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MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XXII. GEOGRAPHIC MAPPING AND DISTRIBUTION OF *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE (*DHFR)* MUTANT ALLELES

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Abstract. Sulfadoxine-pyrimethamine (SP) is still a useful drug to combat chloroquine-resistant *Plasmodium falciparum* malaria in Cameroon. Because of several disadvantages of the *in vivo* test and *in vitro* drug sensitivity assays, molecular assays are an alternative laboratory tool to monitor the evolution of antifolate resistance, especially over the entire country that is characterized by several epidemiologic strata and malaria transmission patterns. In this study, 1,430 blood samples from either symptomatic children or asymptomatic carriers were collected from 14 sites throughout the country between 1999 and 2003 for the analysis of dihydrofolate reductase (*dhfr*) sequence. Of 1,368 samples (95.7%) that were successfully amplified, 1,180 were analyzed by direct sequencing of the polymerase chain reaction product, and 188 were analyzed by restriction enzymes. The prevalences of the wild-type, single Asn-108 mutation, double Arg-59/Asn-108 mutations, double Ile-51/Asn-108 mutations, triple Ile-51/Arg-59/Asn-108 mutations, and mixed alleles were 20.8%, 2.8%, 5.7%, 0.8%, 62.2%, and 7.6%, respectively. The proportions of triple *dhfr* mutations were > 60% at all study sites, with the exception of the eastern province (42% triple mutants in Bertoua in 1999) and the northern provinces (11–35% triple mutants in Ngaoundere, Garoua, and Maroua). In these two provinces, the proportion of mutant parasites increased significantly ($P < 0.05$) over the period of 2–4 years. Furthermore, there was a higher proportion ($P < 0.05$) of wild-type parasites in the northern provinces, compared with the rest of the country. The geographic mapping of molecular markers offers a novel tool for monitoring the epidemiology of drug-resistant malaria.

Molecular markers are one of the available means to describe the epidemiology of drug-resistant *Plasmodium falciparum*. The other methods include the evaluation of clinical and parasitological responses to drug treatment in malaria-infected patients (also called the *in vivo* test) and *in vitro* drug sensitivity assays. The latter 2 methods provide useful data on the drug-resistance status of the parasites but have several disadvantages. The *in vivo* test requires a 14-day to 28-day follow-up of individual patients, and the efficacy of only 1 drug that has been approved for human treatment can be evaluated at a time in each patient.[1] Therapeutic success and therapeutic failure are not synonymous with drug sensitivity and drug resistance, respectively, since a patient may or may not be cured due to drug action alone. Various host factors, such as acquired immunity, pharmacokinetic variations, previous drug treatment, and unreported self-medication during the follow-up period, may influence the clinical and parasitological response. Furthermore, the evaluation of therapeutic efficacy of antimalarial drugs, such as chloroquine and sulfadoxine-pyrimethamine (SP) monotherapies, in young children has become inappropriate in many endemic areas due to the high levels of resistance to these drugs. Current trends towards the generalized use of combination therapies may lead to difficulties in analyzing the degree of resistance to each component of drug combinations if recrudescence occurs after drug therapy.[2] *In vitro* drug assays characterize the phenotype of the parasites isolated from the host. The assay protocol is not standardized, and its performance in the field requires a specialized skill and laboratory equipment. At present, there is no universally accepted threshold that allows a clear-cut distinction between sensitive and resistant isolate for any antimalarial drug. Moreover, the *in vitro* response is not always correlated with the clinical and parasitological responses of patients from whom the parasites were isolated.[3]

