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Evolution of artemisinin partial resistance in Ugandan malaria parasites

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Abstract

BACKGROUND

Partial resistance of *Plasmodium falciparum* to artemisinins, the most important malaria drugs, emerged in Southeast Asia and now threatens East Africa. Partial resistance is mediated principally by mutations in the PfK13 protein. Limited longitudinal data are available on its emergence and spread in Africa.

METHODS

We performed annual surveillance in patients presenting with uncomplicated malaria at 10-16 sites across Uganda from 2016-2021. We sequenced *pfk13*, primarily using molecular inversion probes, and explored relatedness by characterizing microsatellites flanking the PfK13 gene and polymorphisms across the genome. We assessed malaria metrics longitudinally in eight Ugandan districts from 2014-2021.

RESULTS

The PfK13 469Y and 675V mutations were seen at low prevalence in far northern Uganda in 2016-17 and rapidly increased and spread thereafter, reaching high (28-54%) combined prevalence across much of northern Uganda, with recent spread to other regions. The 469F mutation reached 40% prevalence in one district in southwestern Uganda in 2021. The 561H mutation, previously described in Rwanda, was first seen (prevalence 15%) in southwestern Uganda in 2021. Genetic analysis indicated singular emergences of mutant parasites independent of those in Southeast Asia. Although antimalarial resistance has typically been first seen in low transmission regions, emergence in northern Uganda, with historically high transmission, was explained by selection and spread of mutant parasites in districts where effective malaria control had been discontinued, leading to high malaria burdens in populations with relatively low antimalarial immunity.

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CONCLUSIONS

Our results identify rapid emergence of artemisinin partial resistance and offer guidance on conditions favoring resistance selection.

Malaria, in particular disease caused by *Plasmodium falciparum*, remains one of our greatest health problems, with an estimated 241 million cases and 627,000 deaths in 2020.[1] In Africa, where ~95% of malaria cases and deaths occur, the burden has worsened recently, exacerbated by challenges in maintaining control measures, increasing resistance to drugs and insecticides, and disruptions due to the COVID-19 pandemic.[2] Malaria control centers on long-lasting insecticide impregnated bednets (LLINs) and indoor residual spraying of insecticides (IRS) to limit mosquito vectors and efficacious drugs to treat and prevent malaria. Resistance to chloroquine and then antifolates limited malaria treatment efficacy, especially in Africa, where chloroquine resistance likely contributed to millions of excess malaria deaths in the 1980s and 1990s.[3] Early this century, standard treatment shifted to artemisinin-based combination therapy (ACT), which combines a rapidly effective, but short-acting artemisinin with a long-acting partner drug [4]. By 2005 ACTs, primarily artemether-lumefantrine and artesunate-amodiaquine, were the standard of care to treat uncomplicated malaria across Africa.[5]

In southeast Asia, artemisinin partial resistance, manifest clinically as delayed clearance of parasites after treatment with artemisinins and in vitro as enhanced survival of parasites after exposure to an artemisinin pulse, was reported in 2009.[6,7] The primary mediator of partial resistance was shown to be mutations in the propeller domain of a kelch protein (PfK13).[8] Artemisinin partial resistance is of particular importance when it is accompanied by resistance to ACT partner drugs, leading to decreases in efficacies of multiple ACTs in Southeast Asia.[9] Of

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many PfK13 mutations identified in *P. falciparum* around the world, ~20 have been associated with partial resistance.[10] These mutations have been reported at low prevalence in some regions outside Southeast Asia, including Guyana, Papua New Guinea, and India, but to date these appear to have been sporadic outbreaks without stable high PfK13 mutation prevalence or verified artemisinin partial resistance.[11]

Of greatest concern has been emergence or spread of artemisinin partial resistance in Africa, where impacts are expected to be profound. In older studies PfK13 mutations were seen in African parasites at low prevalence, but with few exceptions these were not validated resistance mediators.[5,12] In East Africa, resistance mediating PfK13 mutations[13,14] and in vitro enhanced survival after artemisinin exposure[15] were not seen in isolates collected in various parts of Uganda up to 2017, and resistance mediating mutations were very uncommon in samples collected in Rwanda up to 2015.[16] This situation changed recently. In Rwanda, the PfK13 R561H mutation, a validated resistance mediator, was first seen in samples collected in 2014, had prevalence of ~20% in isolates collected from two sites in 2018, and was associated with delayed parasite clearance in patients treated with artemether-lumefantrine.[17-19] In Uganda, a single isolate with the A675V mutation and enhanced survival after in vitro artemisinin exposure was identified in 2016.[20] Subsequently, the C469Y and A675V mutations were seen at increasing prevalence in northern Uganda,[21,22] and they were associated with partial resistance clinically and in vitro.[23,24]

Resistance to antimalarials has typically emerged in regions of relatively low malaria transmission intensity, presumably facilitated by low population immunity (allowing relatively unfit resistant parasites to spread and increasing drug exposure, as most infections are symptomatic) and low complexity of infection (limiting within-host competition between parasites).[25] It is of interest to determine why artemisinin partial resistance emerged in historically high transmission regions of Uganda. To better characterize the extent of artemisinin partial resistance and explore reasons for resistance selection, we characterized parasite

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genotypes and assessed malaria metrics over the period when resistance emerged and spread in Uganda.

