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Discordant Temporal Evolution of *Pfcrt* and *Pfmdr1* Genotypes and *Plasmodium falciparum In Vitro* Drug Susceptibility to 4-Aminoquinolines after Drug Policy Change in French Guiana

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Analysis of the evolution of drug target genes under changing drug policy is needed to assist monitoring of *Plasmodium falciparum* drug resistance in the field. Here we genotype *Pfcrt* and *Pfdmr1* of 700 isolates collected in French Guiana from 2000 (5 years after withdrawal of chloroquine) to 2008, i.e., the period when the artemether-lumefantrine combination was progressively introduced and mefloquine was abandoned. Gene sequencing showed fixation of the 7G8-type *Pfcrt* SMVNT resistance haplotype and near fixation of the NYCDY *Pfdmr1* haplotype. *Pfdmr1* gene copy number correlated with 50% inhibitory concentrations of mefloquine and halofantrine ($r = 0.64$ and 0.47, respectively, $n = 547$); its temporal changes paralleled changes in *in vitro* mefloquine susceptibility. However, the molecular parameters studied did not account for the regained *in vitro* susceptibility to chloroquine and showed a poor correlation with susceptibility to artemether, lumefantrine, or quinine. Identification of novel markers of resistance to these antimalarials is needed in this South American area.

Plasmodium falciparum drug resistance is a major concern for malaria control. Mutations of the *P. falciparum* chloroquine resistance transporter (*Pfcrt*), a member of the drug metabolite transporter superfamily, have been identified as causal determinants of chloroquine resistance *in vitro* (17, 41) and associated with chloroquine treatment failures (13). Susceptibility to mefloquine is associated with single nucleotide polymorphisms (SNPs) and copy number variation of *Pfmdr1* (*P. falciparum* multidrug resistance protein 1), a P-glycoprotein homolog (34, 35). *Pfcrt* and *Pfmdr1* haplotypes interact to modulate *in vitro* sensitivity to amodiaquine and chloroquine; this interaction is particularly marked with alleles originating from South America (37). Furthermore, *Pfcrt* and *Pfmdr1* haplotypes influence *in vitro* susceptibility to multiple antimalarials (23, 34, 36, 37, 39–41), probably by altering their intracellular transport (38).

Understanding of the evolution of drug target genes under changing drug policy is crucial for drug efficacy monitoring using molecular markers. In Malawi, withdrawal of chloroquine was followed by the expansion of chloroquine-sensitive parasites possessing a wild-type *Pfcrt* allele, apparently better fit than mutant parasites in the absence of chloroquine pressure (26, 32). A similar phenomenon was reported in China (44). Expansion of resistance to mefloquine following its deployment in Thailand was associated with a temporal increase of the frequency of parasites with an increased *Pfmdr1* copy number and harboring specific SNPs (35). Here, we analyze the association of the *Pfcrt* and *Pfmdr1* genotypes and *Pfmdr1* gene copy number with the temporal changes of *in vitro* susceptibility in a series of isolates collected over the years 2000 to 2008 across French Guiana.

Chloroquine was abandoned as a first-line treatment for *P. falciparum* malaria in French Guiana in 1995 for the quinine-doxycycline combination, which was progressively replaced since 2002 by the artemether-lumefantrine combination. Mefloquine or halofantrine was used as a second-line treatment from 1990 to 2002, when it was replaced with atovaquone-proguanil (28). We reported that these changes impacted on in *vitro* drug sensitivity profiles. *In vitro* resistance to chloroquine declined in the years following its withdrawal to reach an approximately 50% frequency. Sensitivity to mefloquine decreased progressively after its introduction. Susceptibility to amodiaquine was correlated positively with susceptibility to chloroquine and more moderately with susceptibility to mefloquine or halofantrine (28).

We analyze here the temporal changes of *Pfcrt* and *Pfmdr1* gene polymorphisms and of *Pfmdr1* gene copy number during the years 2000 to 2008, i.e., the period starting 5 years after the withdrawal of chloroquine and encompassing the implementation of artemether-lumefantrine as a replacement for mefloquine. We report that *Pfmdr1* copy number variation reflected the temporal evolution of *in vitro* mefloquine susceptibility. However, the molecular parameters studied did not account for the regained susceptibility to chloroquine and were poorly associated with responses to quinine, lumefantrine, and artemether.

MATERIALS AND METHODS

***In vitro* drug sensitivity assays.** The *in vitro* susceptibility to a panel of antimalarials of 513 *P. falciparum* isolates collected longitudinally during the years 2000 to 2005 by our reference center has been reported (28). Similar procedures were used for 187 isolates collected in the years 2006 to

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TABLE 1 Overview of *in vitro* susceptibility of isolates from French Guiana[a]

IC_{50} range	% (no.) of isolates						
	CQ	Quin	Mef	Hal	Art	Amo	Lum
Total	100 (700)	100 (698)	100 (684)	100 (690)	100 (600)	100 (370)	100 (280)
High	53.4 (374)	4.7 (33)	20.2 (138)	14.5 (100)	4.8 (29)	4.3 (16)	27 (76)
Intermediate	9.1 (64)	12.8 (89)	21.6 (148)	25.8 (178)	3.4 (20)	5.3 (20)	7.1 (20)
Low	37.4 (262)	82.5 (576)	58.2 (398)	39.7 (412)	91.8 (551)	90.4 (334)	65.8 (184)

