MAJOR ARTICLE

Molecular Markers of In Vivo *Plasmodium vivax* Resistance to Amodiaquine Plus Sulfadoxine-Pyrimethamine: Mutations in *pvdhfr* and *pvmdr1*

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Background. Molecular markers for sulfadoxine-pyrimethamine (SP) resistance in *Plasmodium vivax* have been reported. However, data on the molecular correlates involved in the development of resistance to 4-aminoquinolines and their association with the in vivo treatment response are scarce.

Methods. We assessed *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmdr1* (Y976F and F1076L) mutations in 94 patients who received amodiaquine (AQ) plus SP in Papua New Guinea (PNG). We then investigated the association between parasite genotype and treatment response.

Results. The treatment failure (TF) rate reached 13%. Polymorphisms in *pvdhfr* F57L, S58R, T61M, and S117T/N and in *pvmdr1* Y976F were detected in 60%, 67%, 20%, 40%, and 39% of the samples, respectively. The single mutant *pvdhfr* 57 showed the strongest association with TF (odds ratio [OR], 9.04; $P = .01$). The combined presence of the quadruple mutant *pvdhfr* 57L+58R+61M+117T and *pvmdr1* mutation 976F was the best predictor of TF (OR, 8.56; $P = .01$). The difference in TF rates between sites was reflected in the genetic drug-resistance profile of the respective parasites.

Conclusions. The present study identified a new molecular marker in *pvmdr1* that is associated with the in vivo response to AQ+SP. We suggest suitable marker sets with which to monitor *P. vivax* resistance against AQ+SP in countries where these drugs are used.

More than 50% of all cases of malaria occurring outside Africa are caused by *Plasmodium vivax,* and it is estimated that 70–80 million people are infected each year [1]. *P. vivax* has considerable clinical and socioeconomic influence in countries where this species is endemic, and the resurgence of malaria due to *P. vivax* is mainly attributable to the emergence of parasite resistance to commonly applied therapies [2, 3].

Chloroquine (CQ)–resistant *P. vivax* was first reported in Papua New Guinea (PNG) in 1989 [4]. Thereafter, reduced susceptibility of *P. vivax* to CQ was reported from several countries where this species is endemic [2]. Sulfadoxine-pyrimethamine (SP) has never been recommended for *P. vivax* malaria. Nevertheless, increasing levels of *P. falciparum* resistance to CQ led to the adoption of SP as a cheap and safe alternative first-line option in many countries in Southeast Asia, Central and South America, and Oceania, where both species are endemic, and *P. vivax* resistance to SP developed rapidly in many areas within only a few years after the initial deployment of SP as monotherapy [2, 5, 6].

The hypnozoite, the latent liver stage of *P. vivax,* can give rise to a recurrent intraerythrocytic infection between 3 weeks and several months after the initial infection, depending on the strain. Therefore, in vivo assess-

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ment of drug efficacy is complicated by difficulties in clearly differentiating between treatment failures (TFs; i.e., true recrudescences originating from asexual blood stage parasites), relapses (i.e., red blood cell infection originating from hypnozoites), and newly acquired infections. Moreover, comparison of data is aggravated by the lack of studies that have followed standardized protocols. As in the case of *Plasmodium falciparum* malaria, the assessment of molecular drug-resistance markers could be a valuable complementary tool for the mapping and regular monitoring of drug-resistant *P. vivax* malaria [7, 8]. Several genes related to resistance to commonly used drugs have been described in *P. falciparum.* Orthologous genes of *pfdhfr* (*P. falciparum* dihydrofolate reductase), *pfdhps* (dihydropteroate synthase), *pfcrt* (chloroquine resistance transporter gene), and *pfmdr1* (multiple-drug–resistance gene 1) have been found in *P. vivax,* notably *pvdhfr* [9], *pvdhps* [10], *pvcg10* [11], and *pvmdr1* [12]. Although no evidence could be found for an association between point mutations in both *pvcg10* and *pvmdr1* and CQ resistance in *P. vivax* field isolates [11, 13], several laboratory studies have shown that pyrimethamine resistance is associated with a specific single-nucleotide polymorphism (SNP) accumulation in *pvdhfr* that leads to reduced enzyme affinity to the drug and that corresponds to reduced susceptibility to pyrimethamine in vitro [14–18]. This association could be confirmed in epidemiological studies investigating the association between the genetic *pvdhfr* background and the in vivo response to antifolates [19–21]. Likewise, reduced in vitro susceptibility to sulfadoxine [22, 23] and an association with the clinical response to SP have been shown to be associated with SNPs in *pvdhps* [10, 24].

In PNG, where all 4 *Plasmodium* species affecting humans are found, the majority of infections are caused by *P. falciparum* and *P. vivax,* and mixed infections are common [25]. After a long history of the use of 4-aminoquinolines against malaria, reports of reduced in vivo efficacy of 4-aminoquinolines against *P. falciparum* and *P. vivax* malaria began to accumulate since the mid-1970s and the 1980s, respectively [26, 27]. The extent of this reduction in efficacy became unacceptably great in the 1990s [28, 29]. Despite low levels of SP use in PNG (where it was used only in combination with quinine to treat severe and TF malaria cases), *P. falciparum* resistance to SP, as well as a reduced efficacy of SP against *P. vivax,* has been described in the Madang province [30–33].

