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MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDE, CAMEROON IV. EVOLUTION OF PYRIMETHAMINE RESISTANCE BETWEEN 1994 AND 1998

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Abstract. Pyrimethamine, in combination with sulfadoxine, is currently one of the major alternative drugs used for the treatment of chloroquine-resistant *Plasmodium falciparum* malaria infections in Africa. The mechanism of pyrimethamine resistance has been strongly associated with a single, key point mutation in the dihydrofolate reductase–thymidylate synthase gene, resulting in the substitution of the wild-type allele Ser-108 by either Asn-108 or Thr-108. The pyrimethamine-resistant phenotype and/or genotype were determined in 273 Cameroonian clinical isolates obtained in Yaounde by *in vitro* assays and polymerase chain reaction–restriction fragment length polymorphism over a 5-year period. The *in vitro* assays showed that 42% (18 of 43) and 63% (69 of 110) of the isolates obtained in 1994–1995 and 1997–1998, respectively, were resistant to pyrimethamine (50% inhibitory concentration [IC_{50}] > 100 nM). The polymerase chain reaction showed that 43% (55 of 127) and 59% (50 of 85) of the isolates in 1994–1995 and 1997–1998, respectively, had the mutant Asn-108 allele. The pyrimethamine-resistant genotype (Asn-108) corresponded with the pyrimethamine-resistant phenotype ($IC_{50} \geq 100$ nM) in a large majority (> 95%) of the isolates. The results of our study suggest an increasing prevalence of pyrimethamine resistance in Yaounde. Our study further suggests that pyrimethamine resistance can be monitored by a technique that can be adopted by malaria research centers in Africa.

Pyrimethamine, in combination with sulfadoxine, is one of the major antimalarial drugs currently used in Africa.[1] Pyrimethamine, a 2,4-diaminopyrimidine derivative, was synthesized and tested for antimalarial activity in the United Kingdom during the Second World War.[2] Because of its relatively slow action and the availability of chloroquine, pyrimethamine was mainly used for malaria chemoprophylaxis from the late 1940s to 1960s. Numerous cases of prophylactic failure were reported in the 1950s, usually within 1 or 2 years after pyrimethamine was massively used in an endemic region for monoprophylaxis.[3,4] The discovery of the synergistic effect of pyrimethamine and sulfonamides or sulfones, which can overcome a moderate level of pyrimethamine resistance, led to the use of this combination for both prophylaxis and treatment of chloroquine-resistant *Plasmodium falciparum in* the late 1960s and 1970s.[3] However, resistance to sulfadoxine-pyrimethamine combination became widespread in South America and Southeast Asia in the 1980s, and its use for chemoprophylaxis was discontinued due to the potentially serious side effects associated with sulfonamides.[5] In the 1990s, the spread of chloroquine resistance has led to an increasing use of sulfadoxine-pyrimethamine to treat chloroquine-resistant *P. falciparum* infections in Africa.[6]

Sulfadoxine-pyrimethamine is currently the first-line drug in several east African countries and second-line drug in west and central Africa, including Cameroon. Its characteristic features include a high efficacy against chloroquine-resistant *P. falciparum* parasites, good tolerance, high compliance due to the single-dose therapy, and safety in young children. Pyrimethamine is one of the few antimalarial drugs whose biochemical and genetic mechanism of action and mechanism of resistance have been well established.[7,8] Early biochemical studies in bacteria, mammals, and malaria parasites have shown that pyrimethamine, a structural analog of dihydrofolate, inhibits folate metabolism by binding to dihydrofolate reductase (DHFR) with a high affinity.[9,10] The molecular basis of the selection of pyrimethamine-resistant *P. falciparum* was elucidated when the gene coding for DHFR was isolated, cloned, and sequenced.[11,12] A single point mutation in the amino acid residue 108, resulting in the substitution of the wild-type allele Ser by the mutant allele Asn (or Thr), was shown to be associated with drug resistance.[7,13–15]

In our previous study, we have shown that 43% of the isolates in Yaounde were carriers of the mutant Asn-108 allele and that a similar proportion of the parasites were resistant *in vitro* to pyrimethamine.[16] In the present study, we analyzed the key DHFR allele of the clinical isolates obtained in 1997–1998 with the aim to 1) monitor pyrimethamine resistance by techniques that could be performed in African malaria reference centers and 2) establish whether there is any change in the proportion of the pyrimethamine-resistant isolates in Yaounde.

