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Acta Tropica 95 (2005) 219–225
ACTA TROPICA
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# In vivo chloroquine resistance and prevalence of the *pfcrt* codon 76 mutation in *Plasmodium falciparum* isolates from the Republic of Congo

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Available online 5 July 2005

**Abstract**

Chloroquine (CQ) resistance in *Plasmodium falciparum* has been particularly associated with mutations in the *pfcrt* gene. The present study was carried out in the malaria hyperendemic town of Brazzaville (Republic of Congo, Central Africa) where CQ is still recommended and used as a first-line drug for *P. falciparum* malaria. We assessed the efficacy of CQ in vivo, and the association between *pfcrt* mutation at codon 76 and treatment outcome in 50 children with uncomplicated malaria. The failure rate on day 28 was 95.7% and the *pfcrt* K76T mutation was present in 100% of isolates. No variation in the multiplicity of infection was observed in pre- and post-treatment isolates. In further 87 isolates from uncomplicated patients not treated with CQ, the mutation was detected in 98.5% of isolates. This study confirms the high level of in vivo resistance to CQ and shows the high prevalence of *pfcrt* K76T mutation in the Republic of Congo.


*Keywords:* *P. falciparum*; Chloroquine; Malaria; Congo; *Pfcrt*

## 1. Introduction

Chloroquine (CQ) has been considered as the first-line drug for the prevention and therapy of malaria. In 1950s resistance of *Plasmodium falciparum* strains to this antimalarial was reported from Southeast Asia, South America and sub-Saharan Africa. However, CQ remains the most affordable and widely used antimalarial for many parts of endemic areas in Africa (Trape, 2001; Warhurst, 2001).

Chloroquine resistance (CQR) in *P. falciparum* is conferred by mutations in the parasite *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene, which encodes a putative transporter localized in the digestive

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doi:10.1016/j.actatropica.2005.06.001

vacuole (Sidhu et al., 2002). The substitution from lysine (K) to threonine (T) at amino acid 76 (K76T) in the *pfcrt* protein appears to be a primary genetic mechanism conferring resistance to CQ (Fidock et al., 2000).

Direct evidence for in vivo selection for mutant *pfcrt* has been shown in patients originated from different countries like Cameroon (Basco et al., 2002), Mali (Djimde et al., 2001a), Mauritania (Jelinek et al., 2002) and Gabon (Binder et al., 2002), whereas the results on in vitro evaluation of resistance to CQ are more controversial. Several in vitro studies using field isolates showed high but imperfect association between chloroquine response and the key *pfcrt* mutation (Babiker et al., 2001; Basco and Ringwald, 2001a; Thomas et al., 2002). In vitro resistance of *P. falciparum* strains has been shown to be higher in isolates from patients with severe malaria than those with uncomplicated disease in Nigeria (Olumese et al., 2002). Thus, this codon has been considered as a highly reliable genetic marker for the epidemiologic monitoring of chloroquine resistance (Djimde et al., 2001b).

The detection of high-grade resistance to chloroquine led to a change of national drug policy and the use of sulfadoxine–pyrimethamine (S/P) and/or amodiaquine (AQ) as first-line therapy for uncomplicated *P. falciparum* malaria in Western Africa (Djimde et al., 2004), Eastern Africa (Kublin et al., 2003; Shretta et al., 2000), and Central Africa (Basco et al., 2002; Kremsner et al., 1993, 1994; Winkler et al., 1994). To our knowledge, only one published study (Nsimba et al., 2004) reported the high level of in vivo resistance to CQ in the Republic of Congo. In this work, the level of resistance to CQ was evaluated at 70% using the Lot Quality Standard test of WHO and considering 26 patients with uncomplicated malaria and originated from different parts of Brazzaville, the capital of Congo. As this city is divided into different districts with different levels of transmission (Carme et al., 1993), it seemed important to provide additional data from this area where 30% of the Congolese population is concentrated.

