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SULFADOXINE–PYRIMETHAMINE EFFICACY AND SELECTION OF PLASMODIUM FALCIPARUM DHFR MUTATIONS IN BURKINA FASO BEFORE ITS INTRODUCTION AS INTERMITTENT PREVENTIVE TREATMENT FOR PREGNANT WOMEN

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Abstract. Sulfadoxine-pyrimethamine efficacy was determined with a 28-day follow-up in 97 children between 6 months and 15 years of age. The polymerase chain reaction (PCR)-corrected treatment failure was 8.2% and the uncorrected was 21.6%. The presence of the dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) mutations linked to sulfadoxine-pyrimethamine resistance before and after treatment was determined by PCR-restriction fragment length polymorphism (RFLP) and by a fluorogenic PCR assay. Before treatment, the prevalence of the triple DHFR mutations was higher among the patients having had a recurrent parasitemia (either recrudescence or new infection; 28.6% versus 9.3%), although the difference was not significant (P = 0.1). The double mutation Ala-436/Gly-437 was observed in 67% of samples, whereas no Glu-540 mutation was found. After treatment, the triple DHFR mutation was found in 76.2% of patients with recurrent parasitemia, recrudescence, and new infection alike. Such high prevalence of mutant parasites indicates that sulfadoxine-pyrimethamine should not be used as monotherapy.

INTRODUCTION

In Africa, sulfadoxine-pyrimethamine (SP) has been used as a second-line drug for the treatment of chloroquine-resistant (CQ-R) malaria.1 However, CQ-R is now so widespread that several countries such as Burundi (from CQ to amodiaquine-artesunate), Rwanda (from CQ to amodiaquine-SP), Uganda (from CQ-SP to artemether-lumefantrine), and Zambia (from SP to artemether-lumefantrine) have changed or are about to change their drug policy.2

Pyrimethamine inhibits dihydrofolate reductase (DHFR), whereas sulfadoxine inhibits dihydropteroate synthetase (DHPS).3–5 The understanding of the molecular mechanisms of parasite resistance to anti-folates has led to a proliferation of laboratory and field studies investigating the role of molecular markers in detecting drug resistance. DHFR mutations at Asn-51 to Ile-51 and Cys-59 to Arg-59 in association with Asn-108 were found to be associated with greater resistance to pyrimethamine, whereas P. falciparum strains with variable levels of sensitivity to sulfadoxine had sequence variation in the DHPS gene.5,6

In Burkina Faso, until recently, CQ and SP have been used as first- and second-line drugs, respectively, for the treatment of uncomplicated malaria. However, in vivo tests carried out in 2002 in Nanoro (center of the country) showed clinical failure (early and late) to CQ of > 50% (J. B. Ouédraogo, personal communication). The first SP treatment failure was observed in 19997 and a study conducted in 2001, a time when SP was still used as second-line treatment, reported about 4% resistance (J. B. Ouédraogo, personal communication). Such data have prompted the change of the anti-malarial drug policy, more specifically to the adoption of artemether-lumefantrine as first-line treatment of uncomplicated malaria and to the use of SP intermittent preventive treatment (IPT) during pregnancy instead of CQ weekly.7 Indeed, in Mali, a neighboring country, SP IPT during the second and third trimester was shown to be more efficacious than weekly CQ in preventing malaria infection and its consequences on the mother’s and newborn’s health.8 In Burkina Faso, all studies on SP efficacy conducted thus far used a 14-day follow-up that under-estimates failure rate.9 Considering the high CQ-R in Nanoro and the low SP efficacy in some neighboring countries such as Côte d’Ivoire and Ghana,10,11 the relationship between SP treatment failures (28-day follow-up) and the selection of the DHFR and DHPS mutations in children living in Nanoro was studied before the introduction of SP IPT. Such data might help in estimating the probable impact of SP IPT during pregnancy.

