AMINO ACID MUTATIONS IN _PLASMODIUM VIVAX_ DHFR AND DHPS FROM SEVERAL GEOGRAPHICAL REGIONS AND SUSCEPTIBILITY TO ANTIFOLATE DRUGS

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Abstract. The increasing use of sulfamide-pyrimethamine (SP) for the treatment of chloroquine-resistant _Plasmodium falciparum_ has resulted in increased reports of SP resistance of _P. falciparum_ worldwide. Selection of SP-resistant _Plasmodium vivax_ in areas where _P. falciparum_ and _P. vivax_ co-exist is not entirely clear. We examined the prevalence and extent of point mutations in _pvdhfr_ and _pvdhps_ in 70 _P. vivax_ isolates from China, East Timor, Papua New Guinea (PNG), Philippines, Vanuatu, and Vietnam. Mutations in seven codon positions were found in _pvdhfr_, with the majority of isolates having double mutations (S58R/S117N). The greatest range of mutations was observed in the PNG and Vanuatu isolates, ranging from single to quadruple mutations (F57L/S58R/T61M/S117T). Single mutations in _pvdhps_ were observed only in parasites with mutations in corresponding _pvdhfr_. Parasites with the S58R/S117N _dhfr_ allelic type showed a MIC level for pyrimethamine and cycloguanil comparable to that previously reported, but were susceptible to WR99210.

INTRODUCTION

Of the four species of _Plasmodium_ causing malaria in humans, _Plasmodium vivax_ is widely distributed and accounts for up to 80 million clinical cases annually. Although it causes very little mortality compared with _Plasmodium falciparum_, _P. vivax_ is responsible for considerable morbidity and economic loss in endemic countries. Antifolate-sulfadialarial combinations such as sulfadoxine-pyrimethamine (SP) are not generally recommended for the treatment of _P. vivax_ infections because the combination was reported to be less effective against the parasite. In practice, however, in areas where _P. falciparum_ and _P. vivax_ co-exist, SP is given for any diagnosis of malarial fever. Thus the increasing use of SP for the treatment of chloroquine-resistant _P. falciparum_ malaria exerts a strong selection for SP-resistant _P. vivax_

SP is a combination of pyrimethamine (PYR) and sulfadoxine (SDX). Pyrimethamine targets dihydroreductase (dhfr), and SDX inhibits parasite dihydropterate synthase (dhps). Point mutations in parasite _dhfr_ and _dhps_ genes confer resistance to PYR and SDX in _P. falciparum_. High-level resistance to PYR in _P. falciparum_ results from the accumulation of mutations in _pfdhfr_, principally at codons 16, 51, 59, 108, and 164 (see review by Sibley). These mutations have been shown to alter the PYR-binding sites in _pfdhfr_ and reduce enzyme–drug interactions. Parasites with >3 mutations in _pfdhfr_ have been associated with SP treatment failures. Mutations have also been identified in _P. vivax_ _dhfr_ ( _pvdhfr_ ) at positions 51, 58, and 117 corresponding to mutations at amino acids 50, 59, and 108 in _P. falciparum_, and the existence of such mutants correlates geographically with antifolate use against _P. falciparum_. Because it is difficult to maintain _P. vivax_ in _in vitro_ cultures, _Escherichia coli_ and yeast-expressed _pvdhfrs_ have been used to demonstrate that mutations in the _pvdhfr_ correlate with reductions in susceptibility to PYR. Recently, novel mutations in _pvdhfr_, L57R and T61M, have been reported in combination with S58R/S117T in isolates from West Papua, Indonesia. The _pvdhfr_ containing these four mutations is correlated with treatment failure using combinations with SP.

Genetic mutations at positions 436, 437, 540, 581, and 613 in _dhps_ have been found to result in SDX resistance in _P. falciparum_. Like _P. falciparum_, mutations were also identified in _P. vivax_ _dhps_ at positions 383 and 553, corresponding to mutations of 437 and 581 in _pfdhps_. The amino acid (V585) at the predicted drug-binding site in _pvdhps_ was predicted to affect the binding of SDX to _pvdhps_ and thus be responsible for innate resistance to sulfadoxine. Examination of _pvdhps_ sequences from larger numbers of isolates collected from geographically different regions would provide support for this prediction.

In _P. falciparum_ parasites, development of resistant mutations in _pfdhfr_ and _pfdhps_ is asymmetric (reviewed by Sibley). In the field, mutations in _pfdhfr_ appear to be selected prior to those in _pfdhps_. It is unclear whether a similar selection process also occurs in _P. vivax_ because the sequence of _pvdhps_ has become available only recently and there is no information on mutations in _pvdhfr_ and _pvdhps_ in a single set of parasites.

