MOLECULAR ANALYSIS OF *PLASMODIUM FALCIPARUM* FROM DRUG TREATMENT FAILURE PATIENTS IN PAPUA NEW GUINEA

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**Abstract.** A study was conducted in Papua New Guinea to analyze *Plasmodium falciparum* drug resistance polymorphisms in patients presenting with resistant malaria. One hundred ninety-nine *P. falciparum*-positive patients were recruited at two sites, Madang and Maprik. Exposure to the 4-aminoquinolines chloroquine and amodiaquine was uniformly high, at 84% overall. However, 59% of these were taken in various combinations of sulfadoxine/pyrimethamine and/or primaquine and/or quinine. Two markers for 4-aminoquinoline resistance, *P. falciparum* chloroquine resistance transporter 76T and *P. falciparum* multidrug resistance 1, were fixed in the population and two markers for pyrimethamine resistance, dihydrofolate reductase (dhfr) 59R and 108N, were found at moderate to high levels, overall 60% and 75%, respectively. No polymorphisms in dhps associated with sulfadoxine resistance were present. Differences between the two sites are analyzed. The study period encompasses a change in standard malaria treatment policy. These findings stress the need for regular monitoring of the effects of standard drug treatment of uncomplicated malaria in Papua New Guinea.

**INTRODUCTION**

Although resistance to the 4-aminoquinolines amodiaquine and chloroquine has been well documented in Papua New Guinea, until recently they were still the standard treatment for uncomplicated malaria. The combination of sulfadoxine/pyrimethamine (S/P) (Fansidar®) has not been part of standard treatment for uncomplicated malaria in Papua New Guinea, although it has been the standard second-line treatment, principally in combination with quinine, for the treatment of severe or recrudescent malaria in this country. In spite of the low level of S/P use, resistance both *in vivo* and *in vitro* has been documented for some time. Primaquine and quinine have a long history of use in Papua New Guinea and are well accepted by both health workers and malaria patients. Given the logistical difficulties that exist in all developing countries, treatment is often dependant on availability and cost of drugs rather than their efficacy.

Polymorphisms in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*) gene are central to chloroquine resistance, while high-level chloroquine resistance appears to require alterations in other genes such as *P. falciparum* multidrug resistance 1 (*pfmdr1*). Polymorphisms K76T in *pfcr* and S108N/T and I164L in *pfmdr1* have been identified as being associated with chloroquine resistance, with some geographic variations. In Southeast Asia and Africa, 86Y is the predominant *pfmdr1* chloroquine resistance genotype, while combinations of 1034C, 1042D, and 1246Y are most frequently found in South American chloroquine-resistant *P. falciparum* parasites. More recently, geographic variations in *pfcr* between codons 72 and 76 have been identified in chloroquine-resistant malaria. *P. falciparum* resistance to S/P is associated with combinations of polymorphisms in dihydrofolate reductase (*dhfr*) (sulfadoxine resistance) and dihydropteroate synthetase (*dhps*) (pyrimethamine resistance). Among those currently identified are the polymorphisms S436A/F, A437G, L540E, A581G, and S613A/T in *dhps* and A16V, N51I, C59R, S108N/T, and I164L in *dhfr*. It has been postulated that the 108N polymorphism may be a good indicator of clinical S/P resistance even without polymorphisms in *dhps*.

In this study, we have analyzed *P. falciparum* DNA taken from patients who have re-presented with malaria within 28 days of antimalarial drug therapy in two malaria-endemic areas of Papua New Guinea, Madang in Madang province and Maprik in East Sepik province. During the course of the study, the Papua New Guinea Department of Health revised the standard treatment recommendations for uncomplicated and severe/treatment failure malaria to 4-aminoquinolines plus S/P and S/P plus artemisinin derivatives, respectively. The data presented follows the changes in standard treatment for uncomplicated malaria and the prevalence of molecular markers for 4-aminoquinolines and S/P resistance at health centers in the two areas.

