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, pfmdr1 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.† 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.‡ Chloroquine (CQ) and the antifolates sulfadoxine-pyrimethamine (SP) are the key antimalarial drugs used in this area, largely as monotherapies.§ 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 RBM recommendations.¶ The monotherapeutic use of CQ and SP is expected to trigger drug resistance in both species.** Although studies have shown widespread resistance to CQ and SP in neighboring India and other parts of Asia,†† Information on the molecular epidemiology of antimalarial drug resistance in Pakistan is lacking. We sought to address 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’–70°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. 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.\*

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. Paraite 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. 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, 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, pfmdr1) and to SP (i.e., in dihydrofolate reductase, pfdhfr; and dihydropteroyl 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. Specifically, we analyzed mutations at codon 76 of pfCRT, codons 86, 1042, and 1246 of pfmdr, codons 16, 50, 51, 52, 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, 37, 58, 61, 117, and 173 of pfdhfr and codons 383 and 553 of pfdhps. Codons 58, 117, and 173 of pfdhfr correspond to the three key positions 59, 108, and 164 of the pfmdr1 gene. Mixed mutant/wildtype infections were scored as mutant to reflect the expected phenotype of the infection.

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

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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. 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 *pfldhfr* and, 31.8% of isolates showed the A437G mutation in the *pfldhps* gene (Table 1). The *pfcrt* K76T mutation, a key determinant of CQ resistance, was observed in all (100%) isolates, whereas the *pfdmrd1* N86Y and D1042Y mutations were detected in 32.8% and 8.7% of *P. falciparum* isolates, respectively (Table 1). Mutations were not detected in *pfldhfr* codons 16, 50, 51, and 164, in *pfldhps* codons 436, 540, 581, and 613, and in *pfmdr1* codon 1246. The triple 59R + 108N + 437G mutation haplotype in *pfldhfr/pfdhps* was found in 31.8% isolates, and the *pfmdr1* 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 country showed a wide variation in microscopic results among the health units, with only 65% overall concordance with the

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Codon and mutation*</th>
<th>Percentage</th>
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</thead>
<tbody>
<tr>
<td><em>P. vivax</em></td>
<td><em>Pvdhfr</em></td>
<td>F57L</td>
<td>1.7</td>
</tr>
<tr>
<td>P. falciparum</td>
<td><em>Pfdhfr</em></td>
<td>S58R</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S117N</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C50R</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S108N</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td><em>Pfdhps</em></td>
<td>A437G</td>
<td>31.8</td>
</tr>
<tr>
<td></td>
<td><em>Pfcr</em></td>
<td>K76T</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td><em>Pfmdr1</em></td>
<td>N86Y</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N1042D</td>
<td>8.7</td>
</tr>
</tbody>
</table>

*Mutations were not detected in *Pvdhfr* codons 13, 33, 61, and 173 and in *Pfdhfr* 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 *Pfmdr1* codon 1246 of *P. falciparum*.

Similarly, no mutations were observed in *Pfdhps* codons 383 and 553 of *P. vivax*. *P. falciparum* isolates showed 58R + 117N haplotype detected in all isolates and the triple mutant 59R + 108N + 437G haplotype in 32% isolates. However, 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. Furthermore, consistent with what has been in East Africa, the Middle East, and other parts of Asia, 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. 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. 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.

### Table 2

<table>
<thead>
<tr>
<th>Species Haplotype</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax</em> <em>pvdhfr</em> double 57L + 58R</td>
<td>1.7</td>
</tr>
<tr>
<td><em>P. vivax</em> <em>pfdhfr</em> double 58R + 117N</td>
<td>16.1</td>
</tr>
<tr>
<td><em>P. falciparum</em> <em>pfdhfr</em> double 59R + 108N</td>
<td>100.0</td>
</tr>
<tr>
<td><em>P. falciparum</em> <em>pfdhfr/pfdhps</em> triple 59R + 108N + 437G</td>
<td>31.8</td>
</tr>
<tr>
<td><em>P. falciparum</em> <em>pfmdr1</em> 86Y + 1042D</td>
<td>9.1</td>
</tr>
</tbody>
</table>
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\(^2\) 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 Pakistani in 9 and a unique, mutant 16S + 59R haplotype in 1 of the 10 gene but detected the double mutant 59R + 108N haplotype in 100% samples. In addition, mutations pfmdr1 86Y and 1042N that further enhance CO resistance 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, 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\(^26\)). 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.\(^4\) 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.\(^24\)

Received April 22, 2009. Accepted for publication June 8, 2009.

Acknowledgments: The authors thank the study participants for being involved in the study, the staff of the Bannu Women and Children Hospital Malaria Control Program for their cooperation throughout the study, and two anonymous reviewers whose insightful comments and suggestions helped improve the manuscript.

Financial support: This work was financed by a grant from the Higher Education Commission of Pakistan in support of L.K.’s PhD studies at Quaid-i-Azam University, Islamabad, Pakistan, and a grant (D43 TW001505) from the National Institutes of Health.

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