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Investigation of *Plasmodium falciparum* Resistance Biomarkers among Primary School Children in Western Kenya

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Abstract

Background: A marked decrease in malaria-related deaths worldwide has been attributed to the administration of effective antimalarials against *Plasmodium falciparum*. However, the continuous spread of *P. falciparum* resistance to anti-malarial drugs is raising a serious problem in controlling Malaria to the vulnerable children's immune system. In recent studies, *Plasmodium falciparum* Kelch 13 propeller gene (*Pfk*13) has been reported to develop resistance to artemisinin in South Asia. In this study, we checked *Plasmodium falciparum* chloroquine resistance transporter gene (*Pfcrt*) involved in chloroquine (CQ) resistance. **Method:** In this study, archived 280 samples were collected from Alupe primary school children in Busia, Western Kenya from May, 2016 to November, 2016. Genomic DNA was extracted using the MightyPrep reagent. The samples were investigated for *P. falciparum* positivity out of which 67 of them tested positive giving a prevalence rate of 24%.

The sixty-seven were subjected to PCR amplification for the molecular marker resistance to *Pfcrt*. After PCR amplification, the amplicons were purified and sequenced using Sanger Sequencing. The sequence data were analyzed using BioEdit software to identify point mutations. **Results:** 14 samples sequences were analyzed on Bioedit software giving the following amino acid changes F76C, Y66H, L70A, Y58C, T59V, V65I, P67L, T81L, Y60S, Y66S, P67T and I71F). New mutations have been reported at position 76 leading to an amino acid change, one of Pfcrt gold standard biomarkers. However, amino acid changes Y66H, L70A, Y58C, T59V, V65I, P67L, T81L, Y60S, Y66S, P67T and I71F are newly reported giving an increase in Pfcrt prevalence of concern from zero to 5.0%. A phylogenetic evolutionary relationship was constructed as shown below. Generally, the results showed a continuous resistance of *P. falciparum* to Pfcrt which calls for robust continuous monitoring and surveillance. **Conclusion:** Due to the increase of the resistant Pfcrt gene prevalence, continuous development of new mutants against chloroquine indicates that there is need to repurpose anti-malarial drugs for future partner drugs.

Keywords

P. falciparum, Chloroquine, Drug Resistance, Malaria, *P. falciparum* Chloroquine Resistance Transporter Gene, Mutations

1. Introduction

Despite numerous advances in malaria control strategies, the disease still kills countless children worldwide, mainly in Sub-Saharan African countries. Assessing the genetic diversity of *P. falciparum* populations in different regions may allow tracking malaria parasites circulating across different geographic areas [1]. Malaria continues to cause unacceptably high levels of disease and death, as documented in successive editions of the World malaria report [2]. According to the latest report, there were estimated 229 million cases and 409,000 deaths globally in 2019. Malaria is preventable and treatable, and the global priority is to reduce the burden of disease and death while retaining the long-term vision of malaria eradication [3]. According to the world malaria report 2016, there were 212 million cases of malaria and approximately 438,000 deaths due to malaria infections. 90% of these deaths occur in Sub-Saharan Africa and 70% are children aged below 5 years old. It is the leading cause of morbidity in children with 10% of all deaths among the under-fives which is equivalent to one child in Sub-Saharan Africa dying of malaria every two minutes. In Kenya, more than 4 million cases of malaria are reported annually and *P. falciparum* is the most frequently associated with severe Malaria accounting to 80% - 90% of all the cases in the country. *P. falcciparum* is the cause of high mortality rate in Africa [4] [5] [6]. Five years before, it was reported that malaria caused around 627,000 deaths in 2013, mostly of children aged less than 5 years living in Africa. There are now

a large number of regular prevalence surveys of childhood parasitaemia, as most malaria deaths occur in children, but published surveys of parasitaemia in the general adult population are very scarce [7]. *P. falciparum* had developed high levels of resistance to the available, cheap and safe drugs such as, quinolones derivatives [8]. Chloroquine (CQ) was one of the first safe and effective antimalarials to be employed. It is characterized by potent and selective cytotoxic action against *P. falciparum* and the spread of drug resistance is a major obstacle for this achievement [9] [10] [11] [12]. CQ exerts its antimalarial effect by preventing the polymerization of toxic heme released during proteolysis of hemoglobin in the *P. falciparum* digestive vacuole, hence blocking the hemozoin elongation reaction in the absence of protein causing digestive vacuole swelling and pigment clumping [13] [14] [15]. Molecular markers of drug-resistance are very useful in identifying drug resistant *P. falciparum,* other examples are; quantitative trait locus (QTL), construction of genetic linkage maps, population studies. They are used in epidemiological surveillance of drug-resistance including their emergence and spread monitoring. Molecular markers have been described for many of the common anti-malarial drugs and are constituted of either single nucleotide substitution (SNP) or concatenated SNP in genes involved in parasite interaction with drugs [1]. In addition, long-term monitoring of parasite sensitivity to previously withdrawn anti-malarial drugs, such as CQ, can provide useful surveillance information if these drugs target similar resistance markers to current candidate ACT partner drugs [16].

