Evidence of Partial Artemisinin Resistance in Malaria Endemic Lake Region, Busia County, Western, Kenya

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Abstract

Malaria remains a key health and economic problem particularly in sub-Saharan Africa. The emergence of artemisinin resistance (ART-R) parasite strains poses a serious threat to the control and elimination of this scourge. This is because ART remains the first-line treatment drug in the majority of malaria-endemic regions in Sub-Saharan Africa. *P. falciparum* ART resistance has been linked to mutations in the Kelch – 13 propeller gene (k13) of the *Plasmodium falciparum*. Single nucleotide polymorphisms in the K-13 region have been associated with delayed parasite clearance *in vivo* and *in vitro*. These mutations serve as vital molecular markers for tracking the emergence and dispersion of resistance. Recently, there have been increasing reports of the emergence and spread of *P. falciparum* ART-R parasites in the Eastern Africa region. This necessitates continued surveillance to best inform mitigation efforts. This study investigated the presence of K-13 mutations in the parasite population in Busia County, Kenya, a known malaria-endemic region. Two hundred twenty-six participants with microscopically confirmed uncomplicated malaria were recruited for this study. They were put under directly observed treatment with Artemether-Lumefantrine (AL), and microscopy repeated after 24 hours. *P. falciparum* DNA from samples showing the lowest 24-hour relative parasite clearance underwent targeted amplification of the K-13 gene using a semi-nested PCR approach, followed by Sanger sequencing. The recently validated ART-R nonsynonymous mutation C469Y was identified in 3% (n = 3) of the samples suggesting it could have had an impact on clinical parasite clearance 24 hours post-AL administration. Our findings highlight the need for continuous surveillance of AL resistance in western Kenya and the region to determine the spread of ART-R and inform containment.

BACKGROUND

Malaria remains a major public health challenge, claiming over 619,000 lives and causing more than 249 million clinical cases worldwide in 2022 alone, with most of the burden occurring in Sub-Saharan Africa [1]. Despite substantial efforts to reduce the incidence and mortality associated with malaria, progress has stalled in recent years [2]. This stagnation is particularly pronounced in East Africa, a region striving to control and ultimately eliminate malaria amidst myriad challenges [3, 4]. These include the emergence of drug-resistant malaria parasites, resistance of mosquito vectors to commonly used insecticides, genetic deletions undermining the efficacy of rapid diagnostic tests (RDTs), routine malaria commodity stock-outs, and limitations in the capacity for effective surveillance and response [5, 6]. The parasite *Plasmodium falciparum* accounts for most malarial infections in this region. The first cases of artemisinin resistance (ART-R) were reported in Southeast Asia in 2008 [7] then the *P. falciparum* Kelch 13 (K13) protein mutation, C580Y, was reported to be responsible for this ART-R in 2014 [7]. The K13 C580Y mutation was spread over the years to different parts of Asia [8, 9], and now several mutations in the K13 protein in the BTB-POZ and Propeller domains have been validated to induce delayed parasite clearance and they are defined as ART-R mutations, including Y493H, R539T, I543T, and C580Y [9, 10, 11].

Studies in the Eastern Africa region have reported other K13 ART-R mutations independent from mutations reported in Asia; R561H in Rwanda [12], Tanzania [13], and Democratic Republic of the Congo

[14], R622I in the Horn of Africa [15, 16]; and C469Y in Uganda [17]. There is a strong expectation that these ART-R parasites will spread more due to the open border policies in the region, highly interconnected border communities, and lack of robust border malaria control strategies.

In Kenya, several novel and potential ART-R markers, including R539T, N458T, R561H, N431S, and A675V, have been detected in the malaria-endemic areas of the country [18, 19]. However, most reported markers occur at low allelic frequencies. Recent studies in the coastal and Lake Victoria regions in Kenya reported no evidence of validated K13 resistance markers over a period of 13 years since the introduction of Artemether-Lumefantrine (AL) as the first-line treatment drug for uncomplicated malaria in Kenya [20, 21].

