The emergence of drug-resistant malaria

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SUMMARY

Stochastic processes play a vital role in the early stages of the evolution of drug-resistant malaria. We present a simple and flexible method for investigating these processes and understanding how they affect the emergence of drug-resistant malaria. Qualitatively different predictions can be made depending on the biological and epidemiological factors which prevail in the field. Intense intra-host competition between co-infecting clones, low numbers of genes required to encode resistance, and high drug usage all encourage the emergence of drug resistance. Drug-resistant forms present at the time drug application starts are less likely to survive than those which arise subsequently; survival of the former largely depends on how rapidly malaria population size stabilizes after drug application. In particular, whether resistance is more likely to emerge in areas of high or low transmission depends on malaria intra-host dynamics, the level of drug usage, the population regulation of malaria, and the number of genes required to encode resistance. These factors are discussed in relation to the practical implementation of drug control programmes.

Key words: malaria, drug resistance, population genetics, epidemiology.

INTRODUCTION

Several previous papers have investigated the dynamics underlying the evolution of drug-resistant malaria (Curtis & Otoo, 1986; Dye, 1991, 1994; Dye & Williams, 1997; Hastings, 1997; Mackinnon & Hastings, 1998). In these studies, these dynamics have been assumed either to be deterministic or else the studies have simulated conditions where the frequency of resistance has been sufficiently high that stochastic effects can be ignored. However, in the initial emergence of resistant malaria, the number of resistant genotypes may be low and the likelihood that they become extinct or become established in the population will depend on the stochastic events which surround their transmission. This was examined by Mackinnon (1997) who concluded that resistance is more likely to evolve in areas of intense malaria transmission. In contrast, Hastings (1997) concluded that drug-resistant genotypes at very low frequencies could evolve faster at either high or low transmission levels depending on the assumptions made about the underlying biology. The purpose of this paper is 2-fold: firstly, to show how these differing conclusions can be reconciled by examining the assumptions and parameter spaces examined in the 2 models, and secondly, to present a general and simple method to predict the initial stages of the emergence of drug-resistant malaria. The latter objective is important as it allows the method to be applicable to situations where any number of genes may be required to encode resistance, and to investigate a variety of biological assumptions and processes which underlie the model.

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METHODS

The fate of a single drug-resistant mutant depends on its probability of being transmitted to the next generation. If the mean number of offspring is greater than 1, the mutant has a non-zero probability of surviving and becoming established in the population: this probability depends on the mean and the variance of the number of offspring.

As a concrete example, consider a highly advantageous mutation which is expected to double in frequency each generation, that is each mutation would, *on average*, transmit 2 copies of itself to the subsequent generation. In reality, chance 'stochastic' processes such as death of the mosquito, inoculation into an immune host, and so on, mean that it will not invariably transmit exactly 2 copies but may transmit $0, 1, 2, 3, 4, 5 \ldots$ depending on chance. Assuming a Poisson distribution with mean of 2 it will transmit 0 with probability 0·14, 1 with probability 0·27, 2 with probability 0·27, 3 with probability of 0·18 and so on. The critical point is that even though it is a highly advantageous mutation, it may be lost from the population purely by chance, i.e. leave zero offspring with probability 0·14; even if it successfully transmits 2 copies to the next generation then both may be lost in the subsequent generation with probability $0{\cdot}14^2 = 0{\cdot}02$. There is a standard body of theory which calculates

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the probability of it being lost by chance extinction (and by extension the probability of it surviving) given the expected mean number of transmissions and the variance associated with this mean; this theory is that of 'branching processes' which will be employed later.

