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Selection of *Plasmodium falciparum pfmdr1* Alleles following Therapy with Artemether-Lumefantrine in an Area of Uganda where Malaria Is Highly Endemic

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Polymorphisms in the *Plasmodium falciparum pfmdr1* gene were assayed in pretreatment samples and in samples from patients reinfected following therapy with artemether-lumefantrine. The *pfmdr1* alleles 86N, 184F, and 1246D significantly increased in prevalence after treatment. All samples had a single *pfmdr1* copy. Treatment with artemether-lumefantrine selects for polymorphisms that may alter antimalarial drug response.

Due to widespread resistance of *Plasmodium falciparum* to drugs such as chloroquine and sulfadoxine-pyrimethamine, artemisinin combination therapy (ACT) is currently advocated in Africa as a means of improving treatment efficacy and slowing the spread of resistance. The rationale behind ACT is to rapidly reduce the parasite burden with a short-acting artemisinin compound, leaving a longer-acting partner drug to eliminate the remaining parasites, thus reducing the chance of selection of drug-resistant parasites. In Southeast Asia, where the risk of reinfection following therapy is low, the combination of artesunate and mefloquine has been effective at slowing the spread of resistance (10). It is unclear whether ACT will be as successful in preventing the selection of resistant parasites in Africa, where parasite transmission rates are generally much higher. In a recent study from Tanzania, treatment with the widely advocated ACT artemether-lumefantrine (AL) was associated with selection of newly infecting parasites containing the *pfmdr1* 86N allele (16), which has been associated with decreased in vitro sensitivity to artemisinins and lumefantrine (4, 5).

We recently completed a clinical trial including AL at a rural site in Uganda with extremely high transmission intensity (1). Briefly, children aged 1 to 10 years with uncomplicated falciparum malaria received directly observed therapy and were followed for 28 days. Molecular genotyping techniques were used to distinguish recrudescent from new infections for all patients failing therapy after day 3. Briefly, filter paper blood samples collected on the day of enrollment and the day of failure (late clinical failure or late parasitological failure) were analyzed for polymorphisms in merozoite surface protein 1 (MSP-1) and MSP-2 using nested PCR as previously described (2). An outcome was defined as recrudescence if all MSP-1 and MSP-2 gene alleles present at the time of failure were present at the time of treatment initiation and as a new infection otherwise. To test the hypothesis that AL selects for polymorphisms in the *pfmdr1* gene, we compared the prevalences of key alleles and *pfmdr1* copy numbers between pretreatment isolates and new isolates following treatment with AL.

Polymorphisms studied were *pfmdr1* N86Y, Y184F, N1042D, and D1246Y. Alleles were identified using nested PCR and restriction fragment length polymorphism methods, and copy number was assessed by TaqMan quantitative PCR as previously described (4, 12). Single (3D7)- and three-copy (W2mef) standards were used as positive controls. Reactions were done in quadruplicate and repeated for a change in cycle threshold standard error >0.3 or a copy number between 1.3 and 1.6. A copy number <1.5 was considered single copy, and one ≥1.5 was considered multiple copy (12). Data were entered and verified using SPSS and analyzed using STATA 8.0. Categorical variables were compared using the chi-squared or Fisher's exact test as appropriate. Concordance between polymorphisms in the same sample was assessed using the kappa statistic. A *P* value <0.05 was considered statistically significant.

A total of 202 patients were treated with AL and had complete follow-up. Pretreatment samples were successfully assayed for *pfmdr1* polymorphisms of interest for 201 (99%). A total of 102 patients had recurrent parasitemia within 28 days of treatment. One patient classified as an early treatment failure on day 1; a single patient with a recrudescence (on day 27) and three patients with unsuccessful genotyping were excluded from the analysis. The remaining 97 samples from patients with new infections (identified 19 to 28 days after therapy) were successfully assayed for *pfmdr1* polymorphisms of interest. The prevalence of samples containing only the *pfmdr1* 86N allele increased significantly from 8% in pretreatment samples to 43% in samples from patients with new infections (relative risk [RR] = 5.2, 95% confidence interval [CI], 3.1 to 8.5; *P* < 0.0001). The prevalence of samples containing only the *pfmdr1* 184F allele increased significantly from 4% in pretreatment samples to 14% in samples from patients with new infections (RR = 3.6, 95% CI, 1.6 to 8.4; *P* = 0.001). The prevalence of samples containing only the *pfmdr1* 1246D allele increased significantly from 12% in pretreatment samples to 42% in samples from patients with new infections (RR = 3.4, 95% CI, 2.2 to 5.2; *P* < 0.0001) (Fig. 1). There was significant concordance between the *pfmdr1* 86N and 1246D alleles in both

