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Prevalence and patterns of antifolate and chloroquine drug resistance markers in *Plasmodium vivax* across Pakistan

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Abstract

Background: *Plasmodium vivax* is the most prevalent malaria species in Pakistan, with a distribution that coincides with *Plasmodium falciparum* in many parts of the country. Both species are likely exposed to drug pressure from a number of anti-malarials including chloroquine, sulphadoxine-pyrimethamine (SP), and artemisinin combination therapy, yet little is known regarding the effects of drug pressure on parasite genes associated with drug resistance. The aims of this study were to determine the prevalence of polymorphisms in the SP resistance-associated genes *pvdhfr*, *pvdhps* and chloroquine resistance-associated gene *pvmdr1* in *P. vivax* isolates collected from across the country.

Methods: In 2011, 801 microscopically confirmed malaria-parasite positive filter paper blood samples were collected at 14 sites representing four provinces and the capital city of Islamabad. Species-specific polymerase chain reaction (PCR) was used to identify human *Plasmodium* species infection. PCR-positive *P. vivax* isolates were subjected to sequencing of *pvdhfr*, *pvdhps* and *pvmdr1* and to real-time PCR analysis to assess *pvmdr1* copy number variation.

Results: Of the 801 samples, 536 were determined to be *P. vivax*, 128 were *P. falciparum*, 43 were mixed *vivax/falciparum* infections and 94 were PCR-negative for *Plasmodium* infection. Of PCR-positive *P. vivax* samples, 372 were selected for sequence analysis. Seventy-six of the isolates (23%) were double mutant at positions S58R and S117N in *pvdhfr*. Additionally, two mutations at positions N50I and S93H were observed in 55 (15%) and 24 (7%) of samples, respectively. Three 18 base pair insertion-deletions (indels) were observed in *pvdhfr*, with two insertions at different nucleotide positions in 36 isolates and deletions in 10. Ninety-two percent of samples contained the *pvdhps* (S382/A383G/K512/A553/V585) SAKAV wild type haplotype. For *pvmdr1*, all isolates were wild type at position Y976F and 335 (98%) carried the mutation at codon F1076L. All isolates harboured single copies of the *pvmdr1* gene.

Conclusions: The prevalence of mutations associated with SP resistance in *P. vivax* is low in Pakistan. The high prevalence of *P. vivax* mutant *pvmdr1* codon F1076L indicates that efficacy of chloroquine plus primaquine could be in danger of being compromised, but further studies are required to assess the clinical relevance of this observation. These findings will serve as a baseline for further monitoring of drug-resistant *P. vivax* malaria in Pakistan.

Keywords: *Plasmodium vivax*, Malaria, Pakistan, Drug resistance, Sulphadoxine-pyrimethamine, Chloroquine, *Pvmdr1*, *Pvdhfr*, *Pvdhps*

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Background

Malaria imposes a significant public health burden in Pakistan, causing a reported 1.6 million cases per year according to the World Health Organization (WHO) [1]. *Plasmodium vivax* is the most prevalent species of human malaria in the country, accounting for 200,000 (67%) of all cases in 2011 [2], with *Plasmodium falciparum* malaria accounting for the remaining one-third. Chloroquine plus primaquine is the first-line treatment for *P. vivax* in most of the world, including Pakistan. After the rise of chloroquine resistance in *P. falciparum*, sulphadoxine-pyrimethamine (SP) treatment became widespread throughout Asia and has since been replaced by artesunate plus SP for treatment of falciparum malaria in Pakistan. Despite its prevalence and public health importance, *P. vivax* has not been well-characterized with respect to drug resistance to chloroquine or to SP (to which it may be exposed during presumptive treatment of *P. falciparum*) in Pakistan [3].

