Environmental, pharmacological and genetic influences on the spread of drug-resistant malaria

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Plasmodium falciparum malaria is subject to artificial selection from antimalarial drugs that select for drug-resistant parasites. We describe and apply a flexible new approach to investigate how epistasis, inbreeding, selection heterogeneity and multiple simultaneous drug deployments interact to influence the spread of drug-resistant malaria. This framework recognizes that different human ‘environments’ within which treatment may occur (such as semi- and non-immune humans taking full or partial drug courses) influence the genetic interactions between parasite loci involved in resistance. Our model provides an explanation for how the rate of spread varies according to different malaria transmission intensities, why resistance might stabilize at intermediate frequencies and also identifies several factors that influence the decline of resistance after a drug is removed. Results suggest that studies based on clinical outcomes might overestimate the spread of resistant parasites, especially in high-transmission areas. We show that when transmission decreases, prevalence might decrease without a corresponding change in frequency of resistance and that this relationship is heavily influenced by the extent of linkage disequilibrium between loci. This has important consequences on the interpretation of data from areas where control is being successful and suggests that reducing transmission might have less impact on the spread of resistance than previously expected.

Keywords: Plasmodium falciparum; drug resistance; malaria; prevalence; selection; epistasis

1. INTRODUCTION

Malaria is a major public health concern for the one-third of the human population estimated to be exposed to the threat of the most virulent species, Plasmodium falciparum, with an estimated number of clinical episodes ranging from 300 to 660 million per annum [1]. There is no effective vaccine for this species, and infection is controlled by insecticides targeted at vector mosquito species, and treatment by antimalarial drugs. As might be expected, insecticide and drug resistance rapidly evolved and spread [2–4]. This article focuses on the dynamics of antimalarial drug resistance although the approach we develop herein may be generalized to other control agents such as insecticides, herbicides and anthelmintics.

Plasmodia parasites are haploid and reproduce asexually in humans. Humans often contain several genetically distinct malaria clones acquired from different mosquito bites; the number of clones in a human is called the multiplicity of infection (MOI). MOI is a proxy for transmission intensity as higher transmission intensity increases MOI owing to repeated sequential infection [5]. Malaria parasites undergo an obligate sexual phase in the mosquito before being transmitted back into humans as haplotypes. Mating between gametes from the same clone (selfing) involves sexual recombination between identical haploid genotypes, resulting in clonal reproduction. Mating between different clones (outcrossing) results in genetic re-assortment of malaria genes.

Mating can only occur between clones transmitted from the same human, hence the rate of outcrossing depends on the MOI. Field estimates reveal that outcrossing is relatively common and can occur in more than 50 per cent of matings [6]. It has been postulated that clones within a human compete for resources and transmission and that removal of drug-sensitive clones following treatment allows the surviving resistant clones to garner additional resources and to increase their transmission [7,8], an effect recently termed ‘competitive release’ [9]; the higher the MOI, the larger the potential effect of competitive release. MOI is therefore fundamental in the dynamics of the spread of resistance as it increases the rate of sexual recombination (outcrossing) that allows parasites with different resistance profiles to mate and also breaks down the association between alleles encoding drug resistance.

Mathematical models play an important role in understanding the forces driving resistance and in designing policies to minimize the rates at which resistance arises and spreads (e.g. [10,11]). Their importance arises for three main reasons: first, it is near-impossible to address this issue empirically as antimalarial drug deployments occur on country- and even continent-wide scales, so the effects of local differences in deployment strategies are likely to be swamped by immigration of resistance driven by national deployment policies [12]. Second, it is difficult to generalize the lessons learnt from individual drugs because their dynamics are likely to differ substantially depending on the genetic basis of resistance and the degree of resistance they encode: for example, resistance arises incredibly rarely to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) [12], the two
drugs so far deployed worldwide, but very easily to other
drugs such as atovaquone [13] and pyrimethamine [14],
and at intermediate rates—apparently owing to gene
duplications rather than point mutations—to mefloquine
[15]. In addition, high-level resistance to atovaquone
occurs in a single mutational step, while resistance to
SP requires sequentially accumulation of mutations at
several codons in two genes [16]. Third, basic population
genetics predicts that the alleles encoding resistance
increase in frequency exponentially, so the most import-
tant dynamics occur when resistance is at undetectably
low frequencies, meaning there are few empirical data
on the process.

