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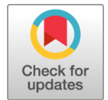

Changing Molecular Markers of Antimalarial Drug Sensitivity across Uganda

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ABSTRACT The potential spread of antimalarial drug resistance to Africa, in particular for artemisinins and key partner drugs, is a major concern. We surveyed *Plasmodium falciparum* genetic markers associated with drug sensitivity on 3 occasions at ~6-month intervals in 2016 and 2017 at 10 sites representing a range of epidemiological settings in Uganda. For putative drug transporters, we found continued evolution toward wild-type sequences associated with increased sensitivity to chloroquine. For *pfcrt* K76T, by 2017 the prevalence of the wild type was >60% at all sites and >90% at 6 sites. For the *pfmdr1* N86Y and D1246Y alleles, wild type prevalence ranged from 80 to 100%. We found low prevalence of *K13* propeller domain mutations, which are associated with artemisinin resistance in Asia, but one mutation previously identified in northern Uganda, 675V, was seen in 2.0% of samples, including 5.5% of those from the 3 northernmost sites. Amplification of the *pfmdr1* and *plasmepsin2* genes, associated elsewhere with decreased sensitivity to lumefantrine and piperaquine, respectively, was seen in <1% of samples. For the antifolate targets *pfdhfr* and *pfdhps*, 5 mutations previously associated with resistance were very common, and the *pfdhfr* 164L and *pfdhps* 581G mutations associated with higher-level resistance were seen at multiple sites, although prevalence did not clearly increase over time. Overall, changes were consistent with the selective pressure of the national treatment regimen, artemether-lumefantrine, with increased sensitivity to chloroquine, and with poor efficacy of antifolates. Strong evidence for resistance to artemisinins was not seen. Continued surveillance of markers that predict antimalarial drug sensitivity is warranted.

KEYWORDS Uganda, antimalarial drug sensitivity, molecular markers

Antimalarial drug resistance is a major concern. In Africa, resistance to chloroquine and antifolates has been widespread for many years (1). In Uganda, the standard therapy for uncomplicated malaria changed from chloroquine to chloroquine plus sulfadoxine-pyrimethamine (SP) in 2000 and then to the artemisinin-based combination therapy (ACT) artemether-lumefantrine in 2004, although implementation of the new regimen was slow (2). Many countries in Africa utilize the ACT artesunate-amodiaquine as first-line therapy. Dihydroartemisinin-piperaquine is an alternative ACT for uncomplicated malaria in some countries and is under evaluation for chemoprevention (3). Amodiaquine plus SP is used for seasonal malaria chemoprevention in parts of west and central Africa (4). SP is the standard for intermittent preventive therapy during pregnancy (IPTp) (5). Resistance to each component of these regimens has been

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detected (6) and the spread of resistance to Africa, in particular that to artemisinins and key partner drugs, may have devastating consequences.

Mechanisms of altered sensitivity to a number of antimalarial drugs are quite well understood. Mutations in genes encoding two putative drug transporters, *Plasmodium falciparum* multidrug resistance protein-1 (*pfmdr1*) and *P. falciparum* chloroquine resistance transporter (*pfcrt*), impact sensitivities to a number of drugs (1). Three mutations that have been common in Uganda, *pfcrt* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y (7), are selected by therapy with artesunate-amodiaquine (8, 9) and associated with decreased sensitivity to aminoquinolines (10). Wild-type sequences at these same alleles are selected by prior therapy with artemether-lumefantrine (11–13), associated with decreased lumefantrine sensitivity (10), and, in a pooled analysis, predicted recrudescence after treatment with artemether-lumefantrine (14).

P. falciparum resistance to artemisinin derivatives, defined as delayed parasite clearance either clinically (15) or in the *in vitro* ring stage survival assay (16), is now widespread in southeast Asia (17, 18). This phenotype is associated with a number of different mutations in the propeller domain of the kelch13 (*K13*) protein (19). To date, 20 *K13* propeller domain mutations in Asia have been associated with delayed clearance (20). *K13* mutations have also been seen in *P. falciparum* isolates from Africa; most of these differ from the mutations associated with delayed clearance, and, based on clinical, parasitological, and molecular data, artemisinin resistance has not clearly been identified in Africa (21–25). Resistance to ACT partner drugs is also of great concern. In southeast Asia resistance to mefloquine, mediated by increased *pfmdr1* copy number (26), and to piperaquine, mediated by increased *plasmepsin2* copy number (27, 28), has been noted. Furthermore, combined resistance to artemisinins and piperaquine that has led to very high rates of treatment failure for dihydroartemisinin-piperaquine in Cambodia is a concern (29, 30).