The first-line policy against uncomplicated malaria in PNG was changed to the combination of amodiaquine (AQ) or CQ plus SP in 2000. To assess the clinical efficacy of the current first-line regimen against *P. vivax* malaria, we conducted in vivo drug efficacy studies in 3 different areas in PNG between 2004 and 2005, using standard clinical classifications set forth by the revised World Health Organization (WHO) protocol [34]. In the present study, we assessed *P. vivax* mutations in pretreatment samples obtained from patients with a monoinfection who received AQ in combination with SP and then investigated the association between parasite genotype and treatment response.

SUBJECTS, MATERIALS, AND METHODS

Subjects and therapeutic classification. In vivo drug efficacy studies performed according to the revised WHO protocol were conducted between October 2004 and April 2005 in the Karimui area (Simbu Province), the South Wosera area (East Sepik Province), and the North Coast area of Madang (Madang Province), as described in detail elsewhere [34].

Patients were classified as TF cases when they (1) experienced clinical deterioration in the presence of *P. vivax* parasitemia, (2) developed parasitemia between 3 and 28 days after treatment initiation and had an axillary temperature of $\geqslant$37.5°C, or (3) developed parasitemia between 7 and 28 days after treatment initiation, irrespective of clinical conditions [35]. Patients without clinical signs and without recurrent asexual parasites up to 28 days after treatment initiation were classified as having an adequate clinical and parasitological response (ACPR).

Laboratory analyses. Blood samples obtained by fingerprick were collected into EDTA Microtainer tubes (Becton Dickinson), and DNA was extracted using the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's instructions.

Detection of SNPs in *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmdr1* (Y976F and F1076L) was performed using a LightCycler system (Roche) and fluorescence resonance energy transfer technology. Primers and probes (table 1) were designed and synthesized by TIB Molbiol (DNA Synthesis Service). The polymerase chain reaction mixture and assay conditions were used as described in detail elsewhere [12, 36]. DNA sequencing was used to confirm the presence of codon L or I at position 57 in *pvdhfr.*

Statistics. Statistical analyses were performed using STATA software (version 8.2; Stata Corporation). The strength of an association was evaluated by calculating odds ratios (ORs). We used a χ^2 test or Fisher's exact test and, where applicable, logistical regression analysis, to assess the relationship between single or multiple mutations and TF, taking into account other variables known to be associated with treatment outcome.

RESULTS

In vivo response to AQ+SP. Baseline characteristics of all children who had a *P. vivax* monoinfection on the day of admission to the health center are presented in table 2. A total of 98 children were treated with AQ+SP. TF was seen in 13 (13.3%) of all 98 children with *P. vivax* monoinfection (table 2). The majority of patients (11/13 [84.6%]) were classified as having parasitological TF at day 28 after treatment initiation, 1 patient experienced treatment failure at day 6, and 1 experienced treatment failure at day 20. There was a significant difference in

Table 1. Sequences of primers and oligonucleotide probes used for the detection of *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmdr1* (Y976F and F1076L) mutations.

Gene, primer or probe	Sequence	Position	SNP
pvdhfr[a]			
Primer			
pvdhfr S	5'-TCTGGGCAATAAGGGGACT-3'	114–132	
pvdhfr A	5'-AGTTTCTACTTAGGCATTCCCTAT-3'	559–536	
Probe			
Sensor 57/8	5'-GTAGGTCGTCACCGAGCTGAAGT FL-3'	189–167	57, 58
Anchor 57/8	5'-CTTCATATCGACGGAGTTGCATTTCCATG PH-3'	165–137	61
Sensor [G]	5'-GATGCTCTCCCAGCTGCTTC FL-3'	363–344	117
Anchor 117	5'-CCCCATGACCACGACGTTTTGCAG PH-3'	342–319	117
Sensor 172V	5'-TGTGCTCCCCCAATGACGA FL-3'	530–512	173
Anchor 172/173	5'-GCATTTGTAGTACTTCAGCTTCTTTAAGAGC PH-3'	510–480	173
pvmdr1[b]			
Primer			
pvmdr1	5'-ATAGTCATGCCCCAGGATTG-3'	2753–2772	
pvmdr1 447AS	5'-ACCGTTTGGTCTGGACAAGTAT-3'	3535–3516	
Probe			
Sensor Phe	5'-CATAAAAATGAAGAACGTTCCGGTC FL-3'	2940–2916	976
Anchor 976	5'-GTACAGCCGCCACGATAGGGCAGAA PH-3'	2914–2890	976
Sensor Leu	5'-AGTGCCCAACTTTTCATTAACAG FL -3'	3217–3239	1076
Anchor 1076	5'-TTGCCTACTGGTTTGGTTCCTTCCT PH-3'	3242–3266	1076

NOTE. *pvdhfr, Plasmodium vivax* dihydrofolate reductase; *pvmdr1, P. vivax* multiple-drug–resistance gene 1; PCR, polymerase chain reaction.

[a] GenBank accession no. X98123 (PCR product, 422 bp).
[b] GenBank accession no. AY618622 (PCR product, 763 bp).

TF rates between sites: 33% in the North Coast area of Madang, 5% in the Karimui area, and 0% in the Wosera area.

