PREVALENT PFMMDR1 N86Y MUTANT PLASMODIUM FALCIPARUM IN MADAGASCAR DESPITE ABSENCE OF PFCRT MUTANT STRAINS

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Abstract. We assessed the status of point mutations associated with chloroquine resistance in pfcrt codon 76 and in pfmdr1 codon 86 among Plasmodium falciparum isolates from symptomatic patients in 3 sites in Madagascar. The in vitro susceptibility of P. falciparum isolates to quinoline-containing drugs was also determined. All isolates (N = 117) successfully typed were pfcrt wild-type, except one from Tsiraoanomandidy (1 of 27). However, 67.5% (95% CI: 58.2–75.9%) of these isolates contained mutant pfmdr1 86Y. The pfmdr1 N86Y mutation is associated with higher mefloquine susceptibility, but it did not affect the sensitivity of parasites to chloroquine or quinine. Our findings demonstrate that pfmdr1 mutant P. falciparum are prevalent in Madagascar and confirm the low prevalence of pfcrt mutant P. falciparum after 60 years of chloroquine use. They provide additional field-based evidence for increased mefloquine susceptibility in pfmdr1 mutant P. falciparum and are suggestive of the intrahost selection of pfmdr1 mutant parasites.

INTRODUCTION

The use of antimalarial drugs for curative and preventive purposes is pivotal in current strategies to control malaria in Madagascar. Chloroquine, first introduced into Madagascar in 1945, has been used as the first-line treatment of uncomplicated malaria.1–3 Between 1949 and 1962, widespread use of chloroquine for malarial chemoprophylaxis, mainly in children, had a major impact on malaria control.4,5 A shortage of chloroquine was one of the main causes of a malaria outbreak that killed thousands of people on the island in the 1980s. After this outbreak, chloroquine was widely distributed through 35,432 dispensaries to ensure its availability in all villages, and this drug was recommended for treatment of presumptive malaria (fever).4,5 Low-grade (R1 or R2) chloroquine resistance and late clinical and parasitological failures have been reported.6,7 Today, chloroquine is also available in groceries, even in rural areas. It remains the most widely used antimalarial drug, with or without medical prescription, and prepackaged chloroquine has been recommended by the Ministry of Health and Family Planning (MoH) since the end of 2003 for treatment at home of fever in children under the age of 5 years.3,8

The MoH and the Institut Pasteur de Madagascar (IPM) created the Réseau d’Etude de la Résistance-Paludisme (RER) in 1999. This national network for the surveillance of malaria resistance was designed to alleviate the lack of medical teams for (a) routine monitoring of the therapeutic effectiveness of antimalarial drugs at peripheral health centers and (b) malaria diagnosis by microscopy. IPM is responsible for malaria parasite phenotyping and genotyping.1,7,9 The Malagasy MoH approved the experimental protocols used for this study. The chloroquine-resistance marker gene, pfcrt, has been typed for clinical Plasmodium falciparum isolates collected in 2001 and 2002. No P. falciparum isolate harboring pfcrt 76T was detected in the seaport towns of Mahajanga (northwestern region), Toamasina (eastern region), Tolagnaro (southern costal region), or Moramanga (eastern foothill region).7,9 The prevalence of mutant pfcrt 76T was very low in Tsiraoanomandidy (western foothill region) and in Andapa (northern coastal region).3 In this context, genotyping of chloroquine-resistance markers remains a useful tool for chloroquine-resistance surveillance.

We report herein the results of RER activities of 2004. The P. falciparum isolates collected from Tsiraoanomandidy, Saharevo, and Sainte Marie were examined to assess their in vitro susceptibility to quinoline-based drugs and to monitor the status of point mutations in pfcrt codon 76 and in pfmdr1 codon 86, which have been associated with chloroquine resistance.10–14

MATERIALS AND METHODS

Study sites and parasite samples. The three study sites—Tsiraoanomandidy, Sainte Marie, and Saharevo—are shown in Figure 1. These sites are part of the national network for drug-resistance surveillance with health workers trained and equipped to diagnose malaria with microscopy. It is worth mentioning that parasitological malaria diagnosis is not possible in most of Madagascar.

