Substantial increase in mutations in the genes *pfdhfr* and *pfdhps* puts sulphadoxine–pyrimethamine-based intermittent preventive treatment for malaria at risk in Burkina Faso

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Abstract

OBJECTIVE Sulphadoxine–pyrimethamine (SP) is widely used as intermittent preventive treatment (IPT) for malaria in pregnant women in Sub-Saharan Africa. There are reports of wide-spread SP resistance in countries where SP had once been used as a first-line treatment. It is unclear whether the development of SP resistance also affects countries where SP is mainly used in the context of IPT, as is the case in Burkina Faso. To assess the efficacy of SP-based IPT, we monitored the prevalence of SP conferring genetic mutations in the genes *dhfr* and *dhps* in *Plasmodium falciparum* populations in a rural area of Burkina Faso over a period of 13 years.

METHODS Molecular epidemiological study consisted of six consecutive cross-sectional surveys of rainy and dry seasons (2009–2012). Data from the rainy season in 2000 served as a baseline. Mutations in *dhfr* and *dhps* associated with SP resistance were analysed by pyrosequencing in 861 parasite-positive samples.

RESULTS The prevalence of the SP resistance conferring triple *dhfr* mutation 51I, 59R, 108N increased from 1.3% in the rainy season of 2000 to 35.3% in 2009, and 54.3% in 2011 (P ≤ 0.001). Comparing rainy and dry seasons, we observed an increasing step-like pattern with higher prevalence of the *dhfr* triple mutant in the respective dry season compared with the preceding rainy season. The proportion of the *dhps* 437Gly mutation in the rainy season of 2000 was 53.2% and subsequently increased to 77.6% in 2009 (P ≤ 0.001).

CONCLUSION The increase in molecular markers linked with SP resistance jeopardises the efficacy of IPTp and the planned IPTi interventions in Burkina Faso, calling for careful monitoring of genotypic resistance markers and *in vivo* validation of IPT efficacy.

KEYWORDS drug resistance, IPT, IPTp, sulphadoxine–pyrimethamine, *pfdhfr*, *pfdhps*, *Plasmodium falciparum*, seasonal variability, Burkina Faso, pyrosequencing

Introduction

Children under age five and pregnant women bear the highest burden of malaria morbidity and mortality in sub-Saharan Africa. Malaria during pregnancy has been associated with fetal growth retardation, pre-term birth, low birth weight, increased perinatal mortality and maternal anaemia (Briand *et al.* 2007; Gosling *et al.* 2010). According to the World Health Organization (WHO), an estimated 35 million pregnant women and up to 26 million infants born per year are at risk of malaria in sub-Saharan Africa and would potentially benefit from intermittent preventive treatment (IPT), (WHO 2013).

Sulphadoxine–pyrimethamine (SP) is recommended as intermittent preventive treatment for all pregnant women (IPTp) at each scheduled antenatal care visit in moderate-to-high malaria transmission settings by the WHO and many Sub-Saharan African countries (Figure 1a) (WHO 2012). However, in 2007, only around 25% of the pregnant women at risk of malaria received one or more doses of IPTp (van Eijk *et al.* 2011). In many East African countries, SP was used as first-line treatment in the period after chloroquine lost its efficacy and before artemisinin combination therapy (ACT) became available (on average 6 years, range 1–13; see Table S1 in the appendix).

Resistance against SP is associated with two gene loci in *Plasmodium falciparum*, the protozoan parasite that causes the most severe form of malaria in humans. A change from serine to asparagine at amino acid position 108 in the dihydrofolate-reductase gene (*dhfr*) confers...
level of resistance (Peterson replacements at positions 51 and 59 further increase the resistance to pyrimethamine, and additional amino acid replacements at positions 51 and 59 further increase the level of resistance (Peterson et al. 1988). The mechanism implicated in resistance to sulphadoxine is more complex. The substitution of alanine by glycine at position 437 within the dihydropteroate synthase (dhps) was reported to confer resistance to sulphadoxine, with an additional mutation at position 540 contributing to the level of resistance (Wang et al. 1997). Mutations in both genes are strong predictors of SP treatment failure (Omar et al. 2001; Kublin et al. 2002).

