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Rapid increase in resistance of *Plasmodium falciparum* to chloroquine-Fansidar in Uganda and the potential of amodiaquine-Fansidar as a better alternative

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Abstract

Combinations of chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) [CQSP] as the first line agents in Uganda have replaced CQ monotherapy. The idea of the combination is to delay the development of malaria resistance to either drug when used alone. We compared the clinical, parasitological and molecular findings of two studies with treatment arms of CQSP, amodiaquine (AQ) plus SP (AQSP) both done in 2003 with a study done 1 year earlier (2002) using SP alone. There was a notable decrease in adequate clinical response (ACR) by day 14 from 92.7% with SP to 80% with the combination CQSP, a year later. AQSP combination was found to have the best effect (94.3% ACR). There were no early treatment failures in the AQSP group. However, treatment failures were recorded at 20% on day 14 and 43% on day 28 for CQSP treatment and 5.7% by day 14 and 28.8% by day 28 in the AQSP group. The number of mutations that are associated with SP resistance increased from 2002 to 2003 at all loci monitored, from 83.8 to 100% at codon 108, 58.7 to 76% at codon 59 in the DHFR gene, and from 58.8 to 86% at codon 437 and 33 to 43% at codon 540 in the DHPS gene. We conclude that there has been a rapid development of resistance since the introduction of the new policy guidelines. AQSP was found to be a superior drug combination compared to CQSP and could be used as a low cost alternative at the moment.
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1. Introduction

Malaria remains one of the most serious global health problems and a leading cause of childhood morbidity and mortality, especially in Africa. Efforts to control malaria in Africa have been severely compromised by the emergence of resistance in *Plasmodium falciparum* to the inexpensive and widely used drugs, chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) (Attaran et al., 2004). In Uganda, chloroquine resistance levels approached a national average of 40% (Kamya et al., 2001, 2002) by the year 2000 and as

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a consequence, even with limited data, the Ugandan Ministry of Health recommended a combination of CQ and SP (Ugandan Ministry of Health, 2000a,b) as an interim policy for the following reasons. Firstly, chloroquine is cheap, available, tolerable and has antipyretic properties regardless of its effect on the malaria parasites (Barat et al., 1998; Brandling-Bennett et al., 1988). Secondly, the long-term effectiveness of SP was debatable since the countries that were using it as first line treatment had began to register high levels of resistance (Nevill and Gardner, 1991; Nwanyanwu et al., 1996; Omar et al., 2001; Verhoeff et al., 1997). Thirdly, while there was only limited data on the other available antimalarial agents in Uganda, earlier studies in Asia and Tanzania (McIntosh, 2001) had showed high efficacy levels with the CQSP combination.

Recently, a number of separate studies have been done to establish the efficacies of drug combinations. In a comparative study done in Kampala, CQ/SP appeared preferable to SP alone in general, but when results were stratified by age, the benefit was only significant in patients below 5 years of age (Gasasira et al., 2003). The AQ/SP combination was much more efficacious and this confirmed earlier studies that had shown AQ to be a better treatment than CQ or SP (Gasasira et al., 2003; Staedke et al., 2001). Another study done in Tororo, Uganda, found AQ/SP 100% efficacious as compared to 93% for CQ/SP and 91% for SP alone. Parasitological failure by day 28, however, occurred in 16, 48 and 61% of the patients in the three treatment arms (AQ/SP, CQ/SP, SP), respectively (Talisuna et al., 2004a). It was found that addition of CQ to SP did not offer any added therapeutic advantage (Talisuna et al., 2004a; Checchi et al., 2004), but whether it would delay the development of resistance to the component drug was not addressed. It is also notable that, since CQ plus SP replaced CQ alone as the first-line antimalarial drug, only few reports have been published from continuous evaluations of clinical, parasitological and molecular profiles of *P. falciparum* resistance patterns.

