PREDICTION OF PLASMODIUM FALCIPARUM RESISTANCE TO SULFADOXINE/ PYRIMETHAMINE IN VIVO BY MUTATIONS IN THE DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHETASE GENES: A COMPARATIVE STUDY BETWEEN SITES OF DIFFERING ENDEMICITY

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Abstract. Plasmodium falciparum resistance to sulfadoxine/pyrimethamine (S/P) is due to mutations in the dihydrofolate reductase (dhfr) and dihydropterotate synthetase (dhps) genes. Large-scale screening of the prevalence of these mutations could facilitate the surveillance of the level of S/P resistance in vivo. The prevalence of mutations in dhfr and dhps in relation to S/P efficacy was studied in four sites of differing endemicity in Sudan, Mozambique, and Tanzania. The sites were organized in order of increasing resistance and a significant increase in the prevalence of triple mutations in codons c51, c59, and c108 of dhfr was observed. A similar trend was observed when dhfr genotypes were combined with c437 of dhps. Since the differences in S/P resistance between the sites were minor, but nevertheless revealed major differences in dhfr genotype prevalence, the role of dhfr as a general molecular marker seems debatable. The differences may reflect variation in the duration and magnitude of S/P usage (or other antifolate drugs) between the sites. Thus, triple dhfr mutations may prove suitable only as a general guideline for detecting emerging S/P resistance in areas where S/P has been introduced recently. However, changes in susceptibility within the same area with moderate levels of resistance may be possible by longitudinal surveillance of a subset of dhfr/dhps mutations that has been associated with S/P resistance in vivo in a defined location.

INTRODUCTION

Sulfadoxine/pyrimethamine (S/P) is the first-line drug in treatment of uncomplicated malaria in countries such as Kenya and Tanzania. However, Plasmodium falciparum resistance to S/P is emerging in these countries and poses a serious threat to control of malaria, since few cheap alternative drugs are available. Monitoring resistance longitudinally and on various locations is therefore of major importance.

In vitro studies have shown a good correlation between mutations in the dihydrofolate reductase (dhfr) and dihydropterotate synthetase (dhps) genes of P. falciparum and resistance to pyrimethamine1–4 and sulfadoxine,5–7 respectively. Detecting the prevalence of mutations in dhfr and dhps, or a selection of these have been suggested as a surveillance tool to monitor the extent of S/P resistance on a large scale.8

Several studies have searched for a common genotypic marker of S/P resistance that enables the prediction of the clinical and/or parasitologic outcome before treatment.9–13 The mutation in codon c108 in dhfr is the initial and most important change developing in response to S/P drug pressure.4 However, the c108 mutation in itself is not a predictive marker of in vivo resistance.14–16 With continuous S/P drug pressure the c51 and c59 mutations of dhfr will eventually follow,4 and infections with triple mutations in dhfr are believed to be a good indicator of clinical and/or parasitologic resistance by some investigators.17 Others believe that a combination of triple mutations in dhfr and mutation(s) in dhps, mainly in c437, are necessary to ensure resistance in vivo.11,18 The question remains whether one unifying marker exists at all since local conditions like malaria intensity as well as host immunity may interfere.

The purpose of the present study was to assess if dhfr/dhps genotypes generally reflect the level of S/P resistance at four sites in three east African countries with varying endemicity and degrees and prevalences of clinical and parasitologic resistance. The four sites were a mesoendemic site in Hag Yousif, Sudan and one holoendemic site in Kibaha, Tanzania, both with low levels of resistance, a hyperendemic site in Matola, Mozambique with a low-to-medium level of resistance, and one holoendemic site in Magoda, Tanzania with a high level of resistance. We have included dhfr/dhps prevalence data from the Magoda site from three consecutive years, but with marked decreasing in vivo resistance over the three-year period. An identical dhfr/dhps genotyping protocol has been performed on all samples in the same laboratory.

MATERIALS AND METHODS

Study sites. Hag Yousif, Sudan. The in vivo trial is described in detail by Khalil and others.16 Hag Yousif is located 11 km east of Khartoum, Sudan. The use and availability of S/P is limited in the area.16 The in vivo sensitivity test was conducted according to guidelines of the World Health Organization (WHO).19,20 Inclusion criteria were P. falciparum monoinfections and asexual parasite densities between 1,000 and 80,000/μL of blood. Patients 10–65 years old (mean ± SD age = 27.7 ± 12.05 years) were included and treated orally under medical supervision with a single dose of S/P (25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine). Venous blood was obtained pretreatment into heparinized vacucontainers. Samples were centrifuged for 10 minutes at 2,000 × g and the plasma was removed. The samples were transported on dry ice to the Institute of Medical Microbiology and Immunology in Copenhagen, Denmark and stored at –80°C for further analysis.

