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Varied Prevalence of Antimalarial Drug Resistance Markers in Different Populations of Newly Arrived Refugees in Uganda

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Newly arrived refugees offer insights into malaria epidemiology in their countries of origin. We evaluated asymptomatic refugee children within 7 days of arrival in Uganda from South Sudan and the Democratic Republic of Congo (DRC) in 2022 for parasitemia, parasite species, and *Plasmodium falciparum* drug resistance markers. Asymptomatic *P. falciparum* infections were common in both populations. Coinfection with *P. malariae* was more common in DRC refugees. Prevalences of markers of aminoquinoline resistance (PfCRT K76T, PfMDR1 N86Y) were much higher in South Sudan refugees, of antifolate resistance (PfDHFR C59R and I164L, PfDHPS A437G, K540E, and A581G) much higher in DRC refugees, and of artemisinin partial resistance (ART-R; PfK13 C469Y and A675V) moderate in both populations. Prevalences of most mutations differed from those seen in Ugandans attending health centers near the refugee centers. Refugee evaluations yielded insights into varied malaria epidemiology and identified markers of ART-R in 2 previously little-studied countries.

Keywords. malaria; drug resistance; refugees; PfCRT; PfMDR1; PfDHFR; PfDHPS; PfK13; Uganda.

Malaria remains a major burden, with approximately 95% of cases and deaths in Africa, where infection with *Plasmodium falciparum* predominates [1]. Malaria control includes use of artemisinin-based combination therapies to treat malaria and the administration of drugs to prevent malaria in high-risk groups, notably sulfadoxine-pyrimethamine (SP) during pregnancy and for intermittent preventive therapy in infants, and SP plus amodiaquine during the transmission season in areas with seasonal malaria [2]. The control of malaria is challenged by worsening resistance to available drugs [3]. Resistance to aminoquinolines, mediated by mutations in the drug transporters PfCRT (K76T) and PfMDR1 (N86Y and D1246Y), is widespread, but decreasing since discontinuation of chloroquine to treat malaria early this century [4]. Resistance to SP, mediated by mutations in the target enzymes dihydrofolate reductase (PfDHFR) and dihydropteroate synthase (PfDHPS), is common, with increasing prevalence of mutations mediating high-level resistance in some areas [4, 5]. Partial resistance to artemisinins was first noted in southeast Asia [6] and has recently been identified in Africa, with independent emergences of parasites expressing multiple mutations in the PfK13 protein that were previously associated with resistance [7]. In Uganda, 5 different PfK13 propeller domain mutations that are validated or candidate resistance mediators have emerged, with spread of 2 mutations, C469Y and A675V, from northern Uganda to other parts of the country [8].

Among populations at greatest risk of malaria are those in conflict zones, where malaria control measures may not be sufficient. Large numbers of refugees from conflict zones are resettled in refugee camps, but studies of malaria in newly arrived individuals have been limited. In a scoping review of refugee populations that departed malaria endemic areas, risks for malaria infection, which was often asymptomatic, were high [9]. In a study of adult refugees from sub-Saharan Africa settled in a nonendemic region of South Africa, about 6% had malaria parasitemia [10].

Uganda is the largest refugee hosting country in sub-Saharan Africa [11, 12]. In the first half of 2023, approximately 48 000 refugees were settled in Uganda, with the largest numbers from South Sudan (19 009) and Democratic Republic of Congo (DRC; 17 693) (https://data.unhcr.org/es/dataviz/68). A recent study found the prevalence of malaria parasitemia

