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Malaria in the Nuba Mountains of Sudan: baseline genotypic resistance and efficacy of the artesunate plus sulfadoxine–pyrimethamine and artesunate plus amodiaquine combinations

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Summary Both northern and southern Sudan are deploying artemisinin-based combinations against uncomplicated *Plasmodium falciparum* malaria (artesunate + sulfadoxine–pyrimethamine [AS + SP] in the north, artesunate + amodiaquine [AS + AQ] in the south). In 2003, we tested the efficacy of 3 day AS + SP and AS + AQ regimens in vivo in the isolated, seasonally endemic Nuba Mountains region (the first study of AS combinations in southern Sudan). We also analysed pre-treatment blood samples for mutations at the *P. falciparum* chloroquine transporter (*Pfcrt*) gene (associated with CQ resistance), and at the dihydrofolate reductase (*Dhfr*) gene (associated with pyrimethamine resistance). Among 161 randomized children under 5 years, PCR-corrected cure rates after 28 days were 91.2% (52/57, 95% CI 80.7–97.1) for AS + SP and 92.7% (51/55, 95% CI 82.4–98.0) for AS + AQ, with equally rapid parasite and fever clearance. The *Pfcrt* K76T mutation occurred in 90.0% (144/160) of infections, suggesting CQ would work poorly in this region. Overall, 82.5% (132/160) carried mutations at *Dhfr* (N51I, C59R or S108N, but not I164L), but triple mutants (more predictive of in vivo SP failure) were rare (3.1%). CQ use should be rapidly discontinued in this region. SP resistance may propagate rapidly, and AS + AQ is likely to be a better long-term option, provided AQ use is limited to the combination.

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1. Introduction

The Nuba Mountains region of central Sudan has an estimated population of 1 million, and has been severely affected by warfare. Since the early 1990s, as a result of heavy fighting, its population has suffered severe nutritional and health crises. Most of the area is currently aligned with the southern-based Sudan People's Liberation Movement (SPLM), but is surrounded by government-controlled territory. A peace settlement has recently been negotiated.

In both government (northern) and SPLM (southern) areas of Sudan, malaria, caused largely by *Plasmodium falciparum*, has until present mainly been treated with chloroquine (CQ). However, falling CQ efficacy has been well documented in northern Sudan (Federal Ministry of Health, 2003), and more recently in southern Sudan (Bachy et al., 2003; Stivanello et al., 2004; van den Broek et al., 2003). Sulfadoxine–pyrimethamine (SP) appears to retain efficacy in most regions of Sudan (Anderson et al., 2003; Bachy et al., 2003; van den Broek et al., 2003), but failure above 50% has been reported on the border with Uganda (Stivanello et al., 2004).

At the time of this study, a move away from CQ and monotherapies, and towards artemisinin-based combinations, was underway, and artesunate + SP (AS + SP) had been selected as first-line antimalarial therapy in government-controlled (northern) Sudan. In SPLM-controlled (southern) Sudan, a recent consensus meeting indicated the combination artesunate + amodiaquine (AS + AQ) as replacement for CQ. Both AS + SP and AS + AQ are recommended artemisinin-based combinations (WHO, 2003a), but high baseline SP or AQ resistance may make them unsuitable (Adjuik et al., 2004).

No data on antimalarial efficacy were available for the Nuba Mountains, and, throughout Sudan (the largest country in Africa, and perhaps among the most malariologically diverse), the evidence basis for selecting AS + SP or AS + AQ relied on a mere handful of studies. In order to identify a suitable artemisinin-based combination for this region, and to add to scarce knowledge of antimalarial efficacy in Sudan, we carried out a randomized, open-label trial of AS + SP and AS + AQ. We also analysed patients' pre-treatment samples for genotypic mutations associated with resistance to CQ or SP.

2. Materials and methods

The trial took place at the basic health care centre in Limun village of Ngorban County (population 22 000, altitude 700 m), operated by the Dutch section of Médecins Sans Frontières (MSF) since 2001. This area was almost completely isolated during the war (1983–2002) but is now accessible by plane or on foot as a result of a ceasefire. Here, malaria transmission is markedly seasonal: in 2003, 93% of cases (all slide- or rapid-test confirmed) occurred between July and December; 69% of inpatient admissions during this period were malaria-related and, of these, 63% were among children under 5 years, suggesting that immunity is somewhat developed among adults. *Plasmodium falciparum* is the dominant species (99%). At the time of the study, the closest informal source of antimalarials aside from the MSF clinic was a market located 2 days' walk from Limun, which reportedly sold only CQ.