MATERIALS AND METHODS

Patients. The study was part of the clinical trials conducted in Yaounde, Cameroon since 1994.[17,18] A total of 273 venous blood samples from malaria-infected Cameroonian patients residing in Yaounde were analyzed. The results of 136 samples collected in 1994–1995 were presented in our earlier work.[16] These data were compared with those of the samples collected during 1997 and 1998. Similar patient populations were studied during the 5-year period by applying the same inclusion criteria to screen symptomatic patients presenting spontaneously at the Nlongkak Catholic missionary dispensary in Yaounde. Inclusion criteria included an age ≥ 5 years old, fever at consultation (or history of fever within the past 24 hr), monoinfection with *P. falciparum* (parasite density > 5,000 asexual parasites/μl of blood), no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result,[19] easy access to health services in Yaounde for daily

monitoring, and informed consent by the patient and/or patient's guardian in the case of children. Our previous studies have shown that clinical parameters and hematologic and blood biochemistry do not differ significantly between the adult population (> 15 years old) and the pediatric population (between 5 and 15 years old); thus, the patients constitute a homogeneous population for our clinical studies.[17,18] Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization,[20] severe anemia (hemoglobin < 5.0 g/dl), and moderate and severe malnutrition, were excluded. The patients were treated with chloroquine, amodiaquine, pyronaridine, or sulfadoxine-pyrimethamine and, for those responding with a therapeutic failure, quinine or halofantrine. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

Parasite DNA. The FCR3/Gambia strain was maintained in a continuous culture according to the standard technique described by Trager and Jensen.[21] This strain was used as a control for the Thr-108 residue in the DHFR-thymidylate synthase (TS) gene since this amino acid residue appears to be a rare mutation in African isolates.[22–25] Clinical isolates of *P. falciparum* were obtained by venipuncture before treatment. Venous blood samples (5–10 ml of whole blood) were collected in a Vacutainer tube coated with an anticoagulant (EDTA) (Terumo Europe N. V., Leuven, Belgium) and washed 3 times in folate- and *p*-aminobenzoic acid-free RPMI 1640 medium by centrifugation (2,000 × *g* for 10 min) within 3 hr after blood collection. An aliquot of 1.5–2 ml of red blood cell pellet was used to extract parasite DNA (contaminated with human leukocyte DNA).

Infected erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centrifuged at 2,000 × *g* for 10 min and the pellet was transferred to a 1.5-ml microfuge tube and suspended in 500 μl of NET buffer. The mixture was treated with 1% N-lauroylsarcosine (Sigma Chemical Co., St. Louis, MO) and RNAse A (100 μg/ml) at 37°C for 1 hr and proteinase K (200 μg/ml) at 50°C for 1 hr. Parasite DNA was extracted 3 times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (v/v/v 25:24:1), and chloroform-isoamyl alcohol (v/v 24:1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20°C until use.

Polymerase chain reaction. The polymerase chain reaction–restriction fragment length polymorphism protocol used in this study was modified from the methods that were previously described.[16,26,27] The entire DHFR domain of the DHFR-TS gene of *P. falciparum* was amplified by the polymerase chain reaction using the PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: approximately 200 ng of genomic DNA, 15 pmol of primers 5′-ATGATGGAACAAGTCTGCGACGTTTTCGAT-3′ (sense) and 5′-TTCATTTAACATTTTATTATTCGTTTTC-TT-3′ (anti-sense), buffer (25 mM KCl, 5 mM $(NH_4)_2SO_4$, 10 mM Tris, pH 8.8), 2 mM $MgSO_4$, 200 μM dNTP, and 1 unit of *Pwo* DNA polymerase (Roche Diagnostics, Meylan, France) in a 50-μl reaction at 94°C for 2 min for the first cycle and 30 sec in subsequent cycles, 50°C for 1 min for the first cycle and 30 sec in subsequent cycles, and 72°C for 1 min in all cycles, for a total of 30 cycles. Five microliters of the amplification product was loaded on a 1.2% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the 708-basepair DNA fragment.