Molecular assays can be performed for a large number of fingerprick capillary blood samples imbibed onto filter papers. The filter papers can be stored for years and transported or sent by ordinary mail with ease. Although numerous polymerase chain reaction (PCR) protocols exist, there is a general consensus on the interpretation of sequence data in relation to drug resistance. The geographic distribution of drug-resistant malaria, as well as its changes over time, can be described and monitored more easily with molecular assays than with *in vivo* test or *in vitro* drug sensitivity assays. The molecular markers for some, but not all, antimalarial drugs have been established. These include *P. falciparum* chloroquine resistance transporter (*pfcrt*) for chloroquine, *P. falciparum*, or *P. vivax* dihydrofolate reductase (*dhfr*) for pyrimethamine, *P. falciparum*, or *P. vivax* dihydropteroate synthase (*dhps*) for sulfonamides and dapsone, and cytochrome *b* gene for atovaquone. *P. falciparum* multidrug-resistance gene 1 (*pfmdr1*) may be associated with resistance to amino alcohols and artemisinin derivatives.[4,5]

The level of chloroquine resistance has attained a high level in many parts of Cameroon.[6,7] SP is still an effective alternative to manage chloroquine-resistant malaria in Cameroon. The current national antimalarial drug policy recommends the use of SP primarily for the intermittent preventive treatment to diminish the prevalence of malaria during pregnancy. Furthermore, the drug is relatively cheap and widely available for self-medication through unofficial outlets in the country.[8] Our previous studies have suggested that triple *dhfr* mutations are associated with both high levels of *in vitro* pyrimethamine resistance and therapeutic failure.[9–11] In the present study, the *dhfr* sequences of field isolates collected from different parts of Cameroon were characterized with the aim to establish the geographic distribution of gene mutations. At some study sites, samples were collected at 2 different time points for comparison.

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MATERIALS AND METHODS

Patients and asymptomatic carriers. Blood samples were collected from symptomatic children aged less than 10 years (majority were aged < 5 years) presenting spontaneously at health care centers in 12 different localities in Cameroon during 1999–2003 (Figure 1). This study was part of the clinical studies to evaluate the efficacy of first-line and second-line drugs.[7] The patients were included in the clinical studies if the following criteria were met: presence of *P. falciparum*, without other *Plasmodium* species, parasitemia ≥ 2,000 asexual parasites per μL of peripheral blood, fever ≥ 37.5°C, hematocrit > 15%, and absence of severe malnutrition, other infectious diseases that may be the origin of fever, and signs and symptoms of severe and complicated malaria. After blood collection, a number of patients were not included in the clinical studies due to insufficient parasitemia, mixed malarial infections, persistent vomiting, or concomitant infectious diseases.[1] Some enrolled patients were excluded from the clinical protocol during the follow-up period due to self-medication with other drugs, development of other infectious diseases, or lost to follow-up. Blood samples from these excluded patients were included in the analysis of molecular markers with the written consent of their parents or guardians.

Fingerprick capillary blood was also collected from asymptomatic schoolchildren, aged between 5 and 10 years, residing in 1 of the study sites where therapeutic efficacy was assessed (Hévécam, near Kribi) and 2 additional sites (Mengang and Sangmélima). These 3 rural sites where asymptomatic carriers were recruited are located in the southern tropical rain forest region where transmission is intense and perennial. The parasitological and entomological surveys in Mengang have been described recently.[12] Thick blood smears of asymptomatic schoolchildren were performed and examined only if the parents signed the written informed consent. At the time of blood examination, body temperature was measured in each of the schoolchildren. Blood samples from schoolchildren who had fever due to an identifiable cause other than malaria or due to unknown origin were excluded from molecular studies.