The trial was approved by the Sudan Relief and Rehabilitation Council, the Nuba Relief, Rehabilitation and Development Organisation, and the Ethical Review Board of MSF. We applied the latest WHO efficacy assessment guidelines (WHO, 2003b). Accordingly, we included children 6–59 months old (screened from the outpatient queue) if they had a *P. falciparum* monoinfection (density 2000–200 000/μl), fever (axillary temperature ≥37.5 °C), weight ≥5 kg, no signs of severe malaria, no concomitant febrile conditions (apart from mild upper respiratory tract infection of presumed viral origin), no reported hypersensitivity to study drugs, and if their guardians provided written informed consent. We aimed for a sample size of 80 in both arms, enough to detect an efficacy of 95% (alpha 5%, precision 5%) with a projected loss of 10% due to non-analysable endpoints.

Arm allocation was randomized by sealed envelopes. Children received either three daily doses of AS (4 mg/kg/d; Arsumax®, Sanofi) plus a single dose of SP (1.25 mg/kg sulfadoxine and 25 mg/kg pyrimethamine; Fansidar®, Roche), or AS as above plus three daily doses of AQ (10 mg/kg/d; Camoquin®, Parke-Davis). We observed all drug intake (days 0, 1, 2), and reassessed children both clinically and parasitologically on days 2, 3, 7, 14, 21 and 28, or any other day in case of illness. We assigned children's therapeutic endpoints according to the WHO classification (Table 1). In case of failure, children received a 7 day course of quinine. We withheld rescue treatment from children with asymptomatic parasitaemia between days 4 and 27 but monitored these children daily.

After thick and thin blood film staining with 10% Giemsa, study slides were read independently by two microscopists, each blinded to the other's result. We reported asexual density as the average of both readings, and resolved positive-negative or

Table 1 Efficacy endpoint classification (WHO, 2003b)

Endpoint	Criteria
Early treatment failure (ETF) (days 1–3)	Parasite density on day 2 > day 0
	Parasite density on day 3 ≥25% of day 0
	Fever in the presence of parasites on day 3
	Severe malaria in the presence of parasites on days 1–3
Late clinical failure (LCF) (days 4–28)	Fever in the presence of parasites on days 4–28
	Severe malaria in the presence of parasites on days 4–28
Late parasitological failure (LPF) (day 28)	Parasites without fever on day 28
Adequate clinical and parasitological response (ACPR) (day 28)	Follow-up completed without meeting any of the above criteria

species discordances by mutual review of the slide in question. For quality control purposes, we sent 100 randomly selected slides to the Kenya Medical Research Institute, where only one re-reading result (1%) caused a change in efficacy endpoint. On day 0 (pre-treatment), and the day of failure endpoint if applicable, we collected a sample of capillary blood on Isocode kits (Schleicher & Schuell BioScience Inc., Dassel, Germany) for PCR analysis. On all pre-treatment samples, we analysed mutations at the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene (K76T, associated with CQ resistance), and at the dihydrofolate reductase (*Dhfr*) gene (N51I, C59R, S108N and I164L, associated with pyrimethamine resistance). Briefly, we detected these mutations through a two-step nested PCR reaction amplifying the target loci, followed by mutation-specific RFLP analysis, as previously described (Djimde et al., 2001a, 2001b; Duraisingh et al., 1998). Furthermore, among patients with a failure endpoint, we compared the pre- and post-treatment genotypes of merozoite surface proteins 1 and 2 (*msp-1* and *msp-2*) to establish whether failure was due to true recrudescence or a novel infection (Snounou et al., 1999). We classified cases as recrudescent or novel infections based on a previously published method (Basco and Ringwald, 2000). We excluded reinfections or indeterminate PCR results from the analysis when calculating efficacy.

We double-entered data on Microsoft Excel®, verified them on EpiData 3.0 (EpiData Association, Odense, Denmark) and analysed them on EpiInfo 6.04 (CDC, Atlanta, GA, USA), using Fisher's exact test for categorical variables and Student's *t* test for continuous variables.

