Am. J. Trop. Med. Hyg., 76(4), 2007, pp. 608–613

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

HALIDOU TINTO, JEAN BOSCO OUÉDRAOGO, ISSAKA ZONGO, CHANTAL VAN OVERMEIR, ERIC VAN MARCK, TINGA ROBERT GUIGUEMDÉ, AND UMBERTO D'ALESSANDRO*

Centre Muraz, Bobo Dioulasso, Burkina Faso; Institut de Recherche en Sciences de la Santé, Bobo Dioulasso, Burkina Faso; University of Antwerp, Antwerp, Belgium; Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium

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,[4] 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 1999[1] 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[9] that under-estimates failure rate.[10] Considering the high CQ-R in Nanoro and the low SP efficacy in some neighboring countries such as Côte d'Ivoire and Ghana,[11,12] 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

* Address correspondence to Umberto D'Alessandro, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. E-mail: udalessandro@itg.be (cc: tintohalidou@yahoo.fr)

a 28-day follow-up, has been considered. Details of the study methodology have been described in detail elsewhere.[1] Briefly, children with fever (axillary temperature ≥ 37.5°C) and a *P. falciparum* infection with a parasite density between 2,000 and 100,000/μL were recruited if the parent or guardian gave informed consent. This study was reviewed and approved by the Center Muraz Ethical Committee.

Treatment and follow-up. The World Health Organization (WHO) 28-day *in vivo* test was carried out.[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.

Definitions of outcomes. Outcomes were defined according to the WHO-modified classification for monitoring antimalarial drug resistance: early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), and adequate clinical and parasitological response (ACPR).[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.

Molecular analysis. All samples from patients with recurrent parasitemia during follow-up were systematically 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.[14]

Detection of the DHFR and DHPS mutations. We analyzed three point mutations both in the *DHFR* gene (108, 51, and 59) and the *DHPS* gene (436, 437, and 540). Details of polymerase chain reaction (PCR) conditions are summarized in Table 1. The detection of mutations in *DHPS* and Asn/Thr-108 in *DHFR* was done using PCR followed by restriction fragment length polymorphism (RFLP).[15,16] The first-round PCR amplification was performed in a 25-μL mixture containing 1× 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 μL of extracted parasite DNA. Nested PCR was performed in a 50-μL mixture containing 1 μL 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 *DHFR* 108 mutations, *Alu*I cuts only the wild-type gene (Ser-108) into 323 and 372 bp, *Bsr*I cuts only the mutant gene (Asn-108) into 328 and 372 bp, and *Scrf*I cuts only the mutant gene (Thr-108) into 324 and 376 bp. For the *DHPS* mutations, *Msp*AI cuts only the mutant gene (Ala-436) into 300 and 851 bp, *Ava*II cuts only the mutant gene (Gly-437) into 303 and 848 bp, and *Fok*I 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 *DHPS* mutations, PS-Mali-clone DNA was used as wild-type control for positions 437 and 540 and mutant control for the position 436, whereas

TABLE 1
Oligonucleotide primer sequences, cycling parameters, and expected fragment sizes

