Emerging Problems in Infectious Diseases

The effect of Point Mutations in Dihydrofolate reductase genes and the Multidrug resistance gene 1-86 on treatment of falciparum malaria in Sudan

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Abstract

Background: This study investigated the prevalence of antimalarial drug resistance markers in *P. falciparum* isolates, involving the detection of mutations at the *mdr1-86*, which associates with amodiaquine resistance, and *dhfr* mutations associated with SP resistances.

Methods: The dot-blot/probe hybridization was used to determine multidrug resistance (*mdr1-86*) and assess the correlation of Amodiaquine (AQ) resistance and PCR/ RFLP was used to determine dihydrofolate reductase (*dhfr)* baseline resistance to Sulphadoxine- Pyrimethamine (SP) resistance in the Nubian region of Southern Sudan.

A randomized open-label trial of Artesunate (AS) + SP and AQ + SP was conducted in children younger than five years. Molecular analysis of the samples was performed to provide a baseline estimate of allele prevalences.

Results: Baseline allele prevalence of the *mdr1*-86 locus in the AS + AQ was successful for 80 isolates: 71(8.11%) carried parasites harbouring the *mdr1*-86 Tyr resistance allele, while 7 (89.19%) carried *mdr1*-86 Asn sensitivity allele, and 2 (2.7%) were of mixed infection, having both resistance and wild type allele. Overall, the prevalence of the *dhfr* point mutation, codon 51, 59 and 108: 82.5% (132/160) carried mutations at *dhfr* (N51I, C59R or S108N), but triple mutants were rare (3.1%) in the AS + SP arm.

Conclusion: The results show that mutations present in *dhfr* and *mdr1*-86 have a significant effect on the type of treatment following SP and AQ chemotherapy. SP resistance may spread rapidly, and AS + AQ is likely a better option, provided AQ use is restricted to the combination.

Key words: Antimalarial drugs, *P. falciparum, dhfr*, *mdr-1*, dot-blot hybridisation technique, PCR/RFLP

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Introduction

Malaria is a public health problem with an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under five years of age. In 2008, 109 countries were endemic for malaria, 45 within Africa [1]. In Africa, approximately 10% of hospital admissions and 20-30% of outpatient visits are due to malaria [2]. One of the major contributors to the increase of disease burden is drug resistance, which has threatened the use of chemotherapy as a weapon against *falciparum* malaria. Several countries have already abandoned Chloroquine (CQ) and Sulfadoxine-Pyrimethamine (SP) (Fansidar®) as monotherapy in favour of Artemisinin combination therapy because of the emergence and worsening rise of CQ and SP resistance [3-6]. However, CQ was being used in Sudan for quite a long time as the second-line treatment for malaria, and SP as the first-line drug for malaria treatment owing to the increasing number of CQ treatment failures. Despite changing treatment policies in favour of SP, the development and rapid spread of SP resistant strains [7,8] has threatened its use and better treatment regimens are being sought to replace SP. Combination therapy (CT), especially Artemisinin based combination therapy (ACT), presently seems to be the best option. The emergence and spread of *Plasmodium falciparum* resistance to antimalarial drugs is now one of the greatest challenges facing the global effort to control malaria. In Africa, until recently, there has been a reliance on the cheap drugs chloroquine and sulfadoxine-pyrimethamine because they were the only safe drugs to prescribe to children and pregnant women. To protect drugs from resistance, there is now clear evidence that combining them can improve their efficacy without increasing their toxicity [9] and with the development of highly effective artemisinin derivatives, there is renewed hope for the treatment of malaria in the form of artemisinin-based combination therapy (ACT). ACTs protect the

individual drugs from resistance by relying on the principle of combining two drugs with different mechanisms of action [10]. The fast-acting artemisinin derivative rapidly clears the main parasite load within the few hours that it remains at therapeutic levels and thus reduces subsequent gametocyte carriage [11], while the partner drug, which is generally longer lasting, remains to clear the rest of the parasites.

