Mutations in *Plasmodium falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase and Epidemiologic Patterns of Pyrimethamine-Sulfadoxine Use and Resistance

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To assess the relationship between mutations in *Plasmodium falciparum* dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) and clinical pyrimethamine-sulfadoxine resistance, polymerase chain reaction surveys and analyses for new mutations were conducted in four countries with increasing levels of pyrimethamine-sulfadoxine resistance: Mali, Kenya, Malawi, and Bolivia. Prevalence of mutations at DHFR codon 108 and a new mutation at DHPS 540 correlated with increased pyrimethamine-sulfadoxine resistance \((P < .05)\). Mutations at DHFR 51, DHFR 59, and DHPS 437 correlated with resistance without achieving statistical significance. Mutations at DHFR 164 and DHPS 581 were common in Bolivia, where pyrimethamine-sulfadoxine resistance is widespread, but absent in African sites. Two new DHFR mutations, a point mutation at codon 50 and an insert at codon 30, were found only in Bolivia. DHFR and DHPS mutations occur in a progressive, stepwise fashion. Identification of specific sets of mutations causing in vivo drug failure may lead to the development of molecular surveillance methods for pyrimethamine-sulfadoxine resistance.

Pyrimethamine-sulfadoxine served as the first line of defense against chloroquine-resistant *Plasmodium falciparum* malaria in South America and Southeast Asia, where clinical resistance to pyrimethamine-sulfadoxine arose rapidly after widespread use began. This combination of antifolate drugs now represents the only currently available, affordable alternative for the treatment of uncomplicated malaria in areas of Africa with extensive chloroquine resistance. Deterring the spread of pyrimethamine-sulfadoxine-resistant malaria is an urgent public health objective, particularly in Africa, and will require the ability to map pyrimethamine-sulfadoxine-resistant malaria quickly and accurately. Neither in vivo surveys nor in vitro chemosensitivity testing methods for measuring drug-resistant malaria are well-suited for large-scale studies. Molecular epidemiologic methods based on detection of mutations in parasite molecules targeted by antimalarial drugs may offer better tools for surveillance [1].

Pyrimethamine binds and inhibits dihydrofolate reductase (DHFR), and sulfas drugs act on dihydropteroate synthase (DHPS). The combination of pyrimethamine and sulfadoxine gives a synergistic action against *P. falciparum* [2]. In vitro resistance of *P. falciparum* to pyrimethamine is due to specific point mutations in DHFR that alter the shape of its active site cavity. A single point mutation causing a Ser-Asn change at codon 108 is linked to pyrimethamine resistance. The addition of Asn->Ile-51 and/or Cys->Arg-59 mutations confers higher-level pyrimethamine resistance. Ser->Thr-108 coupled with Ala->Val-16 is associated with resistance to cycloguanil (the active metabolite of proguanil, a DHFR inhibitor used mainly for prophylaxis). Ile->Leu-164 combined with Asn-108 plus Ile-51 and/or Arg-59 confers high-level resistance to both pyri-
methylene and cycloguanil [3–8]. No mutations in *P. falciparum* DHFR other than these five known to be associated with drug resistance have been found in several prior surveys of strains from a wide range of geographic areas [9–12], although a Val–Leu-140 mutation was recently reported in a single isolate from Vietnam [13].

The gene encoding DHPS has been sequenced in *P. falciparum*, and point mutations have been identified that are associated with in vitro sulfadoxine resistance under low folate testing conditions. Mutations associated with decreased susceptibility to sulfadoxine include Ala→Gly-581, and Ser→Phe-436 coupled with Ala→Thr/Ser-613 [14, 15]. Ser→Ala-436 and Ala→Gly-437 were initially reported not to be associated with resistance, but more recent studies implicate these mutations in sulfadoxine resistance as well [16] (Hyde J, personal communication).

