

Amplification of *pvmdr1* Associated with Multidrug-Resistant *Plasmodium vivax*

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Background. Multidrug-resistant strains of *Plasmodium vivax* are emerging in Southeast Asia.

Methods. In vitro drug susceptibility and *pvmdr1* genotype were determined in *P. vivax* field isolates from Indonesia and Thailand.

Results. Increased *pvmdr1* copy number was present in 21% of isolates from Thailand (15/71) and none from Indonesia (0/114; $P < .001$). Compared with Indonesian isolates, the median IC₅₀ of Thai isolates was lower for chloroquine (36 vs. 114 nmol/L; $P < .001$) but higher for amodiaquine (34 vs. 13.7 nmol/L; $P = .032$), artesunate (8.33 vs. 1.58 nmol/L; $P < .001$), and mefloquine (111 vs. 9.87 nmol/L; $P < .001$). In 11 cryopreserved Thai isolates, those with increased *pvmdr1* copy number had a higher IC₅₀ for mefloquine (78.6 vs. 38 nmol/L for single-copy isolates; $P = .006$). Compared with isolates with the wild-type allele, the Y976F mutation of *pvmdr1* was associated with reduced susceptibility to chloroquine (154 nmol/L [range, 4.6–3505] vs. 34 nmol/L [range, 6.7–149]; $P < .001$) but greater susceptibility to artesunate (1.8 vs. 9.5 nmol/L; $P = .009$) and mefloquine (14 vs. 121 nmol/L; $P < .001$).

Conclusions. Amplification of *pvmdr1* and single-nucleotide polymorphisms are correlated with susceptibility of *P. vivax* to multiple antimalarial drugs. Chloroquine and mefloquine appear to exert competitive evolutionary pressure on *pvmdr1*, similar to that observed with *pfmdr1* in *Plasmodium falciparum*.

Malaria continues to exert a huge global burden both in public health and economic terms. Although *Plasmodium falciparum* infections have been the focus of research and control efforts, there is a growing awareness that the burden of, and morbidity and mortality associated with, *Plasmodium vivax* infections are also substantial [1–4]. Control strategies for both species have been confounded by the emergence of antimalarial drug resistance to multiple drugs. Although chloroquine-

resistant *P. falciparum* was first documented >50 years ago in Cambodia, the first cases of chloroquine-resistant *P. vivax* infection were not reported until 1989 from Papua New Guinea [5] and northern Papua, Indonesia [6]. In the last decade, chloroquine resistance has continued to emerge, with failure rates after monotherapy exceeding 70% in Papua [7–9]. Other reports suggest declining chloroquine efficacy across much of Asia and South America [1, 10].

Few studies have specifically addressed the clinical efficacy of other antimalarial regimens against *P. vivax*, although the available data suggest that in western Indonesia mefloquine retains clinical efficacy [11], whereas amodiaquine efficacy is waning, particularly in the eastern province of Papua [12] and Papua New Guinea [13]. The dearth of clinical studies of *P. vivax* reflects both the inherent difficulties associated with the in vivo test for relapsing malaria [14] and the difficulties in differentiating between reinfection, recrudescence, and relapse after treatment failure [15, 16].

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To better define the drug-susceptibility profile of *P. vivax*, we recently developed a standardized in vitro assay [17] and validated it using isolates of *P. vivax* from Indonesia, where *P. vivax* is known to be highly resistant to chloroquine, and from Thailand, where strains remain susceptible to chloroquine [18]. The aim of the present study was to define the in vitro susceptibility profile of *P. vivax* for a wider range of antimalarial drugs and correlate this with *pvmdr1* copy number and a polymorphism known to be prevalent in both Thailand and Indonesia [18].