More than 90% of malaria cases and the great majority of malaria deaths occur in tropical Africa mainly due to *Plasmodium falciparum* infections. Every year in tropical countries, *P. Falciparum*, the most dangerous of the four species affecting man, causes almost 270 million deaths in Africa [12-14], In Sudan 100% of the population is at risk with 55% at endemic risk. There is therefore need to asses the mutation profiles of the parasites resistant to Sulfadoxine/ Pyrimethamine and Amodiaquine drugs from the endemic area to enhance the prediction of treatment outcomes in a given treatment, including the use of Artemisinin based Combination Therapy (ACT). The Nuba Mountains region of central Sudan has an estimated population of one million, and has been severely affected by warfare. Since the 1990s, as a result of heavy fighting, its population has suffered severe damage, especially in form of nutritional and health crises.

In response to chloroquine resistance, in several areas of Africa first-line antimalarial drug of choice has passed from sulfadoxine-pyrimethamine (S-P) to artemisinin based combination. Since specific mutations in the dihydrofolate reductase (*dhfr*) genes on chromosome 4 of *Plasmodium falciparum* have been associated with resistance to these drugs *in vitro* [15-19], it is hoped that the introduction of molecular screening methods will make surveillance of the extent of resistance as well as the choice of suitable first-line treatment regimens easier. Therefore, pre-treatment screening for dhfr point mutation in *Plasmodium falciparum* infection can be used to predict SP treatment failure [20].

In both government (northern) and (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 (21), and more recently in Southern Sudan [20-22]. Sulfadoxine-Pyrimethamine (SP) appears to retain efficacy in most regions of Sudan [22], but failure above 50% has been reported on the border with Uganda [23].

Materials and methods

In this study, molecular techniques (specifically, PCR followed by Restriction Fragment Length Polymorphism (RFLP) and Dot-Blot Hybridisation) are employed to determine prevalence of point mutation conferring resistance to Amodiaquine and Fansidar. Due to the magnitude of the disease, the dangers posed by the development of drug resistant strains, and the promise of Artemisinin combination therapy, it is important to evaluate the prevalence of resistance in a combination regimen and determine which combination is a viable option for treating uncomplicated *falciparum* malaria at Nuba. A total of 160 field isolate samples and four positive controls were analyzed. Eighty of the samples were tested in the AS/AQ treatment arm and the other 80 were treated with the AS/SP combination. The investigation involved extracting the DNA of *P. falciparum* from blood-spotted Whatman filter papers, then amplifying the extracted DNA using the PCR method and finally analysing the amplified products using a radioisotopic method to determine the prevalence of *mdr1- 86* and restriction enzymes using the RFLP technique to determine the *dhfr 51, 59,108.*

Study site

The study was conducted at the *Medecins Sans Frontieres* (MSF) field station in Ngorban Country, Nuba Mountains, Southern Sudan. The area has one rain season, mainly in June to July. Annual rainfall ranges from 650 mm to 1100 mm. The area experiences high temperatures throughout the year ranging from 28 to 35 degrees Celsius. During the rainy season, there are ample breeding sites for *A. gambiae*, *A. arabiensis* and *A. funestus*, the major malaria vectors in the area. The study was approved by the health authority of the Sudan Relief and Rehabilitation Commission, and by the health secretariat of the Nuba Relief Rehabilitation and Development Association (NRRDA). The study also received ethical approval from the ethics review board of *Medecins Sans Frontieres*. Informed consent was obtained from adults or parents of children aged 6 to 59 months old before they were enrolled in the study according to [24] protocol for monitoring antimalarial drug resistance.

