High Prevalence of Markers for Sulfadoxine and Pyrimethamine Resistance in *Plasmodium falciparum* in the Absence of Drug Pressure in the Ashanti Region of Ghana

Florian Marks, Jennifer Evans, Christian G. Meyer, Edmund N. Browne, Christa Flessner, Vera von Kalkreuth, Teunis A. Eggelte, Rolf D. Horstmann, and Jürgen May*

Department of Molecular Medicine, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; Kumasi Centre for Collaborative Research in Tropical Medicine and Department of Community Health, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana; and Division of Infectious Diseases, Tropical Medicine & AIDS, Academic Hospital, Amsterdam, The Netherlands

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Markers of *Plasmodium falciparum* resistance to chloroquine (CQ) and pyrimethamine-sulfadoxine (PYR-SDX) are widespread in areas where malaria is endemic. In an area where the use of PYR-SDX is negligible, the Ashanti Region of Ghana, West Africa, adult individuals were enrolled in an analysis of CQ- and PYR-SDX-associated molecular resistance markers in 2001 (n = 177) and 2003 (n = 180). Parasite prevalence, as assessed by PCR assays, were 56.5 and 48.8% in 2001 and 2003, respectively. A high frequency of CQ, PYR, and SDX resistance markers was observed, whereby, as a weak trend, the frequency was higher in 2003. The quintuple combination of three *pfdfhr* mutations and two *pfdfhps* mutations has previously been recognized to be the most important determinant of PYR-SDX resistance. Approximately 60% of parasite carriers harbored fourfold mutated parasites, indicative of a considerable risk for a switch to high-level PYR-SDX resistance in an area where the rate of PYR-SDX use is low. Among the factors contributing to the high frequency of PYR-SDX resistance-associated mutations are background use of PYR-SDX, past use of PYR for malaria prophylaxis, cross-resistance of trimethoprim with PYR, and the sufficient biological fitness of resistant parasites in the absence of drug pressure.

Sub-Saharan African countries are confronted with the rapid emergence of resistance against virtually every drug that is used for the treatment of *Plasmodium falciparum* malaria (21). This applies, in particular, to chloroquine (CQ) but also to the synergistic combination of pyrimethamine (PYR) and sulfadoxine (SDX). Drug pressure is considered to essentially promote the emergence of PYR-SDX resistance, which is now widespread in East Africa, but also well recognized in West Africa.

CQ resistance has been attributed to a single mutation at codon 76 in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*, chromosome 7; Lys→Thr [*pfcrt*K76T] (6)). In addition, variants of parasite multidrug resistance transporter genes (*pfdmbr*) have been found to contribute to CQ resistance, albeit inconsistently (23). Resistance to PYR is primarily conferred by a nonsynonymous point mutation at codon 108 and is consecutively enhanced by mutations at codons 51 and 59 of the *P. falciparum* dihydrofolate reductase gene (*pfdfhr*, chromosome 4) (27). The enzyme is part of the folate pathway and, thus, of DNA replication. Selection for the Ser→Asn substitution at codon *pfdfhr*108 (*pfdfhr*S108N) has been shown to be linked to parasite survival after treatment with PYR-containing regimens (5). Accordingly, the high frequencies of resistant parasite populations have been attributed to increased PYR consumption (28). An Asp→Ile substitution at codon *pfdfhps*51 (*pfdfhps*N51I) and/or a Cys→Arg exchange at codon *pfdfhps*59 (*pfdfhps*C59R) appears to enhance PYR resistance if one or both of these occur concurrently with *pfdfhps*S108N. *pfdfhps*S108N-N51I-C59R is the combination of mutations most strongly associated with PYR resistance (36).

Point mutations at codons 437 and 540 of the dihydropterotate synthase gene (*pfdfhs*, chromosome 8) of *P. falciparum* are considered responsible for SDX resistance. *pfdfhs* encodes a key enzyme in the folate pathway, as does *pfdfhr*. The Ala→Gly substitution at position *pfdfhs*437 (*pfdfhs*E437G) is, in general, the first mutation to occur. In Africa this is followed by the Lys→Glu substitution (codon position 540; *pfdfhs*K540E), which confers higher levels of resistance (29). A recent publication shows (16) that the presence of the three *pfdfhr* mutations combined with the two *pfdfhs* mutations (quintuple mutation) is strongly associated with SDX-PYR resistance.