Chloroquine resistance in *P. vivax* has been observed in the Pacific [4], Latin America [5], and parts of Asia [6,7] but has not yet been reported in Pakistan and Afghanistan [8,9], and chloroquine remains effective against *P. vivax* in India [10,11]. Chloroquine resistance in *P. vivax* is thought to be mediated by single nucleotide polymorphisms (SNPs) in the *pvmdr1* gene of *P. vivax* [12-14]. It has been suggested that point mutations at codons Y976F and F1076L of the *pvmdr1* gene may be involved in resistance to 4-aminoquinolones, chloroquine and amodiaquine [12,15]. *In vitro* data show that Indonesian *P. vivax* isolates with a prevalence of 96% for mutation Y976F had a significantly higher IC₅₀ for chloroquine compared to Thai isolates, which had a 25% prevalence of Y976F [16]. Whereas an association of mutation F1076L with chloroquine resistance has not yet been found [16,17], the most common *pvmdr1* haplotypes found in nature are F1076L [18-20], and Y976F plus F1076L [21], with the single mutant Y976F reported only rarely [16]. This observation has given rise to the theory that a two-step mutational trajectory, with mutations at codon F1076L followed by Y976F, may lead to chloroquine resistance [12,22]; however, *in vivo* substantiation of this theory is required. Polymorphisms in copy number of the *pvmdr1* gene may also modulate drug resistance in *P. vivax*, as shown by a link between gene amplification and decreased *in vitro* susceptibility to mefloquine, artesunate, and amodiaquine but increased susceptibility to chloroquine [23].

Although SP has not been indicated for treatment of *P. vivax*, there is evidence that intensive SP use for treatment of *P. falciparum* may also select for resistant *P. vivax* in regions where both species coexist [24-26]. An efficacy study of SP, chlorproguanil-dapsone and chloroquine carried out from 2004 to 2006 suggests that antifolates were effective against *P. vivax* in Afghanistan

and Pakistan at that time [9]. In the absence of more recent data, molecular markers may be used to monitor the effects of SP pressure on *P. vivax*.

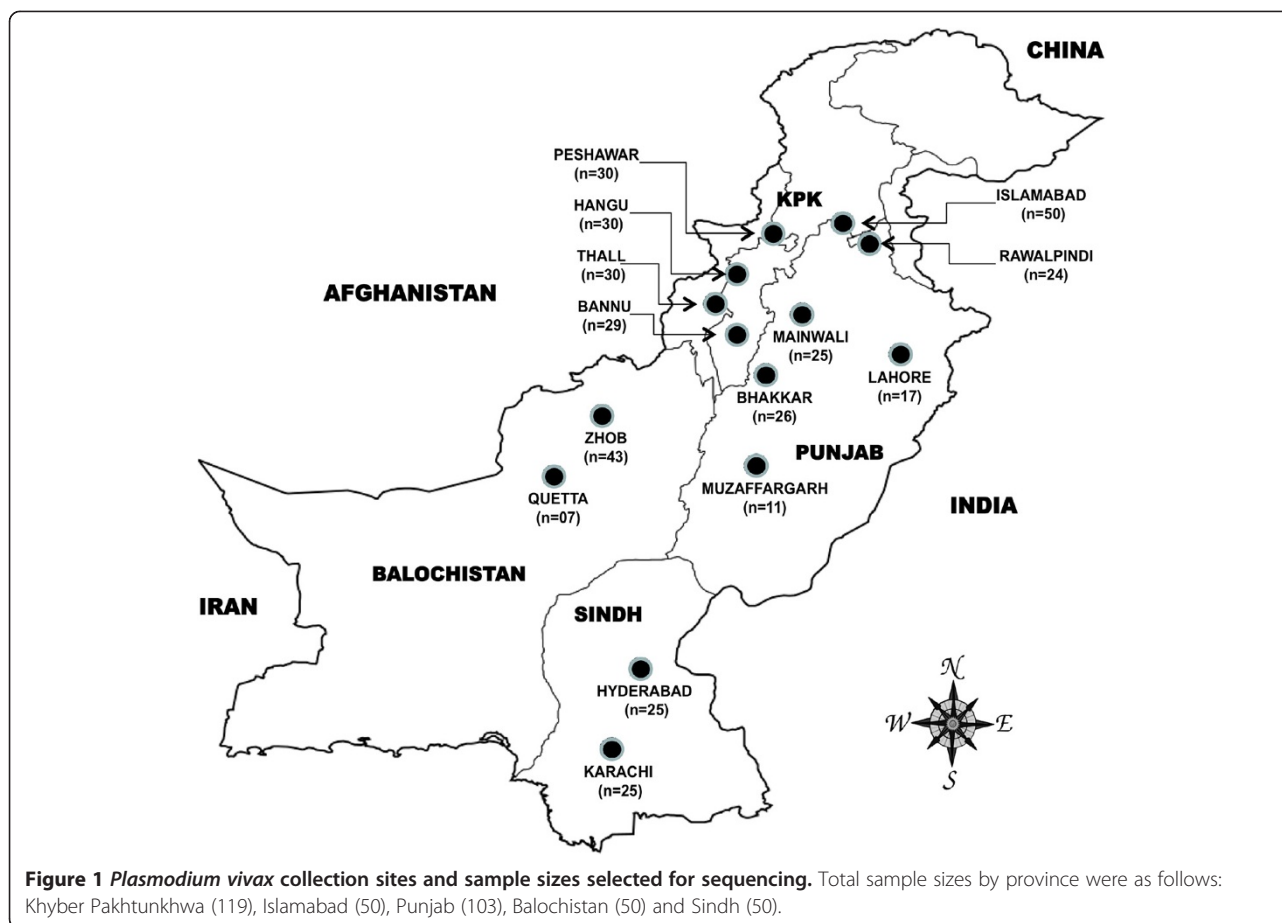
Pyrimethamine and sulphadoxine target the *dhfr* and *dhps* enzymes in *P. falciparum* and point mutations in these genes confer resistance to each drug [27-34]. Mutations in codons I13L, P33L, F57L/I, S58R, T61M, S117N/T and I173L/F of the *P. vivax dhfr* enzyme have been proposed as conferring similar resistance to pyrimethamine in *P. vivax* [19,35]. *In vitro* studies of *pvdhfr* expression in *Escherichia coli*, *P. falciparum* and yeast have shown that *pvdhfr* double and triple mutants S58R + S117N, F57L + S58R, and F57L + S58R + S117T confer significant (up to several hundred-fold) reductions in susceptibility to antifolates [36-39] and are associated with reduced drug affinity to the target enzyme [38]. In *P. vivax dhps*, mutations at codons S382A/C, A383G, K512M/T/E, and A553G/C are hypothesized to confer resistance to sulphadoxine [25,40].