METHODS

GENETIC SURVEILLANCE OF *P. FALCIPARUM* IN UGANDA

At each of 10 (2016-17) or 16 (2018-21) sites, we sequenced *pfk13* in 50 (2016-19) or 100 (since 2020) isolates annually from individuals presenting with uncomplicated malaria at malaria reference centers (MRCs) around the country (Fig. 1 and Table S1); results on a subset of these isolates from 2017-19 were published previously.[21,22] We utilized dideoxy sequencing and molecular inversion probe (MIP) technology, as previously described (Supplemental appendix).[22] For all analyses mixed genotypes at any locus were categorized as mutant. These studies and assessments of malaria metrics 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.

PHYLOGENETIC ANALYSIS OF ISOLATES WITH PARTIAL RESISTANCE MARKERS

To assess the origins of mutant parasites, we genotyped 7 microsatellites flanking *pfk13* in monoclonal Ugandan parasites collected in 2017-21 and generated a neighbor joining tree.[26] We also examined phylogeny using genotypes of mutant Ugandan (collected in 2020) and Southeast Asian (from the MalariaGEN Pf6 repository, collected in 2008-2013) parasites.[27] Ugandan parasites were genotyped using MIPs targeting predicted drug resistance genes and SNPs distributed across the genome.[28] Equivalent variant sites were extracted from Pf6K whole genomes after variant calling using an optimized GATK4 pipeline. After filtering for missingness and coverage, a cladogram was created in R. Detailed methods are in the Supplementary Appendix.

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EVALUATION OF MALARIA METRICS AT SITES ACROSS UGANDA

Enhanced malaria surveillance was established in 2006 and extended to the sites described in this report in 2014.[29] For all patients presenting at each MRC, demographics, diagnoses, and malaria infection status (by rapid diagnostic test or microscopy) were recorded. To evaluate malaria incidence, we considered the monthly number of laboratory-confirmed cases of malaria at surveillance sites (catchment areas are stable, allowing a reasonable estimate of incidence) and the test positivity rate (TPR, percentage of those tested for malaria with a positive test). As an indirect measure of population antimalarial immunity we considered the median age of presentation with malaria. With effective malaria control and decreased force of infection, immunity wanes, and the median age of presentation with malaria increases.[30]

STATISTICAL ANALYSIS

Prevalences of mutations were compared using the two-sided Fisher's exact test and assessed longitudinally using the chi-square test of trend. Prevalences were compared between districts using the Mann-Whitney test. P-values <0.05 were considered statistically significant.

RESULTS

STUDY SITES

Genomic surveillance sites collected isolates from patients presenting with uncomplicated malaria beginning in 2016; malaria metric data were from a subset of these sites with enhanced surveillance data available since 2014 (Fig. 1 and Table S1). Key interventions included universal distributions of LLINs in 2013-14, 2017-18, and 2020-21 at all sites. IRS, the malaria control measure that is most impactful, but limited in scope due to cost, was implemented selectively, with MRCs receiving (a) twice-yearly IRS with the carbamate bendiocarb from 2010-14 plus a single round of the organophosphate pirimiphos-methyl in

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2017, (b) once or twice yearly IRS since 2015 (initially bendiocarb, then pirimiphos-methyl from 2016-19, and clothianidin or clothianidin/deltamethrin in 2020), or (c) no IRS (Fig. 1).[29,31]

PREVALENCE OF RESISTANCE-ASSOCIATED MUTATIONS

Analysis of the oldest available samples identified modest prevalence of both the 469Y and 675V mutations in 2016, particularly in Lamwo District, in far north-central Uganda (Fig. 2, Table 1, and Table S1). Prevalence of these two mutations increased over time in five northern districts (Agago, Kaabong, Katakwi, Koboko, and Lamwo; test of trend $p < 0.05$ for 469Y at three sites and for 675V at four sites; Table 1), with sustained combined prevalence >10% since 2019. These mutations have gradually spread to other regions of the country, appearing at 13 of the 16 surveillance sites. Despite overall increased prevalence, in some cases the identification of mutations was transient. A different mutation at the 469 codon, 469F, was seen in Rukiga District in southwestern Uganda in 2016, and reached high (40%) prevalence in 2021 (low malaria incidence led to few available samples for study at this site in 2017-19); the mutation has been identified sporadically at other sites, predominantly in southern Uganda. The 561H mutation, which has reached high prevalence in Rwanda, was first detected in 2021, also in Rukiga district, at a 15% prevalence. Additional PfK13 propeller domain mutations that are not validated resistance mediators were seen, mostly at prevalence <10%, with some clusters suggesting local emergence and spread (Table S3); mutations outside the propeller domain were common (Table S4). Overall, multiple PfK13 mutations, including four validated resistance markers, have demonstrated increasing prevalence and spread over time.

