Malaria is the most important public health problem in several countries. In Thailand, co-infections of Plasmodium vivax and Plasmodium falciparum are common. We examined the prevalence and patterns of mutations in P. vivax dihydrofolate reductase (Pvdhfr) and P. vivax dihydropteroate synthase (Pvdhps) in 103 blood samples collected from patients with P. vivax infection who had attended the malaria clinic in Mae Sot, Tak Province during 2009 and 2010. Using nested polymerase chain reaction-restriction fragment length polymorphism, we examined single nucleotide polymorphisms-haplotypes at amino acid positions 13, 33, 57, 58, 61, 117 and 173 of Pvdhfr and 383 and 553 of Pvdhps. All parasite isolates carried mutant Pvdhfr alleles, of which the most common alleles were triple mutants (99%). Eight different types of Pvdhfr and combination alleles were found, as follows: 57I/58R/117T, 57I/58R/117T, 57I/58R/117T/N, 57L/58R/117T, 57L/58R/117T, 58R/61M/117N, 58R/61M/117N and 13L/57L/58R/117T. The most common Pvdhps alleles were 57I/58R/117T (77.7%), 57I/58R/117T/N (1%), 57L/58R/117T (5.8%) and 58R/61M/117N (14.5%). The most common Pvdhps alleles were 57L/58R/117T (77.7%), 57I/58R/117T/N (1%), 57L/58R/117T (5.8%) and 58R/61M/117N (14.5%). Additionally, we recovered one isolate of a carrying a quadruple mutant allele, 13L/57L/58R/117T. The most prevalent Pvdhps allele was a single mutation in amino acid 383 (82.5%), followed by the wild-type A383/A553 (17.5%) allele. Results suggest that all P. vivax isolates in Thailand carry some combination of mutations in Pvdhfr and Pvdhps. Our findings demonstrate that development of new antifolate drugs effective against sulfadoxine-pyrimethamine-resistant P. vivax is required.

Key words: Plasmodium vivax - Plasmodium vivax dihydrofolate reductase - Plasmodium vivax dihydropteroate synthase
the prevalence and diversity of \textit{Pvdhfr} and \textit{Pvdhps} mutant alleles in \textit{P. vivax} isolates collected from Mae Sot District, an endemic area of Thailand along the Thai-Myanmar border. This information will assist in the development of effective new antifolates.

**SUBJECTS, MATERIALS AND METHODS**

Study areas and sample collection - A total of 103 blood samples with \textit{P. vivax} mono-infection were collected from patients attending malaria clinics in Mae Sot during 2009 and 2010. Approval for the study protocol was obtained from the Ethics Committees of Mae Sot General Hospital, Tak Province. Blood samples of between 200-300 µL were collected by finger-prick onto filter paper (Whatman 3). Dried filter paper samples were stored in small zipper plastic bags prior to the extraction of parasite DNA for analysis by polymerase chain reaction (PCR). Giemsa-stained thin and thick blood smears were prepared and examined microscopically for the presence of \textit{P. vivax} parasites.

Extraction of parasite genomic DNA - Parasite genomic DNA was extracted from individual dried blood spots on filter paper using the QIAamp DNA Extraction Mini-kit (QIAGEN) and used as the template for PCR amplification.

Amplification of \textit{Pvdhfr} - Point mutations of \textit{Pvdhfr} in all \textit{P. vivax} isolates were investigated by nested PCR-restriction fragment length polymorphism (PCR-RFLP) at seven amino acid positions (13, 33, 57, 58, 61, 117 and 173) according to previously described methods (Imwong et al. 2001, 2003).

Amplification of \textit{Pvdhps} - Point mutations of \textit{Pvdhps} at two amino acid (383 and 553) in all \textit{P. vivax} isolates were investigated by nested PCR-RFLP (Rungsihirunrat et al. 2008).

**RESULTS**

Detection of mutations in the \textit{Pvdhfr} and \textit{Pvdhps} genes - The frequencies of \textit{Pvdhfr} and \textit{Pvdhps} mutations are summarised in Table I. Point mutations in \textit{Pvdhfr} were detected in five of seven amino acids investigated (13, 33, 57, 58, 61 and 117), whereas wild-type alleles were detected at two amino acid positions (33 and 173). All isolates carried mutations at amino acids 58 (58R) and 117 (117N, 117T, 117N/T). One (1%) and 88 (85.4%) isolates carried mutations at amino acids 13 (13L) and 61 (61M), respectively. We recovered two mutations at amino acid 57, 57I and 57L. For \textit{Pvdhps}, the most prevalent allele recovered was 383G (82.5%) and the wild-type allele A383A/A553 (17.5%) alleles.