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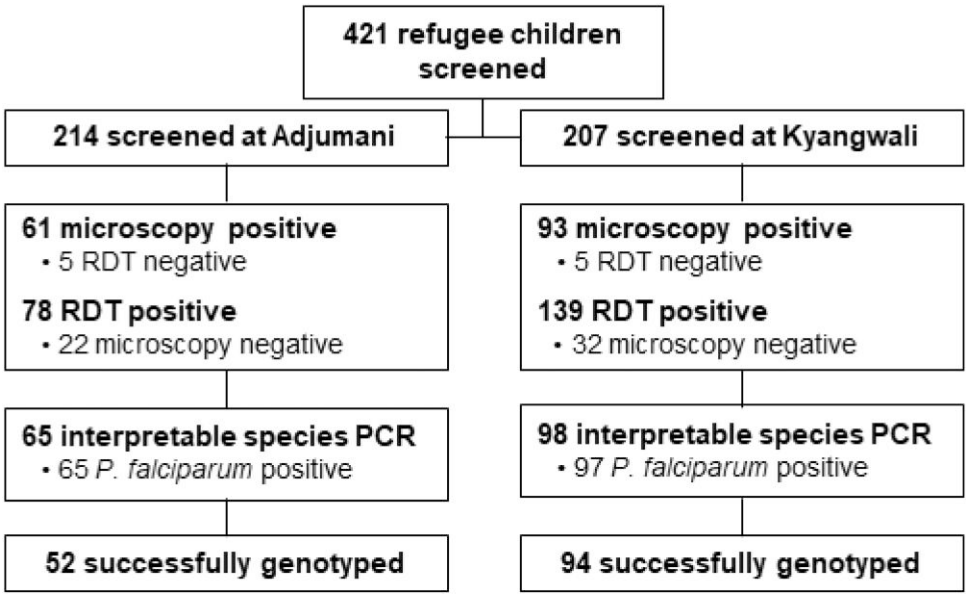

Figure 1. Study flow chart. Abbreviations: PCR, polymerase chain reaction; RDT, rapid diagnostic test.

to be 36.6% in Ugandan refugees, with wide variation between 9 sites [12]. Testing of incoming refugees for malaria parasitemia is only performed when they present with illness. As asymptomatic parasitemia is common in populations living in endemic areas, refugees may frequently harbor parasites, leading to risks of progression to clinical illness and importation of drug-resistant parasites that may infect others. To characterize risks in Uganda, we assessed parasites infecting newly arrived refugee children that originated in 2 neighboring countries.

METHODS

Study Design and Population

We evaluated all newly arrived refugee children aged 0.5–10 years who were registered at reception centers at Adjumani and Kyangwali refugee camps from June 1 to 31 August 2022. Children were enrolled if parents/guardians (1) reported no history of fever in the last 14 days, (2) reported arriving in Uganda within the past 7 days, and (3) provided informed consent (including assent for children over 7 years of age) to participate in the study. These studies were approved by the Makerere University School of Biomedical Sciences Research and Ethics Committee (SBS-2022–132) and the Uganda National Council for Science and Technology (HS2164ES).

Sample and Data Collection

Eligible children were enrolled (Figure 1), and a finger prick blood sample was used to prepare thick smears for microscopy, to perform a malaria rapid diagnostic test (RDT; SD Bioline-Pf/Pan-25), and for storage on filter paper (Whatman 3MM) for molecular analyses. A questionnaire was administered to collect information on age, sex, and country of origin. For comparison of prevalences of drug resistance markers between refugees and Ugandans living nearby, we utilized data from surveillance studies conducted in June 2022 among patients who presented with uncomplicated malaria at nearby health centers, as previously described [8, 13].

Species Determination

For all microscopy- or RDT-positive samples, genomic DNA was extracted using Chelex, as previously described [14]. For species determination, we performed nested polymerase chain reaction (PCR) with primers specific for plasmodial 18S small subunit ribosomal RNA genes and then species-specific primers, as previously described [15]. All reactions included negative (water) and positive (3D7 genomic DNA) controls. Sizes of amplicons were identified using a QIAxcel capillary electrophoresis instrument (Qiagen). Samples with indeterminate or negative results were repeated with twice the DNA template.

Drug Resistance Genotyping

Genomic DNA from all microscopy- or RDT-positive samples was studied using molecular inversion probe (MIP) technology to sequence *pfcrt, pfmdr1, pfdhfr, pfdhps,* and *pfk13,* as previously described [5, 16]. For samples with negative or indeterminate MIP genotyping results, we performed dideoxy sequencing of *pfk13* [5] and ligase detection reaction-fluorescent microsphere analysis of resistance-associated mutations in *pfmdr1, pfcrt, pfdhfr,* and *pfdhps*, as previously described [17]. Mixed genotypes at any locus were categorized as mutant.

