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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 *pfcrt*, *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 artemisinin 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 (*pfcrt*) 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).

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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

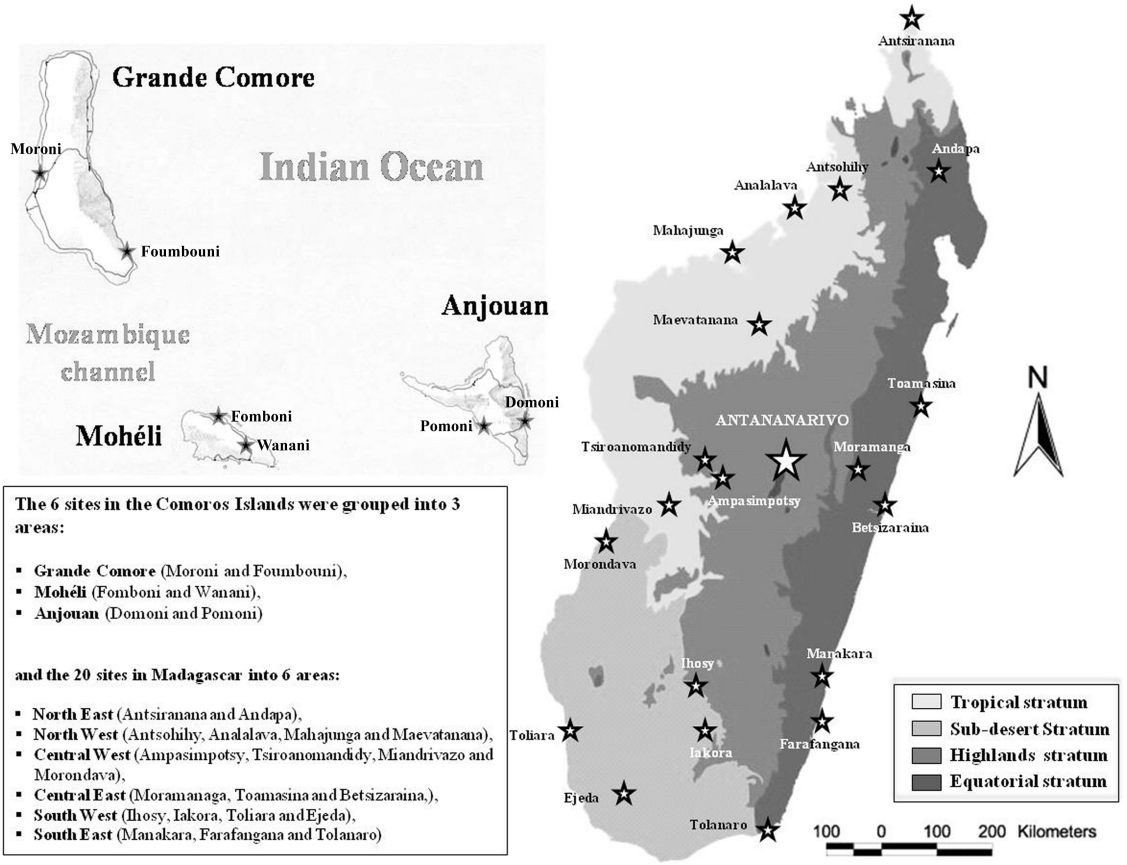

FIG. 1. Map of the southwestern Indian Ocean and locations of the sample collection sites.

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 [^{3}H]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 (IC_{50}), defined as the concentration at which 50% of the level of incorporation of [^{3}H]hypoxanthine was inhibited compared to the level of incorporation in the control wells. The IC_{50} 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 analyzer (Applied Biosystems, Courtaboeuf, France). Electrophoregrams were visualized and analyzed with CEQ2000 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[a]

