PLASMODIUM FALCIPARUM RESISTANT TO CHLOROQUINE AND TO PYRIMETHAMINE IN COMOROS

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Summary:

We report the outcome of chloroquine treatment and the prevalence of mutations at codon 86 of the *pfmdr1* gene, at codon 76 of the *pfcr1* gene, and at codon 108 of the *pfdhfr* gene in clinical isolates of *Plasmodium falciparum* collected from 30 children under 10 years of age living in the Comoros Union. This *in vivo* study was carried out in February and March 2001 in Moroni. Chloroquine treatment failed in 23 children (76.6 %; 95 % confidence interval: 57.7 to 90.1 %). Subsequent genotyping showed that all *P. falciparum* isolates (100 %) harboured a tyrosine residue at position 86 in *pfMDR1*. 83.3 % (25/30) of these isolates harboured a mutation at position 76 in *pfCRT* and half (15/30) of these isolates also harboured a mutation at position 108 in *pfDHFR*. Chloroquine resistance is a real concern in the Comoros Union. The prevalence of *pfDHFR* mutant parasites is alarming. The alternative drugs proposed as a replacement for chloroquine as first-line treatment in Comoros, and the strategy to monitor the drug susceptibility of *Plasmodium* sp in this part of the Indian Ocean sub-region are discussed.

KEY WORDS : Comoros, chloroquine, malaria, *Plasmodium falciparum*, resistance.

Résumé : RÉSISTANCE DE *PLASMODIUM FALCIPARUM* À LA CHLOROQUINE ET À LA PYRIMÉTHAMINE AUX COMORES

Cet article décrit la réponse thérapeutique à la chloroquine dans le traitement du paludisme à *P. falciparum* chez 30 enfants de moins de 10 ans atteints de paludisme, vivant dans l'Union des Comores, et aussi la prévalence des mutations au niveau des marqueurs génétiques de la résistance des parasites à la chloroquine (*pfcr1* K76T, *pfmdr1* N86Y), et de la résistance à la pyriméthamine (*pfdhfr* S108N) chez les isolats de *P. falciparum* prélevés à JO. Lors de l'étude réalisée en février et mars 2001 à Moroni, le traitement par chloroquine en monothérapie a échoué chez 23 enfants (76,6 %; Intervalle de confiance 95 % : 57,7-90,1 %). La détection des mutations par la technique de PCR/RFLP a montré que tous les isolats de *P. falciparum* testés (100 %) contiennent la tyrosine à la position 86 dans *pfMDR1*. 83,3 % (25/30) de ces isolats contenaient des souches mutées à la position 76 pour *pfCRT* et la moitié (15/30) des isolats était mutée asparagine-108 *pfdhfr*. Les résultats des tests *in vivo* et le génotypage montrent que la résistance de *P. falciparum* à la chloroquine est à présent un problème majeur dans l'Union des Comores. La prévalence des parasites mutés pour *pfDHFR*, donc potentiellement résistants à la pyriméthamine, est alarmante. Les propositions des alternatives à la chloroquine pour le traitement de première intention dans l'Union des Comores, et la stratégie de surveillance de la sensibilité/résistance de *Plasmodium* sp. dans cette sous-région de l'Océan Indien sont discutées.

MOTS CLÉS : Comores, chloroquine, paludisme, *Plasmodium falciparum*, résistance.

In Comoros Union, 15 to 30 % of hospital admissions are due to malaria, and malaria is responsible for 15 to 20 % of all deaths registered in paediatric units (Ouledi, 1995). *Plasmodium falciparum* accounts for over 95 % of malaria cases. Chloroquine

has been the recommended first-line treatment for malaria for the last four decades. Local populations with limited income, frequently use a chloroquine auto-medication in case of fever. Recent reports from peripheral health districts of the archipelago (Grande Comore, Anjouan and Mohéli) have shown an increasing rate of chloroquine treatment failure, which now exceeds 30 % (Silai, unpublished data). This probably reflects increasing prevalence of chloroquine-resistant *P. falciparum* parasites in the Comoros. However, in order to change the national treatment policy, evidence-based information is needed.