[a] Shown are percentages of isolates classified as resistant, intermediate, and susceptible based on the IC_{50} of each drug. Three classes were considered for each antimalarial. The IC_{50} cutoff for each class was as follows: CQ, chloroquine (high [resistant], IC_{50} of >100 nM; intermediate, 80 < IC_{50} < 100 nM; low [susceptible], IC_{50} of <80 nM); Quin, quinine (high [resistant], IC_{50} of >500 nM; intermediate, 300 < IC_{50} < 500 nM; low [susceptible], IC_{50} of <300 nM); Mef, mefloquine (high [resistant], IC_{50} of >30 nM; intermediate, 15 < IC_{50} < 30 nM; low [susceptible], IC_{50} of <15 nM); Hal, halofantrine (high, IC_{50} of >8 nM; intermediate, 4 < IC_{50} < 8 nM; low, IC_{50} of <4 nM); Art, artemether (high, IC_{50} of >12 nM; intermediate, 8 < IC_{50} < 12 nM; low, IC_{50} of <8 nM); Amo, monodesthyl-amodiaquine (high, IC_{50} of >60 nM; intermediate, 40 < IC_{50} < 60 nM; low, IC_{50} of <40 nM); Lum, lumefantrine (high, IC_{50} of >150 nM; intermediate, 100 < IC_{50} < 150 nM; low, IC_{50} of <100 nM).

2008. An aliquot of a patient isolate was cultured for 24 h in the absence of drug, frozen at −80°C, and subsequently processed for RNA and DNA extraction.

DNA extraction and genotyping. DNA was extracted as described previously (27). *Pfmsp1* block2 genotyping was carried out as described previously (23, 27). *Pfcrt* exon2 and exon4 amplification was performed as described previously (1, 19). Three overlapping PCR were used to amplify the full-length *Pfmdr1* sequence. The conditions were 1.5 mM $MgCl_2$, 2 μM each primer, and 200 μM deoxynucleoside triphosphate, 1.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in a final volume of 50 μl, with 1 cycle of 94°C for 8 min; 35 cycles of 94°C for 1 min, incubation at the annealing temperature for 1 min, and elongation at 72°C for 2 min; and final extension at 72°C for 10 min (for the primer sequence and annealing temperature, see Table S1 in the supplemental material). PCR products were analyzed on 2% agarose gels and stored at −20°C before being processed for direct sequencing.

mRNA extraction and cDNA synthesis. Following maturation *in vitro*, RNA was extracted at the late trophozoite stage using QIAamp RNA Blood (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. For each isolate, 200 μl of whole blood was treated. Five micrograms of total RNA was reverse transcribed using random hexamer priming and Moloney murine leukemia virus reverse transcriptase (Superscript First-Strand kit; Invitrogen, Cergy Pontoise, France). The full-length *Pfcrt* coding cDNA sequence was amplified by nested PCR as described previously (15).

Direct sequencing of PCR products. PCR products were sequenced using ABI Prism BigDye Terminator chemistry as described previously (16). For the *Pfmdr1* central region, specific internal primers were used to determine the whole sequence on both strands. Sequence analysis was done using the Phred Phrap package. Sequences with segments of ≥1,000 bp called with a quality over 20 per base were retained. Sequences of insufficient quality were either resequenced or rejected. Sequence assembly was done with Seqscape software v.2.0 (Applied Biosystems). SNPs were considered only if observed unambiguously.

Assessment of *Pfmdr1* gene copy number and expression level. *Pfmdr1* (PFE1150w), β-tubulin (PF10_0084), and *maebl* (PF11_0486) copy numbers and mRNA expression were assessed by TaqMan real-time PCR (ABI 7300 real-time PCR systems; Applied Biosystems, Warrington, United Kingdom) (for primer sequences, see Table S2 in the supplemental material). The *Pfmdr1* probes were FAM (6-carboxyfluorescein) labeled at the 5′ end, and the β-tubulin and *maebl* probes were VIC labeled (Table S2). All probes had a TAMRA (6-carboxytetramethylrhodamine) label at the 3′ end. All samples were assayed in triplicate in 96-well optical reaction plates in a 25-μl final volume containing 4 μl genomic DNA or cDNA, 1 μM each primer, and 0.25 μM fluorogenic probe in Universal PCR Master Mix (Applied Biosystems). The amplification conditions for all loci were 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

The relative mRNA levels were quantified and compared as described previously (30). The fold change in gene expression was normalized to the *maebl* or β-tubulin control gene.

Statistical analysis. The association of the 50% inhibitory concentration (IC_{50}) of each antimalarial, considered a quantitative variable with the *Pfmdr1* genotype or gene copy number, was analyzed using the Pearson correlation test. The association of IC_{50} classes (see Table 1) with *Pmdr1* copy number was examined using the Kruskal-Wallis test. The Spearman correlation coefficient was calculated. All tests were performed with SAS, version 9.1 (SAS Institute Inc., Cary, NC). Results were considered significant if the *P* value was below 0.05.

RESULTS

Drug susceptibility and infection complexity. A panel of 700 isolates was collected and tested for *in vitro* drug susceptibility by our reference center during the period 2000 to 2008 (Table 1). IC_{50}s of mefloquine and halofantrine were correlated ($r = 0.63$; $P < 0.0001$); 88% of the isolates with a high IC_{50} of mefloquine (>30 nM) had an elevated IC_{50} of halofantrine. The IC_{50}s of chloroquine and quinine, mefloquine, or doxycycline were unrelated.

