Within-host competition and drug resistance in the human malaria parasite

Plasmodium falciparum

Mary Bushman1,2, Lindsay Morton2, Nancy Duah4, Neils Quashie4,5, Benjamin Abuaku4, Kwadwo A. Koram4, Pedro Rafael Dimbu6, Mateusz Plucinski2,3, Julie Gutman2, Peter Lyaruu7, S. Patrick Kachur2, Jacobus C. de Roode1,† and Venkatachalam Udhayakumar2,†

1Department of Biology, Emory University, Atlanta, GA 30322, USA
2Malaria Branch, Division of Parasitic Diseases and Malaria, Center for Global Health, and 3Epidemic Intelligence Service, Centers for Disease Control and Prevention, Atlanta, GA 30333 USA
4Epidemiology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana
5Centre for Tropical Clinical Pharmacology and Therapeutics, University of Ghana Medical School, Accra, Ghana
6National Malaria Control Program, Luanda, Angola
7Ifakara Health Institute, Dar es Salaam, Tanzania

MB, 0000-0003-0933-5053; ND, 0000-0001-8819-1793

Infections with the malaria parasite Plasmodium falciparum typically comprise multiple strains, especially in high-transmission areas where infectious mosquito bites occur frequently. However, little is known about the dynamics of mixed-strain infections, particularly whether strains sharing a host compete or grow independently. Competition between drug-sensitive and drug-resistant strains, if it occurs, could be a crucial determinant of the spread of resistance. We analysed 1341 P. falciparum infections in children from Angola, Ghana and Tanzania and found compelling evidence for competition in mixed-strain infections: overall parasite density did not increase with additional strains, and densities of individual chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) strains were reduced in the presence of competitors. We also found that CQR strains exhibited low densities compared with CQS strains (in the absence of chloroquine), which may underlie observed declines of chloroquine resistance in many countries following retirement of chloroquine as a first-line therapy. Our observations support a key role for within-host competition in the evolution of drug-resistant malaria. Malaria control and resistance-management efforts in high-transmission regions may be significantly aided or hindered by the effects of competition in mixed-strain infections. Consideration of within-host dynamics may spur development of novel strategies to minimize resistance while maximizing the benefits of control measures.

1. Introduction

The global spread of drug-resistant pathogens is a major threat to the control of infectious disease [1]. The malaria parasite Plasmodium falciparum has developed resistance to every type of antimalarial drug available. Resistance to the former first-line therapies chloroquine and sulfadoxine–pyrimethamine originated in low-transmission settings in Asia and South America [2,3], and then spread via gene flow, ultimately invading most of sub-Saharan Africa. If artemisinin resistance, which recently appeared in Southeast Asia [4,5], continues to follow the same pattern, the world may soon find itself without reliable antimalarial drugs.

One potentially crucial, but frequently overlooked determinant of the evolution of resistance is the occurrence of coinfections, in which different pathogens or different strains of a pathogen infect the same host [6,7]. In the case of P. falciparum, mixed-strain infections are very common, especially in high-transmission areas where infectious mosquito bites occur frequently [8,9].
Within-host competition between strains may result in competitive suppression of drug-resistant parasites [10,11]; theoretical models suggest that this could have dramatic consequences for the evolution of drug resistance [12].

On the one hand, within-host suppression of resistant strains (in untreated infections) could impede the spread of resistance, especially in high-transmission areas where mixed-strain infections are common [13–16]. On the other hand, competitive suppression could be alleviated by treatment, which removes drug-sensitive competitors, leading to increased growth and transmission of resistant parasites—a phenomenon known as competitive release [10,17–19]. This may have the opposite effect, accelerating the spread of drug resistance in high-transmission settings.

The best evidence to date for within-host competition and competitive release comes from mouse models of malaria. In the rodent malaria parasite Plasmodium chabaudi, mixed-strain infections exhibit intraspecific competition, in which growth of each strain is impaired by the others [11]. In this system, intra-host competition reduces the density and transmission of resistant parasites (competitive suppression) [10,20–22], and removal of drug-sensitive strains with antimalarial drug therapy results in competitive release of resistant strains [10,17,18,21,22]. However, evidence for within-host competition and suppression of drug-resistant strains in P. falciparum remains lacking. Important discrepancies between P. chabaudi and P. falciparum, such as order-of-magnitude differences in parasite density [23], preclude generalization from the rodent model to human malaria. It is possible, for example, that P. chabaudi might be limited primarily by erythrocytes, resulting in competition, and P. falciparum by strain-specific immune responses [24], allowing strains to grow independently.