RELATEDNESS OF ISOLATES WITH PARTIAL RESISTANCE MARKERS

We explored the relatedness of recently emerged mutant parasites using two methods. First, we characterized microsatellites flanking the *pfk13* gene in Ugandan parasites (Table S5). Mutant parasites were related, with distinct haplotypes associated with each mutation,

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consistent with singular origins for 469Y and 469F mutant parasites, and one or two origins (one in northern and one in western Uganda) for 675V mutant parasites (Fig. 3A). Second, we characterized polymorphisms across the *P. falciparum* genome in Ugandan and Southeast Asian parasites (Figure 3B). The Ugandan mutant parasites were phylogenetically distant from parasites with the 675V mutation isolated in Southeast Asia (parasites with mutations at the 469 locus were not available from Asia), consistent with local emergence and spread of mutant Ugandan parasites.

EVALUATION OF MALARIA METRICS AT SITES ACROSS UGANDA

We examined monthly malaria burden, measured as case load and TPR, at MRCs from 2014, when surveillance data became available, through 2021, capturing the interval during which artemisinin partial resistance emerged in northern Uganda (Fig. 4). Patterns of malaria incidence were strongly associated with utilization of IRS, a highly effective control measure when effective carbamate or organophosphate insecticides are used.[29] Incidence was cyclic at many sites, likely impacted by seasonal rainfall, but patterns associated with IRS interventions were clearly discernible. Districts with regular IRS from 2010-14 and one additional round in 2017 showed relatively low case loads and TPR after a sustained period of IRS (mostly occurring before enhanced surveillance data were available),[29,32] with marked increases within ~4-8 months after ceasing IRS (Fig. 4A). Districts with IRS beginning only in 2015 experienced marked decreases in case loads and TPR after implementation until a change to different insecticides for IRS and a marked increase in malaria incidence in 2020 (Fig. 4B). Districts with no history of IRS, which include sites with diverse malaria epidemiology, had varied case loads and TPR over time, including transient seasonal increases (Fig. 4C). Evaluation of mean age at malaria presentation as a surrogate for antimalarial immunity generally showed increased median age (decreased immunity) during periods of malaria control, such that populations in

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northern Uganda had relatively low immunity at the time of cessation of IRS and subsequent surges in malaria incidence (Fig. S1).

ASSOCIATIONS BETWEEN MALARIA METRICS AND PREVALENCE OF PARTIAL RESISTANCE MARKERS

In northern Uganda prevalence of the 469Y and 675V mutations increased markedly following malaria resurgences (Fig. 4A). From 2018-21, the prevalence of each mutation was significantly greater in northern districts (Agago, Arua, Kaabong, Katakwi, Koboko, Kole, and Lamwo; mean prevalence 9.5% for 469Y and 9.2% for 675V) and in the subset of districts that stopped IRS after 2014 (16.0% for 469Y and 10.8% for 675V) compared to all other study districts (0.5% and 1.1%, $p < 0.0001$ for both comparisons) including those with regular IRS since 2015 (0.5%, $p = 0.002$ and 0.8% $p = 0.0007$) or no IRS (2.1%, $p < 0.0001$ and 3.8% $p = 0.0003$). In the two studied districts with sustained IRS since 2015, malaria incidence decreased to low levels, with low prevalence of PfK13 mutations from 2015-20, but with steep increases in malaria incidence and modest increases in prevalence of the 469Y and 675V mutations after changes in IRS insecticides (Fig. 4B). In districts that did not undergo IRS, malaria incidence followed expected seasonal patterns, without marked changes over time, and the prevalence of PfK13 mutations was relatively low, without clear patterns of emergence (Fig. 4C). An exception was Rukiga District, a southwestern site that did not receive IRS, which had emergence of high prevalence of both 469Y (an apparent independent emergence, Fig. 2A) and 561H (most likely spread from Rwanda). Unfortunately, malaria enhanced malaria surveillance data were not available from Rukiga, but this is a high elevation site that is epidemic prone,[33] favoring resistance selection independent of malaria control measures.

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DISCUSSION

The emergence of artemisinin partial resistance in *P. falciparum* in East Africa provides one of the most profound challenges to the control of malaria in decades. We demonstrate emergence and spread of four different resistance-mediating PfK13 mutations in Uganda since 2016. By 2021, prevalences of parasites with validated resistance markers reached >20% in five of the 16 districts where surveillance was conducted, with foci of >50% prevalence in both northern and southwestern Uganda. The full clinical consequences of these genetic changes are not yet known, but experience from Southeast Asia suggests that treatment of malaria may be compromised by the identified resistance mutations. Improved characterization of the emergence and spread of resistance and of factors that facilitate spread will assist policymakers in developing strategies to limit the spread and consequences of drug resistance.

