Short Report: Prevalence of Antimalarial Drug Resistance Mutations in *Plasmodium vivax* and *P. falciparum* from a Malaria-Endemic Area of Pakistan

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Abstract

To study drug resistance in Bannu district, a malaria-endemic area in Pakistan, molecular-based analyses were undertaken. In *Plasmodium vivax*, antifolate resistance mutations were detected in *pvdhfr* gene codons 57, 58, and 117, with a 117N mutation frequency of 93.5%. All *P. falciparum* isolates exhibited double 59R + 108N mutations in *pfdhfr*, whereas the triple mutant 59R + 108N + 437G haplotype was found in 31.8% isolates. Furthermore, all (100%) *P. falciparum* isolates exhibited the key chloroquine resistance mutation, *pfcrt* 76T, which is also associated with resistance to amodiaquine. Additionally, *pfmdr*1 86Y and D1042Y mutations were, respectively, detected in 32% and 9% isolates. These results indicate an emerging multi-drug resistance problem in *P. vivax* and *P. falciparum* malaria parasites in Pakistan.

Antimalarial drugs, deployed by ways of chemotherapy, chemoprophylaxis, and intermittent preventive therapy, play a key role among the available malaria control tools worldwide. However, the widespread of antimalarial drug resistance has substantially limited treatment options and is now the greatest obstacle to controlling the disease worldwide. [1] Understanding the epidemiology of drug resistance is vital to effective drug policy.

Pakistan is plagued by both *Plasmodium vivax* and *P. falciparum* malaria. Bannu, where this study was conducted, is among the areas with the highest malaria incidence in Pakistan.[2] Chloroquine (CQ) and the antifolates sulfadoxinepyrimethamine (SP) are the key antimalarial drugs used in this area, largely as monotherapies.[2] Although Pakistan joined the Roll Back Malaria (RBM) initiative in 2001 with the formulation of a 5-year RBM strategy, several of her regions, such as the North West Frontier Province (NWFP), were unable to implement the strategy because of lack of resources, to the effect that, by the end of 2005, there was no evidence of monotherapies being replaced by combination therapies, contrary to RMB recommendations.[2] The monotherapeutic use of CQ and SP is expected to trigger drug resistance in both species.[1,3] Although studies have shown widespread resistance to CQ and SP in neighboring India and other parts of Asia,[1,4-6] information on the molecular epidemiology of antimalarial drug resistance in Pakistan is lacking. We sought to address

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this information gap by determining the prevalence of mutations in genes conferring resistance to CQ and SP in Bannu district, Pakistan.

Bannu district (32°43′–33°06′ N; 70°22′–57′ E) is located in the southwest of the NWFP of Pakistan. Bannu is densely populated (552 persons/km2), and the high influx of Afghan refugees has exacerbated the malaria problem in the area. The district has enormous economic and strategic significance, being the central market of the Southern Region and providing a safe, short route to the Central Asian Markets.[7] Mean daily temperatures range between 10.8°C and 32.9°C. Rains come in March and during July–August (summer monsoon), with peak malaria transmission occurring after the monsoon winds.[8]

Participants were recruited from the Bannu Women and Children Hospital (BWCH). The hospital's catchment area covers the entire district. Inclusion criterion was all consenting, symptomatic malaria patients visiting the Malaria Control Program of BWCH, irrespective of age or sex, whereas exclusion criterion was non-consent.

After obtaining informed consent, ~200 μL of blood was collected on Whatman filter papers by finger-prick method from July through October 2007. At the same time, thick and thin smears were prepared and stained with Giemsa for microscopic examination by technicians trained in malaria diagnosis in line with WHO guidelines.[2] Parasite DNA was extracted from filter papers using the Chelex method and infections diagnosed by a species-specific nested polymerase chain reaction (PCR) method as previously reported.[9] Positive controls were MR4 clones MRA-340G, MRA-343G, 3D7, and HB3, whereas sterile water was used as a negative control. The study was approved by the Institutional Review Board of Quaid-i-Azam University, Pakistan.

Following previously published PCR-restriction fragment length polymorphism (RFLP) protocols,[4,10,11] we screened *P. falciparum* isolates for key mutations in codons associated with resistance to CQ (i.e., in CQ resistance transporter gene, *pfcrt,* and multidrug resistance gene, *pfmdr*1) and to SP (i.e., in dihydrofolate reductase, *pfdhfr,* and dihydropteroate synthetase, *pfdhps,* genes). For *P. vivax*, we examined mutations in *pvdhfr* and *pvdhps* genes, but not in CQ resistance genes because no clear-cut markers for CQ resistance are presently available for this species.[3] Specifically, we analyzed mutations at codon 76 of *pfcrt*, codons 86, 1042, and 1246 of *pfmdr*, codons 16, 50, 51, 59, 108, and 164 of *pfdhfr*, and codons 436, 437, 540, 581, and 613 of *pfdhps* in *P. falciparum.* In *P. vivax,* we analyzed codons 13, 33, 57, 58, 61, 117, and 173 of *pvdhfr* and codons 383 and 553 of *pvdhps*. Codons 58, 117, and 173 of *pvdhfr* correspond to the three key positions 59, 108, and 164 of the *pfdhfr* gene. [12] Mixed mutant/wildtype infections were scored as mutant to reflect the expected phenotype of the infection.[13]