(22) The study was conducted by F.M. as part of his Ph.D. thesis at the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.

**MATERIALS AND METHODS**

**Study population.** Blood samples were collected from 177 (2001) and 180 (2003) healthy adults in an area hyperendemic for *P. falciparum* malaria (Bodomase, Ashanti Region, Ghana) in the course of a biennial study on *P. falciparum* CQ, PYR, and SDX resistance markers. The frequencies of antimalarial self-treatment, visits to health care facilities, and details on socioeconomic status.
were assessed through the administration of questionnaires to the study participants. Ethical clearance for the study was obtained from the Ethics Committee of the School of Medical Sciences, University of Science and Technology, Kumasi, Ghana, and written consent was obtained from each participant. Positivity for parasites was determined by PCR assays designed to amplify genomic fragments of the \textit{pfdhfr} and \textit{pfdhps} genes.

\textit{pfcrt}, \textit{pfdhfr}, and \textit{pfdhps}\textit{ PCR} (20) and \textit{pfdhr} PCR (19) were performed as described previously. For the \textit{pfdhps} PCR, two primers (primer \textit{pfdhps}-F [5’-ATGATCTTTTTTGAGATG-3] and primer \textit{pfdhps}-R [5’-CCAATGTTGATTGTTCAG-3]) were designed to amplify 747 bp of the region exhibiting mutations relevant to SDX resistance. PCR was performed with a volume of 25 \(\mu\)l (each primer at 0.2 \(\mu\)M, deoxynucleoside triphosphates [dNTPs] at 200 \(\mu\)M, and 1 \(\mu\)l of Hotstar-Taq with the appropriate buffer [Qiagen, Valencia, Calif.] with MgCl₂, at a final concentration of 2.0 mM) and approximately 80 ng of template genomic human DNA, with parasite DNA concentrations corresponding to the parasite burden in the individual. After an initial denaturation (15 min at 95°C), 31 cycles of 30 s at 94°C, 40 s at 53°C, and 1 min at 72°C were run. Elongation of the amplicons was completed by a final cycle of 10 min at 72°C.

Subsequently, a nested touchdown PCR (ntPCR) was performed to increase the yields of the specific amplicons. Three microliters of the primary PCR product was used in a reaction volume of 25 \(\mu\)l (each primer at 0.2 \(\mu\)M [primer \textit{pfdhps}-F (5’-GGTTGACCTAAAGCTGCTG-3’) and primer \textit{pfdhps}-R1 (5’-AATTCAACATTTTGATCC-3’)], dNTPs at 200 \(\mu\)M, reaction buffer with MgCl₂, at a final concentration of 2.0 mM, and 1 \(\mu\)l of Hotstar-Taq). In an ntPCR, a high initial annealing temperature (AT), which ensures a high level of specificity of initial primer binding, is followed by a gradual decrease in the AT toward the precalculated optimal AT. The ntPCR parameters consisted of an initial denaturation step (15 min at 95°C) and 43 cycles of 30 s at 94°C, 40 s at 53°C, and 1 min at 72°C. Fragment elongation was performed by use of a cycle of 10 min at 72°C. The amplicons were monitored for quality and the expected size (647 bp) on 1% ethidium bromide-stained agarose gels.

\textbf{Mutation analyses.} Restriction fragment length polymorphism analysis of the \textit{pfcrt} mutation at codon 76 (20) and matrix-assisted laser desorption ionization–time of flight mass spectrometry-based analysis of the three \textit{pfdhfr} single-nucleotide polymorphisms (SNPs) (19) were performed as described previously.

