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Gene Amplification of the Multidrug Resistance 1 Gene of *Plasmodium vivax* Isolates from Thailand, Laos, and Myanmar[▽]

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***Plasmodium vivax mdr1* gene amplification, quantified by real-time PCR, was significantly more common on the western Thailand border (6 of 66 samples), where mefloquine pressure has been intense, than elsewhere in southeast Asia (3 of 149; $P = 0.02$). Five coding mutations in *pvmdr1*, independent of gene amplification, were also found.**

An increase in the copy number of the *Plasmodium falciparum mdr1*gene is the most important determinant of mefloquine resistance in vitro and in vivo (3, 6–8) and is inversely correlated with chloroquine resistance (1). To assess *Plasmodium vivax mdr1* gene amplification, we developed a real-time PCR method and evaluated copy numbers and polymorphisms in *P. vivax* samples from Laos, Myanmar, and Thailand, areas with considerable differences in antimalarial drug usage and *P. falciparum* antimalarial drug susceptibility.

Dry blood samples were collected before treatment from 215 patients with acute vivax malaria from three areas: 66 came from Tak province on the Thai-Myanmar border, 50 were from elsewhere in Thailand, 50 were from Laos, and 49 were from Myanmar. The *pvmdr1* copy number was assessed by a novel real-time PCR method on a Corbett Rotor-Gene 3000 (Corbett Research, Australia). The primers and probes were PVMDR1F (CAGCCTGAAAGATT TAGAAGCCTT), PVMDR1R (CGGCTGTTGGAATCA CTTTGA), PVMDR1probe (FAM-CGGAGGAGTCGAA CGAAGATGGTTTTTCTT-TAMRA), PVTUBULINF (T CGCTTAACGACGTCCCC), PVTUBULINR (TGGAAT GTCACAAACGCTGG), and PVTUBULINprobe (VIC-TTCCGCTTCCCCCTCCACAGG-TAMRA). A QuantiTect Multiplex PCR NoROX (Qiagen, Germany) was used, and the temperature profile was prepared according to the manufacturer's instructions. The calibrator, a single-copy control, is a plasmid that was constructed by the insertion of *pvmdr1* (nucleotides [nt] 1102 to 1993) and *pvtubulin* (nt 14393 to 2354) fragments in a ratio of 1:1 into the pCR2.1 vector using a TOPO TA cloning kit (Invitrogen U.S.A.). β-Tubulin served as an internal control to normalize the amount of sample DNA added to the reactions. The relative amounts of the target genes were calculated by using the comparative C_t method. Copy numbers were calculated as follows: copy number $= 2^{-\Delta\Delta Ct}$. All assays with samples containing two copy numbers *pvmdr1* were repeated five times, and 33% of the single copy number *pvmdr1* analyses were repeated twice. The results were consistent (Fig. 1). Three fragments (496, 590, and 541 bp) of *pvmdr1*, which covered nt 158 to 653, 2752 to 3341, and 3683 to 4223, respectively, were amplified by PCR. Direct sequencing from PCR products was performed by using an ABI automated sequencer.

Double *pvmdr1* copies were significantly more common in Tak province (6 of the 66 samples) than elsewhere in Thailand (0 of 50; Fisher's exact test, $P = 0.03$). In the samples from Laos ($n = 50$) and Myanmar ($n = 49$), a double copy number *pvmdr1* was found in only two and one *P. vivax* isolates, respectively. Comparison of *pvmdr1* sequences with the published wild-type sequence *pfmdr1* (M29154) revealed that the polymorphisms found in *P. falciparum* (at codons 86, 184, 1034, 1042, and 1246) corresponded to homologous mutations in *pvmdr1* (AY618622) at codons 91, 189, 1071, 1079, and 1291, respectively (Table 1). *pvmdr1* sequences were obtained for the 21 different isolates and the Belem laboratory strain. Compared to Sal1 as the reference wild type, 48 mutations were distributed among five positions, but none of these mutations corresponded to those in *pfmdr1*. Four of the mutations in *pvmdr1* were nonsynonymous. These were at codons 133, 139, 976, 1076, and 1261, which are equivalent to codons 128, 134, 940, 1039, and 1216 in *pfmdr1* (Table 1). The recent sequencing of *pvmdr1* has revealed a single open reading frame of 4,392 bp encoding a deduced protein of 1,464 amino acids, with 12 transmembrane segments (2). Brega et al. reported two SNPs (976 and 1076) in the *pvmdr1* gene, which were not associated with chloroquine resistance in *P. falciparum.* Sa et