P. falciparum resistance to SP is mediated by mutations in the target dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes (31). The combination of three mutations in *pfdhfr* (51I, 59R, and 108N) and two in *pfdhps* (437G and 540E) leads to an intermediate level of SP resistance (32). This genotype is common in Uganda and other parts of Africa, although the *pfdhps* 540E mutation is absent in much of west and central Africa (33). Addition of either *pfdhfr* 164L or *pfdhps* 581G leads to higher-level SP resistance (31). These additional mutations have been uncommon in Africa, but some reports have noted moderate prevalence of the *pfdhfr* 164L mutation in parasites from southwestern Uganda (34–36) and of the *pfdhps* 581G mutation in parasites from Uganda and Tanzania (35–37).

Changes in treatment practices appear to have impacted antimalarial drug sensitivity in Africa. In the clearest demonstration of this phenomenon, nonuse of chloroquine in Malawi in the 1990s was followed by reversion of parasites to the wild-type *pfcrt* K76 genotype, with subsequent demonstration of improved chloroquine sensitivity *in vitro* (38) and *in vivo* (39). In Uganda, with increased use of artemether-lumefantrine and decreased use of chloroquine to treat malaria, parasite genotypes have been changing, with increased prevalence of *pfcrt* and *pfmdr1* wild-type alleles (7, 35). Recently, high prevalence of wild-type *pfcrt* K76 parasites and remarkable improvement in *in vitro* sensitivity to chloroquine was observed in Tororo in eastern Uganda (10). Changing parasite sensitivities appear to have clinical consequences, with the efficacy of artesunate-amodiaquine being better than that of artemether-lumefantrine in recent trials, a change from the results of trials conducted about a decade ago (21, 40). For antifolates, resistance-associated mutations are widespread, but SP remains the standard of care for IPTp, and there is concern for selection of more highly resistant parasites. With this background, careful surveillance for established markers of antimalarial drug resistance is a high priority, and so we surveyed the prevalence of key polymorphisms over time at 10 sites across Uganda.

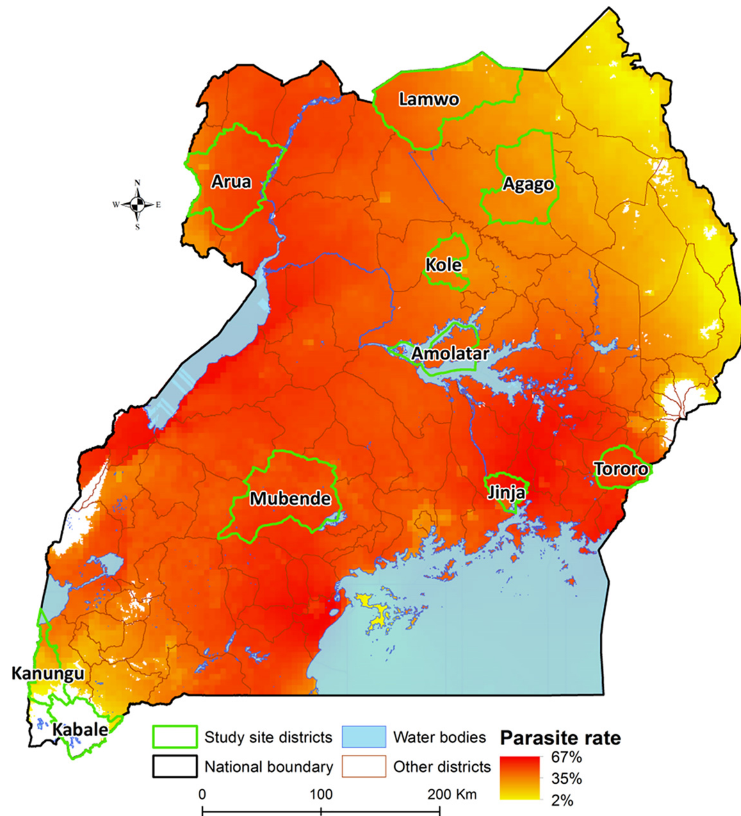

FIG 1 Surveillance sites in Uganda. The map shows study sites and estimated parasite prevalence for children 2 to 10 years of age. Estimates are based on community surveys between 1985 and 2010 under the Malaria Atlas Project. White locations have indeterminate prevalence based on insufficient data; these are primarily high-elevation areas with known low malaria transmission intensity.