A total of 114 participants (72 male, 42 female), 1–60 years of age were enrolled. All were diagnosed as infected by microscopy, with 31.6% (36/114) having *P. falciparum* infections and 68.4% (78/114) having *P. vivax* infections. No mixed infections were detected by microscopy. However, PCR found 14.9% (17/114) to be parasite negative: 1.8% (2/114) with *P. falciparum* only,60.5% (69/114) with *P. vivax* only, and 22.8% (26/114) with both *P. falciparum* and *P. vivax*. Parasite-negative samples identified by PCR were not caused by PCR failure or assay insensitivity because the positive controls worked well, and PCR method should be far more sensitive than microscopic diagnosis.[14,15] The prevalence of *P. falciparum* and *P. vivax* infections was therefore 24.6% (28/114) and 83.3% (95/114), respectively. *P. malariae* and *P. ovale* were not detected. The 17 PCR-negative samples were excluded from further analysis, after remaining negative on repeated DNA extraction and re-amplification.

In *P. vivax*, mutations were detected only in the *pvdhfr* gene, at codons 57, 58, and 117, with the single 117N mutation being most prevalent (93.5%; Table 1). The double mutant 57L + 58R haplotype occurred in only 1.7%, whereas the double mutant 58R + 117N haplotype was detected in 16.1% of the isolates (Table 2). Mutations were not detected in *pvdhfr* codons 13, 33, 61, and 173 and in *pvdhps* codons 383 and 553.

For *P. falciparum*, all (100%) isolates carried C59R and S108N mutations in *pfdhfr*, and 31.8% of isolates showed the A437G mutation in the *pfdhps* gene (Table 1). The *pfcrt* K76T mutation, a key determinant of CQ resistance,[4] was observed in all (100%) isolates, whereas the *pfmdr*1 N86Y and D1042Y mutations were detected in 32.8% and 8.7% of *P. falciparum* isolates, respectively (Table 1). Mutations were not detected in *pfdhfr* codons 16, 50, 51, and 164, in *pfdhps* codons 436, 540, 581, and 613, and in *pfmdr*1 codon 1246. The triple 59R + 108N + 437G mutation haplotype in *pfdhfr/pfdhps* was found in 31.8% isolates, and the *pfmdr*1 mutant haplotype (86Y + 1042D) was detected in 9.1% isolates (Table 2).

The primary objective of this study was to examine the neglected aspects of malaria epidemiology in Pakistan: malaria diagnosis and antimalarial drug resistance. The PCR method confirmed that *P. vivax* and *P. falciparum* co-exist in Bannu district in 22.8% of clinical malaria samples, *P. vivax* being the predominant species (83.3% *P. vivax* versus 24.6% *P. falciparum* infections). The microscopy diagnosis method underestimated the prevalence of *P. vivax* (68.4% *P. vivax* versus 31.6% *P. falciparum* infections). Moreover, microscopy misdiagnosed ~15% of the cases. A recent study in Tanzania involving 3,131 febrile children from 16 health facilities around the country showed a wide variation in microscopic results among the health units, with only 65% overall concordance with the National Reference Laboratory. False-positive smears were frequently reported, betraying a tendency among laboratory staff to report a blood smear as positive when they are in doubt, especially when faced with symptomatic patients.[16] We cannot unequivocally state that the same tendency existed among laboratory workers at BWCH, because some other factors such as inappropriate cleaning of re-usable microscope slides or artifacts may be responsible for such diagnostic errors.[17] Furthermore, consistent with what has been in East Africa, the Middle East, and other parts of Asia,[18] microscopy failed to detect mixed *vivax* and *falciparum* infections, which PCR successfully detected. These findings highlight the challenge of diagnosing and treating malaria, more so in areas where mixed species occur. CQ plus primaquine is the standard treatment of *P. vivax* malaria, whereas SP, and better still, artemisinin combination therapies (ACT) are currently being recommended for *P. falciparum* malaria in the face of CQ resistance. Models predict that treatment of *P. vivax* alone caused by failed diagnosis of a co-infecting, more lethal *P. falciparum* can lead to a rapid surge in *P. falciparum* parasitemia.[19] Inappropriate diagnosis will thus cause higher morbidity and mortality from malaria, enhance the development of drug resistance because of administration of the wrong drugs, and prevent appropriate management of serious fever–causing illness such as bacterial infections, resulting in poor treatment outcomes.[16] Because PCR is untenable as a routine confirmatory diagnostic method in such settings, diagnostic accuracy in this area of mixed endemicity could be improved by supplementing microscopy with rapid diagnostic tests.[20,21]