There is one other methodological caveat. We require both the mean infection level (which determines recombination rate) and the mean number of successful transmissions per host. At equilibrium, these are obviously equal in the discrete generation model employed here. Slight disparities may occur if populations are shrinking as a result of drug application (see later Discussion section of normalization); under these circumstances transmission may be less than infection, but this disparity is unlikely to affect the results presented here. The analyses would, however, be inappropriate in an epidemic situation where low infection rates (typically by a single clone) may be associated with very large transmission rates. On grounds of brevity we refer to 'mean infection rate' as the epidemiological variable rather than its equivalent of 'mean number of successful transmissions'. The former also has the advantage that it can actually be measured (e.g. Hill & Babiker, 1995 and references therein) and used to compare populations.

The following sections describe the mean number of offspring for a resistant malaria parasite as a function of the 3 forces determining mutant survival – drug treatment, mean infection rate and recombination. The methods for calculating survival probability based on the distribution of offspring number are then given followed by a description of some of the assumptions made.

Calculating mean number of resistant haplotypes successfully transmitted from a single resistant haplotype

It will be assumed initially that alleles at either 1 or 2 loci are required to encode drug resistance although the method can be extended to any number of loci. The following derivation is derived intuitively but a more rigorous derivation has been given by Hastings (1997). We assume for clarity that the frequency of resistant clones and alleles are negligible, that natural selection is absent and a constant number, c, of independent clones are present in each individual. (These assumptions may be easily relaxed and do not affect the conclusions below.) Each clone in a host is assumed to have been acquired independently, each successful mosquito bite transmitting only a single clone as assumed in previous studies (Hill & Babiker, 1995; Hill *et al*. 1995; Hastings, 1997; Mackinnon, 1997; Mackinnon & Hastings, 1998). In an untreated host the probability of a resistant haplotype surviving meiosis and being transmitted to the next generation is $\tau = x^2 + 2x(1-x)\,d(n)$ where $x = 1/c$ is the frequency of a resistant clone within the host (which can therefore be adjusted to incorporate the effects of natural selection (Hastings, 1997; Mackinnon, 1997)), and $d(n) = 1/2^n$ is the probability that it survives meiosis with a sensitive clone assuming n loci are required to encode resistance (Hastings, 1997). In the case of 2 unlinked genes encoding resistance, $d(n) = 0{\cdot}25$ so $\tau = 1/2c(1+1/c)$. Now assume that a proportion, T, of the population of hosts are treated with drugs: susceptible haplotypes will, by definition, be eliminated in this group while resistant forms survive and transmit t copies of themselves to the subsequent generation. If the population is assumed to be stable in its total size (see later), the mean number of clones transmitted per infection is c and the expected number of resistant descendants transmitted per resistant haplotype per generation, $E(P')$, is:

$$E(P') = \frac{(1-T)\,c\tau + Tct}{(1-T)}. \qquad (1\,a)$$

If, however, drugs have been only recently introduced and the parasite population size is decreasing as a consequence, we may also need to consider the case when normalization is absent, as assumed by Mackinnon (1997) i.e.

$$E(P') = (1-T)\,c\tau + Tct. \qquad (1\,b)$$

A fuller discussion of when normalization is required is given later.

The parameter t is used in 2 ways here: to investigate the effects of intra-host dynamics (Hastings, 1997) or to investigate the effects of the drug on subsequent transmission (Mackinnon & Hastings, 1998). The nature of the intra-host dynamics is assumed to depend on the mechanism of immune regulation within the infected host. Under a model of generalization immunity (GI) the total level of infection is regulated and a resistant haplotype actively expands to replace any co-infecting sensitive clones killed by drug treatment: its expected transmission from such hosts therefore increases from 1 to c. Under a model of specific immunity (SI), each clone is regulated independently and a resistant haplotype does not expand to replace those killed by drug treatment: its mean transmission to the following generation is 1. Hence setting $t = 1$ or $t = 1/c$ restores the GI and SI models respectively. This analysis assumes that drugs have no effect on subsequent transmission, i.e. the resistant haplotype is completely resistant and its transmission output is unaffected by drugs. Alternatively, t can be considered to be the effect of the drug on transmission, whether this be due to a direct effect of the drug on transmission stages, or varying susceptibility of the mutant to the drug, or through some complex effect of immunity and within-host dynamics following drug treatment. For example $t = 0{\cdot}5$ implies that a treated individual host with a single 'resistant'

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clone transmits half as much as the same individual without drug treatment.