The increasing failure rate of SP for treatment of uncomplicated _P. falciparum_ malaria in many areas has led to the development of new combinations of sulfas and dihydrofolate inhibitors such as LapDap and homologues of WR99210. Although these drugs have been shown to be more potent than SP against _P. falciparum_ with mild resistance to SP, their efficacy in controlling _P. vivax_ is not clear. Because it is inevitable that _P. vivax_ patients will be exposed to antimalarial drugs that are designed for the treatment of _P. falciparum_ in areas where both species co-exist, it would be desirable for the next generation of antifolate drugs to also be effective in treating _P. vivax_ infections. A recent report of
yeast-expressed pvdhfr with quadruple mutations having reduced susceptibility to WR99210\(^\text{14}\) is of particular concern. Molecular surveys will provide the prevalence and distribution of various *pvdhfr* and *pvdhps* allelic types and help to predict the efficacy of LapDap or WR99210 in these regions.

To date, mutations in *pvdhfr* have been examined in parasites from only a very small number of *P. vivax*-endemic countries, namely, India,\(^\text{23}\) Indonesia,\(^\text{11,15,18}\) PNG,\(^\text{18}\) and Thailand.\(^\text{11–13}\) In this paper, we report mutations in both *pvdhfr* and *pvdhps* in *P. vivax* populations from China, East Timor, PNG, the Philippines, Vanuatu, and Vietnam.

**MATERIALS AND METHODS**

*P. vivax*-infected blood samples. Blood samples were collected from patients who acquired *P. vivax* infections in Agusan Del Sur, Mindanao, the Philippines (\(N = 15\)) in 2002 and in Fujian and Jiangsu provinces, China (\(N = 7\)) in 1994. The East Timor (\(N = 18\)) and PNG (\(N = 5\)) samples were collected from malaria patients reporting to the Australian Defence Force health facilities in East Timor during 2001 and in PNG during 1998. The Malo Island, Vanuatu (\(N = 18\)), samples were collected from malaria patients reporting to the U.S. Naval Medical Research Unit 2 (NAMRU-2) field site in 2005, and the Vietnam samples (\(N = 7\)) were collected from Truong Xuan Commune-Quang Binh Province in 2002. Collection of samples in the Philippines, East Timor, Vanuatu, PNG, and Vietnam and use of samples at AMI were approved by the Queensland Institute of Medical Research Human Research Ethics Committee; Division of Health Services, East Timor; Ministry of Health, Government of the Republic of Vanuatu; and Australian Defence Health Research Ethics Committee, respectively. For individuals whose samples underwent *in vitro* susceptibility testing, a treatment history was taken. Individuals who had taken any anti-malarial drugs over the previous 7 days were excluded from *in vitro* testing.

DNA isolation. Blood samples were stored on filter papers or in 6 M guanidine-HCl solution. Genomic DNA was isolated from the samples using previously described methods.\(^\text{24}\)

Confirmation of *Plasmodium* species. A multiplex PCR using conditions previously described by Padley\(^\text{25}\) was performed to confirm the presence of *Plasmodium* species in all isolates collected.

Amplification of *pvdhfr* and *pvdhps*. *pvdhfr* was amplified with primers *pvdhfr1* (5' ATGGAGGACCTTCAGATG-CCACCTTGCTGTAAAC-3') and *pvdhfr2* (5' CCACCTTGCTGTAAC-CAAAGTCCAGAGGAGT-3') using conditions described earlier.\(^\text{15}\) The amplified fragment corresponds to amino acids 30–225. *pvdhps* was amplified using primers and conditions described previously.\(^\text{20}\)

Sequence analysis. DNA sequences were translated into amino acid sequences and were then compared using the GCG program PILEUP (Wisconsin Package v. 8; Genetics Computer Group, Madison, WI) on the Australian National Genomic Information Service (ANGIS).

Field *in vitro* drug-susceptibility tests. *P. vivax* *in vitro* drug-susceptibility tests were performed using a modified microtest method developed by Rieckmann and others.\(^\text{26}\) Briefly, plates were coated with PYR (Hoffmann-La Roche, Basel, Switzerland), CYC (ICI Pharmaceuticals, Macclesfield, Cheshire, United Kingdom), or WR99210 (Jacobus Pharmaceuticals, Princeton, NJ) and then standardized using *in vitro*-cultured chloroquine and PYR-resistant *P. falciparum* strains K1 and FC27, respectively.\(^\text{27}\) An aliquot of 50 \(\mu\)L blood-RPMI 1640 (consisting of 0.0005 mg/L para-aminobenzoic acid, 0.01 mg/L folate, L-glutamine) medium mixture (4% hematocrit) was added to each well and incubated in a culture chamber containing 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\) at 37 °C, until ~50% of the ring-stage parasites had matured to schizonts (24–36 hours). Contents of the wells were transferred onto microscope slides, stained with Giemsa, and examined via microscopy. The MIC of each drug (the minimum concentration at which ~99% of the parasites, relative to the control, were inhibited from developing to schizonts, which are parasites with one or more chromatin dots) was determined for each isolate. Degenerate schizonts (normal appearance except that the chromatin dot appeared smudged and out of shape) were not counted.