Prevalence of and relationship between* pvdhfr *and* pvmdr1 *mutations. Mutation analyses were accomplished for 94 (95.9%) of all 98 samples. Polymorphisms in *pvdhfr* codons F57L, S58R, T61M, and S117T/N and in *pvmdr1* codon Y976F were detected in 60%, 67%, 20%, 40%, and 39% of samples, respectively. Depending on the codon position, a pure mutant allele was found in most of the samples (14%–57%), whereas, in 6%–17% of the samples, a mutant allele was found in conjunction with the wild-type allele. None of the other SNPs (i.e., *pvdhfr* F57I, *pvdhfr* I173F/L, and *pvmdr1* F1076L) was detected

Table 2. Baseline characteristics and treatment outcomes for patients at enrollment who were receiving amodiaquine plus sulfadoxine-pyrimethamine against *Plasmodium vivax* malaria, by study site.

Characteristic	North Coast area[a] (n = 33)	Karimui area[b] (n = 39)	South Wosera area[c] (n = 26)	All (n = 98)
Weight, mean (95% CI), kg	15.9 (8.0–23.8)	12.6 (11.7–13.5)	11.8 (10.3–13.2)	14.1 (11.2–16.9)
Age, mean (95% CI), years	2.3 (1.9–2.8)	3.2 (2.8–3.6)	3.2 (2.5–3.9)	2.9 (2.6–3.2)
Sex, no. of females (%)	20 (60.6)	15 (38.5)	9 (34.6)	44 (45.8)
Temperature, mean (95% CI),°C	37.1 (36.6–37.76)	38.6 (38.3–38.8)	37.0 (36.4–37.7)	37.7 (37.4–38.0)
Hemoglobin level, mean (95% CI), g/dL	10.2 (9.4–11.1)	10.5 (9.8–11.1)	9.3 (8.6–9.9)	10.1 (9.7–10.5)
Parasite density,[d] geometric mean (range)	4688 (300–41280)	3810 (40–36600)	4994 (160–50640)	4930 (40–50640)
Classification, no. (%) of patients				
ACPR	22 (66.7)	37 (94.9)	26 (100)	85 (86.7)
Treatment failure	11 (33.3)	2 (5.1)	0	13 (13.3)

NOTE. ACPR, adequate clinical and parasitological response; CI, confidence interval.

[a] Madang province.
[b] Simbu province.
[c] East Sepik province.
[d] Per microliter.

Table 3. *pvdhfr* haplotypes in 94 pretreatment samples from patients in Papua New Guinea who received amodiaquine plus sulfadoxine-pyrimethamine against *Plasmodium vivax* malaria.

Category	*pvdhfr* polymorphism[a] 57	58	61	117	Samples, no. (%) (n = 94)
Samples with a single *pvdhfr* haplotype (n = 72 [76.6%])	F	S	T	S	28 (29.8)
	F	S	T	**N**	2 (2.1)
	F	**R**	T	S	1 (1.1)
	F	**R**	T	**T**	2 (2.1)
	F	**R**	T	**N**	3 (3.2)
	L	**R**	T	S	22 (23.4)
	L	**R**	T	**T**	1 (1.1)
	L	**R**	**M**	**T**	13 (13.8)
Samples with mixed *pvdhfr* haplotypes (n = 22 [23.3%])	F	S/**R**	T	S	1 (1.1)
	F	S	T	S/**T**	1 (1.1)
	L	**R**	T	S/**T**	4 (4.3)
	L	**R**	T	S/**N**	5 (5.3)
	L	**R**	T/**M**	S/**T**	2 (2.1)
	L	**R**	T/**M**	**T**	1 (1.1)
	F/**L**	S/**R**	T	S	4 (4.3)
	F/**L**	S/**R**	T	S/**N**	1 (1.1)
	F/**L**	S/**R**	T/**M**	S/**N**	1 (1.1)
	F/**L**	S/**R**	T/**M**	S/**T**	2 (2.1)

NOTE. *pvdhfr, P. vivax* dihydrofolate reductase.

[a] Boldfaced letters denote mutated alleles.

as a mutated allele in any of the samples. Eight different *pvdhfr* alleles were observed in single-clone infections (76.7%), with the wild type 57F+58S+61T+117S, the double mutant 57L+58R, and the quadruple mutant 57L+58R+61M+117L/I being the most prevalent haplotypes (30%, 23%, and 14%, respectively). Twenty-two (23.4%) of the samples contained mixed alleles at varying codon positions indicating polyclonal infections (table 3). It is worth mentioning that (1) the mutation F57L was always linked to S58R and (2) the T61M mutation was always linked to the triple mutation F57L+S58R+S117T. In contrast to S117T, which was found in single, double, triple, and quadruple mutations in *pvdhfr,* S117N was observed only in single or double mutations.

Association between* pvdhfr *and* pvmdr1 *alleles and treatment outcome. We pooled the data from all 3 study sites and evaluated the association between infections with single and combined mutant alleles in *pvdhfr* and *pvmdr1* and the response to treatment. All patient isolates were coded according to the presence or absence of mutant alleles, and isolates showing both wild-type and mutant alleles were considered to be mutant. Likewise, infecting genotypes were coded according to the most highly mutated *pvdhfr* and *pvmdr1* alleles present in the sample.

In the present study, 2 variables—fever (axillary temperature, ≤37.5°C) and parasite density on the day of study enrollment—were not associated with an increased risk of TF associated with *P. vivax* malaria. Risk of TF tended to decrease with increasing patient age (OR, 0.55; 95% confidence interval [CI], 0.31–0.97; $P = .02$). Regarding single molecular markers in *pvdhfr,* the presence of mutated codon position 57L, 58R, or 117T was independently associated with an increased risk of TF (table 4). The same observation was made with infections harboring the mutation *pvmdr1* 976F. This relationship was further confirmed by the observation of a decreased risk of TF for infections having the wild-type allele 976Y, although the association did not reach statistical significance.

