

Quantifying the Evolution and Impact of Antimalarial Drug Resistance: Drug Use, Spread of Resistance, and Drug Failure over a 12-Year Period in Papua New Guinea

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Background. Antimalarial use is a key factor driving drug resistance and reduced treatment effectiveness in *Plasmodium falciparum* malaria, but there are few formal, quantitative analyses of this process.

Methods. We analyzed drug usage, drug failure rates, and the frequencies of mutations and haplotypes known to be associated with drug resistance over a 12-year period (1991–2002) in a site in Papua New Guinea. This period included 2 successive treatment policies: amodiaquine (AQ) or chloroquine (CQ) from 1991 through 2000 and their subsequent replacement by sulfadoxine-pyrimethamine (SP) plus AQ or SP plus CQ.

Results. Drug use approximated 1 treatment per person-year and was associated with increasing frequencies of *pfcr* and *pfmdr1* mutations and of treatment failure. The frequency of *pfdhfr* mutations also increased, especially after the change in treatment policy. Treatment failure rates multiplied by 3.5 between 1996 and 2000 but then decreased dramatically after treatment policy change.

Conclusions. With high levels of resistance to CQ, AQ, and SP, the deployment of the combination of both drugs appears to increase clinical effectiveness but does not decelerate growth of resistance. Our estimates of mutation and haplotype frequencies provide estimates of selection coefficients acting in this environment, which are key parameters for understanding the dynamics of resistance.

Mass deployment of antimalarial drugs invariably results in emergence and spread of resistance. The effectiveness of the most widely used antimalarials, chlo-

roquine (CQ) and sulfadoxine-pyrimethamine (SP), was heavily compromised by the emergence and spread of resistant *Plasmodium falciparum* [1]. The genetic basis of this resistance is known in some detail: resistance to CQ and SP is associated with accumulation of mutations in *pfcr*/*pfmdr1* and *pfdhfr*/*pfdhps* genes, respectively [2–4], which have spread within and across continents [5–7]. Here, we use a unique 12-year compilation of clinical records of drug use, molecular analyses of blood samples, and longitudinal morbidity surveillance to quantify the 3 key components of the process leading to drug failure:

1. The driving force for resistance: the level of community drug use.
2. The mediator: the spread of resistance-associated mutations.
3. The outcome: drug failure and reduced effectiveness.

We investigate and quantify the relationship between these 3 factors to investigate the impact of drug pressure

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in driving mutations that encodes CQ and SP resistance in an area of moderate to high malaria transmission in Papua New Guinea (PNG). Rather than considering prevalences of these mutations in patient blood, which is potentially misleading if there are changes in transmission intensity and multiplicity of infection (MOI), we present these analyses of the evolution of resistance in terms of allele and haplotype frequencies [8]. We then proceed to quantify the impact on failure rates and therapeutic effectiveness.

PATIENTS AND METHODS

Patients and follow-up. The study, part of the Malaria Vaccine Epidemiology and Evaluation Project [9–11] in Wosera, focused on the inhabitants of the immediate catchment area of the Kunjingini health center in East Sepik Province, PNG. This health center provided the only source of primary care for this area [12], and identification (ID) numbers, presumptive diagnosis, and treatment were recorded for all patients that presented during the period 1991–2002. ID numbers were then linked to those in the Wosera demographic surveillance system, and reattendance at health facilities within any given period of time was thus recorded.

Patients with fever were treated presumptively for malaria according to the guidelines of the PNG Department of Health [13], which for uncomplicated malaria recommended amodiaquine (AQ) for children <20 kg and CQ for others, until 2000. From 1997 on, to reduce vivax malaria transmission, 1 dose of primaquine (PQ) was combined with AQ or CQ. Second-line treatment was quinine (QN) plus SP. In 2000, the first-line treatment changed to AQ plus SP or CQ plus SP, depending on the patient's weight.

Blood was collected for parasitological and hematological assessment, and samples were frozen in EDTA-Microtainer (Becton Dickinson).

The Medical Research and Advisory Committee of the Ministry of Health in PNG gave scientific approval and ethical clearance, and written informed consent was obtained from patients, parents, or legal guardians before recruitment of each patient.