METHODS

Field location and sample collection. Clinical isolates were collected between 2003 and 2007 from 2 sites, the first in Timika, southern Papua, Indonesia, and the other in Mae Sod on the western border of Thailand. In southern Papua, *P. vivax* demonstrates high-grade clinical resistance to chloroquine, with failure rates >65% on day 28 after chloroquine monotherapy [9]. In contrast, *P. vivax* in Mae Sod was clinically susceptible to chloroquine when last tested in 1999 [19]. The clinical efficacy of other monotherapies against *P. vivax* in either location is unknown.

Patients with symptomatic infections of pure *P. vivax* presenting to an outpatient facility were recruited into the study, and 5-mL blood samples were collected by venipuncture. After removal of host white blood cells using a CF11 column, 2 mL of packed infected red blood cells was divided as follows: 1 mL was cryopreserved in glycerolyte, 200 μ L was spotted onto a filter paper, and 800 μ L was used for the in vitro drug-susceptibility assay. Patients were treated with dihydroartemisinin-piperaquine (Indonesia) [20] or chloroquine (Thailand) [19] according to local guidelines but were not followed up routinely thereafter. The in vitro susceptibility to chloroquine for 124 isolates included in this study has been presented elsewhere [18].

In vitro drug-susceptibility assay. The antimalarial susceptibility of fresh field *P. vivax* isolates was measured using a modified World Health Organization microtest protocol, as described elsewhere [17]. Briefly, 200 μ L of a 2% hematocrit blood-medium mixture made from McCoy's 5A medium and 20% AB Rh+ human serum was added to each well of predosed drug plates. In Indonesia, each drug plate contained 11 serial concentrations of the antimalarials chloroquine (maximum concentration, 5910 nmol/L), amodiaquine (557 nmol/L), artesunate (93 nmol/L), mefloquine (338 nmol/L), and piperaquine (769 nmol/L). In Thailand only, chloroquine was tested from 2003 until 2006, after which all 5 drugs were assayed. A candle jar was used to mature the parasites at 37.5°C (22–42 h). Incubation was stopped when >40% of ring-stage parasites had matured to schizonts in the drug-free control well.

Thick blood films made from each well were stained with 5% Giemsa stain for 30 min and examined microscopically. Differential counts of 200 asexual parasites in the preincubation and test slides were classified into ring-stage parasites (ring-shaped

trophozoites without pigment), mature trophozoites (single or double chromatin dot and hemazoin pigment visible), and schizonts. The number of schizonts (≥ 5 chromatin dots visible) per 200 asexual-stage parasites was determined for each drug concentration and normalized to the control well. The dose-response data were analyzed using nonlinear regression analysis (WinNonLin software; version 4.1; Pharsight), and the IC₅₀ value was derived using an inhibitory sigmoid E_{max} model. In vitro data were only used from predicted curves for which the maximum (E_{max}) and minimum (E_0) effect were within 15% of 100 or 0, respectively. Previous analysis has highlighted a significant stage specificity of drug activity, with chloroquine showing almost no activity against trophozoites stages in *P. vivax* infection [17]. For this reason, even though isolates were processed irrespective of the parasite staging in the initial culture, the analysis of geographical and molecular correlates of in vitro drug susceptibility was restricted to those isolates with predominantly ring-stage parasites initially (ring to trophozoite [RT] ratio >1).

In vitro drug-susceptibility assay in cryopreserved isolates. Because the number of Thai isolates with susceptibility data for drugs other than chloroquine was limited, a separate experiment was conducted using 11 cryopreserved isolates collected in Mae Sod in 2003 [21]. These comprised all 6 available isolates with *pvmdr1* amplification and a random selection of 5 isolates with a single copy of *pvmdr1*. Cryopreserved isolates were thawed and set up in short-term culture, as described elsewhere [21].