In Pakistan, published data are not available on the prevalence of point mutations in *pvmdr1*, and limited information is available regarding the prevalence of mutations in *pvdhfr* and *pvdhps*. In 2009, a study conducted in Bannu district of Pakistan reported a prevalence of 1.7% of *pvdhfr* F57L, 19% for S58R and 93.5% for S117N [3]. In *pvdhps*, a study conducted in 2010 in the Federally Administered Tribal Areas of Khyber Pakhtunkhwa province reported that 15% of samples had A383G and 54% had A553G/C [41]. Because up to date information on clinical efficacy and molecular markers of anti-malarial resistance in Pakistan is not available, this study was designed to evaluate the current prevalence of point mutations in *pvdhfr*, *pvdhps* and *pvmdr1* genes of *P. vivax* in 14 sites around the country. The results presented here provide indications of whether efficacy of current anti-malarials is being maintained against *P. vivax* in Pakistan or requires further assessment.

Methods

Sample collection and ethics

Twenty-five government and private hospitals in the four provinces with the highest malaria burden (Khyber Pakhtunkhwa, Sindh, Balochistan and Punjab) along with the capital city were invited to provide samples collected during routine malaria surveillance. Fourteen facilities responded and were included in this study: Islamabad (the capital), Peshawar, Thall, Bannu and Hangu (Khyber Pakhtunkhwa Province; KPK), Karachi and Hyderabad (Sindh Province), Zhob and Quetta (Balochistan Province), Rawalpindi, Bhakhar, Mainwali, Lahore and Muzaffargarh (Punjab Province) (Figure 1) [42]. Despite the high prevalence of malaria, the Federally Administered Tribal Areas were excluded from this study because political uncertainty and terrorism [43]



make it difficult to establish sample collection. Samples were collected between April and October 2011. A total of 10,782 symptomatic patients of all ages presenting with signs of malaria was screened for malaria infection by microscopic examination of blood by a trained laboratory technician. Approximately 3 ml of venous blood was drawn in EDTA tubes from each patient for suspected cases after obtaining informed consent. Information on patient age, gender, and city of residence was collected. The study was approved by the institutional review board of Quaid-i-Azam University, Islamabad, Pakistan.

