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Review

Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread

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ABSTRACT

The emergence of resistance to former first-line antimalarial drugs has been an unmitigated disaster. In recent years, artemisinin class drugs have become standard and they are considered an essential tool for helping to eradicate the disease. However, their ability to reduce morbidity and mortality and to slow transmission requires the maintenance of effectiveness. Recently, an artemisinin delayed-clearance phenotype was described. This is believed to be the precursor to resistance and threatens local elimination and global eradication plans. Understanding how resistance emerges and spreads is important for developing strategies to contain its spread. Resistance is the result of two processes: (i) drug selection of resistant parasites; and (ii) the spread of resistance. In this review, we examine the factors that lead to both drug selection and the spread of resistance. We then examine strategies for controlling the spread of resistance, pointing out the complexities and deficiencies in predicting how resistance will spread.

1. Introduction

Malaria is the most important parasite of humans, affecting more than 2 billion people and causing hundreds of millions of clinical cases of malaria every year [1,2]. Five species of the malaria parasite cause disease in humans, namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. Of these species, *P. falciparum* causes the most severe disease and is the leading cause of death in children under the age of 5 years in Africa [3]. The discovery in the 1940s that the synthetic drug chloroquine (CQ) could effectively treat individuals safely and cheaply helped spur malaria eradication efforts in the 1950s. However, the emergence of CQ resistance diminished its therapeutic efficacy and doomed initial efforts to eradicate the disease.

The demise of these initial eradication efforts led to a resurgence in the disease and a significant change in the ecology, as CQ-resistant parasites spread from Southeast Asia to Africa [4]. In the ensuing years, CQ was replaced as a first-line drug by sulfadoxine/pyrimethamine (SP), but resistance to SP soon emerged and spread widely [5,6]. Whilst drugs are only one tool in the eradication effort, they are crucial to the effort; thus, in this review we examine the mechanisms that lead to the emergence of resistance and the factors that contribute to its spread. This is of particular importance because in recent years artemisinin class drugs, the current recommended first-line treatment for uncomplicated and severe malaria [7], have become widely available and are being promoted as a significant tool in the renewed fight to eradicate the disease. However, a delayed-clearance phenotype has already been reported both in western Cambodia [8] and Thailand [9]. This delayed-clearance phenotype, whilst not of clinical significance as yet [9], is the first indication that resistance to artemisinin may emerge soon. This has important implications for global eradication efforts, as it will likely be at least a decade before a new compound is capable of replacing the artemisinins [10]. Thus, it is important to understand how resistant parasites are selected, how resistance spreads through a population, and the efficacy of mechanisms for controlling its spread.

2. Drug selection of resistant parasites

Antimalarial drug resistance is mediated by two processes: (i) the rate that *de novo* mutations conferring resistance appear and are selected through drug use within an individual; and (ii) the spread of those resistant alleles to other individuals. CQ, SP and more recently the artemisinin class drugs have been widely adopted as first-line drugs because they are highly efficacious in eliminating *P. falciparum*-infected erythrocytes and they are well tolerated by almost all patients [11,12]. In addition, unlike other drugs such as atovaquone and pyrimethamine (when not combined with sulfadoxine), the rate at which *de novo* mutations conferring resistance occur is low.

Heritable drug resistance is enabled through a number of mechanisms, including reductions in active or passive uptake of a drug,

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abrogation of drug activity by conversion of the drug to an altered form, increased expression of the drug target, or a decrease in the ability of an inhibiting agent to bind due to alterations in the enzyme target [13]. These heritable phenotypic changes, of which more than one is possible, are the result of mutations that can be either single point mutations, alterations to multiple loci, or the result of gene duplication [14].

Because resistance-conferring mutations generally result in significant changes in metabolic pathways [15,16], they are likely to be deleterious in the absence of drug treatment. Evidence for this has been found in *in vitro* experiments where significant growth differentials were observed for specific CQ-resistant point mutations [17,18]. A biological fitness cost also explains the significant reductions in the prevalence of resistant parasites after the removal of CQ as a first-line therapy in Malawi [19,20].