Kibaha, Tanzania. Kibaha is situated in the coastal region 30 km of Dar es Salaam. The use of S/P in the area is restricted (Tarimo D, unpublished data). The in vivo trial was conducted according to WHO guidelines20 and is published.
elsewhere. Children between 12 and 59 months of age with *P. falciparum* monoinfections and parasitemias between 2,000 and 250,000 asexual parasites/μL of blood were included and treated orally with a single dose of S/P (25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine). Venous blood was obtained pretreatment into EDTA-containing vacu-containers. Samples were centrifuged for 10 minutes at 2,000 × g and the plasma was removed. Samples were transported on dry ice to the Institute of Medical Microbiology and Immunology in Copenhagen, Denmark and stored at −80°C for further analysis.

**Matola, Mozambique.** Details of the study area in Matola district are described elsewhere. Briefly, Matola is a peri-urban area located about 15 km north of Maputo, Mozambique. Malaria transmission in this area is seasonal, with the majority of cases occurring during the rainy season from November to April. Sulfadoxine/pyrimethamine has been available for some years in the area and was also used as a first-line drug during the floods in early 2000 as a temporary measure due to the threat of epidemics (Enosse S and Magnussen P, unpublished data). The *in vivo* sensitivity test was conducted from April to June 2000 according to WHO guidelines. Inclusion criteria were children between 6 and 59 months of age with *P. falciparum* monoinfections and parasitemias between 2,000 and 80,000 asexual parasites/μL of blood. Children were treated orally under medical supervision with a single dose of S/P (25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine). Finger prick blood was taken for blood microscopy and an additional 50 μL of blood were collected onto filter paper for genotyping.

**Magoda, Tanzania.** The study population was children between 6 and 59 months of age with uncomplicated *P. falciparum* malaria living in Magoda and Mpapayu villages in Mueza in the northeastern part of Tanzania in July 1997, 1998, and 1999. Sulfadoxine/pyrimethamine was introduced in the villages in 1993 during a study investigating the prophylactic effect of weekly pyrimethamine/dapsone (Maloprim), batch 490B; Wellcome Foundation, Limited, London, United Kingdom) on malaria morbidity. Sulfadoxine/pyrimethamine has been available ever since in the villages (Lemnge M, unpublished data). The *in vivo* sensitivity test was conducted according to WHO guidelines. Children with *P. falciparum* monoinfections and parasitemias between 2,000 and 200,000 asexual parasites/μL of blood were included in the study; 84, 70, and 51 children completed the *in vivo* trial in 1997, 1998, and 1999, respectively. All children were treated orally under medical supervision with a single dose of S/P (25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine). Before treatment, 50 μL of blood was collected onto filter paper for genotyping.

**Ethical review.** The study in Sudan was reviewed and approved by the Institute of National Health and the Research Board of the Faculty of Medicine of the University of Sudan (Khartoum, Sudan). The studies in Tanzania were reviewed and approved by the Commission for Science and Technology and the Tanzanian Ministry of Health. The study in Mozambique was reviewed and approved by the Ethical Review Board of the National Institute of Health-Ministry of Health.

**Extraction of DNA and restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR).** DNA from blood samples collected in EDTA- or heparin-containing tubes was extracted by treatment with phenol-chloroform. Briefly, 50 μL of blood was incubated overnight with 250 μL of a proteinase K solution at 37°C (1 mM EDTA, 15 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulfate, 100 μg/mL of proteinase K). The samples were then extracted with phenol-chloroform and precipitated with ethanol as described by Sambrook and others. The samples from Sudan were collected in heparin and were additionally treated with heparinase I as described by Khalil and others. DNA from filter paper was extracted by the Chelex-100 method as described by Wooden and others. One microliter of the extracted DNA suspension was added to the PCR mixture. For the outer and nested PCRs and RFLPs, the method of Du raisingh and others was used, except that the two outer *dhfr* and *dhps* PCRs were multiplexed into a single reaction. Additionally, a nested *dhps* PCR targeting c540 specifically was designed (c540-K/-PCR product of 126 basepairs, same reaction conditions as the other nested *dhfr* and *dhps* PCRs). The c540 primer sequence was 5’-GCATAAAAGAG-GAAATCCACATAATGGrF3’; the lower case base is one mismatch engineered into the primer to provide a cleavage site for the restriction enzyme Mse I to detect the c540 wild type (Lys). The *P. falciparum* laboratory strain 3d7 was used as a control for the PCR.

**Statistical analysis.** The chi-square test with Yates’ correction or Fisher’s exact test was used to compare the genotypic prevalence. *P* values less than 0.05 were considered significant. All calculations were performed using Sigmasat version 2.03 software (Jandel Scientific, San Rafael, CA).

