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Plasmodium vivax Isolates from Cambodia and Thailand Show High Genetic Complexity and Distinct Patterns of *P. vivax* Multidrug Resistance Gene 1 (*pvmdr1*) Polymorphisms

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Abstract. *Plasmodium vivax* accounts for an increasing fraction of malaria infections in Thailand and Cambodia. We compared *P. vivax* genetic complexity and antimalarial resistance patterns in the two countries. Use of a heteroduplex tracking assay targeting the merozoite surface protein 1 gene revealed that vivax infections in both countries are frequently polyclonal (84%), with parasites that are highly diverse ($H_E = 0.86$) but closely related ($G_{ST} = 0.18$). Following a history of different drug policies in Thailand and Cambodia, distinct patterns of antimalarial resistance have emerged: most Cambodian isolates harbor the *P. vivax* multidrug resistance gene 1 (*pvmdr1*) 976F mutation associated with chloroquine resistance (89% versus 8%, $P < 0.001$), whereas Thai isolates more often display increased *pvmdr1* copy number (39% versus 4%, $P < 0.001$). Finally, genotyping of paired isolates from individuals suspected of suffering relapse supports a complex scheme of relapse whereby recurrence of multiple identical variants is sometimes accompanied by the appearance of novel variants.

INTRODUCTION

Plasmodium vivax accounts for an increasing fraction of malaria cases in Thailand and Cambodia.[1–3] It is now recognized that *P. vivax* poses a greater challenge to malaria elimination in the region, largely caused by its propensity for relapse.[4,5] Little is known about the transmission dynamics of vivax parasites in Southeast Asia and their evolving drug resistance patterns. Molecular tools can provide insights into these processes by evaluating the diversity of parasites and assessing for polymorphisms correlated with drug resistance. They also have the potential to identify genotypic patterns of relapsing parasites.[6] These data can provide a framework for monitoring control efforts and informing interventions targeted at elimination.

The interplay between malaria genetic diversity, multiplicity of infection (MOI), and emergence of drug resistance is complex. The MOI is the number of parasite clones found simultaneously within a host, a measure of within-host genetic complexity. While parasite diversity on a population scale allows for resistant parasite populations to expand under drug pressure, in-host diversity impacts the development of drug resistance by affecting sexual recombination rates and allowing competitive suppression among co-infecting drug-resistant and drug-sensitive clones.[7,8]

Previous studies have shown high genetic diversity but limited MOI among *P. vivax* infections in Southeast Asia.[9–12] However, the genotyping methods used in these studies are often limited in their ability to detect minority clones, leading to an underestimation of MOI.[8] The heteroduplex tracking assay (HTA) is a genotyping technique that has proven adept at uncovering minority variants within malaria infections and in infections with other agents.[13–16] Thus, it provides a more accurate picture of within-host genetic diversity.[17] It also facilitates the tracking of clones within hosts over time, potentially providing a nuanced portrait of relapsing vivax populations.[18]

Additionally, there is concern for emerging chloroquine-resistant vivax in Southeast Asia; however, reports concerning putative vivax drug resistance genes remain limited. Studies of *P. vivax* multidrug resistance gene 1 (*pvmdr1*), the vivax analogue to the multidrug resistance gene found in *P. falciparum*, have linked a polymorphism (Y976F) in this gene to chloroquine resistance, whereas an increased copy number of the gene is associated with increased susceptibility to chloroquine and reduced susceptibility to mefloquine, amodiaquine, and artesunate *in vitro*.[19–22] Variable prevalence of mutant single nucleotide polymorphisms (SNPs) and copy number increases in *pvmdr1* have been reported from Thailand, however little comparative data has been published from Cambodia.[19,21–26] Because this region has played an important role in the evolution of drug-resistant falciparum malaria, a better understanding of the distribution of vivax drug resistance alleles in the parasite populations is needed.

In this report, we use an HTA and nested polymerase chain reaction (nPCR) assay for *P. vivax* merozoite surface protein 1 (*pvmsp1*), a real-time PCR assay for *pvmdr1* copy number, and *pvmdr1* sequencing on parasite isolates from Thailand and Cambodia to determine: 1) the relative genetic diversity and multiplicity of infection of *P. vivax* infections in these regions, 2) differences in the patterns of molecular markers of antimalarial resistance between the two countries, and 3) genotype patterns of paired initial and recurrent vivax infections in Cambodia. Together, these data provide a comparative view of drug resistance patterns and the genetic factors that may shape the future development of drug resistance in these areas.

