Molecular Investigation of Genetic Signatures of Selection in *Plasmodium falciparum* Actin-Binding Protein Coronin, Cysteine Desulfurase, and Plasmepsin 2 Gene in Mbita Field Isolates, Western Kenya

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**Abstract**

**Background:** *Plasmodium falciparum* (*Pf*) resistance to antimalarial drugs is a major impediment to malaria control. The *Pf*Kelch 13 (*PfK13*) gene has been largely reported to be associated with artemisinin resistance. However, recent studies have shown artemisinin resistance without *Kelch13* mutations suggesting the implication of other genes in artemisinin resistance. In this current study, we focused on mutations in *Pf*actin-binding protein coronin, *Pfcysteine desulfurase* and *Pfplasmepsin 2* gene, three putative candidates recently were reported to be involved in artemisinin, lumefantrine and piperazine resistance respectively. **Method:** Archived blood samples previously collected from asymptomatic school children from December 2016 to October 2018 were used in this study. Genomic DNA was extracted using ISOLATE II Genomic DNA kit. After PCR amplification, amplicons were purified and sequenced by capillary sequencing. Reads were analyzed for the identification of point mutations previously reported to be involved in drug selection. **Results:** Mutations R100K, and G50E involved in reduced artemisinin susceptibility were detected in *Pfcoronin*. From 2016/17 to 2018 the allele 100K increased frequency (11.2%); while 50E was only observed in 2018 time point.
reaching 11.1%. Lumefantrine selection marker K65, in codon (K65Q) was observed at 14.2% in *P. falciparum* desulfurase, and the mutant allele 65Q gradually increased frequency from 28.5% in 2016/17 to 57.1% in 2018. *Pfplasmepsin 2* was the less polymorphic gene. Several other polymorphism codons and single nucleotide variants were detected. **Conclusion:** The findings indicate the presence of mutations associated with reduced artemisinin susceptibility and lumefantrine selection marker. Therefore, the results call for continuous monitoring of molecular makers in Mbita parasites.

