A	2	0	0	2	0	0
1	**S**	**I**	C	S	**G**	0	0	0	1	0	0
24	N	–	–	–	–	0	1	17	0	1	13
2	**N**	–	–	S	**G**	0	0	1	0	0	1
3	**N**	–	–	**A**	A	0	0	3	0	0	2
1	**N**	–	–	A	**G**	0	0	1	0	0	1
4	**N**	**I**	C	S	**G**	1	0	1	0	1	2
6	**N**	**I**	**R**	S	A	0	0	1	0	0	1
9	**N**	**I**	**R**	S	**G**	0	0	7	1	0	4
7	**N**	**I**	**R**	**A**	A	0	0	5	0	1	1
1	**N**	**I**	**R**	**A**	**G**	0	0	1	0	0	1

DHFR and DHPS mutations are indicated in bold. *In vitro* test responses are classified as susceptible (S), intermediate (I) and resistant (R).
[a]Mixed genotypes are included as mutant genotypes.
[b]Eleven and 14 isolates did not furnished valid *DHFR* and *DHPS* data, respectively.

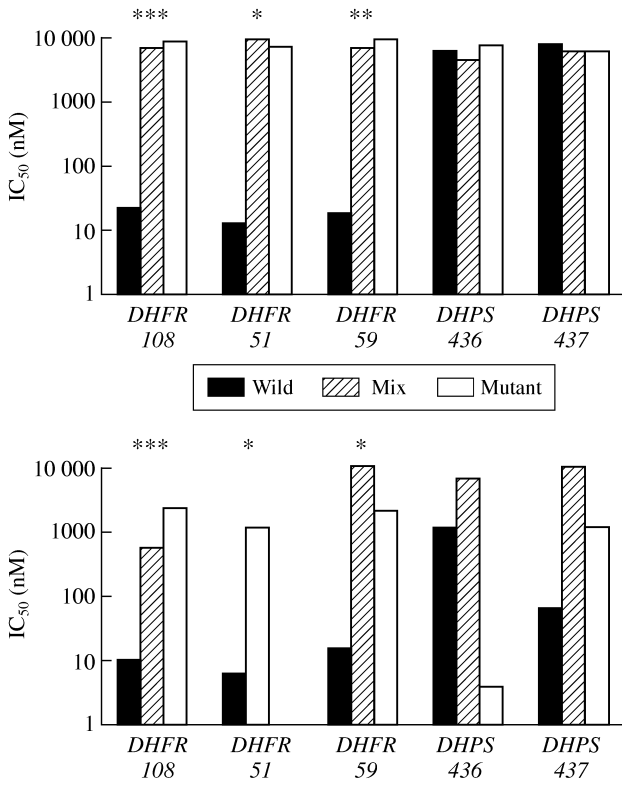

Figure 1. *In vitro* IC_{50} median values of pyrimethamine (Pyr, top) and cycloguanil (Cy, bottom) according to *DHFR* and *DHPS* genotypes. Black bars represent wild genotypes, grey bars mixed genotypes and white bars mutant genotypes. Pyr: $n = 59$ for codon 108, 23 for both codons 51 and 59 of *DHFR*, $n = 29$ for *DHPS* codons. Cy: $n = 54$ for codon 108, $n = 20$ for both codons 51 and 59 of *DHFR*, $n = 24$ for *DHPS* codons. Cy *in vitro* data was not available for mixed *DHFR* N51I isolates. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$ by Kruskal–Wallis test or Mann–Whitney *U*-test.

detected in five samples. No other polymorphism was detected in the $DHPS_{425–542}$ sequenced region. The combined analysis of *DHFR* and *DHPS* genotypes showed that most unmixed isolates (62.0%) with regard to the *DHFR*/*DHPS* loci harboured at least three *DHFR* and one *DHPS* mutations. The most common genotype (43.7%) was N, I, R at positions *DHFR* 108, 51 and 59, and S and G at positions *DHPS* 436 and 437. With the addition of mixed samples at the *DHFR*/*DHPS* loci, 64.3% of samples included genotypes with at least three *DHFR* and one *DHPS* mutation.

