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Supplementary appendix

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SUPPLEMENTAL APPENDIX

Prevalence of mutations associated with artemisinin partial resistance and sulfadoxine-pyrimethamine resistance in 13 regions in Tanzania in 2021: a cross-sectional survey

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SUPPLEMENTAL TEXT

Study Design

Cross-sectional sampling from 100 health facilities was undertaken according to the WHO protocol for surveillance of *Plasmodium falciparum* histidine rich proteins 2 and 3 (*pfhrp* 2/3) deletions.^{[1,2](https://paperpile.com/c/idWs8e/HK2qZ+GjHIs)} Ten health facilities in each of the following regions were used: Dar es salaam, Dodoma, Kagera, Kilimanjaro, Manyara, Mara, Mtwara, Njombe, Songwe and Tabora. Patients that presented with symptoms compatible with uncomplicated malaria were approached and enrolled in the study. Written informed consent was provided by adult patients or parents/guardians for their children. Children aged 7 - 17 years also provided an assent. Enrollment criteria included being 6 months of age or older, meeting the case definition for uncomplicated malaria and residing in the study area. All clinic attendees who were enrolled in the study were provided with two types of malaria rapid diagnostic tests (mRDT), the standard histidine rich protein 2 (HRP2)/pan lactate dehydrogenase (LDH) mRDT used by the national health system and a *P. falciparum* LDH test [#C14RHG25, RapiGEN Inc. BIOCREDIT Malaria Ag Pf (Pf-pLDH), Republic of Korea], according to the WHO protocol and as described by Rogier et al.^{[2](https://paperpile.com/c/idWs8e/GjHIs)} Any patient with positive results by either of the two mRDTs was treated per national guidelines. In addition to mRDTs, all participants provided dried blood spots (DBS). These DBS were used to test for malaria using qPCR and sequencing. Participants who were mRDT were sequenced. In addition, a subset of qPCR positive mRDT negative were included in the study for sequencing.

In addition to health facility sampling, three cross-sectional community surveys were conducted in regions of Tanzania.[3](https://paperpile.com/c/idWs8e/1j4Ia) Malaria epidemiology in these regions has also been previously studied by the National Institute for Medical Research (NIMR).^{[4,5](https://paperpile.com/c/idWs8e/q6sVW+5n7ZT)} Community samples were collected from 3 regions: Kigoma, Ruvuma, and Tanga. In these regions, asymptomatic community members or their guardians provided written consent (assent procedures were done as described above), were tested by an mRDT, and then provided DBS if positive for molecular analysis, including MIP sequencing.^{[3](https://paperpile.com/c/idWs8e/1j4Ia)}

Participant characteristics are shown in **Table S2 p.5**. Age strata were designated based on previous reports.^{[1](https://paperpile.com/c/idWs8e/HK2qZ)}

Molecular Inversion Probes

Molecular surveillance of mutations that impact antimalarial resistance (**Table S1 p. 4**) should rely on platforms that can broadly detect different resistance mutations. Highly multiplex amplicon deep sequencing is one approach that has shown promise. Another approach is molecular inversion probes (MIPs), which have now been used extensively to characterize drug resistance and population structure in parasites in Africa.^{[5–7](https://paperpile.com/c/idWs8e/h07ed+5n7ZT+pKNYC)} The ability to create and combine different highly multiplexed panels for antimalarial resistance mutations, copy number variation, gene deletions and other genome wide polymorphisms to study complexity of infection, parasite relatedness or population structure makes the MIP platform a highly flexible and cost-effective means of conducting malaria molecular surveillance (MMS). The methods used to produce the parasite genotyping reported in this study are summarized as follows (**Figure S2, p.34**): 1.) Chelex purified DNA template was hybridized to long oligos approximately 100 base pairs long, with each oligo containing complementary arms to the targeted capture region, as well as universal primer sequences and a unique molecular identifier (UMI). 2.) Following hybridization, a circularization step was performed using T4 ligase, then non circularized DNA was digested using exonucleases I and III. 3.) The pool of circularized capture sequence was then PCR amplified using universal primers containing illumina adapters to produce an amplicon that can be sequenced on the Illumina NextSeq500 platform (Illumina, San Diego, CA).

Amplicons from each sample contained a dual index that was demultiplexed using Illumina's built-in bcl2fastq2 software (Illumina, San Diego, CA). Resulting fastq data was further processed in MIPtools and variants were called using freebayes (https://github.com/freebayes/freebayes). Downstream filtering exploited the UMI clustering to enable high-confidence calls. Samples with less than 10X coverage at K13 R561H, were rebalanced by re-pooling amplified products to increase the ratio of samples with low coverage and resequenced.

For the MSMT programme, we recognized that the high priority regions neighboring Rwanda needed additional sequencing effort. After an initial pass with all of the MIP probes included in each capture reaction, we found that sample missingness was relatively high. This led to a second round of MIP capture with a greatly reduced probe set designed to capture only key gene fragments associated with well described drug resistance phenotypes (**Table S1, p.4**).

