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Molecular Epidemiology of Malaria in Cameroon. XXVI. Twelve-Year *In Vitro* and Molecular Surveillance of Pyrimethamine Resistance and Experimental Studies to Modulate Pyrimethamine Resistance

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Abstract. *In vitro* pyrimethamine response of *Plasmodium falciparum* isolates and dihydrofolate reductase (*dhfr*) gene sequences were analyzed in 2004–2005 and compared with our previous data. Most isolates (n = 103, all *dhfr* mutants) had 50% inhibitory concentrations (IC_{50}s) ≥ 119 nM, and six isolates had low IC_{50}s (five wild-type or mixed *dhfr*, 0.04–1.37 nM; one triple mutant, 6.4 nM). Of 194 isolates, only 7 had the wild-type *dhfr* and 187 were mutants. The results of the two methods were highly concordant and indicated a significant increase ($P < 0.05$) in the prevalence of mutant, pyrimethamine-resistant *P. falciparum* between 1994 and 2005. The addition of probenecid or sulfinpyrazone to pyrimethamine resulted in a slight-to-moderate decrease in the level of *in vitro* pyrimethamine resistance without rendering the parasites susceptible to pyrimethamine. Analysis of molecular markers may be useful for the long-term surveillance of antifolate-resistant malaria.

INTRODUCTION

Sulfadoxine-pyrimethamine plays an important role in antimalarial chemotherapy in Africa. Due to the widespread occurrence of chloroquine-resistant *Plasmodium falciparum* infections since the 1980s, many African countries have resorted to sulfadoxine-pyrimethamine monotherapy for the first-line or second-line treatment of uncomplicated malaria.[1] In recent years, African countries have been adopting artemisinin-based combination therapy, but this novel strategy to enhance therapeutic efficacy and delay the emergence of drug-resistant parasites is not yet fully implemented in the field. During the transition period towards the generalized use of artemisinin-based combinations in Africa, sulfadoxine-pyrimethamine monotherapy continues to be useful in many African countries for the routine treatment of uncomplicated malaria in children and adults and for the intermittent preventive treatment in infants and pregnant women.[2,3]

The massive use of antifolate drugs leads to a rapid development of drug resistance, as it occurred in southeast Asia.[4] Therefore, sulfadoxine-pyrimethamine monotherapy is not expected to remain highly effective in Africa in the future unless novel therapeutic strategies are applied in the field and target populations for the use of sulfadoxine-pyrimethamine are well defined. Treatment failures after sulfadoxine-pyrimethamine monotherapy have already been reported from many areas in Africa.[5] In this context, it is important to set up a surveillance system for a rapid and appropriate response to the changing epidemiology of antifolate-resistant *P. falciparum*. *In vitro* drug susceptibility assay and molecular analysis of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*), targets of pyrimethamine and sulfadoxine, respectively, are alternative epidemiologic tools to describe the evolution of antifolate resistance. *In vitro* pyrimethamine resistance is strongly associated with a single Ser108Asn substitution in DHFR, and additional substitutions (Asn51Ile, Cys59Arg, and Ile164Leu) increase the level of resistance.[6–8] *In vitro* sulfadoxine resistance is associated with amino acid changes occurring at positions 436, 437, 540, 581, and 613 in DHPS.[9] The first objective of the present study was to determine the *dhfr* sequence and *in vitro* response to pyrimethamine of clinical isolates obtained in 2004–2005 and compare the results with those of the preceding years to analyze the dynamics of the spread of pyrimethamine-resistant *P. falciparum* in Yaoundé, Cameroon.

