Molecular Epidemiology of Malaria in Cameroon. XXX. Sequence Analysis of *Plasmodium falciparum* ATPase 6, Dihydrofolate Reductase, and Dihydropteroate Synthase Resistance Markers in Clinical Isolates from Children Treated with an Artesunate-Sulfadoxine-Pyrimethamine Combination

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Abstract. *Plasmodium falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes are reliable molecular markers for antifolate resistance. The *P. falciparum* ATPase 6 (*pfatp6*) gene has been proposed to be a potential marker for artemisinin resistance. In our previous clinical study, we showed that artesunate-sulfadoxine-pyrimethamine is highly effective against uncomplicated malaria in Yaoundé, Cameroon. In the present study, *dhfr*, *dhps*, and *pfatp6* mutations in *P. falciparum* isolates obtained from children treated with artesunate-sulfadoxine-pyrimethamine were determined. All 61 isolates had wild-type *Pfatp6* 263, 623, and 769 alleles, and 11 (18%) had a single E431K substitution. Three additional mutations, E643Q, E432K, and E641Q, were detected. The results did not indicate any warning signal of serious concern (i.e., no parasites were seen with quintuple *dhfr*-*dhps*, DHFR Ile164Leu, or *pfatp6* mutations), as confirmed by the high clinical efficacy of artesunate-sulfadoxine-pyrimethamine. Further studies are required to identify a molecular marker that reliably predicts artemisinin resistance.

INTRODUCTION

Plasmodium falciparum malaria parasite has become resistant to most affordable antimalarial drugs, such as chloroquine (CQ), amodiaquine (AQ), and sulfadoxine-pyrimethamine (SP).¹ To circumvent the problem of drug resistance, national health authorities of many concerned countries, with the support of the World Health Organization, have resorted to the use of artemisinin-based combination therapies (ACTs) for the first-line treatment of uncomplicated malaria. However, a decrease in the sensitivity to artemisinin has been recently documented in Cambodia, necessitating an increased vigilance in monitoring drug-resistant malaria.²

The mechanism of action of artemisinin is not well understood. One of the hypotheses is based on the specific inhibition of *P. falciparum* ATPase 6 (PfATP6), an orthologue gene product of the mammal sarco-endoplasmic reticulum calcium-dependent ATPase (SERCA).³ Initial laboratory studies have suggested that L263E substitution in PfATP6 affects the active site and induces conformational changes, reducing the affinity between the enzyme and artemisinin.⁴ Subsequent studies carried out on field isolates have shown an association between increased 50% inhibitory concentration (IC₅₀) for artemether and either a single amino acid substitution S769N (in South American strains) or double amino acid substitutions E431K and A623E (in African strains).⁵ Quiescence is another possible mechanism of resistance demonstrated in laboratory-adapted *P. falciparum*. Ring stages of artemisinin-tolerant *P. falciparum* strain may undergo quiescence, i.e. developmental arrest, during exposure *in vitro* to high concentrations of artemisinin derivatives, and pursue normal process of cell cycle once the drug is removed.⁶

The mechanisms of resistance to sulfadoxine and pyrimethamine have been extensively studied. The key amino acid substitutions associated with *in vitro* resistance to sulfadoxine and pyrimethamine are Ala437Gly and Ser108Asn, respectively. However, for clinical resistance to occur, additional mutations, commonly referred to as quintuple dihydrofolate reductase (*dhfr*)-dihydropteroate synthase (*dhps*) mutations, are required.⁷

In this study, we assessed the mutations of *dhfr*, *dhps*, and *P. falciparum* ATPase 6 (*pfatp6*) genes associated with drug resistance in *P. falciparum* isolates obtained from children treated with artesunate-sulfadoxine-pyrimethamine (AS-SP) combination and followed-up for 28 days. We also assessed the usefulness of molecular markers as a complementary tool for the evaluation of therapeutic efficacy of ACT. The efficacy of AS-SP, in relation to other ACTs, has been analyzed in our previous study.⁸

MATERIALS AND METHODS

Patients and blood collection. Blood samples were obtained during February–May 2005 as part of a randomized clinical study that compared the therapeutic efficiency of amodiaquine monotherapy, artesunate-amodiaquine, and AS-SP combinations.⁸ Fingerprick capillary blood was collected on Isocode Stix® filter papers (Schleicher and Schuell, Ecqueville, France) from children less than five years of age who came to the Nlongkak Catholic Missionary Dispensary in Yaoundé, Cameroon. Children were enrolled in the study if they satisfied the following criteria set by the World Health Organization: presence of *P. falciparum* with a parasite density > 2,000 asexual parasites/μL of blood without any other *Plasmodium* species, fever > 37.5°C, hematocrit > 15%, absence of severe malnutrition and other infectious diseases that may be the origin of fever, absence of signs and symptoms of severe and complicated malaria, and a signed written informed consent provided by parents or a legal guardian.⁹