RESULTS

Collection of samples. We set out to collect blood samples from 50 children presenting with malaria at each of 10 sites (Fig. 1) on 3 occasions during 2016 and 2017. A total of 1,459 samples were collected, as follows: 499 from April to June 2016 (collection 1), 491 from November 2016 to January 2017 (collection 2), and 469 from May to June 2017 (collection 3; Table S1); for Kabale, the third collection continued to October 2017 due to the low number of available samples. Fewer than 50 samples per collection were available from Kabale and Tororo during some collection periods due to low incidence of malaria (Table S1). Due to anticipated low prevalence of polymorphisms, sequencing of *K13* and assays for amplification of *pfmdr1* and *plasmepsin2* were only performed on samples from 2017; due to slow collection, these assays were not performed on samples from Kabale. Reported results are for samples that yielded data for at least one polymorphism.

Prevalence of drug resistance-mediating polymorphisms in putative transporters. The prevalence of key *pfcrt* and *pfmdr1* polymorphisms varied across the country, but the temporal trends were similar (Fig. 2). For the *pfcrt* K76T polymorphism, for which the mutant is associated with resistance to chloroquine, wild-type prevalence was higher than that reported in earlier surveys performed at 3 of the sites (7, 35), and this prevalence increased over time, such that, in 2017, >60% of samples were wild type at all sites and >90% were wild type at 6 of the sites. For *pfmdr1* N86Y and *pfmdr1* D1246Y, 80 to 100% of parasites were wild type at all study sites. As seen previously, the *pfmdr1* Y184F allele was polymorphic, but the prevalence of wild-type alleles did not change notably over time.

Prevalence of K13 polymorphisms. Of the 412 samples with successful sequencing of the *K13* propeller domain, 25 carried nonsynonymous polymorphisms, with 7