Molecular analysis. DNA was extracted by the QIAamp DNA kit from a random sample of 50 blood samples per year from cases that presented with >1000 asexual *P. falciparum* parasites/ μ L. Polymorphisms in the *pfcr* (C72S, M74I, K76T, H97Q, T152A, S163R, A220S, Q271E, N326S/D, I356L/T, R371I), *pfmdr1* (N86Y, Y184F, S1034C, N1042D, D1246Y), *pfdhfr* (A16V, N51I, C59R, S108N, I164L), and *pfdhps* (S436A/E, A437G, K540E, A581G, A613T/S, I640E, H645P) genes were analyzed by DNA microarray as described elsewhere [14]. MOI was determined using GeneScan-based *msp2* genotyping [15].

Drug use. Drug pressure was measured as the mean number of treatments per person per year. For 1991–1993, the total

number of treatments dispensed from the clinic was known. The number of treatments in our study area was then obtained by multiplying this total by the proportion of the clinic's catchment falling in our study area (39%, obtained from the total person-time in the demography database for 1999–2002). Our experiences during this period suggest that this catchment had typical levels of patients seeking malaria treatment and that local demographic characteristics did not alter significantly between 1991–1993 and 1997–2002. For 1994–1996, no reliable data on treatments were available. From 1997 onward, treatment data were available from the Institute of Medical Research (IMR) study nurses who recorded the drugs used during their visits. They were not present each day, so the total number of treatments was obtained by dividing the recorded number of IMR treatments by the proportion of days that the IMR study team was present. This calculation relies on the assumption that treatment rates and type were independent of presence of the IMR nurses, because the nurses' role was observational.

Allele and haplotype frequencies. Single allele frequencies of resistance markers were estimated from nonlinear statistical model by accounting for the effects of varying multiplicity of infection and by assuming that resistant and sensitive parasite clones are transmitted independently. The likelihood of a sample containing no resistant clones is $(1 - p)^n$, where p is the frequency for the mutant allele and n is the MOI of the sample. Similarly, the likelihood of a sample containing no wild-type allele is p^n , and for a mixture of both, a wild-type and a resistant allele, is $1 - p^n - (1 - p)^n$. The overall likelihood for p is then the product of this likelihood over all samples, using values of n obtained from *msp2* genotyping results [16]. We used a simple 1-dimensional search routine to maximize this likelihood and obtain estimates of p [17]. This maximum likelihood (ML) analysis is a standard-population genetic approach for samples whose genetic composition is ambiguous (eg, ambiguity arises if a sample has MOI = 4 and contains both mutant and wild-type alleles because it is unclear whether the sample contains 1, 2, or 3 mutant clones). ML emphasizes the probability of observing the entire data set rather than attempting to unambiguously identify the genetic composition of each sample. Consequently, it can include all samples, and we were not forced to discard genetically ambiguous samples when MOI \geq 2. Polymerase chain reaction (PCR) bias and parasite sequestration means that minor clones may be missed in some samples and MOI may be underestimated. However, we are not directly counting the number of clones of each genotype, so this effect is not important. We avoid subsequent problems, such as the most appropriate denominator to use; provided that the probability of detecting clones is unaffected by the genotype being analyzed, then the methodology is robust. The frequencies of distinct haplotypes for *pfdhfr* and *pfdhps* were estimated by ML using the MalHaploFreq program [8]; the ML approach is de-

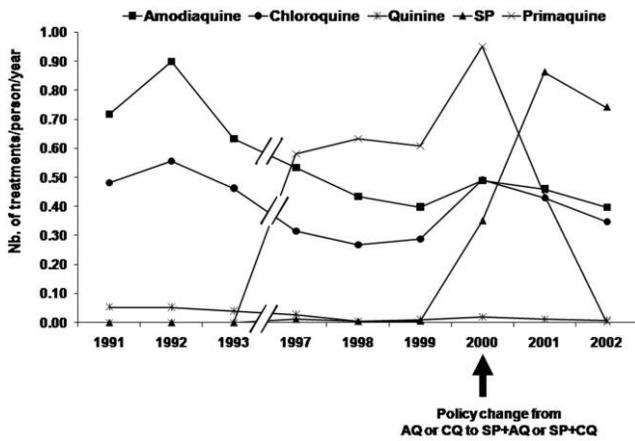


Figure 1. Per capita treatment showing use of various drug combinations, from 1991 through 2002 (but excluding 1994–1996 due to lack of data). AQ, amodiaquine, CQ, chloroquine; SP, sulfadoxine-pyrimethamine.

scribed in more detail in that publication and in the accompanying users' manual.