Microscopy and dried blood spot collection

Thick and thin blood films of patients with suspected malaria were stained with 10% Giemsa solution and slides were examined at 100X under oil immersion of microscope by laboratory technician/technologist trained in malaria diagnosis in line with WHO guidelines [44]. For samples testing positive for malaria by microscopy, 50 μ l blood was applied to Whatman 3MM filter paper. Blood from 30 malaria-negative samples by microscopy was also spotted on filter paper. The remaining blood from all samples was stored on site at -80°C . Filter

papers were air-dried overnight and stored in individual plastic bags with desiccant at room temperature.

DNA extraction and speciation

DNA was isolated from 801 malaria-positive by microscopy and 30 malaria-negative filter paper samples using the QIAmp 96 DNA kit (Qiagen, Valencia, CA, USA). Isolated DNA was stored at -20°C for further analysis. *Plasmodium* species (*P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*) were confirmed by nested polymerase chain reaction (PCR) amplification of the small subunit ribosomal ribonucleic acid (ssrRNA) genes using primers and thermal cycler conditions as previously described [45]. Twenty-seven of the 30 microscopy-negative samples were also subjected to PCR for quality control. PCR products were visualized by 2-2.5% agarose gel electrophoresis using the Bio-Rad gel doc system (Bio-Rad, Hercules, CA, USA). Molecular analysis was conducted at the University of Maryland School of Medicine, Baltimore, USA.

Amplification of *pvdhfr*, *pvdhps* and *pvm-dr1*

Out of 579 PCR-confirmed (536 *P. vivax* and 43 *P. vivax/P. falciparum* mixed infection) samples, 372

isolates were randomly selected for further molecular characterization (Table 1). Target sequences of the *pvdhfr*, *pvdhps* and *pvmdr1* genes harbouring known or putative mutations associated with SP and chloroquine resistance were selected for nested PCR amplification and sequencing. Primers, reagent concentrations and thermal cycling conditions were adapted from those previously published [19] with the following modifications. Denaturation was done at 94°C for 30 sec, 2 µl of DNA template was added to a total reaction volume of 50 µl, and the second round of PCR was carried out for a total of 25 cycles. PCR products were visualized as previously described. The expected band sizes for *pvdhfr*, *pvdhps* and *pvmdr1* were 632, 767 and 547 bp, respectively.

Table 1 Prevalence of multilocus genotypes and single nucleotide polymorphism in *pvdhfr*, *pvdhps* and *pvmdr1* gene of *P. vivax*

Gene	Sample size	Haplotypes*	No of isolates (%)
<i>pvdhfr</i>	336	IPFSTSI (wild type)	151 (45)
		IPFSTNI	100 (30)
		IPFRTNI	76 (23)
		IPLRTSI	5 (1.5)
		IPFRTSI	3 (1.0)
		IPFITNI	1 (0.3)
<i>pvdhps</i>	339	SAKAV (wild type)	330 (97)
		SGKAV	7 (2.0)
		SAKGV	1 (0.3)
		SGKGV	1 (0.3)
<i>pvdhfr + pvdhps</i>	322	IPFSTSI SAKAV (wild type)	143 (44)
		IPFSTNI SAKAV	95 (30)
		IPFRTNI SAKAV	69 (21)
		IPLRTSI SAKAV	5 (1.6)
		IPFRTSI SAKAV	3 (1.0)
		IPFSTNISGKAV	2 (0.6)
		IPFRTNISGKAV	2 (0.6)
		IPFSTSISAKGV	1 (0.3)
		IPFSTSISGKAV	1 (0.3)
		IPFRTNISGKGV	1 (0.3)
<i>pvmdr1</i>	342	YF (wild type genotype)	7 (2.0)
		YL (single mutant genotype)	335 (98)
		FF (single mutant genotype)	0
		FL (double mutant genotype)	0

*Drug resistance conferring point mutations are shown for the following codons: *pvdhfr* 13, 33, 57, 58, 61, 117, 173; *pvdhps* 382, 383, 512, 553, 585; and *pvmdr1* 976, 1076. Mutated amino acids are shown in bold.