Our results demonstrating emergence of artemisinin partial resistance in northern Uganda are remarkable in one important respect. Resistance to antimalarial drugs has typically first emerged in regions of relatively low malaria transmission intensity. This scenario was seen with emergence of resistance to chloroquine, antifolates, mefloquine, and artemisinins, all first in Southeast Asia (and for chloroquine independently in South America). Emergence was followed by spread of chloroquine resistance from Asia to Africa[34] and selective sweeps of antifolate resistance in Africa.[35] Our data and prior data from Rwanda[17,18] and Uganda[23] indicate multiple independent emergences of PfK13 mutations in these regions of relatively high malaria burden, rather than spread of resistant lineages from Asia.

Why has artemisinin resistance emerged and spread in northern Uganda, a region of historically high malaria incidence? We hypothesize that emergence and spread have been facilitated by events that led to high malaria transmission intensity in populations with relatively low malaria immunity. Key features of malaria control in Uganda have included provision of effective antimalarial therapy with ACTs, national distributions of LLINs, and IRS, which is the most effective vector control intervention, but has been limited in scope due to its high cost. IRS

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with highly effective insecticides was instituted in 10 northern districts from 2010-14, accompanied by sustained decreases in malaria test positivity rates and parasite prevalence.[36,32] Subsequent discontinuation of IRS in 2014 was associated with a five-fold increase in malaria incidence within 10 months, and a malaria epidemic was declared by the Ministry of Health in June, 2015.[29,37] This rapid increase in incidence in a population in which antimalarial immunity had declined was accompanied by the emergence and spread of artemisinin partial resistance. More recently, similar events appear to have occurred elsewhere, with increases in resistance mediating PfK13 mutations seen after a change in IRS regimen was accompanied by increased malaria incidence in central and eastern Uganda,[31] and in southwestern Uganda, where malaria transmission is unstable.

Resistance selection in Uganda may also have been facilitated by use of artemisinin monotherapies, with the artemisinin component unprotected by a long-acting partner drug, and increased potential for resistance selection. Use of artemisinin monotherapies to treat uncomplicated malaria has been discouraged for many years, but the practice persists. In Nigeria, oral artemisinin monotherapies made up 2.5% of the market share for studied drug outlets in 2015.[38] Use of intravenous or rectal artesunate for severe malaria should be followed by a full ACT course,[39] but follow-up therapy may be skipped. In addition, use of intravenous artesunate to treat uncomplicated malaria, although not recommended, is a well-known practice, increasing the potential for selection of resistant parasites.[40]

Our results demonstrate worrisome sustained prevalence of *P. falciparum* with artemisinin resistance-mediating PfK13 mutations in Uganda. Remarkably, the 469Y and 675V mutations are extending their range, and two other mutations, 469F and 561H, have emerged in southwestern Uganda. Resistance selection is likely possible under many scenarios, but our results suggest that low population malaria immunity, due to a sustained low malaria burden, followed by a malaria epidemic, stochastically increasing the opportunities for selection, offers a "perfect storm" for resistance selection. In northern Uganda, this scenario was precipitated by

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withdrawal of effective malaria control. Malaria epidemiology in Africa is highly varied, with many regions experiencing major fluctuations in disease incidence, as seen in Uganda. Hence, there is concern for multiple additional emergences of artemisinin partial resistance. Efforts to blunt malaria epidemics, including the maintenance of effective malaria control interventions and prompt attention to regions with increasing incidence, will be of critical importance to forestall a potential drug resistance catastrophe.

