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Resistance to Antimalarials in Southeast Asia and Genetic Polymorphisms in *pfmdr1*

Amy L. Pickard,[1] Chansuda Wongsrichanalai,[2] Anne Purfield,[3] Deborah Kamwendo,[4] Kathryn Emery,[4] Christy Zalewski,[4] Fumihiko Kawamoto,[5] R. Scott Miller,[2] and Steven R. Meshnick[1,3]*

Department of Epidemiology, University of North Carolina School of Public Health,[1] Department of Microbiology and Immunology, University of North Carolina School of Medicine,[3] Chapel Hill, North Carolina; Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[2]; Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan[4]; and Department of International Health, Nagoya University School of Medicine, Nagoya, Japan[5]

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Resistance to antimalarial drugs is a public health problem worldwide. Molecular markers for drug-resistant malaria, such as *pfcrt* and *pfmdr1* polymorphisms, could serve as useful surveillance tools. To evaluate this possibility, sequence polymorphisms in *pfcrt* (position 76) and *pfmdr1* (positions 86, 184, 1034, 1042, and 1246) and in vitro drug sensitivities were measured for 65 *Plasmodium falciparum* isolates from Thailand, Myanmar, Vietnam, and Bangladesh. The *pfcrt* Thr76 polymorphism was present in 97% of samples, consistent with observations that chloroquine resistance is well established in this region. Polymorphisms in *pfmdr1* clustered into four specific patterns: the wild type (category I), a Tyr86 polymorphism only (category II), a Phe184 polymorphism only (category III), and Phe184 in combination with Cys1034 and/or Asp1042 (category IV). Isolates in categories I and III were more sensitive to chloroquine and more resistant to mefloquine, artesunate, and artemisinin than isolates in categories II and IV ($P \leq 0.01$). Mefloquine resistance was significantly more common in category I and III isolates than in category II and IV isolates, with a prevalence ratio of 14.95 (95% confidence interval, 3.88 to 57.56). These categories identified mefloquine resistance with a sensitivity and a specificity of 94 and 91%, respectively. The *pfmdr1* gene copy number was measured by real-time PCR as a ratio of the amount of *pfmdr1* DNA to the amount of lactate dehydrogenase (*ldh*) DNA. Eight samples had *pfmdr1* DNA/*ldh* DNA ratios ≥3. The isolates in all 8 samples fell into categories I and III and were significantly more resistant to mefloquine, quinine, artemisinin, and artesunate and more sensitive to chloroquine than the isolates in the 57 samples with <3 copies of the gene ($P \leq 0.001$). Thus, measurement of *pfmdr1* mutations and gene copy number may be useful for surveillance of mefloquine-resistant malaria in Southeast Asia.

Resistance to antimalarials is spreading throughout the world and is impeding efforts to control malaria, which causes 700,000 to 2.7 million deaths every year (3). Drug-resistant *Plasmodium falciparum* is a particularly serious problem in Southeast Asia, where strains are commonly resistant to chloroquine, antifolates, quinine, and mefloquine (20).

Surveillance for drug-resistant malaria is based at present on strict in vivo criteria for treatment failure and on measurement of the activities of antimalarial drugs against cultured parasites in vitro. Surveillance could be carried out more effectively by using molecular markers, once such markers have been validated. At present, there is good evidence that mutations in two genes (*dhps* and *dhfr*) correlate well with in vitro and in vivo resistance to sulfadoxine-pyrimethamine and that mutations in the gene *pfcrt* (especially at position 76) correlate well with in vitro and in vivo resistance to chloroquine (for a review, see reference 20). There is also evidence that mutations in *pfmdr1* are associated with drug resistance, but the evidence is less conclusive. Many of the studies of this relationship were performed with laboratory strains of *P. falciparum* or with other eukaryotic models, so the results of these experiments might not be generalizable to naturally occurring *P. falciparum* isolates. Also, some field studies have suggested that mutations or amplification of this gene is associated with chloroquine resistance, while others have suggested that mutations or gene amplification is associated with increased chloroquine sensitivity (1, 6). On the other hand, there is more general agreement that mutations in *pfmdr1* are associated with altered sensitivity to mefloquine and artemisinin derivatives in vitro, although the role of gene amplification is not clear (1, 4, 13, 14). However, most previous studies were small ($n < 20$ isolates) or limited to single geographical areas where specific polymorphisms may have been absent. Accordingly, we chose to determine the association of *pfcrt* and *pfmdr1* mutations and *pfmdr1* gene amplification with in vitro sensitivity to antimalarial drugs for a group of isolates from several areas in Southeast Asia where malaria is endemic.

* Corresponding author. Mailing address: Department of Epidemiology, University of North Carolina School of Public Health, Chapel Hill, NC 27599-7435. Phone: (919) 966-7414. Fax: (919) 966-2089. E-mail: meshnick@unc.edu.

