January 2004

Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy: Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase mutations in monitoring in vivo resistance

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Abstract. Prior to the 2001 malarial treatment policy change in Tanzania, we conducted trials to assess the efficacy of sulfadoxine-pyrimethamine (SP) and the usefulness of molecular markers in monitoring resistance. A total of 383 uncomplicated Plasmodium falciparum malaria patients (between 6 and 59 months old) were treated with SP and their responses were assessed. Mutations in the P. falciparum dihydrofolate reductase (pfdhfr) and dihydropteroate synthase (pfdhps) genes in admission day blood samples were analyzed. Results indicated that 85.6% of the patients showed an adequate clinical response, 9.7% an early treatment failure, and 4.7% a late treatment failure. The quintuple mutant genotype (pfdhfr 51 Ile, 59 Arg, and 108 Asn and pfdhps 437 Gly and 540 Glu) showed an association with treatment outcome (odds ratio = 2.1; 95% confidence interval = 0.94–4.48, \(P = 0.045\)). The prevalence of the triple pfdhfr mutant genotype (51 Ile, 59 Arg, and 108 Asn) at a site of high SP resistance (23.6%) was four times higher compared with that observed at sites of moderate SP resistance (6.8–14.4%) (\(P = 0.000001\)). The genotype failure index calculated by using this marker was invariable (1.96–2.1) at sites with moderate SP resistance, but varied (3.4) at a site of high SP resistance. In conclusion, our clinical and molecular findings suggest that SP may have a short useful therapeutic life in Tanzania; thus, its adoption as an interim first-line antimalarial drug. The findings also point to the potential of the triple pfdhfr mutant genotype as an early warning tool for increasing SP resistance. These data form the baseline SP efficacy and molecular markers profile in Tanzania prior to the policy change.

INTRODUCTION

In 2001, the Tanzania mainland adopted sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) as first- and second-line antimalarial drugs, respectively, following increased chloroquine (CQ) resistance (45–70%). The use of SP for first-line purposes is an interim measure while different antimalarial combinations are being evaluated for long-term use. Before this change, SP was used as a second-line antimalarial drug. Several other countries in southern Africa including Kenya, Burundi, Rwanda, the Tanzania islands (Zanzibar), and Malawi have switched to SP, AQ, or artesunate (AS) monotherapies or combination therapies, whereas Uganda opted for the SP/CQ combination following widespread CQ resistance. Sulfadoxine-pyrimethamine is one of the few, cheap, and relatively safe antimalarial drugs that is still effective against CQ-resistant malaria in Africa. Recent studies in southern Africa have recorded high efficacies, ranging from 82% to 92%. However, the fact that Plasmodium falciparum rapidly develops resistance to SP following wide use of the drug poses a serious threat to malarial control efforts in endemic countries. High levels of SP resistance have been recorded in a highly endemic northeastern part of Tanzania where pyrimethamine and sulfadoxine were used at different periods between 1950 and 1994 for prophylactic and therapeutic trials, respectively. In a recent study conducted in this area, 45% of the patients treated with SP failed to clear their parasitemias to below patenty levels on day 7. This failure rate is substantially higher compared with 25% in Ifakara (southeastern Tanzania) and 26% in Kigoma (western Tanzania), both of which are also highly endemic areas in Tanzania, but in which SP had not been widely used. Therefore, it is obvious that following wide use of SP in Tanzania, resistance is likely to increase rapidly. Given appropriate tools, the National Malaria Control Program (NMCP) framework provides a better platform for regularly updating information on antimalarial drug resistance situation in Tanzania. Currently, the in vivo efficacy test is the gold standard method for monitoring antimalarial drug resistance in countries endemic for malaria. However, the method is expensive and complex in terms of interpreting outcomes, especially in high transmission areas where chances of re-infection are high. Thus, the need for a cheap, rapid, and reliable epidemiologic tool for SP surveillance has been recognized.