Determination of *pvmdr1* polymorphisms. Genomic DNA from blood spots and cryopreserved samples was extracted using a QIAamp DNA Mini Kit (Qiagen). *Pvmdr1* single-nucleotide polymorphisms (SNPs) at 976 were detected using a DNA template mismatch primer method [18]. Polymerase chain reaction (PCR) was done in a total volume of 50 μ L, containing 5 μ L of 10 \times PCR buffer, 2.5 mmol/L MgCl₂, 0.2 mmol/L each dNTP, 2 μ mol/L Pvmdr976 forward primer, 1.5 μ mol/L Pvmdr976 reverse primer, 1 μ mol/L Pvmdr976 internal primer, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 μ L of genomic DNA. PCR was performed under the following conditions: 95°C for 10 min followed by 40 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 2 min. The product was separated on 2% agarose gel.

The *pvmdr1* gene copy number was estimated by a quantitative real-time SYBR Green PCR assay, as described elsewhere [18]. In brief, a single-copy gene coding for *P. vivax* aldolase (GenBank accession number AF247063) was used as a reference (normalizer) gene for estimating the *pvmdr1* copy number. PCRs were performed in triplicate or quadruplet and contained 1 \times ABgene Absolute QPCR SYBR Green Mix (catalog number AB-1166/a), 100 nmol/L ROX dye (passive reference dye), 1 μ L of DNA template, and 75 nmol/L concentrations of each primer in a final volume of 25 μ L. Cycling conditions were 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. Plasmids containing the aldolase gene fragment and 1 or 2 copies of the *pvmdr1* gene fragments were used as

controls. Fluorescence data were collected 3 times and averaged at the end of the annealing and extension steps. After the amplification cycles, a melting curve analysis was performed to confirm that the correct products were synthesized. The text report containing the threshold cycle values for every well was exported into Excel (Microsoft) and analyzed. Assessment of copy number was repeated at least twice for all isolates, and the repeatability coefficient determined was as 0.30 (viz., 95% of repeated estimates of *pvmdr1* copy number were within 0.15 of the first).

Data and sequence analysis. Analysis was performed using SPSS for Windows (version 14; SPSS). The Mann-Whitney *U* test and Wilcoxon signed-rank test were used for nonparametric comparisons.

Ethics. Ethical approval for this study was obtained from the ethics committees of the National Institute of Health Research and Development, Ministry of Health, Indonesia; the Menzies School of Health Research, Darwin, Australia; and Mahidol University, Bangkok, Thailand.

RESULTS

In vitro susceptibility profile of Indonesian and Thai Isolates.

Between April 2003 and October 2007, 362 isolates were assayed for *in vitro* susceptibility (237 from Indonesia and 125 from Thailand). A total of 212 isolates were rejected owing to inadequate parasite growth ($n = 84$) or because the initial RT ratio was <1 ($n = 128$) (figure 1). Among the 150 isolates with acceptable data, the median percentage of ring-stage parasites in the initial culture was 84% (range, 50%–100%), with no significant difference in this proportion between Thai and Indonesian isolates.

The median IC_{50} of *P. vivax* among Thai isolates was significantly lower than those among Indonesian isolates for chloroquine (36.7 vs. 114 nmol/L) but was higher for mefloquine (111 vs. 9.87 nmol/L), artesunate (8.33 vs. 1.58 nmol/L), and amodiaquine (34 vs. 13.7 nmol/L) (table 1 and figure 2).

Prevalence of *pvmdr1* amplification and Y976F point mutation. DNA was available for 222 (61%) of all isolates collected. The *pvmdr1* copy number could be successfully quantified in 185 (83%) of these isolates and the 976 polymorphism identified in 211 (95%). In total, 15 (21%) of 71 isolates from Thailand had increased *pvmdr1* copy number (13 with 2 copies, 2 with 3 copies), compared with 0 of 114 from Indonesia ($P < .001$). Conversely, the *pvmdr1* 976 mutant (Y976F) was present in 133 (96.4%) of 138 of Indonesian isolates, compared with 19 (26%) of 73 Thai isolates. In Thailand, all 15 of the isolates with increased copy number had the wild-type allele at codon 976, compared with 36 (67%) of 54 with a single copy of *pvmdr1* ($P = .007$).