Within the frame of a regional network on malaria, the Institut Pasteur de Madagascar participated in an *in vivo* evaluation of chloroquine effectiveness for the treatment of uncomplicated *falciparum* malaria in Moroni (Grande Comore) in 2001. This study was the first step of a regional collaboration between Madagascar and

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Comoros. Above all, the aim was to document chloroquine effectiveness and the prevalence of the *pfCRT* mutation K76T associated in other settings with chloroquine-resistance (Fidock *et al.*, 2000; Djimde *et al.*, 2001).

MATERIALS AND METHODS

IN VIVO TEST AND COLLECTION OF BLOOD SAMPLE

The *in vivo* efficacy of chloroquine treatment in children with uncomplicated *falciparum* malaria was measured in Moroni in March 2001. The study was approved by the Ethics Committee of the Comorian Ministry of Health. Informed consent was obtained from all parents or guardians. Thirty-two children with uncomplicated *P. falciparum* malaria were included. Two were excluded during the follow-up, because they were given a quinine injection on Day 1 or Day 2 by a private health worker in their village with parental consent because they had fever ("hot skin" according to local terminology). Thirty children (nine females and 21 males) aged between eight months and nine years (mean age: 3.6 years; median age: 3.25 years; 95 % confidence interval: 2.78-4.42 years) were monitored for 14 days following WHO protocol (WHO, 1996). Before the administration of chloroquine on Day 0, a fingerprick blood sample was collected on filter paper. The air-dried filter paper was placed in an individual plastic bag and shipped to Institut Pasteur de Madagascar. The samples were kept at -20° C until use.

DNA EXTRACTION AND PCR (POLYMERASE CHAIN REACTION)

Mutations in the *pfCRT* and in *pfmdr1* genes were detected by the Malaria Research Group at the Institut Pasteur de Madagascar in October 2002. *P. falciparum* DNA was extracted from the filter blood spot. A ~ 10 mm square of blood impregnated filter paper was placed in 200 µl of PBS buffer (pH 7.2) containing 0.5 % Tween 20 (*v/v*) for three hours at room temperature. The sample was shaken for 10 seconds every hour by use of a vortex mixer. The tube was then centrifuged at 360 g for two minutes at room temperature. The supernatant was collected and used for phenol-chloroform DNA extraction as described (Ariey *et al.*, 1999). DNA was re-suspended in 30 µl of sterile water.

The *pfCRT*, *pfmdr1* and *pfdhfr* genes were amplified by nested PCR, as described (http://medschool.umaryland.edu/cvd/2002_pcr_asra.html). For each PCR and each digestion, DNA from *P. falciparum* FCM29 (chloroquine-resistant) and 3D7 (chloroquine-sensitive), which are maintained in continuous culture in the laboratory,

was used as a positive control and H₂O as a negative control.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

The *pfCRT* and *pfmdr1* nested PCR products were digested with *ApoI*, with *Afl* III (New England Biolabs, UK) respectively, and *pfdhfr* nested PCR were digested separately with *AluI*, *SrfI* and *BsrI* (New England Biolabs, UK), in a final volume of 25 µl as described (http://medschool.umaryland.edu/cvd/2002_pcr_asra.htm; Djimde *et al.*, 2001; Rason *et al.*, 2002; von Seidlein *et al.*, 1997; Zindrou *et al.*, 1996).