2. Material and Methods

2.1. Ethics Statement

The study was approved by the Jomo Kenyatta University of Agriculture and Technology Institutional Ethics Review Committee (JKUAT-IERC) under the Reference number JKU/IERC/02316/0516. The study was conducted on archived primary school children samples that were positive for *P. falciparum* using a gold standard microscopy, stored at −80˚C at Nagasaki University, Institute of Tropical Medicine based at the Kenya Medical Research Institute (NUITM-KEMRI). The study samples were collected from Busia County primary schools, in Western Kenya with written informed consent from their parents or guardians.

2.2. Prevalence

P. falciparum was extracted from dried blood spots then subjected to direct Polymerase chain reaction. The percentage prevalence was determined using descriptive statistics, where, the number of positive samples was divided by the total number of samples collected from primary school children multiplied by 100%.

$$\text{That is, Percentage prevalence} = \frac{\text{frequency}}{\text{Total number of samples}} \times 100.$$

2.3. Detection of *P. falciparum*

2.3.1. Sample Preparation

280 Dried Blood Spots (DBS) Deoxyribonucleic Acid (DNA) lysates were prepared using the MightyPrep reagent (TAKARA BIO INC, Kusatsu, Shiga Prefecture, Japan; Cat No: 9182) following the manufacture's protocol with slight modifications. One Dried Blood Spot (DBS) was carefully cut and put into a 1.5 ml Eppendorf tube, 100 µl of MightyPrep reagent for DNA was added to the tube, spun at 15,000 rpm for 1 minute and heated at 95˚C for 15 minutes while shaking at 800 rpm and later cooled down on a heat block. The sample was hard vortexed for 1 minute, spun at 15,000 rpm for 2 minutes and stored at −30˚C ready for downstream processing.

2.3.2. Direct PCR Amplication of *P. falciparum*

Polymerase chain reaction (PCR) was performed on the DBS lysate to confirm malaria infection by *P. falciparum*. Primers are specific to *P. falciparum* (Table 1). A master mix was prepared which contained a total of 20 µl per reaction. 5.38 µl water, 10 µl of 2× Buffer V3, 2 µl of 10× additive, 0.12 µl primer mix, 0.5 µl MightAmp V3 and 2 µl STD lysate and water as negative control (NC). The PCR was performed on the following conditions: 98˚C for 2 minutes to inactivate the blocking antibody, 40 cycles, denaturation at 98˚C for 10 seconds, annealing at 60˚C for 15 seconds, extension at 68˚C for 1 minute and 42 seconds max 1.7 kb (CT) (1 min/1kbp), 4˚C for 2 minutes to cool down the sample and left on hold at 4˚C∞.

2.4. Agarose Gel Electrophoresis

The PCR amplicons were subjected to 2.0% agarose S (Lot No. 17047B, Japan). 6x loading dye (Nippon Gene; Cat No: 314-90261) was diluted with sample to make 1x and loaded onto the gel. The 100 bp GelPilot® Ladder marker (Qiagen; Cat No: 239035) was used and run at 100 V for 35 minutes. The gels were stained with 2x GelRed™ Nucleic Acid Gel Stain (Biotium; Cat No: 41003) for one hour on a shaker. The image was viewed using the Ultra Slim Blue Light Transilluminator (Maestrogen).