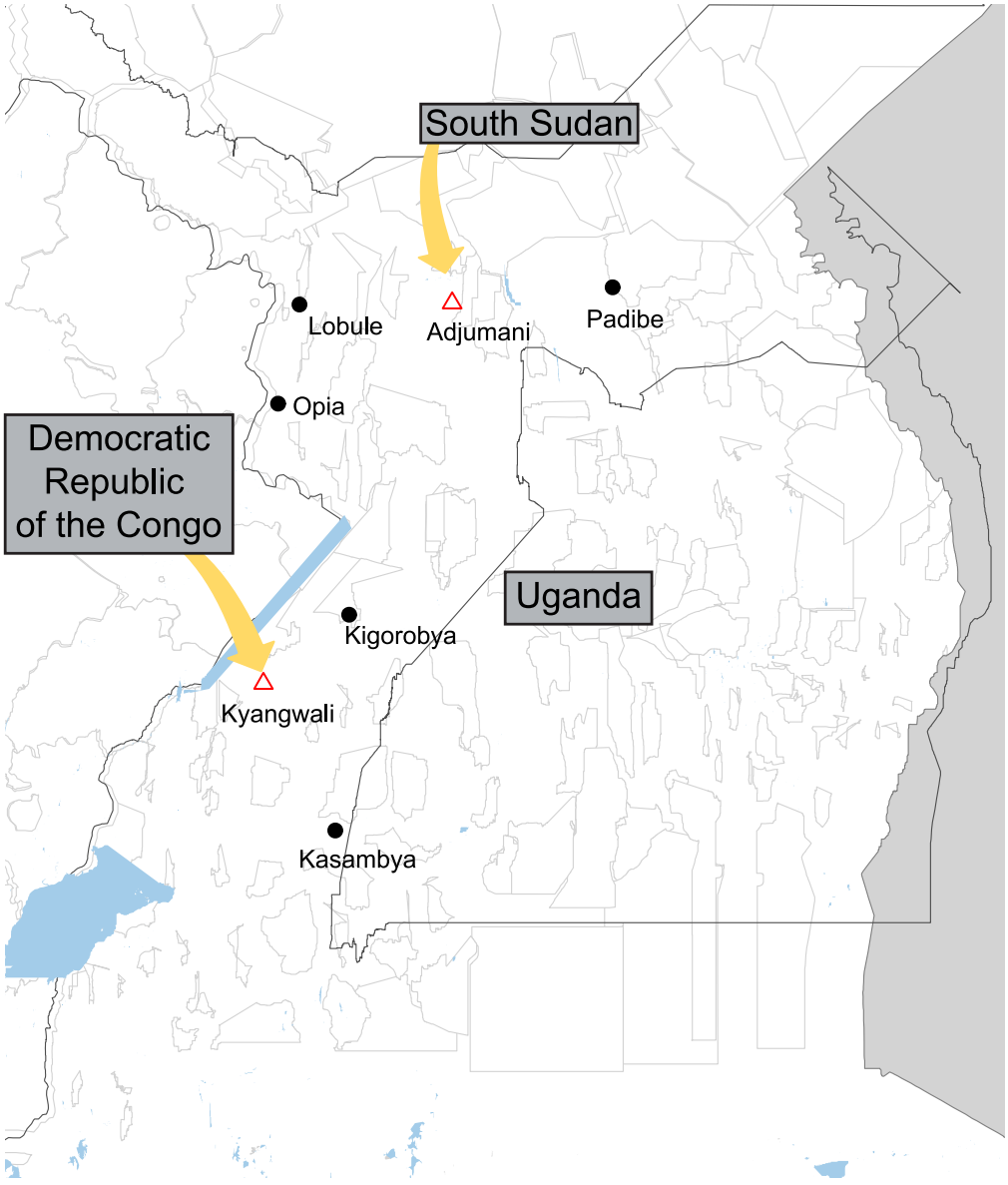

Figure 2. Populations studied. Locations of the 2 refugee camps and nearby health centers that provided samples for this study.