Target gene	SNPs	PCR round	Primer name	Sequence (5′–3′)	Hybridization temp (°C)	Size of PCR product (bp)
pfcrt	Exons 2/3	Outer PCR 1	PfCRT_SeqPCR_F	TTTTGTTTTCTTACAATTAAGGCACT	57	952
			PfCRT_SeqPCR_R	CGACCTTAACAGATGGCTCA		
	Exon 2 (SNPs 72–76 and 97)	Inner PCR 1.1	PfCRT_SeqNest1_F	GGTGGAGGTTCTTGTCTTGG	57	450
			PfCRT_SeqNest1_R	AGCTTCGGTGTCGTTCCTAA		
	Exon 3 (SNPs 144–194)	Inner PCR 1.2	PfCRT_SeqNest2_F	GGAACGACACCGAAGCTTTA	57	447
			PfCRT_SeqNest2_R	TAGATAGAATTTTCTTCTTGTGGTT TCAA		
	Exon 4 (SNPs 220 and 271)	Outer PCR 2	PfCRT2_SeqPCR_F	TCTCGGAGCAGTTATTATTGTTG	57	1,998
			PfCRT2_SeqPCR_R	TCGACGTTGGTTAATTCTCCTT		
		Inner PCR 2	PfCRT2_SeqNest3_F	TGAAATTATCTTTTGAAACACAA GAAG	59	850
			PfCRT2_SeqNest3_R	ATGCTCCGTCACAATCATCA		
pfmdr1	A region (SNPs 86 and 184)	Outer PCR	PfMDR1_SeqPCR_F	TGAAAGATGGGTAAAGAGCAGA	57	896
			PfMDR1_SeqPCR_R	CCATACCAAAAACCGAATGC		
		Inner PCR	PfMDR1_SeqNest1_F	TGAACAAAAAGAGTACCGCTGA	57	549
			PfMDR1_SeqNest1_R	AAATTAACGGAAAAACGCAAG		
	O region (SNPs 1034, 1042 and 1246)	Outer PCR	PfMDR1034-1246_PCR_F	CAAAAGTAAAGAAATTGAGAAAA	50	1,000
			PfMDR1034-1246_PCR_R	AAACTTACTAACACGTTTAACATC		
		Inner PCR	PfMDR1034-1246_Nest_F	CAAGCGGAGTTTTTGCATTT	57	938
			PfMDR1034-1246_Nest_R	CAATGTTGCATCTTCTCTTCCA		
pfdhfr	SNPs 16 to 164	Outer PCR	PfDHFR_SeqPCR_F	CCAACATTTTCAAGATTGATACATAA	52	735
			PfDHFR_SeqPCR_R	ACATCGCTAACAGAAATAATTTGA		
		Inner PCR	PfDHFR_SeqNest1_F	GCGACGTTTTCGATATTTATG	57	600
			PfDHFR_SeqNest1_R	GATACTCATTTTCATTTATTTCTGGA		
pfdhps	SNPs 436 to 613	Outer PCR	PfDHPS_SeqPCR_F	TTGTTGAACCTAAACGTGCTG	54	758
			PfDHPS_SeqPCR_R	TTGATCCTTGTCTTTCCTCATGT		
		Inner PCR	PfMDHPS_SeqNest1_F	TTTGAAATGATAAATGAAGGTGCT	57	724
			PfMDHPS_SeqNest1_R	TCCAATTGTGTGATTTGTCCA		

[a] 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 $MgCl_2$, 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 $MgCl_2$, 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 15 min, followed by 30 to 40 cycles of heating at 94°C for 30 s, 50 to 59°C for 30 s, and 72°C for 90 s (180 s for *pfcrt* exon 4) and a final extension at 72°C for 10 min.

(in which both wild-type and mutant alleles were present) were considered mutants for estimation of the prevalence of the SNPs. Haplotypes for drug resistance markers were reconstructed from the full sequence presenting an unambiguous single allele signal at all positions.

***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 (Rotor-Gene 6000; Corbett Research, Sydney, Australia) relative to the single copy of the ß-tubulin (used as a housekeeping gene), as described previously (26). All samples were run in duplicate in 25-μl reaction mixtures containing JumpStart *Taq* ReadyMix (Sigma, Taufkirchen, Germany), 300 nM each forward and reverse primer, 100 nM each probe, and 4 μl template DNA. Amplifications were performed under the following conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 58°C for 60 s, and 72°C for 60 s. For each run, the *pfmdr-1* copy number was measured relative to the numbers in two standard calibrator parasite clonal lines, the 3D7 Africa line (which has one copy of *pfmdr-1*) and line Dd2 (which has three copies of *pfmdr-1*), by the $\Delta\Delta C_T$ method (where C_T is the threshold cycle) (15). Reference DNA clone W2 (which has three copies of *pfmdr-1*) was used as the quality control in each run.

