PREVALENT PFMDR1 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 pfcr1 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 pfcr1 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 pfcr1 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. Between 1949 and 1962, widespread use of chloroquine for malarial chemoprophylaxis, mainly in children, had a major impact on malaria control. 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). 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.

The MoH and the Institut Pasteur de Madagascar (IPM) created the Réseau d’Étude 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. The Malagasy MoH approved the experimental protocols used for this study. The chloroquine-resistance marker gene, pfcr1, has been typed for clinical Plasmodium falciparum isolates collected in 2001 and 2002. No P. falciparum isolate harboring pfcr1 76T was detected in the seaport towns of Mahajanga (northern coastal region), Toamasina (eastern region), Tolagnaro (southern coastal region), or Moramanga (eastern foothill region). The prevalence of mutant pfcr1 76T was very low in Tsiraoanomandidy (western foothill region) and in Andapa (northern coastal region). 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 pfcr1 codon 76 and in pfmdr1 codon 86, which have been associated with chloroquine resistance.

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 midwestern part of Madagascar, in the foothill area. This region is characterized by meso- to hyperendemic malaria. 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. Tsiraoanomandidy and Sainte Marie are part of health districts in which malaria chemoprophylaxis using chloroquine was actively implemented from 1949 to 1962. 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...
informed consent had been granted, from February to June 2004 in Saharevo (N = 64), Sainte Marie (N = 26), and Tsiraoanomandidy (N = 27). Samples were transported, at +8°C, to the Malaria Research Unit of the IPM at Antananarivo. 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* response of *P. falciparum* isolates was determined by the isotopic method. In at least one of the following drugs: chloroquine, monodesethylamodiaquine, quinine, and mefloquine. All isolates tested met the following criteria: parasitemia ≥ 0.1%, absence of other *Plasmodium* species, no declared antimalarial drug intake during the last 7 days, and transport at +8°C to the IPM at Antananarivo within 48 hours of blood collection. *P. falciparum*-harboring red blood cells were washed with RPMI 1640 (Gibco-BRL Laboratories, Grand Island, NY) and resuspended in complete RPMI 1640 supplemented with 10% (v/v) AB+ human serum (Abcys, Chaussion, Paris), at a hematocrit of 1.5%. In *in vitro* testing was carried out in 96-well plates (200 μL of parasite suspension per well). Parasite growth was assessed 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 modulator incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a gas mixture containing 5% CO₂, 5% O₂, and 90% N₂. 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 (IC₅₀) values were calculated by log-probit approximation. The threshold IC₅₀ values for *in vitro* resistance were 100 nM for chloroquine, 60 nM for monodesethylamodiaquine, 30 nM for mefloquine (although Woodrow and Krishna suggested a threshold at 50 nM), and 800 nM for quinine.

**DNA extraction and PCR/RFLP to detect pfcr 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. The *pfcr* and *pfmdr1* genes of *P. falciparum* were amplified by nested PCR, using a Mastercyber thermal cycler (Eppendorf, Hamburg, Germany) as described at http://medschool.umaryland.edu/cvd/2002_pcr_asra.asp. The *pfcr* and *pfmdr1* nested-PCR products were digested with *ApoI* and with *AluI* (New England Biolabs, Hitchin, UK), respectively. The restricted products (15 μL) were subjected to electrophoresis in a 2% agarose gel, stained with 0.5 g/mL ethidium bromide, and viewed under ultraviolet light. The 145-bp *pfcr* PCR product contains one *ApoI* site if codon 76 of the *pfcr* gene encodes a lysine (K76), resulting in restriction fragments 99 and 46 bp in length. The 291-bp *pfcr* PCR product contains a single *ApoI* site if codon 64 is mutated to encode tyrosine (86Y), resulting in the generation of fragments 165 and 126 bp long. The 291-bp *pfmdr1* PCR product contains a single *AkuI* site if codon 86 is mutated to encode tyrosine (86Y), resulting in the generation of fragments 165 and 126 bp long. If the *pfmdr1* N86Y or the *pfcr* K76T mutation was detected at Mahajanga, Toamasina, or Tolagnaro. The first cases of *P. falciparum* infection were therefore not recorded.

**Data analysis.** Statistical analyses were performed with Epi-Info software. The *in vitro* activity of the drugs was expressed as the mean IC₅₀ for all isolates. Mean IC₅₀ values 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 χ² or Fisher’s exact test. The level of statistical significance was set at *P* = 0.05.

**RESULTS**

**Genotyping.** All 117 nested PCRs for *pfcr* and *pfmdr1* were successful (Table 1). All *pfcr* PCR products were di-
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 pfmdr1 N86Y parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saharivo</td>
<td>Eastern foothill area</td>
<td>64</td>
<td>48 (75.0% of 64)</td>
</tr>
<tr>
<td>Sainte Marie</td>
<td>Eastern coastal area</td>
<td>26</td>
<td>14 (53.8% of 26)</td>
</tr>
<tr>
<td>Tsiroanomandidy</td>
<td>Western foothill area</td>
<td>27</td>
<td>17 (62.9% of 27)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>117</td>
<td>79 (67.5% of 117)</td>
</tr>
</tbody>
</table>

* All isolates were pfcr wild-type K76, except one from Tsiroanomandidy which was pfcr N86 and pfmdr1 86Y.

