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MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. X. EVALUATION OF *PFMDR1* MUTATIONS AS GENETIC MARKERS FOR RESISTANCE TO AMINO ALCOHOLS AND ARTEMISININ DERIVATIVES

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Abstract. Mutations at five positions in the *Plasmodium falciparum* multidrug-resistance gene 1 (*pfmdr1*), initially thought to confer resistance to chloroquine, have been associated with *in vitro* resistance to amino alcohols and artemisinin derivatives in more recent studies. To assess the possible association between drug resistance phenotype and *pfmdr1* polymorphisms and establish the baseline *pfmdr1* sequence data in Yaoundé, Cameroon, the *in vitro* drug sensitivity pattern was determined for 64 clinical isolates by isotopic microtest. The *pfmdr1* alleles were determined by a polymerase chain reaction and automatic sequencing. A large majority of isolates carried Tyr-86 (88%) and Phe-184 (91%) alleles. With the exception of one isolate with mixed codon 1246, all isolates had wild-type alleles Ser-1034, Asn-1042, and Asp-1246. There was no statistical association between codons 86 and 184 and *in vitro* response to chloroquine, amino alcohols, and artemisinin derivatives ($P > 0.05$). Our data do not seem to support the hypothesis that mutations in codons 86 and 184 influence the *in vitro* response to these drugs. Further monitoring of both *in vitro* response and *pfmdr1* polymorphisms is required to evaluate the potential role played by other *pfmdr1* alleles in the determination of drug resistance in Africa.

INTRODUCTION

The global importance of drug-resistant *Plasmodium falciparum* malaria has stimulated the interest of investigators to understand the mechanisms involved in the acquisition of resistant phenotype. A number of candidate genes associated with resistance to chloroquine (*P. falciparum* multidrug-resistance gene 1 [*pfmdr1*], *cg2,* and *P. falciparum* chloroquine resistance transporter [*pfcrt*] gene), sulfonamides (gene encoding dihydropteroate synthase [*dhps*]), and antifolate drugs (gene encoding dihydrofolate reductase [*dhfr*]) have been identified and studied.[1,2] It is now established that mutations occurring at key positions in *pfcrt* and *dhfr* genes and, to a lesser extent, *dhps* gene, are highly correlated with the *in vitro* response of *P. falciparum* to the corresponding drugs.[3–6]

The genetic mechanism of resistance to amino alcohols and artemisinin derivatives has not been totally elucidated. The results of several studies, mostly involving laboratory-adapted *P. falciparum* strains that were subjected to *in vitro* drug pressure, have suggested that amplification of *pfmdr1* may be associated with resistance to chloroquine and/or amino alcohol drugs.[7–12] In some studies, however, the copy number of the *pfmdr1* gene and *in vitro* drug resistance were not associated.[13,14] In addition to gene amplification, the *pfmdr1* gene is known to undergo mutations leading to the substitution of amino acids at five distinct positions: 86, 184, 1034, 1042, and 1246. In earlier studies, the Asn-to-Tyr substitution at position 86 was hypothesized to be the major change that accounts for chloroquine-resistant phenotype in *P. falciparum* strains originating from Asia and Africa, while the triple substitution involving 1034, 1042, and 1246 was suggested to be a potential chloroquine-resistant marker for *P. falciparum* strains in South America.[15,16] However, more recent data have suggested that specific mutations in the *pfmdr1* gene, which were initially thought to be associated with chloroquine resistance, may confer cross-resistance to quinine, mefloquine, halofantrine, and artemisinin derivatives.[17–19] In the face of contradictory data from different studies, we conducted the present study with the aim to assess the possible correlation between *in vitro* drug sensitivity pattern of clinical isolates and *pfmdr1* profile and establish the baseline *pfmdr1* sequence data in Cameroon.