2.5. Amplification of Chloroquine Resistance Transporter Genes (Pfcrt)

Malaria positive samples with *P. falciparum* were subjected to second nested

Table 1. Primers for amplification of *P. falciparum.*

Plasmodium falciparum			
Primers	Sequences	Melting Temperature (TM)	Product Size
rPLU1 *F′*	TCAAAGATTAAGCCATGCAAGTGA	60	1638 bp
rPLU5 *R′*	CCTGTTGTTGCCTTAAACTCC	59	1638 bp

PCR to amplify the amplicons using *P. falciparum* chloroquine resistance transporter genes specific primers (Table 2). A total of 20 µL reaction per PCR was set up as follows; 2 µL of PCR products was added to 18 µL Thermo Scientific™ DreamTaq™ Hot Start Green PCR Master Mix (2x) (Lot No. 00783196). PCR reaction was performed in separate reactions at different conditions. The PCR program was set at initial denaturation at 95˚C for 3 minutes, 35 cycles for both Pfcrt forward and reverse primers 95˚C for 30 seconds, annealing at 58˚C seconds and 72˚C for 60 seconds and final extension at 72˚C for 15 minutes in the SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The PCR products were run on 2.0% agarose gel for 35 minutes at 100 V and the gel was stained with 2x GelRed for 1 hour and visualized using Ultra Slim Blue Light Transilluminator (Maestrogen).

2.6. Gel Extraction and DNA Purification

All the samples that showed bands for Pfcrt gene were excised using a scalpel on an Ultra Slim Blue Light Transilluminator (Maestrogen). The excised gel containing the DNA of interest was placed in a sterile 1.5 eppendorf tube. The samples were purified using QIAquick gel extraction kit (Cat. Number 28704) from Qiagen following the manufacturers' instructions. 3 volumes of buffer QG was added to 1 volume (100 mg gel~100 ul), incubated at 50˚C for 10 minutes. In addition, equal volume of isopropanol to 1 gel volume was added and mixed followed by spinning at 13,000 rpm for 1 minute. Furthermore, 500 ul of QG buffer was added to the sample and spun for 1 minute and 750 ul of PE buffer was added followed by spinning at 13,000 rpm for 1 minute and the final column was transferred to a new Eppendorf tube and finally eluted by adding 50 ul buffer EB and spun at 13,000 rpm for 1 minute.

2.7. Pfcrt Gene Sequence Analysis

The PCR products containing the amplified PCR gene were sequenced using Sanger sequencing for forward primer (Macrogen Europe B.V, Amsterdam Netherland). The samples' sequences were trimmed on a Chromas tool followed by analysis using Bioedit software. The sequences were blasted before multiple sequence alignment was conducted (https://blast.ncbi.nlm.nih.gov).

Table 2. Chloroquine resistance transporter (*Pfcrt*) gene primers.

Plasmodium falciparum chloroquine resistance transporter gene (Pfcrt)			
Primers	Sequences	Melting Temperature (TM)	Size
Pfcrt F′	GTTCTTGTCTTGGTAAATGT	56.6	530 bp
Pfcrt R′	CGGATGTTACAAAACTATAGTT	60.9	530 bp

3. Results

3.1. *P. falciparum* Chloroquine Resistance Transporter Gene Analysis

Figure 1 shows the samples that were amplified for *P. falciparum* using rPLU1 forward and rPLU5 reverse primers. Bands were observed on positive samples, a negative control (NC), a positive control (PC), and a 100 bp ladder at both ends in a 13-well agarose gel with a target size of 1638 bp.

P. faciparum was detected in 280 samples dried blood spot (DBS). Figure 2 shows, second nested detection of amplified PCR products targeting the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*). Sample lane 2, 11, 12, 14, 17, 19, 22 showed clear bands in a 26-well agarose gel at *Pfcr*t gene target size 530 bp.

The gel contains both positive and negative samples for Pfcrt gene. The samples that turned positive for Pfcrt gene are denoted with numbers with positive control (PC) and negative control (NC) as the marker.

Point mutations were identified as a result of substitutions in 14 samples, that is, S017 had point mutations at positions 196 (T196C) and 209 (T209A) with amino acid changes Y66H and L70A respectively. However, S048 had a transitional

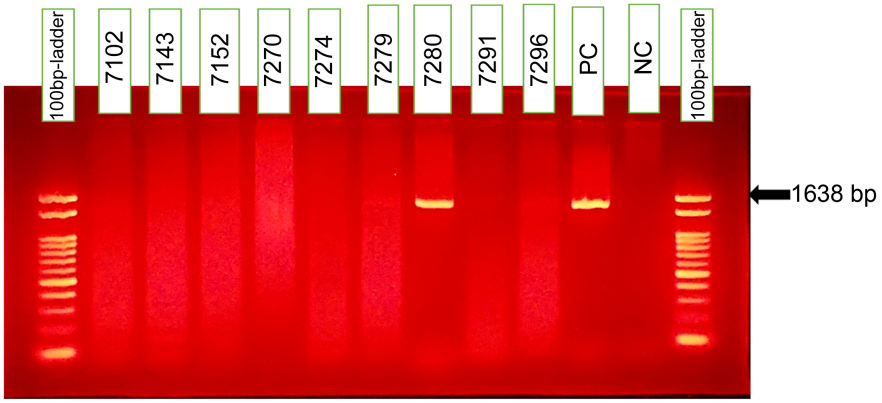

Figure 1. The gel shows the amplification of *Plasmodium falciparum* (The gel shows the positive bands for *P. falciparum* with a size of 1638 bp).