Regular monitoring and reporting of antimalarial drug resistance are essential for treatment recommendations on the national or regional level (Wongsrichanalai et al. 2002). The majority of studies assessing SP resistance trends report data from East African countries, such as Tanzania, Kenya and Mozambique (Raman et al. 2010; Sridaran et al. 2010; Malisa et al. 2011; Iriemenam et al. 2012). The evidence base for West Africa is more limited (Sridaran et al. 2010). West African countries also have a different history of SP usage over time compared with East African countries. With the exception of Nigeria, SP was never introduced as a first-line drug in West Africa – moreover, chloroquine was directly replaced with an ACT (Figure 1b).

In Burkina Faso, chloroquine was recommended as first-line treatment for uncomplicated malaria until 2005, when the policy was officially changed to the ACT Artesunate-Amodiaquine (AS-AQ) (Kouyate et al. 2007) as the efficacy of chloroquine had decreased below 50% (Meissner et al. 2005). After this change, the use of SP most likely increased as the new ACT could not be provided country wide until 2007 (Tipke et al. 2009). Before 2005, SP was actually used as a second-line treatment (Tinto et al. 2002). In a clinical study of children conducted in 2002, late treatment failure was found in one of the 28 children and late parasitological failure (not PCR-corrected) in four of the 28 children (Muller et al. 2004). Since 2010, SP is provided exclusively in the context of IPTp in Burkina Faso.

We monitored the genotypic resistance trends for SP in the Nouna Health District in north-western Burkina Faso from 2009 to 2012 and can refer back to a baseline survey in 2000. Unlike previous studies, our community-based molecular epidemiological study included all age groups and was conducted in a rural setting. Previous studies were carried out in the areas of the two biggest cities Bobo-Dioulasso and Ouagadougou in Burkina Faso (Dokomajilar et al. 2006; Tinto et al. 2007). Furthermore, we collected data twice per year over several years, in the rainy as well as the dry season, to evaluate the influence of seasonality on the prevalence of SP resistance conferring genetic mutations.

Methods

Study area and study design

The community-based study was conducted in Bourasso village, Kossi district, north-western Burkina Faso, approximately 30 km from the district town Nouna. The Health and Demographic Surveillance System at the

Figure 1 Drug policies in Sub-Saharan Africa. (a) time of introduction of SP as IPTp; (b) first-line treatment policy changes over time in Sub-Saharan Africa.

![Image](image-url)
Nouna Health Research Centre currently has 90 000 people in 58 villages under observation. Malaria endemicity is markedly seasonal with a transmission peak at the end of the rainy season (June–October). The parasite prevalence was 35.4% at the end of the rainy season (October 2009–2011) and 30.7% at the end of the dry season (April 2010–2012) (Geiger et al. 2013).

The first cross-sectional survey of the village was carried out in the rainy season of the year 2000 (Stich et al. 2006) and served as a baseline. Starting in October 2009 until April 2012, a series of six surveys was conducted at the end of each rainy and dry season. The study design and the demographics are described in more detail elsewhere (Geiger et al. 2013). In short, a random list of all households was generated using the data from the Health and Demographic Surveillance System (HDSS), and all household members were invited to participate in the study until the target number of participants per survey was achieved (inclusion criterion above 6 months of age). After physical examination, thick and thin blood smears were prepared, fixed in methanol and stained with Giemsa to be analysed on the spot. A drop of blood was applied for the molecular analysis. Individuals with positive blood parasites were treated with AS-PM, Hain Lifesciences, Germany; or Whatman 3 MM chromatography paper, Brentfort, UK). The filter papers were air-dried, stored and taken to the Parasitology Unit at the Department of Infectious Diseases at Heidelberg University Medical Center, for the molecular analysis. Individuals with positive blood smears for Plasmodium parasites were treated with ASQ for free according to national guidelines. Ethical approval was obtained by the ethical review board of Heidelberg University Hospital and the Institutional Review Board in Nouna, Burkina Faso. Written informed consent was obtained from all participants.

Molecular analyses
The genomic DNA of 2009–2011 was extracted from filter papers using the Chelex-100 method (Plowe et al. 1995). The gDNA of the year 2000 had been extracted by the same method and then stored at −20 °C until it was analysed in 2010–2011 together with the samples collected more recently. Of 210 samples stored in 2000, 160 still could be amplified successfully.

The presence of Plasmodium falciparum DNA was confirmed by a species-specific nested PCR (Snounou et al. 1993). Samples that were positive for malaria parasites either by microscopy or PCR were further analysed by pyrosequencing.

