MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDE, CAMEROON II. 
BASELINE FREQUENCY OF POINT MUTATIONS IN THE DIHYDROPTEROATE SYNTHASE GENE OF PLASMODIUM FALCIPARUM

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Abstract. Sulfadoxine-pyrimethamine is one of the alternative antimalarial drugs used to treat chloroquine-resistant Plasmodium falciparum malaria. The molecular target of sulfadoxine, an analog of p-aminobenzoic acid that inhibits the folate biosynthetic pathway, is dihydropteroate synthase (DHPS). The nucleotide sequence of the DHPS gene was determined in 32 clinical isolates obtained in Yaounde, Cameroon, and compared with the sequence of reference clones and Cambodian strains of P. falciparum. Of the 32 Cameroonian isolates, 31 displayed one of the sulfadoxine-sensitive mutation patterns: Ala-436/Ala-437/Ala-581/Ala-613 (n = 20), Ser-436/Gly-437/Ala-581/Ala-613 (n = 6), Ser-436/Ala-437/Ala-581/Ala-613 (n = 4), and Ala-436/Gly-437/Ala-581/Ala-613 (n = 1). One isolate had a sulfadoxine-resistant profile characterized by a double mutation: Phe-436/Ala-437/Ala-581/Ser-613. Although the majority of the isolates had a sulfadoxine-sensitive genetic profile, further studies are needed to correlate the mutation patterns and in vitro and in vivo sulfadoxine sensitivity.

An increasing number of malaria-infected patients are currently being treated with sulfadoxine-pyrimethamine in Cameroon because of the high rate of treatment failure with chloroquine.1 Increasing drug pressure may favor the selection of antifolate-resistant Plasmodium falciparum mutants.2 In fact, our recent studies provide evidence that pyrimethamine-resistant P. falciparum isolates are present in Yaounde.3,4 Pyrimethamine acts synergistically with sulfadoxine, a competitive inhibitor of dihydropteroate synthase (DHPS). This enzyme catalyzes the formation of dihydropteroate from p-aminobenzoic acid (PABA) and 6-hydroxymethyltetrahydropterine pyrophosphate in the folate biosynthetic pathway.5 Dihydropteroate is converted to dihydrofolate, which in turn is transformed into tetrahydrofolate by DHFR, the target enzyme of pyrimethamine.6 Sulfadoxine is a structural analog of p-aminobenzoic acid and, together with pyrimethamine, inhibits the folate biosynthetic pathway in a sequential manner. This results in a marked synergism between the two antifolate drugs.6

The molecular mechanism by which P. falciparum develops resistance to sulfadoxine has not been elucidated. Recent studies on the P. falciparum DHPS gene have suggested that as in the case of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, point mutations at discrete positions may be associated with sulfadoxine resistance.7,8 So far, published data on the DHPS gene have been based on culture-adapted, reference clones of P. falciparum.9,10 The present study was conducted to extend the observations from previous studies and to define the genetic profile of the DHPS gene in fresh clinical isolates of P. falciparum obtained from symptomatic Cameroonian patients in Yaoundé.

MATERIALS AND METHODS

Parasite DNA. Thirty-two clinical isolates of P. falciparum were obtained before treatment from symptomatic Cameroonian patients consulting the Nlongkak Catholic missionary dispensary in Yaoundé in 1994–1995. The study was approved by the Cameroonian National Ethics Committee and the Ministry of Health. Informed consent was obtained before blood extraction. Venous blood samples (5–10 ml of whole blood) were washed three times with RPMI 1640 medium by centrifugation (2,000 × g for 10 min). An aliquot of 1–1.5 ml of red blood cell pellet was used directly for extraction of parasite DNA. In addition, six internationally recognized reference clones from various geographic origins (3D7/unknown origin, D6/Sierra Leone, HB3/Honduras, W2/Indochina, 7G8/Brazil, FCR 3/The Gambia), three African reference clones from our laboratory (L-3/Cote d’Ivoire, L-16/Sierra Leone, FCM 29/Cameroon), and eight Cambodian (uncloned) strains were grown in continuous culture for extraction of DNA. Parasite DNA was extracted as described previously.4

Polymerase chain reaction. The polymerase chain reaction was used to amplify a 708-basepair fragment of the DHPS domain of the bifunctional gene carrying four point mutations identified in previous studies.7,8 The following oligonucleotide primer pairs were used: PDHPS1 5'-GATATTATTGTGAACCTAAACGTG-3' (sense) and PDHPS2 5'-CCACAAATATTATTTTTTATTTT-3' (anti-sense). The reaction mixture consisted of approximately 50–500 ng of DNA, 15 pmol of each primer, 200 μM dNTP, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris buffer (pH 8.4), and 1 unit of Taq DNA polymerase (Boehringer Mannheim, Darnhardt, Germany) in a final volume of 50 μl. The polymerase chain reaction was performed using the PTC-100 thermocycler (MJ Research, Watertown, MA) under the following conditions for a total of 30 cycles: denaturation at 94°C for 5 min for the first cycle and 1 minute in subsequent cycles, annealing at 50°C for 5 min for the first cycle and 1 min in subsequent cycles, and extension at 72°C for 10 min for the first cycle and 1 min in subsequent cycles. One-tenth of the amplified product was loaded on a 1% agarose gel, electrophoresed, and stained with ethidium bromide, to visualize the expected 708-basepair band by ultraviolet transillumination.

