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Informing Drug Policy: Antimalarial Drug Resistance Molecular Marker Surveillance In Tanzania

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Abstract

This study aimed to use molecular markers to do surveillance of antimalarial drug resistance and inform drug policy. The focus was on sulfadoxine-pyrimethamine drug resistance since that was the first-line treatment in Tanzania for an uncomplicated malaria infection of *Plasmodium falciparum*. There are five mutations across two genes that are associated with this drug. On the *dhfr* gene, mutations at the 51 (N51I), 59 (C59R), and 108 (S108N) position are associated with pyrimethamine drug resistance. On the *dhps* gene, mutations at the 437 (A437G) and 540 (K540E) positions are associated with sulfadoxine drug resistance. Samples were collected from Tanzania in 2004, and the blood samples analyzed via high resolution melting (HRM). The peaks of each sample were compared to reference strains to determine the genotype of each sample. The prevalence of 51I, 59R, 108N, 437G, and 540E was 50%, 42.3%, 69.3%, 28.0%, and 0%, respectively. Individual genotypes were reported for those samples that were completed for all the quintuple mutations (n = 50). For the *dhfr* gene, 76% had a mutation at the 108 position, 52% had a mutation at the 51 position, and 42% had a mutant allele at the 59 position. For the *dhps* gene, 28% had a mutant genotype at the 437 position, and 0% had a mutation at the 540 positions. These prevalence results were like those of other studies. However, the individual genotypes differed from other studies. Further research must be conducted to determine the reasons for these differences.

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Introduction

Malaria is a parasitic disease that is transmitted person to person via the bite of an infected female *Anopheles* mosquito. As of 2018, around 228 million cases of malaria exist worldwide [1]. Africa carries a disproportionately high percentage of the global malaria burden. In 2018, Africa represented about 93% of all malaria cases and about 94% of malaria deaths [1]. There are 5 species of the parasite that causes malaria in humans, of which *Plasmodium falciparum* and *Plasmodium vivax* pose the highest risk. *Plasmodium falciparum* is the most virulent strain of *Plasmodium* and is associated with most malaria-related deaths [2]. *P. falciparum* accounted for about 99% of estimated malaria cases in Africa as of 2018 [1]. Certain groups within the population are at a higher risk of becoming infected with malaria and developing severe disease than others. These groups include children under 5 years old, pregnant women, those would are infected with HIV/AIDS, and migrants or travelers to endemic regions [3]. In order to combat a *Plasmodium* infection, antimalarials are used to target proliferative trophozoites and schizonts. The current treatment of choice in endemic areas is artemisinin combination therapies (ACTs). Artemisinin is used since it blocks the ring stage and sexual parasite stage of *Plasmodium* [4].

Drug resistance is defined as the ability of a parasite to survive in the presence of drug concentrations that normally destroy parasites of the same species or prevent multiplication [5]. There are two types of resistance: relative and complete. Relative drug resistance is associated with drug tolerance that will require a higher dose of antimalarials to achieve the same level of parasite death. Complete drug resistance is associated with parasites' survival beyond the highest dosage of antimalarials that can be tolerated by the host. In the absence of drug pressure, drug resistance is thought to emerge *de novo* through spontaneous mutation or gene duplications. In the presence of drug pressure, the mutated parasites have a selection advantage that increases the mutated parasites' population size [6]. There are many mechanisms that drug resistance is achieved through, such as alterations in drug transport and permeability, drug conversion into a form with lower activity, higher expression of the target of the drug, changes to drug target that lowers the binding affinity to inhibitor, and the possibility of entering a quiescent state, so once the drug concentration is cleared, normal cell cycle progression continues [7]. To increase the coverage of larger populations, salt medicated with antimalarial were introduced. This was thought to contribute to the rise of the parasite with increased tolerance to antimalarial drugs since low concentrations of a drug in the population provide optimal conditions for drug resistance to develop. Currently, some resistance exists for every antimalarial drug on the market [8].

There has been a declining response to antimalarial drugs since their implementation. Quinine is one of the oldest antimalarials, and its first use was documented in 1631 [8]. Its responsiveness has steadily been declining from the 1970s to the 1990s [9]. Chloroquine (CQ) was introduced as a treatment in the 1940s. Chloroquine resistance was first recognized at the Thai-Cambodian border in the 1950s and spread to the east coast of Africa in 1978 [10]. Mefloquine was introduced in 1977, with resistance being reported shortly after in 1982 at the Thai-Myanmar and Thai-Cambodian borders [11]. Antifolates, such as pyrimethamine, were used as antimalarials in the late 1940s and resistance documented soon after its introduction [12]. After this, combinations of antifolate drugs began to be used as antimalarials. Sulfadoxine-

pyrimethamine (SP) replaced CQ as resistance to chloroquine increased. However, resistance emerged for SP emerged in Southeast Asia and the Amazon basin in the mid-1970s, and it spread to Africa in the 1990s [13]. With drugs losing their responsiveness to malaria, in 2005, artemisinin-based combination therapies became the first-line treatment for uncomplicated *Plasmodium falciparum* malaria in endemic countries. However, in recent years, artemisinin resistance has been noted in the Greater Mekong sub-region in Southeast Asia [14].

Malaria is endemic to most parts of Tanzania and accounts for over 40% of the disease burden of the world [15]. In 2003, over 95% of the 37.4 million people at Tanzania were at risk for malarial infection [16]. Malaria was also associated with the high rate of death among adults in Tanzania from 2003 to 2007 [17]. More than one-third of deaths among children under the age of 5 years were caused by malaria [18]. Until 2001, chloroquine was used for many years as the first-line treatment for uncomplicated malaria. It was preferred by many people since it was cheap and available, and was used for self-medication in homes since it had minimal side effects [15]. However, the rapid development and transmission of resistance to chloroquine contributed to the change in antimalarial treatment policies to sulfadoxine-pyrimethamine (SP). During the blood sample collection in 2003, the first-line treatment was SP.

Sulfadoxine-pyrimethamine (SP) is an antifolate combination-drug that inhibits two enzymes in the folate pathway. Sulfadoxine inhibits dihydropteroate synthase (DHPS) and targets 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK). Pyrimethamine inhibits dihydrofolate reductase (DHFR) and targets the thymidylate synthase (TS) enzyme. Both sulfadoxine and pyrimethamine are mostly active against the later developmental stage of asexual parasites [19]. The two targeted enzymes targets by the medications are an essential part of the folic acid biosynthesis pathway of the parasite [20]. Resistance to SP is associated with unique point mutations in *P. falciparum* DHPS-PPPK and DHFR-TS genes [21]. Amino acid mutations in the *dhps* gene (S436A, A437G, K540E, & A613T/S) are associated with sulfadoxine resistance [22]. In vitro sulfadoxine resistance is seemly associated with an amino acid mutation at position 437 of the *dhps* domain from alanine (A) to glycine (G) [A437G]. An increase of sulfadoxine resistance is associated with other mutations at positions 540 (lysine to glutamic acid), 436 (serine to phenylalanine), and 613 (alanine to serine) [23]. Amino acid mutations in the *dhfr* gene (N51I, C59R, S108N, & I164L) are associated with pyrimethamine resistance [24]. In vitro resistance, pyrimethamine resistance is more often associated with an amino acid mutation from serine to asparagine at the 108 positions on the *dhfr* domain [S108N]. Higher levels of in vitro resistance are due to mutations at positions 51 (asparagine to isoleucine), 59 (cysteine to arginine), and 164 (isoleucine to leucine) [25]. In contrast, in vivo sulfadoxine-pyrimethamine resistance is associated with a mutation on the *dhfr* domain (C59R) and one on the *dhps* domain (K540E) [26]. In Africa, the presence of the quintuple mutant *P. falciparum* parasites is the best marker for SP resistance in the field. The quintuple mutants carry three mutations in *dhfr* (S108N, C59R, N51I) and two mutations in *dhps* (A437G, K540E) [27].