To achieve this goal we conducted a study in a southern district of Brazzaville. Children with diagnosed malaria were treated with CQ which remains the first-line drug for the treatment of uncomplicated malaria in the country. These children were followed up for 28 days and as additional investigation tool, the genotyping of *pfcrt* mutation K76T in pre- and post-treatment *P. falciparum* isolates was also done. As a secondary objective, the genetic diversity and multiplicity of infections of *P. falciparum* isolates from patients were investigated.

## 2. Materials and methods

### 2.1. Study site

This study was carried out in Brazzaville (Republic of Congo). In this urban area, malaria is highly endemic, stable with a perennial transmission (Trape et al., 1985). Malaria is primarily due to *P. falciparum*. An entomological inoculation rate (EIR) of 22.5 infective bites/person/year was reported (Trape and Zoulani, 1987). Between 1993 and 2002, armed conflicts occurred in the Republic of Congo. During this period, the populations left the city and went to surrounding rural Southern areas where malaria transmission is higher with EIR estimated at one infected bite per person per night in 1985 (Trape et al., 1985). The current study was approved by the Ministry of Public Health of the Republic of Congo.

### 2.2. Enrolment of patients

At the Centre de Santé Intégré (CSI) of Terinkyo Hospital in the south of Brazzaville, children aged 6–60 months presented the following criteria: axillary temperature $\geq 37.5\,^{\circ}C$ measured with an electronic thermometer, parasite density between 2000 and 200,000 $\mu l^{-1}$ of blood, absence of severe malaria symptoms, or febrile conditions caused by other diseases than malaria and ability to come for the stipulated follow up visits were recruited from February to July 2003. Informed consent was obtained from parents or guardians. The recruited children were treated with CQ tablets (Cinpharm, Cameroon, Lot 7.090), according to WHO protocol based on the assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria. Each dose of chloroquine (25 mg/kg of body weight over a three-day period; 10 mg/kg the first day and the second day, and 5 mg/kg the third day). After the administration of the treatment, the children were observed during 30 min for an eventual vomiting

and other side effects. If vomiting occurred the same dose was re-administered. Clinical and parasitological observations were recorded daily for the first four days (0–3) and during follow-up on days 7, 14, 21, and 28. Clinical observations were recorded on days 0, 1, 2, 3, 7, 14, 21, and 28; and parasitological observations 0, 2, 3, 7, 14, 21, and 28 to assess the efficacy of the treatment. Out of this schedule, the parents were advised to bring children to the health center if they do not feel well. The therapeutic response was classified as: (a) early treatment failure (ETF) if children developed symptoms of severe malaria at days 1, 2, or 3; having axillary temperature $\geq$37.5 °C on day 2 with parasitemia > of day 0 count or axillary temperature $\geq$37.5 °C on day 3 in the presence of parasitemia or parasitemia on day 3 $\geq$25% of count on day 0; (b) late clinical failure (LCF) if children have a temperature $\geq$37.5 °C or late treatment failure (LTF) if harbouring parasites in the absence of symptoms of severe malaria from days 4–28; (c) an adequate clinical and parasitological response (ACPR) if children have no parasite on day 28 irrespective of the axillary temperature without symptoms of early or late treatment failure. During the follow-up; children who presented treatment failure were treated according to the standard therapy at the health center with S/P (25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine) and ferrous sulfate tablets for at least 2 weeks. Due to the high number of ETF among the first 50 included patients according to the revised protocol for the assessment of the antimalarial drug resistance (WHO/CDS/CSR/EPH/2002.17, WHO/CDS/RBM/2002.39) (World Health Organisation, 2002), the next 87 patients meeting the inclusion criteria were not treated with CQ but with another antimalarial and blood samples were collected only for determining the prevalence of K76T *pfcrt* mutation and multiplicity of infections in *P. falciparum* isolates.

