Multigenic drug resistance among inbred malaria parasites

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SUMMARY

Recent population genetic studies on the malaria parasite *Plasmodium falciparum* have confirmed that selfing is more frequent where the transmission rate is lower, with inbreeding coefficients estimated to be 0.33 and 0.92 for sites in Tanzania and Papua New Guinea (PNG), respectively. These geographical differences in *Plasmodium* mating patterns have been linked to the rate of spread of chloroquine resistance (CQR) which, according to some measures, has been slower in Tanzania than in PNG. It has been proposed that the former observation explains the latter, although the theoretical argument linking the two is based on limited simulation studies. Taking a more analytical approach here, we first establish the relevant relationship between the coefficient of inbreeding (F, within loci) and the recombination rate (r, between loci), defining an 'effective recombination rate', $\tilde{r} = r(1-F)$. We then show that the emergence of multigenic drug resistance can indeed be slowed (or even quickened) by more outcrossing, but only when resistance is determined by two or more genes, none of which independently confers significant protection. The resistance genes should both be initially rare, and subject to low selection pressure. The analysis does not completely discount the hypothesis that inbreeding significantly influences the spread of CQR, but we show that it can only do so under a restrictive set of conditions, and that these conditions are not satisfied by some laboratory and field data. We discuss some of the wider implications of these results for the evolution of multigenic resistance.

1. INTRODUCTION

The development of polymerase chain reaction (PCR) as a tool to identify the genotypes of diploid malaria parasites in mosquitoes (Ranford-Cartwright *et al.* 1991), together with the expansion of appropriate population genetic and sampling theory (Read *et al.* 1992; Dye & Godfray 1993; Babiker *et al.* 1994; Hill *et al.* 1995), have led to new insights into the mating structure of *Plasmodium* populations. In particular, recent estimates of inbreeding coefficients (F) for *P. falciparum* at Ifakara in Tanzania and Madang in Papua New Guinea (PNG) have confirmed the prediction that selfing is more frequent where the transmission rate is lower (Read *et al.* 1992; Hill *et al.* 1995; Paul *et al.* 1995). The explanation is that individuals who are bitten by fewer infective mosquitoes per unit time are less likely to support, concurrently, genetically different infections acquired from different mosquitoes. So mating between related parasites is more likely.

Prior to these studies, inferences about the genetic structure of *Plasmodium* populations were made largely from calculations of linkage disequilibrium (D), which measures the disproportionate association between alleles at different loci. Linkage disequilibrium has been investigated because genetic analysis could be carried out on haploid parasites taken from people. Inbreeding is expected to facilitate the generation of linkage disequilibrium because the pairing of like gametes tends to produce homozygotes, and recombination merely swaps identical alleles at a locus. However, linkage disequilibrium is a less sensitive measure of selfing than the coefficient of inbreeding (Dye 1994) because D is devalued each generation by a factor equal to the recombination rate (Maynard Smith 1989), whereas persistent selfing produces a steady excess of homozygotes. Not surprisingly, markers of different loci in *Plasmodium* genomes are usually found to be in linkage equilibrium (Carter & McGregor 1973; Joshi *et al.* 1989; Conway & McBride 1991; Paul *et al.* 1995), even though malaria parasites are partially isolated in different hosts, which implies inbreeding.

Paul *et al.* (1995) contend that lower selfing rates in Tanzania are associated with the relatively slow spread of chloroquine resistance (CQR), and they suggest that the former explains the latter. Their proposition raises questions of two kinds. The first is to do with the pattern of spread of CQR. Low-frequency CQR could be observed under a variety of circumstances; for example, from a slow but continuous increase in the

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proportion of infections containing resistant parasites, or from a rapid initial increase to a low but stable steady state. Whilst the proposition made by Paul *et al*. assumes the first of these patterns, Ifakara is more likely to have experienced the second (Koella *et al*. 1990; Koella 1993), and may be typical of areas with high levels of acquired immunity (Björkman 1991). The notion that CQR increased relatively quickly at first in the Ifakara area is consistent with the view (Wernsdorfer 1994) that the spread throughout tropical Africa was more rapid than in South America and parts of south-east Asia.