Here, we make the first report of a K13 C469Y mutation in Kenya, a validated ART-R mutation originally reported from Uganda. This mutation has most likely spread from Uganda, where studies have shown increasing prevalence of this mutation over the past 10 years [22]. Our findings elucidate the need for continuous surveillance of ART-R in this region and to establish systems for controlling the spread of resistance across border points of Kenya.

METHODS

Study site

This study was conducted in Busia County is located on the Kenyan border with Uganda characterized by moderate climatic conditions with average temperatures ranging between 20 to 28 degrees Celsius [23]. Malaria transmission is high and perennial with seasonal peaks during long rains periods in May–June and short rains periods between October and November. The county is classified under lake endemic regions in Kenya, where the high temperature in these zones favors the malaria parasite sporogonic cycle while the rainfall creates the breeding sites for malaria vectors. The population in this region is characterized by scattered homesteads. Despite being one of the smallest counties in Kenya, with a population of approximately 1.3 million people, it is also one of the biggest malaria-endemic areas with a prevalence of 39%. The situation is aggravated by factors such as limited access to healthcare facilities and poverty. In the 2020 Malaria Indicator Survey, malaria prevalence among children aged 6 months to 14 years in the Lake endemic region (where Busia is classified) was 42.4% by rapid diagnostic test (RDT) and 26.7% by microscopy. Notably, Insecticide treated nets (ITNs) coverage in 2020 was reported lower at 78% as compared to 2015 with 84% of Households owning at least one ITN and 66% of persons reporting sleeping under a net the previous night in 2015 compared to 56% in 2020 [24].

Selection of study participants

The study included participants who visited Busia County Hospital presenting with malaria symptoms. Informed consent was obtained from all participants, with participants < 18 years consent obtained from guardians. Patients with febrile clinical illnesses caused by pathogens other than malaria, pregnant, < 6 months, or those who had taken antimalarial medications 24 hrs prior to visiting the health facilities were

excluded from the study. *Plasmodium falciparum* infection was confirmed through microscopy and only patients presenting with ≥ 1,000 parasites/μL were included in the study. Parasitemia was recorded for Day 0, thereafter patients were treated with AL After 24 hours, patients were re-tested for infection, and parasitemia was recorded for Day 1. Both parasitemia levels were used to calculate the parasite percentage clearance by AL.

Sample collection

A hundred microliters of venous blood samples were collected and placed in EDTA tubes. A further 50 μL was obtained from finger pricks and dried blood spots were prepared on the filter papers (Whatman® 903 Proteinsaver cards; Cytiva, Marlborough, US) and appropriately marked with the participant's unique study number. A second set of samples were subsequently collected on day 1 following the initial administration of AL.

DNA extraction

DNA extraction was done using the protocol by Jaturas et al. (2015) [25], with a few modifications. A hundred samples were randomly selected for analysis. A piece of paper 3 mm in diameter was punched from each dried blood spot, immersed in 1 mL of phosphate-buffered saline (PBS) for 30 minutes in a tube, then the tube was centrifuged at 16,000xg for 2 minutes. The supernatant was discarded, and then the papers were washed with PBS. Fifty microliter of nuclease-free water and 20 μL of 20% Chelex 100 were added to the tube and incubated at 99˚C for 10 minutes. Then, the supernatant was recovered after centrifugation at 16,000xg for 1 minute. DNA quantification was done by nanodrop spectrophotometry.

Amplification of P. falciparum K-13 gene

A semi-nested approach was used to target the *P. falciparum* k-13 gene. Primers were designed and their specificity was evaluated using PrimerBlast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table 1 **and** Fig. 1). For the first round of primary PCR (outer nested) 0.5 μL of KOD DNA polymerase (Sigma-Aldrich), 1 μL of 10 μM PfK13.F3 primer, 1 μL of 10 μM PfK13.R3 primer, 1 μL of DMSO, 1.5 μL of 25 mM $MgSO_4$, 2.5 μL of KOD buffer ver2, 2.5 μL of 2 mM dNTPs, and 2 μL of the template DNA solution were mixed and topped up with nuclease-free water to a total reaction volume of 25 μL. The second run (inner nested) utilized primers PfK13.F3 and PfK13.R2 and 1 μL of the primary PCR product solution, with otherwise identical constituents as the first run. The optimized cycling conditions and expected fragment lengths are as shown in Table 1. The amplified PCR products were analyzed and purified in 2% agarose gel.