3. Results

Between 10 September and 10 November 2003, we screened 307 febrile children, of whom 200 had a *P. falciparum* infection, and 161 met inclusion criteria. Baseline characteristics in the two arms were similar (Table 2), although in the AS + AQ arm males were more frequent ($P = 0.09$). All 80 children in the

Table 2 Baseline characteristics of included patients

Characteristic		AS + SP ($n = 81$)	AS + AQ ($n = 80$)
Age (months)	Mean (IQR)[a]	30 (16–36)	31 (15–43)
Gender	Ratio M/F	1.0 (41/40)	1.8 (51/29)
Asexual parasitaemia (/μl)	Geometric mean (IQR)[a]	22 757 (9800–48 279)	21 190 (8202–51 351)
Gametocytes on day 0	*n* (%)	4 (4.9)	4 (5.0)
Temperature (°C)	Mean (range)	38.9 (37.5–40.7)	39.0 (37.5–40.6)
Haemoglobin (g/dl)			
Moderate anaemia (<8)	*n* (%)	13 (16.0)	11 (13.8)
Mild anaemia (8–11)	*n* (%)	42 (51.9)	39 (48.8)
Absence of anaemia (≥11)	*n* (%)	26 (32.1)	30 (37.5)
Middle upper arm circumference (mm)	Mean (SD)	142.2 (12.6)	142.7 (9.4)

[a] IQR: interquartile range.

Table 3 Mutations in codons 51, 59 and 108 of *Dhfr* gene before treatment

Dhfr genotype	Mutation			Frequency	
	N51I	C59R	S108N	*n*	%
No mutations	No	No	No	28	17.5
Single mutations	Yes	No	No	0	0.0
	No	Yes	No	0	0.0
	No	No	Yes	90	56.3
Double mutations	Yes	Yes	No	0	0.0
	Yes	No	Yes	6	3.8
	No	Yes	Yes	31	19.3
Triple mutations	Yes	Yes	Yes	5	3.1
Total				160	100.0

AS + AQ arm completed follow-up; in the AS + SP arm one was lost to follow-up and one withdrawn due to repeated dose vomiting. No significant adverse events were reported.

Overall, 90.0% (144/160, 95% CI 84.3–94.2) of pre-treatment infections carried *Pfcrt* K76T mutations, although 17 of these were mixed with a wild-type genotype. Similarly, 82.5% (132/160, 95% CI 75.7–88.0) of patients carried one or more mutations at *Dhfr*, but none of these were I164L. N51I and C59R mutations occurred exclusively in the presence of S108N (Table 3), but this association was significant only for C59R ($P = 0.214$ for N51I; $P = 0.002$ for C59R). Triple *Dhfr* mutations were infrequent.

In the in vivo follow-up, 96.3% (78/81) and 98.8% (79/80) of children became afebrile by day 2 in the AS + SP and AS + AQ arms respectively, whereas by day 3 98.8% (80/81) and 98.9% (79/80) were slide-negative. Gametocyte carriage remained low throughout follow-up: 5.1% (4/79) on day 14 and 2.9% (2/70) on day 28 in the AS + SP arm; 3.8% (3/80) on day 14 and 2.9% (2/68) on day 28 in the AS + AQ arm. Excluding one novel infection (see below), gametocyte carriage post-treatment occurred only among children who also carried gametocytes on day 0.

A considerable number of late failures occurred, but only nine were PCR-confirmed recrudescences (Table 4): five in the AS + SP arm (three *Dhfr* S108N single mutants, and two C59R and S108N double mutants), and four in the AS + AQ arm (all *Pfcrt* K76T mutants). In both arms, no PCR result was available for eight cases, and the remainder were novel infections, slightly less frequent in the AS + SP arm (14) than in the AS + AQ arm (17). One PCR-confirmed recrudescence (LCF in the AS + SP arm) occurred on day 14; three more (two AS + SP LCF, one AS + AQ LCF) occurred between days 15 and 21, and the remainder (two AS + AQ LPF) on day 28. PCR-adjusted efficacy was thus above 90% in both arms (Table 4), with a non-significant difference.

4. Discussion

This is the first study of malaria in the Nuba Mountains region, and the first trial of AS combinations in southern Sudanese territory. War-affected Sudanese populations have poor access to health care, and malaria control has been particularly neglected. Yet, in our setting, as elsewhere in Sudan, *P. falciparum* malaria is the main health problem for nearly half the year.

Mutations associated with CQ and SP resistance are surprisingly frequent in the Nuba Mountains, despite isolation and apparently limited drug use (apart from 3 years of MSF intervention with CQ, SP and then AS + SP). This finding may have explanations aside from mere drug pressure. Firstly,

Table 4 Efficacy at day 28 after PCR adjustment

Arm	Endpoint at day 28 (PCR-confirmed recrudescences shown in parentheses)				Efficacy		
	ETF	LCF	LPF	ACPR	*n*	%	95% CI
AS + SP	0	15 (4)	12 (1)	52	52/57	91.2	80.7–97.1
AS + AQ	0	17 (2)	12 (2)	51	51/55	92.7	82.4–98.0

a recent influx of returnees from the Khartoum area may have introduced previously absent mutant strains. Secondly, in such a markedly seasonal transmission setting, the ecological balance of strains could be heavily modulated by genetic drift (Anderson et al., 2003). As shown elsewhere in Sudan (Abdel-Muhsin et al., 2004), resistant mutants may survive more easily in the dry season, and could therefore be amplified in the subsequent seasonal peak.