We could discriminate between 14 different *pvdhfr/pvmdr1* genotypes (table 4). Of those genotypes, 7 were observed in association with TF, whereas the remaining 7 were found exclusively in patients with an ACPR. Regarding *pvdhfr* genotypes alone, the risk of TF was clearly associated with the numbers of mutations present in an infection. However, the only significant association with a negative treatment outcome was noted for infecting genotypes having *pvdhfr* quadruple mutations combined with the *pvmdr1* mutation 976F.

We further investigated whether the difference in treatment outcome between study sites was reflected in the drug-resistance marker profile of the corresponding parasite populations. Regarding polymorphisms in *pvdhfr,* there was a marked difference between sites for the mutated positions 57L, 58R, and 117T/N. Similarly, when compared with the prevalence of the 2 sites with lower rates of TF, the mutated locus *pvmdr1* 976F had a significantly higher prevalence in the North Coast area (table 5). Correspondingly, the prevalence of the wild-type allele 976Y was lowest at this site with the highest level of in vivo resistance. A similar finding was noted when the genotype frequencies were compared. The different levels of treatment response were not reflected only in a varying prevalence of the number of mutations in *pvdhfr* ($P = .04$, by χ^2 test). There was a significant difference between sites in terms of the prevalence of the wild-type *pvdhfr/pvmdr1* genotype, which showed a trend toward an increase in prevalence with a decreasing TF rate ($P = .02$, by χ^2 test). The inverse trend was observed with the 2 genotypes having a mutated *pvmdr1* 976F combined with the *pvdhfr* double (57L+58R) or quadruple (57L+58R+61M+117T) mutation, for which prevalences increased with increasing rates of in vivo TF rates ($P = .002$ and $P = .003$, respectively, by χ^2test) (data not shown).

DISCUSSION

After only 4 years of effective implementation in PNG, the efficacy of the new first-line regimen of either AQ or CQ plus SP

Table 4. Association between single-mutated gene loci in *pvdhfr* and *pvmdr1* and infecting *pvdhfr*/*pvmdr1* genotypes and treatment response to amodiaquine plus sulfadoxine-pyrimethamine.

Polymorphism/genotype	Prevalence[a]	Treatment response		OR (95% CI)	*P*
		ACPR	TF		
Polymorphic SNP site[b]					
pvdhfr 57L	56 (59.6)	45 (80.4)	11 (19.6)	9.04 (1.12–73.32)	.01[c]
pvdhfr 58R	63 (67.0)	52 (82.5)	11 (17.5)	6.35 (0.78–51.61)	.03[c]
pvdhfr 61M	19 (20.2)	14 (73.7)	5 (26.3)	3.47 (0.96–12.52)	.07[c]
pvdhfr Mut117[d]	38 (40.4)	31 (81.6)	7 (18.4)	2.30 (0.67–7.89)	.18[c]
pvdhfr 117T	26 (27.7)	19 (73.1)	7 (26.9)	4.64 (1.32–16.32)	.02[c]
pvdhfr 117N	12 (12.8)	12 (100)	0	. . .[e]	.14[c]
pvmdr1 976F[f]	37 (39.4)	29 (78.4)	8 (21.6)	3.66 (1.01–13.18)	.04[c]
pvmdr1 976Y[g]	68 (72.3)	62 (91.2)	6 (8.8)	0.32 (0.09–1.11)	.08[c]
pvdhfr/*pvmdr1* genotype[h]					
Wild-type/976Y[i]	19 (20.2)	18 (94.7)	1 (5.3)	0.29 (0.04–2.40)	.18[j]
Wild-type/976F[k]	9 (9.6)	9 (100)	0	. . .[l]	
Single 117/976Y	2 (2.1)	2 (100)	0	. . .[l]	
Single 117/976F	1 (1.1)	1 (100)	0	. . .[l]	
Single 58/976Y	1 (1.1)	1 (100)	0	. . .[l]	
Single 58/976F	1 (1.1)	1 (100)	0	. . .[l]	
Double 57–58/976Y	16 (17.0)	15 (93.8)	1 (6.2)	0.40 (0.05–3.32)	.34[j]
Double 57–58/976F	10 (10.6)	7 (70.0)	3 (30.0)	3.17 (0.70–14.29)	.16[j]
Double 58–117/976Y	4 (4.3)	4 (100)	0	. . .[l]	
Double 58–117/976F	1 (1.1)	1 (100)	0	. . .[l]	
Triple 57–58-117/976Y	5 (5.3)	4 (80.0)	1 (20.0)	2.17 (0.21–22.57)	.54[j]
Triple 57–58-117/976F	6 (6.4)	5 (83.3)	1 (16.7)	1.60 (0.17–15.59)	.70[j]
Quadruple 57–58-61–117/976Y	10 (10.6)	9 (90.0)	1 (10.0)	0.67 (0.08–5.75)	.70[j]
Quadruple 57–58-61–117/976F	9 (9.6)	5 (55.6)	4 (44.4)	8.56 (1.82–40.24)	.01[j]
Total	94 (100)	82 (87.2)	12 (12.8)		

NOTE. Data are no. (%) of patients, unless indicated otherwise. ACPR, adequate clinical and parasitological response; CI, confidence interval; OR, odds ratio; *pvdhfr*, *Plasmodium vivax* dihydrofolate reductase; *pvmdr1,* multiple-drug–resistance gene 1; SNP, single-nucleotide polymorphism; TF, treatment failure.

