Molecular Epidemiology of Malaria in Yaoundé, Cameroon I. Analysis of Point Mutations in the Dihydrofolate Reductase-Thymidylate Synthetase Gene of Plasmodium falciparum

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Abstract. Resistance to antifolate antimalarial drugs (cycloguanil, a biologically active metabolite of proguanil, and pyrimethamine) is associated with a Ser- to Asn-108 point mutation in the dihydrofolate reductase-thymidylate synthetase gene of Plasmodium falciparum. The frequency of this mutation was studied in 127 clinical isolates obtained in Yaoundé, 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 cycloguanil (IC50 < 50 nM). Fourteen of 15 isolates with the mutant-type Asn-108 codon were resistant to pyrimethamine and cycloguanil. 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 Yaoundé, 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. Yaoundé, 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 Cameroonians in Yaoundé are resistant to pyrimethamine and/or cycloguanil, a biologically active major metabolite of proguanil. Although recent clinical data on the efficacy of sulfadoxine-pyrimethamine are lacking in Cameroon, 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 assays are 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 synthetase (DHFR-TS) is one of the few known molecular targets of antimalarial agents classified as antifolates, otherwise known as DHFR inhibitors. Antimalarial antifolates include pyrimethamine and cycloguanil.

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 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 Yaoundé, Cameroon.

Materials and Methods

Parasite DNA. The study was part of a clinical trial of pyronaridines conducted between 1994 and 1995 at the Nlongkak Catholic missioninary dispensary in Yaoundé. This study was approved by the Cameroon 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 Cameroonians residing in Yaoundé. 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 centrifuged 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% sarcosyl 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-isooamyl alcohol (24:1 [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 oligonucleotide primer pairs: 5'-ATGATGGAAACAGTCTGCGACGTCTTGCA-3' (sense) and 5'-TTCTATTTAACAACATT-TATTATTGCCATT-3' (antisense). These oligonucleotides were designed from the complete DNA sequence of the gene. 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 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 visualized under ultraviolet transillumination to ensure the presence of the 708-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 described by Eldin de Pecoulas and others. 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-isooamyl alcohol (24:1 [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 transillumination. 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. The mutant Asn-108, in contrast, is associated with resistance to both pyrimethamine and cycloguanil. The reliability of the polymerase chain reaction-restriction fragment length polymorphism method was confirmed by comparing the results with DNA sequencing in our previous study. The polymerase chain reaction-restriction fragment length polymorphism technique used in this study and developed by Eldin de Pecoulas and others was subsequently used by other investigators, validating its utility to describe antifolate resistance.

**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. 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 serum (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 isotopic semi-microtest used in this study was described in our previous study. 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. *H*-Hypoxanthine (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., Buckinghamshire, United Kingdom) were added. The incorporation of *H*-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden).

The 50% inhibitory concentration (IC₅₀) values, defined as the drug concentration corresponding to 50% of the uptake of *H*-hypoxanthine measured in the drug-free control wells, were determined by linear regression analysis of logarithm 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 regression. The threshold IC₅₀ values for in vitro resistance to pyrimethamine and cycloguanil were estimated to be > 100 nM and > 50 nM, respectively.
Results

A typical result of the polymerase chain reaction–restriction fragment length polymorphism assay and electrophoresis in a 1.2% agarose gel. 

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 IC₅₀ 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 IC₅₀ 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 IC₅₀ values for pyrimethamine (IC₅₀ > 100 nM) and cycloguanil (IC₅₀ > 50 nM). In contrast, parasites without any point mutation in the gene were sensitive in vitro to pyrimethamine (IC₅₀ < 100 nM) and cycloguanil (IC₅₀ < 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.
...tation of a mixed genotype at codon 108 is not clear since a mixed genotype may be associated with either the sensitive or resistant phenotype. The underlying problem may be related to the proportion of sensitive and resistant parasite populations in a given isolate. The polymerase chain reaction is sensitive enough to amplify both genotypes even if there is an unequal proportion of two parasite populations, thus revealing the presence of the mixed genotype. Conversely, results of the in vitro assay are expected to be dependent on the dominant parasite population, independent of antifolate drug sensitivity, that succeeds in adapting rapidly to in vitro growth conditions. This discrepancy between mixed genotype and drug sensitivity phenotype is one of the limitations of molecular approaches in describing the epidemiology of drug resistance.

Sulfadoxine-pyrimethamine was shown to be fully effective against *P. falciparum* malaria in Yaounde in the late 1980s. 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, 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. 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. Indeed, our molecular studies seem to suggest that recent Cameroonian isolates are generally characterized by a sulfadoxine-sensitive genotype. 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. 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. 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. 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 *P. falciparum* in the Amazon and in West Africa on the basis of amino acid residue 108 of the DHFR-TS gene. 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.

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