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Drug resistance in *Plasmodium falciparum* from the Chittagong Hill Tracts, Bangladesh

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Summary

OBJECTIVE To assess the efficacy of antimalarial treatment and molecular markers of *Plasmodium falciparum* resistance in the Chittagong Hill Tracts of Bangladesh.

METHODS A total of 203 patients infected with *P. falciparum* were treated with quinine 3 days plus sulphadoxine/pyrimethamine (SP) combination therapy, and followed up during a 4-week period. Blood samples collected before treatment were genotyped for parasite mutations related to chloroquine (*pfcrt* and *pfmdr1* genes) or SP resistance (*dhfr* and *dhps*).

RESULTS Of 186 patients who completed follow-up, 32 patients (17.2%) failed to clear parasitaemia or became positive again within 28 days after treatment. Recurring parasitaemia was related to age ($\chi^2 = 4.8$, $P < 0.05$) and parasite rates on admission ($t = 3.1$, $P < 0.01$). PCR analysis showed that some of these cases were novel infections. The adjusted recrudescence rate was 12.9% (95% CI 8.1–17.7) overall, and 16.6% (95% CI 3.5–29.7), 15.5% (95% CI 8.3–22.7) and 6.9% (95% CI 0.4–13.4) in three age groups (<5 years, 5–14, ≥15). The majority of infections carried mutations associated with chloroquine resistance: 94% at *pfcrt* and 70% at *pfmdr*. Sp-resistant genotypes were also frequent: 99% and 73% of parasites carried two or more mutations at *dhfr* and *dhps*, respectively. The frequency of alleles at *dhfr*, *dhps* and *pfmdr* was similar in cases that were successfully treated and those that recrudesced.

CONCLUSIONS The clinical trial showed that quinine 3-days combined to SP is still relatively effective in the Chittagong Hill Tracts. However, if this regimen is continued to be widely used, further development of SP resistance and reduced quinine sensitivity are to be expected. The genotyping results suggest that neither chloroquine nor SP can be considered a reliable treatment for *P. falciparum* malaria any longer in this area of Bangladesh.

keywords malaria, drug efficacy, quinine, sulphadoxine–pyrimethamine, genotyping

Introduction

In Bangladesh, malaria transmission is restricted to the hilly districts in the eastern and northern border areas. The majority of malaria cases are found in three districts of the Chittagong Hill Tracts (CHT). The forested, green hills of CHT are suitable breeding grounds for malaria vectors from the *Anopheles dirus* complex. Malaria in this area is of moderate endemicity, with perennial but seasonally intense transmission (Rosenberg 1982). The Ministry of Health statistics indicates that in 2001, 67% (37 180) of the countrywide laboratory-confirmed malaria cases occurred in this area, which houses only 1% of the country's population. The CHT borders India and Myanmar and has a large indigenous population of Chakma, Marma, Tripura and other ethnic minorities. Health services in the remote and rural areas of CHT were disrupted and are still currently understaffed due to a preceding period of political instability.

In 1999, Médecins sans Frontières (MSF-Holland) started two primary healthcare clinics (with laboratory facilities) in a remote area of the CHT District Khagrachari. Malaria is responsible for one-third of all disease cases presenting to the clinics, with the predominant species being *Plasmodium falciparum*. The nationally recommended first line malaria treatment chloroquine (CQ) was quickly abandoned in the MSF-H clinics due to an unacceptably high number of treatment failures. This was confirmed in studies from other areas of Bangladesh (Rahman *et al.* 1998, 2001) and bordering areas of

Myanmar (Rakhine State; Smithuis *et al.* 1997) and India (MSF-Holland, Assam, India, 2001, unpublished data). Instead, *P. falciparum* patients were treated with the national second line treatment, short course quinine (Q) (3 days) followed by sulphadoxine/pyrimethamine on the fourth day (SP; protocols described in Rahman *et al.* 1996; Montanari *et al.* 2001). However, this treatment appears to fail in some patients returning *P. falciparum* positive within 1 month after treatment, suggesting that resistance to its components Q and/or SP is arising. Indeed, resistance to SP has been documented in Bangladesh (Government of Bangladesh, unpublished report, 1993, quoted in Rahman *et al.* 2001). Q 7-day therapy can result in parasitological failures (Rahman *et al.* 2001). The efficacy of the Q + SP combination was last assessed in 1996–97 in Sadar (Rahman *et al.* 1998) and Ramu (Rahman *et al.* 2001), Cox's Bazar District, and showed parasitological failure rates of 80% and 66%, respectively. In this study, we assessed the clinical efficacy of Q + SP treatment in *P. falciparum* patients in the CHT of Bangladesh.