**MATERIALS AND METHODS**

**Study population and blood sample collection.** On the basis of a positive Optimal® (Diamed, Cressier, Switzerland) test result, 262 patients were recruited between May 2000 and November 2001, 127 from the town clinic in Madang, Madang Province and 145 from the Maprik Hospital in the East Sepik Province of Papua New Guinea as part of a comparative drug trial for resistant malaria (Genton B and others, unpublished data). Both areas are malaria endemic and have had access to anti-malarial drugs, predominantly chloroquine/amodiaquine and quinine, for many years. Patients were recruited if there was a history of anti-malarial drug treatment in the previous 28 days as determined by the patient’s health record book. Overall, 209 were *P. falciparum* positive by the Optimal® test, although only 199 were *P. falciparum* positive by a polymerase chain reaction (PCR). Due to periodic technical difficulties, not all samples were successfully amplified at all polymorphic sites.

At the Madang site, S/P (in combination with 4-aminoquinolines) was introduced as the first-line treatment in late 2000 resulting in changed prior drug exposure of enrolled cases. To better assess the effects of this treatment change, two sets of samples from *P. falciparum*-positive patients, not treated for malaria in the previous 28 days, were included in the analyses. A total of 28 samples from the early study period
necessary.

**Preparation of template DNA.** Packed red blood cells (50 μL) were lysed in 250 μL of 8 M guanidine hydrochloride/0.1 M sodium acetate and stored at 4°C. The DNA was isolated using a variation of the Wizard SV Miniprep system (Promega, Madison, WI). Essentially, 50 μL of red blood cells/guanidine solution was mixed with 250 μL of column wash solution, transferred to the spin column, washed twice with 250 μL of column wash solution, and eluted into a sterile microcentrifuge tube with 50 μL of nuclease-free water.

**Analysis by PCR.** The PCR analysis was performed using published methods for pfmdr1, dhfr, and dhps14,24,25 (oligonucleotides also found under supporting information at www.pnas.org for Mehlotra and others15). For nested PCRs, 3 μL of primary PCR product was used as template for a secondary PCR. All PCRs used one unit of RedTaq polymerase with associated buffer (Sigma-Aldrich, St. Louis, MO) per 50-μL reaction and were performed in an Eppendorf Mastercycler (Eppendorf-Netheler-Hinz, Hamburg, Germany). The PCR products were subjected to electrophoresis on 1.5–2.5% agarose gels (UltraPure; Gibco-BRL, Gaithersburg, MD) and visualized with ethidium bromide in a UVP Imaging System (UVP Inc., Upland, CA).

**Restriction fragment length polymorphism.** Restriction digestions for pfmdr1, dhfr, and dhps were carried out as previously described14,24,25 using the restriction enzymes Afl III, Ava I, BsaW I, Bse I, BstN I, BstUI I, Dde I, Fok I, Mnl I, Mwo I, Nla III, and Xmn I (New England Biolabs, Beverly, MA); Alu I, Bgl II, BseN I (Bsr I), BstI I (Age I), Eco 321 (EcoRV), Taq I (TspE I), Vsp I (Ase I), and Xap I (Apo I) (MBI Fermentas, Vilnius, Lithuania); and Dra I, Hha I, Hind III,MspA, and Xmn I (Promega). The enzyme Vsp I (MBI Fermentas) was used to digest pfcr PCR products. All reactions were carried out for two hours using recommended buffers and conditions. If there was doubt about a complete digestion, reactions were repeated overnight.

**Statistical analysis.** Statistical analyses were done using STATA version 7.0 (Stata Corp., College Station, TX) statistical analyses software. Chi-square tests and where adequate Fisher’s exact tests were used to test for difference in polymorphism rates between different groups.