A key part of malarial control is monitoring the efficacy of antimalarial drugs. Efficacy of antimalarial is assessed through therapeutic efficacy studies (TES). TES is conducted in an environment where drug administration is supervised, and then the results from the blood samples are assessed. Therapeutic efficacy studies are

prospective in terms of evaluating the patients' clinical and parasitological responses after directly observed treatment for uncomplicated malaria. These studies assess resistance that regular intervals at the same sites to allow for early detection. The clinical and parasitological outcomes are assessed on the last day of the study, which would be either 28 or 42 days, depending on the drug's elimination half-life. If infections appear during the follow-up, the samples should be genotyped to determine if the infection are new infections or recrudescence. TES can help to determine the likelihood of antimalarial drug resistance, but additional tools, such as molecular marker analysis, need to be done to confirm the presence of drug resistance [28]. There are four possible outcomes of treatment with antimalarial drugs: early treatment failure, late clinical failure, late parasitological failure, and adequate clinical and parasitological response.

Early treatment failure (ETF) can be suspected in situations that involve several possible conditions. One sign of ETF is the presence of danger signs or severe malaria on Day 1, 2, or 3 with parasitemia still present. Another sign is the existence of higher parasitemia on Day 2 than Day 0, regardless of axillary temperature. Also, the presence of parasitemia on Day 3 with an axillary temperature of greater than or equal to 37.5°C and the existence of parasitemia on Day 3 that is greater than or equal to 25% of the count that was present on Day 0. There is an absolute requirement for a measured fever on Day 3 to classify the response as a failure because a fever of Day 2 happens frequently. If a history of fever in the preceding 24 hours were used as a condition on Day 3, this would lead to an overestimation of the presence of early treatment failure. Further modifications to this definition include the requirement of a blood smear on Day 2. This change was made due to patient safety; waiting until Day 3 to get the first follow-up post-treatment blood smear would be too long and could risk delays in treatment for patients with unresponsive parasitemia [29].

Late clinical failure (LCF) is usually characterized by two main conditions. One is the existence of danger signs or severe malaria in the presence of parasitemia on any day between 4 to 28 (or Day 42, depending on the drug's elimination half-life) in any patient who did not previously meet any of the criteria of early treatment failure. The other condition would be the existence of parasitemia between 4 and 28 days (or Day 42, depending on the drug's elimination half-life) with an axillary temperature of greater than or equal to 37.5°C in patients who did not previously meet any of the criteria of early treatment failure. There are some minor variations because of differences in the recommended duration of follow-up. These differ based on transmission in a specific area. For intense transmission areas, the follow-up is suggested to be 14 days. For low to moderate transmission areas, the follow-up period is 28 days. Also, in low to moderate transmission areas, a history of fever in the previous 24 hours can be used instead of a measured fever if requiring a measured increase in body temperature could cause logistic and financial hardship because of difficulty recruiting and enrolling patients [29].

Late parasitological failure (LPF) is characterized by the presence of parasitemia on any day between 7 to 28 days (or Day 42, depending on the drug's elimination half-life) with an axillary temperature of less than 37.5°C in any patients who did not previously meet any of the criteria of early treatment failure or late clinical failure. There are differences in this definition, depending on the transmission areas. This category is a recent addition. Prior to this, asymptomatic parasitemia after Day 4 was included as part of the adequate clinical and parasitological response, but it was deemed that information

on the parasitological failure frequencies is essential to understanding drug efficacy and should be accounted for [29].

An adequate clinical and parasitological response (ACPR) is characterized by the absence of parasitemia on Day 28 (or Day 42, depending on the drug's elimination half-life) in patients who did not meet any of the criteria for early treatment failure, late clinical failure, or late parasitological failure, regardless of the axillary temperature of the patient [30]. The definition of this treatment outcome reconciles differences that existed in its previous definition by transmission intensity and is applied to only those responses that show both clinical and parasitological clearance by the end of the follow-up period [29].

Some additional important concepts for therapeutic efficacy studies are adequate clinical response (ACR), total failure, and clinical failure. Adequate clinical response (ACR) is equal to the sum of those with an adequate clinical and parasitological response (ACPR) and those with late parasitological failure (LPF). Total failure is equal to the sum of those with early treatment failure (ETF), late clinical failure (LCF), and late parasitological failure (LPF). Clinical failure is equal to the sum of those with early treatment failure (ETF) and late clinical failure (LCF). The cut-off point for policy change using the WHO protocol in high transmission areas is when patients with ACPR is less than 75%, and patients with ACR is less than 85% [29].

Failure in terms of malaria treatment may be because of infection with drug-resistant parasites or bad absorption of the drug by the patient. A key measure of drug efficacy is the 50% inhibitory concentration (IC_{50}). This measure is the concentration at which the antimalarial is able to inhibit the malarial parasite to 50% of its value in an untreated infection [31]. It is a useful parameter for monitoring in vitro resistance. Determining the IC_{50} of various antimalarials could be used to determine if resistance to drugs, especially those that are no longer the first-line treatment, decreases over time. If resistance does decrease, then there could be a possibility of reinstating previous first-line treatments for uncomplicated malaria as treatment options again.

Treatment failure of sulfadoxine-pyrimethamine can be associated with many potential causes. The presence of resistance-conferring mutations in the parasite is one reason. Another is the existence of high host folate levels. In a study where children were given folic acid supplements, these children had a higher treatment failure rate of SP when compared to a group of children who were given a placebo [32]. There may also be differences between individuals in terms of host pharmacokinetics. Variations in host pharmacokinetics include (a) the absorption rate of the drug, (b) where its distributed in the body, (c) how the tissue and plasma protein bind, and (d) the metabolism and excretion rates of the drugs. There all affect drug concentrations at active sites and are essential to determine the individual variability of therapeutic efficacy [33].

In order to track the spread of antimalarial resistant alleles, countries can use molecular marker surveillance. This would allow policymakers to any possible changes before the failures of *in vivo* treatment reach a critical level. However, this is not routinely used. Researchers can also use this information to monitor resistance levels to stop usage when resistance to a particular treatment is high [8]. The sensitivities to various antimalarial drugs can be used to inform treatment as well as mass drug administration [2]. Molecular marker surveillance uses the prevalence of a particular antimalarial resistant alleles to determine the therapeutic efficacy of the antimalarial drug. The goal is

to incorporate what is known about in vivo efficacy with IC_{50} and molecular marker to improve the understanding of antimalarial drug resistance and to inform drug policy better.

Hypothesis 1: When the samples were collected in Tanzania from 2003 to 2004, the first-line treatment of malaria was sulfadoxine-pyrimethamine (SP). Based on this, there may be a high level of prevalence of drug resistance polymorphisms related to the quintuple mutations (N51I, C59R, S108N in the *DHFR* gene and A437G, K540E in the *DHPS* gene).

Hypothesis 2: Tanzania is a highly endemic area for malaria, which means there is an ongoing incidence of malarial infection and transmission in this population. Due to this, the follow-up samples may likely show high rates of clinical failure.

The objectives of this study were to: (1) determine the prevalence of drug resistance polymorphisms at Day 0 in the 2003 – 2004 Tanzania sample, focusing on N51I, C59R, S108N in the *DHFR* gene and A437G, K540E in the *DHPS* gene; and, (2) and through genotyping determine whether the parasitological failure is a result of recrudescence or reinfection. There are three possibilities for the results of the follow-up. The first option is reinfection with a resistant strain. The second is a recrudescence of a resistant strain at a low frequency in the Day 0 population. The final option is the selection of a de novo drug resistance.

Research Design

Table 1 Patient Summary Table: Descriptive statistics of the sample population.

