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MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XI. GEOGRAPHIC DISTRIBUTION OF *PLASMODIUM FALCIPARUM* ISOLATES WITH DIHYDROFOLATE REDUCTASE GENE MUTATIONS IN SOUTHERN AND CENTRAL CAMEROON

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Abstract. The DNA sequence of the dihydrofolate reductase (*dhfr*) gene, a molecular marker for pyrimethamine resistance, was determined for 178 field isolates of *Plasmodium falciparum* collected along the east-west axis in southern Cameroon. The proportion of isolates having the wild-type *dhfr* allele varied from 48.1% in the east (city of Bertoua) to 11.3–15.7% in central provinces (Yaounde and Eseka) and 0% in the littoral region (port city of Douala). Isolates with a single Asn-108 mutation or double mutations (Ile-51 or Arg-59 and Asn-108) constituted approximately 10% of the samples. Isolates with triple mutations (Ile-51, Arg-59, and Asn-108) were present in an equal proportion (48.1%) as the wild-type isolates in the east (Bertoua), while triple mutations predominated in Yaounde (62.3%), Eseka (62.7%), and Douala (78.9%). The distribution of triple *dhfr* mutations along the east-west axis in southern Cameroon suggests the presence of a decreasing gradient from the west coastal region to the central region and then to the east towards the interior of the country.

INTRODUCTION

Recent developments in molecular biology of *Plasmodium falciparum* have shown that analysis of genes encoding the target or transporter of an antimalarial drug may be a reliable method to detect drug resistance.[1] Several candidate enzymes and transporters involved in drug resistance have been identified, including dihydrofolate reductase (bifunctional enzyme DHFR-thymidylate synthase [TS]; for antifolate resistance), dihydropteroate synthase (DHPS; for sulfonamide resistance), Pgh1 encoded by *P. falciparum* multidrug-resistance gene 1 (*pfmdr1*; for resistance to 4-aminoquinolines, amino alcohols, and artemisinin derivatives), CG2 transporter (for chloroquine resistance), *P. falciparum* chloroquine resistance transporter (PfCRT; for chloroquine resistance), and cytochrome bc1 complex (for atovaquone resistance). Among these molecular markers, DHFR is one of the most studied enzyme, not only in *P. falciparum* but also in other organisms. Pyrimethamine specifically inhibits malarial DHFR by occupying the active site of the enzyme. The mechanism of pyrimethamine resistance involves amino acid substitutions at key residues surrounding the active site of the enzyme, leading to decreased affinity between the mutant enzyme and pyrimethamine.[2–4]

Point mutations leading to amino acid substitutions at distinct positions in the *dhfr* gene have been demonstrated to be highly correlated with the *in vitro* response to pyrimethamine in reference clones and field isolates.[5–8] Further evidence that there is a causal relationship between amino acid substitutions and pyrimethamine resistance has been provided by kinetic studies of DHFR expressed in heterologous systems and transfection with plasmids carrying mutant *dhfr*.[4,9,10] These studies have provided strong evidence that a single Ser- to Asn-108 change is associated with pyrimethamine resistance *in vitro*. The presence of other amino acid substitutions (Ala- to Val-16, Asn- to Ile-51, Cys- to Arg-59, and Ile- to Leu-164), in addition to the Ser- to Asn-108 change (Ser- to Thr-108 change in the case of Ala- to Val-16 substitution), increases the level of pyrimethamine resistance.[4,6,8,11,12] Recent studies conducted in sub-Saharan Africa have suggested that multiple *dhfr* mutations are also correlated with the *in vivo* response to the drug combination sulfadoxine-pyrimethamine.[13,14]

Experimental and field data indicate that characterization of *dhfr* sequence in field isolates of *P. falciparum* may provide an additional and alternative source of information in describing the epidemiology of drug-resistant malaria. With this perspective in view, blood samples obtained as part of clinical studies conducted at different sentinel sites located in southern and central Cameroon were analyzed to determine the *dhfr* sequences.