**RESULTS**

**Clinical and parasitologic resistance to S/P in Hag Youssif (Sudan), Kibaha (Tanzania), Matola (Mozambique) and Magoda (Tanzania).** The outcome after S/P treatment from the four sites, including data from three consecutive years of S/P trials in Magoda, is shown in Table 1. The age of the patients in the study in Hag Youssif in Sudan ranged between 10 and 65 years; otherwise, children less than five years old were enrolled. The trials in Hag Youssif, Kibaha, and Matola were carried out as 28-days tests. The trials in Magoda were performed as 14-days tests.

**Frequency of mutations in *dhfr* and *dhps* in relation to *in vivo* outcome of S/P treatment.** The individual prevalence of *dhfr* and *dhps* genotype infections for the patients from the four sites grouped according to parasitologic and clinical outcome is presented at www.cmp.dk/Files/Appendix_03-103.pdf. The frequency of infections with mutations in c51, c59, and c108 of *dhfr* and c436, c437, and c540 of *dhps* according to parasitologic and clinical outcome are summarized in Table 2. For all localities, there was a general tendency for a higher frequency of mutations in the parasitologic or clinical failure groups. However, except for Hag Youssif, no single combination of mutations was found exclusively in these groups. Furthermore, except for Hag Youssif, both parasitologic-sensitive groups and the adequate clinical responder groups had infections with triple mutations in *dhfr* combined with at least a double mutation in *dhps*, except for the parasitologic sensitive group in Magoda in 1997 (www.cmp.dk/Files/Appendix_03-103.pdf). Statistical analysis comparing the frequency of mutant type (including mixed infections) versus the frequency of wild types showed a significant higher frequency of mutations...
at c436 in dhps in Hag Yousif in patients who failed to clear parasites upon treatment with S/P comparing the parasitologically sensitive and the resistant group (P < 0.001). In Matola, c59 of dhfr and c437 of dhps were predictive of parasitologic failure (P/H11505 0.036 and P/H11505 0.011, for c59 and c437, respectively). In Magoda 1997 samples, c437 of dhps was predictive of both parasitologic and clinical failure (P/H11505 0.015 and P/H11505 0.029 for parasitologic and clinical comparisons, respectively).

Prevalence of combinations of mutations in dhfr. The prevalence of combinations of genotypes in dhfr from the four sites is shown in Figure 1. The sites have been listed with increasing S/P resistance in vivo, although the differences were only marginal between Hag Yousif, Kibaha, and Matola. The prevalence of combinations of dhfr genotypes in Magoda in 1997, 1998, and 1999 showed no significant differences, and for the rest of the analysis, the data from the three years were pooled.

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The presence of infections with one or more wild types in c51, c59, and/or c108 decreased significantly as a function of S/P resistance (Hag Yousif, 100% > Kibaha, 67.6% > Matola, 35.3% > Magoda, 9.2%; P < 0.001 for a comparison at all levels). The prevalence of true triple dhfr mutants at c51, c59, and c108 (excluding wild type/mutant [mixed] genotype in-
Infections increased as a function of S/P resistance in vivo (Hag Yousif, 0.0% < Kibaha, 20.6% < Matola, 50.9% < Magoda, 60.5%; $P \leq 0.001$ for a comparison at all levels, except for Hag Yousif versus Kibaha [$P = 0.008$] and Magoda versus Matola [$P = 0.124$]). The prevalence of infections in which all three mutant dhfr codons were present and a wild type in at least one of the codons (mixed genotype infections) was statistically significant higher in Magoda than in the other localities ($P \leq 0.001$, $P = 0.003$, and $P = 0.001$ for Magoda versus Hag Yousif, Kibaha, and Matola, respectively). Fewer mixed infections were found in Hag Yousif versus Kibaha ($P = 0.074$) and Matola ($P = 0.038$). Only wild types in c16 and c164 of dhfr were observed in all four sites.

**Prevalence of combinations of mutations in dhfr and c437 of dhps.** The prevalence of combinations of dhfr genotypes and c437 of dhps from the four sites is shown in Figure 2. The prevalence of combinations of dhfr genotypes including c437 of dhps in Magoda in 1997, 1998, and 1999 showed no significant differences and the data from the three years were pooled.