MATERIALS AND METHODS

Patient isolates. The Armed Forces Institute of Medical Sciences in Bangkok, Thailand, has been carrying out active surveillance for resistance to antimalarials in a variety of provinces of Thailand as well as in neighboring countries since

TABLE 1. Primers and probes used for real-time PCR

Primer or probe	Sequence
Primers	
LDH-F	ACG ATT TGG CTG GAG CAG AT
LDH-R	TCT CTA TTC CAT TCT TTG TCA CTC TTT C
PF-R	TCT CCT TCG GTT GGA TCA TAA AG
PF-F	TTA AGT TTT ACT CTA AAA GAA GGG AAA ACA TAT
Probes	
PF-FAM	FAM-CAT TTG TGG GAG AAT CAG GTT GTG GGA AAT-TAMRA
LDH-FAM	FAM-AGT AAT AGT AAC AGC TGG ATT TAC CAA GGC CCC A-TAMRA

1992. Over 400 patient isolates have been grown in culture, tested for their drug sensitivities in vitro, and archived by storage in liquid nitrogen. This study received ethical clearance from the ethical review boards of the University of Michigan, the Walter Reed Army Institute of Research, and the Thai Ministry of Public Health.

Field isolates were cryopreserved in a dry liquid nitrogen shipping container and transported to the Armed Forces Institute of Medical Sciences, where they were thawed 1 to 3 months later. In vitro drug sensitivity assays were performed by a radioisotope microdilution technique slightly modified from that described previously (16). Briefly, the suspension of the malaria parasite culture (0.5% parasitemia and 1.5% hematocrit, 200 μl/well) was dispensed into the wells of a standard microtiter plate (a 96-well flat-bottom plate) predosed with duplicate serial dilutions of the antimalarial drugs. The microtiter plate was placed in a gas-tight box to maintain an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 and then in an incubator (37°C). At the end of the 24th hour, the plate was temporarily removed for pulsing with [^{3}H]hypoxanthine. The contents of each plate were harvested after 42 to 44 h of incubation. The 50% inhibitory concentration (IC_{50}), IC_{90}, etc., were estimated by nonlinear regression analysis of the incorporated radioactivity data. The antimalarial drugs and the ranges of concentrations used were as follows: chloroquine diphosphate, 7.144 to 457.200 ng/ml; quinine citrate, 21.438 to 1,372.000 ng/ml; mefloquine hydrochloride, 1.948 to 124.700 ng/ml; artesunate, 0.265 to 16.93 ng/ml.

For the present study, 73 stabilates were selected to maximize the statistical power to detect differences in genotypes between sensitive and resistant isolates by achieving an even distribution of mefloquine-resistant and -sensitive isolates. Of these 73 stabilates, cultures were successfully obtained from 63. In addition, two laboratory strains originally obtained from Thai isolates, isolates W2 and PH6, were used. The isolates in these cultures were then retested for their sensitivities to chloroquine, mefloquine, quinine, artesunate, and artemisinin in vitro. There were good correlations between sensitivities before and after cryopreservation for mefloquine, quinine artesunate, and artemisinin, while the correlations were less good for chloroquine. Strains with the following assay results both in the initial test and in the repeat test were considered resistant to antimalarials: mefloquine, $IC_{50} > 20$ ng/ml or $IC_{90} > 80$ ng/ml; quinine, $IC_{50} > 500$ or $IC_{90} > 1{,}000$ ng/ml; and chloroquine, $IC_{50} > 80$.

Genetic polymorphisms. DNA was obtained from each culture by using QIAamp DNA mini kits (Qiagen, Valencia, Calif.). Samples were genotyped for *msp1*, *msp2*, and *glurp* by the methods described by Snounou et al. (15). PCR amplification and direct sequencing were used to determine the sequences at a single *pfcrt* polymorphic site (position 76) and five *pfmdr1* polymorphic sites (positions 86, 184, 1034, 1042, and 1246), as described previously (8). The sequences at all loci could be determined for 61 isolates plus laboratory strains W2 and PH6. For one isolate, the sequence at position 184 could not be obtained, and for a second isolate, the sequences at positions 1034 and 1042 could not be obtained.

Real-time PCR. Real-time PCR was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, Calif.). This system uses a fluorescence-based PCR method that measures the rate of gene amplification; as more amplicons are made, the DNA-bound reporter dye and quencher are separated, generating fluorescence. The rate at which fluorescence increases is related to the number of gene copies initially present (2). Primers and fluorescence-labeled probes were designed by using Primer Express software (version 2.0; Applied Biosystems, Foster City, Calif.) to amplify the *P. falciparum* multidrug resistance (*pfmdr1*) and lactate dehydrogenase (*ldh*) genes (Table 1).