Sample collection

Patients visiting the outpatient clinics in the year 2003-2004 were enrolled in the study if they met the following criteria: informed consent; mono-infection with *P. falciparum* and parasitaemia between 2,000-200,000 parasites/ µl of blood; fever (axillary temperature $\geq$ 37.5°C or with a history of fever); weight $\geq$ 5 kg; no history of antimalarial drug intake during the previous week as confirmed by urine tests (Saker Solomon Test and Bratton-Marshall Test for CQ and SP intake); no signs of severe malaria; no concomitant febrile conditions apart from mild upper respiratory tract infection of presumed viral origin; and no reported hypersensitivity to the study drugs. Patients were excluded from the study if there was administration of any additional antimalarial drugs. Arm allocation was randomized by sealed envelopes. Children received either three daily doses of AS (4mg/kg/day; Arsumax®, sanofi) plus a single dose of SP (1.25 mg/kg sulfadoxine and 25mg/kg pyrimethamine; Fansidar®, Roche), or AS as above plus three daily doses of AQ (10mg/kg/day; Camoquin®, Parke-Davis). Drug-intake was observed on days 0, 1, 2, and children were reassessed both clinically and parasitologically on days 2, 3, 7, 14, 21 and 28, or any other days with evidence of illness. Peripheral blood collected by lancet prick from a fingertip was used for direct microscopic diagnosis. After thick and thin blood film staining with10% Giemsa, study slides were read independently by two microscopists, each blinded to the other's results. For quality control purposes, randomly selected slides were read in the Kenya Medical Research Institute Malaria lab. Some amount of blood was also spotted directly on 3mm Whatman filter paper (Whatman®) [24]. Blood spots were allowed to air-dry, then placed individually in plastic bags and stored at 4°C with a silica gel (Sigma) to prevent dampness until required for DNA extraction and analysis. To optimize for the specificity and sensitivity of the technique, six *P. falciparum* clones, (3D7, 7G8, Dd2, K1, T9-96, W2 and HB3) from WHO/IAEA, which contained known *dhfr* and *Pfmdr1*alleles, were used. These were also used as controls in the scoring of the sample results on the autoradiograph, after radio probing and restriction fragments.

DNA extraction, PCR amplification and product analysis

Molecular analysis of filter paper-preserved blood samples was done in the Malaria Molecular Lab in the Centre of Biotechnology Research and Development, at the Kenya Medical Research Institute. The blood spot samples and clones collected using filter papers were excised from the filter paper and the DNA extracted using Chelex-100® Bio- Rad method as described by Plowe *et al.* [24]. Oligonucleotides primer pairs M1 and M5 were included in a single outer PCR reaction. The nest 1 or outer amplification reaction volume was 20 µl containing a sector of prepared 5 µl DNA from chelex extraction, 0.25µM of each nest 1 primer (Table 1), 1× standard PCR buffer (1.5mM $MgCl_2$, 50mM KCl, 10mM Tris HCl (pH8.3), 0.5% DMSO), 200 µm of each of the dNTPs, and 1 unit of Taq polymerase (Roche, Promega). The reaction was allowed to proceed for one cycle at 94°C for 3 minutes, then 40 cycles at 94 °C for 1 minute, 50°C for 2 minutes, 72°C for 2 minutes and finally 1 cycle at 72°C for 10 minutes. Using the PCR products generated from the primary reaction as templates, a nested PCR was then performed using primers based on conserved sequences, so that the PCR product included codons 108, 51 and 59 in *dhfr*. M3-F/ were the nested primers used for codon 51 and 108 (Asparagine, Threonine), while F-M4 primers were used for codon 59 and 108 (Serine). The nest II reaction consisted of 0.25µM of each nest II primer, standard PCR buffer, 200 µm of each of the dNTPs, 1 unit of Taq polymerase, and 5 µl of nest 1 PCR products as template. Tubes were briefly centrifuged and placed in a thermocycler (Bio-Rad / MJ Research). The reaction was cycled 40 times at 94°C for 1 minute, 45°C for 1 minute, 72° C for 2 minutes and a final extension step of 72°C for 10 minutes. Parasites were examined for antifolate-resistance associated point mutations in the *dhfr* gene on chromosome 4 using a nested PCR/RFLP method described by Duraisingh *et al.* [25]. Restriction digestions were carried overnight at optimum temperatures for each enzyme in the presence of 1× buffer, 1 BSA (where required) 1-2 units of the specific enzyme to a total volume of 15 µl and at optimal conditions as indicated by the suppliers (New England Biolabs, UK). Next, 5µl of the PCR product was used without purification, and when the incubation temperature was above 50°C, samples were overlaid with a few drops of mineral oil. Restriction digestions were carried out overnight at incubation temperatures which differ for different enzymes (Table 2). Control amplification using DNA from *P. falciparum* parasite clones known to contain the possible different alleles at position 108, 51 and

Table 1. PCR primer sequences and reaction conditions.