The relationship between both sets of mutations and in vivo resistance to pyrimethamine-sulfadoxine is not known, and previous studies disagree on the relative importance of in vitro resistance to pyrimethamine versus that to sulfadoxine in causing in vivo pyrimethamine-sulfadoxine failure [17, 18]. Recent in vitro studies of the synergistic action between pyrimethamine and sulfadoxine indicate that pyrimethamine susceptibility is paramount in determining synergy and, therefore, presumably the response to treatment [19]. However, this remains to be confirmed in vivo. Mutation-specific diagnostic polymerase chain reaction (PCR) assays to detect the DHFR 51, 59, 108, and 164 mutations and the DHPS 436, 581, and 613 mutations have been described and can be performed on samples of fingerstick blood dried onto filter paper strips [1, 9, 10, 20–22].

Epidemiologic studies of the prevalence of the DHFR and DHPS mutations in areas with different levels of pyrimethamine-sulfadoxine use, in conjunction with laboratory-based analyses of relationships between parasite genotypes and phenotypes, can aid in identifying the key mutations for clinical pyrimethamine-sulfadoxine resistance, validating molecular assays for resistance, and in screening for new mutations that may contribute to resistance.

**Materials and Methods**

**Study sites.** In Mali, chloroquine remains effective (unpublished data), and national malaria control policy reserves pyrimethamine-sulfadoxine for treatment of cases of chloroquine failure. Of cases of malaria at the study site (n = 218), 99% exhibited a sensitive response to pyrimethamine-sulfadoxine at the time of this study [23]. Resistance definitions (RI, RII, and RIII) are based on those of the World Health Organization: RI is the disappearance of parasitemia after pyrimethamine-sulfadoxine therapy followed by recurrent parasitemia; RII resistance is a ≥ 75% diminution of parasitemia followed by a subsequent rise in parasitemia; and RIII resistance is < 75% or no diminution of parasitemia following therapy. Therapeutic failure refers to RI or RIII resistance and persistence or recurrence of symptoms. Samples were collected in 1995 at two clinics on the outskirts of Bamako, the capital city of Mali.

In Kenya, increasing chloroquine resistance has very recently led to the policy that pyrimethamine-sulfadoxine be used as the first-line antimalarial drug in Malawi, the first African country to make this change [24]. This policy was accompanied by large public education and health worker training efforts, and chloroquine was restricted to prescription use while pyrimethamine-sulfadoxine was allowed to be sold without prescription, resulting in an abrupt and near-total replacement of chloroquine with pyrimethamine-sulfadoxine. Pyrimethamine-sulfadoxine resistance was absent in Malawi in 1990 before pyrimethamine-sulfadoxine became the first-line drug but is now rising, with 14-day recrudescence rates (RI, RII, and RIII combined) of up to 36% and up to 3% RIII in 1995 and 1996 (unpublished data), when samples were collected at a clinic in the town of Karonga.

Pyrimethamine-sulfadoxine became the first-line antimalarial throughout the Amazon region of South America after the failure of chloroquine there and was used heavily in Bolivia throughout the 1980s. The Bolivian Ministry of Health stopped recommending pyrimethamine-sulfadoxine for the treatment of malaria in 1991 because of high treatment failure rates: 5.6% sensitive, 44.4% RI, 27.8% RII, and 22.2% RIII in 1988 (Bolivian Ministry of Health, unpublished data). Despite this policy, the Pan American Health Organization estimates that 550,421 tablets of pyrimethamine-sulfadoxine were consumed in Bolivia in 1995, more than in any other South American country for which figures were available [25]. Samples were collected in the village of Guayaramerin in the department of Beni in northern Bolivia during a malaria outbreak in May and June of 1994. Guayaramerin is near the border with Rondonia, Brazil, where malaria is endemic and pyrimethamine-sulfadoxine use is unrestricted.

On the basis of these drug use policies, histories of pyrimethamine-sulfadoxine use, and the available evidence of in vivo pyrimethamine-sulfadoxine resistance, Mali, Kenya, Malawi, and Bolivia were ranked in that order as sites with lowest to highest levels of in vivo pyrimethamine-sulfadoxine resistance, each at a further point along a time line of pyrimethamine-sulfadoxine use.