MATERIALS AND METHODS

Study sites. Blood specimens were collected from the following urban sites in Cameroon: Yaounde, Eseka, Douala, and Bertoua (Figure 1). Yaounde is the capital of Cameroon and is situated in the tropical rain forest region. The city is on a hilly plateau at an average altitude of 760 meters. Its estimated population is one million inhabitants. Douala is the largest city in Cameroon, with an estimated population of two million. The city is located in the coastal region, approximately 260 km west of Yaounde. Eseka is situated in the Central Province and within the southern belt of tropical rain forest area in Cameroon, approximately 140 km southwest of Yaounde. The population of the city of Eseka is 22,000 inhabitants. Bertoua, with an estimated population of 100,000, is located approximately 350 km east of Yaounde. It lies within the tropical rain forest area, but its geography is intermediate between rain forest and savannah type of forest. In each city, dispensaries and/or district hospitals were selected on the basis of the mean number of consultations of patients with fever presumably due to malaria (more than 50 per month) and the presence of a sufficient number of health personnel, adequate diagnostic facilities, and hospital beds.

These urban sites are easily accessible by road. The east-west axis Bertoua-Yaounde-Eseka-Douala is one of the bus-

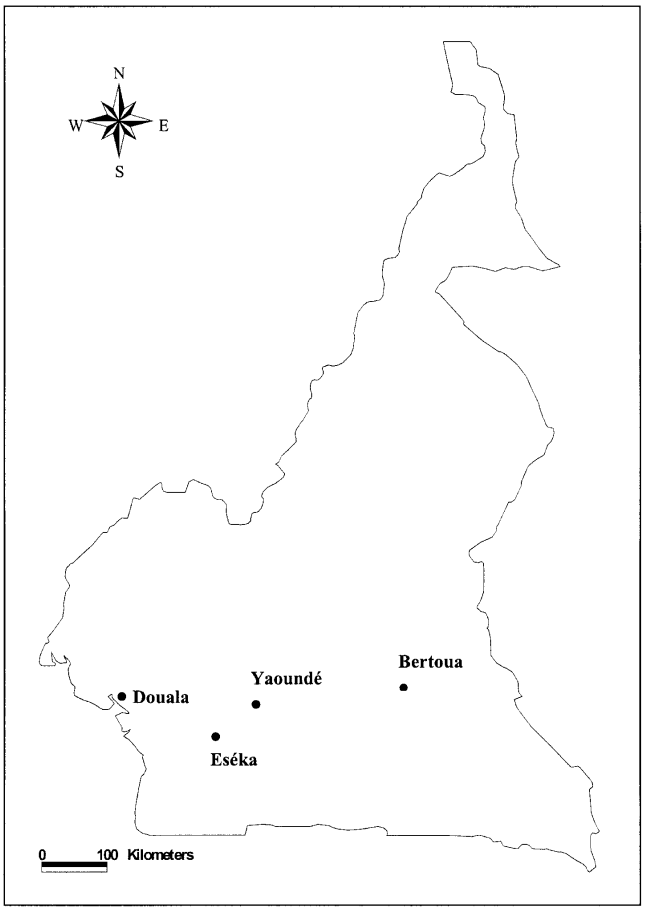

FIGURE 1. Study sites in southern Cameroon.

iest route and provides a vital economic link between the Central African Republic and the port city of Douala. Malaria transmission is intense and continuous throughout the year in rural areas, but is much less intense in urban sites in southern and central Cameroon, with peak seasons corresponding to the rainy seasons (March-May and September-November). Although there are no reliable epidemiologic data on malaria prevalence in Central Africa, it was assumed, for the purpose of this study, that the level of malaria exposure is roughly comparable in these four urban sites. *In vivo*, our recent studies in Yaounde and two additional sites in the central and southern regions have shown a high cure rate (> 90%) with sulfadoxine-pyrimethamine on day 14 in symptomatic young children less than five years old (Soula G, unpublished data).

Patients. Malaria-infected symptomatic children between six months and five years old were enrolled in the clinical studies if the following criteria of the World Health Organization were met: the presence of *P. falciparum* without other *Plasmodium* species, a parasitemia > 2,000 asexual parasites per microliter of peripheral blood, a fever ≥ 37.5°C, a hematocrit > 15%, and the absence of severe malnutrition, other infectious diseases that may be the origin of fever, and signs and symptoms of severe and complicated malaria.[15] After informed consent was obtained, patients were treated with either chloroquine or amodiaquine. The clinical studies were conducted in similar patient populations at different sites in 1999. This study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