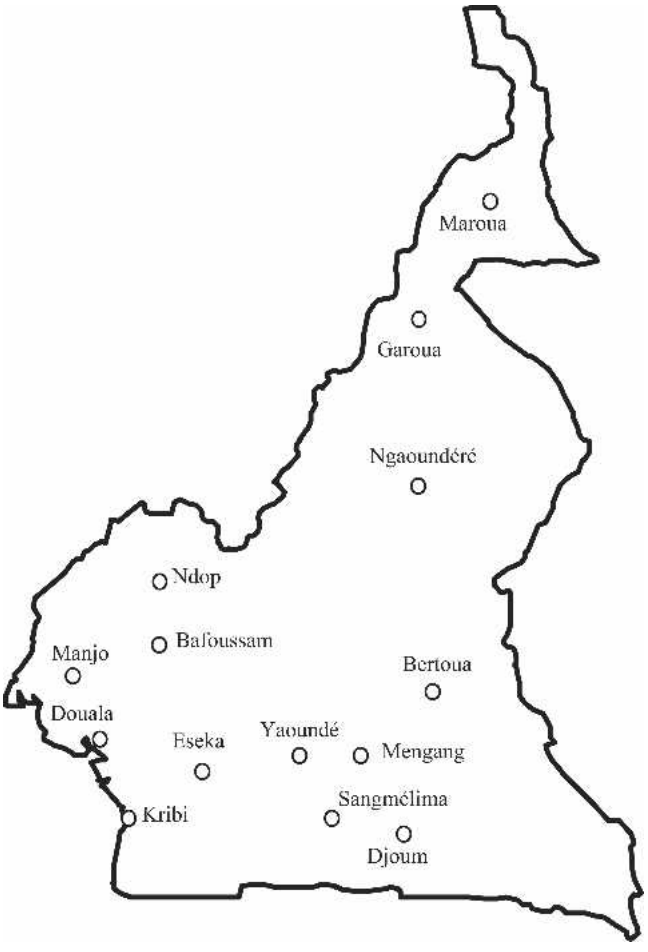

FIGURE 1. Study sites in Cameroon.

All blood samples were collected and imbibed onto Isocode Stix® filter papers (Schleicher & Schuell, Ecquevilly, France). The filter papers were thoroughly dried overnight and stored at room temperature in airtight plastic bags until analysis. The patients were treated with chloroquine, amodiaquine, SP, or amodiaquine-SP combination, depending on the treatment group to which they were assigned. The clinical and parasitological outcomes are described in another paper.[7] Asymptomatic schoolchildren with a positive blood smear were treated with SP. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

Polymerase chain reaction. DNA was extracted from filter papers by the boiling method.[13] 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 were 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.

Because of the small quantity of parasite DNA on filter papers, a nested PCR was required to obtain a sufficient amount of DNA for the sequencing reaction. The primary amplification described later in this article is the same protocol as in our previous studies.[9–11,14] For some samples, the secondary amplification was based on a new set of primer pairs to perform nested PCR, instead of a semi-nested reaction described in our previous studies. In the primary PCR, the entire DHFR domain (708 basepairs) was amplified using the following reaction mixture: genomic DNA (10 μL of supernatant containing parasite and human DNA), 15 pmol of a pair of synthetic oligonucleotides, 5′-ATGATGGAACAAGTCTGCGACGTTTTCGAT-3′ (forward primer, ST1L) and 5′-TTCATTTAACATTTTATTATTCGTTTTCTT-3′ (reverse primer, ST2L), buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM $MgCl_2$, 200 μM of deoxynucleoside triphosphate (mixture of dGTP, dATP, dTTP, and dCTP), and 1 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 for the first cycle and 1 minute in subsequent cycles, 50°C for 90 seconds for 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 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′-ATTTATGCCATATGTGCATGTTGTAAC-3′) and

DHFR-529R (reverse primer, 5′-CTTTTCTAAAAATTCT-TGATAAACAAC-3′) in a 50-μL reaction mixture as described in the previous paragraph. The same thermocycler 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.

Restriction fragment length polymorphism. The PCR products of some samples collected from patients in Bertoua and Garoua in 2003 were analyzed by restriction endonucleases.[15] Briefly, the secondary amplification products (20 μL) were incubated separately with *Alu* I and *Bsr* I (New England Biolabs, Beverly, MA) for 4 hours at 37°C and 65°C, respectively. The digestion products were subjected to agarose electrophoresis. There is a single restriction site for both enzymes. If there was no digestion with either *Alu* I or *Bsr* I, the presence of Thr-108 was suspected and verified by *Scr*F I digestion.

