Tropical Medicine and International Health doi:10.1111/tmi.12305

VOLUME 19 NO 6 PP 690–697 JUNE 2014

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 ($P \leq 0.001$). 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 ($P \leq 0.001$).

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

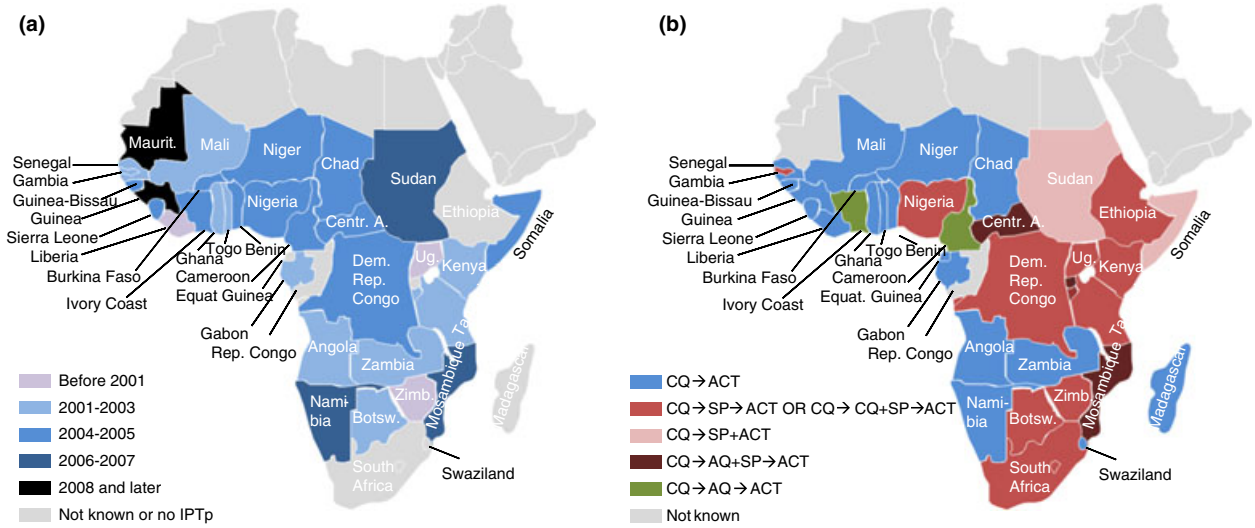

Figure 1 Drug policies in Sub-Saharan Africa. (a) time of introduction of SP as IPTp; (b) first-line treatment policy changes over time in Sub-Saharan Africa.

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

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 55.4% 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 to filter paper (either GenoCard™, 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 AS-AQ 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–e. Graphs were created with Sigma-Plot 11 (Systat Software, Chicago, USA). The logistic regression (Table 1) was performed using R.

Results

Resistance mediated by the dihydrofolate-reductase (dhfr) and dihydropteroate 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

(appendix, Figure S1). 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 *pfdhfr* and *pfdhps* 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 pfdhps 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

