Molecular Epidemiology of Malaria in Yaounde, Cameroon I. Analysis of Point Mutations in the Dihydrofolate Reductase–Thymidylate Synthase Gene of Plasmodium falciparum

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Abstract. Resistance to antifolate antimalarial drugs (cycloguani1, a biologically active metabolite of proguanil, and pyrimethamine) is associated with a Ser to Asn-108 point mutation in the dihydrofolate reductase–thymidylate synthase gene of Plasmodium falciparum. The frequency of this mutation was studied in 127 clinical isolates obtained in Yaounde, Cameroon using a simple and rapid molecular technique based on the polymerase chain reaction and restriction fragment length polymorphism. Of the 127 isolates, pure wild-type Ser-108 codon, pure mutant-type Asn-108 codon, and mixed codons were observed in 66, 55, and six parasites, respectively. The proportion of antifolate-resistant, pure mutant-type codon, with respect to pure wild-type or mixed alleles, was 43% (55 of 127). The results of the molecular assay were compared with those of semimicro isotopic in vitro assay in 34 isolates. All 17 pure Ser-108 isolates and two isolates with mixed alleles were sensitive to both pyrimethamine (50% inhibitory concentration [IC50] < 100 nM) and cycloguani1 (IC50 < 50 nM). Fourteen of 15 isolates with the mutant-type Asn-108 codon were resistant to pyrimethamine and cycloguani1. One isolate with Asn-108 showed a slightly elevated pyrimethamine IC50 (78 nM), which was within the sensitive range. This study provides further evidence that antifolate-resistant P. falciparum isolates are already present in Yaounde, Cameroon.

Malaria is a major public health problem and is associated with high mortality and morbidity rates in Cameroon. Transmission is seasonal in the northern, drier region and occurs throughout the year in the southern half of the country, which is densely covered with tropical rain forest. Yaounde, the capital city of Cameroon, is situated in the southern plateau at an average altitude of 800 meters above sea level. The first-line drug for the treatment of acute, uncomplicated Plasmodium falciparum malaria is chloroquine (or amodiaquine) for the indigenous population, and halofantrine (or sometimes mefloquine or mefloquine-sulfadoxine-pyrimethamine) is often indicated for the nonimmune nonindigenous population. Second- and third-line drugs for resistant cases are sulfadoxine-pyrimethamine and quinine, respectively.

In our previous in vitro study conducted in 1994–1995, it was shown that about 42% of the clinical isolates obtained from symptomatic Cameroonian patients in Yaounde are resistant to pyrimethamine and/or cycloguani1, a biologically active major metabolite of proguanil. Although recent clinical data on the efficacy of sulfadoxine-pyrimethamine are lacking in Cameroon, the in vitro results are alarming. If in vivo evaluation confirms the decreasing efficacy of the drug, the use of this drug combination as the second-line antimalarial drug to treat chloroquine-resistant P. falciparum malaria may be compromised. Since an increasing number of cases are expected to be treated with sulfadoxine-pyrimethamine in Cameroon, it is important to establish the baseline data on the sensitivity of P. falciparum isolates and monitor the evolution of drug sensitivity.

In addition to clinical evaluation, which is usually time-consuming to conduct, in vitro assay is generally accepted as a valuable tool to monitor the drug sensitivity of malaria parasites and assess the epidemiology of drug-resistant malaria. An alternative, novel approach is based on nucleic acid detection of genes that are associated with drug resistance. The bifunctional enzyme dihydrofolate reductase–thymidylate synthase (DHFR-TS) is one of the few known molecular targets of antimalarial agents classified as antifolates, otherwise known as DHFR inhibitors. Antifolate-resistant parasites include pyrimethamine and cycloguani1.

