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

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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 *pfcr* 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 ≤ 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, artesinin, and artesunate and more sensitive to chloroquine than the isolates in the 57 samples with <3 copies of the gene (P ≤ 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 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 artesinin 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.

**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

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

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

Optimized according to the protocol recommended for the TaqMan Universal PCR Master Mix (Applied Biosystems).

RESULTS

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

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 IC50s between the different categories. The IC50 of mefloquine, artemisinin, and...
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 IC50 data did not follow any specific pattern. Thus, isolates in categories I and III are relatively more resistant to mefloquine, artesinin, 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 \( pfcr \) 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 \( \Delta C_T \) values were determined from the concentration-response experiments run with standard DNA (Fig. 1A), yielding a mean \( \Delta C_T \) of 2.82 with a 95% CI of 1.05 to 4.57. \( \Delta C_T \) values that fall below the lower CI are indicative of high \( pfmdr1/ldh \) ratios, \( pfmdr1/ldh \) ratios yielding \( \Delta C_T \) 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 \( \Delta C_T \) 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 \( \Delta C_T \) values are shown in Fig. 1B. The isolates in 8 of 65 samples (12.3%) had \( \Delta C_T \) 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 IC50 values 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 IC50 values of all drugs examined were significantly different across these three categories (Table 4). The mefloquine, quinine, artemisinin, and artesunate IC50 values were

<table>
<thead>
<tr>
<th>Category</th>
<th>Mefloquine</th>
<th>Quinine</th>
<th>Chloroquine</th>
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<tbody>
<tr>
<td>II and IV, no amplification (n = 33)</td>
<td>6.1 (16.86) (11.13–20.45)</td>
<td>15.2 (112.01 (80.98–143.74)</td>
<td>100 (84.37 (68.22–107.12)</td>
</tr>
<tr>
<td>I and III, no amplification (n = 24)</td>
<td>87.5 (53.01 (34.15–62.79)</td>
<td>16.7 (144.35 (71.96–199.15)</td>
<td>91.7 (69.06 (50.87–86.92)</td>
</tr>
<tr>
<td>I and III, amplification (n = 8)</td>
<td>100 (72.13 (64.77–99.21)</td>
<td>37.5 (289.31 (253.44–360.07)</td>
<td>100 (57.55 (43.51–64.92)</td>
</tr>
</tbody>
</table>

\( P \) value for difference\(^b\) \(<0.0001\) \(<0.0001\) \(0.3280\) \(0.3654\) \(0.0029\) \(0.0008\) \(<0.0001\)

\(^a\) % 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 IC50 values.
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<sub>50</sub>s being for isolates in categories I and III with amplification and the highest chloroquine IC<sub>50</sub>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 (Tyr66) 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<sub>50</sub>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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