**Keywords**

*Plasmodium falciparum*, Mutations, Artemisinin, Lumefantrine, Piperaquine

### 1. Background

Malaria is one of the most widespread and deadliest infectious parasitic diseases and it threatens millions of lives worldwide and global public health. The World Health Organization (WHO) estimated a total of approximately 229 million cases of malaria with a global total death of 409,000 [1]. The largest burden of malaria morbidity occurs in Africa with an estimated 94% of cases and 384,000 deaths in 2019 [1]. Children and pregnant women are the most affected by the disease, about 78% of malaria deaths are estimated to be in children aged under 5 years [2] [3]. In Kenya, malaria is the major cause of morbidity and mortality ordinarily affecting children under 5 years of age and pregnant women, accounting for 19% of outpatient attendance to health centres [4]. Clinical cases are estimated at around 6.7 million each year, with 4000 deaths occurring among children, making malaria a major threat in Kenya [5]. It has been estimated that about 74% of Kenya’s population is at risk of malaria infection [6] [7]. Western Kenya and the coastal part bear the highest rate of transmission, where the prevalence of infection ranged from 8% to 15% among children age (<5 years) and (5 - 15 years) respectively, with an incidence of hospitalization respectively bracketed from 64% and 70% [7] [8]. *Plasmodium falciparum* is the predominant species and represents 92% of malaria cases nationally [4]. Artemisinin-based combination therapies (ACTs) are the first-line drugs recommended by WHO for the treatment of uncomplicated *Plasmodium falciparum* malaria in settings of endemicity [9] [10]. ACT consists of the combination of a rapid clearing fast-acting artemisinin derivative with a longer half-life slower-acting partner drug (such as piperaquine, lumefantrine) [9] [11], resulting in potent effective parasite clearance and is supposed to reduce the likelihood of parasites resistance which raises from monotherapy [12]. The use of ACTs has been successful and has contributed to reducing malaria burden. However, this success is threatened by the emergence and spread of *Plasmodium falciparum* artemisinin-resistant parasites, first reported in Cambodia in 2007 and has subsequently spread over Greater Mekong Subregion (GMS) [13] [14] [15], where the former
first-line drugs (chloroquine, and sulfadoxine-pyrimethamine) resistance started and subsequently spread to Africa [16]. Drug resistance remains a major impediment to malaria eradication. To this end, the recent prevalence of resistance to artemisinin and its partner drugs in Africa is highly alarming. Mutations (P553L, M476I, P574L, and R561H) have been detected in Africa, with an increased prevalence of R561H, found in 12.8% in Rwanda, associated with late parasite clearance [17]. Recently, in Uganda, evidence of *P. falciparum* resistance to artemisinin has been reported in patients with *K13* mutations (C469Y and A675V), which were confirmed to be associated with late parasite clearance after evaluating parasite susceptibility by ring-stage survival assay and genotyping [17]. Continuous monitoring of point mutations and temporal trends of mutant frequency in genes associated with *P. falciparum* resistance to artemisinin and its partner drugs is crucial, especially the identification of single nucleotide polymorphism (SNP) markers associated with treatment failure. Mutation in *P. falciparum kelch13* gene located on chromosome 13 is associated with artemisinin resistance in Cambodia. *kelch13* gene point mutations (C580Y, R561H, P553L, R539T, I543T, F446I, N458Y, Y493H and M476I) are providing insight into artemisinin resistance in South East Asia [18] [19] [20] among which five (C580Y, Y493H, R539T, M476I, I543T) have been validated to confer artemisinin resistance [21] [22] [23]. However, recently artemisinin resistance mutations were observed in parasites at other loci, notably a mutation in codon I356T of chloroquine resistance transporter gene (crt), codon V127M in apicoplast ribosomal protein S10 precursor gene (arps10), and codon D193Y in ferredoxin (fd) gene, suggesting that, in addition to the *Kelch13* gene, other genes may be involved in artemisinin and its partner drugs resistance [24]. This was supported by a recent study in which parasites lacking *kelch13* mutations exhibited an increased ring-stage survival phenotype [11] [25]. *Plasmodium falciparum* actin-binding protein coronin is a WD40-propeller domain protein family sharing the β-propeller motif with *Kelch13* protein [26]. Interestingly, mutations (R100K, E107V and G50E) in the *P.f.coronin* gene (PF3D7_1251200) on chromosome 12, were reported to confer reduced artemisinin susceptibility after a CRISPR/Cas9-based mutagenesis study [26], making the *P.f.coronin* gene, a potential target for tracking artemisinin resistance. Although much attention has been focused on *Kelch13* gene, it is of importance to monitor artemisinin-resistant signatures in other putative genes. On the other hand, it is important to also consider artemisinin partner drugs resistance, mainly lumefantrine and piperaquine since Artemether-lumefantrine (AL) and Dihydroartemisinin-piperaquine (DP) are most commonly ACTs used in Africa [27] [28]. Lumefantrine resistance has been associated with *Plasmodium falciparum* multidrug resistance 1 (*Pfmdr1*) gene and the increase of its copy number) [16] [29], and the combination (AL) shown selection for polymorphisms in the 1 (*mdr1*) (N86/184F/D1246-NFD haplotype and chloroquine resistance transporter (crt) (K76) [16] [30]. Furthermore, the cysteine desulfurase gene (*Pfnfs*) (PF3D7_0727200), a novel putative gene for resistance to lumefantrine, located on chromosome 7 has recently shown significant temporal
trend polymorphism in Kenya [16]. Mutation in Pfns in codon K65Q has been reported to have a significant trend pre and post-ACT introduction with a decline in the K65 wild-type allele while the mutant allele (65Q) increased in the Kilifi population since the introduction of artemether-lumefantrine treatment [16]. This calls for further investigation. The same codon, (K65Q) in Pfns has also been found to have strong temporal differentiation 7 years after the introduction of ACTs in Gambian isolates, with significantly higher IC50 (inhibitory concentration) in the wild type allele K65 [31]. Parasites with artemisinin resistance may develop resistance to piperquine, compromising the efficacy of dihydroartemisinin-piperaquine as well as all other combinations [10]. Piperaquine resistance is now reported to be spreading in Cambodia [32], and DHA-PPQ treatment failure has been reported in Ethiopia and in Cameroon [33]. Increased copy number of P. falciparum plasmspin 2 (Pfpm2) gene (PF3D7_1408000), located on chromosome 14, and Pfmdr1 copy number have been associated with resistance to piperquine and lumefantrine respectively [16] [34] [35] [36]. The reported signature of PPQ resistance also includes SNPs in Pfcr [25] [34]. Perpiraquine resistance call for continuous surveillance. Although no evidence of ACT resistance is yet been reported in Kenya, it is crucial to continuously investigate artemisinin and its partner drugs resistance markers in the Kenyan population as artemether-lumefantrine (Coartem™) has been adopted in 2004 [7] and dihydroartemisinin-piperaquine (Artekin®) since 2009 [37] as first-line and second-line drugs in the treatment of uncomplicated malaria respectively. Investigation of genetic mutations associated with antimalarial resistance provides molecular markers for monitoring resistance. To this end, the purpose of this study was to investigate mutations associated with artemisinin, lumefantrine and piperaquine resistance in Plasmodium falciparum actin-binding protein coronin gene, cysteine desulfurase gene and plasmepsin2 gene respectively in Mbíta Sub-county isolates, a region of high infection of malaria where the three putative genes have yet been studied. The use of such information is of paramount importance in the management of antimalarial drugs policy and the adoption of strategies to track the emergence and spread of resistance.