Restriction fragment length polymorphism. Approximately 1–2 μg of the amplified DHFR-TS gene fragment were incubated separately with *Alu* I (New England Biolabs, Beverly, MA), *Bsr* I (New England Biolabs), and *Scr*F I (New England Biolabs) restriction endonucleases in a mixture containing different buffers (for *Alu* I, 5 mM NaCl, 1 mM Tris-HCl, 1 mM $MgCl_2$, and 0.1 mM dithiothreitol, pH 7.9; for *Bsr* I, 10 mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, and 0.1 mM dithiothreitol, pH 7.9; for *Scr*F I, 5 mM potassium acetate, 2 mM Tris-acetate, 1 mM magnesium acetate, and 0.1 mM dithiothreitol, pH 7.9) in a final volume of 50 μl. The reaction mixtures were incubated for 3 hr at 37°C with *Alu* I and *Scr*F I and at 65°C with *Bsr* I. At the end of the incubation period, the restriction enzymes were heat-inactivated for 20 min at 65°C for *Alu* I and *Scr*F I and 80°C for *Bsr* I. Ten microliters of the restriction fragments were loaded on a 1.2% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized by ultraviolet transillumination.

Only a single restriction site for *Alu* I, *Bsr* I, and/or *Scr*F I is present in the 708-basepair polymerase chain reaction product, depending on the amino acid residue at position 108 of the DHFR-TS gene. Thus, the cleavage of the 708-basepair DNA fragment into 2 fragments (324 basepairs and 384 basepairs) by *Alu* I, *Bsr* I, and *Scr*F I indicates the presence of the wild-type codon Ser-108, mutant codon Asn-108, and mutant codon Thr-108, respectively. If 2 or 3 restriction enzymes yielded 2 cleaved fragments, the presence of mixed alleles was deduced. The wild-type Ser-108 allele in the DHFR-TS gene is associated with pyrimethamine sensitivity, while the mutant Asn-108 allele is associated with pyrimethamine resistance.[7,13–16,22,24–27] It has been suggested that the mutant Thr-108 allele may be associated with cycloguanil (active metabolite of proguanil) resistance.[28,29]

***In vitro* assay.** *In vitro* drug sensitivity assays were performed on the clinical isolates without prior adaptation to the *in vitro* culture conditions. Infected erythrocytes were suspended in the complete, folate- and *p*-aminobenzoic acid-free RPMI 1640 medium consisting of 10% non-immune human serum, 25 mM HEPES, 25 mM $NaHCO_3$ at a hematocrit of 1.5% and an initial parasitemia of 0.2–1.0%. If the blood sample had a parasitemia > 1.0%, fresh uninfected, type A^+ erythrocytes were added to adjust the parasitemia to 0.6%.

The isotopic microtest developed by Desjardins and others was used in this study.[30] 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 5% CO_2 for 18 hr. ^{3}H-hypoxanthine (1 μCi/well; Amersham International, Plc., Buckinghamshire, United Kingdom) was added to assess parasite growth. After an additional 48 hr of incubation, the plates were frozen to terminate the *in vitro* drug sensitivity assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell har-

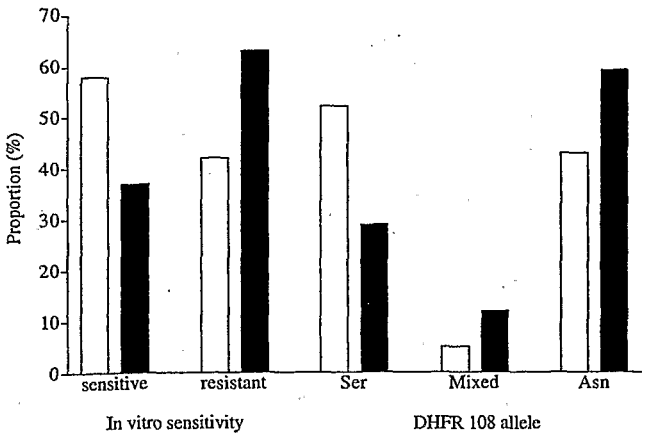

FIGURE 1. Proportions of pyrimethamine-sensitive (50% inhibitory concentration [IC_{50}] < 100 nM) isolates, pyrimethamine-resistant ($IC_{50} \geq 100$ nM) isolates, and parasites with the wild-type dihydrofolate reductase (DHFR) allele Ser-108, mutant allele Asn-108, or mixed alleles (Ser + Asn) in samples of Cameroonian isolates of *Plasmodium falciparum* collected in 1994–1995 (**white columns**) and 1997–1998 (**black columns**).