Pfmsp1 block2 genotyping of a subset of 61 isolates showed a very high rate (91.4%) of single infection, confirming previous studies (27).

***Pfcrt* genotype and cDNA sequence analysis.** The *Pfcrt* exon 2-exon 4 genotype was determined for 628 samples, including 212 chloroquine-sensitive samples. Unambiguous sequencing of 626 isolates showed the same haplotype at positions 72 to 76, 220 coding for SVMNT/S, associated with a monomorphic intron 4 microsatellite $(TAAA)_3(TA)_{14}$ in all isolates. Sequencing of reference alleles from laboratory lines amplified and run in the same experiments ruled out possible experimental contamination artifacts.

To determine whether additional mutations in the *Pfcrt* gene were present in the chloroquine-susceptible isolates, a complete cDNA sequence was determined for 53 samples (25 chloroquine sensitive and 28 chloroquine resistant). All but one cDNA had a C72S K76T A220S N326D I356L protein sequence haplotype, and 6 (11%) had the 72S residue encoded by AGT (i.e., the 7G8 allele, also called SMVNT1), while the other 47 sequences (89%) had a TCT codon (a 7G8 type also called SMVNT2). One susceptible sample, with a chloroquine IC_{50} of 22 nM, carried an additional C350R *Pfcrt* mutation. In brief, the *Pfcrt* genotype of the panel of isolates studied was unrelated to the observed IC_{50} of chloroquine or of any of the molecules from the panel of antimalarials tested.

Pfmdr1 sequencing. A full-length *Pfmdr1* coding sequence was established for 700 isolates. Overall, nine alleles, called FG-A

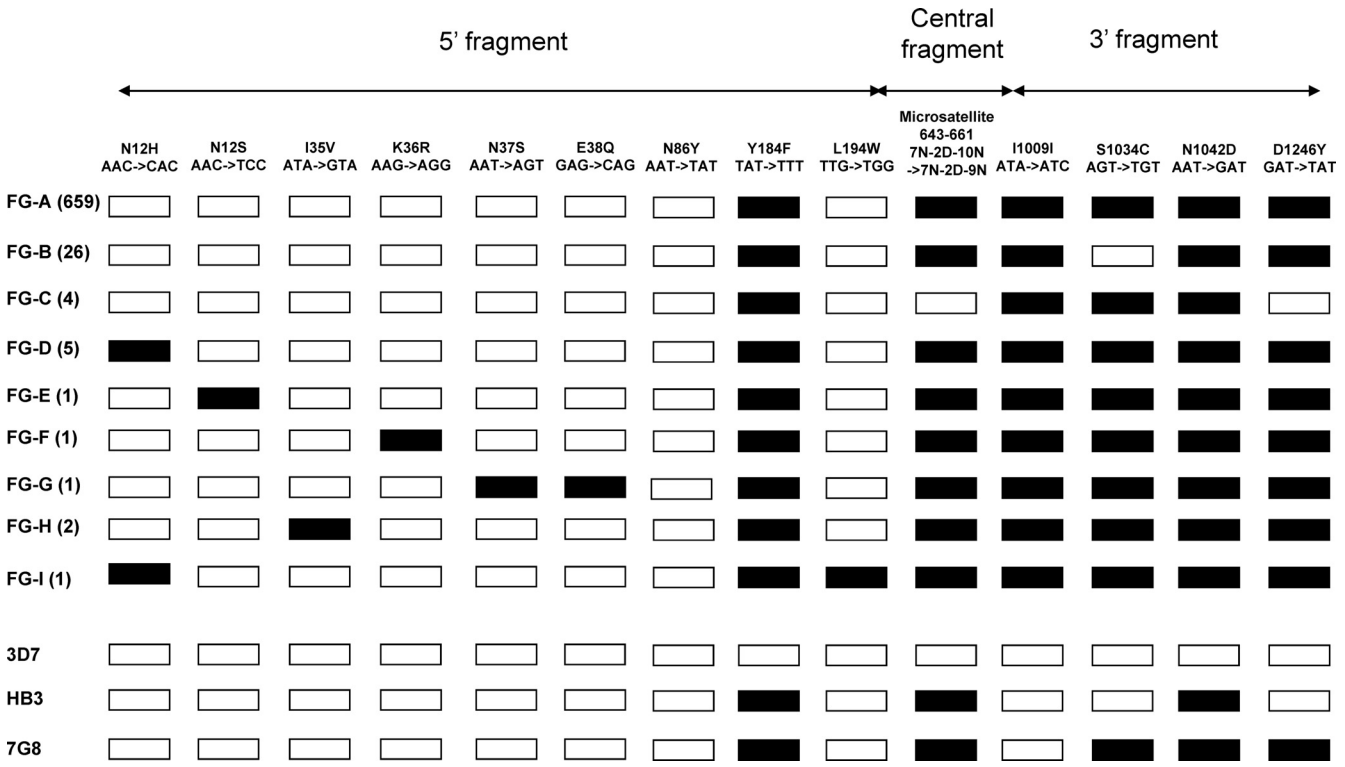

FIG 1 *Pfmdr1* sequence polymorphism of 700 isolates collected in French Guiana during the years 2000 to 2008. Polymorphic codons are indicated. Open symbols denote the wild-type (3D7-type) nucleotide sequence, and black symbols indicate the presence of the mutant sequence shown at the top. The reference alleles 3D7 (PFE1150w, http://www.plasmodb.org/plasmo/), HB3, and 7G8, (http://www.broad.mit.edu/annotation/genome/plasmodium_falciparum_spp/) are shown for comparison. The accession numbers of alleles FG-A to -I, respectively, are HQ215524 to HQ215532. The number of isolates observed for each allele is indicated in parentheses.