MATERIALS AND METHODS

Patients. The study was part of randomized clinical trials conducted at the Nlongkak Catholic missionary dispensary in Yaoundé, Cameroon between 1997 and 2000.[20,21] Patients were enrolled in the study if the following criteria were met: age ≥ five years old, fever at consultation (or history of fever within the past 24 hours), monoinfection with *P. falciparum,* parasite density > 5,000 asexual parasites/μL of blood to allow the performance of *in vitro* assays, easy access to the dispensary for daily monitoring, absence of signs and symptoms of severe and complicated malaria, and no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result.[22] The patients were treated with standard oral doses of chloroquine, amodiaquine, or sulfadoxine-pyrimethamine under supervision. Informed consent was obtained from either the patients or a guardian accompanying the sick children. Venous blood samples (5–10 ml of whole blood) were collected in EDTA-coated Vacutainer tubes (Terumo Europe N. V., Leuven, Belgium) before treatment. Giemsa-stained thin blood film was examined under the microscope to identify the malaria species and determine the parasite density. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

In vitro assay. The test compounds were obtained from the following sources: chloroquine phosphate (Sigma Chemical Co., St. Louis, MO), quinine hydrochloride (Sigma Chemical Co.), mefloquine hydrochloride (Hoffman-La Roche, Basel, Switzerland), halofantrine hydrochloride (Smith Kline Beecham, Hertfordshire, United Kingdom), artesunate (Sanofi Winthrop, Gentilly, France), artemether (Aventis, Antony, France), and dihydroartemisinin (Sapec Fine Chemicals, Lugano, Switzerland). The stock solution of chloroquine was prepared in sterile distilled water. Stock solutions of quinine, mefloquine, halofantrine, and dihydroartemisinin were prepared in methanol. The stock solution of artesunate was pre-

pared in ethanol. Further dilutions and final concentrations of the test compounds were described in our previous studies.[23]

Infected erythrocytes were washed three times in RPMI 1640 medium and suspended in RPMI 1640 plus 10% human serum obtained from European blood donors without a history of malaria, 25 mM HEPES, and 25 mM $NaHCO_3$ at a hematocrit of 1.5% and an initial parasitemia ranging between 0.2% and 1.0%. If the blood sample had a parasitemia > 1.0%, fresh, uninfected type A^+ erythrocytes were added to adjust the parasitemia to 0.6%. The *in vitro* drug sensitivity assay was performed using tritium-labeled hypoxanthine as an indicator of parasite growth, as described in our previous study.[23] Briefly, the suspension (200 μL) was distributed in the 96-well tissue culture plates and incubated at 37°C in 5% CO_2 for 42 hours. The plates were frozen to terminate the *in vitro* assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The incorporation of ^{3}H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1409; Pharmacia, Uppsala, Sweden). The 50% inhibitory concentration (IC_{50}), defined as the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthine measured in the drug-free control wells, was determined by non-linear regression analysis of logarithm of concentrations plotted against growth inhibition. A sigmoid curve was fitted to the plot using the Prism™ software (GraphPad Software, Inc., San Diego, CA). Our correlational study on the *in vivo* and *in vitro* response to chloroquine has shown that the threshold value for *in vitro* resistance to chloroquine is approximately ≥ 100 nM.[24] The threshold IC_{50} values for *in vitro* resistance to quinine, mefloquine, and halofantrine were arbitrarily fixed at ≥ 800 nM, ≥30 nM, ≥ 6 nM, respectively.[23] The threshold for artemisinin derivatives is still undetermined.

Polymerase chain reaction and sequencing. An aliquot of 1.5 ml of red blood cell pellet was used to extract parasite DNA, as described in our previous study.[13,16] Two primer pairs were designed from the complete sequence to amplify *pfmdr1* fragments carrying the five key codons.[7] A 590-basepair fragment was amplified with primer pairs 5′-AGAGAAAAAAGATGGTAACCTCAG-3′ (forward primer) and 5′-ACCACAAACATAAATTAACGG-3′ (reverse primer) to determine the sequences of codons 86 and 184. The second fragment (968 base pairs) was amplified with primer pairs 5′-GCGGAGTTTTTGCATTTAGTTCAGATGATG-3′ (forward primer) and 5′-AGCAGCAAACTTACTAACACGTTTAACATC-3′ (reverse primer) to determine the sequences of codons 1034, 1042, and 1246.