Relation between DHFR and DHPS genotypes, in vitro and in vivo data

The presence of *DHFR* point mutations was linked to *in vitro* resistance to pyrimethamine and cycloguanil. The phenotype of *in vitro* susceptibility to pyrimethamine or cycloguanil was associated with wild *DHFR* genotypes at positions 108, 51, and 59 (χ^2 test, Pyr: $P < 0.0001$ for codons 108 and 59, $P = 0.002$ for codon 51; Cy: $P = 0.0005$ for codon 108, $P = 0.03$ for codon 51 and $P = 0.05$ for codon 59) (Table 2), whereas no relationship was observed with *DHPS* genotypes. Figure 1 shows that low IC_{50} values were associated with wild *DHFR* genotypes, as opposed to mixed and mutant genotypes. Again, no difference was evident with *DHPS* 436 and 437 genotypes. Table 3 shows the different genotypes of all isolates with complete genotyping in relation to *in vivo* and *in vitro* results. As mixed and mutant genotypes showed similar *in vitro* phenotypes (Figure 1), mixed genotypes were considered as mutant for this analysis. Mutations *DHPS* K540E and A581G were not included in this analysis, as all sequenced isolates presented with the wild genotype. All samples exhibiting *in vitro* resistance to pyrimethamine and cycloguanil were associated with the presence of the *DHFR* S108N mutation (Table 2). *In vitro* data for pyrimethamine and cycloguanil were available for 39 and 29 samples, respectively, presenting with this mutation. Among these, 35 and 25 were resistant to the corresponding drug. Figure 2 represents the impact of the increase in the number of *DHFR* mutations on SP treatment outcome, and *in vitro* susceptibility to pyrimethamine and cycloguanil. Such increase was highly correlated to *in vitro* results (Spearman correlation test, $r = 0.84$, $P = 0.0002$ and $r = 0.73$, $P = 0.002$). When *DHFR* and *DHPS* number of mutations were both analysed, the increase was also highly significant (Spearman correlation test, $r = 0.83$, $P = 0.0003$ and $r = 0.76$, $P = 0.0016$ for Pyr and Cy, respectively).

No such correlations were observed with SP treatment outcome, although 10/11 of treatment failures appeared in the presence of parasites with at least three mutations (Table 3). Failure of SP treatment occurred for one child infected by an isolate in which only a wild genotype for both *DHFR* and *DHPS* mutations was detected. *In vitro* susceptibility of this isolate was not determined, but the child presented with low post-treatment plasma concentrations of sulfadoxine (85 μg/mL) and pyrimethamine (98 ng/mL), compared with the mean (±S.E.) concentrations exhibited by the other children (100.0 ± 4.2 μg/mL and 212.0 ± 14.4 ng/mL, respectively).[14] No

Table 3. *DHFR* and *DHPS* genotypes in relation to sulfadoxine–pyrimethamine (SP) combination treatment efficacy, and *in vitro* susceptibility to pyrimethamine and cycloguanil, in *P. falciparum* isolates from Gabonese children

