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Increased prevalence of the *Plasmodium falciparum* Pfmdr1 86N genotype among field isolates from Franceville, Gabon after replacement of chloroquine by artemether–lumefantrine and artesunate–mefloquine

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ABSTRACT

Despite global antimalarial measures, *Plasmodium falciparum* malaria remains a major public health problem. WHO has recommended the use of arteminisin-based combination therapy to limit the emergence of antimalarial drug resistance. However, ACT treatment failures have been linked to the selection of the wild types 86N genotype of *P. falciparum multidrug resistance 1* (*Pfmdr1*) and the 76K genotype of *P. falciparum chloroquine resistance* (*Pfcrt*) genes. The aim of this study was to investigate the molecular impact of widespread implementation of artemether–lumefantrine and artesunate–mefloquine on local parasite population in Franceville, Gabon.

We analyzed 230 pediatric field isolates (96 from 2004 and 134 from 2009). Routine hematological parameters were collected. *Pfmdr1* codons 86 and 1246 and *Pfcrt* codon 76 were genotyped using PCR–RFLP and the prevalence of the genotypes was compared.

The children's mean age did not differ between 2004 and 2009 (respectively 31.8 (6–84) months vs 38.6 (6–84) months, $p = 0.32$), and neither did mean parasitemia [16,750 (1000–96,234) and 14,587 (1093–83,941) parasites/μL, respectively ($p = 0.21$)]. The mean hemoglobin level was higher in 2009 than in 2004 (11.0 ± 2.4 vs 7.8 ± 2.0 g/dL, respectively; $p = 0.04$). More interesting, the prevalence of *Pfmdr1* wild type 86N increased from 15.6% ($n = 15/96$) in 2004 to 31.3% ($n = 42/134$) in 2009 ($p = 0.007$). A significant increase combining pure and mixed genotypes (86N + 86N/Y) was also found between 2004 and 2009 ($p = 0.02$), while the prevalence of genotypes *Pfmdr1* 1246D, *Pfcrt* wild type 76T and all mixed genotypes (*Pfmdr1* 86N/Y and 1246D/Y, and 76K/T) remained stable. The complexity of isolates was high (around 2.9 and 2.4) and the FC27 allele of *Pfmsp2* was more prevalent.

These findings show a substantial benefice of artemether–lumefantrine and artesunate–mefloquine and of new control measures. The selection, in the general population, of wild type *Pfmdr1* 86N, which is associated with antiplasmodial resistance against some drugs, has been induced underlining the need for molecular surveillance of the impact of ACT on antimalarial resistance.

1. Introduction

The fight against malaria has grown more effective in recent years thanks to increased funding, cutting the malaria burden by a factor of 2 (WHO, 2009). However, *Plasmodium falciparum* malaria remains a serious public health problem worldwide. Widespread parasite drug resistance is a major challenge in endemic regions, and new drug combinations have been introduced. Artemisinin-based combination therapy is increasingly recommended because of its high efficacy and low propensity to induce parasite resistance against antimalarial drugs (White, 1999). The concept behind ACT is that artemisinin derivatives quickly reduce parasitemia, leaving

Abbreviations: ACT, artemisinin-based combination therapy; AL, artemether–lumefantrine; AM, artesunate–mefloquine; CHL, centre hospitalier de Libreville; $CI_{95\%}$, confidence interval 95%; CQ, chloroquine; MQ, mefloquine; N86Y, asparagine (N) to tyrosine (Y) substitution; D1246Y, aspartate (D) to tyrosine (Y) substitution; K76T, lysine (K) to threonine (T) substitution; *Pfmdr1*, *P. falciparum multidrug resistance 1* gene; *Pfcrt*, *P. falciparum chloroquine resistance transporter* gene; *Pfmsp2*, *P. falciparum merozoite surface protein 2* gene; PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism; SNP, single nucleotide polymorphism; OR, odds ratio; WHO, World Health Organisation.

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the partner drug, which has a long elimination half-life, to eradicate a smaller residual parasite population, thereby minimizing the risk of selecting parasites resistant to the partner drug (Nosten et al., 2000). However, reinfecting resistant parasites may be selected during this treatment. The period during which the partner drug alone is present in the body represents the weak link in the chain of the ACT use. Some ACT failures have been observed in endemic areas. For instance, the failure rate after artemether–lumefantrine (AL) and artesunate–mefloquine (AM) treatments were about 18% in Tanzania and 2.4% in Thailand, respectively (van Vugt et al., 2002; Khim et al., 2005; Alker et al., 2007; Humphreys et al., 2007). Studies of the molecular mechanisms of plasmodial drug resistance are urgently needed.

