PYRIMETHAMINE-SULFADOXINE EFFICACY AND SELECTION FOR MUTATIONS IN PLASMODIUM FALCIPARUM DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHASE IN MALI

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Abstract. To assess pyrimethamine-sulfadoxine (PS) efficacy in Mali, and the role of mutations in Plasmodium falciparum dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) in vivo PS resistance, 190 patients with uncomplicated P. falciparum malaria were treated with PS and monitored for 56 days. Mutation-specific polymerase chain reactions and digestion with restriction endonucleases were used to detect DHFR and DHPS mutations on filter paper blood samples from pretreatment and post-treatment infections. Only one case each of RI and RII level resistance and no cases of RIII resistance or therapeutic failure were observed. Post-PS treatment infections had significantly higher rates of DHFR mutations at codons 108 and 59. No significant selection for DHPS mutations was seen. Pyrimethamine-sulfadoxine is highly efficacious in Mali, and while the low level of resistance precludes assessing the utility of molecular assays for in vivo PS resistance, rapid selection of DHFR mutations supports their role in PS failure.

Resistant Plasmodium falciparum has rendered the anti-folate combination pyrimethamine-sulfadoxine (PS) useless in parts of South America and Southeast Asia, and threatens its utility in Africa, where PS has become the first-line drug in areas with widespread chloroquine resistance. In Mali, where malaria is the leading cause of death, PS is recommended for cases of chloroquine failure. Although chloroquine remains effective here, PS is available in pharmacies in urban and periurban areas. This is the first study of PS efficacy in Mali.

Simple and reliable methods for drug resistance testing are needed in this setting, but both standard in vivo and in vitro methods have several disadvantages, many of which are overcome by molecular assays for resistance. These polymerase chain reaction (PCR) and restriction digestion assays detect point mutations in parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), the enzymes targeted by pyrimethamine and sulfadoxine, respectively. Briefly, DHFR Ser→Asn 108 causes moderate pyrimethamine resistance in vitro, with higher level resistance resulting from Asn→Ile 51 and/or Cys→Arg 59, and the addition of Ile→Leu 164 causes high level resistance to both pyrimethamine and cycloguanil, the active metabolite of proguanil. A five amino-acid insert at codon 30 and point mutations at codons 50 and 140 have recently been discovered in areas with widespread in vivo PS resistance, but not yet confirmed to play a role in resistance to the DHFR inhibitors. The DHPS mutations associated with in vitro resistance to sulfadoxine include Ser→Phe 436 paired with Ala→Thr/Ser 613, Ser→Ala 436, Ala→Gly 437, Lys→Glu 540, and Ala→Gly 581. Digital puncture blood samples blotted onto filter paper can be analyzed by mutation-specific PCR and restriction digestion to detect all of these mutations.

Because the DHFR Ser→Asn-108 mutation is associated with PS use and resistance and is selected for by PS treatment, it has been used as a marker for PS resistance. Prevalence rates of this and other DHFR and DHPS mutations are associated with higher levels of PS use and resistance, and one study has suggested that various combinations of both DHFR and DHPS mutations can account for persistent or recurrent infection following PS treatment. However, no specific set of mutations predictive of therapeutic failure of PS has been identified, nor have assays for any specific mutation or mutations been validated as tools for surveillance of PS resistance.

The relative importance of DHFR versus DHPS mutations in PS resistance is also controversial. Transfection studies have proven that DHFR mutations cause in vitro pyrimethamine resistance. The role of DHPS mutations in sulfadoxine resistance is less certain, in part because in vitro assays for sulfadoxine resistance have been done under sub-physiologic folate conditions, and also because some parasite isolates appear to be able to use host folate to antagonize sulfa drugs irrespective of their DHPS and DHFR genotype. It is also difficult to extrapolate from in vitro studies to clinical efficacy of PS: some studies suggest that pyrimethamine resistance alone is sufficient to cause PS failure and that DHPS mutations do not play a role, while other studies support the view that resistance to both pyrimethamine and sulfadoxine is necessary. Here we report a prospective study evaluating PS efficacy in Mali and attempting to test the association between DHFR and DHPS mutations and the parasitologic and therapeutic outcome of PS treatment.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Plasmodium falciparum malaria is mesoendemic in the study area with seasonal increases in transmission. Parasitemia prevalence rates in children six months to nine years of age range from 20% to 30% during the December–May low transmission season to 40–50% during the June–November high transmission season (Doumbo O, unpublished data). From July to December 1995, persons who presented to the Point G National Hospital in Bamako and to a village clinic