	DHFR genotypes			*DHPS* genotypes		*In vivo* response to SP (*n*/total)			*In vitro* susceptibility to pyrimethamine (*n*/total)			*In vitro* susceptibility to cycloguanil (*n*/total)		
$n^{a,b}$ (total = 82)	108	51	59	436	437	ACPR	ETF	LTF	S	I	R	S	I	R
2	S	N	C	S	A	–	–	1/1	1/1	–	–	–	1/1	–
3	S	N	C	**A**	A	3/3	–	–	2/2	–	–	2/2	–	–
2	S	N	C	S	**G**	1/1	–	–	2/2	–	–	2/2	–	–
2	S	**I**	C	**A**	A	1/1	–	–	2/2	–	–	2/2	–	–
1	S	**I**	C	S	**G**	1/1	–	–	ND	ND	ND	1/1	–	–
1	**N**	N	C	**A**	A	1/1	–	–	ND	ND	ND	ND	ND	ND
9	**N**	**I**	C	S	**G**	7/7	–	–	1/2	–	1/2	–	1/3	2/3
2	**N**	N	**R**	S	**G**	–	–	1/1	ND	ND	ND	ND	ND	ND
6	**N**	**I**	**R**	S	A	4/4	–	–	–	–	1/1	–	1/1	–
16	**N**	**I**	**R**	**A**	A	11/13	–	2/13	–	–	5/5	–	1/2	1/2
32	**N**	**I**	**R**	S	**G**	26/31	1/31	4/31	–	–	8/8	1/6	–	5/6
6	**N**	**I**	**R**	**A**	**G**	4/6	–	2/6	ND	ND	ND	ND	ND	ND

DHFR and DHPS mutations are indicated in bold. *In vivo* test responses are classified as adequate clinical and parasitological response (ACPR), early (ETF) or late (LTF) treatment failure. *In vitro* test responses are classified in susceptible (S), intermediate (I) and resistant (R). ND, not determined.

[a]One isolate presented the genotype **N**, **I**, C, S, A, but treatment outcome and *in vitro* susceptibility were not determined in this case.

[b]Mixed genotypes are included as mutant genotypes.

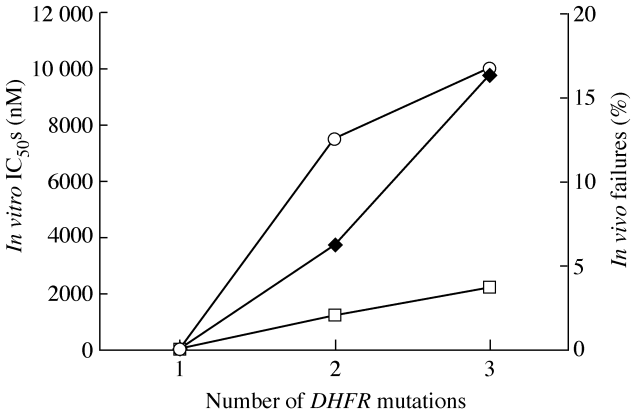

Figure 2. Relation between the number of *DHFR* mutations (whatever the *DHPS* genotype), *in vivo* failures to SP combination treatment in Gabonese children (open circles), and *in vitro* IC_{50} values for pyrimethamine (Pyr, filled diamonds) and cycloguanil (Cy, open squares). One *DHFR* mutation was any of S108N, N51I, or C59R. *In vivo* failures included ETF, LPF and LCF.

other *in vivo* failure arose in children infected with isolates presenting with less than two mutations at *DHFR* loci (in the presence or not of *DHPS* mutations). One late failure was due to an isolate presenting the genotype mutant at codons 59 and 108 of *DHFR*, and 437 of *DHPS*. The other failures occurred in children infected by isolates presenting three *DHFR* mutations concomitant to one or two *DHPS* mutations. Early treatment failure occurred in the presence of a single isolate (among 31) presenting mutations S108N, N51I and C59R of the *DHFR* gene and A437G of the *DHPS* gene. However, infection by six isolates presenting three *DHFR* mutations and two *DHPS* mutations did not lead to early treatment failure. In addition, infections by isolates presenting three *DHFR* and one or two *DHPS* mutations led most often (82.0%) to treatment success (Table 2). Unfortunately, our isolates did not include a genotype mutant at both 436 and 437 *DHPS* positions, and wild at *DHFR* locus, that would have allowed us to analyse the consequences of dual *DHPS* mutation on the response to SP treatment.