Multiplicity of infection and prevalence of monoclonal infections. The multiplicity of infection, defined as the highest number of alleles detected at either of the two loci, was estimated by using an allelic family-specific nested PCR (MAD20, K1, and RO33 for *pfmsp-1* and 3D7 Africa and FC27 for *pfmsp-2*), as described previously (40). All PCR amplifications contained a positive control (genomic DNA from strains W2, HB3, and 3D7 Africa) and a negative control (no target DNA). Multilocus genotype analysis for drug resistance markers was performed with monoclonal isolates, i.e., isolates in which a single *pfmsp-1* and/or *pfmsp-2* allelic form was detected.

Assessment of antimalarial drug pressure and population mobility. Between April and May 2006, cross-sectional surveys were carried out in Madagascar. Thirty households from each of the seven sites were randomly selected. Three randomly selected individuals in each household were interviewed about their recent travel and antimalarial drug consumption, as described by Gardella et al. (8). Data collected from the individuals and the household were secondarily aggregated to characterize each site.

Statistical analysis. EpiInfo software (version 6.0; CDC, Atlanta, GA) and MedCalc software (version 9.1.0.1; Mariakerke, Belgium) were used for data analysis. Categorical variables were compared by using the χ^2 or Fisher's exact test, and continuous variables were compared by using the independent-sample *t* test. Data from the in vitro assays were analyzed after logarithmic transformation and were expressed as geometric means with 95% confidence intervals. A *P* value of <0.05 was considered statistically significant.

Ethical approval. The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health of Madagascar (approval number 007/SANPF/2007; registration number ISRCTN36517335). Informed written consent was provided by all patients or their parents/guardians before inclusion in the study.

Nucleotide sequence accession numbers. The exact sequence of each new mutant allele has been submitted to the GenBank database (accession numbers FJ941830 to FJ941841).

RESULTS

***P. falciparum* dihydrofolate reductase (*pfdhfr*).** A 600-bp fragment (codon 6 to 206 of the *pfdhfr* gene) was sequenced

TABLE 2. Prevalence of point mutation haplotype in the *P. falciparum* dihydrofolate reductase, dihydropteroate synthetase, CQ resistance transporter, and multidrug resistance 1 genotypes among clinical samples collected from the Comoros Islands and Madagascar in 2006, 2007, and 2008

Gene (amino acid positions)	Comoros Islands		Madagascar			
	Amino acids[a]	Prevalence (%) of alleles in 2006	Amino acids	Prevalence (%) of the alleles in:		
				2006	2007	2008
pfdhfr (51, 59, 108, 164)[b]	**IRN**I	45.0	NCSI	69.2	59.5	55.7
	NCSI	33.8	**IRN**I	27.7	34.1	33.3
	N**R**N**I**	9.3	NCS**L**	1.0	5.5	8.8
	NC**N**I	6.3	NC**N**I	2.1	0.8	1.1
	IC**N**I	3.3	**I**C**N**I	0	0	1.1
	ICSI	1.1	**I**CSI	0	0	0
	IRNL	1.1	N**R**N**I**	0	0	0
	NCS**L**	0	**IRNL**	0	0	0
pfdhps (436, 437, 540, 581, 613)[c]	SAKAA	88.5	SAKAA	69.2	51.2	
	S**G**KAA	5.9	S**G**KAA	35.8	46.2	
	CAKAA	3.3	**F**AKAA	0	1.6	
	AAKAA	1.1	**C**AKAA	0	0.5	
	FAKAA	0.7	**F**AKA**T**	0	0.5	
	S**GE**AA	0.4	**A**AKA**S**	0.3	0	
	AAKA**S**	0	**A**AKA**T**	0.3	0	
	AAKA**T**	0	S**G**K**G**A	0.3	0	
	FAKA**T**	0	**A**AKAA	0	0	
	S**G**K**G**A	0	S**GE**AA	0	0	
pfcrt (74, 75, 76, 220)[d]	NMKA	41.7	NMKA	99.3	99.7	
	IETS	36.1	**IET**A	0.7	0	
	NMK**S**	15.0	NMK**S**	0	0.3	
	IETA	7.1	**IETS**	0	0	
pfmdr-1 (86, 184, 1246)[e]	**YFY**	66.1	N**F**D	21.8	30.0	
	YYY	11.1	**YFY**	24.1	21.0	
	YYD	11.1	**YF**D	20.1	15.5	
	YFD	9.2	**Y**YD	17.2	11.1	
	NYD	1.8	NYD	8.0	14.6	
	N**F**D	0.4	N**FY**	4.0	2.9	
	N**FY**	0.4	**YYY**	2.9	4.1	
	NY**Y**	0	NY**Y**	1.7	0.9	