Probes were synthesized by Applied Biosystems and labeled in the standard manner with a reporter dye (6-carboxyfluorescein [FAM]) at the 5′ end and a quencher dye (6-carboxytetramethylrhodamine [TAMRA]) at the 3′ end. The primers were obtained from Qiagen. The primer and probe concentrations were optimized according to the protocol recommended for the TaqMan Universal PCR Master Mix (Applied Biosystems).

The reagents used for each unknown sample or standard were 1× TaqMan Universal Master Mix (2×; Applied Biosystems); 300 nM forward primer; 300 nM reverse primer; 250 nM TaqMan probe; and distilled, sterile water (Sigma, St. Louis, Mo.). The total reaction volume was 50 μl. The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold was set to optimize the threshold cycle (C_T) value for the first standardization reaction, and all subsequent C_T values were obtained by using the same threshold value.

In order to determine the reproducibility of the real-time PCR, 160 amplifications of each gene were performed with *P. falciparum* strain 3D7 DNA, which was extracted and purified from culture in phosphate-buffered saline with Qiagen DNA Mini kits, as stated in the spin protocol for blood and body fluids. The *P. falciparum* gene copy number was calculated to be 8,000 copies/ng of DNA on the basis of a genome size of 1.06×10^6 bp (www.ncbi.nlm.nih.gov). Five 10-fold serial dilutions of *P. falciparum* 3D7 DNA were made, so that there were 8, 80, 800, 8,000, or 80,000 copies of genome per reaction mixture. The *ldh* DNA and *pfmdr1* DNA in paired standard wells were amplified 40 times (quadruplicate reactions per plate times 10 reaction plates) for each of the four DNA concentrations (80 to 80,000 genome copies). Amplifications were successful for 157 of 160 reactions. The C_T values for the reactions with *ldh* and *pfmdr1* were calculated with ABI Prism 7000 SDS software, with a threshold value of 0.2000 and a reference baseline reading of cycles 6 to 15. The change in the C_T value (ΔC_T) was calculated by subtracting the C_T for a given *ldh* DNA concentration from the paired C_T for the same *pfmdr1* DNA concentration. (Since the C_T is proportional to the log of the concentration, ΔC_T is equivalent to the log of the ratio.) A histogram (Fig. 1A) of the ΔC_Ts was made and used to calculate standard deviations and means by using SAS software (version 8; SAS, Inc., Cary, N.C.). The distribution shown is due to assay variability.

Since the ratio of the *pfmdr1* gene copy number to the *ldh* gene copy number (*pfmdr1*/*ldh* ratios) is the ultimate end point, calculations were made to determine the minimal detectable ratio. ΔC_Ts were calculated for different *pfrmdr1*/*ldh* ratios (10,000:1, 1,000:1, 100:1, 10:1, 1:1) by pairing and subtracting the C_T for a low concentration of *ldh* from the C_T for a high concentration of *pfmdr1* by using the same data obtained as described above plus the C_T data for eight copies of *ldh*. For each ratio, mean ΔC_Ts were plotted against the copy number ratio and were found to fit to the equation $\Delta C_T = -1.6145 \ln(\text{copy number ratio}) + 2.8565$ by using Excel Office 2000 software.

The *pfmdr1* and *ldh* amplification reactions were run in duplicate by using 1-μl aliquots of DNA from each clinical sample. ΔC_Ts were calculated as described above, and the duplicate values were averaged. ΔC_Ts which were more than 2 standard deviations beyond the mean (95% confidence interval [CI]) calculated from the data in Fig. 1A were considered significantly different from 1.

Statistical analysis. Statistical analyses were conducted with SAS software (version 8; SAS, Inc.). Nonnormally distributed IC_{50} data were assessed by Wilcoxon rank sum tests. Group differences in the percentage of resistant isolates and IC_{50}s were assessed by the chi-square test. Prevalence ratios were estimated for the association between genetic markers and drug susceptibility phenotypes.

RESULTS

Polymorphisms. Of the 65 isolates successfully cultured, amplified, and sequenced, 39 were from Thailand, 14 were from