Correlation of *pvmdr1* amplification and Y976F point mutation with *in vitro* susceptibilities in fresh field isolates. Compared with isolates with the wild-type allele, those with the *pvmdr1* Y976F allele had reduced susceptibility to chloroquine

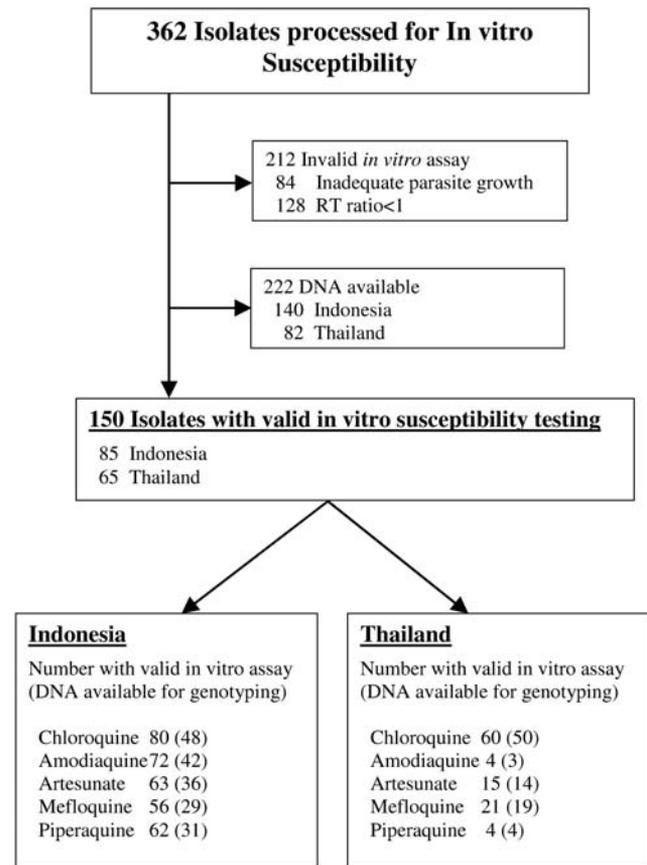


Figure 1. Selection of samples analyzed. RT ratio, ring to trophozoite ratio.

(154 vs. 34.0 nmol/L; $P < .001$) but increased susceptibility to mefloquine (14.0 vs. 121 nmol/L; $P < .001$) and artesunate (1.8 vs. 9.52 nmol/L; $P < .001$) (table 2). The association between *pvmdr1* mutation and IC_{50} remained after controlling for *pvmdr1* amplification.

Because *pvmdr1* amplification was absent in Indonesian isolates, the correlation with the *in vitro* response was restricted to Thai isolates. There was no significant difference between the chloroquine IC_{50} for 35 isolates with a single copy of *pvmdr1* (median, 33.3 nmol/L [range, 6.7–239]) and that for the 12 isolates with *pvmdr1* amplification (40.5 nmol/L [range, 11.1–99.4]; $P = .772$). The median mefloquine IC_{50} among single-copy Thai isolates was 77.0 nmol/L (range, 30.1–163 nmol/L), compared with 177 nmol/L and 137 nmol/L for the 2 Thai isolates with *pvmdr1* amplification. Statistical comparison could not be made for the other drugs, because only 1 isolate with *pvmdr1* amplification had a valid assay for artesunate (9.5 nmol/L), and none with *pvmdr1* amplification had *in vitro* data on amodiaquine or piperaquine available. The median IC_{50} for isolates with a single copy of *pvmdr1* was 8.3 nmol/L (range, 2.38–16.2 nmol/L) for artesunate, 28.2 nmol/L (range, 20.1–41.6 nmol/L) for amodiaquine, and 27.7 nmol/L (range, 20.9–47.6 nmol/L) for piperaquine.

Table 1. In vitro susceptibility of isolates from Thailand and Indonesia.