DNA sequencing. The secondary amplified product was purified using the High Pure PCR Purification kit (Roche Diagnostics) and subjected to electrophoresis on an agarose gel. The concentration of the purified amplification product was determined by comparing the intensity of its band with that of molecular weight markers with known quantity. The 499-base pair product was marked with fluorescent nucleotides in the following mixture: 200 ng of amplified product, 4 μL of Terminator Ready Reaction Mix (Perkin Elmer Corp., Les Ulis, France), 80 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 3.2 pmol of primer DHFR-31, in a final volume of 20 μL. The PTC-100 thermal cycler was programmed as follows: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes, for 25 cycles. Residual dye terminators were removed by precipitation with ethanol. The extension product was sequenced using the ABI Prism automated DNA sequencer (Perkin Elmer Corp.).

Data analysis. Electropherogram data were viewed and analyzed using the Editview sequence analysis software (Perkin Elmer Corp.). An isolate was considered to be of wild-type if the following amino acid residues were present: Ala-16, Asn-51, Cys-59, Ser-108, and Ile-164.[16–18] 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. For amplification products analyzed by restriction endonucleases, *Alu* I and *Bsr* I are specific for the wild-type Ser-108 and the mutant Asn-108 codons, respectively. The presence of 2 digested bands with both *Alu* I and *Bsr* I denotes mixed Ser- and Asn-108 codons. Mixed codons were considered to be mutant.

The sequence data of isolates from Eseka in 1999 ($N = 51$), Bertoua in 1999 ($N = 53$), and Douala in 1999 ($N = 22$) were already published in our previous work.[14] These data were included in the present work to provide the global analysis of the epidemiology of antifolate resistance in Cameroon. The sample population of patients in Yaoundé in 1999 ($N = 60$ patients treated with SP) presented in the present work is different from that of the previous published data from Yaoundé in 1999 ($N = 53$ patients treated with amodiaquine).[14]

Proportions were compared by the Fisher's exact test. The significance level was fixed at 5%.

RESULTS

The following numbers of samples were available for analysis from the study sites (year of collection): Yaoundé/1999, 60 (data from 53 additional isolates previously published were included);[14] Yaoundé/2003, 61; Eseka/1999, 51; Bertoua/1999, 53; Bertoua/2003, 172; Douala/1999, 22; Ndop/2000, 68; Bafoussam/2000, 105; Maroua/2000, 107; Mengang/2001, 25; Hévécam/2001, 126 symptomatic patients and 75 asymptomatic carriers; Sangmélima/2001, 59; Djoum/2001, 67; Garoua/2001, 64; Garoua/2003, 123; Ngaoundéré/2001, 70; and Manjo/2002, 69. Of the total of 1,430 samples analyzed for *dhfr*, 62 (4.3%) failed to yield the amplification product. Most of these samples (36 of 62) for which PCR failed were from a single study site in Hévécam in 2001. The most probable underlying reason is DNA degradation due to an incomplete airdrying of filter papers in a highly humid atmosphere. Of 1,368 samples that were successfully amplified, 1,180 were sequenced, and 188 (samples from Bertoua and Garoua in 2003) were analyzed by enzymatic digestion specific for codon 108.

