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Mutations Associated with Sulfadoxine-Pyrimethamine and Chlorproguanil Resistance in *Plasmodium falciparum* Isolates from Blantyre, Malawi

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We conducted a prevalence study of mutations in *Plasmodium falciparum* that are associated with antifolate resistance in Blantyre, Malawi. The dihydrofolate reductase 164-Leu mutation, which confers resistance to both pyrimethamine and chlorproguanil, was found in 4.7% of the samples. Previously unreported mutations in dihydropteroate synthase were also found.

Sulfadoxine-pyrimethamine (SP) is the first-line antimalarial in much of Africa. Resistance to SP in *Plasmodium falciparum* is increasing and is conferred by mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). A combination of five mutations ("quintuple mutant": DHFR-51, -59, and -108 and DHPS-437 and -540) strongly predicts clinical outcome (10, 13). Mutations in DHFR-164, DHPS-581, and DHPS-613 develop later and are associated with increased SP resistance (14).

One proposed alternative to SP is chlorproguanil-dapsone (Lapdap). Both chlorproguanil and pyrimethamine target DHFR, and changes at codon 164 confer resistance to both drugs (7). This cross-resistance might limit the usefulness of Lapdap in areas where SP has been used heavily (11). Currently in Africa, DHFR-164 mutations are rare. Nevertheless, this mutation should be monitored where Lapdap is being considered as a candidate therapy.

In Malawi, SP has been the first-line agent for malaria since 1993. The quintuple mutation is widespread: 78% of samples contained the quintuplet mutation in the Salima district in 2001 (5). However, subsequent mutations (DHPS-613, DHPS-581, and DHFR-164) have not been previously reported. The purpose of this study is to determine the prevalence of mutations in DHFR and DHPS in Blantyre, Malawi.

Clinical samples of *Plasmodium falciparum* were acquired from pregnant women at Queen Elizabeth Hospital (Blantyre, Malawi) who were enrolled in a study investigating mother-to-child transmission of human immunodeficiency virus (12). Venous blood was taken at enrollment and frozen at −80°C. Malaria was screened for using thick-blood-smear microscopy. A random sample of the *Plasmodium falciparum*-positive specimens was included in this study. Twenty-two samples were transferred to filter paper and processed as previously described (1). Sixty-eight additional samples were shipped frozen to the University of North Carolina at Chapel Hill (UNC-Chapel Hill), where DNA was extracted using the Qiamp DNA minikit (Qiagen). The samples for this study were collected between March 2001 and May 2003. In this subgroup, 15.7% report taking SP within the last 2 weeks while 85.4% report taking SP at some point during their pregnancy. No one reported using Lapdap. Institutional review boards at UNC-Chapel Hill and the University of Malawi College of Medicine approved all research.

All samples were genotyped for codons 51, 59, and 108 in the DHFR gene and codons 437, 540, 581, and 613 in the DHPS gene, as previously described (1). The DHFR/DHPS genotypes for five of the blood spot samples were reported previously (1).

The samples were genotyped at DHFR-164 using a new real-time PCR assay with minor groove binder (MGB) probes (Table 1). The primers and probes were synthesized by MWG Biotech (High Point, NC) and Applied Biosystems (ABI), respectively. PCRs were carried out in duplicate in 25 μl reaction mixtures containing 12.5 μl Universal PCR Master Mix (ABI), 2 μl DNA, forward primer at 300 nM, reverse primer at 500 nM, and both probes at 200 nM. All reactions were run on an ABI PRISM 7000 (ABI); reaction mixtures were initially denatured at 95°C for 10 min and cycled 45 times, with each cycle consisting of 92°C for 14 s and 60°C for 60 s. The results were analyzed as previously described (1). A sample was considered mixed if there was an increase in the signal for both the wild-type and mutant probes.

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TABLE 1. Sequences of primers and probes used in real-time PCR for the DHFR-164 genotyping assay

Type	Sequence (5′→3′)[a]
Forward primer	ATC ATT AAC AAA GTT GAA GAT CTA ATA GTT TTA C
Reverse primer	TCG CTA ACA GAA ATA ATT TGA TAC TCA T
Wild-type probe (isoleucine)	6FAM-ATG TTT TAT TAT AGG AGG TTC CGT T-MGB
Mutant probe (leucine)	VIC-ATG TTT TAT TTT AGG AGG TTC CGT T-MGB

[a] FAM, carboxyfluorescein.