**Sample collection and DNA extraction.** Fingerstick blood from persons presenting to clinics with uncomplicated, microscopically confirmed *P. falciparum* malaria was collected onto filter paper strips, and DNA was extracted by use of a chelating resin (Chelex-100; Bio-Rad, Richmond, CA) as described [1].

**Mutation-specific PCR.** Nested mutation-specific PCR was done as previously described for analysis of the DHFR 108 and 164 mutation sites [1, 20]. Amplified DHFR domain from the first round of PCR, which used the oligonucleotide primer pair AMP1 and AMP2 as described [1], was used in mutation-specific second-round PCR to assess the DHFR mutation sites 51 and 59. For codon 51, mutation-specific sense primers FR51w (5’-TTACCA-TGGAAATGTAA-3’) and FR51m (5’-TTACCA-TGGAAATGTAT-3’) [22], which is specific for the mutant codon...
ATT (Ile), were paired with the common antisense primer SP2 [20], for a final product of 514 bp. Taq DNA polymerase (Life Technologies GIBCO BRL, Gaithersburg, MD) was used with the manufacturer’s buffer containing 2.5 mM MgCl2, primers were used at 1 μM, and other reaction conditions were as described [1] with these cycling parameters: initial denaturation at 95°C for 3 min, followed by 15 cycles of denaturation at 92°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s, and a final extension at 72°C for 3 min. For analysis of the DHFR 59 codon, the antisense primers FR59w (5'-ATGGTGAATGGCACAC-3'), specific for the TGT (Cys) wild type and FR59m (5'-ATGGTGAATGGCACCCG-3') [22], specific for the CGT (Arg) mutant, were paired with the common sense primer SP1 [20], to generate a final product of 190 bp. For this reaction, 1.5 mM MgCl2 was used, and cycling conditions were identical to those for codon 51 with the exception of an annealing temperature of 54°C.

Screening for the previously known DHPS mutations was done as described [21] with the following modifications. Five microliters of the chelating resin–extracted DNA was amplified in a 50-μL reaction using primers M3717 and 186 and conditions as described [21] but with the following cycling parameters: initial denaturation for 3 min at 95°C; 45 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 45 s; and a final extension of 3 min. Depending on product yield as determined showing 849- and 303-bp digestion products; lane 5

**Figure 1.** A. Restriction digestion method for detecting dihydrofolate reductase (DHFR) Arg-50 mutation. Lane 1 = 720-bp polymerase chain reaction (PCR)–amplified DHFR domain from sample containing wild type Cys-50, showing no digestion by Tacl; lane 2 = DHFR from sample with Arg-50 mutation showing 540-bp Tacl digestion product; lane 3 = DHFR from sample containing both wild and resistant genotypes. B, PCR size polymorphism method for detecting DHFR Bolivia repeat mutation. Lane 1 = 190-bp product from sample containing wild type Bolivia repeat; lane 2 = 205-bp product from sample containing Bolivia repeat. C, Restriction digestion method for detecting dihydropteroate synthase (DHPS) 540 and 437 mutations. Lane 1 = 1152-bp PCR-amplified DHPS domain negative control; lane 2 = DHPS from sample containing wild type Ala-437, showing no digestion by Avall; lane 3 = DHPS from sample containing wild type Lys-540, showing no digestion at mutation site by FokI (864- and 188-bp products result from FokI digestion at other sites in DHPS); lane 4 = DHPS from sample containing Gly-437 mutation, showing 849- and 303-bp digestion products; lane 5 = DHPS from sample containing Glu-540, showing 188-, 25-, and 539-bp digestion products; lane 6 = 100-bp ladder.

**DNA sequencing.** DNA sequencing of both TA-cloned and uncloned PCR products (SP1/SP2–amplified DHFR domain and primers 185/218–amplified DHPS domain) was done by the Biopolymer Laboratory of the University of Maryland School of Medicine.