The reaction mixture consisted of approximately 200 ng of genomic DNA, 15 picomole of forward and reverse primers, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP), and one unit of *Taq* DNA polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 μL. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for 2 min for the first cycle and 30 sec in subsequent cycles, 50°C for 1 min for the first cycle and 30 sec in subsequent cycles, and 72°C for 1 min for all cycles, for a total of 30 cycles, followed by a 15 min extension step at 72°C. The amplified DNA fragments were resolved by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination. The amplified products were marked with fluorescent nucleotides by re-amplifying the fragments with a single primer (5′-TTTGTATGTGCTGTATTATCAGG-3′ for the 590-basepair fragment and 5‘-GAAAAAGCTATTGATTATAAAAATAAAGG-3′ for the 968-basepair fragment), and sequenced by an automatic DNA sequencer (ABI Systems, Perkin Elmer, Les Ulis, France). The results were interpreted as follows: codon 86 (wild-type Asn; mutant Tyr), 184 (wild-type Tyr, mutant Phe), 1034 (wild-type Ser, mutant Cys), 1042 (wild-type Asn, mutant Asp), and 1246 (wild-type Asp, mutant Tyr).[15] The *pfmdr1* sequence of the chloroquine-sensitive 3D7 reference clone was used to define wild-type codons.

Statistical analysis. The IC_{50}s were expressed as the geometric mean and range. Correlation coefficients (r) between the IC_{50}s of different drugs were calculated by Spearman rank correlation. Quantitative variables were compared by the unpaired *t*-test. Proportions were compared by Fisher's exact test. The level of significance was set at 0.05.

RESULTS

A total of 64 Cameroonian isolates were used to characterize the *in vitro* drug sensitivity pattern and analyze the *pfmdr1* polymorphisms. The complete *in vitro* drug sensitivity pattern for chloroquine, quinine, mefloquine, halofantrine, and artemisinin derivatives was characterized for 31 clinical isolates. For 33 additional isolates, the *in vitro* response data were available for chloroquine and quinine. Of 64 isolates, 26 (41%) were chloroquine-sensitive (geometric mean IC_{50} = 33.4 nM, range = 14.6–61.1 nM), and 38 (59%) were chloroquine-resistant (geometric mean = 246 nM, range = 111–586 nM). All isolates were sensitive to quinine (n = 64; geometric IC_{50} = 166 nM, range = 31.6–591 nM), mefloquine (n = 31; geometric mean IC_{50} = 9.81 nM, range = 2.90–29.8 nM), and halofantrine (n = 31; geometric mean IC_{50} = 1.40 nM, range = 0.560–5.35 nM). The IC_{50} values for artemether (n = 8), artesunate (n = 14), and dihydroartemisinin (n = 9) ranged from 0.290 to 6.60 nM, with geometric means of 1.81 nM, 0.940 nM, and 1.07 nM, respectively. The IC_{50} values for individual isolates (n = 31) tested against the complete panel of antimalarial drugs are shown in Table 1. The *in vitro* responses between chloroquine and quinine (r = 0.407), quinine and mefloquine (r = 0.405), mefloquine and halofantrine (r = 0.704), mefloquine and artemisinin derivatives (r = 0.368), and halofantrine and artemisinin derivatives (r = 0.504) were significantly correlated ($P < 0.05$).

The majority of isolates displayed mutant codons Tyr-86 and Phe-184. Fifty-six of 64 isolates (88%) carried the mutant codon Tyr-86, while seven (11%) carried the wild-type codon Asn-86 and one had mixed codons. Fifty-eight of 64 had the mutant codon Phe-184, five had the wild-type codon Tyr-184, and one (isolate no. 70/00) had mixed codons. The geometric mean IC_{50} values for chloroquine were 83.4 nM (range = 14.6–279 nM) in parasites carrying Asn-86 allele (n = 7) and 115.6 nM (20.8–586 nM) in those carrying Tyr-86 allele (n = 56; one mixed isolate was excluded from this analysis) ($P > 0.05$). Taken individually, Tyr-86 and Phe-184 were not associated with *in vitro* resistance to chloroquine ($P > 0.05$). For quinine, the geometric mean IC_{50} values were 107 nM (range = 31.6–369 nM) in parasites with Asn-86 allele (n = 7) and