Table 1
Nucleotide sequences of the oligonucleotide primers and optimized conditions for the amplification of Pf-k13 gene

Reaction	Primer name	Primer sequence 5' –> 3'	PCR product length	Cycling conditions
First round PCR (outer nested)	*Pf-k13*.F3	AGTGGAAGACATCATGTAACCAG	1143 bp	Denaturation 94˚C for 5 minutes, then 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, 68˚C for 1 minute 30 seconds, and a final elongation of 68˚C for 5 minutes.
	Pf-k13.R3	TGTGCATGAAAATAAATATTAAAGAAG		
Second round PCR (inner nested)	*Pf-k13*.F3	AGTGGAAGACATCATGTAACCAG	1021 bp	
	Pf-k13.R2	CGGAGTGACCAAATCTGGG		
Sequencing primers	*PfK13*.F1	GCCTTGTTGAAAGAAGCAGAA	849 bp	
	PfK13.R1	GCCAAGCTGCCATTCATTTG		

Pf-k13 gene sequencing

Sanger sequencing was done using PfK13.F1 and PfK13.R1 primers (Table 1 **and** Fig. 1) on the 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) platform with a 2x coverage for both forward and reverse reads. Chromas software Ver 2.6.6 (Technelysium Pty Ltd, Australia) was used for quality control. Multiple sequence alignment was then performed against the 3D7 K13 reference sequence (PF3D7_1343700) using MUSCLE software [26].

Ethical considerations

Ethical approval for the research was obtained from the Mount Kenya University Institutional Scientific and Ethics Review Committee (MKUISERC), and Research Ethics Committee, Institute of Tropical Medicine, Nagasaki University (Approval number 211216271).

RESULTS

Participant characteristics

A total of 226 participants were recruited for this study from Alupe Level 4 Hospital, Busia County, western Kenya, between October to December 2022 (Fig. 2). These participants tested positive for *P. falciparum* and exhibited parasitemia levels above 1000 parasites/μL on Day 0 baseline and therefore enrolled to the study (Fig. 3A**)**. Majority of the participants were children below 5 years (41%) while those between 6–10 years represented 18% of those enrolled (Fig. 3B**)**. Participants older than 30 years exhibited lower infection rates, likely due to naturally acquired immunity acquired after prolonged

exposure to *P. falciparum*. Within this cohort, 63 individuals (27.9%) showed slow parasite clearance, failing to eliminate at least 90% of the parasite within 24 hours following AL administration (Fig. 4).

Analysis of Pf-K13 gene

We sought to determine if any of the previously validated SNPs in the *Pf-K13* gene that associated with ART-R was present in the population (Fig. 1). Analysis of multiple sequence alignment revealed a nonsynonymous mutation, a change of Cystine to Tyrosine at codon position 469 (C469Y) (Fig. 5). This mutation has been validated as a marker for ART-R in the Ugandan parasite population [17][22]. The mutation occurred due to a SNP at position 1406 of the Kelch 13 gene, replacing codon TGC to TAC. This was observed in 3% of the isolates from participants who had presented with delayed parasite clearance on day 1.

DISCUSSION

Ugandan-type K13 C469Y ART-R mutation was detected in Kenyan *P. falciparum* patients in Busia county in 2022. Busia county is a malaria-endemic region bordering Uganda. Cross-border interactions between Kenyans and Ugandans could be a key factor in the possible spread of *P. falciparum* resistant mutants from Uganda into Kenya. There is a need to further characterize the parasites to determine their lineage, which will enhance our understanding of the evolution and spread of ART-R. This comprehensive approach will ensure more effective malaria control and prevention strategies.

