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Changing Antimalarial Drug Sensitivities in Uganda

By

Stephanie A. Rasmussen

A culminating thesis submitted to the faculty of Dominican University of California in partial fulfillment of the requirements for the degree of Master of Science in Biology

San Rafael, CA

December 2017

This thesis, written under the direction of the candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

Stephanie A. Rasmussen Candidate	December 15, 2017
Maggie C. Louie, PhD MS Biology Program Director	December 15, 2017
Roland A. Cooper, PhD, MPH Thesis Advisor	December 15, 2017
Philip J. Rosenthal, MD Secondary Thesis Advisor	December 15, 2017

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List of Abbreviations

ACT: Artemisinin-based combination therapy

AL: Artemether-lumefantrine

ASAQ: Artesunate-amodiaquine

DHA: Dihydroartemisinin

DP: Dihydroartemisinin-piperaquine

IC_{50}: Half-maximal inhibitory concentration

PfCRT: *Plasmodium falciparum* chloroquine resistance transporter

PfMDR1: *Plasmodium falciparum* multi-drug resistance transporter 1

SNP: Single nucleotide polymorphism

WHO: World Health Organization

Abstract

Dihydroartemisinin-piperaquine (DP) has demonstrated excellent efficacy for the treatment and prevention of malaria in Uganda. However, resistance to both components of this regimen has emerged in Southeast Asia. The efficacy of artemether-lumefantrine, the first-line regimen to treat malaria in Uganda, has also been excellent, but continued pressure may select for parasites with decreased sensitivity to lumefantrine. To gain insight into current drug sensitivity patterns, *ex vivo* sensitivities were assessed and genotypes previously associated with altered drug sensitivity were characterized for 58 isolates collected in Tororo, Uganda from subjects presenting in 2016 with malaria from the community or as part of a clinical trial comparing DP chemoprevention regimens. Compared to community isolates, those from trial subjects had lower sensitivities to the aminoquinolines chloroquine, monodesethyl amodiaquine, and piperaquine, and greater sensitivities to lumefantrine and mefloquine, consistent with DP selection pressure. Compared to results for isolates from 2010-13, sensitivities of 2016 community isolates to chloroquine, amodiaquine, and piperaquine improved (geometric mean IC_{50}s 248, 76.9, and 19.1 nM in 2010-13 and 33.4, 14.9, and 7.5 nM in 2016, respectively, $P<0.001$ for all comparisons), sensitivity to lumefantrine decreased (IC_{50} 3.0 nM in 2010-13 and 5.4 nM in 2016, $P<0.001$), and sensitivity to dihydroartemisinin was unchanged (IC_{50} 1.4 nM). These changes were accompanied by decreased prevalence of transporter mutations associated with aminoquinoline resistance and low prevalence of polymorphisms recently associated with resistance to artemisinins or piperaquine. Antimalarial drug sensitivities are changing in Uganda, but novel genotypes associated with DP treatment failure in Asia are not prevalent.

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Background

Malaria Burden

Malaria has been evolving with humans for thousands of years, yet it remains a serious public health concern. According to the World Health Organization (WHO), there were approximately 216 million new cases and approximately 445,000 deaths from malaria worldwide in 2016. Eighty percent of the worldwide cases and 91% of the deaths occurred in Sub-Saharan Africa, with 99% of the estimated cases caused by *Plasmodium falciparum*, the most lethal of the human malaria species. Pregnant women and children under the age of five are the primary groups affected by malaria (1). Unfortunately, the majority of malaria cases are located in developing countries, where lack of resources hinders eradication efforts.

Human Malaria Life Cycle

Malaria is a disease caused by single-cell protozoa from the phylum Apicomplexa, genus *Plasmodium*. There are five different species of *Plasmodium* that can infect and cause malaria in humans – *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, all of which require a mosquito host for sexual reproduction and a human host for asexual reproduction (Figure 1). Only female *Anopheles* species are suitable mosquito hosts for human malaria species. Once inside a mosquito the malaria gametocytes develop into gametes. A microgamete fuses with a macrogamete to produce a zygote. The zygote develops into a motile ookinete that penetrates the mosquito's gut wall and develops into an oocyst on the hemocoel side of the gut. Via mitosis and meiosis, the oocyst turns into

sporoblasts, haploid nucleated masses, that divide to form motile sporozoites (2). The sporozoites break out of the oocyst, travel to the mosquito's salivary glands, and lead to human infection when the mosquito takes a blood meal, injecting the sporozoites. The entire development in the mosquito takes approximately 10-12 days.

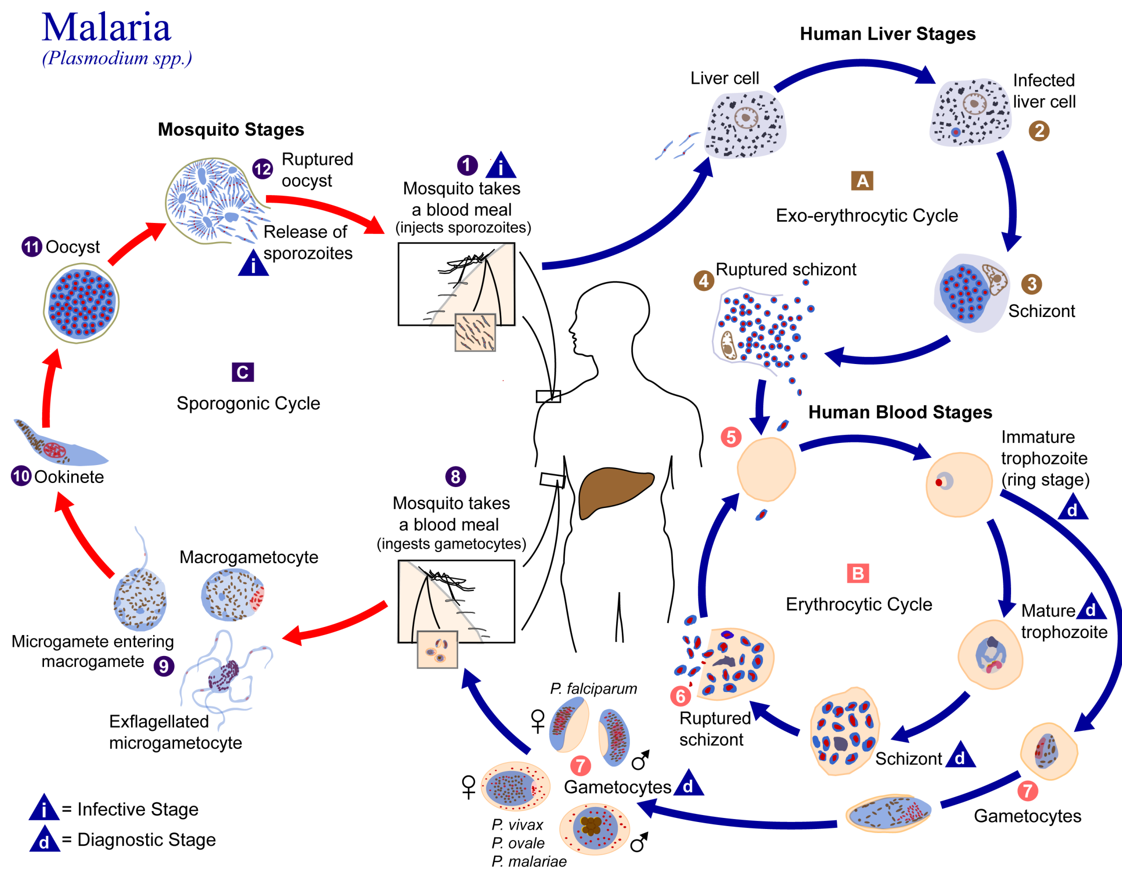

Figure 1. The life cycle of human malaria species. The life cycle involves sexual reproduction in a female *Anopheles* mosquito and asexual replication in a human host. Image taken with permission from the CDC – DPDx Public Health Image Library (3).