Sequencing

Amplified secondary PCR products of *pvdhfr*, *pvdhps* and *pvmdr1* genes were purified using Millipore® filtration plates (EMD Millipore, Billerica, MA, USA). The sequencing reaction for all three genes was performed in the forward and reverse directions using secondary PCR primers. The BigDye terminator v3.1 kit (ABI) was used for each sequencing reaction in the following ratios: BigDye 1 µl, buffers 1 µl, 2 µmol of nested PCR forward or reverse primers, purified PCR products 2 µl and ultra pure water 5 µl in a volume of 10 µl. The thermal cyclers were programmed as follows: 94°C for 20 min, 25 cycles of 94°C for 20 sec, 50°C for 5 sec, 48°C for 5 sec, 60°C for 3 min, and final extension at 60°C for 5 min. Sequencing was performed using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA) and sequence analyses and alignment was performed using Sequencher 4.10.1 software (Gene Codes Corp., Ann Arbor, MI, USA). Sequences of a *P. vivax* ARI/Pakistan isolate (Gen-Bank accession no. X98123), Brazilian clinical isolate (accession no. AY186730) and Sal-1 (Gen-Bank accession no. AY618622) were used as references for polymorphisms in *pvdhfr*, *pvdhps* and *pvmdr1*.

Confirmation of rare variants

Rare or novel SNP variants were ruled out as PCR or sequencing errors as follows. SNPs occurring in a single sample and those not previously reported were re-amplified by PCR and sequenced a second time to confirm presence of the polymorphism. Original and re-sequencing reactions were both conducted in the forward and reverse directions (2X coverage) to reduce the likelihood of sequencing errors.

Quantification of *pvmdr1* copy number

Quantification of *pvmdr1* copy number was assessed using previously described primers, probes and thermal cyclers conditions [19] on a TaqMan 7700 real-time PCR machine (Applied Biosystems, Warrington, UK). *Pvaldolase* was used as the endogenous control and a plasmid containing a single and double copy number insert of *pvmdr1* in pCR2.1 vector was used as calibrator. All samples were run in triplicate. *pvmdr1* copy number values between 0.5 and 1.3 were considered as single copy, between 1.6 and 2.3 as two copies and between 2.6 and 3.3 as three copies. Assays were repeated if copy number values were between 1.3 and 1.6 and 2.3 and 2.6. Assays were also repeated if the Ct value exceeded 35 or the standard deviation for replicates of *pvmdr1* or *pvaldolase* copy number exceeded 0.2. Copy number of *pvmdr1* was

estimated by using the following formula:

$$\Delta\Delta Ct = \frac{(Ct_{pvmdr1} - Ct_{pvaldolase})_{\text{sample}} - (Ct_{pvmdr1} - Ct_{pvaldolase})_{\text{calibrator}}}{\text{calibrator}}$$

Results

Of 801 malaria-positive isolates by microscopy, PCR analysis revealed that 536 (76%) isolates were positive for *P. vivax*, 128 (18%) were *P. falciparum*, and 43 (4%) were mixed infections of both *P. falciparum* plus *P. vivax*. None of the samples was positive for *P. malariae* or *P. ovale* and 94 samples were PCR-negative for *Plasmodium* DNA. Results discordant with microscopic diagnosis were re-tested by PCR for confirmation, and a further comparison of microscopy and PCR results is presented in a separate manuscript [42]. Age among the 801 subjects ranged from two months to 75 years, with a median of 24 years. 64% of samples were collected from males and 36% from females. The gender distribution was identical and age distribution nearly identical in the subset of 372 of 579 *P. vivax*-positive samples by PCR that were selected for sequencing (age range = two months to 70 years; median = 25 years). Of these, sequencing was successful in 90% (336/372) for *pvdhfr*, 91% (339/372) for *pvdhps* and 92% (342/372) for *pvmdr1*. A subset of 120 PCR-positive *P. vivax* isolates was randomly selected for the quantification of *pvmdr1* copy number variation, and the assay was successful in 108 (90%) of isolates.