#### 2.2.1. Blood samples

At inclusion thick blood films were prepared from each patient to determine the parasite density and the *Plasmodium* species, and three drops of blood from each patient were blotted onto 3MM Whatman filter paper dried and stored in individual sealed envelopes until being use for DNA extraction. The slides were prepared as follows: 10 μl of blood were evenly distributed on a 10 mm × 18 mm area of a microscope slide (drawn on paper underneath the slide), dried and stained (10% Giemsa (Sigma Chemical, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), pH 7.2, 15 min). The parasite density was measured by counting the number of asexual parasites per 200 leukocytes under Giemsa-stained thick blood smears, based on a mean count of 8000 leukocytes per microliter of blood. A slide was declared negative after microscopic fields corresponding to at least 500 leukocytes had been checked. Two experienced technicians performed the microscopic independently, each time comparing their results. During the follow-up of patients, blood samples were collected onto filter paper if necessary.

### 2.3. Extraction of parasite DNA

Parasite genomic DNA was extracted from blood samples collected on filter paper using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Then the DNA was recovered in 100 μl of elution buffer from the kit. All parasite DNA extracted were stored at −20 °C until use.

#### 2.3.1. MSP-1 and MSP-2 genotyping of P. falciparum isolates

The highly polymorphic loci, merozoite surface protein-1 (MSP-1) block 2 and merozoite surface proteine-2 (MSP-2) central region, were used to genotype the *P. falciparum* parasite isolates by polymerase chain reactions (PCRs). The K1, MAD20, and RO33 allelic families of MSP-1, and the FC27 and 3D7 allelic family of MSP-2 were analysed. Primers sequences and PCR conditions were described elsewhere (Ntoumi et al., 2000). Allele-specific positive controls and DNA-free negative controls were included in each set of reactions. Eight microliters of each of the PCR products were electrophoresed on a 1.5% agarose gel (PeqLab, Erlangen, Germany), and visualized under ultraviolet (UV) transillumination after staining with ethidium bromide.

#### 2.3.2. Analysis of point mutation in codon 76 in pfcrt gene

The lysine to threonine mutation in codon 76 of *pfcrt* was detected by nested mutation specific

restriction enzyme digestion PCR reactions using the method of Djimde et al. (2001a) in a volume of 50 μl in a Biometra Uno II Thermal cycler (Biometra, Göttingen, Germany). Each PCR mixture contained 200 μM deoxynucleoside triphosphates, 1.5 U of Taq polymerase (Qiagen), and 2.5 mM $MgCl_2$ (Qiagen). In this method, the second round of amplification produces a 134 bp amplicon containing codon 76. Then, 5 μl of the amplicon from the second PCR product were digested with 0.5 U of the restriction enzyme *Apo*1 (New England Biolabs, Beverly, MA) during six hours incubation at 50 °C according to the manufacturer's protocol. In all PCRs and digests, genomic DNA from the laboratory-adapted chloroquine-resistant strain Dd2 containing T76 mutation and chloroquine-sensitive strain HB3 without this mutation were used as positive controls while, water was used as negative control. The PCR products and the digest were run onto 2% agarose gel containing ethidium bromide and visualized under UV transillumination. The K76T mutation results in the loss of an *Apo*1 recognition site (Mayor et al., 2001) so that mutated (K76T) DNA samples remain undigested while those in which the mutation is absent yield two fragments of 100 and 34 bp. Point mutation in the *pfcrt* gene was analysed in overall isolates before treatment and after treatment failure for the first 50 patients treated with CQ.

### 2.4. Data and statistical analysis

The multiplicity of infection was defined as the minimum number of *P. falciparum* genotypes per infected subject and estimated by dividing the number of amplified PCR fragments reflecting parasite genotypes by the number of positive samples. The chi2 test was used to compare proportional data. Mean values of temperature and parasite density were determined. MSP-1 and MSP-2 allele frequency was calculated as the proportion of alleles found for the allelic family out of all alleles detected in isolates. The distribution of MSP-1 and MSP-2 allelic families was estimated considering overall isolates before treatment. The Mann–Whitney *U*-test was used to compare the parasite density and the age between two groups (multiple and single infected). Difference was considered statistically significant when $P \leq 0.05$ (IC 95%).