Extraction of DNA. Fingerprick capillary blood was obtained to prepare thick blood smears, and approximately 5–10 μL of blood were blotted onto either Whatman (Hillsboro, OR) 3MM filter paper or Isocode Stix® (Schleicher & Schuell, Ecquevilly, France). Blood samples were dried overnight and sealed in a plastic bag for conservation until use. The DNA was extracted from filter papers according to the methods described by Long and others[16] and Henning and others.[17] Briefly, a 3 mm × 3 mm piece of Whatman 3MM filter paper was cut and fixed with methanol for 5 min (fixation is unnecessary for the Isocode Stix® filter). After drying, the filter paper was placed into a 0.5-mL microtube to which 50 μL of sterile distilled water were added. The filter paper was incubated at 99°C for 30 min and agitated for a few seconds. The supernatant (10 μL) was used directly for amplification of the *dhfr* gene fragment. Both types of filter papers yielded similar quantity of amplified gene fragments.

Polymerase chain reaction. Because of the small quantity of parasite DNA on filter paper, a semi-nested polymerase chain reaction was required to obtain a sufficient amount of DNA for the sequencing reaction. In the primary polymerase chain reaction, the entire DHFR domain (708 basepairs) of the *dhfr-ts* gene 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 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 two minutes for the first cycle and 30 seconds in subsequent cycles, 50°C for one minute for the first cycle and 30 seconds in subsequent cycles, and 72°C for one minute in all cycles, for a total of 30 cycles.

In the secondary polymerase chain reaction, a 595-basepair fragment was amplified from the primary amplification product by using the primer pairs ST1L and DHFR-595R (5′-CTGGAAAAAATACATCACATTCATATGTAC-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 1.8% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the 595-basepair DNA fragment.

Sequencing of DNA. 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 595-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

$MgCl_2$, and 3.2 pmol of primer 5′-ATGATGGAACAAGTCTGCGACGTTTTC-3′, 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 four 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 wild-type if the following amino acid residues were present: Ala-16, Asn-51, Cys-59, Ser-108, and Ile-164.[5–8,11,12] 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. Descriptive analysis of the proportion of wild-type and different combination of mutant *dhfr* alleles was performed for each study site.

RESULTS

A total of 178 blood samples were available for DNA analysis: 53 from Yaounde, 51 from Eseka, 52 from Bertoua, and 22 from Douala. The number of samples was relatively small in Douala, as compared with other urban sites, because the clinical study with chloroquine was terminated prematurely due to a high therapeutic failure rate. The sampling method, called double lot quality assurance method, allowed the termination of the clinical study on the basis of the initial results obtained in a small sample size if a very high level of treatment failure was observed. With the exception of three successive samples from Douala, all other samples (n = 175; 98%) yielded sufficient amplified *dhfr* fragment for DNA sequencing. The reason for the failure to obtain amplified products from three samples from Douala is not clear, but it may be due to inadequate drying of samples before storage or poor conservation of filter papers.

The frequency distribution of all observed combinations of *dhfr* alleles in the study sites is shown in Figure 2. Bertoua had the highest proportion of wild-type *dhfr* allele (48.1%) compared with the other urban sites (Yaounde, 11.3%; Eseka, 15.7%; Douala, 0%). Only a small proportion, ranging from 1.9% in Bertoua to 5.3% Douala, had a single Asn-108 mutant allele. Similarly, a relatively small proportion of isolates (from 5.3% in Douala to 9.8% in Eseka) had double mutations. Except for Bertoua, where an almost equal proportion of isolates carried wild-type alleles (48.1%) and triple mutant alleles (42.3%), triple mutants predominated in Yaounde (62.3%), Eseka (62.7%), and Douala (78.9%).

Overall, the wild-type *dhfr* allele represented 22.3% (39 of 175) of samples collected in southern and central Cameroon. Isolates with a single Ser- to Asn-108 substitution (n = 5; 2.9%) or double mutations (Asn-108 and either Ile-51 [n = 1; 0.6%] or Arg-59 [n = 12; 6.9%]) constituted approximately 10% of the samples. The majority of isolates carried the triple mutations Ile-51, Arg-59, and Asn-108 (n = 102; 58.3%). The remaining isolates (n = 16) had mixed alleles with at least one mutant allele (Asn-108), with or without mutant alleles Ile-51 and/or Arg-59. None of the isolates had mutant allele Val-16, Thr-108, or Leu-164. Other *dhfr* polymorphisms reported from South America, such as Cys-50 and Bolivia repeat, were not found in any of our samples. No new mutation was identified within the 595-basepair fragment analyzed in this study.