	Sample Size	Median	Mean	Standard Deviation	Lower 95% CI	Upper 95% CI
Age (years)	88	4.29	7.90	9.05	5.99	9.82
Parasitemia (%)	93	2.00	2.85	2.83	2.27	3.43
Parasite Density (parasite/μL)	96	16,200	37,191.25	38,630.54	29,363.98	45,018.52

	Male	Female
Sex	36%	64%

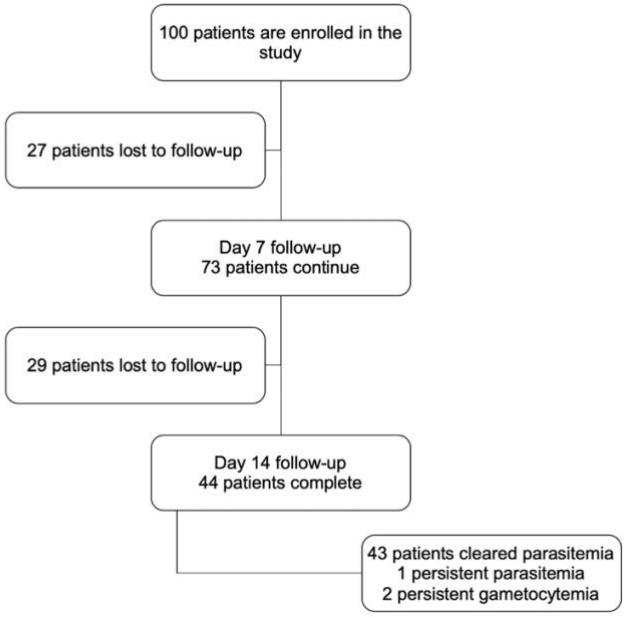
Study Population

The samples for this study were collected from the Mlandizi Health Centre in the Kibaha coastal region in Tanzania. The region is about 40 kilometers northwest of Dar es Salaam. Malarial transmission in this area is perennial, so the incidence peaks occur during the end of the long rains, which is from May to July, and the short rains, which is

from December to January. Individuals who came to the center for treatment of uncomplicated *Plasmodium falciparum* malaria during 2003 to 2004 were tested using microscopy. Patients who tested positive and aged between six months and fifty-two years old were enrolled in the study. The Tanzanian Commission for Science and Technology approved these studies (Permit No. 2003-207-CC-2003-102). Information on follow-up and patient data can be found in Supplemental Table 1 in the Appendix.

The sample consisted of 64% females and 36% males. The sample size of the ages was 88, and the median age was 4.29 years old. The average age of the participants was 7.9 years, with a standard deviation of 9.05 years of age (95% CI: 5.99, 9.82). The sample size of the parasitemia was 93, and the median of the parasitemia was 2%. The average parasitemia, or the percentage of red blood cells infected with the parasite, was 2.85%, with a standard deviation of 2.83% (95% CI: 2.27%, 3,43%). The sample size of the parasite densities was 96, and the median parasite density was 1.6×10^4 parasites per one microliter of blood. The average parasite density was about 3.7×10^4 parasites per one microliter of blood, with an estimated standard deviation of 3.8×10^4 parasites per microliter (95% CI: 2.9×10^4, 4.5×10^4). These descriptive statistics of the enrolled study population can be found in Table 1.

Figure 1 Patient Flowchart: This figure shows the patients who were enrolled in the in vivo study, including those who were lost to follow-up and continued through to the end of the study.



Sample Collection

After patients consented to enroll in the study, blood samples were collected from finger-pricks and stored on Whatman FTA filter papers before patients were treated with sulfadoxine-pyrimethamine (SP). Patients were asked to return to the clinic on Day 7 and Day 14 for follow-up or unscheduled if malaria-like symptoms returned. In the in vivo study, there were one hundred patients enrolled. On Day 7, twenty-seven patients were lost to follow-up, and seventy-three patients continued in the study. By day 14, an

additional 29 patients were lost to follow-up, and forty-four patients completed the study. On those who finished the study, forty-three cleared their parasitic load, and one patient had persistent parasitemia. Two of the forty-four patients also presented with persistent gametocytaemia. Figure 1 depicts the enrollment of the study. For the molecular marker study, seventy-seven Day 0 samples and thirty-five follow-up samples were to be used in this study. However, there were varying numbers of participants for each mutation, depending on the completion of HRM, and this can be found in Tables 2 and 3.

DNA Extraction

The filter paper was used to extract genomic DNA via the QIAamp DNA Mini Kit (Qiagen) method. The protocol for filter paper samples was followed for all the samples in the study. 3 mm circles were punched out of from a dried blood spot on filter paper and added to a microcentrifuge tube with Buffer ATL, which lyses cells for use in nucleic acid purification. The tube was then incubated for at 85°C to ensure complete lysis of the cells. Proteinase K was added, vortexed, and incubated at 56°C. Proteinase K is used to digest contaminating proteins and degrade nucleases that may be present. This is to protect nucleic acids from being degraded by the nucleases. Buffer AL was then added to the sample to promote the lysis of the cell membrane and promote DNA isolation. Next, ethanol was added to the sample to remove the solvation shell around the DNA and allow for the precipitation of DNA in the pellet form. It also promotes the aggregation of DNA. The contents of the microcentrifuge tube were then added to a spin column and were centrifuged. Spin columns contain a silica resin that selectively binds DNA. The contents in the collection tube were discarded after centrifugation. Buffer AW1 was added to the spin column, and the tube was centrifuged. The purpose of Buffer AW1 is to denature proteins, so they are able to pass through the filter in the spin column. The contents of the collection tube were discarded again. Then Buffer AW2 was added to the spin column and centrifuged. Buffer AW2 is used to wash out the salts. The collection tube was discarded and replaced with a new one. The sample was centrifuged again to ensure that any possible Buffer AW2 carryover is eliminated. The spin-column was then placed in a microcentrifuge tube, and Buffer AE was added. The tube and spin-column were centrifuged. Buffer AE elutes DNA from the spin column membrane to the microcentrifuge tube to allow for the stable storage of the DNA. This step was repeated for a second elution. The extracted DNA for both Day 0 and follow-up samples were stored at –20°C until tested by high resolution melting (HRM).

High-Resolution Melting (HRM)

High resolution melting (HRM) is a method for single-nucleotide polymorphism (SNP) genotyping and mutation and sequence scanning in DNA samples. This technology characterizes nucleic acid samples based on how the double-stranded DNA separate and how the specific differences in a polymerase chain reaction (PCR) amplified sequences are detected by melting. Samples can also be distinguished by sequence length, GC content, and strand complementarity.

HRM analysis is a post-PCR method. A specific region of interest within the DNA sequence is first amplified using PCR. Special saturation dyes are added to the reaction,

so the only florescence present is of the double-stranded DNA. These dyes are called intercalating dyes. The amplified region of interest is called the amplicon. The amplicon concentration in the reaction tube increases as fluorescence releases by the double-stranded amplified product increases. After the PCR is complete, HRM analysis starts. The amplicon DNA is heated up slowly from 50°C to about 95°C. At the temperature increases, the melting point of the amplicon is reached. When this point is reached, the DNA sample starts to denature, and the fluorescence from the dye is released, and it begins to fade away. At the start of the HRM analysis, there is a high level of fluorescence in the samples because there are many copies of the amplicon. However, as the sample continues to heat up, the two strands denature, so there is no more double-stranded DNA present, and fluorescence decreases. The HRM machine has a camera that measures the fluorescence while this process is happening. The process is then plotted on a graph known as a melt curve that shows the level of fluorescence versus temperature.

The temperature at which the DNA denatures is predictable. It is dependent on the sequence of the DNA bases. When comparing two samples, the shape of the melt curve should be exactly the same. However, a single base change in the sample DNA sequence can cause a difference in the HRM curves. Because different DNA sequences melt at different rates, these curves can be viewed and compared to determine similarities to a control.

This technique is very useful in a highly polygenomic population, such as Tanzania, for a variety of reasons. This method is accurate and has the ability to detect minor alleles [34]. HRM is also able to identify new genetic variants [35]. These variants could then be sent for sequencing to confirm that they are indeed new variants. HRM is a great tool for genotyping on a large scale because it is quick, inexpensive, and easy to deploy in the field.

The reaction was performed on a LightScanner 96 using primers and probes for each specific mutation on a particular gene. A 96-well plate was used with a final well volume of 10 μL. All reactions were done using 2.5 x LightScanner Master Mix, forward primers at a final concentration of 0.05 μM, reverse primers at a final concentration of 0.25 μM, specific probes at a final concentration of 0.2 μM, and 1 μL of genomic DNA. The list of primers and probes can be found in Supplemental Table 2.

Table 2 Reference Strains for HRM: Summary of reference strains used into assess the presence of each mutation.