Gene fragment	**Primer name**	**Primer sequence**
dhfr Outer PCR	M1 M5	5'TTTATGATGGAACAAGTCTGC3' 5'AGTATATACATCGCTAACAGA3'
dhfr Nested PCR	M3 F/ F M4	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTttTA3' 5'GAAATGTAATTCCCTAGATATGgAATATT3' 5'TTAATTTCCCAAGTAAAACTATTAGAgCTTC3'
Mdr1-86	A1 A3 A4 A2	5'TGTTGAAAGATGGGTAAAGAGCAGAAAGAG3' 5' TACTTTCTTATTACATATGACACCACAAAC3' 5'AAAGATGGTAACCTCAGTATCAAAGAAGAG3' 5'GTCAAACGTGCATTTTTTATTAATGACCATTTA3'

Table 2. Restriction digest enzymes.

Codon	**Amplification primers**	**Restriction enzyme**	Incubation **temp°C**	**Product fragment**
Pfcrt 76	TCRD1/2	ApoI	50	126 & 73 bp for wild
dhfr 51	M3-F/	Tsp509l	65	154 & 64bp for wild
dhfr 59	F-M4	XmnI	37	163,137,26bp-Mutant 189,137 bp Wild
dhfr 108	F-M4 M3-F/ M3-F/	AluI BsrI BstNI	37 65 60	Ser Asn Thr

Table 3. Positive and negative control parasite clones for the DHFR locus.

Parasite clones	***dhfr***						
	Codon 108			**Codon 51**		**Codon 59**	
	Ser (W)	Asn(M)	Thr (M)	Asn (W)	Ile (M)	Cys (W)	Arg (M)
3D7	**positive**	negative	Negative	**Positive**	Negative	**Positive**	negative
Dd2	negative	**positive**	Negative	Negative	**Positive**	Negative	**positive**
HB3	negative	positive	Negative	Positive	Negative	Positive	negative
V1/S	negative	negative	Negative	negative	Positive	Negative	positive

Table 4. Baseline characteristics of included patients.

Characteristics		**AS + SP (n = 80)**	**AS + AQ (n = 80)**
Age	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 (9800-48279)	21 190 (8202-51351)
Gametocytes on day 0	n (%)	4 (4.9)	4 (5.6)
Temperature (ºC)	Mean (range)	38.9 (37.5-40.7)	39.0 (37.5-40.6)

Table 5. Mutations in codon 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

59 of *dhfr* were used to ensure specificity and sensitivity of the technique (Table 3).