MATERIALS AND METHODS

Patient samples. Filter paper blood spots from vivax patients were acquired from clinical studies carried out in northwestern Thailand and southern Cambodia. Vivax malaria cases occur throughout the year in both locales, but most cases

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occur during the rainy season. Thai samples ($N = 50$) were collected from patients presenting to public health clinics in the Mae Sod district of Tak Province between February and June 2009. Cambodian samples were collected between August 2006 and December 2007 as part of a chloroquine treatment trial at the Chumkiri health center in Kampot Province.[27] Details of the study have previously been published. Anti-relapse therapy with primaquine was not given. One hundred and ten (110) subjects were enrolled, 45 of whom developed recurrent infection within the 42-day follow-up. One hundred and nine (109) enrollment isolates and 44 recurrent isolates were available for study.

Ethics statement. Written informed consent was obtained from all participants, with approval from the ethics committees of Walter Reed Army Institute of Research, the Naval Medical Research Unit 2 (Jakarta, Indonesia), the Ministry of Public Health in Thailand (Bangkok, Thailand), the National Ethics Committee for Health Research (Phnom Penh, Cambodia), and the University of North Carolina.

HTA tracking assay and nPCR of *pvmsp1*. The strategy used to develop the *pvmsp1* HTA was based on that previously used to develop HTAs for *Plasmodium falciparum*.[13,17] Four radiolabeled probes targeting 500 bp of interspecies conserved blocks (ICB) 5&6 of the *Pvmsp1* gene and the variable region in between were developed—two based on *P. vivax* strains found in the MR4 repository (Nicaragua, Pakchong), and two generated from Thai clinical samples (Th04, Th06). The PCR products amplified using the primers and conditions listed in Table 1 were blunt-end cloned into the pT7Blue vector (Novagen, Inc., Madison, WI) and sequenced to confirm *pvmsp1* insertion. Ten micrograms (10 μg) of each *pvmsp1* ICB5-6 pT7Blue construct was then digested, end-labeled, and digested again to release the ^{35}S-labeled probes as previously described. Heteroduplexes containing ^{35}S-labeled probe and 8 μL of PCR-amplified ICB5-6 of *Pvmsp1* from patient samples were formed in annealing reactions and separated by electrophoresis on a 6% polyacrylamide gel, and then exposed to BioMax MR x-ray film (Eastman Kodak, Rochester, NY) for 24–72 hours at room temperature.

The HTA bands were considered unique *pvmsp1* sequence variants if they were not in the single-stranded probe or probe homoduplex lanes (Figure 1A). Because all four probes were able to distinguish multiple minority variants in the clinical samples, the Nicaragua probe (NIC) (GenBank accession no. JN674534) was chosen for application of the HTA to the study samples, as it was expected to contain the sequence that was the most different from the Thai/Cambodian samples. Supplemental Figure 1 provides a comparison of the *pvmsp1* ICB5-6 probe sequence to the Sal1 and Belem strains and several Thai sequences from GenBank.

A previously published nPCR assay targeting ~700 bp of the same ICB5-6 region was selected as a comparison assay for measuring MOI.[29,30] The assay was carried out as previously described using Hotstar master mix (Qiagen, Valencia, CA), 800 nM of the forward and reverse primers, and 2 μL of parasite DNA in each reaction. The PCR products were run on a 1.8% agarose gel with the presence of mixed genotypes determined by size polymorphisms (Figure 1B and C). Randomly selected products were also run on a 3.0% agarose gel but did not result in a greater number of distinguished bands per sample. Plasmid DNA containing the cloned *Pvmsp1* fragment of the Nicaragua strain (cat. MRA-340; MR4, Manassas, VA) was used as a positive control for all assays.

Measuring MOI and genetic diversity. The MOI was defined as the number of genotypic clones detected per patient isolate by counting either the number of size variants detected by nPCR or the number of sequence variants detected by HTA (Figure 1). The MOI was determined by both assays on all enrollment samples (Thai [$N = 50$] and Cambodian [$N = 109$]). To distinguish allelic variants in the HTA, the distance migrated by each *pvmsp1* variant was measured and assigned a relative migration index (R_f). The R_f units were divided among 20 bins of 0.05 R_f units, a method that has previously been validated.[17] When more than one band fell within the same bin, they were assigned the same R_f. Allele frequencies were calculated as the prevalence of a clone with a unique R_f among the total number of clones within each geographical cohort. The genetic diversity within each population was estimated by calculating the virtual heterozygosity (H_E).[8] In this context, H_E gives the average probability that two clones taken at random from the population are different. The genetic divergence between populations was estimated by calculating Nei's genetic distance (similar to the fixation index or FST) using Genetic analysis in Excel (GenAlEx) software v6.1.[31,32] Recurrent isolates were not included in these calculations to avoid sampling bias.