Mutation	Reference Strain	DNA Genotype	Type
DHFR 51 (N51I)	3D7	A	Wild-type
	DD2	T	Mutant
	7G8	T	Mutant
	TM90	A	Wild-type
DHFR 59 (C59R)	3D7	T	Wild-type
	DD2	C	Mutant
	7G8	T	Wild-type
	TM90	C	Mutant
DHFR 108 (S108N)	3D7	G	Wild-type
	DD2	A	Mutant

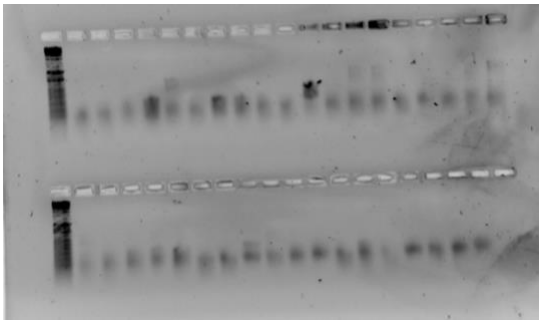
	FCR3	C	Mutant
DHPS 437 (A437G)	3D7	C, G	Wild-type
	DD2	T, G	Mutant
	FCR3	C, C	Mutant
DHPS 540 (K540E)	3D7	A	Wild-type
	VS1	A	Wild-type

The standard software included with the LightScanner 96 was used to compare unknown probe peaks from samples with known probe peaks from reference strains. The reference strains differ depending on the gene. The experiments to assess the presence of the *dhfr* 51 and 59 mutations were combined. Four reference strains with known peaks were used: 3d7, Dd2, 7g8, and Tm90. For the presence of a mutation on *dhfr* 108, 3d7, Dd2, and Fcr3 were used as reference strains. To assess the existence of a mutation on *dhps* 437, 3d7, Dd2, and Fcr3 were also used as reference strains. As for *dhps* 540, 3d7 and Vs1 were used as a reference. Table 2 shows the reference strains used for each experiment and the corresponding nucleic acid genotype.

The melting peaks, that are based on different melting temperatures related to base pairing, were analyzed to call the specific genotypic mutations. Since the peaks of the reference strains are known, the sample peaks are compared to them. If the peaks match the wild-type reference strain, that sample is thought to have the wild-type genotype. If the peaks match the mutant strains, that sample is thought to have the mutant genotype. However, if the peaks match some combination of the two, that sample is thought to have a mixed genotype. At times, some peaks may not match any of the peaks; this could be an indication of a new mutation. HRM of the sample would then be repeated twice to ensure that this was a unique peak. After that, the sample would then be sent for sequencing to confirm if this was an indication of a new mutation.

PCR Positivity by Plasmodium genus Primers

Figure 2 Gel Electrophoresis of Follow-Up Samples: Gel of follow-up samples from Tanzania



18s rRNA gene was amplified using nested PCR. The PCR was carried out with a total volume of 20 μL. This final volume included 10 μL of GoTAQ, 1 μL of each of the primers (PLU5 and PLU6), 6 μL of reagent grade water, and 2 μL of genomic DNA. The products were then analyzed on a gel, and their bands were compared. The reference strain 3D7 and reagent grade water was used as a positive control and negative control,

respectively. If the samples had the presence of bands similar to the reference strain, they were separated and tested by HRM to determine its drug resistance polymorphism.

A 1% gel was made with agarose and TBE buffer and was left to set. Once the gel had been set, the DNA samples were loaded into wells. The power is then turned on, and the DNA fragments migrate through the gel based on their size, with larger fragments traveling a shorter distance than smaller fragments. Once the run has ended, the gel is placed under a UV light source that will display the DNA fragments at the bands for that distinct length. Figure 2 shows a gel that was run with the follow-up samples from Tanzania. There were thirty-five total samples run with two wells filled with the ladder located in the first well for each of the rows. However, positive and negative controls were not used. Based on where the band would have been had a positive control been used, there was one sample that was positive for parasites. This gel would need to be redone using both the positive and negative controls to confirm this result.

Results

Table 3 Prevalence in Day 0 Samples for *dhfr* Gene: This tables shows the percent prevalence of each mutation on the *dhfr* gene. At position 51, the *N51* denotes the presence of asparagine (N), which is the wild-type or the one that normally occurs in nature at that locus. *51I* represents the presence of isoleucine (I), which is the mutant or the non-standard allele at position 51. *N51+51I* denotes a mixed infection at position 51, which means there was some combination of both the wild-type and mutant alleles. At position 59, the *C59* denotes the presence of cysteine (C), which is the wild-type. *59R* represents the presence of arginine (R), which is the mutant. *C59+59R* denotes a mixed infection. At position 108, the *S108* denotes the presence of serine (S), which is the wild-type. *108N* represents the presence of asparagine (N), which is the mutant. *S108+108N* denotes a mixed infection.

Gene	Allele	Prevalence in Population	
		Proportion	Percentage
DHFR 51 (N = 52)	N51	9/52	17.3%
	51I	26/52	50.0%
	N51 + 51I	17/52	32.7%
DHFR 59 (N = 52)	C59	14/52	26.9%
	59R	22/52	42.3%
	C59 + 59R	16/52	30.8%
DHFR 108 (N = 75)	S108	18/75	24.0%
	108N	52/75	69.3%
	S108 + 108N	5/75	6.7%

The percent prevalence in completed Day 0 samples for the *dhfr* gene was found and can be viewed in Table 3. The prevalence of the asparagine to isoleucine mutation at the 51 position was 50% (N51I). This is associated with a DNA mutation of A to T. The prevalence of asparagine at the 51 position, the wild-type genotype, was 17.3%. The presence of mixed infections at the 51 position on the *dhfr* gene was 32.7%. The

prevalence of the cysteine to arginine mutation at the 59 position was about 42% (C59R). This corresponds to a mutation in DNA from T to G. The prevalence of a cysteine at the 59 position, the wild-type genotype, was 26.9%. The presence of mixed infections at the 59 position on the *dhfr* gene was 30.8%. The prevalence of the serine to asparagine mutation at the 108 positions was about 70% (S108N). This corresponds to a DNA mutation for G to either A or C. The prevalence of a serine at the 108 position, the wild-type genotype, was 24.0%. The presence of mixed infections at the 108 position on the *dhfr* gene was 6.7%.

Table 4 Prevalence in Day 0 Samples for *dhps* Gene: This tables shows the percent prevalence of each mutation on the *dhps* gene. At position 437, the *A437* denotes the presence of alanine (A), which is the wild-type. *437G* represents the presence of glycine (G), which is the mutant. *A437+437G* denotes a mixed infection. *Undetermined* is used to denote samples whose peaks did not match either the wild-type or mutant and needed to be sent for sequencing. At position 540, the *K540* denotes the presence of lysine (K), which is the wild-type. *540E* represents the presence of glutamic acid (E), which is the mutant. *K540+540E* denotes a mixed infection.

Gene	Allele	Prevalence in Population	
		Proportion	Percentage
DHPS 437 (N = 50)	A437	17/50	34.0%
	437G	14/50	28.0%
	A437 + 437G	12/50	24.0%
	Undetermined	7/50	14.0%
DHPS 540 (N = 50)	K540	50/50	100%
	540E	0/50	0%
	K540 + 540E	0/50	0%

The percent prevalence in completed Day 0 samples for the *dhps* gene was found as well and can be viewed in Table 4. The prevalence of the alanine to glycine mutation at the 437 position was 28% (A437G). This mutation corresponds to a DNA mutation of either C or G to T or C. The prevalence of an alanine at the 437 position, the wild-type genotype, was 34.0%. The presence of mixed infections at the 437 position on the *dhps* gene was 24.0%. 14.0% of the samples that had undetermined peaks at the 437 position, which were then sent for sequencing. The prevalence of the lysine at the 540 position was 100% (K540E). There were no samples with the lysine to glutamic acid mutation at the 540 position on the *dhps* gene. This is associated with a DNA mutation of A to T. There were also no samples with a mixed infection.

Table 5 Prevalence in Day 0 Samples for *Kelch* Gene: This tables shows the percent prevalence of each mutation on the *Kelch* gene. At position 580, the *C580* denotes the presence of cysteine (C), which is the wild-type. *580Y* represents the presence of tyrosine (Y), which is the mutant. *C580+580Y* denotes a mixed infection.

Gene	Allele	Prevalence in Population (N = 46)	
		Proportion	Percentage
Kelch 580	C580	46/46	100%

	580Y	0/46	0%
	C580 + 580Y	0/46	0%

The percent prevalence of completed Day 0 samples for the *Kelch* gene was determined and can be view in Table 5. the prevalence of the cysteine to tyrosine mutations at the 580 positions was 0%. This corresponds to a mutation in DNA from G to A. The presence of cysteine at the 580 position on the *Kelch* gene is 100%. There were also no samples with a mixed infection.

