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First evidence of *pfcrt* mutant *Plasmodium falciparum* in Madagascar

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Summary

The island of Madagascar, lying in the Indian Ocean approximately 250 miles from the African coast, has so far remained one of the few areas in the world without noticeable *Plasmodium falciparum* high-grade chloroquine (CQ) resistance. Here we report genotyping data on *pfcrt* in Madagascar. The *pfcrt* K76T mutation, which is critical for resistance to CQ, was detected in six (3.3%) of 183 *P. falciparum* isolates screened, within the mutant haplotypes CVIET and CVIDT. This is the first observation of *pfcrt* mutant parasites on the island. The current massive distribution of CQ for in-home management of fever in children will promote the dissemination of these mutant CQ-resistant parasites. In this context, genotyping of *pfcrt* remains a useful tool for CQ resistance surveillance as the prevalence of *pfcrt* mutations is far from saturation in Madagascar.

Keywords

Malaria; *Plasmodium falciparum*; Chloroquine; *pfcrt*; Resistance; Madagascar

1. Introduction

The island of Madagascar, lying in the Indian Ocean approximately 250 miles from the African coast, has so far remained one of the few areas in the world without noticeable *Plasmodium falciparum* high-grade chloroquine (CQ) resistance. In Madagascar, the use of CQ in public and private health facilities and at the community level is part of the national strategy to control malaria. Since 1945, CQ has been used as the front-line drug to treat uncomplicated malaria (Randrianarivelojosia et al., 2002). Thus far, low-grade CQ resistance, categorised as R1 or R2, or late clinical and late parasitological failures, has been reported (Ariey et al., 2002; Milijaona et al., 1998). Previously, the CQ resistance marker gene *pfcrt* in clinical *P.*

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Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

falciparum isolates was typed. In these studies, no mutant *P. falciparum* strains harbouring *pfcrt* 76T were detected in four different sites (Ariey et al., 2002; Rason et al., 2002).

Documentation of the susceptibility and resistance of *P. falciparum* to drugs is vital to generate useful and usable data to guide changes in national antimalarial policies. To alleviate the lack of medical teams to monitor routinely the therapeutic effectiveness of antimalarial drugs at peripheral health centres throughout the country, the Ministry of Health and Family Planning (MoH) and the Institut Pasteur de Madagascar (IPM) created the Réseau d'Etude de la Résistance-paludisme (RER) in 1999 to serve as a national network for malaria resistance surveillance. The IPM is mandated to run malaria parasite phenotyping and genotyping (Ariey et al., 2002; Randrianarivelojosia et al., 2002; Rason et al., 2002). As part of the RER activities, *P. falciparum* isolates collected from Andapa and Tsiroanomandidy were examined to monitor the status of point mutations in the *pfcrt* gene.

2. Materials and methods

2.1. Ethical approval

The study protocol related to the surveillance of malaria resistance was submitted to the national ethical committee recently reconstituted in 2003 and obtained ethical clearance.

2.2. Parasite samples from Tsiroanomandidy

Tsiroanomandidy is in the western region of the foothill areas of Madagascar. Sampling was undertaken in November and December 2002 by the RER correspondent at the urban primary health centres. Malaria was diagnosed by microscopy. Malaria patients were treated according to the national recommendation. Clinical *P. falciparum* isolates ($N = 51$) were collected by venipuncture of 2–5 ml samples into EDTA-coated tubes from consenting symptomatic outpatients >2 years old with uncomplicated malaria. Isolates were transported at 8°C to the Institut Pasteur de Madagascar in Antananarivo within 48 h of blood collection and kept at −20°C until use.

2.3. Parasite samples from Andapa

Andapa is situated in the wet northern region of Madagascar. Sample collection was undertaken from October 2001 to March 2002. During this period, microscopy was used to detect the presence of malaria parasites in pregnant women seen at health centres for pre-natal consultation and also in women who delivered at the maternity ward. *Plasmodium falciparum* isolates were collected by venipuncture of 2–5 ml samples into EDTA-coated tubes from consenting women. Samples (106 isolates from pregnant women and 28 from post-partum women) were frozen and sent to the Institut Pasteur de Madagascar in Antananarivo in cold chain and kept at −20°C until use. Malaria patients were treated according to the national recommendation. There was no follow-up.