Molecular markers of SP resistance are considered to be a cheap and less complex candidate tool for in vivo SP resistance surveillance. There is a large body of data showing that a combination of mutations in the P. falciparum dihydrofolate reductase (pfdhfr) (51 Ile, 59 Arg, and 108 Asn) and dihydropteroate synthase (pfdhps) (437 Gly and 540 Glu) genes might constitute a useful marker for field surveillance of SP resistance in Africa. However, the usefulness of these markers remains controversial because other investigators did not establish an association with treatment outcome. Furthermore, some new mutations in the pfdhfr gene have been discovered; thus, their roles in vivo resistance must be assessed. New approaches for understanding the relationship between mutations and antimalarial drug resistance have been suggested. The genotype resistance index (GRI) and the genotype failure index (GFI) concepts have been pointed out as practical models using a pfcr 76 Thr mutation.
in the surveillance of CQ resistance. There is a need to verify such models (by using the \textit{pfdhfr} and \textit{pfdhps} gene markers) in areas where SP is used as the first-line antimalarial drug.

As a preparation for the policy change, we conducted studies to determine SP efficacy and prevalence of SP resistance molecular markers (\textit{pfdhfr} and \textit{pfdhps} gene mutations) in Tanzania. We also assessed the applicability of these markers in monitoring SP resistance. The findings presented here form the baseline SP efficacy and molecular markers profile for Tanzania and support the decision made by the Ministry of Health to adopt SP as an interim first-line antimalarial drug. Our findings also present evidence of association between treatment failure and quintuple mutant genotype. The prevalence of mutant genotypes and GFI values in high versus moderate resistance sites point to the potential of the triple \textit{pfdhfr} mutant genotype as an early warning tool for increasing SP resistance in Tanzania. Nonetheless, we recommend further studies, at both community and health facility levels, to verify the usefulness of \textit{pfdhfr} and \textit{pfdhps} genotypes in estimating SP resistance.

**MATERIALS AND METHODS**

**Study sites.** These trials were carried out in Butimba, Kyela, Masasi, Mkuzi, and Mlimba Rural Health Centers in Tanzania. These areas are antimalarial drug resistance surveillance sites of the NMCP, classified epidemiologically as mesoendemic (Kyela and Butimba) or holoendemic (Mkuzi, Mlimba, and Masasi), and are located in different geographic areas in the country (Figure 1). The catchment areas for these health facilities are rural-based communities of similar socioeconomic background.

**Recruitment of study subjects.** All patients between 6 and 59 months old who reported to the health centers were evaluated and considered for recruitment by the study team. Detailed medical histories were obtained and clinical examinations were conducted. Thick and thin smears were made from finger prick blood and stained with Giesma for parasite detection and identification by microscopy. Patients were eventually recruited for study if they had an axillary temperature $\geq 37.5\,^\circ{C}$, microscopically confirmed \textit{P. falciparum} monoinfection.
fections (parasitemia between 2,000 and 100,000 asexual stage parasites/μL), no history of antimalarial use in the last 14 days prior to the episode, an absence of co-infection with other diseases, and consent from parents or guardians. Patients who had mixed *Plasmodium* spp. infections, severe malaria or danger signs, history of allergy to sulfa drugs, or other chronic infections were not recruited for study but, respectively, were given appropriate treatment by the study team.

**Treatment of patients.** Recruited patients were treated with SP (Fansidar®, 500 mg of sulfadoxine and 25 mg of pyrimethamine; Roche, Basel, Switzerland) in a single oral dose of 1.25 mg/kg of pyrimethamine and 25 mg/kg of sulfadoxine and observed for 30 minutes. If vomiting occurred within this period, a replacement dose was administered and again observed for an additional 30 minutes. Further vomiting led to exclusion of the patient from the study. These patients were rescued by parenterally administered quinine (nine doses of 30 mg/kg). Parents or guardians of recruited children were asked to return to the health centers for response evaluation on days 2, 3, 7, and 14 post-treatment. In addition, they were advised to return at any other (unscheduled) day if temperature or sickness persisted or relapsed. Patients who did not turn up for scheduled follow-ups were visited at home by a member of the study team. Clinical and parasitologic examinations were conducted on each follow-up day. A patient was withdrawn from the study if any of the following occurred during the follow-up period: development of a concurrent infection, treatment with another antimalarial drug, the patient could not be traced at a home visit on a scheduled day or the day after, or the parent/guardian requested that the patient be withdrawn from the study. Treatment responses were classified as an adequate clinical response (ACR), an early treatment failure (TF), and a late treatment failure (LTF) as described in the 1996 World Health Organization (WHO) *in vivo* efficacy testing protocol for areas of intense transmission. Patients who failed to respond were treated with amodiaquine (10 mg/kg for dose 1 and 2 and 5 mg/kg for dose 3). At the end of the study, 414 patients were recruited (67 in Butimba, 70 in Kyela, 78 in Masasi, 133 in Mkuzi, and 66 in Mlimba). Thirty-one cases were either lost to follow-up or excluded from the study during follow-up. Thus, 383 patients completed the study or were followed-up to day of failure. The study was reviewed and approved by both the institutional (Ifakara Health Research and Development Centre [IHRDC] Ethics Committee) and national (Medical Research Coordinating Committee) authorities and consent was obtained from parents or guardians prior to recruitment of each patient.