**RESULTS**

*pvdhfr* alleles and their prevalence. All isolates were confirmed as *P. vivax* by only multiplex PCR (data not shown). Amino acid comparison of *pvdhfr* from 70 samples originating from 6 countries revealed changes at several amino acid positions. These changes and corresponding positions of mutations observed in *pvdhfr* are shown in Table 1. Various numbers of wild-type alleles were identified from parasites originating from all countries, with frequencies ranging from 5.6% in Vanuatu to 71.4% in China (Table 1). Overall, the proportion of wild-type *pvdhfr* was identified in 26% of the samples (18/70); the remaining 74% of isolates carried a mutant *pvdhfr*. The mutant *pvdhfr* isolates from China, East Timor, Philippines, Vanuatu, and Vietnam had either a single mutation of S117N or S58R or a combination of two mutations: S58R/S117N. This double-mutant allele plus F57L/S117N and F57L/S58R were observed in PNG isolates. Alleles with triple mutations S58R/T61M/S117T and F57L/S117T/I173F were identified in isolates from Vanuatu. Quadruple mutant alleles of F57L/S58R/T61M/S117T and S58R/T61M/S117T/I173F were identified in isolates from PNG and Vanuatu, respectively. Among all countries examined, isolates from Vanuatu had the highest frequency (95%) and greatest range of mutant *pvdhfr* alleles (single, double, triple, and quadruple mutations).

Variation in repeats in *pvdhfr*. Different numbers of an 18-bp repeat (6 amino acids) were observed in the *pvdhfr* gene between amino acid positions 92 and 118, which is consistent with previous reports.\(^\text{11,18}\) The majority of the isolates sequenced (86%) contained two 18-bp repeats. A small number of isolates (13%) contained one 18-bp repeat. One isolate from PNG was found to have three 18-bp repeats.
also found to have mutations in corresponding \( pvdhfr \), and 87% (26/30) of isolates with wild-type \( pvdhps \) were found to have mutations in the corresponding \( pvdhfr \) (Table 1).

**In vitro drug susceptibility.** Four of the nine \( P. vivax \) isolates from East Timor that were sequenced were tested for their susceptibilities to PYR, CYC, and WR99210. Three isolates had double mutations of S58R/S117N, and one isolate had a single mutation of S117N in \( pvdhfr \). The MIC values to PYR and CYC for all four isolates tested were greater than 5.0 and 2.0 pmol/well, respectively. The isolates were all susceptible to WR99210 at 0.025 pmol or less per well. Two of the four isolates from Vanuatu that were sequenced and found to have double mutations of S58R/S117N in their \( pvdhfr \) were also tested. Both isolates showed MIC levels to PYR and CYC greater than 10 and 2 pmol/well, respectively. In contrast, \( P. vivax \) parasites from China, East Timor, the Philippines, and Vietnam were found to carry, at most, double \( dhfr \) mutations similar to those reported for samples from South America and Africa. The wild-type \( dhfr \) allele is present at a relatively high proportion in \( P. vivax \) parasites from these countries. The difference in the prevalence of mutant \( pvdhfr \) alleles reflects the selection pressure exerted by the antifolate drug in these countries.

In this study, we examined genetic mutations in \( dhfr \) and \( dhps \) in \( P. vivax \) samples from six Asian-Pacific countries and showed that PYR-resistant mutations were present in samples collected from each country, with parasites from the South West Pacific countries of PNG and Vanuatu carrying the highly resistant \( dhfr \) allelic types with triple or quadruple mutations. The prevalence and distribution of the resistant allelic types in these two countries are similar to those reported earlier from PNG, Indonesia, and Thailand. In contrast, \( P. vivax \) parasites from China, East Timor, the Philippines, and Vietnam were found to carry, at most, double \( dhfr \) mutations similar to those reported for samples from South America and Africa. The wild-type \( dhfr \) allele is present at a relatively high proportion in \( P. vivax \) parasites from these countries. The difference in the prevalence of mutant \( pvdhfr \) alleles reflects the selection pressure exerted by the antifolate drug in these countries.

