**PLASMODIUM FALCIPARUM: SELECTION OF SERINE 108 OF DIHYDROFOLATE REDUCTASE DURING TREATMENT OF UNCOMPPLICATED MALARIA WITH CO-TRIMOXAZOLE IN UGANDAN CHILDREN**

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**Abstract.** *In vivo* testing for resistance of *Plasmodium falciparum* to co-trimoxazole (trimethoprim/sulfamethoxazole) was performed in Uganda in 41 children with uncomplicated malaria, and blood samples were screened before and after treatment for polymorphisms in the antifolate target genes for dihydrofolate reductase (DHFR) and dihydropyrimidinase (DHPS). Selection towards a specific genotype at some codons of the DHFR and DHPS genes was observed in samples collected after exposure to co-trimoxazole drug pressure. The alleles 51-isoleucine, 59-arginine, and 108-serine of DHFR were significantly associated with clinical resistance, as was allele 581-alanine of DHPS. Resistance against antifolate combinations probably requires resistance-related polymorphisms in both the DHFR and the DHPS genes. In addition, it appears that the trimethoprim-resistant DHFR genotype differs from that for pyrimethamine at residue 108.

Falciparum malaria and acute respiratory tract infection, two of the most common causes of childhood morbidity and mortality in sub-Saharan Africa, show an overlap of symptoms in a high proportion of cases. The World Health Organization (WHO) recommends co-trimoxazole (trimethoprim and sulfamethoxazole) as treatment for children in malaria-endemic areas presenting with fever and respiratory symptoms. This drug has been shown in the past to be effective against malaria as well as pneumonia. The components of co-trimoxazole target selectively dihydrofolate reductase (DHFR) and dihydropyrimidinase synthetase (DHPS), the same folate pathway enzymes as pyrimethamine/sulfadoxine, which is used in many African countries for treatment of chloroquine-resistant infections with *Plasmodium falciparum*. *In vitro* drug resistance of *P. falciparum* to pyrimethamine has been associated with point mutations in the gene coding for DHFR, in particular with the presence of asparagine in position 108. Likewise, polymorphisms at 5 highly conserved positions within DHPS have been reported in sulfadoxine-resistant isolates of *P. falciparum*: codons 436, 437, 581, 613, and 540. Unlike DHFR, no single polymorphism has been associated with all resistant strains and only limited data are available on the global distribution of these polymorphisms. In *in vivo* testing for resistance to co-trimoxazole was performed in rural health centers in the 2 districts of Kabarole and Bundibugyo in western Uganda. Results of the clinical and parasitologic outcomes have been reported elsewhere. Forty-one children with symptomatic malaria from the Kabarole and Bundibugyo Districts of western Uganda were recruited for the study. Data were obtained about polymorphisms in the DHFR and DHPS genes that have been associated with antifolate resistance by extraction and amplification of parasite DNA from filter paper and thick blood films. We report on a comparison of the prevalence of polymorphisms of the antifolate target genes at days 0 and 3/7 and their association with *in vivo* results in one subsample of these children.

**Patients, Materials, and Methods**

Forty-one children (24 boys) 6 months to 5 years of age (mean ± SD age = 1.4 ± 1.0 years) with uncomplicated malaria were enrolled. Inclusion criteria were mono-infection with *P. falciparum* with at least 300 parasites/μl, no intake of sulfa drugs during the last 4 weeks, no signs of complications, no history of allergic reactions to sulfonamides, and informed consent of a parent or guardian. The study was reviewed and approved by the University of Munich and the Ministry of Health of Uganda. Co-trimoxazole tablets (Sanavita, Weiterstadt, Germany) containing 100 mg of sulfamethoxazole and 20 mg of trimethoprim were used. Treatment was given over a 5-day period in two daily doses according to age (6–18 months = 4 tablets/day, 18 months–3 years = 6 tablets/day; 3–4 years = 8 tablets/day). Based on the weight range of these children derived from earlier surveys in the area, the dosage was estimated to be 40–60 mg/kg of body weight/day of sulfamethoxazole and 8–12 mg/kg of body weight/day of trimethoprim. In accordance with current recommendations of WHO, only the first dose was given under supervision of health staff. However, mothers were encouraged to comply and questioned with respect to compliance during subsequent visits. Cases that needed a change of treatment during follow-up were treated with a standard oral dose of quinine over a 7-day period. Children were seen again on days 3, 7, and 14 and on any other day in between in case of symptoms. At each visit, the occurrence of fever and other symptoms during past 24 hr were recorded and temperature and parasite density were measured. Children whose parents admitted having given other antimalarials during the period of study where excluded. Similarly, patients who developed signs of complications were immediately transferred for adequate treatment and excluded from the study. There was no active follow-up of patients at their homes but a small incentive was given if follow-up was completed. Thick blood films were prepared from fingerprick blood, stained, immediately read by an experienced laboratory assistant, and later re-read. Outcome of
treatment was defined following recommendations by WHO\(^2\) in four classes: early treatment failure (parasite density on day 3 > 25% of day 0 with or without measurable fever or signs of complicated malaria on or before day 3), late treatment failure (fever or history of fever associated with parasitemia any time after day 3), asymptomatic resistance (after clinical cure at day 3 no fever or history of fever [24 hr] and persisting or reappearing parasites present on day 14), and sensitive (after clinical cure [day 3] no fever or history of fever [24 hr] and no parasites on day 14). At the first visit of each child, before initiation of treatment, 10 \(\mu\)l of blood was dotted on Whatman (Maidenstone, United Kingdom) 3MM\(^2\) filter paper and air-dried at room temperature.