2.4. DNA extraction and PCR/RFLP to detect *pfcrt* K76T mutation

Parasite DNA was extracted from 200 μl of red blood cell pellets by phenol—chloroform purification (Ariey et al., 1999). The *pfcrt* gene of *P. falciparum* was amplified by nested PCR as described (http://medschool.umaryland.edu/cvd/2002_pcr_asra.htm) using a Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany). The *pfcrt* nested PCR products were digested with *Apo*I (New England Biolabs, Hitchin, UK) in a final volume of 25 μl according to the manufacturer's recommendations. The digested products (15 μl) were subjected to electrophoresis in a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide and visualised under UV light. The 145 bp *pfcrt* PCR product contains one *Apo*I site when codon 76 of the *pfcrt* gene codes for lysine (K76), which is visualised by the presence of 99 bp and

46 bp restriction fragments. For each PCR and each digestion, DNA from *P. falciparum* strains FCM29 (CQ resistant) and 3D7 (CQ sensitive), which are maintained in continuous culture in the laboratory, were used as positive controls, and H_2O was used as a negative control. When the presence of mutant *pfcrt* K76T was detected in a sample, a blinded technician repeated the whole process from DNA extraction to digestion of the PCR products.

2.5. Sequencing

Sequencing was done at Genomex (Grenoble, France) or at the Albert Einstein College of Medicine in New York for isolates containing mutant *pfcrt* K76T detected by PCR/RFLP (*N* = 6). Twelve randomly selected samples of wild-type *pfcrt* K76 were also sequenced. Primary PCR products were sent with the primers for the nested PCR to Genomex to sequence the fragment of interest surrounding codon 76 of *pfcrt*. Genomic DNA samples were sent to the Albert Einstein College of Medicine. Our counterparts in charge of sequencing were not informed of the PCR/RFLP results. Sequencing was performed as described (Fidock et al., 2000). Sequences were cross-analysed at the Albert Einstein College of Medicine and at Institut Pasteur de Madagascar.

3. Results

All nested PCRs for *pfcrt* were successful except for two samples from pregnant women from Andapa (Table 1). PCR/RFLP results were all confirmed by sequencing. Sequences of *pfcrt* codons 72–76 indicate that all 12 wild-type parasites (K76) sequenced were of CVMNK haplotype, as is the reference strain of CQ-sensitive *P. falciparum* 3D7.

Of the 51 clinical samples examined from Tsiroanomandidy, one of the *pfcrt* PCR products (1.96%, 95% CI 0.05–10.4%) was incompletely digested by *Apo*I, indicating that it harboured parasites that were mutant at codon 76. Interestingly, the sequence for codons 72–76 indicates the presence of the CVIDT haplotypes (Table 2). Of the successfully typed samples (104/106) from pregnant women seen at the pre-natal clinic in Andapa, PCR/RFLP showed that two isolates (1.92%) contained *pfcrt* mutant parasites. Sequencing indicated that both of them harboured mixed infections, with mutant parasites of the CVIDT haplotype and wild-type parasites of the CVMNK haplotype. The proportion of mutant parasites detected among women who delivered at the maternity ward appeared higher. Of the 28 isolates examined, 3 (10.7%) harboured CQ-resistant parasites of the CVIET haplotype. Since the difference in the prevalence of mutant *pfcrt* in the two groups of women in Andapa was not statistically significant, we considered the isolates from these women as a single sample. In the Andapa isolates, mutant parasites were detected in 5/132 samples (3.8%, 95% CI 1.2–8.6%). Officially, CQ is recommended for malaria prophylaxis in pregnant women, but of these five women with mutant parasites three denied using chemoprophylaxis, one reported irregularly taking three tablets of CQ per week, and no information was given by the last patient.

4. Discussion

In this study, we report the detection of malaria parasites containing mutations in *pfcrt* for the first time in Madagascar. Among the six mutant parasites, two haplotypes occurred: the CVIDT initially described in Cambodia, and the CVIET commonly documented in Africa and Asia (Ariey et al., 2002; Lim et al., 2003). Irrespective of the results reported herein, Madagascar is facing decreasing therapeutic efficacy of CQ, and the need to identify an effective replacement therapy has been previously proposed (Milijaona et al., 1998).

Although there is ongoing debate on the role of *pfcrt* mutations in mediating clinical responses to CQ therapy in the field, the detection of mutant parasites must be considered within the context of national malaria control in Madagascar, a country of limited resources. Previous

screening studies in Madagascar for the *pfcrt* K76T mutation in *P. falciparum* have demonstrated the absence of mutant parasites among examined samples (Ariey et al., 2002; Rason et al., 2002). Paradoxically, unpublished data from the malaria department of the MoH highlight that massive use of CQ, called nivaquinisation or chloroquinisation campaigns, were part of the operational approaches to fight malaria in Madagascar. The most important episodes are the chemoprophylaxis of children from the 1950s to 1970s and the large distribution of CQ by 40 000 community dispensers to treat fever in efforts to combat a malaria outbreak in the 1980s (Lepers et al., 1989).