There is currently only indirect evidence to support within-host competition in P. falciparum: one study observed longitudinal infection dynamics consistent with competition between different Plasmodium species [25], while another study observed an effect of treatment on placental parasitaemia consistent with competitive release of resistant parasites [26]. Here, we describe a study of naturally acquired P. falciparum infections in which we sought to determine whether within-host competition occurs in mixed-strain infections and whether drug-resistant parasites suffer competitive suppression.

2. Material and methods

(a) Focus on chloroquine resistance

We chose to focus on resistance to chloroquine, a largely retired antimalarial drug, for several reasons. The genetic basis of chloroquine resistance is straightforward and well characterized [27], and chloroquine-resistant genotypes are present at intermediate frequencies in many parts of the world, making it possible to obtain sufficient numbers of mixed-genotype infections. By contrast, resistance to another older drug, sulfadoxine–pyrimethamine, can be complex (multiple mutations in two different genes, with varying degrees of resistance [28]), while resistance to modern artemisinin-based drugs is still quite rare. We expect findings related to within-host competition to be generalizable to other forms of resistance (see Discussion).

(b) Sample collection and processing

Blood spots on filter papers were collected from children (ages 6–59 months in Ghana and Tanzania and 6–108 months in Angola) with symptomatic but uncomplicated microscopy-confirmed P. falciparum infection (electronic supplementary material, table S1). All samples were obtained from children enrolled in antimalarial therapeutic efficacy studies conducted in accordance with the guidelines of the World Health Organization [29]. Samples used in this study were collected prior to treatment. Samples from Ghana were collected between 1999 and 2010, samples from Tanzania in 2011, and samples from Angola in 2013 [30]. Electronic supplementary material, figure S1, shows study locations and malaria prevalence. All available samples from these clinical efficacy studies were included; sample sizes were not pre-determined to ensure statistical power. Investigators were blinded to patient clinical data in both experimental and analytical phases of the study. Parents or guardians gave informed consent on behalf of enrolled children.

DNA was extracted from blood spots using QIAamp DNA Mini Kit (Qiagen) and eluted in 150 μl buffer AE (Qiagen). For samples from Ghana, each blood spot was excised entirely, cut up into pieces and used for DNA extraction. The average blood spot was about 1 cm in diameter (approx. 50 μl), but there was some variation in size, and therefore in volume. This variation is expected to increase the variance in parasite density, but is not expected to result in bias because parasite density and chloroquine resistance genotype should not influence the size of blood spots. For samples from Tanzania and Angola, a consistent amount of each blood spot was used for DNA extraction: for Tanzania, three triangles (3 mm each side) were cut out of each spot, while for Angola, three 3 mm hole punches were used. The pieces excised from each blood spot were pooled for DNA extraction. The different volumes used for DNA extraction (approx. 50 μl for Ghana, 8.3 μl for Tanzania and 13 μl for Angola) were corrected for when calculating parasite densities.

(c) Quantification of drug-sensitive and drug-resistant parasites

We measured parasite densities by using extracted DNA in a quantitative real-time PCR assay which amplified codons 72–76 of the PfCRT gene on chromosome 7 (electronic supplementary material, tables S2 and S3). TaqMan probes (Life Technologies) were designed to bind to two different genotypes, one encoding the amino acid sequence C72V73I74E75T76, which is chloroquine-sensitive (CQS), and the other encoding C72V73I74D75T76 which is chloroquine-resistant (CQR). Previous studies have shown that these genotypes dominate most countries in Africa, including Ghana, Tanzania and Angola [31–33]. Owing to reports of a third genotype, S72V73M74N75T76, circulating in Angola [34], samples from Angola were analysed with a third probe designed to bind to SVMNT. Samples positive for SVMNT (n = 13) were excluded from analysis.

A full description of the methods for quantifying CQS and CQR can be found in the electronic supplementary material.