Genetic variation associated with both CQ and SP resistance can be monitored with specific molecular markers. Position 76 in the *Pfcrt* gene which is putatively associated with *P. falciparum* resistance to CQ was found to be 100% mutated in Kampala (Dorsey et al., 2001; Kyosiimire-Lugemwa et al., 2002) and we recently confirmed these findings by similar analysis of over 200 isolates from patients at Kasangati Health Centre (results not shown). Therefore, in the present study, we did not study the *Pfcrt* gene. *P. falciparum* resistance to SP has been reported to be associated with point mutations in genes that encode two key enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), which are the targets for pyrimethamine and sulfadoxine, respectively (Wang et al., 1997). The accumulation of mutations in DHFR (108-Asn, 51-Ile, 59-Arg) and DHPS (437-Gly, 540-Glu) appears be valuable in detecting emerging resistance before clinical treatment failure is evident (Hastings et al., 2002; Kyabayinze et al., 2003). Some studies found mutations at codons 108 and 51 too frequent to be practical predictors of resistance and only three mutations (DHFR Arg 59, DHPS 437 and 540) were identified as helpful in correlating with clinical outcome (Kyabayinze et al., 2003; Staedke et al., 2004; Talisuna et al., 2003). Another study has revealed that mutations at DHFR codons 108 and 59 together with DHPS 540 are related to parasitological failure (Talisuna et al., 2004b). Based on the above recent findings, we examined four mutations at DHFR 108 and 59 and at DHPS 437 and 540 as the markers to monitor the rate of development of *P. falciparum* resistance to SP. Most clinical studies of malaria drug resistance, which apply the WHO guidelines of assessing clinical or parasitological outcome (WHO, 2001), are limited as they do not distinguish recrudescence of resistant parasites from re-infection by new parasite strains. For this, a simple genotyping system using only merozoite protein 2 gene (MSP 2) to type infecting parasites adequately was described recently (Cattamanchi et al., 2003).

In this report, subsequent to the recent policy change from CQ alone to CQ plus SP as first-line therapy for uncomplicated malaria in Uganda, we compared the clinical efficacies of CQSP and AQSP combination therapies with SP alone while we monitored parasitological outcomes and development of gene mutations associated with *P. falciparum* SP resistance.

2. Materials and methods

2.1. Subject recruitment

Studies were conducted at Kasangati Health Centre, an outreach health unit for Makerere University

Institute of Public Health and a district referral hospital for Wakiso district. Kasangati is 20 km Northeast of Kampala is a peri-urban locality with much lower numbers of drug kiosks and health care services than urban Kampala. Malaria is meso-endemic in Kasangati and peak transmission occurs after each of two rainy seasons in a year.

Results of three studies are reported here. The first study was done in 2002 before Uganda's malaria policy was fully implemented and, in this, we estimated the efficacy of SP alone. In 2003, two consecutive studies were done during the same transmission period and we estimated the efficacies of the combinations CQSP and AQSP, respectively. All studies were done using the same inclusion criteria. Patients who presented with symptoms suggestive of malaria had a blood smear undertaken and were later considered for recruitment if they fulfilled the following inclusion criteria:

1. 6–59 months of age;
2. positive blood smears with *P. falciparum* mono-infection and parasite density more than 1000 asexual parasites per microliter;
3. uncomplicated malaria with absence of symptoms of severe malaria or danger signs e.g. convulsions, excessive vomiting, drowsiness or inability to feed;
4. axillary temperature of 37.5 °C and above or history of fever in the last 48 h;
5. absence of history of allergy to sulfa drugs;
6. residence within an accessible address and agreement to return to the clinic for follow-up;
7. provision of informed consent.

Clinical history was taken from the recruited patients including anti-malarial therapy in the last 72 h. A physical examination was done and the temperature was measured using a clinical thermometer. Fever was defined as axillary temperature of 37.5 °C and above.