To initiate the human phase of the life cycle, an infected mosquito injects sporozoites via its saliva into the bloodstream while taking a blood meal. After the first

twenty-four hours in the blood stream, the sporozoites migrate to the liver where they invade hepatocytes in a ligand-receptor-mediated fashion (4). Once inside the hepatocyte, the malaria parasite goes through asexual reproduction, forming thousands of merozoites that exit the liver cells in about seven to ten days, depending on the species of malaria. The merozoites leave the liver and invade erythrocytes to initiate the asexual blood stage infection. Inside the erythrocyte, the parasite goes through a round of asexual replication that takes approximately 48 hours for *P. falciparum* and results in the rupture of the infected red blood cell, releasing up to 32 newly formed merozoites that subsequently infect new erythrocytes. The erythrocytic cycle is responsible for all clinical manifestations of malaria (5) and persists indefinitely. Some erythrocytic parasites develop into the sexual stages, the microgametocyte and the macrogametocyte. When a mosquito takes a blood meal from a human host that contains these gametocytes, the *Plasmodium* life cycle enters the invertebrate, sexual stage.

Clinical Falciparum Malaria

Patients with malaria can have varying degrees of symptoms, ranging from asymptomatic to severe, life-threatening complications. In uncomplicated malaria, patients exhibit nonspecific symptoms such as anemia, fever, malaise, headaches, body aches, and nausea (5). However, complicated or severe malaria can have lethal consequences because patients can develop metabolic acidosis, severe anemia, and cerebral malaria. *P. falciparum* is the most dangerous human malaria species because of its ability to cause complicated malaria by causing severe anemia and cytoadherance of infected red blood cells to the brain, heart, lungs, liver, kidneys, and placenta (6).

Even though the mechanism by which uncomplicated malaria progresses to complicated malaria is not fully understood, the ability of *P. falciparum*-infected erythrocytes to adhere to tissues appears to play a key role. Sequestration of infected red blood cells in the placenta can lead to hazardous conditions for the fetus and mother including maternal anemia, reduced birth weight, premature birth, and increased mortality of the newborn. Additionally, sequestration of infected red blood cells in the brain is hypothesized to play a role in cerebral malaria, which often leads to coma, brain damage, and/or death (7). Because of the danger *P. falciparum* poses to human health, it is imperative that useful, effective antimalarials are available for the treatment of the disease.

Drug Resistant *P. falciparum*

It is essential to have potent, safe, and inexpensive antimalarials available for the treatment of malaria. Chloroquine, an antimalarial that gained popularity in 1950, was a game-changing drug for the treatment of malaria because it had minimal side effects, was efficacious, and was affordable. However after only a decade of widespread use, resistance originated from independent foci in Asia and South America that later spread throughout the world, rendering the drug useless (8, 9). Resistance to chloroquine and the structurally related drug amodiaquine, both of which are in the 4-aminoquinoline drug class, has been linked to mutations in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) at position 76 and *Plasmodium falciparum* multi-drug resistance transporter 1 (PfMDR1) at positions 86 and 1246, which are both transmembrane proteins on the parasite digestive vacuole hypothesized to mediate the influx and efflux

of drugs (8, 10). In an effort to find a useful replacement, artemisinin-based combination therapies (ACTs) were developed (11).

Currently, the first line therapies for uncomplicated *P. falciparum* infections are ACTs (12). Unlike chloroquine, the ACTs combine a fast acting artemisinin derivative with a slow acting partner drug, such as piperaquine, lumefantrine, mefloquine, amodiaquine, or pyronaridine, that work in combination to eradicate the malaria parasites in a patient. The artemisinin derivative is very potent and fast acting, so it kills the majority of the parasites in a patient's blood stream quickly while the slow acting partner drug with a longer half-life stays in the patient longer and kills any remaining parasites (Figure 2) (13, 14). ACTs have proven to be efficacious and have a good safety record (1, 12).

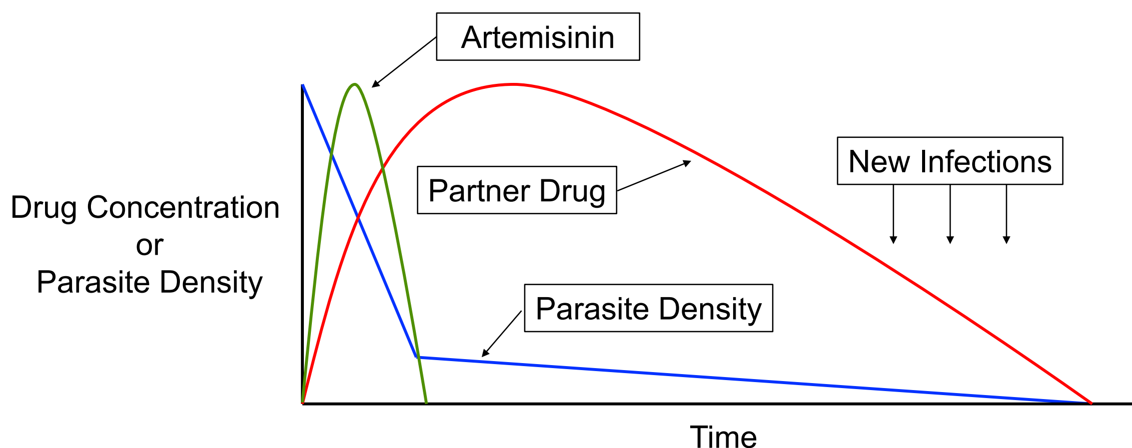

Figure 2. Mechanism of action of ACTs. ACTs work by combining a fast acting artemisinin derivative with a slow acting partner drug to effectively kill all the parasites in a patient. Initially, the artemisinin kills the majority of the parasites in the patient and the partner drug kills the surviving parasites. New infections may occur once the slow-acting partner drug concentration drops to sub-therapeutic levels.

Unfortunately, resistance to the artemisinin component of ACTs has developed in Southeast Asia (15, 16). Clinical artemisinin resistance is defined as a delayed clearance phenotype, meaning that it takes significantly longer for the drug to clear parasites. In a region of Cambodia with artemisinin resistance, it took on average 36 more hours to clear parasites with artesunate monotherapy compared to a region of Thailand without artemisinin resistance (16). The delayed clearance phenotype has been linked to single nucleotide polymorphisms (SNPs), specifically C580Y, R539T, Y493H, M476I, and I543T, in the kelch propeller domain of the protein encoded by the kelch *k13* gene (15, 17, 18). Because artemisinins are now failing in Southeast Asia, it is even more crucial to use combination therapies for the treatment of malaria.

The partner drugs in ACTs can kill the parasites when the artemisinin fails, so combination therapies are a useful way to prevent drug resistance from emerging. Two or more drugs that target different biochemical pathways in the parasite would likely slow down the acquisition of drug resistance. Nevertheless, with ACTs the risk of developing resistance to the slow acting partner drug is serious if patients get re-infected during a time when there are sub-therapeutic levels of the partner drug present in their blood (11) (Figure 2). Additionally, now that artemisinin resistance is present in Southeast Asia, there is even more selective pressure on the partner drugs, and currently, drug resistance to the partner drugs piperaquine, mefloquine, and amodiaquine is present in Southeast Asia (19-22).

