Plasmodium falciparum Drug Resistance in Madagascar: Facing the Spread of Unusual pfdhfr and pfmdr-1 Haplotypes and the Decrease of Dihydroartemisinin Susceptibility

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The aim of this study was to provide the first comprehensive spatiotemporal picture of Plasmodium falciparum resistance in various geographic areas in Madagascar. Additional data about the antimalarial resistance in the neighboring islands of the Comoros archipelago were also collected. We assessed the prevalence of pfert, pfmdr-1, pfdhfr, and pfdhps mutations and the pfmdr-1 gene copy number in 1,596 P. falciparum isolates collected in 26 health centers (20 in Madagascar and 6 in the Comoros Islands) from 2006 to 2008. The in vitro responses to a panel of drugs by 373 of the parasite isolates were determined. The results showed (i) unusual profiles of chloroquine susceptibility in Madagascar, (ii) a rapid rise in the frequency of parasites with both the pfdhfr and the pfdhps mutations, (iii) the alarming emergence of the single pfdhfr 164L genotype, and (iv) the progressive loss of the most susceptible isolates to artesinin derivatives. In the context of the implementation of the new national policy for the fight against malaria, continued surveillance for the detection of P. falciparum resistance in the future is required.

In recent decades, the emergence and subsequent spread of Plasmodium falciparum chloroquine (CQ)- and sulfadoxine-pyrimethamine (SP)-resistant parasites across areas where malaria is endemic have been a challenge to malaria control programs (41, 44). Substantial advances toward gaining an understanding of the genetic basis of antimalarial drug resistance have been made (14). Molecular evolutionary studies have concluded that the CQ-resistant P. falciparum chloroquine resistance transporter (pfert) and high-level pyrimethamine-resistant dihydrofolate (pfdhfr) alleles have emerged in a limited number of independent foci, from which they have rapidly spread in the local vicinity and have then invaded areas continent-wide and transferred between continents (1, 36). These lessons of the past have, first, stimulated changes in antimalarial treatment policies by introducing combinations of drugs that act on different targets and, second, resulted in the implementation of effective monitoring systems to detect as early as possible the emergence of resistant parasites on the basis of the assessment of the therapeutic efficacies of antimalarials (25, 46), determination of the decreased sensitivity of the parasites to drugs in vitro (4), and the detection of an increasing prevalence of molecular markers related to drug resistance (24).

According to data published from 2002 to 2006, the epidemiological features of P. falciparum CQ and SP resistance differ considerably between Madagascar and the Comoros Islands, two countries located close to each other in the southwestern Indian Ocean (43). In vitro CQ resistance was moderate in Madagascar (29, 33, 45), although the level of therapeutic efficacy was declining. During that time, the rate of CQ resistance was high in the Comoros Islands (22, 23, 30). Likewise, pyrimethamine resistance was absent in Madagascar (28, 32) but was present at high levels in the Comoros Islands (23). The most recent in vivo data obtained on the basis of the WHO 28-day follow-up protocol, conducted in 2006 and 2007 at multiple sites, have confirmed that resistance to all antimalarials except CQ in Madagascar remains rare. Indeed, the prevalence of the clinical failure of treatment with amodiaquine, SP and the combination artesunate and amodiaquine was <5%, while the rate of failure of treatment with CQ was 44% (19). However, the recent demonstration of the introduction of multidrug-resistant P. falciparum parasites into Madagascar from the Comoros Islands (18) and the emergence of the uncommon dihydrofolate reductase I164L genotype in P. falciparum parasites (17) suggest that the situation is currently changing in Madagascar.

In this context and in order to help with the rationalization of the malaria elimination policy recently launched by the Malagasy government (withdrawal of CQ in favor of the combination of artesunate plus amodiaquine as first-line treatment...
and SP usage for intermittent preventive treatment for pregnant women), a large-scale survey was designed and carried out between 2006 and 2008. The aim was to provide a comprehensive spatiotemporal picture of *P. falciparum* resistance in several geographic areas of Madagascar. We report here the prevalence of *P. falciparum* parasites harboring mutations correlated with resistance to some quinolines, namely, *pfcrt* and *P. falciparum* multidrug resistance gene 1 (*pfmdr-1*), or SP resistance (*pfdhfr*, *pfdhps*) or presenting an increased *pfmdr-1* gene copy number, along with the in vitro responses of the parasites to a panel of drugs, including CQ, mefloquine (MF), amodiaquine, quinine (QU), and artemisinin derivatives. In addition, information related to the risk factors that contribute to the spread of antimalarial drug resistance, such as antimalarial resistance in the neighboring islands of the Comoros archipelago, drug pressure, and population movement in Madagascar, was collected (8, 42).