The wild-type *dhfr* allele was present in 246 of 1,180 (20.8%) sequenced samples. The prevalences of a single Asn-108 mutation (2.8%), double Arg-59/Asn-108 mutations (5.7%), and double Ile-51/Asn-108 mutations (0.8%) were relatively low. The triple Ile-51/Arg-59/Asn-108 mutant allele predominated (62.2%) in Cameroon. Mixed alleles were observed in 7.6% of sequenced samples. The following mutations were not observed in the isolates included in the present study: Val-16 (sequenced in 820 Cameroonian isolates; in additional 360 isolates, the use of nested PCR, instead of semi-nested PCR, did not allow the determination of the sequence of codon 16), Arg-50 (sequenced in 1,180 Cameroonian isolates), Thr-108 (sequenced in 1,180 Cameroonian isolates), and Leu-164 (sequenced in 1,180 isolates). The prevalence of the wild-type and triple mutant alleles showed marked geographic differences (Figure 2). Except in the eastern province (Bertoua) in 2001 (42% triple mutants)[14] and in the northern provinces (Ngaoundéré, Garoua, and Maroua), where the prevalences of triple mutants were 11–35%, the proportion of triple *dhfr* mutations surpassed 60% in the rest of the country. The difference in the prevalence of triple *dhfr* mutants in the eastern (in 2001) and northern provinces, compared with the rest of the country, was statistically significant ($P < 0.05$). An extreme situation was found in Manjo where all isolates were either double ($N = 1$) or triple ($N = 68$) mutants and in Djoum where all but one isolate were double ($N = 2$) or triple mutants ($N = 63 + 1$ with mixed allele). Our data from Hévécam, where blood samples were collected from both symptomatic children aged less than 5 years and asymptomatic schoolchildren aged 5–10 years during the same year, suggest that there is no significant difference ($P > 0.05$) in the prevalence of *dhfr* mutations in parasites infecting these 2 human populations.

The prevalence of *dhfr* mutations was compared at 2 time-points in 3 study sites in the central (Yaoundé, $N = 174$), eastern (Bertoua, $N = 219$), and northern (Garoua, $N = 180$) provinces (Figure 3). The comparison was based on the proportion of isolates with Asn-108 or mixed Asn- and Ser-108 versus pure Ser-108 determined by either restriction enzymes (Bertoua and Garoua in 2003, $N = 188$) or direct sequencing of PCR products. There was no statistically significant difference ($P > 0.05$) in the proportion of mutant Asn-108 allele in