**Molecular modeling.** A computer-generated model of the energy-minimized tertiary structure (TS) of DHFR was created using InsightII (Biosym Technologies, San Diego). The primary sequence of the P. falciparum DHFR-TS was aligned with the extracted sequence from the Leishmania major DHFR-TS [26], and homology modeling and energy minimization were performed on an Iris indigo XZ4000 computer (Silicon Graphics, Mountain View, CA).

**Data analysis.** Prevalence rates for each mutation represent the number of infections at a site containing the mutant genotype, divided by the total number of infections from that site analyzed for the presence of that mutation. Mutations were determined to be present or absent on the basis of the presence or absence of the expected bands on ethidium-stained agarose gel analysis of diagnostic PCR product and restriction digestions. Gels were recorded by video photography.

**Statistical analysis.** Spearman correlation coefficients between rank of sites by pyrimethamine-sulfadoxine resistance and rank of prevalence for each mutation were determined with SPSS for Windows 6.1 (SPSS, Chicago). Two-tailed significance was tested at P < .05.

**Results**

**Prevalence of DHFR and DHPS mutations.** Prevalence rates of previously known mutations are shown in figures 2

Clonal analysis of PCR products. The TA cloning system (Invitrogen, San Diego) was used to analyze individual PCR products of blood samples containing mutations of interest. Isolated colonies of transformed bacteria were transferred to 20 μL of sterile water and heated to 95°C for 5 min, debris was pelleted by microcentrifugation, and 0.1–0.2 μL of the supernatant was directly added to a 25-μL PCR reaction for analysis of the mutation site of interest.
and 3. DHFR Asn-108, which must be present before additional mutations conferring higher-level pyrimethamine resistance can occur [27], is present at all sites and correlates with increased pyrimethamine-sulfadoxine use and resistance (r = 1.00, P < .05). DHFR Ile-51 (r = 0.95, P = .05) and Arg-59 (r = 0.40, P = .60) show a similar trend, with the exceptions that Ile-51 is universal in both Malawi and Bolivia and Arg-59 is less prevalent in Bolivia than in the African sites. DHFR Leu-164 (r = 0.78, P = .23), which has not previously been reported outside of Southeast Asia, and DHPS Gly-581 (r = 0.78, P = .23), were observed at high frequencies in Bolivia but were not found in any of the African countries surveyed. DHPS Gly-437 (r = 0.80, P = .20), while more frequent in Mali than in Kenya, otherwise tended to increase with increased in vivo resistance, but the paired DHPS mutations at positions 436 and 613 (r = −0.78, P = .23) were rare in Africa and absent in Bolivia. The Ala-436 DHPS mutation (r = −1.00, P < .05) was inversely associated with drug use and in vivo resistance. The DHFR Thr-108 mutation associated with cycloguanil resistance was not detected in samples from any site.

**New DHFR mutations.** On the basis of the finding of a high prevalence of previously identified DHFR and DHPS mutations in Bolivia, PCR-amplified DHFR from 3 Bolivian samples containing multiple DHFR mutations (as detected by diagnostic nested PCR) was cloned, and a total of 7 clones were sequenced to determine if additional new mutations had appeared in this setting. One sample was determined to harbor a new single base alteration causing a Cys−Arg change at DHFR codon 50, and 2 samples contained an in-frame 15 bp repeat inserted between aa 30 and 31, termed the Bolivia repeat (figure 4). A computer-generated model of the tertiary protein structure of *P. falciparum* DHFR suggests that the Bolivia repeat lies adjacent to the active site of DHFR (figure 5).

Methods were devised to identify both new DHFR mutations and used to screen additional samples from all four study sites for their presence. The Arg-50 mutation was found in 52.5% (n = 40) of Bolivian samples tested, and its presence was confirmed by direct sequencing of PCR-amplified DHFR from 3 of these samples. The Bolivia repeat was detected in 43.8% (n = 48) of Bolivian samples and confirmed by direct sequencing of PCR product in 1 of these. Both new DHFR mutations were absent in the three African countries surveyed (figure 6). No additional new DHFR mutations were found among a total of 8 Bolivian samples and 6 Malawian samples sequenced.