MATERIALS AND METHODS

Study sites. The study was carried out in 2003 in Nanoro, situated in the center of Burkina Faso at 85 km from Ouagadougou, the capital city. The rainy season occurs from June to October (average rainfall: 700 mm/yr; mean temperature > 30°C), and it is followed by a cold dry season from November to February (minimum temperature, 15°C) and a hot dry season from March to May. The population was estimated at 136,209 inhabitants in 2001; 19% of them are children < 5 years of age. Malaria is hyperendemic with seasonal transmission. The entomological inoculation rate (EIR) is estimated at 50–60 infective bites/man/yr (A. Diabate, personal communication). The most common vectors are Anopheles gambiae, A. funestus, and A. arabiensis. Plasmodium falciparum is the predominant malaria parasite. Malaria is the main reason for consultations at health facilities all year round, with a peak between September and December. In 2000, malaria represented 34% (21,039/62,400) of the total consultations in the district (District’s health statistics).

Study population. This is part of a larger study that investigated CQ and SP efficacy in children between 6 months and 15 years of age in Burkina Faso. In the following analysis, only SP efficacy in Nanoro, the only site where we conducted

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a 28-day follow-up, has been considered. Details of the study methodology have been described in detail elsewhere.\textsuperscript{1} Briefly, children with fever (axillary temperature $\geq 37.5^\circ\text{C}$) and a \emph{P. falciparum} infection with a parasitemia density between 2,000 and 100,000/\text{\mu L were recruited if the parent or guardian gave informed consent. This study was reviewed and approved by the Center Muraz Ethical Committee.}

\textbf{Treatment and follow-up.} The World Health Organization (WHO) 28-day \textit{in vivo} test was carried out.\textsuperscript{13} SP was administered orally and according to body weight (25 mg/kg of sulfadoxine stat). All doses were given under direct supervision. Children were observed for at least 30 minutes, and a replacement dose was given when vomiting occurred. When vomiting persisted, children were withdrawn from the study. Axillary temperature and clinical information were collected at days 0, 1, 2, 3, 7, 14, 21, and 28 after treatment. Thick and thin blood films were collected at days 0, 3, 7, 14, 21, and 28.

\textbf{Definitions of outcomes.} Outcomes were defined according to the WHO-modified classification for monitoring anti-malarial drug resistance: early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), and adequate clinical and parasitological response (ACPR).\textsuperscript{13} Late treatment failure (LTF) is defined as the sum of LCF and LPF, whereas total treatment failure (TTF) is defined as the sum of ETF, LCF, and LPF.

\textbf{Molecular analysis.} All samples from patients with recurrent parasitemia during follow-up were genetically genotyped in addition to about one half of randomly selected ACPRs. Blood samples for the molecular analysis were collected on filter paper (Whatman 3, Whatman International, Ltd., Maidstone, UK) at day 0 before treatment and at the time of parasitemia reappearance. DNA was extracted using the Chelex-100 method.\textsuperscript{14}

Detection of the DHFR and DHPS mutations. We analyzed three point mutations both in the \textit{DHFR} gene (108, 51, and 59) and the \textit{DHPS} gene (436, 437, and 540). Details of polymerase chain reaction (PCR) conditions are summarized in Table 1. The detection of mutations in \textit{DHFR} and \textit{DHPS} and Asn/Thr-108 in \textit{DHFR} was done using PCR followed by restriction fragment length polymorphism (RFLP).\textsuperscript{15,16} The first-round PCR amplification was performed in a 25-\text{\mu L mixture containing 1\times PCR buffer (Promega, Madison, WI), 2.5 mmol/L MgCl$_2$ (Promega), 0.2 mmol/L of dNTPs, 0.5 pmol of each primer, 1 U of Taq polymerase (Promega), and 1 \mu L of extracted parasite DNA. Nested PCR was performed in a 50-\text{\mu L mixture containing 1 \mu L of primary PCR product. The amplified DNA fragments were separated by electrophoresis in a 2\% agarose gel. The nested PCR product was subjected to enzyme (New England Biolabs) digestion for the \textit{DHFR} 108 mutations, \emph{Ala1} cuts only the wild-type gene (Ser-108) into 323 and 372 bp, \emph{Bor1} cuts only the mutant gene (Asn-108) into 328 and 372 bp, and \emph{Scr1} cuts only the mutant gene (Thr-108) into 324 and 376 bp. For the \textit{DHPS} mutations, \emph{Msp1} cuts only the mutant gene (Ala-436) into 300 and 851 bp, \emph{Av1II} cuts only the mutant gene (Gly-437) into 303 and 848 bp, and \emph{FokI} cuts only the mutant gene (Glu-540) into 538 and 326 bp. For each series of samples, water was used as a negative control. For the detection of Asn-108, 3D7-clone DNA was used as the wild-type control and Dd2-DNA was used as the mutant control. For the detection of \textit{DHPS} mutations, PSMali-clone DNA was used as wild-type control for positions 437 and 540 and mutant control for the position 436, whereas
PS-Peru-clone DNA was used as wild-type control for the position 436 and mutant control for positions 437 and 540.