[a] Amino acids conferring resistance are shown in boldface and underlined.
[b] $n = 269$ for the Comoros Islands in 2006 and $n = 292$, 361, and 273 for Madagascar in 2006, 2007, and 2008, respectively.
[c] $n = 269$ for the Comoros Islands in 2006 and $n = 293$ and 377 for Madagascar in 2006 and 2007, respectively.
[d] $n = 266$ for the Comoros Islands in 2006 and $n = 293$ and 400 for Madagascar in 2006 and 2007, respectively.
[e] $n = 271$ for the Comoros Islands in 2006 and $n = 174$ and 343 for Madagascar in 2006 and 2007, respectively.

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, $n = 1$; K72I, $n = 13$; D91H, $n = 1$) or shared by both countries (N88S, 6 Comorian and 4 Malagasy isolates). The four major SNPs related to pyrimethamine resistance (N51I, C59R, S108N, I164L) were observed, with there being eight different alleles overall (Table 2). In 2006, four alleles (NC**N**I, **I**C**N**I, N**R**N**I**, and **IRN**I, where the boldface and underscore indicate amino acids conferring resistance; Table 2) were more frequent in the Comoros Islands than in Madagascar ($P < 0.05$). The allele with quadruple mutations (**IRNL**), which conferred a high level of resistance to antifolates, was found at a low frequency (3/269 [1.1%]) in the Comoros Islands, whereas the single 164L mutation (NCS**L**) was observed in three isolates in Madagascar (3/292, 1.0%). The spatial-temporal distribution of the two most prevalent alleles (**IRN**I and NCS**L**) 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 **C**AKAA allele was more frequent in the Comoros Islands, whereas the S**G**KAA 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 S**G**KAA 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.