The second question concerns the theory linking inbreeding to the spread of drug resistance which, in the case of *Plasmodium*, is based on limited simulation studies. Curtis & Otoo (1986) investigated how and when recombination between loci can slow the build up of resistance to a mixture of antimalarial drugs, in which each drug selects for a single, different resistance gene. An extension of this work (Dye 1991, 1994) showed how the effective recombination rate is influenced by the mating structure of the parasite population, and made explicit the relationship between inbreeding and the build up of resistance. Although these studies were concerned principally with the use of drug mixtures, their results can be applied to the case of a single drug-inducing multigenic resistance, as is likely to be true for chloroquine (Newbold 1990; Rubio & Cowman 1996). But both explored the problem under a restricted set of conditions. They did not consider, for example, the possibility of functional interactions between loci leading to cross-resistance against the different drugs used in a mixture.

In this paper we describe a theoretical investigation which is more analytical in its approach, and wider in scope, than the previous simulation studies. Our aims are to specify more precisely the conditions under which outcrossing could delay the evolution of drug resistance, and to ask how well these conditions are met by known multigenic resistance mechanisms, paying special attention to CQR.

2. A MODEL OF MULTIGENIC DRUG RESISTANCE

The simplest possible model of multilocus drug resistance requires two alleles at each of two loci, that is, A/a and B/b. Malaria parasites in their human hosts are haploid, and here there are four possible genotypes, *Ab*, *ab*, *AB* and *aB*. Alleles *a* and *b* confer resistance to an antimalarial drug (such as chloroquine) or drug mixture, and we assume at first that parasites which carry both are entirely invulnerable to drug treatment (i.e. 100% survival), whilst parasites which carry one or neither allele are entirely vulnerable (100% mortality).

The change in genotype frequencies from one generation to another is governed by two processes: selection and recombination. (We do not consider migration.) To calculate the effect of drug treatment, we must selectively kill, and then rescale so that genotype frequencies sum to 1. The changes are laid out in table 1, in which p_{ij} are the genotype frequencies (i is either a or A, j is either b or B), and δ is the death rate attributable to drug treatment, its magnitude being determined by the fraction of infected persons who are treated, dosage, and so on.

We are interested primarily in the change in p_{ab} through time. Rows 1 and 4 of table 1 show that the change due to selection is

$$p'_{ab} \approx \frac{p_{ab}}{1-\delta} \approx p_{ab} + \delta p_{ab}(1-p_{ab}), \tag{1}$$

assuming that $\delta p_{ab} \ll (1-\delta)$ and $\delta \ll 1$. The prime denotes genotype frequency after selection, and the third term shows that growth due to selection is roughly logistic.

Following selection, parasites are acquired by mosquitoes, and genotypes recombine during sexual reproduction. In the process of recombination, *ab* genotypes can arise in two ways: from ab/ij matings in which no recombination takes place, and from aj/ib matings in which recombination does take place (Maynard Smith 1989). If r is the recombination rate, which takes a value between 0 and 0.5, we have the standard result

$$p''_{ab} = (1-r)\,p'_{ab} + r p'_a p'_b, \tag{2}$$

where the double prime denotes genotype frequency after recombination.

