Artemisinin Resistance in Africa: *Plasmodium falciparum kelch13* R561H spread and emergence of other artemisinin partial resistance mutations in Rwanda

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B.S. Biology, B.A. Computer Science, University of North Carolina Chapel Hill, 2021

Thesis

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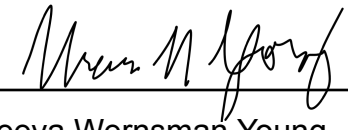

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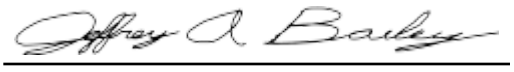
Date April 27, 2023 Signature: 

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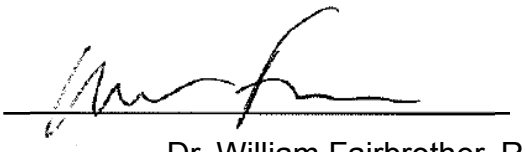
Date April 11 2023 Signature: 

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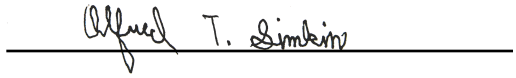
Date 04/30/2023 Signature: 

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Abstract

Abstract of "Artemisinin Apocalypse: *Plasmodium falciparum kelch13* R561H spread and emergence of other artemisinin partial resistant mutations in Rwanda" by Neeva N. Wernsman Young, ScM, Brown University, May, 2023.

First detected in Rwanda, recent monitoring has detected multiple emerging Plasmodium falciparum kelch13 (K13) propeller gene mutations across East Africa. Mutations, such as R561H in Rwanda and C469Y/F, and A675V in Uganda, have been increasing and are associated with artemisinin partial resistance mainly manifesting as delayed clearance after treatment with ACTs and recrudescence. Coordinated surveillance efforts are necessary to track these K13 mutants and inform control efforts. Here we introduce a new method of collection: liquid blood drops, and apply pooled sequencing to provide a rapid initial assessment of resistance mutation allele frequency at multiple collection sites across Rwanda. Whole blood samples (n=2,713) from malaria-positive patients were collected from 20 Rwandan health centers from May to December 2022. Equal volumes of samples were pooled, stratified by site and month to generate 104 pools. DNA was extracted using magnetic beads and genotyped using molecular inversion probe targeted sequencing of key drug resistance genes. Site allele frequencies were calculated weighted by the number of samples in a given pool. From two rounds of sequencing, R561H was detected at 16 of 18 sites with an average frequency of 18.0% (0.8% to 49.1%). The higher site frequencies clustered in the center of the country near regions of high transmission. In Bugaragara, in northeast Rwanda, we found 49.1% average frequency that progressively increased monthly from October to December (0.0% to 94.1%). Notably, A675V, previously seen in Uganda, was found at 6.0% across 13 sites. Additionally, C469F, recorded in southern Uganda, was observed in 5 sites, with the highest frequency in Mushubati (10.6%), near the DRC border. Concerningly, G449A was at 1.1%, a WHO candidate resistance mutation never before reported in Africa but associated with a two-fold prolonged parasite clearance half-life in Asia. It was detected at 3 sites including Tanda (5.5%) where a large outbreak has been occurring. Overall, it appears multiple K13 mutations are rapidly expanding in Rwanda, further endangering control efforts and treatment efficacy with the potential of engendering partner drug resistance.

Introduction

Malaria is a life-threatening disease responsible for 247 million cases and 619,000 deaths in 2021, with most occurring in Africa.[1] The majority of African infections can be attributed to the parasite *Plasmodium falciparum* (*Pf*), transmitted by mosquitoes.[2] Symptoms begin usually within two weeks of receiving a bite and often present as a fever, headache and chills.[2] These common symptoms create difficulty for monitoring efforts, further challenged by the fact that these symptoms become more mild in people with a history of previous *Pf* infections.[2] Malaria treatments are often selected case by case because regional drug resistance has been increasing over the last 50 years. As new antimalarials have been introduced, *Pf* mutates and resistance appears.[3] Chloroquine (CQ) was introduced for commercial use in the 1940s and heavy usage resulted in the rapid spread of resistant parasites across endemic areas.[3] As new antimalarials have been introduced, they continue to be challenged as parasites evolve resistance within an average of 20 years from drug deployment.[3]

In the 1990s, artemisinin (ART) was recognized as the last effective frontline drug for malaria.[4] ART is extremely efficacious as it rapidly reduces parasitemia approximately 10,000-fold every 48 hours.[5,6] In response to rapid parasite evolution, the World Health Organization (WHO) recommended Artemisinin-based combination therapies (ACT) in 2001 in an effort to preserve ART as a frontline anti-malarial. ACTs have been adopted globally and remain the current standard of treatment.[1,2] ACTs have provided a highly effective method for treatment and combat increasing resistance to partner drugs, with nearly all African countries employing ACTs as front line defense after WHO recommendation.[4,7] ACTs combine an initial 3 day treatment period of ART

derivative (artemether, artesunate (AS), or dihydroartemisinin (DHA)) to quickly lower the majority of parasites, followed up with a longer-acting partner drug (lumefantrine or amodiaquine) that clears remaining parasites.[5]

Despite these efforts, ART resistance was first documented already widespread across Cambodia in 2006 and quickly moved across Southeast Asia (SEA) by 2018.[8–10] This was reported as single nucleotide polymorphisms (SNPs) appearing on the *kelch13* (*PfK13*) gene.[10] In the last few years, mutations in *PfK13* have also been found in Africa and show association with ART partial resistance *in vitro*.[11] Partial resistance is characterized as a failure for ART to sufficiently reduce parasite burden before a partner drug is administered.

To combat resistance, there has been a concerted global effort to genotype *P. falciparum* in search of SNPs or other genomic markers of resistance.[12] Since the identification of the *PfK13* gene in 2014, multiple molecular markers for resistance have been determined.[8,13] Drug resistant markers for ART are defined by the WHO, candidate mutations must show ART resistance *in vitro* (enhanced survival) or show statistically significant delay in parasite clearance in at least 20 clinical cases.[13] If both aspects are met, the mutation's status is validated.[13,14]

PfK13 is divided into three regions: the upstream region, the Pox virus and Zinc finger (POZ) domain, and the kelch propeller. The majority of SEA mutations are within the propeller region (SF 4).[15] From a mechanistic approach, *PfK13* mutations have been induced *in vitro* to determine the effect these mutations have in parasites. While the exact function of *PfK13* is still not known, mutated parasites exhibited ring-stage dormancy and could not compete in growth assays, but tolerated oxidative stress

induced from ART drugs.[16] As expected from these *in vitro* results, *PfK13* is highly conserved along the propeller and POZ domain across other *Plasmodium* species.[15] *PfK13* clearly plays an essential role within parasite development and mutations impact parasite fitness. Logic from these *in vitro* results would suggest that non-synonymous mutations would not evolve naturally in parasite populations as they come at such a fitness cost, indicating that these molecular markers are likely increasing due to ART drug selection pressure.

In regard to the application of ACT in Africa, the appearance of *PfK13* mutations represents a major threat as decreased sensitivity to ART is thought to have expedited partner drug resistance and led to ACT treatment failure in SEA.[17] While monitoring ART resistance is critical to maintaining the viability of ACTs, monitoring partner drug usage and resistance is also imperative. There are four common ACT formulations employed in Africa and SEA: artemether-lumefantrine (AL), artesunate-amodiaquine (ASAQ), artesunate-sulfadoxine-pyrimethamine (ASP) and dihydroartemisinin-piperaquine (DP). AL therapy is the most widely deployed drug in Africa and has remained relatively effective as lumefantrine has never been available as a monotherapy.[4,18] Lumefantrine resistance has been correlated to mutations in the *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*) and *P. falciparum* chloroquine resistance transporter (*Pfcrt*).[19,20] Specifically *Pfcrt* 76T, and the combined haplotype on *Pfmdr1* of N86, 186F and D1246.[21,22] The other notably endangered ACT is ASP, as SP is often still employed as a monotherapy during pregnancy.[4] This increases the selective pressure for parasites resistant to SP, so as ART resistance also appears there is increased likelihood resistant parasites will come in contact. Parasites carrying *Pfdhfr* (dihydrofolate

reductase) I146L are associated with delayed clearance when treated with pyrimethamine. Africa has managed to avoid ACT resistance through continued partner drug efficacy. Only reporting delayed clearance in Rwanda, Uganda and Eritrea, making monitoring efforts vital to inform appropriate responses in these and nearby areas.[13,18]