to FG-I, were observed (Fig. 1). Interestingly, all contained an as-yet-undescribed I1009I synonymous mutation. One highly dominant haplotype was observed (allele FG-A, 94% of the isolates) that differed from the 7G8-type haplotype at position 1009. A double mutant S1034C codon was observed in all samples, except 26 isolates harboring a wild-type codon 1034 (allele FG-B), which all originated from a single village (Papaïchton) and were collected in the years 2000 and 2001. The remaining seven alleles (FG-C to FG-I) were rare.

The *Pfmdr1* allelic type was unrelated to the IC_{50} of any of the antimalarials tested. As all isolates but one harbored the same mutant *Pfcrt* haplotype, the combined *Pfmdr1*/*Pfcrt* genotype was also unrelated to any of the IC_{50}s.

***Pfmdr1* copy number and its relationship with *in vitro* resistance.** The *Pfmdr1* copy number was determined in 547 samples using β-tubulin or *Pfmaebl* as a reference. Identical estimates were obtained for both. The *Pfmdr1* copy number varied from 1 to 12 copies, and 39% of the samples had ≥2 copies (Fig. 2). Amplification was observed for the FG-A, FG-D, FG-G, FG-H, and FG-I alleles.

Figure 3 shows the distribution of the IC_{50}s of the antimalarials by *Pfmdr1* gene copy number. Chloroquine and amodiaquine susceptibility was unrelated to the *Pfmdr1* copy number ($P = 0.16$ and 0.12, respectively, Kruskal-Wallis test), in contrast to the susceptibility to mefloquine, halofantrine, quinine, and lumefantrine ($P = 0.0013$, 0.026, 0.009, and 0.049, respectively). A low correlation with an artemether IC_{50} of >12 nM was also observed ($P = 0.031$). The presence of multiple (i.e., ≥2) *Pfmdr1* copies positively correlated with *in vitro* resistance to mefloquine and halofantrine and was moderately associated with *in vitro* resistance to quinine, lumefantrine, and artemether (Table 2).

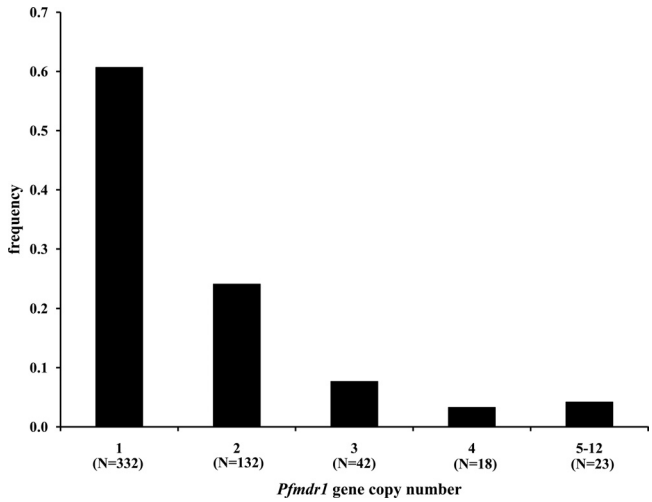

FIG 2 Frequency distribution of *Pfmdr1* gene copy numbers in 547 isolates collected in French Guiana during the years 2001 to 2008. The number of isolates per group is shown under each column. The 5-to-12 group includes 9, 8, 3, 1, 1, and 1 isolates with 5, 6, 7, 8, 9, and 12 copies, respectively.