One of the experimental strategies to circumvent drug resistance is to combine an antimalarial drug with a pharmacological agent (calcium channel blockers, tricyclic antidepressants, tricyclic antihistamines, phenothiazines, and others), collectively called a modulator, which modifies drug transport and increases the intracellular concentration of the antimalarial drug.[10] Methotrexate is a DHFR inhibitor commonly used as an anticancer agent. One of the proposed mechanisms of methotrexate resistance in human cancer cells is an enhanced drug efflux mediated by multidrug resistance protein 1 (MRP1), which is an ATP-binding cassette drug transporter.[11,12] Methotrexate resistance can be modulated *in vitro* in mammalian cells by probenecid and sulfinpyrazone.[13] Based on these observations, Nzila and others[14] have shown that the addition of probenecid (50 μM) results in a sevenfold increase in pyrimethamine susceptibility in a highly resistant laboratory-adapted *P. falciparum* strain. If this experimental approach is validated, pyrimethamine resistance may be circumvented. The second objective of the present study was to evaluate the effectiveness of this experimental approach in clinical isolates collected in the field.

PATIENTS AND METHODS

Patients. Venous blood samples (5–10 mL) were collected from symptomatic children ≥ 12 years of age and adults seeking consultation at the Nlongkak Catholic Missionary Dispensary in Yaoundé, Cameroon during 2004–2005 after informed consent was obtained. The following criteria were used: the presence of *P. falciparum* at a parasitemia ≥ 0.1% and a negative Saker-Solomons urine test result for 4-aminoquinolines.[15] Patients with severe anemia (hematocrit < 15%), pregnant women, and those with signs and symptoms of se-

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vere and complicated malaria were excluded. All enrolled patients were treated with amodiaquine, amodiaquine–sulfadoxine-pyrimethamine combination or an artesunate-amodiaquine combination. The study was reviewed and approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

***In vitro* drug assay.** Venous blood samples were collected and transported to our laboratory within two hours after venipuncture. Blood samples were washed three times by centrifugation with *p*-aminobenzoic acid (PABA)– and folic acid–free RPMI 1640 culture medium (Sigma Chemical Co., St. Louis, MO). Washed erythrocytes were resuspended in complete PABA- and folic acid–free RPMI 1640 medium containing buffers (25 mM HEPES and 25 mM $NaHCO_3$) and 10% fetal bovine serum (batch no. 5-41201; Integro b. v., Amsterdam, The Netherlands) 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, probenecid, and sulfinpyrazone were obtained from the Sigma Chemical Co. Stock solutions and dilutions of pyrimethamine, probenecid, and sulfinpyrazone were prepared in absolute ethanol. Drug solutions were distributed in duplicate in 96-well culture plates and dried. The final concentrations of pyrimethamine, probenecid, and sulfinpyrazone ranged from 0.0488 nM to 51,200 nM (in four-fold dilutions), 6.25 to 6,400 μM (in two-fold dilutions), and 0.625 to 640 μM (in two-fold dilutions), respectively. Fixed concentrations of probenecid (25 μM or 50 μM) or sulfinpyrazone (12.8 μM) were added to the pyrimethamine assay plate and dried.

In vitro isotopic drug assays were performed as previously described.[16,17] Two hundred microliters of the suspension of infected erythrocytes were distributed in each well of the 96-well tissue culture plates. The parasites were incubated at 37°C in an atmosphere of 5% CO_2 for 42 hours. ^{3}H-hypoxanthine (1 μCi/well; Amersham International, Little Chalfont, United Kingdom) was added at the beginning of the incubation period to measure parasite growth. The plates were frozen to terminate the 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 vials, and 2 mL of scintillation cocktail (Organic Counting Scintillant®; Amersham International) were added. The incorporation of ^{3}H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1409; Pharmacia, Uppsala, Sweden). The 50% inhibitory concentration (IC_{50}), defined as the drug concentration at which 50% of the incorporation of ^{3}H-hypoxanthine is inhibited compared with that of drug-free control wells, was determined by nonlinear regression analysis using the Prism™ software (GraphPad Software, Inc., San Diego, CA).