2.2. Treatment

In the SP study, the patients received a single dose of 25 mg/kg sulfadoxine plus 1.25 mg/kg pyrimethamine (Fansidar, Roche). In the CQSP study, SP was given as above together with CQ (Pharco Pharmacueticals, Egypt), 25 mg/kg in three divided doses given on day 0, 1, and 2. In the AQSP study, SP was given on day 0 as above in addition to AQ (Kampala Pharmeceuticals, Uganda), which was given at 15 mg/kg on days 0, 1 and 2. Drugs were administered under direct observation, such that if a patient vomited within 30 min, the drug was re-administered. Those who vomited the drugs more than once were excluded from the study and were given parenteral quinine (QNN). Patients who failed treatment were given 10 mg/kg QNN every 8 h for 7 days. Those who developed severe malaria were referred to Mulago Hospital.

Patients were asked to return for follow-up on days 1, 3, 7 and 14 for the SP study and on days 1, 2, 3, 7, 14, 21 and 28 in the CQSP and AQSP combination therapy studies. Confirmatory thin and thick smears were done, and heamoglobin was estimated on day 0, and days 7 and 21. Follow-up consisted of taking the patients history plus a physical exam. The outcomes were registered on a case record form labeled with the patients study identification number. On the follow-up days described above and any other day when patients returned to the clinic with fever within the follow-up period, a thick blood smear was done to assess parasitemia. In case of a positive smear, a full heamogram was done as well. Patients who failed to return were picked by our social worker/health visitor and brought back to the health unit. Thick smears were stained with 10% Giemsa stain for 10 min, and we then counted the number of asexual parasite forms seen against every 200 white blood cells (WBCs). The count was then multiplied by 40 in order to obtain number of circulating parasites per microliter of blood, assuming a WBC count of 8000/μl. Some of the blood collected on day 0 and on the follow-up days was spotted on Whatman filter paper for molecular analysis.

2.3. Molecular methods

Parasite DNA was extracted from filter paper using the chelex extraction method (Plowe et al., 1997). The DNA was amplified by PCR using conditions described earlier (Duraisingh et al., 1998). However, for a set of samples in the SP study, the PCR method above failed to work and a new set of primers, developed by modifying the protocol, was used for examining the codons encoding DHFR residues 108 and 59. Table 1 shows the PCR conditions and the modifications described.

Mutations were determined by digestion of nested PCR products using restriction enzymes from New

Table 1
Details of PCR conditions for parasite DHFR and DHPS DNA amplification[a]

Reaction and conditions	Old primers
DHFR primary amplification	M1 5′-TTTATGATGGAACAAGTCTGC-3′
Denaturing: 94 °C, 3 min	M5 5′-AGTATATACATCGCTAACAGA-3′
Denaturing: 94 °C, 60 s	
Annealing: 50 °C, 2 min	648 bp
Extension: 72 °C, 2 min	
Cycles: 40	
Final extension: 72 °C, 10 min	
DHFR secondary amplifications for codon 59 and 108	F 5′-GAAATGTAATTCCCTAGATATGgAATATT-3′
Denaturing: 94 °C, 2 min	M4 5′TTAATTTCCCAAGTAAAACTATTAGAgCTTC-3′
Denaturing: 94 °C, 60 s	
Annealing: 45 °C, 2 min	326 bp
Extension: 72 °C, 2 min	
Cycles: 5	
Denaturing: 94 °C, 60 s	
Annealing: 45 °C, 1 min	
Extension: 72 °C, 1 min	
Cycles: 35	
Final extension: 72 °C, 10 min	

Reaction and conditions	Modified primers
DHFR primary amplification	A_1 5′-TTTATGATGGAACAAGTCTGCGACGTTTTC 3′
Denaturing: 94 °C 3 min	A_2 5′-AATTTGATACTCATTTTCATTTATTTCTGG-3′
Denaturing: 94 °C 1 min	622 bp
Annealing: 54 °C 1 min	
Ext:72 °C 2 min 30 s	
Cycles 5	
Denaturing: 94 °C 45 s	
Annealing: 56 °C 1 min	
Ext: 72 °C 2 min	
Cycles 30	
Final extension: 72 °C, 10 min	
DHFR secondary amplification for codons 59, 108	A_3 5′-GTCTGCGACGTTTTCGATATTTATGC3′
Denaturing: 94 °C, 3 min	F/5′-AAATTCTTGATAAACAACGGAACCTttTA-3′
Denaturing: 94 °C, 60 s	
Annealing: 50 °C, 1 min	506 bp
Extension: 72 °C, 2 min	
Cycles: 5	
Denaturing: 94 °C, 45 s	
Annealing: 54 °C, 1 min	
Extension: 72 °C, 1 min	
Cycles: 30	
Final extension: 72°	

