Complexity of *Plasmodium falciparum* Infections and Antimalarial Drug Efficacy at 7 Sites in Uganda

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Malaria infections in Africa frequently include multiple parasite strains. We examined the relationship between the number of infecting *Plasmodium falciparum* strains and the responses to 3 different combination therapies in 3072 patients with uncomplicated malaria at 7 sites in Uganda. Patients infected with ≥3 strains had almost 3 times the odds of treatment failure (odds ratio, 2.93 [95% confidence interval, 2.51–3.43]; $P < .001$), compared with those infected with 1 or 2 strains. Our data suggest that efforts to reduce the complexity of infection in highly endemic areas through the use of intermittent presumptive therapy, improved case management, and reduction in transmission intensity may improve the efficacy of antimalarial therapies.

The response to antimalarial therapy involves a complex interaction between the infecting parasite strains, host immune response, and drug pharmacokinetics and pharmacodynamics [1]. A better understanding of the factors involved in the response to antimalarial therapy should improve efforts to control malaria. In malaria-endemic areas of Africa, *Plasmodium falciparum* infections exhibit a wide range of antigenic diversity, and individuals are commonly infected with multiple strains [2]. Little is known about the relationship between the num-

ber of strains infecting a patient and the response to antimalarial therapy. A higher number of strains could increase the probability of there being parasite clones containing resistance-conferring mutations and/or evading host immune responses, leading to an increased risk of treatment failure. In the present study, we examined the independent effect of complexity of infection (number of infecting strains) on antimalarial drug efficacy across a range of treatment regimens and transmission intensities in Uganda.

Patients, materials, and methods. Patients were enrolled in clinical trials of antimalarial drug efficacy at 7 sites in Uganda that have varying transmission intensity, by use of a common protocol. Details of the clinical trials have been published elsewhere [3, 4]. Briefly, patients aged 6 months or older with uncomplicated falciparum malaria and parasite densities of 2000–200,000 parasites/μL were randomized to receive chloroquine (CQ) plus sulfadoxine-pyrimethamine (SP) or amodiaquine (AQ) plus SP at all 7 sites, with the addition of an AQ plus artesunate (AS) arm at 4 sites. Patients were given directly observed therapy and were followed for 28 days. Treatment outcomes were classified, in accordance with the 2003 World Health Organization guidelines for areas of intense transmission, as adequate clinical and parasitological response, early treatment failure, late clinical failure, or late parasitological failure [5].

Blood smears were stained with 2% Giemsa for 30 min. Parasite densities were determined from thick blood smears by counting the number of asexual parasites per 200 white blood cells (WBCs) (or per 500 WBCs, if the count was <10 parasites/200 WBCs), under the assumption of a WBC count of 8000 cells/μL. A smear was considered to be negative if no parasites were seen after review of 100 high-powered fields. Molecular genotyping, based on polymorphisms in the merozoite surface protein–2 *(msp2)* gene, was used to determine pretreatment complexity of infection and to distinguish recrudescence from new infection for all episodes of recurrent parasitemia (late clinical failure or late parasitological failure) identified on days 4–28. Briefly, the polymorphic block 3 region of *msp2* was amplified by nested polymerase chain reaction (PCR) using primers exactly as described elsewhere [6]. Nested PCR products were analyzed by electrophoresis using 2% agarose. Gel images were digitized and molecular weights assigned to bands by use of GelCompar II software (Applied Maths). Each band assigned a molecular weight was considered to be an individual strain. Strains were considered to be the same if their molecular weights were within 10 bp of each other. For this analysis,

treatment failure was defined as all early treatment failures (presenting on days 1–3) and episodes of recurrent parasitemia in which any parasite strain present on the day of failure was also present in pretreatment samples.

The mean complexity of infection across the sites was compared using a t test. Risks of treatment failure were estimated using the Kaplan-Meier product limit formula with censoring for new infections. Associations between complexity of infection and treatment failure were assessed using a logistic regression model controlling for age and pretreatment parasite density. The complexity of infection was analyzed as a dichotomous variable, using the median value as a cutoff (1 or 2 strains vs. ⩾3 strains), and as an ordinal variable. All data were double-entered and verified using EpiInfo (version 6.04; Centers for Disease Control and Prevention) and analyzed using Stata (version 8.0; Stata Corporation).

