High prevalence of sulfadoxine/pyrimethamine-resistant alleles of *Plasmodium falciparum* isolates in pregnant women at the time of introduction of intermittent preventive treatment with sulfadoxine/pyrimethamine in Gabon

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**Objectives:** The frequency of *dhfr* and *dhps* point mutations was assessed in *Plasmodium falciparum* isolates from pregnant women in Libreville.

**Methods:** PCR–restriction fragment length polymorphism of polymorphic codons of the *dhfr* gene (51, 59 and 108) and the *dhps* gene (436, 437 and 540) was performed in matched peripheral and placental blood samples.

**Results:** The proportion of multiple mutations was high (98%), and was not different between women with and without a history of intermittent preventive treatment with sulfadoxine/pyrimethamine (IPTp/SP). The prevalence of triple *dhfr* mutation was 80%, and that of quadruple and quintuple mutations was 53% and 22%, respectively. The Glu540 mutation was present in two isolates. The concordance of resistant alleles in matched peripheral and placental isolates was >90% for both genes.

**Conclusions:** These findings underline the need for a regular assessment of the relationship between the presence of resistant isolates and *in vitro/in vivo* IPTp/SP efficacy, and evaluation of an alternative drug.

**Keywords:** malaria, drug resistance, pregnancy

**Introduction**

The WHO recommends the use of intermittent preventive treatment (IPTp) during pregnancy, along with insecticide-treated bednets (ITNs) and prompt case management to avoid deleterious effects of pregnancy-associated malaria (PAM). IPTp with sulfadoxine/pyrimethamine (IPTp/SP), which was implemented in Gabon in 2005, reduces the burden of the disease. Treatment failures of between 12% and 14% with sulfadoxine/pyrimethamine in children aged <10 years have been described in the country. ¹,² Resistance to this drug is conferred by mutations in the genes encoding dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes. Resistance occurs when at least two *dhfr* mutations and one *dhps* mutation are present. ¹,³ In using IPTp, the antimalarial is administered to women irrespective of their parasitaemia, thus the drug concentration confronted by the biomass of parasites is sometimes low, allowing selection of resistant parasites. Therefore, pregnant women could be considered as an important contributor to the spread of antimalarial drug resistance. Indeed, the placenta is the site of parasite sequestration, and appears to facilitate the multiplication of parasites that escape the immune response against surface-expressed antigens in women who have not had malaria during a previous pregnancy, thereby increasing the probability of resistant parasite selection. Furthermore, the introduction of sulfadoxine/pyrimethamine in the main lines of malaria treatment, including IPTp, is associated with an increase in the prevalence of *dhfr* and *dhps* point mutations and treatment failures. ³,⁴

With the aim of providing baseline data on molecular markers of sulfadoxine/pyrimethamine resistance at the time of IPTp/SP implementation, *dhfr* and *dhps* mutant alleles in maternal peripheral and placental *Plasmodium falciparum* isolates and their association with a history of sulfadoxine/pyrimethamine intake during the pregnancy were studied.
**Patients and methods**

**Patients and samples**

The study was carried out from September 2005 to January 2006 in Gabon where malaria transmission is perennial; prevalence of microscopic peripheral and placental *P. falciparum* infection was 12% and 23%, respectively.\(^5\) Peripheral and placental blood samples were collected from pregnant women delivering at the Centre Hospitalier de Libreville, who provided signed informed consent. History of IPTp/SP use during pregnancy was recorded. Blood samples were collected in EDTA tubes.

Diagnosis of *P. falciparum* infection was done by microscopic examination of thick blood smears according to the method of Lambaréné as detailed in Planche et al.\(^6\) Infected samples were selected for molecular analysis.

The study was reviewed and approved by the Ministry of Public Health of Gabon.

**Analysis of dhfr and dhps genes**

Parasite DNA was extracted from blood pellets according to the manufacturer’s recommendations (QIAamp DNA Mini Kit; Qiagen, Germany). PCR-restriction fragment length polymorphism of polymorphic codons of the *dhfr* gene (51, 59 and 108) and the *dhps* gene (436, 437 and 540) was performed as described by Duraisingh et al.\(^7\)

Mutations at codon positions 51, 59, 108, 436, 437 and 540 were detected by digesting the PCR products with the restriction enzymes Tsp509I, XmnI, AluI, MspA1I, AvaII and FokI (New England Biolabs, Beverly, MA, USA), respectively. PCR products and digested products were subjected to electrophoresis on 1.5% and 2.0% agarose gels, respectively, and visualized under UV light after ethidium bromide staining. Digested products were compared with those of reference isolates that were known to have wild-type and mutant alleles at the codon positions listed, kindly provided by Dr Menard from the Institut Pasteur in Madagascar.

