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MAJOR ARTICLE

Dihydrofolate Reductase Mutations in *Plasmodium vivax* from Indonesia and Therapeutic Response to Sulfadoxine plus Pyrimethamine

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Background. The target enzyme of pyrimethamine is dihydrofolate reductase (DHFR), but little is known about allelic variants of *dhfr* in *Plasmodium vivax* populations. Still less is known about associations between specific alleles and the failure of sulfadoxine/pyrimethamine (S/P) to clear the erythrocytic stages of *P. vivax* in vivo.

Methods. We studied *P. vivax dhfr* mutations in 24 patients who received S/P therapy in Papua or Central Java, Indonesia, and we measured the resistance of the alleles in vitro in a *dhfr* yeast expression assay.

Results. Fourteen (58%) of 24 patients had an inadequate therapeutic response. Two of 6 alleles that were identified were novel. One allele that expressed 4 point mutations (57L+58R+61M+117T) correlated with a high risk of therapeutic failure. The 9 patients infected by *P. vivax* carrying this allele proved 23 times more likely to experience early therapeutic failure, compared with patients infected by *P. vivax* carrying other alleles ($P = .003$; 95% confidence interval, 2–450). This allele also conferred high levels of pyrimethamine resistance in vitro. The experimental antifolate WR99210 inhibited the allele in this system.

Conclusions. The present study identified a strong correlation between specific mutations in *P. vivax dhfr* and S/P treatment failure. Our results suggest that WR99210 could provide effective therapy for S/P-resistant *P. vivax*.

Plasmodium vivax infects an estimated 80 million people each year, causing severe and debilitating febrile illness. The risk of infection by this parasite is increasing in areas of endemicity, and the geographic range of risk has expanded rapidly during the past 10 years [1, 2]. Resistance to widely available and applied therapies, such as chloroquine, may substantially contribute to this problem [3]. Fansidar, a fixed combination of sulfadoxine and pyrimethamine (S/P), is not recommended for therapy of *P. vivax* malaria (vivax malaria) [4], on the basis of its historically poor demonstrated efficacy [5–9]. However, in areas of endemicity, S/P is widely used to treat chloroquine-resistant malaria caused by *Plasmodium falciparum*. Where these species occur together, *P. vivax* may often be treated with S/P, either because of mixed infection, inaccurate diagnosis, or economic issues. The risk of therapeutic failure associated with the use of S/P for *P. vivax* affects strategies for malaria-treatment policies. Molecular probes that reliably predict susceptibility of *P. vivax* to S/P would provide information supporting rational policy decisions and practice with regard to malaria treatment.

S/P resistance in *P. vivax* also poses important questions with regard to the development of new antimalarial drugs. Drugs that are effective against both *P. falciparum* and *P. vivax* offer obvious advantages in areas where these infections occur together—notably, in southern Asia and South America. The most conspicuous advantage is that appropriate therapy would not hinge on an accurate diagnosis of the parasite species. The dihydrofolate reductase (DHFR; E.C. 1.5.1.3)

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enzyme of plasmodia is the therapeutic target of the pyrimethamine component of S/P [10]. Extensive laboratory and field studies of *P. falciparum* have demonstrated that resistance to pyrimethamine is caused by *dhfr* alleles that carry specific point mutations [11–17]. In *P. falciparum,* clinical resistance to S/P apparently requires an accumulation of ⩾3 mutations in *dhfr* [18], and the resistance is augmented by additional mutations in dihydropteroate synthase, the target of sulfadoxine [19–21]. DHFR in *P. vivax* has not been evaluated as thoroughly as that in *P. falciparum,* but mutant alleles are prevalent in Southeast Asia [7, 22–27], and mutations at positions 57, 58, and 117 have been associated with antifolate resistance [7, 26]. One study suggested that parasites that carry the wild-type *dhfr* allele are susceptible to S/P, as are parasites with alleles that contain 1 or 2 mutations [7]. WR99210 is a novel antifolate drug with potent activity against S/P-resistant *P. falciparum* [28], and analogues of WR99210 currently are in the preclinical phase of development. If drugs of this class exert similar activity against S/P-resistant *P. vivax,* this finding would greatly increase their potential clinical usefulness [29, 30]