AL is the first line of defense against symptomatic malaria in the East African region of Africa [1]. Despite the high efficacy presented by the drug studies, clinical cases of delayed parasite clearance have been reported over time, creating a need for continued therapeutic efficacy studies. A rigorous investigation of clinical resistance to ART should use only ART for treatment. However, to minimize the possibility of ART-R emergence, ART-derivatives are currently administered with a partner antimalarial drug. We hypothesized that the effect of artemether on *P. falciparum* parasitemia one day after AL administration would be stronger than that of lumefantrine, based on the fact that the blood concentration of artemether increases rapidly, while that of lumefantrine, a slowly absorbed long-acting drug, increases slowly. To increase the likelihood of finding ART-R parasites, we examined the reduction rate of parasitemia on day 1 after drug administration. Although we found 3 samples with the ART-R type K13 sequence, the sample number is insufficient to statistically assess if our strategy was feasible. To verify whether our strategy is more likely to find ART-R parasites, further study is required by increasing the number of samples.

The discovery of ART-R mutations in Uganda[17, 27], Tanzania, and Rwanda [12, 27, 28] was a warning sign of ART R in the East African region. Based on the proximity of these countries to Kenya, the spread of these mutants into Kenya was imminent. From the molecular analysis of the K13 sequences, we confirm that the mutant C469Y, initially reported in Uganda, is now present in Kenya. The occurrence of this mutation in Kenya serves as a wake-up call for increased surveillance studies not only in the border region with Uganda, but also with Tanzania and Rwanda.

CONCLUSION

This study confirms the presence of the C469Y mutation in the K13 gene in Kenyan isolates, with no Asian mutations detected in any of the samples. This finding underscores the importance of addressing the presence of this mutation in Kenya. Policymakers must take appropriate actions.

Further molecular and phenotypic surveillance studies are essential in malaria-endemic regions of the country to inform policy-making and implementation, providing a comprehensive understanding of ART-R in Kenya. Additionally, implementing cross-border malaria surveillance is critical for monitoring and managing the spread of resistant strains between neighboring countries.

Moreover, there is a need to further characterize the parasites to determine their lineage, which will enhance our understanding of the evolution and spread of ART-R. This comprehensive approach will ensure more effective malaria control and prevention strategies.

Declarations

Contributions

JG and OK conceived and designed the experiments, MaM, BK, CM, MiM, HA, HW, ET, FA and performed the experiments, MaM, BK, OK and JG analysed the data, MaM, BK, OK and JG wrote the paper. All authors read and approved the final manuscript.

Ethical approval

for the research was obtained from the Mount Kenya University Institutional Scientific and Ethics Review Committee (MKUISERC), and Research Ethics Committee, Institute of Tropical Medicine, Nagasaki University (Approval number 211216271) and performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all study participants at enrolment.

Consent for publication

Not applicable.

Competing interests

We declare no competing interest.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