In this study we want to explore the way in which the growth rate of genotype *ab* depends not only on the recombination rate, but also on the coefficient of inbreeding, F. F is the probability that two alleles at a (diploid) locus are identical by descent. In general, inbreeding increases the frequency of homozygotes in a population, so that recombination more frequently swaps identical alleles at a locus. The importance of inbreeding for multilocus drug resistance is that it prevents recombination from separating two or more alleles conferring drug resistance. This speeds up the growth rate of genotype *ab* as follows (see Appendix):

$$p''_{ab} = (1-r(1-F))\,p'_{ab} + r(1-F)\,p'_a p'_b. \tag{3}$$

Table 1. *Effect of drug treatment on genotype frequencies*

initial frequencies	p_{ab}	p_{aB}	p_{Ab}	p_{AB}
after selection	p_{ab}	$(1-\delta)\,p_{aB}$	$(1-\delta)\,p_{Ab}$	$(1-\delta)\,p_{AB}$
after rescaling (exact)	$\dfrac{p_{ab}}{1-\delta+\delta p_{ab}}$	$\dfrac{(1-\delta)\,p_{aB}}{1-\delta+\delta p_{ab}}$	$\dfrac{(1-\delta)\,p_{Ab}}{1-\delta+\delta p_{ab}}$	$\dfrac{(1-\delta)\,p_{AB}}{1-\delta+\delta p_{ab}}$
after rescaling (approximate)	$\dfrac{p_{ab}}{1-\delta}$	p_{aB}	p_{Ab}	p_{AB}

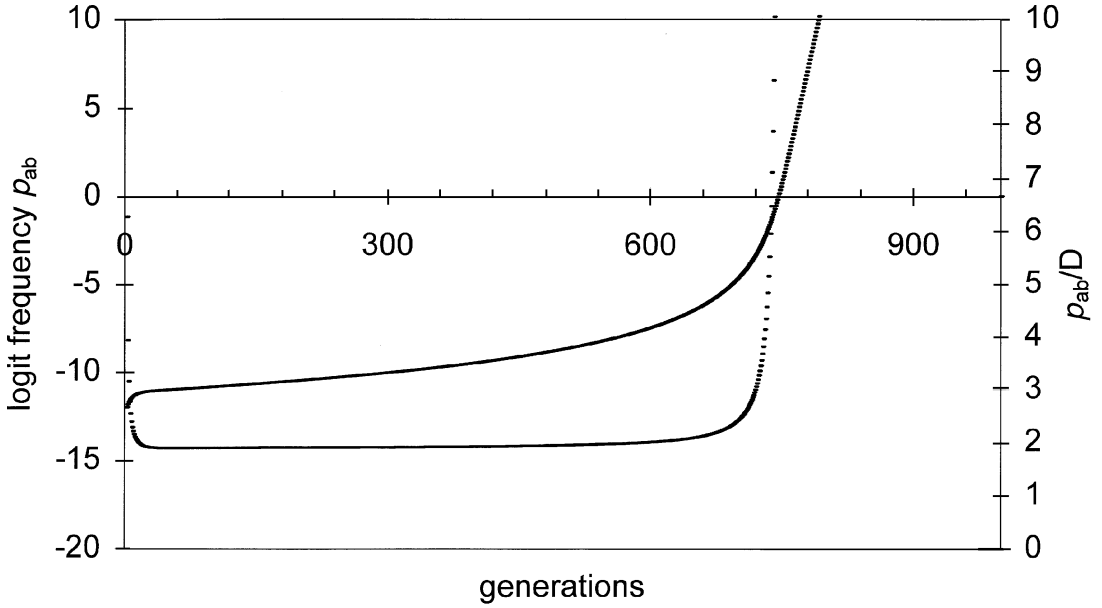

Figure 1. A simulation to illustrate the general form of p_{ab} growth through time (measured in parasite generations), using equations 5 and 8 and the transitions in table 1. Upper line: p_{ab}, expressed in logits. Lower line: the ratio of p_{ab}/D. Initial values of variables: $p_a(0) = p_b(0) = 0.0025$, $D(0) = 0$; with $\tilde{r} = 0.3$ and $\delta = 0.2$.