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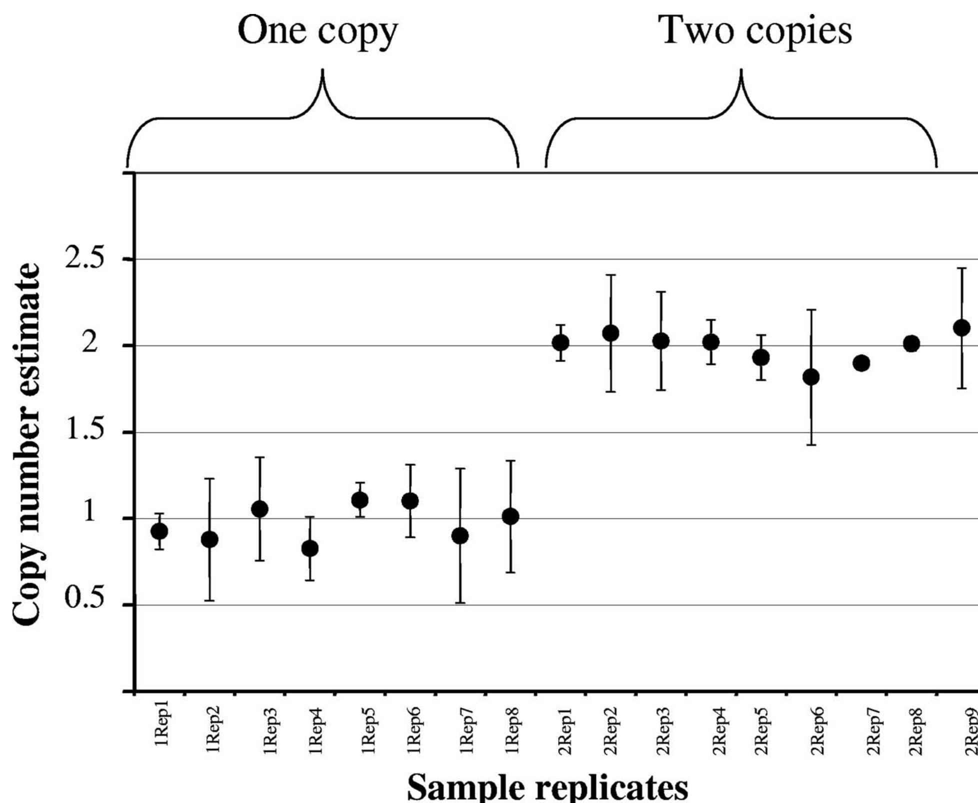

FIG. 1. Copy number estimates (with indicated confidence intervals) for parasites with one or two copies.

al. compared *pvmdr1* from 10 isolates with different levels of chloroquine sensitivity and did not find a correlation between codon mutations and resistance (9).

Gene amplification is a potentially important resistance mechanism. This study confirms *mdr1* amplification does occur in *P. vivax*, and this varied between geographical locations differing in their use of antimalarial drugs. In Tak province, on the Thai-Burmese border, mefloquine alone or in combination has been used for 23 years. In *P. falciparum* intense and sustained mefloquine pressure has been associated with high rates

TABLE 1. Mutations present in the *pvmdr1* genes amplified from *P. vivax* isolates