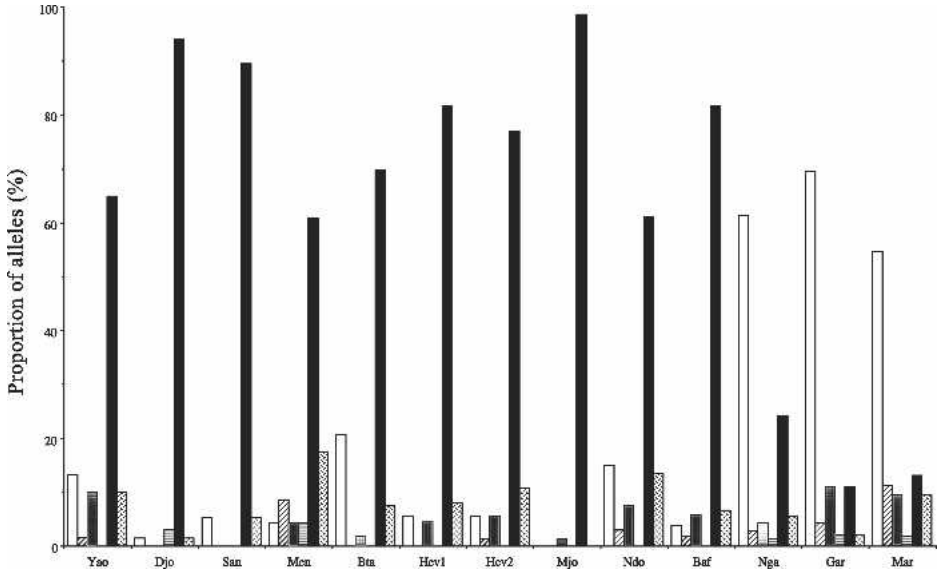

FIGURE 2. Prevalence of wild-type *dhfr* allele (**white bars**), a single Asn-108 mutation (**bars with diagonal lines**), double Arg-59/Asn-108 mutations (**gray bars**), double Ile-51/Asn-108 mutations (**bars with horizontal lines**), triple Ile-51/Arg-59/Asn-108 mutations (**black bars**), and mixed alleles (**cross-hatched bars**) in Cameroon between 1999 and 2003. Central and southern provinces: Yao, Yaoundé in 1999; Djo, Djoum; San, Sangmélima; Men, Mengang. Eastern province: Bta, Bertoua in 2003. Littoral provinces: Hev, Hévécam (Hev 1 refers to symptomatic patients; Hev 2 denotes asymptomatic schoolchildren); Mjo; Manjo. Western provinces: Ndo, Ndop; Baf, Bafoussam. Northern provinces: Nga, Ngaoundéré; Gar, Garoua in 2001; Mar; Maroua. Results on the isolates collected in Eseka, Bertoua in 1999, and Douala have been published.[14]

1999 (88%) and 2003 (90%) in Yaoundé, where a large majority of isolates were mutants. By contrast, there was a significant increase ($P < 0.05$) in the proportions of mutant isolates between 1999 (52%) and 2003 (79%) in Bertoua and between 2001 (45%) and 2003 (63%) in Garoua.

DISCUSSION

The descriptive molecular epidemiology of antifolate resistance presented here is one of the first studies conducted at the national level over an extensive geographic area covering 475,442 km^2. Our results constitute the baseline data for mapping the distribution of *dhfr* alleles in Cameroon. For consistency, parasites were collected from patient populations with similar clinical and parasitological characteristics residing in different parts of the country and from asymptomatic carriers in high-transmission rural areas in the southern and central provinces. In Hévécam, an area of intense malaria transmission, the prevalence of *dhfr* mutations in *P. falciparum* infecting both young patients aged less than 5 years and asymp-

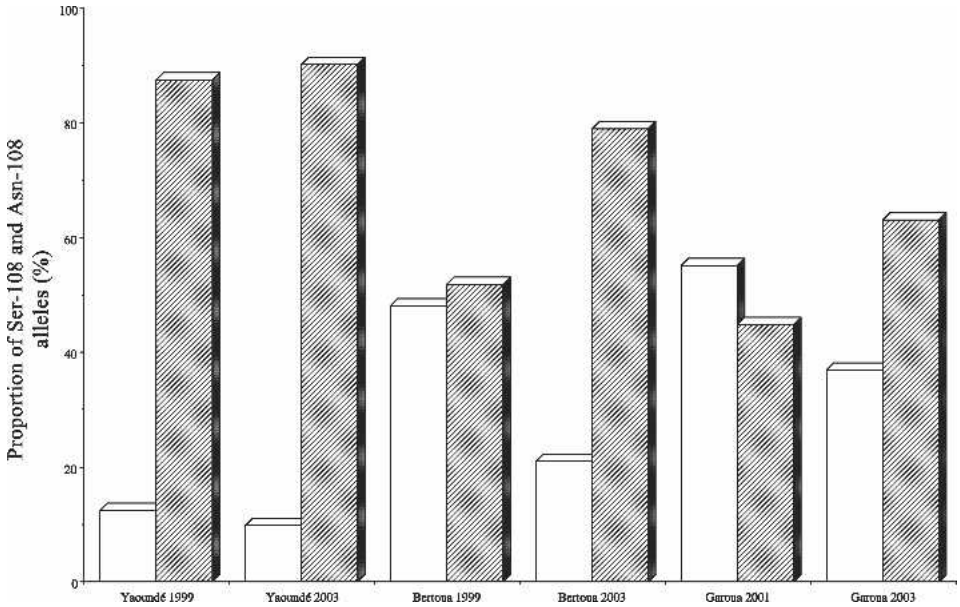

FIGURE 3. Comparison of the proportions of isolates with wild-type (**white bars**) and mutant (**hatched bars**) *dhfr* at 2 timepoints in Yaoundé (central province), Bertoua (eastern province), and Garoua (northern province). The increase in the prevalence of the mutant Asn-108 allele was statistically significant ($P < 0.05$) in Bertoua and Garoua.

tomatic carriers aged between 5 and 10 years was similar. This finding implies that asymptomatic carriers are important sources of parasites, including mutants characterized by antifolate resistance, which are transmitted to the human hosts.