2. Method

2.1. Ethical Approval

The study was performed under the granted approval for the original study (KEMRI/RES/7/3/1) provided by the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU). All experiments were conducted consistently with good laboratory practice regulations and relevant guidelines.

2.2. Study Site

The study was conducted in Mbíta sub-county located on the shores of Lake Victoria in Homa Bay County, western Kenya [6]. The district is bordered by
Lake Victoria to the north, west and south and situated between longitudes 34˚04’ and 34˚24’ East and latitudes 0˚21’ and 0˚32’ South [38]. It is located about 400 km west of Nairobi and has an area of 163.28 km² [6]. Mbita sub-county has a population of approximately 115,896 [6]. The area experiences two rainy seasons annually from March to June and October to November [39], with an average annual rainfall estimated at 1300 mm and daily temperatures ranging between 26˚C to 34˚C [40]. Malaria transmission is intense among the local population with P. falciparum prevalence ≥ 40% [41] [42] [43]. The high infection rate of malaria among the residents makes the disease a burden for health care in the region.

2.3. Sample Collection

The study used archived samples previously collected over two years from December 2016 to October 2018 in the context of a study evaluating symbiotic microbes and mosquito vector ability using membrane feeding assay in the bid to characterize candidates that potentially block malaria transmission. The samples were collected from asymptomatic schools children (male and female) of various ages ranging from 5 to 15 years. Eligibility criteria for study participants included the bracket age (5 - 15 years) and no malaria infection symptoms. Only participants’ parents/legal guardians and participants above 12 years or older who provided written consent were recruited. Samples collection was carried out at the St. Jude’s Clinic located within the Thomas Odhiambo Campus of the International Centre for Insect Physiology & Ecology (ICIPE) in Mbita Town. The venous blood samples (4 mL), and drops of blood (collected through a finger prick) on filter paper (Whatman 3 MM; Whatman, Maidstone, UK) were obtained from each individual. Blood samples were screened for Plasmodium parasite carriage using rapid diagnostic tests (SD Bioline malaria Ag Pf/Pan (HRP-II/pLDH)) and microscopy. Blood smears were prepared, stained with 10% Giemsa for 10 minutes, and examined by trained microscopists. Plasmodium-positive by RDT and by microscopy observation were enrolled and stored at −80˚C until usage. Overall, 85 venous blood collected in EDTA tubes were randomly selected for this study.

2.4. Genomic DNA Extraction, PCR, and Sanger Sequencing

DNA was extracted from 85 frozen blood samples using ISOLATE II Genomic DNA kit (Bioline, Meridian Bioscience, UK) as per the manufacturer’s protocol. Amplicons were obtained from the three target genes (Pfcoronin, Pfnsf and Pfpm2) using gene-specific primers targeting the regions of interest. All primers used were designed in this study (Table 1) using the primer3plus tool, (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the online OligoAnalyzer tool (Integrated DNA Technology) for quality control, (https://www.idtdna.com/pages/tools/oligoanalyzer). Subsequently, Primer-BLAST was used to check oligo-sequences specificity. We performed HotStart
Table 1. PCR primer.