Results. Of the 3138 subjects who were enrolled in the clinical trials and had measurable treatment outcomes, 3072 (98%) had complete genotyping data and were included in this analysis (table 1). The mean complexity of infection was significantly higher ($P < .001$) in the 2 sites with the highest transmission intensity than in the other sites. There was an inverse relationship between complexity of infection and pretreatment parasite density (log transformed) at the 2 sites with the highest transmission intensity (correlation coefficient, -0.15; $P < .001$). No relationship was observed between complexity of infection and parasite density at the sites with lower transmission intensity. The risks of treatment failure at the 7 sites ranged from 57% to 84% for CQ plus SP, from 20% to 47% for AQ plus SP, and from 9% to 56% for AQ plus AS. Patients infected with ⩾3 parasite strains had almost 3 times the odds of treatment failure, compared with patients infected with 1 or 2 strains, after age and pretreatment parasite density were controlled for (odds ratio [OR], 2.93 [95% confidence interval {CI}, 2.51–3.43]; $P < .001$). The odds of treatment failure were consistently higher (OR, >1) in patients infected with ⩾3 strains than in patients infected with 1 or 2 strains, for all 3 treatment arms across all 7 sites (figure 1). The association between a higher complexity of infection and an increased risk of treatment failure remained significant (OR, 2.36 [95% CI, 2.02–2.75]; $P < .001$) when we repeated our analysis using outcomes unadjusted by genotyping. Examining complexity of infection as an ordinal variable resulted in a significant increase in the odds of treatment failure (OR, 1.40 [95% CI, 1.34–1.46]; $P < .001$) for each unit increase in the number of infecting strains. When the estimated risks of treatment failure are examined, patients infected with ⩾3 parasite strains had a significantly higher risk of treatment failure than did those infected with 1 or 2 strains, in the CQ plus SP (79% vs. 55%; $P < .001$), AQ plus SP (44% vs. 19%; $P < .001$), and AQ plus AS (43% vs. 14%; $P < .001$) treatment groups.

Discussion. Previous research on the response to antimalarial therapy has primarily focused on mechanisms by which parasites mediate drug resistance [7]. However, other factors, such as age (a surrogate for acquired immunity) and parasite density, are also associated with the response to antimalarial treatment [8]. To our knowledge, this is the first study to demonstrate that a higher complexity of infection is independently associated with a greater risk of treatment failure. We found this association to hold across a wide range of transmission intensities and with 3 different treatment regimens.

A potential bias in our study is the genotyping method used to distinguish recrudescence from new infection and to determine the complexity of each infection. We used a single genetic marker to define complexity of infection, because, with the use of multiple markers, it is only possible to define the minimum complexity of infection. However, this method may have underestimated infection complexity, because of the limited diversity of *msp2* alleles in our population and the failure of PCR to detect minority strains. In addition, we cannot rule out the possibility that newly infecting parasite strains could have been misclassified as recrudescent strains. Therefore, samples with a higher complexity of infection may have been more likely to be misclassified as treatment failures. However, this possibility is unlikely to explain our findings, since there was a high diversity of *msp2* genotypes in our population, and the probability of 2 independent strains having the same genotype was ⩽5% across all sites (table 1). In addition, the association between

Table 1. Characteristics of study subjects, stratified by study site.

Characteristic	Kanungu	Mubende	Kyenjojo	Jinja	Arua	Tororo	Apac
Entomological inoculation rate, infectious bites/person/year	7	3	8	6	393	591	1564
Patients with treatment outcomes, no.	357	351	349	514	522	515	530
Patients included in molecular analyses,[a] no. (%)	353 (99)	344 (98)	339 (97)	497 (97)	505 (97)	512 (99)	522 (98)
Complexity of infection, mean (SD)	2.64 (1.49)	3.01 (1.61)	2.79 (1.65)	2.63 (1.59)	2.16 (1.04)	4.44 (2.02)	4.21 (1.82)
Age, median (range), years	1.9 (0.5–65)	1.8 (0.5–52)	4.3 (0.5–65)	3.8 (0.5–65)	1.5 (0.5–30)	1.3 (0.5–56)	1.8 (0.5–47)
Parasite density, geometric mean, parasites/μL	24,238	20,493	26,059	35,665	23,586	18,345	11,627
Strain diversity[b]	4.8	4.2	4.8	4.9	4.9	5.0	5.0