(d) Neutral microsatellite genotyping and analysis

To examine within-host diversity beyond PfCRT, six microsatellites (electronic supplementary material, table S4) were genotyped for all samples from Angola. These microsatellites consist of tandem repeats of 1–6 bp and exhibit considerable polymorphism in repeat number. Each microsatellite was amplified by PCR (electronic supplementary material, tables S5–S6) and analysed by fragment electrophoresis on a capillary sequencer (ABI 3130xl Genetic Analyzer) to determine amplicon size(s). Sequence data were read manually using GeneMapper (Life Technologies) by counting the number of distinct size variants of each microsatellite for each sample. MOI (multiplicity of infection based on microsatellites) is defined as the maximum number of variants identified at any of the six microsatellites...
for a given sample and provides a lower bound on the number of strains in the sample.

(e) Statistical analysis
All analyses were carried out in the statistical software R v. 3.1.2 [35]. Parasite densities were log_{10}-transformed to meet the assumptions of normality of errors and homogeneity of variance. Unless noted otherwise, significance of fixed effect terms was determined by removal of the term followed by model comparison using the command anova [36].

(i) Prevalence of mixed-genotype infections
To determine whether allele frequencies and the prevalence of mixed-genotype infections varied by country, we used χ^2 tests for equality of proportions. One χ^2 test compared the frequencies of single-genotype (CQS-only and CQR-only) and mixed-genotype (CQS+CQR) infections between the three countries, while another test compared the frequencies of CQS-only and CQR-only infections between the countries.

(ii) Factors affecting total parasite densities
We carried out two analyses to identify factors affecting total parasite densities.

In the first analysis, which included the samples from all three countries, we used a linear mixed-effects model (using the function lmer in the R package lme4 [37]) to analyse the effects of infection type (single- versus mixed-genotype) and country (Angola, Ghana or Tanzania), as well as site within country (see electronic supplementary material, table S1, for study sites within each country). Infection type and country were modelled as fixed effects while site was modelled as a random effect.

The second analysis was restricted to the samples from Angola, which were genotyped for microsatellite markers and for which patient age data were available. We analysed the effects of age, infection genotype (CQS, CQR or CQS+CQR) and MOI_{mix} on overall parasite density. We began with a linear mixed-effects model (using lmer) and included study site (Uige or Zaire) as a random effect; however, site did not explain significant variation in parasite density. We therefore analysed the effects of age, infection genotype and MOI_{mix} (and their two- and three-way interactions) using a linear model. Finally, we analysed the relationship between patient age and MOI_{mix} using a linear model, with patient age serving as the explanatory variable.

(iii) Within-host competition
If within-host competition occurs in mixed-genotype infections, then the density of an individual genotype should be reduced in mixed-genotype infections compared with single infections. We therefore analysed differences in parasite densities of each genotype (CQS or CQR) between single- and mixed-genotype infections.

As above, we carried out two analyses. First, using a linear mixed-effects model—again with lmer—we analysed the effects of infection type (single versus mixed), parasite genotype (CQS versus CQR), country (Angola, Ghana or Tanzania) and site within country (see electronic supplementary material, table S1) on parasite density. Again, site was treated as a random effect, while all other factors and their interactions were treated as fixed effects. The interaction between infection type, genotype and country was statistically significant; therefore, the significance of other terms could not be assessed by deletion and model comparison using anova. Instead, we re-ran the model using the lme function in the package nlme [38] to obtain approximate p-values for the remaining terms in the model.

The second analysis, which included patient age as an additional explanatory variable, was restricted to samples from Angola (the only samples for which age data were available). We started with a linear mixed effects model (using lmer), which included infection type (single versus mixed), parasite genotype (CQS versus CQR) and patient age—plus all two- and three-way interactions—as fixed effects, while site (Uige or Zaire) was included as a random effect. Because site did not explain significant variation in parasite density, we excluded it from the model, and analysed the effects of infection type, parasite genotype, age and their interactions using a linear model instead.

Finally, to check the assumption that mixed-genotype infections contained more strains than single-genotype infections, we used a Mann–Whitney U-test to compare MOI_{mix} between single- and mixed-genotype infections from Angola (the only samples for which MOI_{mix} data were available).