Drug	Indonesian isolates		Thai isolates		P
	No.	IC ₅₀ , nmol/L	No.	IC ₅₀ , nmol/L	
Chloroquine	80	114 (4.6–3506)	60	37 (6.7–1264)	<.001
Amodiaquine	72	14 (0.4–125)	4	34 (20–42)	.032
Artesunate	63	1.6 (0.04–14.3)	15	8.3 (2.4–23)	<.001
Mefloquine	56	9.9 (0.8–138)	21	111 (30–194)	<.001
Piperaquine	62	19 (1.8–120)	4	28 (21–48)	.119

NOTE. Data for IC₅₀ are median (range) values.

Correlation between *pvmdr1* amplification and in vitro susceptibility among thawed isolates. To investigate the role played by *pvmdr1* amplification in drug susceptibility, 11 cryopreserved Thai isolates were thawed and assayed for in vitro susceptibility. Mefloquine was assayed for all isolates and artesunate for the 6 isolates for which there was sufficient sample to test a second drug. All assays undertaken were successful in generating IC₅₀ data. The median IC₅₀ for mefloquine among the 6 isolates with *pvmdr1* amplification was 78.6 nmol/L (range, 56.5–188 nmol/L), compared with 38.0 nmol/L (range, 8.23–44.5 nmol/L) among the 5 isolates with a single copy of *pvmdr1* ($P = .006$) (figure 3). The corresponding figures for artesunate were 12.01 nmol/L (range, 6.69–17.3 nmol/L) for the 2 isolates with increased *pvmdr1* copy number and 2.57 nmol/L (range,

1.58–7.42 nmol/L) for the 4 isolates with a single copy of *pvmdr1* ($P = .165$).

DISCUSSION

Antimalarial resistance to most classes of antimalarial drugs has evolved, with the underlying resistance mechanisms derived mainly from mutations in the genes encoding target enzymes or transporters [22, 23]. In *P. falciparum*, the *pfmdr1* gene on chromosome 5 encodes a P-glycoprotein pump that affects the intraparasitic concentrations of several important antimalarial drugs [24]. Point mutations in *pfmdr1* are associated with decreased susceptibility to chloroquine and increased susceptibility to mefloquine, artesunate, and lumefantrine [25, 26]. Conversely, amplification of wild-type *pfmdr1* is associated with