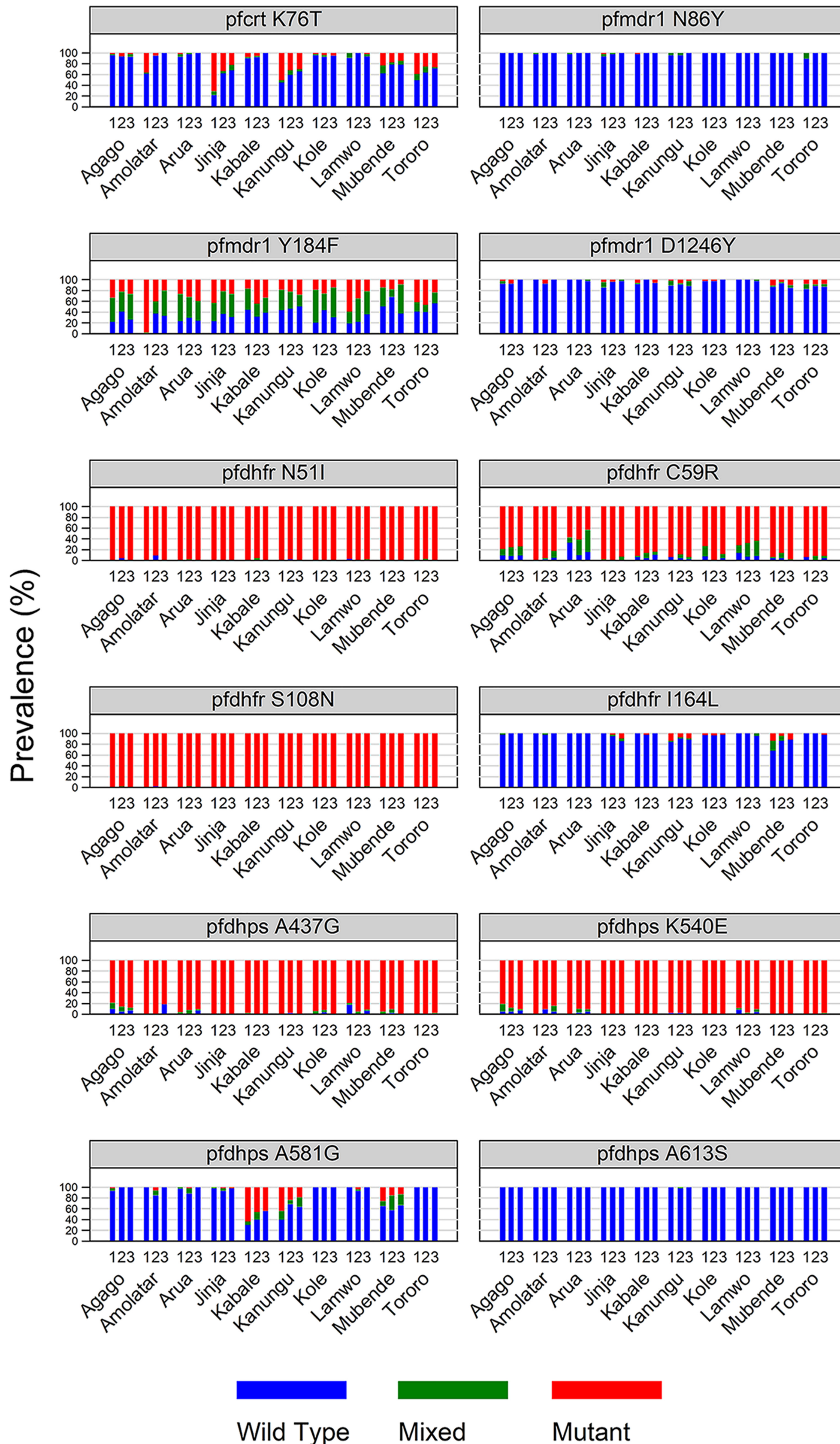

FIG 2 Prevalence of wild-type, mixed, and mutant alleles at the named sites over the indicated survey periods. The numbers above the site names represent survey periods (1, April 2016 to June 2016; 2, November 2016 to January 2017; 3, May 2017 to June 2017).

TABLE 1 Prevalence of K13 and copy number polymorphisms at the different sites in 2017

Site	N[a]	K13 candidate artemisinin resistance markers						Increased copy number		
		PfK13 A675V			*PfK13* C469Y					
		Wild type (%)	Mixed (%)	Mutant (%)	Wild type (%)	Mixed (%)	Mutant (%)	N	*pfmdr1* (%)	*plasmepsin2* (%)
Agago	42	40 (95.2)	1 (2.4)	1 (2.4)	40 (95.2)	1 (2.4)	1 (2.4)	30	2 (6.7)	1 (3.3)
Amolatar	48	48 (100)	0	0	48 (100)	0	0	41	0	0
Arua	43	42 (97.7)	0	1 (2.3)	43 (100)	0	0	37	0	0
Jinja	48	48 (100)	0	0	48 (100)	0	0	37	0	1 (2.7)
Kanungu	48	48 (100)	0	0	48 (100)	0	0	45	0	0
Kole	47	46 (97.9)	0	1 (2.1)	46 (97.9)	0	1 (2.1)	31	0	0
Lamwo	43	39 (90.7)	0	4 (9.3)	41 (95.4)	1 (2.3)	1 (2.3)	35	0	0
Mubende	45	45 (100)	0	0	44 (97.8)	1 (2.2)	0	39	0	0
Tororo	48	48 (100)	0	0	48 (100)	0	0	40	0	0

[a]*N*, number of samples successfully evaluated.

different mutations observed (Tables S2 and S3). Two single-nucleotide polymorphisms (SNPs) had a prevalence of >1%, A675V (2.0%) and C469Y (1.4%); both alleles were seen primarily at sites in northern Uganda (Table 1). The 675V mutation was seen in 7/128 (5.5%) samples from the 3 northernmost sites. The most common mutation previously reported in Africa, 578S, was seen in 3 (0.7%) samples.

