**PLASMODIUM FALCIPARUM RESISTANCE TO SULFADOXINE/PYRIMETHAMINE IN UGANDA: CORRELATION WITH POLYMORPHISMS IN THE DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHETASE GENES**

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**Abstract.** The efficacy of sulfadoxine/pyrimethamine (S/P) in treatment of uncomplicated falciparum malaria in Africa is increasingly compromised by development of resistance. The occurrence of active site mutations in the *Plasmodium falciparum* gene sequences coding for dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) is known to confer resistance to pyrimethamine and sulfadoxine. This study investigated the occurrence of these mutations in infected blood samples taken from Ugandan children before treatment with S/P and their relationship to parasite breakthrough by day 7. The results confirm the occurrence of mutations in DHFR and DHPS that were significantly selected under S/P pressure at day 7: a combination of alleles 51-isoleucine and 108-asparagine in DHFR, and 436-serine, 437-alanine, 540-lysine and 581-alanine in DHPS, appears to play a major role in the development of in vivo resistance in *P. falciparum* strains against S/P. Therefore, earlier results derived from isolates from hyperendemic areas in Tanzania were confirmed by this investigation.

The antifolate combination of sulfadoxine/pyrimethamine (S/P) is experiencing an increasingly widespread use in Africa, making it to one of the major first- and second-line drugs. After replacement of chloroquine, clinical resistance to S/P has arisen rapidly in other areas of the world. Monitoring and, if possible, delaying the spread of S/P-resistance is therefore a major public health objective in affected African countries. All efforts to control resistance will require methods that make it possible to map S/P-resistant malaria quickly and accurately in epidemiologic studies. Currently used *in vitro* and *in vivo* methods for the measurement of drug resistance in *Plasmodium falciparum* are not well-suited for fast epidemiologic surveillance. Techniques that facilitate screening of a large number of samples without unduly straining scarce financial resources are needed.

Drug resistance of *P. falciparum* to pyrimethamine has been associated with point mutations in the gene coding for dihydrofolate reductase (DHFR), in particular with the occurrence of asparagine in position 108. Polymorphisms at 5 highly conserved positions within dihydropteroate synthetase (DHPS) have been reported in sulfadoxine-resistant isolates of *P. falciparum*: codons 436, 437, 581, 613 and also at 540. Recently, alteration of 437-alanine to glycine has been identified as key residue on DHPS for the development of sulfadoxine resistance.

The occurrence of resistance of *P. falciparum* to pyrimethamine was first reported from east Africa in 1954. In the same area in northern Tanzania, resistance to pyrimethamine was observed after 5 months of prophylactic use of weekly doses in 1957. Studies on S/P resistance in Uganda have been much more recent, generally showing very high rates of sensitivity. As part of a survey of antimalarial susceptibility, *in vivo* testing for resistance to S/P was performed in a rural health center in the 2 districts of Kabarole and Bundibugyo in western Uganda. We report on associations between *in vivo* results and polymorphisms of the antifolate target genes in a subsample of these children.

**Patients, Materials, and Methods**

**In vivo testing.** One hundred twenty-two children 6 months to 5 years of age with uncomplicated malaria were enrolled. Inclusion criteria were monoinfection 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. All enrolled children were treated with a single dose of sulfadoxine-pyrimethamine tablets (Fansidar®; F. Hoffman LaRoche, Basel, Switzerland), following the dosage advice of the manufacturer. Cases that needed a change of treatment during follow-up were treated with standard oral dose of quinine over a 7-day period. Children were seen again on days 3, 7, 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 them other antimalarials during the period of study were 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 (a large bar of soap) was given if follow-up was completed. Thick blood films were prepared from fingerprick blood, stained with Giemsa, immediately read by an experienced laboratory assistant, and later re-read. Outcome of treatment was defined following recommendations of the World Health Organization (WHO) into four classes: early treatment failure (E; 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 (L; fever or history of fever associated with parasitemia any time after day 3), asymptomatic resistance (R; 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 (S; 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 μl of blood was dotted on Whatman (Maidstone, United Kingdom) 3MM® filter paper and air-dried at room temperature.