Gene	Primers	Sequences (5′_3′)	Size (bp)	Reaction conditions
DHFR				
First round	AMP1	5′-TTTATATTTTCTCCTTTTTTA-3′	720	95°C × 3 minutes; 92°C × 30 seconds, 45°C × 45 seconds, 72°C × 45 seconds,
	AMP2	5′-CATTTTATTATTCG-TTTTCT-3′		× 45; 72°C × 3 minutes
Second round	SP1	5′-ATGATGGAACAAGTCTGCGAC-3′	700	94°C × 5 minutes; 95°C × 30 seconds, 52°C × 1 minute, 72°C × 1 minute,
108	SP2	5′-ACATTTTATTATTCGTTTTC-3′		× 35; 72°C × 8 minutes
Second round	FRET3-rox	5′-GGAAATA-AAGGAGTATTACAATGGAAA-3′		95°C × 3 minutes; 92°C × 30 seconds, 45°C × 30 seconds, 72°C × 30 seconds,
51 & 59	FRET2	5′-TATAAACATCTCATCAAAATCTTC-3′		× 30; 72°C × 3 minutes
DHPS				
First round	186	5′-GTTTAATCACATGTTTGCACTTTC-3′	1,330	95°C × 3 minutes; 92°C × 30 seconds, 50°C × 45 seconds, 72°C × 1 minute,
	M3717	5′-CCATTCCTCATGTGTATACACAC-3′		× 30; 72°C × 3 minutes
Second round	185	5′-TGATACCCGAATATAAGCATAATG-3′	1,115	95°C × 3 minutes; 92°C × 30 seconds, 48°C × 30 seconds, 72°C × 30 seconds,
	218	5′-ATAATAGCTGTAGGAAGCAATTG-3′		× 30; 72°C × 3 minutes
MSP1				
First round	O1	5′-CACATGAAAGTTATCAAGAACTTGTC-3′	500–700	94°C × 3 minutes; 94°C × 25 seconds, 50°C × 35 seconds, 68°C × 2 minutes 30 seconds,
	O2	5′-GTACGTCTAATTCATTTGCACG-3′		× 30; 72°C × 3 minutes
Second round	N1	5′-GCAGTATTGACAGGTTATGG-3′	400–600	94°C × 3 minutes; 94°C × 25 seconds, 50°C × 35 seconds, 68°C × 2 minutes 30 seconds,
(nested)	N2	5′-GATTGAAAGGTATTTTGAC-3′		× 30; 72°C × 3 minutes
MSP2				
First round	S3	5′-GAAGGTAATTAAAACATTGTC-3′	450–800	94°C × 3 minutes; 94°C × 25 seconds, 42°C × 1 minute, 65°C × 2 minutes,
	S2	5′-GAGGGATGTTGCTGCTCCACAG-3′		× 30; 72°C × 3 minutes
Second round	S1	5′-GAGTATAAGGAGAAGTATG-3′	400–700	94°C × 3 minutes; 94°C × 25 seconds, 50°C × 1 minute, 70°C × 2 minutes,
(nested)	S4	5′-CTAGAACCATGCATATGTCC-3′		× 30; 72°C × 3 minutes

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 *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′-CACAAAAATATTTCATATCTAGGGAATTACAT-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 heatings (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) (*Tm:* 62.4°C) was used as the wild-type control, and FR-V1S, with the genotype 50 Cys (TGT)-51 Ile (ATT)-59 Arg (CGT) (*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 *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 the day of parasitemia reappearance were used to genotype parasite strains. Nested PCR[18] was adopted for the analysis of two polymorphic genetic markers from *P. falciparum*: merozoïte surface proteins 1 and 2 (MSP1 and MSP2; Table 1). A recrudescent infection was defined as one that showed match in size of at least one allele for both the *MSP1* and *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 recrudescent 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. χ^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 2
Treatment outcome and DHFR–DHPS mutations before treatment % (n/N)

		DHFR (n = 53)			DHPS (n = 51)		
	Wild-type	S108N	Double mutation (108-51)	Triple mutation (108-051-59)	A437G	S436A-A437G	A437G-K540E
TTF	12.5 (1/8)	25.0 (2/8)	25.0 (2/8)	37.5 (3/8)	25.0 (2/8)	75.0 (6/8)	0.0 (0/8)
New infection	53.8 (7/13)	23.0 (3/13)	0.0 (0/13)	23.0 (3/13)	7.7 (1/13)	84.6 (11/13)	0.0 (0/13)
ACPR	43.7 (14/32)	56.2 (18/32)	15.6 (5/32)	9.3 (3/32)	70.9 (22/31)	54.8 (17/31)	0.0 (0/31)

Values are percentasge (*n/N*).
TTF, total treatment failures; ACPR, adequate clinical and parasitological response.

higher among the patients having had a recurrent parasitemia (either recrudescence or new infection; 28.6% versus 9.3%), but the difference was not significant ($P = 0.1$; Table 2).