With no inbreeding, $F = 0$, and we return to equation 2. With complete inbreeding, $F = 1$, and p_{ab} does not change from one generation to another. Letting $\tilde{r} = r(1-F)$, which we call the 'effective recombination rate', equation 3 can be rewritten

$$p''_{ab} = (1-\tilde{r})\,p'_{ab} + \tilde{r}p'_a p'_b. \tag{4}$$

Using the exact transitions from p_{ab} to p'_{ab} (selection: row 1 to row 3 in table 1), and from p'_{ab} to p''_{ab} (recombination: equation 4), we can plot the growth in the frequency of genotype *ab* through time. Figure 1 illustrates the general form of this growth curve, which has three phases. There is (a) rapid adjustment to (b) a pseudo-equilibrium in which the ratio of p_{ab} to linkage disequilibrium (p_{ab}/D) remains almost constant (figure 1, lower line), until (c) p_{ab} eventually breaks away into logistic growth (linear in the logits), heading for fixation. By contrast with what appears to be true in some regions of high malaria endemicity (Koella *et al.* 1990; Koella 1993), this model has no truly stable equilibrium at $p_{ab} < 1$, provided there is some selection.

In the pseudo-equilibrium, the increase in p_{ab} due to selection is almost exactly balanced by the decrease due to recombination, and this phase can be characterized as follows. Linkage disequilibrium, D, is the difference between the observed and expected frequencies of *ab* under linkage equilibrium,

$$D = p_{ab} - p_a p_b. \tag{5}$$

When p_{ab} is small and growing slowly, changes in $p_a p_b$ are negligible. The increase in D due to selection in each generation is almost entirely due to the change in p_{ab} (table 1) so,

$$D' \approx D + \frac{p_{ab}}{1-\delta} - p_{ab} = D + \frac{p_{ab}\,\delta}{1-\delta}. \tag{6}$$

Substituting equation 5 in equation 4 (whilst appropriately adjusting primes) gives the usual expression for the decrease in D due to recombination in each generation (Maynard Smith 1989),

$$D' = D(1-\tilde{r}). \tag{7}$$

Thus, the combined effect of selection followed by recombination is

$$D' \approx \left(D + \frac{p_{ab}\,\delta}{1-\delta}\right)(1-\tilde{r}). \tag{8}$$

In the pseudo-equilibrium state $D' \approx D$, and by rearrangement,

$$\frac{p_{ab}}{D} \approx \frac{\tilde{r}(1-\delta)}{\delta(1-\tilde{r})}. \tag{9}$$

This is the value taken by the lower line in figure 1 when almost horizontal: for $\tilde{r} = 0.3$ and $\delta = 0.2$, equation 9 gives $p_{ab}/D \approx 1.7$.

Once the pseudo-equilibrium breaks down, the rate of increase of p_{ab} towards fixation is relatively rapid. We are mainly interested here in the time taken for genotype *ab* to become common as a function of different effective recombination rates and different selection pressures, and we measure this time as the number of parasite generations required for p_{ab} to reach a frequency of 0.5 (t_{50}, when logit $p_{ab} = 0$, figure 1, upper line). There is apparently no simple, general expression (or even a good approximation) for t_{50} in terms of the model's parameters and variables, so the results below have been obtained numerically. However, when $\tilde{r} = 0$, the pseudo-equilibrium is by-passed and p_{ab} grows more or less logistically from the outset (equation 1),

$$p_{ab}(t) \approx p_{ab}(0)\,e^{\delta t}, \tag{10}$$

so that $t_{50} \approx \ln(0.5/p_{ab}(0))/\delta$, which provides a useful check on simulation results.

3. DRUG RESISTANCE AND THE EFFECTIVE RECOMBINATION RATE

Figure 2 has been drawn by repeating the simulation shown in figure 1 for numerous different parameter values. When $\tilde{r}$ is assumed to change (as a result of changes in either r or F) independently of δ, more outcrossing generally prolongs the time taken for the doubly-resistant genotype *ab* to become common. With $\delta = 0.15$ and $p_a(0) = p_b(0) = 0.005$, t_{50} increases 15-fold as $\tilde{r}$ increases from 0 to 0.5 (line 1). Assuming ten generations of *P. falciparum* per year, this is the difference between 7 years and 97 years. The effect is sensitive to selection pressure (δ, compare lines 1 and 2) and to the initial frequencies of alleles *a* and *b* (compare line 2 with lines 3 and 4), being less marked when these are higher.