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District	Locus	2016	2017	2018	2019	2020	2021	p
Agago	C469Y	0/30 (0%)	3/42 (7%)	1/31 (3%)	9/40 (23%)	30/79 (38%)	8/50 (16%)	<0.001
	A675V	0/31 (0%)	2/42 (5%)	1/31 (3%)	5/35 (14%)	13/80 (16%)	7/52 (13%)	0.04
Amolatar	A675V	0/30 (0%)	0/48 (0%)	0/27 (0%)	0/19 (0%)	0/85 (0%)	1/50 (2%)	0.22
Arua	C469Y	0/31 (0%)	0/45 (0%)	0/20 (0%)	0/21 (0%)	0/81 (0%)	2/68 (3%)	0.12
	A675V	0/33 (0%)	1/45 (2%)	0/30 (0%)	0/24 (0%)	0/81 (0%)	1/68 (3%)	0.99
Hoima	C469Y	-	-	0/42 (0%)	0/48 (0%)	8/92 (9%)	0/82 (0%)	0.61
	A675V	-	-	0/45 (0%)	0/47 (0%)	6/92 (7%)	0/82 (0%)	0.63
	C469F	-	-	1/42 (2%)	0/47 (0%)	0/92 (0%)	0/82 (0%)	0.08
Kaabong	C469Y	-	-	1/29 (3%)	0/32 (0%)	12/74 (16%)	7/50 (14%)	0.03
	A675V	-	-	3/34 (9%)	8/31 (26%)	14/75 (19%)	10/59 (17%)	0.59
	C469F	-	-	0/29 (0%)	0/32 (0%)	0/74 (0%)	3/57 (5%)	0.04
Kapchorwa	C469Y	-	-	-	0/16 (0%)	-	1/77 (1%)	NA
	A675V	-	-	-	0/15 (0%)	-	3/71 (4%)	NA
Katakwi	C469Y	-	-	2/40 (5%)	1/39 (3%)	11/87 (13%)	7/58 (12%)	0.09
	A675V	-	-	1/45 (2%)	4/37 (11%)	6/88 (7%)	9/58 (16%)	0.04
Kanungu	C469F	0/37 (0%)	0/48 (0%)	1/36 (3%)	0/40 (0%)	3/91 (3%)	2/46 (4%)	0.07
Koboko	C469Y	-	-	0/23 (0%)	0/4 (0%)	2/56 (4%)	1/46 (2%)	0.54
	A675V	-	-	0/34 (0%)	0/5 (0%)	5/64 (8%)	5/44 (11%)	0.04
Kole	C469Y	1/23 (4%)	1/47 (2%)	0/36 (0%)	2/41 (5%)	9/85 (10%)	0/54 (0%)	0.45
	A675V	0/26 (0%)	1/47 (2%)	1/34 (3%)	2/41 (5%)	10/86 (12%)	2/58 (3%)	0.08
Lamwo	C469Y	2/32 (6%)	3/44 (7%)	6/32 (19%)	4/31 (13%)	28/84 (33%)	16/51 (31%)	<0.001
	A675V	2/37 (5%)	4/44 (9%)	1/36 (3%)	5/31 (16%)	16/84 (19%)	12/53 (23%)	0.002
Mubende	C469Y	0/26 (0%)	0/46 (0%)	0/38 (0%)	0/22 (0%)	3/87 (3%)	0/19 (0%)	0.14
	A675V	0/28 (0%)	0/46 (0%)	0/41 (0%)	0/24 (0%)	6/87 (6%)	0/19 (0%)	0.03
	C469F	0/26 (0%)	0/46 (0%)	4/38 (11%)	0/22 (0%)	0/87 (0%)	0/19 (0%)	0.40
Rukiga	A675V	0/42 (0%)	-	-	1/14 (7%)	1/39 (3%)	2/82 (2%)	0.57
	C469F	7/41 (17%)	-	-	0/14 (0%)	11/39 (28%)	29/72 (40%)	0.002
	R561H	0/41 (0%)	-	-	0/14 (0%)	0/39 (0%)	10/64 (15%)	0.001
Tororo	C469Y	0/35 (0%)	0/48 (0%)	0/31 (0%)	0/44 (0%)	0/40 (0%)	3/73 (4%)	0.03
	A675V	0/37 (0%)	0/48 (0%)	0/43 (0%)	0/44 (0%)	0/41 (0%)	3/74 (4%)	0.03
	C469F	1/35 (3%)	1/48 (2%)	0/31 (0%)	0/44 (0%)	0/40 (0%)	0/73 (0%)	0.66
Kanungu	C469F	0/37 (0%)	0/48 (0%)	1/36 (3%)	0/40 (0%)	3/91 (3%)	2/46 (4%)	0.07

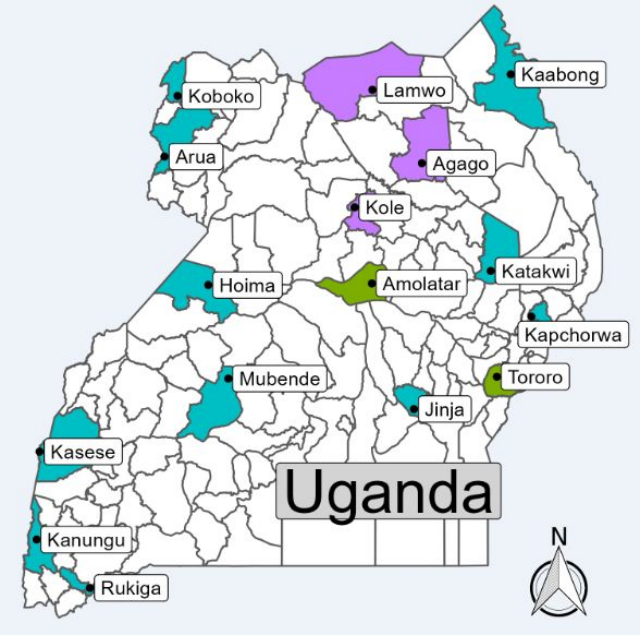

Figure 1. Map of Uganda and study districts. Study districts are labelled and the locations of study clinics are shown as black dots. The color code indicates study districts that received IRS every 6 months from 2010-14 and once in 2017 (purple), received regular IRS beginning in 2015 (green), and did not receive IRS (blue).