The *DHPS* mutation Gly-437 was observed in 78.4% (40/51) of samples analyzed, most of them (77.5%) being mixed infections with both the mutant and wild alleles (Ala-437). 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.[19] Despite its long use as a second-line drug, SP resistance in Burkina Faso was low, often < 1%.[1] However, patients had always been followed only up to day 14 after treatment, under-estimating the level of treatment failures.[10] 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.[1] Nevertheless, SP is still relatively efficacious in Burkina Faso[20–22] and in several other West African countries (treatment failure < 4%),[23,24] although a few areas of high resistance, particularly in forest areas, exist (parasitological resistance > 20%).[11,12] 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[25] 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,[26–28] 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.[22,29] 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.[30] Considering the importance of this mutation for the development of pyrimethamine resistance,[20,31,32] 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,[30,33] 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.[22,34] 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.[35–38] Although the triple mutation was observed only in a minority of treatment failures, this was observed in almost all samples col-

TABLE 3

DHFR pre-treatment/post-treatment and *DHPS* pre-treatment genotypes of *P. falciparum* isolates in treatment failures

	Pre-treatment					Post-treatment			
	DHFR			DHPS		DHFR			
Samples	108	51	51/59	436	437	108	51	51/59	MSP1/2 profile
N55	***Asn***	***Ile***	***Ile/Arg***	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	***R***
N62	Ser/***Asn***	Asn	Asn/Cys	Ser/***Ala***	Ala	***Asn***	***Ile***	—	***R***
N203	Ser/***Asn***	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	—	***R***
N434	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	***R***
N510	Ser/***Asn***	***Ile***	Asn/Cys	Ser/***Ala***	***Gly***	***Asn***	***Ile***	***Ile/Arg***	***R***
N551	***Asn***	—	***Ile/Arg***	Ser/***Ala***	Ala	***Asn***	—	***Ile/Arg***	***R***
N605	Ser/***Asn***	***Ile***	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	***R***
N638	***Asn***	—	***Ile/Arg***	Ser/***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	***R***
N100	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	NI
N246	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	—	NI
N283	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	NI
N318	Ser/***Asn***	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	NI
N351	Ser/***Asn***	Asn	Asn/Cys	Ser/***Ala***	***Gly***	***Asn***	***Ile***	***Ile/Arg***	NI
N362	Ser	Asn	Asn/Cys	Ser/***Ala***	***Gly***	***Asn***	—	***Ile/Arg***	NI
N386	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	NI
N441	Ser/***Asn***	Asn	***Ile/Arg***	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	NI
N505	Ser	Asn	Asn/Cys	***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	NI
N515	Ser/***Asn***	Asn	Asn/Cys	***Ala***	Ala/***Gly***	***Asn***	—	***Ile/Arg***	NI
N567	***Asn***	—	***Ile/Arg***	Ser	Ala	***Asn***	***Ile***	—	NI
N573	Ser	Asn	Asn/Cys	Ser/***Ala***	Ala/***Gly***	***Asn***	***Ile***	***Ile/Arg***	NI
N598	Ser/***Asn***	Asn	***Ile/Arg***	Ser	***Gly***	***Asn***	***Ile***	—	NI

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.[22–24,26–39] It is interesting to note that *DHFR* mutants were also selected in treatment failures classified later as new infections. Despite the limitation of the PCR technique used[16] 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 lower[22,30] 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.[36] 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.[34,39] We were unable to observe this; no sample carried the *DHPS* Glu-540 mutation,[29] 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.

Received July 20, 2005. Accepted for publication March 13, 2006.

Acknowledgments: The authors thank the parents of the children included in this study for participation. We also thank the health staff of Nanoro for collaboration. Many thanks to Saskia Decuypere for her suggestions and MR4 for providing the controls.

Financial support: We thank the International Atomic Energy Agency (IAEA) for its financial support for the field study and the Belgium cooperation for supporting the laboratory work through a training grant for Halidou Tinto. The FRET assay was supported by the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO).