We also assessed the status of *P. falciparum* resistance in CHT by molecular investigation of infected blood samples from the same patients. We genotyped the following mutations in loci that are known to confer resistance to CQ, Q and SP: (i) the point mutation K76T in the CQ resistance transporter (*Pfcrt*) on chromosome 7, a major determinant for both *in vitro* and *in vivo* CQ resistance (Fidock *et al.* 2000; Djimde *et al.* 2001); (ii) the N86Y mutation in multidrug resistance gene *pfmdr1* on chromosome 5 associated with, but not essential for, CQ resistance (Foote *et al.* 1990); (iii) mutations in dihydrofolate reductase (*dhfr*) on chromosome 4 (51I, 59R, 108N, 164L), coding for a stepwise increase in SP resistance; and (iv) mutations in dihydropteroate synthase (*dhps*) on chromosome 8 (436A, 437G, 540E, 581G), known to further enhance SP resistance (Plowe *et al.* 1998; Kublin *et al.* 2002).

Patients and methods

The study was performed from August to November 2002, in the MSF-clinics of Baghaichara, Dighinala upazilla and Pujgang, Panchari upazilla, situated at a distance of 25 km from each other, in Khagrachari District near the Indian border. The population within the catchment area, estimated at 100 000, comprises a mix of indigenous and Bengali people. The rainy season is from May to October. The two MSF clinics were attended by a total of 57 000 patients from March 2000 till October 2002. About 60% of these patients had fever or fever complaints and therefore had their blood checked for malaria (by microscopy). Forty-four per cent of all slides were positive, 84% with *P. falciparum* mono-specific or mixed species infections, 15% *P. vivax* and 1% *P. malariae*. Infections were distributed quite evenly across the population. During the first 9 months of 2002, we tested 15 268 patients and found malaria infections in 42% of children under 5 years ($n = 1805$), in 48% of children 5–14 years old ($n = 2691$) and in 41% of adults 15 years and older ($n = 2189$). Around 46% of *P. falciparum* patients were females (similar in the three age groups).

Patients were selected for the study according to the following criteria: at least 1 year old, *P. falciparum* infection (mono or mixed) with more than 1000 asexual malaria parasites microlitre, fever (axillary temperature of 37.5 °C or higher) or a history of fever. Patients with a parasitaemia higher than 100 000 but not above 250 000 parasites per microlitre were included only when no additional general danger signs were present. Parasitaemia of 250 000/µl (or 5% of red blood cells infected) was considered a sign of severity and treated with parenteral drugs in accordance with the national guidelines. Excluded were pregnant women, patients with signs or symptoms of severe malaria or with another febrile disease requiring treatment. Patients fitting these criteria and not living too far from the clinic were given standard information on the nature and purpose of the study. The patient was included only after he/she (or their parent or caregiver) gave verbal informed consent.

All patients received the same treatment: Q, three times daily at 30 mg base/kg/day for 3 days followed by a single dose of sulphadoxine (25 mg/kg) combined with pyrimethamine (1.25 mg/kg) on the fourth day. Q administration was semi-observed on a daily basis: one dose of Q was taken in the clinic and two doses were given to take at home, with comprehensive explanation before and questioning afterwards; SP treatment was given in the clinic under observation.

The study procedures were based on WHO guidelines to monitor antimalarial drug efficacy (WHO 1996, 2002). Patients were asked to return on day 3, 7, 14, 21 and 28, and any other day when feeling ill. They were traced in their homes when absent. Blood slides were collected at each visit. If present, *P. falciparum* trophozoites were counted using the parasite/white blood cell (WBC) count, assuming a standard density of 8000 WBC/µl of blood. Slide results were cross-checked by experienced malaria microscopists of MSF and an external reference laboratory (Chittagong Medical College Hospital). On days 0 and 14, the haemoglobin value of the blood was checked with the Lovibond-comparator method.

The efficacy of treatment was evaluated by the patients' parasitological response, and fever or fever complaints were carefully recorded to give an indication of clinical response. Cumulative failure rates were calculated at

days 14 and 28. Following WHO guidelines for classification of early treatment failures, patients with moderate levels of parasites in their blood (below 25% of that on day of admission) on day 3 but no fever were not considered as treatment failures. Patients with parasites in their blood before day 7 and fever, as well as patients with parasitaemia at any day after day 7 irrespective of axillary temperature, were retreated with Q 30 mg/kg/day for 7 days. Data were analysed with SPSS (SPSS Inc., Chicago, IL, USA) and Epi Info (version 6; CDC, Atlanta, GA, USA). Continuous data were analysed by *t*-test and proportions were assessed by the chi-square test with Yates correction or Fisher's exact test.