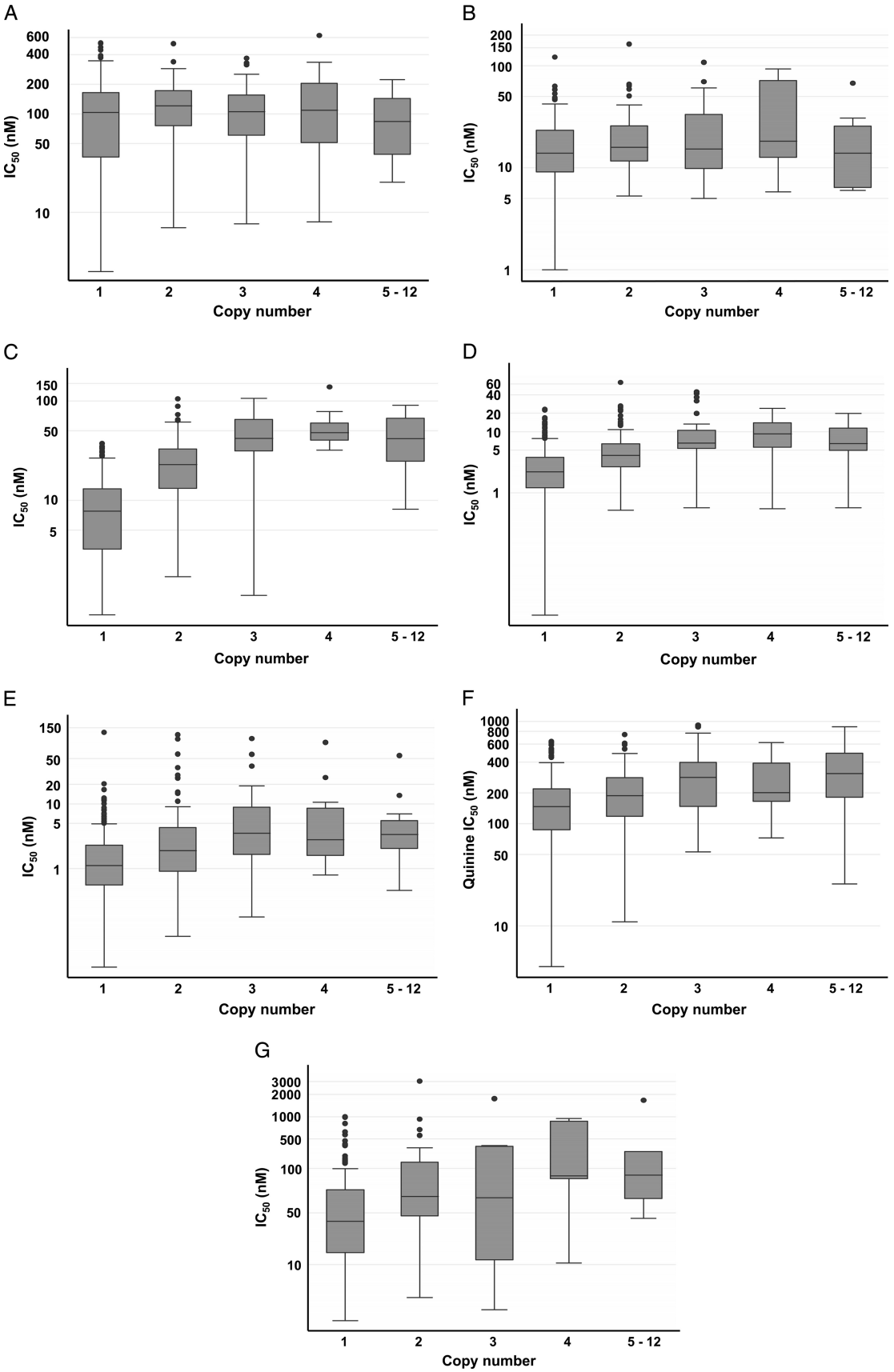

FIG 3 Distribution of IC_{50}s of chloroquine (A), amodiaquine (B), mefloquine (C), halofantrine (D), artemether (E), quinine (F), and lumefantrine (G) by *Pfmdr1* gene copy number in 547 isolates collected in French Guiana during the years 2001 to 2008. The number of isolates per group is as in Fig. 2. Shown is a boxplot representation of the IC_{50} of each antimalarial. The boundaries of the boxes indicate the 25th (Q1) and 75th (Q3) percentiles, the line in each box indicates the median, and the whiskers indicate the IC_{50} range corresponding to $Q_1 - 1.5 \times (Q_3 - Q_1)$ and $Q_3 + 1.5 \times (Q_3 - Q_1)$ for the lower whisker and upper whisker, respectively. The outlying dots show values exceeding this range.

TABLE 2 Spearman correlation coefficients of increased (≥2) *Pfmdr1* copy number and *in vitro* resistance

Molecule	Spearman correlation coefficient (r)	*P* value
Chloroquine	0.06	0.17
Quinine	0.30	<0.0001
Mefloquine	0.64	<0.0001
Halofantrine	0.47	<0.0001
Artemether	0.34	<0.0001
Atovaquone	−0.04	0.46
Amodiaquine	0.11	0.05
Lumefantrine	0.32	<0.0001

The association between *Pfmdr1* amplification and elevated IC_{50}s of mefloquine and halofantrine was also reflected by their parallel temporal fluctuation during the 2002 to 2008 period, corresponding to the progressive replacement of mefloquine for artemether-lumefantrine (Fig. 4). Interestingly, the proportion of isolates with multiple copies decreased in the years 2005 to 2008, when *in vitro* mefloquine resistance declined ($P < 0.01$).

***Pfmdr1* gene expression and its relationship with *in vitro* resistance.** The expression of *Pfmdr1* was determined in 35 isolates harboring an FG-A haplotype (16 isolates with 1 copy and 19 isolates with 2 to 4 *Pfmdr1* gene copies). The level of *pfmdr1* mRNA of each isolate was compared to its level in 3D7, which harbors a single *Pfmdr1* gene copy. The *pfmdr1* mRNA levels of the various isolates did not linearly correlate with their *Pfmdr1* gene copy numbers (Fig. 5A), with 11 isolates displaying discordant gene copy number to mRNA level ratios.

Pfmdr1 gene expression showed a very strong correlation with the IC_{50} of mefloquine (Fig. 5B) (Spearman rank correlation coefficient $r = 0.89$; $P < 0.0001$). With the exception of one isolate with 4 gene copies, there was a perfect match between a ≥2-fold increased expression level and a resistance threshold of mefloquine IC_{50}s above 30 nM *in vitro*. This correlation was stronger than the association with the *Pfmdr1* copy number ($r = 0.74$ in this panel of isolates; $P = 0.0001$). A similar trend was observed for the *in vitro* response to halofantrine ($r = 0.61$; $P = 0.0001$), with a higher IC_{50} being associated with increased expression levels, but this was less clear-cut than for mefloquine (Fig. 5C). *Pfmdr1* gene expression was unrelated to any of the other antimalarials tested (not shown).