Calculation of survival probabilities from the distribution of the number of offspring

The distribution of offspring from each resistant clone can be assumed to be the weighted sum of 2 Poisson distributions (or negative binomial distributions, as in Mackinnon (1997)), 1 with mean $c\tau/(1-T)$ and 1 with mean $c/(1-T)$ with frequencies $(1-T)$ and T respectively for the GI model. An analogous procedure is used to obtain the distribution of offspring in the SI model. Using branching process theory, the probability-generating function of this mixed distribution is then solved to obtain the probability of survival of the mutant (Crow & Kimura, 1970).

RESULTS

The values of $E(P')$ assuming that the population has stabilized before the mutant arises (Equation 1 *a*) and that 2 loci jointly confer resistance are shown on Fig. 1 A for the GI and SI models. The curve shows that for the GI model the mean is higher at both low ($c < 1{\cdot}5$) and high infection rates ($c > 3$) than at intermediate infection rates, thus producing the interesting situation where it is true that both low transmission rates and high transmission rates favour mutant survival. The reason why this 'valley' effect occurs (i.e. increasing survival probabilities as transmission both decreases and increases) in the GI case is because of the interplay between 2 processes. Recombination breaks up resistant haplotypes more frequently as infection rate increases. If this, plus drug selection, were the only forces operating then a plot of $E(P')$ against infection rate would be qualitatively indistinguishable from the SI curve on Fig. 1 A. The reason why high infection levels may result in higher rates of evolution in the GI model lies in the model of intra-host dynamics. Transmission from all hosts is assumed to be equal so, effectively, a resistant haplotype in a treated host expands to replace to co-infecting clones killed by drugs; for example if mean infection (and hence transmission) rate is 2·0 a single resistant haplotype in a treated host would leave 2 offspring (i.e. double in number), if mean infection/transmission rate was 3·0, it would leave 3 offspring and treble in number, and so on. At high infection rates the number of co-infecting clones is higher and so expansion is greater and the increase in transmission higher; thus a linear increase in $E(P')$ is superimposed on the loss due to recombination, resulting in the type of GI curve shown in Fig. 1 A. Under an SI model this expansion does not occur and there is no advantage at higher infection rates, i.e. low transmission rates always favour mutant survival. The choice between GI and SI is problematic and this is not the place to attempt a resolution; it is merely necessary to note that both models are plausible but result in profoundly different dynamics: resistance will only spread faster in areas of high transmission if the term cTt dominates Equation 1 *a*, i.e. if the intrahost dynamics are close to a GI model or if the drug causes little reduction in transmission from hosts carrying the mutant, i.e. t is close to 1 (see later). The values of T and t for which this valley in survival probability occurs are given later. Importantly, the valley in survival probabilities never occurs in the absence of normalization (as in Mackinnon, 1997) or in the case of a single locus encoding resistance because whenever $E(P') > 1$, it is always increasing with c.

Since survival probability is greater than zero whenever $E(P') > 1$, similar qualitative results for survival probabilities are found (Fig. 1 B). The results from Fig. 1 A do not translate directly onto Fig. 1 B; for example under GI, $E(P') = 1{\cdot}11$ for transmission rates of 1·0 and 4·5 but the survival probability is markedly lower for the latter. This occurs because the dynamics are composed of 2 distributions and most (90 % in this case) transmissions occur from untreated hosts. When infection rates are high, the probability of loss (via meiosis) in untreated hosts is larger and the overall probability of a resistant clone leaving zero progeny is higher (0·33 and 0·46 for transmission rates of 1·0 and 4·5 respectively).