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Patients were treated with artesunate at a dose of 4 mg/kg of body weight administered per day on days 0, 1, and 2. The standard dose of SP (25 mg/kg of body weight for sulfadoxine and 1.25 mg/kg of body weight for pyrimethamine) was administered in a single dose. Patients were followed-up on days 1, 2, 3, 7, 14, 21, and 28, as recommended in the 2003 World Health Organization protocol.⁹ Each dose of antimalarial drugs was administered under supervision during the visits. Patients who failed to respond to the assigned drug were treated with oral quinine (25 mg/kg of body weight/day for 5 days) or the standard dose of artemether-lumefantrine, one of the first-line ACTs in Cameroon. In case of treatment failure, the polymorphic merozoite surface antigen-1, merozoite surface antigen-2, and glutamine-rich protein genes of the pre-treatment and recrudescence samples were compared by agarose gel electrophoresis to distinguish between recrudescence and reinfection, as recommended by a group of malaria experts.¹⁰

DNA extraction and polymerase chain reaction. Parasite DNA was extracted from filter paper by the boiling method as recommended by the manufacturer of the Isocode Stix® filter papers (Schleicher and Schuell). Briefly, after rinsing the filter paper once in 500 µL of sterile distilled water, the filter paper was placed into a 0.5-mL microtube into 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. Ten microliters of the supernatant was used to amplify *pfdhfr*, *pfdhps*, and *pfatp6* gene fragments.

The *pfdhfr* mutations at codons 51, 59, 108, and 164 and *pfdhps* mutations at codons 436, 437, and 540 were determined by nested polymerase chain reaction, followed by enzymatic digestion, as described by Eldin de Pécoulas and others¹¹ and Duraisingh and others.¹² The DHFR amino acid residue 16 and DHPS amino acid residues 581 and 613 were not analyzed in this study because mutations at these positions are rare in African isolates and the Ala16Val substitution in DHFR occurs with the rare Ser108Thr substitution.⁷

For *pfatp6* mutation analysis, a gene fragment of 1,793 base-pairs corresponding to exon 1 of the coding region was amplified by the nested polymerase chain reaction (PCR) protocol described in our previous study.¹³ Amplification products were sequenced from the 5'- and 3'-ends by using an automated DNA sequencer (ABI Prism; Perkin Elmer Corp., Les Ulis, France) to determine the codons that were reported to be associated with artemisinin resistance (amino acid residues 263, 431, 623, and 769) and possible novel mutations in the *pfatp6* gene.^{4,5} On the basis of these studies, the wild-type haplotype was defined as LEAS. Known *pfatp6* mutants include the West African double mutant type (E431K + A623E) and the South American single mutant type (S769N).

Statistical analysis. Fisher's exact test was used to analyze the relationship between the clinical outcome of AS-SP treatment and sequence polymorphisms of drug resistance markers. The significance level was fixed at 0.05.

RESULTS

Sixty-one isolates obtained on day 0 from all patients treated with AS-SP combination were included in the present study. The *pfdhfr* and *pfdhps* fragments of all 61 isolates were successfully amplified. All 61 isolates were triple mutants, i.e., carried mutant 51, 59, and 108 *dhfr* alleles (Asn51Ile, Cys59Arg, Ser108Asn; haplotype IRN). On the basis of results of *dhfr*

and *dhps* sequences, 42 (69%) isolates were classified as quadruple mutants, i.e., triple *dhfr* mutation (Asn51Ile, Cys59Arg, Ser108Asn) and Ala437Gly *dhps* mutation. There was no quintuple mutant (quadruple mutation + *dhps* mutant allele Lys540Gln). Mutation was not detected at DHFR-164.

On the basis of amino acid substitutions at positions 263, 431, 623, and 769 that have been linked to artemisinin resistance in previous studies,^{4,5} the mutant haplotype LKAS was observed in 18% of isolates. The E432K mutation was present as a pure allele in two isolates. Two non-synonymous *pfatp6* mutations (E641Q, n = 3 isolates and E643Q, n = 1 isolate) and three synonymous mutations (440, 594, and 621 in three different isolates) were also found. The *pfatp6* haplotypes (positions 263, 431, 432, 623, and 769) are summarized in Table 1. Because of the low rate of *pfatp6* mutations among 61 isolates obtained from patients treated with AS-SP and that pure or mixed E431K allele occurred only in patients with an adequate clinical and parasitologic response outcome, possible associations between molecular markers and clinical outcome were not analyzed.