Discussion

Our study combined *in vitro* and *in vivo* tests, as well as molecular genotyping at *DHFR* and *DHPS* loci. SP treatment failed in 14.0% of children, whereas 75.0% of isolates were resistant *in vitro* to pyrimethamine and 65.5% to cycloguanil, and whereas 64.3% presented the triple mutant *DHFR* genotype combined with a *DHPS* 436 and/or 437 mutation. Molecular and *in vitro* data were strongly related, whereas both methods rarely reflected *in vivo* data.

SP combination was efficient in treating malaria attacks in young Gabonese children, although most isolates were *in vitro* resistant to pyrimethamine. A similar discrepancy between *in vivo* and *in vitro* results was observed in Cameroon,[21] where SP treatment failure rate was 12.1%, and 60.5% of isolates were *in vitro* resistant to pyrimethamine. Several factors may explain these disparities, such as the use of pyrimethamine only for carrying out the *in vitro* tests whereas both pyrimethamine and sulfadoxine are given simultaneously as treatment. Unfortunately, *in vitro* testing of sulfadoxine gives inconsistent results.[22] Secondly, treatment failures reflect the combination of several parameters, including parasite resistance to the drug, drug level achieved in the host, and action of the host immune response

(although this latter parameter is likely not to play a major role in this study, given the young age of our study population).

The triple *DHFR* mutant at codons 108, 51 and 59 was highly prevalent (71.8%) among our isolates. Such high prevalence rates above 50% were also reported in Vietnam,[10,12] Malaysia[23] and Brazil.[24] In East Africa, this rate reaches around 30%.[25] Our method did not allow detection of *DHFR* S108T mutant isolates. However, a previous study in a nearby area of Gabon detected this mutation in a single sample among 81, and as in our study, did not reveal any *DHPS* mutations at codons 540 and 581.[20] In the Bakoumba area, the common *DHPS* 436 and 437 mutations were frequent, particularly at codon 437. Double *DHPS* mutation was detected in two isolates only, resulting in the high prevalence (64.3%) of the genotype presenting with at least three *DHFR* mutations and one *DHPS* (436 or 437) mutation.

Our results confirm *DHFR* S108N is a key mutation for *in vitro* resistance to pyrimethamine and cycloguanil,[5,6,8,9] as all *in vitro* resistant isolates presented the mutant N genotype. Conversely, among samples presenting this mutation and for which *in vitro* data are available, one of 16 was susceptible to pyrimethamine and one of 12 was susceptible to cycloguanil. PCR-based methods do not detect minor clones in a mixed population, but although a wild-type clone may remain undetected, this is unlikely for *in vitro* susceptibility, as IC_{50} mainly reflects the susceptibility of the major clone(s) present in the blood sample. Mutations *DHFR* N51I and C59R are thought to increase *in vitro* resistance to both drugs.[7] However, in Papua New Guinean isolates, the presence of mutant genotypes at both codons 59 and 108 did not imply pyrimethamine or cycloguanil *in vitro* resistant phenotype.[8] Similarly, the three Gabonese isolates presenting concomitant mutations at S108N and N51I (in the absence of the C59R mutation) of the *DHFR* gene included isolates that were either *in vitro* susceptible or resistant to pyrimethamine, and either intermediate or resistant to cycloguanil. Although four samples presented with the three *DHFR* mutations at codons 108, 51 and 59 and no *DHPS* mutation, the *in vitro* activity of pyrimethamine and cycloguanil against these was determined in a single sample, which was highly resistant to pyrimethamine and presented an intermediate susceptibility to cycloguanil. The triple *DHFR* mutant at positions 108, 51 and 59 has been strongly associated with *in vitro* resistance to pyrimethamine,[13,26] whereas other point mutations, *DHFR* S108T plus A16V, and I164L are also thought to be of importance for *in vitro* resistance to both cycloguanil and pyrimethamine. Although these mutations have mostly been detected in South America and southeast Asia,[7,10,12,27,28] the latter mutation I164L in combination with two or three other *DHFR* point mutations including S108N, has been shown to be associated with high *in vitro* resistance levels to pyrimethamine, and to a lesser extent to cycloguanil.[28] *DHFR* V16 plus T108 mutation seems to confer more specifically resistance to cycloguanil.[27,28,30]