The detection of DHFR Ile-51 and Arg-59 mutations was done by using a fluorogenic PCR assay that combines fluorescence–resonance energy transfer between fluorophores present on a probe and a PCR primer and a melt-curve analysis.\(^{17}\) The basis of this assay is fluorescence resonance energy transfer (FRET), which requires two fluorophores referred to as donor and acceptor. On hybridization of the fluorogenic probes on the amplicon, these fluorophores are brought into close proximity. The fluorescence emitted from the donor is then absorbed by the acceptor resulting in fluorescence emission by the acceptor, which can be detected. Progressive increase of the temperature during melt curve analysis leads to the dissociation of the fluorogenic probes from the target at specific temperatures, resulting in the loss of fluorescent signal visualized on a melt curve. The specific temperature of probe melting depends on the thermodynamic stability and allows discrimination of perfectly complementary and mismatched probe-target duplexes. The assay allows 1) the identification of the genotype at multiple codons simultaneously in a mutation hot spot region (codons 50-60) of the \textit{P. falciparum} DHFR gene and 2) the quantification of different genotypes present in a polyclonal malaria infection. For the detection of DHFR Ile-51 and Arg-59 mutations, it allows the identification of the genotype at double codons Ile-51/Arg-59 simultaneously (when existing) or one of the two single mutations alone.

The second-round PCR amplification was performed in a 50-μL mixture containing 1 μL of primary PCR product resulting from amplification with AMP1 and AMP2, 1× PCR buffer iQ Supermix (Bio-Rad), 500 nmol/L of primer FRET3 (Rox), and 100 nmol/L of primer FRET2. The probe DHFR (FAM): 5′-CACAAAAATATTTCATATCTAGGGAT-TACAT-3′ was then added immediately after amplification, in a final concentration of 160 nmol/L. The iCycler (Bio-Rad, Hercules, CA), a standard PCR cycler equipped with an optical module, was used to perform the melt-curve analysis (MCA) as described by Decuypere and others.\(^{17}\) The MCA protocol consisted of two steps: 1) 1 minute at 94°C and 2) 110 repeated heating (each for 30 seconds), starting at 48°C and with increments of 0.3°C. Change in fluorescence appears as a positive peak on a plot of the first negative derivative of the fluorescence/temperature function. All experiments using FRET/MCA were performed in triplicate to ensure reproducibility. Plasmids containing a DHFR insert with a known genotype were obtained from MR4/American Type Culture Collection. FR-3D7, with the genotype 50 Cys (TGT)-51 Asn (AAT)-59 Cys (TGT) (\(\text{Tm}: 52.8°C\)) was used as the mutant control. Four other controls (N2, N5, N7, and N15) defined on the basis of significant and reproducible (inter-experiment SD < 0.4°C) differences in \(\text{Tm}\) were also used.\(^{17}\)

**Genotyping to distinguish between recrudescence and new infection.** If a patient had recurrent parasitemia, blood samples from day 0 and from that day the parasitemia reappearance were used to genotype parasite strains. Nested PCR\(^{18}\) was adopted for the analysis of two polymorphic genetic markers from \textit{P. falciparum}: merozoite surface proteins 1 and 2 (MSP1 and MSP2; Table 1). A recrudescence infection was defined as one that showed match in size of at least one allele for both the \textit{MSPI} and \textit{MSP2} genes between day 0 and the day of recurrent parasitemia.