**Blood sample collection, extraction of DNA, and mutation analysis.** Before treatment of recruited patients, finger prick blood was spotted onto filter paper (3MM; Whatman International Ltd., Maidstone, United Kingdom), air-dried, transported to the IHRDC laboratory, and stored dry in self-sealing plastic bags at room temperature until required for extraction of DNA. The DNA was extracted from the filter paper using the Chelex extraction method previously described.** Polymorphisms in *pfdhfr* codons 51, 59, 108, and 164 and *pfdhps* codons 436, 437, 540, 581, and 613 were determined by performing primary and nested polymerase chain reaction (PCR) amplifications with subsequent restriction fragment length polymorphism (RFLP) analysis of the nested PCR products as described in detail elsewhere.** The RFLP products were resolved by electrophoresis on 10% polyacrylamide gels, stained with ethidium bromide, photographed, and scored. A 2 × 2 chi-square table was used to analyze associations between clinical and molecular data and Epitable in Epi-Info (Centers for Disease Control and Prevention, Atlanta, GA and World Health Organization, Geneva, Switzerland) and was used to compare differences in the prevalence of SP resistance and molecular markers in the study sites. *P* values < 0.05 (and confidence intervals [CIs] > 1 for odds ratio [OR]) were considered significant. The GFI was calculated as the ratio of the prevalence of resistant genotype to the prevalence of drug failure, and the variability of the values among study sites was assessed by linear regression.

**RESULTS**

**Treatment outcome for SP and association with the quintuple mutant genotype.** Of 383 SP treated patients, 328 (85.6%) showed ACR with highest level of efficacy (93%) being recorded in Butimba and the lowest (76.4%) in Mkuzi. Fifty-five (14.4%) cases did not respond to SP treatment of which 37 (9.7%) and 18 (4.7%) were ETF and LTF cases, respectively (Table 1). There was no significant difference in the prevalence of SP treatment failure in Butimba, Kyela, Masasi, and Mlimba (χ² = 2.52, degree of freedom [df] = 3, *P* = 0.4723), but significant difference was observed (χ² = 15.06, df = 4, *P* = 0.0046) when the Mkuzi Health Center was included in the analysis. Table 2 relates the clinical and molecular data for the SP-treated patients. Of 55 treatment failure cases 12 (22%) and 43 (78%) carried parasites with quintuple and non-quintuple genotypes, respectively. Of the 328 patients who showed ACR, 39 (12%) and 289 (88%) individuals harbored the quintuple and non-quintuple (any other combination of genotypes apart from quintuple) genotypes, respectively. Statistical analysis showed association between the quintuple mutant genotype and SP treatment failure (OR = 2.1, 95% CI = 0.94-4.48, *P* = 0.045). Although the lower 95% CI was slightly less than 1, a Pearson chi-square test (χ² = 4.0) indicated that this represented a statistically significant association (Table 2). In a separate analysis, the triple *pfdhfr* mutant and the double *pfdhps* mutant genotype did not show a predictive value for SP treatment failure.