The dried blood spots were transported to the laboratories of the London School of Hygiene and Tropical Medicine and stored at \(-20^\circ\)C before further analysis. Preparation of DNA from filter paper was performed as previously described in detail.\(^2\)\(^2\) Following a modified procedure used for DNA extraction from material derived from Egyptian mummies,\(^2\)\(^4\) blood slides taken at days 3 or 7 after treatment were incubated in 10 mM Tris-\(\mathrm{HCl}\), 2 mM EDTA, 0.1\% sodium dodecyl sulfate, 10 mg/ml of dithiothreitol, and 0.5 mg/ml of proteinase K for 16 hr at 37\(^\circ\)C. Subsequently, the dissolved material was extracted with phenol-chloroform. A nested polymerase chain reaction (PCR) protocol was used in all samples. For the first round, the following primer pairs were used: M1 (5\'-TTT-ATG-ATG-GAA-CAA-GTC-TGC-3') and M5 (5\'-AGT-ATA-TAC-ATG-AGA-3') for the DHFR gene and R2 (5\'-AAC-CTA-AGC-GTC-TTC-AA-3') and R/(5\'-ATT-TGT-GTT-ATT-TGT-CTG-GAC-3') for the DHPS gene. Denaturation at 94\(^\circ\)C was performed for 3 min, followed by 40 cycles of denaturation at 94\(^\circ\)C for 1 min, annealing at 50\(^\circ\)C for 2 min, and extension at 72\(^\circ\)C for another 2 min, with a final extension at 72\(^\circ\)C for 10 min.

For the second round of PCR, the DHFR gene, 2 \(\mu\)l of DNA by the M1-M5 primer pair was added to each of 2 PCR mixtures: F (5\'-GAA-ATG-TAA-TTC-CCT-AGA-TAT-Gga-A-TA-TT-3') and M4 (5\'-TAA-ATT-TCC-CAA-GTA-AAA-CTA-TTA-GAG-CTT-3') or M3 (5\'-TTT-ATG-GAA-CAA-GAC-Tgg-GAC-GTT-TTC-AA-3') and R/(5\'-ATT-TGT-GTT-ATT-TGT-CTG-GAC-3') for the DHPS gene. Denaturation at 94\(^\circ\)C was performed for 3 min, followed by 5 cycles of denaturation at 94\(^\circ\)C for 1 min, annealing at 50\(^\circ\)C for 2 min, and extension at 72\(^\circ\)C for 2 min. The PCR mixture, 1 unit of Taq DNA polymerase was used per 50 \(\mu\)l with 1.5 mM MgCl\(_2\) and 200 \(\mu\)M of each dNTP. Primers were used at a concentration of 0.25 \(\mu\)M. The products of the second round were confirmed by electrophoresis in an ethidium bromide–stained 1% agarose gel prior to restriction digestion.