Reaction and conditions	Primers
DHPS primary amplification	R2 5′-TTTATGATGGAACAAGTCTGC-3′
Denaturing: 94 °C, 3 min	R/5′-AATTGTGTGATTTGTCCACAA-3′
Denaturing: 94 °C, 60 s	
Annealing: 45 °C, 2 min	710 bp
Extension: 72 °C, 2 min	
Cycles: 5	
Denaturing: 94 °C, 60 s	
Annealing: 45 °C, 1 min	
Extension: 72 °C, 1 min	
Cycles: 35	
Final extension: 72 °C, 10 min	

Table 1 (*Continued*)

Reaction and conditions	Old primers
DHPS secondary amplifications for codons 437, 540	K 5′-TGCTAGTGTTATAGATATAGGatGAGcATC-3′
	Or
Denaturing: 94 °C, 3 min	J 5′TGCTAGTGTTATAGATATAGGTGGAGAAagC-3′
Denaturing: 94 °C, 60 s	K/5′-CTATAACGAGGTATTgCATTTAATgCAAGAA-3′
Annealing: 45 °C, 1 min	
Extension: 72 °C, 1 min	438 bp
Cycles: 40	
Final extension: 72 °C, 10 min	

[a] The primary reactions, carried out in 50 μl, contained 5–10 μl DNA extract, 1x PCR buffer (67 mM Tris–HCl [pH 8.8], 2–4 mM $MgCl_2$), 200 μM dNTP, 0.25 μM of each primer, and 1 U Taq polymerase. The secondary reactions had the same composition except that the DNA from the primary reaction was reduced to 0.5–1 μl.

England Biolabs, Mass, USA as recommended by the manufacturer. In order to analyze the *pfdhfr* single nucleotide polymorphisms (SNPs), restriction enzyme *Alu*I was employed for detection of Ser108 and *XmnI* for Arg59. For the analysis of the two *pfdhps* SNPs, *AvaII* and *Fok*I were used to examine the codons for Gly437 and Glu540, respectively. All restriction products were separated using 2% agarose gel electrophoresis and observed under UV light.

The polymorphic regions of *msp*-2 were identified using PCR primers designed from the conserved sequences flanking these regions in the primary amplification (Zwetyenga et al., 1998). Secondary amplification was based on the IC3D7 and FC27 allelic families and compared to genomic DNA from HB3 and 3D7 laboratory strains as controls (Fenton et al., 1991). Genotyping and interpretation of the results for distinguishing recrudescence from re-infection were carried out as described recently (Cattamanchi et al., 2003).

2.4. Outcome measures

Our primary outcome end points for all studies were clinical and parasitological outcome on day 14. The secondary end points for the CQSP and AQSP studies were clinical and parasitological outcomes at day 28. Clinical outcomes were classified as adequate clinical response (ACR, early treatment failure (ETF) or late treatment failure (LTF). Parasitological outcomes were described as sensitive (S) or resistant (RI, RII, RIII), according to the WHO clinical and parasitological classification system (WHO, 2001). In order to categorize treatment failures, in day-28 follow-up studies, as either due to recrudescence or due to new-infection, the corresponding rates of failure were adjusted by the assessed percentages of recrudescence and re-infection as described recently (Cattamanchi et al., 2003).

