Abstract. Clinical observations have shown that pyrimethamine resistance develops rapidly in endemic countries where antifolate drugs are used massively for the treatment of *Plasmodium falciparum* infections. To analyze this phenomenon, the *in vitro* response of clinical isolates to pyrimethamine and the dihydrofolate reductase (*dhfr*) gene sequence were analyzed in 2000–2001 and compared with the results obtained since 1994 in Yaounde, Cameroon. Of 139 samples obtained in 2000–2001, 10 (7.2%) isolates were of the wild-type, 116 had pure mutant alleles (2 [1.4%] with a single mutation, 11 [7.9%] with double mutations, and 103 [74.1%] with triple mutations), and 13 (9.4%) had mixed alleles. With the exception of a single isolate with triple mutations (50% inhibitory concentration [IC50] = 84.3 nM), all isolates with pure wild-type *dhfr* alleles (IC50 < 100 nM) and those with pure mutant *dhfr* alleles (between 1 and 3 point mutations; IC50 ≥ 100 nM) were clearly distinguished by *in vitro* drug sensitivity assays. The results of the two methods are highly correlated, and both methodologic approaches indicate an increasing proportion of pyrimethamine-resistant isolates in Yaounde over the past eight years (42–45% in 1994–1995, 63–67% in 1997–1998, and 88–92% in 2000–2001). At present, clinical isolates carrying triple *dhfr* mutations predominate in Yaounde. This situation calls for a regular surveillance of the efficacy of antifolate drugs by all available means, including clinical evaluation, *in vitro* drug sensitivity assays, molecular markers, and pharmacologic studies.

INTRODUCTION

Clinical studies conducted in Asia and South America in the 1950s and 1960s had shown that *Plasmodium falciparum* and *P. vivax* rapidly acquire resistance to pyrimethamine administered as monotherapy.1,2 The use of the synergistic sulfadoxine-pyrimethamine combination initially overcame moderate pyrimethamine resistance and improved the cure rate in the late 1960s and 1970s, but within a few years, antifolate-resistant malaria spread to the extent that this antifolate drug combination is no longer recommended for the treatment of *P. falciparum* malaria in Southeast Asia and for the treatment of *P. vivax* in endemic areas.2,3 In the African continent, drug-resistant *P. falciparum* has emerged and spread several decades after South America and Asia. At present, because of the high prevalence of chloroquine-resistant *P. falciparum*, some east African countries have adopted the use of sulfadoxine-pyrimethamine for the first-line treatment to combat chloroquine-resistant malaria. In other African countries, antifolate drugs are usually reserved for the second-line treatment of chloroquine-resistant infections. As a result of the increasing use of sulfadoxine-pyrimethamine, a decreasing clinical efficacy of antifolates has been recently reported from east Africa.4,5

The direct relationship between the use of antifolate drugs and their decreasing efficacy a few years after their massive distribution has been largely documented by clinical observations. An alternative approach to analyze this phenomenon of an ever increasing level of antifolate resistance includes molecular markers for drug resistance and *in vitro* drug sensitivity assay. *In vitro* pyrimethamine resistance is strongly associated with a single Ser108Asn substitution in dihydrofolate reductase (DHFR), and additional substitutions (Asn51Ile, Cys59Arg, and Ile64Leu) further increase the level of resistance.6–8 These amino acid substitutions occurring at key residues surrounding the active site of the enzyme lead to a decreased affinity between the mutant enzyme and pyrimethamine, therefore to a decreased efficacy of the drug to inhibit parasite enzyme activity. In the present study, we analyzed the *dhfr* sequence and *in vitro* response to pyrimethamine of clinical isolates obtained in 2000–2001 and compared the results with those of the preceding years to analyze the dynamics of the spread of pyrimethamine resistance in Yaounde, Cameroon.

MATERIALS AND METHODS

Patients. Venous blood samples (5–10 mL) were obtained from symptomatic older children (≥12 years old) and adults seeking consultation at the Nlongkak Catholic Missionary Dispensary in Yaounde, Cameroon after informed consent was obtained if the following criteria were met: the presence of *P. falciparum* at a minimal parasitemia of 0.1% and negative Saker-Solomons urine test for 4-aminoquinolines. Patients with severe anemia (hematocrit < 15%), pregnant women, and those presenting signs and symptoms of severe and complicated malaria were excluded. All enrolled patients were treated with oral amodiaquine, the first-line drug for uncomplicated falciparum malaria in Cameroon. This study was reviewed and approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

*In vitro* isotopic drug assay. Blood samples were washed three times by centrifugation with p-aminobenzoic acid (PABA)- and folic acid–free RPMI 1640 culture medium (Sigma, St. Louis, MO) within less than two hours after venipuncture. Washed erythrocytes were resuspended in complete PABA- and folic acid–free RPMI 1640 medium containing buffers (25 mM HEPES and 25 mM NaHCO3) and 10% human serum at a hematocrit of 1.5%. The initial parasitemia was adjusted to 0.6% by the addition of uninfected erythrocytes if the parasitemia was ≥1.0%.