The presence of infections with one or more wild types in c51, c59, c108, and/or c437 decreased as a function of S/P resistance (Hag Yousif, 100% > Kibaha, 94.1% > Matola, 62.9% > Magoda, 17.9%; $P \leq 0.001$ for a comparison at all levels, except for Hag Yousif versus Kibaha; $P = 0.332$). The prevalence of true triple dhfr mutants at c51, c59, c108, and c437 in dhps increased as a function of S/P resistance (Hag Yousif, 0.0% = Kibaha, 0.0% < Matola, 27.6% < Magoda, 41.1%; $P \leq 0.001$ for a comparison at all levels, except for Hag Yousif versus Kibaha; $P = 1.0$ and Magoda versus Matola; $P = 0.024$). The presence of mixed genotype infections in c51, c59, c108, and/or c437 showed significantly more mixed infections in Magoda versus the other sites ($P \leq 0.001$). No apparent trend was observed when examining triple mutations in dhfr in combination with either c436 (only expressing Ala or Ser at all four sites) or c540 of dhps.

**DISCUSSION**

A number of studies have investigated the association between mutations in dhfr and dhps genes and the parasitologic and/or clinical response to S/P treatment at an individual level. The actual associations were poor or none existed. The present study investigated the frequency of mutations in the two genes at four distinct sites in east Africa and compared the frequency of mutations between in vivo sensitive and resistant outcomes that were parasitologically and/or clinically defined. There was a general tendency at all four sites for a higher frequency of mutations in the groups of resistant outcomes. However, it is possible that due to few patients in some of the groups, only mutations at c436 in dhps in Hag Yousif, Sudan and c59 in dhfr and c437 of dhps in Matola, Mozambique was significantly predictive of parasiticologic failure. In Magoda, Tanzania, c437 in dhps was predictive of both parasitologic and clinical failure in 1997, but not in 1998 and 1999. These results indicate that PCR analysis of mutations in dhfr and dhps cannot be used to predict infection outcome at an individual level. However, the high frequency of mixed genotype infections found in Kibaha, Matola, and Magoda may be an important factor that affects the predictive usefulness of the mutations. The study also examined the relationship between dhfr genotypes either alone or in combination with dhps genotypes and the prevalence of S/P resistance by comparing the four distinct sites with variable endemicity, transmission intensity, and probably S/P usage.

The four sites were organized in order of increasing parasitologic and clinical resistance, although the differences between Hag Yousif, Kibaha, and Matola were marginal. As a function of increasing resistance in vivo, the prevalence of true triple mutations in dhfr increased significantly. Furthermore, the prevalence of infections with one or more wild...
types in c51, c59, and/or c108 decreased significantly. When dhfr genotypes in c51, c59, and c108 were combined with c437 in dhps, similar differences between the sites were found, although not as marked as when examining dhfr genotypes alone. In other words, inclusion of c437 in dhps does not contribute significantly to the relationship and, consequently, this suggests that dhfr genotypes alone may be a suitable marker of the overall resistance level. However, combinations of dhfr and dhps mutations may still prove to be important mainly in areas of low endemicity, as has been observed by others.9,12,16

Despite our results, it should be emphasized that the usefulness of dhfr/dhps genotypes as a marker of S/P resistance is still highly debatable. In Magoda, the in vivo resistance decreased between 1997 and 1999 (Lemnge M, unpublished data), while the dhfr/dhps genotypes changed only marginally. Thus, the applicability of dhfr/dhps mutations in this area remains limited. Moreover, the evident differences in dhfr/dhps prevalence between the three sites with relatively low levels of resistance (Hag Younis, Kibaha, and Matola) reflected only minor differences in resistance in vivo. Perhaps the differences in dhfr/dhps prevalence reflect the duration and magnitude of S/P usage (or antifolate drugs in general), rather than the differences in resistance in vivo between these three areas. Conversely, the in vivo test defined by WHO20 may not be equally suitable in all endemic areas, especially if it is conducted with the 14-day version.28

Immunity and transmission intensity may be important confounders that reduce the predictive usefulness of dhfr/dhps genotypes. In hyperendemic or holoendemic areas, partially resistant parasites may be cleared by immunity rather than by treatment, even in children, resulting in discrepancies between dhfr/dhps genotypes and in vivo outcome.8,20 The rate of immunity acquisition depends on transmission intensity and may therefore play a varying role in treatment outcome between different sites. For instance, in mesoendemic Sudan, the role of immunity may be insignificant and the treatment outcome may be more dependent on the genotypic profile of the parasitic infection. High transmission intensity may result in multilocional infections.29 As expected, Magoda showed a significantly higher prevalence of mixed genotype infections compared with the other sites. Treatment outcome is probably dependent on the initial density of wild type versus the mutant type population. Therefore, the impact of multilocional infections may likewise play a varying role in treatment outcome between different localities. Based on our data, triple dhfr mutations may only prove to be suitable as a general guideline for detecting emerging S/P resistance in areas where S/P recently has been introduced. However, changes in susceptibility within the same area with moderate levels of resistance may be detectable by longitudinal surveillance of a subset of dhfr/dhps mutations that has been associated with S/P resistance in vivo in a defined location.

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