The likelihood that a specific mutation conferring resistance will be present in a treated individual is a function of the mutation rate and the biological fitness cost of the mutation [21]. If resistance requires more than one mutation, then the initial frequency of resistance will be less. For example, if the frequencies of two necessary resistance-conferring mutations are both 0.01%, then parasites with both mutations will have an initial frequency in an infection of 0.0001%. This process underlies the recommendation that all malaria infections should be treated with combinations of two or more drugs [22–24]. Assuming that each drug requires a single non-identical mutation in order for the parasite to acquire resistance, then the initial frequency of multidrug-resistant parasites will have a similar calculation as above.

The total parasite load also plays an important role. The malaria parasite life cycle requires a mosquito in which meiosis occurs, but the vast majority of cell divisions in its life cycle occur mitotically in the human host and thus is the more likely place where resistance mutations arise [25]. Although the density at which symptoms occur can vary widely depending on the immune status of the individual, they are generally associated with blooms in parasite biomass. In non-immune individuals, symptoms may occur at densities of ca. 50 parasites/μL of blood, or between 10^8 and 10^9 asexual parasites (children who have less total blood volume have correspondingly lower total parasites). Clinically immune individuals (see below) may tolerate higher parasite loads, but parasite loads above 10 000 parasites/μL, or ca. 10^{11} parasites, are typically symptomatic regardless of immune status [26].

Clinical attacks are short, lasting on average only a couple of days. However, infections can persist for a long time without further symptoms. Estimates suggest that infections last on average ca. 200 days [27,28] but can last significantly longer. Thus, most individuals harbouring parasites at any one time are asymptomatic, with low levels of parasitaemia. However, because individuals that are symptomatic have such high levels of parasitaemia, the majority of malaria parasites in the world at any one time are likely in individuals that are symptomatic [25], suggesting that symptomatic individuals are more likely to harbour resistant parasites [29].

The appearance of de novo mutations is only important for drug selection if individuals harbouring these mutants use drugs. Increased drug use within a population thus leads inexorably to a greater probability of resistant mutants being drug-selected (i.e. all the sensitive parasites being eliminated, leaving only resistant parasites), a relationship that has been well documented both in models and experimentally [30]. However, widespread use of drugs has significant benefits both for the individual—reduced likelihood of morbidity and mortality—and the population—as a treated individual is less likely to transmit an infection. Thus, decisions about the distribution and use of drugs must balance the positive benefits of current treatment with the negative externality of an increased likelihood of drug selection for resistance and reductions in the future effectiveness of the drug [31].

Drug selection for resistant mutants at the individual level depends on the concentration of drug over time in the blood (pharmacokinetics) and the inhibitory effects on the malaria parasite at those concentrations (pharmacodynamics). Together, the pharmacokinetics and pharmacodynamics give the concentration and length of exposure to a drug that parasites will face; however, antimalarial drugs differ significantly in the length of time they are maintained in the body. Some drugs, such as CQ, have long half-lives (1–2 months), whilst others such as artemisinin have much shorter half-lives (1 h) [32]. As the concentration of a drug falls, its therapeutic efficacy also falls. If the dosing is incomplete, meaning it fails to effectively eliminate all of the parasites, either because of non-compliance or too low a dosage, parasites that may be inhibited at higher concentrations could survive and recrudesce. Alternatively, new infections may be exposed to subtherapeutic levels of drugs due to a long drug half-life [33], or because of prophylactic use [34], or because the drug was substandard.[1]

There have been some cases of high-level drug resistance arising in individual infections during therapy [35,36]; however, in general, the mechanism by which resistant parasites are drug-selected is believed to be through subtherapeutic drug levels [33]. At low drug concentrations, parasites with resistance mutations are able to survive and over time increase their fitness through compensatory mutations [37]. A similar phenomenon is known to occur in resistance evolution in bacteria [38]. Studies suggest that large proportions of the population in sub-Saharan Africa may have subtherapeutic concentrations of CQ in their blood [39] and that these are often associated with resistance [40].