(iv) Fitness cost of resistance in mixed-genotype infections
A null model was developed to calculate the expected proportion CQR in mixed-genotype infections in the absence of a fitness cost of resistance. This model was based on population-level CQS and CQR allele frequencies and information about within-host strain diversity; a second version incorporated estimates of fitness costs of resistance based on PfCRT qPCR results (a full description of the model can be found in the electronic supplementary material). For each country, a one-sample, two-sided t-test was used to determine whether the observed proportions of CQR in mixed-genotype infections (logit-transformed to correct for non-normality) differed from the expected values.

(v) Temporal dynamics of resistance in Ghana
Patient samples from Ghana were collected between 1999 and 2010, while chloroquine was retired in favour of artemisinin-based therapies in early 2005. The prevalence of the CQR genotype in Ghana over time was analysed using a generalized linear mixed-effects model with binomial errors (using the function glm in the R package lme4). In this model, the frequency of CQR was modelled as a bivariate variable in which the numbers of CQR-positive and CQR-negative samples for each location and time point were column-bound (using the R command cbind). Time was included as a continuous fixed effect, and site (Hoheo, Navrongo, Sunyani or Yendi) as a random effect. We also included data point (each measurement of CQR prevalence for a given site and time point being one data point) as a random effect to account for overdispersion of the data [39].

3. Results
Using quantitative real-time PCR, we were able to accurately determine the density of CQS and CQR P. falciparum in patient samples (for full description of qPCR methods see electronic supplementary material, figures S3–S4). Infections with either CQS or CQR were classified as single-genotype and infections with both were classified as mixed-genotype.

(a) Variation in PfCRT genotype frequencies
The three countries differed significantly in the relative frequencies of the CQS and CQR alleles (χ^2 = 326.97, p < 0.0001), but not in the relative frequencies of single-genotype and mixed-genotype infections (χ^2 = 2.89, p = 0.24; electronic supplementary material, figure S1, table S7).

Infection genotype frequencies (CQS, CQR and CQS+CQR) did not vary significantly between the sites in Angola (χ^2 = 4.452, p = 0.108). There was significant variation among the sites in Ghana (χ^2 = 20.5, p = 0.002); however, this was probably the result of different sampling schedules for the various sites, combined with longitudinal change in
The total parasite density of mixed-genotype infections was roughly the same as, or lower than, that of single-genotype infections (figure 1). The linear mixed-effects model that analysed data from all three countries indicated a significant three-way interaction between infection type (single- versus mixed-genotype), parasite genotype (CQS versus CQR) and country ($\chi^2 = 6.18, p = 0.045$). This interaction is apparent in figure 3: although both CQS and CQR strains had much lower density in mixed- than single-genotype infections (approximated $p$-value $< 0.0001$), and CQR generally had lower density than CQS in both single and mixed infections (approximated $p$-value $< 0.0001$), in Tanzania, CQR had similar density to CQS in single infections, but much lower density in mixed infections. This suggests that, in Tanzania, CQR parasites were competitively suppressed to a greater degree than in the other two countries, and to a larger extent than CQS.

We used a linear model to examine the effects of patient age, infection type (single versus mixed) and genotype on parasite density in the samples from Angola (the only samples for which age data were available). This analysis showed that, although parasite density did decrease with age (F$_{1,444} = 11.3$, $p = 0.0009$), the significant effect of infection type was retained ($F_{1,444} = 65.8, p < 0.0001$), as was the effect of genotype ($F_{1,444} = 9.67, p = 0.002$). None of the two- or three-way interaction terms in the model were significant. Thus, irrespective of age, both parasite genotypes had lower densities in mixed-genotype infections than they did in single-genotype infections; CQS parasites also consistently had higher densities than CQR parasites.