2.5. Statistical analysis

Sample size calculation for the SP study was based on difference in the level of clinical SP resistance in Kampala (10%) (Kamya et al., 2001) an urban setting, and the level of resistance in the rural settings (2%) (Ndyomugyenyi and Magnussen, 1997) in Uganda. For the drug combination studies, the corresponding calculations were based on the reported differences between CQSP resistance (7%) and SP resistance (10%) and between AQSP (0%) and SP resistance in Uganda (Gasasira et al., 2003). Consequently, in order to accomplish statistical confidence intervals of 95% and power of 80% in the 14-day follow-up studies, we aimed to recruit a minimum of 130 patients for the SP treatment study, 150 patients for the CQSP and 50 for AQSP treatment groups, considering 10–15% potential loss to follow-up. A p-value of ≤ 0.05 was assumed to be significant.

2.6. Limitations of the studies

The studies were done as cross sectional studies whereby a year after the SP investigation was completed, the two drug combination studies (SP + CQ compared to SP + AQ) were undertaken within one transmission period. This may not provide the clear benefits of a blinded randomized study. The results pre-

sented below show a progression of resistance and indicate a clear relationship with earlier published studies.

3. Results

Uganda changed her first-line treatment guideline for uncomplicated malaria from CQ to CQSP in 2000 but this policy amendment was not widely implemented till well after 2002. In this report, we present the results of two studies, which we undertook during this crucial phase so as to inform policy on the available low-cost treatment alternatives. In the first study, which we conducted within year 2002, we examined the efficacy of SP alone. One year later, in 2003, we carried out two investigations, one of CQSP and the other of AQSP, within the same malaria transmission season. Consecutive enrolled patients were alternately assigned to CQSP or AQSP. In these parallel evaluations (CQSP versus AQSP), the reporting physician was blinded to the drug allocations. Fig. 1 shows the profiles for screening, recruitment and follow-up the patients who were studied in the three (SP, CQSP and AQSP) treatment groups. A total of 500 patients were screened in the SP study from whom 139 were recruited and 126 completed the 14-day follow-up. The other 13 patients were excluded for the following reasons: taking non-study medication (8), development of danger signs or severe malaria (2), withdrawal of consent (1) and failure to return to clinic or to locate patients' addresses (2). Another 712 patients were screened for the CQSP group. Of these, 146 patients were recruited and 128 completed treatment and follow-up to day 14 while 123 were successfully followed up to day 28. We

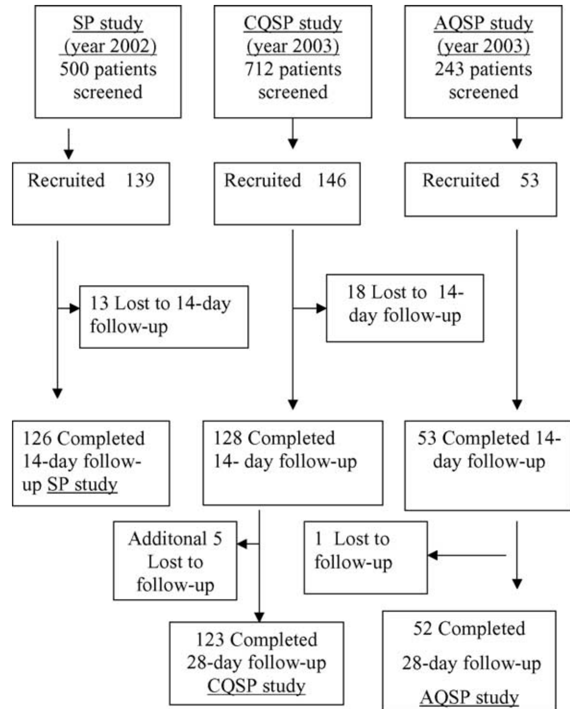

Fig. 1. Enrolment and follow-up of patients in the three-arm drug efficacy study.

screened 243 patients for the AQSP study, of whom 53 were recruited. All 53 patients were successfully followed up to day 14 whereas one did not complete the subsequent follow-up to day 28. Table 2 shows the baseline characteristics (at day 0) of the patients for whom a final treatment outcome could be assigned. All study participants in the two 2003 (CQSP and AQSP) studies were below 5 years of age as per the study protocols, but in the SP- study, only 86.3% were actually below