### RESULTS

**Prior drug exposure.** There were a limited number of drugs prescribed by health officers and nurses over the course of this study: amodiaquine, chloroquine, S/P, primaquine, and quinine. Of the 199 P. falciparum-positive patients, the 28-day anti-malarial pretreatment history of 187 was known, 75 of 77 from Madang and 112 of 122 from Maprik. There were 14 different drug combinations recorded in this study. Table 1 shows only the more common treatments. A total of 82% (153 of 187) had exposure to 4-aminoquinolines, although treatment with 4-aminoquinolines alone was twice as prevalent in Maprik (41% versus 21%; \( P = 0.005 \); Table 1). Patients in Madang were twice as likely to be treated with S/P in any combination than patients in Maprik (35% versus 17%; \( P = 0.006 \)). In May 2000, the government of Papua New Guinea changed the standard treatment for uncomplicated malaria from 4-aminoquinolines plus pyrimethamine to 4-aminoquinolines plus S/P. The introduction of this treatment was relatively prompt in Madang, with the first recorded patient in this study receiving this treatment in November 2000. This resulted in a significant increase in S/P exposure (4% to 41%; \( P = 0.001 \)) and an equivalent decrease in primaquine use (63% to 18%; \( P = 0.001 \), Table 1). No patients from Maprik received the standard treatment of 4-aminoquinolines plus S/P. However, treatment with S/P in any combination doubled in Maprik from the first to the second half of the study period (10% versus 21%; \( P = 0.17 \), Table 1).

**Polymorphism rates.** The number of patients carrying parasites with the threonine polymorphism at pfcr K76T was 96% (75 of 78) in Maprik and 97% (30 of 31) in Madang (Figure 1). Although PCR products were examined for polymorphisms in pfmdr1 at N86Y, S1034C, N1042D, and DI246Y, the only polymorphism observed was at 86Y. This polymorphism was significantly more frequent in Madang than Maprik (96% [67 of 70] versus 84% [82 of 98]; \( P = 0.02 \)). In Maprik there was a significant association between use of 4-aminoquinolines and the pfmdr1 86Y, with polymorphism rates of 88% (66 of 75) and 68% (17 of 22) (\( P = 0.03 \)) in exposed and non-exposed patients, respectively. This association was not found in Madang.

Two polymorphisms in dhfr associated with pyrimethamine resistance, C59R and N108S, were observed in both Maprik and Madang (Figure 1). Polymorphism rates in Madang and Maprik were significantly different for 59R (74% [54 of 73] versus 51% [55 of 108]; \( P = 0.02 \)), but not for 108N (82% [63 of 77] versus 72% [77 of 101]; \( P = 0.17 \)). No polymorphisms were observed at codons 16, 51, or 164 of dhfr. Likewise, no

### Table 1

Comparison of major treatment combinations for Plasmodium falciparum–positive recruits treated in the previous 28 days for the first (May 2000–January 2001) and second (February–October 2001) half of the study period*

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<tbody>
<tr>
<td>Total 4-A (all combinations)</td>
<td>64 (84%)</td>
<td>22 (88%)</td>
<td>42 (84%)</td>
<td>89 (79%)</td>
<td>30 (77%)</td>
<td>59 (81%)</td>
</tr>
<tr>
<td>4-A alone</td>
<td>16 (21%)†</td>
<td>4 (16%)</td>
<td>12 (24%)</td>
<td>46 (41%)†</td>
<td>17 (44%)</td>
<td>29 (40%)</td>
</tr>
<tr>
<td>Total S/P (all combinations)</td>
<td>26 (35%)†</td>
<td>2 (8%)</td>
<td>24 (47%)</td>
<td>19 (17%)‡</td>
<td>4 (10%)</td>
<td>15 (21%)</td>
</tr>
<tr>
<td>4-A + S/P</td>
<td>22 (29%)§</td>
<td>1 (4%)</td>
<td>21 (41%)§</td>
<td>7 (6%)</td>
<td>0</td>
<td>7 (10%)</td>
</tr>
<tr>
<td>4-A + Pr</td>
<td>24 (32%)</td>
<td>15 (63%)§</td>
<td>9 (18%)§</td>
<td>40 (36%)</td>
<td>12 (31%)</td>
<td>28 (38%)</td>
</tr>
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*4-A = 4-aminoquinolines; S/P = sulfadoxine/pyrimethamine; Pr = primaquine.
† \( P = 0.005 \).
‡ \( P = 0.006 \).
§ \( P = 0.001 \).
polymorphisms associated with sulfadoxine resistance were observed in *dhps* at codons 436, 437, 540, 581, or 613. There was no significant association between recent S/P exposure and *dhfr* polymorphisms.