**MATERIALS AND METHODS**

**Collection of *P. falciparum* isolates.** *P. falciparum* isolates were obtained from febrile patients seeking treatment for malaria at public health centers. Comorian isolates were collected from blood from finger pricks placed on filter paper at six different sites from May to June 2006, and those from Madagascar were collected from blood obtained by venipuncture at sites involved in the antimalarial drug resistance surveillance network from 2006 to 2008 (Fig. 1). Blood samples collected in EDTA tubes were transported to Antananarivo, Madagascar, at +4°C within 24 to 48 h of collection. Giemsa-stained blood smears were examined to check for monoinfection with *P. falciparum*, and the parasite density was determined. The samples were processed for in vitro assays, and aliquots were stored at −20°C before genomic DNA extraction.

**In vitro susceptibility assays.** In vitro assays were performed by using the classical isotopic 48-h test, as described previously (34). The final drug concentrations ranged from 12.5 to 1,600 nM for CQ, 2.5 to 320 nM for monodesethylamodiaquine (MDA), 25 to 3,200 nM for QU, 0.25 to 40 nM for dihydroartemisinin (DHA), and 3.1 to 400 nM for MF. For each drug tested, three control wells were drug free, and each concentration (nine for CQ and eight for each of the other drugs) was studied in duplicate or triplicate. Tests were considered interpretable if the level of [3H]hypoxanthine incorporation was >1,000 cpm in the drug-free wells. The validity and the reproducibility of the assays were controlled by using *P. falciparum* reference lines (3D7 Africa and FCM29 Cameroon). The results were expressed as the 50% inhibitory concentration (IC50), defined as the concentration at which 50% of the level of incorporation of [3H]hypoxanthine was inhibited compared to the level of incorporation in the control wells. The IC50 was calculated by using a probit/logit regression of the percentage of growth inhibition for each drug.

**DNA extraction, PCR amplification, and sequencing.** Parasite DNA was extracted from blood spots with Instagene matrix (Bio-Rad, Marnes la Coquette, France), according to the manufacturer’s instructions, or directly from 100 μl infected blood by the phenol-chloroform method (27). The parasite species was confirmed by real-time PCR, as described by de Monbrison et al. (5). The primers and PCR conditions used are detailed in Table 1. Sequencing reactions were carried out with a ABI Prism BigDye Terminator cycle sequencing ready reaction kit and were run on a model 3730 xl genetic analysis system software (Beckman Coulter, Villepinte, France). The amino acid sequences were compared with the wild-type amino acid sequences (GenBank accession numbers, AF030694 for *pfcrt*, XM_001351751 for *pfmdr-1*, XM_001351443 for *pfdhfr*, and Z30654 for *pfdhps*). The presence of single nucleotide polymorphisms (SNPs) was confirmed by reading both the forward and the reverse strands. Parasites with mixed alleles...
TABLE 1. Sequences of primers used to amplify the pfcrt (exons 2 to 4), pfmdr-1, pfdhfr, and pfdhps genes in Plasmodium falciparum isolates collected in the Comoros Islands and Madagascar, 2006 to 2008