Cameroon has a unique geography ranging from dry sahelian savannah to mountain ranges, plateaus, littoral areas, and dense tropical rain forest. Geographic and climatic variations are associated with several epidemiologic strata and malaria transmission patterns. Surveillance of drug resistance by molecular assays is particularly useful and adapted in this epidemiologic context. By contrast, future surveillance of SP efficacy by *in vivo* tests poses an ethical problem since the current drug of choice for the management of uncomplicated malaria in Cameroon is artesunate-amodiaquine combination, the only officially recommended use of SP is for the intermittent preventive treatment in pregnant women, and early and late treatment failures may occur in 8–12% of Cameroonian patients treated with SP monotherapy.[7,19] Surveillance by *in vitro* drug sensitivity assays would be impractical and too cumbersome to organize at different sentinel sites in the country. In addition, *in vitro* assays are either redundant due to the high correlation between *in vitro* pyrimethamine response and *dhfr* mutations or of limited interest due to the technical difficulties in performing assays for sulfadoxine.[9–11,20]

Our preliminary molecular data have suggested that there may be an east-to-west gradient of increasing proportions of triple *dhfr* mutations along Bertoua–Yaoundé–Eséka–Douala axis (i.e., from the interior of the country towards the coastal area in the southern and central regions of Cameroon).[14] The present data further suggest the existence of a marked difference in the epidemiology of antifolate resistance along the north-south axis in Cameroon. The northern half of the country is generally characterized by a sahelian geography, tropical climate with 2 distinct seasons, and seasonal malarial transmission periods, whereas the southern half of the country is generally characterized by humid, tropical rain forest and stable malaria transmission.

At 2 sites in the eastern (Bertoua) and northern (Garoua) provinces where the proportions of *dhfr* mutants were compared at an interval of 2–4 years, our results suggested an evolution towards an increased proportion of *dhfr* mutants. At both sites, the initial proportion of *dhfr* mutants was close to 50%. In the present study, there was no observable difference in the proportions of *dhfr* mutants between 1999 and 2003 in Yaoundé, where a high proportion of *dhfr* mutants has been attained. The evolution of antifolate resistance has been monitored regularly in Yaoundé since 1994. For our longitudinal studies, *P. falciparum* isolates obtained from symptomatic children aged more than 12 years and adults have been analyzed. Our results showed that the proportion of *dhfr* mutants (pure Asn-108 and mixed Ser-108 and Asn-108) has increased from 48% in 1994–1995 to 71% in 1997–1998 and 93% in 2000–2001.[21,22] These data are supported by *in vitro* pyrimethamine responses that are highly correlated with the number of *dhfr* mutations. Although more longitudinal data are required for a more conclusive evidence in Bertoua and Garoua, an even higher proportion of *dhfr* mutants may be expected in the coming years unless the national antimalarial drug policy is rapidly implemented throughout the country.

Several studies have suggested that triple *dhfr* mutations, with or without additional mutations in the *dhps* gene, are associated with clinical resistance to SP in Africa.[11,23,24] Recent molecular studies conducted in East Africa have suggested that the combined presence of triple *dhfr* mutations and double Gly-437/Glu-540 *dhps* mutations, referred to as the "quintuple genotype," may be associated with a high risk of SP treatment failure.[25–29] However, in other regions of the African continent, Glu-540 does not appear to be common at present. In our series of studies in Cameroon, this *dhps* mutation has not been detected. Yet, SP treatment failure due to recrudescence has been observed in our studies. These observations suggest that further clinical and molecular monitoring of SP efficacy in different African countries, in particular where SP is currently recommended for intermittent preventive treatment in pregnant women, is required to define the *dhfr* and *dhps* mutations that should be monitored for the changing patterns of antifolate resistance.

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