Real-time PCR for *Pvmdr1* copy number. A novel multiplex Taqman assay was developed to estimate *pvmdr1* copy

TABLE 1

Primers and probe sequences used in the heteroduplex tracking assay (HTA) and *Pvmdr1* assays

Genetic marker	Primer/probe sequence	Amplification program
Pvmsp1		
HTA forward*	5′-GATGGTCCTCAAAAGGGAAA	95°C × 15′; 35 cycles of
HTA reverse*	5′-GACATGCGTAAGCGGATTTT	94°C × 1′, 55°C × 2′, and 72°C × 2′; 72°C × 5′
Pv aldolase		
Forward	5′-GACAGTGCCACCATCCTTACC	
Reverse	5′-CCTTCTCAACATTCTCCTTCTTTCC	
Probe	5′-VIC-ATTCCATCAATGCGTTAGGC-MGB	
Pvmdr1		
Copy number forward	5′-CGATTGAAGATTCCGAAGTTG	50°C ×2′; 95°C × 10′; 40
Copy number reverse	5′-TTAGAACCCACCAGCGTTTC	cycles of 95°C × 15″ and
Copy number probe	5′-6FAM-GAAGGTGCTGATCCACGATT-MGB	60°C × 1′
Sequencing forward	5′-ATAGTCATGCCCCAGGATTG	95°C × 15′; 35 cycles of
Sequencing reverse	5′-CCTTTCGAAGGACAGCTTTG	95°C × 15″, 55°C × 30″, and 72°C × 1′; 72°C × 10′

*Primers modified from Maestre and others.[28]

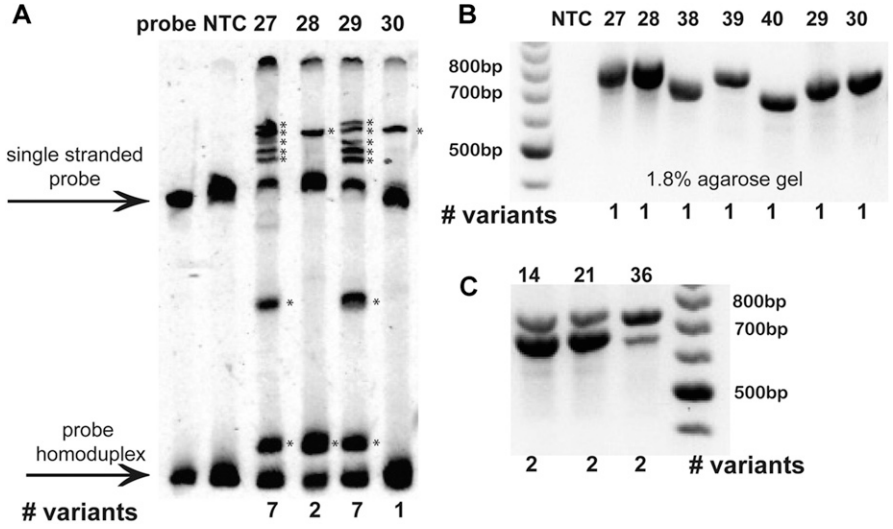

FIGURE 1. Representative *pvmsp1* heteroduplex tracking assay (HTA) (**A**) and nested polymerase chain reaction (nPCR) (**B**, **C**) for selected Thai samples. Panel A shows a representative HTA. Each lane contains bands representing the single-stranded probe and probe homoduplex (seen in the NTC and probe alone lanes). Patient numbers are indicated at the top of each lane. Unique *pvmsp1* sequence variants were identified by counting bands that were not in the single-stranded probe or probe homoduplex lanes. These represent heteroduplexes formed between the probe and amplified PCR products from each patient sample. Heteroduplexes can migrate both above and below the single-stranded probe and are marked with asterisks in the figures. Panel B shows the corresponding nested PCRs for the patient samples shown in the HTA, demonstrating that a higher number of variants was detected by HTA in most of these samples (except patient 30 who had a single variant by each method). Panel C shows a representative gel of patient samples containing multiple variants by nPCR.