In fact, $\tilde{r}$ is unlikely to change independently of δ. Infected individuals living in areas of higher transmission tend to support more genotypes of *P. falciparum* on average (Paul *et al.* 1995). Consequently, a larger fraction of people carry the genotype *ab*, and the selection pressure on this genotype will be higher, even if the proportion of infected people taking anti-malarial drugs does not change. A comparison of lines 1 and 2 in figure 2 illustrates how t_{50} could actually be smaller in areas of higher transmission (Dye 1991; Mackinnon & Hastings 1996). The horizontal line indicates, for example, that $t_{50} = 450$ generations when $\delta = 0.15$ and $\tilde{r} = 0.2$ (intersection with line 1). If $\tilde{r}$ increases to 0.33 in an area of higher transmission, t_{50} will also increase provided δ remains less than 0.2 (intersection with line 2), but decrease if δ exceeds 0.2.

Whether t_{50} increases or decreases as a result of changing $\tilde{r}$, the magnitude of the change depends most sensitively (in this model) on the assumption that genes *a* and *b* confer no protection against drug treatment when present alone. In figure 3 we apply mortality rates δ_1 to genotype *AB*, and δ_2 to genotypes *aB* and *Ab*. Line 1 is identical to line 1 of figure 2 because $\delta_1 = \delta_2 = 0.15$. Line 2 has been drawn with $\delta_2 = 0.145$, so that genotypes *aB* and *Ab* suffer a mortality rate in the presence of a drug which is 97 % that of *AB*. This has the effect of reducing t_{50} by as much as 63 % at $\tilde{r} = 0.5$. When $\delta_2 = 0.14$ and $\tilde{r} = 0.5$, the reduction in t_{50} is 75 % (line 3). When δ_2 is half the size of δ_1, the increase in t_{50} with $\tilde{r}$ is imperceptible (line 4). The inset to figure

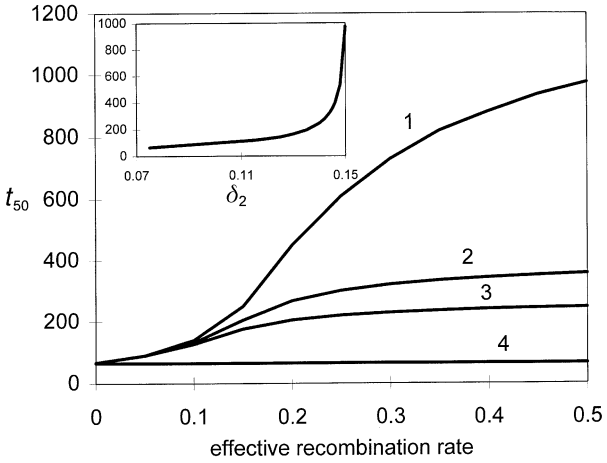

Figure 3. As figure 2, but allowing for different selection pressures on genotypes *aB*, *Ab* (δ_1) and *AB* (δ_2). Line 1: $\delta_1 = \delta_2 = 0.15$. Line 2: $\delta_1 = 0.15$, $\delta_2 = 0.145$. Line 3: $\delta_1 = 0.15$, $\delta_2 = 0.14$. Line 4: $\delta_1 = 0.15$, $\delta_2 = 0.075$. Inset: t_{50} against δ_2 for $\tilde{r} = 0.05$. In all cases $p_a(0) = p_b(0) = 0.005$.