DISCUSSION

Until recently, the epidemiology of drug-resistant malaria was based on the collection of *in vivo* data from asymptomatic

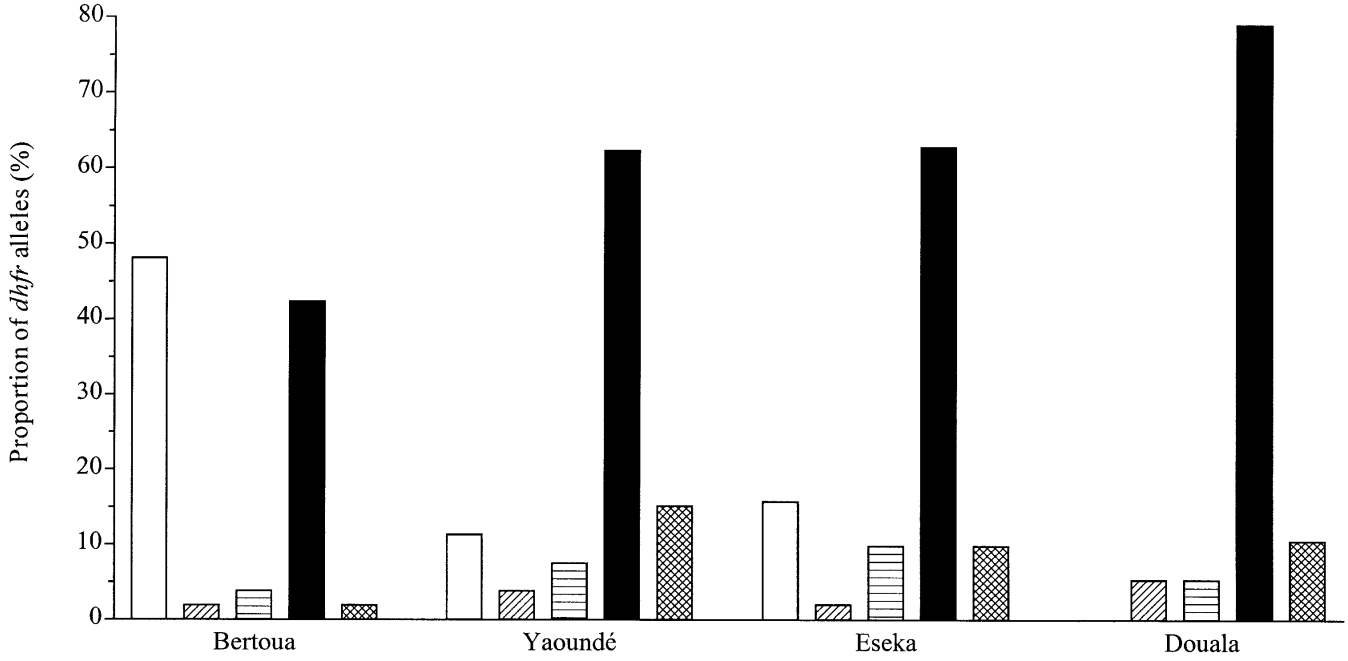

FIGURE 2. Proportions of *Plasmodium falciparum* isolates with wild-type dihydrofolate reductase (*dhfr*) (**white bars**), a single Ser- to Asn-108 *dhfr* mutation (**bars with diagonal lines**), double Arg-59 and Asn-108 *dhfr* mutations (**bars with horizontal lines**), triple Ile-51, Arg-59, and Asn-108 *dhfr* mutations (**black bars**), and mixed *dhfr* alleles (**cross-hatched bars**) at different study sites.