All models make simplifying assumptions, but the pri-
mary one we relax and investigate here is the assumption
that infections bearing a ‘resistant’ genotype always sur-
vive drug treatment. Empirical evidence shows that the
fate of a resistant infection, survival or death is much
more probabilistic and depends critically on the human
‘environment’ within which drug treatment and selection
occurs. Human immunity plays a huge role and drugs
may be highly effective in semi-immune adults but
highly ineffective in non-immune infants [17,18]. Simi-
larly, ‘sensitive’ infections may survive treatment in
humans where drug levels are suboptimal either through
poor compliance with the drug regimen or because their
pharmacogenetics means that drugs are poorly absorbed
or rapidly eliminated [19], while resistant infections
may be eradicated in humans with high drug levels. It is trivial
to incorporate these effects into a single-locus model (we
simply assign a probability of survival in a treated individ-
ual), but dynamics become more realistic and complex in
situations where two (or more) loci are required to encode
resistance.

When more than one locus is involved in drug resist-
ance, it is important to quantify the association between
resistance alleles at different loci. Linkage disequilib-
num (LD), the non-random association of resistance alleles at
different loci, has been shown to be a critical factor influ-
encing the rate of spread of resistance [20]. Many genetic
models have ignored LD and those that have measured it
assumed complete epistasis between the mutations (i.e.
only infections with mutations at all resistance loci
would survive treatment), with the result that LD was
always positive between mutations. We allow three differ-
ent models of parasite genetic interaction: ‘full epistasis’,
where, as before, resistant mutation at both loci are
required for the infection to survive; ‘asymmetric epista-
sis’, where one locus has more impact on survival, so
resistance is determined primarily by that locus irrespec-
tive of the allele at the second locus; and ‘duplicate
gene action’, where a resistant mutation at either locus
will allow survival. It is axiomatic among geneticists that
the mode of gene action is not fixed but depends on the
environment in which they are expressed, and this is
what we address here: infections encountering ‘strong’
selection environments (humans with high levels of immu-
nity and/or drug) may require full epistasis to survive, while
as the environment becomes ‘weaker’ (less immunity and/
or drug), then asymmetric and eventually duplicate gene
action will best describe the fate of resistant mutations.
These modes of selection are summarized in table 1.

Herein, we present a deterministic population genetics
model of the spread of drug-resistant malaria using
computational simulations to investigate how these key
factors affect the spread of drug resistance (i.e. selection
heterogeneity, number of resistance loci) within the
context of local malaria epidemiology and local drug
policies (for example, whether different drugs are
codeployed to reduce selection for resistance or whether
they are rotated such that one is used until it fails and then
replaced). The model presented is sufficiently general to
study many different parameters, but we will present results
for scenarios of most practical importance and realism for
P. falciparum population biology and drug-deployment
strategies. Practical policy considerations are discussed
as we try to understand the implications of control and
elimination measures for the spread of resistance.

2. MODEL AND METHODS

We make the following assumptions in line with most pre-
vious modelling efforts: that clones co-infecting the same
human are genetically unrelated; that clones have equal
infectivity and mate at random so that if there are n
clones in a human, then selfing rate is 1/n and outcrossing
rate 1 − 1/n; and that competitive release occurs.
The spread of drug resistance is tracked using a time scale
of parasite generations (a generation is a parasite reproduc-
tion cycle from host to host, which is likely to be
around 5 per year). Loci are assumed to be physically
unlinked, as is the case for most loci known to be involved
in malaria drug resistance [21], and each locus can have
two alleles: resistant and sensitive. A resistant allele will
incur a fitness penalty in the absence of the drug and all
mutations are assumed to have the same fitness penalty.
Genotypes with multiple mutations suffer a multiplicative
fitness penalty.