The local community and authorities were informed on the procedures of the study. Patients enrolled in the study were given full clinical attention and treated according to the usual protocols similar to regular clients; they came back for follow-up visits entirely voluntarily but received a small financial incentive to compensate for travel-cost and time lost.

Finger prick blood samples were collected on filter paper (*c*. 30 μl) for genotyping by PCR from all patients at day 0 before taking treatment, and again in case of treatment failure. Recurrent infections were identified by PCR analysis using methods described elsewhere (Brockman *et al.* 1999). For genotyping parasite mutations in drug resistance genes, DNA was prepared from blood spots using the Gentra systems extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA extractions were concurrently prepared with plain Whatman paper discs to control for contamination during DNA preparation: these samples were also used as negative controls during PCR. We genotyped the amino acid K76T mutation in *pfcrt* and the N86Y mutation in pfmdr using a simple PCR restriction digest assay and fluorescent detection of products. We amplified a 132-bp section of *Pfcrt* and a 216-bp section of *pfmdr* using semi-nested PCR reactions, digested the PCR products with *Apo*I, and measured the size of the digestion products on an ABI 3100 capillary sequencer. DNA from both CQ resistant (7G8) and sensitive (3D7) parasites was used as positive controls (Anderson *et al.* 2003). For *dhfr* and *dhps*, we used a rapid primer extension technique to genotype all five mutations in each gene simultaneously (Nair *et al.* 2002).

Results

During the period from August to October 2002, a total of 203 patients were enrolled in the study, 107 from Panchari clinic and 96 from Dighinala clinic. The baseline characteristics of the patients admitted to the study are shown in Table 1. The age/sex distribution of these groups was similar to the *P. falciparum* patients normally presenting to the two clinics. In children (under 15 years), the parasitaemia at the day of admission was significantly higher than in the adult group (*t*-test log-transformed parasite densities, $t = 2.5$, $P < 0.05$). The children in the age groups from 1 to <5 years and 5 to 14 years also had significantly higher body temperatures than the adults (*t*-test: $t = 6.4$, $P < 0.001$) and lower haemoglobin values ($t = 6.9$, $P < 0.001$).

Of 203 patients admitted to the study, 17 were lost during follow-up (8.4%), 10 in Panchari and seven in Dighinala. Altogether, 186 patients completed the study, of whom only seven missed one visit.

Parasitological response

The crude parasitological failure rate was 17.2% at day 28 (Table 2). Dighinala and Panchari clinics showed similar results. Failures were related to parasitaemia on admission (*t*-test: $t = 3.1$, $P < 0.01$); the geometric mean parasitaemia of infections for which treatment failed was 17 677 parasites/μl *vs*. 12 210 parasites/μl in successfully treated cases. The risk of treatment failure in patients with a density over 100 000 parasites/μl ($n = 7$) was significantly higher than in patients with a lower parasitaemia [57% *vs*. 16%, RR = 3.63 (95% CI 1.76–7.51)]. Younger infants and children were found to be more at risk of *P. falcipa-*

Table 1 Baseline characteristics of patients admitted to the study

	<5 years	5–14 years	≥15 years	Total
Number of patients	34	103	66	203
Sex: number of F/M (% F)	13/20 (39)	41/62 (40)	27/39 (41)	81/122 (40)
Age (years)*	2.8 (1–4.5)	8.7 (5–14)	24.8 (15–72)	13.1 (1–72)
Weight (kg)*	11.5 ± 2.2 (7.5–16.0)	22.7 ± 8.3 (12–50)	47.4 ± 6.9 (29–75.0)	28.8 ± 15.2 (7.5–75)
Haemoglobin (g/dl)*	9.5 ± 1.9 (5.3–11.7)	11.1 ± 1.8 (6.7–16.7)	12.7 ± 2.0 (6.7–16.7)	11.3 ± 2.2 (5.3–16.7)
Temperature (°C)*	38.0 ± 1.1 (36.4–40.0)	38.1 ± 1.2 (35.6–40.6)	36.9 ± 0.9 (35.0–39.6)	37.7 (35–40.6)
Parasite density (per μl)†	17 501 (1236–135 944)	14 328 (963–255 250)	9414 (1043–87 553)	12 965 (963–255 250)

* Values are given as mean ± SD (range).
† Values representing geometric mean (range).