Pyrimethamine was obtained from Sigma. A stock solution and four-fold dilutions were prepared in absolute ethanol, distributed in duplicate in 96-well culture plates, and dried. The final concentrations ranged from 0.0488 nM to 51,200...

MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON.

XVI. LONGITUDINAL SURVEILLANCE OF *IN VITRO* PYRIMETHAMINE RESISTANCE IN *PLASMODIUM FALCIPARUM*

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nM. In vitro isotopic drug assays were performed as previously described. The 50% inhibitory concentration (IC\textsubscript{50}), defined as the drug concentration at which 50% of the incorporation of tritium-labeled hypoxanthine is inhibited, compared with that of drug-free control wells, was calculated by plotting parasite growth against drug concentration and tracing the best-fitting sigmoid curve with Prism\textsuperscript{TM} software (GraphPad Software, Inc., San Diego, CA).

**Sequencing of DNA.** Parasite DNA was extracted from a red blood cell pellet (1–2 mL) as previously described. The entire DHFR domain (708 basepairs) of the \(dhfr\)-thymidylate synthase \((dhfr-ts)\) gene was initially amplified by a polymerase chain reaction in a mixture consisting of genomic DNA (approximately 50–100 ng), 15 picomole of forward primer ST1L, 5'-ATGATGGAACCAATCTGCAGGACTTTTCTGAT-3' and reverse primer ST2L, 5'-TTCTTTAACAATTTTATTTTCTC-3', buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM MgCl\textsubscript{2}, 200 \(\mu\)M of deoxynucleoside triphosphates (mixture of dGTP, dATP, dTTP, and dCTP), and one unit of Taq DNA polymerase (Roche Diagnostics, Meylan, France) in a total volume of 50 \(\mu\)L. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for two minutes in the first cycle and 30 seconds in subsequent cycles, 50°C for one minute in the first cycle and 30 seconds in subsequent cycles, and 72°C for one minute in all cycles, for a total of 30 cycles. The same program was used to perform the secondary, semi-nested polymerase chain reaction with primers ST1L and DHFR-595R (5'-CTGGAAAAATACATCACATTCATATG-3') in a 50-\(\mu\)L reaction mixture. The final amplification product was purified using the High Pure PCR Purification kit (Roche Diagnostics) and subjected to electrophoresis in an agarose gel to estimate the concentration of the purified amplification product by comparing the intensity of its band with that of molecular weight markers with known quantity. The 595-basepair product was marked with fluorescent nucleotides in the following mixture: 200 ng of amplified product, 4 \(\mu\)L of Terminator Ready Reaction Mix (Perkin Elmer Corp., Les Ulis, France), 80 mM Tris-HCl, pH 9.0, 2 mM MgCl\textsubscript{2}, 3.2 picomole of primer 5'-ATGATGGAACCAATCTGCAGGACTTTTCTGAT-3', in a final volume of 20 \(\mu\)L. The PTC-100 thermal cycler was programmed as follows: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for four minutes for 25 cycles. Residual dye terminators were removed by the ethanol precipitation method. The ABI Prism automated DNA sequencer (Perkin Elmer Corp.) was used to sequence the extension product.

**Data analysis.** Electropherogram data were analyzed by using the Editview sequence analysis software (Perkin Elmer Corp.). An isolate was considered to be the wild-type if the following amino acid residues were present: Ala-16, Asn-51, Cys-59, Ser-108, and Ile-164. An isolate was considered to be mutant if at least one of these codons was mutated: Ala- to Val-16, Asn- to Ile-51, Cys- to Arg-59, Ser- to either Asn-108 or Thr-108, and Ile- to Leu-164. As in a previous study, the *in vitro* threshold for pyrimethamine resistance was arbitrarily fixed at \(\cong 100\) nM.\textsuperscript{9}

The proportions of pyrimethamine resistance, based on either an IC\textsubscript{50} \(\cong 100\) nM or the presence of Asn-108 mutant allele, at different time periods were compared by the chi-square test. Isolates with mixed \(dhfr\) alleles were not included in the statistical analysis because their *in vitro* response is unpredictable (IC\textsubscript{50} range in the present study = 0.011–5.750 nM). The significance level was fixed at \(P < 0.05\).