Development of drug resistance can be seen as a two-
step process, the de novo emergence of the resistant
mutation and its subsequent spread. Existing research
shows that, for most drugs, resistance emerged extremely
infrequently [22,23], the notable exceptions being atova-
quone [24] and pyrimethamine [15]. Understanding the
appearance of de novo mutations is an important topic,
discussed elsewhere (e.g. [25,26]), so we assume that resistance alleles already exist at very low frequencies at the onset of the simulation and focus on understanding their subsequent spread.

The model is designed to study the spread of drug-resistant malaria alleles subjected to different drug-deployment policies and a mathematical formalization of the model is provided in the electronic supplementary material. There are evidently a very large number of parameter combinations that can be explored. Here, we concentrate on seven illustrative scenarios, named and described below.

—**Single locus**. A single drug is deployed with resistance encoded at a single locus. There are two human environments: treated and untreated individuals. Resistant parasites survive in both environments (but may pay a fitness penalty in untreated humans), while sensitive parasites survive in untreated humans but are cleared in treated humans. This is obviously the simplest case, explored elsewhere, but is included as a baseline simulation.

—**Full epistasis**. A single drug is deployed with resistance encoded by two loci. Two environments are present, untreated and treated, and survival in the treated humans requires resistance at both loci (i.e. full epistasis).

—**Duplicate gene function** (DGF). A single drug is deployed with resistance encoded by two loci. Two environments are present, untreated and treated, with selection in the latter sufficiently weak that resistance alleles at either locus can encode survival.

—**Asymmetric epistasis**. A single drug is deployed where resistance is encoded by two loci. Two environments are present, untreated and treated. The first locus is necessary and sufficient to encode resistance in treated individuals and the second is irrelevant. This scenario is, therefore, functionally identical to the single-locus scenario, but asymmetric epistasis serves as a useful model of resistance when used in more complex and realistic environments, in conjunction with full epistasis.

Asymmetric epistasis mimics SP resistance where the *dhps* resistance allele involved in the de novo folate production pathway cannot fully replace the *dhfr* gene involved in exogenous folate usage. This model is also applicable for the asymmetric importance of the supplementary *mdr* gene to *crt* in CQ-based resistance [27].

—**Full epistasis + DGF**. A single drug is deployed with resistance encoded by two loci. There are three environments: untreated, well treated (treatment is sufficiently effective that full epistasis is required for survival) and poorly treated (treatment is suboptimal so that resistance alleles at the more important locus can encode survival). The name of this scenario comes from the drug SP where resistance may be encoded by alleles at *dhfr* alone or, in well-treated individuals, may require resistant alleles at both *dhfr* and *dhps* loci.

—**Two drugs**. Two drugs are deployed separately (i.e. the parasites never encounter both simultaneously in the same generation) with two loci for each drug. There are five environments: untreated, well treated with drug 1, well treated with drug 2, poorly treated with drug 1 and poorly treated with drug 2. Full epistasis is required for survival in well-treated individuals while asymmetrical epistasis determines survival in poorly treated ones.

In scenarios with more than one treatment environment (i.e. the last three scenarios), it is assumed that there are equal proportions of each environment among the treated infections.

### 3. RESULTS

The following outcomes are possible for each locus in a simulation: (i) one allele (sensitive or resistant) tends towards fixation or (ii) allele frequencies stabilize at intermediate levels. We opt to describe the dynamics of the fully sensitive form (i.e. with no mutations) in order to simplify the presentation of results.

Figure 1 plots the frequency of sensitive parasites and its rate of change computed as the proportionate change per generation (fitness penalty = 0.3 and drug rate = 0.6). The frequency of sensitive genotypes is depicted in dashed lines and the rate of change in solid lines. The rate is below 1 as resistance is increasing. At low levels of frequency (which are important for policy decision), bigger MOIs entail a faster decline of sensitive forms. The single-locus model is shown. Solid line, MOI 1 rate; dashed-dotted line, MOI 1 frequency; solid line with circles, MOI 2 rate; dashed-dotted line with circles, MOI 2 frequency; solid line with plus symbols, MOI 4 rate; dashed-dotted line with plus symbols, MOI 4 frequency.
The spread of drug-resistant malaria