The stage of asexual parasite development (Fig. 1) also impacts the efficacy of a drug [45,46], with the maximum inhibitory effect of the drug typically occurring in the late ring and early trophozoite stages [45]. The artemisinin compounds are active during a broader time window of development [45], which is the likely reason they tend to clear parasites at a faster rate than other drugs [47–49]. However, the artemisinin delayed-clearance phenotype reported [8,9,50] is believed to be the result of reduced efficacy of the drug against ring-stage parasites [51].

Lastly, immunity to malaria, which develops with exposure [52] and age [53], plays a role in selection for resistance. Immunity to malaria provides protection against clinical manifestations of the disease only and not against further asymptomatic infections [52]. Clinically immune individuals also respond better to treatment [54], with shorter treatment courses necessary to generate good therapeutic results even when drug resistance is present [33]. Thus, even though individuals who are clinically immune tend to support higher parasite loads without showing symptoms—which increases the possibility that they may harbour a resistant parasite—the immune system is more likely to eliminate mutant resistant parasites.

3. Spread of resistance

3.1. Emergence

Once the sensitive parasites have been eliminated from an individual through drug treatment, to become a population problem the resistant parasites must be transmitted. Transmission of a *de novo* resistant mutant out of the primary host is conceivably the largest hurdle that resistant parasites face. The parasite first must survive the immune system's response long enough to produce

[1] Substandard drugs encompass both counterfeit drugs that have been deliberately manufactured with insufficient active ingredients as well as products that have degraded. Drugs with no active ingredient, whilst significantly impacting the individual taking the medication, have no impact on resistance.