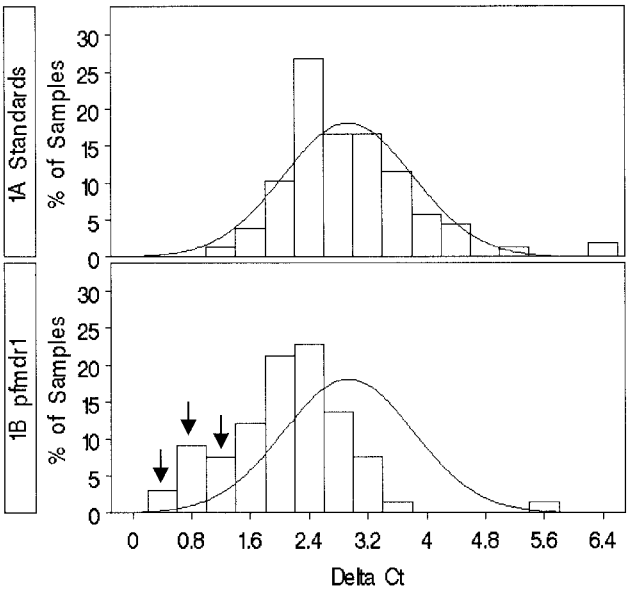

FIG. 1. Frequency distribution of ΔC_Ts calculated from real-time PCR amplifications of *pfmdr1* and *ldh* genes. (A) Histogram of 157 ΔC_Ts calculated from standard DNA, showing a normal distribution; (B) histogram of isolates. The arrows point to samples which are more than 2 standard deviations below the mean.

Myanmar (18), 7 were from Bangladesh (11), and 5 were from Vietnam (19). There were no associations between the *msp1*, *msp2*, or *glurp* types of the strains or the degree of clonality and drug resistance (data not shown).

Mutations in both *pfcrt* and *pfmdr1* were quite common (Fig. 2). Ninety-seven percent (63 of 65) of the samples contained the Thr76 polymorphism in *pfcrt*. *pfmdr1* polymorphisms Tyr86 and Phe184 were each present in about one-third of the samples. Cys1034 and Asp1042 were present in 9 and 15% of the samples, respectively, while no polymorphisms were found at position 1246. None of the amplicons gave mixed sequences at any of these loci.

TABLE 2. Patterns of mutations observed in *pfmdr1*

Category	Codon at position[a]				No. of isolates
	86	184	1034	1042	
I	Asn	Tyr	Ser	Asn	17
II	**Tyr**	Tyr	Ser	Asn	23
III	Asn	**Phe**	Ser	Asn	15
IV	Asn	**Phe**	**Cys**	**Asp**	10
	Asn	**Phe**	Cys	Asn	
	Asn	**Phe**	Ser	**Asp**	

[a] The mutated amino acids (in boldface) and the number of isolates in each category are shown.

Polymorphisms in *pfmdr1* clustered into four specific patterns without variation. We arbitrarily named these specific patterns (Table 2). The wild-type pattern (Asn86, Tyr184, Ser1034, Asn1042) was classified as category I. Isolates which had the Tyr86 polymorphism (and which had no polymorphisms at any of the other sites) were classified as category II. Isolates with the Phe184 polymorphism alone were classified as category III. The only multiple polymorphism observed among these isolates was Phe184 in combination with Cys1034 and/or Asp1042; isolates with this pattern were classified as category IV (Table 2). The two laboratory strains, strains W2 and PH6, fit into categories II and IV, respectively. Both isolates with incomplete sequences had the Tyr86 polymorphism, and no other polymorphism was detected. Thus, they were both presumed to be in category II.

There were significant differences in the IC_{50}s between the different categories. The IC_{50}s of mefloquine, artemisinin, and

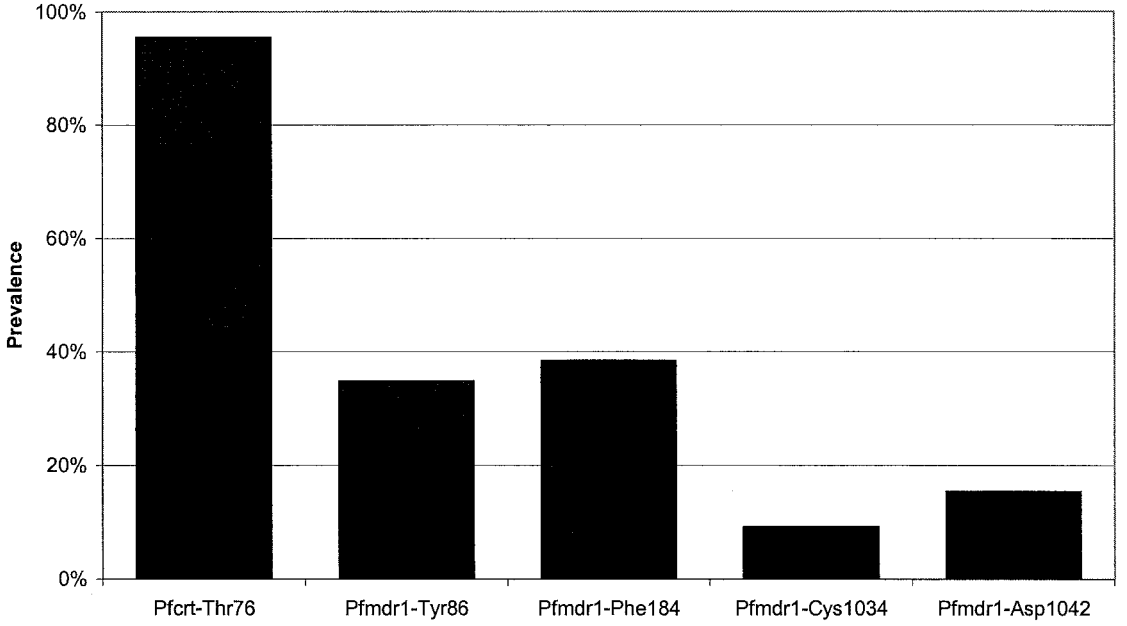

FIG. 2. Prevalence of specific point mutations in *pfcrt* and *pfmdr1*. No mutations were observed at *pfmdr1* position 1246 (data not shown).