The *dhfr* gene from *P. falciparum* can be expressed in yeast that lack endogenous DHFR activity. These engineered yeast lines exhibit in vitro susceptibility to antifolate drugs that reflects the susceptibility of the parasite from which the *dhfr* allele was isolated [31, 32]. We have previously used this assay system to evaluate several alleles of *P. vivax dhfr* for susceptibility to pyrimethamine, chlorcycloguanil, and WR99210 [30]. The *P. vivax dhfr* alleles evaluated in the present study were cloned and sequenced from the blood of patients in Indonesia who were treated with S/P and observed for 28 days. Some alleles were also generated by site-directed mutagenesis, to assess the relative impact of individual mutations in alleles with multiple mutations. This approach permitted both assessment of the efficacy of S/P treatment for *P. vivax* infections and linkage of in vitro findings with therapeutic outcomes, thus allowing for a rational assessment of the potential therapeutic efficacy of WR99210 in the treatment of infection due to S/P-resistant *P. vivax.*

SUBJECTS, MATERIALS, AND METHODS

Subjects and therapeutic classification. The parasites evaluated in the present study were obtained from a subset of samples from clinical studies conducted in the Armopa region of northeastern Papua, Indonesia (from 1996 through 1999), and in Purworejo in southern Central Java, Indonesia (in 2000). These studies have been described elsewhere [33, 34]. The parasites evaluated in the present study were obtained from available samples from all subjects who received standard S/P therapy for slide- and polymerase chain reaction (PCR)–proven *P. vivax* infection. S/P therapy was not indicated for vivax malaria in these studies, but the subjects received it as a result of either an error in initial diagnosis by microscopic examination or the therapeutic precedence of mixed infection with *P. falciparum,* according to study protocol.

Subjects in the clinical studies were followed, by microscopic examination, for 28 days (with such examinations performed on days 0–4, 7, 11, 14, 18, 21, and 28, or on any day when subjects were ill), after they received directly observed S/P therapy (single dose: [25 mg sulfadoxine + 1.25 mg pyrimethamine]/kg body weight). We classified therapeutic outcomes as (1) "susceptible," if parasitemia cleared by day 4 and did not reappear by day 28; (2) "responsive," if parasitemia cleared by day 4 but reappeared between days 8 and 28; and (3) "resistant," if parasitemia either failed to clear by day 4 or cleared but then reappeared by day 7. Susceptible and resistant classifications represented firm, but not entirely unambiguous, characterizations of the therapeutic response to S/P. Rapid clearance of parasitemia and failure of parasitemia to reappear by day 28 were consistent with a susceptible response, but they did not preclude the possibility of recrudescence (as subpatent parasitemia) beyond day 28. Failure of parasitemia to clear by day 4 or reappearance of parasitemia by day 7 was consistent with therapeutic failure but did not preclude emergence of parasitemia caused by reinfection or relapse in subjects exposed to continuous risk of infection. Infections classified as responsive were considered to be either moderately, but not completely, susceptible to S/P or confounded by reinfection and relapse.

Cloning and sequencing of DHFR. Genomic *P. vivax* DNA was extracted from dried blood blots on filter paper, by use of the blood blot DNA extraction protocol from a commercial kit (QIAamp DNA Mini Kit; Qiagen). The *dhfr* gene was then amplified by PCR in a 100-μL reaction, with 2 μL of *Taq* polymerase (Promega), 10 μL of 10× PCR buffer, 8 μL of 25 mmol/L $MgCl_2$, 1 μL of 10 μmol/L each primer, 1 μL of 10 mmol/L each dNTP, 10 μL of template DNA, and 67 μL of PCR H_20. The primers used were as described elsewhere [30]. The 5′ end of each primer is complementary to the sequence of the shuttle plasmid at the desired insertion position, to facilitate homologous recombination in yeast [35, 36]. The cycling parameters were as follows: (1) initial denaturation at 94°C for 3 min, followed by 5 cycles of denaturation at 94°C for 1 min, elongation at 60°C for 1 min, and extension at 72°C for 1 min; (2) 5 cycles of denaturation at 94°C for 1 min, elongation at 55°C for 1 min, and extension at 72°C for 1 min; and (3) 20 cycles of denaturation at 94°C for 1 min, elongation at 55°C for 1 min, and extension at 72°C for 1 min.