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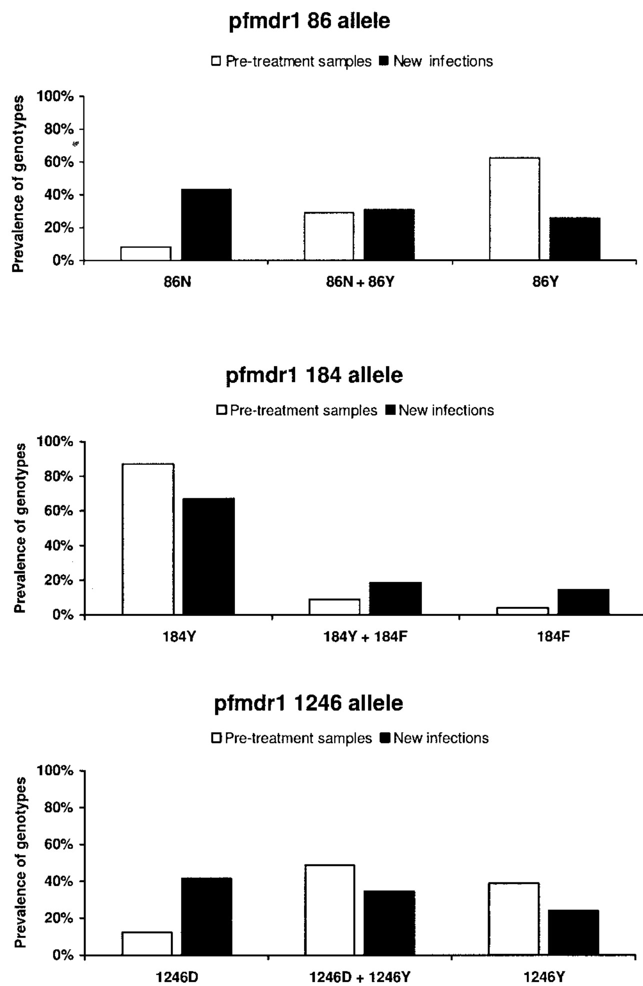

FIG. 1. Prevalence of *pfmdr1* alleles in pretreatment samples and samples from newly infected patients following therapy with artemether-lumefantrine. Alleles typically classified as wild type are on the left, mixed infections are in the middle, and those classified as mutant are on the right.

pretreatment (kappa statistic = 0.63, $P < 0.0001$) and posttreatment (kappa statistic = 0.64, $P < 0.0001$) samples. However, lack of change in the kappa statistic argues for independent selection of these two alleles. None of samples assayed contained the *pfmdr1* 1042D allele. Results for *pfmdr1* copy number were successfully obtained from 195 of 202 pretreatment samples and 86 of 97 new infections; all isolates had a single copy of the gene.

Our results are consistent with those from a recent study in Tanzania (16), where the prevalence of the *pfmdr1* 86N allele increased from 15% in pretreatment samples to 41% in posttreatment new infections. In addition, we identified selection for two other alleles, 184F and 1246D. An important distinction between these two studies is that in Tanzania (8) the risk of new infection following therapy with AL was only 5% after 28 days compared to almost 50% in our study. In absolute terms this translates to a 2% risk of new infections containing the *pfmdr1* 86N allele for all patients treated with AL in Tanzania versus a 21% risk at our high-transmission study site. Thus, polymorphisms in the *pfmdr1* gene are highly selected in parasites emerging during the elimination phase of lumefantrine (half-life 3 to 6 days), and the proportion of treated patients selecting for such parasites increases as transmission intensity increases. However, it is not clear what effect drug selective pressure may have at the population level, where one must also consider the overall proportion of circulating parasites that are exposed to subtherapeutic drug levels (6).

Two of the polymorphisms we observed have been previously associated with altered parasite drug sensitivity in vitro. The *pfmdr1* 86N allele was associated with lower in vitro susceptibility to lumefantrine, halofantrine, and mefloquine (4, 11, 12). The *pfmdr1* 1246D allele was associated with decreased sensitivity to mefloquine and halofantrine in two transfection studies (14, 15). Interestingly, these alleles have been associated with an increased sensitivity to chloroquine (CQ) and are generally considered the wild-type alleles (4, 14). The most likely explanation for our findings is that extended exposure to lumefantrine selected for new infections with parasites with the observed polymorphisms. An alternative explanation is that the higher prevalence of the wild-type alleles following therapy with AL was due to improved fitness of parasites containing these alleles after removal of the selective pressure of CQ (7). It should be noted that we used a highly specific definition for recrudescence and cannot rule out the possibility that some of the outcomes classified as new infections were actually due to recrudescences. A significant number of recrudescences occurring following AL therapy would be of concern, especially if they were associated with *pfmdr1* polymorphisms from pretreatment samples. Little association with drug susceptibility has previously been seen with polymorphisms at the *pfmdr1* 184 locus, but our results suggest that this allele may also play a role in mediating resistance to some antimalarials. Increase in *pfmdr1* copy number has been shown to be an important cause of mefloquine resistance (3, 12) and a predictor of treatment failure following four-dose AL therapy (13). In this study we found no evidence of selection of newly infecting parasites with increased *pfmdr1* copy number following AL therapy. However, increases in *pfmdr1* copy number have been demonstrated elsewhere in Africa despite limited drug pressure (17), and preliminary data suggest that increased *pfmdr1* copy number may arise more rapidly than point mutations in natural populations (9).

We identified independent selection of three polymorphisms in the *pfmdr1* gene following administration of AL in a region of Africa where malaria is highly endemic. These polymorphisms were not associated with clinical treatment failure but are evidence for the ability of this drug combination to drive selection of parasites toward resistant phenotypes. Uganda and several other African countries are currently changing to AL as the first-line treatment for malaria. It will be important to monitor the efficacy of AL closely, especially in high-transmission areas where drug selection pressure may be the greatest. The possible role of the *pfmdr1* 86N, 184F, and 1246D alleles and *pfmdr1* copy number as molecular markers of AL resistance should be further investigated.

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