## 3. Results

### 3.1. In vivo evaluation of the efficacy of chloroquine for the treatment of uncomplicated malaria

In vivo outcomes for the 50 children who were followed-up for 28 days or until they reached an outcome are reported in Table 1. The geometric mean parasite density was 38,858 parasites/μl of blood (ranged from 2130 to 200,000 parasites/μl). Only two children were successfully treated with CQ. The proportion of treatment failure at days 14 and 28 was 60.9 and 95.7%, respectively. Early treatment failure was observed in 21 children, whereas late treatment failure at days 14 and 28 were observed in 7 and 16 patients, respectively.

### 3.2. Analysis of the K76T mutation and multiplicity of infection in pre and post-treatment samples

Genotyping of *pfcrt* K76T mutation was performed on the 50 isolates collected before treatment and the 23 post-treatment isolates from children who presented with LTF (Table 1). All of the pre- and post-treatment isolates produced the expected 134 bp amplicon, and none were cut by *ApoI* suggesting the presence of K76T mutation. Control HB3-strain parasites demonstrated the wild-type sequence with all sets of digestion. MSP-1 and MSP-2 genotyping was used to identify

Table 1
Therapeutic outcomes with chloroquine and, presence of *pfcrt* T76 mutation in pre and post-treatment Congolese isolates

| | No. | % |
|---|---|---|
| Total number of subjects | 50 | |
| Lost | 4 | 8 |
| Adequate response | 2 | 4.4 |
| Treatment failure at day 14 | 28 | 60.9 |
| Treatment failure at day 28 | 44 | 95.7 |
| ETF | 21 | 45.7 |
| LTF days 4–14 | 7 | 15.2 |
| LTF days 15–28 | 16 | 34.8 |
| T76 mutation in pre-treatment isolates | 50 | 100 |
| T76 mutation in post-treatment (LTF) isolates | 23 | 100 |

No.: number of patients; ETF: early treatment failure; LTF: late treatment failure; LTF days 4–14: late treatment failure observed between days 4 and 14; LTF days 15–28: late treatment failure observed between days 15 and 28.

recrudescent and new infections and to determine the multiplicity of infection. Sixteen pre- and post-treatment paired samples were considered. We found that nine isolates (69.6%) presented identical parasite genotypes suggesting recrudescent infection, 5 (21.7%) had different patterns (probably re-infection) and 2 (87%) presented the profile corresponding to both recrudescence and new infection. The multiplicity of infections was 1.76 in both pre- and post-treatment isolates.

### *3.3. Characterization of P. falciparum infections in 87 isolates from children with uncomplicated malaria and not treated with CQ*

As specified in Section 2, due to the high treatment failures rate, 87 children with uncomplicated malaria aged from 6 months to 5 years were recruited but were not followed with CQ. Before antimalarial treatment, blood samples were collected and parasite DNA was genotyped for MSP-1 and MSP-2 genes. The geometric mean parasite density was 4625 parasites/μl ranged from 60 to 200,000 parasites/μl).

The efficiency for MSP-2 gene amplification reactions with family-specific primers was 80%. We found 19 distinct alleles with 11 and 8 alleles belonging to FC27 (78% of overall detected MSP-2 alleles) and 3D7 (20%) type, respectively. Three genotypes could not be assigned to any family. MSP-1 gene was amplified with an efficiency of 94% and among the 21 different alleles detected, 12, 8 and 1 were attributed to K1 (43% of overall detected MSP-1 alleles), Mad20 (14%) and RO33 (40%) family. Four fragments remained non identified.

The multiplicity of infection in this group was 2.1. Out of 87 samples, 62 (72%) harboured more than one parasite genotype. The number of infected samples with more than one parasite genotype was influenced neither by age nor by parasite density.

### *3.4. Analysis of the K76T mutation in 87 Congolese children with uncomplicated malaria*

Analysis of the *pfcrt* K76T mutations indicated that 85 of the 87 (98%) samples had the mutant allele at codon 76 while the wild-type allele and both mutant and wild-type alleles were detected in two different isolates, respectively.