number using *P. vivax* aldolase (*Pv aldolase*) as the reference gene. Aldolase primers were previously described.[19] The other primers and probes were designed with Primer Express Software v3.0 (Applied Biosystems, Foster City, CA) (Table 1). Real-time PCR was performed on an ABI 7300 Real-Time PCR system (Applied Biosystems) in duplicate 20 μL reactions containing Roche FastStart Universal Probe Master mix, 800 nM of *pvmdr1* forward and reverse primers, 400 nM of *pvaldolase* forward and reverse primers, 100 nM of each probe, and 2 μL of parasite DNA, with cycling conditions listed in Table 1. Plasmids containing the cloned fragments of *pvmdr1* and *pvaldolase*, developed using the TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA), were used as positive controls in every experiment. Threshold cycle (Ct) values were exported and analyzed in Microsoft Excel using the Pfaffl method to determine copy number.[33] Copy number was determined by rounding to the nearest integer and was considered increased if > 1.

Sequencing for *Pvmdr1* mutant SNPs. Primers were designed to amplify a 647 bp region of the *pvmdr1* gene containing the Y976F SNP and the F1076L SNP (Table 1). The PCR amplification was carried out in 50 μL reactions containing 5 μL of 10× PCR buffer, 20 μM of forward and reverse primers, of 0.2 mM dNTP, 0.25 μL of Qiagen Hotstar taq, and 5 μL of parasite DNA with cycling conditions listed in Table 1. The amplified products were directly sequenced by Eton Bioscience, Inc. (Research Triangle Park, NC) (GenBank accession nos.: JQ925719–JQ925865).

Statistical analysis. Median MOI was compared between the two *Pvmsp1* genotyping assays (HTA and nPCR) using the Wilcoxon rank-sum test. Possible associations between MOI and clinical features including age, gender, baseline parasitemia, parasite clearance time in days, and *pvmdr1* copy number were investigated using the Wilcoxon rank-sum test or linear regression as appropriate. The proportion of samples harboring increased *pvmdr1* copy number or the Y976F and F1076L mutations were compared between the Thai and Cambodian study populations using the Pearson χ^2 or Fisher's exact tests. Genotypes of initial and recurrent isolates were compared for relatedness, and for MOI and *pvmdr1* copy number status using paired *t* tests. Statistical analysis was done using STATA v.12.0 (STATA Corp, College Station, TX).

RESULTS

Multiplicity of infection. Compared with nPCR, the HTA detected more polyclonal infections, revealing a high multiplicity of infection in both Thailand and Cambodia (Figure 1). Of the 47 Thai isolates that successfully amplified in both assays, 39 (83%) displayed multiple clones by HTA, whereas only 6 (13%) displayed more than one size polymorphism by nPCR Figure 2A, Table 2. Similarly, among the Cambodian enrollment isolates, 92 (84%) were multiclonal by HTA versus 27 (25%) by nPCR (Figure 2B). The HTA detected up to 7 clones within single individuals, whereas nPCR very rarely detected more than 2 clones (only 1 of 156 isolates had 3 variants). The mean MOIs for the Thai and Cambodian samples as determined by HTA were very similar: 3.0 and 2.9, respectively, with a combined mean MOI of 2.9 (interquartile range [IQR] 2–4). Among the Cambodian trial participants, no association was found between MOI at enrollment and age, gender, baseline parasitemia, parasite clearance time, or *pvmdr1* copy number.

***Pvmsp1* sequence variation and allelic diversity.** In total, 15 distinct *pvmsp1* alleles and 30 unique genotypes (representing combinations of allelic clones) were detected among the 47 Thai isolates, whereas 16 unique alleles and 66 unique genotypes were detected among the 109 Cambodian isolates (Figure 3). Five common allelic variants accounted for 75% of