The *Saccharomyces cerevisiae* yeast strain that was used lacks endogenous DHFR activity and has been described elsewhere [30, 32]. Yeast were transformed using a high-efficiency lithium acetate protocol [37]; they then were plated onto medium that lacked tryptophan, to select for the plasmid, and that also lacked deoxythymidine monophosphate, to select for functional DHFR

activity. *Escherichia coli* strain DH5α was used for propagation and preparation of the shuttle plasmid from the transformed yeast. The plasmid was isolated and purified from the bacteria by use of a commercial kit (QIAprep Spin Miniprep Kit; Qiagen). Sequencing was conducted by fluorescent dye chemical analysis (MegaBACE; Amersham Pharmacia Biotech), and sequences were analyzed using Sequencher software (Gene Codes). At least 4 isolates from each patient sample were sequenced to check for mixed genotypes; if the 4 isolates were not all in agreement, then an additional 4–7 isolates were sequenced to verify the mixed genotype. Site-directed mutagenesis (QuikChange; Stratagene) was used to engineer mutations into the wild-type *dhfr* sequence, for generating alleles not found in the patient samples.

The work with human subjects in this study was reviewed (protocols DoD #30820 and DoD #30839) and approved by US Navy and Indonesian institutional review boards. All subjects provided written informed consent to participate in the study, in accordance with US Navy regulations governing the use of human subjects for medical research (SECNAVINST 3900.39C).

In vitro susceptibility classification. The IC_{50} assays were performed as described elsewhere [38], to obtain quantitative measures of drug susceptibility. The growth of the yeast in this assay depends on the antifolate resistance of the *dhfr* allele expressed. In the present study, an "IC_{50}" is defined as the concentration of drug at which the growth of the yeast culture is inhibited by 50% relative to that of the untreated control. Yeast transformed with *dhfr* from *P. vivax* were grown overnight in a 96-well culture dish in complete medium (yeast extract, peptone, and dextrose [YEPD] broth) lacking dTMP. The growth of the yeast in each well was assessed by spectrophotometry after incubation at 30°C for ~24 h, which is the amount of time required for the untreated control yeast to reach mid-log phase. The average reading for each allele at each drug concentration was then used to plot the percent growth relative to that of the control. The numerical IC_{50} value was calculated from the slope and the intercept of the line defined by the 2 data points that bracket 50% relative growth. Comparisons of the IC_{50} values of the mutant alleles with those of the wild-type allele were used to assess the relative drug-resistance level of each allele. IC_{50} assays were performed at least twice for each allele, to determine the SD and to ensure reproducibility.

Assessment of the therapeutic risk of DHFR mutations. The odds ratio (OR) for therapeutic failure, relative to the *dhfr* allele, was calculated by standard χ^2 analyses. We report 95% confidence intervals (CIs) for the OR and a *P* value calculated by 2-tailed Fisher's exact test, by use of EpiInfo software, version 7.0. (public domain software; US Centers for Disease Control and Prevention). $P < .05$, along with a 95% CI that excludes 1.0, was considered to be consistent with an association between an allele and therapeutic outcome.

RESULTS

Parasitologic response to S/P. The primary goal was to classify the therapeutic response to S/P treatment for infections with *P. vivax*. Table 1 classifies the responses of the 24 subjects evaluated. Ten subjects exhibited responses that were consistent with S/P susceptibility, 5 subjects had recurrent parasitemia that was classified as responsive, and 9 subjects had S/P-resistant infections.

dhfr *Alleles.* Six alleles, including wild-type alleles, were isolated from the samples collected immediately before S/P therapy was administered (table 1). Five (21%) of the 24 patient samples contained *P. vivax* with mixed *dhfr* genotypes, indicating a polyclonal infection. Three of the alleles (58R+117N+99S, 58R+117N+199V, and 57L+58R+61M+117T) are novel for this species or have only very recently been described. The presence of 99S and 199V mutations has not been previously reported. The 2 most prevalent alleles in this sample were 58R+117N and 57L+58R+61M+117T; these alleles account for 22 of the 29 alleles (13 of the alleles were 58R+117N, and 9 were 57L+58R+61M+117T). The wild-type allele was found only once, whereas the 58R, 58R+117N+199V, and 58R+117N+99S alleles were each observed twice. The double-mutant allele isolated from sample UW16 had an additional silent mutation (GGT→GGC) at codon 101; this was the only silent change that was observed. We observed 3 different codons that all encoded the arginine amino-acid change at position 58. The AGC→AGA mutation was present in all 58R single-mutant alleles and 58R+117N+199V triple-mutant alleles, as well as in 1 double-mutant allele found in a mixed sample with a 58R+117N+199V allele (UW19). The double-mutant allele isolated from sample UW14 carried an AGC→CGC mutation at codon 58. All 58R+117N+99S and 57L+58R+61M+117T alleles shared the AGC→AGG mutation at position 58.