Genetic Relatedness

To assess *pfk13* haplotypes, we genotyped 7 microsatellites flanking *pfk13*, as previously described [8]. Relatedness was evaluated in samples that had genotypes called for at least 6 of the 7 loci and had only 1 microsatellite allele called for each locus. A neighbor-joining tree was inferred from a distance matrix generated in the R package polysat [18].

Data Analysis

Analysis was done in RStudio using paired *t* tests, Fisher exact tests, or Wilcoxon tests, depending on data distribution. A *P* value ≤.05 was considered statistically significant.

RESULTS

Study Populations

We studied samples from 214 refugee children from South Sudan who were processed at Adjumani, in northwestern Uganda, and 207 refugee children from DRC who were processed at Kyangwali, in western Uganda, in June to August 2022 (Figure 2). Characteristics of the populations are shown in Table 1. All the refugees had entered Uganda within 7 days prior to enrollment, such that circulating malaria parasites can be considered to have been acquired in their home countries. For comparison with local populations, we assessed data from 494 subjects presenting with symptomatic malaria at nearby

health centers, including 98 from Opia, Arua District; 100 from Lobule, Koboko District; and 100 from Padibe, Lamwo District, all near Adjumani; and 96 from Kigorobya, Hoima District and 100 from Kasambya, Mubende District, both near Kyangwali (Figure 2) [8].

Malaria Parasitemia in Refugees

The prevalence of malaria parasitemia was high at both refugee camps: 36.4% and 28.5% at Adjumani and 67.1% and 44.9% at Kyangwali by RDT and microscopy, respectively (Table 1). Prevalences by RDT were greater, consistent with the greater sensitivity of this diagnostic test. Considering *Plasmodium* species, the majority of infections were with *P. falciparum*, but infections with *P. malariae* and *P. ovale* were also seen, in nearly all cases as mixed infections with *P. falciparum* (Table 2). *P. malariae* infection, always seen as coinfection with *P. falciparum,* was significantly more common in samples from Kyangwali (20.4%) compared to Adjumani (1.5%, $P < .001$).

Table 1. Characteristics of Study Participants

Variable	Adjumani	Kyangwali
No. of children screened	214	207
Microscopy-positive samples, No. (%)	61 (28.5)	93 (44.9)
RDT-positive samples, No. (%)	78 (36.4)	139 (67.1)
Microscopy-positive/RDT-negative samples	5	5
Microscopy-negative/RDT-positive samples	22	32
Among microscopy-positive subjects		
Age, y, mean (SD)	5.3 (2.7)	4.8 (2.8)
Days in Uganda, median (IQR)	2.5 (2.0–3.5)	1.0 (1.0–4.0)
Sex, No. (%)		
Male	27 (44.3)	57 (61.3)
Female	34 (55.7)	36 (38.7)
Country of origin	South Sudan	DRC
Parasite density per µL, geometric mean	4441	11 851

Abbreviations: DRC, Democratic Republic of Congo; IQR, interquartile range; RDT, rapid diagnostic test.

Comparison of Prevalences of Drug Resistance Markers Between Refugee Populations

The prevalences of key resistance markers varied greatly between the 2 refugee populations (Figure 3 and Table 3). Prevalences of mutations that mediate resistance to the aminoquinolines chloroquine and amodiaquine (PfCRT K76T and PfMDR1 N86Y) were much higher in samples from Adjumani than Kyangwali. In contrast, prevalences of 3 common mutations that mediate resistance to SP (PfDHFR C59R, PfDHPS A437G, and K540E), and in addition the PfDHFR I164L and PfDHPS A581G mutations, which mediate high-level resistance, were much higher in samples from Kyangwali. PfK13 mutations that have been

Table 2. Species Identified in Study Participants With Parasitemia

Species	Adjumani	Kyangwali
P. falciparum	60 (92.3)	72 (73.5)
P. falciparum, P. malariae	1 (1.5)	12 (12.2)
P. falciparum, P. ovale	4 (6.2)	5 (5.1)
P. falciparum, P. ovale, P. malariae	0 (0)	8 (8.2)
P. ovale	0 (0)	1 (1.0)

Data are No. (%).