reversed when resistant parasites approach fixation. This reveals that the speed of spread is frequency dependent and, consequently, that the total time to resistant fixation is a bad proxy of the speed of spread at low frequencies. This occurs because the total time in most cases is more influenced by what happens at high resistance frequencies rather than the dynamics at lower frequencies; it is the latter part of the dynamics, when resistance is starting to spread and cause drug treatment failures, that has the most implications for drug policy choice. Understanding the spread of resistance at important frequencies therefore requires analysis of the whole behaviour of the model and not just the time until the final outcome.

Figure 2 shows LD, $r$, for the four scenarios where a single drug is deployed and resistance is encoded by two loci. Where full epistasis is required, $r$ is positive (in line with comparable results in Dye & Williams [20] and Hastings [28]). This arises because genotypes $c_{0,1}$ and $c_{1,0}$ (a description of the genotype notation is presented in the electronic supplementary material) provide no advantage in any environments as they are not resistant to treatment and are less fit than the sensitive clones in untreated individuals. With DGF, one mutation is sufficient to confer resistance and furthermore two simultaneous mutations are never advantageous, and $r$ becomes negative. When more than one treated environment is available, as the full-epistasis plus DGF scenario and in the SP-based scenario where asymmetrical epistasis plus DGF occur, the opposing effects tend to cancel each other out and LD is low. From a qualitative point of view, epistasis has a tremendous impact on both signal and amplitude of LD.

Figures 3 and 4 illustrate how MOI and LD affect the relationship between the prevalence of a resistant mutation and its underlying allele frequency. The prevalence at a single locus, $P$, is given by the binomial distribution: $1 - (1 - F)^i$, and is plotted in figure 3 for various MOI. A bigger MOI implies a bigger prevalence for the same frequency. The difference between frequency and prevalence is not maximized at the extreme frequencies (i.e. near 0 or 1.0), but at intermediate frequencies. We note that prevalence is inevitably higher than frequency unless the MOI is 1. This has implications for the interpretation of field data and is discussed below. It is quite easy to compute the frequency of resistance for the single-locus model given the prevalence and the MOI; for more realistic models with two or more loci, the frequency also depends on LD, which is itself dependent on assumptions about epistasis. We can repeat the analysis of the relationship between frequency and prevalence considering one drug with two resistant loci. The prevalence is given by $1 - (1 - F_{1,1})^i$ for full epistasis (this is because only $c_{1,1}$ clones are resistant) and $1 - (1 - (F_{1,1} + F_{1,0} + F_{0,1}))^i$ for DGF ($c_{0,1}$, $c_{1,0}$ and $c_{1,1}$ are all resistant). Assuming equal frequencies for both resistant alleles (a realistic assumption for all epistasis models except asymmetry), the frequency of resistant alleles is given by the binomial distribution: $1 - (1 - F_{1,1})^i$, and is plotted in figure 3 for various MOI. A bigger MOI implies a bigger frequency for the same prevalence. The difference between frequency and prevalence is not maximized at the extreme frequencies (i.e. near 0 or 1.0), but at intermediate frequencies. We note that prevalence is inevitably higher than frequency unless the MOI is 1. This has implications for the interpretation of field data and is discussed below. It is quite easy to compute the frequency of resistance for the single-locus model given the prevalence and the MOI; for more realistic models with two or more loci, the frequency also depends on LD, which is itself dependent on assumptions about epistasis. We can repeat the analysis of the relationship between frequency and prevalence considering one drug with two resistant loci. The prevalence is given by $1 - (1 - F_{1,1})^i$ for full epistasis (this is because only $c_{1,1}$ clones are resistant) and $1 - (1 - (F_{1,1} + F_{1,0} + F_{0,1}))^i$ for DGF ($c_{0,1}$, $c_{1,0}$ and $c_{1,1}$ are all resistant). Assuming equal frequencies for both resistant alleles (a realistic assumption for all epistasis models except asymmetry), the frequency of resistant

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infections can be easily calculated for both models as a function of frequency of mutation of one gene and the LD measure \( r \). The relation between frequency of a locus involved in resistance and prevalence as a function of MOI and \( r \) is shown in figure 4 for an \( r \) of 0 and 0.5 for full epistasis. As expected, higher \( r \) entails a bigger proportion of individuals harbouring a resistant infection in a full-epistasis scenario (the converse is expected and observed for DGF). In all cases, higher MOI implies a larger proportion of individuals harbouring one resistant infection, but the behaviour of the function is difficult to quantify with precision.