[a] Prevalence of polymorphic SNP site or genotype.
[b] In *pvdhfr* and *pvmdr1*.
[c] Determined by standard χ^2 analysis or Fisher's exact test.
[d] Mut117, either 117T or 177N.
[e] 117N was not found in patients who experienced TF.
[f] 976F denotes a mutated allele.
[g] 976Y denotes a wild-type allele.
[h] The genotype is assigned according to the mutated alleles (i.e., a mixed allele is coded as a mutant). Presentation is *pvdhfr* mutation/*pvmdr1* polymorphism.
[i] Wild-type allele.
[j] Determined by likelihood ratio test.
[k] Mutated allele.
[l] These genotypes were not found in patients who experienced TF.

against uncomplicated malaria has reached unacceptably low levels in both species [34]. In the present study, we investigated the association between drug-resistance markers in *pvdhfr* and *pvmdr1* in patients with a *P. vivax* monoinfection and the therapeutic outcome achieved with the newly introduced combination regimen. We measured high prevalences of mutated key markers in both genes and demonstrated an association between the infecting *pvdhfr*/*pvmdr1* genotype and the in vivo treatment response. Furthermore, the different TF rates observed at different study sites were reflected in the genetic drug-resistance profile of the corresponding parasite populations. This is probably the most important finding, because it validates the usefulness of molecular markers in monitoring *P. vivax* resistance to antimalarial drugs to aid policy makers in the development of rational treatment strategies.

Regarding all SNPs analyzed in *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmdr1* (Y976F and F1076L), we found a high prevalence of infections harboring parasites with mutated gene loci (i.e., 20%–67% of samples, depending on the locus analyzed). Furthermore, we observed a high degree of diversity of different *pvdhfr* genotypes in our sample set, which derived from 3 different areas within the same country. In most

Table 5. Prevalence of polymorphisms in *pvdhfr* and *pvmdr1* and corresponding treatment failure (TF) rates for amodiaquine plus sulfadoxine-pyrimethamine at 3 different study sites in Papua New Guinea.

Finding	North Coast area ($n = 29$)	Karimui area ($n = 39$)	South Wosera area ($n = 26$)	P[a]
Mutated SNP site				
pvdhfr 57L	82.8	46.2	53.9	.01
pvdhfr 58R	89.7	53.9	61.5	.01
pvdhfr 61M	27.6	10.3	26.9	.13
pvdhfr Mut117[b]	55.2	25.6	46.2	.04
pvdhfr 117T	41.4	18.0	26.9	.10
pvdhfr 117N	13.8	7.7	19.2	.39
pvmdr1 976F	69.0	25.6	26.9	<.001
Wild-type SNP site				
pvdhfr 57F	31.0	59.0	53.9	.06
Pvdhfr 58S	24.1	53.9	46.2	.05
pvdhfr 61T	86.2	92.3	76.9	.21
pvdhfr 117S	72.4	87.2	65.4	.10
pvmdr1 976Y	48.3	82.1	84.6	.002
TF rate	33.3	5.1	0.0	<.001

NOTE. Data are percentages denoting either the prevalence of polymorphisms or the TF rate in each area. *pvdhfr, Plasmodium vivax* dihydrofolate reductase; *pvmdr1,* multiple-drug–resistance gene 1; SNP, single-nucleotide polymorphism.

[a] As determined by $\chi^2_{(2)}$ analysis.
[b] Mut117, either 117T or 117N.

of the samples, we detected *pvdhfr* wild-type alleles (30% of samples) and double (33%) and quadruple mutations (20%), whereas the rates of single and triple mutations were lower (5% and 12%, respectively). Of the genotypes with double mutations, the allelic variant 57L+58R occurred most often in PNG (84% of genotypes). The prevalence of infections with the mutation Y976F in *pvmdr1* was also high (39% of samples), and the mutation was found in all possible combinations with the different genotypes detected in *pvdhfr*.

Our observations are consistent with the results of similar studies performed in different countries where *P. falciparum* and *P. vivax* are sympatric and where increasing levels of CQ resistance have led to a policy change involving a switch to the alternative low-cost option of using SP. In these regions, in vivo *P. falciparum* resistance to SP developed rapidly after the initial deployment of SP as monotherapy [2, 5], and it was paralleled by the development of in vivo *P. vivax* resistance [6, 21]. Results were further corroborated by the recent demonstration of a similar molecular mechanism of antifolate resistance in both species, one that is conferred by single point mutations in the target enzymes of antifolates and is driven by exertion of selective drug pressure [10, 14, 17, 37]. Different epidemiological studies determining the molecular *pvdhfr* background in field isolates originating from various regions worldwide, such as Thailand [7, 20], Indonesia [15, 19, 21], Cambodia [38], Myanmar [39], India [40, 41], and Ethiopia [42], have shown that previous SP use is correlated with the prevalence rates of resistant *pvdhfr* alleles. Moreover, the association between infecting *pvdhfr* alleles and the treatment outcome associated with SP monotherapy could be demonstrated in Thailand [20] and Indonesia [19, 21].