dhfr *Alleles and clinical response to S/P.* Table 1 lists the *dhfr* genotype(s) and therapeutic response of each isolate evaluated. Of the 9 patients with parasites that contained quadruple-mutant *dhfr* alleles, 7 showed responses consistent with S/P resistance, whereas the remaining 2 showed responses that were fully susceptible. Of the infections associated with the 15 isolates with only wild-type, single, double, or triple mutations in *dhfr*, only 2 infections showed therapeutic responses that were consistent with resistance to S/P. Of parasites from 9 infections that were classified as resistant, 7 carried the quadruple mutation. Three of 5 parasites classified as being responsive to treatment carried either a triple- or quadruple-mutant allele, and only 3 of 10 classified as susceptible carried either a triple- or quadruple-mutant allele. Parasites that carried highly mutant alleles of *dhfr* appeared to be more resistant to S/P treatment. Table 2 compares the risk of therapeutic failure when parasites carry the quadruple-mutant allele relative to the risk of therapeutic failure when parasites carry any other allele. Polyclonal

Table 1. The *dhfr* genotypes of and therapeutic responses for 24 patients from Papua and Central Java, Indonesia, who received therapy with sulfadoxine/pyrimethamine for *Plasmodium vivax* infection.

Patient	Location	No. of clones sequenced	Genotypes[a]	Therapeutic response[b]
UW06	Papua	11	Wild type	Susceptible
UW24	Java	10	58R+117N+99S and 58R+117N	Susceptible
UW05	Papua	4	58R+117N	Susceptible
UW12	Papua	5	58R+117N	Susceptible
UW13	Papua	4	58R+117N	Susceptible
UW18	Papua	4	58R+117N	Susceptible
UW20	Papua	10	58R+117N	Susceptible
UW27	Java	5	58R+117N	Susceptible
UW07	Papua	7	57L+58R+61M+117T	Susceptible
UW23	Papua	4	57L+58R+61M+117T	Susceptible
UW25	Java	5	58R+117N+99S	Responsive
UW19	Papua	10	58R+117N+199V and 58R+117N	Responsive
UW10	Papua	13	58R+117N	Responsive
UW11	Papua	4	58R+117N	Responsive
UW17	Papua	8	58R+117N	Responsive
UW02	Papua	8	58R+117N+199V and 58R	Resistant
UW14	Papua	5	58R+117N	Resistant
UW16	Papua	10	57L+58R+61M+117T and 58R+117N	Resistant
UW01	Papua	10	57L+58R+61M+117T and 58R	Resistant
UW03	Papua	11	57L+58R+61M+117T	Resistant
UW04	Papua	9	57L+58R+61M+117T	Resistant
UW08	Papua	4	57L+58R+61M+117T	Resistant
UW09	Papua	5	57L+58R+61M+117T	Resistant
UW15	Papua	5	57L+58R+61M+117T	Resistant

[a] Mixed genotypes resulted from polyclonal infection. The codon position of each mutation and the amino acid encoded by the mutation are given; standard amino-acid abbreviations are used.

[b] "Resistant" denotes either failure of parasitemia to clear by day 4 or clearance of parasitemia by day 4, followed by reappearance by day 7; "responsive" denotes clearance of parasitemia by day 4, followed by reappearance between days 8 and 28; and "susceptible" denotes clearance of parasitemia by day 4 and no reappearance of parasitemia by day 28.

infections were classified according to the most highly mutated *dhfr* allele present. Patients with the quadruple-mutant allele were 23 times more likely to show a poor therapeutic response, compared with patients with other *dhfr* alleles (table 2; $P = .003$; 95% CI, 2–450). Similarly, when polyclonal infections were excluded from analysis, patients with the quadruple-mutant allele were 25 times more likely to show a poor therapeutic response ($P < .01$; 95% CI, 2–347).

Yeast expression system susceptibility. Susceptibility of *P. vivax* to antifolates cannot be easily assayed in vitro. We created, as a surrogate, a series of yeast lines that each depend on a different vivax *dhfr* allele to assess relative in vitro levels of sensitivity to pyrimethamine and the experimental DHFR inhibitor WR99210. Table 3 presents the mean IC_{50} values ($\pm$ SD) for pyrimethamine and WR99210, in addition to the ratio of resistance, compared with that of the wild type, for each mutant allele.

The yeast line expressing the quadruple-mutant allele 57L+58R+61M+117T did not demonstrate any significant inhibition of growth at drug concentrations of up to 500 μmol/L pyrimethamine. Therefore, the enzyme encoded by this quadruple-mutant allele is >500 times more resistant to pyrimethamine than is the wild type. To investigate whether the novel 61M mutation contributes to this high level of resistance, we constructed the triple-mutant allele 57L+58R+117T (which lacks the 61M mutation) by use of site-directed mutagenesis. This allele was significantly less resistant than the quadruple-mutant allele (270-fold vs. >500-fold). We also constructed alleles with the 58R+117T mutations and the 117T mutation alone. Both showed relatively modest resistance to pyrimethamine (40–43-fold greater than resistance of the wild type).