Sequenced regions of each gene were monoclonal with no evidence of mixtures of SNPs among any of the samples, allowing the investigators to identify haplotypes. For codons I13L, P33L, F57L/I, S58R, T61M, S117N/T and I173L/F of *pvdhfr*, 151 (45%) of the isolates had the wild-type haplotype IPFSTSI. One hundred isolates (30%) had the S117N single mutation, 76 (23%) harboured S58R plus S117N double mutants, five isolates had the F57L plus S58R double mutant and one sample had the S58I plus S117N double mutant. The two mutations responsible for conferring pyrimethamine resistance *in vitro*, S58R and S117N, [46], were found in 84 (25%) and 177 (53%) of isolates, respectively. The *pvdhfr* S58R plus S117N double mutant haplotype was most prevalent among isolates in Sindh (6.8%) and Khyber Pakhtunkhwa province (6.5%) (Table 2). Although the *pvdhfr* double mutant was more common in infected patients over the age of 12 years than in children aged 12 and under prevalence of 24% versus 15%, the difference was not statistically significant (chi-square = 1.87; $p = 0.17$). Similarly, the double mutant was present in 28% of infections in females and 20% of infections in males but the difference was not significant (chi-square = 2.67; $p = 0.10$). Eight additional non-

synonymous SNPs were detected, corresponding to amino acids N50I, S57R, S58R, S58I, S93R, S93H, S117N and N123K. Two of these, S93R and K123N, have not been reported previously. Non-synonymous and synonymous mutations are given in tabulated form in Additional file 1.

Based on the use of *P. vivax* strain ARI/Pakistan (GenBank accession no X98123) as reference for *pvdhfr*, three indels were detected in the study, all 18 base pairs (bp) in size (Table 3). Seven isolates had an 18 bp repeat insertion between amino acid positions 91 and 92, and 21 samples had an 18 bp repeat insertion between codons 103 and 104. Ten isolates were discovered with an 18-bp deletion between amino acids 92 and 97.

The majority of samples were wild-type for *pvdhps* point mutations associated with sulphadoxine resistance, with the exception of seven samples carrying the A383G mutation, one carrying the A553G mutation, and one carrying the A383G plus A553G double mutation (Table 1). Four of the samples carrying the A383G mutation were from Khyber Pakhtunkhwa while the other three were found in each of three provinces, and the A383G plus A553G double mutant was found in Punjab (Table 2). Six non-synonymous mutations were detected (Additional file 1), four of which have not been previously reported.

The three most prevalent *pvdhfr* plus *pvdhps* haplotypes across all sites were wild-type (44%), single *pvdhfr* mutant I₁₃P₃₃F₅₇S₅₈T₆₁N₁₁₇I₁₇₃/S₃₈₂A₃₈₃K₅₁₂A₅₅₃V₅₈₅ (30%) and double *pvdhfr* mutant I₁₃P₃₃F₅₇R₅₈T₆₁N₁₁₇I₁₇₃/S₃₈₂A₃₈₃K₅₁₂A₅₅₃V₅₈₅ (21%).

All isolates were wild type at *pvmdr1* codon Y976F while 335 (98%) isolates carried the point mutation at codon F1076L (Table 1). One hundred and ten, or 32% of all isolates with *pvmdr1* F1076L, were found in Khyber Pakhtunkhwa and 84 (25%) were found in Punjab province. Three other non-synonymous SNPs (Y963C, N1010S and S1071G) not previously reported and a single synonymous SNP were also found (Additional file 1). All 108 isolates tested carried a single copy of the *pvmdr1* gene.

Discussion

Pakistan adopted artesunate plus SP combination therapy as a first line treatment against uncomplicated *P. falciparum* in 2008 [47]. Chloroquine is no longer indicated for treatment of *P. falciparum* but is recommended in combination with primaquine for treatment of *P. vivax*. Although the nature of anti-malarial use in Pakistan is not well-characterized, the availability of SP and other drugs as monotherapy has been documented [48] and along with misdiagnosis of mixed infections [49] and presumptive treatment [50], likely results in *P. vivax* infections being frequently treated

Table 2 Prevalence and distribution of *pvdhfr*, *pvdhps* and *pvmdr1* alleles by province