Gabon is a hyperendemic country where resistance to CQ, amodiaquine, and sulfadoxine–pyrimethanine is widespread (Aubouy et al., 2003; Ndong et al., 2003; Ramharter et al., 2004; Nsimba et al., 2008). Thus, CQ and other monotherapies have been withdrawn, and ACTs were adopted to treat uncomplicated malaria in 2003, in line with WHO recommendations. During the meeting of consensus, artesunate–amodiaquine and AL were adopted as first line and second line of treatment of uncomplicated malaria, respectively. But, according to the easy formulations (coartesiane® syrup and artequin® blister), physicians use largely AL and AM in opposite to the recommendations of health ministry. These combination therapies were only implemented country-wide in 2005. Subsequently, the prevalence of malaria among febrile children fell from 30% to 13% between 2004 and 2008, and the mean age of children with malaria rose from 24 to 41 months at the country's biggest hospital, Centre Hospitalier de Libreville (CHL) (Bouyou-Akotet et al., 2009). However, some ACT treatment failures have been observed at Libreville, where the efficacy of AL and AM were about 90% after PCR-correction (Kombila et al., unpublished data). In Franceville, a town of 60,000 inhabitants situated in south-eastern Gabon (Fig. 1), few drug resistance data are available. One report showed prevalence rates of drug resistance of roughly 50% for CQ, 21.1% for MQ and 0% for quinine (Ndong et al., 2003), but *Pfcrt* and *Pfmdr1* genotypes were not documented. As in the rest of Gabon, CQ was effectively withdrawn and the use of ACT and impregnated bednets has intensified since 2005. In 2009, uncomplicated malaria was treated with ACT (AL and AM) that were largely used.

P. falciparum drug resistance is associated with certain parasite genotypes (Wang et al., 1997; Basco et al., 1998; Duraisingh et al., 2003). Two major genes have been implicated in quinoline resistance, namely *Pfcrt* (*P. falciparum chloroquine resistance transporter*) and *Pfmdr1* (*P. falciparum multi-drug resistance 1*). Single-nucleotide polymorphisms (SNPs) in these genes are associated with the development of resistance to antimalarial drugs both *in vitro* and *in vivo* (Cowman, 1991; Adagu and Warhurst, 1999; Mayor et al., 2001; Wongsrichanalai et al., 2002). Genetic cross-analysis has shown that chloroquine (CQ) resistance is associated with SNP 76T in *Pfcrt* (Perozzo et al., 2002). This mutation has been suggested to play a role in mefloquine (MQ) susceptibility (Sidhu et al., 2002; Cooper et al., 2002). Moreover, several data have shown that *Pfmdr1* SNPs influence the *in vitro* response to various antimalarial drugs, such as artemisinin derivatives, arylaminoalcohols, lumefantrine, MQ, and halofantrine. Mutation 86Y in *Pfmdr1* is associated with increased CQ resistance but with increased sensitivity to MQ and artemisinine (Lopes et al., 2002; Duraisingh et al., 2003).

A given patient can harbour several parasite clones, and *Pfmsp2* genotyping is used to characterize *Plasmodium* multiplicity of infection. Two distinct forms of *Pfmsp2* have been described, defining two allelic families (FC27 and 3D7) (Smythe et al., 1990).

The spectacular results have been yielded about the selection of genotypes. Firstly, the selection of wild type 86N was reported after AL treatment failures in Zanzibar (Sisowath et al., 2005). Secondly this result was confirmed concomitantly with the report of selection of wild type 76K was found in Tanzania (Sisowath et al., 2009). Recently, the selection of both wild type genotypes was reported after AL treatment failures in Burkina Faso and in Swaziland (Dlamini et al., 2010; Some et al., 2010). So, it is important to study the impact of ACT on general parasite populations, in the regions ACTs are used. The aim of this study was to analyze changes in the frequencies of the main polymorphisms associated with drug resistance, namely N86Y and D1246Y in *Pfmdr1* and K76T in *Pfcrt*, among field isolates in Franceville, Gabon, between 2004 and 2009.

Fig. 1. Map of Gabon, location of Franceville.

2. Materials and methods

2.1. Patients

Children were enrolled at the two hospitals in Franceville (Hôpital de l'amitié Sino-Gabonaise and Centre Hospitalier Regional Amissa Bongo) in 2004 (June–September) and 2009 (May–December). Ethical clearance was received from the Gabonese Ministry of Health. All children presenting during the study periods with fever (tympanic temperature $\geq 37.5\ ^{\circ}C$) or history fever less than 24 h preceding the consultation were screened for malarial parasites. After obtaining informed consent from their parents or guardians, 230 children diagnosed with *P. falciparum* malaria were included in this study.

The inclusion criteria were age 6–84 months, uncomplicated malaria, and monospecific *P. falciparum* parasitemia exceeding 1000 parasites/μL.

As recommended by the Gabonese National Malaria Control Program, each episode of uncomplicated malaria was treated with AL for three days.

2.2. Hematological cell tests

Routine hematological tests were done with an automated blood cell counter (STKS, Coulter Corporation, USA). Blood (5 mL)