TABLE 1

In vitro drug sensitivity profile and *pfmdr1* polymorphisms in Cameroonian isolates

Isolate	IC_{50} (nM)*					Amino acid residues†				
	CQ	QU	MQ	HF	QHS	86	184	1034	1042	1246
108	44.0	163	12.0	2.80	3.20‡	Tyr	Phe	Ser	Asn	Asp
111	32.0	55.0	6.60	1.70	3.50‡	Tyr	Phe	Ser	Asn	Asp
116	60.0	90.0	4.20	0.70	1.20‡	Tyr	Phe	Ser	Asn	Asp
117	35.0	87.0	5.60	1.20	1.50‡	Tyr	Phe	Ser	Asn	Asp
1001	31.5	43.8	4.32	1.13	0.65§	Tyr	Phe	Ser	Asn	Asp
1002	23.8	52.4	20.2	2.79	0.82§	Asn	Phe	Ser	Asn	Asp
1008	30.3	31.6	20.7	1.84	1.12§	Asn	Tyr	Ser	Asn	Asp
1010	39.8	505	25.9	3.55	3.85§	Tyr	Phe	Ser	Asn	Asp
1012	39.1	207	7.07	1.07	1.02§	Tyr	Phe	Ser	Asn	Asp
1016	20.8	227	6.67	0.70	0.65§	Tyr	Phe	Ser	Asn	Asp
1063	37.0	183	29.8	5.35	2.29¶	Tyr	Phe	Ser	Asn	Asp
1074	25.0	282	17.9	2.72	0.77¶	Tyr	Phe	Ser	Asn	Asp
1075	43.8	283	13.2	2.88	0.29¶	Tyr	Phe	Ser	Asn	Asp
3006	52.7	353	21.8	2.73	2.16§	Tyr	Phe	Ser	Asn	Asp
71/97	215	170	9.47	0.68	1.18§	Tyr	Phe	Ser	Asn	Asp
77/97	167	110	9.16	0.63	0.50§	Tyr	Phe	Ser	Asn	Asp
110	574	342	16.0	3.00	6.60‡	Tyr	Phe	Ser	Asn	Asp
113	586	274	7.50	1.50	2.00‡	Tyr	Phe	Ser	Asn	Asp
115	367	115	2.90	0.70	0.40‡	Tyr	Phe	Ser	Asn	Asp
118	279	94.0	7.10	1.30	1.10‡	Asn	Tyr	Ser	Asn	Asp
1006	321	591	13.9	0.88	0.76§	Tyr	Phe	Ser	Asn	Asp
1019	287	300	16.7	1.15	0.53§	Tyr	Phe	Ser	Asn	Asp
1020	343	139	10.5	1.26	1.34§	Tyr	Phe	Ser	Asn	Asp
1023	343	145	3.18	0.66	0.95§	Tyr	Phe	Ser	Asn	Asp
1025	241	216	4.63	0.72	0.42§	Tyr	Phe	Ser	Asn	Asp
1061	271	187	16.8	0.78	1.53¶	Tyr	Phe	Ser	Asn	Asp
1062	164	325	26.1	2.77	2.04¶	Tyr	Phe	Ser	Asn	Asp
1065	253	203	9.71	2.30	1.03¶	Tyr	Phe	Ser	Asn	Asp
1076	228	333	8.30	1.50	0.97¶	Tyr	Phe	Ser	Asn	Asp
1085	308	316	6.39	1.13	1.33¶	Tyr	Phe	Ser	Asn	Asp
3049	187	138	5.02	0.56	0.86¶	Tyr	Phe	Ser	Asn	Asp

* 50% inhibitory concentration (IC_{50}) for chloroquine (CQ), quinine (QU), mefloquine (MQ), halofantrine (HF), and artemisinin derivatives (QHS). One of the following artemisinin derivatives was tested for each isolate: ‡artemether, §artesunate, ¶dihydroartemisinin.

†Codon 86, wild-type Asn (AAT), mutant tyr (TAT); codon 184, wild-type Tyr (TAT), mutant Phe (TTT); codon 1034, wild-type Ser (AGT), mutant Cys (TGT); codon 1042, wild-type Asn (AAT), mutant Asp (GAT); codon 1246, wild-type Asp (GAT), mutant Tyr (TAT).