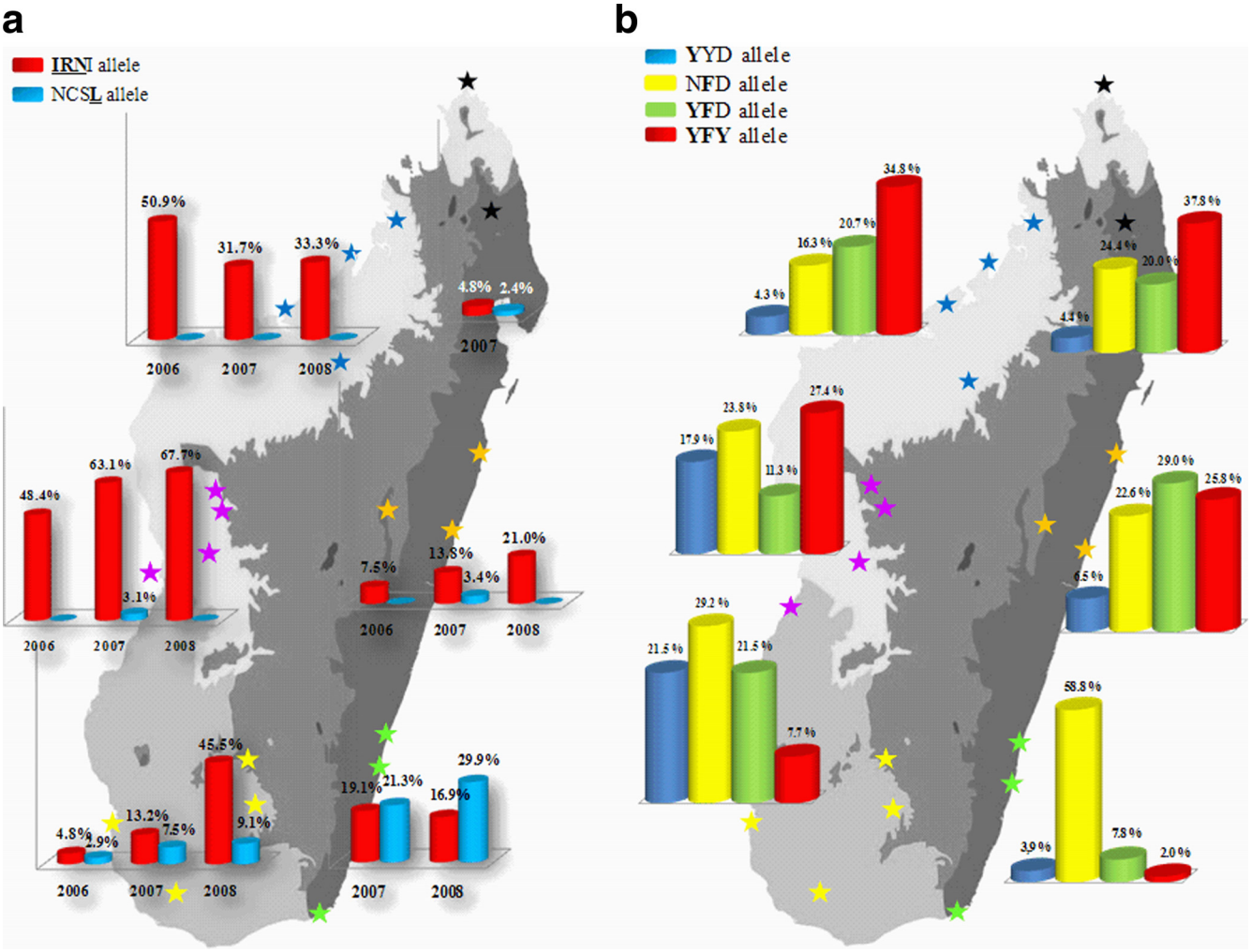

FIG. 2. Spatial-temporal distribution of the most prevalent *P. falciparum* dihydrofolate reductase and multidrug resistance gene 1 haplotypes at the sites involved in the antimalarial drug resistance surveillance network in Madagascar, 2006 to 2008. The 20 sites in six areas are presented as colored stars: black for the northeast (Antsiranana, Andapa), blue for the northwest (Antsohihy, Analalava, Mahajunga, Maevatanana), orange for the central east (Toamasina, Moramanga, Betsizaraina), purple for the central west (Tsiroanomandidy, Ampasimpotsy, Miandrivazo, Morondava), green for the southeast (Tolanaro, Farafangana, Manakara), and yellow for the southwest (Ihosy, Iakora, Toliara, Ejeda). (a) The total numbers of isolates analyzed in 2006, 2007, and 2008 were as follows: 0, 42, and 0, respectively, in the northeast; 53, 60, and 42, respectively, in the northwest; 40, 29, and 81, respectively, in the central east; 95, 130, and 62, respectively, in the central west; 0, 47, and 77, respectively, in the southeast; and 104, 53, and 11, respectively, in the southwest. The **IRN**I allele with triple mutations was particularly frequent in the northwest and the central west of Madagascar, displaying the same prevalence found in the Comoros Islands. Between 2006 and 2007, its prevalence significantly increased in the central west ($P = 0.04$), and between 2007 and 2008 its prevalence significantly increased in the southwest ($P = 0.03$), supporting its spread in the western part of Madagascar. However, the allele with the single 164L mutation was essentially observed in the south of Madagascar: it was first detected in 2006 in the southwest and increased in prevalence between 2007 and 2008 ($P = 0.04$); this allele remains the most frequently observed allele in the southeast and represented approximately one-third of the alleles in 2008. (b) The total numbers of isolates analyzed in 2006 and 2007 were 45 in the northeast, 92 in the northwest, 31 in the central east, 168 in the central west, 51 in the southeast, and 130 in the southwest. No significant differences in the proportions of alleles were found between 2006 and 2007; but the distribution of the three most frequent alleles was heterogeneous: the N**F**D allele was significantly more frequent in the southeast than in the other areas ($P < 0.05$); the **YFY** allele was significantly more frequent in the north (>30%), intermediate in frequency in the central west/east (>20%), and low in frequency in the south (<10%); and the **Y**YD allele was the most prevalent in the central or southwest (>15%) compared to its prevalence in the other parts of the country (<7%).