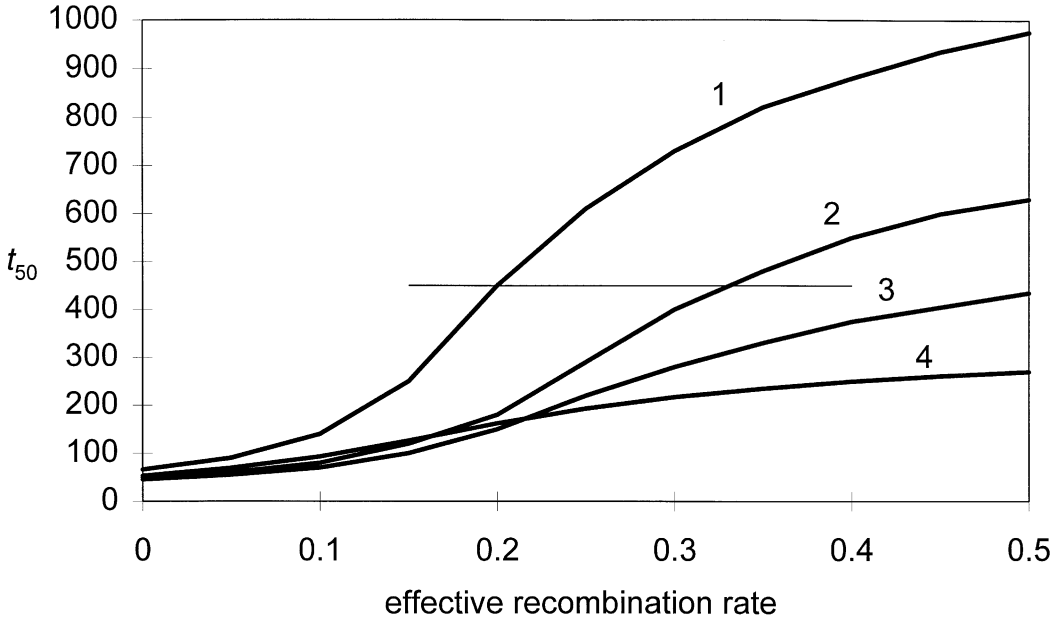

Figure 2. The number of parasite generations taken for genotype *ab* to reach a frequency of 0.5 under different assumptions about selection pressure, effective recombination rate, and initial allele frequencies. Heavy lines, 1: $p_a(0) = p_b(0) = 0.005$, $\delta = 0.15$. Line 2: $p_a(0) = p_b(0) = 0.005$, $\delta = 0.2$. Line 3: $p_a(0) = p_b(0) = 0.0075$, $\delta = 0.2$. Line 4: $p_a(0) = 0.05$, $p_b(0) = 0.005$, $\delta = 0.15$. The horizontal line illustrates what happens if $\tilde{r}$ and δ change together: comparing lines 1 and 2, if the effective recombination rate $\tilde{r}$ increases from 0.2 to 0.33 as a result of a change in transmission rate, t_{50} also becomes larger provided δ remains less than 0.2 (t_{50} stays above line 2), but smaller if δ exceeds 0.2 (see text).

3 displays this sensitivity of t_{50} to δ_2 in a different way, making it plainer still that t_{50} drops sharply as δ_2 falls below δ_1. In short, even a marginal increase in the relative survival rates of *aB* and *Ab* over *AB* eliminates the effect of outcrossing (or conversely inbreeding) on the growth of resistance.

