# BRIEF REPORT

# Changing Antimalarial Drug Resistance Patterns Identified by Surveillance at Three Sites in Uganda

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We assessed *Plasmodium falciparum* drug resistance markers in parasites collected in 2012, 2013, and 2015 at 3 sites in Uganda. The prevalence and frequency of parasites with mutations in putative transporters previously associated with resistance to aminoquinolines, but increased sensitivity to lumefantrine (*pfcrt* 76T; *pfmdr1* 86Y and 1246Y), decreased markedly at all sites. Antifolate resistance mutations were common, with apparent emergence of mutations (*pfdhfr* 164L; *pfdhps* 581G) associated with high-level resistance. K13 mutations linked to artemisinin resistance were uncommon and did not increase over time. Changing malaria treatment practices have been accompanied by profound changes in markers of resistance.

**Keywords.** Malaria; *Plasmodium falciparum*; drug resistance; artemisinin; chloroquine; artemether; lumefantrine; artesunate; amodiaquine; dihydroartemisinin; piperaquine; pfcrt; pfmdr1; K13.

Drug resistance challenges the treatment and control of malaria. In Africa, use of artemisinin-based combination therapies (ACTs) has become standard to treat uncomplicated malaria, accompanied by changes in the selective pressure for resistance. In Uganda, the first-line regimen to treat uncomplicated malaria changed from chloroquine to chloroquine + sulfadoxine/pyrimethamine (SP) in 2000 and artemether-lumefantrine (AL) in 2004, although implementation was slow. Elsewhere in Africa, artesunate-amodiaquine (AS/AQ) is first line to treat malaria in many countries and dihydroartemisinin-piperaquine (DHA/PQ) is under study for

chemoprevention. SP remains the standard-of-care to prevent malaria in pregnant women.

Our understanding of antimalarial drug resistance is incomplete, but some *Plasmodium falciparum* genetic polymorphisms are clearly important. The 76T mutation in the putative transporter PfCRT is linked to decreased sensitivity to the aminoquinolines chloroquine and amodiaquine, and *pfcrt* mutations are selected in new infections that occur soon after treatment with aminoquinolines [1, 2]. Mutations in *pfmdr1*, which encodes another putative transporter, the p-glycoprotein homologue, are also associated with altered drug sensitivity. In Africa, the *pfmdr1* 86Y and 1246Y mutations are common, associated with decreased sensitivity to aminoquinolines, and selected by prior treatment with AS/AQ and DHA/PQ [1, 3]. Interestingly, wild-type sequences at these same alleles are associated with decreased sensitivity to lumefantrine and selected by recent treatment with AL, demonstrating opposite effects of the same polymorphisms on sensitivity to different drugs [1, 2].

Resistance to SP is well characterized, with 5 mutations in dihydrofolate reductase (51I, 59R, and 108N) and dihydropteroate synthetase (437G and 540E) now common in much of Africa and associated with an intermediate level of resistance [4]. Additional mutations, notably *pfdhfr* 164L and *pfdhps* 581G, lead to high-level resistance. These mutations have been rare in African surveys, but recent studies have suggested emergence in some areas [5, 6].

Changing malaria treatment practices may lead to changes in drug sensitivity. In Malawi, the replacement of chloroquine with SP was followed by widespread *pfcrt* wild-type parasites and excellent clinical efficacy of chloroquine [7]. In Uganda, increased prevalence of wild-type *pfcrt* K76 and *pfmdr1* N86 and D1246 sequences was seen in Tororo from 2003 to 2012 [2, 8], although analyses were complicated by numerous mixed genotypes. In ex vivo studies, increasing sensitivity to chloroquine and decreasing sensitivity to lumefantrine were documented [2]. Consistent with these changes, and in contrast with older studies that showed superiority of AL, in a recent 3-site trial, treatment with AS/AQ was followed by decreased recurrent malaria compared with AL [9]. Thus, parasites in Uganda are changing, and these changes appear to have clinical consequences. However, improved measures of parasite trends will be helpful, in particular using randomly collected samples rather than those available from drug efficacy trials, and including analyses of frequency, which circumvent the complexities of polyclonal infections. We now report the prevalence and frequency of key polymorphisms using samples collected by probability sampling.