Operationally, our findings imply that: (1) CQ would work very poorly here and should be discarded as soon as possible (Djimde et al., 2001a, 2001b); and (2) SP resistance mutations, already present, could rapidly become dominant, especially if SP monotherapy is more widely used (Abdel-Muhsin et al., 2004; Anderson et al., 2003). Multiple (and particularly triple) mutations at *Dhfr* are reasonably predictive of in vivo SP failure (Kublin et al., 2002). Presently these are rare, implying that the drug remains useful in Nuba. The high AS + SP efficacy found in our in vivo trial corroborates this prediction, as high baseline SP resistance should also have resulted in a disappointing AS + SP cure rate (Priotto et al., 2003). In this study we did not analyse sulfadoxine resistance mutations at the dihydropteroate synthetase (*Dhps*) gene. These seem less strongly associated with SP failure (Kublin et al., 2002), although some studies have suggested that they may play an important role, especially in individuals with low immunity (Khalil et al., 2002). As the case may be, such an analysis would have provided a better overall prediction of SP resistance in Nuba.

These data only reflect mutation prevalences among children with malaria, who may not be representative of the general population. Nevertheless, our findings are corroborated by studies with different designs from both northern and southern Sudan (Abdel-Muhsin et al., 2003; Anderson et al., 2003; Babiker et al., 2001; van den Broek et al., 2003; Epicentre unpublished data from Marial Lou, Bahr el Ghazal).

Our in vivo trial shows that both AS + SP and AS + AQ are efficacious options for uncomplicated malaria in Nuba Mountains, providing rapid and sustained fever and parasite clearance, as well as gametocyte suppression (gametocyte carriage post-treatment was associated with that at baseline, as in Price et al., 1999). Our findings thus confirm previous experience with AS combinations, including a recent study in Malakal, Upper Nile State, 300 km from our site (Adjuik et al., 2004; van den Broek et al., 2005). Despite efficacious treatment, however, 21% of children experienced a novel infection within a month of follow-up. This suggests that, during the malaria season in Nuba, appropriate treatment should be coupled with an aggressive case-finding approach, and ideally be supplemented by vector control measures. The high reinfection rates also resulted in lower than expected sample sizes, given that novel infections were excluded from the analysis. Trials using PCR correction in high transmission settings (especially if designed to detect superiority), and adhering to present WHO guidelines, should therefore anticipate an effective sample size loss of at least 25–30%. Due to expected difficulties in recruiting a sufficient number of children in this remote setting, we did not attempt a comparative trial (a very large sample size would probably have been required for this, as both AS + SP and AS + AQ were expected to be highly efficacious). Nevertheless, such a design would have better informed the choice between the two combinations.

Recently, AS + AQ has been selected as replacement for CQ in SPLM (southern Sudan) areas. A move to AS + AQ would bring the Nuba Mountains in line with this choice, although it should be recognized that, depending on the outcome of currently ongoing peace negotiations, a joint government/SPLM health policy would suddenly be confronted with conflicting antimalarial treatment guidelines (AS + SP in the north, AS + AQ in the south). Of the two combinations, AS + AQ is likely to have a longer therapeutic lifespan given global SP resistance trends (Roper et al., 2004). AQ resistance seems to develop more slowly according to in vivo observations (East African Network for Monitoring Antimalarial Treatment, 2003). The genotypic basis of AQ resistance, however, is yet to be elucidated, representing a crucial missing element in decisions about which artemisinin-based combinations can be deployed in any given setting. The *Pfcrt* K76T mutation has previously been associated with AQ failure in vivo (Ochong et al., 2003) but, in our trial, even a 90% prevalence of K76T mutants failed to significantly affect AS + AQ efficacy.

It is nevertheless clear that any future use of AQ, in Sudan as elsewhere, should occur strictly in a combination regimen, preferably prescribed in age-specific blister packs. For the Nuba Mountains region, we recommend an urgent policy shift from CQ, a now virtually useless drug, to AS + AQ. Aid agencies involved in post-war relief should actively participate in this process. Donor assistance is essential to make the combination accessible to this population. More generally, malaria control should be a major health priority in the Nuba Mountains and, indeed, in all war-affected areas of Sudan.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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