**Prevalence of multiple *pfdhfr* and *pfdhps* mutant genotypes and estimated GFI values.** The prevalence of SP resistance and *pfdhfr* and *pfdhps* genotypes is summarized and shown in Figure 1. Mkuzi showed highest prevalence of triple *pfdhfr* (80.3%) and double *pfdhps* (32.3%) mutant genotypes, while

**TABLE 1**

<table>
<thead>
<tr>
<th>Site</th>
<th>No.</th>
<th>ETF</th>
<th>LTF</th>
<th>Overall TF</th>
<th>ACR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butimba</td>
<td>57</td>
<td>4</td>
<td>0</td>
<td>4 (7%)</td>
<td>53  (93%)</td>
</tr>
<tr>
<td>Kyela</td>
<td>67</td>
<td>5</td>
<td>3</td>
<td>8 (12%)</td>
<td>59  (88%)</td>
</tr>
<tr>
<td>Masasi</td>
<td>73</td>
<td>4</td>
<td>1</td>
<td>5 (6.8%)</td>
<td>68  (92.9%)</td>
</tr>
<tr>
<td>Mkuzi</td>
<td>127</td>
<td>21</td>
<td>9</td>
<td>30 (23.6%)</td>
<td>97  (76.4%)</td>
</tr>
<tr>
<td>Mlimba</td>
<td>59</td>
<td>3</td>
<td>5</td>
<td>8 (13.5%)</td>
<td>51  (86.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>383</td>
<td>37</td>
<td>18</td>
<td>55 (14.4%)</td>
<td>328 (85.6%)</td>
</tr>
</tbody>
</table>

*ETF = early treatment failure; LTF = late treatment failure; TF = treatment failure; ACR = adequate clinical response.*
Table 2
Assessment of association between pf dhfr and pf dhps genotypes and treatment outcome

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TF</th>
<th>ACR</th>
<th>OR (95% CI)</th>
<th>Chi-square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quintuple mutants</td>
<td>12</td>
<td>39</td>
<td>2.1 (0.94-4.48)</td>
<td>4.0</td>
<td>0.045</td>
</tr>
<tr>
<td>Non-quintuple</td>
<td>43</td>
<td>289</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>328</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a pf dhfr = Plasmodium falciparum dihydrofolate reductase; pf dhps = P. falciparum dihydrololate synthase; TF = Treatment failure; ACR = adequate clinical response; OR = odds ratio; CI = confidence interval; quintuple = pf dhfr 108 Ame, 51 Le, 59 Arg and pf dhps 164 Leu mutations in any of 123, 51, 59, and 164 respectively.*

Table 3
GFI s calculated by using different combinations of mutations in pf dhfr and pf dhps as markers of SP resistance in five sentinel sites in Tanzania

<table>
<thead>
<tr>
<th>Site</th>
<th>Overall TF (%)</th>
<th>GFI* triplet ahy</th>
<th>GFI* Double ahy</th>
<th>GFI* Double ahy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butimba</td>
<td>7</td>
<td>2.1</td>
<td>9.64</td>
<td>2.76</td>
</tr>
<tr>
<td>Kyela</td>
<td>12</td>
<td>1.99</td>
<td>5.3</td>
<td>1</td>
</tr>
<tr>
<td>Masasi</td>
<td>6.8</td>
<td>1.98</td>
<td>5.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Mkuzi</td>
<td>23.6</td>
<td>3.4</td>
<td>4.4</td>
<td>1.37</td>
</tr>
<tr>
<td>Mlimba</td>
<td>13.5</td>
<td>1.96</td>
<td>6.4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*TF = Treatment failure; GFI = genotype failure index (subscripts are markers used to calculate the GFI). For definitions of other abbreviations, see Tables 1 and 2.

Mkuzi showed the lowest prevalences of 18.6% and 3.4%, respectively. There was no difference in the prevalence of the triple pf dhfr mutant genotype (χ² = 0.12, df = 3, P = 0.9893) at the Butimba, Kyela, and Masasi, and Mkuzi sites. However, a significant difference (χ² = 131, df = 4, P = 0.000001) is observed when the Mkuzi site was included in the analysis. Conversely, the prevalence of the double pf dhfr mutant genotype was significantly different (χ² = 12, df = 3, P = 0.0074) at the Butimba, Kyela Masasi, and Mkuzi sites and more so (χ² = 39, df = 4, P = 0.00012) when Mkuzi was included in the analysis. Similarly, the prevalence of pure wild pf dhfr and pf dhps genotypes was different among the low resistance sites (χ² = 12.3, df = 3, P = 0.006345 and χ² = 49.4, df = 3, P = 0.00011, respectively). Using the prevalence of different combinations of mutations in pf dhfr and pf dhps as a marker for SP resistance, we calculated the GFI and observed that only the triple pf dhfr mutant genotype generated invariable indices (ranging from 1.96 to 2.1) in moderate resistance areas (Butimba, Kyela, Masasi, and Mlimba), suggesting a relationship between the marker and SP treatment failure. The GFI observed in Mkuzi (a high resistance area) was 3.4, which was different from that observed in other sites. Indices derived by other markers (combination of triple and double pf dhfr or double pf dhps mutant genotypes) are highly variable (Table 3) and do not suggest any relationship with treatment failure. We did not detect pf dhps 436 Ala/Phe, 581 Gly, and 613 Thr/Ser and pf dhfr 164 Leu mutations in any of our study sites.