(c) Within-host competition

As mean total parasite density of mixed-genotype infections was less than or equal to that of single-genotype infections, it follows that each genotype should have lower density in mixed infections than in single infections. This is clearly supported by the data: for both CQS and CQR, mean parasite density in mixed-genotype infections was reduced by over 50% compared with single-genotype infections (figure 3). The linear mixed-effects model that analysed data from all three countries indicated a significant three-way interaction between infection type (single- versus mixed-genotype), parasite genotype (CQS versus CQR) and country ($\chi^2 = 6.18, p = 0.045$). This interaction manifests as reduced parasite density. An alternative explanation is that both MOI$_{\text{mix}}$ and acquired immunity increase with exposure, such that, as more strains are acquired, immunity becomes more effective at reducing parasite density. As our linear model showed, total parasite density did decrease with age (a reasonable proxy for exposure); however, an additional linear model showed that MOI$_{\text{mix}}$ was not significantly associated with age ($F_{1,380} = 0.0061, p = 0.94$; electronic supplementary material, figure S2); these results do not support the alternative explanation.

(d) Fitness cost of resistance

This study also provided an opportunity to explore the fitness of CQS and CQR parasites in vivo. Epidemiological evidence suggests that chloroquine resistance carries a fitness cost in the absence of chloroquine, but the underlying population dynamics are unknown [40]. As mentioned above, CQR parasites consistently had lower densities than CQS parasites. This suggests a fitness cost of chloroquine resistance which manifests as reduced parasite density.

Comparing parasite densities of two genotypes in mixed infections can be complicated by infections harbouring multiple strains of a given genotype; in such cases, a one-to-one ratio of the two genotypes is no longer an adequate null hypothesis. We therefore compared the frequencies of CQS and CQR parasites in mixed-genotype infections against
predictions from a null model which takes these complications into account (see the electronic supplementary material). At the within-host level, CQR was proportionally less abundant than predicted by the null model in all three countries (figure 4). In Tanzania, the average proportion CQR was even less than predicted by a modified null model incorporating the fitness differences observed in single infections ($p = 0.0008$; figure 4), suggesting that in some but not all cases, the fitness cost of resistance may be amplified by competition. This result is in agreement with the finding of a significant interaction effect of infection type, genotype and country on parasite density, which suggested disproportionate competitive suppression of CQR parasites in Tanzania.

(e) Temporal dynamics of resistance in Ghana

Samples from Ghana were collected between 1999 and 2010; chloroquine was the first-line treatment for uncomplicated malaria in Ghana until 2005, when it was retired in favour of artesunate-based combination therapy. Following this change, the proportion of infections harbouring CQR parasites rapidly decreased throughout the country ($\chi^2 = 10.6, p = 0.001$). This decline, along with similar declines observed elsewhere [42,43], make it clear that CQR parasites are selected against in the absence of...
the spread of resistance. This may help to explain the puzzling observations regarding the fitness costs of resistance are specific to chloroquine; our results suggest that the selective disadvantage is probably due to, at least in part, the fact that CQR parasites do not reach densities as high as those achieved by their CQS counterparts.

4. Discussion

The findings presented here provide compelling evidence for within-host competition in *P. falciparum*. The observation that total parasite density is roughly constant with respect to the number of strains in a host suggests that different strains compete for a shared niche [8]. The mechanism responsible for competition is unknown, but possibilities include resource limitation, strain-transcending immunity and direct interference between parasites [44–47].

The important corollary of within-host competition is the finding that, when hosts are co-infected with CQS and CQR parasites, the different genotypes are both competitively suppressed. Such competition is particularly important for drug-resistant parasites. Resistant parasites, when first emerging, are rare, and therefore proportionally more likely than drug-sensitive strains to be found in mixed-genotype infections (meaning there will be few hosts with purely resistant infections, some with mixed-genotype infections, and many with purely drug-sensitive infections) [48]. Therefore, newly emerged resistant strains may suffer more competitive suppression overall than sensitive strains. In addition, antimalarial therapy, by clearing drug-sensitive parasites from mixed infections, may result in competitive release of resistant strains.

Competitive suppression probably inhibits transmission of resistant parasites to new hosts. Although our study did not examine transmission or transmission potential, previous studies have found parasite density to be positively correlated with gametocytaemia (density of transmission stages) and infectivity to mosquitoes [49,50], and competitive suppression has also been shown to reduce gametocytaemia and transmission success in *Plasmodium chabaudi* [10,11,21]. Therefore, in high-transmission settings, where mixed-strain infections occur frequently [8], within-host competition may impede the spread of resistance. This may help to explain the puzzling fact that resistance to antimalarial drugs emerges readily in low-transmission areas, but not in high-transmission settings [2,3].