Previous studies based on reference clones as well as clinical isolates of P. falciparum from various geographic origins have provided strong evidence that in vitro antifolate resistance is associated with point mutations in the DHFR domain of the DHFR-TS gene. Among several point mutations that may occur in the gene, a Ser to Asn-108 point mutation is considered to be the key mutation that confers resistance to antifolate drugs. This mutation can be detected with ease by polymerase chain reaction and restriction fragment length polymorphism. Due to the unavailability of restriction enzymes, the other point mutations associated with high levels of pyrimethamine resistance (codons 51, 59, and 164 of the DHFR-TS gene) were not studied. We have conducted the present study with the aim of confirming our previous observations, which were based on in vitro assays, using a simple and rapid molecular technique that is suitable for field use in Africa and to establish the correlation between in vitro antifolate resistance and DHFR-TS gene mutations that occur in clinical isolates obtained in Yaounde, Cameroon.

Materials and Methods

Parasite DNA. The study was part of a clinical trial of pyronaridine conducted between 1994 and 1995 at the Nlongkak Catholic missionary dispensary in Yaounde. This study was approved by the Cameroonian National Ethics Committee and the Ministry of Health. One hundred twenty-seven clinical isolates of P. falciparum were obtained by venipuncture before treatment from symptomatic Cameroonian patients residing in Yaounde. Informed consent was obtained before blood extraction. Venous blood samples (5–10 ml of whole blood) were washed three times in 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 for extraction of parasite DNA.

Infected erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centri
gufuged at 2,000 × g for 10 min and the pellet was transferred to a 1.5-ml microfuge tube and suspended in 500 µl of NET buffer. The mixture was treated with 1% sarkosyl and RNase A (100 µg/ml) at 37°C for 1 hr and proteinase K (200 µg/ml) at 50°C for 2 hr. Parasite DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform-isooamyl alcohol (25:24:1 [v/v/v]), and chloroform-isoamyl alcohol (24:1 [v/v/v]) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried in a hood and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20°C until use.

**Polymerase chain reaction.** The entire DHFR domain of the DHFR-TS gene of *P. falciparum* was amplified by the polymerase chain reaction using the following oligonucleo
tide primer pairs: 5′-ATGATGGAAATGCAGTCGGA-
CGTTTTCGAT-3′ (sense) and 5′-TTCAATTAACACATT-
TATTATCTGTTT-3′ (antisense). These oligonucleo
tides were designed from the complete DNA sequence of the gene.14, 15 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 poly
erase 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 min 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 (5 µl) was loaded on a 1.2% agarose gel, electrophoresed, stained with ethidium bromide, and visu
calized under ultraviolet transillumination to ensure the presence of the 708-basepair (bp) DNA fragment.

**Restriction fragment length polymorphism.** The technique combining the polymerase chain reaction and enzymatic digestion with restriction endonucleases to detect point mutations in the *P. falciparum* DHFR-TS gene was first de
cribed by Eldin de Pecoulas and others.11 Approximately 1–2 µg of the amplified DHFR-TS gene fragment were incubated separately with *Alu* I (2 units; Gibco-BRL, Paisley, United Kingdom) and *Bsr* I (2 units; New England Biolabs, Beverly, MA) restriction endonucleases in a mixture containing 5 mM Tris buffer (1 mM MgCl2) and 1 mM Tris buffer (1 mM MgCl2, 15 mM KCl), respectively, in a final volume of 50 µl. The reaction mixtures were incubated for 3 hr at 37°C with *Alu* I and at 65°C with *Bsr* I. At the end of the incubation period, the restriction enzymes were either heat-inactivated or extracted by chloroform:isoamyl alcohol (24:1 [v/v/v]). Ten microliters of the restriction fragments were loaded on a 1.2% agarose gel, electrophoresed, stained with ethidium bromide, and visualized by ultraviolet transillumin
ation. The cleavage of the original 708-bp DNA fragment into two fragments (324 bp and 384 bp) by *Alu* I indicated the presence of the wild-type codon Ser-108. If the enzyme *Bsr* I cleaved the original DNA fragment into two fragments (324 bp and 384 bp), the presence of the mutant codon Asn-108 was deduced. If both endonucleases yielded two cleaved fragments, the presence of mixed codons was deduced. The wild-type Ser-108 residue in the DHFR-TS gene is associated with sensitivity to the DHFR inhibitors, pyrimethamine, and cycloguanil.8, 12 The mutant Asn-108, in contrast, is associated with resistance to both pyrimethamine and cycloguanil. The reliability of the polymerase chain reaction–re
striction fragment length polymorphism method was con
firmed by comparing the results with DNA sequencing in our previous study.8 The polymerase chain reaction–restrict