The newly developed GenoSNIP kit (Bruker Daltonics, Bremen, Germany) was used to analyze the two SNPs relevant to SDX resistance. The amplified \textit{pfdhfr} ntPCR products were treated with shrimp alkaline phosphatase to remove extant dNTPs. The subsequent primer extension reaction depends on the known variability of template DNA, reflected in the content of stop nucleotides (didencyclo nucleotides) in the reaction mixture. Here, ddCTP was used to detect the \textit{pfdhfr} mutation and ddATP was used to identify the \textit{pfdhps} mutation in an antisense reaction and a sense reaction, respectively. Extension primers tagged with a 5’-biotin anchor (primer \textit{pfdhfr}-A [5’-biotin-5’-GATTGATCTTTTTTGAGATG]) and primer \textit{pfdhps}-B [5’-biotin-5’-GAGGA AATCCACATAATGGATAT-3’) and an o-nitrobenzyl moiety (L) replacing a nucleotide allow selective photocleavage. The 16-\(\mu\)l reaction mixture for the primer extension reaction with the \textit{pfdhfr} and \textit{pfdhps} segments consisted of each primer at 0.5 \(\mu\)M, ddGTP at 200 \(\mu\)M, 1 U of Sequenase (Termipol; Solis BioDyne, Tartu, Estonia), and 11 \(\mu\)l of the PCR product. The reaction conditions were an AT of 56°C for mixtures with ddCTP (200 \(\mu\)M) (\textit{pfdhfr}) and an AT of 62°C (\textit{pfdhps}) for mixtures with ddATP (100 \(\mu\)M); enzyme activation at 95°C for 2 min; and 42 cycles of 20 s at 94°C, 30 s at the AT, and 40 s at 72°C, with a final step of 10 min at 72°C.

The elongated oligonucleotides were purified (Genostrep kit; Bruker Daltonics) and eventually cleaved by exposure to UV light (wavelength, 360 nm), which provided shortened low-molecular-mass fragments of the elongated oligonucleotide appropriate for SNP detection.

\textbf{Plasma drug levels.} Residual plasma drug levels were determined by an immunoassay, as described previously (9). The detection limits of PYR, SDX, and CQ were 10 ng/ml, 1 to 2 \(\mu\)g/ml, and 2 \(\mu\)g/ml, respectively.

\textbf{Statistics.} Statistical analyses (\(\chi^2\) tests) were performed by the use of STATATA software (version 8.2; Stata Corp., College Station, Tex.).

\section*{RESULTS}

Blood samples from 177 and 180 clinically healthy adults in an area hyperendemic for the transmission of \textit{P. falciparum} were collected in 2001 and 2003, respectively. The aim was to screen for the mutation associated with CQ resistance, three SNPs of the \textit{pfdhfr} gene associated with PYR resistance, and two SNPs associated with SDX resistance and to assess possible differences in the mutation patterns between the two years.

As determined from the positive signals provided by the \textit{pfcrt}, \textit{pfdhfr}, or \textit{pfdhps} PCR assays, in 2001, 56.5\% of the healthy individuals were identified to be asymptomatic parasite carriers, and in 2003, 48.8\% were carrying parasites. The number of individuals with positive PCR results varied due to the different efficiencies of amplification of the \textit{pfcrt}, \textit{pfdhfr}, and \textit{pfdhps} fragments. The patterns of the SNPs linked to CQ, PYR, and SDX resistance and the combinations of mutations were not significantly different when parasites from both years were compared. The frequencies of the mutations identified are given in Table 1. As a trend, the frequency of mutations associated with CQ and PYR resistance increased from 2001 to 2003. In the following text we refer to the relative frequencies of the different variants and their combinations in parasites from carriers as determined in 2003.

The \textit{pfcrt}K76T mutation was identified in 65.5\% of the individuals in 2003. The frequency of the \textit{pfdhfr}S108N mutation, which so far is most strongly associated with PYR resistance, was 88.1\%. The relative frequencies of the \textit{pfdhfr}S511T and the \textit{pfdhfr}S596R mutations were 66.7 and 79.7\%, respectively. \textit{pfdhps}A437G occurred in parasites from 89.3\% of individuals,
while *pfdhps* K540E was observed only once (1.3%). All samples with the exclusive *pfdhfr* N51I wild-type variant also had the wild
types for *pfdhfr* S108N and *pfdhfr* A437G, indicating a strong linkage of these variants to wild-type *pfdhfr*.

The triple *pfdhfr* variant, almost exclusively in combination with the *pfdhps* A437G variant (quadruple variants), occurred in parasites from 58.3% of the individuals; triple *pfdhfr* variants without additional *pfdhps* variants were found in parasites from only 2.8% of the individuals. Combined triple and double mutations were always characterized by the presence of the *pfdhfr* S108N mutation and various accompanying mutations (triple combinations [26.4%] *pfdhfr* S108N-N51I-pfdhps A437G, and *pfdhfr* S108N-C59R-pfdhps A437G and double mutations [5.5%] *pfdhfr* S108N-N51I, *pfdhfr* S108N-C59R, and *pfdhfr* S108N-pfdhps A437G). The quintuple combination *pfdhfr* S108N-N51I-C59R-pfdhps A437G-K540E was observed only once.