Table 2
Baseline characteristics

Characteristic	SP, $n = 126$	CQ-SP, $n = 128$	AQ-SP, $n = 52$
Female	66 (53.2%)	56 (43.7%)	20 (38.5%)
Age in months			
Mean	40.65	30.6	27.45
Range	6–132	6–60	6–60
Median	26	24	24
Age <5yrs	107 (86.3%)	129 (100%)	52 (100%)
History of anti-malarial use in previous 72 h	20 (16.1%)	24 (19.5%)	14 (26.9%)
Temperature range (°C)	36.2–39.9	36.2–40.1	37.5–39.8
Mean parasite count	33689	93908	47982

Table 3
Clinical and parasitological outcomes

	SP	CQSP		AQSP	
	n = 126 day 14	n = 128 day 14	n = 123 day 28	n = 53 day 14	n = 52 day 28
Clinical outcome					
ACR	115 (92.7%)	104 (80%)	71 (57%)	50 (94.3%)	37 (71.2%)
ETF	5 (4%)	8 (7%)	8 (7%)	0	0
LTF	4 (3.2%)	16 (13%)	44 (36%)	3 (5.7%)	15 (28.8%)
Parasitological outcome					
S	93 (75%)	102 (79.4%)	69 (56.2%)	48 (90.6%)	37 (71.2%)
RI	16 (13%)	9 (6.6%)	37 (29.8%)	5 (9.4%)	15 (28.8%)
RII	6 (4.8%)	8 (6.6%)	8 (6.6%)	0	0
RIII	8 (6.5%)	9 (7.4%)	9 (7.4%)	0	0

5 years. Another difference was that mean parasitemia at recruitment was highest in the CQSP combination study.

Table 3 shows the summary of clinical outcomes for the three studies. The SP study, which was done in 2002, showed adequate clinical response (ACR) in 92.7% of participants while there was a notable decrease in the treatment response to CQSP (80%) in the subsequent study done in 2003. The combination AQ plus SP gave us the best results, with 94.3% ACR (Fig. 2). Early treatment failure rate (ETF) was 4% with SP alone but increased to 7% with CQSP, about a year later. No patient given AQ plus SP had early treatment failure. In the two groups (CQSP and AQSP) where follow-up lasted 28 days, treatment failures were noted on day 14 and their numbers more than doubled by day 28 (Table 3 and Fig. 2). Thus, in the CQSP group, LTF was 13% by day 14 and increased to 36% by day 28, while in the AQSP group, LTF rates were 5.7% by day 14 and rose to 28.8% by day 28 (Table 3). Parasitological outcome was comparable to clinical outcome. In the 14-day follow-up, drug sensitivity was recognized in 75, 79.4 and 90.6% of patients receiving SP, CQSP and AQSP, respectively (Fig. 3). On the other hand, in the 28-day studies, the infections were drug sensitive in 56.2% of patients treated with CQSP and 71.2% of patients treated with AQSP. The levels of parasitological (RII and RIII) resistance were similar in the SP and CQSP treatment groups (Table 3), with the SP group showing 4.8% RII and 6.5% RIII. The corresponding analysis for CQSP showed RII = 6.6% and RIII = 7.4%. Interestingly, there were no RII or RIII resistant patterns observed for parasites from the patients given AQ plus SP.