TABLE 3. Median IC_{50}s and percent resistance for isolates in four catagories

Category	Mefloquine		Quinine		Chloroquine		Median IC_{50} (interquartile range)[b]	
	% R[a]	Median IC_{50} (interquartile range)	% R	Median IC_{50} (interquartile range)	% R	Median IC_{50} (interquartile range)	Artemisinin	Artesunate
I (n = 17)	100	60.68 (49.64–69.90)	35	188.62 (104.44–271.88)	76	56.40 (47.08–73.92)	1.74 (1.10–2.04)	4.51 (2.47–4.84)
II (n = 23)	0	15.79 (11.43–19.56)	4	94.68 (77.79–107.83)	91	88.19 (73.09–107.83)	0.73 (0.54–1.40)	1.43 (1.06–2.69)
III (n = 15)	80	59.51 (30.87–73.96)	31	148.87 (70.07–239.88)	81	70.92 (19.38–92.08)	2.57 (1.80–3.85)	2.57 (1.80–3.85)
IV (n = 10)	20	19.44 (10.01–24.70)	11	184.36 (141.28–233.41)	89	70.10 (63.69–98.97)	0.97 (0.80–1.30)	0.85 (0.64–0.93)
P value for difference[c]	<0.001	<0.001	0.266	0.002	<0.001	0.007	<0.001	0.011

[a] % R, percentage of resistant isolates.
[b] For artemisinin and artesunate, there are no established in vitro cut offs for resistance and sensitivity.
[c] P-values were determined by chi-square analysis for percentage of resistant isolates and analysis of variance for IC_{50}s.

artesunate were significantly higher for the isolates in categories I and III than for the isolates in categories II and IV (one-sided P values, <0.0001, 0.0006, and <0.0001, respectively) (Table 3). The opposite was true for chloroquine (P = 0.007), while the quinine IC_{50} data did not follow any specific pattern. Thus, isolates in categories I and III are relatively more resistant to mefloquine, artemisinin, and artesunate and relatively more sensitive to chloroquine.

We reasoned that under mefloquine pressure, isolates in the wild-type category (category I) could have developed the category II mutation; in the same way, the isolates with the category III mutation present under chloroquine pressure could have developed additional polymorphisms under mefloquine pressure, resulting in the category IV pattern. Because the isolates in categories I and III showed similar resistance patterns, while the isolates in categories II and IV also had similar resistance patterns, we collapsed these two sets of categories to test the hypothesis that there is an association between *pfmdr1* polymorphisms and drug resistance.

The estimated risk of resistance was higher for isolates in categories I and III than for isolates in categories II and IV (Table 3), with prevalence ratios of 14.95 (95% CI, 3.88 to 57.56) for mefloquine and 1.44 for quinine (95% CI, 0.51 to 4.08). In contrast, the estimated risk of resistance to chloroquine was lower for isolates in categories I and III than for isolates in categories II and IV (prevalence ratio, 0.94; 95% CI, 0.86 to 1.03). Thus, there was a strong association between mutation category and the prevalence of mefloquine resistance and a weaker association between mutation category and the prevalence of quinine and chloroquine resistance.

Only two isolates (3%) had the wild-type *pfcrt* genotype (Lys76). Both of these isolates were chloroquine sensitive, quinine sensitive, and mefloquine resistant. One isolate fell into *pfmdr1* category I, and the other isolate fell into category III.

Real-time PCR to determine *pfmdr1*/*ldh* ratio. A total of 157 ΔC_Ts were determined from the concentration-response experiments run with standard DNA (Fig. 1A), yielding a mean ΔC_T of 2.82 with a 95% CI of 1.05 to 4.57. ΔC_T values that fall below the lower CI are indicative of high *pfmdr1*/*ldh* ratios. *pfmdr1*/*ldh* ratios yielding ΔC_T values that fall within the CI are not considered statistically significantly different from 1. Only *pfmdr1*/*ldh* ratios of 3.06 or more would yield clinical ΔC_T values less than 1.05. Thus, the sensitivity limit of this assay is a *pfmdr1*/*ldh* ratio of 3.