Prevalence of parasites with increased *pfmdr1* and *plasmepsin2* gene copy numbers. Of 335 samples successfully assessed for copy number variation, only 2 (0.6%), both from Agago, had an increased *pfmdr1* copy number and 2 (0.6%; one each from Agago and Jinja) had increased *plasmepsin*2 copy numbers (Table 1). Thus, consistent with prior reports (10), increased *pfmdr1* and *plasmepsin2* copy numbers were uncommon in Ugandan isolates.

Prevalence of drug resistance-mediating polymorphisms in folate pathway enzymes. As seen previously (7, 35), the prevalences of 5 mutations in *pfdhfr* (51I, 59R, and 108N) and *pfdhps* (437G and 540E) were high across Uganda (Fig. 2). Additional mutations associated with higher-level antifolate resistance (*pfdhfr* 164L and *pfdhps* 581G) were seen, with the prevalence of mixed or mutant *pfdhps* 581G at ~25 to 60% at sites in central and southwestern Uganda. However, the prevalences of *pfdhfr* 164L and *pfdhps* 581G mutant parasites did not increase over time at most sites.

DISCUSSION

In Uganda, treatment of malaria primarily with artemether-lumefantrine for the last decade has been associated with marked changes in *P. falciparum* genetic markers associated with drug sensitivity. To gain insight into recent trends across the country, we performed surveillance for relevant markers on 3 occasions in 2016 and 2017 at 10 sites representing a range of epidemiological settings. We found continued evolution toward wild-type transporter sequences, low prevalence of *K13* mutations or amplified *pfmdr1* or *plasmepsin2*, and high prevalence of antifolate mutations. These results suggest increasing sensitivity of *P. falciparum* to chloroquine, a lack of resistance to artemisinins or major ACT partner drugs, and continued poor antimalarial efficacy of SP. Thus, in Uganda, *P. falciparum* appears to remain sensitive to the ACTs available to treat malaria, but the utility of antifolates to prevent malaria is in question.

The current evolution of transporter gene sequences in Uganda is not surprising. In Malawi, withdrawal of chloroquine in the 1990s was followed by increased prevalence of parasites with the wild-type *pfcrt* K76 allele, accompanied by improved chloroquine sensitivity (38, 39). Similar changes have been documented in other African countries, including Kenya (41) and Tanzania (42). In Uganda, reversion to wild-type *pfcrt* K76 and *pfmdr1* N86 and D1246 alleles was initially slow following chloroquine withdrawal, perhaps due to continued usage of chloroquine in the community and reasonably strong fitness of chloroquine-resistant parasites. Prior analyses showed a <10% prevalence of parasites with wild-type *pfcrt* K76 until 2012 in Tororo (7), but a steady increase in prevalence of the wild type in Tororo and two other sites since that time

(35). Reversion to wild-type *pfmdr1* N86 and D1246 alleles was also seen, with changes more rapid than those for *pfcrt* K76T (7, 35). Our new results show continued selection of parasites that have wild-type sequences at key transporter alleles. Consistent with this finding, parasites collected in Tororo demonstrated increasing chloroquine *ex vivo* sensitivity over time (10). Remarkably, recent results suggest that chloroquine may soon again be a highly effective antimalarial in Uganda, although widespread use would likely reselect for resistant parasites.

Resistance to artemisinins, manifested as delayed parasite clearance after therapy or *in vitro*, is associated with polymorphisms in the *K13* gene. A total of 20 different *K13* propeller domain mutations have been associated with delayed clearance in southeast Asia (20), with resistance documented across the Greater Mekong Subregion (17, 18). In Africa, delayed clearance after therapy with ACTs (43) or when measured *in vitro* (22) appears to be very uncommon. Multiple *K13* mutations have been seen at low prevalence in African parasites, but many, including the most common polymorphism reported in Africa, A578S, have not been associated with delayed clearance (20). One mutation that has been associated with delayed clearance in southeast Asia, 675V, was seen in 2.0% of our study samples. This mutation was also noted in one sample collected in Rwanda in 2015 (44) and in one sample that showed delayed clearance *in vitro* in a recent study from northern Uganda (45). The clinical significance of this finding is uncertain, but in our study the polymorphism was geographically clustered in northern Uganda.