There were 12 examples in 11 patients in Maprik and one example in Madang where the presence of both the wild-type and polymorphic variant were present at the same allele of an infection. Of these, seven were at S108N and two were at N86Y in Maprik during the first half of the study period. In addition, there was one example each of S108N and N86Y in Maprik and one N86Y in Madang during the second half of the study period.

From the first half (May 2000–January 2001) to the second half (February–October 2001) of the study period, polymorphism rates for *dhfr* 59R in Madang were static (*P* > 0.5; Figure 2). However, there was a significant decrease in the rate of *dhfr* 108N polymorphisms (from 96% [23 of 24] to 75% [38 of 51]; *P* = 0.03). In Maprik, there was an increase in the rate for *dhfr* 59R (from 38% [15 of 39] to 57% [30 of 69]; *P* = 0.07; Figure 2), while rates for 108N mutations were unchanged (*P* > 0.5). No significant changes were observed in rates of *pfcrT* 76T and *pfmdr1* 86Y mutations at either site.

In samples of *P. falciparum*-positive patients who had not been treated with antimalarials in the previous 28 days at the start (May–July 2000) and end (October 2001–January 2002) of the study period, in Madang there was a significant increase in both *dhfr* 59R mutations (from 45% [13 of 29] to 96% [22 of 23]; *P* < 0.001) and in *dhfr* 108N mutations (from 69% [20 of 29] to 96% [23 of 24]; *P* < 0.05). However, rates of *pfcrT* 76T and *pfmdr1* 86Y mutations remained stable.

**DISCUSSION**

This study enrolled patients who had been treated for malaria in the 28 days prior to their current malaria diagnosis. Their previous drug treatment, as shown in the patient’s health record book, was recorded to ascertain drug pressure on the parasite population. Over the period of the study, Madang had a higher exposure rate to S/P than Maprik. This correlates with the results of the molecular analysis, which show that polymorphic rates for *dhfr* 59R and 108N are both higher in Madang than Maprik.

The data show that Madang adapted more rapidly to the change in government standard treatment for uncomplicated malaria from the 4-aminoquinolines plus primaquine combination therapy to 4-aminoquinolines plus S/P. As a consequence, there was a marked increase in S/P and reduction in primaquine exposure between the first and second halves of the study period. While the Maprik site did not adopt the 4-aminoquinolines plus S/P combination therapy, there was a general shift to S/P in other combinations. This shift was accompanied by an increase in *dhfr* 59R polymorphism rates in Maprik, but rather unexpectedly, by a significant decrease in *dhfr* 108N rates at the Madang site. This decrease is even more puzzling given the observed increase in both *dhfr* 59R and 108N polymorphisms in non-treatment failure cases following the introduction of S/P at the Madang site. It is therefore highly unlikely that the introduction of 4-aminoquinolines plus S/P by itself had a suppressive effect on the 108N polymorphism in resistant cases. In contrast, there was a significant association of the 108N mutations with prior primaquine exposure (Casey GJ, unpublished data) and the decrease in 108N mutation rates parallels the reduction in primaquine use. However, given the proposed mode of action of primaquine, this association is unlikely to be a causal one. Further studies are needed to clarify these observations.

**FIGURE 1.** Overall polymorphism rates for Maprik and Madang, Papua New Guinea, May 2000–November 2001. *pfcrT* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; *dhfr* = dihydrofolate reductase. *P* = 0.02.

**FIGURE 2.** Comparison of polymorphism rates between May 2000–January 2001 and February–October 2001 study periods for Madang (A) and Maprik (B), Papua New Guinea. *pfcrT* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; *dhfr* = dihydrofolate reductase. *P* = 0.03, **P** = 0.07.
higher total polymorphism rates in Madang are likely due to Madang having easier access to antimalarials over many years and a population that is more able to afford drugs. Drugs are also available from non-pharmaceutical outlets leading to self-treatment and non-compliance with treatment dosage. As a result, there would be increased drug pressure on resistance polymorphisms. This, coupled with long-term exposure to 4-aminoquinolines at both sites, has lead to the pfcrt 76T and pfmdr1 86Y polymorphisms becoming fixed in the parasite populations of both Madang and Maprik as shown by the high rate of polymorphisms and the low rate of mixed alleles at these two loci.