Tsiraoanomandidy is in the southwestern part of Madagascar, in the foothill area. This region is characterized by meso- to hyperendemic malaria.15 The island of Sainte Marie is a tourist site on the rainy eastern coast, where most of the local population is poor. This region is characterized by hyperendemic malaria.15 Tsiraoanomandidy and Sainte Marie are part of health districts in which malaria chemoprophylaxis using chloroquine was actively implemented from 1949 to 1962.2 Chloroquine is still used in these areas.

Saharevo is a village in the Moramanga district. A primary health care center has been built in this village, and permanent drug pressure related to chloroquine use has been recorded. The population of Saharevo (~ 300 inhabitants) was subjected to mass annual chloroquine treatment from 1995 to 2000. During this period and up to 2003, chloroquine was the systematic first-line antimalarial treatment of confirmed, uncomplicated malaria cases.

Clinical P. falciparum isolates were obtained in venous blood collected into EDTA-coated tubes. Blood samples were obtained from patients over the age of 2 years, after
in vitro The values for *P. falciparum pfcrt* PCR product contains one testing was carried out in 96-well plates (200 *pfmdr1* K76T and 2 *pfcrt* PCR products were di-

or Fisher’s exact test. The 50 were amplified by nested PCR, using a Mastercy-

*pfmdr1* and /H11505 Plas-

18,19 species, no declared antimalarial drug intake during N86Y mutations.

*pfcrt pfmdr1* /H11505 *P. falciparum* *pfcrt* gene encodes a lysine (K76), resulting in re-

/H11505 *pfmdr1* and /H9262 

Afl re-

in vitro for all isolates. Mean IC

an in vitro

for all isolates. Mean IC

of the drugs was ex-

Tests were considered to be interpretable if tritium-labeled 

The 145-bp *pfcrt* PCR product contains one *Apo* I site if codon 76 of the *pfcrt* gene encodes a lysine (K76), resulting in re-

the laboratory, were used as positive controls. H$_2$O was used as a negative control.

**Data analysis.** Statistical analyses were performed with Epi-Info software. The *in vitro* activity of the drugs was expressed as the mean IC$_{50}$ for all isolates. Mean IC$_{50}$ were compared by *t* tests. We analyzed the frequency of mutants with mixed genotypes (wild-type and mutated gene both present), which were counted as “mutation present.” Frequencies were compared, using *χ*² or Fisher’s exact test. The level of statistical significance was set at *P* = 0.05.

**RESULTS**

**Genotyping.** All 117 nested PCRs for *pfcrt* and *pfmdr1* were successful (Table 1). All *pfcrt* PCR products were di-

sessed by adding tritium-labeled hypoxanthine (Amersham Bioscience, Saclay, France) to the culture medium at a concentration of 0.5 μCi per well. Parasitemia ranged from 0.1% to 0.5% (high parasitemia was adjusted to 0.5% by adding freshly prepared uninfected erythrocytes). Plates were incubated at 37°C for 42 hours in a humidified MIC-101 modula-

or incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a gas mixture containing 5% CO$_2$, 5% O$_2$, and 90% N$_2$. Plates were then frozen and defrosted, and the contents of each well were harvested on fiberglass paper (Wallac, Turku, Finland). Tritium-labeled hypoxanthine incorporation was determined with a beta counter (Wallac, model 1450). Tests were considered to be interpretable if tritium-labeled hypoxanthine incorporation gave > 1,000 counts per minute in the drug-free wells. Growth curves were obtained, and 50% inhibitory concentration (IC$_{50}$) values were calculated by log-

probit approximation. The threshold IC$_{50}$ values for *in vitro* resistance were 100 nM for chloroquine, 60 nM for monodes-

ethylamodiaquine, 30 nM for mefloquine (although Wood-

row and Krishna suggested a threshold at 50 nM$^{19}$), and 800 nM for quinine.$^{18,19}$

DNA extraction and PCR/RFLP to detect *pfcrt* K76T and *pfmdr1* N86Y mutations. Red blood cell pellets from each sample were kept at −20°C until use. Parasite DNA was extracted from 200 μL of red blood cell pellets by phenol-

chloroform purification.$^{20}$ The *pfcrt* and *pfmdr1* genes of *P. falciparum* were amplified by nested PCR, using a Mastercyc-

elar thermal cycler (Eppendorf, Hamburg, Germany) as de-

scribed at http://medschool.umaryland.edu/cvd/2002_pcr-

asra.asp.