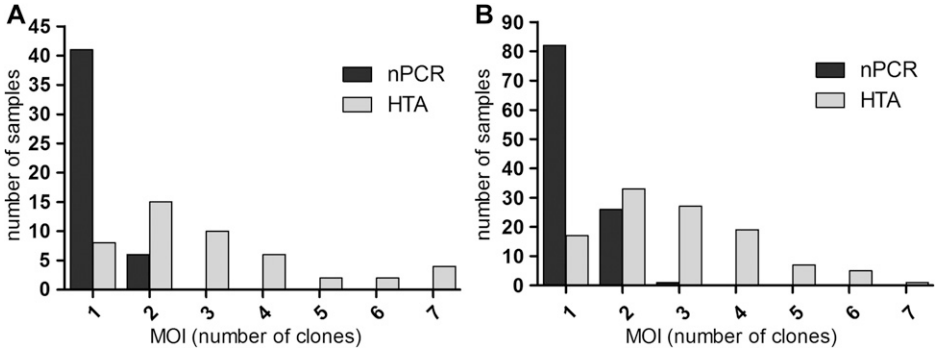

FIGURE 2. Distribution of multiplicity of infection. Number of coinfecting *Pvmsp1* clones per isolate detected by nested polymerase chain reaction (nPCR) versus heteroduplex tracking assay (HTA) among the Thai samples ($N = 47$) (**A**) and Cambodian enrollment samples ($N = 109$) (**B**).

Thai clones, whereas six common variants accounted for 87% of Cambodian clones. The distribution of alleles appeared similar between the two regions. Individual allele frequencies ranged from 0.2% (occurring once) to 28%. Based on these allele frequencies, H_E at the *pvmsp1* locus was 0.86 in Thailand and 0.86 in Cambodia, whereas Nei's genetic distance between the two populations was 0.18.

***Pvmdr1* copy number and SNP analysis.** Thai isolates were more likely to display increased *pvmdr1* copy number compared with the Cambodian isolates (39% versus 4%, $P < 0.001$). Among the Thai samples, 61% (30 of 49) contained one copy of *pvmdr1*, 33% (16 of 49) had two copies, and 6% (3 of 49) had three copies. In contrast, only 4% (4 of 109) of the Cambodian enrollment samples displayed increased copy number (Figure 4). However, the 976F mutation was much more prevalent among the Cambodian isolates. Eighty-nine percent (83 of 93) of the enrollment samples harbored the Y976F mutant SNP versus just 8% (2 of 26) of the Thai samples ($P < 0.001$) (Figure 4). Most of the 83 Cambodian samples that were positive for the Y976F mutation also carried the F1076L mutation, making them double mutants; only two samples were mutant at codon 976 but wild-type at 1076. On the other hand, 62% (16 of 26) of Thai samples were single 1076L mutants, remaining wild-type at codon 976 while carrying the F1076L mutation.

Recurrent and relapsing genotypes. When sampling recurrent infections from an endemic area, it is impossible to distinguish with confidence which recurrences are relapses versus reinfections or recrudescence from treatment failure. However, we have previously reasoned that the majority of the 44 recurrent parasitemias from the Cambodian study represent relapses.[18] Previously, we reported the use of HTA in this cohort to identify variants specifically associated with relapse. However, this does not provide a complete picture of the potentially complex nature of recurrent parasitemias. Therefore, here we provide a more detailed examination of the genotypic patterns associated with these recurrences as determined by HTA. Paired isolates were classified into different categories of relatedness based on the number of shared variants between the initial and recurrent sample. Recurrent genotypes were "related" if at least one of the variants detected initially was also observed in the recurrent isolate and "different" if none of the variants detected initially was observed in the recurrent sample. A genotype was considered "novel" if at least one variant detected in the recurrent sample was not detected initially. Based on this classification system, adapted from other studies,[12] the vast majority of paired infections were related (41 of 44 or 93%), whereas only 3 of 44 (6.8%) pairs were different (Figure 5). Among the related pairs, 13 of 41 recurrent genotypes were identical, 18 of 41 were not identical but did not contain any novel variants, and 10 of 41 contained novel variants. In 29 of 41 (71%) of these related pairs, both initial and recurrent genotypes were multiclonal.

In most of the 41 genotypic pairs that were related, at least half of the variants in the recurrent genotype were also detected in the initial isolate, making them highly related.

TABLE 2

MOI of Thai and Cambodian vivax samples detected by HTA versus nPCR assays for *Pvmsp1**

		Multiplicity of infection†			% Multiclonal	
Sample origin	No.	HTA	nPCR	*P* value‡	HTA	nPCR
Thailand	47	3.0 (2–4)	1.1 (1–1)	< 0.001	83%	13%
Cambodia	109	2.9 (2–4)	1.3 (1–1)	< 0.001	84%	25%
Combined	156	2.9 (2–4)	1.2 (1–1)	< 0.001	84%	21%

*MOI = multiplicity of infection; HTA = heteroduplex tracking assay; nPCR = nested polymerase chain reaction.
†Reported as mean MOI (interquartile range).
‡*P* value comparing MOI of HTA versus nPCR by Wilcoxon rank-sum test.