**In vitro drug sensitivity assay.** An in vitro assay was performed with 34 isolates that were randomly selected from adult patients with no previous antimalarial drug intake. Blood samples from patients whose urine test showed recent intake of antimalarial drugs were not used to assess drug sensitivity.16 Infected erythrocytes were washed three times in RPMI 1640 medium and suspended in the complete RPMI SP241 medium (with low folate and p-aminobenzoic acid concentrations; Gibco-BRL) consisting of 10% human se
rum (obtained from European blood donors without previous history of malaria), 25 mM HEPES, and 25 mM NaHCO3 at a hematocrit of 1.5% and an initial parasitemia of 0.2–1.0%. If the blood sample had a parasitemia > 1.0%, fresh uninfected, type A+ erythrocytes were added to adjust the parasitemia to 0.6%.

The isotope semi-microtest used in this study was de
scribed in our previous study.2 Seven hundred microliters of the suspension of infected erythrocytes were distributed in each well of the 24-well tissue culture plates. The parasites were incubated at 37°C in 5% CO2 for 18 hr. *3H-Hypoxan
thine* (specific activity 5 Ci/mmol, 1 µCi/well; Amersham, Buckinghamshire, United Kingdom) was added to assess parasite growth. After an additional 24 hr of incubation, the plates were frozen to terminate the in vitro drug sensitivity assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant®; Amersham International, Plc., Buck
inghamshire, United Kingdom) were added. The incorpora

tion of *3H-hypoxanthine* was quantitated using a liquid scintill
ation counter (Wallac 1410; Pharmacia, Uppsala, Swe
den).

The 50% inhibitory concentration (*IC*50) values, defined as the drug concentration corresponding to 50% of the up

take of *3H-hypoxanthine* measured in the drug-free control wells, were determined by linear regression analysis of log-
arithmetic of concentrations plotted against the logit of growth inhibition. The logit of p is defined as $y = \ln [p/(1 − p)]$. The logit transformation gives the method of logistic re

gression. The threshold *IC*50 values for in vitro resistance to pyrimethamine and cycloguanil were estimated to be > 100 nM and > 50 nM, respectively.19
Figure 1. Enzymatic digestion of amplification products using endonucleases Alu I (A) and Bsr I (B) in the polymerase chain reaction–restriction fragment length polymorphism assay and electrophoresis in a 1.2% agarose gel. Alu I cleaves the dihydrofolate reductase–thymidylate synthase gene fragment into two fragments in the presence of a wild-type Ser-108 codon associated with antifolate sensitivity (isolates 3, 5, and 6). Bsr I cleaves the DNA fragment if it carries a mutant-type Asn-108 codon associated with antifolate resistance (isolates 1, 2, 4, and 7). The 708-basepair bands represent undigested DNA fragment. L = 1-kilobase DNA ladder. Values on the left are in basepairs.