Specific Whole Genome Amplification

Specific whole genome amplification (sWGA) is a technique that uses primers that more commonly or specifically bind to the pathogen of interest than to DNA in the background (e.g. human DNA). This method has been extensively used in malaria genomics to enrich parasite DNA from clinical samples to allow for whole genome sequencing. The sWGA reaction was carried out as previously described.^{[8](https://paperpile.com/c/idWs8e/EEki)} However, each sample was amplified in triplicate and the sWGA reactions pooled together prior to library preparation for whole genome sequencing.

Genomic architecture of artemisinin resistance polymorphisms from Southeast Asia

To investigate associations with mutations previously found to occur in drug resistant parasites in Southeast Asia in combination with K13 mutations, we used the MIP panel genotypes and quantified mutations occurring in the same isolates.^{[9](https://paperpile.com/c/idWs8e/usCW5)} None of the following mutations were seen in K13 561H isolates:

- *Plasmodium falciparum* chloroquine resistance transporter (CRT) 326S and 356T
- *Plasmodium falciparum* multidrug resistance protein 2 (MDR2) 484I
- *Plasmodium falciparum* protein phosphatase (PPH) 1157L
- putative phosphoinositide-binding protein (PIB7) 1484F

However, ferredoxin (FD) 193Y polymorphisms were found in 13 K13 561H isolates in Kagera (**Table S4, p.9**).

Table S1. Antimalarial resistance polymorphisms for partner and chemoprevention agents reported (Adapted from [10–12](https://paperpile.com/c/idWs8e/VgOVX+xGLsQ+fL2bJ) **)**

Table S2. Participant characteristics.

* Age Distributions: Young Children <5 years of age; School Children 5-16 years of age; Adults >16 years of $age¹$ $age¹$ $age¹$

Table S3. Public genomes used in the analysis.

Southeast Asia Samples	Rwanda Samples
PD0068-C	ERX4235773
PD0079-C	ERX4235772
PD0126-C	ERX4235771
PD0481-C	ERX4235770
PD0495-C	ERX4235769
PD0562-C	ERX4235768
PD0565-C	ERX4235767
PD0799-C	ERX4235766
PD0800-C	ERX4235765
PD0819-C	ERX4235764
PD0822-C	ERX4235763
PD0823-C	ERX4235762
PD0824-C	ERX4235761
PD0826-C	ERX4235760
PD0828-C	ERX4235759
PD0832-C	ERX4235758
PD0876-C	ERX4235757
PD0877-C	ERX4235756
QC0184-C	ERX4235755
QC0185-C	ERX4235754
QC0250-C	ERX4235753
RCN03272	ERX4235752
RCN03319	ERX4235751
RCN03339	ERX4235750
RCN03348	ERX4235749
RCN03388	
RCN03502	
RCN03510	
RCN03535	
RCN09301	
RCN09302	
RCN09312	
RCN09313	
RCN09314	
RCN09316	
RCN09317	
RCN09319	

Table S4. Mutations in antimalarial resistance genes. Only mutations with a UMI coverage of 10, a UMI alternate count of 3, and presence in at least three samples are represented here. The first row for each mutation is a prevalence fraction showing the number of samples with alternate UMIs above the alternate UMI threshold (numerator), and the total number of samples with a coverage above the coverage UMI threshold (denominator). The second row is a percentage representation of the fraction from the first row. The third row is a 90% confidence interval for the percentages of the second row.

Figure S1. Regions with sampling in the study. The regions of Tanga, Ruvuma, and Kigoma involved community survey samples. The remaining regions involved sample collections in health facilities.

Figure S2. Schematic of Molecular Inversion Probe Method. UMI: Unique Molecular Identifier. The 3'-end of the molecular inversion probe ends in the extension arm where polymerase primes and extends while the 5' end of the MIP represents the ligation arm where after extension the circle is ligated closed. Both arms hybridized based on complementary sequence to the sequence flanking the target. Generated with Biorender academic license to University of North Carolina**.**

Figure S3. Extended haplotype comparison between TZ2 and Southeast Asian (SEA) isolates. R561**H** mutant parasites collected in SEA from Pf7K (blue box) are compared to TZ2 isolates (red box) from this study. Extended haplotypes show two distinct groups.

Figure S4. Inheritance by descent relatedness among R561H parasite infection in Kagera. IBD ranges from 0 (completely unrelated) to 1 (identical) for infections. Panel A and B show networks of parasites with 0.5 and 0.25 IBD, respectively. Isolates are colored by the district that they are from.

Figure S5. IBD relatedness of R561H infections between regions. At an IBD level of 0.25, the two parasite isolates from Tabora cluster with isolates from Kagera. The isolates in other regions remain not connected to the network demonstrating lower levels of relatedness.

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