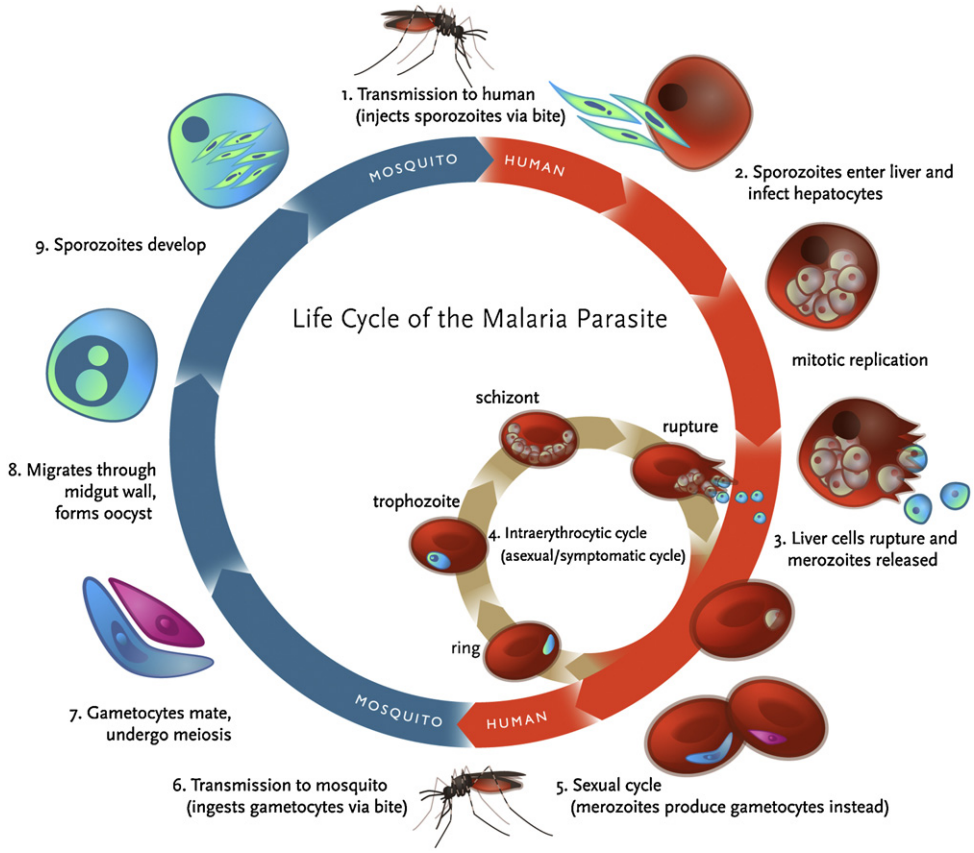

Fig. 1. Life cycle of the malaria parasite. Transmission of malaria occurs through a vector, the mosquito, that ingests gametocytes—the sexual form of the parasite—when feeding on an infected human. Gametocytes, which are both male and female, mate within the gut of the mosquito and undergo meiosis and then migrate through the midgut wall of the mosquito and form an oocyst [41], within which thousands of sporozoites develop [42]. These are then injected into a human during the next blood meal(s), where they rapidly make their way to the liver and infect hepatocytes and begin asexually (mitotically) replicating [43]. After a period of ca. 6–15 days, the liver schizonts rupture, releasing thousands of merozoites into the blood where they invade red blood cells. Over the next ca. 48 h, the parasite begins replicating mitotically, progressing through a set of stages (ring, trophozoite and schizont), and produces an average of 16 new daughter merozoites per schizont [44]. The schizonts then burst in near synchrony with other parasites, producing the characteristic fever cycle that embodies the clinical manifestations of the disease. With each replication, some of the merozoites, instead of producing new merozoites, develop into gametocytes, which can then infect susceptible mosquitoes, bringing the transmission cycle full circle.

infective gametocytes that are transmitted to a mosquito vector. Within the mosquito, the resistant mutation must not be lost during meiosis, and the mosquito must survive sporogony and transmit a viable infection to a new individual.

Despite its importance, owing to the sheer numbers of cases of malaria each year, many of which go unreported, it is unlikely that the first cases of resistant parasites being selected by drugs will be observed. Thus, the rate at which resistance emerges defines the time after a drug is introduced into a population until a specific proportion of clinical infections are caused by resistant parasites. This measure implicitly assumes both the initial drug selection of resistance mutations and their subsequent spread within a population, and is important, as this measure is a large determinant of when to switch first-line drugs [55,56]. The rate that resistance emerges depends in part on how resistance is encoded. Polygenically encoded resistance means that there is a reduced likelihood of selecting a resistant parasite, but it can also impact the emergence rate, depending on the relationship between the genes. If the effects of the mutations are additive, i.e. each subsequent mutation increases the tolerance or competitive ability of the parasite, then resistance will emerge faster than if every mutation is needed for resistance [57–59].

For a number of reasons, low and unstable transmission favours the faster emergence of resistance. First of all, the starting frequency of resistance is likely higher owing to higher biomass infections (i.e. a larger percentage of infections result in parasite blooms) [25]. This is due to lower levels of clinical immunity in these areas, thus each infection is more likely to result in a higher parasite load. Second, because immunity is less developed in low-transmission areas, mutant parasites are more likely to survive the host immune response and be subsequently spread [60,61]. Third, there is more drug treatment per parasite in low-transmission areas compared with high-transmission areas [21,23,61]. Since individuals in low-transmission areas are less likely to have immunity, they are more likely to become symptomatic and to treat each infection; it is thus more likely a resistant parasite will encounter drugs. Whilst there are more clinical infections overall in higher transmission areas, because individuals in these areas are constantly infected and re-infected, the per infection drug rate is actually lower. Fourth, competition within the host between drug-resistant and drug-sensitive parasites, which increases as transmission increases, plays a significant role in the spread of resistance [62]. In low-transmission areas, individuals tend to be concurrently infected by fewer genetically distinct parasites, so resistant parasites face lower competition within the host and an increased probability of transmission success. Higher multiplicity of infection also increases the probability that multilocus resistant genotypes will be broken up by the action of Mendelian segregation during the sexual stage of the life cycle [63]. Fifth, the lower drug use in clinically immune individuals creates a reservoir for drug-sensitive parasites [64]. The more clinically immune individuals there are, the larger this reservoir and the faster a drug-treated

individual will become re-infected with a drug-sensitive parasite. Thus, the higher the transmission rate, the shorter the period that individuals with resistant parasites harbour them without competition after drug treatment. This in turn abrogates the ability of a resistant parasite to spread and reduces the emergence rate of resistance.