One striking result is shown in Table 2 where isolates presenting with two or three *DHFR* mutations, whatever *DHPS* genotype, were preferentially associated with both *in vitro* resistant parasites and treatment failure in children. At such a level of mutations, most isolates (100.0% and 66.7% for pyrimethamine and cycloguanil, respectively, in the case of at least three *DHFR* mutations) were *in vitro* highly resistant but were originating from treatment failures in a minority of children (16.7% in the case of at least three *DHFR* mutations). In Malawi, Kublin *et al.*[31] demonstrated a strong correlation between SP treatment failure and the *DHFR* triple mutant. Nevertheless, *DHPS* 437 and 540 mutations had a great importance in their study, as both double *DHPS* mutant and quintuple *DHFR* and *DHPS* mutants were highly correlated with SP treatment failure. Furthermore, in areas of such endemicity as southeast Gabon, parasites need to combine point mutations with other mechanisms to escape host regulation of infection, essentially immune mechanisms. The parasite may acquire more easily an efficient mechanism of resistance to drugs than to immune response, which may explain part of the *in vivo* and *in vitro* result disparities.

Interestingly, the infection by the single mutant isolate at codons 59 and 108 (and not 51) of *DHFR*, and 437 of *DHPS*, gave treatment failure, whereas all seven isolates presenting mutations at codons 51 and 108 (and not 59) of *DHFR*, and 437 of *DHPS*, gave treatment success. This result stresses the importance of the mutation C59R for SP treatment outcome. *DHPS* mutations are known to have an effect on sulfadoxine resistance, as proven by genetic crossbreeding between sensitive and resistant sulfadoxine parasites.[10,32] In humans, many studies reported the poor predictive value of *DHPS* mutations for SP treatment failure.[13,33] SP treatment gave treatment success in all three children infected with isolates that were triple *DHFR* mutant and double *DHPS* wild-type. However, the unbalanced numbers of genotypes does not allow us to draw conclusions on the impact of *DHPS* mutations.

We conclude that failure of SP treatment in this area of Gabon is related to the combination of at least two *DHFR* (C59R and S108N) and one *DHPS* mutations (S436A or A437G). However, such mutations were not sufficient to lead to SP treatment failure in most Gabonese children. *In vitro*, the three *DHFR* mutations conferred pyrimethamine and to a lesser extent, cycloguanil resistance. The increase in the number of *DHFR* and *DHPS* mutations was strongly correlated to resistance to pyrimethamine and cycloguanil. Further studies are needed to determine the precise incidence of the combination of *DHFR* and *DHPS* mutations on SP treatment outcome and *in vitro* resistance to antifolates. However, the poor success rate of *in vitro* tests to pyrimethamine and cycloguanil (as compared to schizontocidal drugs), as well as the high prevalence of site-specific *DHFR* and *DHPS* genotypes, make difficult the precise analysis of the role of each genotype on the *in vivo* and *in vitro* parasite susceptibilities. In many areas where chloroquine is not effective anymore, SP has been proposed, and used in several African countries, as first-line treatment for malaria attacks. Although SP appears to be effective in treating falciparum malaria attacks in children from Gabon, the high prevalence among the parasite populations of *in vitro* resistance to both pyrimethamine and cycloguanil, and of *DHFR*- and *DHPS*-encoding gene mutations is alarming. Changes in antimalarial policies in favour of the use of SP in this area of Gabon, are likely to increase SP drug pressure, and the clinical efficacy of SP may rapidly wane. New antimalarial combinations should be tested in order to have other effective treatments available.

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