Polymerase chain reaction (PCR) and DNA sequencing. Venous blood (50 μL) was imbibed onto Isocode Stix® filter papers (Schleicher and Schuell, Ecquevilly, France). The filter papers were dried overnight in an incubator at 37°C and stored at –20°C in airtight plastic bags until analysis. Parasite DNA was extracted from filter papers by the boiling method.[18] Briefly, after rinsing once in 500 μL of sterile distilled water, the filter paper was placed into a 0.5-mL microtube to which 75 μL of sterile distilled water was added. The filter paper was incubated at 100°C for 20 minutes and agitated for a few seconds. The supernatant (10 μL) was used directly for amplification of *dhfr* gene fragment.

The entire DHFR domain (708 basepairs) of the *dhfr*-thymidylate synthase (*dhfr-ts*) gene was initially amplified by the primary PCR in a mixture consisting of genomic DNA (10 μL of supernatant containing parasite and human DNA), 15 pmol of a pair of synthetic oligonucleotides (forward primer ST1L, 5′-ATGATGGAACAAGTCTGCGACGTTTTC-GAT-3′ and reverse primer ST2L, 5′-TTCATTTAACATTT-TATTATTCGTTTTCTT-3′), buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM $MgCl_2$, 200 μM 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 μL. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for 2 minutes in the first cycle and 1 minute in subsequent cycles, 50°C for 90 seconds in the first cycle and 1 minute in subsequent cycles, and 72°C for 1 minute in all cycles, for a total of 30 cycles. In the secondary nested PCR, a 499-basepair fragment was amplified from the primary amplification product (0.75–1 μL) by using the primer pairs DHFR-31 (forward primer, 5′-ATTT-ATGCCATATGTGCATGTTGTAAC-3′) and DHFR-529R (reverse primer, 5′-CTTTTCTAAAAATTCTTGAT-AAACAAC-3′) in a 100-μL reaction mixture. The same thermal cycler program was used. Five microliters of the final amplification product was loaded on a 1.5% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the 499-basepair DNA fragment. The PCR products were sequenced using the ABI Prism automated DNA sequencer (Perkin Elmer Corp., Les Ulis, France).

Data analysis. Electropherogram data were viewed and analyzed using the Editview sequence analysis software (Perkin Elmer Corp.). An isolate was considered to be wild-type if the following haplotype was observed: Asn-51, Cys-59, Ser-108, and Ile-164.[6,7] An isolate was considered to be mutant if at least one of these codons was mutated: Asn- to Ile-51, Cys- to Arg-59, Ser- to either Asn-108 or Thr-108, and Ile- to Leu-164. Previous studies have shown that Ser- to Thr-108 change occurs with Ala- to Val-16 substitution.[6,7] The use of the internal primer DHFR-31, which includes codon 16, did not enable determination of the sequence of this codon in the present study. However, our previous studies using semi-nested PCR showed that none of the Cameroonian isolates have the mutant Val-16 codon.[17,19]

As in our previous study, the *in vitro* threshold for pyrimethamine resistance was arbitrarily fixed at ≥ 100 nM.[17] This threshold was estimated from the results of the assays performed with RPMI–10% human serum. The use of RPMI–10% fetal bovine serum, as in the present study, results in an average three-fold decrease in the pyrimethamine IC_{50}.[20] The adjusted threshold for pyrimethamine assays using RPMI–10% fetal bovine serum is expected to be ≤ 30 nM. The proportions of pyrimethamine resistance, based on either an IC_{50} ≥ 100 nM (or 30 nM) or the presence of Asn-108 mutant allele, at different time periods were compared by the chi-square test for trend. Isolates with mixed *dhfr* alleles were not included in the statistical analysis because their *in vitro* response is unpredictable. The significance level (P) was fixed at 0.05.

RESULTS

A total of 194 isolates were collected for *in vitro* assays in 2004–2005. Pyrimethamine response was determined in 137 isolates. Assays were not performed for 57 isolates because of the difficulties in procuring a suitable batch of human serum or serum substitute that supports parasite growth without interfering with antifolate activity. Furthermore, some of the collected isolates were only used for experimental chloroquine assays.[21] Of 137 isolates, 28 did not yield an interpretable assay for pyrimethamine (chloroquine assays performed with the standard RPMI–10% human serum were successful with these 28 isolates) due to poor growth (n = 22 tested with 2 batches of fetal bovine serum) or insufficient growth inhibition even at the highest drug concentration (n = 6 tested with the same batch of human serum as for chloroquine).