DISCUSSION

The first remarkable finding of this work is the fixation of the SMNVT *Pfcrt* resistance haplotype and the quasifixation of a single, multiple mutant NYCDY *Pfmdr1* haplotype in this parasite population. The most frequent polymorphism was the variation of *Pfmdr1* gene copy number. *Pfmdr1* copy number was associated with *in vitro* susceptibility to mefloquine and halofantrine, although the *Pfmdr1* expression level was more informative than the copy number in the subset of isolates analyzed. *Pfmdr1* copy number showed a modest association with susceptibility to quinine, lumefantrine, and artemether but no association with *in vitro* chloroquine or amodiaquine susceptibility profiles in this population. The restricted polymorphism of both loci indicates that additional loci modulate *in vitro* susceptibility to these antimalarials, which presented a relatively large dynamic range. Thus, *Pfcrt* and *Pmdr1* gene/protein characteristics can no longer serve as molecular markers of *in vitro* susceptibility to chloroquine in this parasite population and moreover are of limited value for quinine, lumefantrine, and artemisinin derivatives. Novel markers need to be developed for improving monitoring of resistance to these antimalarials in this area.

In 1995, when chloroquine use for *P. falciparum* malaria was

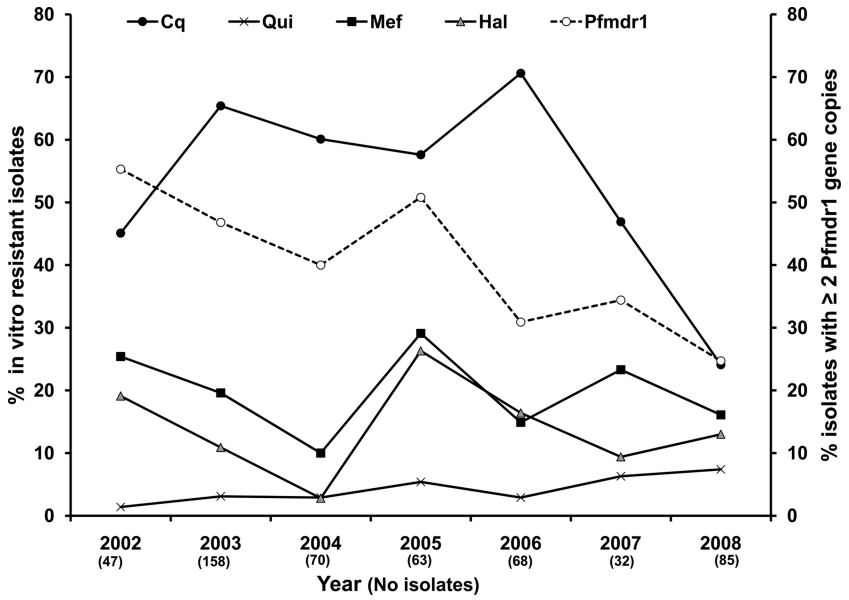

FIG 4 Temporal evolution of *in vitro* chloroquine (Cq), mefloquine (Mef), halofantrine (Hal), and quinine (Qui) resistance and frequency of *Pfmdr1* gene amplification. The median chloroquine IC_{50}s were 104, 118, 123, 123, 178, 70.5, and 80.1 nM in 2002, 2003, 2004, 2005, 2006, 2007, and 2008, respectively. The median quinine IC_{50}s were 150.4, 179, 202, 194, 107.5, 163.5, and 170.6 nM in 2002, 2003, 2004, 2005, 2006, 2007, and 2008, respectively. The median mefloquine IC_{50}s were 14.3, 13.9, 13.3, 14.4, 7.8, 4.3, and 2.5 nM in 2002, 2003, 2004, 2005, 2006, 2007, and 2008, respectively. The median halofantrine IC_{50}s were 3.6, 3.4, 3.3, 3.8, 2.8, 2, and 1.9 nM in 2002, 2003, 2004, 2005, 2006, 2007, and 2008, respectively. The number of isolates studied each year is indicated in parentheses.