Today, the national strategy to roll back malaria in Madagascar still recommends in-home case management with pre-packaged CQ in children under 5 years. At the time of writing of this paper, three million free doses of CQ (of eight million doses already available at the MoH) will already have been given to mothers as standby treatment for their children to treat clinically suspected cases of malaria empirically, and two million doses of another brand of pre-packed CQ are also available at low cost in the villages. The consequent drug pressure following such practice will result in the selection and dissemination of mutant CQ-resistant parasites, which will ultimately lead to failure of the strategy currently in place for controlling malaria in Madagascar. If low-grade CQ resistance reflected by treatment failure occurred in Madagascar despite the low frequency of *pfcrt* mutations, this would suggest a genetic predisposition to CQ resistance among malaria parasites. Therefore, the impact of the spread of *pfcrt* mutations on the resistance profile in Madagascar may be unpredictable. The main road network has been improved from the north (where Andapa is located) to the south, thus likely exchanges between regions will increase. Even if our findings could not be generalised for the whole country of 587 000 km^2, the national strategy for the surveillance of drug-resistant malaria parasites should be adjusted to anticipate the worst-case scenario of a rapid spread of *pfcrt*-mediated CQ resistance.

Unfortunately, in most countries where *P. falciparum* is endemic, identification of *pfcrt* as the CQ resistance marker (Bray et al., 2005; Fidock et al., 2000) came too late to be used effectively as a surveillance tool to prevent the spread of CQ resistance. *pfcrt* typing in these areas has ironically confirmed that genetically CQ-resistant *P. falciparum* was already highly prevalent — from 15–100% of the total parasite population. For example, in the Indian Ocean subregion, the prevalence of mutant *pfcrt* has reached saturation in Comoros Union as well as in various parts of eastern mainland Africa (Ariey et al., 2002; Randrianarivelojosia et al., 2004; Rason et al., 2002). With the high prevalence of the *pfcrt* K76T mutation, high-grade CQ resistance occurs (R3 and early CQ treatment failure) (Ariey et al., 2002; Djimde et al., 2001; Kyosiimire-Lugemwa et al., 2002; Mayor et al., 2001; Rason et al., 2002; Wilson et al., 2005). In these areas, the utility of the *pfcrt* K76T marker for CQ resistance is limited to the molecular verification of CQ-resistant parasites rather than the prevention of country-wide dissemination of CQ resistance.

Here we provide further evidence indicating the need to replace the front-line treatment of CQ monotherapy with combination therapy before CQ resistance becomes widespread in Madagascar. Since CQ-resistant parasites were found in pregnant women, the recent replacement of CQ prophylaxis in pregnant women by intermittent preventive treatment with sulfadoxine/pyrimethamine is a sound choice (Randrianasolo et al., 2004), although vigilant surveillance is needed to detect the potentially rapid appearance and spread of resistance to this drug. As far as we know, no robust method is yet available to date the introduction and/or occurrence of mutant parasites. Thus, resistance surveillance must be maintained and focused on the established in vitro methods as well as on the gold standard of in vivo CQ treatment efficacy to anticipate and prevent the spread of CQ resistance in Madagascar, especially since CQ resistance mechanisms might also affect the susceptibility of parasites to other quinoline antimalarials. Unlike in other endemic countries in Southeast Asia or East Africa, the

prevalence of drug-resistant *P. falciparum* parasites in Madagascar is still low enough to allow for effective tailoring of national policy to prevent a malaria crisis.

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Table 1

Detection of the *pfcrt* K76T mutation by use of PCR/RFLP method in *Plasmodium falciparum* in Madagascar

Study site	Patients	Number of successful PCRs	Number of isolates containing mutant *pfcrt* K76T parasites (%)
Tsiroanomandidy	Clinical cases	51	1 (1.96%)
Andapa	Pregnant women	104[a]	2 (1.92%)
	Women at delivery	28	3 (10.7%)

[a] PCR was unsuccessful for two samples.

Table 2

Haplotypes of six *Plasmodium falciparum* harbouring mutant *pfcrt* detected in Andapa and Tsiroanomandidy

Study site	Number of isolates	pfCRT haplotypes	Codon sequences				
			72	73	74	75	76
Tsiroanomandidy	1	CVMNK and CVIDT[a]	TGT	GTA	ATG/ATT	AAT/GAT	AAA/ACA
Andapa	2	CVMNK and CVIDT[a]	TGT	GTA	ATG/ATT	AAT/GAT	AAA/ACA
	1	CVMNK and CVIET[a]	TGT	GTA	ATG/ATT	AAT/GAA	AAA/ACA
	2	CVIET	TGT	GTA	ATT	GAA	ACA

[a]Haplotypes for mixed infections (wild/mutant) might be debated as suggestive, but sequencing confirmed the mutation of the 74 and 75 codons and also the occurrence of the key mutation K76T.