Table 2 Assessment of quinine–sulphadoxine/pyrimethamine treatment failure rate

	<5 years		5 to <15 years		≥15 years		Total	
	n	% (95% CI)	*n*	% (95% CI)	*n*	% (95% CI)	*n*	% (95% CI)
Number of patients	34		103		66		203	
Dropout	3	8.8	6	5.8	8	12.1	17	8.4
Failure rate day 14	5/32	15.6 (3.0–28.2)	2/102	2.0 (0–4.7)	1/60	1.7 (0–5.0)	8/194	4.1 (1.3–6.9)
Failure rate day 28	8/31	25.8 (21.0–41.2)	19/97	19.6 (11.7–27.5)	5/58	8.6 (1.4–15.8)	32/186	17.2 (11.8–22.6)
PCR corrected day 28*	5/31	16.6 (3.5–29.7)	15/97	15.5 (8.3–22.7)	4/58	6.9 (0.4–13.4)	24/186	12.9 (8.1–17.7)

* Missing PCR data indicate failures.

rum recrudescence after Q–SP treatment than adults: crude failure rates at day 14 were significantly higher in infants than in older children [RR = 7.97 (1.62–39.1), $\chi^2 = 9.2$, $P < 0.01$] and in the adult group [RR = 9.38 (1.14–76.9), $\chi^2 = 6.7$, $P < 0.01$]. Furthermore, at day 28 the failure rate was higher in infants than in adults [RR = 2.99 (1.07–8.37), $\chi^2 = 4.8$, $P < 0.05$].

During follow-up, 16 of 186 patients (9%) developed *P. vivax* infections (at days 21–28). Except for the one mixed *Pf/Pv* infection, these patients were treated with CQ. They were kept in the study group, but the CQ may have interfered with the outcome of their follow-up, as none failed.

PCR-corrected response

Genotyping of 101 blood samples with *P. falciparum* infections yielded 132 genotypes, reflecting a wide degree of genetic diversity in the parasite population, based on the three gene loci examined (merozoite surface proteins-1 and -2, and glutamate rich protein). The average number of *P. falciparum* genotypes per infection was 1.3. The most common genotype was recorded in six of 132 genotypes, giving a probability of detecting the same genotype both pre- and post-treatment by chance alone of 0.045. This justified the assumption that identical three-locus genotypes occurring pre- and post-treatment in the same patient must be recrudescent.

Of the 32 patients who returned with a *P. falciparum* infection, 28 paired samples were collected. Of these 28 samples, six failed to produce a result due to inability to amplify DNA in the post-treatment sample. The results of the 22 remaining samples showed eight novel infections and 14 recrudescent infections. Table 2 shows the PCR-corrected day 28 cumulative failure rates, assuming that samples without PCR results were failures. Corrected failure rates no longer showed a significant difference among age groups.

The parasite and gametocyte rates during the time of follow-up are shown in Figure 1. At day 3 after treatment, 14 patients (7.3%) still had parasitaemia, but only one

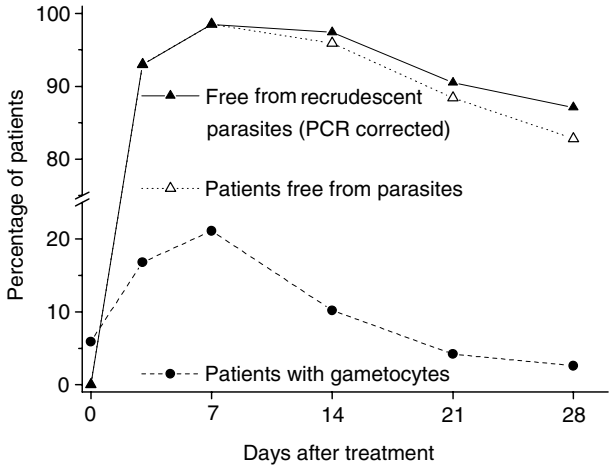

Figure 1 Parasitological and gametocidal response during 4 weeks after quinine–sulphadoxine/pyrimethamine treatment.

patient had fever, and was therefore classified as a failure and retreated. The other 13 all cleared their parasites by day 7 without additional treatment. Within 4 weeks, 31 patients became positive for *P. falciparum*, with eight of 186 PCR-confirmed novel infections (4%).

Gametocytes were found in few patients at admission (6.2%). During follow-up, another 53 patients (28%) had gametocytes in their blood at one or more follow-up visits. The gametocyte rate rose at days 3 and 7 to 21%, but declined after that (Figure 1).

Clinical response

Of all 32 failures (in 186 cases), 17 cases had fever or a history of fever and were categorized as clinical failures: one early clinical failure (0.5%) and 16 late clinical failures (8.6%), and the remaining 15 cases were late parasitological failures (8.1%).