## METHODS

### Cross-Sectional Surveys and Sample Collection

Cross-sectional surveys were conducted in 200 randomly selected households at each of 3 sites in 2012, 2013, and 2015 as previously described [10]. Samples were collected during the same period for each year of the study, January–February in Nagongera, Tororo District, in eastern Uganda near Kenya; March–April in Walukuba, Jinja District, in south-central Uganda on Lake Victoria; and May–June in Kihihi, Kanungu District, in southwestern Uganda. The sites varied greatly in malaria transmission intensity (annual entomological inoculation rates of 3.8, 26.6, and 125.0 infectious bites per person-year for Walukuba, Kihihi, and Nagongera, respectively). Households were randomly selected as described, and finger prick blood samples were collected on filter paper from all children aged <15 years and 1 randomly selected adult from each of 5 age categories; samples were collected regardless of whether symptoms were present [10].

### Assessment of *Plasmodium falciparum* Polymorphisms

Parasite DNA was extracted from dried blood spots from samples positive by microscopy, and sequences of alleles of interest in *pfcrt*, *pfmdr1*, *pfdhfr*, and *pfdhps* were determined using a ligase detection reaction-fluorescent microsphere assay, as previously described [11], with minor modifications, including nested polymerase chain reaction amplifications of templates, as described [12]. The K13 gene propeller domain was amplified and sequenced as previously described [13].

### Frequency and Linkage Analyses

Parasite population frequencies were estimated using all 1466 samples and a previously described model that accounts for mixed infections [14]. Linkage disequilibrium was estimated for all samples, but results from alleles with mixed or missing genotyping outcomes were omitted from the analysis (see Supplementary Methods for details).

## RESULTS

### Prevalence of Resistance-Mediating Polymorphisms in Putative Transporters

A total of 1486 microscopy-positive samples were collected and analyzed. Sequences at all alleles of interest were classified as wild type (identical in sequence to the reference 3D7 strain), mutant, or mixed for the 1466 samples that yielded data for at least 1 polymorphism. Considering the key transporter polymorphisms *pfcrt* K76T, *pfmdr1* N86Y, and *pfmdr1* D1246Y, the prevalence of parasites with mutant sequences decreased steadily from 2012 to 2015 (Figure 1A). Results were similar at the 3 sites. For another polymorphic allele, *pfmdr1* Y184F, the prevalence of parasites with mutant alleles increased in Jinja, but was stable at other sites. The *pfmdr1* 1034C and 1042D

mutations, generally seen only outside Africa, were identified rarely.

### Prevalence of Resistance-Mediating Polymorphisms in Folate Pathway Enzymes

The prevalence of parasites with 5 mutations (*pfdhfr* 51I, 59R, 108N; *pfdhps* 437G, 540E) that were common in prior surveys remained high (Figure 1A). In addition, 2 mutations that have previously been rare in most surveys from Africa were seen, with *pfdhfr* 164L in Kanungu and *pfdhps* 581G at all 3 sites in 2015.

### Frequency of Resistance-Mediating Polymorphisms and Haplotype Analysis

Prevalence data did not take into account multiplicity of infection (MOI) and were complicated by many samples containing both wild-type and mutant sequences. Therefore, we used a statistical model to estimate parasite population frequencies for the studied polymorphisms. Frequency results were broadly similar to those based on prevalence; point estimates for the mutant transporter alleles *pfcrt* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y decreased markedly from 2012 to 2015 at all 3 sites (Figure 1B). Estimates of linkage disequilibrium suggested linkage between *pfmdr1* 86, *pfmdr1* 184, *pfmdr1* 1246, and *pfcrt* 76; between *pfmdr1* 1246 and *pfcrt* 76; and between *pfdhps* 437 and *pfdhps* 540 (Figure 2). Considering haplotypes, Y184F did not appear to impact trends, with increases in both NYD and NFD haplotypes over time (Supplementary Figure 1); there was a marked increase in frequency of the *pfmdr1* N86/D1246 haplotype, suggesting that parasites with both wild-type alleles have a selective advantage (Supplementary Figure 2); and the wild type *pfmdr1* N86/D1246 haplotype was associated with both *pfcrt* K76T alleles, whereas K76 was associated only with N86/D1246 (Supplementary Figure 3).