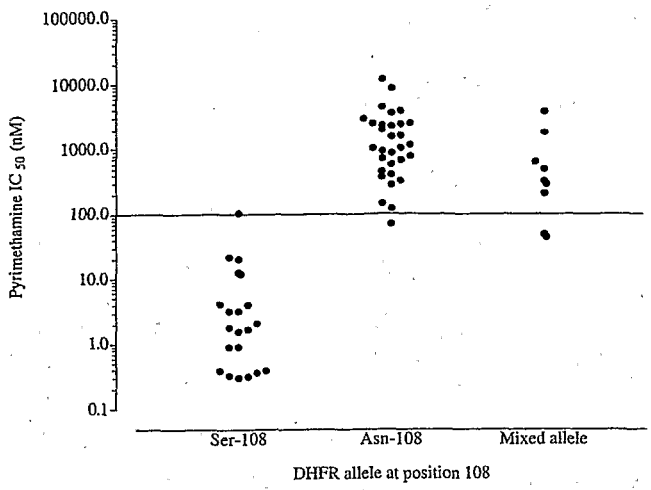

FIGURE 2. Distribution of the 50% inhibitory concentrations (IC_{50}s), expressed in nM, for the Cameroonian *Plasmodium falciparum* isolates carrying the wild-type dihydrofolate reductase (DHFR) allele Ser-108, mutant allele Asn-108, and mixed alleles (Ser + Asn). The threshold IC_{50} value for pyrimethamine resistance was arbitrarily defined as ≥ 100 nM in our previous studies.[31,32]

vester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant®; Amersham International, Plc.) 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 corresponding to 50% of the uptake of ^{3}H-hypoxanthine measured in the drug-free control wells, was determined by nonlinear regression analysis of the logarithm of concentrations plotted against the parasite growth inhibition. The best-fitting sigmoid curve was derived by using the Prism® software (GraphPad Software, Inc., San Diego, CA). The pyrimethamine IC_{50} values were classified as sensitive (< 100 nM) or resistant (≥ 100 nM), as in our previous studies.[31,32]

Sampling and statistical tests. The method of selection of blood samples collected during 1994 and 1995 for either genotypic (n = 127), phenotypic (n = 43), or both genotypic and phenotypic studies (n = 34) was described in our previous study.[16] For samples collected during 1997 and 1998, all available fresh blood samples (n = 110) were tested for *in vitro* pyrimethamine sensitivity, regardless of the treatment that the patient received. Both phenotypic and genotypic markers were determined for 58 of 110 samples. These correspond to samples from patients treated with sulfadoxine-pyrimethamine. The genotype, but not the phenotype, of 27 additional samples obtained from adult patients treated with amodiaquine was determined. Samples from children treated with amodiaquine, chloroquine, or pyronaridine were not tested for genotypic analysis. The statistical significance of the difference in the proportions of pyrimethamine resistance, determined by *in vitro* assays (n = 153) and molecular techniques (n = 196), between the 2 time periods was evaluated by the chi-square test. Samples with mixed Ser and Asn-108 alleles (n = 16) were not taken into consideration in the statistical analysis.

RESULTS

The *in vitro* pyrimethamine sensitivity was determined in 153 clinical isolates during 1994–1995 and 1997–1998. Twenty-five (58%) of 43 isolates obtained in 1994–1995 and 41 (37%) of 110 isolates obtained in 1997–1998 were sensitive to pyrimethamine (IC_{50} < 100 nM) (Figure 1). The amino acid residue of the key allele at position 108 of the DHFR-TS gene was determined in a total of 212 Cameroonian isolates obtained in Yaounde. Of 127 isolates obtained in 1994–1995, 66 (52%), 55 (43%), and 6 (5%) presented the wild-type allele Ser-108, mutant type allele Asn-108, and mixed alleles Ser-108 + Asn-108, respectively (Figure 1). Of 85 isolates collected in 1997–1998, 25 (29%), 50 (59%), and 10 (12%) isolates had Ser-108, Asn-108, and mixed alleles, respectively. For both phenotypic and genotypic markers of pyrimethamine resistance, there was a statistically significant increase ($P < 0.05$) in the proportion of drug resistance in 1997–1998 compared with 1994–1995. Except for the polymerase chain reaction product amplified from the FCR3 strain, none of the 85 samples obtained in 1997–1998 yielded a digestion product after treatment with *Scr*F I, indicating the absence of the Thr-108 amino acid residue among our clinical isolates.