Clonal analysis revealed that the two new DHFR mutations do not occur together in the same gene, although they can occur together in a single, polyclonal field sample. In PCR analysis of cloned field-derived DHFR, both new mutations were found to occur only in combination with the previously identified Ile-51 mutation but not with the Arg-59 mutation.
The Bolivia repeat was found only in combination with the Leu-164 mutation in 25 clones from 5 samples (5 clones analyzed per sample), while Arg-50 occurred with the wild type Ile-164 in 23 of 24 clones analyzed. One of 24 clones was confirmed by sequencing to contain the Arg-50 mutation with the mutant Leu-164.

New DHPS mutation. DHPS from 4 Bolivian samples of \textit{P. falciparum} DNA was amplified by PCR and directly sequenced, and a new point mutation causing a Lys→Glu change at codon 540 was detected in all 4 of these. Restriction digestion methods were used to screen samples for the presence of this new mutation and to determine its prevalence at all four study sites. The presence of this mutation (and absence of other new mutations) was confirmed by direct sequencing of PCR-amplified DHPS domains from 3 Malawian samples determined by restriction digestion methods to contain Glu-540. The Glu-540 DHPS mutation was strongly correlated with in vivo resistance \( (r = 1.00, P < .05; \text{figure } 6) \). This mutation has been independently discovered in \textit{P. falciparum} isolates from Thailand [28].

PCR analysis of individual clones showed that both the Gly-581 and the new Glu-540 mutations only occur in the presence of the Gly-437 and that Ala-436 and Gly-437 are rarely present on the same molecule: Only 3 of 91 DHPS clones derived from African samples that harbored either Ala-436 or Gly-437 contained both mutations. Ala-436 was never observed to occur with the Glu-540 and Gly-581 mutations.

Discussion

Despite an increasingly precise understanding of the molecular basis of in vitro resistance of \textit{P. falciparum} to the antifolate drugs, the determinants of therapeutic failure of the combination of pyrimethamine and sulfadoxine remain unknown. The studies reported here identify those mutations most likely to be important in clinical antifolate resistance, demonstrate the feasibility of using simple molecular methods to conduct surveillance for drug resistance mutations on a large scale, and identify new mutations in both DHFR and DHPS that may contribute to in vivo pyrimethamine-sulfadoxine resistance.

All of the DHFR mutations grow more prevalent in areas with increased pyrimethamine-sulfadoxine use and resistance, with the exception that Arg-59 was less prevalent in Bolivia, where clinical resistance is highest. However, this may be explained by the two new DHFR mutations unique to Bolivia, both of which were found only in DHFR with wild type Cys-108, Ile-51, and Arg-59 mutations with pyrimethamine-sulfadoxine use and in vivo resistance at the sites in Africa suggests that pyrimethamine-sulfadoxine treatment is selective for these mutations, but their high prevalence in these settings, where therapeutic pyrimethamine-sulfadoxine failure remains rare, supports the view that these three mutations are insufficient for high-level in vivo pyrimethamine-sulfadoxine resistance. The DHFR Leu-164 mutation was found only in Bolivia, where high-level in vivo resistance is common. Leu-164 has previously been reported only in \textit{P. falciparum} strains from Southeast Asia [6, 11], another area of widespread pyrimethamine-sulfadoxine resistance, suggesting that this mutation appears late in the course of development of pyrimethamine-sulfadoxine resistance and is likely to play a role in therapeutic failure.

Our analyses are consistent with earlier studies [6, 11, 12, 27] confirming that the DHFR mutations occur in a stepwise fashion. Initial mutations conferring low-level pyrimethamine resistance must be present for high-level resistance mutations to appear. Some mutations appear to be mutually exclusive, an observation that may be explained by deleterious effects of certain combinations of mutations on enzyme function.