Genetic and epidemiological evidence supports the role of low and unstable areas in generating resistance that then spreads to other areas. CQ resistance was first noted around 1960 in western Cambodia [4], a low-transmission area [65]. Resistance to CQ also arose independently in low-transmission areas of South America around the same time [4]. Genetic evidence suggests that resistance genes from these founder events (and from one additional site in Papua New Guinea) then spread to other regions, including sub-Saharan Africa [66–68].

SP resistance [69,70] as well as mefloquine resistance [71–73] also arose in low-transmission areas of Southeast Asia in the 1970s and 1990s, respectively. Thus, unsurprisingly, *P. falciparum* parasites with reduced in vivo susceptibility to artemisinin derivatives were first reported in 2008 in western Cambodia [50,74].

Resistance to CQ did not emerge as high-level resistance but instead was marked by recrudescence following treatment. Accumulation of additional mutations over time led to the ability to survive higher drug concentrations [4]. A similar stepwise accumulation of mutations has been associated with increased resistance to SP as well [75]. Emergence of resistance to artemisinin appears to be following a similar pattern, with the primary manifestation currently being described as delayed clearance [8,76]. In addition, whilst the described phenotype has not been associated with adverse clinical effects as yet [9], some isolates have exhibited increased tolerance [i.e. higher minimum inhibitory concentration (IC_{50}) values] of artemisinin [77], suggesting that the parasite is developing full-blown resistance, or the capacity to grow in the presence of drug, rather than just a dormancy stage that allows it to persist whilst the drug is present.

3.2. Spread between populations

Spread between populations is mediated by the movement of individuals and vectors. Whilst flight distances of some species of *Anopheles* can be significant [78], in general they only average around a kilometre from breeding sites [79]. Local-scale movement patterns of mosquitoes are due to searches for hosts and aquatic environments for oviposition [80], but long-distance dispersal can occur due to natural air movement or by riding along with humans [78]. Whilst there are reports of mosquitoes being swept on long-distance journeys of a couple of hundred miles by natural winds or at the fore of a front (see [78] for a review), the inherent brutality of the trip likely results in significant population loss, suggesting that this is not a major source of resistance spread. On the other hand, conveyance of mosquitoes in vehicles between countries and across oceans, and the subsequent spread of malaria, has been an issue since at least the 1800s [78].

Moreover, the short life span of the mosquito compared with the average length of infection in the human host (10 days vs. 200 days) also suggests that human movement plays the more important role in the spread of resistance. And evidence from the 1950s found that resistance to pyrimethamine spread rapidly from village to village along well-travelled trade routes [35]. As the speed of travel has increased, the rate that resistance can spread has also increased. For instance, there have been numerous instances of individuals living near airports in non-malarious countries who became sick with malaria despite not travelling to a malarious region [81].

The rate that drug resistance will spread between populations is a function of the frequency that resistance is introduced into the new population (see travel above) combined with the probability of the resistant parasite becoming established. The probability of resistance becoming established in a population is determined by the drug usage rate and the transmission rate. As the transmission rate increases, resistant parasites will face increased within-host competition, thus in order to become established they must be able to compete effectively within a host (i.e. they must have a lower biological cost of resistance compared with low/unstable transmission settings).