In addition to the findings related to within-host competition, we made several observations regarding the fitness of CQR parasites. CQR parasites were less abundant than their CQS counterparts in both single- and mixed-genotype infections, suggesting a fitness cost of resistance which manifests, at least in part, as impaired growth in the erythrocytic stage of infection. These findings help to explain the rapid decline of CQ resistance following the termination of chloroquine as a first-line drug in Ghana and elsewhere [42,43]. Interestingly, we found only limited evidence in support of the idea that fitness costs of resistance will be amplified by competition, as has often been assumed (e.g. [13,51]); evidence for disproportionate competitive suppression of resistant parasites was observed only in Tanzania. One possible explanation is that the fitness difference between CQS and CQR strains is larger in Tanzania due to the genetic background(s) of one or both genotypes (epistatic effects on the fitness cost of resistance) [52,53].

Our findings relating to the fitness costs of resistance will help to develop appropriate models that can project the decline of resistance following retirement of a failing drug. Information on the fitness effects of resistance to various antimalarial drugs is likely to be extremely useful for prevention of multi-drug resistance, drug cycling and, potentially, strategies that exploit fitness costs to combat the spread of resistant strains [54]. We emphasize, however, that our observations regarding the fitness cost of resistance are specific to chloroquine. The existence, magnitude and manifestation of fitness costs will depend on the drug. Our observation that CQR parasites are competitively suppressed, however, should readily generalize to other forms of resistance. This is because competitive suppression is not the result of fitness differences, but rather the inevitable result when two populations compete for a shared niche.

Within-host competition (and fitness costs, at least in the case of chloroquine) has probably played an important role in the evolution of resistance. In particular, competitive suppression may have slowed the spread of drug-resistant...
strains in sub-Saharan Africa, where intense transmission means that mixed-strain infections, and therefore within-host competition, are prevalent. It should be noted, however, that the effects of competition on resistant strains probably depend strongly on the amount of antimalarial drug use. Competitive suppression of resistant strains can only occur where drug-sensitive competitors are present (i.e. in untreated infections). Treatment may reverse suppression of resistant parasites by killing off the competitors, with the resistant strain not only surviving but expanding, increasing its transmission potential; this is known as competitive release [10]. The balance between competitive suppression and competitive release determines the rate at which resistance will spread. When relatively few infections are treated, as is probably the case in most settings due to high prevalence of asymptomatic infections [55], competitive suppression will dominate, inhibiting the spread of resistance in high-transmission settings [13]. At higher rates of treatment, however, competitive release may have the opposite effect, causing resistance to spread more rapidly in a high-transmission setting than in a low-transmission area under similar drug pressure [14–16].

The risk of widespread competitive release suggests that broad application of antimalarial drugs, such as in mass drug administration, should be approached with caution in high-transmission settings, perhaps by employing aggressive transmission control measures in conjunction with mass drug administration in order to reduce opportunities for transmission of resistant strains. It has been suggested that competitive release of resistant parasites could be avoided by subcurative drug treatment that eliminates enough sensitive parasites to alleviate symptoms, but leaves enough to maintain competitive suppression of the resistant strain [51]. Such approaches have been demonstrated to work for the rodent malaria parasite Plasmodium chabaudi [17,21,56], but whether they can be safely and effectively applied to P. falciparum in humans is an open question [6,57,58].

In summary, our findings support an important role for within-host competition in the evolution of drug resistance in P. falciparum. These findings will improve modelling and practical management of resistance to maximize the useful lifespan of existing antimalarial drugs.

Ethics. The efficacy studies in Ghana were approved by the IRB of Noguchi Memorial Institute for Medical Research, University of Ghana. CDC IRB approved the studies in Angola and Tanzania, which were also approved, respectively, by the National Malaria Control Program in Angola and the National Institute for Medical Research in Tanzania.


Data accessibility. Data are available through Dryad (http://dx.doi.org/10.5061/dryad.qb814). R code for analysis can be found on GitHub at https://github.com/falciparum/Competition-Analysis.

Competing interests. The authors declare that they have no competing interests.

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References