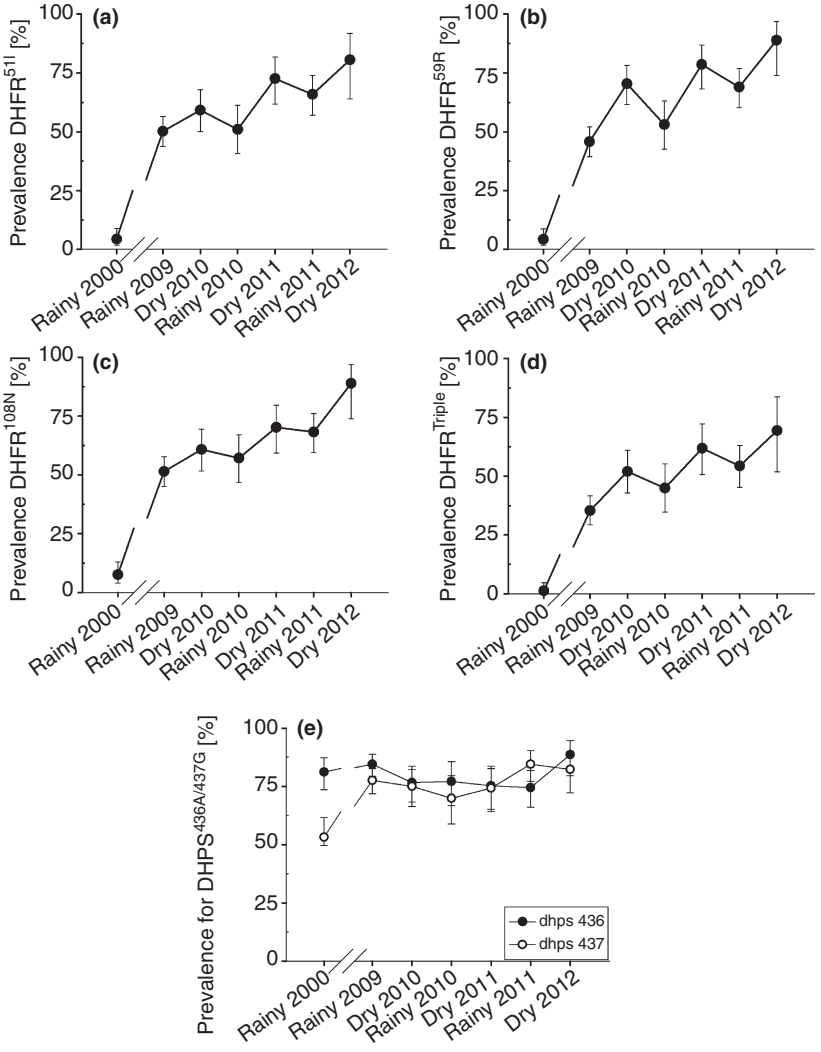

Figure 2 Prevalence of molecular markers of the genes *pfdhfr* and *pfdhps*, comparing rainy and dry seasons over time (2000 *vs.* 2009–2012). (a–c) prevalence of the *dhfr* 51I, 59R and 108N mutations: (d) prevalence of the *dhfr* triple mutant; (e) prevalence of mutant *dhps* 436A and 437G.

Table 1 Logistic regression on *dhfr* resistance mutations in Bourasso, Nouna, Burkina Faso

	Multivariate OR (*P*-value)			
	dhfr51I	dhfr59R	dhfr108N	dhfr triple mutation
Sex				
Female	Reference	Reference	Reference	Reference
Male	0.98 (0.613)	1.00 (0.967)	1.07 (0.105)	0.99 (0.914)
Age group				
0–4	Reference	Reference	Reference	Reference
5–14	0.93 (0.288)	0.92 (0.165)	0.93 (0.238)	0.92 (0.254)
15–24	0.82 (0.043)	0.85 (0.093)	0.88 (0.216)	0.87 (0.192)
25–44	0.89 (0.180)	0.99 (0.895)	0.93 (0.454)	0.99 (0.918)
≥45	0.87 (0.346)	0.97 (0.841)	1.19 (0.258)	0.89 (0.466)
Season				
Rainy	Reference	Reference	Reference	Reference
Dry	0.96 (0.468)	0.89 (0.025)	0.99 (0.874)	0.95 (0.329)
Year				
2009	Reference	Reference	Reference	Reference
2010	0.95 (0.418)	1.02 (0.784)	1.03 (0.688)	1.03 (0.649)
2011	1.10 (0.090)	1.17 (0.009)	1.11 (0.087)	1.13 (0.050)
2012	1.22 (0.040)	1.26 (0.023)	1.43 (0.002)	1.19 (0.133)
Parasite density				
0	Reference	Reference	Reference	Reference
1–1000	1.02 (0.732)	1.01 (0.828)	1.00 (0.971)	1.06 (0.380)
1001–2500	1.14 (0.080)	1.01 (0.713)	0.97 (0.743)	1.12 (0.161)
2501–5000	1.10 (0.300)	1.09 (0.330)	1.00 (0.963)	1.16 (0.105)
5001–10000	1.03 (0.736)	1.11 (0.266)	0.98 (0.874)	1.09 (0.385)
>10000	0.96 (0.700)	1.00 (0.943)	0.98 (0.810)	1.04 (0.740)