174 nM (42.4–591 nM) in those with Tyr-86 (n = 56) ($P > 0.05$). Although the number of isolates with Asn-86 was small (n = 3) when compared with those with Tyr-86 (n = 28), there was no significant difference ($P > 0.05$) between the geometric mean IC_{50} values for mefloquine (14.4 nM, range = 7.1–20.7 nM versus 9.41 nM, range = 2.90–29.8 nM) and halofantrine (1.88 nM, range = 1.30–2.79 nM versus 1.36 nM, range = 0.560–5.35 nM).

The distribution of chloroquine and quinine IC_{50} values in relation to the allelic combinations at positions 86 and 184 is shown in Figure 1. The geometric mean IC_{50}s for chloroquine were 73.0 nM (Asn-86/Tyr-184, n = 4), 99.5 nM (Asn-86/Phe-184, n = 3), 31.1 nM (Tyr-86/Tyr-184, n = 1), and 115 nM (Tyr-86/Phe-184, n = 54; two isolates with mixed alleles were excluded from analysis). The geometric mean IC_{50}s for quinine were 82.3 nM (Asn-86/Tyr-184), 153 nM (Asn-86/Phe-184), 130 nM (Tyr-86/Tyr-184), and 171 nM (Tyr-86/Phe-184). There was no statistical association between these two codons and *in vitro* response to chloroquine and quinine ($P > 0.05$). All 64 isolates had the wild-type codons Ser-1034, Asn-1042, and Asp-1246, except for one isolate (no. 70/00), which had mixed Asp and Tyr-1246 codons and relatively high IC_{50} values for chloroquine (561 nM) and quinine (568 nM). However, another isolate with the highest IC_{50} value for quinine in the present study (591 nM) displayed wild-type codons at these three positions.

DISCUSSION

The spread of resistance to chloroquine and sulfadoxine-pyrimethamine in some endemic areas has led to an increased reliance upon drugs belonging to amino alcohols and sesquit-

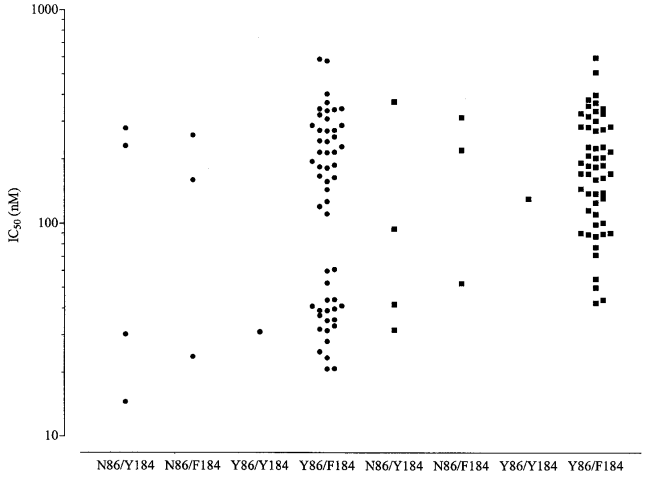

FIGURE 1. *In vitro* response to chloroquine **(black circles)** and quinine **(black squares)** in relation to *pfmdr1* allelic combinations in codon 86 (N = Asn; Y = Tyr) and codon 184 (Y = Tyr; F = Phe). IC_{50} = 50% inhibitory concentration.

erpene lactone to treat *P. falciparum* infections. Despite the restricted use of these recently commercialized antimalarial drugs in Cameroon, it is well known that quinine is being overprescribed and underdosed for the unofficial first-line treatment of uncomplicated malaria in most of sub-Saharan Africa.[25] Even though quinine has a short elimination half-life, such a continuous, high-level drug pressure on the parasites may lead to the selection of quinine-resistant strains, which in turn may lead to the selection of parasites that are cross-resistant to other amino alcohol drugs and artemisinin derivatives.[26]

