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# Molecular Epidemiology of Malaria in Cameroon and Côte d'Ivoire. XXXI. Kelch 13 Propeller Sequences in *Plasmodium falciparum* Isolates before and after Implementation of Artemisinin-Based Combination Therapy

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*Abstract.* Artemisinin-resistant malaria has not been reported from Africa, but resistance can possibly spread from Asia or arise independently in Africa. The emergence of artemisinin resistance in Africa can be monitored by molecular assay of Kelch 13 (K13) propeller sequences. A total of 251 archived DNA samples of *Plasmodium falciparum* isolates collected in 2002, 2003, and 2006 in Yaounde, Cameroon, and 47 samples collected in 2006 and 2013 in Abidjan, Côte d'Ivoire, were analyzed for K13-propeller sequence polymorphism. Only one isolate carried a mutant K13-propeller allele (E602D). None of the isolates carried the key mutant alleles (Y493H, R539T, I543T, and C580Y) associated with artemisinin resistance in Cambodia. The presence of the mutant allele was not correlated with in vitro response to dihydroartemisinin determined by the classical hypoxanthine incorporation assay. There was no evidence of K13 mutations associated with artemisinin resistance before and soon after the introduction of artemisinin-based combination therapies in Cameroon and Côte d'Ivoire.

The emergence of malaria parasites resistant to the majority of conventional antimalarial drugs impedes disease control and compromises malaria elimination programs. For more than a decade, antimalarial chemotherapy has relied on artemisinin-based combination therapies (ACTs) to prevent the spread of drug resistance. The rationale for using ACTs lies in the rapid action of artemisinin derivatives to reduce intraerythrocytic parasite load, followed by the elimination of residual parasites by a slower acting drug partner with longer elimination half-life.[1]

In Cameroon and Côte d'Ivoire, artesunate–amodiaquine and artemether–lumefantrine are the first-line drugs to treat uncomplicated malaria since 2004. These two ACTs have been shown to be highly effective against acute uncomplicated falciparum malaria in these two countries.[2,3] Elsewhere in sub-Saharan Africa, high cure rates, often attaining > 95% after polymerase chain reaction (PCR) correction to exclude reinfections, have been reported with nearly all ACTs.[4]

Artemisinin-resistant *Plasmodium falciparum* was first reported from Cambodia in 2008.[5] Clinical resistance to artemisinin is characterized by delayed parasite clearance, and *P. falciparum* isolates from patients responding with a long parasite clearance half-life are characterized by high survival rates in vitro using ring-stage survival assay ($RSA_{0-3h}$).[6] Several subsequent studies have shown that resistance to artemisinin has spread throughout southeast Asia.[7,8] In Africa, artemisinin resistance has not been reported despite the massive use of ACTs.

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Several amino acid substitutions in the PF3D7_1343700 gene encoding Kelch propeller domain (K13 propeller) in parasites from western Cambodia have been reported to be associated with delay in parasite clearance after treatment with artemisinin and increased ring-stage survival rate when exposed in vitro to artemisinin.[9] Subsequent studies support the direct association between artemisinin resistance and K13-propeller mutations.[10,11] Molecular surveillance of K13 propeller may be useful to detect the emergence of artemisinin-resistant *P. falciparum* in Africa.[12] The aim of the present study was to analyze the sequence polymorphism in K13-propeller gene of *P. falciparum* isolates that were collected before and after the effective implementation of the new drug policy based on ACT in Cameroon and Côte d'Ivoire.

Febrile patients aged ≥ 12 years consulting at the Nlongkak Catholic Missionary Dispensary in Yaounde, Cameroon, were screened for the presence of malaria parasites in 2002, 2003 (pre-ACT period), and 2006 (post-ACT period). Symptomatic malaria-infected patients aged > 6 months consulting spontaneously at El-Rapha d'Abobo dispensary, Abidjan, Côte d'Ivoire, were recruited in 2006 (pre-ACT period) and 2013 (post-ACT period). Venous blood samples (5 mL) were collected from patients who fulfilled the following inclusion criteria: presence of *P. falciparum* with parasitemia > 0.1%, absence of other *Plasmodium* species, and denial of recent self-medication with an antimalarial drug confirmed by the Saker–Solomons urine test.[13] Pregnant women and patients with signs and symptoms of severe and complicated malaria were excluded. The enrolled patients were treated with either amodiaquine monotherapy or amodiaquine–sulfadoxine–pyrimethamine combination in 2002–2003 and artesunate–amodiaquine in 2006 in Cameroon and with amodiaquine or artesunate monotherapy in 2006 or artesunate–amodiaquine in 2013 in Côte d'Ivoire. The study was reviewed and approved by the Cameroonian National Ethics Committee and

Cameroonian Ministry of Public Health for the studies conducted in Cameroon and by the Ivorian National Ethics Committee and Ivorian Ministry of Health for those performed in Côte d'Ivoire.