When drug resistance mutations first appear, they are generally associated with significant fitness costs that are ameliorated over time owing to compensatory mutations (e.g. CQ-resistant parasites have been shown to have compensatory mutations that reduce their fitness cost [37]). This further explains why it is more likely that resistant parasites will emerge in a low-transmission area and spread to a high-transmission area. However, because the transmission rate is higher, once parasites have acquired compensatory mutations allowing them to compete more effectively within the host, drug resistance will spread through the population much faster than in a low-transmission setting [64].

These theoretical results are consistent with the emergence of CQ resistance along the Thai–Cambodia border, a low-transmission area, in the late 1950s and its slow spread across the continent that took until the late 1970s. CQ resistance was not detected in Africa until the late 1970s, but then spread across the continent within a decade [66].

Initial reports of delayed clearance following treatment with artemisinin were from western Cambodia; however, more recently, delayed clearance of parasites following treatment with artemisinin has been described in western Thailand [9,82]. This new focus may be due to spread of resistance from western Cambodia or may be due to a separate *de novo* mutation. As there have also been reports of elevated artemisinin tolerance due to drug misuse [83], as well as possible artemisinin-related mutations in other areas [84], it is possible that artemisinin resistance may emerge in multiple locations. Thus, malaria control strategies must continue to target resistance evolution (i.e. drug selection) as well as strategies that slow the spread of resistance where it has arisen. Part of this must be through increased surveillance to identify new pockets of resistance as well as the rate that resistance already identified is spreading [85,86].

4. Controlling the spread of resistance

When resistance to CQ spread, countries shifted to SP as their nationally recommended treatment, and as resistance to SP spread, countries again shifted to artemisinin combination therapies (ACTs)—the World Health Organization (WHO)-recommended standard. As resistance to artemisinin class drugs has now been reported [9,50,74,87], signalling the possible emergence of resistance, developing and instituting control strategies to delay its spread is important for future malaria control efforts, particularly as it will likely be at least a decade before a new compound is capable of replacing the artemisinins [10]. Mathematical models of malaria have been important for studying the evolution of drug resistance as they provide a means of integrating and synthesising the results of studies done in many different academic disciplines to understand better how drug resistance emerges and spreads through and between populations. They are also useful for predicting the impact, feasibility and cost of control strategies.

Modelling of malaria has a long history dating back to the first descriptions of the life cycle of the disease by Sir Ronald Ross in 1908 [88]. Ross demonstrated mathematically that to eliminate malaria one only needed to reduce the number of anopheline mosquitoes below a critical threshold, not eliminate all mosquitoes. This simple finding, later confirmed and elaborated by Macdonald [89], formed

the basis for the eradication effort of the 1950s based on widespread application of insecticides, which was hugely successful in many parts of the world but failed due to operational constraints and lack of funding rather than any inherent flaws in the theory [90].

More recently, mathematical models of antimalarial drug resistance played a significant role in policy adoption of ACTs as the recommended first-line drugs for combating malaria. Although the theoretical benefit of using multiple drugs dates back to the early 20th century [91], mathematical models showed that not only would ACTs delay the evolution of resistance, but that they could also slow the spread of resistance once it had emerged [92,93]. These results helped underpin the decision to subsidise the widespread adoption of ACTs through the implementation of the Affordable Medicines Facility–malaria (AMFm).

Despite the useful role played by mathematical models in understanding the emergence and spread of drug resistance, there is still significant progress to be made, particularly in devising strategies to maintain the effectiveness of artemisinins. Mathematical models of antimalarial drug resistance have elucidated important aspects of resistance evolution, particularly regarding the role of transmission intensity, superinfection (i.e. multiple simultaneous malaria infections [94]) and clinical immunity [21,64,95–99]. However, these models also make some simplifying assumptions that limit their usefulness for predicting how control strategies will work. One of the primary issues is the mechanism by which resistance evolves. As noted above, the stepwise accumulation of resistance genes, or tolerance, is the presumed mechanism by which most antimalarials, including artemisinin, will acquire high-level resistance. Thus, factors such as the frequency of drug use and the number of individuals with subtherapeutic drug concentrations [59,100] play a crucial role in the spread of resistance and need to be considered in future models to understand better how the current artemisinin-tolerant phenotype may progress to high-level resistance, and what strategies would be most appropriate in delaying increasing tolerance as well as the spread of resistance.