**Statistical analysis**

All data were entered and cleaned using Epi-info version 3.3.2 (2005; CDC, Atlanta). Analysis was performed with the Statview 5.0 software (SAS Institute, Cary, NC, USA). All variables were compared using the \(\chi^2\) or Fisher’s exact test.

**Results**

Overall, 101 pairs of matched peripheral and placental samples were collected and 51 samples (22 pairs, 2 single peripheral blood samples and 5 single placental blood samples) were microscopically infected with *P. falciparum*. Parasitaemia levels ranged from 22 to 2302 parasites/\(\mu\)L with a median (IQR) of 35 (28–276) parasites/\(\mu\)L and from 22 to 2702 parasites/\(\mu\)L with a median (IQR) of 28 (22–40) parasites/\(\mu\)L for peripheral and placental blood, respectively.

*dhfr* and *dhps* typing was achieved in all the infected samples. The prevalence of mutations at codons 108, 59 and 51 was 96% (23/24), 88% (21/24) and 92% (22/24) in peripheral isolates and 93% (25/27), 74% (20/27) and 89% (24/27) in placental isolates, respectively. The Arg59 mutation was significantly more frequent in peripheral isolates (\(P=0.02\)). Multiple mutations in the *dhfr* gene were frequent: 86% of the isolates had at least two mutations and 80% had a triple mutation (Asn108/Arg59/Ile51) (Table 1). *dhps* gene analysis showed that 50% (12/24) of the peripheral isolates exhibited the Asn436 mutation, 63% (15/24) the Gly437 mutation and 4% (1/24) the Glu540 mutation. In placental isolates, the prevalences were 44% (12/27), 67% (18/27) and 4% (1/27) for the same codons, respectively (\(P<0.01\)). Globally, a single *dhps* mutation (codon 436 or 437) was observed in 37 (73%) samples, but no triple mutation was detected. Multiple mutations were present in most of the isolates (n=50/51; 98%). The proportion of triple (86%) and quadruple mutations (53%) was high, and quintuple mutants were present in 22% of the samples, all having mutations at positions 51, 59, 108 and 437 combined with mutations at either position 436 or 540 (Table 1). Among the 22 matched peripheral/placental samples, the Glu540 mutation was detected in one pair. Concordance of 100% was observed for the *dhfr* gene; 86% (19/22) of the pairs carried a triple mutation. *dhps* allele comparison in both compartments showed a concordance of 91% (n=20) for the 22 pairs (Table 2).

History of IPTp/SP use did not select for *dhfr* and *dhps* mutant alleles. The proportion of triple and quadruple mutations did not differ among women with or without IPTp/SP use in peripheral and placental isolates (\(P=0.7\)). Indeed, triple mutation was detected in 83% of peripheral isolates in both groups and, among placental isolates, 86% in the IPTp/SP group versus 85% in the group without previous IPTp/SP use (\(P=0.5\)). Likewise, the proportions of quadruple mutation were 50% versus 56% in peripheral isolates (\(P=0.5\)) and 71% versus 50% (\(P=0.6\)) in placental isolates from women with and without IPTp/SP use, respectively.

**Discussion**

This study, the first analysing the molecular markers of sulfadoxine/pyrimethamine resistance in *P. falciparum* isolates collected from pregnant women in Gabon, shows an increase in the prevalence of *dhfr* and *dhps* mutations compared with previous data.\(^1,8\) The proportions of single and triple *dhfr* mutations in peripheral and placental isolates were higher than those observed in pregnant women from Ghana.\(^9,10\) Twenty-two percent of isolates carried a *dhps* double mutation, and the rare *dhps* 540 mutation was found for the first time in Gabon.

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**Table 1. Distribution of multiple genotype mutations in all of the isolates**

<table>
<thead>
<tr>
<th>Mutated codons</th>
<th>Number of positive isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S108N C59R N51I S436A A437G K540G</td>
<td></td>
</tr>
<tr>
<td>+ + + + + +</td>
<td>0 (0)</td>
</tr>
<tr>
<td>+ + + + + -</td>
<td>9 (17)</td>
</tr>
<tr>
<td>+ + + - + +</td>
<td>2 (4)</td>
</tr>
<tr>
<td>+ + + + - -</td>
<td>8 (16)</td>
</tr>
<tr>
<td>+ + + - + -</td>
<td>19 (37)</td>
</tr>
<tr>
<td>+ + + - - -</td>
<td>3 (6)</td>
</tr>
<tr>
<td>+ - - - + +</td>
<td>2 (4)</td>
</tr>
<tr>
<td>+ - - - - +</td>
<td>1 (2)</td>
</tr>
<tr>
<td>+ + + + - -</td>
<td>3 (6)</td>
</tr>
<tr>
<td>+ - - - - -</td>
<td>2 (4)</td>
</tr>
</tbody>
</table>