MOI and epistasis influence whether stabilization of resistance occurs at intermediate frequencies. Table 2 presents the proportion of scenarios that stabilize at intermediate frequencies for the scenarios considered (the electronic supplementary material provides details on the parameter ranges investigated). Single-locus and full-epistasis models are included for comparison as they have been widely studied before (e.g. [20,28]). Our results are consistent with those studies as both scenarios tend to fixation in a very large part of the search space. DGF, by making all parasites with two mutations less fit than parasites with just a single mutation even in treated individuals, has a much bigger portion of the search space where stability occurs at intermediate frequencies. This is analogous to the situation of over-dominance in diploids that is known to produce stable allele frequencies. In this model, two non-mutated loci are bad for the parasite (it is drug sensitive), one mutated locus is optimal (resistance) while two mutated loci pay a double fitness penalty: they are the least competitive genotype in untreated hosts and less fit than single-mutated parasites in treated hosts. In all models, increasing MOI increases the size of the parameter space where stable intermediate frequencies occur.

We also investigated a single-locus scenario where the frequency of resistance was started at 99 per cent and no drugs were used. This allows us to isolate and investigate the effect of untreated individuals on the spread of drug resistance and provides insight into the likely effect of removing a drug from circulation. In the simple situation of MOI = 1, then the frequency of resistant infections remains unchanged even if a drug is removed, because the fitness penalty was modelled as competition within the human host (electronic supplementary material, equation S1); this assumption was made for convenience, but an additional factor \( s(i) \) could be added to this equation to incorporate other factors such as increased parasite clearance or lower gametocyte densities that may occur and be independent of MOI. When MOI is bigger than 1, it becomes an important factor in the loss of resistance when a drug is removed. Higher MOI increases the rate of spread of sensitive parasites and this effect is stronger at higher frequencies of resistance (data not shown). This arises because both factors increase the probability that a sensitive clone will compete with resistant clones within untreated humans; the formers’ superior competitive ability (they lack fitness penalties associated with resistant mutations) within humans helps drive their spread through the population.

### Table 2. Percentage of scenarios that stabilize at intermediate frequencies of resistance by MOI (MOI affects both effective fitness costs and the level of sexual recombination.)

<table>
<thead>
<tr>
<th>model</th>
<th>MOI 1</th>
<th>MOI 2</th>
<th>MOI 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>single locus</td>
<td>0</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>full epistasis</td>
<td>0</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>DGF</td>
<td>0</td>
<td>53</td>
<td>70</td>
</tr>
<tr>
<td>full epistasis + DGF</td>
<td>0</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>SP-based</td>
<td>0</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>two drugs</td>
<td>2</td>
<td>13</td>
<td>27</td>
</tr>
</tbody>
</table>