Of 109 isolates with interpretable assays, most (n = 103, 94%) had an $IC_{50} \geq 119$ nM, indicating *in vitro* pyrimethamine resistance (Figure 1). The geometric mean pyrimethamine IC_{50} (95% confidence intervals) of the resistant isolates was 1,980 nM (1,580–2,480 nM). All 103 isolates were either pure double (Arg-59/Asn-108, n = 5) or pure triple (Ile-51/Arg-59/Asn-108, n = 97) *dhfr* mutants or mixed isolates with Ile-51/Arg-59/Ser- and Asn-108 (n = 1). Six isolates with low IC_{50}s had the following values: 0.04 nM (extrapolated value due to an almost complete growth inhibition at the lowest drug concentration used; wild-type *dhfr*), 0.19 nM (wild-type *dhfr*), 0.42 nM (mixed *dhfr* alleles with Asn- and Ile-51/Cys- and Arg-59/Ser- and Asn-108), 0.49 nM (wild-type *dhfr*), 1.37 nM (wild-type *dhfr*), and 6.4 nM (triple *dhfr* mutant).

The intrinsic antimalarial activity of probenecid was determined in 19 isolates using RPMI–10% fetal bovine serum. The geometric mean IC_{50} (range) was 53.8 μM (3.19–273 μM) (Table 1). Initial experiments in four isolates cultivated in RPMI–human serum with three fixed concentrations of probenecid (5 μM, 25 μM, and 50 μM) added to the pyrimethamine assay plate showed slight to no effect with 5 μM probenecid and 12–36% decrease in pyrimethamine IC_{50} with 25 μM probenecid. Subsequent experiments were performed with isolates cultivated in RPMI–fetal bovine serum with either 25 μM or 50 μM probenecid added to the pyrimethamine plate. A mean decrease of 19% (maximum = 73%) in pyrimethamine IC_{50} was obtained in 25 isolates by adding 25 μM probenecid. In five isolates tested with 50 μM probenecid, the mean decrease in pyrimethamine IC_{50} was 23%. However, in our experimental conditions, the fixed concentration of 50 μM seemed too close, and even above, to the intrinsic activity of probenecid for some isolates. The modulator activity of probenecid was effective in rendering a pyrimethamine-resistant isolate (assuming a cut-off ≥ 100 nM) pyrimethamine-susceptible in only three isolates (06/04, 12/04, and 17/04), all of which had a low level *in vitro* pyrimethamine resistance (IC_{50}s = 119–236 nM). Experiments on pyrimethamine combined with a single fixed concentration (12.8 μM) of sulfinpyrazone showed a decrease in pyrimethamine IC_{50} in a few isolates (Table 2). However, the

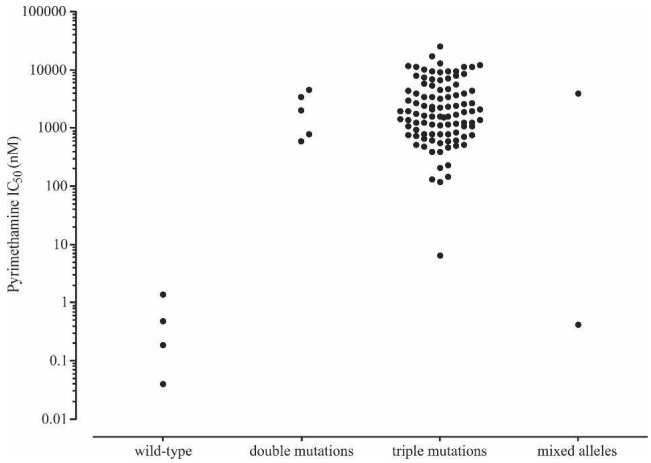

FIGURE 1. *In vitro* response to pyrimethamine in relation to the number of dihydrofolate reductase (*dhfr*) mutations in 109 clinical isolates of *Plasmodium falciparum* collected in Yaoundé, Cameroon in 2004–2005. Wild-type *dhfr* allele indicates the absence of a mutation (Asn-51/Cys-59/Ser-108), double mutations indicate Asn-51/Arg-59/Asn-108, and triple mutations indicate Ile-51/Arg-59/Asn-108. IC_{50} = 50% inhibitory concentration.

