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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Received 25 September 2002; returned 7 March 2003; revised 17 March 2003; accepted 22 April 2003

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-...
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 *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 1164L, A613ST, 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 mero- 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 of a village located in southeast Gabon in the Haut-Ogooué France 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 NaHCO₃, and 0.2% hypoxanthine (specific activity 5 Ci/mmol, Amersham). The *in vitro* drug sensitivity assay was assessed by the isotopic semi-microtest as described.

The 50% inhibitory concentration (IC₅₀) values were calculated, defined as the drug concentration corresponding to 50% of the uptake of [³H]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 IC₅₀ 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 IC₅₀ values and 25th–75th percentiles.

**DNA extraction and 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 deacons method19 was used to study the DHFR S108N mutation in all isolates with the following primers: 5′ TGTGGATAATGTAATGATATGCC 3′ (upper) and 5′ CATTATTCTCATTTGCTTAAAGGTT 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 DHFR 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′ CACATTTTAGGTCTCTGAGATAAAGAGGA 3′; DHFR 51R lower: 5′ TCAATTCTTCTTTTTTTTTTTTTTTTTTTCAC 3′; DHPS upper: 5′ TTGTGTTAACCTAAGCTGGTCT 3′; DHPS lower: 5′ GCCCTGGCAATCCTAATCCCAA 3′), 200 µM of dNTPs, buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1 mM MgCl₂), 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...
Cetux, 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 χ² 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. 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

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Wild</th>
<th>Mutant</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR</td>
<td>108</td>
<td>246</td>
<td>43 (17.5)</td>
<td>155 (63.0)</td>
</tr>
<tr>
<td>DHFR 51</td>
<td>90</td>
<td>12 (13.3)</td>
<td>75 (83.3)</td>
<td>3 (3.3)</td>
</tr>
<tr>
<td>DHFR 59</td>
<td>90</td>
<td>24 (26.7)</td>
<td>61 (67.8)</td>
<td>5 (5.6)</td>
</tr>
<tr>
<td>DHPS 436</td>
<td>110</td>
<td>72 (65.5)</td>
<td>31 (28.2)</td>
<td>7 (6.4)</td>
</tr>
<tr>
<td>DHPS 437</td>
<td>110</td>
<td>40 (36.4)</td>
<td>63 (57.3)</td>
<td>7 (6.4)</td>
</tr>
<tr>
<td>DHPS 540</td>
<td>55</td>
<td>55 (100)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DHFR and DHPS mutations are indicated in bold. In vitro test responses are classified as susceptible (S), intermediate (I) and resistant (R).

Mixed genotypes are included as mutant genotypes.

Eleven and 14 isolates did not furnish valid DHFR and DHPS data, respectively.

DHFR and DHPS mutations were considered as mutant for this analysis. Mutations in relation to wild susceptibility to pyrimethamine (Pyr) and cycloguanil (Cy) were associated with the presence of 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 vivo data was not available for mixed DHFR N51I isolates. ***P < 0.0001, **P < 0.001, *P < 0.05 by Kruskal–Wallis U-test.

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 vivo 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).
DHFR and DHPS genotypes of P. falciparum isolates

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

<table>
<thead>
<tr>
<th>DHFR genotypes</th>
<th>DHPS genotypes</th>
<th>In vivo response to SP (n/total)</th>
<th>In vitro susceptibility to pyrimethamine (n/total)</th>
<th>In vitro susceptibility to cycloguanil (n/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=82</td>
<td>108 51 59 436 437</td>
<td>DCP 436 437</td>
<td>S I R</td>
<td>S I R</td>
</tr>
<tr>
<td>2</td>
<td>S N C S A</td>
<td>– – 1/1</td>
<td>1/1 – – – 1/1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S N C A A</td>
<td>3/3 – – 2/2 – – 2/2 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S N C S G</td>
<td>1/1 – – ND ND ND 1/1 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S I C S G</td>
<td>1/1 – – ND ND ND 1/1 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N N C A A</td>
<td>1/1 – – ND ND ND 1/1 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N N R S G</td>
<td>1/1 – – ND ND ND 1/1 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>N I R S A</td>
<td>1/13 – – 1/13 – – 1/13 – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>N I R S S</td>
<td>26/31 1/31 4/31 – – 8/8 1/6 5/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>N I R A G</td>
<td>4/6 – – ND ND ND 1/1 – –</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

1One isolate presented the genotype N I C S A, but treatment outcome and in vitro susceptibility were not determined in this case.
2Mixed genotypes are included as mutant genotypes.

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, 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. 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.
The double 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 Malaysia22 and Brazil.23 In East Africa, this rate reaches around 30%.24 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 IC50 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 H164L are also thought to be of importance for in vitro resistance to both pyrimethamine 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 endemcity 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.30,32 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.

Acknowledgements

We are grateful to the children who participated in the study, as well as to their mothers and guardians. We thank J. Bourgeais, SODEPAL, for logistical support in Bakoumba, Zorica Jesic for help in molecular genotyping, as well as Bernard Mbomat and Jean Ruffin Makita for technical help. This work was supported by the French Ministry of Research (VIHPAL grant) and by the Fondation pour la Recherche Médicale. A. Aubouy was the recipient of a fellowship from the French Ministry of Research. S. Jafari was the recipient of a fellowship grant from WHO.
**DHFR and DHPS genotypes of P. falciparum isolates**

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