A similar development of *P. vivax* resistance to SP seems to have taken place in PNG, although SP was introduced in combination with 4-aminoquinolines. The low susceptibility to SP noted in both species in PNG has been documented elsewhere [32] and most probably has arisen because of former drug pressure exerted by mass treatment campaigns with pyrimethamine (in combination with CQ) in the late 1960s and 1970s and because of the use of SP in combination with quinine as a second-line regimen against TF and severe malaria. Therefore, the high frequency of pyrimethamine-specific molecular markers that we measured in *P. vivax* populations is not surprising. Moderate rates of mutation of resistance markers in *P. falciparum dhfr* (i.e., S108N and C59R [43]), which had reached almost fixed levels in 2003 [44], provide further evidence supporting the hypothesis that moderately resistant *dhfr* alleles had already occurred before the effective implementation of SP as part of the standard first-line treatment and that the rapid emergence of alleles with high-level resistance could not be curbed by combining SP with AQ or CQ.

Supplementation of in vivo efficacy data with molecular correlates could be a valuable tool in monitoring *P. vivax* resistance, particularly because unambiguous determination of TF rates is aggravated by difficulties in distinguishing relapses and new infections. Recent advances in the understanding of the mechanisms underlying SP resistance in *P. vivax* have paved the way for the molecular monitoring of resistance against antifolates in this species. However, molecular resistance markers for 4-aminoquinolines have not been reported for *P. vivax* up to the present. Although orthologous genes for *pfcrt* and *pfmdr1*, 2 important genes involved in CQ-resistant falciparum malaria (reviewed in [45] and [46]), have been discovered, and although nonsynonymous point mutations have been described, an association between in vivo resistance and these SNPs or other genetic alterations, such as gene amplification or varying levels of expression, could not be established until now [10–12].

To suggest useful markers for the molecular monitoring of *P. vivax* resistance to AQ+SP, we performed a baseline assessment of the molecular profile in *P. vivax dfhr* and *mdr1,* and we investigated the association between infecting *pvdhfr*/*pvmdr1* genotypes and the in vivo treatment response. Regarding *pvdhfr,* the single-point mutations 57L, 58R, and 117T, as well as the total number of mutations, were all independently associated with an increased risk of TF. These results are in concordance with previous results showing that (1) the parasite reduction ratio noted 48 h after initiation of treatment with SP was lower in patients harboring triple *dhfr* mutants than in those harboring double mutants [20], and (2) individuals infected with quadruple *dhfr* mutants had a higher risk of experiencing TF when receiving SP

[19, 21]. Regarding the combinations of mutations in *pvdhfr,* the observations that (1) mutation 117N was never observed in *dhfr* triple or quadruple mutants, (2) triple and quadruple mutants always had the mutation 117T, (3) 57L was always linked to 58R, and (4) 61M was seen only in quadruple mutants were all in agreement with previous data. The most prevalent *dhfr* genotypes reported in previous studies included single 117, double 58+117, triple 117+58+57, and quadruple 117+61+58+57 mutants, and, because of this frequently observed allele structure, the stepwise accumulation of mutations in *pvdhfr* was suggested to be similar to that in *P. falciparum*, in which low-level pyrimethamine resistance is conferred by the single *pfdhfr* mutation 108N (corresponding to *pvdhfr* 117N) and in which drug selection processes leading to the addition of 59R and/or 51I (corresponding to *pvdhfr* 58R and 57L/I) increase resistance to SP [20, 21, 47]. Our data from PNG that showed a high frequency of infections with the double mutant 57L+58R seem to be inconsistent with this hypothesis. Nevertheless, the same double mutant has been previously described in Thai and Indian field isolates [20, 40], and Hastings et al. [15] reported prevalence rates of 5.3% in the Wosera area in 1999 and 8% in the North Coast area in 2000. Moreover, using a yeast expression system for the investigation of in vitro drug susceptibility of different allelic *pvdhfr* variants, the same authors showed that individuals with the double mutant 57L+58R were less susceptible to pyrimethamine than the population expressing the wild-type allele.

Regarding *pvmdr1,* we could confirm previous results that demonstrated the presence of the polymorphic *mdr1* locus Y976F in field isolates [12]. Furthermore, we found the mutation to be a strong independent predictor of TF associated with the use of AQ+SP. To our knowledge, these are the first data to indicate that *pvmdr1* plays an important role in mediating in vivo drug resistance in *P. vivax.* This finding is in contrast with recent studies, in which an association of *pvmdr1* polymorphisms with *P. vivax* resistance to CQ and mefloquine could not be demonstrated [13, 48]; the most likely reason for this inability to demonstrate an association is that these studies used small sample sizes and were not specifically designed to demonstrate an association between *pvmdr1* polymorphisms and the in vivo treatment response. However, the role of *pvmdr1* in conferring resistance to different drugs remains to be clarified. The situation may be equally as complex as that associated with falciparum malaria, in which different SNPs and/or gene amplification was shown to be associated with resistance to 4-aminoquinolines, amino alcohols, and artemisinin derivatives, respectively [49, 50].

The good predictive value of the single markers analyzed in both genes was further confirmed by investigation of the association between the combined *pvdhfr*/*pvmdr1* genotype and the treatment response, for which the highest risk of TF was found to be significantly associated with an infecting genotype with a quadruple mutation in *pvdhfr* plus a 976F mutation in *pvmdr1.* The observation of TF occurring in association with infections involving wild-type alleles may well be a consequence of technical constraints resulting from limitations in differentiating true recrudescences from relapses and/or new infections, which may have lead to an overestimation of true TF rates.