Selection coefficients were calculated for each allele and haplotype by plotting $\ln(R/S)$ against time, where R and S are the frequency of resistant and sensitive alleles or haplotypes, re-

spectively [18]. Time was converted to parasite generations that assumed 6 *P. falciparum* generations per year. The selection coefficient for each single-nucleotide polymorphism (SNP) or haplotype was the slope obtained by regression analysis for the 12-year period. Annual selection coefficients were also calculated for correlation with contemporaneous drug usage.

Treatment effectiveness. Treatment failure rates were calculated by year as the proportion of patients with confirmed malaria administered an antimalarial treatment course who represented with a subsequent episode of confirmed malaria within 8–28 or 29–42 days of start of treatment [19]. Treatment courses were provided at health facilities, and subsequent patient compliance was not noted. Hence, our failure rates reflect drug effectiveness. It is important not to confuse effectiveness with drug efficacy measures that arise from clinical trials. The latter provide drugs in idealized conditions (typically giving exact doses based on weight and observing compliance) to confirm that the drug will work in principle, rather than in practice. Our use of drug failure to measure effectiveness is more appropriate for 2 main reasons. First, it reflects the real-life conditions under which people use (and misuse) the drugs. Second, the impact of mutations depends on their environment: in principle, they might not compromise drug efficacy (mutant parasites are always killed by correct treatment) but

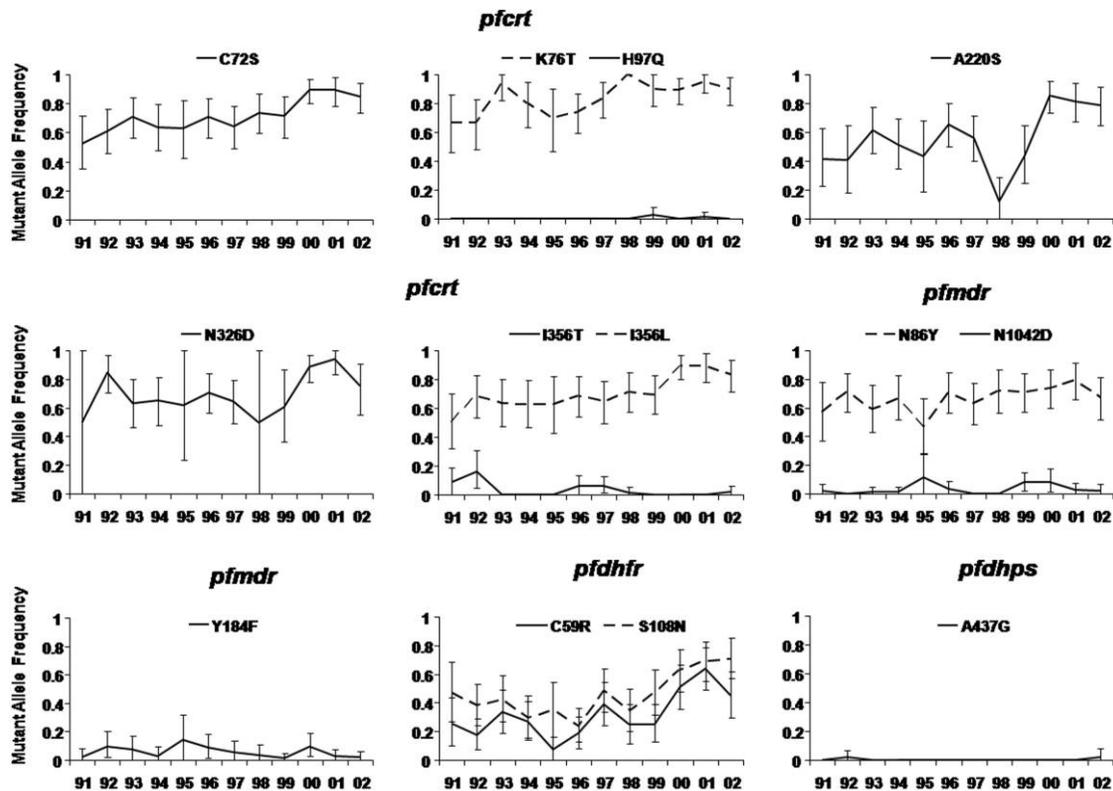


Figure 2. Changes in mutant allele frequency from 1991 through 2002 as a result of use of sulfadoxine-pyrimethamine in combination with chloroquine or amodiaquine.