4. DISCUSSION

The result that outcrossing can delay the onset of resistance conforms with two earlier simulation studies (Curtis & Otoo 1986; Dye 1994). It is tempting to use the recently-obtained estimates of F to calculate effective recombination rates, $\tilde{r}$, and put these directly on to figure 2. Hill *et al.* (1995) give $F = 0.33$ for *P. falciparum* in the holoendemic area of Ifakara, Tanzania. Paul *et al.* (1995) found that $F = 0.92$ for the area of lower transmission at Madang, PNG, which is even higher than Read's (1992) prediction of 0.64 from sex ratio theory. Assuming that different alleles conferring drug resistance are not closely linked ($r = 0.5$), the effective recombination rates for parasites taken from these sites in Tanzania and PNG are $\tilde{r} = 0.335$ and $\tilde{r} = 0.043$, respectively. Using figure 2, it is possible to imagine that more outcrossing is indeed responsible for the relatively low frequency of chloroquine resistance observed in Tanzania (Paul *et al.* 1995).

But there are two important caveats First, the interpretation of data on CQR spread in PNG and Tanzania assumes that resistance genes, having emerged, will continue to increase in frequency towards fixation. If CQR prevalence at Ifakara is now fluctuating around a low, steady state (Koella *et al.* 1990; Koella 1993), indicating a balanced polymorphism, figure 1 suggests comparing the pre-equilibrium growth rates of resistance. The available data provide no evidence that the pre-equilibrium growth rate has been slower at Ifakara than in Madang, and some authors imply that it could have been faster (Koella 1993; Wernsdorfer 1994).

Secondly, the results in figures 2 and 3 show that outcrossing can slow the spread of resistance under a restricted set of conditions. We must assume that more outcrossing (higher $\tilde{r}$) in areas of higher transmission is not accompanied by fully compensating increases in the selection pressure (higher δ, figure 2; Dye 1991; M. J. Mackinnon & I. M. Hastings unpublished data). Resistance must also be determined by two unlinked genes, neither of which confers any significant resistance on its own (no epistasis, figure 3). Moreover, outcrossing is more effective when both resistance genes are simultaneously rare and subject to low selection pressure (figure 2).

Whether all these conditions can be satisfied by any mechanism of resistance to a drug or drug combination is unclear, partly because most resistance mechanisms are imperfectly understood. Regarding chloroquine, the fact that resistance did not appear anywhere for about 10 years after the drug became widely available does point to multigenic resistance, and is consistent with the idea that two or more resistance genes are required to protect parasites from the drug (Cowman & Foote 1990). There are two good candidate loci: the rapid-efflux mechanism in CQR, which pumps the drug out of cells, is evidently controlled by a gene on chromosome 7 (Wellems *et al.* 1990, 1991); in addition, resistance has been associated with mutations at the *pfmdr 1* locus on chromosome 5, mainly because verapamil partially reverses CQR *in vitro* (Rubio & Cowman 1996). A crucial question posed by the present analysis is whether CQR, to any degree, always requires at least two genes at different loci. Ward *et al.* (1995) believe not: they conclude from pharmacological studies that there are at least two phenotypically distinct resistance mechanisms: the verapamil-sensitive mechanism, which is responsible for the shift from moderate to high resistance, and a verapamil-insensitive mechanism, which causes the initial loss of sensitivity. This conforms with the evidence for some chloroquine resistant rodent malarias: resistance in the murine plasmodia is multigenic, but crossing experiments show that some resistance can be conferred by single genes acting independently (Rosario 1976; Padua 1981). Under these circumstances, we would expect almost no effect of inbreeding on the build up of resistance. It is conceivable, too, that single genes protect parasites confronted by low drug doses, which is an additional argument for ensuring that patients take complete courses of a drug.

Our conclusion that the two or more resistance genes need to be simultaneously rare, and that selection pressure should be weak, could have been predicted from the related analysis of Curtis & Otoo (1986), who were interested in the problem of whether multidrug resistance is more effectively delayed by using two new drugs in a mixture, rather than in sequence. Reciprocally, we can use our results to comment, by analogy, on their conclusion that a mixture always performs as well as or better than a sequence, and that the difference in performance increases with the recombination rate. This result must be sensitive to the assumption that, under pressure from a mixture of drugs, parasites with just one resistance gene have no survival advantage over parasites with no resistance genes; in other words, that there is no significant cross-resistance to the different drugs used in a mixture.