Authors' addresses: Halidou Tinto, Chantal Van Overmeir, and Umberto D'Alessandro, Institut of Tropical Medicine, Nationalestraat 155 Antwerp B-2000, Belgium, E-mail: udalessandro@itg.be. Halidou Tinto, Issaka Zongo, Jean-Bosco Ouédraogo, and Tinga Robert Guiguemdé, Centre Muraz/Institut de Recherche en Sciences de la Santé, BP 153 Bobo Dioulasso, Burkina Faso, E-mail: tintohalidou@yahoo.fr. Halidou Tinto and Eric Van Marck, University of Antwerp, Faculty of Medicine, Universiteitsplein 1, B-2610 Antwerp, Belgium, E-mail: Eric.Van.Marck@uza.be.

Reprint requests: Umberto D'Alessandro, Institute of Tropical Medicine, Nationalestraat 155, Antwerp B-2000, Belgium. E-mail: udalessandro@itg.be (cc to tintohalidou@yahoo.fr).

REFERENCES

1. Tinto H, Zoungrana EB, Coulibaly SO, Ouédraogo JB, Traoré M, Guiguemdé TR, Van Marck E, D'Alessandro U, 2002. Chloroquine and sulphadoxine efficacy for uncomplicated malaria treatement and haematological recovery in children in Bobo Dioulasso, Burkina Faso during a 3-year period 1998–2000. *Trop Med Int Health 7:* 925–930.
2. Shretta R, Omumbo J, Rapuoda B, Snow RW, 2000. Using evidence to change antimalarial drug policy in Kenya. *Trop Med Int Health 5:* 755–764.
3. Bzik DJ, Li WB, Horii T, Inselburg J, 1987. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase–thymidylate synthase gene. *Proc Natl Acad Sci USA 84:* 8360–8364.
4. Biswas S, Escalante A, Chaiyaroj S, Angkasekwinai P, Lal AA, 2000. Prevalence of point mutations in the dihydrofolate reductase and dihydrofolate synthetase genes of *Plasmodium falciparum* isolates from India and Thailand: a molecular epidemiologic study. *Top Med Int Health 10:* 773–743.
5. Triglia T, Cowman AF, 1994. Primary structure and expression of the dihydropteroate synthetase gene of *Plasmodium falciparum*. *Proc Natl Acad Sci USA 91:* 7149–7153
6. Brooks DR, Wang P, Read M, Watkins WM, Sims PFG, Hyde JE, 1994. Sequence variation of the dihydromethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance sulfadoxine. *Eur J Biochem 224:* 397–405.
7. Sirima SB, Sawadogo R, Moran AC, Konate A, Diarra A, Yameogo M, Parise ME, Newman RD, 2003. Failure of a chloroquine chemoprophylaxis program to adequately prevent malaria during pregnancy in Koupela district, Burkina Faso. *Clin Infect Dis 36:* 1374–1382.
8. Kayentao K, Kodio M, Newman RD, Maiga H, Doumtabe D, Ongoiba A, Coulibaly D, Keita AS, Maiga B, Mungay M, Parise ME, Doumbo O, 2005. Comparison of intermittent preventive treatment with chemoprophylaxis for the prevention of malaria druing pregnancy in Mali. *J Inf Dis 191:* 109–116.
9. World Health Organization, 1996. *Assessment of Therapeutic Efficacy of Antimalarial Drugs for Uncomplicated Falciparum Malaria in Areas with Intense Transmission.* Geneva: World Health Organization.
10. Stepniewska K, Taylor WRJ, Mayxay M, Price R, Smithuis F, Guthmann JP, Barnes K, Myint HY, Adjuik M, Olliaro P, Pukrittayakamee S, Looareesuwan S, Hien TT, Farar J, Nosten F, Day NPJ, White NJ, 2004. *In vivo* assessment of drug efficacy against *Plasmodium falciparum* malaria: duration of follow-up. *Antimicrob A Chemother 11:* 4271–4280.
11. Driessen GJA, Van Kerkhoven S, Schouwenberg BJJ, Bonsu G, Verhave JP, 2002. Sulfadoxine/pyriméthamine: an appropriate first-line alternative for the treatement of uncomplicated *falciparum* malaria in Ghanaian children under 5 years of age. *Trop Med Int Health 7:* 577–583.
12. Henry MC, Niangue J, Kone M, 2002. Quel médicament pour traiter le paludisme simple quand la chloroquine devient inefficace dans l'ouest de la Côte d'Ivoire? *Med Trop 62:* 55–57.
13. World Health Organization, 2002. *Monitoring Antimalarial Résistance*. Geneva: World Health Organization.
14. Plowe CV, Djimdé A, Bouare M, Doumbo O, Wellems TE, 1995. Pyrimethamine and proguanil resistance-confering mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg 52:* 565–568.
15. Ranford-Cartwright LC, Johnston KL, Abdel-Muhsin AA, Khan B, Babiker HA, 2002. Critical comparison of molecular genotyping methods for detection of drug resistant *Plasmodium falciparum. Trans R Soc Trop Med Hyg 96:* 568–572.
16. Duraisingh MT, Curtis J, Warhurst DC, 1998. *Plasmodium falciparum:* Detection of polymorphisms in the Dihydrofolate re-