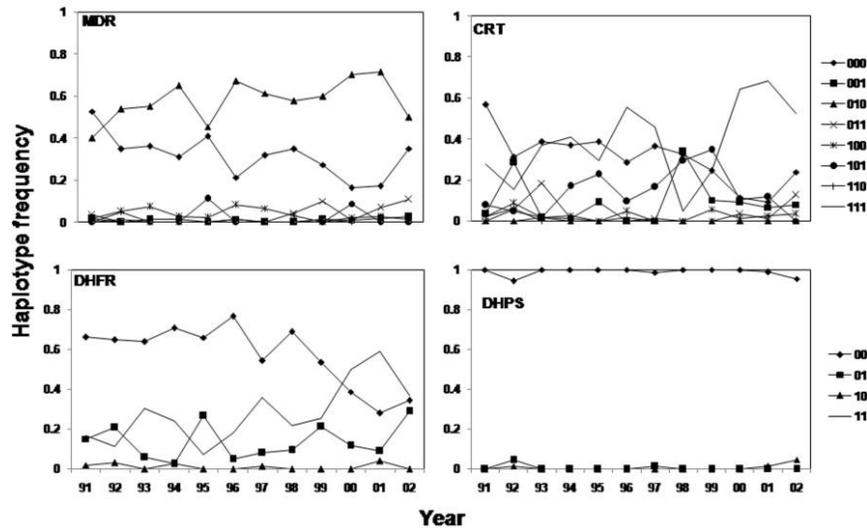


Figure 3. Changes in haplotype frequency from 1991 through 2004.

may reduce effectiveness (mutant parasites may survive the suboptimal treatments that inevitably occur in practice). Failure rates were not PCR-corrected, so they include both recrudescence infections that survived treatment and reinfections. Both are important for effectiveness; mutations to resistance increase the probability of surviving treatment and also allow parasites to tolerate high drug levels, thus enabling them to establish new infections in people more rapidly after treatment [20]. Thus, the absence of PCR correction is not problematic for the study.

RESULTS

Drug use. A total of 7612 AQ, 5179 CQ, 337 QN, 2571 SP, and 4430 PQ treatments were delivered at the Kunjingini Health Center to patients from the core villages from 1991 through 2002, corresponding to an average of 0.81 treatment courses per person-year. From 1991 through 1993, only AQ, CQ, and QN were prescribed. Per capita treatment decreased for AQ and CQ between 1991 and 1999 (with the proviso that data for 1994–1996 were not available), remaining relatively stable thereafter (Figure 1), but was always higher for AQ than for CQ. QN use was low during the entire 12-year period. PQ use commenced in 1997 with 0.58 treatments per person, increased to 0.95 in 2000, and then decreased to 0 in 2002 (Figure 1). SP use started in 1997, with 0.01 treatments per person and remained low until 1999. SP was then combined with CQ or AQ as recommended first-line drug, increasing rapidly to 0.86 and 0.74 treatments per person-year in 2001 and 2002, respectively (Figure 2). Slides collected by the IMR team during their visits showed that 56% of the (presumptively) treated patients did not have *P. falciparum* (data not shown), with this percentage stable over time.

Genetic analyses. MOI showed no significant trend over time, averaging 1.4 ± 0.15 *P. falciparum* clones per sample, with the lowest MOI in 1991 (1.21) and the highest in 2000 (1.78). Thus, 98% of samples could be genotyped (588 samples), with polymorphisms found in *pfprt* codons C72S, K76T, H97Q, A220S, N326D, I356L, and I356T, *pfmdr1* codons N86Y, Y184F, and N1042D, *pfdhfr* codons C59R and S108N, and *pfdhps* codon A437G. The changes in SNP and haplotype frequencies are shown in Figures 2 and 3, respectively.

Most mutant allele frequencies increased over the 12-year period, except *pfmdr1* Y184F and N1042D, *pfprt* I356T and H97Q, and *pfdhps* A437G, which were at low frequencies and showed no discernible trend. Mutant gene frequencies in *pfprt*, already frequent (>0.4) in 1991 (Figure 2), increased over the 12-year period (for *pfprt* I356L, C72S, and K76T multiplying by 1.67, 1.63, and 1.34, respectively). The *pfmdr1* N86Y frequency was multiplied by 1.18 (Figure 3). The mutant allele frequencies of *pfdhfr* C59R and S108N decreased between 1991 and 1995, from 0.47 to 0.24 for S108N and 0.26 to 0.07 for C59R. From 1995 onward, the frequencies of these alleles multiplied by 3 and 6.4, respectively.