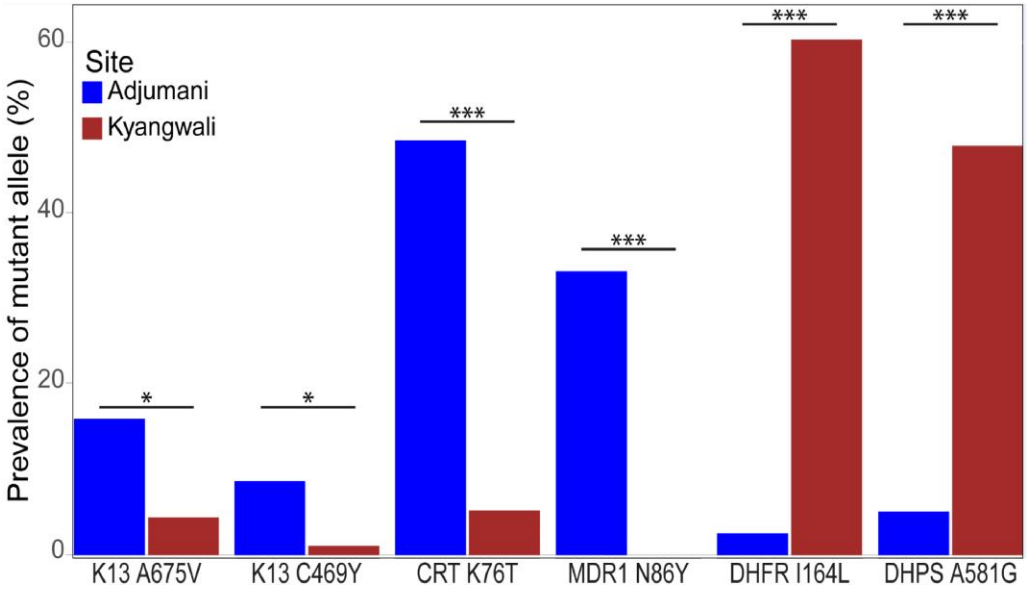

Figure 3. Comparative prevalences of key mutations in newly arrived refugees at 2 refugee camps. Prevalences of selected mutations are shown; full data are in Supplementary Table 3. $*P \leq .05$, $***P < .001$.