The *in vitro* activity of pyrimethamine and DHFR allele were determined for 58 of 85 isolates collected during 1997 and 1998. The distribution of the pyrimethamine IC_{50} value in relation to the key DHFR allele 108 is shown in Figure 2. Nineteen of 20 isolates carrying the wild-type Ser-108 allele were pyrimethamine sensitive, and 30 of 31 isolates carrying the mutant Asn-108 allele were pyrimethamine resistant. One pyrimethamine-sensitive isolate (IC_{50} = 104 nM) and 1 pyrimethamine-resistant isolate (IC_{50} = 71 nM) displayed discordant IC_{50} values, but these values were close to the borderline value. Except for one polyclonal isolate, clinical isolates with mixed alleles (n = 7) were resistant to pyrimethamine.

DISCUSSION

Based on earlier clinical studies in the 1950s and 1960s, pyrimethamine resistance has been known to emerge rapidly in malaria endemic areas where pyrimethamine alone had been distributed in a large scale.[3] Selection of resistant parasites under massive drug pressure has been hypothesized to be the underlying cause of this phenomenon. The results of recent molecular studies on field isolates originating from various geographic regions strongly indicate that the first and key genetic event involved in the acquisition of pyrimethamine-resistant phenotype is a single Ser-to-Asn point mutation at position 108 of the gene.[16,22,24–27,33–35] Additional mutations at positions 51, 59, and/or 164 are associated with a higher level of pyrimethamine and/or cycloguanil resistance.[28,29] Further experimental proofs have been provided by gene expression in a heterologous system and transfection of malaria parasites with DHFR-TS genes carrying different combinations of mutations.[36–40] These observations suggest that the genetic basis of the rapid spread of pyrimethamine resistance is associated with the relative ease in selecting parasites carrying Asn-108 allele in an endemic area where pyrimethamine (or trimethoprim), alone or in combination with sulfonamides, is used for antimalarial (or antibacterial) treatment.

The key amino acid substitution at position 108 can be detected rapidly with precision using a combination of polymerase chain reaction and restriction fragment length polymorphism.[16,26,27,41,42] Using this technique, we have demonstrated the presence of pyrimethamine-resistant clinical isolates in Yaounde. As in our previous studies,[16] there was a strong association between the key DHFR-TS allele at position 108 and *in vitro* sensitivity and resistance to pyrimethamine. With the exception of a few isolates, the threshold IC_{50} value of 100 nM for pyrimethamine allowed the separation of 2 discrete populations of clinical isolates, those with a Ser-108 allele and those carrying an Asn-108 allele. However, parasites with mixed alleles cannot be readily assigned to one of these groups. In our previous studies, there were as many mixed populations of parasites with both alleles that were sensitive and resistant to pyrimethamine.[16,22,27] The IC_{50} values of mixed populations may be determined by the phenotype of dominant subpopulations. Unless quantitative polymerase chain reaction or cloning of the initial isolate is performed, we cannot determine the approximate proportion of Asn-108 carriers in relation to Ser-108 carriers in a given isolate.

Our comparative study over a 5-year period in similar patient populations in Yaounde revealed an alarming trend. Our *in vitro* surveillance showed an increase in the proportion of pyrimethamine-resistant isolates, from 42% in 1994–1995 to 63% in 1997–1998. In parallel, our polymerase chain reaction–based technique showed a similar increase (from 43% in 1994–1995 to 59% in 1997–1998) in the proportion of isolates carrying the mutant Asn-108 allele. We may deduce from our data that there is an increasing prevalence and spread of pyrimethamine resistance in Yaounde during the 5-year period. Our data are also in agreement with the intense drug pressure exerted in Cameroon, as suggested by the volume of sales of antimalarial drugs containing pyrimethamine that are imported in the country (Ducret J-P, unpublished data). Thus, our study seems to reveal the dynamic evolution of the development of pyrimethamine resistance, which may reflect the underlying genetic cause involved in the past, rapid development of pyrimethamine resistance in Asian and South American continents.

At present, pyrimethamine resistance detected in Cameroon by both *in vitro* and molecular assays has not yet been correlated with *in vivo* resistance. There is a clear need to establish whether there is a direct correspondence between *in vitro* and *in vivo* tests for pyrimethamine resistance. Two possible factors, however, may confound the results of these studies in the field. First, the synergistic effect of pyrimethamine-sulfadoxine combination may overcome drug resistance and mask low to moderate level of pyrimethamine resistance.[3] Second, semi-immune patients may be capable of eliminating residual, drug-resistant parasites by mounting an effective immune response. Despite these potential problems in the interpretation of results, additional molecular, *in vitro,* and *in vivo* surveillance is required to gain further insight into the dynamics involved in the spread of antifolate resistance.

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