463
Isolation and amplification of DNA. The DNA was isolated from either filter paper spots or thick films. Isolation of DNA from filter paper was performed as described in detail.14,15 Following a modified procedure used for DNA extraction from material derived from Egyptian mummies,16 blood slides taken at day 7 were incubated in 10 mM Tris-HCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate, dithiothreitol (10 mg/ml), and proteinase K (0.5 mg/ml) for 16 hr at 37°C. Subsequently, the dissolved material was extracted with phenol-chloroform. A nested polymerase chain reaction (PCR) method was used for all samples. Two microliters of purified DNA were added to a final reaction volume of 50 µl. In the first round, a 648-basepair (bp) portion of the DHFR gene was amplified by use of the primers M1 (5'-TTT-ATG-ATG-GAA-CAA-GTC-TGC-3') and M5 (5'-AGT-ATA-TAC-ATC-GCT-AAC-AGA-3'). Similarly, a 710-bp portion of the DHPS gene was amplified by use of R2 (5'-AAC-CTA-AAC-GTG-CTG-TTC-AA-3') and R/ (5'-AAT-TGT-GTG-ATT-TCT-GCA-AA-3'). For the second round of the DHFR gene, 2 µl of amplified DNA from the M1-M5 primer pair were added to each of 2 PCR mixtures: F/ (5'-GAA-ATG-TAA-TCT-AGA-TAT-GgA-ATA-TT-3') (lower case letters indicate variable nucleotides) and M4 (5'-TTA-ATT-TCC-CAA-GTA-AAA-CTA-TTA-GAg-CTT-C-3') to detect 59-arginine, 108-serine, and 108-threonine, or M3 (5'-TTT-ATG-ATG-GAA-GaC-TgG-GAG-GTT-3') and F/ (5'-AAA-TTC-TTG-GTA-AAC-AAC-GGA-ACC-Ttt-TA-3') to amplify the fragments containing 16-alanine, 51-arginine, 108-asparagine, and 164-leucine. Similarly, for the DHPS gene, DNA amplified with the R2/R/ primer pair was added to each of 2 PCR mixtures: K (5'-TGC-TAG-TGT-TAT-AGA-TAT-AGG-atG-AgC-ATC-3') and K/ (5'-CTA-CAA-CGA-GGT-ATT-CAA-AAT-gCA-AGA-A-3') to detect 436-serine, 436-alanine, 437-glycine, 437-alanine, 540-glutamic acid, or L (5'-ATA-GGA-TAC-TAT-TTG-GTA-AGc-GAA-GTA-3') and L/ (5'-TAT-TAC-AAC-ATT-GTC-ATT-Cgc-GAA-ACC-GG-3') for 581-alanine, 581-glycine, 613-threonine, 613-serine, and 613-alanine. The primer pair K/ and J (5'-TGC-TAG-TGT-TAT-AGA-TAT-AGG-TGG-AGA-Aag-C-3') was used to detect 436-phenylalanine. Positive and negative controls were included in all amplification procedures.

Restriction fragment length polymorphisms (RFLPs). Depending on the quality of the bands in the control gel, 2–5 µl of PCR product were incubated with restriction enzymes according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). The 326-bp product of the primer pair F-M4 was digested with Alu I at codon 108 (serine). In the same PCR product, Bst NI detected the 108-threonine mutation and Xml I detected 59-arginine. The amplified product of the M3-F/ primer pair (522 bp) was digested with Dra I to detect 164-leucine and with Bsr I to detect 108-asparagine. A restriction site for Nla III in this PCR product was destroyed by the mutation from 16-alanine to 16-valine and the restriction site for Tsp 509I at 51-asparagine was destroyed by the mutation to 51-isoleucine. For the DHPS gene, the 438-bp product of K-K/ was digested with Mnl I to identify 436-serine, Msp Al to detect 436-alanine, Ava II to detect 437-glycine, Mwo I to detect 437-alanine, and Fok I to detect 540-glutamic acid. The 161-bp product of L-L/ was digested with Bst UI to detect 581-alanine, Bst I to detect 581-glycine, Mwo I to detect 613-alanine, and Age I to detect 613-threonine or Bsa WI to detect 613-threonine and serine. All digested products were separated by electrophoresis in a 1% SeaKem® plus 1% NuSieve® (both from FMC BioProducts, Rockland, ME) gel. The DNA from established laboratory strains of *P. falciparum* served as controls of PCR and enzyme digests. The strains used were K1 (Thailand), FC27 (Papua New Guinea), FCR3 (The Gambia), W2 (Indochina), V1/S (Vietnam), and 7G8 (Brazil).