Oligonucleotides primer pairs *mdr*/A1 and *mdr*/A3 were included in a single outer PCR reaction. Using the PCR products generated from the primary reaction as templates, a nested PCR was then performed using primers *mdr*/A4 and *mdr*/A2. Control amplification using DNA from *P. falciparum* parasite clones known to contain the possible different alleles at position 86 of *mdr1* were used to ensure specificity and sensitivity of the technique. 3D7 and 7G8 were used as wild types while K1 and Dd2 as mutant types. In the primary PCR amplification, the reaction took place in a total volume of 20 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µm each of dNTPs (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µm each and one unit of Taq DNA polymerase. Five microlitres of parasite DNA was used as a template in the primary reaction. The reaction was allowed to proceed for one cycle at 95°C for 3 minutes, then 30 cycles at 94°C for 30 seconds, 50°C for 1 minute, 65°C for 2 minutes and finally one cycle at 65°C for 8 minutes. In the nested PCR amplification, the reaction took place in a total volume of 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µm each of dNTPs (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µm each and one unit of Taq DNA polymerase. Two microlitres of primary PCR was used as template in the nested PCR reaction. The reaction was allowed to proceed for one cycle at 95°C for 3 minutes, then 30 cycles at 94°C for 30 seconds, 50°C for 1 minute, 65°C for 2 minutes and finally one cycle at 65°C for 8 minutes and holds at 4°C. Five microlitres of nested PCR were mixed with a 1-2 µl of 10× loading dye and analysed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.8 µg/ml) in Tris-acetate buffer-EDTA buffer. DNA was visualized by ultraviolet transilluminator and the expected nested PCR product band sizes determined by comparison with a standard 100-base pair DNA ladder. Preparation of dot blotting hybridisation was done as described by Abdel Muhsin [22]. Probes for all the possible alleles in each of the codons were labelled, such as Asn and Tyr probes (MWGBiotech) for codon 86 of the *Pfmdr1* gene. The radioisotope used was 1 µl of [γ-^{32}P] dATP (Amersham Biosciences, UK: Redivue [γ-^{32}P] ATP, 3000Ci/mmol: Cat No. AA00068).

After the restriction digest, the restriction fragments that resulted were used to score the isolates based on the controls used. After radio probing, parasite clones and isolates were scored either as wild types or mutants compared to the specific probe used in a given blot or as indicated by the controls. Data recording was done on MS Excel sheets, MS Access database, and also on the laboratory workbook. Epi-Info computer software was used to calculate the P values of mutations in codons 51, 59 and 108 of the *dhfr* gene before treatment.

Results

Screening of 307 febrile children was done between 10 September and 10 November 2003, of whom 200 had a *P. falciparum* infection, and 161 met inclusion criteria. Baseline characteristics in the two arms were similar (Table 4), although in the AS + AQ arm males were more frequent ($P = 0.09$). All 80 children in the AS + AQ arm completed follow-up; in the AS + SP arm one was lost to follow-up. No significant adverse events were reported. In the *in vivo* follow-up, 98.8% from both arms became afebrile by day two, whereas by day three, 98.8% (80/80) and 98.9% (78/80) were slide-negative. Gametocyte carriage remained low throughout follow-up: 5.1% (4/79) on day 14 and 2.9% (2/70) on

Figure 1. Prevalence of Point Mutations in Dihydrofolate reductase genes (Pfdhfr).

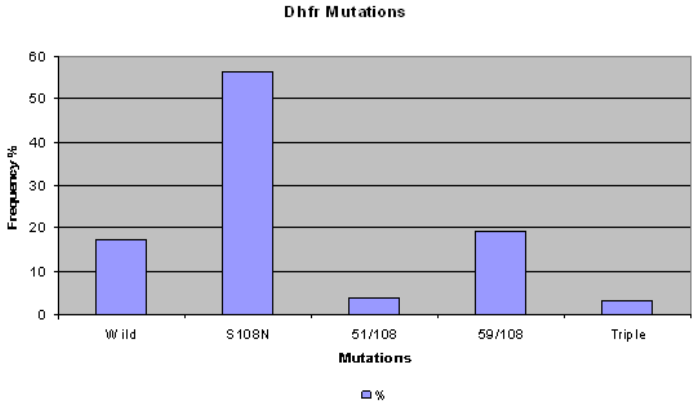
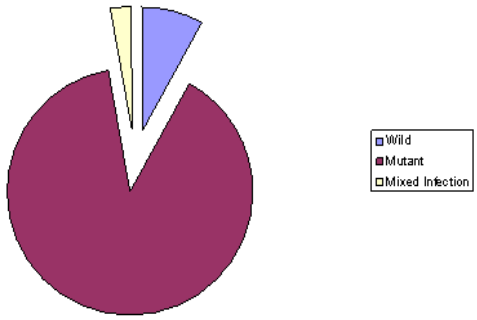


Figure 2. Multidrug resistance gene (Pfmdr 1-86) in a combination regimen in Nuba Mountains.



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.