In spite of these limitations and the fact that a drug-resistant *P. vivax* phenotype is most likely mediated by multigenic processes, we think that the set of SNPs included in our study is sufficient to monitor parasite resistance under the current first-line regimen. The difference in TF rates between sites was not only reflected in the different prevalence rates of key markers that have shown an association with treatment response, but it was also reflected in the different frequencies of highly mutated and/or wild-type *pvdhfr/pvmdr1* genotypes circulating at the respective sites. These findings strongly support the usefulness of molecular markers in monitoring the dynamics of *P. vivax* resistance and, thus, their important role in complementing in vivo efficacy data to determine the most appropriate and feasible drug policy against vivax malaria. For the time being, we propose to use polymorphisms in *pvdhfr* F57L, S58R, and S117T/N and in *pvmdr1* Y976F for the molecular assessment of *P. vivax* resistance to AQ+SP. However, the inclusion of other SP-relevant markers (e.g. polymorphisms in *pvdhps*) and as-yet-unidentified markers involved in resistance to other antimalarials may become necessary for the longitudinal monitoring of resistance in the future, in particular when a policy change will recommend new classes of drugs.

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References

1. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg **2001**; 64:97–106.
2. Baird JK. Chloroquine resistance in *Plasmodium vivax.* Antimicrob Agents Chemother **2004**; 48:4075–83.
3. Sattabongkot J, Tsuboi T, Zollner GE, Sirichaisinthop J, Cui L. *Plasmodium vivax* transmission: chances for control? Trends Parasitol **2004**; 20: 192–8.
4. Whitby M, Wood G, Veenendaal JR, Rieckmann K. Chloroquine-resistant *Plasmodium vivax.* Lancet **1989**; 2:1395.
5. Peters W. Drug resistance in malaria parasites of animals and man. Adv Parasitol **1998**; 41:1–62.
6. Pukrittayakamee S, Chantra A, Simpson JA, et al. Therapeutic responses to different antimalarial drugs in vivax malaria. Antimicrob Agents Chemother **2000**; 44:1680–5.
7. Plowe CV. Monitoring antimalarial drug resistance: making the most of the tools at hand. J Exp Biol **2003**; 206:3745–52.