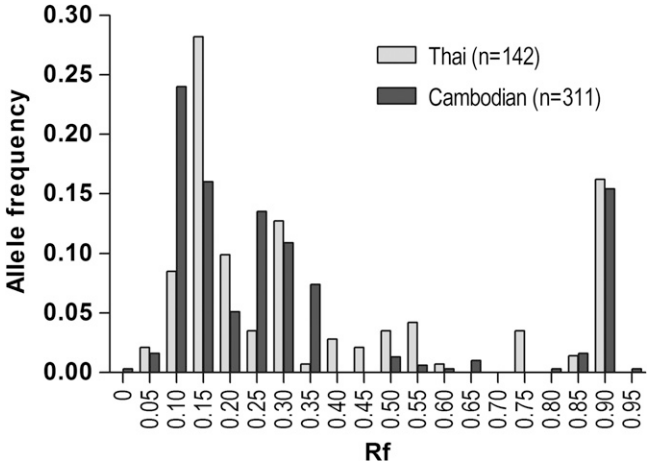

FIGURE 3. Frequency of *pvmsp1* sequence variants in enrollment samples by heteroduplex tracking assay (HTA) migration index (R_f). Total $N = 453$ clones (142 Thai and 311 Cambodian) corresponding to 156 vivax infections. Five common variants accounted for 75% of Thai clones, whereas six common variants accounted for 87% of Cambodian clones.

FIGURE 4. Proportion of Thai versus Cambodian isolates with *pvmdr1* Y976F and F1076L mutants (**A**, **B**) and increased *pvmdr1* copy number (**C**, **D**). $N = 121$ (26 Thai and 122 Cambodian) for sequencing data and $N = 158$ (49 Thai and 150 Cambodian) for copy number data. Thai isolates were more likely to display increased copy number (39% of Thai versus 4% of Cambodian isolates with CN > 1), whereas Cambodian isolates were more likely to carry the 976 mutant allele (89% versus 8% of Thai isolates).

Twenty-six of 38 (68%) of these highly related recurrences were multiclonal, with multiple clones matching the initial genotype, making it unlikely that they represent reinfections. All of these pairs, suspected of being relapses, showed either no change or a reduction in MOI from the initial to recurrent sample.

DISCUSSION

Our study offers several important observations about the genetic diversity, drug resistance patterns, and relapse patterns of *P. vivax* infections in Southeast Asia. First, despite being a region of relatively low transmission, most infections (84%) were polyclonal with a mean MOI of 2.9, a finding consistent between Thailand and Cambodia. This is comparable to estimates of MOI in children in a hyperendemic region of Papua New Guinea (74% multiclonal infections, mean MOI of 2.7) determined by similarly discriminatory methods.[34] This finding supports growing evidence that *P. vivax* complexity rivals that of *P. falciparum* in areas where the species are co-endemic.[34,35] Previous estimates of the prevalence of mixed strain genotypes in Thailand have come close to this level, but have relied on typing multiple microsatellite markers, whereas the HTA sensitively detects multiple alleles or clones at a single locus. A limitation of the HTA is its reliance on the migration index (R_f) to indirectly discriminate between different sequence variants. The genetic relatedness between variants cannot be deduced as may be possible with microsatellite variants with similar numbers of repeats. Furthermore, it is hard to precisely compare multi-allelic genotypes across different gels. We grouped the *pvmsp1* variants into 20 different bins based on their R_f, but there were instances when more than one unique band was found within one bin. Although these bands likely represented unique clones, we assigned them the same genotype. This binning system has the potential to underestimate MOI and allelic diversity, but we were still able to show a great range of *pvmsp1* diversity consistent with other studies.[35]

Why might MOI of *P. vivax* in a hypoendemic region of Southeast Asia reach the same levels as that seen in the hyperendemic region of Papua New Guinea? Transmission intensity is an important determinant of MOI, but factors relating to the intrinsic biology of *P. vivax* likely also contribute to higher MOI in hypoendemic regions. These factors include the contribution of vivax relapses, with the potential for reactivation of multiple heterologous hypnozoites at the same time. The production and circulation of gametocytes early in *P. vivax* infection also leads to 1) more opportunities for recombination when multiple gamete clones are present and 2) individuals with multiclonal infections becoming infectious before they receive antimalarial treatment that quickly eliminates drug-sensitive clones and reduces MOI.[4,12,36] The transmission of multiple gametocyte clones at one time has been shown in *P. falciparum* and is expected to be an important contributor to MOI in areas of low transmission.[37–39] Because increased MOI facilitates recombination, these forces could allow *P. vivax* to maintain high genetic diversity despite lower levels of transmission.