Figure 3 Summary of Mutations for *Kelch*, *dhfr*, and *dhps* Genes: This graph shows the percentage composition of the genotypes at each location. For *dhfr51*, the wild-type genotype is N51, mutant genotype is 51I, and mixed genotype is N51+51I. For *dhfr59*, the wild-type genotype is C59, mutant genotype is 59R, and mixed genotype is C59+59R. For *dhfr108*, the wild-type genotype is S108, mutant genotype is 108N, and mixed genotype is S108+108N. For *dhps437*, the wild-type genotype is A437, mutant genotype is 437G, and mixed genotype is A437+437G. Undetermined is used to denote samples whose peaks did not match either the wild-type or mutant. For *dhps540*, the wild-type genotype is K540, mutant genotype is 540E, and mixed genotype is K540+540E. For *Kelch 580*, the wild-type genotype is C580, a mutant genotype is 580Y, and the mixed genotype is C580+580Y.

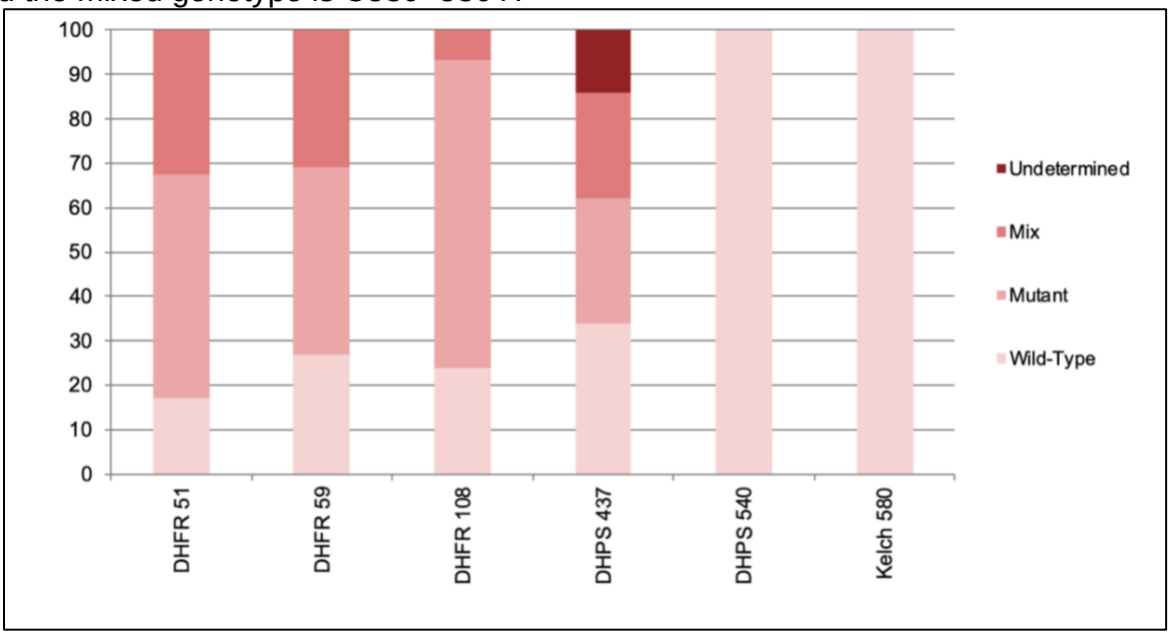


Figure 3 illustrates a summary of the mutations for the *Kelch*, *dhfr*, and *dhps* genes. In descending order, the highest rates of the wild-type genotypes were from *Kelch* 580, *dhps* 540, *dhps* 437, *dhfr* 59, *dhfr* 108, and, finally, *dhfr* 51. The greatest rate of the mutant genotype was at *dhfr* 108, followed by *dhfr* 51, then *dhfr* 59, and, finally, *dhps* 437. In terms of mixed infections, the highest rates in descending order of the completed samples were for *dhfr* 51, *dhfr* 59, *dhps* 437, and *dhfr* 108. *dhps* 437 was the only mutation that had samples that needed to be further sequenced in a lab to determine if that sample had a novel mutation. Both *dhps* 540 and *Kelch* 580 had no mutant alleles at their position in any of the samples that were analyzed by HRM.

Table 6 Patient-Specific Genotypic Variation of Mutation Site (n = 50): This table provides a patient by patient view of the total mutants present of the quintuple mutations. Only patients' samples that were tested for all five of the mutations were included in this table. For each mutation, the first letter represents the wild-type amino acid at that location. The number in the middle denotes the position where the mutation is located on the gene. The last letter indicates the mutant amino acid at that location. For example, for S108N, the location of the mutation is the 108 position on the dhfr gene. The wild-type amino acid at that location is serine (S), and the mutant amino acid is asparagine (N). Patients with *mix* showed peaks that aligned with both the wild-type and mutant alleles. *Mix mut* was used to distinguish patients whose samples' peaks aligned with two different mutant alleles. Patients with *triple mix* showed peaks that aligned with wild-type and two mutant alleles. *Other* was used to classify those patients whose samples' peaks did not align with any of the wild-type or mutant alleles. The mutant alleles for each patient were then summed and placed in the total mutations column.

Sample	DHFR			DHPS		Total Mutations
	S108N	N51I	C59R	A437G	K540E	
MLD 002	N	I	C	Other	K	2
MLD 004	N	Mix Mut	Mix Mut	Mix	K	4
MLD 005	Mix	I	R	Mix	K	4
MLD 007	N	I	R	A	K	3
MLD 013	N	I	R	A	K	3
MLD 014	N	I	R	A	K	3
MLD 017	N	I	R	G	K	4
MLD 018	Mix	Mix	Mix	Other	K	3
MLD 019	Mix	Mix	C	A	K	2
MLD 020	N	N	C	G	K	2
MLD 021	N	I	R	A	K	3
MLD 022	N	I	R	Triple Mix	K	4
MLD 023	N	I	C	G	K	3
MLD 024	S	N	C	Other	K	0
MLD 025	N	I	R	A	K	3
MLD 026	N	I	C	A	K	2
MLD 028	N	Mix	Mix	A	K	3
MLD 029	N	Mix	Mix	Mix	K	4
MLD 030	Mix	N	Mix Mut	Mix	K	3
MLD 031	N	I	R	G	K	4
MLD 034	N	I	C	A	K	2
MLD 035	N	Mix	Mix	Mix	K	4
MLD 036	N	I	R	G	K	4
MLD 037	N	Mix	Mix	Other	K	3
MLD 038	N	I	R	Mix	K	4
MLD 039	N	I	R	G	K	4
MLD 040	N	Mix	Mix	Mix	K	4

MLD 041	N	I	R	G	K	4
MLD 043	N	I	R	A	K	3
MLD 044	S	N	C	G	K	1
MLD 049	N	I	R	Mix Mut	K	4
MLD 050	S	N	C	Other	K	0
MLD 051	N	Mix Mut	Mix Mut	A	K	3
MLD 052	N	I	C	G	K	3
MLD 054	Mix	I	R	A	K	3
MLD 057	S	N	C	A	K	0
MLD 063	N	I	R	Mix	K	4
MLD 065	N	I	R	Mix	K	4
MLD 069	S	Mix	Mix	A	K	2
MLD 070	N	I	R	Mix Mut	K	4
MLD 071	S	N	G	G	K	2
MLD 077	N	Mix	Mix	Other	K	3
MLD 078	N	Mix	Mix	G	K	4
MLD 080	N	Mix	Mix	A	K	3
MLD 081	S	Mix	Mix	G	K	3
MLD 082	N	I	R	Mix	K	4
MLD 096	N	N	R	G	K	3
MLD 097	N	Mix	Mix	G	K	4
MLD 098	N	Mix	Mix	Other	K	3
MLD 099	N	I	R	A	K	3