**Statistical methods.** Data were entered in Excel version 97 and analyzed using STATA 8 (Stata Corp. 2003). Children were considered not to be clinical failures if their parasitemia between day 14 and day 28 was classified as a new rather than recrudescence infection. Frequencies of DHFR point mutations Asn-108, Ile-51, and Arg-59 were determined before and after treatment (the latter for recrudescent and new infections), whereas those of the DHPS point mutations Ala-436, Gly-437, and Glu-540 were determined only before treatment. \(\chi^2\) test was used to test for significant differences between categorical variables. A value of \(P < 0.05\) was considered statistically significant.

### RESULTS

Of 673 children screened, 63% (424) had a microscopically confirmed malaria infection, and of these, 110 were enrolled in the SP treatment group. The mean age of the children was 51.5 months (95% CI, 45.3–57.7), 60% (66/110) of them being < 5 years of age. Outcome at day 28 is known for 97 (88.2%) patients who completed the follow-up (8 were lost to follow-up, 1 developed another illness, 1 had severe malaria, and 3 withdrew the informed consent). There was no ETF. Up to day 14, only two (2%) patients had recurrent parasitemia; 19 (19.6%) additional recurrent parasitemias were observed between day 15 and 28. All these infections were successfully genotyped, and eight (42.1%) of them were classified as recrudescence so that PCR-corrected TTF was 8.2% (8/97) and the ACPR was 91.8% (Table 2).

The DHFR gene was successfully genotyped for the detection of the Asn-108 mutation in 53 samples: the 21 recurrent parasitemias and 32 randomly selected ACPRs. Twenty-two (41.5%) samples did not carry any DHFR mutation (14 ACPR, 7 new infections, and 1 TTF; Table 2). Before treatment, the prevalence of the triple DHFR mutations was

<table>
<thead>
<tr>
<th>Treatment outcome and DHFR–DHPS mutations before treatment</th>
<th>DHFR ((n = 53))</th>
<th>DHPS ((n = 51))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTF</td>
<td>12.5 (1/8)</td>
<td>25.0 (2/8)</td>
</tr>
<tr>
<td>New infection</td>
<td>53.8 (7/13)</td>
<td>23.0 (3/13)</td>
</tr>
<tr>
<td>ACPR</td>
<td>43.7 (14/32)</td>
<td>56.2 (18/32)</td>
</tr>
</tbody>
</table>

\(^{1}\)Values are percentage (n/N).

TTF, total treatment failures; ACPR, adequate clinical and parasitological response.
Asn Nevertheless, SP is still relatively efficacious in Burkina Faso — parasitological resistance > 20%.

The double mutation Ala-436/Gly-437 was observed in 67% (34/51) of samples, and its prevalence was significantly higher among patients having had recurrent parasitemia than in those classified as ACPR ($P = 0.005$). No Glu-540 mutation was observed.

After treatment, 76.2% (16/21) recurrent parasitemias carried the pure triple DHFR mutation, recrudescence, and new infection alike (Table 3).

**DISCUSSION**

The first two cases of in vivo resistance to SP in Burkina Faso were reported in 1990 and 1991 from Bobo Dioulasso. Despite its long use as a second-line drug, SP resistance in Burkina Faso was low, often < 1%. However, patients had always been followed only up to day 14 after treatment, under-estimating the level of treatment failures. Indeed, in this study, the PCR-corrected failure rate at day 28 was much higher than that observed at day 14, confirming that SP resistance in Burkina Faso is probably higher than that reported from previous studies. Nevertheless, SP is still relatively efficacious in Burkina Faso and in several other West African countries (treatment failure < 4%), although a few areas of high resistance, particularly in forest areas, exist (parasitological resistance > 20%). Such patchy distribution and the relative low SP resistance could be explained by several factors, more specifically by the limited drug pressure. This situation might rapidly change as the result of increased SP use, a consequence of high CQ resistance. A new drug policy adopted in February 2005 recommends the use of the combination artemether-lumefantrine as first-line treatment. In this context, SP, one of the few anti-malarials that can be safely used during pregnancy, could be deployed exclusively as IPT, minimizing the drug pressure and the selection of resistant parasites.