The high rates of pfcrt and pfmdr1 polymorphisms together with the significant presence of two polymorphisms in dhfr implies that it is the sulfadoxine component in association with acquired immunity that is providing most therapeutic protection. Planned in vivo efficacy studies with immunologically naive children may clarify the degree to which acquired immunity protects the broader population from treatment failure. This suggests that there will be strong selection pressure on the dhps gene leading to the development of resistance polymorphisms and an increase in treatment failure. Sulfadoxine/pyrimethamine is also part of the new standard second-line treatment in combination with artemisinin derivatives. Thus, if S/P becomes ineffective it leaves the artemisinin derivatives as the only effective standard anti-malarial treatment in Papua New Guinea. It is certainly an advantage that S/P is a single-dose treatment that can be administered on day 0 at the health clinic. However, with the availability of antimalarials at non-pharmaceutical outlets, underdosing will remain a reality. As has been pointed out previously,27 when S/P is used judiciously, resistance develops rapidly.

While the high prevalence of pfcrt and pfmdr1 polymorphisms are consistent with the increasing levels of in vivo resistance to 4-aminoquinolines observed in Papua New Guinea since the 1980s,28 the high level of dhfr polymorphisms in both Madang and Maprik are surprising considering that S/P was used only as a second-line drug (in combination with quinine) before 2000. However, pyrimethamine (in combination with chloroquine) had been widely used in mass drug administration campaigns during the malaria eradication/ control era of the 1960s and early 1970s,29 and a high level of in vitro resistance to pyrimethamine was present by 1980,30 The complete absence of dhps polymorphisms at both field sites indicates long-term persistence of dhfr polymorphisms selected for by past pyrimethamine use rather than recently arisen polymorphisms as the primary origin of dhfr polymorphisms in Papua New Guinea. Such long-term persistence of dhfr polymorphisms is also found in Southeast Asia where high levels of dhfr and dhps polymorphisms persist even though S/P has long been replaced by other antimalarials as first-line treatment.31

The two dhfr polymorphisms that were observed in this study may not be sufficient for clinical resistance. However, they do indicate that the parasite population has the potential to evolve into the dhfr/dhps quintuple mutant polymorphism regarded as a strong predictor of S/P resistance.32 Given the observed fixed levels of chloroquine resistance polymorphisms in pfmdr1 and pfcrt, the current treatment strategy may only be effective for a short time. Keeping in mind that drugs of suboptimal efficacy are more effective in older people who have developed a degree of acquired immunity than in children who are more likely to be immunologically naive, it may be pertinent to question whether young children in malaria-endemic areas of Papua New Guinea should be administered amodiaquine with S/P. It may well be worth reassessing the options for combination chemotherapy of uncomplicated malaria in Papua New Guinea for this section of the population. Further studies currently planned to investigate the molecular status of parasites in treatment naive children with uncomplicated malaria may help clarify the role of acquired immunity in S/P efficacy. Monitoring of the status of dhps alleles remains a high priority as a predictor of developing clinical resistance.

Received May 27, 2003. Accepted for publication November 4, 2003.

Acknowledgments: We thank the people of East Sepik and Madang who made themselves available for this study. Thanks are also given to Inoni Betuela for assistance in setting up the comparative drug trial from which this data was taken, Eric Kum, Jacob Dago, and Roslyn Kilungo for assistance with patient recruitment, and the many Papua New Guinea Institute of Medical Research staff in Madang and Maprik who provided invaluable assistance with this project.

Financial support: This investigation was supported by the Broken Hill Proprietary Community Trust of Australia. The Papua New Guinea Institute of Medical Research Maprik site receives general support from the Australian Agency for International Development.

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