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 woman 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

was not yet readily available (Tinto *et al.* 2007; Tipke *et al.* 2008). SP might still be used by the population as it is available in the private market in Burkina Faso (A. Sie, personal communication).

It is worth noting that the prevalence of the *dhps* mutants was already elevated in the baseline data of the year 2000 (Figure 1e), while the prevalence of the *dhfr* mutants was still comparably low (Figure 1a–d). A similar pattern had been reported by other researchers in Burkina Faso (Diallo *et al.* 2007; Tinto *et al.* 2007) and the use of the widespread antibiotic drug cotrimoxazole was proposed as a possible explanation (Diallo *et al.* 2007). Cotrimoxazole shares the same mechanism of action with SP, although there is evidence that the cross-resistance is incomplete (Manyando *et al.* 2013).

We observed a step-like pattern of increasing prevalence of the resistant *dhfr* genotype from one rainy to the next dry season (Figure 2d). A study from Sudan comparing dry and rainy seasons between 1998 and 2001, reported a similar pattern for *dhfr* with a rise in the prevalence for mutant alleles by approximately 30% from one rainy to the next dry season (Abdel-Muhsin *et al.* 2004; Babiker *et al.* 2005) and attributed this finding to an elevated drug pressure during the high transmission season extending into the subsequent dry season. In Sudan, the sampling took place at the beginning of the respective dry season. The data obtained in our study show that the prevalence of *dhfr* resistance mutations at the end of the dry seasons was still significantly increased compared with the preceding rainy season. An alternative explanation involves the fitness cost associated with drug resistance mutations (Osman *et al.* 2007). Parasites with a lower propensity to cause symptomatic disease due to slower multiplication rates (associated with resistance) could have an evolutionary advantage in the dry season. The variability observed between rainy and dry season for *dhfr* is robust, as the same pattern could be observed for three *dhfr* mutations and the triple mutant. However, this pattern could not be observed for *dhps* – either because the prevalence of resistance mutations was already at a high level to start with, or because the selection for resistant alleles is caused by different mechanisms in these two genes.

Our findings are based on data from a rural area of north-western Bukina 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 (Sridaran *et al.* 2010; Amor *et al.* 2012). We also included both children and adults regardless of the presence of symptoms suggestive of clinical malaria.

In summary, the results of our study indicate that the efficacy of SP for IPTp might be compromised because of parasites carrying mutant *dhfr* and *dhps* alleles associated with resistance to SP. Pregnant women and their unborn children are more vulnerable to malaria should the efficacy of IPTp be decreased. Thus, careful monitoring of genotypic resistance markers and *in vivo* validation of IPT efficacy is warranted.

Acknowledgements

The study and CG were supported by the German Research Foundation (DFG), PhD programme GRK 793 'Epidemiology of communicable and chronic, non-communicable diseases and their interrelationships' and the SFB 544 'Control of Tropical Infectious Diseases'. We express our sincere gratitude to the study team of the Nouna Health Research Centre (CRSN) and to all the inhabitants of Bourasso village. We thank Dr. Konrad Bode (Heidelberg University Hospital, Department of Infectious Diseases, Microbiology) for the use of the Pyromark Q96 Device for Pyrosequencing, and Hani Kartini Agustar (Heidelberg University Hospital, Department of Infectious Diseases, Parasitology) for her help with the gDNA extraction.

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