The PCR product was incubated with restriction enzymes according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). The product of the primer pair F-M4 was digested with \(\text{Alu}\) I at codon 108 (serine). In the same PCR product, \(\text{Bst}\) NI detected the 108-threonine mutation and \(\text{Xmn}\) I detected 59-arginine. The amplified product of the M3-F/primer pair was digested with \(\text{Dra}\) I to detect 164-leucine and with \(\text{Bsr}\) I to detect 108-asparagine. A restriction site for \(\text{Nla}\) III in this PCR product was destroyed by the mutation from 16-alanine to 16-valine and the restriction site for \(\text{Tsp}509I\) at 51-asparagine was destroyed by the mutation to 51-isoleucine. For the DHPS gene, the product of K-K/ was digested with \(\text{Mnl}\) I to identify 436-serine, \(\text{Msp}\) I to detect 436-alanine, \(\text{Ava}\) II to detect 437-glycine, \(\text{Mwo}\) I to detect 437-alanine, and \(\text{Fok}\) I to detect 540-glutamic acid. The product of L-L/ was digested with \(\text{Bsp}\) UI to detect 581-alanine, \(\text{Bst}\) I to detect 581-glycine, \(\text{Mwo}\) I to detect 613-alanine, and \(\text{Age}\) I to detect 613-threonine or \(\text{Bsa}\) WI to detect 613-threonine and serine. The DNA from established laboratory strains of \(P.\) falciparum served as controls of the PCR and enzyme digests.

RESULTS

Among the 41 children recruited to this survey, median parasite density before treatment was 3,460 parasites/\(\mu\)l (range = 640–407,200). Twenty-three children (56.1\%) presenting as treatment failures were classified as being infected with resistant strains of \(P.\) falciparum. Of these, 5 children were diagnosed as early failures at day 3 and 18 others were classified as resistant at day 7. In all of these cases, the median parasitemia was 5,880/\(\mu\)l (range = 1,800–62,400/\(\mu\)l) at days 3/7 after treatment. Co-trimoxazole failures were treated with quinine, and there were no treatment failures on this drug.

We compared prevalence of polymorphisms at day 0 and days 3/7 in resistant cases. Twenty-nine (70.7\%) of the 41 \(P.\) falciparum samples isolated at day 0 showed multilocal infection with at least two clones present. These mixed samples complicated interpretation of the data since polymorphisms were frequently present simultaneously, especially in samples selected on day 0 before drug selection pressure was applied to the parasite populations. No polymorphisms were observed in codons 16 and 164 of DHFR and in 613 of DHPS, and 436-phenylalanine in DHPS or 108-threonine in DHFR was not detected. In samples collected after exposure to co-trimoxazole drug pressure, selection towards a specific genotype at some codons of the DHFR and DHPS genes could be observed (Table 1). The high number of multilocal infections in the investigated population (36 of the 41 samples showed different alleles in at least on codon) accounts for double entries at most codons. Therefore, added percentages of most codons at day 0 are > 100\%. At days 3/7, drug pressure obviously led to selection of a single genotype in relevant codons. In contrast to day 0, all 23 individual samples from resistant cases consisted of a single type at the loci studied. Prevalence of 51-asparagine, 59-cysteine, and...
108-asparagine of DHFR and 581-glycine of DHPS had decreased to zero ($P < 0.001$ in all cases, by chi-square test with Yates’ correction). The prevalence of the alternative alleles at these loci, i.e., 51-isoleucine, 59-arginine, and 108-serine of DHFR and 581-alanine of DHPS consequently became 100% (Figures 1 and 2). In addition, the prevalence of 437-glycine was decreased significantly ($P = 0.0266$, by Yates’ corrected chi-square test) in resistant isolates, indicating selection for alanine at this position. The prevalence of 108-serine in DHFR was significantly lower ($P = 0.004$, by Fisher’s exact test) in pretreatment samples from cases responding to co-trimoxazole. Conversely, the prevalence of 581-glycine in DHPS was higher ($P < 0.001$, by Yates’ corrected chi-square test) in these cases (Table 1 and Figure 2).