## 4. Discussion

This study reports the evaluation of the efficacy of chloroquine for the treatment of uncomplicated malaria in a suburb of Brazzaville, Republic of Congo. Clinical assessment and parasitological monitoring of the patients confirmed the alarming situation already reported by Nsimba et al. (2004). The low efficacy of CQ can be explained by the frequent and uncontrolled use of the drug outside the official health system (Wernsdorfer, 1994).

Here, only 50 patients were treated with CQ face to the observed high rate of treatment failures. It would have been unethical to continue the recruitment in the same way. Blood samples were collected from the next Congolese patients before treatment with S/P, for investigating the K76T mutation in *pfcrt* gene as a molecular tool for surveillance of chloroquine resistance. Studies conducted in several African areas provided a direct evidence for in vivo selection of mutant allele at codon 76 in *pfcrt* gene (Basco et al., 2002; Binder et al., 2002; Djimde et al., 2001a). Analysis of this parasite mutation in Congolese isolates showed the presence of mutant type in all pre- and also post-treatment isolates.

The sensitivity for detection of in vivo resistance to CQ associated the presence of the mutation was 96% (44/46). The two children who were successfully treated with CQ harboured also parasites with *pfcrt* K76T mutation. In areas of intense transmission, where immunity to clinical malaria is developed earlier and faster, it has been reported that some patients were able to clear their parasitemia even in the presence of the *pfcrt* K76T mutation (Dorsey et al., 2001). No variation in the multiplicity of infection was observed in pre- and post-treatment isolates. This might reflect the absence of parasite clearance after the chloroquine ingestion and confirmed the recrudescence of parasites instead of new infection. The multiplicity of infection in clinical Congolese isolates was concordant with reports from other areas of Central Africa (Basco and Ringwald, 2001b; Kun et al., 1998).

The investigation carried out in the 87 isolates before antimalarial treatment showed a prevalence of 98% of this mutation in *P. falciparum* isolates. In Gabon, the presence of this mutation was associated with in vivo and in vitro findings during the re-assessment of CQR in isolates from patients with uncomplicated malaria (Binder et al., 2002; Borrmann et al., 2002). In regard

to that, our results strongly suggest that chloroquine would be associated with high treatment failure rates reflecting the high level of CQ resistance in Brazzaville.

The present study confirmed the substantial polymorphism of the merozoite surface proteins 1 and 2 of *P. falciparum* isolates from an area with high malaria transmission (Basco and Ringwald, 2001b; Ntoumi et al., 1995; Ranford-Cartwright et al., 1997). Using another strategy based on microsatellite, a similar observation was done in Pointe Noire, Republic of Congo (Durand et al., 2003). Here FC27/MSP-2 allelic family and MSP-1 allelic families belonged to K1 and RO33 were found to be the most predominant allelic families in *P. falciparum* isolates from Brazzaville as reported in other sites in Central Africa (Aubouy et al., 2003; Kun et al., 1998; Ntoumi et al., 1996, 2000). In Uganda, Mad20/MSP-1 alleles have been associated with sensitivity to CQ (Jelinek et al., 1999) and the low prevalence of Mad20/MSP-1 alleles in Congolese isolates is an argument in favour of such association.

This study confirms the high level of in vivo resistance to CQ and shows the high prevalence of *pfcrt* K76T mutation in the Republic of Congo.

## Acknowledgements

We thank the children who participated to this study and the staff of the Health center of Terinkyo. We thank Dr. Mavoungou for the critical reading of the manuscript. PIM and DMM were fellow of DAAD and MIM/TDR ID A30031 and RCS ID A20789, respectively. FN received financial support from the Alexander von Humboldt Foundation and is member of MIMPAC network. MN received financial support of RCS/TDR ID A20789 and from PAL+ (action 2002), Ministère français de la Recherche. This study received financial support under the Multilateral Initiative on Malaria (MIM) project A30031 through the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and under RCS ID A20789.

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