A pfdnrl PCR product digested totally or partly by AIII/III 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 mutant; they contained both mutant pfmdr1 86Y parasites and parasites with an AIII-insensitive product. The frequency of isolates harboring pfmdr1 N86Y mutant parasites was 3.7% (95% CI: 0.09–18.9%) in Tsiroanomandidy.

A pfmdr1 PCR product digested totally or partly by A/III 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 mutant; they contained both mutant pfmdr1 86Y parasites and parasites with an AIII-insensitive product. The frequency of isolates harboring pfmdr1 N86Y mutant parasites was 75% (48 of 64) for Saharivo, 53.8% (14 of 26) for Sainte Marie, and 62.9% (17 of 27) for Tsiroanomandidy.

**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.

**Monodesethylamodiaquine and quinine.** The successfully tested isolates were all susceptible to quinine (*N = 71*) and monodesethylamodiaquine (*N = 71*). The mean IC₅₀ of quinine was 70.6 nM (95% CI: 59.2–82.0 nM), with a median of 64.6 nM. The mean IC₅₀ 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 IC₅₀ 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 Saharivo. The IC₅₀ 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 IC₅₀ 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 Saharivo and had an IC₅₀ 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 *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 IC₅₀ 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 IC₅₀s, *P. falciparum* isolates harboring mutant parasites were 2.13 times more sensitive to mefloquine than were wild-type parasites in *in vitro*, and mutant parasites

Table 2: Correlation between presence of pfmdr1 mutant parasites and *in vitro* susceptibility of *P. falciparum* isolates to quinoline-based antimalarial drugs in Madagascar in 2004

<table>
<thead>
<tr>
<th>Drug</th>
<th>pfmdr1 codon 86 profile</th>
<th>Wild-type pfmdr1 N86</th>
<th>Presence of mutant N86Y</th>
<th>IC₅₀ ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td></td>
<td>36</td>
<td>74</td>
<td>0.92</td>
</tr>
<tr>
<td>No. of tested samples</td>
<td></td>
<td>25</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Mean IC₅₀ in nM (95% CI)</td>
<td>28.3 (19.8–36.8)</td>
<td>30.8 (24.5–37.1)</td>
<td>4.8–94.9</td>
<td></td>
</tr>
<tr>
<td>Monodesethylamodiaquine</td>
<td></td>
<td>31</td>
<td>61</td>
<td>2.13</td>
</tr>
<tr>
<td>No. of tested samples</td>
<td></td>
<td>31</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Mean IC₅₀ in nM (95% CI)</td>
<td>14.7 (10.3–19.1)</td>
<td>6.9 (5.9–7.9)</td>
<td>5.3–54.6</td>
<td></td>
</tr>
<tr>
<td>Mefloquine</td>
<td></td>
<td>31</td>
<td>61</td>
<td>2.13</td>
</tr>
<tr>
<td>No. of tested samples</td>
<td></td>
<td>31</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Mean IC₅₀ in nM (95% CI)</td>
<td>14.7 (10.3–19.1)</td>
<td>6.9 (5.9–7.9)</td>
<td>5.3–54.6</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td></td>
<td>36</td>
<td>76</td>
<td>0.73</td>
</tr>
<tr>
<td>No. of tested samples</td>
<td></td>
<td>25</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Mean IC₅₀ in nM (95% CI)</td>
<td>71.2 (49.5–92.9)</td>
<td>70.2 (56.9–83.5)</td>
<td>13.8–282.2</td>
<td></td>
</tr>
</tbody>
</table>

* IC₅₀ ratio = mean IC₅₀ for pfmdr1 wild-type isolates divided by mean IC₅₀ for pfmdr1 mutant-harboring isolates.
† Presence of mutant parasites, including both mutant and mixed (mutant/wild) isolates.
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 *pfcr* 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 *vivo*, and *pfcr* mutant parasites are at low prevalence. It is worth mentioning that, in our previous work, we reported the occurrence of few *pfcr* mutants in Tsiranoamandidy (1 of 51) as in 2001.3 Consequently, detection of the *pfcr* 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 *vivo* 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 *vivo* 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 artesinin.26 The tyrosine-86 allele of the *pfmdr1* gene of *P. falciparum* is associated with greater susceptibility to artesinin.13,27 Amplification of *pfmdr1* has been shown to be associated with mefloquine treatment failure and in *vivo* resistance.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 artesinin in *vivo*.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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**Table 3**

*In vitro* susceptibility of reference strains of *P. falciparum* to quinoline-based antimalarial drugs (*n* = 4)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC₅₀ in nM (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. falciparum</em> 3D7</td>
</tr>
<tr>
<td></td>
<td>(wild-type <em>pfmdr1</em> and <em>pfcr</em>)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>3.5 (1.5–5.5)</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>40.7 (32.7–48.7)</td>
</tr>
<tr>
<td>Monodesethylamodiaquine</td>
<td>6.8 (4.0–9.6)</td>
</tr>
<tr>
<td>Quinine</td>
<td>103.5 (87.5–119.4)</td>
</tr>
</tbody>
</table>
REFERENCES