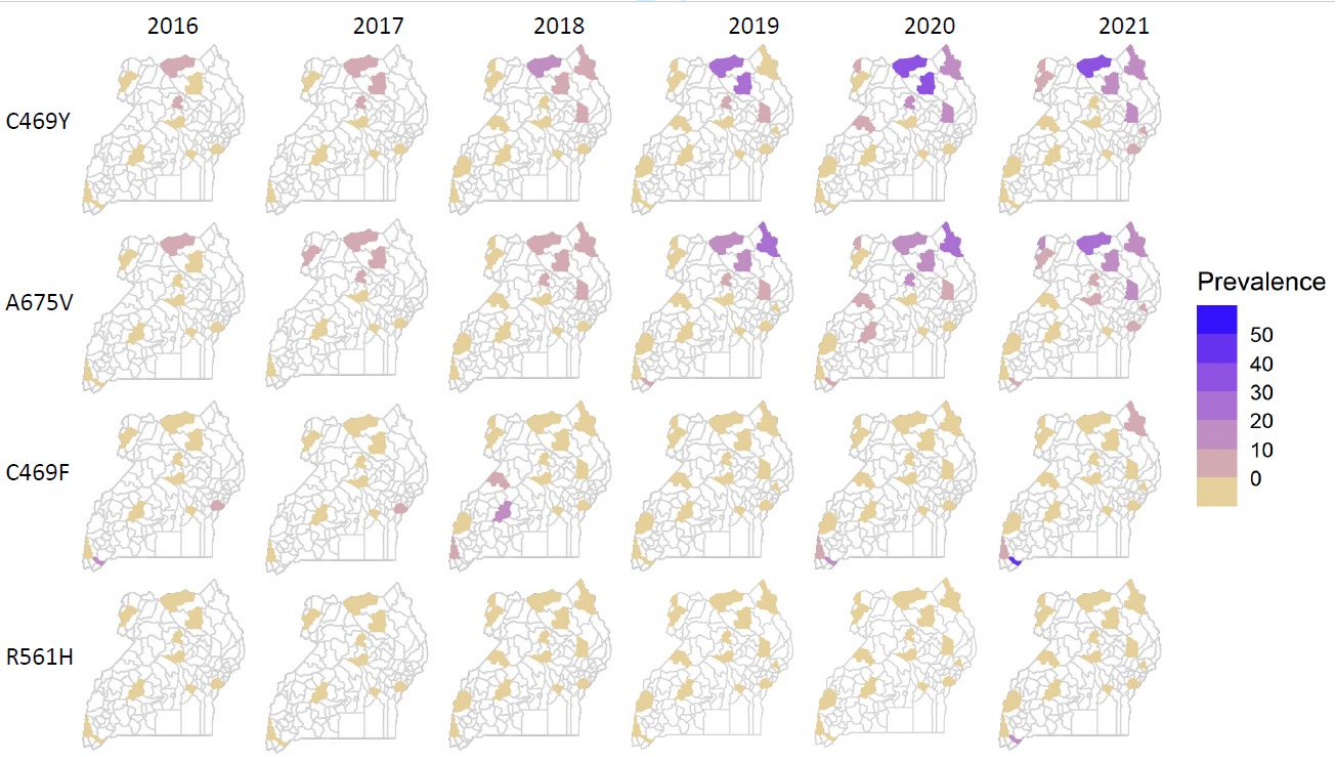

Figure 2. Prevalence of indicated PfK13 mutations in studied Ugandan districts.

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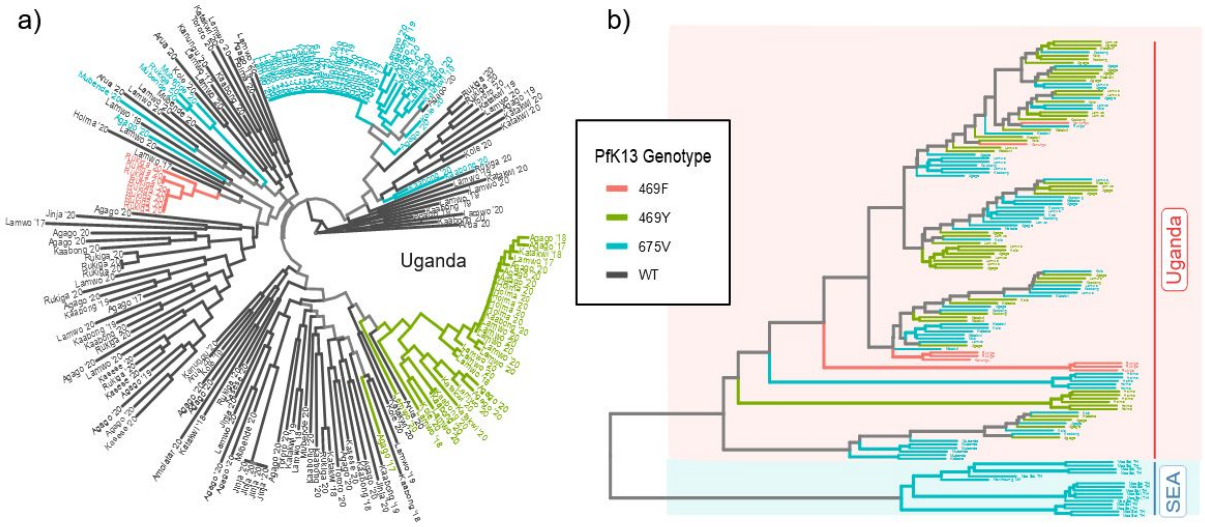