### Prevalence of K13 Polymorphisms

We randomly selected 20 samples from each site and year for analysis. Of the 153 sequences obtained, 4 samples had nonsynonymous polymorphisms, with a total of 5 mutations, each identified once (V555A from Kihihi in 2012; M472V from Nagongera in 2013; A569S from Nagongera in 2015; K563E and A578S in a single sample from Walukuba in 2013). K13 mutations were seen in samples from all 3 sites and all 3 years of study. Mutations at 4 of the loci were reported previously in Africa, from Niger (M472I), Rwanda (V555A), Kenya and Cameroon (A569S), and 7 different countries, including Uganda (A578S), but to our knowledge K563E has not been reported (http://www.wwarn.org/molecular-surveyor-k13).
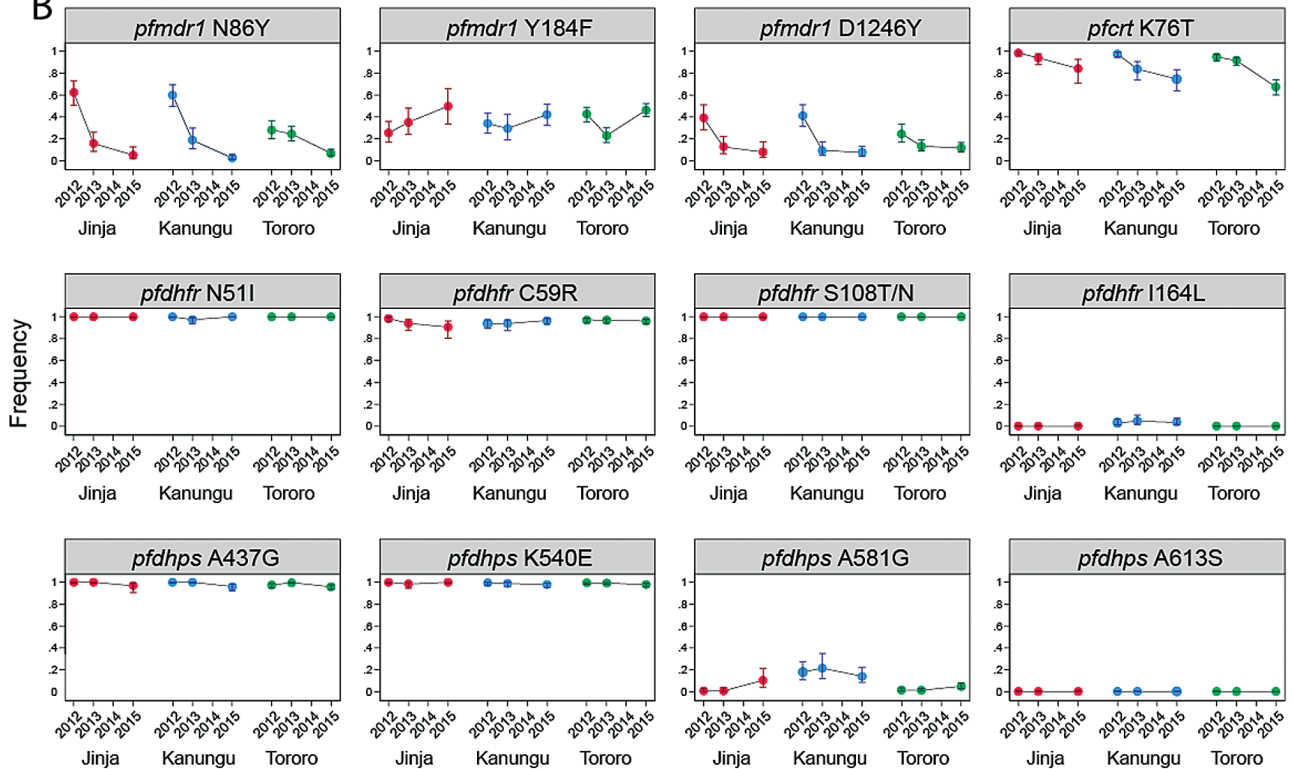
## DISCUSSION