TABLE 1

In vitro activities of pyrimethamine (PYR) alone and in combination with probenecid*

Plasmodium falciparum isolate	IC_{50} (nM) PYR	PYR + 25 μM probenecid	PYR + 50 μM probenecid	Probenecid
06/04†	236	152	99.2	123,000
12/04†	119	89.2	NI	85,900
17/04†	146	98.1	–	87,000
27/04†	391	346	–	63,700
34/04	799	767	–	70,100
37/04	788	638	–	109,000
38/04	601	530	–	91,600
42/04	1,600	1,580	–	51,300
43/04‡	1,990	1,500	–	30,900
49/04‡	5,850	3,100	–	16,800
53/04	744	776	–	76,200
54/04	568	398	–	91,900
55/04	1,090	1,040	–	89,500
57/04	800	843	–	94,600
63/04‡	0.416	0.405	–	17,600
64/04	1,990	2,320	–	104,000
70/04‡	2,650	–	NI	52,700
71/04‡	622	–	NI	25,300
72/04‡	2,580	–	1,850	3,190
77/04‡	1,740	–	NI	25,800
78/04	1,177	–	771	273,000
79/04	632	–	498	152,000
80/04	392	–	300	83,900
81/04	3,420	–	3,120	–
83/04	601	361	–	–
85/04	2,150	2,840	–	–
86/04	1,140	756	–	–
87/04	1,950	521	–	–
88/04	394	212	–	–
94/04	4,000	3,040	–	–
101/04	3,250	2,800	–	–
108/04	483	300	–	–
112/04	3,440	2,720	–	–
114/04	1,400	1,370	–	–
115/04	717	683	–	–
116/04	1,030	505	–	–
119/04	675	424	–	–

* IC_{50} = 50% inhibitory concentration; NI = not interpretable due to curve-fitting failure; – = not done.

† Isolates tested with RPMI–10% human serum. Initial experiments with PYR + 5 μM probenecid showed slight to no effect in isolates 06/04 (208 nM) and 12/04 (123 nM). Other isolates were assayed with RPMI1640–10% fetal bovine serum.

‡ These isolates had IC_{50} values for probenecid that were close to, or lower than, the fixed concentration added to PYR.

TABLE 2

In vitro response to pyrimethamine combined with a fixed concentration of sulfinpyrazone*

Plasmodium falciparum isolate	IC_{50} (nM)		
	Pyrimethamine	Pyrimethamine plus 12.8 μM sulfinpyrazone	Sulfinpyrazone
01/05†	–	–	35,600
02/05†	–	–	86,600
03/05†	–	–	81,500
08/05	844	1,456	36,200
09/05	4,400	3,190	64,700
10/05	521	205	37,600
12/05	1,530	1,840	36,500
14/05	935	1,290	229,000
17/05	1,090	870	38,800
18/05	2,320	2,040	111,000
26/05	1,260	1,000	50,300

* IC_{50} = 50% inhibitory concentration.
† Initial experiments were performed to determine the intrinsic antimalarial activity of sulfinpyrazone. All assays were performed with *p*-aminobenzoic acid and folic acid-free RPMI1640–10% fetal bovine serum.