Figure 3. Phylogenetic relatedness of Ugandan parasites. Dendrograms demonstrate relatedness of Ugandan wild type and PfK13 mutant parasites based on characterization of 7 microsatellite loci flanking PfK13 (A) and relatedness of Ugandan and Southeast Asian mutant isolates based on polymorphic loci distributed across the genome (B).

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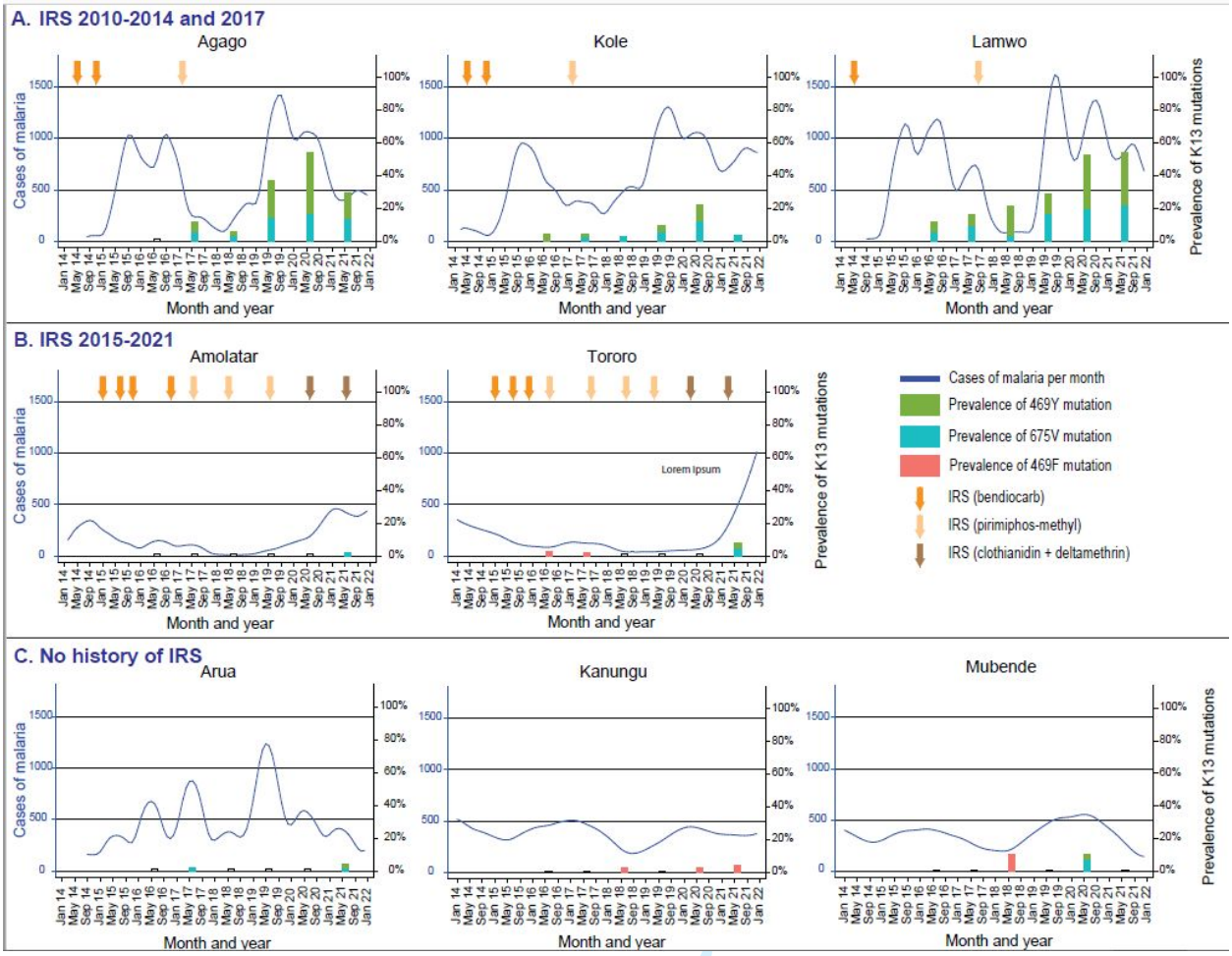

Figure 4. Relationship of malaria metrics and prevalence of PfK13 mutations. Malaria incidence and mutation prevalence are shown for sites that received IRS every 6 months from 2010-14 and once in 2017 (Agago, Kole, and Lamwo Districts, A), received IRS beginning in 2015 (Amolatar and Tororo Districts, B), and did not receive IRS (Arua, Kanungu, and Mubende Districts, C). The metrics shown were assessed monthly and are displayed with lowess smoothing. Orange arrows indicate times of IRS (light orange indicates an ineffective insecticide). Histogram bars indicate prevalence of PfK13 mutations. Other malaria metrics (TPR and median age of presentation with malaria) are shown in Fig. S1.