The conditions under which the valley occurs for the 2-locus case in which normalization is used are quantified as follows. The exact level of drug pressure (T) and effect on transmission (t) for which it is true that survival probabilities increase as transmission rate decreases from a value of c to c^* is found from Equation 1 *a* to be:

$$c < 2, \quad \frac{1}{2tcc^*+1} > T > \frac{c^*-1}{2tc^{*2}-1+c^*}, \quad t > 0.$$

This equation describes 3 regions, as shown in Fig. 2 for the cases of $c = 1{\cdot}1$ or $c = 1{\cdot}5$ and $c - c^*$ is small, i.e. small reductions in transmission rate. The upper (left) boundary for T represents the value above which the mutant will have a strictly decreasing probability of survival as transmission decreases. For example, in a GI model ($t = 1$), when the prevailing value of $c = 1{\cdot}5$ and whenever drug pressure is greater than $T = 0{\cdot}18$, reductions in transmission will always lead to a slower spread of resistance. The lower boundary represents the value of T below which survival probability will either remain zero or increase with decreasing transmission below the value of c, i.e. reducing transmission rate will favour the spread of resistance. To continue the example, for $c = 1{\cdot}5$, further decreases in trans-

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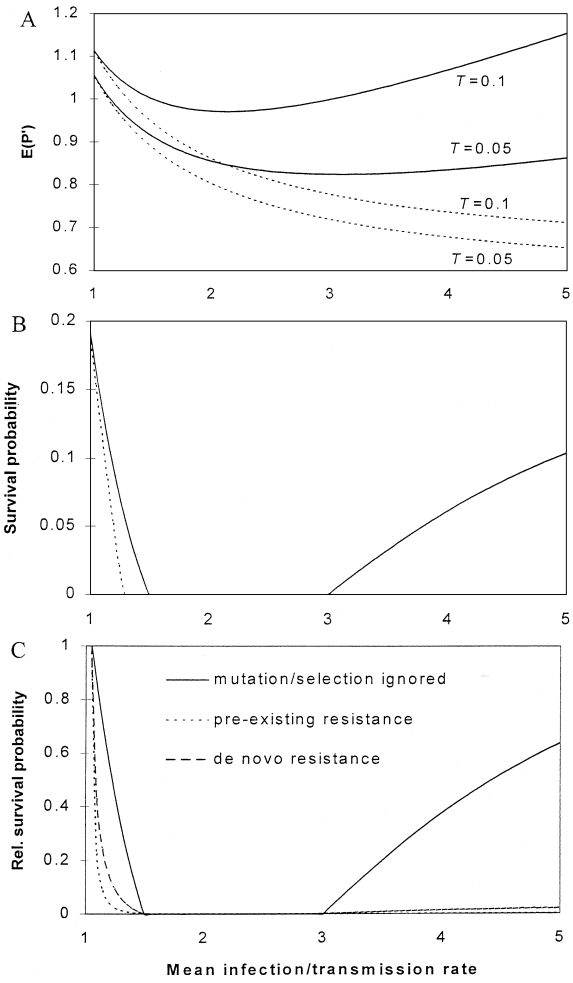

Fig. 1. How survival of drug-resistant malaria varies with infection and transmission rates (which are assumed to be identical and constitute the independent, epidemiological variable plotted along the x axis; see under Methods section for more details). (A) The expected mean number of resistant haplotypes transmitted to the next generation by each resistant haplotype, $E(P')$, under a GI (——) or SI (·····) model with drug treatment rates, T, of 0·05 or 0·1. (B) How this expected mean number translates into the probability of survival, assuming $T = 0{\cdot}1$. (C) The effects of incorporating the frequency of resistance alleles expected under mutation/selection balance assuming GI with $T = 0{\cdot}1$; results are expressed relative to survival probability expected when mean infection rate is 1·05.

mission will not lead to faster spread of drug resistance when $T < 0{\cdot}1$ because survival probability remains zero. Between these boundaries for T, survival probability will increase as transmission is reduced below c, i.e. the valley exists. For a prevailing transmission rate of $c = 1{\cdot}1$, the valley exists between $T = 0{\cdot}04$ and $T = 0{\cdot}29$. This range extends as t decreases, i.e. moving towards an SI model, but remains within the intermediate range of drug pressure for most values of c and t. Note also that the valley never occurs when $c > 2$.