The levels of CQ, PYR, and SDX were measured in the plasma samples obtained in 2003. CQ was detectable (>2 ng/ml) in the plasma of 27.6% of the participants; 4.4% had plasma CQ levels greater than 100 ng/ml. In contrast, 1.1 and 2.2% of the study participants had low, yet detectable subtherapeutic traces of PYR (>10 ng/ml) and SDX (>1 µg/ml), indicating prior usage of PYR-SDX. The high levels of PYR and SDX in the plasma of one individual (149 ng/ml and 105 µg/ml, respectively) indicated recent PYR-SDX intake.

Among the 66% of individuals who reported self-treatment prior to the attendance of medical professionals, 6.7% reported regular use of CQ and 78.6% reported an occasional use of CQ; 14.7% denied CQ use. Rare self-treatment with PYR-SDX was communicated by 9.5% of the participants, and no PYR-SDX use at all was reported by the remaining 90.5%. Better socioeconomic status, evident from the possession of distinct items such as television sets, radios, and refrigerators, as well as of the availability of electricity, was positively correlated with earlier attendance at health care facilities (P < 0.03). This possible confounder was not associated with the frequency of drug resistance markers or plasma drug levels.

**DISCUSSION**

This study was designed to assess the frequency of markers associated with resistance to CQ, PYR, and SDX in an area with high CQ but scarce PYR and SDX use. Testing for markers of drug resistance was performed with samples collected in 2001 and 2003, with the large majority of study participants taking part in both years. The relevant results of this study may be summarized as follows: (i) approximately 50% of the adults tested were asymptomatic carriers of *P. falciparum*, as assessed by PCR assays designed to amplify fragments of the pfcrt, *pfdhfr*, and *pfdhps* genes; (ii) high frequencies of the pfcrt K76T, *pfdhfr* S108N, *pfdhfr* N51I, *pfdhfr* C59R, and *pfdhps* A437G mutations and of combinations of the last four mutations were observed; (iii) the *pfdhps* K540E variant and, thus, quintuple *pfdhfr*-pfdhps combinations were identified only rarely; (iv) residual CQ levels were detected in a considerable proportion of the study group, while residual PYR and SDX levels were observed in a negligible proportion of individuals; and (v) information on antimalarial drug use, provided through questionnaires, corresponded largely to the residual drug levels detected.

The high degree of *P. falciparum* resistance to CQ in sub-Saharan Africa has been the subject of an enormous body of literature, with resistance to CQ in Ghana first being discussed as early as 1968 and confirmed in 1988 (2, 17). PYR, a monoprophylactic agent used for the treatment of *P. falciparum* infections, was introduced in East Africa in 1953, and the development of resistance was already suspected during the first clinical trials (4). PYR was introduced in 1975 (3) into Ghanian malaria control programs and continued to be used for approximately two decades. The first reports on PYR resistance in Ghana date from 1988 (17) and have since been confirmed. Nevertheless, in 2002 PYR-SDX was proposed to be an appropriate alternative to CQ for the first-line treatment of malaria in Ghanian children (7). Meanwhile, and on the basis of several lines of evidence indicating the rapid emergence of PYR resistance, followed by SDX resistance several years later and the sustained maintenance of PYR resistance for many years (18), the official recommendation for first-line treatment of malaria has consequently been changed to arteunate-amodiaquine (26).

The development of resistance and the failure of PYR treatment are a result of the initial and crucial *pfdhfr* S108N mutation, which leads to a moderate degree of resistance to PYR, which is enhanced by the subsequent *pfdhfr* N51I and *pfdhfr* C59R mutations (34). Triple mutations are associated with 60 to 70% rates of treatment failure (16). Notably, *pfdhps* K540E was identified only once in our study, suggesting that SDX sensitivity is widely maintained.