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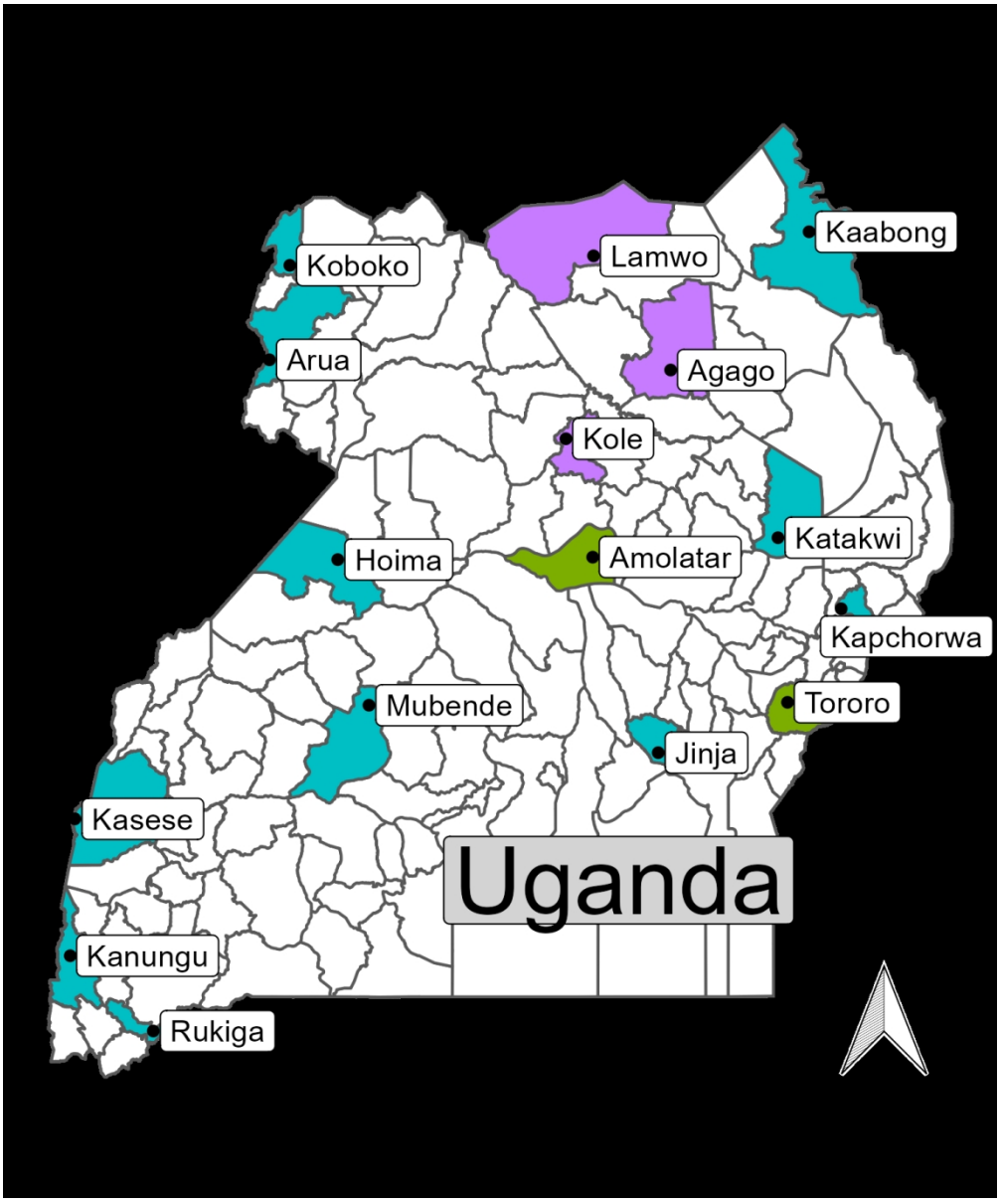

Figure 1. Map of Uganda and study districts. Study districts are labelled and the locations of study clinics are shown as black dots. The color code indicates study districts that received IRS every 6 months from 2010-14 and once in 2017 (purple), received regular IRS beginning in 2015 (green), and did not receive IRS (blue).

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Figure 2. Prevalence of indicated PfK13 mutations in studied Ugandan districts.

338x190mm (96 x 96 DPI)

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