Rwanda presents unique geography in East Africa and represents a potential hotspot for the development of mutations. Drug resistance tends to develop in low transmission regions where there is less competition between wildtype (WT) parasites and mutant parasites.[23,24] These regions allow for mutant parasites to arise, survive, and transmit.[17] Rwanda possesses high-elevation regions in the north with as porous volcanic soil that drains away standing water, additionally reducing larval habitat for mosquitoes, resulting in very low malaria transmission. As the landscape transitions to low lying grasslands in the south, decreased altitude and increased stagnant water creates favorable vector conditions and leads to high malaria transmission.[25]

Rwanda shifted to AL in 2006.[26] *PfK13* mutants were first reported from Rwanda in 2020 in Masaka, finding R561H in 7.4% of samples collected in 2014.[11] More comprehensive retrospective analysis by Kirby et al. utilized Demographic Health Survey (DHS) samples from 2014 to capture R561H in two other southwestern districts. Notably, phylogenetic analyses of these R561H samples demonstrated that these strains had a shared haplotype that differed from SEA suggesting that the mutation arose locally, and was not transmitted from SEA.[11] Multiple studies have confirmed that *PfK13* polymorphisms arising in Africa appear to be indigenous.[11,15] The wide variety of mutants further implies these have developed on their own, independent of resistance in SEA.[11,15] Bergmann et al. reported an increase in R561H when they sampled in the

Huye district in 2019, finding mutant parasite in 12.1% of infections.[27] The objective of our study is to provide a rapid view of the current state of R561H across Rwanda, while simultaneously monitoring K13 for additional mutations.

Large scale studies to effectively monitor resistance are often intensive and require significant investment, making them inaccessible to low-resource countries. Traditional methods of obtaining genomic data at such scale requires whole genome sequencing (WGS) or amplicon sequencing. WGS requires significant infrastructure and costly resources. While large scale amplicon panels, such as CleanPlex from Paragon Genomics, are becoming more accessible and can provide sufficient genome coverage, they are still subject to primer interaction and amplicon dropout.[28] These limitations are relatively inherent and can only be improved to a certain extent as variation in target DNA will always yield different conditions that may be unfavorable to valuable amplicons contained in a multiplexed assay. In this study, we combine a new method of sample storage and extraction with subsequent sequencing using molecular inversion probes (MIPs). MIPs are capable of generating wide genomic coverage that can be applied to the same analyses as WGS, as several thousand MIP captures can be pooled into one assay.[29] To maximize sensitivity, we used a panel designed to capture 250 bp fragments from 121 sites across 10 drug resistance genes.

We also introduce the collection of liquid blood drops (LBD) as an alternative to the standard dried blood spot (DBS) used in many large-scale malaria studies. DBS can encounter variable storage conditions as they are often regarded as a robust method. This can lead to many potential conditions for contamination if samples are not adequately climate controlled or separated.[30,31] DBS extraction also poses challenges,

requiring significant labor as samples must be hole punched and low blood volume stored in DBS yields low purity DNA extractions. Additionally, the widely accepted cost-effective DBS extractions uses Chelex which can inhibit PCR and downstream sequencing if preparation does not sufficiently remove Chelex.[32] This requires repetitive optimization when switching between different applications. DBS and Chelex extraction also significantly fragments DNA which makes these extractions less suitable for long-read sequencing platforms. We have deployed LBD as a competitive cost-effective method for collection and extraction in Rwanda to find R561H which reduces many of the shortcomings associated with DBS and Chelex.

Methods

Ethics

Ethical approval for this study was obtained from the Rwanda National Ethics Committee (reference 12/RNEC/2022) and Institutional Review Board at Brown University (RI, USA). Written informed consent was obtained from participants or guardians of eligible children in the local language (Kinyarwanda).

Sample Collection

Sample collection occurred across 20 health centers in Rwanda from May to December 2022, flanking the short rainy season occurring from September to November. Malaria positive patients (n=2713) were identified by microscopy or rapid diagnostic test (RDT). Trained technicians subsequently collected whole blood from patients, adding 3-4 drops (100 µl) of blood into 2mL O-ring microcentrifuge tubes

pre-filled with 100 µl of DNA/RNA Shield from Zymo Research (Irvine, CA). Collection tubes were vigorously shaken to mix and stored at room temperature for 2 weeks or less before being transported to the main lab at INES-Ruhengeri University where they were stored at -20° C (Fig. XX). Individual samples were grouped by location and storage date (two week intervals) for pooling. If a site provided 8 or fewer samples at a given storage date, multiple dates were combined and the most occurring date was assigned to the pool. 6 µl of each sample was pooled into a single aliquot then vortexed thoroughly before 100 µl was taken for nucleic acid extraction. Pools with insufficient volume (<=16 samples) were brought to 100 µl using 1:1 DNA/RNA shield and MGW. Data regarding region and pooling is provided in Table 1.

Figure 1: Liquid Blood Drop Collection and Pooling Schema

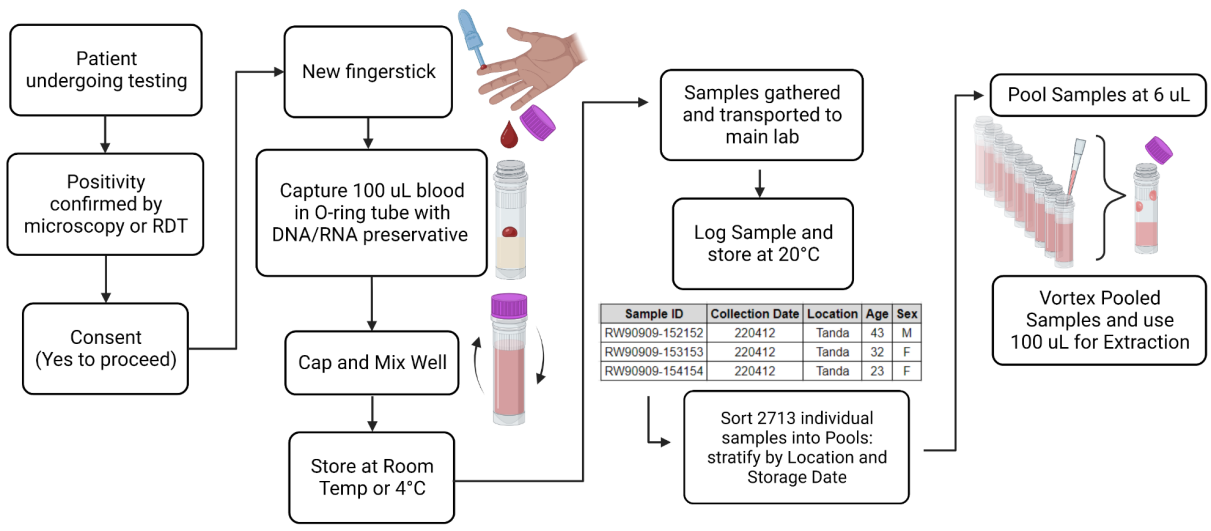


Table 1: Sample Pooling Breakdown

Origin	# of Samples	# of Pools
Bugaragara	50	3
Busanza	238	8
Byahi	27	1
Gahanga	50	5
Gishubi	66	5
Giti	117	5
Jenda	113	3
Kibingo	16	1
Mareba	30	4
Masaka	145	8
Mushubati	176	5
Ngoma	51	4
Nkanka	19	1
Ntarama	68	5
Nyacyonga	234	8
Nyamagabe	117	6
Nyamata	108	8
Rukara	22	1
Solace	230	8
Tanda	836	15
Total	**2713**	**104**

DNA Extraction

Frozen samples were brought to room temperature, spun down and combined with 100 µl Lysis Buffer composed of (80 mM DTT, 2M Guanidine thiocyanate, 25 mM Sodium Citrate Dihydrate, 0.1 M Tris HCl, Proteinase K and DiH_2O). This was incubated at 65° C for 10 minutes and allowed to cool before the addition of 3 µl of RNase A (Invitrogen Cat: 12091021), 400 µl of absolute ethanol, and 15 µl of Spherotech SPHERO Silica Super Paramagnetic Beads (Lake Forest, IL) with mixing between. Samples were allowed 10 minutes on a hula mixer, then placed on a magnet.