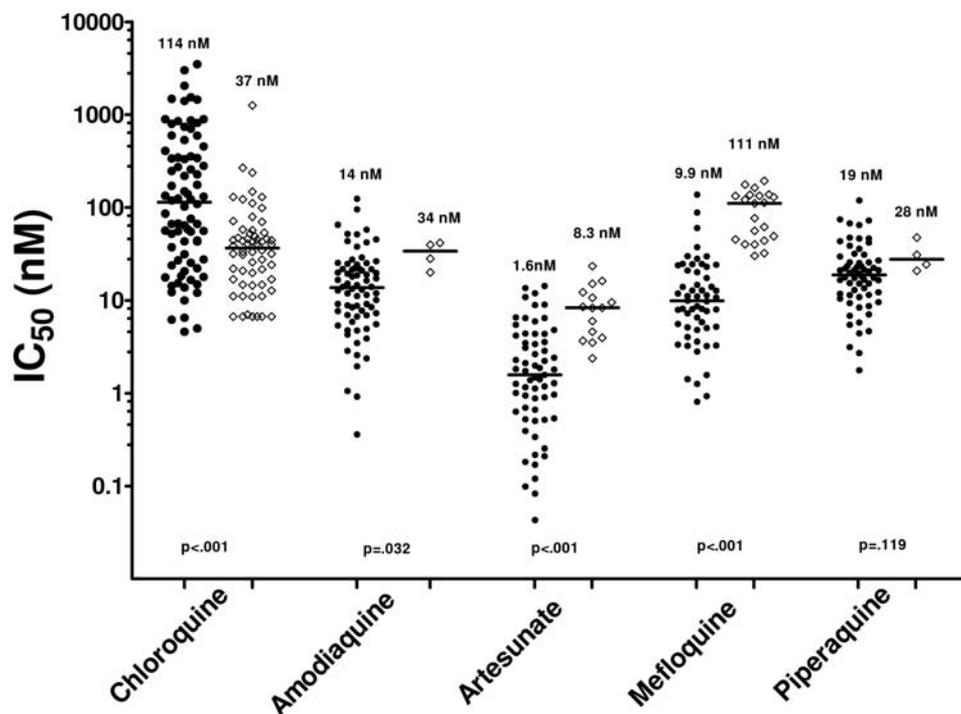


Figure 2. In vitro drug-susceptibility profiles for Indonesian (circles) and Thai (diamonds) isolates. The nos. above each group and the horizontal bars indicate median values. P values are for the comparison between Indonesian and Thai isolates.

Table 2. Relationship of the *pvmdr1* 976 mutation and in vitro susceptibility of isolates from Thailand and Indonesia.

Drug, isolates	Mutant		Wild type		P
	No.	IC ₅₀ , nmol/L	No.	IC ₅₀ , nmol/L	
Chloroquine					
All	58	154 (4.6–3506)	40	34 (6.7–149)	<.001
Indonesian	47	274 (4.6–3506)	1	103	...
Thailand	11	42.8 (6.7–239)	39	33 (6.7–149)	.527
Amodiaquine					
All	43	14.0 (0.4–125)	2	35 (28–42)	...
Indonesian	42	13.7 (0.4–125)	0
Thailand	1	20.1	2	35 (28–42)	...
Artesunate					
All	41	1.80 (0.04–13.6)	9	9.5 (3.7–16)	<.001
Indonesian	36	1.53 (0.04–13.6)	0
Thailand	5	3.96 (2.4–5.97)	9	9.5 (3.7–16)	.009
Mefloquine					
All	34	14.0 (0.9–138)	14	121 (32–177)	<.001
Indonesian	29	12.8 (0.9–138)	0
Thailand	5	49.2 (30–122)	14	121 (32–177)	.064
Piperaquine					
All	31	20.9 (1.8–120)	4	28 (15–48)	.195
Indonesian	30	19.6 (1.8–120)	1	15.2	...
Thailand	1	21	3	31 (25–48)	...

NOTE. Data for IC₅₀ are median (range) values.

reduced in vitro and in vivo susceptibility to structurally unrelated antimalarial drugs, such as mefloquine, artesunate, lumenfantrine, and quinine [25, 27–29].

The molecular basis of antimalarial resistance in *P. vivax* has been less intensely studied. Although the *pfprt* gene is the main determinant of chloroquine resistance in *P. falciparum*, polymorphisms of