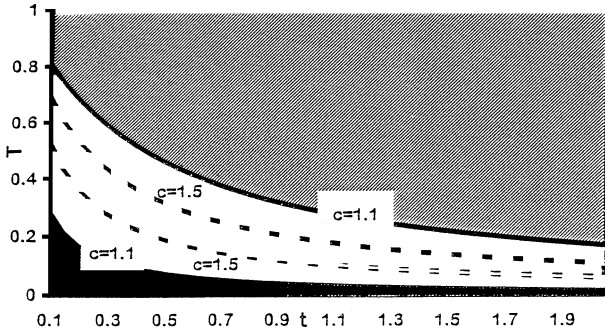

Fig. 2. Values of drug treatment rate (T) and drug effect on transmission (t) for which decreasing transmission below the value of c ($c = 1{\cdot}5$ or $c = 1{\cdot}1$, as shown) has different consequences. In the light shaded area, reducing transmission below c will lead to a decrease in survival probability; in the dark shaded area, it will lead to zero change in survival probability. In the white area, survival probability will increase as transmission rate declines below c, but will become positive again at high values of c, i.e. the valley behaviour exists. See text for details.

DISCUSSION

Consider the fate of resistant haplotypes under 2 conditions: first, where they arise in the population once drug use has stabilized the population (*de novo* mutations), and second, where they pre-exist in the population prior to drug use being commenced.

De novo *mutations*

Infectiousness and prevalence are likely to be saturated for most transmission intensities in areas of stable malaria (see e.g. Gupta & Snow, 1996) so that if drug use does not drive the parasite population to extinction, its size will eventually stabilize despite continued drug usage. This stability is assumed to occur because, although a proportion, T, of clones are killed each generation, each surviving clone (including, by definition, resistance clones) will transmit on average $1/(1-T)$ clones to compensate for this loss. This case can also be argued from another perspective. The population is at equilibrium so an infection of c clones will transmit, on average, c clones to the next generation. However this average will consist of 2 types of infection, those which are treated (frequency T) and transmit zero offspring, and those which are untreated (frequency $1-T$) and transmit $c/(1-T)$: hence $T \times 0 + (1-T)\,c/(1-T) = c$. Since, by definition, infections containing a drug-resistant clone survive then they each leave on average $c/(1-T)$ offspring. This is the reason why the normalization factor $(1-T)$ is included in Equation 1a. Note that this normalizing constant is absent in standard branching theory (e.g. Crow & Kimura, 1970) because the population size is

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unaffected by the presence of a single novel gene. Because $c\tau$ in Equation 1*a* can never exceed unity, the positive survival probabilities which occur at low transmission rates will always be missed in the absence of normalization. Thus this assumption is critical to the outcome of the analysis.

Pre-existing mutations

Once drugs are applied to a population, stability will eventually be reached but there are problems in deciding how quickly the population size will stabilize. These problems are discussed below.