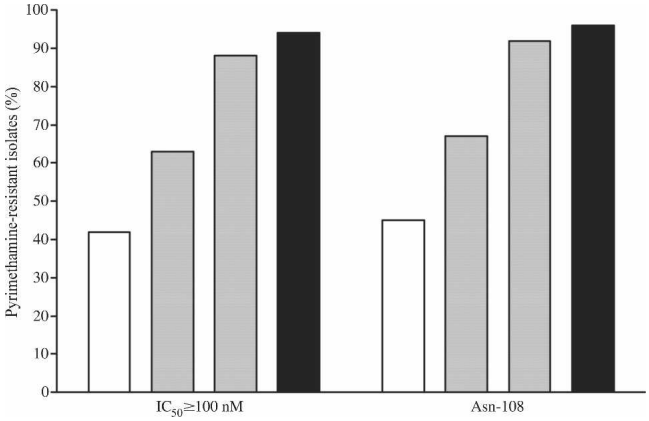

FIGURE 2. Prevalence of pyrimethamine resistance between 1994 and 2005 in Yaoundé, Cameroon determined in 1994–1995 (first column), 1997–1998 (second column), 2000–2001 (third column), and 2004–2005 (fourth column). Pyrimethamine resistance was defined as a 50% inhibitory concentration (IC_{50}) ≥ 100 nM for *in vitro* drug assays performed with RPMI 1640 medium–human serum (left set of columns) or the presence of Asn-108 in the *Plasmodium falciparum* dihydrofolate reductase gene (right set of columns). Data from 1994–2001 were previously published.[22–24]

effect was inconsistent in other isolates. These experiments were not pursued after obtaining the results from 11 isolates.

The prevalence of *dhfr* mutations was assessed in all isolates (n = 194) collected during the study period of 2004–2005. Only 7 (3.6%) had the wild-type *dhfr*. Mutations occurred in two or three alleles: codons 108 and 51 and/or 59. Other mutant codons reported in field isolates (Arg-50, Thr-108, and Leu-164) were not observed. Few were double mutants, with either Arg-59/Asn-108 (n = 7, 3.6%) or Ile-51/Asn-108 (n = 1, 0.5%). Most of the isolates were triple *dhfr* mutants (n = 176, 90.7%). Three isolates had mixed *dhfr* alleles.

Although RPMI–10% fetal bovine serum lowers pyrimethamine IC_{50}s compared with RPMI–10% human serum, the responses of the isolates studied in 2004–2005 can be classified as ≥ 119 nM (n = 103) and ≤ 6.4 nM (n = 6). The isolates in the former group were all *dhfr* mutants or mixed alleles, and those in the latter group were wild-type or mixed alleles, with the exception of one isolate (IC_{50} = 6.4 nM) that had triple mutations. Based on this observation, the arbitrary criterion used to distinguish between pyrimethamine-susceptible isolates (IC_{50} < 100 nM) and pyrimethamine-resistant isolates (IC_{50} ≥ 100 nM) in our previous studies was also applied in the present study for comparison with our results from 1994 to 2001.[22–24] The evolution of pyrimethamine resistance in Yaoundé between 1994 and 2005 (n = 401 for *in vitro* assays and 516 for *dhfr*) showed an increasing prevalence ($P < 0.05$) of pyrimethamine-resistant and *dhfr*-mutant *P. falciparum* isolates (Figure 2). The baseline prevalence of pyrimethamine resistance determined in Yaoundé in 1994–1995 was 41% and 43% (48% including mixed alleles) using *in vitro* assay and PCR, respectively. The prevalence of pyrimethamine resistance increased steadily during the past 12 years to attain 94% and 95% (96% including mixed alleles) using *in vitro* assay and PCR, respectively.