	<i>*pvdhfr</i>		<i>pvdhps</i>		<i>pvmdr1</i>	
	Haplotypes	No of isolates (%)	Haplotypes	No of isolates (%)	Haplotypes	No of isolates (%)
Balochistan	IPFSTSI (wild type)	19 (5.7)	SAKAV (wild type)	44 (13)	YF (wild type)	2 (0.6)
	IPFSTNI	18 (5.4)	SGKAV	1 (0.3)	YL	38 (11)
	IPFRTNI	6 (2)				
Total sequenced	43/50		45/50		40/50	
Islamabad	IPFSTSI	26 (7.7)	SAKAV	47 (14)	YF	3 (1.0)
	IPFRTNI	14 (4.2)			YL	47 (14)
	IPFSTNI	10 (3.0)				
Total sequenced	50/50		47/50		50/50	
Khyber Pakhtunkhwa	IPFSTSI	37 (11)	SAKAV	108 (32)	YF	5 (2.0)
	IPFSTNI	50 (15)	SAKGV	1 (0.3)	YL	110 (32)
	IPFRTNI	22 (6.5)	SGKAV	4 (1.2)		
	IPFRTSI	2 (0.6)				
	IPLRTSI	1 (0.3)				
Total sequenced	112/119		113/119		115/119	
Punjab	IPFSTSI	55 (16)	SAKAV	89 (26)	YF	5 (2.0)
	IPFRTNI	12 (3.6)	SGKAV	1 (0.3)	YL	84 (25)
	IPFSTNI	12 (3.6)	SGKGV	1 (0.3)		
	IPLRTSI	4 (1.2)				
	IPFRTSI	1 (0.3)				
Total sequenced	84/103		91/103		89/103	
Sindh	IPFSTSI	14 (4.2)	SAKAV	42 (12)	YF	2 (0.6)
	IPFRTNI	23 (6.8)	SGKAV	1 (0.3)	YL	46 (14)
	IPFSTNI	10 (3.0)				
Total sequenced	47/50		43/50		48/50	

* Drug resistance-conferring point mutations are shown for the following codons: *pvdhfr* 13, 33, 57, 58, 61, 117, 173; *pvdhps* 382, 383, 512, 553, 585; and *pvmdr1* 976, 1076. Mutated amino acids are shown in bold.

with AS + SP or SP alone. This study sought to characterize the current distribution of chloroquine resistance-associated polymorphisms in *pvmdr1* and SP resistance-associated point mutations in *pvdhfr* and *pvdhps* in Pakistan.

Resistance of *P. falciparum* to chloroquine is nearly fixed in Pakistan [3,51], and the emergence of chloroquine-resistant *P. vivax* in neighbouring India [52-54] along with established resistance in Thailand [55,56], Indonesia [57,58] and Vietnam [59] suggests that the treatment of *P. vivax* with chloroquine in Pakistan could be in danger of being compromised. Clinical trials conducted during 2004-2006 indicate that chloroquine was still efficacious against *P.*

vivax in Pakistan [9]. As a more recent follow-on to clinical evidence, this study presents the first report of *pvmdr1* gene polymorphisms in Pakistan. Nearly all isolates were wild type at *pvmdr1* Y976F, suggesting that chloroquine resistance in *P. vivax* has not emerged, but many carried the F1076L mutant. These results are consistent with previous studies showing the rise of *pvmdr1* F1076L prior to Y976F [12,23,60]. If the hypothesis that the two-step trajectory of mutations at codon F1076L followed by Y976F may lead to chloroquine resistance [12] is correct, then *pvmdr1* F1076L may provide an early signal of emerging chloroquine resistance in Pakistan prior to the appearance of the drug resistance phenotype in the population [22], particularly in

Table 3 Insertions and deletions in *pvdhfr* gene of *Plasmodium vivax*

Insertions/Deletions	Codons	No of bp	Sequence	No of samples
Insertion A	91,92	18 bp	ACACACGGTGGTGACAAC	7
Insertion B	103,104	18 bp	ACACACGGTGGTGACAAT	29
Deletion	92,97	18 bp	AGCGGTGGTGACAACACA	10

Khyber Pakhtunkhwa where its prevalence is highest. Polymorphisms in *pvmdr1* should continue to be monitored in case the mutation at position Y976F appears. Amplification of the *pvmdr1* gene was not detected in this study, reflecting the absence of mefloquine drug pressure in Pakistan. Regions with a history of mefloquine use for *falciparum* malaria have shown amplification of both *pfmdr1* in *P. falciparum* and *pvmdr1* in *P. vivax*, likely caused by inadvertent drug exposure [17,23].