8. World Health Organization (WHO). Guidelines for the treatment of malaria. WHO/HTM/MAL/2006.1108. Geneva: WHO, **2006**.
9. Eldin de Pécoulas P, Basco LK, Tahar R, Ouatas T, Mazabraud A. Analysis of the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene sequence. Gene **1998**; 211:177–85.
10. Korsinczky M, Fischer K, Chen N, Baker J, Rieckmann K, Cheng Q. Sulfadoxine resistance in *Plasmodium vivax* is associated with a specific amino acid in dihydropteroate synthase at the putative sulfadoxine-binding site. Antimicrob Agents Chemother **2004**; 48: 2214–22.
11. Nomura T, Carlton JM, Baird JK, et al. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J Infect Dis **2001**; 183:1653–61.
12. Brega S, Meslin B, de Monbrison F, et al. Identification of the *Plasmodium vivax mdr*-like gene *(pvmdr1)* and analysis of single-nucleotide polymorphisms among isolates from different areas of endemicity. J Infect Dis **2005**; 191:272–7.
13. Sá JM, Nomura T, Neves J, Baird JK, Wellems TE, del Portillo HA. *Plasmodium vivax:* allele variants of the *mdr1* gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol **2005**; 109:256–9.
14. de Pécoulas 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.
15. Hastings MD, Maguire JD, Bangs MJ, et al. Novel *Plasmodium vivax dhfr* alleles from the Indonesian Archipelago and Papua New Guinea: association with pyrimethamine resistance determined by a *Saccharomyces cerevisiae* expression system. Antimicrob Agents Chemother **2005**; 49: 733–40.
16. Hastings MD, Sibley CH. Pyrimethamine and WR99210 exert opposing selection on dihydrofolate reductase from *Plasmodium vivax.* Proc Natl Acad Sci U S A **2002**; 99:13137–41.
17. 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.
18. Tahar R, de Pécoulas 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.
19. Hastings MD, Porter KM, Maguire JD, et al. Dihydrofolate reductase mutations in *Plasmodium vivax* from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. J Infect Dis **2004**; 189:744–50.
20. 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.
21. 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.
22. Chotivanich K, Udomsangpetch R, Chierakul W, et al. In vitro efficacy of antimalarial drugs against *Plasmodium vivax* on the western border of Thailand. Am J Trop Med Hyg **2004**; 70:395–7.
23. Russell BM, Udomsangpetch R, Rieckmann KH, Kotecka BM, Coleman RE, Sattabongkot J. Simple in vitro assay for determining the sensitivity of *Plasmodium vivax* isolates from fresh human blood to antimalarials in areas where *P. vivax* is endemic. Antimicrob Agents Chemother **2003**; 47:170–3.
24. Imwong M, Pukrittayakamee S, Cheng Q, et al. Limited polymorphism in the dihydropteroate synthetase gene *(dhps)* of *Plasmodium vivax* isolates from Thailand. Antimicrob Agents Chemother **2005**; 49:4393–5.
25. Müller I, Bockarie M, Alpers M, Smith T. The epidemiology of malaria in Papua New Guinea. Trends Parasitol **2003**; 19:253–9.
26. Grimmond TR, Donovan KO, Riley ID. Chloroquine resistant malaria in Papua New Guinea. P N G Med J **1976**; 19:184–5.
27. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance to chloroquine? Lancet **1989**; 2:1183–4.
28. al-Yaman F, Genton B, Mokela D, Narara A, Raiko A, Alpers MP. Resistance of *Plasmodium falciparum* malaria to amodiaquine, chloroquine and quinine in the Madang Province of Papua New Guinea, 1990–1993. P N G Med J **1996**; 39:16–22.
29. Genton B, Baea K, Lorry K, Ginny M, Wines B, Alpers M. Parasitological and clinical efficacy of standard treatment regimens against *Plasmodium falciparum, P. vivax* and *P. malariae* in Papua New Guinea. P N G Med J **2005**; 48:141–50.
30. Al-Yaman F, Genton B, Mokela D, Alpers MP. Resistance of *Plasmodium falciparum* in vivo to 3 days' treatment with quinine and single-dose Fansidar. P N G Med J **1994**; 37:54–6.
31. Darlow B, Vrbova H, Gibney S, Jolley D, Stace J, Alpers M. Sulfadoxine-pyrimethamine for the treatment of acute malaria in children in Papua New Guinea. I. *Plasmodium falciparum.* Am J Trop Med Hyg **1982**; 31: 1–9.
32. Darlow B, Vrbova H, Gibney S, Jolley D, Stace J, Alpers M. Sulfadoxine-pyrimethamine for the treatment of acute malaria in children of Papua New Guinea. II. *Plasmodium vivax.* Am J Trop Med Hyg **1982**; 31:10–3.
33. Lamont G, Darlow B. Comparison of in vitro pyrimethamine assays and in vivo response to sulphadoxine-pyrimethamine in *Plasmodium falciparum* from Papua New Guinea. Trans R Soc Trop Med Hyg **1982**; 76: 797–9.
34. Marfurt J, Müeller I, Sie A, et al. Low efficacy of amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against *Plasmodium falciparum* and *P. vivax* malaria in Papua New Guinea. Am J Trop Med Hyg **2007**; 77:947–54.
35. World Health Organization (WHO). Monitoring antimalarial drug resistance: report of a WHO consultation. WHO/CDS/RBM/2002.39. Geneva: WHO, **2001**.
36. Brega S, de Monbrison F, Severini C, et al. Real-time PCR for dihydrofolate reductase gene single-nucleotide polymorphisms in *Plasmodium vivax* isolates. Antimicrob Agents Chemother **2004**; 48:2581–7.
37. Imwong M, Pukrittayakamee S, Rénia 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.
38. de Pécoulas PE, Tahar R, Yi P, Thai KH, Basco LK. Genetic variation of the dihydrofolate reductase gene in *Plasmodium vivax* in Snoul, northeastern Cambodia. Acta Trop **2004**; 92:1–6.
39. Na BK, Lee HW, Moon SU, et al. Genetic variations of the dihydrofolate reductase gene of *Plasmodium vivax* in Mandalay Division, Myanmar. Parasitol Res **2005**; 96:321–5.
40. Kaur S, Prajapati SK, Kalyanaraman K, Mohmmed A, Joshi H, Chauhan VS. *Plasmodium vivax* dihydrofolate reductase point mutations from the Indian subcontinent. Acta Trop **2006**; 97:174–80.
41. Valecha N, Joshi H, Eapen A, et al. Therapeutic efficacy of chloroquine in *Plasmodium vivax* from areas with different epidemiological patterns in India and their *Pvdhfr* gene mutation pattern. Trans R Soc Trop Med Hyg **2006**; 100:831–7.
42. Schunk M, Kumma WP, Miranda IB, et al. High prevalence of drug-resistance mutations in *Plasmodium falciparum* and *Plasmodium vivax* in southern Ethiopia. Malar J **2006**; 5:54.
43. Reeder JC, Rieckmann KH, Genton B, Lorry K, Wines B, Cowman AF. Point mutations in the dihydrofolate reductase and dihydropteroate synthetase genes and in vitro susceptibility to pyrimethamine and cycloguanil of *Plasmodium falciparum* isolates from Papua New Guinea. Am J Trop Med Hyg **1996**; 55:209–13.
44. Marfurt J, Müller I, Sie A, et al. The usefulness of twenty-four molecular markers in predicting treatment outcome with combination therapy of

amodiaquine plus sulphadoxine-pyrimethamine against falciparum malaria in Papua New Guinea. Malar J **2008**; 7:61.
45. Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA. Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. Mol Microbiol **2005**; 56:323–33.
46. 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.
47. Sibley CH, Hyde JE, Sims PF, et al. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum:* what next? Trends Parasitol **2001**; 17: 582–8.
48. Picot S, Brega S, Gérôme P, et al. Absence of nucleotide polymorphism in a *Plasmodium vivax* multidrug resistance gene after failure of mefloquine prophylaxis in French Guyana. Trans R Soc Trop Med Hyg **2005**; 99:234–7.
49. Duraisingh MT, Cowman AF. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. Acta Trop **2005**; 94:181–90.
50. Duraisingh MT, Refour P. Multiple drug resistance genes in malaria—from epistasis to epidemiology. Mol Microbiol **2005**; 57:874–7.