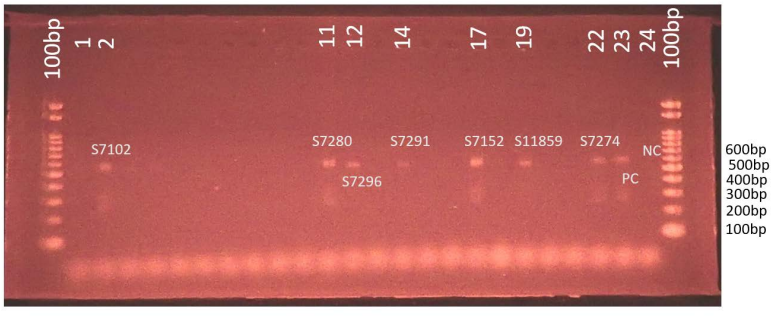

Figure 2. Shows, second detection of amplified PCR products targeting the *P. falciparum* chloroquine resistance transporter gene (Pfcrt). Sample lanes 2, 11, 12, 14, 17, 19, 22 showed clear bands in a 26-well agarose gel at Pfcrt gene target size of 530 bp.

substitution at position A173G, A175G, G193A, C200T, T241C with amino acid changes Y58C, T59V, V65I, P67L, T81L and substitution by transversion at position A179C, A197C, C199A, A211T, T227G with amino acid changes Y60S, Y66S, P67T, I71F and F76C. No amino acid changes were found at positions C198T, T222C and T234C.

3.2. Evolutionary Relationships of Pfcrt Gene among Sample

Using the NCBI Reference Sequence NC_004328, a phylogenetic tree was constructed showing evolutionary relationship among 14 samples (S17, S38, S42, S45, S47, S48, S50, S56, S58, S59, S61, S62, S65 and S67) using Protdist Neighbor-Joining method as shown in **Figure 3** below.

4. Discussion

CQ resistance is strongly associated with a single nucleotide polymorphism (SNP), resulting in a Lys76Thr change in the pfcrt gene, which encodes the CQ resistance transporter [17]. In Africa, the pfcrt Lys76Thr change appears to be a reliable marker for CQ resistance [17]. This study detected 2 samples which conferred *P. falciparum* CQ resistance transporter (Pfcrt) gene leading to the following amino acid changes Y66H, L70A, Y58C, T59V, V65I, P67L, T81L, Y60S, Y66S, P67T, I71F and F76C as shown in (**Table 3**). The percentage prevalence of pfcrt marker in Busia County was 5.0%, this showing in every 20 samples, one patient had developed resistance. Studies from Western Kenya demonstrated that in 2008, the prevalence of parasites with the Pfcrt genotype was 72.4%, which was shown to decline to 32.1% by 2011, 7% by 2013, 18% in 2010 to 0% by 2013 [18]. However, our study showed an increase of Pfcrt gene to 5.0% from 0% according to Luchi *et al*., study reported in 2015. High prevalence of Pfcrt mutant gene in this present study could be as a result of the treatment of malaria infection with the use of chloroquine in which long time exposure of the parasite to the drug could bring about mutation leading to development of the Pfcrt mutant genes [19]. The simultaneous presence of very low and high prevalence of CQ could be related to a between-regions difference of CQ pressure and

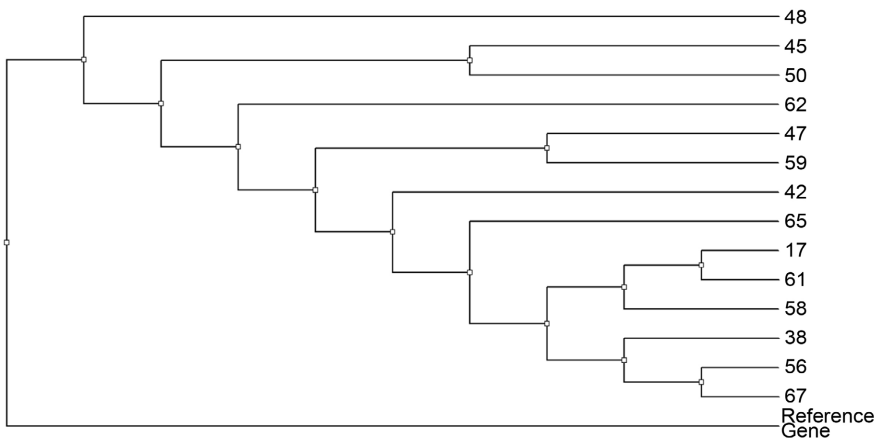

Figure 3. Phylogenetic tree using protdist neighbour-phylogenetic tree.