<table>
<thead>
<tr>
<th>Target gene</th>
<th>SNPs</th>
<th>PCR round</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Hybridization temp (°C)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfcrt</td>
<td>Exons 2/3</td>
<td>Outer PCR 1</td>
<td>PfCRT_SeqPCR_F, PfCRT_SeqPCR_R</td>
<td>TTGTTGTCTTTACAAATTAAGGC</td>
<td>57</td>
<td>952</td>
</tr>
<tr>
<td></td>
<td>Exon 2 (SNPs 72–76 and 97)</td>
<td>Inner PCR 1.1</td>
<td>PfCRT_SeqNest1_F, PfCRT_SeqNest1_R</td>
<td>GCACCTAAAGACTTGCC</td>
<td>57</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Exon 3 (SNPs 144–194)</td>
<td>Inner PCR 1.2</td>
<td>PfCRT_SeqNest2_F, PfCRT_SeqNest2_R</td>
<td>GGTGGAGGTCTTGTCTGGG</td>
<td>57</td>
<td>447</td>
</tr>
<tr>
<td></td>
<td>Exon 4 (SNPs 220 and 271)</td>
<td>Outer PCR 2</td>
<td>PfCRT2_SeqPCR_F, PfCRT2_SeqPCR_R</td>
<td>AGCTCGGAGTTATATTGTG</td>
<td>57</td>
<td>1,998</td>
</tr>
<tr>
<td></td>
<td>Exons (codons 6 to 206)</td>
<td>Inner PCR 2</td>
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<td>TACAA</td>
<td>59</td>
<td>850</td>
</tr>
<tr>
<td>pfmdr1</td>
<td>A region (SNPs 86 and 184)</td>
<td>Outer PCR</td>
<td>PfMDR1_SeqPCR_F, PfMDR1_SeqPCR_R</td>
<td>TCAA</td>
<td>57</td>
<td>896</td>
</tr>
<tr>
<td></td>
<td>O region (SNPs 1034, 1042 and 1246)</td>
<td>Inner PCR</td>
<td>PfMDR1_SeqNest1_F, PfMDR1_SeqNest1_R</td>
<td>GCACCTAAAGACTTGCC</td>
<td>57</td>
<td>549</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Outer PCR</td>
<td>PfMDR1034–1246_SeqPCR_F, PfMDR1034–1246_SeqPCR_R</td>
<td>CCAAAGTGAAAGAAATGGAAA</td>
<td>50</td>
<td>1,000</td>
</tr>
<tr>
<td>pfdhfr</td>
<td>SNPs 16 to 164</td>
<td>Outer PCR</td>
<td>PfDHFR_SeqPCR_F, PfDHFR_SeqPCR_R</td>
<td>CAACTCTCAGTACTATGAA</td>
<td>57</td>
<td>938</td>
</tr>
<tr>
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<td>Inner PCR</td>
<td>PfDHFR_SeqNest1_F, PfDHFR_SeqNest1_R</td>
<td>ATCTGTTGTCATCTCTG</td>
<td>57</td>
<td>735</td>
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<tr>
<td>pfdhps</td>
<td>SNPs 436 to 613</td>
<td>Outer PCR</td>
<td>PfDHPS_SeqPCR_F, PfDHPS_SeqPCR_R</td>
<td>TCAAGTATCTTAAATGACTG</td>
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<td>Inner PCR</td>
<td>PfDHPS_SeqNest1_F, PfDHPS_SeqNest1_R</td>
<td>CAACTCTCAGTACTATGAA</td>
<td>57</td>
<td>548</td>
</tr>
</tbody>
</table>

* Primary PCR amplifications were performed with a 25-μL reaction mixture containing 0.5 μL DNA, 0.25 to 0.5 μM each primer, 250 μM each deoxynucleoside triphosphate, 2.5 mM MgCl₂, and 1.25 U HotFirePol DNA polymerase (Solis Biodyne, Tartu, Estonia). Secondary, internal amplifications were done with a 55-μL reaction buffer with 2 μL of PCR products, 0.4 μM each primer, 250 μM each deoxynucleoside triphosphate, 2.5 mM MgCl₂, and 1.25 U HotFirePol DNA polymerase. The primers used are indicated. PCR amplifications were performed under the following conditions: heating at 94°C for 10 min, followed by 30 cycles of heating 94°C for 15 s, 58°C for 60 s, and 72°C for 60 s. For each run, the multiplicity of infection and prevalence of monoclonal infections. The multi-

P. falciparum Pfmdr-1 copy number determination by real-time PCR. The P. falciparum Pfmdr-1 copy number was measured by TaqMan real-time PCR (Ro-