Baseline allele prevalence

A total of 160 samples for molecular analysis were collected from the study site. These were all pre-treatment blood samples collected on Whatman filter paper. These were all positive cases according to the microscopists at the study site. Mutations in *dhfr*, 82.5% (132/160, 95% CI 75.7—88.0) of patients carried one or more mutations at *dhf* but none were I164L. N51I and C59R mutations occurred exclusively in the presence of S108N (Table 5), but this association was significant only for C59R (Fisher's exact test, $P = 0.214$ for N51I; $P = 0.002$ for C59R) (Figure 1). Triple *dhfr* mutations were infrequent. Baseline dot-blot hybridization of the *mdr1*-86 locus was successful for 160 isolates: 143 (89.2%) carried parasites harbouring the *mdr1*-86 Tyr resistance allele, while 13 (8.1%) carried *mdr1*-86 Asn sensitivity allele and 4 (2.7%) were of mixed infection, having both resistance and wild type allele (Figure 2).

Presentation alleles and treatment outcomes

Using parasitological failure by day 28 as the outcome measure, we analyzed the baseline samples stratified by treatment group. A considerable number of late failures occurred, but only nine were PCR-confirmed recrudescences: 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.

Discussion

Several studies associating mutations in *dhfr*, *dhps*, *Pfcrt* and *mdr1* genes with treatment outcome following the treatment of patients with SP, AQ and CQ respectively have been done. However, no data on *in vitro* antimalarial efficacy were available for the Nuba Mountains and policy shift was done on an *in vivo* outcome. War-affected Sudanese populations have poor access to health care, and malaria control has been particularly neglected. As elsewhere in Sudan, *P. falciparum* malaria is the main health problem and mutations associated with SP resistance are frequent in the Nuba Mountains. Regardless of the isolation and limited drug use, apart from three years of MSF involvement with CQ, SP and then AS + SP, this finding may have explanations aside from mere drug pressure. Nuba Mountain is just below the border of Northern Sudan, and the return of internal people displaced by the civil war from the Khartoum area may have introduced previously absent mutant strains. Additionally, the mountain conditions give a markedly seasonal transmission setting; the ecological balance of strains could be heavily altered by genetic drift [26]. As shown elsewhere in Sudan [27], resistant mutants may survive more easily in the dry season, and could therefore be amplified in the subsequent seasonal peak.

The spread of resistance of *P. falciparum* to chloroquine prompted several African countries to identify alternative drugs as first-line antimalarial treatment, mostly considering their activity against local parasite strains but also their tolerability, ease of administration, and cost [28,29]. Due to the high cost of artemisinin, many African countries currently use a sulfadoxine- pyrimethamine combination (SP) or amodiaquine (AQ) to treat uncomplicated *P. falciparum* malaria. Both drugs represent the least inexpensive alternative to chloroquine. Several studies have indicated that AQ is effective in treating chloroquine-resistant *P. falciparum* malaria parasites [12,30], and recent studies report high cure rates in Africa, [23-25], despite concerns over a few cases of

adverse effects reported during prophylactic use of this drug [31]. Two alleles of *Pfmdr1* previously were claimed to be associated with amodiaquine resistance; one was characterized by Y86, and the other by 1034C, 1042D and 1246Y where most field studies have investigated the Y86 allele, which was associated with chloroquine and amodiaquine resistance in some studies [32- 34] but not in others [35- 39]. It is likely that the AQ component of the drug combination is responsible for treatment outcome or success. In support of this hypothesis prior to the introduction of AQ/AS as the first-line drug, CQ treatment failure rates were in excess of 30% [40], whereas the combination drug gave an adequate clinical response of 92%. Of the two combinations, AS + AQ is likely to have a prolonged therapeutic lifespan given global SP resistance trends [41]. AQ resistance seems to develop more slowly according to *in vivo* observations [42]. The genotyping 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. It is nevertheless clear that any future use of AQ, in Sudan as elsewhere, should occur strictly in a combination regimen.