Clinical dihydroartemisinin-piperaquine (DP) failure has recently developed in Southeast Asia and has been associated with increased *plasmepsin 2* copy number in two studies and with an E415G encoding mutation in an exonuclease gene in one study (23,

24). DP failure is especially concerning because it has been shown to have excellent efficacy for the treatment of malaria and for malaria chemoprevention in both children and pregnant women in Africa (25-29). DP works well as a chemoprevention therapy because piperaquine has a long half-life (23 days [range 19-28 days] in adults and 14 days [range 10-18 days] in children (30)), which helps protect patients from new malaria infections for a longer amount of time (Figure 2). Piperaquine, a bisquinoline, is an interesting drug because it is structurally similar to the 4-aminoquinolines chloroquine and amodiaquine, but its mechanism of action and selective pressures are not as well understood as they are for the other 4-aminoquinolines.

Resistance to the partner drug mefloquine, an arylaminoalcohol, was first reported in 1982 (31), five years after it was introduced, and has been associated with increased *pfmdr1* copy number in both *in vitro* and *in vivo* experiments (21, 32-34). Interestingly, resistance to the partner drug lumefantrine, which is an arylaminoalcohol-containing fluorene, has not been described to date. However similar to mefloquine, increased *pfmdr1* copy number has been associated with decreased sensitivity to lumefantrine (21, 32-34). Oppositely, an N86Y mutation in PfMDR1 has been shown to be associated with increased sensitivity to both of these drugs (35). Even though the exact mechanism by which these drugs act is uncertain, *in vivo* and *in vitro* studies have shown that decreased sensitivity to both drugs is mediated by the wildtype genotype at position 76 in PfCRT and positions 86 and 1246 in PfMDR1, which for the 4-aminoquinolines is associated with increased sensitivity (35-40). The arylaminoalcohols and 4-aminoquinolines exert opposite selective pressures on mediators of *P. falciparum* drug resistance.

Antimalarial Use in Africa

Historically, chloroquine was used to treat uncomplicated malaria in Africa, but it has been replaced by the ACTs artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) in most African countries. These ACTs are highly efficacious in Africa, with efficacy rates above 97% from 2010-2016 (1, 12). However, considering the fact that the vast majority of the world's malaria and falciparum malaria cases occur in this region, it is pertinent to continue surveillance efforts to ensure the swift detection of resistance if or when it develops in Africa to facilitate the subsequent change of drug therapy policies.

Hypothesis and Rationale

The work contained in this thesis was focused on studying the phenotypic and genotypic mechanisms of drug resistance present in clinical *P. falciparum* isolates from the Tororo District of Uganda (Figure 3). The Tororo District, located in the Southeastern part of Uganda, has a high burden of malaria and people were bitten by an average of 125 infective mosquitoes per year in 2011-2012 (41). Uganda recently widely implemented AL as the national therapy for uncomplicated malaria after switching from chloroquine in 2006. Our group collected a large amount of *ex vivo* drug sensitivity data and genetic data on *P. falciparum* clinical isolates from Tororo between 2010-2013 (40). Unsurprisingly because clinical chloroquine resistance was widespread, the study showed that a large proportion of *P. falciparum* isolates had decreased sensitivity to the 4-aminoquinolines and a high prevalence of SNPs associated with resistance to the 4-aminoquinolines.

When we returned to Tororo in 2016, we sought to study the current drug sensitivity phenotypes and genotypes present in *P. falciparum* isolates from the same location in the new setting of decreased chloroquine use and widespread AL use. We hypothesized that parasites in 2016 would differ significantly from those studied between 2010-2013 because of the different drug therapies now used in the community.

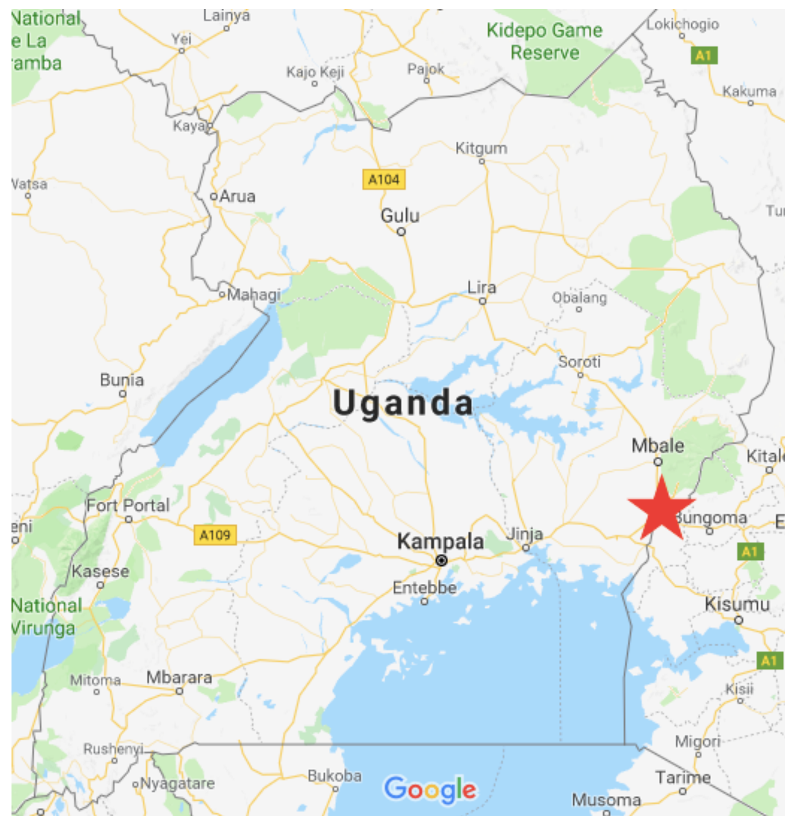

Figure 3. Map of Uganda. The red star indicates the location of the study site Tororo. Image taken with permission from Google Maps (Map data ©2017 Google).

Paper

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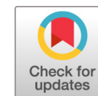

Changing Antimalarial Drug Sensitivities in Uganda

Stephanie A. Rasmussen,[a] Frida G. Ceja,[a] Melissa D. Conrad,[b] Patrick K. Tumwebaze,[c] Oswald Byaruhanga,[c] Thomas Katairo,[c] Samuel L. Nsobya,[c,d] Philip J. Rosenthal,[b] Roland A. Cooper[a]

Department of Natural Sciences and Mathematics, Dominican University of California, San Rafael, California, USA[a]; Department of Medicine, University of California, San Francisco, California, USA[b]; Infectious Diseases Research Collaboration, Kampala, Uganda[c]; Department of Pathology, Makerere University, Kampala, Uganda[d]