Prevalence rates of the DHPS mutations are less clearly correlated with pyrimethamine-sulfadoxine resistance. The high prevalence of Gly-581 in Bolivia and the correlation between prevalence of Gly-437 and Glu-540 and in vivo resistance are consistent with a role in therapeutic pyrimethamine-sulfadoxine failure for these mutations. However, despite their association with in vitro sulfadoxine resistance, the mutations at positions 436 and 613 do not correlate with in vivo pyrimethamine-sulfadoxine resistance. This disparity may be because the sub-physiologic folate and PABA conditions used in sulfadoxine in vitro susceptibility studies do not reflect in vivo conditions.
Figure 6. Prevalence of new dihydrofolate reductase (DHFR) and dihydropterate synthase (DHPS) mutations at 4 sites with increasing levels of in vivo pyrimethamine-sulfadoxine resistance, determined by restriction digestion (DHFR Arg-50, DHPS Glu-540) or polymerase chain reaction size polymorphism (DHFR Bolivia repeat). Nos. of samples tested are indicated.

Several lines of reasoning support the hypothesis that the new DHFR Bolivia repeat and Arg-50 mutations are involved in resistance. First, these mutations are found only in an area of the world with very high rates of in vivo pyrimethamine-sulfadoxine resistance, where they are highly prevalent. Second, all previously identified naturally occurring DHFR mutations examined confer resistance, and finally, the Arg-50 mutation is located at the active site pocket of DHFR, where drug binding occurs [3], and molecular modeling suggests that the Bolivia repeat mutation also lies adjacent to this site (figure 5). The strong correlation between prevalence of the new DHPS Glu-540 mutation and in vivo pyrimethamine-sulfadoxine resistance suggests that it too contributes to resistance.

We hypothesize that RI, RII, and RIII levels of in vivo pyrimethamine-sulfadoxine resistance are due to the progressive accumulation of DHFR and DHPS mutations. For example, infections with mutations at DHFR-108 and DHPS-437, which are common in Mali, where pyrimethamine-sulfadoxine resistance is rare, are likely to exhibit a sensitive response to pyrimethamine-sulfadoxine treatment; RI and RII resistance may arise when various combinations of DHFR-51 and DHFR-59 mutations and/or the DHPS-540 mutation are added; and RIII resistance may require the additional presence of DHFR-164, the Bolivia repeat and/or DHPS 581. Because of the polyclonal nature of natural P. falciparum infections [29] and the impact of other factors, such as immunity [30] and host folate levels [31], on pyrimethamine-sulfadoxine treatment outcome, there will likely be overlap among the categories of resistance seen with each combination of mutations.

Proof that specific DHFR and DHPS mutations cause in vivo pyrimethamine-sulfadoxine resistance will require prospective studies of the relationship between parasite genotype and clinical outcome in individual infections treated with pyrimethamine-sulfadoxine. If a limited set of DHFR and DHPS mutations can be identified as predictive of in vivo pyrimethamine-sulfadoxine resistance, it will be possible to conduct broad surveillance for pyrimethamine-sulfadoxine resistance. This capability will be particularly important in sub-Saharan Africa, where the question of whether and when to use pyrimethamine-sulfadoxine is becoming increasingly urgent. However, molecular methods for monitoring pyrimethamine-sulfadoxine resistance will also be useful in settings such as Bolivia, to determine whether, once established, high levels of resistance will subsequently fall to levels allowing the reintroduction of pyrimethamine-sulfadoxine. A number of antifolate compounds and combination drugs are under consideration as potential replacements for pyrimethamine-sulfadoxine, and defining the relationship between DHFR and DHPS mutations and resistance to these drugs will aid in selecting the most suitable candidates among them. Continued surveillance for and analysis of new mutations in target molecules will be key to the successful development and deployment of molecular assays for drug-resistant malaria.

Acknowledgments

We thank the residents and health workers at all the study sites for participating in these studies, Thomas Wellems, David Peterson and Louis Miller for critical review of the manuscript, John Hyde and Terrie Taylor for helpful discussions, Steven Wasserman for statistical advice, and Lisa Sadzewicz for assistance with DNA sequencing.
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