Although the molecular basis of *P. falciparum in vitro* resistance to the anti-folate drugs is well established, the relationship between the identified molecular markers and therapeutic failure is not a linear one. Indeed, it can vary according to the geographical situation and/or level of resistance. High prevalence of the DHFR Asn-108 point mutation was strongly associated with high SP use. In our study, despite low SP resistance, more than one half of the pre-treatment samples carried the Asn-108 mutation, a frequency similar to that observed in some high SP resistance areas.

Considering the importance of this mutation for the development of pyrimethamine resistance, such high prevalence might indicate a rapid development of anti-folate resistance in Burkina Faso. We did not find any Thr-108 mutation, a result consistent with earlier studies confirming the rarity of this mutation in Africa.

The use of the fluorogenic PCR assay, allowing the identification of the double mutation Ile-51/Arg-59, showed that the Arg-59 mutation was always associated with Ile-51 mutation, confirming the stepwise accumulation of DHFR mutations in the following order: Asn-108 → Ile-51 → Arg-59. The prevalence of these two mutations was relatively low, about twice than that of SP resistance, indicating its possible link with SP resistance, at least in places where this is low. The DHFR triple mutation, with or without mutant DHPS alleles, has been associated with SP resistance. Although the triple mutation was observed only in a minority of treatment failures, this was observed in almost all samples col-

<table>
<thead>
<tr>
<th>Samples</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
<td>51</td>
<td>51/59</td>
</tr>
<tr>
<td>N55</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>N62</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N203</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N434</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N510</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N551</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>N605</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N638</td>
<td>Asn</td>
<td>—</td>
</tr>
<tr>
<td>N100</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N246</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N283</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N318</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N351</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N362</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N386</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N441</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N505</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N515</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>N567</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>N573</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td>N598</td>
<td>Ser/Asn</td>
<td>Asn</td>
</tr>
</tbody>
</table>

Mutant genotypes are indicated in bold italics. R, recrudescence; NI, new infection.
lected after treatment, confirming that SP treatment selects these specific mutations, regardless of the outcome. It also confirms that the DHFR triple mutation is necessary but not sufficient for SP treatment failure.22–24,26–29 It is interesting to note that DHFR mutants were also selected in treatment failure classes later as new infections. Despite the limitation of the PCR technique used16 and the possibility of misclassification, this indicates that resistant parasites can be selected in newly established infections if these occur during the time SP plasma levels are at sub-therapeutic concentrations. Therefore, DHFR mutations can rapidly arise and spread as soon as SP use becomes common.40

Resistance to sulfadoxine has been associated to several mutations in the DHPS gene.5,6 In our study, no Glu-540 mutation was found, and only Ala-436 and the Gly-437 mutations were found, confirming earlier findings that Ala-436 is most common where SP use and resistance is lower22,28 and that it occurs without the Glu-540 mutation.22 Nevertheless, unlike previous observations,22 in our study, the Ala-436 mutation was found in most isolates with the Gly-437 mutation. It has been suggested that the Gly-437 mutation is the first of the DHPS mutations, a situation comparable with the Asn-108 mutation in the DHFR gene.41 However, our results show that the frequency of the Gly-437 mutation was similar to that of the Ala-436 mutation, not supporting this hypothesis. It has also been suggested that the DHFR Arg-59 and the DHPS Glu-540 mutations can predict SP treatment failure.54,39 We were unable to observe this; no sample carried the DHPS Glu-540 mutation,59 possibly because of the low SP resistance. This might indicate that the DHPS mutations play a secondary role in determining treatment failure,37 although the DHFR triple mutations are necessary but not sufficient for SP resistance.

In conclusion, our results show that SP is still highly efficacious in Burkina Faso. However, considering the relatively high prevalence of the DHFR and DHPS mutations and, more importantly, their selection after treatment, SP should not be used as monotherapy for uncomplicated malaria but should be specifically targeted to pregnant women as IPT. The choice of SP as IPT in pregnancy is unavoidable because alternatives are not available yet. It still needs to be seen what will be the influence of this policy on the selection of mutant parasites and eventually on the SP efficacy in preventing malaria during pregnancy.

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