Table 1

Comparison of the amino acid residue in position 108 of the dihydrofolate reductase–thymidylate synthase gene of Plasmodium falciparum and in vitro antifolate drug sensitivity

<table>
<thead>
<tr>
<th>Amino acid residue (number of isolates)</th>
<th>Pyrimethamine IC_{50} (nM)*</th>
<th>Mean</th>
<th>Range</th>
<th>Cycloguanil IC_{50} (nM)*</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser (n = 17)</td>
<td>14.3 ± 3.5–53</td>
<td>7.9</td>
<td>2.5–28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn (n = 14)</td>
<td>78.0 ± 22.0</td>
<td>4.730</td>
<td>179–15,130</td>
<td>671</td>
<td>76–2,500</td>
<td></td>
</tr>
<tr>
<td>Ser plus Asn (n = 2)</td>
<td>19.1 ± 13–28</td>
<td>11.8</td>
<td>9.3–15</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Geometric mean 50% inhibitory concentration (IC_{50}). Threshold IC_{50} values for resistance to pyrimethamine and cycloguanil are >100 nM and >50 nM, respectively.
† One mutant isolate was sensitive to both pyrimethamine and cycloguanil.

RESULTS

A typical result of the polymerase chain reaction–restriction fragment length polymorphism is presented in Figure 1. Of the 127 isolates, 66 were carriers of pure wild-type Ser-108 codon, and 55 isolates displayed pure mutant-type Asn-108. Six isolates showed digested fragments with both Alu I and Bsr I enzymes, indicating mixed alleles. If the number of isolates with either pure wild-type allele or mixed alleles is pooled (n = 72), the proportion of pure, mutant-type associated with antifolate-resistance, would be 43% (55 of 127).

An in vitro drug assay was carried out with 34 clinical isolates to determine the correlation between the IC_{50} values for pyrimethamine and a point mutation in codon 108. The amino acid residue 108 of the DHFR-TS gene of these 34 isolates is summarized in Table 1. All 17 Ser-108 isolates were sensitive to both pyrimethamine and a point mutation in codon 108. The amino acid residue 108 of the DHFR-TS gene of these 34 isolates is summarized in Table 1. All 17 Ser-108 isolates were sensitive to both pyrimethamine (geometric mean IC_{50} = 14.3 nM) and cycloguanil (IC_{50} = 7.9 nM). Of the 15 isolates with Asn-108, 14 were resistant in vitro to both pyrimethamine (IC_{50} > 100 nM) and cycloguanil (IC_{50} > 50 nM). One isolate with Asn-108 had IC_{50} values that were within the sensitive range for both pyrimethamine (IC_{50} = 78 nM) and cycloguanil (IC_{50} = 22 nM). Two isolates with mixed alleles were sensitive to both antifolate drugs.

DISCUSSION

The results of the present study confirm the presence of pyrimethamine-resistant P. falciparum isolates among symptomatic Cameroonian patients in Yaounde and are in agreement with our in vitro results. Of the 127 clinical isolates studied, 43% presented pure mutant-type allele in codon 108. These parasites are expected to be resistant to pyrimethamine and cycloguanil, as suggested by elevated IC_{50} values in Asn-108–carrying isolates. The proportion of Asn-108 isolates in this study (55 of 127 or 43%) is comparable with that of our previous study in which 18 of 43 Cameroonian isolates (42%) were resistant in vitro to pyrimethamine and cycloguanil. Moreover, the results of the present study on isolates in Yaounde as well as those based on a collection of various African isolates of P. falciparum support the relevance of our in vitro threshold values for resistance to antifolate drugs, which were initially calculated on the basis of statistical analysis of the IC_{50} values. In our previous in vitro studies, almost all clones and isolates with a point mutation in codon 108 of the DHFR-TS gene had increased IC_{50} values for pyrimethamine (IC_{50} > 100 nM) and cycloguanil (IC_{50} > 50 nM). In contrast, parasites without any point mutation in the gene were sensitive in vitro to pyrimethamine (IC_{50} < 100 nM) and cycloguanil (IC_{50} < 50 nM). A similar recent study in Papua New Guinea, correlating the presence of point mutations in the DHFR-TS gene and in vitro pyrimethamine resistance is in agreement with our results.