Typing analyses for SP resistance mutations in *P. vivax* in the present study neither showed mutations at codons 13, 33, 61, 173 of *pvdhfr* nor at 383, 553 of *pvdhps*, unlike what has been reported in other Asian countries like Thailand where SP resistance is highly prevalent.[5] We observed mutations only in the *pvdhfr* gene, at codons 57, 58 and 117, with two-mutation haplotypes in < 20% *P. vivax* isolates. Codon 117 in *pvdhfr* is homologous with codon 108 in *P. falciparum*,[12] and the high prevalence of mutations at this codon mirrors that observed in *P. falciparum*. Whereas the observed 117N mutation has been associated with reduced *in vitro* susceptibility to pyrimethamine, previous studies found clinical *P.*

vivax resistance to SP to be mainly associated with the *pvdhfr* 117T mutation,[22] which we did not detect in the present study. Moreover, while double mutants such as 58R + 117N may be associated with delayed parasite clearance following SP treatment, clinical SP treatment failure is more likely in patients carrying *P. vivax* parasites with at least four mutations in the *dhfr* gene (see review by Hawkins and others[12]), and goes up even more if there are additional mutations in the *dhps* gene.[23] Indeed, recent clinical trials indicate that SP is effective against *P. vivax* in NWFP,[24] which is consistent with our molecular evidence.

Unlike in *P. vivax*, mutations were detected in both *pfdhfr* and *pfdhps* genes in *P. falciparum*, with a double mutant 59R + 108N haplotype detected in all isolates and the triple mutant 59R + 108N + 437G haplotype in 32% isolates. However, *pfdhfr* codons 16, 50, 51, and 164 and *pfdhps* 436, 540, 581, and 613 were all wild-type. In their 1997 multi-country study, Wang and others[25] similarly found no mutation in the *pfdhps* gene but detected the double mutant 59R + 108N haplotype in 9 and a unique, mutant 16S + 59R haplotype in 1 of the 10 Pakistani *P. falciparum* isolates they analyzed. Profound SP resistance occurs where the 164L mutation or quadruple or more mutations occur.[1,25] It therefore seems that, like in the case of *P. vivax*, *P. falciparum* has not yet reached critical SP resistance levels in our study area. On the other hand, the *pfcrt* 76T mutation, which has been linked to resistance to amodiaquine, and has been strongly associated with CQ resistance in *P. falciparum* isolates from various parts of Asia, Papua New Guinea, Africa, and South America,[1,4] was detected in 100% samples. In addition, mutations *pfmdr*1 86Y and 1042N that further enhance CQ resistance[4] were detected, although to a lesser extent. It is therefore apparent that CQ can no longer be relied on for treating *P. falciparum* malaria in this area.

Although we did not screen for CQ mutations in *P. vivax* for absence of reliable markers,[3] results from recent clinical trials in NWFP show that *P. vivax* is still sensitive to CQ,[24] which is encouraging. *P. vivax* is known to remain sensitive to CQ, even where *P. falciparum* has become resistant to the drug (see review by Leslie and others[24]). The apparent effectiveness of SP against both species in this area is advantageous, especially in the face of uncertainties in microscopic diagnosis of mixed infections. Nevertheless, the presence of mutations that have also been linked to amodiaquine resistance present early indicators of an emerging multi-drug resistance problem in this area. Moreover, monotherapies are still being used in NWFP.[2] The continued use of monotherapies will likely hasten the dangerous development of high levels of antifolate resistance by both species. The enforcement of lucid drug use to minimize drug pressure, and the use of antifolates, together with artemisinin, would therefore be a highly desirable therapeutic policy for this area.[24]

Acknowledgments

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Table 1

Prevalence of mutations conferring resistance to sulfadoxine-pyrimethamine in *P. vivax* and *P. falciparum* and chloroquine in *P. falciparum* isolates from Bannu district, Pakistan

Species	Gene	Codon and mutation[*]	Percentage
P. vivax	*Pvdhfr*	F57L	1.7
		S58R	19.0
		S117N	93.5
P. falciparum	*Pfdhfr*	C59R	100.0
		S108N	100.0
	Pfdhps	A437G	31.8
	Pfcrt	K76T	100.0
	PfmdrI	N86Y	32.8
		N1042D	8.7

[*] Mutations were not detected in *Pvdhfr* codons 13, 33, 61, and 173 and in *Pvdhps* codons 383 and 553 of *P. vivax*. Similarly, no mutations were observed in *Pfdhfr* codons 16, 50, 51, and 164, in *Pfdhps* codons 436, 540, 581, and 613, and in *PfmdrI* codon 1246 of *P. falciparum*.

Table 2

Frequency of antimalarial drug resistance mutation haplotypes in *P. vivax* and *P. falciparum* isolates collected from Bannu district, Pakistan

Species	Haplotype	Percentage
P. vivax	*pvdhfr* double 57L + 58R	1.7
	pvdhfr double 58R + 117N	16.1
P. falciparum	*pfdhfr* double 59R + 108N	100.0
	pfdhfr/pfdhps triple 59R + 108N + 437G	31.8
	pfmdr 1 86Y + 1042D	9.1