Among haplotypes, the frequency of *pfdhfr* is the most interesting. The wild-type frequency did not vary significantly between 1991 and 1998, but it dramatically decreased thereafter. The *pfdhfr*108 single-mutant haplotype did not change much in frequency over the entire period. There was no clear trend for *pfdhfr* double-mutant haplotype 59R/108N between 1991 and 1997, but after 1998 it increased to a frequency that was 2.5 times higher in 2001 than in 1998 (Figure 3).

The selection coefficients for all the mutations are listed in Table 1. There was significant selection for the *pfprt* SNPs C72S and I356L ($P \leq .05$) and the *pfdhfr* SNPs C59R (and S108N

Table 1. Analysis of the Spread and Consequences of Single-Nucleotide Polymorphism (SNPs) and Haplotypes in *pfprt*, *pfmdr1*, and *pfdhfr*

SNP or haplotype	Selection coefficient, % (95% CI)	Correlation between selection coefficient and drug usage					Correlation between SNP/haplotype frequency and drug failure rates	
		CQ	AQ	QN	PQ	SP	ETF	LTF
Gene <i>pfprt</i> . (haplotypes defined at codons 76, 220, and 356)								
SNP C72S	2.60 ^a (1.47 → 3.73)	-0.05	-0.62	-0.68 ^a	0.45	N/A	0.33	0.05
SNP K76T	4.08 (-4.90 → 13.05)	-0.56	-0.43	-0.55	0.36	N/A	0.65 ^b	0.01
SNP H97Q	6.13 (-2.54 → 14.80)	-0.27	-0.41	-0.42	0.27	N/A	0.63 ^c	0.77 ^a
SNP I356L	2.53 ^a (1.31 → 3.75)	0.04	-0.50	-0.61	0.45	N/A	0.21	0.07
SNP I356T	-4.94 (-19.3 → 09.4)	-0.003	0.45	0.37	-0.41	N/A	-0.23	-0.14
SNP A220S	2.15 (-0.82 → 5.12)	0.35	-0.20	-0.11	0.07	N/A	-0.38	-0.13
SNP N326D	1.70 (-0.73 → 4.13)	0.47	0.01	-0.12	0.18	N/A	-0.50	0.02
Haplotype 000	-0.025 ^a (-0.04 → -0.01)	-0.02	0.48	0.60	-0.54	N/A	-0.21	-0.24
Haplotype 001	0.01 (-0.11 → 0.13)	0.23	0.04	-0.13	-0.11	N/A	0.15	-0.15
Haplotype 010	-0.03 (-0.10 → 0.04)	0.21	0.18	0.29	-0.37	N/A	-0.18	-0.02
Haplotype 011	0.03 (-0.11 → 0.17)	0.45	0.18	0.13	-0.24	N/A	-0.27	-0.05
Haplotype 100	-0.01 (-0.13 → 0.11)	0.6	0.27	0.27	-0.43	N/A	-0.28	0.18
Haplotype 101	-0.04 (-0.13 → 0.05)	-0.05	0.11	0.11	0.55	N/A	0.62 ^c	0.4
Haplotype 110	-0.10 ^a (-0.19 → -0.005)	0.65 ^b	0.88 ^d	0.82 ^d	-0.56	N/A	-0.37	-0.13
Haplotype 111	0.02 (-0.01 → 0.05)	0.26	-0.24	-0.10	0.11	N/A	-0.27	-0.02
Gene <i>fmdr1</i> . (haplotypes defined at codons 86, 184, and 1042)								
SNP N86Y	1.03 (-0.04 → 2.10)	-0.06	-0.37	-0.58	0.53	N/A	0.49	-0.19
SNP Y184F	-1.27 (-3.73 → 1.20)	0.60	0.55	0.47	0.11	N/A	-0.82 ^d	0.04
SNP N1042D	3.27 (-8.76 → 15.31)	0.16	-0.36	-0.17	0.03	N/A	-0.17	0.63 ^c
Haplotype 000	-0.02 ^a (-0.03 → -0.002)	0.003	0.45	0.54	-0.69 ^a	N/A	-0.16	-0.04
Haplotype 100	0.01 (-0.002 → 0.02)	-0.09	-0.43	-0.51	0.75 ^a	N/A	0.24	-0.04
Haplotype 101	0.12 ^a (0.01 → 0.24)	-0.37	-0.64 ^b	-0.62	0.26	N/A	0.78 ^a	0.51
Gene <i>pfdhfr</i> (haplotypes defined at codons 59 and 108)								
SNP C59R	2.25 ^a (0.09 → 4.41)	N/A	N/A	N/A	N/A	0.83 ^d	-0.26	0.35
SNP S108N	1.95 ^a (0.34 → 3.57)	N/A	N/A	N/A	N/A	0.92 ^e	-0.97	-0.92
Haplotype 00	-1.76 ^a (-3.27 → -0.23)	N/A	N/A	N/A	N/A	-0.90 ^e	0.97	0.63
Haplotype 01	0.11 (-2.45 → 2.69)	N/A	N/A	N/A	N/A	0.02	-0.25	-0.80
Haplotype 11	2.15 ^a (0.03 → 4.27)	N/A	N/A	N/A	N/A	0.79 ^d	-0.23	0.38