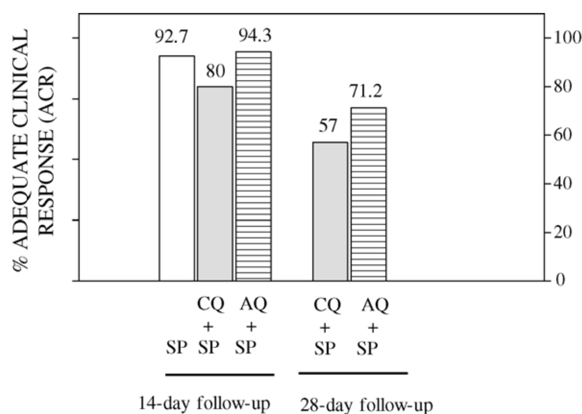

Fig. 2. Adequate clinical response (ACR) in the three treatment groups (SP alone, CQ + SP and AQ + SP). The SP treatment was studied for up to 14 days while CQSP and AQSP were studied using both 14-day and 28-day follow-up protocols.

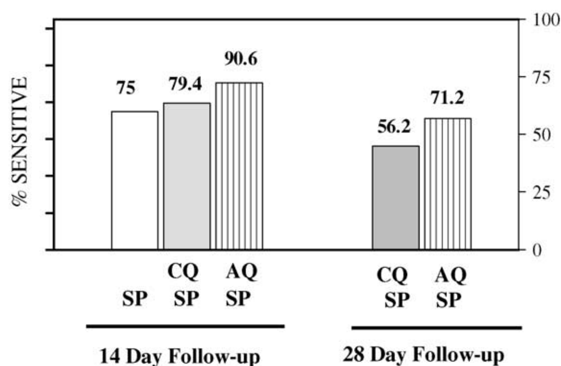

Fig. 3. Parasite sensitivity to SP, CQSP and AQSP.

Table 4
Changes in heamoglobin concentration after treatments with either CQSP or AQSP combination therapies

	CQSP	AQSP
Hb 0		
Mean	9.49	9.08
Range	5.20–11.9	5.7–10.7
Hb 7		
Mean	10.47	8.92
Range	6.2–14.6	7.7–10
Hb 28		
Mean	10.8	9.74
Range	8.4–12.6	7.9–11.3

Haemoglobin concentrations were not measured for the SP study.

We found a hemoglobin concentration increase (Table 4) in the CQSP group from an average of 9.49 on day 0 to 10.8 by day 28, as compared to an increase from 9.08 to 9.74 in the AQSP although the differences were not statistically significant.

Molecular typing of DHFR and DHPS mutations were only performed on samples collected on day 0 for the three study groups. In the analysis, we combined the results from the CQSP and AQSP groups to represent the genotype pattern in 2003, while the results from the SP showed the corresponding pattern in 2002. In this comparison, we could then monitor the general trend in the rate of development of resistance markers. Tables 5 and 6 illustrate the rapid accumulation of resistance markers in the parasite population. The already high frequency of mutations at codon 108 (83.8% recorded in 2002), increased to 100% in year 2003. Also mutations at codon 59 increased from 58.7 to 76%. Similar results were found for DHPS with mutations increasing from 58.8 to 86% at codon 437 and from 33 to 43% at codon 540. These percentages include all samples with mixed infections added to those with pure mutant genotype. We did not find a significant correlation of the molecular markers with clinical outcome.

The MSP 2 genotyping analysis showed that of 58 samples with successful results on both day 0 and day of failure, 35 (60.3%) were new infections and 23 (39.7%) represented treatment failure due to parasite recrudescence. When percentages of treatment failure (Table 3) are adjusted by these genotyping results (Cattamanchi et al., 2003), we see that AQSP is more effective both in limiting the levels of parasite recrudescence (CQSP = 16.3% versus AQSP = 11.4%) and in preventing re-infections (proportion of re-infections: CQSP = 24.7% versus AQSP = 17.4%).

4. Discussion

The studies presented here were done to evaluate the effect of the change in national drug policy to use CQSP as the first line drug for treatment of uncomplicated malaria. Two results are clear, first that the efficacy of CQSP is not improved compared to SP alone, second that the frequencies of mutations indicative of SP resistance have increased dramatically over a short period of time.