Distribution of \textit{Pvdhfr} and \textit{Pvdhps} combination alleles - Table II summarises the distribution of eight different combinations of alleles recovered from our isolates of \textit{Pvdhfr} (5 different alleles) and \textit{Pvdhps} (2 different alleles). The most common \textit{Pvdhfr} alleles were the triple mutants 57L/58R/117T (77.7%), 57I/58R/117T/N (1%), 57L/58R/117T (5.8%) and 58R/61M/117N (14.5%); only one isolate carried a quadruple mutation (13L/57L/58R/117T). The most prevalent genotype recovered was the triple mutant \textit{Pvdhfr} 57I/58R/117T with the single mutant \textit{Pvdhps} 383G (64.1%), followed by the triple mutant \textit{Pvdhfr} 57I/58R/117T with a wild-type \textit{Pvdhps} allele (13.6%) and finally the triple mutant \textit{Pvdhfr} 58R/61M/117N with the single mutant \textit{Pvdhps} 383G (12.6%). We did not recover any isolates that carried wild-type alleles of both genes.

**TABLE I**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Amino acid position</th>
<th>SNPs</th>
<th>Isolates n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Pvdhfr}</td>
<td>13</td>
<td>I (wild-type)</td>
<td>102 (99)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>P (wild-type)</td>
<td>103 (100)</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>F (wild-type)</td>
<td>15 (14.6)</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>T (wild-type)</td>
<td>15 (14.6)</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>S (wild-type)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>173</td>
<td>I (wild-type)</td>
<td>103 (100)</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>\textit{Pvdhfr} alleles</th>
<th>\textit{Pvdhps} alleles</th>
<th>Isolates n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>57I/58R/117T</td>
<td>A383G</td>
<td>A (wild-type)</td>
<td>14 (13.6)</td>
</tr>
<tr>
<td>57I/58R/117T</td>
<td>A553G</td>
<td>A (wild-type)</td>
<td>66 (64.1)</td>
</tr>
<tr>
<td>57L/58R/117T/N</td>
<td>57I/58R/117T</td>
<td>G (mutant)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>57L/58R/117T</td>
<td>A383G</td>
<td>A (wild-type)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>58R/61M/117N</td>
<td>A553G</td>
<td>A (wild-type)</td>
<td>2 (1.9)</td>
</tr>
<tr>
<td>58R/61M/117N</td>
<td>A383G</td>
<td>G (mutant)</td>
<td>13 (12.6)</td>
</tr>
<tr>
<td>13L/57L/58R/117T</td>
<td>A (wild-type)</td>
<td>A (wild-type)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>
DISCUSSION

In 2005, we conducted a study in the same area (Mae Sot District) and collected a total of 32 isolates that were found to contain a combination of Pvdhfr mutant alleles (81.3% quadruple, 12.5% triple, 6.2% double) with a single or double mutant allele of Pvdhps (codons 383 and 553) (Rungsihirunrat et al. 2007). In the earlier study and in the study presented here, we did not detect any isolate carrying wild-type alleles of both genes. In fact, mutations at amino acids 58 and 117 of Pvdhfr were found in 100% of isolates. Another recent study of P. vivax isolates from the same area found that 57.1% of all isolates contained a 57L/58R/61M/117T triple mutation in Pvdhfr and a 383G/553G double mutation in Pvdhps (Lu et al. 2010). Of the five Pvdhfr amino acids (57, 58, 61, 117, 173) under investigation in our study, single (57L), double (58R, 117N) and quadruple (57L/58R, 61M, 117T) mutations were found in 3.6%, 10.7% and 85.7% of the isolates, respectively. In Pvdhps sequences, wild-type, single (383G) and double (383G and 553G) mutant alleles were found at frequencies of 3.6%, 14.3% and 82.1%, respectively. All these data suggest that prevalence and patterns of mutant Pvdhfr and Pvdhps alleles in P. vivax samples isolated from the same area may vary over time in response to changing degrees of drug pressure. In Mae Sot, P. vivax and P. falciparum co-infect in an equal ratio. SP was used as the first-line treatment for P. falciparum in Thailand from 1972-1982 leading to a progressive selection of SP-resistant alleles in P. vivax (Imwong et al. 2003). Monitoring of P. vivax isolate drug sensitivity in vitro (Russell et al. 2003, Kosaisavee et al. 2006) in parallel with genotyping for markers of antimarialar resistance is essential for guiding effective treatment of P. vivax infection with antifolate drugs. In vitro sensitivity data obtained from a yeast expression system has been shown to provide correlated sensitivity data for P. vivax and may be more applicable for this purpose (Rungsihirunrat et al. 2007). We observed an increase in geometric mean IC50 values of antifolates with an increasing number of Pvdhfr mutations, from double to quadruple (Rungsihirunrat et al. 2007). Quadruple mutant alleles confer decreased sensitivity to pyrimethamine but retain sensitivity to WR99210 (Rungsihirunrat et al. 2007). Further examination of the association between different patterns of Pvdhfr and Pvdhps mutation and in vitro sensitivity of P. vivax isolates using the yeast expression system is needed.