Mutations in *dhfr* are clearly linked to resistance to pyrimethamine and to SP. The key mutation at position 108 (Serine to asparagines) was present in all patients in whom treatment was unsuccessful. In pre-treatment with SP/ AS, only a few patients (17.5%) had parasite with the wild type allele *dhfr* 108Ser. Two other mutations in *dhfr* in codon 51 and 59 have been linked to SP resistance. For codon 51, the majority of parasites typed had the wild form encoding 51Asn. Infections with purely the mutant form of codon 51 (51Ile) were found in 8% of pretreatment samples from patients in whom SP/AS failed to clear parasitaemia. Mixtures of wild type (51 Asn) and mutant (51 Ile) parasites were found in two samples, one pretreatment isolate in the SP/AS arm, and one isolate in the AQ/AS arm. A pure wild infection was found in 80% of patients with AQ/AS post-treatment and in 50% in SP/AS treatment failure. Because the majority of infections carried only the wild allele, there was significant association between the presence of different pre-treatment *dhfr* 51 allele and the treatment combination failure in AQ/AS and SP/AS treated patients. For codon 59, the frequencies of wild type (59Cys) and mutant (59Arg) alleles were more evenly balanced. Infections with only the wild type allele or only the mutant allele were found in a small number of pre-treatment patients in both treatment regimes. Analysis of combination of drugs to recrudescence showed that patients with parasites carrying pure *dhfr* 59Arg in D0 sample recrudesced significantly earlier than those with a mixture of *dhfr* 59Cys + 59Arg or those with pure *dhfr* 59Cys. This was true only for the SP arm of the study; it was not observed with the AQ/AS arm of the study, possibly because of the low numbers of parasites recrudescing early (< 14 days) in the latter arm. Patients carrying infections of pure *dhfr* 108Asn/59Arg double mutant parasites had significantly earlier recrudescence following SP treatment than those with other alleles. There was significant difference in the patients treated with AS/AQ, possibly because very few patients in this drug treatment arm had early treatment failure with recrudescence before day 14 post-treatment. Patients carrying infections of pure triple mutant (dhfr108asn/ 51Ile/59Arg) recrudesced significantly earlier than patients with other infections in the SP/AS drug treatment arm, but not AQ/AS treated patients, for the same reasons discussed above. For the SP/AS-true recrudescence subset, even with the low numbers, there was still an association with the triple mutant and early recrudescence.

This study attempted to investigate the role of double and triple mutant forms of *dhfr* in clinical treatment failure. The results show that parasite infections of pure double mutant (*dhfr* 108Asn/59Arg) are associated with significantly earlier recrudescence following treatment with SP. Infections with parasites carrying the triple mutant form alone (*dhfr* 108Asn/51Ile/59Arg) are even more significantly associated with early treatment failure. The research presented suggests that the number of mutations present in *dhfr* has a significant effect on the type of treatment failure following SP chemotherapy. Mutations in *dhps* have also been shown to be implicated in resistance to SP [43]. In this study we did not analyse sulfadoxine resistance mutations at the dihydropteroate synthetase (*dhps*) gene. As the case may be, such an analysis would have provided a better overall prediction of SP resistance in Nuba. The finding as discussed above will help in laying strategies that will lead to the delay of the spread of antimalarial drug resistance in Sudan. Operationally, the findings imply that SP resistance mutations are present and may become dominant, especially if SP monotherapy is widely used. Multiple and particularly triple mutations at *dhfr* are reasonably predictive of *in vivo* SP failure.

Presently these are not rare, implying that the drug should not be used in Nuba. Of the two combinations, AS + AQ is likely to have a longer therapeutic lifespan.

Recommendation

In vivo clearance of resistant parasites may involve host factors. Identifying these factors will improve our understanding of the immunological events and will have great implication for predicting resistance. Development of molecular markers/tests for Coartem resistance (and other ACTs) is difficult because there are no known resistant lab lines for positive controls and therefore resistance to artemisinin combination could only be predicted as a result of the other combined drug; for example, there is high chance of resistance to Lumefantrine rather than the artemether in Coartem combination.

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