We surveyed the prevalence and calculated the frequency of key drug resistance polymorphisms in *P. falciparum* isolates
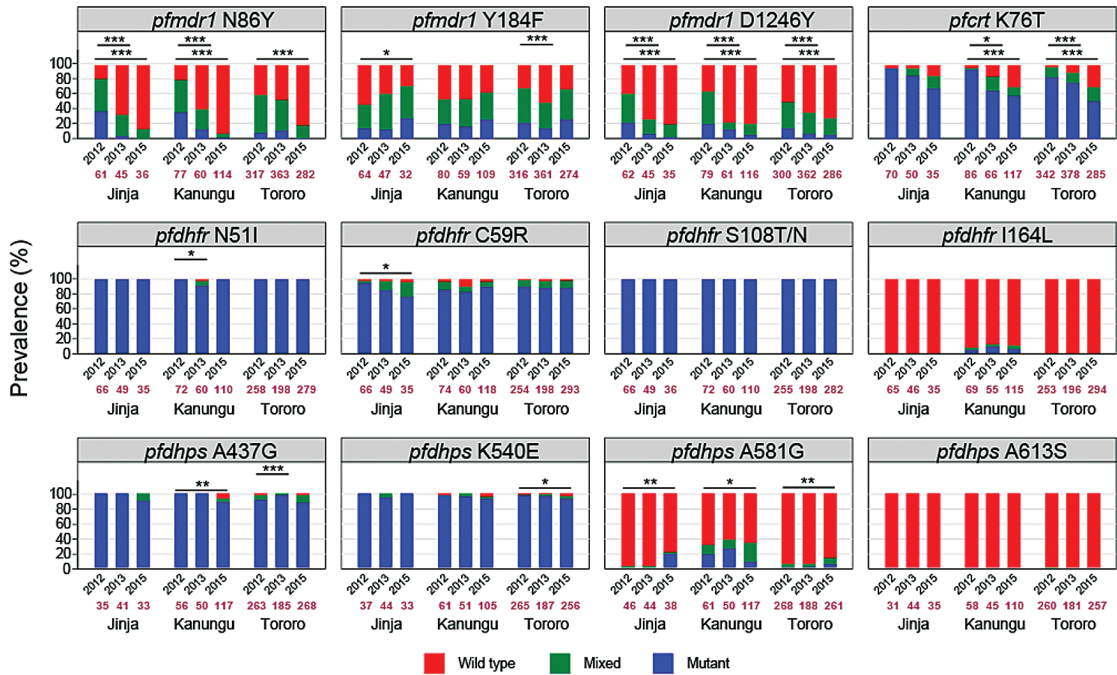
**Figure 1.** *A*, Prevalence of wild-type, mixed, or mutant sequences at alleles of interest at the indicated sites and years. The numbers under the dates represent the number of samples generating results. Asterisks represent *P* values based on comparisons with pure wild-type infections for *pfmdr1* and *pfcrt* and pure mutant infections for *pfdhfr* and *pfdhps* using the Fisher's exact test (*** <.001, ** <.01,* <.05). *B*, Frequency of mutant sequences. Error bars represent 95% credible intervals.

collected at 3 sites in Uganda from 2012 to 2015. This study improved on prior evaluations by studying randomly collected isolates, rather than those collected in the context of a clinical trial, and by evaluating the frequency of polymorphisms, circumventing confounding by varied MOI and prevalence of mixed infections at different sites. We identified important changes over time. Notably, with increasing use of AL to treat malaria, the prevalence and frequency of mutant sequences at 3
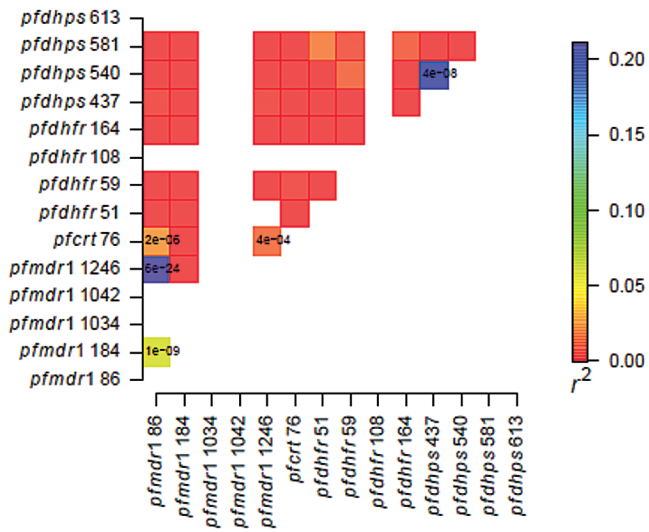
**Figure 2.** Plot of linkage disequilibrium for pairwise comparisons of all studied *pfcrt, pfmdr1, pfdhfr,* and *pfdhps* polymorphisms. Linkage disequilibrium, *r2*, captures the nonrandom association of alleles within 1 gene or in different genes, with values ranging from 0 (no correlation) to 1 (perfect correlation). Colors indicate *r²* values (see Supplementary Materials for details), calculable for polymorphic markers only; white denotes nonpolymorphic markers. *P* values of statistically significant *r²* values are shown. These were calculated using Fisher's exact test, with a Bonferroni correction for 45 tests over 10 polymorphic markers, placing significance at .001.

key *pfcrt* and *pfmdr1* alleles decreased, consistent with decreasing sensitivity to lumefantrine but increasing sensitivity to aminoquinolines. In addition, mutations in *pfdhfr* and *pfdhps* that mediate high-level antifolate resistance appear to be emerging.