Several studies have investigated the prevalence and diversity of Pvdhfr and Pvdhps in P. vivax isolates from different malaria endemic areas of Thailand. In two studies conducted during 1992-1998 (Imwong et al. 2001, 2005) of patients admitted to the Bangkok Hospital for Tropical Diseases, double and triple Pvdhfr mutant alleles (amino acids 57, 58, 117) were found in almost all (n = 99.99%) of the isolates collected, the majority of which consisted of the triple mutant 57L/58R/117N allele (53%) (Imwong et al. 2001). Wild-type, single and double mutant alleles of amino acids 383 and 553 in Pvdhps were detected among 33 isolates (Imwong et al. 2005). Nine isolates carried four (57L/58R/61M/117T) or more (13L/57L/58R/61M/117T) point mutations in Pvdhfr, together with two point mutations in Pvdhps (Imwong et al. 2005). Previously, we described a collection of 160 isolates from 10 malaria endemic areas (Thai-Myanmar border, 117 isolates, Thai-Cambodian border, 13 isolates, and Thai-Malaysian border, 30 isolates) in which we detected wild-type, single, double, triple and quadruple mutant Pvdhfr alleles in 1.9%, 0.6%, 35.6%, 2.5% and 59.4% of all isolates, respectively. Only 1.3% of isolates were wild-type Pvdhps at amino acids 383 and 553 while single mutants at amino acid 383 corresponded to 26.9% and double mutant alleles at both amino acids corresponded to 60% of all isolates.

The prevalence and patterns of Pvdhfr and Pvdhps mutations vary not only over time in the same malaria endemic area, but also across geographical areas. The common Pvdhfr alleles 58R and 117T/N were found in the southeast Asian countries of East Timor (de Almeida et al. 2010), Thailand (Imwong et al. 2001, Lu et al. 2010), Myanmar (Lu et al. 2010), Vietnam and the Philippines (Auliff et al. 2006), in India (Kaur et al. 2006, Alam et al. 2007), in the African countries of French Guinea (Barnadas et al. 2009) and Madagascar (Barnadas et al. 2008). Mutants in Pvdhps at amino acids 383 and 553 were found at low prevalence in most geographic regions including East Timor (de Almeida et al. 2010), Korea (Lu et al. 2010), Iran (Zakeri et al. 2009) and Pakistan (Zakeri et al. 2011), whereas they were found at high prevalence in Thailand (Imwong et al. 2005, Rungsihirunrat et al. 2008). Isolates carrying Pvdhps double mutations co-existing with multiple mutations (4 or more amino acids) of Pvdhfr are being reported with increasing frequency (Imwong et al. 2005). Moreover, quadruple mutant alleles of Pvdhfr associated with SP treatment failure were reported in Myanmar (Na et al. 2005), India (Kaur et al. 2006), Papua New Guinea, Vanuatu (Auliff et al. 2006) and Thailand (Rungsihirunrat et al. 2007). Increasing treatment failure with SP for uncomplicated P. falciparum malaria in several areas has led to the development of new combinations of antifolates, sulfas and dihydrofolate inhibitors such as LapDapTM and WR99210. In vitro sensitivity data has revealed that the quadruple mutant alleles of Pvdhfr confer resistance of P. vivax to pyrimethamine but retain sensitivity to WR99210 (Rungsihirunrat et al. 2007).

REFERENCES