The 117T allele conferred slight (1.6-fold) resistance to WR99210, whereas the 58R+117T double-mutant allele conferred a 10-fold increase in resistance to WR99210. The addition of the 57L mutation to create the 57L+58R+117T allele decreased resistance to WR99210 (5-fold more than the wild type). Finally, the quadruple-mutant allele 57L+58R+61M+117T, which is essentially insensitive to pyrimethamine in this system, showed an ~90-fold increased level of resistance to WR99210, compared with the wild type.

We determined the in vitro sensitivities to pyrimethamine and WR99210 of the novel triple-mutant alleles (58R+117N+99S and 58R+117N+199V), relative to those of the double-mutant allele (58R+117N). All 3 alleles show high levels of resistance to pyrimethamine, ranging from 80-fold to 240-fold resistance, compared with the wild type. The addition of the 99S mutation to the 58R+117N allele did not increase resistance to pyrimethamine, but the addition of the 199V mutation produced an allele with an IC_{50} value that was more than twice that of the double mutant. All 3 alleles were more sensitive to WR99210 than was the wild-type allele (table 3). The 58R single-mutant allele was slightly more resistant to pyrimethamine (1.3-fold more resistant) than was the wild-type allele in the yeast system, whereas it was 8.5-fold more resistant to WR99210 than was the wild type.

DISCUSSION

Six different alleles of *dhfr* were found in this sample of 24 isolates of *P. vivax* from Indonesia. Subjects infected by parasites

Table 2. Risk analysis for the genotype and therapeutic response of each isolate associated with infection, including mixed-genotype infections, in 15 patients.

	Therapeutic response[b]				
Genotype[a]	Resistant	Susceptible and/or responsive	OR[c]	95% CI[c]	P[d]
Quadruple mutant	7	2			
			23	2–450	.003
Single, double, or triple mutant or wt	2	13			

NOTE. CI, confidence interval; OR, odds ratio; wt, wild type.

[a] The genotype classification is assigned according to the most highly mutated allele in the sample.

[b] "Resistant" denotes either failure of parasitemia to clear by day 4 or clearance of parasitemia by day 4, followed by reappearance by day 7; "responsive" denotes clearance of parasitemia by day 4, followed by reappearance between days 8 and 28; and "susceptible" denotes clearance of parasitemia by day 4 and no reappearance of parasitemia by day 28.

[c] Calculated by standard χ^2 analysis.

[d] Calculated by Fisher's exact test.

that carried the quadruple-mutant allele 57R+58L+61M+117T were 23 times more likely to experience therapeutic failure, compared with subjects infected by parasites that carried only wild-type, single-, double-, or triple-mutant alleles of *dhfr*. Two subjects infected by parasites that carried the quadruple-mutant allele had a good therapeutic response, and this may reflect the operation of host factors (e.g., immunity) as contributing determinants of therapeutic outcomes. Of the 15 subjects who carried only parasites with wild-type, single, double, or triple mutations in *dhfr*, only 2 showed therapeutic responses that were consistent with resistance. Those exceptional outcomes could be the result of one of any number of explanations, such as poor absorption of drug or efficient use of exogenous folate by the parasites. In addition, like most studies evaluating the efficacy of drugs against *P. vivax*, accurate designation of clinical outcomes as treatment failures was limited by the possibility of early relapse and reinfection in evaluated cases. The trends detected in our sample nonetheless point to *dhfr* genotype in *P. vivax* as a probable determinant of therapeutic outcome with S/P.

The poor therapeutic response to S/P by *P. vivax* was documented in early studies [5, 9]. S/P is not recommended for treatment of vivax malaria [4]. Recent studies in Thailand, which demonstrated clinical efficacy of just 40% and 31%, corroborate that view [6, 7]. An intervention employing mass administration of S/P and primaquine in northern Sumatra, Indonesia, sharply reduced falciparum malaria but not vivax malaria [8]. Although such findings are often attributed to an inherent tolerance of pyrimethamine by *P. vivax* [5, 9], recent studies point to *dhfr* mutations as the more likely explanation [7, 26]. This is an important point that bears directly on approaches to discovery of drugs for the treatment of vivax malaria. If the molecular basis of poor therapeutic response is understood, it may be addressed in drug design and in vitro assessment. This was the rationale behind our assessment of *dhfr* alleles in a yeast expression system allowing relative measures of DHFR inhibition by antifolates.