Table 6 describes the genotypic differences of each patient for each mutation on the *dhfr* and *dhps* gene. This table shows only those patients whose samples were able to be analyzed by HRM for all of the quintuple mutations. For the *dhfr* gene, 10% of the dataset had a wild-type allele at the 108, 51, and 59 positions. 90% of the samples had mutations at least one of these three locations. For the *dhps* gene, 32% of the dataset had a wild-type allele at the 437 and 540 positions. The remaining samples (68%) had mutations at the 437 position of the *dhps* gene. 0% of the samples had mutations at the 540 position of *dhps* gene. For the samples that were complete, only 2% of the dataset had a wild-type allele for every mutation of the quintuple mutations. The rest of the samples (98%) had at least one mutation or other alleles for any of the five mutations. 34% of the samples had at least four mutant alleles, 40% of the samples had three mutant alleles, 14% had two mutant alleles, and 2% had one mutant alleles. 36% of the samples completed were *dhfr*-triple mutant (three mutations present on the *dhfr* gene). 16% of the sample were *dhfr*-double mutants, and 26% were *dhfr*-single mutants. Finally, 18% were considered to be *dhfr*-wild-types. In terms of the *dhps* gene, 28% of the population were *dhps*-single mutants, and 32% were *dhps*-wild-types. In the completed samples, 42% had a mutant allele at the 59 position for the *dhfr* gene. 0% of the completed samples had a mutant allele at the 540 position on the *dhps* gene. A total of 0% of the completed sample had these two mutations. In the completed samples, there were about 70% of samples with a mutant allele at the 108 position of the *dhfr* gene. 28% of the completed samples

had a mutant allele at the 437 position of the *dhps* gene. A total of 22% of the completed sample had these two mutations.

Discussion

At the end of the study, there was one patient who was positive for gametocytaemia among the completed samples. Gametocytemia is the presence of gametocytes in the patient's blood. Gametocytes are the form of the parasite that is able to transmit malaria to the mosquito vector [36]. A study conducted by Robert et al. found that post-therapeutic gametocytaemia was greater in patients that were treated with SP than those treated with chloroquine in terms of both prevalence and density [37]. With that in mind, it was not surprising to see one patient who was gametocyte-positive.

In a review done by Naidoo et al., the uses and prevalence of resistant genotypes of sulfadoxine-pyrimethamine in Africa were discussed [38]. The review identified almost 300 surveys that aimed to conduct surveillance of one or more of the *dhfr* or *dhps* mutations. The quintuple mutations (*dhfr* N51I, C59R, S108N, and *dhps* A437G and K540E) were the most common mutations surveyed. The prevalence of each mutation in Tanzania was provided. The prevalence of 51I, 59R, 108N, 437G, and 540E were about 85%, 80%, 100%, 60%, and 50%, respectively. As stated above, the results of the current study differed from the review. For every mutation, the percentage in the review was greater than the percentage in the current study. However, the trends were similar, in that the review percentage was 1.5 to 2 times that of the current study. The current study experienced 108N genotype having the highest percentage of 69%, the review observed a 1.5 times greater value of 100%. 51I and 59R genotypes were close to each other and held the second and third highest percentages at 50% and 42.3%, respectively. The percentages in the review were about 1.7 to 1.9 times greater, at 85% and 80%, correspondingly. The 437G genotype was considered to be the second lowest percentage for the current study at 28%. The review, however, observed that 60% prevalence of this mutation, which was about double that of the current study. The review stated the 540E genotype had the lowest percentage at 50%, but it was non-existent in the completed samples of the current study. A reason for the existence of discrepancies between the studies is the number of samples in the studies. The review was using upwards of 4,000 samples to determine the prevalence of each mutation; the current study used at most 75 samples.

Mutations in the *Kelch* gene are associated with artemisinin combination therapies (ACTs), which is the current first-line of treatment for uncomplicated malaria caused by the *P. falciparum* parasite by the World Health Organization (WHO) [39]. Since ACTs were not implemented in Tanzania as the first-line treatment until 2006, it would be expected that there should no mutations found. This would be due to the fact that there was no selective drug pressure for the ACT resistant genotype in 2003 when the samples were collected. Consequently, there were no 580Y genotypes in the completed samples.

In a 2004 study, the therapeutic efficacy of sulfadoxine-pyrimethamine was assessed in Tanzania. High levels of SP resistance had already been documented in the northeast part of Tanzania since sulfadoxine was used as a therapeutic drug in 1994 [40].

In the same region, pyrimethamine had also been used as a prophylactic in the 1950s [41]. 45% of patients in that region who were treated with SP failed to clear their parasitemia to low levels, which was a higher failure rate than other Tanzanian regions [42]. This provides a backdrop that suggested that after the implementation of SP as the first-line treatment in 2001, it would be likely that resistance would increase quickly. The results of this study showed that there was an association between treatment outcome (adequate clinical response, early treatment failure, late treatment failure) and the quintuple mutant genotype. The prevalence of the three mutation on the *dhfr* gene was four time greater at sites with high SP resistance than those with moderate SP resistance. The authors concluded that SP not be useful as the first-line treatment for malarial infection for a long period of time and that the triple *pfdhfr* mutant genotype may be used as an indicator of increasing resistance to SP [43].

The 2004 study also looked at molecular markers in five different regions of Tanzania: Butimba, Kyela, Mlimba, Masasi, and Mkuzi. Mkuzi is the region that would be the closest to where the current study's samples were gathered from. In Mkuzi, there were 127 samples collected. After molecular marker analysis, 80.3% of the population had three mutant alleles on the *dhfr* gene, 11.9% had two mutant alleles, and 2.4% had a single mutant. For the *dhps* gene, 32.3% of the population samples had two mutant alleles, and 13.4% had a single mutant. For wild-type alleles, 54.3% of the population possessed them on the *dhps* gene, and 5.5% possessed them on the *dhfr* gene. As mentioned earlier, the results in the current study concur with the 2004 study in that the largest percentage for the *dhfr* gene was for triple mutants. The double mutant percentage on the *dhfr* gene of both of the studies is comparable, with the current having a slightly higher percentage. However, the differences between the studies dominate. There was a greater percentage of single mutants on both the *dhfr* and *dhps* genes of the current study compared to the 2004 study. The percentage of wild-types on the *dhfr* gene in the current study was three times that on the study from 2004. The wild-type percentage of the *dhps* gene in the 2004 study were about one-third greater than that of the current study. These could be due in fact to the inability to finish conducting the molecular marker assays for the entire Day 0 samples. Another limitation could be the comparison between the Mkuzi region in the 2004 study and the Kibaha region in the current study. Although both regions are thought to have low malarial transmission, there are differences between the two regions in terms of parasite prevalence [44]. Perhaps this could explain the differences between the two studies. Additionally, the 2004 study did not include patients with mixed infections, which could have made a difference in the number of mutants present.

In 2003, a study was conducted to assess the efficacy of SP in Tanzania as the first-line treatment for uncomplicated malaria [45]. On Day 14 of the study, the treatment failure rates were lower than the overall treatment failure rate prior to 2001. This study then extended the period of follow-up (28-day) in three sites in Tanzania. This resulted in about 50% of patients failing treatment. The authors also concluded that SP does not have high therapeutic value in Tanzania and recommended changing the first-line treatment to artemether + lumefantrine combination therapy. They also stressed that policy change in highly endemic areas should consider clinical and parasitological responses beyond 14 days. A limitation of the current study is that the follow-up points were Day 7 and Day 14. With an extended follow-up period of 21- or 28-days, the results

could have differed for this sample. The follow-up samples could have possibly shown more resistance has the number of days increased.

A 2002 study aimed to move molecular assays to the forefront of tools for antimalarial drug resistance surveillance by accurately finding a subgroup of mutations that served as a reliable marker to predict SP treatment failure [46]. These researchers analyzed *dhps* and *dhfr* genotypes and related them to the treatment outcomes of patients. They found that a *dhfr* triple-mutant and *dhps* double-mutant were associated with SP treatment failure. In vivo resistance was associated with mutations at the 59 position on the *dhfr* gene (C59R) and the 540 position on the *dhps* gene (K540E). However, as stated above, the current study did not have any samples that possessed both of those mutations. Additional research would be required to compare the two studies further. Treatment outcomes of the patients in the current study, including ETF, LCF, LPF, and ACPR, would need to be analyzed and compared to their *dhps* and *dhfr* genotypes. Also, since the entire sample could not be completed, it is difficult to make any conclusions at this time.