In summary, whilst our results do not completely discount the hypothesis that inbreeding significantly increases the rate of spread of CQR, they show that there are restrictive conditions under which it can do so, and that these conditions are not satisfied by some of the available laboratory and field data. They highlight difficulties in interpreting the incomplete information about the mechanisms and rate of spread of CQR, and emphasize the importance of testing the hypothesis against a clearly defined model of the evolution of resistance.

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APPENDIX

Inbreeding and the effective recombination rate

Here we justify our definition of the effective recombination rate, $\tilde{r} = r(1-F)$. If mating is random within the whole parasite population, the frequency of *ab* gametes arising by recombination is just $rp_a p_b$ (equation 2). For our purposes, we want to distinguish recombinant *ab* gametes which arise via inbreeding from *ab/ib* or *ab/aj* matings, and those which arise via outcrossing from *aj/ib* matings. In doing so, we must also allow for the fact that pairing *Plasmodium* gametes will be more or less closely related depending on whether they come from the same oocyst, the same mosquito, or from other mosquitoes.

Following Dye & Godfray (1993), we assume that each infected person supports gametes from n mosquitoes, that each mosquito carries m oocysts, and that each oocyst produces b gametes; m and n generally lie in the range 1–10, whilst b is several orders of magnitude greater. Columns 2 and 3 of table A 1 show the relatedness of a focal gamete to gametes from all three possible sources under inbreeding and outcrossing. This focal gamete is expected to encounter, and pair with, gametes from each of these sources according to their frequencies in the gamete population; these frequencies are the number of gametes from each source (column 4) divided by the total number of gametes, bmn, giving the quantities in column 5. To get the total number of new *ab* gametes which arise from inbreeding we take, for each row, the product of $p_{ab} r$ and the entries in columns 2 and 5, and then sum down rows:

$$\frac{p_{ab} r(1+F)}{2mn} + \frac{p_{ab} rF(m-1)}{mn}. \qquad \text{(A 1)}$$

Similarly, the total number which arise by outcrossing come from the product of $p_a p_b r$ and the entries in columns 3 and 5,

$$\frac{p_a p_b r(1-F)}{2mn} + \frac{p_a p_b r(1-F)\,(m-1)}{mn} + \frac{p_a p_b r(n-1)}{n}. \qquad \text{(A 2)}$$

Table A 1. *Sources of recombinant gametes, their abundance and frequency, and their relatedness (inbreeding) and unrelatedness (outcrossing) to a focal gamete.*

(Gametes from other mosquitoes (*) are assumed to be unrelated.)

source of gametes	relatedness: inbreeding	unrelatedness: outcrossing	number of gametes	proportion of all gametes
same oocyst	$(1+F)/2$	$(1-F)/2$	$b-1 \approx b$	$1/mn$
same mosquito	F	$(1-F)$	$(m-1)\,b$	$(m-1)/mn$
other mosquitoes	0*	1	$(n-1)\,mb$	$(n-1)/n$

Adding together both recombinant (A 1+A 2) and non-recombinant ($p_{ab}(1-r)$) *ab* gametes gives the total number at the start of the next generation,

$$p'_{ab} = (1-r(1-\tilde{F}))\,p_{ab} + r(1-\tilde{F})\,p_a p_b, \qquad \text{(A 3)}$$

in which $\tilde{F}$ is related to the coefficient of inbreeding by

$$\tilde{F} = \frac{1+F(2m-1)}{2mn}. \qquad \text{(A 4)}$$

Dye & Godfray (1993) also showed, with the above definition and assumptions, that

$$F = \frac{1}{2m(n-1)+1}. \qquad \text{(A 5)}$$

Equation (A 5) can be used to eliminate m and n from (A 4), revealing that $\tilde{F} = F$. Thus equation (A 3), with an extra set of primes indicating changes due to recombination rather than selection, is the same as equation 3 in the main text.