*Deceased.
in Sotuba, 5 km east of Bamako, were recruited for a prospective study of PS efficacy. Patients more than two years of age with positive malaria smears and either fever (axillary temperature ≥ 37.5°C) or a screening parasitemia ≥ one parasitized erythrocyte per 100× field on a thin smear were eligible for enrollment. Exclusion criteria included known pregnancy, a hematocrit < 15%, a parasitemia > 10%, prostration, respiratory distress, shock, bleeding, persistent vomiting, convulsions, and signs of altered consciousness or neurologic impairment. Those with uncomplicated, microscopically confirmed *P. falciparum* malaria who agreed to participate were treated with PS as a single oral dose: adults received three tablets of Fansidar™ (25 mg of pyrimethamine and 500 mg of sulfadoxine per tablet; F. Hoffman LaRoche, Basel, Switzerland); children received one tablet per 20 kg of body weight. If vomiting occurred within 30 min, the dose was repeated; if within 30–60 min, one half the dose was repeated; if persistent vomiting occurred the patient was not enrolled in the study and was referred for alternative therapy. Symptoms, prior antimalarial drug use, and physical examination data were recorded on standard forms.

**Efficacy of PS.** With enrollment and treatment occurring on day 0, subjects were followed actively on days 2, 7, 14, 28, 42, and 56 by clinical evaluation and microscopic examination of blood for parasites. The extended period of follow-up was chosen based on the long post-PS treatment period of selective pressure for pyrimethamine resistance.¹⁹ Passive surveillance was conducted by continuous availability of study clinicians to evaluate subjects who felt ill. RI resistance was defined as disappearance of parasitemia by day 7 after PS therapy, followed by recurrent parasitemia by day 14; RII resistance as a ≥ 75% diminution of parasitemia by day 7 followed by a subsequent increase in parasitemia; and RIII resistance as < 75% or no diminution of parasitemia following PS therapy. Therapeutic failure was defined as RII or RIII resistance accompanied by recurrent or persistent symptoms of malaria. Symptomatic parasitemias occurring during the follow-up period, as well as asymptomatic parasitemias > 0.5% or 20,000/mm³, were to be treated with standard doses of oral chloroquine. Parasitemias were determined by standard microscopic methods from thick blood smears assuming a leukocyte count of 7,500/mm³ and parasites/mm³ = number of parasites per 300 leukocytes × 25. Digital blood was collected onto filter paper strips as described²⁰ at the time of enrollment and at all scheduled and unscheduled follow-up times.

**Mutation-specific analysis.** Filter paper strips were air-dried and transported at room temperature, and the DNA was extracted by boiling with a chelating resin as described.²⁰ The resulting supernatant was used as template for nested mutation-specific PCR and/or restriction digestion using published methods.⁷⁻²⁰ Assays to detect the DHFR mutations at codons 108, 51, 59 and 164 and DHPS mutations at codons 436, 437, 540, 581 and 613 were done on 48 randomly selected pretreatment infections and on all infections occurring more than two days after PS treatment.

**Ethical review.** Study protocols were reviewed and approved by Institutional Review Boards at the University of Mali and the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Informed consent was obtained from all study subjects or their parents or guardians.

**Statistical analysis.** Fisher’s exact test and the chi-square test for two-tailed significance at *P* = 0.05 were used to analyze differences between mutation prevalence rates using EpilInfo Version 6.0 (Centers for Disease Control and Prevention, Atlanta GA).

**RESULTS**

Twelve (12.9%) of 93 subjects enrolled in the study at Hospital Point G and 16 (12.8%) of 125 subjects enrolled at the Sotuba clinic were lost to follow-up during the 14 day period during which *in vivo* resistance levels were determined. Most subjects lost to follow-up had left the study area, and did not significantly differ from subjects remaining in the study with respect to age, sex, area of residence, presence of fever or level of parasitemia at enrollment (*P* > 0.05, by Fisher’s exact test). The median age of subjects was 12 years; 56.4% were male and 43.6% were female. Initial infections were *P. falciparum* in all but two cases, both of which were mixed infections with *P. falciparum* and *P. malariae*. Treatment with PS was well tolerated with no episodes of repeated vomiting and no adverse reactions.