The *pfcrt* and *pfmdr1* nested-PCR products were digested with *Apo* I and with *AflIII* (New England Biolabs, Hitchin, UK), respectively. The restricted products (15 μL) were sub-

jected to electrophoresis in a 2% agarose gel, stained with 0.5 μg/mL ethidium bromide, and viewed under ultraviolet light. The 145-bp *pfcrt* PCR product contains one *Apo* I site if codon 76 of the *pfcrt* gene encodes a lysine (K76), resulting in re-

striction fragments 99 and 46 bp in length. The 291-bp *pfmdr1* PCR product contains a single *AflIII* site if codon 86 is mu-

tated to encode tyrosine (86Y), resulting in the generation of fragments 165 and 126 bp long. If the *pfmdr1* N86Y or the *pfcrt* K76T mutation was detected in a sample, a technician repeated the entire process, blind, from DNA extraction to PCR product analysis by digestion and electrophoresis. For each PCR and each digestion, DNA from *P. falciparum* strains FCM29 (chloroquine-resistant) and 3D7 (chloroquine-

susceptible), which are maintained in continuous culture in the laboratory, were used as positive controls. H$_2$O was used as a negative control.

**Data analysis.** Statistical analyses were performed with Epi-Info software. The *in vitro* activity of the drugs was expressed as the mean IC$_{50}$ for all isolates. Mean IC$_{50}$ were compared by *t* tests. We analyzed the frequency of mutants with mixed genotypes (wild-type and mutated gene both present), which were counted as “mutation present.” Frequencies were compared, using *χ*² or Fisher’s exact test. The level of statistical significance was set at *P* = 0.05.

**RESULTS**

**Genotyping.** All 117 nested PCRs for *pfcrt* and *pfmdr1* were successful (Table 1). All *pfcrt* PCR products were di-

informed consent had been granted, from February to June 2004 in Saharevo (N = 64), Sainte Marie (N = 26), and Tsirnomomandidy (N = 27). Samples were transported, at +8°C, to the Malaria Research Unit of the IPM at Antanan-

arivo. The medical teams at the local health centers were responsible for all decisions concerning treatment of their patients. Patients were not followed up, and treatment outcomes were therefore not recorded.

**In vitro testing of antiplasmodial agents.** The *in vitro* re-

sponse of *P. falciparum* isolates was determined by the iso-

topic method.$^{19,20}$ We tested 114 isolates against at least one of the following drugs: chloroquine, monodesethylamodi-

aquine, quinine, and mefloquine. All isolates tested met the following criteria: parasitemia ≥ 0.1%, absence of other *Plas-

modium* species, no declared antimalarial drug intake during the last 7 days, and transport at +8°C to the IPM at Antanan-

arivo within 48 hours of blood collection. *P. falciparum* harboring red blood cells were washed with RPMI 1640 (Gibco-BRL Laboratories, Grand Island, NY) and resus-

pended in complete RPMI 1640 supplemented with 10% (v/v) AB+ human serum (Abcys, Chausson, Paris), at a hematocrit of 1.5%. *In vitro* testing was carried out in 96-well plates (200 μL of parasite suspension per well). Parasite growth was as-

Figure 1. Map of Madagascar showing parasite collection sites. Legend: Black circles indicate the study sites involved in surveillance of malaria-drug resistance in 2004 reported herein. Black rhombi indicate sites where occurrence of *pfcrt* mutants has already been assessed. No *pfcrt* mutant was detected at Mahajanga, Toamasina, or Tolagnaro. The first cases of *pfcrt* K76T mutant *P. falciparum* were detected among samples collected in 2001 in Tsirnomomandidy (1 of 51 samples of CVIET haplotype) and in Andapa (5 of 132 samples; both CVIET and CVIET mutant haplotypes were present). Institut Pasteur de Madagascar is located at Antananarivo.
gested by ApoI, indicating that the *P. falciparum* isolates tested were all wild-type for *pfcr* codon 76 (K76) except for one isolate (1 of 27) from Tsiraoanomandidy (76T). Thus among examined samples, the frequency of isolates harboring *pfcr* 76T mutant parasites was 3.7% (95% CI: 0.09–18.9%) in Tsiraoanomandidy.