The in vitro yeast expression assay system made it possible to dissect the contributions of specific mutations to DHFR inhibition and, presumably, therapeutic outcome. We detected 58R alleles in parasites from 2 clinically resistant infections mixed with triple- or quadruple-mutant alleles and, thus, could not assess the role of the 58R mutation in that therapeutic outcome. However, the 58R mutation had a negligible effect on pyrimethamine sensitivity in the yeast system. The 99S mutation did not confer additional resistance to pyrimethamine, compared with that conferred by the double-mutant allele 58R+117N. This finding is consistent with the fact that the mutation occurred in a region that is prone to insertion and deletion events [22, 27] (M.D.H., unpublished data) and, apparently, is located peripheral to the folate-binding pocket of the enzyme (C.H.S., unpublished data). In contrast, the in vitro analyses revealed that the 61M mutation is an important contributing factor to DHFR inhibition by pyrimethamine: 57L+58R+117T showed 270-fold resistance, whereas 57L+58R+61M+117T showed >500-fold resistance.

The in vitro analyses of the inhibition of *dhfr* mutants by WR99210 point to a high probability of therapeutic efficacy against S/P-susceptible and -resistant *P. vivax*. The poor therapeutic response of *P. vivax* to S/P is evidently linked to the quadruple-mutant allele of the *dhfr* gene, and these data, along with the data from several recent studies, suggest that this quadruple-mutant allele may be widespread in Southeast Asia [26, 27]. The quadruple-mutant allele also showed the highest level of in vitro resistance to WR99210, relative to the other 5 alleles. However, the IC_{50} value for this allele was only 92 times greater than the IC_{50} value measured for the wild type, whereas no other allele was >10-fold more resistant than the wild type. S/P was still clinically effective against parasites carrying alleles that were 80–240-fold more resistant to pyrimethamine than was the wild type. If this ratio of effectiveness is also observed for drugs of the WR99210 class, even parasites that carry the quadruple-mutant allele might be successfully treated with a drug of this kind. As development of these drugs proceeds, testing their effectiveness against strains of *P. vivax* that are highly resistant to pyrimethamine should be a high priority.

The clinical findings reported in the present study provide a benchmark for correlation with findings of the in vitro yeast expression assay [30]. The in vitro inhibition of expressed DHFR mutants correlated well with the clinical outcomes of parasite populations carrying the corresponding alleles. For example, the *dhfr* double-mutant allele 58R+117N was 100-fold more resistant to pyrimethamine than the was wild-type allele in the in vitro assay, and the triple-mutant allele 58R+

Table 3. *Plasmodium vivax dhfr* alleles and IC_{50} values measured in the yeast expression system.

	IC_{50} ± SD, μmol/L		Impact of mutation on IC_{50}[b]	
dhfr Allele[a]	Pyrimethamine	WR99210	Pyrimethamine	WR99210
Wild type	1.0 ± 0.3	0.58 ± .08	1.0	1.0
117T	41 ± 2	0.91 ± .06	40	1.6
58R+117T	44 ± 2	6.0 ± 1.4	43	10
57L+58R+117T	270 ± 10	2.9 ± 1.3	260	5.0
57L+58R+61M+117T	>500	54 ± 16	>500	92
58R	1.4 ± 0.8	4.9 ± 3.0	1.3	8.5
58R+117N	110 ± 28	0.26 ± .02	100	0.44
58R+117N+99S	84 ± 5.5	0.41 ± .04	81	0.70
58R+117N+199V	250 ± 11	0.44 ± .003	240	0.76

[a] The codon position of each mutation and the amino acid encoded by the mutation are given; standard amino-acid abbreviations are used.
[b] Fold wild-type IC_{50} value.

117N+99S had a similar resistance level; clinical isolates that carried these alleles were generally susceptible or responsive to S/P treatment. In contrast, the quadruple mutant was >500 times more resistant to pyrimethamine in yeast and exhibited very poor clinical response to S/P treatment. These data will allow an estimation of the in vivo S/P susceptibility of *P. vivax* parasites that carry a novel allele of *dhfr,* by determining the relative level of pyrimethamine resistance in the yeast expression system.