RESULTS

Among the 122 children recruited into this survey, the mean parasite density before treatment was 10,086 parasites/µl (range = 464–49,600). Fifteen children (12.3%) who presented as treatment failures were classified as being infected with resistant strains of *P. falciparum*. Of these, 3 children were diagnosed as early failures at day 3, 7 were classified as resistant at day 7, and 5 at day 14. In all these cases, the mean parasitemia was 1,814/µl (range = 20–17,520/µl) at days 3/7 after treatment. The S/P failures were treated with quinine and there were no treatment failures with this drug.

At day 0 before treatment, only 21 (17.2%) of the 122 *P. falciparum* isolates presented with a homogenous allelic picture: all other samples showed polymorphisms in at least one codon of the DHFR or DHPS genes. Various alleles were observed at 51-asparagine/isoleucine, 59-cysteine/arginine, and 108-serine/asparagine on the DHFR gene and at 436-alanine/serine, 437-alanine/glycine, 540-lysine/glutamic acid on the DHPS gene (Table 1). Similar to previous studies in other areas of east Africa,17,18 no polymorphisms were detected at codons 16-alanine and 164-isoleucine of DHFR and at codons 581- and 613-alanine of DHPS. At day 0, significant associations between polymorphisms on either the DHFR or the DHPS gene (or a combination of these) and clinical S/P resistance were not apparent (Table 1). The high number of multyclonal infections in the investigated population accounts for double entries at most codons. Therefore, added percentages of most codons at day 0 are above 100% in Table 1.

The DNA from thick blood smears from day 3 and/or 7 after S/P treatment were examined for genetic polymorphisms and results were compared with the filter spots from day 0. Of the 15 isolates that were defined as resistant, 8 (53.3%) had monoclonal infections at day 0. Although the allelic distribution was highly uniform in most isolates, significant differences to S/P-sensitive samples with monoclonal infections were observed at day 3 (Table 2). At days 3/7, drug pressure lead to selection of a single genotype in relevant codons. In contrast to day 0, all 15 individual samples from resistant cases consisted of a single type at 66.7% of the loci studied. The prevalence of 51-asparagine and 108-serine of DHFR and 436-alanine and 581-glycine of DHPS had decreased to zero while 59-arginine of DHFR and 437-alanine and 540 glutamic acid of DHPS were reduced to one sample each (Table 1).

DISCUSSION

Changing drug resistance patterns in *P. falciparum* strains and diminishing public health resources in Africa require the
development of fast, reliable, and affordable methods for the determination of drug resistance. A technique that is able to determine developing, but not yet clinically apparent, drug resistance could be of great value in deciding on regional drug policies. A series of studies concerning the determination of genetic changes as the basis of clinical resistance have been conducted. The combination of S/P is widely used in Africa and has been promoted by WHO as an alternative to chloroquine for the rapid treatment of uncomplicated malaria. The combination of S/P is widely used in Africa and has been promoted by WHO as an alternative to chloroquine for the rapid treatment of uncomplicated malaria. As an epidemiologic approach, the determination of polymorphisms in various codons of the DHFR and DHPS genes has been previously used to estimate the amount of parasite resistance against pyrimethamine in west Africa, and this method has been proposed for surveillance purposes. As shown in Tables 1 and 2, the comparison of resistant and sensitive isolates in monoclonal and multiclonal infections on day 0 produced no significant differences in allelic prevalence. These findings correlate with previous findings from studies on S/P resistance in Tanzania and cast a shadow on the predictive value of statements on evolving drug resistance in hyperendemic areas that are solely based on the prevalence of DHFR and DHPS polymorphisms. Under S/P drug pressure, selection towards multiclonal infections were present in the majority of samples: only 21 isolates (17.2%) presented with a homogenous genotype in the investigated codons. This is consistent with previous findings from highly endemic areas, where multiclonal infections seem to be the rule rather than the exception. As an epidemiologic approach, the determination of polymorphisms of the DHFR gene has been previously used to estimate the amount of parasite resistance against pyrimethamine in west Africa, and this method has been proposed for surveillance purposes. As shown in Tables 1 and 2, the comparison of resistant and sensitive isolates in monoclonal and multiclonal infections on day 0 produced no significant differences in allelic prevalence. These findings correlate with previous findings from studies on S/P resistance in Tanzania and cast a shadow on the predictive value of statements on evolving drug resistance in hyperendemic areas that are solely based on the prevalence of DHFR and DHPS polymorphisms. Under S/P drug pressure, selection towards a certain genotype became obvious when results from isolates from days 3/7 were compared with those obtained before treatment (Table 1). The findings of this study, taking into account changes in consecutive years and the influence of treatment on DHFR and DHPS polymorphisms, suggest that alleles 51-isoleucine, 59-cysteine, and 108-asparagine on DHFR and 436-serine, 437-glycine, 540-glutamic acid, and 581-alanine on DHPS play a major role in the development of in vivo resistance in P. falciparum strains against S/P. This confirms previous observations on laboratory strains and field isolates. 