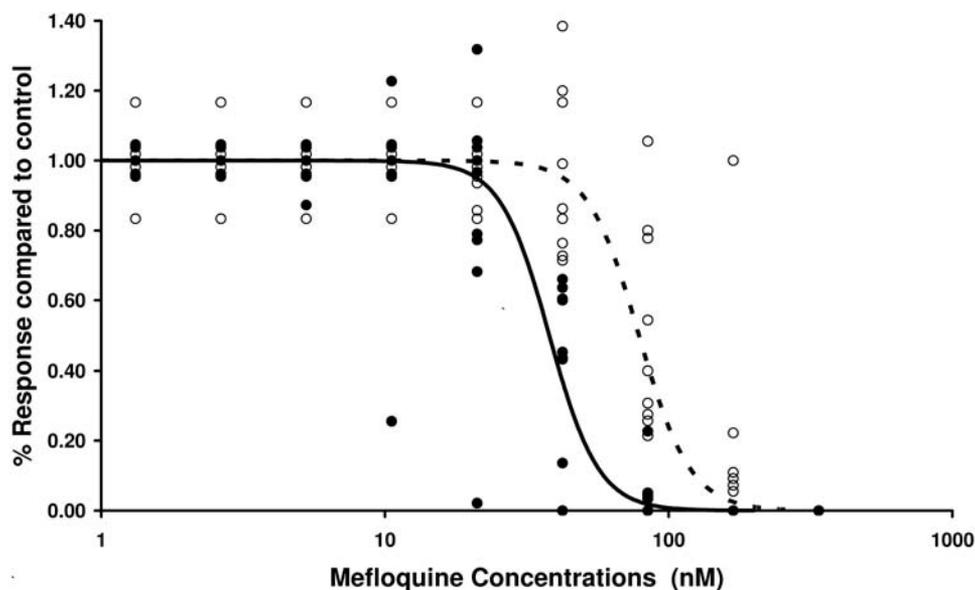


Figure 3. In vitro susceptibility to mefloquine in thawed Thai isolates according to *pvmdr1* genotype: single copy of *pvmdr1* (black circles, solid line) vs. amplified *pvmdr1* (white circles, dashed line). Data points represent assay wells for 11 isolates with 8 dilutions of mefloquine. The predicted population curves were calculated using the formula $Y = 1 - [X^\gamma / (X^\gamma + IC_{50}^\gamma)]$, where Y is the response compared with the control well, X is the mefloquine concentration, and IC_{50} and γ are the median values for the IC_{50} and slope, respectively, derived for each isolate by regression analysis.

pvcr1, the orthologue in *P. vivax*, do not appear to affect susceptibility to chloroquine [18, 30]. However, our recent study suggested a role for the *pvmdr1* gene, the orthologue of *pfmdr1*, with a tyrosine for phenylalanine substitution at position 976 (Y976F) associated with a 2-fold increase in chloroquine IC₅₀ [18]. This article also noted the presence of gene amplification of *pvmdr1* in Thai isolates. More recently, we have reported that the initial stage of the parasites before culture is a major confounding factor for in vitro parasite susceptibility [17] and that this varies considerably in isolates collected at different locations. Therefore, in the present study, we investigated the prevalence of *pvmdr1* amplification and polymorphisms and their associated correlation with in vitro susceptibility in 2 geographically distinct sites, applying a more stringent assay restriction to isolates with a majority of ring-stage parasites before culture.

In Indonesia, high-grade resistance to chloroquine has emerged in *P. vivax*, and resistance in *P. falciparum* is mainly limited to chloroquine and sulfadoxine-pyrimethamine [9]. Before 2006, the mainstay of treatment for uncomplicated malaria in this region was chloroquine plus sulfadoxine-pyrimethamine; mefloquine was not available, and the use of the artemisinin derivatives was limited to clinical trials. On the western border of Thailand, *P. vivax* remained generally susceptible to chloroquine when last tested in 1999 [19], although in *P. falciparum* significant resistance has long been present to a wide range of drugs, including mefloquine, chloroquine, sulfadoxine-pyrimethamine, halofantrine, and lumefantrine [31]. In western Thailand, a combination of mefloquine and artesunate is used to treat *P. falciparum*, and chloroquine monotherapy is still recommended for *P. vivax*.

There were significant differences in the prevalence of *pvmdr1* amplification and a SNP. In Papua, all isolates had single copies of the gene, with the Y976F allele present in 96.4% of isolates. In Thailand, on the other hand, the Y976F allele was present in only 26% of isolates, with *pvmdr1* amplification present in 21%. The difference in allele frequencies was mirrored in the in vitro drug-susceptibility profiles. In Indonesia, isolates were highly resistant to chloroquine but had low IC₅₀ values for mefloquine, amodiaquine, and artesunate. Conversely, in Thailand, isolates demonstrated greater susceptibility to chloroquine but had reduced susceptibility to mefloquine, amodiaquine, and artesunate. These drug-susceptibility profiles, presented in table 1, concur with findings in other studies of Thai isolates [32].