The *pfmdr1* and *ldh* genes were successfully amplified from all DNA samples. The frequency distributions for ΔC_T values are shown in Fig. 1B. The isolates in 8 of 65 samples (12.3%) had ΔC_T values which were below the 95% confidence limit (i.e., less than 1.05), which is indicative of *pfmdr1*/*ldh* ratios between 3 and 4.2. Five of these isolates were in category I, and three were in category III. The IC_{50}s were compared for three groups: categories I and III with amplification, categories I and III without amplification, and categories II and IV without amplification. The IC_{50}s of all drugs examined were significantly different across these three categories (Table 4). The mefloquine, quinine, artemisinin, and artesunate IC_{50}s were

TABLE 4. Median IC_{50}s and percent resistance for isolates categorized by *pfmdr1* gene amplification

Category	Mefloquine		Quinine		Chloroquine		Median IC_{50} (interquartile range)	
	% R[a]	Median IC_{50} (interquartile range)	% R	Median IC_{50} (interquartile range)	% R	Median IC_{50} (interquartile range)	Artemisinin	Artesunate
II and IV, no amplification (n = 33)	6.1	16.86 (11.13–20.45)	15.2	112.01 (80.98–143.74)	100	84.37 (68.22–107.12)	0.82 (0.64–1.09)	1.32 (0.83–2.13)
I and III, no amplification (n = 24)	87.5	53.01 (34.15–62.79)	16.7	144.35 (71.96–199.15)	91.7	69.06 (50.87–86.92)	1.18 (0.74–1.92)	2.48 (1.71–4.60)
I and III, amplification (n = 8)	100	72.13 (64.77–99.21)	37.5	289.31 (253.44–360.07)	100	57.55 (43.51–64.92)	1.89 (1.28–2.76)	5.63 (3.33–8.33)
P value for difference[b]	<0.0001	<0.0001	0.3280	0.0010	0.3654	0.0029	0.0008	<0.0001

[a] % R, percentage of resistant isolates.
[b] P values were determined by Fisher's exact chi-square test for percentage of resistant isolates and the Wilcoxon rank sum test for IC_{50}s.

the highest for isolates in categories I and III with amplification, moderate for isolates in categories I and III without amplification, and lowest for isolates in categories II and IV without amplification (P values, <0.0001, 0.001, 0.0008, and <0.0001, respectively) (Table 4). Chloroquine exhibited the opposite pattern, with the lowest chloroquine IC_{50}s being for isolates in categories I and III with amplification and the highest chloroquine IC_{50}s being for isolates in categories II and IV without amplification.

Evaluation for the presence of category I and III genotypes may be useful tool in surveillance for mefloquine resistance. The presence of these genotypes has a positive predictive value of 91% for this group of isolates, with a sensitivity of 94% and a specificity of 91% for the detection of in vitro mefloquine resistance. For quinine resistance and chloroquine sensitivity, on the other hand, the values are much lower. Because all isolates whose DNA was amplified were mefloquine resistant, the *pfmdr1* gene copy number has a positive predictive value and a specificity of 100% for the detection of mefloquine resistance; however, it is less useful than the genotype for surveillance because of a sensitivity of only 26%.

DISCUSSION

Among 65 cultured isolates from Southeast Asia, almost all contained the *pfcrt* polymorphism at position 76. Polymorphisms were found at four loci in the *pfmdr1* gene and occurred in four specific patterns (Table 2). The isolates with two patterns (categories I and III) tended to be more resistant to mefloquine, artesunate, and artemisinin, while the isolates with two others (categories II and IV) tended to be more resistant to chloroquine. Our data also demonstrate that isolates with increased *pfmdr1* copy numbers tend to be more resistant to mefloquine, quinine, artesunate, and artemisinin and more sensitive to chloroquine than isolates without increased *pfmdr1* copy numbers.

An association between the polymorphism that comprised category II (Tyr86) and mefloquine susceptibility had previously been suggested, but the strength of this association was never precisely quantified (for a review, see reference 20). Attempts to answer the question related to *pfmdr1* gene amplification also have a history of conflicting results. Some studies have reported increased copy numbers with selection for mefloquine resistance (5, 12), while others have not (9, 10).

In this study, isolates that were in categories I or III were 15-fold more likely to be mefloquine resistant in vitro. This is the strongest association ever reported between *pfmdr1* and resistance to antimalarials. The presence of these genotypes correctly identifies 94% of the mefloquine-resistant isolates in vitro, while the absence of these genotypes correctly identifies 91% of the mefloquine-sensitive isolates in vitro.