DISCUSSION

Recent evaluation of the therapeutic efficacy of sulfadoxine-pyrimethamine monotherapy has shown an overall failure rate of 9.9% on day 14 in pediatric patients in Cameroon.[25] Many of the failures were caused by early treatment failure requiring alternative treatment on or before day 3 after sulfadoxine-pyrimethamine treatment. In one of our previous studies conducted in an area of intense transmission,[26] the failure rates (uncorrected by PCR) in young children less than five years of age were 0% on day 14 and 13.6% on day 28. Sulfadoxine-pyrimethamine monotherapy is no longer highly effective for the routine treatment of uncomplicated malaria in Cameroonian children. Further prospective studies on sulfadoxine-pyrimethamine monotherapy are not planned in Cameroonian children for several reasons, including the existence of recent clinical data, current recommendation to use artemisinin-based combination therapies, and ethical concerns. Nonetheless, because of the pivotal role that sulfadoxine-pyrimethamine plays in the national antimalarial drug policy, notably for the intermittent preventive treatment in pregnant women, regular surveillance of antifolate resistance by alternative means is required.

Our previous studies, as well as the present study, have demonstrated the high correlation between *in vitro* pyrimethamine assay and molecular analysis of *dhfr*.[22–24] This finding implies that the two methods do not need to be performed in parallel for further monitoring. *In vitro* assay is more labor-intensive and more cumbersome to perform in aseptic conditions in the field. It also requires expensive equipment and is best performed in a well-equipped laboratory. The successful rate of interpretable assays may be low (< 80%), especially in populations in whom self-medication is a common practice. Moreover, *in vitro* assay for pyrimethamine is difficult to standardize because of the interference of PABA and folic acid, which are present in the standard RPMI 1640 medium, human serum, and erythrocytes, with drug action. In contrast, a large number of finger prick capillary blood samples can be collected on filter papers with more ease and more rapidly in the field. The success rate of PCR is usually very high (> 95%), even with samples from patients who have self-medicated before consultation and from patients with low parasitemia (< 0.1%). A high output

nested PCR can amplify the target gene of up to 96 samples simultaneously in a single PCR plate in one day. These technical advantages indicate that further surveillance of pyrimethamine resistance in Cameroon can be entirely based on molecular studies.

Molecular surveillance of antifolate resistance should include the search for triple *dhfr* mutations, which are a necessary but insufficient condition for clinical resistance to sulfadoxine-pyrimethamine monotherapy, and the appearance of mutations that are associated with a high level of pyrimethamine resistance, such as Ile164Leu,[27] but are not presently encountered in central Africa. Furthermore, because pyrimethamine (or biguanides such as proguanil and chlorproguanil) is always administered with either sulfones or sulfonamides, a concomitant search for amino acid substitutions in DHPS, in particular Ala437Gly and Lys540Glu, which with the triple *dhfr* mutations (called quintuple mutations) are associated with therapeutic failure to sulfadoxine-pyrimethamine in east Africa, is required to provide complementary information for epidemiologic studies and for monitoring sulfadoxine-pyrimethamine resistance within the context of intermittent preventive treatment of pregnant women in Africa.[28]

Our series of studies using two complementary methodologic approaches[22–24] have shown an increasing prevalence of pyrimethamine resistance in Yaoundé since 1994. The increasing prevalence is largely explained by the steady replacement of wild-type isolates (52% in 1994–1995 and only 3.6% in 2004–2005) by triple *dhfr* mutants during the 12-year period in Yaoundé. Single and double *dhfr* mutants constitute only a few isolates. Longitudinal and cross-sectional studies at other sites in Cameroon showed a similar pattern of *dhfr* allelic distribution.[19]

A model that may explain this phenomenon is an introduction into Cameroon of an original triple mutant *P. falciparum*, possibly from southeast Asia via east Africa in the late 1980s or early 1990s,[29] and the subsequent genetic transmission of triple *dhfr* mutations to new generations of parasites that were under increasing drug pressure. This transmission is probably non-clonal because of the high rate of multiple parasite populations within a single clinical isolate, which favors recombination during the sporogonic phase in mosquitoes, and a wide polymorphism of the *P. falciparum* isolates circulating in southern Cameroon.[30] A single or double mutant, or mixed *P. falciparum* co-existing with the triple mutant, may have been diluted within a large parasite population of triple *dhfr* mutants.