Table 3. Prevalence of Drug Resistance Mutations

Genotype		Refugee Camps						
		Adjumani	Kyangwali	*P*	Northern Uganda Health Facilities	*P*	Western Uganda Health Facilities	*P*
PfCRT K76T	WT	20 (51.3)	73 (94.8)	<.001	129 (87.2)	<.001	129 (100)	.018
	Mut	19 (48.7)	4 (5.2)		19 (12.8)		0 (0)	
PfMDR1 N86Y	WT	26 (66.7)	77 (100)	<.001	169 (100)	<.001	149 (100)	1.0
	Mut	13 (33.3)	0 (0)		0 (0)		0 (0)	
PfMDR1 Y184F	WT	12 (30.8)	25 (32.1)	1.0	65 (34.4)	.714	68 (41.7)	.160
	Mut	27 (69.2)	53 (67.3)		124 (65.6)		95 (58.3)	
PfMDR1 D1246Y	WT	35 (89.7)	78 (98.7)	.040	133 (99.3)	.010	120 (100)	.397
	Mut	4 (10.3)	1 (1.3)		1 (0.7)		0 (0)	
PfDHFR N51I	WT	1 (2.6)	0 (0)	.336	0 (0)	ND	0 (0)	ND
	Mut	38 (97.3)	77 (100)		184 (100)		157 (100)	
PfDHFR C59R	WT	14 (35.9)	1 (1.3)	<.001	11 (6.0)	<.001	0 (0)	.329
	Mut	25 (64.1)	76 (98.7)		173 (94.0)		157 (100)	
PfDHFR S108N	WT	0 (0)	0 (0)	ND	0 (0)	ND	0 (0)	ND
	Mut	39 (100)	75 (100)		169 (100)		112 (100)	
PfDHFR I164L	WT	38 (97.4)	30 (39.0)	<.001	133 (85.3)	.053	73 (58.4)	.009
	Mut	1 (2.6)	47 (61.0)		23 (14.7)		52 (41.6)	
PfDHPS A437G	WT	11 (28.9)	0 (0)	<.001	5 (3.1)	<.001	1 (0.7)	1.0
	Mut	27 (71.1)	77 (100)		158 (96.9)		147 (99.3)	
PfDHPS K540E	WT	15 (38.5)	0 (0)	<.001	8 (4.5)	<.001	0 (0)	1.0
	Mut	24 (61.5)	76 (100)		169 (95.5)		154 (100)	
PfDHPS A581G	WT	37 (94.9)	41 (51.9)	<.001	170 (92.4)	.744	112 (69.6)	.010
	Mut	2 (5.1)	38 (48.1)		14 (7.6)		49 (30.4)	
PfDHPS A613S	WT	39 (100)	78 (100)	ND	171 (100)	ND	118 (100)	NA
	Mut	0 (0)	0 (0)		0 (0)		0 (0)	
PfK13 469Y	WT	46 (92.0)	89 (98.9)	.055	158 (89.8)	.79	148 (100)	.378
	Mut	4 (8.0)	1 (1.1)		18 (10.2)		0 (0)	
PfK13 675V	WT	44 (84.6)	87 (95.6)	.030	161 (91.0)	.202	126 (82.9)	.004
	Mut	8 (15.4)	4 (4.4)		16 (9.0)		26 (17.1)	

Data are No. (%).

Results for northern (Lobule, Opia, and Padibe) and western (Kasambya and Kigorobya) health facilities represent means for the different study sites. WT and Mut prevalences are shown. *P* values for northern health facilities represent comparisons with Adjumani and those for western health facilities represent comparisons with Kyangwali.

Abbreviations: Mut, mutant/mixed; ND, *P* values could not be determined; NA, not applicable; WT, wild type.

associated with artemisinin partial resistance in Uganda [8] were seen in both refugee populations; the prevalence of both the C469Y and A675V mutations was greater in samples from Adjumani than in samples from Kyangwali. Prevalences for many of the mutations studied differed greatly between the refugee camp populations and Ugandan patients presenting with malaria at nearby sites (Table 3).

Relatedness of Parasites With Artemisinin Partial Resistance Markers

We characterized microsatellites flanking *pfk13* and used neighbor-joining trees to compare relatedness with samples from Ugandan populations (Figure 4). As previously described [8], mutant parasites from Uganda generally showed distinct haplotypes associated with each mutation, consistent with a singular origin for PfK13 C469Y and a predominant lineage for most A675V mutants. All C469Y alleles in refugee samples clustered within the Ugandan C469Y clade, indicating a shared origin. For A675V, 5 of 7 alleles in refugee samples clustered within the primary Ugandan A675V clade. Secondary clades were seen for some A675V mutant samples, suggesting that this allele may have multiple origins or may have been circulating for an extended period.

DISCUSSION

We studied malaria parasites infecting refugees who had recently arrived in Uganda from South Sudan and DRC. Asymptomatic refugee children from both countries were commonly parasitemic. As the *Plasmodium* life cycle requires over a week from an infectious mosquito bite to the development of parasitemia, we can be confident that the infections in refugees were acquired in their countries of origin. Remarkably, the prevalences of multiple mutations associated with drug resistance differed greatly between South Sudan and DRC refugee populations. These data offer important information on resistance prevalence and other features of malaria in difficult-to-study conflict regions.