We also found a robust association between *pfmdr1* copy number and drug resistance. Increased *pfmdr1* copy numbers were found only among isolates in the two categories (categories I and III) in which isolates already displayed increased mefloquine resistance. Within categories I and III, isolates with *pfmdr1* amplification were significantly more resistant to mefloquine, quinine, artemisinin, and artesunate than isolates without *pfmdr1* amplification. This suggests that *pfmdr1* amplification may occur secondarily as a way to achieve greater levels of resistance. However, *pfmdr1* amplification appears to be less useful as a surveillance tool, with a sensitivity of only 26%. One possible reason for this is the fact that our real-time PCR assay could only reliably detect *pfmdr1*/*ldh* ratios ≥ 3. Improvements to the assay might improve its sensitivity.

The distribution of *pfmdr1* polymorphisms found here can be compared constructively with those found in a previous study, in which the *pfmdr1* genotypes of 54 isolates from the northwestern border of Thailand and Myanmar were reported (13). Of the 54 sequences published, 52 fit into the categories described in this paper: 28 (54%) were category I, 5 (10%) were category II, and 19 (37%) were category III or IV. That study did find increased *pfmdr1* copy numbers in mefloquine-resistant parasites; similar to the present study, isolates with increased copy numbers were all wild type at position 86 (corresponding to possible category I or III isolates) (13).

The lack of a polymorphism at position 1246 in this study supported previous reports from Asia, suggesting that this polymorphism may not be important for isolates from this region (4, 13, 17). Also, the coexistence of elevations in mefloquine, artemisinin, and artesunate IC_{50}s for the same categories of isolates is also consistent with previous observations (1, 7, 16, 21).

Since the isolates in category IV contain the one mutation found among the isolates in category III plus other mutations, category IV isolates could have derived from category III isolates. Different geographic distributions were found for the different classes and may support this idea. The most striking geographic differences were between Yala (in southern Thailand) and Borai (on the Thai-Cambodian border): eight of eight isolates in samples selected from among those that originated in Yala were category II, while five of five isolates in samples from Borai were category III or IV. Circumstantial support for the evolution of genotype is offered by the only two chloroquine-sensitive isolates, which were mefloquine resistant and which were in categories I and III.

The interpretation of our results must bear certain considerations. First, isolates were chosen to provide the broadest range of in vitro sensitivities to mefloquine. Thus, the power for discriminating between mefloquine-sensitive and -resistant isolates was high. This study was designed to test the hypothesis that there is an association between mefloquine resistance and the *pfmdr1* genotype. Because of this, the prevalence of sensitive and resistant isolates in this study does not represent the prevalence in the Southeast Asian populations from which they were drawn. Our observation that polymorphisms were more significantly associated with resistance to mefloquine than with resistance to other drugs may simply be due to our selection criterion. Second, the observation that polymorphisms fell into only four patterns may not be generalizable; studies with larger numbers of samples from Southeast Asia or samples from other regions might reveal other patterns. Third, like other studies in Southeast Asia, this study was not able to assess the relative importance of *pfcrt* mutations, given the high percentage of isolates with the Thr76 polymorphism. Fourth, it is possible that *pfmdr1* mutations may not be responsible for mefloquine resistance but may only be in linkage disequilibrium with other determinants that do cause resistance. Finally, the in vitro resistance studied here may not correlate with in vivo resistance. Future studies are needed to determine the

association between *pfmdr1* polymorphisms and in vivo resistance.

In summary, our data suggest that categories of polymorphisms in *pfmdr1* exist in Southeast Asia and that isolates resistant to mefloquine and chloroquine have opposite genetic patterns. Chloroquine use may have selected for *pfmdr1* polymorphism categories II and IV, but when mefloquine became widely used, the selection pressure was put on isolates in categories I and III. The strong association observed suggests that categories I and III, along with *pfmdr1* gene amplification, could be key determinants of resistance to mefloquine in Southeast Asia. Future studies should more rigorously study this association. Furthermore, the patterns of the polymorphisms reported here have high degrees of sensitivity and specificity for the detection of mefloquine resistance and might serve as useful epidemiological tools.