***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 **IET**A 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 (**IET**A) 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 (NMK**S**) 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 mutation ($n = 1$). 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 **YYY** and **YFY** alleles were more common in the Comoros Islands ($P<0.05$), while the NYD, **YF**D, and N**FY** 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 significantly different between the two countries (172/472 [36.4%] in Madagascar and 36/86 [41.8%] in the Comoros Islands; $P = 0.40$). The results of multilocus genotype analysis of 75 isolates that had a single allelic form and that were considered monoclonal are presented in Fig. 3.

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 summarized 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 observed 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}s in some isolates but, rather, was associated with a drastic reduction 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 practitioners (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 prescribed, despite the official recommendations. Among the 37% (range, 19% to 51%) of individuals who had taken an antimalarial 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 traveled 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 antimalarial 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 observed in the Comoros Islands.

Comparative analysis of the prevalence of the *pfdhfr* SNPs showed the rapid emergence and spread of the **IRN**I 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 indicated that resistant *P. falciparum* parasites were introduced into Madagascar by travelers coming from the Comoros Islands (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 detected 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 serious 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 recombination 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

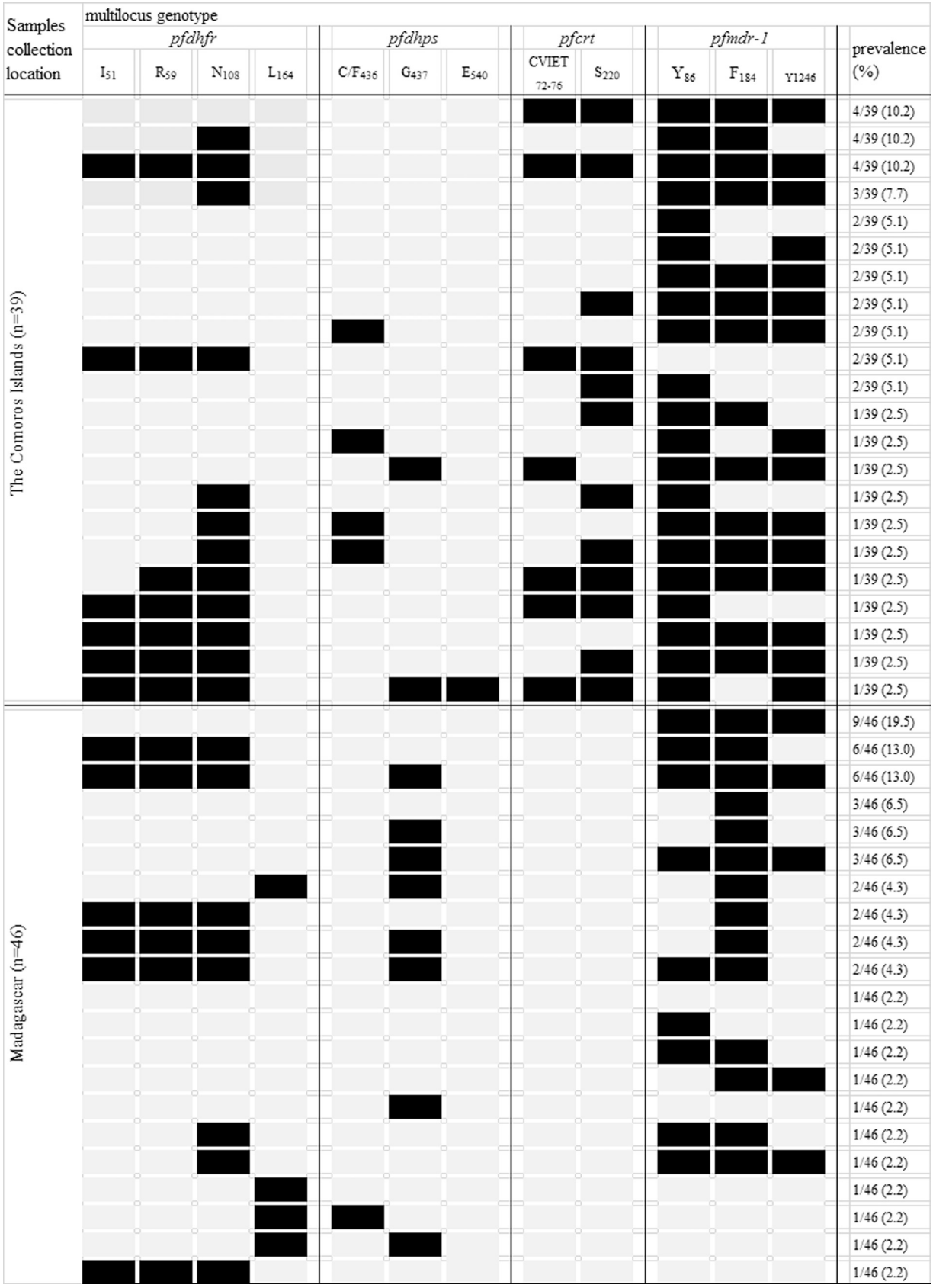

FIG. 3. Multilocus genotypes in *P. falciparum* isolates from Madagascar (2006 to 2007) and the Comoros islands (2006). Wild-type amino acids are indicated in gray, and mutated amino acids are indicated in black.

TABLE 3. In vitro susceptibility and prevalence of resistance to antimalarial drugs of *Plasmodium falciparum* isolates collected from patients with uncomplicated malaria in the sentinel sites of the antimalarial surveillance network, Madagascar, 2006 to 2008[a]

Drug	Period	No. of isolates tested	IC_{50} (nM)			Resistant isolates		
			Geometric mean	95% confidence interval	Range	No.	%	95% confidence interval
CQ	2006	196	18.6	15.6–22.3	1–142	8	4.1	1.8–7.9
	2007	125	21.3	17.7–25.6	1–142	4	3.2	0.9–8.0
	2008	51	12.8	9.3–17.5	1–77	0	0	0–7.0
	Total	372	18.5	16.4–20.9	1–142	12	3.2	1.8–5.7
MDA	2006	197	7.6	6.8–8.6	1–140	1	0.5	0–2.8
	2007	125	8.9	7.9–10.2	1–72	1	0.8	0–4.4
	2008	51	9.7	8.0–11.8	1–35	0	0	0–7.0
	Total	373	8.3	7.7–9.0	1–140	2	0.5	0.1–2.1
QU	2006	197	49.4	42.2–57.8	1–712	0	0	
	2007	125	59.1	50.6–69.0	5–435	0	0	
	2008	51	58.4	44.7–76.4	3–296	0	0	
	Total	373	53.7	48.4–59.6	1–712	0	0	
DHA	2006	195	0.76	0.65–0.88	0.1–6.2	0	0	
	2007	124	1.02	0.87–1.20	0.1–6.2	0	0	
	2008	51	1.83	1.53–2.19	0.6–8.1	0	0	
	Total	370	0.95	0.86–1.05	0.1–8.1	0	0	
MF	2006	162	6.2	5.4–7.2	0.5–30.9	1	0.6	0–3.4
	2007	113	7.0	6.3–7.9	1.0–26.0	0	0	
	2008	6	14.3	9.7–21.1	10.0–23.0	0	0	
	Total	281	6.7	6.1–7.3	0.5–30.9	1	0.4	0–2.0

[a] The threshold IC_{50}s 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 IC_{50}s ± 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.