When the in vitro response was correlated directly with molecular polymorphisms, the Y976F *pvmdr1* mutant was associated with a 4-fold higher chloroquine IC₅₀ (similar to our findings reported elsewhere [18]) but a 5–8-fold lower IC₅₀ for artesunate and mefloquine (table 2). However, when only Thai isolates were selected, the correlation between chloroquine IC₅₀ and the Y976F mutation was no longer significant, although the trend remained similar (table 2). It is possible that the current sample size no longer had the necessary power to determine a significant association, and a study with larger sample size may

clarify the relationship between this mutation and drug susceptibility. Alternatively, the difference we reported in another study could have been attributable in part to the differences in the stage of the isolates between locations. In either case, although the 976 mutant may prove to be a useful population marker of emerging chloroquine-resistant *P. vivax*, alternative molecular mechanisms need to be evoked to account for the significant variation in IC₅₀ values in wild-type *pvmdr1* alleles.

The presence of *pvmdr1* amplification in Thai but not Papuan isolates is intriguing, particularly because mefloquine is not a standard treatment for *P. vivax* in either area. Studies from Thailand recently showed that almost 70% of *P. falciparum* isolates from infections recurring after treatment with mefloquine had amplified *pfmdr1* [33]. In practice, *P. vivax* and *P. falciparum* are often not discriminated between at diagnosis, and thus *P. vivax* infections are likely to be frequently treated with a mefloquine regimen. If the selective pressure on *mdr1* amplification that is apparent in *P. falciparum* occurs in *P. vivax*, this would account for the high prevalence of *pvmdr1* amplification present in Thailand even after inadvertent drug exposure. In Papua, where mefloquine has not been deployed, none of the isolates tested had increased *pvmdr1* copy number. This hypothesis is supported by recent data showing a greater prevalence of *pvmdr1* amplification among *P. vivax* isolates from Thailand than among isolates from other regions of Southeast Asia with less mefloquine exposure [34].

Unfortunately, apart from chloroquine, in vitro drug-susceptibility assays were available only for 2 field isolates with an increased *pvmdr1* copy number. Interestingly, these isolates had a high IC₅₀ for both mefloquine (177 and 137 nmol/L) and artesunate (9.5 nmol/L). To assess this further, 11 cryopreserved isolates were thawed, and the synchronous culture was tested against mefloquine and, when there was sufficient sample volume, artesunate. Despite the small numbers of isolates tested, *pvmdr1* amplification was associated with a 2-fold increase in the median mefloquine IC₅₀ (38–79 nmol/L; *P* = .006) and a 4.6-fold increase in the median artesunate IC₅₀ (2.6–12.0 nmol/L; *P* = 17).

The association of a higher IC₅₀ for both mefloquine and artesunate with increased *pvmdr1* copy number suggests important similarities between the species with respect to the mechanism of multidrug resistance. Our results with *P. vivax* also suggest that chloroquine and mefloquine exert competitive evolutionary pressure on *pvmdr1*, as has been found elsewhere with *P. falciparum*. In Papua, chloroquine rather than mefloquine provides the main force for drug selection; hence, the observed high prevalence of single-copy *pvmdr1* Y976F allele. Conversely, in Thailand, mefloquine has been used for many years for the treatment of falciparum malaria, and its widespread use has selected for amplification of *pvmdr1*, which occurs only in parasites with the wild-type allele at 976. Therefore, amplification of *pvmdr1* may provide a counterbalance to chloroquine pressure, providing a plausible explanation for

the low prevalence of Y976F and the retention of chloroquine-susceptible *P. vivax* in Thailand.

In conclusion, both a SNP and amplification of *pvmdr1* are associated with variation in the in vitro susceptibility of *P. vivax*, similar to that associated with *pfmdr1* in *P. falciparum*. Further studies are needed to confirm the correlation between such *pvmdr1* polymorphisms and clinical response.

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