ABSTRACT Dihydroartemisinin-piperaquine (DP) has demonstrated excellent efficacy for the treatment and prevention of malaria in Uganda. However, resistance to both components of this regimen has emerged in Southeast Asia. The efficacy of artemether-lumefantrine, the first-line regimen to treat malaria in Uganda, has also been excellent, but continued pressure may select for parasites with decreased sensitivity to lumefantrine. To gain insight into current drug sensitivity patterns, *ex vivo* sensitivities were assessed and genotypes previously associated with altered drug sensitivity were characterized for 58 isolates collected in Tororo, Uganda, from subjects presenting in 2016 with malaria from the community or as part of a clinical trial comparing DP chemoprevention regimens. Compared to community isolates, those from trial subjects had lower sensitivities to the aminoquinolines chloroquine, monodesethyl amodiaquine, and piperaquine and greater sensitivities to lumefantrine and mefloquine, an observation consistent with DP selection pressure. Compared to results for isolates from 2010 to 2013, the sensitivities of 2016 community isolates to chloroquine, amodiaquine, and piperaquine improved (geometric mean 50% inhibitory concentrations [IC_{50}] = 248, 76.9, and 19.1 nM in 2010 to 2013 and 33.4, 14.9, and 7.5 nM in 2016, respectively [$P < 0.001$ for all comparisons]), the sensitivity to lumefantrine decreased (IC_{50} = 3.0 nM in 2010 to 2013 and 5.4 nM in 2016 [$P < 0.001$]), and the sensitivity to dihydroartemisinin was unchanged (IC_{50} = 1.4 nM). These changes were accompanied by decreased prevalence of transporter mutations associated with aminoquinoline resistance and low prevalence of polymorphisms recently associated with resistance to artemisinins or piperaquine. Antimalarial drug sensitivities are changing in Uganda, but novel genotypes associated with DP treatment failure in Asia are not prevalent.

KEYWORDS *Plasmodium falciparum*, Uganda, artemether-lumefantrine, dihydroartemisinin-piperaquine, drug resistance, *ex vivo*, *k13*, *pfcrt*, *pfmdr1*, *plasmepsin 2*

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Address correspondence to Roland A. Cooper, roland.cooper@dominican.edu.

With widespread resistance to older drugs, the World Health Organization currently recommends artemisinin-based combination therapies (ACTs) as first-line treatments for uncomplicated malaria in Africa, where ACTs remain highly efficacious against *Plasmodium falciparum*, the most lethal and prevalent of the human malaria species (1). ACTs combine a potent and fast acting artemisinin derivative with a longer-acting partner drug, notably lumefantrine, piperaquine, amodiaquine, mefloquine, or pyronaridine (2). Because the partner drugs circulate long after short-acting artemisinins have been cleared, resistance may be selected if patients are reinfected soon after therapy.

For many years, malaria was treated principally with the aminoquinoline chloroquine. Resistance to chloroquine and its analogue amodiaquine is strongly associated

with mutations in two putative drug transporters: PfCRT 76T and PfMDR1 86Y and 1246Y. Treatment with chloroquine and amodiaquine selects for these same mutations (3, 4). Until recently, nearly all *P. falciparum* strains isolated in Uganda demonstrated chloroquine resistance, as evidenced by poor chloroquine treatment outcomes (5), high *ex vivo* half-maximal inhibitory (IC_{50}) values (6, 7), and the near universal prevalence of the transporter mutations noted above (8). In 2004, Uganda established the ACT artemether-lumefantrine (AL) as the national therapy for uncomplicated malaria; implementation was slow, but AL use is currently widespread, and it has demonstrated excellent antimalarial efficacy (9, 10). AL selects for wild-type sequences at the same alleles selected for mutations by aminoquinolines, and these wild-type sequences have been associated with moderately decreased lumefantrine sensitivity (7, 11–13). Thus, aminoquinolines and lumefantrine exert opposite selective pressures on *P. falciparum* drug resistance mediators.

Although it is structurally related to chloroquine and amodiaquine, mediators of resistance and selective effects of the bisquinoline piperaquine, a component of the ACT dihydroartemisinin-piperaquine (DP), are less certain. In Africa, DP has shown excellent efficacy for the treatment of malaria (14, 15) and for malaria chemoprevention in children (16, 17) and pregnant women (18). For chemoprevention, DP benefits from the long half-life of piperaquine, but the impacts of this regimen on the selection of drug resistance are uncertain. Use of DP as therapy (19) or chemoprevention (20) was not associated with selection of the *pfcrt* and *pfmdr1* mutations selected by other aminoquinolines in Burkina Faso, but in Uganda, DP treatment (12) and chemoprevention (7, 17) was followed by selection of parasites with increased prevalence of aminoquinoline resistance-mediating mutations, although selection of different mutations varied among the studies.

Artemisinin resistance emerged in Southeast Asia over the last decade, manifesting as delayed parasite clearance after therapy and causally linked to mutations in the *k13* gene (PF3D7_1343700) (21–23). More recently, resistance to piperaquine also emerged in Southeast Asia, linked to an increased *plasmepsin 2* (PF3D7_1408000) copy number (24, 25) and, in one study, an E415G mutation in an exonuclease (encoded by PF3D7_1362500) (24). In the same region, amplification of the *pfmdr1* gene has been linked to decreased sensitivity to mefloquine and lumefantrine but increased sensitivity to aminoquinolines (26, 27). However, *pfmdr1* gene amplification appears to be very uncommon in *P. falciparum* in Africa.

Considering recent changes in malaria treatment practices in Uganda, we investigated changes in drug sensitivity. We analyzed the drug susceptibility phenotypes and genotypes of clinical *P. falciparum* isolates collected from two sources in 2016 and compared them to the characteristics of isolates collected in prior years. We found marked changes in drug susceptibilities over time that were consistent with decreased selective pressure from chloroquine and/or increased selective pressure from the national treatment regimen AL.

RESULTS

***P. falciparum* isolates.** Isolates were collected from May to July 2016 from subjects with uncomplicated malaria from two sources: 29 children and adults from the community (mean age, 4.7 years; range, 1 to 21 years) diagnosed at Tororo District Hospital and 29 children (mean age, 1.4 years; range, 1.2 to 1.5 years) enrolled in a trial comparing two different regimens of DP (monthly or every 3 months) for the prevention of malaria.

Comparative *ex vivo* drug sensitivities. We compared *ex vivo* drug sensitivities between samples from community members and trial subjects. Samples from trial subjects were significantly less sensitive to the aminoquinolines chloroquine, monodesethyl amodiaquine (the active metabolite of amodiaquine), and piperaquine; were significantly more sensitive to lumefantrine and mefloquine; and had no difference in sensitivity to dihydroartemisinin (DHA), atovaquone, or pyronaridine (Table 1). These results were consistent with selection by DP in trial subjects for decreased aminoquino-

TABLE 1 *Ex vivo* drug sensitivity of *P. falciparum* isolates collected from 2010 to 2013 and in 2016 from trial and community patients[a]