The mean (SD) haemoglobin values showed a small increase after treatment: from 11.3 (2.2) g/dl at day 0 to 11.7 (1.6) g/dl at day 14. There was no clear difference

between the haemoglobin change of patients with treatment failures and those without.

Patients complained mainly of mild symptoms, such as dizziness, headache, nausea, itching, which may have been an effect of the treatment and disease alike. Three patients reported loss of hearing. One patient had an allergic reaction to Q: a few large bluish-black patches (drug eruptions) appeared symmetrically on arms and legs at day 2 of Q treatment but no other signs of allergy were seen and the patches disappeared after Q treatment was stopped. Twenty patients received additional non-malaria treatment for diseases diagnosed during the follow-up period (respiratory tract infections, ear infection, eye infection, worms).

Drug resistance markers

Of the 203 samples, three were not genotyped for *dhfr*, while four, five and 16 samples were not genotyped for *pfcrt*, *dhps* and *pfmdr*, respectively. Low levels of multiple infections were observed: 12 of 199 samples (6%) carried multiple genotypes at *pfcrt*, 14 of 187 (7%) had multiple infections at *pfmdr*, while 18 of 200 (9%) and 22 of 198 (11%) had multiple bases present in at least one of the five sites in *dhfr* and *dhps*. We analysed only infections carrying single alleles for each locus.

The prevalence of the CQ resistance-related *Pfcrt* and *Pfmdr* mutant alleles was particularly high: 94% of parasites (176/187) carried the 76T mutation at *pfcrt*, while 70% (121/173) carried the 86Y mutation at *pfmdr*. In addition, key mutations for SP resistance at *dhfr* and *dhps* were very common. At *dhfr*, six different alleles were identified: no parasites contained sensitive, wild-type *dhfr* alleles, 1% (1/182) had a single resistance mutation, whereas 74% (135/182) had double mutations, 25% (45/182) triple mutations and 1% (1/182) quadruple mutations. The mutation 164L, which results in high-level resistance to pyrimethamine, was found in 7% of cases (12/186). Eight *dhps* alleles were identified: 5% (9/176) of parasites were wild type, 22% (39/176) had one mutation and 36% (62/176) had two mutations, while 38% (66/176) had three mutations. The genotype data are summarized in Table 3(a).

Table 3 Allele frequencies of mutations in *dhfr*, *dhps*, *pfcrt* and *pfmdr*: (a) of all samples analysed and (b) of samples from patients with known outcome of treatment, i.e. 'sensitive' or 'recrudescent' infections

	(a) Result of samples of all patients ($n = 203$)*			(b) Result related to clinical response to Q + SP	
	Amino acids†	Count‡	Frequency‡	Sensitive ($n = 162$)*	Recrudescent ($n = 16$)*
DHFR	A***IRNL***	1	0.01	0.01	0
	A***IRN***I	34	0.19	0.15	0.20
	A***ICN***I	5	0.03	0.03	0.07
	AN***RN***I	130	0.71	0.74	0.67
	AN***RNL***	11	0.06	0.07	0.07
	ANC***N***I	1	0.01	0.01	0
	ANCSI	0	0	0	0
DHPS	S***GEG***A	2	0.01	0.01	0
	AGEAA	64	0.36	0.39	0.15
	S***G***K***G***A	45	0.26	0.22	0.46
	AGKAA	4	0.02	0.02	0
	S***GE***AA	12	0.07	0.05	0.23
	AA***E***AA	1	0.01	0.01	0
	S***G***KAA	39	0.22	0.25	0.15
	SAKAA	9	0.05	0.05	0
PfCRT-76	***T***	176	0.94	0.95	0.93
	K	11	0.06	0.05	0.07
PfMDR-86	***Y***	121	0.70	0.73	0.57
	N	52	0.30	0.27	0.43

* Total numbers are given above, but numbers genotyped for each locus may be lower (see text).

† The five letter codes for *dhfr* show amino acid residues at positions 16, 51, 59, 108 and 164, while codes for *dhps* show amino acids at positions 436, 437, 540, 581 and 613, respectively. Amino acids conferring resistance are shown in bold italic. Alleles are listed in order of decreasing resistance.

‡ Counts and frequencies are based only on samples with single alleles, multiple infections were excluded from analyses.

Relation of molecular markers with clinical response to Q–SP therapy

There were 32 patients who had a *P. falciparum* infection in the follow-up period and 154 who remained negative following treatment. We were able to identify eight novel and 14 recrudescent infections by PCR analysis. Another two early failures at days 3 and 7 are presumed recrudescent as well. This divides the patients in two groups: 162 'sensitive' infections (treatment success or coming back with new infection) and 16 'recrudescent' ones (identified by PCR or early failure).