(i) *Slow stabilization*. In this model, the fitnesses of the sensitive and resistant parasites are as assumed by Mackinnon (1997), namely, in untreated hosts, the mean number of offspring is 1 for both sensitive and resistant parasites and in treated hosts is, respectively, zero and c (i.e. the normalization factor $1-T$ is omitted (Equation 1*b*). The mean number of sensitive offspring is $1-T$ so, as there are only a few mutants in the population, this means that the total parasite population size is decreasing, at least in the immediate term, due to drug killing. Note that this decrease in population size does not violate the assumptions of branching process theory as long as the population remains large such that there is zero probability that the total population goes extinct. In the absence of normalization, the population is predicted to shrink rapidly, e.g. if drug use is 20% then population size falls 20% each 'generation'; if we assume 5 generations/year then after 2 years population size is $0{\cdot}8^{10} = 0{\cdot}1$ of the original and after 5 years population size is $0{\cdot}8^{25} = 0{\cdot}004$ of the original. We do not, of course, argue that this will in fact happen, or has been observed. What we argue is that this model adequately describes the first few critical generations of drug use, during which period (provided $E(P')$ is significantly above 1) stochastic processes determine whether the pre-existing mutant will survive. This assumption has a critical consequence, as it is only under this model of complete non-normalization that the peak in expected rate of evolution of drug resistance predicted at low infection rates does not translate into a positive survival probability, i.e. the model predicts that drug-resistant haplotypes would always be lost through stochastic processes in populations with low infection rates (as stated by Mackinnon, 1997).

(ii) *Rapid stabilization*. An alternative to the slow stabilization model can be justified by considering the factors which result in stabilization of population size. This is an ecological concept which may be more easily understood by analogy. Consider a bird population which only nests in defined territories and that the number of territories determines the breeding size of the population. If, for example, 10% of the birds are artificially removed each year by an over-zealous ecology student, then the reproductive excess inherent in most species means that the vacated territories will be utilized next breeding season and stabilization of population size occurs within a generation. If, however, the territories disappear, for example due to fire, then the population will only re-stabilize once the vacated territories become habitable again and it may take several generations for the vegetation to regenerate. In other words, population size will stabilize on a time-scale determined by how quickly the vacated territories can be re-colonized. Once stabilization has occurred, a normalizing factor has to be included as in Equation 1*a*. The analogy should now be clear: in malaria populations, the breeding territory is humans and population stability is reached once haplotypes can be transmitted to territories (hosts) left vacant by the sensitive haplotypes killed by drugs. It seems reasonable to suppose that the reason why resistant haplotypes cannot *immediately* occupy these vacant niches once drugs are applied is residual host immunity and that the time-scale to stabilization depends on the length of this immunity. The question is therefore to determine how long this immunity lasts, i.e. once a niche is 'cleared' by drugs how long does it remain inaccessible to resistant forms? Some estimates of this come from field studies where individuals are cleared by drugs and their subsequent susceptibility to re-infection is measured (see, for example, Hoffman *et al*. 1987; Alonso *et al*. 1994). The results of these studies suggest that re-infection may be possible after a matter of weeks/months i.e. on the time-scale of malaria 'generations'. Since the epidemiological factors such as bite rate etc. presumably do not change then we can conclude that stabilization may be rapid. This line of argument predicts that normalization is immediately required and Equation 1*a* can be used to obtain survival probabilities as in Fig. 1B.

The critical differences in the 2 models of pre-existing mutation is the time taken for this stabilization to occur. Epidemiological models based on the prevailing conditions would need to be used to predict whether the time taken for such an adjustment would be relatively 'rapid' or 'slow'. These are not, of course, the only possibilities but are ends of a spectrum represented algebraically by full and no normalization respectively. It is worth re-iterating that only under a model of complete non-normalization (as described by Mackinnon (1997)) does the peak in expected rate of evolution at low infection rate shown on Fig. 1A fail to translate into a positive survival probability.

The method presents a simple means of calculating survival probabilities and has the advantage of being able to easily incorporate differing numbers of genes required to encode resistance. In the above cal-

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culation it was assumed that 2 genes were required but the method is general: if n genes are required then $d(n) = 1/2^n$ (Hastings, 1997) in Equation 1. In the case of a single gene required for resistance then loss of drug resistance through recombination is absent and $d(n) = 1/2$ so $c\tau = 1$; substituting this into Equation 1 shows that under a SI model, $E(P')$ (and hence probability of survival) is independent of infection rate while under a GI model, $E(P')$ (and survival probability) is an increasing function of infection rate. More interesting is the case where $n > 2$ as many putative vaccines have several components, for example $n = 4$ in the case of the recent anti-malaria vaccine trial of SPF66 (Patarroyo *et al*. 1988; Alonso *et al*. 1994). There is a close correspondence underlying the evolution of drug resistance and vaccine insensitivity: in the former, alleles become insensitive to the drug (e.g. by a conformational change in their active sites) while in the latter, alleles arise whose conformations have changed such that they are not recognized by the antibodies elicited by the vaccine. The results when $n > 2$ are qualitatively similar to the case of $n = 2$ for both the GI and SI models, the only difference being that the period where survival probability is zero which separates the 2 modes in the GI model become longer (results not shown).