NOTE. Only the main haplotypes and their constituent SNPs are included, identified in the table using “0” to indicate a wild type and “1” to indicate a mutant. Hence, *pfprt* haplotype 001 is wild type at positions 76 and 220 and mutant at position 356; *pfmdr1* haplotype 011 is wild type at position 86 and mutant at positions 184 and 1042; and so on. The Table provides 3 key factors describing the spread of resistance: (1) selection coefficients for CQ and SP resistance-related molecular markers over the whole study period assuming 6 generations of *Plasmodium falciparum* per year. (2) The correlation between these selection coefficients (a measure of how rapidly the mutations are spreading or disappearing) and level of individual drug use. This was done on an annual basis (ie, each year constitutes 1 data point), excluding years 1994–1996 in which there was no quantification of drug usage. (3) The correlation between the allele/haplotype frequency and early and late treatment failure rates analyzed on an annual basis. In the case of treatment failures and *pfprt* and *pfmdr1*, we only include 1991–1999 (because failure rates are not directly comparable in the periods before and after the introduction of SP as their partner drug). Similarly, SP was only deployed for 3 years, 2000–2002, so correlation was only over 3 time points. AQ, amodiaquine; CI, confidence interval; CQ, chloroquine; ETF, early treatment failure (between days 8 and 28); LTF, late treatment failure (between days 29 and 42); N/A, not applicable; QN, quinine; PQ, primaquine; SP, sulfadoxine-pyrimethamine.

^a $P \leq .05$.

^b $P = .06$.

^c $P = .07$.

^d $P \leq .01$.

^e $P \leq .001$.

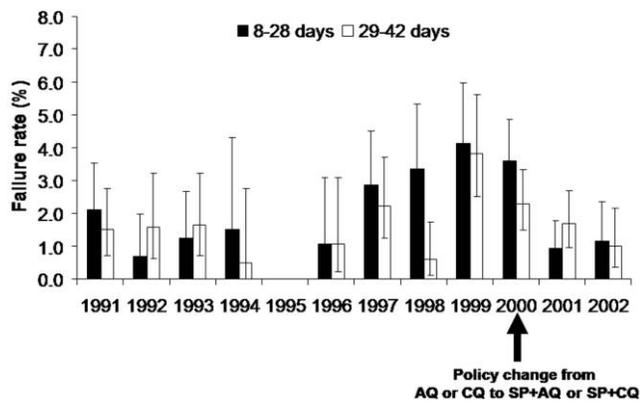


Figure 4. Treatment failure rates in relation to changes in treatment policy. AQ, amodiaquine; CQ, chloroquine; SP, sulfadoxine-pyrimethamine.

($P \leq .05$). Among the *pfcr*t haplotypes, there was low but significant selection against the triple wild-type haplotype and against the double-mutant 76/220 haplotype. In *pfmdr*1, there was significant selection against the triple wild-type haplotype and positive selection for the double-mutant 86/1042 haplotype. There was significant selection for the *pfdhfr* double-mutant haplotype and significant selection against the *pfdhfr* 59/108 wild-type haplotype. In particular, the selection coefficient for the *pfdhfr* 59/108 double mutant after 1999 (ie, after the change to SP plus AQ or SP plus CQ as a first drug in 2000) was 4.87% (data not shown), but it was not significant (−14.34% to 24.07%).