The Q–SP recrudescent strains all had multiple mutations at *dhfr* and one or more mutations at the *dhps* site. However, the frequency of the various allele combinations found in *dhfr*, *dhps*, *pfcrt* and *pfmdr* sites did not differ significantly between sensitive and recrudescent infections (Table 3b). Presumed SP refractory genotypes with triple or quadruple mutations in *dhfr* were not more frequent in the recrudescent infections (31%, 4/13) than the sensitive ones (23%, 33/145; Fisher's exact test, two-tailed, $P = 0.50$), neither were the SP resistance-enhancing double or triple mutations at *dhps*, which were found in 83% (10/12) of recrudescent and in 70% (99/141) of sensitive strains (Fisher's exact test, $P = 0.51$). Even in the more vulnerable groups, young children and patients with high-density infections, we could not find a relationship between the molecular markers of resistance in *dhfr* and *dhps* and treatment failures. Gametocytaemia at any day during the follow-up period, known to be enhanced by *dhfr* and *dhps* mutations (Mendez *et al.* 2002), also appeared unrelated to the number of mutations at these loci (*dhfr* single or double *vs*. more than two mutations: $\chi^2 = 1.76$, $P = 0.18$; *dhps* wild type or single mutations *vs*. two or three mutations: $\chi^2 = 1.18$, $P = 0.27$).

Discussion

The genotyping of isolates from the 203 patients' pre-treatment blood samples showed that the majority of patients had a genetically CQ-resistant *P. falciparum* infection: 94% had the 76T mutation in *pfcrt* and 70% 86Y at *pfmdr1*. High prevalence of *pfcrt* mutations has been reported from various South-east Asian countries, whereas *pfmdr* mutation rate is generally lower than what was found here (Labbe *et al.* 2001; Berens *et al.* 2003; Ngo *et al.* 2003; Pickard *et al.* 2003). Patients carrying parasites with the 76T *pfcrt* mutation have a 30–50% chance of CQ treatment failure, and probably even more when the 86Y *pfmdr* mutation is also present (Djimde *et al.* 2001; Jelinek *et al.* 2002). This is consistent with the high *in vitro* and *in vivo* CQ resistance reported in Bangladesh (Rahman *et al.* 1998, 2001; Noedl *et al.* 2003).

At present CQ is still used as a first line antimalarial in many treatment facilities in Bangladesh. The large group of patients returning for second line treatment normally receives short course Q + SP combination therapy. Our results show that the Q + SP combination treatment for *P. falciparum* malaria patients is still relatively effective in the CHT of Bangladesh. The failure rate in this 28-day follow-up survey was 12.9% when new infections identified with PCR were taken into account. Nevertheless, there are several reasons for concern when Q + SP therapy is continued to be used as common second line treatment in Bangladesh.

First, parasites in this region have clearly developed resistance to SP. In Bangladesh resistance to SP has been documented previously (in unpublished data, Government of Bangladesh 1993, quoted in Rahman *et al.* 2001). The genotyping data reported here indicate that resistant alleles at both *dhps* and *dhfr* have spread to Bangladesh as a result of SP use, as recently described for other SE Asian countries (Nair *et al.* 2003; Ngo *et al.* 2003). Single or double mutant *dhfr* generally have increased parasite clearance times and higher post-treatment gametocyte carriage than wild-type parasites, while parasites with three or four resistance mutations are refractory to SP treatment. Mutations in *dhps* play a limited role on their own, but in the presence of mutations in *dhfr*, resistance to SP is enhanced (Plowe *et al.* 1998; Kublin *et al.* 2002). Hence, we can expect that at least the quarter of the parasite population that shows triple or quadruple mutations in *dhfr* will not respond adequately to SP treatment, in particular the 20% also carrying one or more mutations at *dhps*. Another factor contributing to the rapid spread of antifolate resistance is the high level of gametocytes after SP treatment. Gametocytaemia is enhanced by SP and this might increase malaria transmission and promote the spread of drug resistance (von Seidlein *et al.* 2001; Mendez *et al.* 2002). In our clinical trial on Q–SP efficacy, SP-resistant genotypes were not more frequently observed in recrudescent than in sensitive infections, although the differences were in the predicted direction. This suggests that failures of this therapy may not result from SP resistance alone.