**DISCUSSION**

Since co-trimoxazole has been used for decades in African countries for treatment of lower respiratory infections, it was likely that resistant clones of *P. falciparum* had evolved in some areas. Therefore, an assessment of the prevalence of resistance appears mandatory before this drug is widely used for malaria treatment. Several studies focusing on the determination of genetic changes as the basis of clinical resistance have been conducted. Polymorphisms in various codons of the DHFR and DHPS genes have been associated with in *vivo* resistance to antifolate drug combinations in laboratory clones and field isolates.\(^5\) The mutation from serine to asparagine at position 108 of DHFR has been shown to be selected during treatment *in vitro* with sulfadoxine and pyrimethamine.\(^6\)

In this study of 41 children with symptomatic malaria from the Kabarole and Bundibugyo Districts of western Uganda, data were obtained about polymorphisms in the DHFR and DHPS genes that have been associated with antifolate resistance \(^5\,5\,14\,16\,18\,27\) by extraction and amplification of parasite DNA from filter paper and thick blood films. We report on a comparison of prevalence of polymorphisms at day 0 and days 3/7 in resistant cases. In samples collected after exposure to co-trimoxazole drug pressure, selection towards a specific genotype at some codons of the DHFR and DHPS genes could be observed (Table 1 and Figures 1 and 2). The alleles 51-isoleucine, 59-arginine, and 108-serine in DHFR were significantly associated with clinical resistance, as was allele 581-alanine, and to a lesser extent, 437-alanine in DHPS. From a consideration of the shift of allelic frequency after treatment within the parasite population investigated in this study, we infer selection. These findings suggest that resistance against combinations of antifolates probably requires a range of resistance-related polymorphisms in both the DHPS and the DHFR genes. According to this rationale, isolated polymorphisms in one of these genes will not necessarily lead to clinical resistance to a drug combination. The sulfadoxine-resistant strain K1 has DHPS glycine-581 and DHFR 108-asparagine, 51-asparagine, and 59-arginine.\(^7\) This suggests that polymorphisms determining resistance to different sulfonamides may vary, possibly due to relevant structural differences between sulfadoxine and sulfamethoxazole. More interestingly, the 108-serine of DHFR, selected by co-trimoxazole, is recognized as indicative of sensitivity to pyrimethamine. However, an early clinical study comparing the quinine-, chloroquine-, pyrimethamine-, and proguanil-resistant Camp strain from Southeast Asia in sensitivity to pyrimethamine alone and sulfadoxine alone appeared not to be absolute.\(^9\) There was a marked reduction in sensitivity to pyrimethamine alone and sulfadoxine alone in the chloroquine-, pyrimethamine-, and sulfadoxine-resistant Thailand K1 strain compared with the sensitive F32 Tanzanian strain, while sensitivities to trimethoprim and sulfamethoxazole in the two strains were similar. In excluding patients with multiclonal infections from data...
evaluation, 14 samples were left. Among these, the presence of glycine-581 in DHPS was significantly associated with sensitivity to co-trimoxazole ($P = 0.0143$, by Mantel-Haenszel test). Supporting this observation, the alternative allele 581-alanine, if present alone, was significantly associated with resistance (Table 2). The detection of only 581-alanine proved to be a predictor of in vivo resistance in this group with high sensitivity (100%, positive predictive value = 93.3%). Due to the high number of multiclonal infections in the investigated population, this result was clouded by a larger group of samples exhibiting both alleles. However, in studies among patients from hypoendemic and mesoendemic areas, where a far lower number of multiclonal infections is to be expected, the detection of 581-alanine on DHPS could prove to be a very useful to in predicting resistance.

Our observations strengthen and give a rationale for previous findings, since they indicate that use of trimethoprim is likely to be more successful where resistance to pyrimethamine is prevalent. However, the results presented here need to be confirmed by DNA sequencing of the DHFR and DHPS genes in resistant samples. Recent work shows that there is also a possibility that resistance to different sulfonamides may relate to the presence of different alleles of DHPS. Indeed, the DHFR genotype 59-arginine and 108-serine has been reported as anomalous, albeit, with a 16-serine. However, we believe that 51-isoleucine/59-arginine/

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108-serine is the genotype selected by co-trimoxazole in this study. Transfection of P. falciperum with this genotype, which is now feasible, could confirm its role in co-trimoxazole resistance. Examination of DHFR and DHPS codon polymorphisms by nested PCR and subsequent restriction fragment length polymorphism appears to be a useful approach for the detection of P. falciperum resistance against co-trimoxazole. Analysis of individual samples can give an understanding of combinations of allelic differences that might be important for development of resistance. However, in an area of high transmission, the high number of multiclonal infections that can be expected makes prediction of the outcome of individual cases difficult, although it should be very useful in population based surveys in areas with lower endemicity as an indicator of for first- and second-line drug recommendations.

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