Several studies have established the utility of molecular techniques in predicting the in vitro drug sensitivity phenotype of field isolates. These techniques can clearly distinguish resistant isolates from sensitive parasites when the genotype is pure at codon 108. However, interpre-
Sulfadoxine-pyrimethamine was shown to be fully effective against \textit{P. falciparum} malaria in Yaounde in the late 1980s.\textsuperscript{22} Although the first case of resistance to the drug combination in Cameroon was reported in 1987 in a non-immune traveler on chemoprophylaxis with sulfadoxine-pyrimethamine, who also failed to respond to the same drug combination given at the therapeutic dose,\textsuperscript{23} more recent clinical data on its effectiveness in Cameroon are not available. However, the presence of pyrimethamine-resistant isolates, demonstrated both by in vitro studies and genetic analysis, is consistent with the massive drug pressure exerted in Cameroon for almost a decade.

Although recent, confirmatory clinical data are not available, we surmise that based on our daily personal experience at a dispensary in Yaounde, sulfadoxine-pyrimethamine is generally effective in curing acute uncomplicated malaria. In neighboring countries such as Gabon and Nigeria, this drug combination has been an effective alternative antimalarial.\textsuperscript{24, 25} Thus, it seems that in contrast to the expected in vivo efficacy, our results suggest the presence of a considerable number of pyrimethamine-resistant isolates. The discrepancy between the apparent clinical efficacy of sulfadoxine-pyrimethamine and our results based on in vitro assay and polymerase chain reaction–restriction fragment length polymorphism can be attributed to several factors. First, even if an isolate has a pyrimethamine-resistant phenotype, sulfadoxine and pyrimethamine inhibit the parasites’ folate biosynthetic pathway synergistically.\textsuperscript{26} Indeed, our molecular studies seem to suggest that recent Cameroonian isolates are generally characterized by a sulfadoxine-sensitive genotype.\textsuperscript{27} Second, the relatively long elimination half-lives of the two drugs, together with immune response in African patients, may favor the complete elimination of the parasites, even in the presence of low-level drug-resistant parasite populations.\textsuperscript{1} Third, although there are insufficient data, our previous studies based on full-length DNA sequencing of the DHFR domain of the DHFR-TS gene have suggested that pyrimethamine-resistant African isolates tend to have a lower number of point mutations (usually 1–3) as compared with multidrug-resistant Cambodian isolates, which possessed three or four point mutations.\textsuperscript{5, 28} Our studies have also suggested that the increasing number of point mutations in the DHFR-TS gene is associated with a higher level of antifolate resistance.

The molecular technique described in this paper is relatively easy and rapid to perform, even in the field. When necessary precautions are taken to prevent cross-contamination of the DNA samples, several dozens of samples may be handled simultaneously. If a rapid DNA extraction method is used, the results can even be obtained within 12 hr, much faster than what the in vitro assay would take. Additional advantages include the use of less expensive equipment and nonisotopic reagents. Comparison of the technique described here and in vitro assay showed high correlation.\textsuperscript{12, 20} In previous studies, a similar molecular approach using allele-specific oligonucleotides in nested polymerase chain reactions has shown the usefulness of novel nucleic acid detection methods in defining the epidemiology of pyrimethamine-resistant \textit{P. falciparum} in the Amazon and in West Africa on the basis of amino acid residue 108 of the DHFR-TS gene.\textsuperscript{21, 29, 30} However, it is not yet known to what extent the high correlation between the in vitro drug assay and polymerase chain reaction-restriction fragment length polymorphism is relevant to clinical response. To address this problem, it would be necessary to conduct clinical studies in parallel with in vitro assay and molecular techniques, including molecular analysis of clinical isolates before treatment and after recrudescence due to drug resistance and cloning of field isolates, followed by separate genotypic analysis of individual clones. Further studies are under way in Yaounde to compare the in vivo response to sulfadoxine-pyrimethamine and the presence of point mutations in the DHFR-TS gene.

Acknowledgments: We express our gratitude to Sister Solange and her nursing and laboratory staff at the Nlongkak Catholic missionary dispensary for their precious aid in screening patients for this study. Financial support: This study was supported in part by Zeneca Pharma. Leonardo K. Basco received a grant from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

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