Although PYR-SDX resistance in areas devoid of drug pressure has been documented previously (22) and such a scenario has also been described for mefloquine (8), the extreme prevalence of resistance-associated *pfdhfr* and *pfdhps* mutations and the high proportion of individuals carrying parasites with these variations are surprising and may not be explained solely by the selection of resistant parasites on the basis of the prior use of PYR as monophrophylaxis and the present low rate of PYR-SDX use. Several reasons may, in addition, account for the high prevalence of the mutations.

Cross-resistance between PYR-SDX and trimethoprim (TMP)-sulfamethoxazole (SMX) has been described (13), *pfdhfr* S108N, *pfdhfr* N51I, and *pfdhfr* C59R strains have been shown to be less susceptible to both PYR and TMP than wild-type isolates (14), and a significant rate of bacterial resistance, e.g., by *Salmonella*, to TMP-SMX has been observed in Ghana (24, 25). Intermittent use of these substances could contribute to PYR-SDX resistance, even if the rate of PYR-SDX use is low, as assessed by residual levels in plasma and interviews with the participants in this study. The use of TMP-SMX as prophylaxis against human immunodeficiency virus (HIV) infection-associated opportunistic infections most likely does not make an essential contribution to PYR-SDX resistance, in view of the still comparatively low rate of HIV infection in the Ghanian population. Cross-resistance between PYR-SDX and TMP-SMX appears to be a contributing factor rather than the exclusive factor responsible for the high prevalence of resistant parasites.

On the basis of evolutionary theories, biological disadvantages are expected for parasites carrying resistance-mediating mutations in the absence of drug pressure. The fitness deficit conferred by the *pfdhfr* S108N mutation in the absence of PYR use is considered quite low. Enduring resistance in the absence
of strong drug pressure implies that the expected decline in the prevalence of resistant parasites is balanced by mechanisms that confer biological advantages with regard to survival fitness, replication and transmission probability, infection, reproduction, and vector properties that favor transmission.

Limited information only is available on the fitness of PYR-resistant parasites that occur alone and those that occur concomitantly and with and compete with sensitive parasites, and most studies have focused on the fitness deficits or benefits of CO-resistant parasites devoid of drug pressure. The central determinant of parasite fitness is transmission efficiency. Early studies have shown that the transmission of CQ-resistant parasites occurs efficiently and, in terms of fitness deficits, is not costly (30, 35). This view has been confirmed by more recent observations that indicate that although CO-resistant parasites grow more slowly, they reach their reproductive stages earlier and to a higher extent than nonresistant parasites (12, 15, 31, 32) and their degree of infectivity to mosquitoes exceeds that of nonresistant parasites (11). This is in line with the observation that, in certain vectors, some CO-resistant P. falciparum strains may reproduce more efficiently and produce substantially higher numbers of oocysts than CO-sensitive strains do (15, 37). Thus, a fitness benefit may, most likely, be attributed to CO-resistant parasites, and similar mechanisms might also apply to PYR-SDX-resistant parasites. It has been shown that apicomplexan Toxoplasma gondii dhfr-S108N mutants (the nomenclature is according to that for P. falciparum dhfr mutations) have no significant fitness defects in vitro (10), and similar conditions might also apply to P. falciparum.

It has been demonstrated with a PYR-resistant Plasmodium berghei line that resistant and sensitive lines were very similar in terms of fitness, although sporogony was slower in the resistant line (33). These observations and the restricted drug pressure exerted through the rare use of PYR-SDX and the intermittent use of TMP-SMX suggest that resistant parasites would not be subject to severe disadvantages. It remains to be shown whether this also applies to PYR-SDX-resistant P. falciparum parasites, but a high degree of PYR-SDX resistance is consistent with natural selection and the lack of substantial disadvantages.

In analogy to the stochastic model that has been suggested by Ariey and Robert (1) for the development of resistance in areas where malaria is holo- and hypoenemic, a stepwise increase in resistant mutants is also conceivable under the conditions of malaria transmission in an area of hyperendemicity, like those present in our study area. As a consequence, the model then suggests eventual high frequencies of mutant parasites.

The main conclusions drawn from our observations, namely, that mutations that confer PYR-SDX resistance can be widely maintained in the absence of drug pressure, have the advantage of being only hypothetical. If, however, they should hold true and apply to other antimalarial drugs as well, the dramatic situation of malaria control in Africa may be foreseen to deteriorate still more.

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