The restricted products (15 µl) were subjected to electrophoresis in a 2 % agarose gel, stained with 0.5 µg/ml ethidium bromide and visualized under ultraviolet light. The 145-bp *pfCRT* PCR product contains one *ApoI* site when the codon 76 of the *pfCRT* gene codes for a lysine (K76), visualised by presence of a 99-bp and a 46-bp restriction fragments. The 291-bp *pfmdr1* PCR product contains a single *Afl*III site when codon 86 is a mutant-type coding for tyrosine (86Y), resulting in generation of a 165-bp and a 126-bp fragment. The 256-bp *pfdhfr* PCR products contained one restriction site for either *AluI*, *BsrI* or *SrfI*, depending on the amino acid residue at position 108 of the *pfdhfr* gene. *AluI*, *BsrI* and *SrfI* each cleaved the PCR product into one 210-bp product and one 46-bp product. *AluI* cut the product when the wild-type codon was present at position 108 (serine-108), *BsrI* cut the product when an asparagine codon was present at position 108 (asparagine-108) linked to pyrimethamine resistance and *SrfI* cut the product when a threonine codon was present at position 108 (threonine-108) linked to cycloguanil resistance (Bzik *et al.*, 1987; Cowman *et al.*, 1988).

RESULTS

OUTCOME OF CHLOROQUINE TREATMENT

On Day 0, parasitaemia ranged from 1,560 to 101,000 trophozoites per 8,000 white blood cells (geometric mean parasitaemia: 12,336; 95 % confidence interval: 13,800-33,852). There were 21 clinical failures, and two parasitological failures on Day 14 (Table I). Thus, the overall chloroquine treatment failure rate was 76.6 % (95 % confidence interval: 57.7-90.1 %). For 15 of 21 children aged below five years (71.4 %), a clinical failure was recorded and in eight cases, treatment failed at an early stage. All children in whom the initial chloroquine treatment failed were subsequently treated successfully either with quinine or with sulfadoxine-pyrimethamine (data not shown).

Chloroquine treatment outcome	Number of children	Children carrying parasites with mutation at	
		codon 76 in <i>pfcr</i> t	codon 108 in <i>pf</i> dhfr
Overall clinical failure*	21	18	10
Parasitological failure on Day 14	2	2	1
Adequate clinical and parasitological response**	7	5	4
Total	30 (100 %)	25 (83.3 %)	15 (50 %)

* : Overall clinical failure combines early and late treatment failure.

** : Clinical and parasitological response means clinical cure and absence of parasitaemia.

Table I. – Outcome of chloroquine treatment in Comorian children and prevalence of *Plasmodium falciparum* with mutation at codon 76 in *pfcr*t (samples were collected on Day 0).

GENOTYPING

The 30 nested PCRs for *pfcr*t, *pfmdr*1 and *pf*dhfr were successful. All of the examined isolates of *P. falciparum* collected on day-0 yielded a *pfmdr*1 PCR product that was digested with *A*/III. This indicated that all isolates contained the *pfmdr*1 tyrosine-86 (86Y) mutation. Only two of 30 isolates were mixed *i.e.* contained in addition to the mutant *pfmdr*1 86Y parasites, parasites that had an *A*/III-insensitive product. Twenty-five (83.3 %) of the *pfcr*t PCR products were not cut by *Apo*I, indicating that they harboured a mutation at codon 76. Fifteen (50 %) of the 30 typed isolates contained the *pf*dhfr asparagine-108 mutation, and 11 of these 15 isolates harbouring asparagine-108 *pf*dhfr harboured also a mutation at *pfcr*t codon 76.

DISCUSSION

We found that the chloroquine treatment failure rate was unacceptably high (76.6 %) in children. The situation seen in Moroni can probably be generalised throughout the archipelago due to the population dynamics between islands. Almost 20 years ago, a case of chloroquine treatment failure was reported in Germany in a patient who had recently visited Comoros (Eichenlaub & Pohle, 1980). In the 1980s and early 1990s, the prevalence of chloroquine resistance was considered to be low in Comoros even though RIII resistance cases had been recorded (Blanchy & Benthein, 1989; Feillet *et al.*, 1993). The selection of chloroquine-resistant *P. falciparum* is probably related to the high chloroquine consumption by the local inhabitants (Feillet *et al.*, 1993). This probably explains why the treatment failure rate increases in general population (Blanchy & Benthein, 1989; Arie *et al.*, 2002).