Drug	Source and/or study period (yr)	No. of samples	Geometric mean IC_{50} (nM)	95% CI (nM)	IC_{50} range (nM)	*P* value		
						2010 to 2013 vs 2016 trial	2010 to 2013 vs 2016 community	2016 trial vs 2016 community
Chloroquine	Dd2	7	209	180–242	155–240			
	3D7	7	9.2	7.9–10.8	6.7–11			
	2010–2013	408	248	223–275	31.0–1,400	<0.001	<0.001	<0.001
	2016, trial	25	57.1	32.7–99.6	12.4–727			
	2016, community	24	33.4	19.8–56.2	8.7–318			
Monodesethyl amodiaquine	Dd2	7	35.5	25.8–48.8	25–61			
	3D7	7	5.4	4.7–6.3	4.1–6.2			
	2010–2013	421	76.9	70.2–84.1	12.5–565	<0.001	<0.001	<0.001
	2016, trial	20	20.6	15.0–28.4	7.5–91.8			
	2016, community	24	14.9	10.6–21.0	4.6–70.4			
Piperaquine	Dd2	7	4.4	2.6–7.3	1.8–9.6			
	3D7	7	3.7	2.6–5.2	2–6.4			
	2010–2013	381	19.1	17.1–21.4	3.1–189	<0.001	<0.001	0.05
	2016, trial	25	8.6	6.5–11.2	1.8–26.6			
	2016, community	25	7.5	6.0–9.3	2.7–20.5			
Lumefantrine	Dd2	7	1.8	1.4–2.4	1.3–2.7			
	3D7	7	3.4	3.0–3.7	2.7–3.7			
	2010–2013	378	3.0	2.6–3.3	0.4–24.4	0.52	<0.001	<0.001
	2016, trial	24	3.4	2.6–4.5	0.6–11.6			
	2016, community	25	5.4	4.3–6.9	1.8–23.7			
Dihydroartemisinin	Dd2	7	0.8	0.5–1.3	0.5–2.1			
	3D7	7	0.9	0.6–1.5	0.6–2.2			
	2010–2013	442	1.4	1.3–1.5	0.3–16.9	0.52	0.94	0.55
	2016, trial	25	1.7	1.4–2.2	0.5–4.4			
	2016, community	24	1.4	1.2–1.7	0.4–3.9			
Atovaquone	Dd2	7	0.2	0.1–0.3	0.1–0.3			0.97
	3D7	7	0.1	0.1–0.2	0.1–0.2			
	2016, trial	18	0.5	0.4–0.6	0.2–1.0			
	2016, community	22	0.5	0.4–0.6	0.1–1.0			
Mefloquine	Dd2	7	10.2	7.2–14.4	6.7–20			<0.001
	3D7	7	9.0	6.9–11.6	6.4–14			
	2016, trial	20	18.8	15.6–22.8	5.9–33.6			
	2016, community	24	21.2	18.5–24.4	8.9–41.7			
Pyronaridine	Dd2	7	1.8	0.9–3.8	0.7–6.0			0.69
	3D7	7	1.0	0.8–1.4	0.6–1.6			
	2016, trial	24	2.5	1.8–3.5	0.7–29.1			
	2016, community	23	2.2	1.5–3.3	0.2–10.1			

[a]Geometric mean IC_{50}s for each group were compared using *t* tests. Dd2 and 3D7 are laboratory control strains. Data from 2010 to 2013 were published previously (7).

line sensitivity in parasites that emerged despite chemoprevention. We then compared results for samples collected in 2016 to those for samples collected at the same study site from 2010 to 2013 (7). Parasites collected from the community in 2016 were more sensitive to the tested aminoquinolines but less sensitive to lumefantrine than the samples collected earlier (Table 1).

Because the standard 72-h IC_{50} assay does not identify DHA resistance in Southeast Asian parasites (28), the *ex vivo* ring-stage survival assay, in which parasites are exposed to a 6-h pulse of 700 nM DHA, was performed on 16 samples collected from the community and 16 samples collected from trial subjects in 2016. Seven of the community samples had undetectable parasites at 72 h, and the remaining nine samples had median parasitemia of 0.5% that of controls. For the trial samples, seven had undetectable parasites at 72 h, and the remainder had median parasitemias that were 0.6% those of controls. Our results were similar to those for parasites collected in

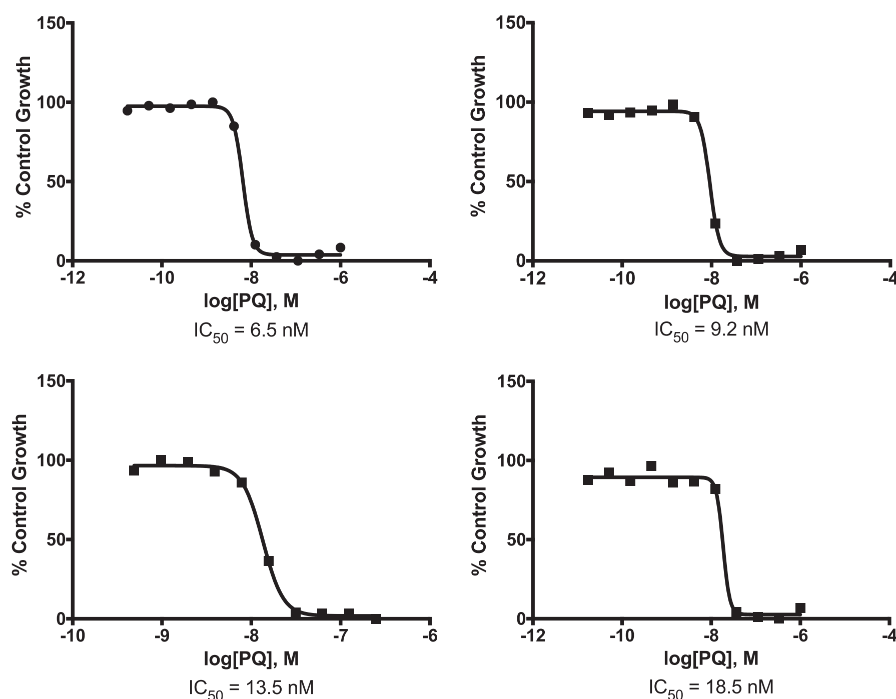

FIG 1 Representative piperaquine (PQ) growth inhibition curves for four different *P. falciparum* isolates collected in 2016.

Kampala, Uganda, in 2014 (29) and contrasted with those for DHA-resistant parasites from Southeast Asia, in which 72-h parasitemias were much higher (median 13.5% that of controls in isolates from a region of Cambodia with frequent artemisinin resistance [30]). Thus, we did not see evidence for *ex vivo* artemisinin resistance in Ugandan parasites.

Piperaquine resistance in Southeast Asia has been accompanied by irregular dose-response curves, necessitating establishment of novel methods for determining *ex vivo* drug sensitivity (31). It was thus important to confirm reliable determination of piperaquine sensitivity by our methods. Standard 72-h growth inhibition assays yielded sigmoidal dose-response curves that were reproducible and readily interpretable (Fig. 1). Thus, in contrast to the case for Southeast Asian isolates resistant to piperaquine, standard IC_{50} determinations were deemed valid for the Ugandan isolates.

Polymorphisms in parasite drug resistance markers. Prevalences of polymorphisms in putative transporters that are associated with sensitivity to a number of antimalarials were compared between *P. falciparum* isolates from 2010 to 2013 and isolates from 2016. Consistent with previous trends (7, 8, 12, 32), genotypes have changed markedly in recent years, with reversion to wild-type sequences at *pfcrt* K76T ($P < 0.001$) and *pfmdr1* N86Y ($P < 0.01$) and D1246Y ($P = 0.095$) (Fig. 2). Nonsignificant trends toward greater prevalence of wild-type *pfcrt* K76 and *pfmdr1* N86 sequences in community compared to trial samples were also seen, an observation consistent with selective pressure from DP in trial subjects. Sensitivity to some drugs is also altered by amplification of *pfmdr1*, but this phenomenon has been uncommon in African parasites. All 58 samples collected in 2016 had only one copy of *pfmdr1*.