Study Limitations

A limitation of this study is the lack of extended follow-up. The World Health Organization now recommends follow-up for 28 to 42 days to determine the efficacy of the antimalarial drug. Since SP has a long half-life, the recommended follow-up is 28 days. Since the efficacy assessment was only based on 14 days in the current study, the level of parasite resistance could be underestimated. There was also no active follow-up, so there was a total of fifty-six patients lost to follow-up. One of the main reasons that patients may drop out of this study could be that after six days (for the 7-day follow-up) or thirteen days (for the 14-day follow-up), patients began to feel better, so they felt they did not need to come into the clinic to receive treatment anymore. Losses to follow-up are an issue because this reduces the effective sample size since the outcome measures of those who are lost are missing.

Another limitation is the age of the sample of patients in the current study. The median age in Tanzania, as of 2020, is 18.2 years [47]. However, the median age of the present study was 4.29 years, which is not an accurate representation of the population of Tanzania. There were only thirteen participants above the age of eighteen in the present study. This would make it difficult to generalize the results of this study. The average age of the samples was 7.9 years old. In Tanzania, the age distribution of children is zero to fourteen years [48]. In the present study's sample, this age range makes up about 85%. With that in mind, these patients would not be able to come into the clinic on their own to receive the antimalarial treatments. They would be required to have an adult with them to receive treatment. This could be another explanation for why many patients were lost to follow-up.

The sex ratio for the current study is also a possible limitation. Tanzanian sex ratio as of 2020 for the male to female was 1.02, 1.01, and 1.01 for the 0 to 14, 15 to 24, and 25 to 54 age groups [47]. Conversely, the overall male to female ratio of the present study was about 0.5. The ratio for 0 to 14 years was about 0.75. For both the 15 to 24 and 25 to 54 age groups, there was no males present. Since the 0 to 14 age group males to female ratio of the current study were less than that of the corresponding overall

Tanzanian age group, this indicated that the samples had an abundance of females participating in the study. Considering that, it would be difficult to generalize the results to the whole Tanzanian population.

COVID-19 Pandemic Situation

The novel coronavirus was declared a Public Health Emergency of International Concern on January 30, 2020, by the WHO. This led to many states in the U.S. implementing shelter-in-place as a preventative measure. Yale University implemented university-wide closure on March 10th, citing that only essential personnel would be allowed on campus. At this time, the study was in the process of completing HRM of the Tanzanian Day 0 samples as well as conducting PCR and running a gel on the follow-up samples. This presented a challenge since the university closure led to the inability to access the laboratory to complete the HRM for all Day 0 samples and PCR-positive follow-up samples.

Conclusions

This study attempts to track the spread of antimalarial resistant alleles of sulfadoxine-pyrimethamine through molecular marker surveillance. Blood samples of patients from Tanzania were analyzed using high resolution melting (HRM), and their peaks were compared to reference strains. This would assist in determining the genotype of the mutations and its prevalence. The prevalence of 51I, 59R, 108N, 437G, and 540E was 50%, 42.3%, 69.3%, 28.0%, and 0%, respectively. Individual genotypes were reported for those samples that were completed for all of the quintuple mutations (n = 50). For the *dhfr* gene, 76% had a mutation at the 108 position, 52% had a mutation at the 51 position, and 42% had a mutant allele at the 59 position. For the *dhps* gene, 28% had a mutant genotype at the 437 position, and 0% had a mutation at the 540 positions. Further research would be needed to be conducted to see if this aim is possible.

Future Steps

Next steps for further research include the completion of HRM for the quintuple mutation polymorphisms on all of the blood samples in the 2004 Tanzania cohort as well as testing for PCR positivity for all of the follow-up samples. Based on the results of the PCR, barcoding or *msp* typing could be used to determine the genotype of the follow-up sample, depending on if the sample is monogenomic or mixed infection, respectively. This drug resistance typing will be used to determine if the resistance profiles of the follow-up samples differ from the respective Day 0 samples. Follow-up samples for the patients resulted in two patients with persistent parasitemia.

There would have been two possible reasons for the follow-up samples' results: recrudescence or reinfection. Recrudescence is the presence of the same parasitic genotype as the Day 0 sample. This could be due to treatment failure because of a variety of reasons, such as the difference in drug metabolism or patient compliance with the drug was too low to make it effective [49]. Although directly observed treatment is used to

improve compliance, patients can possibly skip coming into the clinic for their regular dosage. This would lead to a reduction in compliance. To evaluate this, the percent of follow-up completed would be analyzed for each patient that has had persistent parasitemia. If the percentage of follow-up completed was low, this could be an indication of decreased drug effectiveness due to the lack of compliance by the patient. Individual differences in drug metabolism could lead to limited metabolism, and therefore, decreased effectiveness of the antimalarial drug. To determine if this was the cause of recrudescence, the plasma clearance of each patient would be needed for analysis [50].

Recrudescence could also occur because of antimalarial drug resistance. Compliance may contribute to drug resistance in that the lower drug levels in the blood allow the parasite to adapt and develop resistance to the antimalarial drug [51]. The presence of antimalarial drug resistance for these two samples would be tested by using HRM. As stated above, the primers and the probes for each mutation would be used to determine the genotype of the follow-up sample at each locus. This would be done by comparing the peaks of the follow-up strains to the reference strains to conclude if the sample had a wild-type, mutant, or mixed genotype. The follow-up samples' genotypes would be compared to its corresponding Day 0 genotype. If the genotype types are similar, this would be an indication of recrudescence due to drug resistance.

Reinfection is the identification of a different genotype from the Day 0 sample. The follow-up samples could have also been used to determine if the patients had early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR). A sample would be considered to be LCF if its follow-up sample at Day 7 or Day 14 contained the presence of parasitemia, and the patient had a fever or signs of severe malaria. If a follow-up sample at Day 7 or Day 14 contained the presence of parasitemia, but the patient had no fever, this would be an indication of LPF. Prediction of ETF and ACPR would be difficult since the former would require a follow-up sample on Day 3, and the latter would require a follow-up sample on Day 28.

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Appendix or Appendices

Supplemental Table 1 Patient Data (n = 100): Provides individual patient information.

Sample	Sex	Age (years)	Parasitemia (%)	Parasite density (parasites/μL)	Day 7	Day 14
MLD 001	M	1.42	0.30%	2,280	---	---
MLD 002	M	0.83	1.06%	7,440	✓	---
MLD 003	F	2.00	nr	5,920	✓	✓
MLD 004	F	4.00	1.60%	25,440	✓	✓
MLD 005	M	6.50	3.80%	6,000	✓	✓
MLD 006	F	40.00	0.00%	4,800	✓	---
MLD 007	F	7.00	7.10%	160,000	---	---
MLD 008	F	3.00	4.10%	128,000	✓	✓
MLD 009	M	1.50	3.50%	88,000	---	---
MLD 010	F	1.75	5.80%	100,000	✓	✓
MLD 011	F	5.17	4.80%	68,160	✓	✓
MLD 012	F	4.50	2.20%	8,000	✓	✓
MLD 013	F	13.00	1.60%	44,720	✓	✓
MLD 014	F	20.00	1.03%	13,120	✓	✓
MLD 015	F	4.00	2.20%	16,000	---	---
MLD 016	M	7.00	0.30%	5,200	---	---
MLD 017	M	2.50	3.00%	33,600	✓	---
MLD 018	F	0.75	2.10%	80,000	✓	✓
MLD 019	F	3.00	1.70%	60,000	✓	✓
MLD 020	F	52.00	0.45%	4,280	---	---
MLD 021	M	4.25	2.00%	88,000	✓	---
MLD 022	F	14.00	1.30%	9,600	---	---
MLD 023	M	1.00	3.30%	13,680	---	---
MLD 024	F	3.33	7.10%	76,000	✓	---
MLD 025	F	17.00	2.20%	9,200	---	---
MLD 026	F	3.00	1.70%	16,000	✓	✓
MLD 027	F	3.00	2.30%	17,040	✓	---
MLD 028	F	17.00	1.30%	20,080	✓	✓
MLD 029	F	2.25	11.00%	60,000	✓	✓
MLD 030	M	6.00	3.10%	16,000	✓	---
MLD 031	M	4.50	1.90%	48,000	✓	---
MLD 032	M	0.58	na	43,520	---	---
MLD 033	F	1.75	10.00%	120,000	---	---
MLD 034	M		2.80%	52,000	---	---