Even though different studies have concluded that there is not a perfect correlation between the presence of the mutation at position 76 of *pfcr*t and the clinical response to chloroquine treatment (Djimde *et al.*,

2001; Kyosiimire-Lugemwa *et al.*, 2002; Talisuna *et al.*, 2002), the prevalence of chloroquine treatment failure is consistently higher when the prevalence of the *pfcr*t threonine-76 mutation is high. Such situation is now occurring in Comoros. Prior the discovery of *pfcr*t, the majority of research into the genetic basis of *P. falciparum* chloroquine-resistance focused on *pfmdr*1 (Foote *et al.*, 1990). But it was also reported later that there is no strong correlation between point mutations in *pfmdr*1 gene and clinical response to therapy with chloroquine (Dorsey *et al.*, 2001). Although the role of mutations in known resistance genetic markers in mediating responses after chloroquine therapy remains uncertain, both *in vivo* test and genotyping reported herein indicate a high prevalence of chloroquine resistance in Comoros. Given this dramatic situation, there is an urgent need to reconsider the national malaria control policy and introduce the use of preferment therapy based on the use of antimalarial drugs other than chloroquine.

For many years, sulfadoxine-pyrimethamine has been advocated as the second line treatment in Comoros. This drug has been widely used with or without medical prescription in Moroni (and elsewhere in Comoros). Our data indicate that a large number of *P. falciparum* isolates (15/30) already harboured an asparagine-108 *pf*dhfr allele that is associated with pyrimethamine resistance (Peterson *et al.*, 1888; Cowman *et al.*, 1988; Hyde, 1990; Ranford-Cartwright *et al.*, 2002). We would not give much weight to the prevalence of asparagine-108, as this single mutation has not been found to be predictive of sulfadoxine-pyrimethamine treatment failure, and the quintuple DHFR/DHPS mutant is the most strongly associated with sulfadoxine-pyrimethamine failure. (Kublin *et al.*, 2002) But for a regional system for malaria resistant surveillance, the occurrence of prevalent asparagine-108 mutant parasites is alarming. An area with ~ 50 % asparagine-108 is generally likely to have more sulfadoxine-pyrimethamine resistance sooner or later. Thus, whatever the profiles (wild or mutant) of the other codons known as pyrimethamine resistance markers in *pf*dhfr or sulfadoxine resistance

markers in *pfdbhps*, this presence of prevalent asparagine-108 *pfdbhfr* in Comoros is not in favour of a switch from chloroquine to sulfadoxine-pyrimethamine alone (monotherapy) as first line treatment in this archipelago. Therefore, it was decided by Comorian health policy makers that combination therapies will be tested to determine an effective replacement for chloroquine. Fight against malaria should not be limited to a single country. In contrast with the situation in Comoros, *P. falciparum* isolates carrying *pfcr* and *pfdbhfr* mutations have been absent (or might be present at very low prevalence) in Madagascar and the clinical responses to chloroquine and to sulfadoxine-pyrimethamine are satisfactory (Ariey *et al.*, 2002; Randrianariveლოსია *et al.*, 2002a, 2002b, 2002c; Rason *et al.*, 2002). The dissemination of resistant parasites from an area of resistance to an area of sensitivity is a factor leading to the spread of resistance. Thus, the biggest danger for Madagascar may be importation of resistant malaria in view of the improving commercial exchange between neighbouring islands in this part of the Indian Ocean. However, the Malagasy recording system does not yet allow us to document cases of malaria imported into Madagascar, while for example, it has been proven that many cases of malaria are introduced into France from Comoros (Minodier *et al.*, 1999).

Regional surveys involving the Comoros archipelago and Madagascar should be strengthened to generate up-to-date and comparative *in vivo* and *in vitro* data to allow the design of regional and national evidence-based strategies for controlling malaria.

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