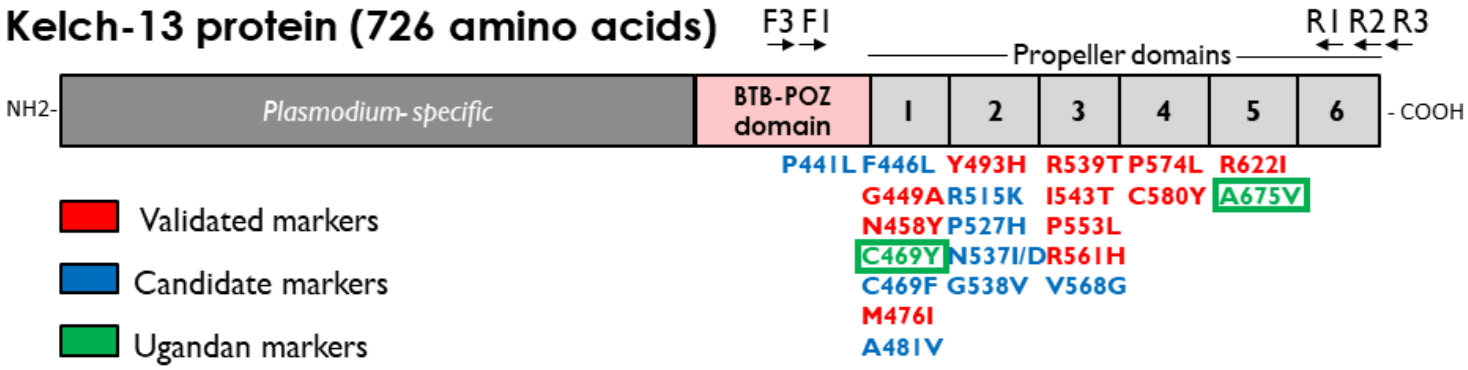

Figure 1

Schematic image of the Kelch 13 protein with *Plasmodium*-specific sequence, a BTB-POZ domain, and 6 propeller domains. Mutations confirmed to be related to artemisinin resistance (red), mutations suspected to be related (blue), and the location of confirmed artemisinin resistant mutations found in Uganda (green) are shown.

Figure 2

Geographical location of Busia County in Kenya. This is a malaria endemic region neighboring Uganda to the west. This serves as a critical surveillance point based on cross-border interactions between citizens of both countries.