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The multi-
from 1,195 clinical isolates (269 Comorian and 926 Malagasy isolates collected from 2006 to 2008). Seven novel mutations were detected in 30 samples (2.5%): two synonymous mutations (codon G26G, n = 2; codon L73L, n = 1) and five nonsynonymous mutations, either specific for Madagascar (G26R, n = 2; K27E, n = 1; K27I, n = 13; D91H, n = 1) or shared by both countries (N88S, 6 Comorian and 4 Malagasy isolates). The four major SNPs related to pyrimethamine resistance (N88S, 6 Comorian and 4 Malagasy isolates) were detected and 164L mutation (NCS1 [1.1%]) in the Comoros Islands, whereas the single 164L mutation (NCSL) was observed in three isolates in Madagascar (3/292, 1.0%). The spatial-temporal distribution of the two most prevalent alleles (IRNI and NCSL) in 2006 to 2008 is given in Fig. 2a.

**P. falciparum dihydropteroate synthetase (pfdhps).** The sequence of a 690-bp fragment (codons 419 to 649 of the pfdhps gene) was generated for 939 clinical isolates (269 Comorian and 670 Malagasy isolates collected in 2006 and 2007). Eight SNPs related to sulfadoxine resistance (S436A, S436C, S436F, A437G, K540E, A581G, A613S, A613T) were detected and were displayed by 10 alleles (Table 2). The 540E mutation was found only in the Comoros Islands, whereas the 581G, 613S, and 613T mutations were observed only in Madagascar. In 2006, the CAKAA allele was more frequent in the Comoros Islands, whereas the SGKAA allele was more frequent in Madagascar ($P < 0.05$). Although it was predominant mainly in the northwest in 2006 (11/54, 20.7%), the frequency of the SGKAA allele increased in 2007 in the central west (34/95 [35.8%] to 90/136 [66.2%]; $P < 10^{-4}$) and the southwest (40/104 [38.5%] to 34/58 [58.6%]; $P = 0.02$), reaching a prevalence >50% in the western part of Madagascar.
*P. falciparum* chloroquine resistance transporter (*pfcrt*). Three fragments of 450 bp (from codon 37 in exon 2 to codon 125 in exon 3), 447 bp (from codon 121 in exon 3 to codon 211 in exon 4), and 850 bp (from codon 199 in exon 3 to codon 316 in exon 7) located in the *pfcrt* gene were sequenced from 959 clinical isolates (266 Comorian and 693 Malagasy isolates collected in 2006 and 2007). Four SNPs related to CQ resistance (N74I, M75E, K76T, A220S) and four alleles were observed. Among the isolates from Madagascar, the mutant-type IETA allele was very rare compared to its incidence among the isolates from the Comoros Islands ($P = 0.05$) (Table 2). Interestingly, the 76T mutation, which is key for CQ resistance, was observed in an allele with triple mutations (IETA) and an allele with quadruple
mutations (IETS), while the 220S mutation, which acts in
synergy with 76T for the establishment of CQ resistance,
was observed as an allele with a single mutation (NMKS) as
well as an allele with quadruple mutation (IETS).

*P. falciparum* multidrug resistance gene 1 (*pfmdr-1*). Two
fragments of 549 bp (from codons 23 to 206) and 938 bp (from
codons 966 to 1278) were sequenced from 788 clinical isolates
collected in 2006 and 2007 (271 from the Comoros Islands and
517 from Madagascar). Five novel mutations were detected in
10 Malagasy samples (1.9%): four synonymous mutations
(codon G102G, n = 1; codon 182G, n = 1; codon T1069T, n = 1;
codon E1095E, n = 6) and the S112L nonsynonymous muta-
tion (n = 1). Among the previously described SNPs, only
N86Y, Y184F, and D1246Y were found in samples from both
countries, with there being eight alleles overall (Table 2). In
2006, the **YY** and **YFF** alleles were more common in the
Comoros Islands (P < 0.05), while the NYD, YFD, and NFD
alleles were more common in Madagascar (P < 0.05). The
spatial distribution of the major alleles observed in Madagascar
in 2006 to 2007 is presented in Fig. 2b.