Sequencing of the propeller-encoding domain of *k13*, where certain mutations are strongly associated with artemisinin resistance in Southeast Asia, identified a single-nucleotide polymorphism (SNP) in 1 of 29 community samples that corresponded to an A578S mutation and a mixed wild-type/A578S genotype in 1 of 27 trial samples. The A578S mutation has been described in isolates from Uganda and other African countries, and it has not been associated with artemisinin resistance (29, 32, 33).

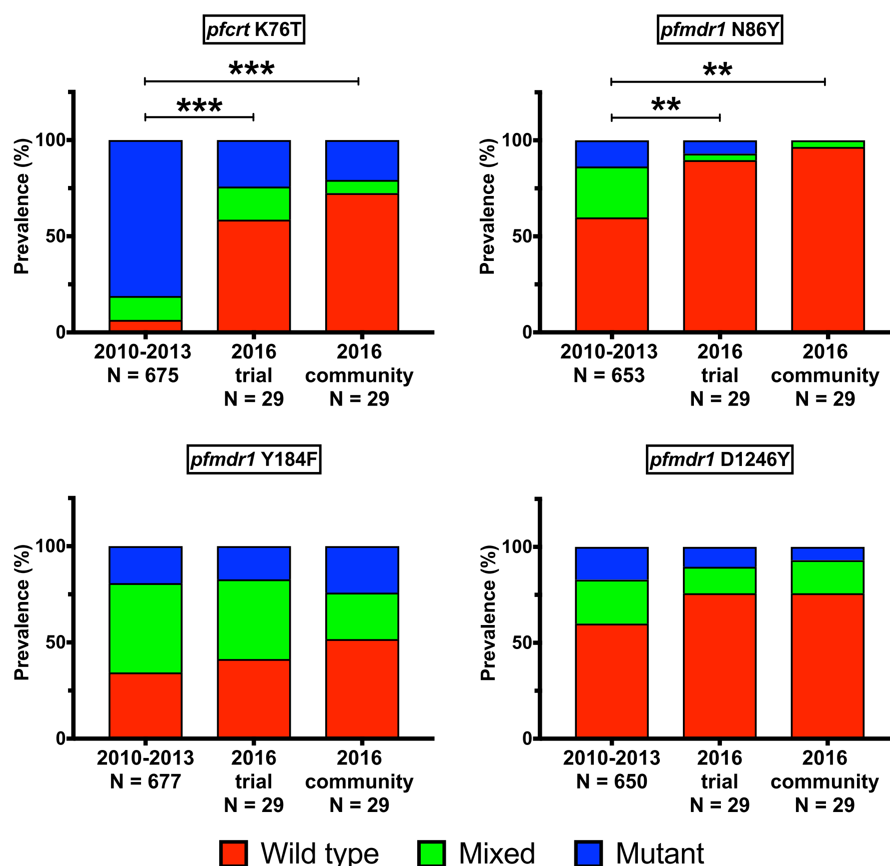

FIG 2 Allele prevalence over time. Wild-type, mixed, and mutant allele prevalences are indicated for the 2010 to 2013 trial and the 2016 trial and community samples. Univariate analysis using generalized estimating equations with exchangeable correlations was used to determine significance of genotype changes over time. Asterisks indicate significance of comparisons between wild-type and mixed/mutant prevalences for the indicated samples ($P < 0.01$ [**] and $P < 0.001$ [***]). Allele prevalences from 2010 to 2013 were published previously (7).

Recent reports have identified amplification of a gene encoding plasmepsin 2 and a SNP in an exonuclease gene (PF3D7_1362500) that encodes an E415G mutation as markers of piperaquine resistance in Southeast Asia (24, 25). We assessed the prevalence of these polymorphisms in Ugandan parasites. First, we assessed the *plasmepsin 2* (PF3D7_1408000) copy number in samples collected in 2010 to 2013, when piperaquine sensitivity varied more widely in Tororo (Table 1) (7), stratifying parasites with piperaquine IC_{50}s above or below 50 nM. Second, we assessed the *plasmepsin 2* copy number in samples collected in 2016. For all three sets of samples, we identified increased copy number in 10 to 14% of samples, but there was no apparent association between increased copy number and piperaquine sensitivity (Table 2). Third, we

TABLE 2 *plasmepsin 2* copy number among samples collected from 2010 to 2013 and in 2016[a]

Sample period (yr)	*n*	PQ IC_{50} (nM)	No. of samples (%) with increased *plasmepsin 2* copy no.
2010–2013	36	$<$50	4 (11.1)
	22	$>$50	3 (13.6)
2016, trial	29	$<$50	3 (10.3)
2016, community	29	$<$50	4 (13.8)

[a]The copy number was considered increased when measured at $>$1.6 copies. *n*, total number of samples; PQ, piperaquine.

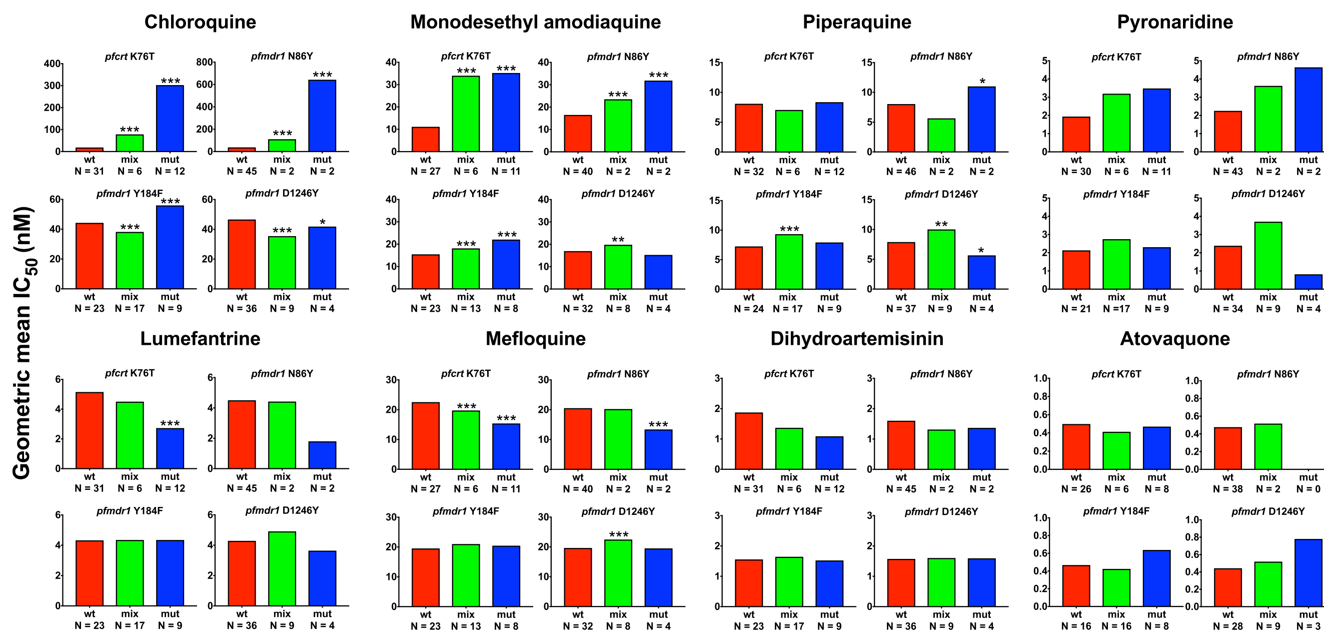

FIG 3 Associations of *ex vivo* geometric mean IC_{50}s with the indicated polymorphisms. "N" represents the number of samples with wild-type (wt), mixed (mix), or mutant (mut) genotypes. The geometric mean IC_{50}s for the wild-type genotype were compared to other genotypes using *t* tests, with the significance noted ($P < 0.05$ [*], $P < 0.01$ [**], and $P < 0.001$ [***]).

sequenced the exonuclease gene in 29 community samples and 27 trial samples collected in 2016, as well as the same 58 samples from 2010 to 2013 for which we assessed *plasmepsin 2* copy number. The exonuclease E415G mutation was not seen in any of the samples. However, two different SNPs were detected in the 2010–2013 samples, and five different SNPs were detected in the 2016 samples. Five of the seven polymorphisms were nonsynonymous, encoding (i) a D360E mutation (from 2012), (ii) a Y365C mutation (2016 community), (iii) a V352A mutation (2016 trial), (iv) a set of seven nonsynonymous SNPs (N369D, K371N, V372D, N373K, and N374V mutations; 2016 trial), and (v) a 24-base-pair insertion corresponding to 8 amino acids (DNDKVN NN), starting at position 376 (2016 community).