Table 3. Point mutation and amino acids changes.

Gene		Samples	
		S017	S048
Plasmodium falciparum 3D7 genome assembly, chromosome: 7 Pfcrt (PF3D7_0709000)	Point mutations	T196C T209A	A173G, A175G, A179C, G193A, A197C, C198T (No Change), C199A, C200T, A211T, T222C (No Change), T227G, T234C (No Change), T241C
	Amino Acids (AA) Changes	Y66H L70 STOP	Y58C, T59V, Y60S, V65I, Y66S, Y66Y (No Change), P67T, P67L, I71F, F74F (No change), F76C, F78F (No Change), F81L

also to the effect of selection for CQ resistance depending on the genetic structure of parasite populations, which have been shown to vary significantly across the country [20].

This study reported new mutational changes which might be as a result of the high levels of gene flow between lowland and highland populations facilitating the introduction of new alleles from endemic lowland sites. Lack of genetic structure between highland and lowland populations, as determined by the analysis of molecular variance may result from human travel between highland and lowland sites [21]. Busia, Western Kenya borders Uganda on the West. ACT rarely reaches such remote areas of the county, hence many results to over-the counter medicine to the old drugs AQ and SP.

There was no statistical difference in the mutational frequencies at F76C, such a high frequency of the Pfcrt K76T mutation suggests that the fitness costs associated with this mutation is not high enough to cause dramatic reduction in its frequency [21]. Alternatively, infrequent but continuous use of AQ, a 4-aminoquinoline compound related to CQ and artemisinin or in combination with SP or artesunate to treat uncomplicated malaria infections in children might have been the reason for CQ resistance recrudescence. These quinolines-based compounds like amodiaquine, mefloquine, primaquine, and piperaquine share a similar structure [22] Resistance also develops more quickly where a large population of parasites are exposed to drug pressure removing sensitive parasites, while resistant parasites would survive [23]. The study showed novel nucleotide substitutions not reported before.

The phylogenetic relationship showed that the reference gene (accession number MW275076), the root for the samples has a common ancestral origin with the samples resistance to Pfcrt. The topology of the dendrogram is a rooted

cladogram structure and is well defined in samples S17, S38, S42, S45, S50, S56, S58, S59, S61, S65 and S67 have the most recent common ancestry which can form a clade. Sample S45 and S50 are sisters with same evolutionary relationship origin, the same applies to samples S47 and S59, and S17, S61, S58, S38, S56 and S67. Sample 48 has a longer branching, and the longer the branching the more the genetic change divergence.

5. Conclusion and Recommendation

The study found novel and synonymous mutations in CQ resistance transporter gene coupled with increased SNPs. In this view continuous surveillance is required to monitor of prospect related drugs such as AQ and 4-aminoquilones. Findings suggest that SNPs in Pfcrt genes could be as a result of the related drugs recrudescence. However, the impact of *P. falciparum* chloroquine resistance transporter gene mutation requires further study with its related drugs altogether.

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Authors' Contribution

Conceptualization, Methodology, Investigation, Manuscript review, O. P. S: Methodology, Manuscript review T.T.N, R.M.I: Supervision, R.W.W: Investigation, M.M.M, P.K.R, C.W.N, A.W.M, J.J.Y, N.S.T, D.K.O, G.N.K, P.M.N: Conceptualization, Methodology, Investigation, Manuscript review, Supervision S.M.N.

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Competing Interest

The authors declare that there are no competing interests.

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Abbreviations

Pfcrt: *Plasmodium falciparum* chloroquine resistance transporter; PCR: Polymerase Chain Reaction; SNPs: Single Nucleotide Polymorphism; DBS: Dried Blood Spot; CQ: Chloroquine; DR: Drug Resistance; ACT: Artemisinin based Combination Therapy; AQ: Amodiaquine; SP: Sulfadoxine pyrimethamine.