Supernatant was removed and the Paramagnetic Beads were washed twice with 80% Ethanol. After the last wash, the sample was removed from the magnet, allowed to dry briefly, then eluted with 60 µl of 1X TE Low EDTA (Thermo J75793.AP) heated to 65° C. The beads were resuspended in the low EDTA, incubated at 65° C for 10 minutes and returned to the magnet. Once beads pellet to the magnet, the elution was removed and a random subset was quantified with Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA, USA) for quality check. The DNA concentration of quantified samples (n=39) ranged between 0.1 and 38.6 ng/µl. Extracted DNA was subsequently stored at -20° C to await Molecular Inversion Probe Genotyping.

Molecular Inversion Probe (MIPs) Genotyping

Extracted DNA underwent capture, exonuclease treatment, PCR and sequencing as detailed by Aydemir et al. using the MIP panel described as DR1.[29] Our panel targets 121 SNPs across the genome associated or suspected to be associated with drug resistance.

Library Preparation

After PCR, all 104 pools were combined into a single tube at 5 µl each with 2 µl of two positive controls and four negative controls and cleaned prior to sequencing to remove lower weight non-specific PCR products. To prepare libraries for sequencing, a 1.2x solid phase reversible immobilization (SPRI) bead cleanup was performed on the final library and eluted in 50 µl TE low EDTA pH=8 (Thermo Fisher Scientific, Waltham MA, USA). The amplicons were resolved on a 1.0% agarose gel. Fragments of the correct size were excised and DNA was purified using the NEB DNA Gel Extraction kit T1020S (Ipswich, MA, USA). Library quality was assessed using a fragment analyzer

(Agilent, Santa Clara, CA), the library pooled with other libraries and sequenced on an Illumina Nextseq 550 (Illumina, San Diego, California) using a 300-cycle mid output kit based on the manufacturer's instructions.

Repooling

Samples that did not reach 1,000 Unique Molecular Indexes (UMI) for the three target mutations (G449A, R561H, A675V) on the first pass of sequencing (n=96) were repooled, SPRI cleaned, gel extracted (NEB) and resequenced on Illumina NextSeq. Samples that still did not cumulatively reach the UMI threshold were recaptured and sequenced.

Analysis

Samples were demultiplexed using MIPtools software available at https://github.com/bailey-lab/MIPTools, previously described by Verity et al.[33] Downstream analysis was performed using R version 4.2.3. UMIs were used to cluster sequences and link amplified SNPs occurring from the same probe to properly downsample sequencing data. Frequency data for different polymorphisms were summarized using ggplot2 and dplyr packages in R version 3.6.2 software. Map figures were created in RStudio using spatial data from rnaturalearth, sf and ggplot2.

Results

Magnetic bead extraction provides high molecular weight DNA and successfully amplifies

Our LBD collection method resulted in an average qbit value of 3.7 ng/μL, extraction quality remained independent of the number of samples pooled. These extractions successfully amplified with MIPs and produced the expected band around 400bp (SF 1b). Fragment analysis further confirms size specificity with 420 bp concentration measuring at 1.96 ng/μL (SF 1a).

Table 2: DNA Extraction Quantification

Pool Name	Quantification (ng/μL)	# of Samples
10101D	0.1	6
10101A	4	9
30303B	0.3	9
11011A	0.1	9
15015A	0.2	10
30303G	1.9	10
20202E	0.1	11
50505C	0.1	11
17017C	0.2	13
121012A	0.1	13
90909K	1.4	15
60606C	38.6	16
20202A	4.6	16
60606A	8	16
13013B	22.2	17
80808D	0.5	17
70707E	2.3	17
30303H	1	18
80808H	0.1	18

19019A	0.2	19
40404H	1.1	21
40404F	1.4	22
11011C	0.6	24
80808C	1.2	27
11011E	0.1	29
14014C	0.4	29
70707C	1.3	30
80808B	4.7	32
90909N	1.7	36
40404C	3	40
14014B	0.1	45
17017B	0.9	48
90909H	6.5	52
70707A	3	56
90909D	0.4	71
90909J	1.5	73
90909C	0.7	83
90909E	26	88
90909B	1.8	88
Average	**3.7**	**29.8**

Pooled sequencing effectively provides a rapid method to overall frequency for mutation monitoring of R561H

We use pooled sequencing which allows us to represent a larger number of samples in initial collection/clinic site frequency estimates, with lower reagent consumption on a faster timeline. We made 104 pools from 2713 samples with our average pool size falling around 26 (ST 1). Two rounds of sequencing yielded data for 94 pools (90.4%) that passed our quality filters. Unsuccessful pools can be attributed to poor balancing and low DNA input. These pools can be sent to resequencing and

recapture to improve yield. Pooled sequencing yields a frequency for each pool which was weighted by the number of samples it represented. The average of these weighted frequencies represents a country-wide frequency for these mutations. Based on previous reports, our main target for surveillance, R561H, was most wide spread across 16 sites with an overall frequency of 18.0% (Table 3).[11,31]

Table 3: WHO Validated/Candidate *PfK13* Mutation Frequencies

Mutation	Overall Frequency	Samples Represented	Sites	WHO Status
P441L	0.9%	2385	7	Candidate
G449A	1.1%	2385	3	Candidate
C469F	1.0%	2385	5	Candidate
R561H	18.0%	2444	16	Validated
P574L	0.8%	2444	3	Validated
A675V	6.0%	2555	13	Candidate

Targeted sequencing finds 17 PfK13 *mutants and the first evidence of G449A in East Africa*

In addition to R561H, other notable WHO validated or candidate mutations we found were: P441L at a low overall frequency of 0.9% from 7 sites, G449A was found in 3 neighboring sites, reaching 1.1%, C469F in 5 sites at an overall frequency of 1%, P574L appeared at 0.8% and A675V was found at 6.0%, but spread across 13 sites (Table 3). As for unvalidated mutations, there were 2 other mutations found in the propeller region and 7 mutations found in the upstream region that reached < 0.5% frequency nationally (ST 2, SF 4).