A *pfmdr1* PCR product digested totally or partly by AflIII was obtained with 79 of the 117 isolates of *P. falciparum*. Thus, 67.5% (95% CI: 58.2–75.9%) of the tested isolates contained the *pfmdr1* tyrosine-86 (86Y) mutation. Twenty-one isolates (17.9%) were mixed; they contained both mutant *pfmdr1* 86Y parasites and parasites with an AflIII-insensitive product. The frequency of isolates harboring *pfmdr1* N86Y mutant parasites was 75% (48 of 64) for Saharevo, 53.8% (14 of 26) for Sainte Marie, and 62.9% (17 of 27) for Tsiraoanomandidy.

**In vitro testing of antimalarial drugs.** We tested 114 *P. falciparum* isolates with at least one of the following drugs: chloroquine, monodesethylamodiaquine, quinine, and mefloquine. On average, about 65% (74 of 114) of tests could be interpreted. Because preliminary data analysis indicated no significant differences between sites, the results obtained for the three study sites were considered together.

### Table 1
Detection of *pfmdr1* N86Y mutation by PCR/RFLP in clinical *P. falciparum* isolates collected from Madagascar in 2004*

<table>
<thead>
<tr>
<th>Study site</th>
<th>Geographical zone</th>
<th>No. of examined isolates</th>
<th>No. of isolates containing mutant <em>pfmdr1</em> N86Y parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saharevo</td>
<td>Eastern foothill area</td>
<td>64</td>
<td>48 (75.0%)</td>
</tr>
<tr>
<td>Sainte Marie</td>
<td>Eastern coastal area</td>
<td>26</td>
<td>14 (53.8%)</td>
</tr>
<tr>
<td>Tsiraoanomandidy</td>
<td>Western foothill area</td>
<td>27</td>
<td>17 (62.9%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>117</td>
<td>79 (67.5%)</td>
</tr>
</tbody>
</table>

* All isolates were *pfcr* wild-type K76, except one from Tsiraoanomandidy which was *pfcr* 76T and *pfmdr1* 86Y.

**Monodesethylamodiaquine and quinine.** The successfully tested isolates were all susceptible to quinine (*N* = 71) and monodesethylamodiaquine (*N* = 71). The mean IC\(_{50}\) of quinine was 70.6 nM (95% CI: 59.2–82.0 nM), with a median of 64.6 nM. The mean IC\(_{50}\) of monodesethylamodiaquine was 10.8 nM (95% CI: 9.2–12.4 nM), with a median of 9.4 nM.

**Chloroquine and mefloquine.** The mean IC\(_{50}\) of chloroquine was 29.9 nM (*N* = 74; 95% CI: 24.9–34.9 nM), with a median of 22.8 nM. All but one of the successfully tested isolates (1.4%) were sensitive to chloroquine. The chloroquine-resistant isolate was from Saharevo. The IC\(_{50}\) of chloroquine was 106.4 nM for this strain, which was susceptible to the other drugs tested. It is of *pfcr* wild-type. The mean IC\(_{50}\) of mefloquine was 9.8 nM (*N* = 59; 95% CI: 7.8–11.8 nM), with a median of 7.3 nM. All but one of the successfully tested isolates were mefloquine-sensitive. The mefloquine-resistant isolate was from Saharevo and had an IC\(_{50}\) of 54.6 nM for mefloquine. It was of *pfmdr1* wild-type and showed susceptibility to the other drugs tested. However, our results indicate that mefloquine was the most potent of the four quinoline-containing drugs tested in *vitro*, with activity levels 3 times higher than those of chloroquine (*P < 0.001*) and 7.2 times higher than those of quinine (*P < 0.001*).

The *in vitro* susceptibility of reference strains of *P. falciparum* to quinoline-based antimalarials is reported in Table 3.

**Correlation between *in vitro* response of *P. falciparum* isolates to drugs and presence of mutant *pfmdr1* N86Y parasites.**

The presence or absence of mutant *pfmdr1* N86Y parasites did not affect the *in vitro* susceptibility of *P. falciparum* isolates to chloroquine and quinine (Table 2). The situation was different for mefloquine. The mean IC\(_{50}\) of mefloquine was 14.7 nM for the wild-type parasites (*pfmdr1* N86) versus 6.9 nM for their counterparts containing mutant *pfmdr1* 86Y. Based on mean IC\(_{50}\)s, *P. falciparum* isolates harboring mutant parasites were 2.13 times more sensitive to mefloquine than were wild-type parasites *in vitro*, and mutant parasites...
were 1.38 times less sensitive to monodesethylamodiaquine, although differences were not statistically significant.