MLD 035	F	13.00	1.70%	16,400	✓	✓
MLD 036	M	3.00	2.90%	80,080	✓	✓
MLD 037	M	11.00	3.20%	80,000	---	---
MLD 038	M	1.42	2.20%	9,200	---	---
MLD 039	F	3.50	2.10%	96,000	✓	---
MLD 040	F	22.00	1.10%	6,000	✓	---
MLD 041	M		2.00%	6,400	✓	---
MLD 042	M	8.00	0.02%	7,200	---	---
MLD 043	F	6.00	5.00%	80,000	---	---
MLD 044	F	11.00	0.90%	7,200	✓	✓
MLD 045	F		0.01%	22,400	---	---
MLD 046	F	10.00	0.80%	4,880	✓	---
MLD 047	F	5.17	4.60%	100,000	✓	---
MLD 048	F	25.00	1.20%	8,000	✓	---
MLD 049	F	14.00	0.40%	16,000	✓	---
MLD 050	M	1.58	1.10%	10,000	---	---
MLD 051	F	32.00	1.00%	6,000	✓	✓
MLD 052	F	3.00	7.50%	108,000	✓	✓
MLD 053	F	2.00	15.80%	68,000	---	---
MLD 054	F	22.00	1.80%	6,000	---	---
MLD 055	F	14.00	0.80%	4,080	---	---
MLD 056	F	7.00	4.20%	17,600	✓	---
MLD 057	F	4.00	9.00%	120,000	✓	---
MLD 058	F	18.00	0.50%	2,800	✓	✓
MLD 059	F	2.00	0.40%		---	---
MLD 060	F	3.42	3.70%	11,440	✓	---
MLD 061	F	2.50		120,000	---	---
MLD 062	F	6.00	2.10%	6,480	✓	---
MLD 063	M		1.00%	4,960	✓	✓
MLD 064	F	18.00	0.10%	10,000	✓	---
MLD 065	M	4.00	12.40%	100,000	---	---
MLD 066	F			34,000	---	---
MLD 067	M	0.50		60,000	✓	---
MLD 068	F	20.00	0.01%	27,360	✓	---
MLD 069	F	25.00	0.01%	4,640	✓	---
MLD 070	M	7.00	1.70%	25,600	✓	---
MLD 071	F	5.00	1.40%		✓	---
MLD 072	M	4.00	7.00%	48,000	✓	---
MLD 073	F	2.50		56,000	✓	✓

MLD 074	M	4.33	2.90%	5,360	✓	---
MLD 075	F	5.00	3.70%	72,000	---	---
MLD 076	M	1.00	4.00%	60,000	✓	✓
MLD 077	M	1.00	4.80%	80,000	✓	---
MLD 078	F	1.00	2.10%	8,000	---	---
MLD 079	M		6.60%	100,000	✓	✓
MLD 080	M	1.50	1.70%		✓	✓
MLD 081	F	4.50	1.80%		✓	✓
MLD 082	M		1.00%	23,200	✓	✓
MLD 083	M	3.50	5.20%	120,000	✓	---
MLD 084	F	18.00	3.50%	6,120	✓	✓
MLD 085	F		1.00%	60,000	✓	✓
MLD 086	F		2.50%	9,040	✓	✓
MLD 087	M	4.00	1.20%	50,240	✓	✓
MLD 088	M	5.50	0.60%	14,720	✓	✓
MLD 089	F	19.00	1.90%	23,040	✓	✓
MLD 090	F			6,240	✓	✓
MLD 091	M	0.50	0.70%	5,200	✓	✓
MLD 092	F		3.50%	6,000	✓	✓
MLD 093	F	3.25	2.40%	8,000	✓	✓
MLD 094	F	3.25	7.50%	6,400	✓	✓
MLD 095	M	1.75	2.60%	9,040	✓	✓
MLD 096	M	6.00	1.10%	7,440	✓	✓
MLD 097	F	2.00	1.50%	23,280	✓	✓
MLD 098	F		2.00%	4,400	✓	✓
MLD 099	F	6.50	1.70%	16,000	✓	✓
MLD 100	M	9.83	1.10%	5,840	✓	✓

Supplemental Table 2 Primers and Probes for HRM: These are the DNA sequences of the primers and probes used the HRM experiments. The 3' bold base pairs in the probes represent mismatched bases to ensure that amplification using the probe as a template does not occur.

Gene	Mutation		Sequence (5'→ 3')
DHFR	S108N	Forward Primer	CTGTGGATAATGTAAATGATATGCCTAATTCTA
		Reverse Primer	GACAATATAACATTTATCCTATTGCTTAAAGGT
		Probe	GGAAGAACAAgCTGGGAAAGCAT**GA**
	N51C/ C59R	Forward Primer	ACATTTAGAGGTCTAGGAAATAAAGGAGT
		Reverse Primer	ATATTTACATCTCTTATATTTCAATTTTTCATATTTTGATTCATTCAC
		Probe	AAATGTAaTTCCCTAGATATGAAATATTTTtGTGCAG**CC**
	I164L	Forward Primer	ACAAAGTTGAAGATCTAATAGTTTTACTTGGG
		Reverse Primer	CTGGAAAAAATACATCACATTCATATGTACTATTTATTCTA
		Probe	AATGTTTTATTaTAGGAGGTTCCG**CC**
DHPS	A437G	Forward Primer	GAATGTTTGAAATGATAAATGAAGGTGCTA
		Reverse Primer	CAGGAAACAGCTATGAC GAAATAATTGTAATACAGGTACTACTAAATCTCT
		Probe	ATCCTCTGGTCCTTTTGTTATACC**GG**
	K540E	Forward Primer	GTGTTGATAATGATTTAGTTGATATATTAAATGATATTAGTGC
		Reverse Primer	GTTTATCCATTGTATGTGGATTTCCTCTT
		Probe	TAATCCAGAAATTaTAAAATTATTAAAAAAAAAAAAC**GG**
	A581G	Forward Primer	CTTGTATTAAATGGAATACCTCGTTATAGGA
		Reverse Primer	AGTGGATACTCATCATATACATGTATATTTTGTAAG
		Probe	TTGGATTAGGATTTGcGAAGAAACATGATCA**CC**
	A613T/S	Forward Primer	CTCTTACAAAATATACATGTATATGATGAGTATCCACTT
		Reverse Primer	CATGTAATTTTTGTTGTGTATTTATTACAACATTTTGA
		Probe	AAGATTTATTgCCCATTGCATGA**CC**
CRT	H97Q	Forward Primer	TTTGCTAAAAGAACTTTAAACAAAATTGGTAACTA
		Reverse Primer	ATTTATCTTACTTTTGAATTTCCCTTTTTATTTCCA
		Probe	CATACAAATAAAGTTgTGAGTTTCGGATGTTAC**CC**
	K76T	Forward Primer	GTAAAACGACGGCCAGTTTCTTGTCTTGGTAAATGTGCTCA
		Reverse Primer	CAGGAAACAGCTATGACCGGATGTTACAAAACTATAGTTACCAAT
		Probe	GTGTATGTGTAATGAATAAAATTTTTG**AC**
	A220S	Forward Primer	gctcaggtcgTTGAAACACAAGAAGAAAATTCTATC
		Reverse Primer	gctcaggtcgAAACAAAGTTTAAGTGTTAATATATATTAAATATTAC

		Probe	GTCTTAATTAGTgCCTTAATTGTA**CT**
	N326S/D	Forward Primer	cgagcaTTTTTTAGAAAACCTTCGCATTGT
		Reverse Primer	TTCATCCTTTTTATTCTTACATAGCTG
		Probe	CTTCTTTgaCATTTGTGATAATT**CC**
	I356T/L	Forward Primer	gctcaggtcgAAATTTTCTACCATGACATATACTATTG
		Reverse Primer	cacctgactgaTTTATATATTTATATCTTTTTAATTCTTACGGC
		Probe	GTCCAGCAacAGCAATTGCT**CC**
Kelch	C580Y	Forward Primer	GGCACCTTTGAATACCC
		Reverse Primer	CATTAGTTCCACCAATGACA
		Probe	AGCTATGTGTATTGCTTTTGAT