The single-nucleotide polymorphisms in the pfdhfr and pfdhps genes were analysed by pyrosequencing according to the protocol by Zhou et al. (Zhou et al. 2006), with the exception that the concentration of deoxynucleoside triphosphates used for the PCR was increased to 0.5 mM. The laboratory strains HB3 (chloroquine sensitive) and Dd2 (chloroquine resistant) were used in each reaction as positive controls. A component mix without DNA was run as a negative control.

Statistical analysis
Statistical analysis was performed using STATA 12 (Stata Corporation, Duxbury, USA) and R 2.15 (R Foundation for Statistical Computing, Vienna, Austria). A t-test for proportions was used (‘prtest’ in STATA) to assess the difference between prevalence rates for mutant alleles. Binomial exact two-sided confidence intervals were calculated for Figure 2a–c. Graphs were created with Sigma-Plot 11 (Systat Software, Chicago, USA). The logistic regression for Figure 2a and statistical analysis for Figure 2b, 2c were performed using R.

Results
Resistance mediated by the dihydrofolate-reductase (dhfr) and dihydropterotate synthase (dhps) gene
The baseline study conducted in the rainy season of 2000 included 1561 individuals. Genomic DNA was available from 210 representative patient samples, of which 160 could be successfully amplified for the molecular analysis. In the subsequent surveys between October 2009 and April 2012, a total of 1767 patient samples were collected – of which 925 (52.3%) were positive for Plasmodium by microscopy or PCR. For the years 2009–2012, 873 samples were successfully genotyped for dhfr (51I, 59R, 108N) and 891 for dhps (436A, 437G). The numbers broken down by year and season are shown in Table S2 in the appendix.

To evaluate the increase in the prevalence of SP resistance conferring mutations over time, we compared the results from the rainy season in 2000 with the rainy seasons of 2009–2012. During the rainy season in the year 2000, the mutant dhfr alleles 51I, 59R and 108N were present in 4.4%, 4.3% and 7.6% of the population. By 2009, the prevalence of the mutant alleles had increased almost 10-fold to 50.2%, 45.8% and 51.4% of the cases (P < 0.001) and continued in the following years (2010–2011). The same tendency was observed for the dhfr triple mutant 51I, 59R and 108N that increased from 2.0% in 2000 to 35.3% in 2009 (P < 0.001), 45.8% in 2010 and 55.0% in 2011 (Figure 2a–d). The increasing overall trend between 2009 and 2012 can be confirmed via a fitted regression line with P = 0.022 for 51I, 0.046 for 59R, 0.009 for 108N and 0.020 for the triple mutation...
In a multivariate regression, the influence of the year (2009–2012) is significantly adjusted for sex, age group and parasite density (Table 1).

The mutant dhps genotypes 436A and 437G were already present at high frequencies in the year 2000 (81.2% and 53.2%, respectively). In the case of the mutant dhps 437G, the increase between the rainy seasons in the years 2000 (53.2%) and 2009 (77.6%; \( P < 0.001 \)) is still statistically significant (Figure 2e).

Variability of molecular markers for pf dhfr and pf dhps between rainy and dry seasons (2009–2012)

A stepwise pattern of increasing prevalence of resistance mutations was observed in the dhfr resistance mutations from one rainy season to the subsequent rainy season (Figure 2a–d) – for example, rainy season 2009, 35.3%, the following dry season 2010, 52.0%, rainy season 2010, 44.9%, the following next dry season 2011, 61.9%, etc. For the mutant alleles of pf dhps 436A and 437G, this pattern of variation between rainy and dry season was not observed (Figure 2e). Season, as a covariate, was statistically significantly associated with resistance only in the case of the dhfr 59R mutation (Table 1). The same pattern with increased resistance in the dry season, although less pronounced, was seen for dhfr 51I, 108N and the triple dhfr mutation (Figure 2a–d).