R561H and other WHO mutations cluster near high-transmission regions in Rwanda

Figure 2: Spatial Distribution of WHO mutations

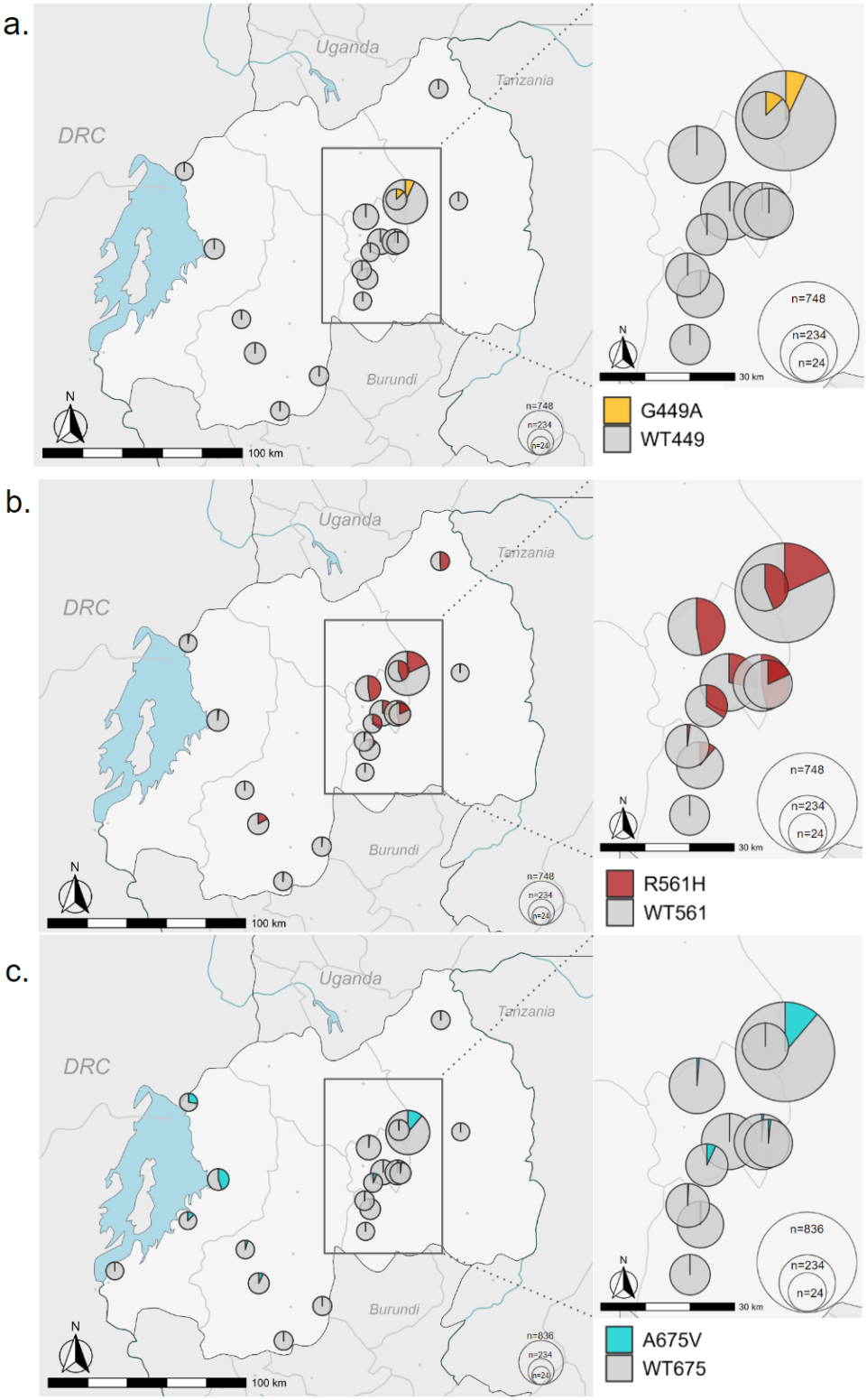


Figure 2: Geographic distribution for 4 WHO mutations are mapped to the collection center pools were sourced from. Pie chart size depicts the number of samples represented in the frequency measure while color depicts frequency of mutation (a) K13 G449A, (b) K13 R561H, (c) K13 A675V.

R561H was found throughout Rwanda with notable clustering in the central high transmission region near Tanda, which experienced an outbreak throughout sample collection (Fig. 2b). R561H frequency reached the highest level in Bugaragara at 49.1% (ST 3). A similar clustering trend was maintained in the other 2 WHO identified mutations. G449A appeared in neighboring Giti (12.7%) and Tanda (6.9%) (Fig. 2a). A675V appears in high frequency near the border of DRC at Mushubati (44.6%), our second outbreak site. A675V was found in 13 sites but only 6 of these sites were recorded above 5% (Fig. 2c, ST 3). Maps of the other three WHO mutations are provided in SF 3, as national frequencies less than 1% showed relatively similar clustering. P441L, C469F and P574L were all present in Masaka, and similarly clustered nearby at Solace. C469F appeared highest in the east in Mushubati at 10.6% and P574L was highest in the south at 9.1% in Nyamagabe (ST 3). P441L reached the highest frequency in Masaka (7.7%), with the other 6 sites with P441L mutant clustered near the high frequency sites in central Rwanda, but were all at frequencies less than 2.5% (SF 3).

Temporal analysis reveals a potential seasonal trend & shows heterozygosity in R561H at one site

Figure 3: Bimonthly frequency of WHO mutations

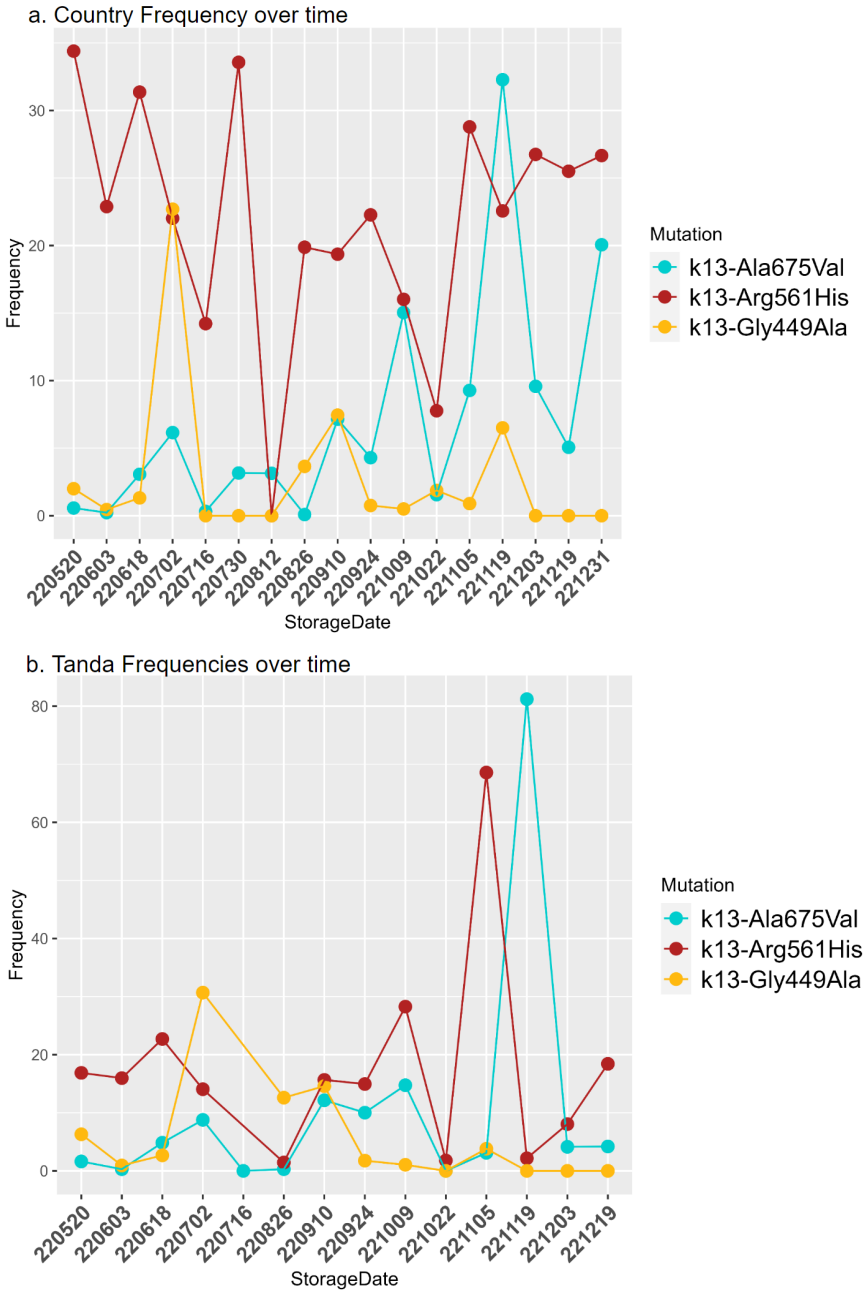


Figure 3: (a) Averaged pools based on the most occurring storage date in each pool to develop a country-wide frequency measure over the collection period. (b) One site, Tanda, had a notable pattern when frequency was graphed over the available collection points, seeing all 3 *PfK13* occur simultaneously at multiple dates.

Temporal analysis shows heterozygosity within the country at various time points. Most notably between all three mutations on 220910 (Fig. 3a). R561H appears above 30% when sampling starts, this drops almost below 20% over August to October, increasing again in November. These fluctuations reflect seasonality - the long dry season (June to August), lowers transmission and the rise of R561H in November corresponds with the effect of the short rainy season (September-November). Country-wide temporal trends are helpful to analyze seasonality, but does not account for distance in determining if mutants are interacting. To account for this, individual graphs were developed for every site. Most interesting of which came from Tanda, where we see distinct fluctuations between dominant mutations as G449A is replaced by R561H then superseded by A675V (Fig. 3b).