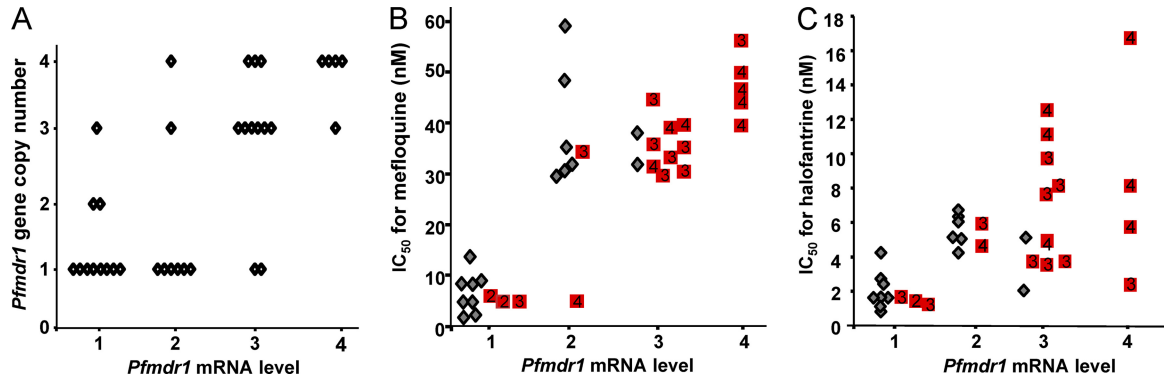

FIG 5 *Pfmdr1* gene expression levels (A) and their association with *in vitro* mefloquine (B) and halofantrine (C) resistance. Gray diamonds indicate isolates with one *Pfmdr1* gene copy. Red squares indicate isolates with multiple gene copies (the number of gene copies is shown in each square).

stopped in French Guiana, all isolates were resistant to chloroquine *in vitro*. *In vitro* resistance to chloroquine has declined since 2000 from 100% resistant isolates in 1999 to 24% resistant isolates in 2008 (28; this work). We show here that this temporal change cannot be attributed to the expansion of parasites carrying a wild-type *Pfcrt* allele, as previously observed in Africa (26, 32) and China (44). Indeed, as *Pfcrt* resistance alleles were fixed in this population at the time chloroquine was replaced, there were no wild-type alleles to compete against chloroquine resistance alleles and re-expand in the population. Furthermore, chloroquine has not been discontinued in the area, as it is still used to treat *P. vivax* malaria, which accounts for approximately half of the malaria cases in French Guiana (5). As a consequence, the bystander pressure on *P. falciparum* parasites may contribute to the maintenance of chloroquine-resistant *Pfcrt* in the population. Chloroquine sensitivity was unrelated to the *Pfmdr1* sequence type, copy number, or gene expression level. In particular, the 86Y mutation associated with the *in vitro* resistance of field isolates to chloroquine in some studies (9, 14) was absent from the panel of isolates studied here. The 184F, 1034C, 1042D, and 1246D mutations associated with *in vitro* resistance to chloroquine of field isolates in some studies (18, 24) but not in allelic exchange experiments (36, 40) were almost at fixation and again unrelated to the IC_{50} of chloroquine. Therefore, neither the *Pfcrt* nor the *Pfmdr1* locus accounts for the *in vitro* sensitivity of isolates collected in recent years across French Guiana. Importantly, such discordant genotypes were also observed in French Guiana isolates adapted to long-term *in vitro* culture (42). This indicates that additional loci govern the regained *in vitro* susceptibility to chloroquine in the parasite population from French Guiana. Although particularly striking in French Guiana due to the quite restricted polymorphism of the two loci reported to control resistance to chloroquine, the situation does not seem to be unique. Several recent studies reported a quasifixation of the *Pfcrt* resistance alleles in field populations from Southeast Asia, India, and East Africa and a lack of correlation with *in vitro* susceptibility to chloroquine and other antimalarials (3, 6, 7, 12).

In the panel of 700 isolates studied here, *in vitro* susceptibility to amodiaquine or chloroquine was not dependent on the interaction of the 7G8-type *Pfcrt* and 7G8-type *Pfmdr1* alleles shown to modulate resistance to the two molecules in the 7G8 × GB4 genetic cross (37). Irrespective of their *in vitro* susceptibility profile, a large number of isolates harbored a 7G8-type *Pfcrt* haplotype associated with a *Pfmdr1* allele identical to the 7G8 sequence, except for one synonymous mutation at position 1009. Although the possibility exists that the I1009I mutation has phenotypic repercussions, as observed for the human MDR protein (25), its fixation in French Guiana rules out an impact on the *in vitro* susceptibility profile. Our conclusion is that, in this parasite population, the interaction between the 7G8-type *Pfcrt* (SMVNT1 or SMVNT2), and 7G8-type *Pfmdr1* haplotypes does not account for the *in vitro* susceptibility patterns to chloroquine. Amodiaquine IC_{50}s varied over a 2-log concentration range, although 90% of the isolates were classified as presenting low or no resistance. This relative phenotypic homogeneity regarding amodiaquine is consistent with the restricted *Pfcrt* and *Pfmdr1* polymorphism, although Sá et al. (37) reported that the specific 7G8 *Pfcrt*/ 7G8 *Pfmdr1* haplotype combination and the 7G8 *Pfmdr1* haplotype (NFCDY) were associated with resistance to amodiaquine. Reduced amodiaquine sensitivity in French Guiana was rather associated with an increased *Pfmdr1* copy number, although the correlation was modest. It is worth noting that the 86Y *Pfmdr1* mutation, associated with amodiaquine resistance in other settings (11, 21, 33), was totally absent from the French Guiana isolates studied here and that the NFY *Pfmdr1* haplotype has been associated with an adequate treatment response in African settings where the *Pfcrt* allele was present at a very high frequency (11, 21). Unfortunately, most of the field studies conducted so far to investigate the relationship between amodiaquine susceptibility and *Pfmdr1* polymorphisms did not investigate the full gene sequence and ignored polymorphic positions such as 1034 and 1042, precluding definitive comparisons with the data presented here. Nevertheless, our data indicate that apart from the *Pfmdr1* gene copy number variation, no single mutation, haplotype, or combination showed a strong association with the observed variation of the amodiaquine IC_{50}.