Discussion

We report a substantial increase in the prevalence of mutant alleles of dhfr 51, 59 and 108 between the years

![Figure 2](https://example.com/figure2.png)

**Figure 2** Prevalence of molecular markers of the genes *pf dhfr* and *pf dhps*, comparing rainy and dry seasons over time (2000 vs. 2009–2012). (a–c) prevalence of the *dhfr* 51I, 59R and 108N mutations; (d) prevalence of the *dhfr* triple mutant; (e) prevalence of mutant *dhps* 436A and 437G.
- 2000 and 2009–2011 (as evaluated in the respective rainy seasons). The prevalence of the dhfr triple mutant IRN rose from 1.96% in 2000 to 55.0% in 2011. This substantial increase seems to have taken place quite recently as the prevalence of the dhfr 51I, 59R and 108N triple mutant in 2009 only amounted to 35.3%. Furthermore, the prevalence of the mutation at position 108, which supposedly increases first in the process of developing resistance, is higher than that at the other locations.

- A similar rise of mutant alleles in dhfr was seen in south-eastern Tanzania after the first-line treatment recommendation was changed to SP in the year 2001. Within the first 5 years after introduction of SP as first-line treatment, the prevalence for mutant alleles in dhfr increased from 32% to 75%. This steep increase happened even though SP had been used as second-line treatment for 18 years previously in Tanzania (Malisa et al. 2011). Comparable findings were also reported from other East African countries, where due to the use of SP as first-line treatment, resistance markers remain prevalent at around 90–100% in the parasite populations (Menegon et al. 2009; Sridaran et al. 2010; Raman et al. 2011). Considering that Burkina Faso never officially recommended SP as a first-line treatment (Figure 1b), the increase in the prevalence of the dhfr triple mutation is surprising. The dhfr triple mutation has been linked with treatment failure (Omar et al. 2001). Efficacy studies of SP reported treatment failure rates of 12.9% in pregnant women in Burkina Faso in 2003 (Coulibaly et al. 2006) and of 12.3% in pregnant women in Ghana in 2007 (Tagbor et al. 2010). The increase in the dhfr triple mutation is alarming in the context of treating symptomatic malaria with SP, but does not directly translate into a loss of efficacy for preventive use. In the absence of alternative drugs to be used for IPT, the risk of reduced efficacy due to increased genotypic resistance needs to be further validated.

- In 2011, 59123 doses of SP were distributed to pregnant women in the Nouna health district (G. Compaoré, personal communication), which is equivalent to approximately 9850 women receiving a two course treatment (approximately 3% of the total district population). It remains to be discussed whether the use of SP in the context of IPTp can explain the observed increase in resistant dhfr and dhps mutants. Other possible contributing factors include the use of SP as second-line treatment before 2005, and the increased use of SP after chloroquine was discontinued in 2005 when the ACT

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Logistic regression on dhfr resistance mutations in Bourasso, Nouna, Burkina Faso</th>
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<tr>
<td></td>
<td>dhfr51I</td>
</tr>
<tr>
<td>Sex</td>
<td>Reference</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.98 (0.613)</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td></td>
</tr>
<tr>
<td>5–14</td>
<td>0.93 (0.288)</td>
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<tr>
<td>15–24</td>
<td>0.82 (0.043)</td>
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<tr>
<td>25–44</td>
<td>0.89 (0.180)</td>
</tr>
<tr>
<td>≥45</td>
<td>0.87 (0.346)</td>
</tr>
<tr>
<td>Season</td>
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</tr>
<tr>
<td>Rainy</td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>0.96 (0.468)</td>
</tr>
<tr>
<td>Year</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>0.95 (0.418)</td>
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<tr>
<td>2011</td>
<td>1.10 (0.090)</td>
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<tr>
<td>2012</td>
<td>1.22 (0.040)</td>
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<tr>
<td>Parasite density</td>
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<tr>
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<tr>
<td>1–1000</td>
<td>1.02 (0.732)</td>
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<tr>
<td>1001–2500</td>
<td>1.14 (0.080)</td>
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<tr>
<td>2501–5000</td>
<td>1.10 (0.300)</td>
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<tr>
<td>5001–10000</td>
<td>1.03 (0.736)</td>
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<tr>
<td>&gt;10000</td>
<td>0.96 (0.700)</td>
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Our findings are based on data from a rural area of north-western Burkina Faso, which is different from previous studies in more urban areas (Ouagadougou and Bobo-Dioulasso). We used a prospective study design with random sampling of households. We were able to cover time trends over a period of thirteen years including the dynamics between subsequent rainy and dry seasons between 2009 and 2012 – an approach that has rarely been applied in other studies in West Africa.
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