The *pfmdr-1* copy number was determined for 233 Comorian
and 350 Malagasy isolates. The mean copy numbers were 0.96
and 0.92 in the Comoros Islands and Madagascar, respectively.
When the value was rounded to the nearest integer, no *pfmdr-1*
amplification was observed.

**Prevalence of monoclonal infections.** Multiplicity of infection,
analyzed for a subset of randomly chosen isolates (472 from
Madagascar and 86 from the Comoros Islands), showed that
the proportion of monoclonal infections was not signifi-
cantly different between the two countries (172/472 [36.4%] in
Madagascar and 86/1246 [41.8%] in the Comoros Islands;
P < 0.05), while the NYD, YFD, and NFD
alleles were more common in Madagascar (P < 0.05). The
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and 0.92 in the Comoros Islands and Madagascar, respectively.
When the value was rounded to the nearest integer, no *pfmdr-1*
amplification was observed.

**In vitro assays.** Among the 420 Malagasy *P. falciparum*
isolates tested, 372 (88.6%), 373 (88.9%), 373 (88.9%), 370
(88.1%), and 281 (66.9%) were successfully assayed for their
susceptibilities to CQ, MDA, QU, DHA, and MF, respectively.
The in vitro activities of the five antimalarial drugs are sum-
malated in Table 3. The prevalence of isolates classified as
having in vitro resistance to CQ, MDA, or MF was low; and
there was no indication of temporal fluctuations. In contrast,
a significantly decreased in vitro susceptibility to DHA was ob-
served between 2006 and 2008 (0.76 nM, 1.02 nM, 1.83 nM in
2006, 2007, and 2008, respectively; P < 10^−3), as shown in Fig.
4. Interestingly, this was not associated with increased IC_{50}
but, rather, was associated with a drastic reduc-
tion in the number of isolates presenting a very low IC_{50}
for DHA.

**Antimalarial drug pressure and population mobility.** Among
the 713 individuals interviewed (mean age, 23.4 years:
age range, 1 to 90 years), 18% (range, 8% to 30%) declared that
they had been prescribed an antimalarial treatment at the
health center or hospital in the previous 30 days. Surprisingly,
QU was the antimalarial most commonly prescribed by prac-
titioners (48%; range, 28% to 77%), followed by CQ (34%;
range, 8% to 53%) and SP (16%; range, 11% to 24%). The
combination of artesunate plus amodiaquine was not pre-
scribed, despite the official recommendations. Among the 37%
(range, 19% to 51%) of individuals who had taken an antima-
larial drug at home, 80% (range, 65% to 88%) declared that
they had used CQ, 15% (range, 2% to 35%) declared that they
had used SP, and 4% (range, 0% to 10%) declared that they
had used QU.

A population mobility survey showed that one-third of the
individuals (range, 13% to 48%) lived at another site 1 year
before the interview. The proportion of individuals who trav-
eled outside the site in the previous 30 days was estimated to
be 6.6% (range, 2% to 15%).

**DISCUSSION**

The present study provides the first comprehensive picture
of antimalarial drug resistance in Madagascar at a time that
coincides with the implementation of the new national policy
on antimalarial use (20) and completes previous results on in
vivo drug efficacy (19). The work conducted within the anti-
malarial drug resistance surveillance network in Madagascar
involved in vitro assays and sequencing of key loci implicated
in drug resistance. A large number of *P. falciparum* isolates
collected at health centers from multiple sites between 2006
and 2008 were studied. Hitherto, Madagascar was relatively
unaffected by multiresistant *P. falciparum* parasites, especially
parasites with the *pfdhfr* and *pfCRT* mutations (28, 29), and drug
resistance in Madagascar displayed unique and uncommon
features and markedly differed from the drug resistance ob-
served in the Comoros Islands.