Associations of *ex vivo* drug sensitivity with transporter polymorphisms. We and others have previously shown that polymorphisms in the putative transporters *pfcrt* and *pfmdr1* are associated with altered *ex vivo* drug sensitivity (7, 12, 34–38). We tested for associations between *pfcrt* K76T and *pfmdr1* N86Y, Y184F, and D1246Y polymorphisms, and the *ex vivo* drug sensitivities were determined for samples collected in 2016 (Fig. 3). Consistent with previous reports (7, 34, 35), decreased sensitivity to chloroquine, monodesethyl amodiaquine, and pyronaridine and increased sensitivity to lumefantrine and mefloquine were associated with the mutant *pfcrt* 76T and *pfmdr1* 86Y sequences (Fig. 3) (36). Interestingly, consistent with prior reports (7, 37), the associations seen for other aminoquinolines were not seen for piperaquine. Associations with other polymorphisms and for other drugs were mostly not significant.

DISCUSSION

We characterized *ex vivo* drug sensitivities and molecular markers associated with drug sensitivity for *P. falciparum* isolates collected in Tororo, Uganda, in 2016. Sensitivities to aminoquinolines and other components of standard ACTs differed between isolates from the community and those receiving regular DP in a chemoprevention trial, suggesting selective pressure of piperaquine for aminoquinoline resistance. More notably, sensitivities to these drugs differed markedly compared to results for isolates collected in 2010 to 2013 (7). Specifically, sensitivities to aminoquinolines have increased and sensitivity to lumefantrine has decreased, which is consistent with the

selective pressure of AL, the national malaria treatment regimen. These changes were accompanied by decreased prevalence of well-characterized transporter mutations (PfCRT 76T, PfMDR1 86Y, and 1246Y) associated with aminoquinoline resistance but not by an increased prevalence of polymorphisms recently associated with resistance to artemisinins or piperaquine. Thus, antimalarial drug sensitivities are changing in Uganda, most remarkably with reversion to aminoquinoline sensitivity, and genotypes associated with ACT treatment failure in Asia are not prevalent.

Uganda changed from chloroquine-based regimens to AL for the treatment of malaria, with implementation beginning in 2006 (39). Replacement of chloroquine by other regimens has been followed by changes in drug sensitivity in other countries. In Malawi, after discontinuation of chloroquine, the prevalence of the PfCRT 76T mutation, which is the primary mediator of chloroquine resistance (38), decreased markedly (40), followed by excellent treatment efficacy for chloroquine (41). In Uganda, *ex vivo* evidence of chloroquine resistance ($IC_{50} > 50$ to 100 nM) was common in isolates collected from 2006 to 2008 in Kampala (6) and from 2010 to 2013 in Tororo (7), findings consistent with a high prevalence of the PfCRT 76T mutation (3, 42). This situation changed in Tororo most notably beginning in 2012, with increasing prevalence of the *pfcrt* K76 wild-type genotype and decreasing chloroquine *ex vivo* IC_{50}s (7, 8). Our data from 2016 show that this trend has continued, with—based on both parasitological and genetic assessments—the majority of parasites being sensitive to chloroquine and amodiaquine. The replacement of aminoquinoline-resistant parasites by sensitive parasites has been accompanied by reemergence of wild-type sequences at *pfmdr1* 86 and 1246 and by decreases in the *ex vivo* sensitivity to lumefantrine. However, the majority of malaria infections in Uganda are likely polyclonal. Minority clones may persist, allowing rapid selection of parasites with altered sensitivity when certain drugs are used, as seen in Malawi with the discontinuation of chloroquine (40) and in Uganda where treatment with artesunate-amodiaquine or AL selected for different genotypes in new infections emerging after therapy (43). The changes happening in Uganda have likely been driven by both decreased selective pressure from chloroquine and increased selective pressure from lumefantrine.

For the aminoquinolines chloroquine and amodiaquine, resistance is linked to mutations in *pfcrt* and, to a lesser extent, *pfmdr1*, and use of these drugs selects for the same mutations (3, 4, 34, 35, 44). However, associations are less straightforward for the related bisquinoline piperaquine. With the use of DP for the treatment or prevention of malaria in Uganda, selection in parasites that emerged after therapy was consistently seen for PfMDR1 86Y, but selection at other alleles was inconsistent (7, 12, 17). In the present study, samples from the DP chemoprevention trial had decreased sensitivity to chloroquine, monodesethyl amodiaquine, and piperaquine, increased sensitivity to lumefantrine and mefloquine, and an increased prevalence of the PfCRT 76T and PfMDR1 86Y mutations; all of these findings are consistent with a selective pressure of piperaquine, as seen previously for chloroquine (38) and amodiaquine (4, 45). However, in contrast to the results for chloroquine and amodiaquine, the *ex vivo* drug sensitivities for piperaquine were not clearly linked to the prevalences of *pfcrt* and *pfmdr1* polymorphisms (7; the present study). The reasons for the differences in results for different aminoquinolines are uncertain; the larger size of piperaquine may lead to decreased impact of transporter mutations on sensitivity to this drug. In any event, it appears that the widespread use of DP for malaria chemoprevention will select for parasites with decreased sensitivity to aminoquinolines but increased sensitivity to lumefantrine.

Of great concern is the identification of artemisinin resistance within the last decade and of piperaquine resistance very recently in Southeast Asia (22, 24, 25, 46). Important advances from groups in Asia have identified parasitological (30, 31) and molecular (21, 23–25) markers of resistance to these drugs which, when seen together, have been accompanied by high rates of treatment failure with DP (31, 47). We utilized these new tools to characterize parasites circulating in Tororo in 2016. As seen in prior studies from Uganda, occasional *k13* mutations were seen (12, 29, 33), but these were not the mutations associated with artemisinin resistance in Southeast Asia, and prevalence was

not associated with drug sensitivity or recent drug pressure. In addition, as seen recently in parasites from Kampala (29), the *ex vivo* DHA ring survival assay did not suggest artemisinin resistance in Ugandan parasites. Similarly, an increase in *plasmepsin 2* copy number, a newly identified marker associated with piperaquine resistance, was also seen occasionally in Ugandan isolates, but it was not associated with piperaquine sensitivity in recent isolates or in a comparison of older isolates with relatively high or low piperaquine sensitivity. Overall, we did not see evidence suggesting that resistance to artemisinins or piperaquine has emerged in Uganda.