As in other studies using sequence analysis of *dhfr,* in the present study, we detected novel alleles [26, 27]. The diversity of mutations in *dhfr* is apparently rich in natural parasite populations, and allele-specific methods do not reveal this diversity. For example, position 117 of *P. vivax dhfr* corresponds to position 108 in the *P. falciparum* enzyme. In *P. falciparum,* highly pyrimethamine-resistant alleles carry an asparagine at this position. Therefore, the frequent finding of the threonine mutation in the *P. vivax* isolates evaluated in the present study was quite unexpected. An allele-specific PCR protocol was initially used to characterize *P. vivax dhfr* alleles in Thailand, on the basis of a *P. falciparum* model, under the assumption that triple mutants that did not carry the wild-type serine at position 117 had a mutation to asparagine [7]. When the entire coding sequence was determined, it was found that these alleles, in fact, carried a threonine at position 117 [27]. This difference emphasizes the importance of direct sequence analysis of *P. vivax dhfr,* because *P. vivax* has greater allelic diversity than is found in *P. falciparum.*

In summary, of the 6 different alleles that were detected in 24 patient samples from Papua and Central Java, Indonesia, only the quadruple-mutant allele was linked to a high risk of therapeutic failure of S/P. The yeast in vitro expression system allowed us to examine, in detail, the response of the various alleles to both S/P and WR99210, and there was a strong correlation between the therapeutic response to S/P and the susceptibility of yeast lines carrying the various alleles. The in vitro system also allowed us to predict that the experimental DHFR inhibitor, WR99210, is likely to demonstrate good clinical efficacy against *P. vivax* parasites carrying the S/P-resistant quadruple mutant reported in the present study. Furthermore, WR99210 may be particularly effective against parasites carrying the double- and triple-mutant alleles. The effectiveness of WR99210 against the alleles of *P. falciparum dhfr* that are most resistant to pyrimethamine is well established [31, 39], and extending that effectiveness to treatment of *P. vivax* infections is an exciting possibility [29].

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References

1. Campbell CC. Malaria: an emerging and re-emerging global plague. FEMS Immunol Med Microbiol **1997**; 18:325–31.
2. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg **2001**; 64:97–106.
3. Baird JK. Resurgent malaria at the millennium: control strategies in crisis. Drugs **2000**; 59:719–43.