Comparative analysis of the prevalence of the *pfdhfr* SNPs
showed the rapid emergence and spread of the *IRNI* allele in
Madagascar, where parasites with *pfdhfr* mutations had not
previously been detected (28). These findings are consistent
with those presented in our previous report, in which we indi-
cated that resistant *P. falciparum* parasites were introduced
into Madagascar by travelers coming from the Comoros Is-
lands (18). Resistant parasites were highly prevalent in the
north-central western part of Madagascar in 2006 (>50%), and
we observed their rapid spread along the north-to-south axis,
reaching first the southwest (10-fold increase in 3 years) and
then the eastern part of the country (1.5-fold increase in the
central east and 3-fold increase in the southeast). These results
are also concordant with those of in vivo efficacy studies, which
showed that SP treatment failures were more frequent in the
west (12.5%) and in the south (6.0%) (19). We suppose that
the dissemination of the *pfdhfr* allele with triple mutations has
been accelerated since 2006 by the massive use of SP, because
of its better fitness compared to that of the wild type (38)
and/or because of human population movements. A rapid rise
in the prevalence of parasites with the single 164L mutation
was observed in the south-to-north direction and is a worrying
development. This single *pfdhfr* mutation was previously de-
scribed in the south of Madagascar in 2006 (17). Although it is
rare in Africa (12, 16), it is obvious that this single *pfdhfr*
164L allele, which has not been described before, represents a seri-
ous threat that may lead to the emergence of a mutant with
quadruple mutations, which will render SP ineffective. The
mutant with quadruple mutations could arise locally by recom-
bination between mutant parasites with triple mutations and
parasites with the single 164L mutation (especially in areas
with high rates of transmission, such as the southeast, where
the prevalence of the both genotypes is high), or by stepwise
FIG. 3. Multilocus genotypes in *P. falciparum* isolates from Madagascar (2006 to 2007) and the Comoros islands (2006). Wild-type amino acids are indicated in gray, and mutated amino acids are indicated in black.
The quality controls for the batches of plates tested with the reference lines were not significantly different over the period from 2006 to 2008. The results were as follows (geometric mean IC₅₀s and 95% confidence intervals, which were 0.65 to 0.88 nM in 2006, 0.87 to 1.05 nM in 2007, and 0.91 to 1.05 nM in 2008 were 195, 124, and 51, respectively. The bars show the 95% confidence intervals of the reference lines were not significantly different over the period from 2006 to 2008 (P < 0.05). The results were as follows (geometric mean IC₅₀s ± standard deviations): for 3D7 Africa (n = 5), CQ, 19 nM ± 5 nM in 2006, 14 nM ± 3 nM in 2007, and 16 nM ± 2 nM in 2008; MDA, 16 nM ± 4 nM in 2006, 15 nM ± 4 nM in 2007, and 20 nM ± 2 nM in 2008; QU, 104 nM ± 14 nM in 2006, 100 nM ± 32 nM in 2007, and 102 nM ± 20 nM in 2008; DHA, 2.4 nM ± 0.8 nM in 2006, 2.2 nM ± 0.5 nM in 2007, and 2.5 nM ± 0.3 nM in 2008; and MF, 11.9 nM ± 2.6 nM in 2006, 15.3 nM ± 1.2 nM in 2007, and 11.2 nM ± 4.0 nM in 2008; for FCM29 Cameroon, CQ, 216 nM ± 19 nM in 2006, 222 nM ± 4 nM in 2007, and 238 nM ± 8 nM in 2008; MDA, 43 nM ± 6 nM in 2006, 37 nM ± 4 nM in 2007, and 42 nM ± 5 nM in 2008; QU, 222 nM ± 10 nM in 2006, 204 nM ± 16 nM in 2007, and 215 nM ± 18 nM in 2008; DHA, 3.7 nM ± 0.5 nM in 2006, 4.2 nM ± 1.1 nM in 2007, and 4.4 nM ± 0.7 nM in 2008; and MF, 21.1 nM ± 3.8 nM in 2006, 24.2 nM ± 2.0 nM in 2007, and 23.1 nM ± 4.3 nM in 2008.