Cloning. The amplified product was purified by chlorormisonamyl alcohol (24:1 [v/v]) extraction and approximately 50–100 ng of the polymerase chain reaction product was ligated into 50 ng of pMOSBlue T-vector (Amersham International, PLC, Buckinghamshire, United Kingdom) fol-
Table 1

Amino acid residues of dihydropteroate synthase gene in reference strains of Plasmodium falciparum

<table>
<thead>
<tr>
<th>Parasite*</th>
<th>Amino acid residue</th>
</tr>
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<tbody>
<tr>
<td>3D7/unknown origin</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>D6/Sierra Leone</td>
<td>Ala Ala Ala Ala</td>
</tr>
<tr>
<td>HB3/Honduras</td>
<td>Ser Ala Ala Ala</td>
</tr>
<tr>
<td>W2/Indochina</td>
<td>Phe Gly Ala Ser</td>
</tr>
<tr>
<td>7G8/Brazil</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>FCR3/Gambia</td>
<td>Ser Ala Ala Ala</td>
</tr>
<tr>
<td>L-3/Cote d'Ivoire</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>L-16/Sierra Leone</td>
<td>Ser Ala Ala Ala</td>
</tr>
<tr>
<td>FCM29/Cameroon</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>K2/Cambodia</td>
<td>Ala Gly Ala Ala</td>
</tr>
<tr>
<td>K4/Cambodia</td>
<td>Ser Gly Gly Ala</td>
</tr>
<tr>
<td>K7/Cambodia</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>K8/Cambodia</td>
<td>Ser Gly Gly Ala</td>
</tr>
<tr>
<td>K9/Cambodia</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>K10/Cambodia</td>
<td>Ser Gly Ala Ala</td>
</tr>
<tr>
<td>K11/Cambodia</td>
<td>Ser Gly Gly Ala</td>
</tr>
<tr>
<td>L14/Cambodia</td>
<td>Ser Gly Ala Ala</td>
</tr>
</tbody>
</table>

* The 3D7 and FCR3 strains are sensitive in vitro to sulfadoxine; the W2 clone is sulfadoxine-resistant.14

Table 2

Point mutations in the dihydropteroate synthase gene of 32 Cameroonian clinical isolates of Plasmodium falciparum

<table>
<thead>
<tr>
<th>Sulfadoxine sensitivity</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive 1</td>
<td>Ala Ala Ala Ala</td>
</tr>
<tr>
<td>Sensitive 2</td>
<td>Ala Gly Ala Ala</td>
</tr>
<tr>
<td>Sensitive 3</td>
<td>Ala Gly Ala Ala</td>
</tr>
<tr>
<td>Resistant</td>
<td>Ala Ala Ala Ser</td>
</tr>
</tbody>
</table>

* The criteria for sulfadoxine sensitivity based on genetic profiles of the dihydropteroate synthase gene were established by Brooks and others3 and Triglia and Cowman.9

RESULTS

Most of the nucleotide variations were found in codons 436 and 437. Except for three Cambodian strains with the mutant Gly-581, all parasites had a wild-type codon Ala-581. The amino acid residue at position 613 was also wild-type codon Ala-613 in all parasites, except for W2 clone and one African isolate with the mutant Ser-613.

The DNA sequences of the four variable DHPS codons in reference clones and Cambodian strains are presented in Table 1. Four clones (3D7, 7G8, L3, and FCM29) presented the 3D7-type sulfadoxine-sensitive profile Ser-436/Gly-437/Ala-581/Ala-613. Its close FCR3-type sulfadoxine-sensitive variant, Ser-436/Ala-437/Ala-581/Ala-613, was observed in three clones (HB3, FCR3, and L16). The D6 clone displayed the sensitive-profile Ala-436/Ala-437/Ala-581/Ala613 (D6-type profile). The W2 clone had a unique sulfadoxine-resistant profile Phe-436/Gly-437/Ala-581/Ser-613. Highly variable patterns were observed among the Cambodian strains. Two strains had the 3D7-type (L14) or FCR3-type (K7) profile. Three strains (K2, K9, and K10) were characterized by the Tak 9/6-type sensitive profile Ala-436/Gly-437/Ala-581/Ala-613. Three other strains (K4, K8, and K11) displayed the K1-type sulfadoxine-resistant pattern with a single point mutation in codon 581 (Ser-436/Gly-437/Ala-581/Ala-613).