Second, we have shown that despite similar allelic diversity and close kinship measured at the *pvmsp1* locus within Thailand and Cambodia, the parasites display distinct drug resistance patterns. The H_E was high for both locations, whereas genetic divergence between the two was low, suggesting similar genetic backgrounds. However, *pvmdr1* resistance patterns were markedly different: a substantial proportion of parasites from northwestern Thailand displayed increased copy number but few carried the 976F mutant allele, whereas most Cambodian parasites carried the mutant allele without increased copy number. Sampling of the Thai and Cambodian samples in our study was not concurrent, with the Cambodian

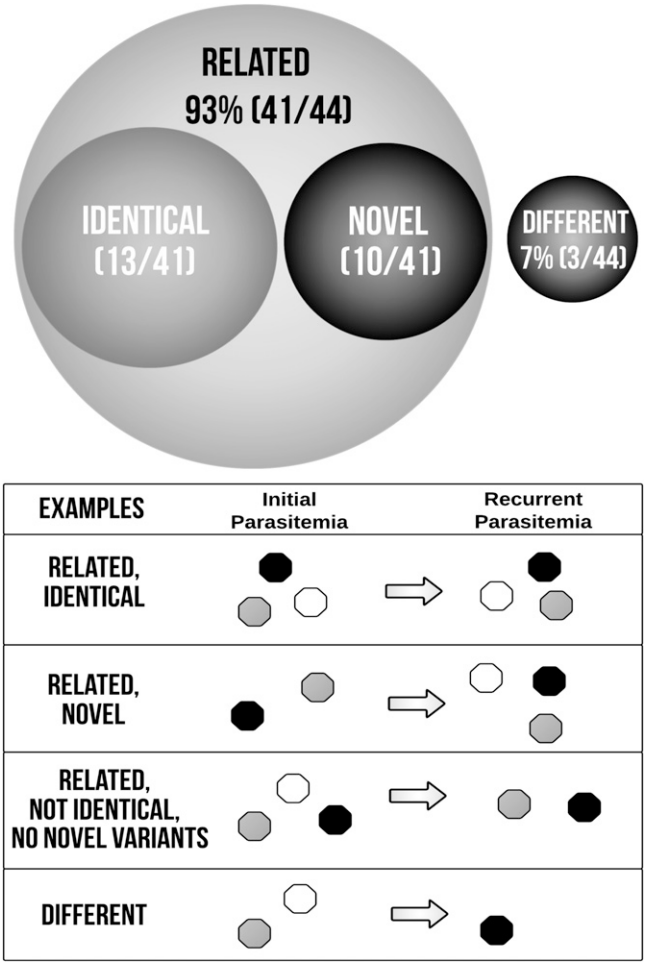

FIGURE 5. Categories of relatedness for 44 paired initial and recurrent vivax parasitemias. Genotypic patterns of 44 paired isolates from persons with recurrent vivax infection were categorized as related (at least one of the variants detected in the initial sample was observed in the recurrent sample), different (none of the variants detected in the initial sample was observed in the recurrent sample), or novel (at least one variant detected in the recurrent sample was not detected in the initial sample). The majority of genotypic pairs were related (93% or 41 of 44). In 29 of 41 (71%) of these related pairs, both initial and recurrent genotypes were multiclonal. Representative examples are diagrammed in the lower panel.

samples collected 2–3 years before the Thai samples. However, the prevalence of 976F mutants in our 2006–2007 Cambodian cohorts closely matched the prevalence found in samples collected in 2008 from an area just north of Chumkiri.[25] No prior studies measuring *pvdmr1* copy number in Cambodia have been reported. Our Thai copy number data show a slightly higher proportion of increased copy number isolates (39%) than previous studies in which 7–23% (cumulative 23 of 164 or 14%) of western Thai isolates collected from 2003 to 2007 displayed increased copy number[19,21,22,24]; however, our isolates were also collected later in time. The same studies reported 9–25% prevalence of the 976F mutant, slightly greater than the 8% prevalence in our sample. Interestingly, *P. falciparum* isolates from Thailand show a synonymous pattern of contrasting *Pfmdr1* drug resistance profiles at the Thai-Myanmar versus Thai-Cambodian borders, with western isolates having higher *mdr1*copy numbers, and eastern isolates on the Cambodian border carrying the mutant 184F allele.[40,41]