4. World Health Organization (WHO). The use of antimalarial drugs: report of a WHO informal consultation. Geneva: WHO, **2001**.
5. Young MD, Burgess RW. Pyrimethamine resistance in *Plasmodium vivax* malaria. Bull World Health Organ **1959**; 20:27–36.
6. Wilairatana P, Silachamroon U, Krudsood S, et al. Efficacy of primaquine regimens for primaquine-resistant *Plasmodium vivax* malaria in Thailand. Am J Trop Med Hyg **1999**; 61:973–7.
7. Imwong M, Pukrittakayamee S, Looareesuwan S, et al. Association of genetic mutations in *Plasmodium vivax dhfr* with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother **2001**; 45:3122–7.
8. Doi H, Kaneko A, Panjaitan W, Ishii A. Chemotherapeutic malaria control operation by single dose of Fansidar plus primaquine in North Sumatra, Indonesia. Southeast Asian J Trop Med Public Health **1989**; 20:341–9.
9. Doberstyn EB, Teerakiartkamjorn C, Andre RG, Phintuyothin P, Noeypatimanondh S. Treatment of vivax malaria with sulfadoxine-pyrimethamine and with pyrimethamine alone. Trans R Soc Trop Med Hyg **1979**; 73:15–7.
10. Peters W. Chemotherapy and drug resistance in malaria. 2nd ed. London: Academic Press, **1987**.
11. Peterson DS, Walliker D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase–thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA **1988**;85: 9114–8.
12. Plowe CV, Kublin JG, Doumbo OK. *P. falciparum* dihydrofolate reductase and dihydropteroate synthase mutations: epidemiology and role in clinical resistance to antifolates. Drug Resist Updat **1998**; 1:389–96.
13. Sibley CH, Hyde JE, Sims PFG, et al. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum:* what next? Trends Parasitol **2001**; 17:582–8.
14. Sirawaraporn W, Yongkiettrakul S, Sirawaraporn R, Yuthavong Y, Santi DV. *Plasmodium falciparum:* asparagine mutant at residue 108 of dihydrofolate reductase is an optimal antifolate-resistant single mutant. Exp Parasitol **1997**; 87:245–52.
15. Wang P, Lee CS, Bayoumi R, et al. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. Mol Biochem Parasitol **1997**; 89:161–77.
16. Hyde JE. Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. Microbes Infect **2002**; 4:165–74.
17. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase–thymidylate synthase gene of *Plasmodium falciparum.* Proc Natl Acad Sci USA **1988**; 85:9109–13.
18. Mutabingwa T, Nzila A, Mberu E, et al. Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania. Lancet **2001**; 358:1218–23.
19. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum.* Proc Natl Acad Sci USA **1997**; 94: 13944–9.
20. Wang P, Read M, Sims PFG, Hyde JE. Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. Mol Microbiol **1997**; 23:979–86.
21. Kublin JG, Dzinjalamala FK, Kamwendo DD, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. J Infect Dis **2002**; 185:380–8.
22. de Pecoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco LK. Sequence variations in the *Plasmodium vivax* dihydrofolate reductase–thymidylate synthase gene and their relationship with pyrimethamine resistance. Mol Biochem Parasitol **1998**; 92:265–73.
23. de Pecoulas PE, Basco LK, Tahar R, Ouatas T, Mazabraud A. Analysis of the *Plasmodium vivax* dihydrofolate reductase–thymidylate synthase gene sequence. Gene **1998**; 211:177–85.
24. Leartsakulpanich U, Imwong M, Pukrittayakamee S, et al. Molecular characterization of dihydrofolate reductase in relation to antifolate resistance in *Plasmodium vivax.* Mol Biochem Parasitol **2002**; 119:63–73.
25. Tahar R, de Pecoulas PE, Basco LK, Chiadmi M, Mazabraud A. Kinetic properties of dihydrofolate reductase from wild-type and mutant *Plasmodium vivax* expressed in *Escherichia coli.* Mol Biochem Parasitol **2001**; 113:241–9.
26. Tjitra E, Baker J, Suprianto S, Cheng Q, Anstey NM. Therapeutic efficacies of artesunate-sulfadoxine-pyrimethamine and chloroquine-sulfadoxine-pyrimethamine in vivax malaria pilot studies: relationship to *Plasmodium vivax dhfr* mutations. Antimicrob Agents Chemother **2002**; 46:3947–53.
27. Imwong M, Pukrittayakamee S, Renia L, et al. Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: evidence for sequential selection by drug pressure. Antimicrob Agents Chemother **2003**; 47:1514–21.
28. Milhous WK, Weatherly NF, Bowdre JH, Desjardins RE. In vitro activities of and mechanisms of resistance to antifol antimalarial drugs. Antimicrob Agents Chemother **1985**; 27:525–30.
29. Ridley RG. Chemotherapeutic hope on the horizon for *Plasmodium vivax* malaria? Proc Natl Acad Sci USA **2002**; 99:13362–4.
30. Hastings MD, Sibley CH. Pyrimethamine and WR99210 exert opposing selection on dihydrofolate reductase from *Plasmodium vivax.* Proc Natl Acad Sci USA **2002**; 99:13137–41.
31. Hankins EG, Warhurst DC, Sibley CH. Novel alleles of the *P. falciparum dhfr* highly resistant to pyrimethamine and chlorcycloguanil, but not WR99210. Mol Biochem Parasitol **2001**; 117:91–102.
32. Wooden JM, Hartwell LH, Vasquez B, Sibley CH. Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of *Plasmodium falciparum.* Mol Biochem Parasitol **1997**; 85:25–40.
33. Maguire JD, Lacy MD, Sururi, et al. Chloroquine or sulfadoxine-pyrimethamine for the treatment of uncomplicated, *Plasmodium falciparum* malaria during an epidemic in Central Java, Indonesia. Ann Trop Med Parasitol **2002**; 96:655–68.
34. Krisin, Basri H, Fryauff DJ, et al. Malaria in a cohort of Javanese migrants in Indonesian Papua. Ann Trop Med Parasitol **2003**; 97:543–56.
35. Sikorski RS, Hieter P. A system or shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics **1989**; 122:19–27.
36. Cortese JF, Plowe CV. Antifolate resistance due to new and known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. Mol Biochem Parasitol **1998**; 94:205–14.
37. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol **1983**; 153:163–8.
38. Sibley CH, Brophy VH, Cheesman S, et al. Yeast as a model system to study drugs effective against apicomplexan proteins. Methods **1997**; 13:190–207.
39. Ferlan JT, Mookherjee S, Okezie IN, Fulgence L, Sibley CH. Mutagenesis of dihydrofolate reductase from *Plasmodium falciparum:* analysis in *Saccharomyces cerevisiae* of triple mutant alleles resistant to pyrimethamine or WR99210. Mol Biochem Parasitol **2001**; 113:139–50.