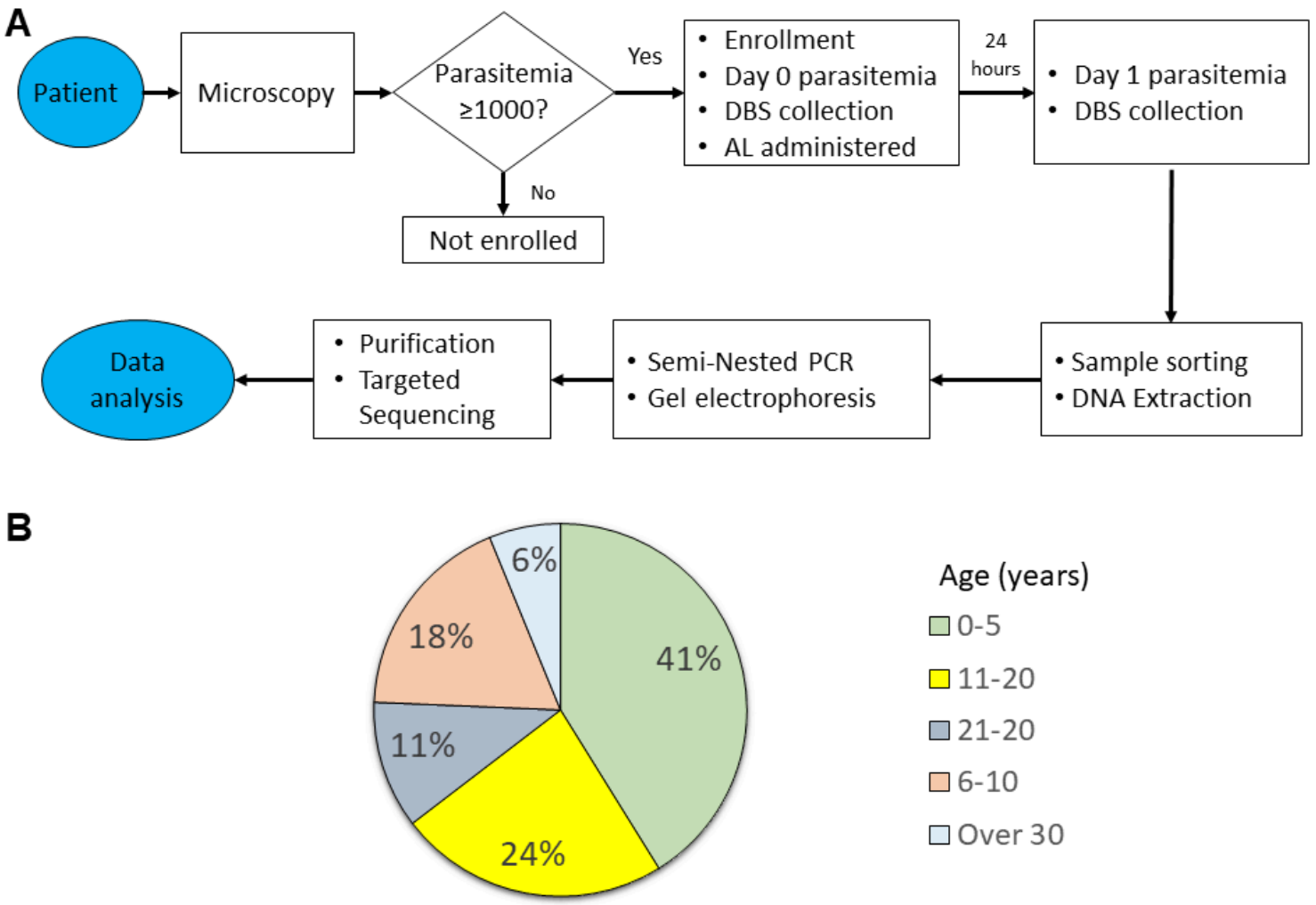

Figure 3

(**A**) The workflow used to select the study participants. (**B**). The study cohort stratifiedaccording to age groups. The majority of Plasmodium falciparum infections occurred in children under 5 years of the age in this region.