Secondly, the Q short course (3 days) component of the Q–SP combination therapy may be insufficiently effective. So far, no clinical failures from Q (7 days) were found in Bangladesh, but parasitological failures have been reported (Rahman *et al.* 2001), as well as a low level of *in vitro* resistance to Q (Noedl *et al.* 2003). Hence, Q resistance may also contribute to the patterns observed. Furthermore, SP-resistant *P. falciparum* strains may not be adequately

eliminated by the Q + SP combination, because Q is a slow-acting drug and therefore unable to kill most parasites in 3 days (White 1998). Moreover, some patients may not have complied with the complete Q treatment, because we were only able to give one of the three daily doses under observation in the clinic.

Thirdly, children and patients with high parasitaemia are at increased risk of failure from Q + SP treatment. We found that the proportion of children with parasites in the blood within 28 days after treatment is higher than that of adults, which is attributable to recrudescent and novel infections. Treatment failures were related to higher parasite densities. Patients with a parasitaemia above 100 000/μl appeared to be at a much higher risk of failure. The parasite rates in children (up to 15 years) were generally higher than in the adults.

Fourthly, the actual percentage of patients with recurring infections after Q + SP treatment may be higher than indicated in our 28-day study. Failures from SP can occur 4–6 weeks after treatment (Mabuza *et al.* 2001). Higher failure rates of Q + SP treatment than our study have been documented in Bangladesh, Cox's Bazar District, previously. Crude parasitological failure rates of 20% were observed in a 14-day assessment (Rahman *et al.* 1998) and 34% in a 28-day assessment (Rahman *et al.* 2001), without PCR analyses to distinguish novel and recrudescent infections. In our study, the crude parasitological failure rate at day 28 was 17%. PCR analyses identified one-fourth of recurrences as 'novel' infections. However, there remains a possibility of underestimating the true recrudescence rate if a post-treatment strain was present but not amplified in the pre-treatment sample, which can occur if the density was below the limit of PCR detectability (Brockman *et al.* 1999).

We suggest a change in malaria treatment protocols in the CHT region of Bangladesh. Artemisinin combination therapy is now advocated by WHO and the worldwide scientific malaria community and is the aim for all MSF projects in *P. falciparum* endemic areas. Combination therapies including artemisinin or its derivatives have proven to be excellent drugs for the treatment of malaria. These drugs also reduce post-treatment levels of gametocytes, thereby reducing transmission and protecting partner drugs from developing resistance. The radical treatment and reduced transmission by artemisinin have shown to substantially reduce the incidence of malaria (Price *et al.* 1996; White 1998; White *et al.* 1999; Nosten *et al.* 2000). We predict that combination therapies including artemisinin derivatives would result in improved treatment efficacy and decreased mortality of *P. falciparum* infected patients in the CHT region of Bangladesh. However, combinations with SP should not be adopted, as levels of resistance to these drugs are already high.

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References

Anderson TJ, Nair S, Jacobzone C, Zavai A & Balkan S (2003) Molecular assessment of drug resistance in *P. falciparum* from Bahr El Gazal Province, Sudan. *Tropical Medicine and International Health* **8**, 1068–1073.

Berens N, Schwoebel B, Jordan S *et al.* (2003) *Plasmodium falciparum*: correlation of in vivo resistance to chloroquine and antifolates with genetic polymorphisms in isolates from the south of Lao PDR. *Tropical Medicine and International Health* **8**, 775.

Brockman A, Paul R, Anderson T *et al.* (1999) Application of genetic markers to the identification of recrudescent *Plasmodium falciparum* infections on the northwestern border of Thailand. *American Journal of Tropical Medicine and Hygiene* **60**, 14–21.

Djimde A, Doumbo OK, Steketee RW & Plowe CV (2001) Application of a molecular marker for surveillance of chloroquine-resistant falciparum malaria. *Lancet* **358**, 890–891.

Fidock DA, Nomura T, Talley AK *et al.* (2000) Mutations in the *P. falciparum* digestive vacuole trans-membrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell* **6**, 861–871.

Foote SJ, Kyle DE, Martin RK *et al.* (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* **345**, 255–258.

Jelinek T, Aida AO, Peyerl-Hoffmann G *et al.* (2002) Diagnostic value of molecular markers in chloroquine-resistant *falciparum* malaria in Southern Mauritania. *American Journal of Tropical Medicine and Hygiene* **67**, 449–453.

Kublin JG, Dzinjalamala FK, Kamwendo DD *et al.* (2002) Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *Journal of Infectious Diseases* **185**, 380–388.

Labbe AC, Bualombai P, Pillai DR *et al.* (2001) Molecular markers for chloroquine-resistant *Plasmodium falciparum* malaria in Thailand and Laos. *Annals of Tropical Medicine and Parasitology* **95**, 781–788.