**RESULTS**

Of 139 isolates collected during 2000–2001, 17 (12%) were pyrimethamine-sensitive (IC\textsubscript{50} < 100 nM), with a geometric mean IC\textsubscript{50} of 0.063 nM (range = 0.011–84.3 nM), and 122 (88%) were resistant to pyrimethamine *in vitro* (IC\textsubscript{50} \(\cong 100\) nM), with a geometric mean IC\textsubscript{50} of 1.510 nM (range = 136–17,100 nM). One isolate (22/00), which was considered to be pyrimethamine sensitive (IC\textsubscript{50} = 84.3 nM) on the basis of the arbitrary cut-off point of 100 nM, displayed a high IC\textsubscript{50} value compared with the other 16 pyrimethamine-sensitive isolates (geometric mean = 0.040 nM, range = 0.011–0.307 nM).

The complete \(dhfr\) sequence was determined for all isolates (n = 139). There were 10 (7.2%) isolates with wild-type \(dhfr\) alleles, 116 with pure mutant alleles (2 \(\%\) with a single mutation, 11 \(\%\) with double mutations, and 103 \(74.1\%\) with triple mutations), and 13 \(9.4\%\) with mixed alleles. Among the isolates characterized to be pyrimethamine sensitive (IC\textsubscript{50} < 100 nM), 10 were wild-type \(dhfr\) and 6 carried mixed \(dhfr\) alleles. Isolate 22/00 (IC\textsubscript{50} = 84.3 nM) had triple mutations, which indicate that this isolate should have been classified as pyrimethamine resistant and that the threshold level for pyrimethamine resistance is slightly lower than 100 nM. Of the isolates classified as pyrimethamine resistant on the basis of an IC\textsubscript{50} \(\cong 100\) nM, all had at least one mutation (Asn-108, n = 115) or had mixed alleles (n = 7). Two isolates with a single Asn-108 mutation displayed IC\textsubscript{50}s of 570 nM and 551 nM. All 11 isolates with double mutations had Arg-59 and Asn-108 mutant allelic combinations (none with the other possible combination: Ile-51 and Asn-108 mutant alleles). The geometric mean IC\textsubscript{50} for pyrimethamine were 559 nM for isolates with double mutations and 1,720 nM for isolates with triple mutations (Ile-51, Arg-59, and Asn-108; n = 103, including isolate 22/00) (Figure 1). None of the isolates displayed the other mutant alleles reported in the literature among naturally occurring *P. falciparum* isolates: Val-16, Arg-50, Thr-108, and Leu-164.

The evolution of *in vitro* pyrimethamine resistance over an eight-year period in Yaounde is shown in Figure 2. There is a clear tendency towards an increasing proportion of pyrimethamine-resistant isolates in Yaounde, as determined by the *in vitro* drug sensitivity assay (\(P < 0.05\)). Likewise, sequence analysis of the \(dhfr\) gene of these isolates showed a similar trend towards an increasing proportion of mutant isolates (\(P < 0.05\)).

**DISCUSSION**

In studies dating from the pre-molecular era, the increasing level of antifolate resistance on the national or regional level was documented by observing the decreasing clinical efficacy of either pyrimethamine alone or sulfadoxine-pyrimethamine combination.\textsuperscript{1–3} In more recent studies that compared the \(dhfr\) gene sequence of pre-treatment and post-treatment isolates, it has been shown that treatment with antifolate antimalarial drugs favors the selection of \(dhfr\) (and dihydropteroate synthase) mutants among recrudescence parasites over a period of 1–4 weeks.\textsuperscript{4,5,10,11} The present study goes
beyond these recent short-term observations and demonstrates the increasing prevalence of pyrimethamine resistance at the study site where in vitro antifolate resistance has been monitored since 1994 by using two complementary methodologic approaches. Moreover, this surveillance of antifolate resistance was based on the analysis of in vitro pyrimethamine activity and dhfr gene sequences since previous studies have suggested that clinical resistance to sulfadoxine-pyrimethamine therapy is essentially associated with triple dhfr mutations, with or without additional mutation(s) in the gene encoding dihydropteroate synthase.\textsuperscript{12}