The 70 isolates screened for \( pvdhfr \) mutations, 52 were identified as having a mutant \( pvdhfr \) allele, of which 30 isolates (58%) had double mutations of S58R/S117N. The high prevalence of double mutations compared with other mutant alleles suggests that the combination of S58R/S117N may be the result of primary drug selection toward the development of resistance to SP. \( pvdhfr \) alleles with greater than triple mutations were observed in tandem with only S117T, not S117N, suggesting S117T is a key mutation in the development of high resistance to antifolate drugs, consistent with previous findings. Although a significant number of \( P. vivax \) isolates were identified to carry double mutations in their \( dhfr \), it is unlikely that these parasites will fail SP treatment because failure with SP is only significantly associ-

### DISCUSSION

Genetic mutations in \( pf dhfr \) and \( pf dhps \) and their correlations with PYR and SDX resistance *in vivo* and *in vitro* have been widely reported from many locations in various countries and regions (see review by Sibley). As a result, the prevalence and extent of antifolate resistance in *P. falciparum* populations is comprehensive. In comparison, the prevalence and extent of antifolate resistance in *P. vivax* has been much less investigated.

In this study, we examined genetic mutations in \( dhfr \) and \( dhps \) in *P. vivax* samples from six Asian-Pacific countries and showed that PYR-resistant mutations were present in samples collected from each country, with parasites from the South West Pacific countries of PNG and Vanuatu carrying the highly resistant \( dhfr \) allelic types with triple or quadruple mutations. The prevalence and distribution of the resistant allelic types in these two countries are similar to those reported earlier from PNG, Indonesia, and Thailand. In contrast, *P. vivax* parasites from China, East Timor, the Philippines, and Vietnam were found to carry, at most, double \( dhfr \) mutations similar to those reported for samples from South America and Africa. The wild-type \( dhfr \) allele is present at a relatively high proportion in *P. vivax* parasites from these countries. The difference in the prevalence of mutant \( pvdhfr \) alleles reflects the selection pressure exerted by the antifolate drug in these countries.
ated with isolates having a quadruple mutant pvdhfr allele.\textsuperscript{14,15}

The \textit{in vitro} drug-susceptibility assay demonstrated that the parasites carrying the double-mutant pvdhfr allele had MIC values for PYR and CYP at > 5 pmol/well (100 nM), comparable to those (≥ 150 nM) reported by de Pecoulas et al. for \textit{P. vivax} isolates carrying identical double mutations.\textsuperscript{11} However, it is not yet clear how these MIC values will compare with the \textit{P. vivax} parasites that have wild-type \textit{dhfr}. Our \textit{in vitro} cultures were performed in the field prior to determination of the parasite \textit{dhfr} mutant status by PCR and sequencing; it was unfortunate that all of the parasites that we tested in the field happened to have mutant \textit{dhfr}. Further studies are required to determine the susceptibility of \textit{P. vivax} isolates with wild-type pvdhfr to antifolates. \textit{In vitro} drug-susceptibility testing has also shown that isolates from East Timor carrying double or single mutations in pvdhps were highly susceptible to WR99210. This is consistent with results from experiments conducted in a yeast system\textsuperscript{17} showing that WR99210 is an extremely effective inhibitor of the pvdhps. However, whether \textit{P. vivax} with quadruple-mutant \textit{dhfr} is still susceptible to WR99210 requires further investigation.

A small number of mutations were detected in pvdhps. Of the pvdhps gene from 37 \textit{P. vivax} isolates from the 6 countries, only 7 were found to carry a single mutation of A383G or A553G, corresponding to A347G and A581G in \textit{P. falciparum}, respectively. A great majority (87\%) of pvdhps wild-type parasites had mutations in corresponding pvdhfr, and all pvdhps mutant parasites also had mutations in corresponding pvdhfr, supporting the conclusion that the asymmetric selection process observed in \textit{P. falciparum} also occurs in \textit{P. vivax}. Note that all of the pvdhps that were examined had V585, providing further support to our prediction that this amino acid is associated with innate resistance to SDX in \textit{P. vivax}. Addition of mutations to pvdhps may enhance innate resistance to SDX and confer resistance to other sulfa drugs.

In conclusion, our data demonstrate that \textit{P. vivax}-carrying mutant \textit{dhfr} allelic types are present in all six Asian-Pacific countries examined in this study, with the highly resistant mutant allelic types being more prevalent in Western Pacific countries. Because chloroquine-resistant \textit{P. vivax} is also prevalent in the region,\textsuperscript{28–30} the need to develop alternative drugs and strategies for effectively treating \textit{P. vivax} infections is urgent.

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The DNA sequences of pvdhfr and pvdhps have been deposited in the GenBank database (accession numbers DQ244102-DQ244124 and DQ379957-DQ379962, respectively).

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