To validate this assay, genomic DNA (Dd2, HB3, W2, FCR3, K1, and VI/S from the Malaria Reagent Repository Resource [http://www.malaria.mr4.org/]) was genotyped for DHFR-164. The assay correctly identified VI/S as mutant and the rest as wild type. Clinical samples were sequenced for further validation. The amplified product from the real-time reaction was purified using CentriSpin-10 columns (Princeton Separations) and then sequenced as previously described (1). To check for contamination, samples with the DHFR-164 mutation were genotyped for PFCRT-76, which is mutant in VI/S but wild type in Malawian samples (9, 17).

Eighty-seven out of the 90 samples were genotyped successfully for all codons. Of these samples, 93.2% contained the quintuple mutation (Fig. 1). One sample (1.1%) contained a DHPS-613 mutant component, while three different samples (3.4%) contained a DHPS-581 mutant component. These mutations were only observed in mixed infections.

Genotyping for DHFR-164 was successful for 85 out of 90 samples. Four samples contained the DHFR-164-Leu mutation, which results in a prevalence of 4.7% (95% confidence interval: 0, 9.2%). One sample contained only the mutant genotype, while the rest contained a mixture of wild-type and mutant genotypes. The genotypes of the fully mutant sample and two wild-type samples were confirmed with sequencing. The four samples with the DHFR-164 mutation also contained the quintuple mutation but were wild type at DHPS-581 and DHPS-613. These four samples were wild type at PFCRT-76, which suggests the DHFR-164 mutation was not due to contamination.

Malawi was one of the first African countries to adopt SP as the first-line agent against *Plasmodium falciparum* malaria.

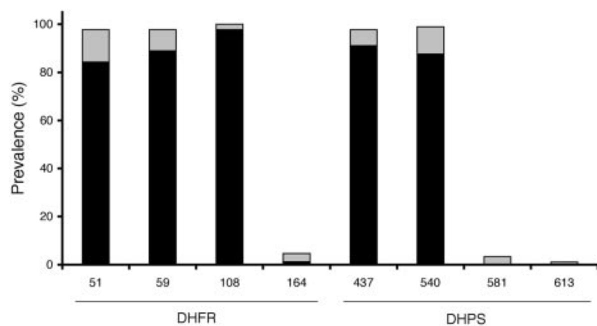

FIG. 1. The prevalence of mutant (black) and mixed (gray) genotypes in samples from pregnant women with malaria in Blantyre, Malawi. A mutant genotype is defined as encoding DHFR-51-Ile, DHFR-59-Arg, DHFR-108-Asn/Thr, DHFR-164-Leu, DHPS-437-Gly, DHPS-540-Glu, DHPS-581-Gly, and DHPS-613-Ser/Thr. The sample size is 89 for all codons except for the DHFR-59 ($n = 90$), DHPS-437 ($n = 90$), and DHFR-164 ($n = 85$) codons.

This is the first report of the DHFR-164, DHPS-581, and DHPS-613 mutations in clinical samples from Malawi. The presence of these mutations suggests SP resistance may be increasing. Mutations in DHFR-164 and DHPS-581 and DHPS-613 have been associated with high-level SP resistance in Southeast Asia and Latin America (3, 4, 14). The observation of these mutations in Malawi suggests that *P. falciparum* strains with high-level SP resistance may be occurring in Africa, too. The presence of the DHFR-164-Leu mutation is troubling because it confers cross-resistance to Lapdap. In Africa, the DHFR-164 mutation has rarely been found: previous reports of this mutation were from travelers and people treated with Lapdap, and it was also found as a rare component of samples (6, 8, 15). In contrast, we report the DHFR-164 mutation as a major component of samples in African women who report not taking Lapdap.

Our screening for DHFR and DHPS mutations was restricted to pregnant women in Blantyre with previous SP exposure, and therefore the prevalence of these mutations in the general population is uncertain. In fact, Lapdap has been efficacious in recent clinical trials in children in Blantyre (2, 16), suggesting that the DHFR-164 mutation remains rare. Nevertheless, continued surveillance for these mutations is needed, as is confirmation that these mutations are associated with high levels of clinical SP and Lapdap resistance. New technologies for SNP detection, such as real-time PCR, enable rapid low-cost screening of a large number of samples and can be employed for the monitoring of parasite mutations associated with drug resistance.

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