**Efficacy of PS.** No therapeutic failures or RIII resistance was observed, and only two cases of parasitologic resistance occurred in two siblings, one case each of RI and RII level resistance, for a resistance rate of 1.1% (*n* = 190) (Table 1). The RI case had asexual *P. falciparum* parasite counts of 15,700/mm³ on day 0 (the day of treatment), 0/mm³ on day 2, 4,725/mm³ on day 7, and 42,050/mm³ on day 14. The RII case had parasite counts of 45,750/mm³ on day 0, 9,150/mm³ on day 2, 950/mm³ on day 7, and 18,125/mm³ on day 14. Because both of these cases had asymptomatic parasitemias below the treatment threshold, neither was treated on day 7. Although the protocol had called for treating uncomplicated PS-resistant cases with chloroquine, the study physician made a clinical judgment to re-treat both resistant cases with standard doses of intramuscular PS on day 14. Both remained parasite-free for the remaining six weeks of the study. All other subjects were parasite-free by day 7 and remained so on day 14. No cases of recurrent malaria were detected by passive surveillance between days 15 and 20. Between days 21 and 56, 14 new episodes of parasitemia were detected among 13 individuals who had an initial sensitive response to PS, representing a rate of either new infection or late recrudescence of 6.9% among PS-sensitive infections (*n* = 188). All of these new episodes of parasitemia were asymptomatic and detected by active surveillance.

**Genetic analysis.** Both resistant cases were genetically mixed infections at day 0, carrying both wild type DHFR, and DHFR with the mutations Asn-108 and Arg-59, and the
same mixed genotype was detected in all post-treatment samples from these two cases. No other DHFR or DHPS mutations were detected in any pretreatment samples from these two cases. The RI infection had the DHPS Phe-436 and Ser-613 mutations on day 7, but reverted back to wild-type DHPS on days 14 and 21. Several PS-sensitive infections also had DHFR and DHPS mutations at Day 0. These included two sensitive infections that were wild type at DHFR codon 108 but mutated at codons 51 and/or 59, and one with the DHFR 108/51/59 triple mutant.

As shown in Figure 1, PS treatment resulted in a more than three-fold increase in the prevalence of DHFR mutations Asn-108 (pretreatment 14.9%, n = 47; post-treatment 55.0%, n = 20; P < 0.05) and Arg-59 108 (pretreatment 12.5%, n = 48; post-treatment 55.0%, n = 20; P < 0.05). The DHFR Ile-51 mutation was rare both pretreatment and post-treatment, and the Leu-164 mutation was absent in all samples. The DHPS Ala-436 mutation was seen in 55.3% (n = 47) of pretreatment sensitive infections but in only 21.1% (n = 19) of post-treatment infections (P < 0.05). The prevalence rates of DHPS mutations Phe-436, Thr/Ser-613, and Ala-437 were not significantly different in pretreatment versus post-treatment infections. The DHPS Glu-540 and Gly-581 mutations were absent in all samples.

**DISCUSSION**

Pyrimethamine-sulfadoxine remains highly effective for the treatment of uncomplicated *P. falciparum* malaria in these urban and peri-urban settings in Mali. Because chloroquine is still effective here, PS is reserved as the second-line drug for chloroquine failures. Although available at most pharmacies, PS is perceived to be a strong medicine and most pharmacies, PS is perceived to be a strong medicine and is used mainly by relatively affluent adults.

Figure 1. Prevalence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) mutations in pretreatment (Rx) and post-

pyrimethamine-sulfadoxine (PS) treatment infections. Following PS treatment, DHFR mutations Arg-59 and Asn-108 are more common and the DHPS mutation Ala-436 is less common (P < 0.05).

Due to the low level of *in vivo* PS resistance, this study could not address the hypothesis that specific combinations of DHFR and/or DHPS mutations are predictive of therapeutic PS failure. A variety of DHFR and DHPS mutants were seen among infections that cleared with PS treatment, including one with the DHFR 108/51/59 triple mutant form that has been proposed to be sufficient for PS failure. However, *in vitro* synergy studies suggest that pyrimethamine susceptibility and DHFR genotype are the main determinants of PS treatment outcome.

A recent study in Peru found that a constellation of mutations at DHFR 108/51/164 and DHPS 437/540/581 was highly correlated with *in vivo* PS resistance. Many of these mutations were not found in Mali in the present study, although all but the DHFR Leu-164 mutation have been observed elsewhere in Africa. To identify a set of DHFR and/or DHPS mutations that are predictive of clinical PS failure and useful for differential genetic fingerprinting would not necessarily rule out recrudescence of a minute subpopulation of the initial infection under drug pressure. In either case, the higher prevalence of DHFR mutations in the post-treatment infections indicates that drug pressure selects for mutant parasites, whether in new or recrudescent infections.

The episodes of recurrent parasitemia occurring after day 20 could represent either new or recrudescent infections. More detailed genetic typing using other markers might help to distinguish reinfection from recrudescence, although a different genetic fingerprint would not necessarily rule out recrudescence of a minute subpopulation of the initial infection under drug pressure. In either case, the higher prevalence of DHFR mutations in the post-treatment infections indicate that drug pressure selects for mutant parasites, whether in new or recrudescent infections.
for surveillance of PS resistance, it will be necessary to repeat similar studies in areas of Africa with higher rates of in vivo PS resistance.

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