[a] Subjects with data for pretreatment complexity of infection (no. of infecting strains) and treatment outcomes adjusted by genotyping.
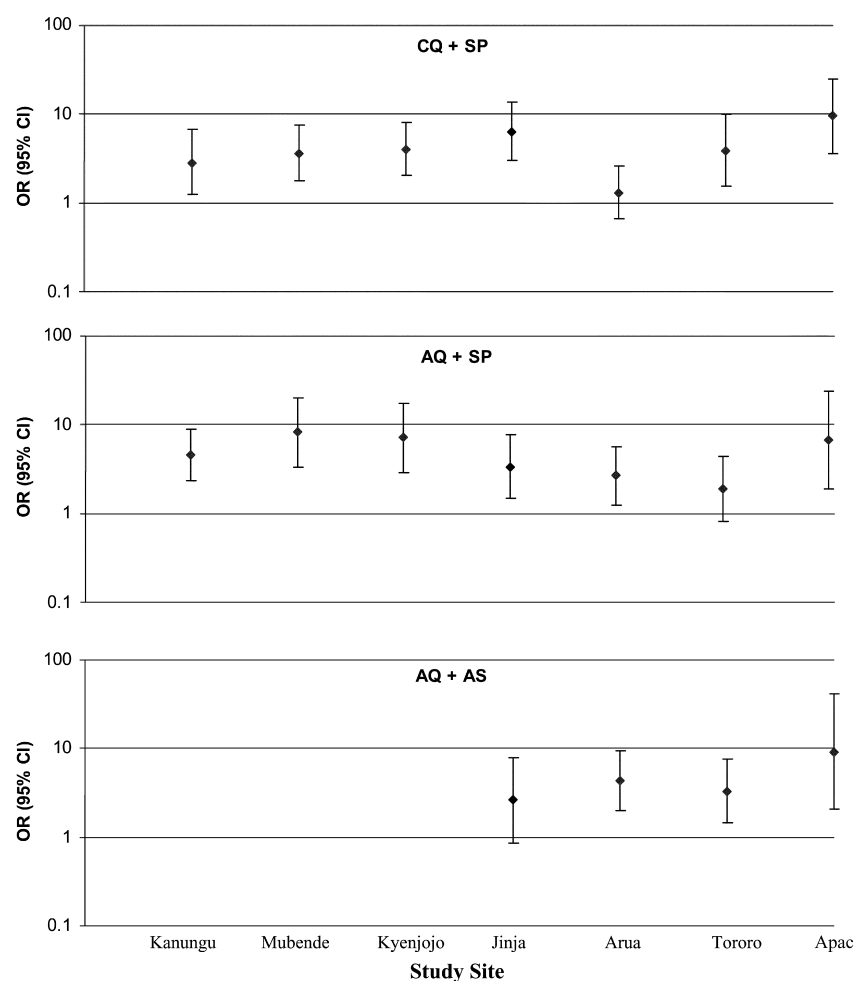[b] Mean probability that 2 independent strains were the same.

Figure 1. Odds ratios (ORs) and 95% confidence intervals (CIs) of treatment failure for infections with ⩾3 strains vs. 1 or 2 strains of *Plasmodium falciparum,* after controlling for age and pretreatment parasite density across 7 study sites in the chloroquine plus sulfadoxine-pyrimethamine (CQ + SP), amodiaquine plus sulfadoxine-pyrimethamine (AQ + SP), and amodiaquine plus artesunate (AQ + AS) treatment arms.

a higher complexity of infection and an increased risk of treatment failure was significant even when we considered outcomes that were adjusted to distinguish recrudescence from new infection, as well as those that were unadjusted.

These findings add new insight to our understanding of the epidemiology of antimalarial drug resistance in highly endemic areas. A higher complexity of infection would be expected to increase the probability of harboring a strain with resistance-conferring mutations and/or with the ability to evade the host immune response. Therefore, decreasing the complexity of infection should improve antimalarial treatment responses independently of other parasite and host factors. Multiple approaches may decrease the complexity of infections, thereby potentially improving outcomes. First, improving therapeutic efficacy should reduce the number of parasite strains surviving treatment, which would reduce the complexity of subsequent infections. Second, intermittent presumptive therapy will likely reduce the complexity of in-

fections that eventually progress to symptomatic disease. Third, vaccines with even partial efficacy have been shown to reduce the complexity of infection in some [9, 10], but not all, studies [11]. Finally, vector control measures aimed at reducing transmission intensity will likely reduce the complexity of infection, since a correlation between transmission intensity and complexity has been observed in this and other [2] studies.

Our data suggest that, at least in the setting of significant levels of drug resistance, measures that decrease the complexity of malaria infections will lead to improved efficacy of antimalarial therapies. The results thus support the use of a multifaceted approach to malaria control that provides both effective therapy and measures to reduce malaria transmission and complexity of infection. In addition, our findings suggest that a comparison of results from antimalarial drug efficacy studies conducted at different sites should, ideally, adjust for pretreatment complexity of infection.

Acknowledgments

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