Table 5
Frequencies of DHFR and DHPS resistance markers for 97 (day 0) samples from the SP study

Result/codon[a]	DHFR59 (%)	DHFR108 (%)	DHFR437 (%)	DHFR540 (%)
WT	40 (41.2)	6 (6.2)	40 (41.2)	65 (67)
MX	27 (27.8)	7 (7.2)	22 (22.7)	28 (28.9)
M	30 (30.9)	84 (86.6)	35 (36.1)	4 (4.1)

[a] WT = wild type; MX = mixed; M = mutant.

Table 6
Frequencies of DHFR and DHPS resistance markers for 183 (day 0) samples from the CQSP and AQSP studies

Result/codon[a]	DHFR59 (%)	DHFR108 (%)	DHPS437 (%)	DHPS540 (%)
WT	47 (24)	0	26 (14)	27 (15)
MX	56 (29)	0	27 (14)	49 (28)
M	93 (47%)	183 (100)	134 (72)	101 (85)

[a] WT = wild type; MX = mixed; M = mutant.

As one measure of efficacy, the recorded ACR dropped from 92.7% for SP alone to 80% for CQSP (Fig. 2), confirming that the addition of CQ to SP did not improve efficacy (Talisuna et al., 2004a; Checchi et al., 2004). If we compare these findings with a study done in Kampala in 2002 (Gasasira et al., 2003) we also note a rapid deterioration of the efficacy from 7 to 20% treatment failures. Due to the very high rates of resistance to CQ noted earlier (Dorsey et al., 2001; Kyosiimire-Lugemwa et al., 2002), we assume that the reported deterioration is due to increasing resistance to SP alone. Patients in the CQSP and AQSP groups were followed up to 28 days (Table 3 and Fig. 2). Treatment failures increased dramatically by day 28 in both treatment groups, but mostly in the CQSP, showing AQSP to be a superior drug. Even with AQSP, however, we noted an increase of treatment failures from 0% (1% parasitological failure RII) in Kampala by day 14 (Gasasira et al., 2003) to 5.7% at day 14 and 28.8% at day 28 in the present studies in Kasangati. Our results mean that failure of CQSP therapy in this study area has already reached the WHO alert stage, but we also show that AQSP therapy is more efficacious and could be considered as the replacement therapy after CQSP. AQ and CQ belong to the same class of 4-aminoquinoline anti-malarials, so it was assumed that the superiority of AQ, which was associated with less previous use, would be short-lived as cross-resistance from chloroquine could develop shortly after its introduction (Bloland and Ruebush, 1996). This assumption is not proven with the current results and was not found in earlier studies (Geary and Jensen, 1983; Smrkovski et al., 1985). The decrease in efficacy could, however, reflect a worrying shift in potency of AQSP as well.

Our second observation was the high frequencies of mutations in the DHFR and DHPS genes (Table 5). The mutation frequencies in the DHFR gene have been steadily increasing compared to our earlier study done in 2000–2001 (Sendagire et al., 2005). In the earlier study, however, we demonstrated higher levels of mutations specifically in the DHPS gene. There is no obvious reason for this high frequency but it could reflect use of sulfonamide containing drugs for treatment of bacterial infections. Our present results suggest that during the years 2002–2003, all DHFR and DHPS resistance markers dramatically increased in frequency. While coartem (artemisinin plus lumefantrine) has been the policy drug for uncomplicated malaria in Uganda since 2004, it is too expensive (>US$ 7 for a complete treatment dose) and unaffordable locally. In addition, the regional supplies of coartem are yet limited compared to CQ, SP and AQ which are readily available and much more affordable. The present results indicate that AQSP may have a potential role as a low cost alternative anti-malarial in Uganda. However, the observed increases in resistance to SP as well as CQSP and rise in levels of the associated gene mutations are troubling. Rapid rises in frequency of resistance-associated gene mutations could be associated with recent increases in use of SP as part of CQSP, which has been commonly used over the last decade and is now widely administered locally for intermittent presumptive treatment (IPT) of pregnancy malaria. Therefore, regular monitoring of the usefulness of SP and aminoquinoline-SP combinations for IPT and affordable management of uncomplicated malaria is important.

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