DISCUSSION

The results of the present study showed that all isolates are triple *dhfr* mutants and wild-type or single *dhps* mutant at position 437. The role of *dhps* mutant alleles Ser436Ala or Ser436Phe in African isolates is not well known. The predominance of triple *dhfr* mutants and the absence of Ile164Leu in DHFR and Lys540Gln in DHPS in isolates from Cameroon are consistent with that of our previous studies on isolates obtained during 1999–2004, in which we observed a steady

TABLE 1
Plasmodium falciparum dhfr, pfdhps, and pfatp6 sequence polymorphisms of isolates and clinical response of children treated with artesunate-sulfadoxine-pyrimethamine combination, Cameroon*

No. of isolates (%)	DHFR	DHPS	PFATP6	Outcome
5 (8.2)	IRN	SA	LEEAS	ACPR
1 (1.6)	IRN	SA	LEEAS	LPF†
7 (11.5)	IRN	A/FA	LEEAS	ACPR
3 (4.9)	IRN	A/FA	LKEAS	ACPR
2 (3.3)	IRN	S + A/FA	LEEAS	ACPR
1 (1.6)	IRN	A/FA	LEEAS	LPF‡ (ACPR)
1 (1.6)	IRN	SA/G	LEEAS	ACPR
1 (1.6)	IRN	SA/G	LKEAS	ACPR
13 (21.3)	IRN	SG	LEEAS	ACPR
1 (1.6)	IRN	SG	LEEAS	LPF†
1 (1.6)	IRN	SG	LEEAS	LPF‡ (ACPR)
3 (4.9)	IRN	SG	LEEAS	LCF‡ (ACPR)
3 (4.9)	IRN	SG	LEEAS	Lost
1 (1.6)	IRN	SG	LEKAS	ACPR
1 (1.6)	IRN	SG	LE/KEAS	ACPR
3 (4.9)	IRN	SG	LKEAS	ACPR
6 (9.8)	IRN	A/FG	LEEAS	ACPR
1 (1.6)	IRN	A/FG	LEKAS	ACPR
1 (1.6)	IRN	A/FG	LKEAS	ACPR
1 (1.6)	IRN	A/FG	LKEAS	Lost
1 (1.6)	IRN	A/FG	LE/KEAS	ACPR
4 (6.6)	IRN	S + A/FG	LEEAS	ACPR

* *dhfr* = dihydrofolate reductase; *dhps* = dihydropteroate synthase; *atp6* = ATPase 6; ACPR = adequate clinical and parasitologic response; LPF = late parasitologic failure; LCF = late clinical failure; Lost = lost-to-follow-up. Mutant alleles are indicated in **bold**. Triple *dhfr* mutant is defined by the haplotype IRN (Ile-51, Arg-59, Asn-108). Quadruple mutant is defined by the *dhfr-dhps* haplotype IRN + G (triple *dhfr* mutant + Gly-437). All isolates carried the wild-type DHFR Ile-164 and DHPS Lys-540 alleles. In the presence of Ala-436 or mixed Ser- and Ala-436 in DHPS, the presence or absence of Phe-436 cannot be established with restriction enzymes *Mnl* I and *Msp* A1 I.

† Recrudescence.

‡ Reinfection (classified as ACPR after polymerase chain reaction correction).

replacement of wild-type parasites (52% in 1994–1995 and only 3% in 2004–2005) by triple *dhfr* mutants.¹⁴

Studies conducted with large numbers of *P. falciparum* isolates in eastern and western Africa and Asia (China, Cambodia) have shown E431K, N569K, and A630S substitutions (also additional rare mutations) in the *pfatp6* gene but did not find any mutant with L263E, A623E, or S769N, despite the fact that artemisinin derivatives have been used extensively in Asia.^{15–19} Only one African isolate carrying the South American-type S769N substitution has been reported, but its low dihydroartemisinin IC₅₀ (0.83 nM) suggested full *in vitro* sensitivity.²⁰ In isolates from Cameroon, only E431K, E432K, E641Q, and E643Q changes have been observed.¹³ The novel E432K mutation, which occurred in the background of a wild-type LEAS haplotype, was also observed in our earlier study of isolates from Cameroon obtained during 2001–2006.¹³ The presence of these mutations did not influence the level of dihydroartemisinin IC₅₀. The *pfatp6* gene seems to show polymorphic patterns depending on the geographic origin of parasites, but these mutations, including changes in amino acid residues 263, 431, 623, and 769, have not been consistently associated with changes in artemisinin IC₅₀ level or poor clinical response to ACT.

The AS-SP combination was shown to be highly effective in Cameroon. Elsewhere in Africa, where SP is less efficacious, AS-SP is not the recommended ACT. This discordance may be explained, at least in part, by the absence of additional *dhfr* and *dhps* mutations known to increase the level of antifolate resistance (DHFR-164 and DHPS-540) in *P. falciparum* isolates from Cameroon and lack of molecular evidence for artemisinin resistance on the basis of *pfatp6* analysis. In the present study, recrudescence occurred in patients infected with triple or quadruple *dhfr-dhps* mutant and wild-type *pfatp6* parasite, and patients infected with quadruple *dhfr-dhps* mutants were cured with AS-SP. These results, and those of other studies, suggest that mutations in genetic markers for drug resistance are necessary but not sufficient cause that leads to treatment failure. Field isolates of *P. falciparum* remain highly sensitive *in vitro* to artemisinin derivatives. However, the use of *pfatp6* as a molecular marker and conventional *in vitro* assays may not be appropriate tools to detect artemisinin resistance. If quiescence mechanism is demonstrated in naturally occurring *P. falciparum* isolates,⁶ new alternative laboratory tools are required to determine artemisinin-resistant phenotype.

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