Although the full-length dhfr gene sequence was not determined in the 1994–1995 samples, the complete sequence was determined for 90 isolates collected in 1997–1998.\textsuperscript{12} In Yaounde, the decreased proportion of wild-type parasites (25.6% in 1997–1998 to 7.2% in 2000–2001) was largely explained by the increased proportion of triple mutants (43.3% in 1997–1998 to 74.1% in 2000–2001). The dhfr mutants carrying a single Asn-108 mutation (4.4% in 1997–1998 and 1.4% in 2000–2001) or double Arg-59/Asn-108 mutations (10.0% in 1997–1998 and 7.9% in 2000–2001) constituted a minority of parasites during these study periods. The remaining parasites carried mixed alleles (16.7% in 1997–1998 and 9.4% in 2000–2001). This phenomenon seems to suggest that in the pres-

**FIGURE 1.** In vitro response to pyrimethamine in relation to the number of dihydrofolate reductase mutations in 136 clinical isolates of *Plasmodium falciparum* collected in Yaounde, Cameroon in 2000–2001. Wild-type = absence of a mutation; N108 = a single Asn-108 mutation; R59-N108 = double Arg-59/Asn-108 mutations; I51+R59+N108 = triple Ile-51/Arg-59/Asn-108 mutations; mixed = presence of at least Ser- and Asn-108 alleles, with or without both alternative codons at positions 51 and/or 59, in the same isolate. The dashed line indicates the arbitrary threshold value for pyrimethamine resistance (50% inhibitory drug concentration [IC\textsubscript{50}] \geq 100 nM).

herence of triple mutants in an endemic zone, the parasites rapidly acquire a high-level pyrimethamine resistance by inheriting three dhfr mutations at once, rather than developing resistance in a stepwise fashion by acquiring one mutation at a time (i.e., first acquiring Asn-108, followed by Arg-59 and then Asn-51, culminating with the acquisition of Leu-164 in highly resistant zones). This tendency has also been observed in other study sites in southern and central Cameroon, where triple dhfr mutants predominate.13

It may be hypothesized that once a triple mutant is selected or introduced into a zone, the use of antifolates doubly favors the transmission of mutants. First, any residual or recrudescent parasites surviving the therapeutic attack, especially in semi-immune patients, have a high probability of being mutants. This phenomenon of parasite selection under drug pressure has already been documented in several recent studies.4,5,10,11 These parasite survivors may be passed on to the mosquito for the spread of mutants to other human hosts. The long elimination half-life of sulfadoxine-pyrimethamine combination may be another contributing factor for selecting mutant parasites during the sub-therapeutic elimination phase. Second, several clinical studies have shown that antifolate treatment enhances gametocytogenesis, notably when the parasites are antifolate resistant.14,16 If these gametocytes were normally infective to mosquitoes, it may be surmised that antifolate resistance may spread rapidly in vector and human populations. However, it is still not clear whether post-therapeutic gametocytemia following pyrimethamine administration is associated with normal or enhanced mosquito infectivity or possibly with reduced infectivity due to the purported sporontocidal effects of pyrimethamine.14,17 The persistence of triple mutations has an obvious biologic advantage for the survival of the parasites under continuous drug pressure, especially because these mutations do not seem to affect the affinity between the mutant enzyme and natural substrates and are associated with a normal folate metabolism.8 However, further experimental studies are needed to estimate the biologic cost of maintaining mutant DHFR in natural parasite populations and study whether mutant parasites are maintained in natural populations if drug pressure is removed.

In a previous study,9 Basco and Ringwald reported a significant increase in the proportion of pyrimethamine-resistant P. falciparum isolates during the period spanning from 1994 to 1998. Additional data from the present study confirm this trend towards an even higher proportion of pyrimethamine-resistant parasites. In contrast to this dramatic increase in antifolate resistance over an eight-year period, the annual proportion of chloroquine-resistant isolates has remained relatively stable at approximately 50–60% in Yaoundé.18 The molecular basis underlying the different rate of acquisition of resistance to pyrimethamine and chloroquine is probably related to the requirement of only a single point mutation in dhfr for antifolate resistance, in contrast to the requirement of either an accumulation of several distinct point mutations in a single chloroquine resistance gene candidate or mutations (and possibly other genetic changes) in multiple genes all of which have not yet been identified.19

A constant drug pressure is mounted against the parasites in central Africa. As more east African countries resort to sulfadoxine-pyrimethamine for the first-line treatment of uncomplicated malaria and as this trend spreads to central and west Africa in the coming years, the increased reliance on this antifolate drug combination may become widespread on the African continent. History may repeat itself in Africa, with a rapid decrease in the clinical efficacy of sulfadoxine-pyrimethamine, as it was observed 40 years ago in Southeast Asia and, to a lesser extent, in the Amazonian basin. Before this scenario becomes a reality, it is urgently needed to implement novel therapeutic strategies, including new antifolate drugs, combinations with drugs from other chemical classes, preferably those with gametocytocidal action, and development of new drugs.

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