Refugees at the Adjumani camp, originating from South Sudan, had high prevalence of transporter mutations associated

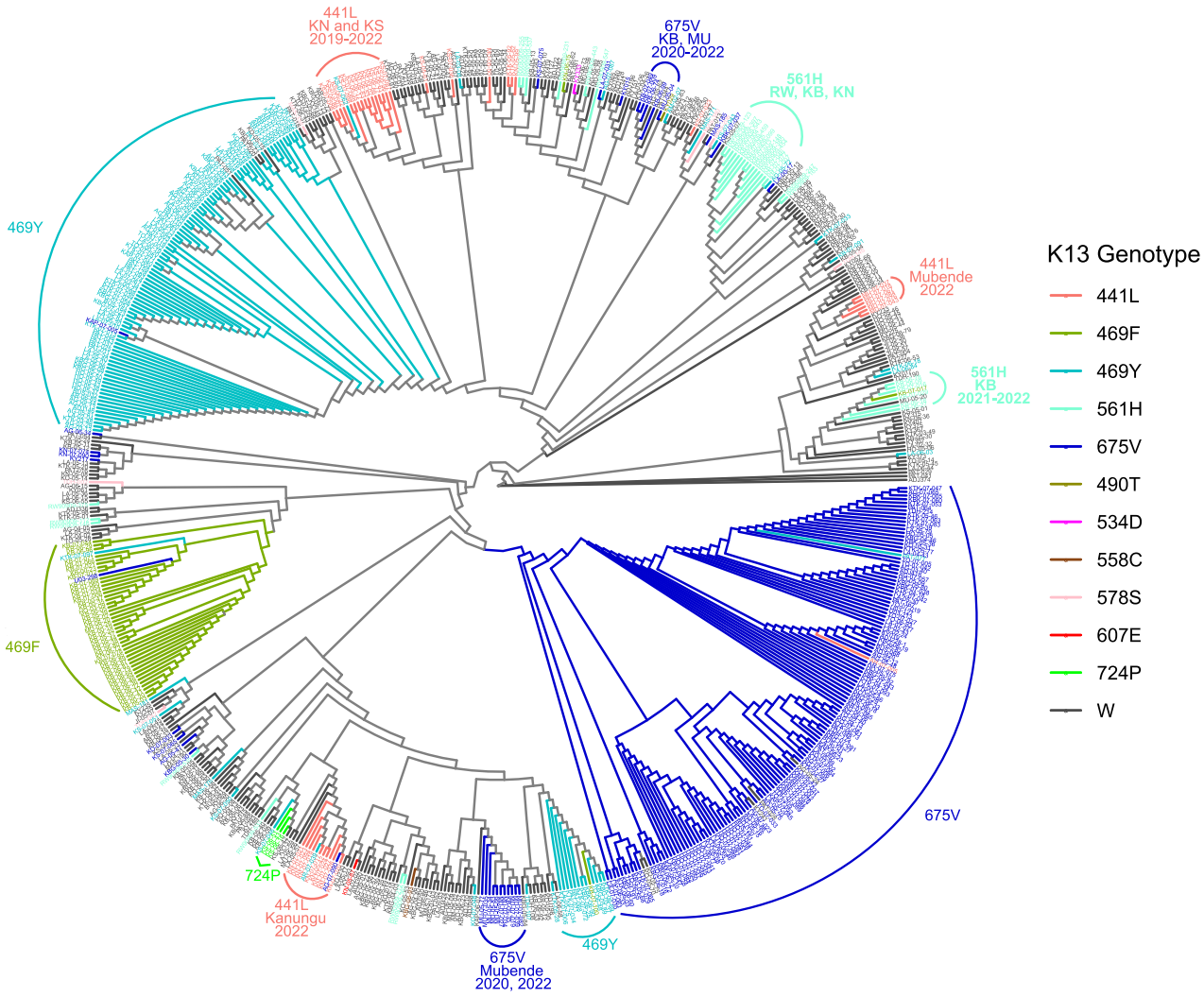

Figure 4. Genetic relatedness of *pfk13* haplotypes from refugee children and Ugandan populations. Samples from Ugandan populations (malaria reference centers [MRC] samples) were collected from 16 health facilities across Uganda from 2016 to 2022. Data were filtered to include only monoallelic samples with data for at least 6 of the 7 microsatellite loci, omitting samples with mixed genotypes and/or missing data at more than 1 locus. Abbreviation: WT, wild type.