4. DISCUSSION

The methodology described above shows that it is conceptually straightforward to incorporate a flexible genetic basis of resistance into models of antimalarial drug resistance, and that this may have important qualitative implications for important factors, such as the rate at which resistance evolves and whether it may stabilize at intermediate frequencies. This entails a slight redefinition of the concept of ‘resistance’ away from hard-wired genetic determinism and towards a more subtle realization that drug resistance is determined by both the parasite genome, and the ‘environment’ of the infected host: two hosts might carry infections with exactly the same genotype but have different treatment outcomes depending on their immune status, pharmacogenetics and/or compliance to the recommended drug regimen. CQ provides a useful illustration of this. There was widespread resistance in Guinea-Bissau, but when that country adopted a policy of doubling the CQ dosages, the problem of resistance largely disappeared [29]: the mutations, probably in \( c rt \), encoded resistance to normal levels of CQ but remained drug sensitive when exposed to high levels. The presence of multiple epistasis environments in the host population (table 1) reflects the importance of human immunity and pharmacological variables, such as drug quality and correct dosage. A key strength of this approach lies in its flexibility, as it can also easily be used to incorporate other factors besides the main ones of immunity, pharmacogenetics and compliance explicitly discussed above. Other factors could be human genetic variation in malaria susceptibility and the role of residual drugs in driving resistance. Most antimalarial drugs have long half-lives and are frequently taken in many areas to presumptively treat any fever, with the consequence that a large proportion of people (up to 80%, e.g. [30]) carry ‘residual’ levels of drugs from previous treatments. Most antimalarial drugs do not affect malaria during its initial incubation in the liver, and parasites emerging from the liver may encounter drug and be subject to ‘accidental’ drug action, which may be an important driver of resistance [31,32]. It is easy to envisage a plausible situation where full epistasis may describe survival to direct therapeutic treatment, while in the bloodstream, asymmetric epistasis may describe resistance to high residual drug levels encountered on emergence from the liver and DGF describes survival to low levels of drug encountered after liver emergence. It would be straightforward to extend our approach to this situation by constructing a new scenario similar to those described above.

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Fixation of sensitive alleles is far more likely to occur in the ‘two drugs’ scenario (data not shown). This occurs because mutations encoding resistance to one drug offer no protection against the second drug but are deleterious in untreated individuals. If we extrapolate this result and assume that the sensitive alleles are more likely to be fixed if more drugs are used, and that the resistant mechanisms to all drugs involved are independent, then it should be possible to treat higher number of individuals before resistance spreads. This is consistent with the results presented elsewhere [10,33].

Simulations of drug removal following fixation of resistance allele illustrate the impact of untreated infections in the spread of drug resistance. Untreated infections delay the spread of drug resistance because sensitive infections are favoured in these hosts. This effect is more pronounced in higher transmission settings as MOI is high, increasing competition between clones, and is especially significant with higher frequencies of resistance. This highlights, and quantifies, a fundamental conundrum in drug deployment: an individual-centred, medical approach dictates that symptomatic patients be identified and cured, while drug policies aim to reduce the overall amount of drug used to minimize selection for resistance. These are not entirely incompatible considerations, for example, better diagnostics can reduce drug usage without reducing patient care, but it is an important component of models that they can quantify the impact of changes in drug-deployment policies. Population genetics models also clearly highlight the tension between measures for control (defined as the reduction in incidence of the disease) and elimination (the reduction to zero of the incidence in a specific human population) of malaria. If control is the main objective, semi-immune, asymptomatic hosts provide a reservoir of sensitive parasites, where these are fitter than resistant parasites. From a control perspective, having a reservoir of sensitive parasites is a positive development that slows the spread of resistance and increases the time a drug will remain effective. From an elimination perspective, asymptomatic reservoirs have to be treated in order to completely remove the parasite from the host population, and are a source of concern as they are difficult to detect in the human population. Any attempt at elimination, if unsuccessful, will probably have negative consequences for long-term control, as selection against sensitive parasites in elimination policies will most probably increase the frequency of resistance against any drugs used in the elimination phase.

It is generally assumed that mutations encoding drug resistance pay a fitness penalty and are deleterious in the absence of the drug [34,35]. Consequently, the frequency of resistant parasites should fall once that drug is removed from general use. This effect is important as reintroduction of the drug might be considered if the frequency of resistance to a certain drug drops to very low levels. For example, CQ reintroduction as a partner drug in combination therapy, probably with artesunate, has been considered in Malawi [36]. Our results (data not shown) suggest the rate of fall will be faster in high-transmission settings as a consequence of higher MOI and hence higher competition within hosts. This can be observed in the field where the return of CQ-sensitive parasites was observed after the introduction of the replacement drug SP, especially in high-transmission areas like Malawi [37] and to some degree in Gabon [38], but less in low-transmission areas like Colombia and Venezuela [39], suggesting that the intensity of transmission might be a factor in decreasing levels of drug resistance. However, these areas differ in many other aspects besides MOI (for example, migration from border countries or provinces with different drug-deployment policies), and the results need to be interpreted with caution; for example, the fall in resistance was faster in areas of low MOI in Yunnan province, China [40], but the area shows highly heterogeneous patterns of transmission, so other factors might have a substantial impact.