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REFERENCES

1. **Basco, L. K., J. Le Bras, Z. Rhoades, and C. M. Wilson.** 1995. Analysis of pfmdr1 and drug susceptibility in fresh isolates of Plasmodium falciparum from sub-Saharan Africa. Mol. Biochem. Parasitol. **74:**157–166.
2. **Bell, A. S., and L. C. Ranford-Cartwright.** 2002. Real-time quantitative PCR in parasitology. Trends Parasitol. **18:**337–342.
3. **Breman, J. G.** 2001. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am. J. Trop. Med. Hyg. **64:**1–11.
4. **Chaiyaroj, S. C., A. Buranakiti, P. Angkasekwinai, S. Looressuwan, and A. F. Cowman.** 1999. Analysis of mefloquine resistance and amplification of pfmdr1 in multidrug-resistant Plasmodium falciparum isolates from Thailand. Am. J. Trop. Med. Hyg. **61:**780–783.
5. **Cowman, A. F., D. Galatis, and J. K. Thompson.** 1994. Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc. Natl. Acad. Sci. USA **91:**1143–1147.
6. **Duraisingh, M. T., L. V. von Seidlein, A. Jepson, P. Jones, I. Sambou, M. Pinder, and D. C. Warhurst.** 2000. Linkage disequilibrium between two chromosomally distinct loci associated with increased resistance to chloroquine in Plasmodium falciparum. Parasitology **121**(Pt 1)**:**1–7.
7. **Huong, N. M., S. Hewitt, T. M. Davis, L. D. Dao, T. Q. Toan, T. B. Kim, N. T. Hanh, V. N. Phuong, D. H. Nhan, and L. D. Cong.** 2001. Resistance of Plasmodium falciparum to antimalarial drugs in a highly endemic area of southern Viet Nam: a study in vivo and in vitro. Trans. R. Soc. Trop. Med. Hyg. **95:**325–329.
8. **Ittarat, W., A. L. Pickard, P. Rattanasinganchan, P. Wilairatana, S. Looareesuwan, K. Emery, J. Low, R. Udomsangpetch, and S. R. Meshnick.** 2003. Recrudescence in artesunate-treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. Am. J. Trop. Med. Hyg. **68:**147–152.
9. **Lim, A. S., D. Galatis, and A. F. Cowman.** 1996. Plasmodium falciparum: amplification and overexpression of pfmdr1 is not necessary for increased mefloquine resistance. Exp. Parasitol. **83:**295–303.
10. **Mungthin, M., P. G. Bray, and S. A. Ward.** 1999. Phenotypic and genotypic characteristics of recently adapted isolates of Plasmodium falciparum from Thailand. Am. J. Trop. Med. Hyg. **60:**469–474.
11. **Noedl, H., M. A. Faiz, E. B. Yunus, M. R. Rahman, M. A. Hossain, R. Samad, R. S. Miller, L. W. Pang, and C. Wongsrichanalai.** 2003. Drug-resistant malaria in Bangladesh: an in vitro assessment. Am. J. Trop. Med. Hyg. **68:**140–142.
12. **Peel, S. A., P. Bright, B. Yount, J. Handy, and R. S. Baric.** 1994. A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (pfmdr) of Plasmodium falciparum in vitro. Am. J. Trop. Med. Hyg. **51:**648–658.
13. **Price, R. N., C. Cassar, A. Brockman, M. Duraisingh, M. van Vugt, N. J. White, F. Nosten, and S. Krishna.** 1999. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob. Agents Chemother. **43:**2943–2949.
14. **Reed, M. B., K. J. Saliba, S. R. Caruana, K. Kirk, and A. F. Cowman.** 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature **403:**906–909.
15. **Snounou, G., X. Zhu, N. Siripoon, W. Jarra, S. Thaithong, K. N. Brown, and S. Viriyakosol.** 1999. Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand. Trans. R. Soc. Trop. Med. Hyg. **93:**369–374.
16. **Webster, H. K., S. Thaithong, K. Pavanand, K. Yongvanitchit, C. Pinswasdi, and E. F. Boudreau.** 1985. Cloning and characterization of mefloquine-resistant Plasmodium falciparum from Thailand. Am. J. Trop. Med. Hyg. **34:**1022–1027.
17. **Wilson, C. M., S. K. Volkman, S. Thaithong, R. K. Martin, D. E. Kyle, W. K. Milhous, and D. F. Wirth.** 1993. Amplification of pfmdr1 associated with mefloquine and halofantrine resistance in Plasmodium falciparum from Thailand. Mol. Biochem. Parasitol. **57:**151–160.
18. **Wongsrichanalai, C., K. Lin, L. W. Pang, M. A. Faiz, H. Noedl, T. Wimonwattrawatee, A. Laoboonchai, and F. Kawamoto.** 2001. In vitro susceptibility of Plasmodium falciparum isolates from Myanmar to antimalarial drugs. Am. J. Trop. Med. Hyg. **65:**450–455.
19. **Wongsrichanalai, C., T. D. Nguyen, N. T. Trieu, T. Wimonwattrawatee, P. Sookto, D. G. Heppner, and F. Kawamoto.** 1997. In vitro susceptibility of Plasmodium falciparum isolates in Vietnam to artemisinin derivatives and other antimalarials. Acta Trop. **63:**151–158.
20. **Wongsrichanalai, C., A. L. Pickard, W. H. Wernsdorfer, and S. R. Meshnick.** 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. **2:**209–218.
21. **Wongsrichanalai, C., T. Wimonwattrawatee, P. Sookto, A. Laoboonchai, D. G. Heppner, D. E. Kyle, and W. H. Wernsdorfer.** 1999. In vitro sensitivity of Plasmodium falciparum to artesunate in Thailand. Bull. W. H. O. **77:**392–398.