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**Note:** dhfr and dhps mutations in isolates from Gabonese pregnant women.
Quadriple and quintuple mutations, associated with intense sulfadoxine/pyrimethamine resistance, were highly prevalent, with good concordance of alleles in matched peripheral and placental isolates as detected in Ghanaian women. Genotyping of maternal peripheral blood samples may be sufficient to give an estimate of the prevalence of mutant alleles in parasites infecting pregnant women. The high proportion of multiple mutations (98%) could be due to the drug pressure; in Senegal, the prevalence of dhfr gene mutant alleles increased a few months after sulfadoxine/pyrimethamine introduction despite a controlled use of the drug. At the time of the study, IPTp/SP was infrequent in Gabon, but sulfadoxine/pyrimethamine was recommended for home-based management of childhood fever. When taking into account its impact on gametocytogenesis and the perennial transmission of malaria, resistant parasites could have been easily spread widely in Gabon. However, in the present study, no link was found between sulfadoxine/pyrimethamine use during pregnancy and the high proportion of mutant alleles, most probably due to the small sample size; nevertheless, in Ghana, the prevalence of multiple mutations also did not differ among isolates from delivering women with and without IPTp. Other factors, such as the fitness of parasites and the use of trimethoprim/sulfamethoxazole for prophylaxis in HIV-positive patients (data not available for this study), could influence the emergence and the spread of resistant parasites.

It is reasonable to speculate that at the time of IPTp/SP implementation in Gabon, some circulating P. falciparum isolates were already at higher risk of being less susceptible to sulfadoxine/pyrimethamine, compromising in the near future the efficacy of this drug for the prevention of maternal and fetal outcomes during PAM. Indeed, it is established that the risk of treatment failure increases by 2- to 5-fold in the presence of triple to quintuple mutations. However, until now, no relationship was found between the frequency of multiple mutant alleles and the efficacy of this drug in reducing maternal and placental infection. The in vivo response to sulfadoxine/pyrimethamine varies according to entomological aspects, epidemiological settings and, in particular, immunity. Studies evaluating its impact on the reduction of the frequency of PAM outcomes have shown that this drug still has good efficacy when used for prevention. Nevertheless, the public health benefits of IPTp will probably decline due to sulfadoxine/pyrimethamine resistance. Intensifying the use of ITNs, research on vaccine candidate development and alternative drugs for IPTp become priorities in malaria endemic areas. Controlled trials comparing sulfadoxine/pyrimethamine versus mefloquine or amodiaquine have shown equivalent or even higher efficacy of those molecules, but their tolerability was lower. Considering the lack of a potential anti-malarial to replace sulfadoxine/pyrimethamine, IPTp/SP should be continued until a more potent drug is available.

The present data also emphasize the need for rigorous and regular assessment of resistant P. falciparum isolates in pregnant women through epidemiological surveys based on analysis of molecular markers and their association with in vitro/in vivo sulfadoxine/pyrimethamine efficacy.

**Acknowledgements**

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**Table 2. Comparison of maternal peripheral and placental dhps and dhfr genotypes**

<table>
<thead>
<tr>
<th>dhfr alleles</th>
<th>Peripheral isolates, n</th>
<th>Placental isolates, n</th>
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<tbody>
<tr>
<td>Wild-type m108</td>
<td>m 108</td>
<td>2</td>
</tr>
<tr>
<td>Wild-type m51</td>
<td>m 51</td>
<td>1</td>
</tr>
<tr>
<td>Wild-type m59</td>
<td>m 59</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Wild-type m51/108</td>
<td>m 51/108</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Wild-type m51/59/108</td>
<td>m 51/59/108</td>
<td>19 (86.4)</td>
</tr>
<tr>
<td>Wild-type dhps</td>
<td>m 436</td>
<td>1</td>
</tr>
<tr>
<td>Wild-type m437</td>
<td>m 437</td>
<td>5</td>
</tr>
<tr>
<td>Wild-type m540</td>
<td>m 540</td>
<td>9</td>
</tr>
<tr>
<td>Wild-type m437/540</td>
<td>m 437/540</td>
<td>1</td>
</tr>
<tr>
<td>Wild-type m436/437</td>
<td>m 436/437</td>
<td>4</td>
</tr>
</tbody>
</table>

m, mutated codon.
dhfr and dhps mutations in isolates from Gabonese pregnant women

Transparency declarations
None to declare.

References