A large body of existing research predicts that once drug resistance arises, it will spread rapidly to fixation. Hastings [28] postulated that forces driving resistance (genetic recombination, intra-host dynamics, natural selection) vary with frequency and could cancel out to allow stable intermediate frequencies; he used a model of full epistasis and showed that stable intermediate frequencies could occur, but was fairly uncommon, especially for lower MOI. By contrast, many field studies suggest stabilization is relatively common; for example, for SP in Malawi [41] and Tanzania [42] and for CQ in eastern Sudan [43] and Guinea-Bissau [29]. The more biologically realistic ‘full epistasis + DGF’ and ‘SP-based’ models of host heterogeneity described here exhibit an increase in intermediate stable frequencies, especially with lower MOI (table 2). In summary, realistic modes of gene interaction are more compatible with field observations, demonstrating the importance of accurately modelling gene interactions and also suggesting the need of further empirical research on the basis of drug resistance.

Previous models based on an assumption of full epistasis always predict strong positive LD [20]. Here, we show that LD depends on the mode of gene action (figure 2), which is not well understood for most drugs and which will, as stressed above, depend on the selection environment of the treated human. This has implications for interpretation of field data. Studies have investigated LD between loci in the belief that significant positive LD would be indicative that both loci are important for encoding resistance, the most obvious examples being mdr and ctt loci in CQ resistance. This is true if full epistasis is required, but figure 2 shows that ‘negative’ results (i.e. absence of LD or negative LD) cannot be taken as evidence that they do not have a joint role in determining resistance. LD also has a strong impact on genotype inference and on the ability to accurately calculate the frequency of resistance from prevalence (figure 4). Many clinical studies present the prevalence or frequencies of each locus separately, and normally no attempt is made to report multi-locus genotypes because it is often impossible to determine a multi-locus genotype (i.e. the linkage phase in the population genetic terminology) when MOI is greater than 1. The frequency of multi-locus resistance genotypes, and the direction and extent of LD between the loci, provides clues as to the underlying genetic mechanisms of resistance, so a strong case could be made for future studies to attempt genotype inference and LD estimation from multiple infections [44]. Intensity of transmission determines MOI, which determines the proportion of individuals carrying
resistant infections (i.e. the prevalence of resistance). This has two main implications for comparative analysis of clinical studies from areas with different transmission intensities, or for temporal studies from the same area if the transmission rate has changed.

—The potential confusion between frequency and prevalence of resistance should be avoided. Different transmission settings change the relationship between frequency and prevalence and higher MOI clearly entails higher prevalence for the same frequency of resistance.

—For longitudinal studies where transmission has decreased, an observed drop in prevalence of resistance does not always reflect decreased frequency of resistance. Any conclusion that cutting transmission decreases the frequency of resistance should be carefully evaluated as such observation could be explained, partially if not totally, by a lower MOI generating a smaller prevalence for the same frequency (figure 3). The relationship becomes much more complex when more than one locus is involved, as LD and epistasis also affect prevalence (figure 4).

Our theoretical conclusions on the relationship between transmission, frequency and prevalence are consistent with a recent study [45] that analysed field data from Tanzania and Papua New Guinea.

Malaria biology has several features that preclude simple analysis using standard population genetics equations. Studying the implications of parasite inbreeding, epistasis between drug resistance loci and heterogeneity in human host environments will allow a better understanding of the dynamics of resistance spread of *Plasmodium falciparum*. This explicit and flexible framework of gene action can be used in the future to study deployment strategies for the new generation of antimalarial drugs, the artemisinin combination therapy for uncomplicated malaria in southern Cambodia. Malar. J. 8, 10. (doi:10.1186/1475-2875-8-10)


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