with resistance to chloroquine and amodiaquine. These mutations were previously highly prevalent across Africa, but prevalence decreased greatly after discontinuation of chloroquine-based treatment regimens [4]. Prevalences of the key PfCRT K76T and PfMDR1 N86Y mutations are now very low in many areas of Africa, but prevalences were higher in refugees from South Sudan compared to those from DRC, perhaps because artesunate-amodiaquine is the first-line treatment for uncomplicated malaria in South Sudan, while in Uganda and most nearby countries, the first-line therapy is artemether-lumefantrine [1]. In contrast, refugees originating in South Sudan had relatively low prevalence of the PfDHFR I164L and PfDHPS A581G mutations, which mediate high-level resistance to SP, a key component of malaria prevention regimens. Prevalence of these 2 mutations was previously shown to be high in eastern DRC [16] and in regions of Uganda bordering DRC [5]. Results from refugees who recently departed DRC were consistent with this trend, with remarkably high prevalence of the I164L and A581G mutations.

Of particular interest is artemisinin partial resistance, which has emerged in multiple foci in eastern Africa. Six validated or candidate PfK13 mutations (P441L, C469F, C469Y, R561H, R622I, and A675V) initially associated with artemisinin partial resistance in Asia have emerged in eastern Africa. However, limited studies from DRC or South Sudan have either not identified validated/candidate PfK13 mutations [7], or, in 1 report for samples collected in DRC in 2020–2021, seen them at very low prevalence (P441L and R561H, each at <1% prevalence) [19]. Our data show that the mutations that are now at high prevalence across much of northern Uganda, C469Y

and A675V [8], are also present at moderate prevalence in South Sudan and in DRC. Thus, our study of refugees has expanded the map of regions of Africa with noteworthy prevalence of molecular markers associated with artemisinin partial resistance.

We also used molecular assays to evaluate for plasmodial species. We found moderate prevalence of *P. ovale* and *P. malariae* infections, almost always as mixed infections with *P. falciparum*, and with greater prevalence of *P. malariae* infections in refugees originating from DRC.

This study had a number of limitations. First, its budget and scope limited assessments to fairly small numbers of refugee children at 2 camps. Assessment of larger populations of refugees from multiple countries will improve our understanding of malaria infections across eastern Africa, and this is planned. Second, information on the places of origin of refugees was limited to the country. Thus, the specific prevalence of mutations of interest in any particular region of South Sudan or DRC is unknown. More detailed data from refugees and/or studies conducted in the countries of interest will be required to better characterize malaria across these countries, and to explain the remarkable differences in prevalences between refugee populations. Third, evaluations were limited to a small panel of known resistance markers. Broader consideration of potential markers in these populations will be informative. Fourth, the percentage of successfully genotyped samples was greater for samples from Kyangwali compared to those from Adjumani, likely because parasite densities were higher in the Kyangwali samples. Fifth, due to logistical constraints, mutation prevalences were compared between asymptomatic refugee and symptomatic Ugandan populations, but the marked differences in results between refugee and nearby Ugandan populations nonetheless offer assurance that the refugee results were representative of their places of origin.

Our data demonstrate remarkable differences in the prevalences of antimalarial drug resistance markers in refugee populations originating from South Sudan and DRC, with prevalence near 50% for the 2 main markers of aminoquinoline resistance in South Sudan, high prevalence of markers of high-level antifolate resistance in DRC, and the presence of 2 markers of artemisinin partial resistance that were previously identified in Uganda in both countries. These data offer a window into malaria in regions that are difficult to study. Furthermore, they suggest that it is important to better control malaria in populations moving across Africa, and strategies including mass drug administration or targeted treatment of infected individuals may be appropriate to both improve refugee health and limit the spread of drug or diagnostic resistance.

Notes

Acknowledgments. We thank the participants and refugee reception center staff members of the facilities who contributed to this study. Control DNA samples (*P. falciparum* genomic DNA and plasmids containing the *P. vivax, P. ovale,* and *P. malariae* 18S genes) were obtained from the Malaria Research and Reference Reagent Resource Center.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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