Resistance mutations associated with partner drugs appear localized in the same regions

Figure 4: Partner Drug resistance distribution

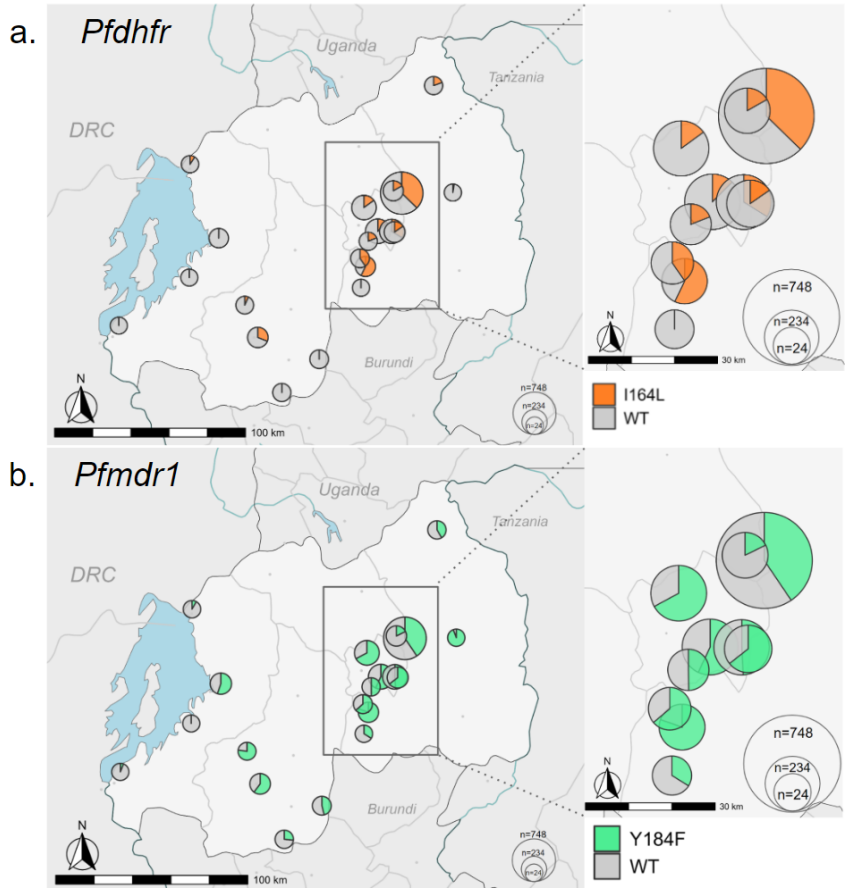


Figure 4: Depicts frequencies of a) *Pfdhfr* I164L b) *Pfmdr1* Y184F according to collection center pools were sourced from. Size of pie charts corresponds to the number of samples represented from the center. Frequencies are available in supplemental table 4.

In an effort to determine the specific threat to ACTs, we investigated mutations located on *Pfdhfr* and *Pfmdr1*, associated with pyrimethamine and lumefantrine resistance. Mutations on both genes were colocalized in high transmission regions that show elevated *PfK13* mutations (Fig. 4). The highest recorded frequency for *Pfdhfr* I164L came from Nyamata at 57.0%. In this site *Pfmdr1* Y184F reached 80.4%. *Pfmdr1* Y184F was highest in Rukara at 94.3% (ST 4).

Discussion

The emergence of *PfK13* mutations in Rwanda has serious implications for malaria control efforts as ACTs could quickly become ineffective, requiring the innovation of more efficient strategies for surveillance. We pioneered a cost-effective rapid method for sample collection and DNA isolation combined with MIP genotyping, providing a rapid initial assessment within 3 months of the final collection that can inform future sequencing targets and studies. LBD provides an efficient method of sampling in-country and magnetic bead extractions yield extraction output (ng/ul), comparable to the commercial standard while lower cost (Table 2).[34] Additionally, LBD provides high quality DNA extract that is directly transferred to multiple amplification protocols, reducing the time to result by avoiding the need for optimization. This makes LBD + magbead extraction a more robust method compared to DBS + chelex, reducing failed sequencing due to chemical contamination. LBD additionally has the potential for high quality RNA. Our sample collection cost requires $0.30 per sample and $0.80 per extraction which makes it significantly more affordable than current DBS kits that provide comparable DNA output.[34,35] The advantage of our pooling method is represented by the rapid delivery of this paper. Pooling, extraction, sequencing and analysis was completed within one month. Due to the large scale of this sample collection, individual sample processing and analysis for these samples will likely require a year before sample level information can be delivered for publication. ACT mutation surveillance can only inform treatment and vector management effectively if the data is disseminated within a timely manner while it is still reflective of current parasite populations.

To place our findings in context, various low level mutations across the *PfK13* have been reported previously in Rwanda.[11,31] Uwimana et al. provide the first report of R561H from samples collected in 2014 from Masaka.[11] Kirby et al, report finding additional R561H in 2014 samples near Kirehe and Ngoma.[31] When samples from 2018 were tested, prevalence of R561H increased by more than 10% in the same regions.[14] In surveying these 2022 samples for R561H, we found R561H has expanded from these regions into the district of Gicumbi (Fig. 2b). R561H was present in the two sites sampled from this district as well as 14 others spread across the country. Finding R561H at an overall frequency of 18.0% prompted us to search for other markers of resistance. Concerningly, five other WHO designated mutations were found at lower frequencies, (<10%) but spatial and temporal analysis makes their presence all the more concerning. *PfK13* P441L, G449A, C469F, P574L and A675V were all located at or near sites that also harbored R561H frequencies >20% (ST 3). Temporal analysis shows that not only are multiple ART mutations appearing in the same place, they are also occurring at the same time (Fig. 3a). This has significant implications as recombination events are likely and could lead to a heterozygous parasite with significant ART resistance.

Our temporal analysis also showed an interesting interaction between the three *PfK13* mutations. In Tanda, we see trade off between the most prevalent mutations over time. This suggests some amount of competition between parasites containing G449A and R561H. Potentially related, Tanda experienced a malaria outbreak throughout the duration of our study providing nearly 200 samples per month during peak transmission. This isolation and the possible fitness cost visible in this data warrants further

investigation of these parasites. Contextual analysis of country-wide frequency data shows correlating patterns with climate and vector life cycles, as an increase of R561H occurs simultaneously with the start of the rainy season, which is to be expected from geographic and transmission patterns associated with mosquito breeding (Fig. 3a).

V555A, a mutation not currently flagged by the WHO, has been reported at various sites in 2012, 2014, 2015 and 2019.[11,13,27,36] We also found V555A at 0.7% across 8 sites and report 16 mutations found within *PfK13* (ST 2). Eight of these mutations lie within the propeller region - two representing validated targets and four candidate mutations (ST 2, SF 4). Unvalidated mutations, like V555A, should continue to be monitored and investigated further for potential ART resistance association. In a site level comparison with previous literature, at Masaka in 2014 (n=58), R561H occurred at 12.07%. In 2015 this was reported at 6.03% (n=199).[11] By 2021 rates from Masaka were reported to increase to 20% (n=51).[14] We found R561H at 18.1% in Masaka (n=129), showing a dynamic landscape and demonstrating the need for consistent sampling as long gaps may interfere with appropriate clinical response.

Lastly, our concern for ACT resistance stems from the overlap between the occurrence of partner drug resistance and WHO designated ART resistant mutations. *Pfdhfr* I146L is associated with delayed clearance when treated with pyrimethamine.[4,22] I146L was found clustered in the high transmission region near Tanda, as well as the northern site Bugaragara and the southern site Nyamagabe both of which harbored R561H. While ASP is not used nationally in Rwanda, SP can be found on the private market and this may be increasing selection pressure for *Pfdhfr* I164L mutant parasites.

With colocalization introducing ART and SP resistant populations, recombination could yield a parasite resistant against ASP.

Similarly, *Pfmdr1* haplotypes can be an indicator of Lumifantrine resistance. A haplotype of wild type at N86 and D1246 combined with a mutation at 184F has been associated with recrudescence following AL treatment.[21,22] As a result of our pooling method, we are unable to look at specific haplotypes but can observe the distribution of mutant Y184F. Y184F was present in all but one site that successfully sequenced (Fig 4b, ST 4). Rwanda's malaria management strategy relies on AL for treatment. Considering that wildtype alleles for N86 and D1246 are likely in higher frequency than mutant, there is a clear threat to AL efficacy. This finding represents the biggest threat to Rwandan malaria management and calls for investigation into individual data to find haplotype prevalence and determine the severity of the situation.