The correlations between selection coefficients and drug use patterns were nonsignificant for nearly all the *pfcr*t and *pfmdr*1 mutations and QN, AQ, and CQ use, except for *pfcr*t C72S and QN use, and *pfcr*t 76/220 haplotype and AQ and QN use (Table 1). In contrast, the correlations between selection coefficients of *pfdhfr* SNPs and haplotypes and SP use were significantly positive.

Treatment effectiveness. Overall, 148 (2.2%) of 6678 treated patients were reinfected during the 8–28-day period and an additional 122 (1.8%) patients were reinfected during the 29–42-day follow-up period. The treatment failure rate for the 28-day follow-up period did not change significantly between 1991 and 1995, but it gradually increased from 1996 until 1999; treatment failures were 3.8 times more common in 1999 than in 1996 (Figure 4). Patient follow-up was not recorded in 1995. After 1999, the treatment failure rate decreased dramatically, resulting in a treatment failure rate that was 3.5 lower in 2002 than it was in 1999 (Figure 1). There was no significant overall trend for treatment failure rates over the 29–42-day follow-up period. Nevertheless, treatment failure during that period was 3.8 times more likely in 2002 than in 1991 (Figure 4).

DISCUSSION

Over the 12-year study period, annual drug use was ~1 treatment per person, consistent with the area being of moderate to high intensity of transmission, with little drug access outside the formal health sector [10]. There was a gradual decline in deployment of AQ and CQ, whereas use of SP was high, following its introduction in 2000. Despite little use of SP before 2000, *pfdhfr*108 and *pfdhfr*59 mutations were already present in 1991, and there was significant selection for these mutations even without evident SP use (Table 1 and Figures 2 and 3). Several explanations are possible: first, trimethoprim-sulfamethoxazole, an antifolate used mainly for its antibacterial effect, could select for *pfdhfr* mutations [21]. Second, SP was not systematically recorded when it was given with QN as a second-line treatment. Third, resistant parasites could immigrate from neighboring areas, where SP resistance was higher. Fourth, effects of antifolate pressure exerted by mass drug administration of pyrimethamine in the 1960s and 1970s were persistent [22]. Any of these explanations would be compatible with the model that the acquisition of mutations enabling parasites to become tolerant takes much longer than subsequent acquisition of solid resistance [23]. The haplotypes of genes, rather than polymorphisms at individual codons, are the basic unit of drug selection. Evolution of resistance may proceed through a series of haplotype turnovers, some haplotypes initially spreading before being replaced in turn by more-resistant haplotypes [23]. Interestingly, our analyses suggest that resistance is in very different stages of development (Figure 3) in the 4 genes; *pfdhfr* is represented essentially by only wild-type haplotypes. Mutants occur only on a single codon 86 in *pfmdr*1, although there was a late appearance of a double *pfmdr*86/1042 haplotype. In *pfcr*t the wild-type haplotype was almost eliminated and replaced by the 76/356 and 76/220/356 mutant haplotypes, with the triple mutant eventually largely displacing the double mutant.

In *pfdhfr*, the wild-type haplotype shows a steady decline with the 108 single-mutant haplotype, initially being slowly displaced by the 59/108 double-mutant haplotype but increasing rapidly after the introduction of SP as a first-line drug in 1999. The estimated selection coefficient was 4.9% after this introduction, and although the sample size was tiny (3-year points), resulting in a large confidence interval (−14% to 24%), it is interesting that this estimate is almost identical to that of 5% reported by Anderson and Roper [18], despite their work being performed in a low-transmission area and examining a more highly resistant *pfdhfr* triple mutant haplotype, which would have been displacing the double.

Note that these selection coefficients are overall values, which reflect both the advantage of the mutations in the presence of a drug and their possible disadvantage in untreated individuals [24–28]. The selective disadvantage of *pfcr*tK76T in the absence

of drug treatment was previously estimated at -5% [27] in Malawi, so making an illustrative assumption that the advantage in the presence of the drug is $+25\%$ and that 30% of infections are treated would give an overall selection coefficient of $(0.3 \times 0.25) + (0.7 \times -0.05) = 0.04$. Obviously, as drug use increases or decreases, this overall selection coefficient will change; hence, the importance of examining the correlation between the 2 parameters is clear (Table 1). In principle, it should be possible to disentangle the 2 effects by regressing the selection coefficient against drug use and finding where the regression cuts the Y axis (when drug use = 0), which would give the selective disadvantage in the absence of the drug, but in practice the relationship was so weak as to preclude this calculation.