DISCUSSION

Our findings provide additional field-based evidence for the greater susceptibility to mefloquine of pfmdr1 mutant P. falciparum and demonstrate above all that pfmdr1 mutant P. falciparum is present at a high prevalence despite the absence of pfcr1 mutant strains in Madagascar. The occurrence of pfmdr1 mutants probably results from the cumulative effects of drug pressure and the intrahost selection of pfmdr1 mutant parasites, related to the use of chloroquine over the last 60 years. Previously, the sequencing of pfmdr1 in 31 P. falciparum isolates collected in 2001 from Tolagnaro, in the southern costal area of Madagascar, showed that mutant pfmdr1 N86Y was present in 25 (80.6%) of these isolates, in the absence of the mutation at codon 1042 (Ariey, unpublished).

The situation in Madagascar looks unique and unusual as most parasites isolates are sensitive to chloroquine in vitro, and pfcr1 mutant parasites are at low prevalence. It is worth mentioning that, in our previous work, we reported the occurrence of few pfcr1 mutants in Tsiranoamandidy (1 of 51) as in 2001.3 Consequently, detection of the pfcr1 mutant P. falciparum in Tsiranoamandidy (1 of 27 isolates) in our current results indicates circulation of mutant parasites in this region.

The low frequency of isolates resistant to chloroquine in vitro contrasts interestingly with the non-negligible level of overall crude chloroquine treatment failure of 15–40% detected within a 14-day follow-up (late clinical or parasitological failure in almost all cases) recorded over the last few years in Madagascar and at the 3 study sites investigated here.7,21–23

Basco et al. reported a positive correlation between the asparagine to tyrosine mutation at position 86 (N86Y) in pfMDR1 and chloroquine resistance in vitro in Sub-Saharan Africa.24 These findings contrast with our results. Regardless of the status of the pfmdr1 codon 86, isolates from Madagascar do not display high levels of resistance to chloroquine, as does the reference strain P. falciparum FCM29. This would suggest that the genetic background of most P. falciparum parasites in Madagascar is not yet favorable for development of a high level of chloroquine resistance; the N86Y mutation of pfmdr1 is also not sufficient itself to generate measurable levels of resistance to chloroquine.

Our current results indicated that all the isolates tested were susceptible to quinine (the drug recommended for the management of severe malaria in Madagascar) and to monodesethylamodiaquine (a combination of amodiaquine and artesunate) is recommended in the recently revised malaria therapy policy for Madagascar). Mefloquine is recommended for malaria prevention for travelers to Madagascar (http://www.pasteur.mg/prevpal.html) but is very rarely used by local people because of its high cost.25 Our current study shows that P. falciparum is potentially susceptible to mefloquine in Madagascar, and this is reassuring to the health authorities. In vitro monitoring for assessing or predicting the susceptibility of malaria parasites to drugs is required to generate useful and usable information. The detection of parasites highly resistant to mefloquine in a country like Madagascar, where most parasites are mefloquine-susceptible, should facilitate identification of new genetic markers of resistance for this drug.

The triple mutations S1034C/N1042D/D1246Y in pfmdr1, highly prevalent in South America, have been shown to increase parasite susceptibility to mefloquine, halofantrine, and artemisinin.26 The tyrosine-86 allele of the pfmdr1 gene of P. falciparum is associated with greater susceptibility to artesunate.14,28,29 Polymorphisms at amino-acid residues 86, 184, 1034, 1042, and 1246 have been associated with changes in susceptibility to chloroquine, quinine, mefloquine and artemisinin in vitro.25,30 A recent study on P. falciparum from Papua New Guinea31 suggested that pfmdr1 N86Y mutation plays a compensatory role in chloroquine-resistant isolates under chloroquine pressure, also increasing the level of chloroquine resistance in K76T parasites to a small extent. In Madagascar, with its virtually isolated malaria, a shift toward the use of ACT (artesunate + amodiaquine) is planned in the newly revised policy for treating malaria. Chloroquine will eventually be withdrawn at national level.32 Thus, chloroquine pressure will decrease while ACT pressure will increase. These findings on pfmdr1 in isolates from different malaria-infested continents suggest that the spatial and temporal monitoring of pfmdr1 (mutation and expression) would help to track the evolution of Malagasy P. falciparum in the era of ACT use.

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