Amplification of *pfmdr1* has been associated with decreased sensitivity of *P. falciparum* to mefloquine (26) and lumefantrine (46) and amplification of *plasmepsin2* with decreased sensitivity to piperaquine (27, 28). Amplification of *pfmdr1* (47, 48) and *plasmepsin2* (10) has been uncommon in previous studies from Uganda, as also seen in our new results. These results are reassuring, as they suggest continued strong efficacy of important ACT partner drugs, consistent with excellent efficacy for leading ACTs in recent trials (21, 49).

SP was abandoned as a treatment for malaria due to widespread resistance in *P. falciparum*, mediated by well-characterized mutations in the *pfdhfr* and *pfdhps* genes (31), however, SP remains the standard of care for IPTp in areas of Africa where malaria is endemic (5). SP is also increasingly used for seasonal malaria chemoprophylaxis, whereby treatment courses of SP plus amodiaquine are provided monthly during the rainy season in parts of west and central Africa (4). We found that all 5 *pfdhfr* and *pfdhps* mutations commonly associated with SP resistance in Africa remain widespread in Uganda. In addition, the *pfdhfr* 164L and *pfdhps* 581G mutations, which predict higher-level resistance, were seen in samples from many sites. These results suggest that, in Uganda, the antimalarial efficacy of SP for IPTp or other indications is poor, consistent with results of recent clinical studies (5, 36, 50). Furthermore, while SP plus amodiaquine appears to be efficacious for malaria chemoprevention in areas where the *pfdhps* 540E mutation is absent (4), this regimen is unlikely to be effective in Uganda. Other regimens for chemoprevention, in particular the ACT dihydroartemisinin-piperaquine, are under study for intermittent preventive therapy (IPT) in pregnancy (50, 51) and in children (3, 52). Our data suggest continued good antimalarial activity of dihydroartemisinin-piperaquine in Uganda, although loss of activity of this regimen in Cambodia (29, 30) is concerning.

Our study had important limitations. First, we studied convenience samples collected across Uganda; we cannot be sure that our results are representative of all parasites from the study areas. Second, we assessed only polymorphisms already associated with resistance to antimalarials. Additional genetic changes in *P. falciparum* likely impact sensitivity to various antimalarial agents. Although it is difficult to identify new resistance mediators in highly diverse clinical isolates, broader deep sequencing approaches should shed light on additional polymorphisms contributing to drug sensitivity. Third, for some uncommon markers we only evaluated the most recent available samples, so were unable to characterize temporal trends.

In summary, in studies from a range of sites in Uganda, we identified consistent

changes in *P. falciparum* genetic markers associated with drug sensitivity over time. Importantly, markers associated with resistance to artemisinins or key ACT partner drugs were not seen. Markers indicating resistance to antifolates had continued high prevalence. These findings suggest that continued use of leading ACTs to treat malaria in Uganda is warranted, but that continued surveillance for markers associated elsewhere with ACT resistance is a high priority.

MATERIALS AND METHODS

We selected 10 sites to represent different regions of Uganda with varied malaria transmission intensity and epidemiology (Fig. 1). At each site, as part of routine care, health care personnel evaluated children 6 months to 10 years of age with clinical syndromes suggestive of malaria using either Giemsa-stained blood smears or histidine-rich protein 2 (HRP2)-based rapid diagnostic tests, following national guidelines and depending on local availability of these tests. Consecutive children diagnosed with malaria and their parents or guardians were approached for enrollment, and, if consent was obtained, blood was collected as 4 blood spots dried on Whatman 3MM filter paper. Filter paper samples were stored in zipper storage bags with desiccant at room temperature and transported to our laboratory in Kampala for evaluation. This study was approved by the Makerere University Research and Ethics Committee, the Uganda National Council of Science and Technology, and by the University of California, San Francisco Committee on Human Research.

Genomic DNA was extracted from blood spots using Chelex 100, as previously described (53). *Pfmdr1, pfcrt, pfdhfr*, and *pfdhps* polymorphisms of interest were characterized by PCR and ligase detection reaction-fluorescent microsphere assays, as previously described (54), with minor modification to incorporate nested PCR (48). Copy number variations for *pfmdr1* and *plasmepsin2* were assessed by quantitative PCR (qPCR), and the *K13* propeller domain was amplified and sequenced, all as previously described (10).

Data availability. Nucleotide sequences are available in the GenBank database under the accession numbers MH788997 to MH789408.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01818-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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