The sign and magnitude of these selection coefficients, s , is a key parameter in much of population genetic theory. For example, the magnitude of s affects the following:

1. The rate at which mutations and haplotypes migrate across geographic regions. Classically, the rate is $\sigma\sqrt{2s}$, where σ is the standard deviation of parasite dispersal via host movement ([23]; but see the brief discussion in the Appendix of [29]).
2. The chance of new mutations surviving the first few generations after their first appearance or introduction (see box 2 of [30]).
3. The mutation/selection balance and hence the frequency of the mutation that would be present before drugs are deployed [31].
4. The size of the selective sweep in the genome surrounding a mutation being selected for resistance [32].

The estimates for s given in Table 1 can therefore be used to calibrate and inform broader discussions of the population genetics of emergence and spread of resistance. They can also be used to validate models of the spread of resistant mutations, which should predict selection coefficients similar to those observed in the field. Although the estimates of s are imprecise, the results are remarkably consistent (Table 1). Despite the central role of the selection coefficient in understanding the dynamics of resistance, in addition to the value of 5% estimated for *pfdhfr* by Anderson and Roper [18], the only other estimate of selection coefficients acting on *P. falciparum* drug-resistant mutations/haplotypes appears to be that of Nair et al [33]. They used the decline in clinical effectiveness of SP rather than direct frequency estimation to assess the selection coefficient acting on *dhfr* resistance as 11%. The results summarized in Table 1 are therefore a substantive advance.

The increasing treatment failure rate between 1996 and 1999 can be ascribed to the increasing parasite resistance to standard treatment with CQ and AQ [34, 35], consistent with increasing

frequencies of the CQ resistance-associated mutant alleles in *pfcr* and *pfmdr1*. Although AQ in combination with SP is efficacious in some areas where both drugs have previously been used in monotherapy [36, 37], this drug combination is already failing in PNG [38–40]. Our data have shown that resistant *pfdhfr* allele frequencies already began to increase in 1998 before the large-scale use of SP. The addition of SP to AQ or CQ in 2000 improved the effectiveness of the standard treatment but did not prevent the increase in 4-aminoquinolines resistance in this situation where resistant alleles were already frequent. Treatment failure rates by day 28 with combination therapy reached 16% in Wosera in 2003 [38]. Later molecular data have also confirmed that resistance to SP, as measured by *pfdhfr* S108N and C59R frequencies, continued to increase steadily from 1999 onward [39, 40].

Few previous studies have assessed the relationships between therapeutic effectiveness and molecular resistance markers over extended periods [41–43]. The only other study, in Senegal [19], to correlate these with drug pressure found results similar to ours. The frequencies of both resistance markers and clinical failure to both SP and CQ increased rapidly despite restriction of antimalarial treatments to parasitologically confirmed cases [19].

We conclude by considering how closely the 3 parts of the process leading to resistance interrelate. There were strong correlations between SP use and *pfdhfr* selection coefficients but little correlation between drug use and selection coefficients in either *pfcr* or *pfmdr1*, probably because mutations known to be associated with resistance (*pfcr*K76T and *pfmdr*N86Y) were already present at high frequencies. There also appears to be a closer relationship between mutations in *pfdhfr* and SP resistance than there is between *pfmdr1/pfcr* mutations and CQ/AQ failures. The weak correlations between individual mutation/haplotype frequency and CQ/AQ failure rates very likely reflect other phenomena, such as external factors that include pharmacogenetics, compliance, and host immunity.

The genetic component was the most illuminating. The genetic basis of resistance is known for several drugs, and there is increasing interest in using molecular monitoring as a tool to assess resistance [44–46] and to influence treatment policy change [47, 48]. We demonstrated the ability to use high-throughput screening to simultaneously genotype *P. falciparum* at numerous loci and could estimate frequencies of individual alleles and haplotypes, which are the basic units of drug selection, and selection coefficients (consistently in the range of 1%–4%). These analyses provide a valuable resource to inform genetic modeling of drug resistance and, more generally, contribute to effective and appropriate use of genetic surveillance to support and guide antimalarial drug deployment policy.

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