In vitro susceptibility to mefloquine and, to a lesser extent, halofantrine was associated with an increased *Pfmdr1* copy number, which itself was modestly correlated with susceptibility to quinine, lumefantrine, or artemether. This is consistent with reports from Southeast Asia (7, 29) and with data from laboratory lines selected for resistance to mefloquine *in vitro* (10, 34). The temporal trends of *Pfmdr1* amplification paralleled *in vitro* susceptibility to mefloquine, and in particular, the mean copy number decreased in the years following the switch from mefloquine monotherapy to the artemether-lumefantrine combination.

These data are consistent with *Pfmdr1* gene amplification driven by mefloquine monotherapy, deamplification upon cessation of mefloquine use, and reversal to susceptibility to mefloquine as observed by others (8).

The pilot study performed on a set of isolates harboring the FG-A allele outlined a very strong correlation between the IC_{50} of mefloquine and the *Pfmdr1* transcript level. Elevated *Pfmdr1* mRNA levels were observed in all isolates displaying mefloquine IC_{50}s of >30 nM, commonly used as the cutoff for mefloquine resistance. Furthermore, the association with mefloquine resistance was stronger for an increased expression level than for an increased gene copy number. This suggests that increased *Pfmdr1* transcript abundance indeed contributes to *in vitro* resistance to mefloquine in these isolates, in contrast to a trend toward higher *Pfmdr1* expression levels in mefloquine-sensitive isolates in Thailand (6). We did not observe a linear relationship between transcript levels and gene copy numbers in the field isolates studied here, consistent with data from Thailand (6). This differs from observations of laboratory lines (10, 34). Further studies are needed to identify the factors governing *Pfmdr1* mRNA expression and/or stability in field isolates and how they impact on *Pfmdr1* protein levels and/or cellular localization and on profiles of resistance to mefloquine, halofantrine, and possibly lumefantrine.

The population characteristics of French Guiana isolates differ from those of isolates from neighboring areas, suggesting different gene flow dynamics. Although fixed as in other South American areas (reference 20 and references therein), the *Pfcrt* 76T mutation was harbored by two synonymous alleles, in contrast to neighboring Guyana or Brazil, where up to five distinct alleles were reported (4, 31, 43). The 7G8 type (also called SMVNT1) is rare in French Guiana (11.3%) and in Brazil (6.9%), where the synonymous SMVNT2 allele predominates (4, 31), in contrast to one of two studies from Guyana (4, 31). The other resistance haplotypes described in South and Central America (see reference 20) were totally absent from French Guiana. Regarding *Pfmdr1*, the frequency of the 7G8 *Pfmdr1* (FG-A) and FG-B alleles resembles the distribution described in Brazil (19, 31) and Guyana (31), where the rare FG-E, -F, -G, -H, and -I alleles have not been observed (4, 19, 31). In contrast, minor alleles harboring the wild type N1042 codon (4, 19) or the mutant 86Y codon (19, 31) observed in the neighboring countries were not observed in our sample of French Guiana isolates. Interestingly, the FG-B allele observed here with a 3.7% frequency had a 10-fold higher frequency in Venezuela (20) and allele FG-C has been repeatedly observed in Peru (2). *Pfmdr1* gene amplification was observed at a higher rate in French Guiana (a mean of 40%) than, e.g., the 12% rate observed in Venezuela during a period where mefloquine monotherapy was used (20), in stark contrast to the absence of *Pfmdr1* gene amplification in the Peruvian Amazon region, where artesunate-mefloquine has been the first line treatment since 2001 (2). As amodiaquine and the artemether-lumefrantrine combination have been reported to exert opposite selective pressures on the *Pfmdr1* gene (22), we are currently pursuing monitoring of the *Pfmdr1* gene copy number to investigate whether the *Pfmdr1* gene copy number continues to decline as the number of years of artemether-lumefantrine use increases.

In conclusion, withdrawal of chloroquine in French Guiana was not followed by expansion of wild-type parasites, as reported elsewhere (26, 32, 44), and none of the molecular features studied here accounted for the regained susceptibility to chloroquine seen during the last decade. The proposed interaction between the specific *Pfcrt* and *Pfmdr1* 7G8-type alleles for determining susceptibility to chloroquine and amodiaquine (37) did not apply to the parasite population of French Guiana. Cessation of mefloquine monotherapy was rapidly followed by reduction of the prevalence of isolates with multiple *Pfmdr1* copies without impacting on the haplotype frequency. The strong association of mefloquine susceptibility with the *Pfmdr1* expression level is interesting and certainly deserves further studies to evaluate its usefulness as a molecular marker of susceptibility to mefloquine and possibly other antimalarials, including halofantrine, quinine, lumefantrine, or artemisin derivatives. It is worth noting that about 90% of isolates seem to have good sensitivity to artemether and amodiaquine. The sensitivity profile of lumefantrine, with only 65.8% of isolates susceptible, is a concern and needs to be actively monitored, as the artemether-lumefantrine combination is being used as a first-line treatment for malaria in the area. Novel molecular markers of susceptibility to chloroquine and lumefrantrine are urgently needed in this area. These could be identified using whole-genome sequencing or expression profiling. The limited polymorphism of the French Guiana parasites will facilitate the identification of loci modulating susceptibility to these molecules and understanding of the molecular basis of parasite evolution following changes in treatment policy.

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