An aliquot of 1.5 mL of red cell pellet was used for DNA extraction by phenol–chloroform method. A 771-bp fragment of the K13-propeller gene was amplified by PCR as described by Ariey and others.[9] PCR products were sequenced from both the 5′ and 3′ ends by using an automated DNA sequencer. The procedures for conducting $^{3}$H-hypoxanthine-based in vitro drug sensitivity assay for dihydroartemisinin and the calculation of 50% inhibitory concentration ($IC_{50}$) were described in our previous study.[14] The deduced K13-propeller amino acid sequences of the isolates were compared with those of Cambodian *P. falciparum* isolates reported by Ariey and others.[9] The following amino acid substitutions were considered to be key codons: Y493H, R539T, I543T, and C580Y. Moreover, the same study reported that continuous cultivation of F32-Tanzania *P. falciparum* strain exposed to increasing concentrations of artemisinin resulted in the acquisition of M476I mutant allele in K13.

A total of 251 Cameroonian *P. falciparum* isolates collected between 2001 and 2006 were used to assess the sequence polymorphism in the K13-propeller region. Sequence data and dihydroartemisinin $IC_{50}$ values were available from all 251 isolates. The geometric mean $IC_{50}$ (95% confidence interval [CI]; range) for dihydroartemisinin was 0.940 nM (0.809–1.09 nM; 0.074–13.1 nM; $N$ = 200) for isolates collected in 2002–2003 and 3.49 nM (2.86–4.27 nM; 0.635–18.4 nM; $N$ = 51) for isolates collected in 2006, respectively ($P < 0.05$; $t$ test). The higher geometric mean $IC_{50}$ of isolates collected in 2006 was due to the change in the batch of pooled human serum, rather than due to decreased in vitro sensitivity to dihydroartemisinin.

In Côte d'Ivoire, 31 of 41 isolates collected in 2006 were tested for in vitro response to dihydroartemisinin. The geometric mean $IC_{50}$ (95% CI; range) for dihydroartemisinin was 2.7 nM (1.45–3.99 nM; 0.7–16.3 nM; $N$ = 31). In 2013, 16 blood samples collected from patients who responded to artesunate–amodiaquine treatment with adequate clinical and parasitological response on day 28 were not tested for in vitro response to dihydroartemisinin.

A single-nucleotide polymorphism (SNP; E602D) was found in the 771-bp fragment of K13-propeller domain in a single isolate, Yaounde 117/03, collected in 2003. The $IC_{50}$ of this isolate for dihydroartemisinin was 1.121 nM. Of 251 isolates, 250 (99.6%) had wild-type K13-propeller alleles, defined as the nucleotide sequence in 3D7 reference clone. All 47 Ivorian samples had the wild-type K13 alleles.

Until 2004, amodiaquine monotherapy and sulfadoxine–pyrimethamine were the first- and second-line antimalarial drugs for the treatment of uncomplicated malaria in Cameroon, respectively. Artesunate–amodiaquine was officially adopted as the first-line drug in 2004, followed by artemether–lumefantrine as an alternative ACT in 2006. Despite the official change in national antimalarial drug policy, the actual implementation of the policy went into effect nationwide in 2006 at subsidized low prices with the aid of the Global Fund. During the transition period between 2004 and 2006, various formulations of ACTs, as well as artesunate monotherapy, were widely available through official outlets. Therefore, drug pressure due to an uncontrolled use of artesunate monotherapy and ACTs, often used for self-medication by part of the local population, was present in Yaounde during the study period. However, the extent of drug pressure cannot be quantified, and its possible impact on selection of parasites is unknown. In Côte d'Ivoire, artesunate–amodiaquine was officially adopted in 2005 but fully implemented from 2007.

The present data demonstrate that all isolates collected in Cameroon and Côte d'Ivoire before the era of ACT were of wild type and that K13-propeller mutants did not emerge among the isolates collected in 2006 (in Cameroon) or in 2013 (in Côte d'Ivoire), soon after the official adoption of ACTs as the first-line drugs. Although a single SNP was observed in one Cameroonian isolate, the role of this amino acid substitution in artemisinin resistance is undetermined. As expected from previous studies,[6] dihydroartemisinin $IC_{50}$ was not correlated with K13-propeller sequence variation. In vitro, RSA data would have added more pertinent information on the susceptibility level of these isolates to dihydroartemisinin. The lack of RSA data of our isolates collected between 2002 and 2013 is a major limitation of the present study. Overall, the absence of K13 mutations in Cameroonian and Ivorian isolates collected between 2002 and 2013 is compatible with the more recent findings that there is currently no molecular evidence for artemisinin resistance in Africa.[15]

In a recent study, it was also reported that among 11 samples collected in Buea, western Cameroon, in 2013–2014, SNPs were not observed.[16] However, a high level of diversity in K13-propeller sequence was found among 2,000 African isolates across 12 countries, possibly resulting from genetic drift and/or positive selection.[15,16] Most of the mutations occurred in single parasite populations and were different from those found earlier in Cambodian isolates. In general, the allelic frequencies were < 6% in west and east Africa. Some mutant alleles occurred at a high rate (up to 36%) among parasite populations collected in 2005–2007 in Democratic Republic of Congo, Central Africa. Given favorable conditions, including massive use of ACTs exerting constant drug pressure on local parasite populations, improper use of ACTs, and intense transmission, artemisinin-resistant *P. falciparum* may emerge independently in the African continent.

Our data suggest that despite intense malaria transmission and uncontrolled use of artesunate monotherapy and ACTs in Cameroon and Côte d'Ivoire during the early period of ACT era, K13 mutations have not been selected. Further molecular and in vitro surveillance is needed to follow the current epidemiological trend in Cameroon and Côte d'Ivoire.

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