Plasmodium vivax is likely exposed to SP pressure (either alone or in combination with artesunate) in a bystander effect, particularly because it is found alongside *P. falciparum* in regions with mixed-species infections such as Balochistan (10%), Khyber Pakhtunkhwa (8%), and Punjab (5%) [42], that are vulnerable to misdiagnosis of *Plasmodium* species [3,41,49,51]. Exposure of *P. vivax* parasites to SP pressure, with likely subtherapeutic levels in some patients, could favour the emergence of SP resistance [61].

In this study, the *pvdhfr* double mutant S58R and S117N was found in 20% of isolates. The double mutant has been reported previously from one site in Pakistan [3] as well as in neighbouring India [62] and Iran [63], and is associated with *in vitro* pyrimethamine resistance [64]. In contrast, point mutations in *pvdhps* A383G and A553G were detected in a very small number of samples, confirming similar results from a previous report in Bannu district in 2007 [3]. These mutations have also been reported as rare in Iran [63] and Afghanistan [65], suggesting that markers associated with sulphadoxine resistance in *P. vivax* remain rare in this region. Because SP treatment failure is closely associated with infections carrying four or more mutations in *pvdhfr* [35] and additional *pvdhps* mutations [66], it appears that SP likely remains efficacious against *P. vivax* in Pakistan as previously reported [9].

The prevalence of *pvdhfr* S117N single and S58R plus S117N double mutants was highest in Sindh and Khyber Pakhtunkhwa provinces. Khyber Pakhtunkhwa had the second highest prevalence of mixed species and *P. falciparum* infections in this survey [42], indicating that perhaps regions with *P. falciparum* experience higher SP use (with or without artesunate) that also exerts pressure on co-occurring *P. vivax* infections. Khyber Pakhtunkhwa province also had the highest prevalence of *pvmdr1* F1076L mutants, and may be especially vulnerable to the influx or emergence of drug-resistant parasites because of the large-scale migration of Afghan refugees and internally displaced people (IDPs) from conflict areas to this region, along with conditions of underdevelopment that may prevent adequate diagnosis and treatment of malaria [67].

Conclusions

This is the first comprehensive report on molecular patterns of drug resistance in *P. vivax* in Pakistan. The

major chloroquine resistance-mediating mutation was not detected in this study; however continued molecular monitoring of polymorphisms in *pvmdr1* is crucial to detect the potential emergence of chloroquine-resistant *P. vivax* in Pakistan. Although some evidence of pyrimethamine selective pressure was observed, polymorphisms associated with sulphadoxine resistance were rare, and markers of clinically relevant SP resistance in *P. vivax* were not present. Thus, the data on molecular markers presented here suggest that efficacy of both chloroquine and SP against *P. vivax* is currently high, and can serve as a baseline reference for regional studies of *P. vivax* drug sensitivity in Pakistan.

Additional file

Additional file 1: Synonymous and non-synonymous SNPs detected in *pvdhfr*, *pvdhps* and *pvmdr1*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AAK designed study, carried out the laboratory experiments, and performed data analysis. AAK and MV drafted the manuscript. AO and LJK contributed to laboratory experiments and sequence analysis. MFN and FN participated in sample collection and microscopy. MV, LK, SAM and CVP provided guidance and coordination for study design, genotyping, and data analyses, and edited and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors also thank Matthew Adams, Malathi Vadla and Sudhaunshu Joshi at the University of Maryland for their technical assistance and Muhammad Shahid, Ismail Jalil, Ifthikhar Ahmed, Adeel Riaz, Muhammad Adnan, Ihsan Khan and Noor Rehman in Pakistan for help with sample collection. This work was supported by a grant from the Higher Education Commission of Pakistan in support of AAK's PhD studies at Quaid-i-Azam University, Islamabad, Pakistan, and was supported in part by the Howard Hughes Medical Institute at the University of Maryland School of Medicine, Baltimore, MD, USA.

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Received: 30 May 2013 Accepted: 29 August 2013

Published: 5 September 2013

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doi:10.1186/1475-2875-12-310

Cite this article as: Khattak et al.: Prevalence and patterns of antifolate and chloroquine drug resistance markers in *Plasmodium vivax* across Pakistan. *Malaria Journal* 2013 **12**:310.

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