An alternative model is an independent lineage of mutant parasites arising from either single, double, or triple mutations occurring at once or a gradual acquisition and stepwise accumulation of *dhfr* mutations (from a single Ser108Asn replacement to one of the alternative double substitutions [Asn51Ile–Ser108Asn or Cys59Arg–Ser108Asn], then to triple [Asn51Ile–Cys59Arg–Ser108Asn] *dhfr* mutations) by the local *P. falciparum* parasite in central Africa. The latter hypothesis of multiple, independent origins of *dhfr* mutant *P. falciparum* is supported by one recent study based on the microsatellite analysis of east African malaria parasites.[31] These two models are not necessarily contradictory.

The results of other microsatellite studies support the epidemiologic model of regional expansion of *dhfr* mutant *P. falciparum* at the original foci of antifolate-resistant malaria (southeast Asia and the Amazon basin).[32,33] Further studies using microsatellite markers would be required to advance an evidence-based hypothesis that may explain the spread of antifolate-resistant malaria parasites in Cameroon and to determine whether the original *dhfr* mutant *P. falciparum* was introduced from outside or emerged locally.

It cannot be predicted whether antifolate drugs will regain their clinical efficacy if they were entirely withdrawn for several years from a stable malaria-endemic area. Such a scenario would be unlikely because sulfadoxine-pyrimethamine is officially recommended for the intermittent preventive treatment of pregnant women and generic and counterfeit drugs are widely circulating through unofficial outlets in central Africa.[34] One possible strategy would involve the use of a modulator to enhance the efficacy of pyrimethamine. Based on the initial *in vitro* findings of the modulator effect of probenecid on pyrimethamine,[14] several clinical studies were conducted in Nigeria to assess the efficacy of sulfadoxine-pyrimethamine-probenecid combination.[35–37] This combination was reported to be well-tolerated and more effective than sulfadoxine-pyrimethamine alone, but probenecid had no effect on sulfadoxine-pyrimethamine–induced gametocytogenesis. Although previous experimental data suggested that probenecid decreases folate uptake into malaria-infected erythrocytes, the exact underlying mechanism of action of sulfadoxine-pyrimethamine–probenecid combination in malaria parasites is unknown.[14] In our present *in vitro* study, the modulator effect of probenecid on pyrimethamine activity seemed inadequate to reverse pyrimethamine resistance. Our results may suggest that altered drug transport may not be the major mechanism of resistance involved or that *in vitro* culture in PABA- and folic acid–deficient medium may alter drug and/or substrate transport. Further *in vitro* experiments are required to elucidate whether a homolog of human MRP1 exists in *P. falciparum* and how probenecid enhances pyrimethamine activity.

The present longitudinal study demonstrates the rapid replacement of wild-type *P. falciparum* by triple *dhfr* mutants in Yaoundé over a 12-year period. The increasing prevalence of *dhfr* mutants is strongly supported by *in vitro* drug susceptibility assays performed in parallel with PCR and DNA sequencing. Based on the evaluation of clinical efficacy during the 14-day follow-up period, sulfadoxine-pyrimethamine monotherapy remained moderately effective in the clinical studies conducted between 1999 and 2004.[25] The prevalence of *in vitro* pyrimethamine resistance increased during the same period.[24] However, molecular analysis of Cameroonian *P. falciparum* isolates indicated the absence of quintuple mutations (*dhfr* and *dhps*) that have been suggested to predict clinical failure after sulfadoxine-pyrimethamine monotherapy. The limited data on the modulator effect of probenecid on pyrimethamine preclude further clinical studies on this combination until more data on the mechanism of action, pharmacodynamics, pharmacokinetics, and toxicologic studies are available. The apparent discordance between molecular and *in vitro* assays and *in vivo* response is probably related to multiple factors, including the synergistic action of pyrimethamine and sulfadoxine, the absence of quintuple mutations and Ile164Leu DHFR substitution in Cameroonian isolates, and acquired immunity. Nevertheless, analysis of molecular markers is an alternative method that characterizes

the *dhfr* alleles of field isolates and may be useful for the long-term monitoring of antifolate-resistant malaria.

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