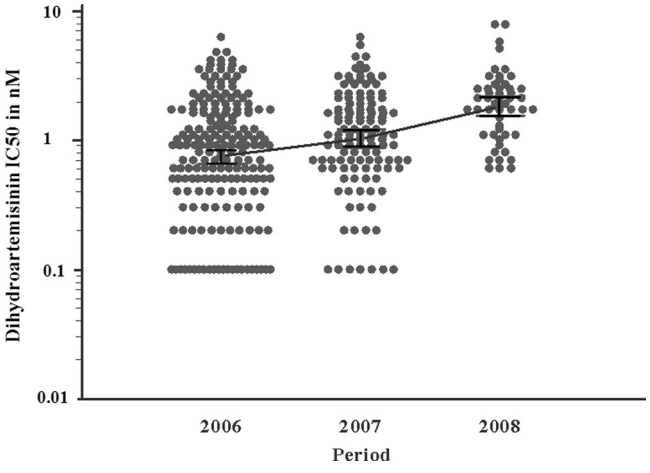

FIG. 4. Evolution of the DHA geometric mean IC_{50}s in *Plasmodium falciparum* isolates collected from 2006 to 2008 in patients with uncomplicated malaria at the sentinel sites of the antimalarial drug resistance surveillance network, Madagascar. The total numbers of isolates analyzed in 2006, 2007, and 2008 were 195, 124, and 51, respectively. The bars show the 95% confidence intervals, which were 0.65 to 0.88 nM in 2006, 0.87 to 1.20 nM in 2007, and 1.53 to 2.19 nM in 2008. The DHA geometric mean IC_{50}s ± SDs of the reference lines were not significantly different over the period from 2006 to 2008 ($P < 0.05$): for 3D7 Africa ($n = 5$), 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; for FCM29 Cameroon, 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.

selection of the 51I, 59R, and 108N mutations from the mutant parasite with the single 164L mutation, as suggested by our recent detection of a mutant parasite with a double mutation, NC**NL** (unpublished data). This adds to the threat of the possible spread of the mutant with quadruple mutations from the Comoros Islands.

Of the *pfdhps* point mutations related to sulfadoxine resistance, only A437G was prevalent in both countries, confirming previous findings of studies performed in the Comoros Islands (23). This SNP, which had never previously been described in Madagascar, showed the same prevalence and spatiotemporal evolution as the *pfdhfr* triple mutation. The rapid, concomitant rise in the frequencies of mutant parasites with both the *pfdhfr* and *pfdhps* mutations is challenging the current recommendation to use SP for intermittent preventive treatment of pregnant women (13).

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 *pfcrt* mutations responsible for the failures of CQ therapy?

To evaluate the contribution of *pfmdr-1* to the unusual CQ susceptibility profiles in Madagascar, we analyzed *pfmdr-1* SNPs and gene copy number, as Pgh-1 (encoded by *mdr-1*) is known to modulate *P. falciparum* drug sensitivity (37). This showed that SNPs at codons 86, 184, and 1246 in *pfmdr-1* were highly prevalent, likely reflecting the widespread use of quinolines, such as CQ and QU. The elevated frequency of *pfmdr-1* mutant alleles (**Y**YD, N**F**D, **YF**D, and **YFY**) associated with CQ resistance in some studies (6, 7, 35), in a context of a low frequency of *pfcrt* mutant genotypes, may account for the high rate of late clinical failure of CQ treatment in the absence of in vitro CQ resistance. Further field studies are needed to clarify this issue and obtain a better understanding of the usefulness of the *pfmdr-1* marker for predicting in vivo CQ resistance in Madagascar. *pfmdr-1*gene amplification has been associated with MF resistance in vivo (26) and in vitro (39). Interestingly, there was no evidence for any increase in *pfmdr-1* copy number in Madagascar or in the Comoros Islands in the context of a lack of MF usage. In line with this, all the IC_{50}s for MF were in the range classified as susceptible. Our observation that QU was the most frequently prescribed drug in the public sector in 2006 is informative and may point to the specific selective pressures exerted on the parasite population in Madagascar. Additional factors contributing to the uncommon drug resistance situation in Madagascar may be its geographical isolation, the broad range of malaria epidemiological strata in which the four *Plasmodium* species are present, and the admixture of inhabitants with multiple ethnic origins.

Finally, concern is raised by our observations of a significant decrease in DHA susceptibility between 2006 and 2008, since the implementation of the combination of artesunate plus amodiaquine in Madagascar. In the present study, we witnessed a progressive loss of the most susceptible isolates during 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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