Judging from the results of this study, examination of DHPS codon polymorphisms by the nested PCR and sub-

**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Codon</th>
<th>Sensitive (%) (n = 167)</th>
<th>Resistant (%) (n = 17)</th>
<th>Total (%) (n = 184)</th>
<th>After Days 3/7 (%) (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR</td>
<td>51-asparagine</td>
<td>22 (13.7)</td>
<td>1 (6.7)</td>
<td>23 (13.7)</td>
<td>15 (100)</td>
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<tr>
<td></td>
<td>51-isoleucine</td>
<td>100 (95.3)</td>
<td>15 (100)</td>
<td>115 (95.3)</td>
<td>15 (100)</td>
</tr>
<tr>
<td></td>
<td>59-cysteine</td>
<td>97 (90.7)</td>
<td>14 (93.3)</td>
<td>111 (91)</td>
<td>14 (93.3)</td>
</tr>
<tr>
<td></td>
<td>59-arginine</td>
<td>39 (36.4)</td>
<td>4 (26.7)</td>
<td>43 (35.2)</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td></td>
<td>108-serine</td>
<td>38 (35.5)</td>
<td>5 (33.3)</td>
<td>43 (35.2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>108-asparagine</td>
<td>99 (92.5)</td>
<td>15 (100)</td>
<td>114 (93.4)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>DHPS</td>
<td>436-serine</td>
<td>101 (94.4)</td>
<td>15 (100)</td>
<td>116 (95.1)</td>
<td>15 (100)</td>
</tr>
<tr>
<td></td>
<td>436-alanine</td>
<td>16 (14.9)</td>
<td>4 (26.7)</td>
<td>20 (16.4)</td>
<td>0</td>
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<tr>
<td></td>
<td>437-serine</td>
<td>46 (43.0)</td>
<td>4 (26.7)</td>
<td>50 (41.6)</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td></td>
<td>437-glycine</td>
<td>102 (95.3)</td>
<td>14 (93.3)</td>
<td>116 (95.1)</td>
<td>14 (93.3)</td>
</tr>
<tr>
<td></td>
<td>540-lysine</td>
<td>103 (95.3)</td>
<td>14 (93.3)</td>
<td>117 (95.9)</td>
<td>14 (93.3)</td>
</tr>
<tr>
<td></td>
<td>540-glutamic acid</td>
<td>73 (58.2)</td>
<td>20 (13.3)</td>
<td>76 (62.3)</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td></td>
<td>581-alanine</td>
<td>100 (93.5)</td>
<td>15 (100)</td>
<td>115 (94.3)</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>
sequent RFLP appears to be a useful approach for the detection of
P. falciparum resistance against S/P in areas with lower endemicity. The approach of predicting evolving S/P resistance by results from prevalence studies on polymorphisms on the DHFR and DHPS genes is unreliable in hyperendemic areas. Also, the high number of multicolon infections that can be expected in any semi-immune population in such areas makes an individual diagnosis by this method difficult. However, it should be helpful in population-based surveys in areas with lower endemicity as an indicator of for first- and second-line drug recommendations.

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