ductase and Dihydropteroate synthetase genes by PCR and restriction digestion. *Exp Parasitol 89:* 1–8.
17. Decuypere S, Elinck E, Van Overmeir C, Talisuna AO, D'Alessandro U, Dujardin JC, 2003. Pathogen genotyping in polyclonal infection: application of a fluorogenic polymerase-chain-reaction assay in malaria. *J Infect Dis 188:* 1245–1249.
18. Ranford-Cartwright LC, Taylor J, Umasunthar T, Taylor LH, Babiker HA, Lell B, Schmidt-Ott JR, Lehman LG, Walliker D, Kremsner PG, 1997. Molecular analysis of recrudescent parasite in a *Plasmodium falciparum* drug efficacy trial in Gabon. *Trans R Soc Trop Med Hyg 91:* 719–724.
19. Guiguemdé TR, Aouba A, Ouédraogo JB, Lamizana L, 1994. Ten-year surveillance of drug-resistant malaria in Burkina Faso (1982-1991). *Am J Trop Med Hyg 50:* 699–4.
20. Curtis J, Duraisingh MT, Trigg JK, Bwana H, Warhurst DC, Curtis CF, 1996. Direct evidence that asparagine at position 108 of the *Plasmodium falciparum* dihydrofolate reductase is involved in resistance to antifolate drugs in Tanzania. *Trans R Soc Trop Med Hyg 90:* 678–680.
21. Deloron P, Mayombo J, Le Cardinal A, Mezui-Me-Ndong J, Bruz-Baert C, Lekoukou F, Elissa N, 2000. Sulfadoxine-pyrimethamine for treatment of *Plasmodium falciparum* malaria in Gabonese children. *Trans R Soc Trop Med Hyg 94:* 188–190.
22. Plowe CV, Cortese JF, Djimde A, Nwanyanwu O, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avila JC, Cespedes JL, Carter D, Doumbo OK, 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J Infect Dis 176:* 1590–1596.
23. Diourté Y, Djimdé A, Doumbo OK, Sagara I, Coulibaly Y, Dicko A, Diallo M, Diakité M, Cortese JF, Plowe CV, 1999. Pyrimethamine-sulfadoxine efficacy and selection for mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase in Mali. *Am J Trop Med Hyg 60:* 475–478.
24. Von Seidlein L, Ouilligan P, Pinder M, et al., 2000. Efficacy of artesunate plus pyrimethamine-sulphadoxine for uncomplicated malaria in Gambian children: A double-blind, randomised, controlled trial. *Lancet 355:* 352–357.
25. Programme National de Lutte contre le Paludisme (PNLP): Rapport de la réunion de consensus sur le changement de politique de traitement du paludisme au Burkina Faso. Ouagadougou 21–23 Février 2005; 29 pp.
26. Foote SJ, Galatis D, Cowman AF, 1990. Amino acids in the dihydrofolate reductase- thymidilate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. *Proc Natl Acad Sci USA 87:* 3014–3017.
27. Peterson DS, Milhous WK, Wellems TE, 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum*. *Proc Natl Acad Sci USA 87:* 3018–3022.
28. Aubouy A, Jafari S, Huart V, Migot-Nabias F, Mayombo J, Durand R, Bakary M, Lebras J, Deloron P, 2003. DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. *J Antimicrob Chemother 52:* 43–49.