carriers or symptomatic patients to whom different antimalarial drugs were administered and, to a lesser extent, on *in vitro* drug sensitivity assays. The present study evaluated the feasibility and utility of the third approach, based on the analysis of molecular targets, for the epidemiologic description of drug-resistant malaria. Our data suggest a heterogeneous distribution of *dhfr* polymorphisms associated with pyrimethamine resistance along the east-west axis in southern and central Cameroon. In the east, near the border with Central African Republic, there is an almost equal proportion of wild-type allele and triple mutant alleles, with a few single or double mutant alleles. The situation in the central part of the country, represented by Yaoundé and Eseka, seems to be more homogeneous, with similar proportions of different alleles at both sites. Further west towards the littoral region, there seems to be an extreme situation at Douala with no wild-type allele and a high proportion of triple mutants. It is open to speculation whether these results reflect the presumed level of drug pressure due to the consumption of sulfadoxine-pyrimethamine, as well as antibiotics containing antifolates (e.g., co-trimoxazole), and the relative financial ease in Douala, the economic capital of the country, compared with other smaller cities along the east-west axis in southern and central Cameroon. The heavy circulation of people along this route also needs to be taken into consideration for the further understanding of the spread of antifolate resistance in this region. However, our data on the geographic distribution of *dhfr* polymorphisms need to be interpreted with some caution since the number of samples from Douala was limited, compared with the other sites. Nevertheless, our data suggested the possible existence of a gradient, with decreasing proportion of isolates carrying triple *dhfr* mutations from the western coastal area (Douala) to the interior of the country. This hypothesis may provide a useful starting point to guide other epidemiological studies in Cameroon.

One of the unexpected trends in our data was the predominance of triple mutant alleles, followed by wild-type alleles in Yaounde, Eseka, and Douala. In a somewhat similar way, isolates were predominantly wild-type or triple mutants in Bertoua. Thus, there seemed to be a leap from a wild-type allele to triple mutations, rather than a gradual accumulation of mutations in the *dhfr-ts* gene. Part of the explanation may lie in the presence of mixed alleles, which represent the transition state. To obtain a clearer understanding of the dynamics of the spread of antifolate resistance, further studies are needed, for example, in asymptomatic carriers who may serve as reservoirs of malaria transmission, mosquito infectivity of gametocytes carrying different *dhfr* alleles, and the fate of mixed alleles in multiple *P. falciparum* populations during the passage in mosquitoes.

Several advantages of the molecular approach were observed during our study. In contrast to *in vivo* tests, field collection of blood samples for molecular analysis can be completed within a few days or weeks by health personnel without advanced medical training. Samples blotted onto filter papers can be stored at room temperature and sent to reference centers, even through the local post office. The collection of samples in the field is thus greatly simplified and can be done by personnel at local primary and secondary health centers. The expenditures for field work involved in data collection should therefore be considerably reduced, compared with the mobilization of a team of experienced clinical workers to conduct *in vivo* tests in far-fetched regions.

As for the laboratory analysis of samples, the molecular approach has several advantages over the *in vitro* drug sensitivity assay. First, several dozens of isolates can be simultaneously treated for amplification reactions. Second, *in vitro* drug assays based on the incorporation of tritium-labeled hypoxanthine require a minimal initial parasitemia of 0.1%. Under optimal conditions, a nested polymerase chain reaction can amplify the target gene even if the parasitemia is less than 0.001%.[18] Third, parasites obtained from patients who self-medicated before consultation usually do not grow *in vitro*, leading to uninterpretable drug assays. In contrast, DNA extracted from patients who self-medicated and who still have a positive blood smear does yield amplification product for further sequence analysis (Basco LK, unpublished data). Fourth, the use of isotopes can be avoided by molecular techniques.

Despite these advantages, the molecular approach should probably be considered an experimental procedure reserved for reference laboratories in Africa at the present time. From the technical viewpoint, there is still no single, universally accepted polymerase chain reaction protocol to amplify the target genes. Likewise, several techniques have been proposed to determine DNA sequences, including hybridization with DNA probes, allele-specific nested polymerase chain reaction, polymerase chain reaction coupled with restriction fragment length polymorphism, and direct sequencing of amplified products. In addition, technical performance of molecular approaches requires skilled technicians and/or trained scientists, and most required equipment and reagents are expensive. Nevertheless, these obstacles will probably be surmounted in the near future in Africa. As more data become available from different epidemiologic settings and molecular markers are validated so that both *in vitro* and *in vivo* responses to antimalarial drugs can be predicted accurately by the characterization of genetic profile of target genes, molecular approaches to describe the epidemiology of drug-resistant *P. falciparum* can be expected to become a valuable tool and may even supplant other existing methods.

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