Of the 32 Cameroonian clinical isolates, 20 had the sulfadoxine-sensitive profile Ala-436/Ala-437/Ala-581/Ala-613 (Table 2). Three variant sulfadoxine-sensitive profiles were observed: Ser-436/Gly-437/Ala-581/Ala-613 (n = 6), Ser-436/Ala-437/Ala-581/Ala-613 (n = 4), and Ala-436/Gly-437/Ala-581/Ala-613 (n = 1). One isolate had a sulfadoxine-resistant profile with a double mutation Phe-436/Ala-437/Ala-581/Ser-613.

DISCUSSION

The P. falciparum DHPS-7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK) gene was recently cloned and sequenced by Brooks and others3 and Triglia and Cowman.4 The size of the coding region of the bifunctional gene is 2.1 kilobases, of which approximately 1 kilobase codes for the DHPS domain of the gene. Using reference clones of P. falciparum, these two studies have identified four codon sites within the coding region of the DHPS domain that may undergo mutation. To identify the codon sequences, Wang and others8 have described a polymerase chain reaction protocol using allele-specific oligonucleotides. Although a similar polymerase chain reaction strategy has been used successfully to detect point mutations in the P. falciparum DHFR-TS gene,12-15 in our experience, this technique requires a highly reliable thermal cycler and needs fine adjustment of oligonucleotide primer length, annealing temperature, and MgCl2 concentration to optimize amplification
conditions that allow discrimination of a single-base mismatch in the target alleles. We preferred using a more reliable DNA sequencing to establish the baseline genetic profile of clinical isolates in Cameroon.

In this study, the DNA sequences of four variable codons of the DHPS gene were determined in 32 Cameroonian isolates and compared with those of the reference clones of *P. falciparum*. The sequences of 3D7, W2, 7G8, and FCR3 determined in our study corresponded exactly with those that were published previously.² ³ The DHPS genetic profile of HB3/Honduras was not published in the previous studies. Our sequence of the D6 clone (Ala-436/Ala-437/Ala-581/Ala-613) does not agree with the published sequence (Phe-436/Ala-437/Ala-581/Ser-613). The underlying reason may involve cross-contamination of the stock parasites and/or independent mutations arising in different subclones grown in various laboratories.

Based on the criteria proposed by Brooks and others⁷ and Triglia and Cowman,⁷ 31 of 32 Cameroonian isolates obtained from symptomatic indigenous patients residing in Yaoundé were sensitive to sulfadoxine. Only one Cameroonian isolate displayed a sulfadoxine-resistant genetic profile similar to that of W2/Indochina clone. This isolate exhibited the pyrimethamine-sensitive phenotype Ser-108 in the DHFR-TS gene and was sensitive in vitro to both pyrimethamine (50% inhibitory concentration [IC₅₀] = 12 nM) and cycloguanil (IC₅₀ = 6.5 nM). Among the resistant parasites, the K1/Thai-land-type pattern was observed in three Cambodian strains. This profile has so far been noted exclusively in parasites originating from Southeast Asia. The W2-type was seen in only one African isolate.

Unlike the case of the enzyme DHFR, the crystal structure of DHPS of any organism has not been determined. It is therefore not known at present whether the four variable codons of the malarial DHPS gene modify or not the conformation of the active site. Sulfadoxine resistance is probably not associated with gene amplification.⁷ ⁸ Other possible mechanisms of sulfadoxine resistance that need to be explored include diminution of drug uptake and increased de novo synthesis of p-aminobenzoic acid.¹⁶ ¹⁸

In this study, the sulfadoxine sensitivity of the Cameroonian isolates was not determined in vitro or in vivo since the objective of our analysis was to establish the baseline sequence data. The absence of the sensitivity data does not allow us to draw a definite conclusion regarding the correlation between the genetic profile and drug sensitivity in the Cameroonian isolates. Since sulfadoxine-pyrimethamine is one of the most promising candidates to replace chloroquine in Africa, the importance of further studies on the genetic pattern of the DHPS gene cannot be underestimated.

**Addendum.** In a recent publication,¹⁹ the criteria of in vitro sulfadoxine resistance were modified. Sulfadoxine sensitivity is defined by Ser-136 (or Ala-436), Ala-437, Ala-581, and Ala-613 (FCR3 type pattern). Moderate sulfadoxine resistance is now defined by a 3D7 type pattern with a single Ala-to-Gly mutation in codon 437. Higher levels of sulfadoxine resistance are associated with double (Ser-436, Gly-437, Gly-581, Ala-613; K1 type) or triple (Phe-436, Gly-437, Ala-581, Ser-613; W2 type) DHPS mutations. According to the revised classification, seven Cameroonian isolates presented in Table 2 (Ser- or Ala-436, Gly-437, Ala-581, Ala-613) are moderately sulfadoxine resistant, while one Cameroonian isolate (Phe-436, Ala-437, Ala-581, Ser-613; W2 type) is highly sulfadoxine resistant.

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