While this analysis has many advantages over traditional approaches, there are also multiple limitations. The pools that have been developed in this study do not consider parasitemia per sample. Subsequently, when samples with wide-ranging parasitemia values are pooled together, high parasitemia samples are likely to dominate sequencing reads in each pool. Considering known data regarding transmission and mutation emergence, mutant and low variant samples are suppressed in high transmission regions like Tanda.[25,37] Conversely in low transmission regions, where mutant parasites have reduced competition and can reach high concentrations, mutations may be overrepresented.

We should also consider the possibility of incomplete DNA purification. Table 2 provides qbit values for the extractions completed on these pools. Comparable

commercial kits, like Dynabead from ThermoFisher, characterizes a standard range of 1-5 ng/µl DNA.[34] For samples that quantify below this concentration (n=18, 46%), there is potential for disproportionate extraction of all the samples input to the pool. This is balanced in our analysis here by weighting pools based on the number of samples instead of relying on UMI ratios.

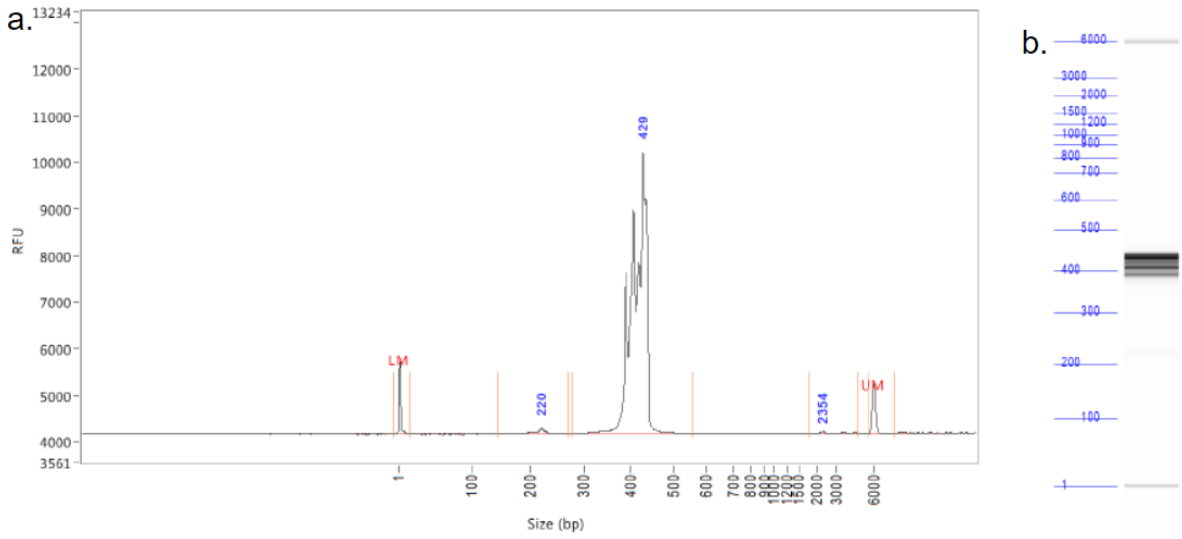
An inherent weakness in the temporal analysis provided is the assumed date of collection. These dates are recorded based on intervals where technicians went to health centers to gather samples, not the date of collection. This stratifies the samples into two week intervals, but is a low resolution representation of temporal trends. While the pooled strategy provides an estimate of current frequency, it appears that mutations in Rwanda have risen to a level where this strategy is only effective as an initial assessment. Pooling restricts the variance research that can be performed. Mixed infection, haplotype and identity by descent analyses all require individual level data to provide reliable results. WGS of these pooled samples are unlikely to yield any further meaningful results and focus should shift to sequencing and analysis of individual isolates. In depth-study of individual sample results can incorporate more specific geographic data, allowing for a more detailed study of each district. Ultimately if G449A and V555A continue to spread, further therapeutic efficacy studies should be conducted on Rwandan strains to determine the severity of this threat.

Conclusion

This work characterizes a new method for sample collection and pooled extraction and MIP sequencing that reduces barriers to the characterisation and surveillance of *PfK13* ART resistant parasites. We apply this method in Rwanda as previous studies have characterized the development of R561H across the country as early as 2014 which represents a clear threat to ACT efficacy.[11,31] Using a rapid pooling strategy, this study shows increasing R561H that clusters in high-transmission regions at a national frequency of 18.0%, with temporal frequency patterns that correspond to seasonality. We also identify five other *PfK13* propeller mutations, including G449A which has not yet been reported in East Africa, all of which should be included in future investigations. This data provides an important update on the state of R561H and a way forward for accessible informative monitoring studies.

Supplementary

Supplementary Figure 1



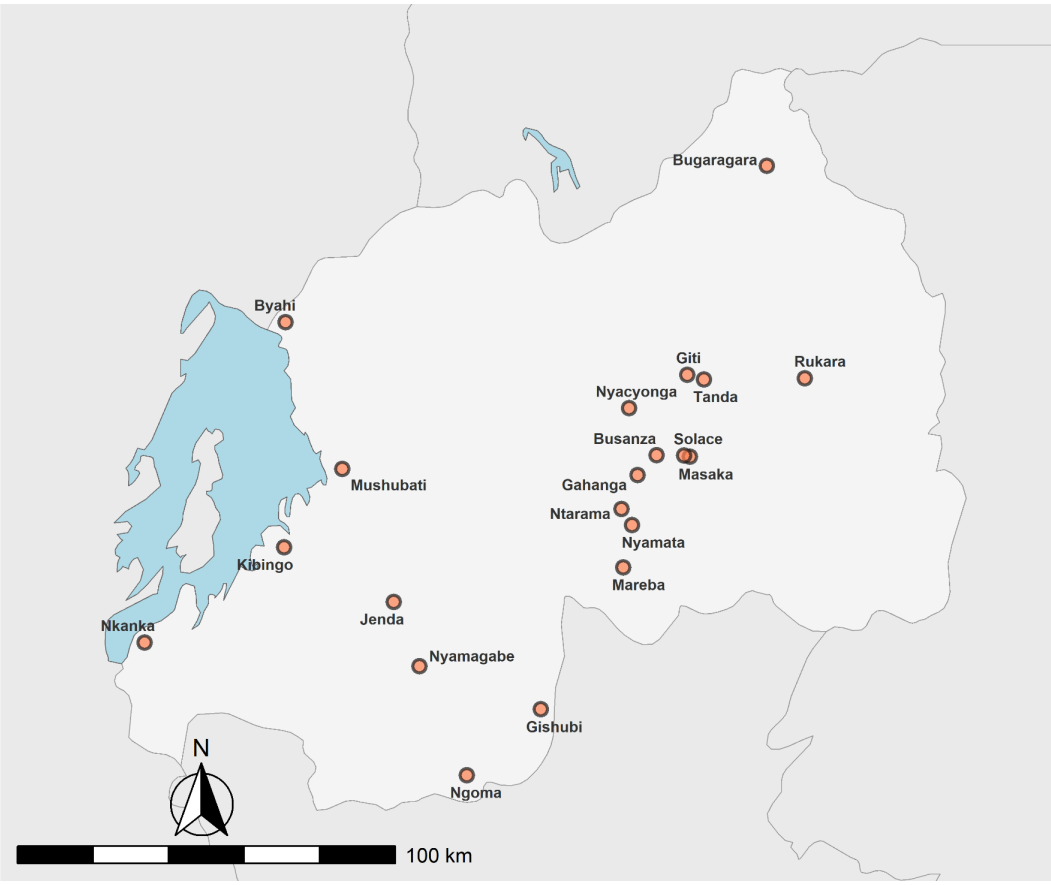
Supplementary Table 1

Pool Name	Origin	Number of Samples	Storage Date	Pool Name	Origin	Number of Samples	Storage Date
2020A	Bugaragara	18	221009	19019A	Nkanka	19	2022
2020B	Bugaragara	16	221105	20202A	Ntarama	16	220603
2020C	Bugaragara	16	221231	20202B	Ntarama	21	220618
40404A	Busanza	40	220603	20202C	Ntarama	13	220716
40404B	Busanza	32	220716	20202D	Ntarama	7	220826
40404C	Busanza	40	220826	20202E	Ntarama	11	220910
40404D	Busanza	40	220910	80808A	Nyacyonga	56	220520
40404E	Busanza	24	221009	80808B	Nyacyonga	32	220618
40404F	Busanza	22	221105	80808C	Nyacyonga	27	220730
40404G	Busanza	19	221203	80808D	Nyacyonga	17	220826
40404H	Busanza	21	221231	80808E	Nyacyonga	28	220924
18018A	Byahi	27	2022	80808F	Nyacyonga	34	221022
50505A	Gahanga	14	220520	80808G	Nyacyonga	22	221105
50505B	Gahanga	10	220603	80808H	Nyacyonga	18	221219
50505C	Gahanga	11	220702	13013A	Nyamagabe	12	221009