Isolate	Origin	Residue at codon[a]:										*Pvmdr1* copy no.
		86N/ 91N	128*K/ 133K	134*S/ 139S	184Y/ 189Y	940*Y/ 976Y	1034S/ 1071S	1039*L/ 1076F	1042N/ 1079N	1216Q/ 1261K	1246D/ 1291D	
Belem	Brazil	.	.	.	.	.	.	.	.	E	.	1
PV3217	Thailand	.	.	.	.	.	.	.	.	E	.	1
PV3247	Thailand	.	.	.	.	.	.	.	.	E	.	1
PV3224	Thailand	.	N	R	.	.	.	.	.	E	.	1
PV3265	Thailand	.	.	.	.	.	.	L	.	E	.	1
PV3251	Thailand	.	.	.	.	F	.	L	.	E	.	1
L20	Laos	.	.	.	.	.	.	L	.	E	.	1
Lao 2003 15	Laos	.	N	R	.	.	.	L	.	E	.	1
Lao 2003 32	Laos	.	N	R	.	.	.	L	.	E	.	1
P122	Myanmar	.	.	.	.	.	.	.	.	E	.	1
P137	Myanmar	.	.	.	.	.	.	.	.	E	.	1
P90	Myanmar	.	.	.	.	F	.	L	.	E	.	1
P143	Myanmar	.	.	.	.	F	.	L	.	E	.	1
P152	Myanmar	.	.	.	.	F	.	L	.	E	.	1
PV3230	Thailand	.	.	.	.	.	.	L	.	E	.	2
PV3222	Thailand	.	.	.	.	.	.	L	.	E	.	2
PV2677	Thailand	.	.	.	.	.	.	L	.	E	.	2
PV2667	Thailand	.	.	.	.	.	.	L	.	E	.	2
PV3232	Thailand	.	.	.	.	.	.	L	.	E	.	2
PV3246	Thailand	.	.	.	.	.	.	L	.	E	.	2
L10	Laos	.	.	.	.	.	.	L	.	E	.	2
L27	Laos	.	.	.	.	.	.	L	.	E	.	2

[a] *, No mutant type was found at codons 128, 134, 940, and 1039 in *pfmdr1*, Wild-type residues (position number and residue) are as indicated in the subheadings in the form: *P. falciparum* (accession no. M29154)/*P. vivax* (accession no.618622). A period (.) in the table indicates no change from the wild-type sequence.

of selection for *pfmdr1* amplification (13). In other areas of Thailand, Myanmar, and Laos, there has been less exposure of parasites to mefloquine. Gene amplifications of *pvmdr1* were significantly more common in isolates from Tak (6 of the 66 samples) than in those from patients from other provinces of Thailand, Myanmar, and Laos ($P = 0.02$), suggesting that there is a relationship between the *pvmdr1* copy number and widespread deployment of mefloquine. The degree of amplification is low currently (no more than two copies), whereas in *P. falciparum* up to five copies of *pfmdr1* have been observed in this region. Point mutations at codons 133, 139, 976, 1076, and 1261 of the *pvmdr1* gene (corresponding to codons 128, 134, 940, 1039, and 1216, respectively, in *pfmdr1*) were found in this study. Mutations at codons 133, 139, and 1261 are located outside the transmembrane segment. Two point mutations at codons 976 and 1076 are located in the putative transmembrane segments X and XI. Double mutations (976 and 1076) in these segments were found in the samples from three areas (31%, $n = 13$). The Y976F mutation in *P. vivax* has been correlated with reduced susceptibility to chloroquine (10). This mutation, seen in 1 of 11 Thai and 3 of 5 Myanmar isolates examined here, may affect chloroquine efficacy against *P. vivax* in this region and is worthy of further exploration. Mutations in *pvmdr1* were independent of *pvmdr1* amplification. Sal1, a laboratory-adapted *P. vivax* strain usually regarded as the wild type, was found to possess residue F at position 1076, while the residue L was identified at the correspond codon in *pfmdr1* (position 1039, accession no M29154; strain FC27, D10) and *pcmdr1* (AY123625), suggesting that the residue L might be a neutral allele. None of the corresponding mutations in *pfmdr1* were observed in *pvmdr1*. This suggests that there may be different mechanisms conferring chloroquine resistance in *P. vivax* compared to *P. falciparum*.

Amplification of *pfmdr1* has occurred multiple times in *P. falciparum* (5, 12). This is evident from the ready intrahost selection, the patterns of polymorphism in flanking microsatellite markers, and from the different-sized fragments of chromosome 5 regions containing *pfmdr1* that are amplified in different isolates. In contrast, the fact that all *P. vivax* isolates carrying multiple copies of *pvmdr1* have the same point mutations suggests that this gene amplification has a single origin.

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