Mabuza A, Govere J, Durrheim D *et al.* (2001) Therapeutic efficacy of sulfadoxine-pyrimethamine in uncomplicated *Plasmodium falciparum* malaria 3 years after introduction in Mpumalanga. *South African Medical Journal* **91**, 975–978.

Mendez F, Munoz A, Carasquilla G *et al.* (2002) Determinants of treatment response to sulfadoxine-pyrimethamine and

subsequent transmission potential in falciparum malaria. *American Journal of Epidemiology* **156**, 230–238.

Montanari RM, Bangali AM, Talukder KR *et al.* (2001) Three case definitions of malaria and their effect on diagnosis, treatment and surveillance in Cox's Bazar district, Bangladesh. *Bulletin of the World Health Organisation* **79**, 648–656.

Nair S, Brockman A, Paiphun L, Nosten F & Anderson TJ (2002) Rapid genotyping of loci involved in antifolate drug resistance in Plasmodium falciparum by primer extension. *International Journal of Parasitology* **32**, 852–858.

Nair S, Williams JT, Brockman A *et al.* (2003) A selective sweep driven by pyrimethamine treatment in SE Asian malaria parasites. *Molecular Biology and Evolution* **20**, 1526–1536.

Ngo T, Duraisingh M, Reed M, Hipgrave D, Biggs B & Cowman AF (2003) Analysis of pfcrt, pfmdr1, dhfr, and dhps mutations and drug sensitivities in *Plasmodium falciparum* isolates from patients in Vietnam before and after treatment with artemisinin. *American Journal of Tropical Medicine and Hygiene* **68**, 350–356.

Noedl H, Faiz MA, Yunus EB *et al.* (2003) Drug-resistant malaria in Bangladesh: an in vitro assessment. *American Journal of Tropical Medicine and Hygiene* **68**, 140–142.

Nosten F, van Vugt M, Price R *et al.* (2000) Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. *Lancet* **356**, 297–302.

Pickard AL, Wongsrichanalai C, Purfield A *et al.* (2003) Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. *Antimicrobial Agents and Chemotherapy* **47**, 2418–2423.

Plowe CV, Kublin JG & Doumbo OK (1998) *P. falciparum* dihydrofolate reductase and dihydropteroate synthase mutations: epidemiology and role in clinical resistance to antifolates. *Drug Resistance Updates* **1**, 389–396.

Price RN, Nosten F, Luxemburger C *et al.* (1996) Effects of artemisinin derivatives on malaria transmissibility. *Lancet* **347**, 1654–1658.

Rahman MR, Faiz MA, Yunus EB, Mujibul Hoq AJM & Chowdhury MK (1996) Malaria: new clinical case definitions and treatment guidelines. *Journal of the Chittagong Medical College Training Association* **7**, 75–82.

Rahman MR, Hassan MR, Faiz MA, Paul B & Jalil MA (1998) Monitoring efficacy of commonly used antimalarials by a 14-day in-vivo test in a new settler's camp in endemic zone at Cox's Bazar. *Bangladesh Medical Research Council Bulletin* **24**, 67–74.

Rahman MR, Paul DC, Rashid M *et al.* (2001) A randomised controlled trial on the efficacy of alternative treatment regimens for uncomplicated *falciparum* malaria in a multidrug-resistant falciparum area of Bangladesh – narrowing the options for the National Malaria Control Programme? *Transactions of the Royal Society of Tropical Medicine* **95**, 661–667.

Rosenberg R (1982) Forest malaria in Bangladesh. III. Breeding habits of *Anopheles dirus*. *American Journal of Tropical Medicine and Hygiene* **31**, 192–201.

von Seidlein L, Drakeley C, Greenwood B, Walraven G & Targett G (2001) Risk factors for gametocyte carriage in Gambian children. *American Journal of Tropical Medicine and Hygiene* **65**, 523–527.

Smithuis FM, Moni F, Grundl M *et al.* (1997) *Plasmodium falciparum*: sensitivity in vivo to chloroquine, pyrimethamine/sulfadoxine and mefloquine in western Myanmar. *Transactions of the Royal Society of Tropical Medicine* **91**, 468–472.

White NJ (1998) Preventing antimalarial drug resistance through combinations. *Drug Resistance Update* **1**, 3–9.

White NJ, Nosten F, Looareesuwan S *et al.* (1999) Averting a malaria disaster. *Lancet* **353**, 1965–1967.

WHO (1996) Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense transmission. WHO, Geneva. WHO/MAL/96.1 077.

WHO (2002) Monitoring antimalarial drug resistance. WHO, Geneva. WHO/CDS/RBM/2003.39.

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