Our previous *in vitro* studies on African isolates have suggested that cross-resistance may occur between chloroquine and quinine and between amino alcohols and artemisinin derivatives.[27–30] A similar trend of cross-resistance patterns was observed in the present study. *In vitro* cross-resistance between these drugs has also been confirmed in other independent studies in Senegal and Thailand.[26,31] In the present study, the resistant phenotype was not correlated with the *pfmdr1* polymorphisms. In our earlier studies based on the determination of codon 86 of the *pfmdr1* gene, we have already observed the predominance of Tyr-86 allele (110 of 129 isolates, 85%) in the clinical isolates obtained in Yaoundé in 1994–1996.[32,33] This predominance of Tyr-86 (56 of 64 isolates, 88%) was reconfirmed in the present study for Cameroonian isolates obtained more recently (1997–2000) at the same study site. The presence of Tyr-86 allele in Cameroonian isolates was not correlated with chloroquine resistance *in vitro* or *in vivo*.[32,33]

Although it was suggested that a close link exists between mutations at codons 184 and 1042 and *in vitro* response to amino alcohols and artemisinin derivatives, the same study also suggested that the presence of Tyr-86 allele, and not Phe-184 and/or Asp-1042, was associated with increased sensitivity to these drugs in other reference clones.[17] In another study based on plasmid construction and transfection, the triple 7G8-like *pfmdr1* mutations, Cys-1034, Asp-1042, and Tyr-1246, were suggested to determine resistance to quinine, increased sensitivity to amino alcohols and artemisinin derivatives, and no effect on chloroquine sensitivity.[19] In studies involving field isolates, Tyr-86 was associated with increased sensitivity to mefloquine, halofantrine, and artemisinin derivatives and resistance to chloroquine and quinine in Gambian isolates, but the same mutation was associated with increased sensitivity to mefloquine alone in Thailand.[18,26] Thus, it seems that, at present, there is still no single *pfmdr1* allelic profile that clearly distinguishes between sensitive and resistant parasites.

In Cameroon, the parasites have been subjected to intense pressure from underdosed quinine treatment but have been generally spared from pressure due to synthetic amino alcohols and artemisinin derivatives. The Tyr-86 mutant allele was present in a large majority of isolates (166 of 193 isolates [86%], including those analyzed in our previous study).[33] This disproportionate presence of Tyr-86 may partly explain the lack of correlation between the *pfmdr1* polymorphisms and drug resistance. If Tyr-86 were a marker for increased sensitivity to synthetic amino alcohols and artemisinin derivatives, as suggested by Duraisingh and others,[18] it may explain the absence of Cameroonian isolates that are resistant *in vitro* to these drugs, but it does not explain the concomitant increased resistance to chloroquine and quinine observed in the Gambian isolates, in particular the presence of 22 (39%) of 56 Cameroonian isolates carrying Tyr-86 that were sensitive *in vitro* to chloroquine. This discordance between the Gambian and Cameroonian isolates may be due to the difference in the epidemiology of drug-resistant *P. falciparum* or difference in the *in vitro* assay methodology. It was reported from The Gambia that the proportions of isolates that are resistant *in vitro* to chloroquine, quinine, mefloquine, and halofantrine are 7.4%, 0%, 14.8%, and 32.1%, respectively.[18] In contrast, the corresponding proportions in Cameroonian isolates in the present study were 59%, 0%, 0%, and 0%. Another potential source of discordance is multiclonality of field isolates which may affect IC_{50} values. At our study site, 57% of clinical isolates are multiclonal.[34] However, the extent to which multiple parasite populations in a given isolate influence *in vitro* response to amino alcohols and artemisinin derivatives is probably limited in Yaoundé, where regular *in vitro* monitoring of drug response has shown their high activity.[23,29,30]

In conclusion, it does not seem to be clear at present whether there is a distinct set of *pfmdr1* mutations that are strongly associated with resistance to amino alcohols and artemisinin derivatives in field isolates in Africa. The main reason may be related to the high clinical efficacy of these drugs in Africa, in contrast to Southeast Asia where clinical failure is frequently observed when monotherapy with quinine, mefloquine, or halofantrine is administered.[35] Further studies are needed to assess the utility of *pfmdr1* allelic profiles in describing and following the evolution of the epidemiology of drug-resistant *P. falciparum* in Africa.

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