The threshold IC₅₀ used for in vitro resistance or reduced susceptibility were >100 nM for CQ, >60 nM for MDA, >800 nM QU, >10.5 nM for DHA, and >30 nM for MF (23). The quality controls for the batches of plates tested with the reference lines were not significantly different over the period from 2006 to 2008 (P < 0.05). The results were as follows (geometric mean IC₅₀ ± standard deviations): for 3D7 Africa (n = 5), CQ, 19 nM ± 5 nM in 2006, 14 nM ± 3 nM in 2007, and 16 nM ± 2 nM in 2008; MDA, 16 nM ± 4 nM in 2006, 15 nM ± 4 nM in 2007, and 20 nM ± 2 nM in 2008; QU, 104 nM ± 14 nM in 2006, 100 nM ± 32 nM in 2007, and 102 nM ± 20 nM in 2008; DHA, 2.4 nM ± 0.8 nM in 2006, 2.2 nM ± 0.5 nM in 2007, and 2.5 nM ± 0.3 nM in 2008; and MF, 11.9 nM ± 2.6 nM in 2006, 15.3 nM ± 1.2 nM in 2007, and 11.2 nM ± 4.0 nM in 2008; for FCM29 Cameroon, CQ, 216 nM ± 19 nM in 2006, 222 nM ± 4 nM in 2007, and 238 nM ± 8 nM in 2008; MDA, 43 nM ± 6 nM in 2006, 37 nM ± 4 nM in 2007, and 42 nM ± 5 nM in 2008; QU, 222 nM ± 10 nM in 2006, 204 nM ± 16 nM in 2007, and 215 nM ± 18 nM in 2008; DHA, 3.7 nM ± 0.5 nM in 2006, 4.2 nM ± 1.1 nM in 2007, and 4.4 nM ± 0.7 nM in 2008; and MF, 21.1 nM ± 3.8 nM in 2006, 24.2 nM ± 2.0 nM in 2007, and 23.1 nM ± 4.3 nM in 2008.

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The situation with regard to in vitro CQ resistance and CQ resistance-associated mutations in Madagascar is unique. The scarcity of parasites with the pfcrt mutation (3/693 isolates; Table 2) and isolates resistant to CQ in vitro (3.2%; Table 3) is striking in view of the high rate of CQ treatment failures (44%) observed in our survey of the efficacy of antimalarial drug treatment (19). Thus, CQ resistance in Madagascar is different from that in other African countries where malaria is endemic and the Comoros Islands. CQ treatment failure was
first described in 1975 (9) and reached Madagascar early, at the same time that it reached East Africa (3). Since then and despite the massive use of CQ, the rate of CQ resistance has remained stable over the past 30 years, with the incidence of treatment failures ranging from 30% to 50% (31). This unique epidemiological situation, despite the regular introduction of pfcrt mutant-type alleles from the Comoros Islands (18), raises several questions. (i) Why is the spread of parasites with the pfcrt mutation rare in Madagascar, despite the huge pressure from the use of CQ? (ii) Is monitoring of isolates for the presence of the pfcrt mutant allele the best way to assess the incidence of CQ resistance in Madagascar, as suggested by Ariey et al. (2)? (iii) Are mechanisms unrelated to the incidence of CQ resistance in Madagascar, as suggested by SNPs and gene copy number, as Pgh-1 (encoded by Plasmodium falciparum pfpg1 gene) (34)?

Additional factors contributing to the uncommon drug resistance situation in Madagascar may be its geographical isolation, the broad range of malaria epidemiological strata in Madagascar, the geographical isolation of the antimalarial drug resistance patterns in Madagascar are rapidly changing because of gene flow from the Comoros Islands to Madagascar. In the present study, we witnessed a very short period of time. Close monitoring of in vitro susceptibility to artesunate and its companion drugs should be actively pursued, along with an exploration of the molecular markers including pfcrt and pfmdr-1 associated with some failures of treatment with artemisinin combined therapies in East Africa (10, 11, 21).

In conclusion, the work reported here provides evidence that the antimalarial drug resistance patterns in Madagascar are rapidly changing because of gene flow from the Comoros Islands and the local emergence and spread of polymorphisms. These changes reflect the efficiency of antimalarial drugs at eliminating susceptible parasites, but with the dark side of the coin being that the parasite population gets progressively enriched in less susceptible individuals. In this context, the establishment of regional and global monitoring networks such as the Worldwide Antimalarial Resistance Network (www.wwarn.org) is crucial to scaling up surveillance, rapidly sharing information on drug resistance, and helping with minimizing delays in implementing changes to antimalarial drug policies.

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REFERENCES