A second issue is the role of within-host competition due to superinfection. Most epidemiological models of drug resistance have ignored the role of superinfection. The one exception [101] used a formulation that allowed co-existence of drug-resistant and drug-sensitive parasites under mathematical conditions that were not biologically realistic [62,95]. A consequence of not accounting for superinfection is that many models consistently predict that once resistance emerges it will rapidly spread to reach 100% [21,58,96,102–104], despite empirical evidence showing that resistance does not always fix [95]. Including complex within-host competition provides one means of explaining this result [95],[2] but a better understanding of how competition within the host impacts resistance is still needed. This is particularly important, because as CQ resistance spread through Africa, there were reports of increased clinical cases of malaria [106–108] and increased morbidity and mortality risks [109,110] that were not attributable to treatment failure. In other words, there may be a genetic linkage between drug resistance and increased morbidity and mortality. Since an increase in clinical disease would be associated with an increase in drug usage, this could generate a significant advantage for drug-resistant parasites in competition with drug-sensitive parasites. A clearer understanding of how superinfection and within-host competition affect the relationship between drug resistance and virulence is needed to better predict how this relationship may drive the spread of drug resistance.

Lastly, a more complete understanding of how heterogeneity in vector movement and biting patterns impacts the spread of resistance is needed. Studies have shown that mosquito biting patterns are highly heterogeneous owing to differences in size (i.e. children receive fewer bites than older individuals) [111], ecology [112–114], host infection status [115] and innate differential attraction [116–118]. In addition, movement patterns both by mosquitoes and hosts can localise biting intensity even further [119]. Estimates suggest that heterogeneity in biting results in ca. 20% of the individuals receiving 80% of the bites [27,120,121].

Heterogeneity in biting patterns can slow the rate at which resistance emerges in a population, as an individual infected with a resistant parasite is either going to be: (a) an individual with a high biting rate and thus a high concurrency of infections and thus have a high level of within-host competition; or (b) an individual who does not get bitten often. In either case, drug-selected resistant parasites are less likely to spread from these individuals, reducing the emergence rate [122]. However, once resistance becomes established (i.e. reaches an appreciable level above which it is unlikely to die off stochastically), resistance will spread faster because once resistant parasites start spreading from individuals with high biting rates, they will spread rapidly to everyone [122]. Models of control need to take account of these factors, particularly at the regional spatial scale that informs where resistance is likely to spread.

5. Summary

Whilst the holy grail for malaria eradication is the development of a cheap, effective vaccine, currently none exists and the most promising candidate to date has, at best, only a moderate impact on the incidence of malaria [123]. Despite this, significant progress against morbidity and mortality is possible with the tools we already have. However, the emergence of an artemisinin-resistant phenotype threatens one of the key components of elimination and eradication plans, and new control strategies are urgently needed. Whilst eliminating the parasite from the area where resistance has emerged has been suggested [124], the history of malaria elimination suggests that this is an unlikely scenario. Whilst more surveillance and epidemiological studies are needed to help determine the extent of the problem as well as the effectiveness of interventions [85,86], a better understanding of how drug resistance emerges and spreads is urgently needed to devise other control strategies. Determining what type of intervention to implement, as well as when and where and how much it will cost, requires a detailed understanding of how drug use, clinical immunity, superinfection and heterogeneous biting impact the spread of resistance. Addressing these issues will allow for a better, more robust understanding of how to control this urgent challenge.

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[2] Heterogeneous drug use due to clinical immunity can also generate co-existence [64,99]. Alternatively, co-existence can occur if the *de novo* emergence rate of resistance is high and resistance is continually introduced into the population [105], although this is not a particularly likely scenario for important first-line drugs.

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