DISCUSSION

In 2001, Tanzania-mainland adopted SP as an interim, first-line antimalarial drug. As a preparation for this policy change, we conducted studies to establish the baseline SP efficacy and prevalence of SP resistance molecular markers (pf dhfr and pf dhps mutations) in this country. We have established that SP was effective against uncomplicated malaria when the treatment failure rate by day 14. In previous studies in Uganda and Malawi, stronger associations (OR > 10) between treatment failure and pf dhfr 59 Arg and pf dhps 540 Glu mutations (the quintuple mutant predictors) were observed. The smaller OR value observed in our study is partly attributable to a shorter (14 days) follow-up period used in this study. The majority of the SP treatment failure cases are known to occur beyond day 14. Therefore, extended follow-ups with subsequent distinction of recrudescence by genotyping would have provided more reliable interpretation of treatment response and improved the association. In addition, inclusion of in vitro data would have been of paramount importance in elucidating the reason for the smaller OR value and providing a wider SP efficacy baseline data for Tanzania.

Our study has established that the prevalence of the triple pf dhfr mutant genotype was four times higher in an area of high SP resistance compared with areas of moderate SP resistance. This observation clearly suggests a relationship between the marker and SP resistance, and points to the potential of this genotype in the development of a reliable early warning tool for escalating SP resistance in Tanzania. The GFI calculated by using this marker also varied between high (3.4) and moderate SP resistance (1.96-2.1) sites. Nonetheless, values observed in the later sites are invariable and comparable with those observed using pf dhfr 59 Arg and
The PfDHFR 164 Leu alleles in sub-Saharan Africa because its appearance, through importation or otherwise, and subsequent spread would compromise the useful therapeutic life (ULT) of other alternative antimalarial drugs such as chlorproguanil-dapsone.

These findings constitute the baseline data on SP efficacy and prevalence of PfDHFR and PfDHPS genotypes in Tanzania. The clinical and molecular information gained from these studies signal that SP may have a short UTL in Tanzania, the basis for adoption of SP as an interim, first-line antimalarial drug. Thus, there is a need to advocate for rational use of the drug and conduct regular surveillance to monitor resistance concurrent with accelerated evaluation of different alternative treatments, especially combination antimalarial therapies. These data provide preliminary evidence suggesting that the triple PfDHFR mutant genotype may form a suitable early warning tool for increasing SP resistance in Tanzania. Further studies need to be done, at both community and health facility levels, to verify the usefulness of PfDHFR and PfDHPS genotypes in estimating SP resistance.

Received November 17, 2003. Accepted for publication June 16, 2004.

Acknowledgments: We thank the Tanzania Ministry of Health, the NMCP, and the East African Network for Monitoring Antimalarial Treatment (EANMAT) for coordinating the study in the sentinel sites. The sample collection exercise in some sites was also part of EANMAT activities of antimalarial sensitivity testing. We are grateful to the Swiss Tropical Institute for facilitating acquisition of molecular biological reagents and chemicals. We also thank the laboratory team at the IHDRD, including John Malugu, Magdalena Kiulugo, and Selina Churu for their hard working spirit. Lastly, we thank the individual clinical and field officers who performed the on site duties and the parents/guardians of all children who volunteered and consented to participate.

Financial support: These studies were supported by Multilateral Initiative on Malaria–United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases (TDR). The IHDRD received financial support from Swiss Agency for Development and Cooperation. The International Atomic Energy Agency and the Swiss Tropical Institute provided laboratory equipment and personnel training. The PhD program of Kefas Mugittu was supported by the TDR.

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