Changes in antimalarial drug use have been accompanied by marked changes in the drug sensitivity of malaria parasites around the world. Our new results demonstrate marked recent changes in Ugandan parasites with, for the most part, a return to sensitivity to chloroquine and amodiaquine. Parasites have also shown some loss of sensitivity to lumefantrine, a component of the national treatment regimen, but it is unlikely that this slight change, although statistically significant, will impact AL treatment efficacy. Considering markers for resistance to artemisinins and piperaquine, our results are reassuring, without evidence for emergence of the worrisome ACT resistance seen in southeast Asia. Resistance to different antimalarial regimens has developed more slowly in Africa than in other regions, likely due to strong antimalarial immunity in Africans living in high-transmission areas, and yet it is extremely important to limit resistance spread. Our results are consistent with other recent studies in suggesting that the continued use of AL to treat malaria and the institution of DP for chemoprevention will exert opposite selective resistance pressures and thus may offer an optimal means for maintaining antimalarial treatment and chemopreventive efficacy while limiting the spread of drug resistance.

MATERIALS AND METHODS

Sample collection. *P. falciparum* isolates were obtained from May to July 2016 from two sources in Tororo, Uganda. First, children and adults presenting at the Tororo District Hospital outpatient clinic with malaria (temperature ≥ 37.5°C axillary or history of fever in the previous 24 h and a positive Giemsa-stained blood smear for *P. falciparum*) and without signs of severe disease were enrolled after informed consent. Second, children enrolled in a clinical trial (registered at ClinicalTrials.gov [NCT02163447]) comparing monthly versus every 3-month intermittent therapy with DP to prevent malaria provided samples if they presented to the study clinic with uncomplicated malaria (defined as above). The studies were approved by the Makerere University Research and Ethics Committee, the Uganda National Council for Science and Technology, and the University of California, San Francisco Committee on Human Research. All subjects were treated with AL after sample collection.

Sample collection and parasite culture. Blood was collected before therapy in a heparinized tube. Parasitemia was determined with Giemsa-stained thin smears. Samples containing only *P. falciparum* and with a parasitemia of ≥1% were selected for culture. Initiation of culture was performed as previously described (6), with slight modifications. Briefly, blood was centrifuged, plasma and buffy coat were removed, and the erythrocyte pellet was washed three times with RPMI 1640 medium prewarmed to 37°C. The pellet was resuspended in complete media (RPMI 1640 medium supplemented with 25 mM HEPES, 0.2% $NaHCO_3$, 0.1 mM hypoxanthine, 10 μg/ml gentamicin, and 0.5% AlbuMAX II serum substitute) to produce a 50% hematocrit. Culture aliquots were spotted onto Whatman 3MM filter paper for molecular studies.

Ex vivo determination of drug susceptibilities. Drug susceptibilities were assessed immediately or from samples stored at 4°C for a maximum of 18 h. Drug sensitivities were determined for chloroquine (Sigma-Aldrich), monodesethyl amodiaquine (BD Gentest), piperaquine (Jinan Jiaquan International Trade Co., Ltd.), lumefantrine (Sigma-Aldrich), dihydroartemisinin (DHA; TCI Tokyo Chemical Industry), mefloquine (Sigma-Aldrich), atovaquone (Sigma-Aldrich), and pyronaridine (Santa Cruz Biotechnology) using a 72-h, 96-well microplate fluorescence assay with SYBR green I detection as described previously (48). Drugs were validated by regular IC_{50} assessment with laboratory strains Dd2 and 3D7, which yielded results similar to those seen previously (7). Drugs were prepared as 10 mM stocks in dimethyl sulfoxide or water and stored at −20°C. For assays, fresh drug stocks were prepared in complete media by diluting chloroquine to 20 μM; piperaquine, monodesethyl amodiaquine, and mefloquine to 4 μM; lumefantrine to 0.8 μM; and dihydroartemisinin, atovaquone, and pyronaridine to 0.4 μM. Drugs were serially diluted 3-fold in complete medium in 96-well microplates, with inclusion of drug-free control wells, to final volumes of 50 μl/well. Parasite culture was added for a total volume of 200 μl/well with a 0.2% parasitemia at 2% hematocrit. Plates were maintained under a gas mixture of 5% CO_2, 5% O_2, and 90% N_2 for 72 h at 37°C in a modular incubator (Billups Rothenberg, Del Mar, CA). Plates were frozen at −80°C and thawed, wells were mixed, and 100 μl from each well was transferred to a black 96-well plate containing 100 μl/well SYBR green lysis buffer (20 mM Tris buffer, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, and 0.2 μl/ml SYBR green I). Plates were incubated for 1 h in the dark at room temperature, and the fluorescence was measured with a FLUOstar Omega plate reader (BMG LabTech, Inc., Cary, NC;

485-nm excitation/530-nm emission). IC_{50}s were derived by plotting percent growth against log drug concentration and fitting the data by variable slope, sigmoidal curve fit in Prism 6.0 (GraphPad Software, San Diego, CA). DHA drug susceptibility was measured with an *ex vivo* ring stage survival assay as previously described (29).

Characterization of parasite polymorphisms. Parasite DNA was extracted from filter paper blood spots using Chelex-100, genes of interest were amplified with nested PCR, and polymorphisms in *pfcrt* and *pfmdr1* were evaluated using a ligase detection reaction-fluorescent microsphere assay, all as previously described (12, 49). *pfmdr1* copy number was determined in quadruplicate using a TaqMan real-time PCR assay with 3D7 and Dd2 strain standards, as previously described (12, 50). The *k13*-propeller-encoding domains (codons 440 to 726; PF3D7_1343700) (21) and an exonuclease gene (PF3D7_1362500) (24) were amplified and sequenced using previously described methods and primers. For the exonuclease gene, samples that failed the initial round of PCR were amplified using nested PCR with sequencing primers as previously described (24). Sequences were aligned with the 3D7 sequence (PlasmoDB.org) using MacVector v.15 (MacVector, Inc., Apex, NC). SNPs were confirmed by inspection of individual chromatograms. The *plasmepsin 2* copy number was quantified using a previously published quantitative PCR method (25). Amplification of *plasmepsin 2* was defined as $>$1.6 copies.

Statistical methods. Data analysis was performed using Stata v.14 (StataCorp LLC, College Station, TX). Significant differences between *ex vivo* IC_{50}s were characterized with *t* tests. Univariate analysis using generalized estimating equations with exchangeable correlations was used to determine significance of genotype changes over time. Associations between genotype and drug sensitivity were determined by comparing wild-type and mixed/mutant genotypes using *t* tests. Differences were considered significant at *P* values of $\leq$0.05.

Accession number(s). Nucleotide sequence data are available in the GenBank database under accession numbers MF477020 to MF477187.

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Concluding Remarks

The drugs used to treat uncomplicated falciparum malaria impact the drug sensitivities and genotypes of the malaria parasites infecting humans. Our results from Uganda show that sensitivity to the 4-aminoquinolines is returning while sensitivity to the arylaminoalcohols has decreased. The decreased sensitivity to the arylaminoalcohols does not appear to be clinically relevant. Importantly, there is no apparent resistance to ACTs. Having potent and efficacious drugs is crucial, so it is important to continue parasite surveillance efforts to track changing drug susceptibility and molecular markers of resistance among field isolates. This information will help determine if current and past drug therapies are becoming more or less potent over time, which will aid decision making on what drug therapies should be used. Parasite surveillance is a key method to managing the ever-present threat of antimalarial drug resistance.

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