29. Wang P, Lee CS, Bayoumi R, Djimde A, Doumbo O, Swedberg G, Duc Dao L, Mshinda H, Tanner M, Watkins WM, Sims PFG, Hyde JE, 1997. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol Biochem Parasitol 89:* 161–177.
30. Mawili-Mboumba DP, Ekala MT, Lekoulou F, Ntoumi F, 2001. Molecular analysis of DHFR and DHPS genes in *P. falciparum* clinical isolates from the Haut-Ogooué region in Gabon. *Acta Trop 78:* 231–240.
31. Sirawaraporn W, Sathitkul S, Sirawaraporn R, Yuthavong Y, Santi DV, 1997. Antifolate-resistant mutants of Plasmodium falciparum dihydrofolate reductase. *Proc Natl Acad Sci USA 94:* 1124–1129.
32. Basco LK, Ringwald P, 1998. Molecular epidemiology of malaria in Yaounde, Cameroon I. Analysis of point mutations in the dihydrofolate reductase-thymidylate synthetase gene of *Plasmodium falciparum. Am J Trop Med Hyg 58:* 369–373.
33. Parzy D, Doerig C, Pradines B, Rico A, Fusai T, Doury JC, 1997. Proguanil resistance in *Plasmodium falciparum* African isolates: Assessment by mutation-specific polymerase chain reaction and in vitro susceptibility testing. *Am J Trop Med Hyg 57:* 646–650.
34. Talisuna AO, Nalunkuma-kazibwe A, Langi P, Mutabinga TK, Watkins WW, Van Mark E, Egwang TG, D'Alessandro U, 2004. Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated falciparum malaria. *Infect Genet Evol 4:* 321–327.
35. Watkins WM, Mberu EK, Winstanley PA, Plowe CV, 1997. The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses. *Parasitol Today 13:* 459–464.
36. Nzila AM, Mberu EK, Sulo J, Dayo H, Winstanley PA, Sibley CH, Watkins WM, 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum:* genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Antimicorb. Agent Chemother 44:* 991–996.
37. Basco LK, Tahar R, Keundjian, Ringwald P, 2000. Sequence variations in the genes encoding dihydropteroate synthase and dihydrofolate reductase and clinical response to sulfadoxine-pyrimethamine in patients with acute uncomplicated falciparum malaria. *J. Inf. Diseases 182:* 624–628.
38. Bates JS, Winstanley PA, Watkins WM, Alloueche A, Bwika J, Happi TC, Kremsner PG, Kublin JG, Premji Z, Sibley CH, 2004. Rare, highly pyrimethamine-resistant alleles of the *Plasmodium falciparum* dihydrofolate reductase gene from 5 African sites. *J Infect Dis 190:* 1783–1792.
39. Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam RAG, Rogerson SJ, Lescano AG, Molyneux ME, Winstanley PA, Chimpeni P, Taylor TE, Plow CV, 2002. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis 185:* 380–388.
40. Doumbo OK, Kayentao K, Djimde A, Cortese JF, Diourte Y, Konare A, Kublin JG, Plowe CV, 2000. Rapid selection of *Plasmodium falciparum* dihydrofolate reductase mutants by pyrimethamine prophylaxis. *J Infect Dis 182:* 993–996.