The results examine the fate of a resistant clone once it has arrived in the population but ignore the biological and epidemiological processes which give rise to them. One possibility is that resistant clones enter a population through migration, either of infected people or infected mosquitoes, which presumably occurs at a rate independent of transmission rate. The other plausible possibility is that resistant clones are formed by recombination bringing together the 2 alleles necessary to encode resistance. Such alleles presumably exist in the population at a frequency determined by the forces of mutation and natural selection, the so-called mutation/selection balance. Pre-existing resistant clones will occur at frequency p^2 where p is the frequency of resistance alleles. Once drug treatment has started they may arise during recombination between different clones (which occurs at the rate $(1-1/c)$ so the *de novo* rate of appearance is $(1-1/c)p^2$). Under a simple model of between-clone competition, it can be shown that p is much higher at low rates of infection (Hastings, 1997) and it is informative to incorporate the effects of mutation/selection balance into the calculation on survival probability by multiplying their expected frequency by their probability of survival. Since between clone competition is absent when $c = 1$, the results on Fig. 1C are shown only for $c \geqslant 1{\cdot}05$ and scaled to the survival probability at this level of infection.

A further epidemiological consideration of the factors promoting drug resistance is the variability of transmission. In this paper we assumed that the variability in the mean number of offspring was equal to the mean, i.e. the distribution of the number of offspring was Poisson. However, if the variability is higher than the mean, the mutant is at more risk of extinction and so survival probability is reduced (Crow & Kimura, 1970; Mackinnon, 1997). However, the qualitative conclusions of this paper do not change as variability in transmission varies because $E(P')$ is unaffected.

It is impossible to ascertain the past level of drug treatment in specific areas which may have been very high, for example when drugs were routinely added to table salt. Under intermediate levels of drug use in stabilized populations, multilocus resistance may arise and spread faster in areas of high or low transmission. In any case such *post hoc* investigations are not very useful. What is important is how these results can be used to guide current drug treatment strategies and how to minimize the risk of resistance evolving. It is clear that overall drug use must be minimized and preferably (if possible) restricted to life-threatening infections. Since these form only a small part of the total infections (1–2% in Gambia according to Greenwood *et al*. (1991)) then usage of novel drugs should, under these conditions, be relatively low. Under a model of specific immunity (SI), or strong effects of the drug in reducing transmission output (low values of t), control measures can run the risk of increasing the rate of evolution of drug resistance. If resistance is encoded by more than 1 locus, the situation under generalized immunity (GI) in stabilized populations is more complex: at low levels of drug pressure, in areas where transmission is above the 'valley' of minimum transmission control measures to reduce transmission rate will be beneficial provided they do not lower mean infection rate below the critical level corresponding to the valley minimum, while in areas of transmission lower than this, control measures will increase the risk of resistance appearing. In unstabilized populations, or where resistance is controlled by a single locus, this is not a concern, i.e. control measures will hinder the spread of resistance. Thus an understanding of the underlying biology is required in designing a control strategy. Essentially we need to know (i) the effects of immunity on intra-host dynamics (GI versus SI), (ii) the effect of drugs on subsequent transmission, t, (iii) the conditions under which normalization is appropriate or inappropriate, and (iv) the number of independent loci controlling resistance. While answers to these problems are only partly known, this paper at least points out the important factors to be considered in the implementation of control programmes.

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