Changes in the prevalence of parasites with drug resistance polymorphisms were not unexpected. In Malawi, discontinuation of chloroquine as the first-line antimalarial was accompanied by loss of the *pfcrt* 76T mutation and regaining of chloroquine antimalarial efficacy [7]. Multiple studies showed selection for mutant genotypes by amodiaquine-containing regimens and for wild-type genotypes by AL [1]. In Uganda, wild-type sequences at 3 key transporter alleles are increasingly common. Most parasites studied in Tororo were mutant at these 3 alleles through about 2010 [8], but wild-type sequences have been increasingly prevalent since that time. The rate of change toward wild-type transporter sequences was greater in a cohort treated with AL for every episode of malaria compared with a cohort treated with DHA/PQ, documenting the contribution of selective pressure from AL to this process [12].

Mutations in *pfdhfr* and *pfdhps* have been common in Uganda for at least a decade. Use of SP to treat malaria, WHO-recommended SP for intermittent preventive therapy in pregnant women, antifolates to treat bacterial infections, and trimethoprim-sulfamethoxazole in human immunodeficiency virus–infected individuals likely all offer continued selective pressure for antifolate resistance. It was thus of interest to see whether additional mutations that have been seen primarily outside Africa [4] are emerging in Uganda. The *pfdhfr* 164L mutation was identified in an earlier survey in southwestern Uganda [5], and it was present in Kanungu, also in southwestern Uganda, over the course of our study. The *pfdhps* 581G mutation, which has been noted in Tanzania [6], was detected at all 3 study sites. These additional mutations will probably render SP useless for the treatment or control of malaria. Consideration of other regimens for the prevention of malaria, notably DHA/PQ, which recently showed outstanding efficacy in children and pregnant women [3, 15], is an urgent priority.

Frequency analyses clarified results by accounting for MOI and mixed infections, and were consistent with prevalence results. Linkage analyses demonstrated linkage between the transporter polymorphisms *pfmdr1* 86, *pfmdr* 184, *pfmdr* 1246, and *pfcrt* 76. Haplotype analyses demonstrated an apparent selective advantage of parasites with the wild-type alleles *pfmdr1* N86 and *pfmdr1* D1246 and that wild-type *pfcrt* K76 was present almost exclusively with a background of *pfmdr1* N86/D1246. As *pfcrt* 76T is the main mediator of resistance to chloroquine, but decreased sensitivity to lumefantrine is linked to all 3 of these polymorphisms [1, 2], it seems likely that the evolution of transporter polymorphisms has been driven both by decreasing use of chloroquine and increasing use of AL over time.

Resistance to artemisinins, mediated principally by mutations in K13, is of great concern, but resistance does not appear to have yet spread to Africa. We identified a handful of K13 propeller domain mutations but no evidence of geographic differences in prevalence or changes over time. K13 polymorphisms may be under selection from ACT use in Uganda, but they do not yet appear to be mediating artemisinin resistance.

Our study had some limitations. First, we studied only 3 sites over 4 years; important additional trends may be underway but not evident over this short interval. Second, sample sizes were fairly small, especially for Jinja, where decreasing prevalence limited available samples over time. Third, we considered only a small number of well-characterized resistance-mediating polymorphisms. Consideration of full sequences of genes of interest or of whole genomes might identify additional important trends in the evolution of drug resistance.

In summary, surveillance for *P. falciparum* drug resistance markers in Uganda has demonstrated marked changes in recent years, with a return to wild-type transporter sequences that likely mediate decreased sensitivity to AL, the national regimen to treat malaria, and emergence of mutations that mediate high-level antifolate resistance but no convincing evidence of artemisinin resistance. Continued surveillance for mediators of antimalarial drug resistance is warranted. Furthermore, as selective pressures of AS/AQ and DHA/PQ differ from those of AL [1–3, 9, 12], we suggest consideration of rotating treatment regimens to delay emergence of resistance.

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