Selection for different *Pvmdr1* haplotypes has likely been shaped by different drug policies in the two countries. Chloroquine monotherapy was still the official drug policy for *P. vivax* in Cambodia until very recently, whereas in Thailand a combination of chloroquine and primaquine has been the recommended regimen for over 30 years. Because primaquine also has efficacy against blood-stage vivax parasites,[42] the Thai drug policy aimed at radical cure may have delayed the development of chloroquine resistance by administering combination therapy. Additionally, a longer history of mefloquine therapy in Thailand, with a gradual switch from mefloquine monotherapy to artemisinin-based combination therapy with artesunate-mefloquine from 1995 to 2005, may have prevented the accumulation of 976F mutants. The increased *pvmdr1* copy number of Thai vivax parasites suggests that they have responded to mefloquine drug pressure in the same fashion as their falciparum counterparts, likely in the context of highly prevalent mixed falciparum/vivax infections.[2]

The *pvdmr1* profile of the Cambodian parasites in our study resembles that of multidrug-resistant vivax in Papua New Guinea. However, the *in vivo* trial from which our samples were collected identified no vivax recurrences before Day 28 that would be suggestive of treatment failure. Unfortunately, there is not enough evidence to determine whether the high prevalence of double 976F/1076L *pvmdr1* mutants in the Cambodian parasites heralds increasing tolerance to chloroquine. It is also important to note that because all current *pvmdr1* assays are PCR based, they cannot account for the presence of multiple clones that may have varying degrees of drug resistance; sequences and copy numbers are likely a reflection of the dominant clones existing within samples. As a result of increasing evidence of rising clinical chloroquine resistance in Cambodia, as of 2012, dihydroartemisinin-piperaquine has been adopted by the National Malaria Program for treatment of all malaria species, whether *P. falciparum* or *P. vivax*.

As opposed to prior studies of vivax relapses, which have concluded that approximately two-thirds of vivax relapses in adults display *different* genotypes from the initial primary infection,[12,29,43] we found high degrees of relatedness in paired initial and recurrent isolates from Cambodia when accounting for minority variants at a single locus. The HTA was highly discriminatory at *pvmsp1* (H_E 0.86), but still showed frequent recurrence of multiple variants matching the initial genotype. Similarly, when de Araujo and others[44] accounted for rare alleles detected by sequencing 10 microsatellite markers, they found that 88% of relapses met their definition of homologous relapse. Our relapse data are limited by the inability to distinguish with confidence which recurrences are relapses versus reinfections or recrudescence when sampling persons in an endemic setting. However, as discussed elsewhere in more depth, the lack of primaquine dosing in these persons, combined with the timing of recurrences after treatment (between Day 35 and Day 42), and the overall low incidence of malaria in the area lead us to believe that relapses comprised a significant proportion of the recurrences we studied.[18]

If the recurrence of multiple identical variants matching the initial genotype identifies a relapse as we suspect, our findings support a highly complex scheme of relapse. In initial infections with multiple genetically distinct vivax clones, all or a

subset of these multiple clones can reactivate simultaneously to cause relapse. At the same time, 30% of recurrent samples showed novel variants, suggesting that latent hypnozoites unrelated to the initial infection may also be reactivated at the time of relapse. Together, a picture of reactivation of multiple homologous hypnozoites, sometimes accompanied by heterologous hynozoites, emerges. This schema of relapse could help explain the high MOI we observed among vivax parasites in the region.

CONCLUSIONS

We have used a range of molecular assays to characterize *P. vivax* populations from Thailand and Cambodia, including those causing relapse. Together, these assays have drawn a portrait of 1) high genetic diversity both within and among hosts, rivaling that of *P. falciparum*; 2) different resistance profiles among parasites in Thailand versus Cambodia shaped by unique drug pressures within the two countries; and 3) frequent relapses caused by multiclonal populations that are related to preceding infections. These findings expand our knowledge of the unique factors influencing the development of drug-resistant vivax and provide insight into potential barriers to malaria elimination in Southeast Asia.

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Note: Supplemental figure appears at www.ajtmh.org.

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