50505D	Gahanga	8	220730	13013B	Nyamagabe	17	221022
50505E	Gahanga	7	221009	13013C	Nyamagabe	23	221104
15015A	Gishubi	10	220924	13013D	Nyamagabe	18	221119
15015B	Gishubi	10	221009	13013E	Nyamagabe	19	221203
15015C	Gishubi	29	221022	13013F	Nyamagabe	28	221219
15015D	Gishubi	8	221119	30303A	Nyamata	27	220603
15015E	Gishubi	9	221203	30303B	Nyamata	9	220618
11011A	Giti	9	220924	30303C	Nyamata	17	220730
11011B	Giti	28	221022	30303D	Nyamata	9	220812
11011C	Giti	24	221119	30303E	Nyamata	9	220910
11011D	Giti	27	221203	30303F	Nyamata	9	221009
11011E	Giti	29	221231	30303G	Nyamata	10	221105
14014A	Jenda	39	221022	30303H	Nyamata	18	221219
14014B	Jenda	45	221119	101010A	Rukara	22	2022
14014C	Jenda	29	221231	70707A	Solace	56	220520
16016A	Kibingo	16	2022	70707B	Solace	54	220618
10101A	Mareba	9	220702	70707C	Solace	30	220730
10101B	Mareba	5	220826	70707D	Solace	10	220826
10101C	Mareba	10	220924	70707E	Solace	17	220910
10101D	Mareba	6	221009	70707F	Solace	28	221105
60606A	Masaka	16	220520	70707G	Solace	16	221119
60606B	Masaka	16	220618	70707H	Solace	19	221219
60606C	Masaka	16	220702	90909A	Tanda	66	220520
60606D	Masaka	24	220826	90909B	Tanda	88	220603
60606E	Masaka	17	220910	90909C	Tanda	83	220618
60606F	Masaka	17	221105	90909D	Tanda	71	220702
60606G	Masaka	21	221203	90909E	Tanda	88	220716
60606H	Masaka	18	221219	90909F	Tanda	42	220826
17017A	Mushubati	40	221022	90909G	Tanda	46	220910
17017B	Mushubati	48	221104	90909H	Tanda	52	220910
17017C	Mushubati	13	221203	90909I	Tanda	36	220924
17017D	Mushubati	40	221219	90909J	Tanda	73	221009
17017E	Mushubati	35	221231	90909K	Tanda	15	221022
121012A	Ngoma	13	221022	90909L	Tanda	40	221105
121012B	Ngoma	14	221104	90909M	Tanda	64	221119

121012C	Ngoma	9	221119	90909N	Tanda	36	221203
121012D	Ngoma	15	221231	90909O	Tanda	36	221219
Total Samples = 2713				**Average Pool Size = 26**			

Supplemental Figure 2



Supplementary Table 2

Mutation	Country-wide Frequency (%)	# of Samples represented
Non-Validated Mutations		
k13-Asn142del	8.0	2085
k13-Asn142dup	4.1	2085
k13-Lys189Thr	35.9	2313
k13-Ile205Thr	1.0	2313

k13-Asn217His	2.2	2396
k13-Asp247Tyr	0.9	2444
k13-Leu258Met	5.4	2444
k13-Asn264Ser	5.4	2444
k13-Val555Ala	0.7	2444
k13-Pro667Arg	1.7	2555
k13-Pro667Ser	0.8	2555
WHO Validated/Candidate Mutations		
k13-Pro441Leu	0.9	2385
k13-Gly449Ala	1.1	2385
k13-Cys469Phe	1.0	2385
k13-Arg561His	18.0	2444
k13-Pro574Leu	0.8	2444
k13-Ala675Val	6.0	2555

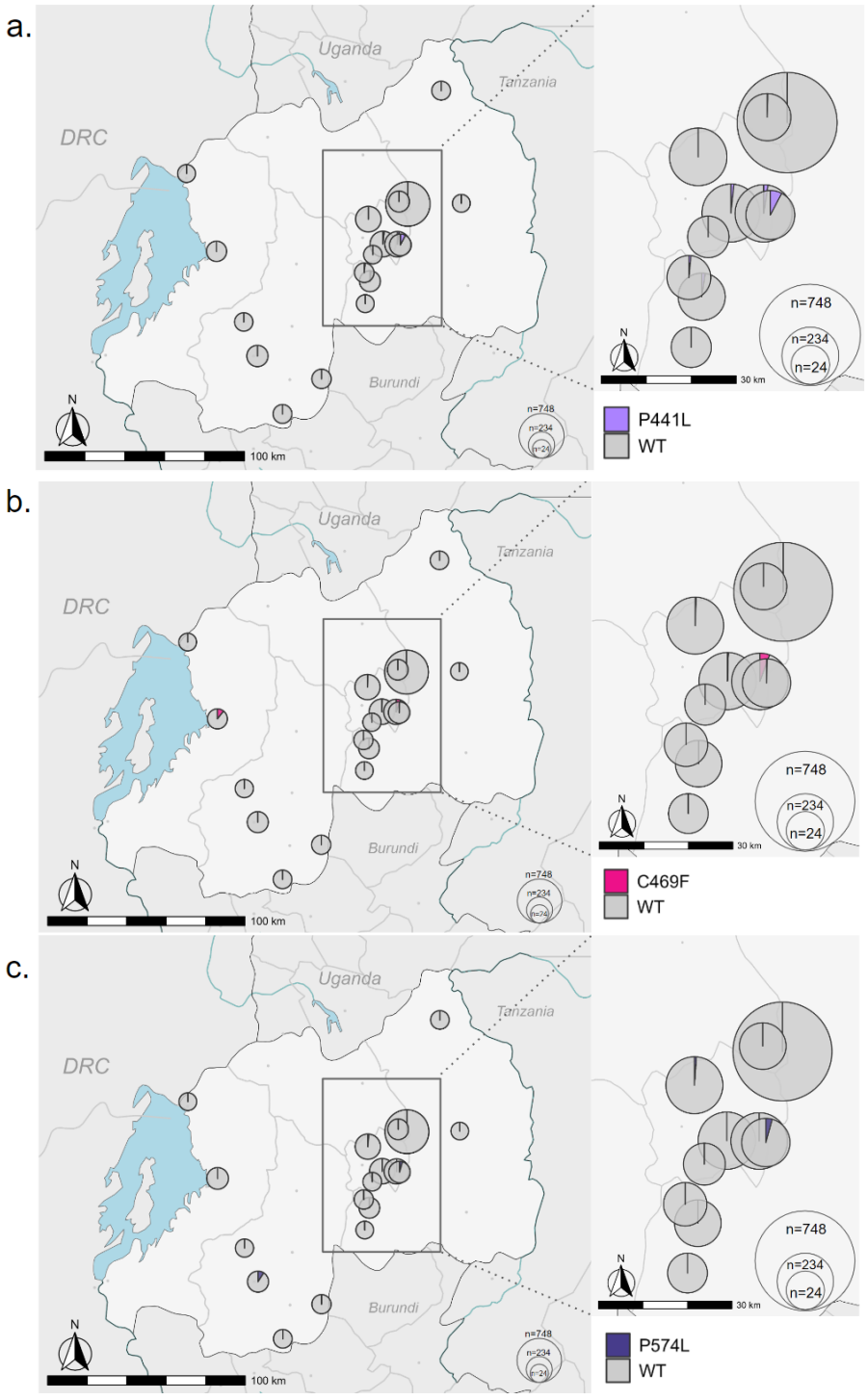
Supplemental Table 3

Origin	% G449A Frequency	# of Samples	% R561H Frequency	# of Samples	% A675V Frequency	# of Samples
Bugaragara	0.0	50	49.1	50	0.0	50
Busanza	0.0	238	28.3	238	0.2	238
Byahi	0.0	26	2.7	26	27.3	26
Gahanga	0.0	39	34.5	50	6.9	50
Gishubi	0.0	66	1.1	66	0.0	56
Giti	12.7	108	43.8	108	0.0	108
Jenda	0.0	39	0.0	39	4.4	39
Kibingo	NA	NA	NA	NA	12.2	15
Mareba	0.0	24	0.0	24	0.0	24
Masaka	0.0	129	18.1	129	1.5	129
Mushubati	0.0	88	1.8	136	44.6	136
Ngoma	0.0	51	0.9	51	0.0	51
Nkanka	NA	NA	NA	NA	0.0	18
Ntarama	0.0	68	2.1	68	0.7	68
Nyacyonga	0.0	234	47.2	234	1.4	234