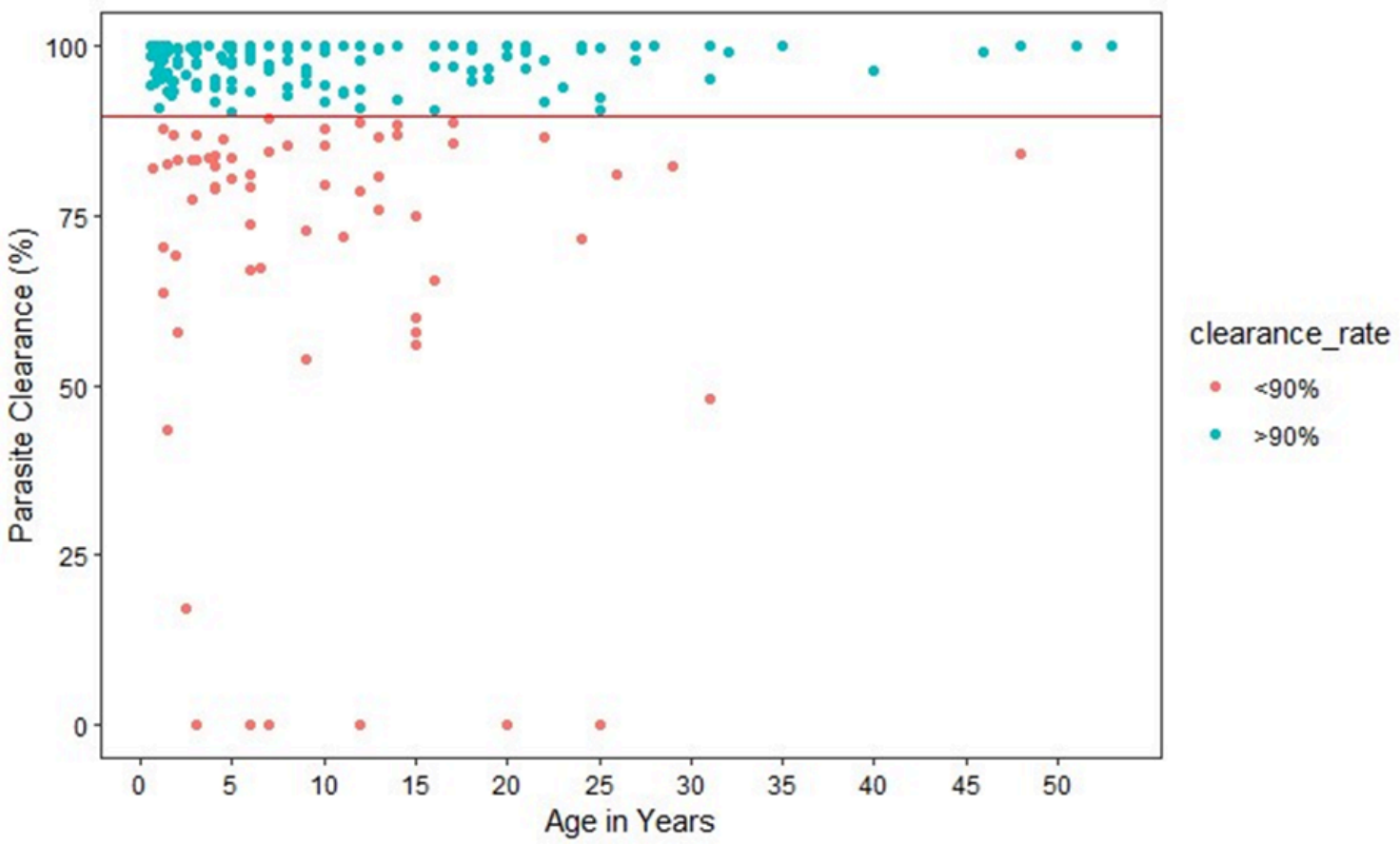

Figure 4

Parasite clearance per individual. The red line indicates the average clearance for the whole lot. The red labeled individuals did not clear >90% of the parasite. These individuals exhibiting clearance below the threshold were prioritized for sequence analysis.

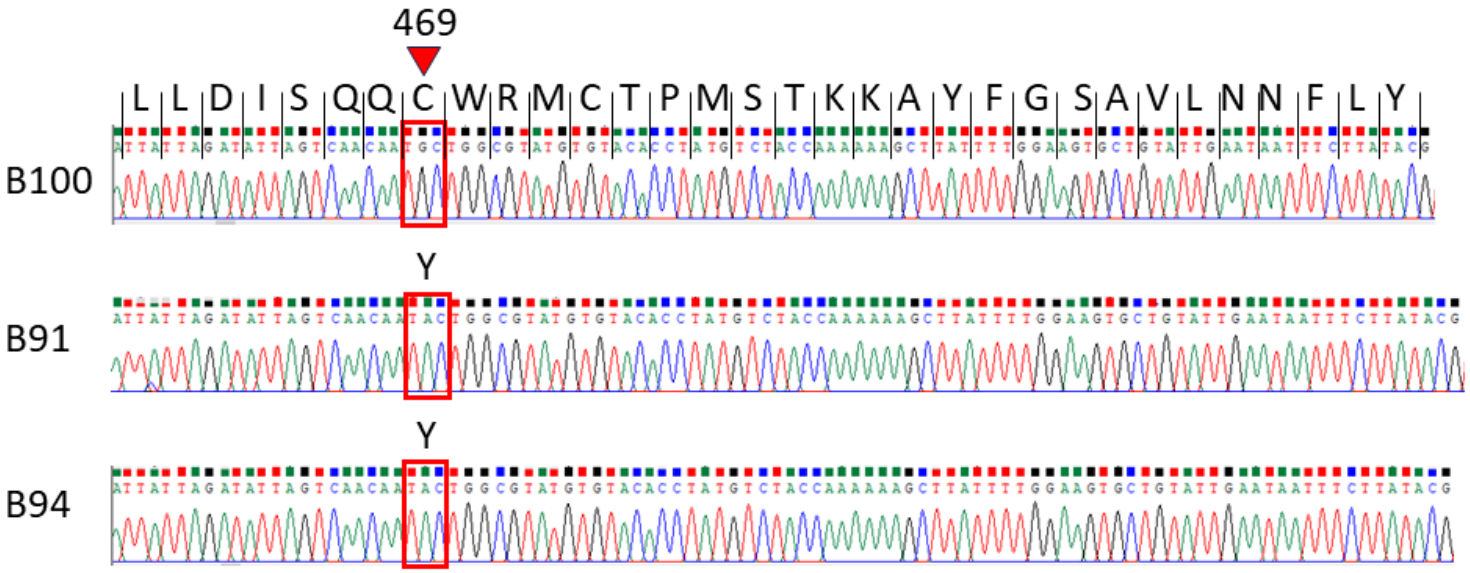

Figure 5

Sequencing chromatograph showing the mutation from TGC (wild-type sample B100) to TAC (artemisinin-resistant-type samples B91 and B94) representing the amino acid change from cysteine (C) to Tyrosine (Y), respectively. B100, B91 and B94 represent the internal sequenced sample ID.