Nyamagabe	0.0	117	17.0	117	6.8	117
Nyamata	0.4	108	11.4	108	0.6	108
Rukara	0.0	22	0.8	22	0.0	22
Solace	0.0	230	46.6	230	1.1	230
Tanda	6.9	748	18.0	748	11.3	836
Average/ Total	1.1%	2385	18.0%	2444	6.0%	2555

Origin	% P441L Frequency	# of Samples	% C469F Frequency	# of Samples	% P574L Frequency	# of Samples
Bugaragara	0.0	50	0.0	50	0.0	50
Busanza	1.7	238	0.5	238	0.0	238
Byahi	0.0	26	0.0	26	0.0	26
Gahanga	0.0	39	0.0	39	0.0	50
Gishubi	0.0	66	0.0	66	0.0	66
Giti	0.4	108	0.0	108	0.0	108
Jenda	0.0	39	0.0	39	0.0	39
Mareba	0.0	24	0.0	24	0.0	24
Masaka	7.7	129	0.3	129	4.3	129
Mushubati	0.0	88	10.6	88	0.0	136
Ngoma	0.0	51	0.0	51	0.0	51
Ntarama	1.5	68	0.0	68	0.0	68
Nyacyonga	0.0	234	0.5	234	1.2	234
Nyamagabe	0.0	117	0.0	117	9.1	117
Nyamata	2.3	108	0.0	108	0.0	108
Rukara	0.0	22	0.0	22	0.0	22
Solace	2.4	230	5.5	230	0.0	230
Tanda	0.1	748	0.0	748	0.0	748
Average/ Total	0.9%	2385	1.0%	2385	0.8%	2444

Supplementary Figure 3



Supplemental Table 4

Origin	% dhfrI164L Frequency	# of Samples	% mdrY184F Frequency	# of Samples
Bugaragara	19.5	50	41.4	50
Busanza	12.4	238	57.0	238
Byahi	9.3	26	8.3	26
Gahanga	19.0	50	49.8	50
Gishubi	0.0	66	46.1	66
Giti	16.7	108	17.7	108
Jenda	6.6	39	77.3	39
Kibingo	0.0	15	0.0	15
Mareba	0.0	24	33.9	24
Masaka	15.3	129	64.4	129
Mushubati	0.3	88	55.3	136
Ngoma	0.0	51	26.6	51
Nkanka	0.0	18	6.1	18
Ntarama	40.4	68	63.9	68
Nyacyonga	14.9	234	67.1	234
Nyamagabe	31.1	117	60.2	117
Nyamata	57.0	108	80.4	108
Rukara	2.5	22	94.3	22
Solace	33.9	230	49.1	230
Tanda	37.4	748	40.6	748
Average/Total	15.8%	2429	47.0%	2477

Supplementary Table 5

Molecular markers of resistance to antimalarial drugs for *P. falciparum*

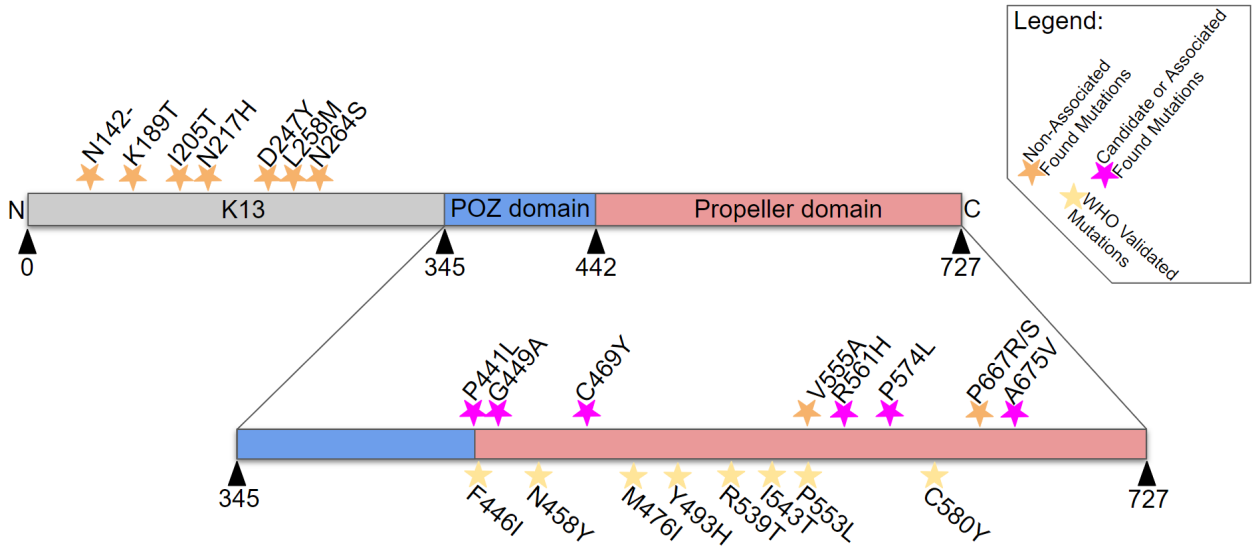
Drug	Molecular markers		References
	Gene	Mutation	
4-aminoquinolines			
Chloroquine	*Pfcrt*	K76T + different sets of mutations at other codons (including C72S, M74I, N75E, A220S, Q271E, N326S, I356T and R371I)	*(46-49)*
	Pfmdr1 (in combination with *Pfcrt* mutations only)	N86Y, Y184F, S1034C, N1042D and D1246Y	*(50-52)*
Amodiaquine (mannich base)	Yet to be validated	Studies show that amodiaquine selects for *Pfmdr1* mutations (86Y)	*(53-57)*
Piperaquine	*Pfpm2–3*	*Pfpm2–3* increased copy number	*(44)*
	Pfcrt	Detected in vivo: T93S, H97Y, F145I, I218F and C350R	*(58-62)*
		Detected in vitro: T93S, H97Y, F145I, I218F, M343L and G353V	
Antifolates			
Pyrimethamine	*Pfdhfr*	N51I, C59R, S108N and I164L	*(63, 64)*
Sulfadoxine	*Pfdhps*	S436A/F, A437G, K540E, A581G and A613T/S	*(64)*
Proguanil	*Pfdhfr*	A16V, N51I, C59R, S108N and I164L	*(65)*
Amino-alcohols			
Lumefantrine	Yet to be validated	Studies show that lumefantrine selects for *Pfmdr1* mutations (N86)	*(54, 55, 57, 66)*
Mefloquine	*Pfmdr1*	*Pfmdr1* increased copy number	*(51)*
Quinine	Yet to be validated		
Mannich base			
Pyronaridine	Yet to be validated		
Naphthoquinone			
Atovaquone	*Pfcytb*	Y268N/S/C	*(65, 67)*
Sesquiterpene lactones			
Artemisinin and its derivatives	*PfK13*	List of candidate and validated markers developed (see Table 4)	*(13, 68)*
Antibiotics			
Doxycycline	Resistance not documented		
Clindamycin	Resistance not documented		
8-aminoquinolines			
Primaquine	Resistance not documented		
Tafenoquine	Resistance not documented		

Current list of validated and candidate or associated *PfK13* mutations

Validated		Candidate or associated	
F446I	**P553L**	P441L	N537I/D
N458Y	**R561H**	G449A	G538V
M476I	**P574L**	C469F/Y	V568G
Y493H	**C580Y**	A481V	R622I
R539T		R515K	A675V
I543T		P527H	

Taken from WHO[13]

Supplementary Figure 4



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