| Gene      | Primer                        | Tm    | Ta    | Product length |
|-----------|-------------------------------|-------|-------|----------------|
| *PfCorin* | F: 5‘-TTAGCTGATCCGTCAAATAACC-3’ | 58.4˚C | 56.3˚C | 986 bp         |
|           | R: 5‘-TACGCTTCATCATATAATGTTG-3’ | 58.4˚C |       |                |
|           | F: 5‘-TGGTATAGCTTGTAGGTGCTGA-3’ | 60.3˚C | 56˚C  | 547 bp         |
|           | R: 5‘-CAGAAGAATCAAAAAGGTAGATG-3’ | 60.1˚C |       |                |
| *Pfnfs*   | F: 5‘-TGTCGTACCTATGAACATATCAG-3’ | 59.3˚C | 56.6˚C | 924 bp         |
|           | R: 5‘-GACTTTTCCAGCAGCTTG-3’     | 58.4˚C |       |                |
| *PfPmII*  | F: 5‘-CTCTTTTGATGTAGTATGTTG-3’  | 60.1˚C | 56.7˚C | 914 bp         |
|           | R: 5‘-AAGTCAGTGGTACAGTATGG-3’   | 58.4˚C |       |                |

F and R represent forward and reverse primers respectively. Tm = melting temperature. Ta = annealing temperature.

PCR system in a final volume of 20 µL containing 0.3 µM of each primer (Macrogen, South Korea), 5.5 µL of 5X Hot FIRE-Pol Master Mix (Solis BioDyne, Estonia), 3 µL template DNA, and 10.9 µL water, PCR grade (Sigma-Aldrich, Merck, Germany). The PCR reaction was set up in a Kyratec SuperCycler (Kyratec, Australia) using the following conditions: initial denaturation (one cycle) at 95˚C for 15 min, followed by 35 cycles, 95˚C for 30 sec, 56.7˚C for 30 sec, 72˚C for 45 sec, final elongation, 72˚C for 7 min. In each reaction, a non-template reaction was run in parallel as negative control. Amplicons were visualized in 2% agarose gels using GelRed(R) nucleic acid gel stain (Biotium, California). Successful PCR products were cleaned using ExoSAP-IT (Thermo Fisher Scientific) and transferred into 1.5 mL microcentrifuge tubes, labelled and shipped for Capillary Sanger sequencing facility (Macrogen, Netherlands). PCR products were read in an ABI 3730XL DNA Analyser (Applied Biosystems) using the BigDye® terminator V3.1 cycle sequencing chemistry. PCR primers were used for DNA sequencing.

3. Data Analysis

Sequencing results were processed and analyzed in Geneious 10.2.3 software (Biomatters, New Zealand). The sequences were trimmed, edited, and BLAST (Basic Local Alignment Search Tool) alignment was performed in NCBI (National Center for Biotechnology Information), to check the per cent identity of the sequences vis à vis the corresponding reference genes. We used *Plasmodium falciparum* 3D7 strain as the reference genome. The sequences were subsequently assembled in Geneious, and aligned using ClustalW alignment. Alignment output was mapped to the corresponding reference genes for variants calling. The Geneious software automatically assigns a P-value to the variant, if the same variant is commonly observed (SNP that are observed in more than one isolate (Table 2, Table 3). The rare single point mutations (Tables 2-4) were recovered without a P-value assigned based on, if the base quality was ≥20 (the Phred quality score), and if the point mutation does not contain mixed peaks.
All variants that had mixed peaks were removed from the data. To reduce noise in SNP calling, we set up SNPs calling parameters such that only highly significant SNPs ($P < 10^{-6}$) were filtered out. Statistical analyses were carried out using GraphPad Prism 9.0.0. In order to compare changes in mutation frequencies over time, we defined two-time points (2016/17 and 2018). Only five samples collected from December 2016 were used, and because of the closing time (from December 2016 to January 2017), thus we assumed that no significant change

| Codon | Nucleotide | Wild type | Mutant | Type | N/S | 2016/2017 | 2018 | P-value |
|-------|------------|-----------|--------|------|-----|-----------|------|---------|
| 125   | 375        | K [G]     | K [A]  | Trans| S   | 50 (16)   | 50 (16)| $6.3 \times 10^{-20}$|
| 100   | 299        | R [G]     | K [A]* | Trans| N   | 44.4 (18) | 55.6 (18)| $5 \times 10^{-16}$|
| 96    | 286        | D [G]     | N [A]  | Trans| N   | 44.4 (18) | 55.6 (18)| $1 \times 10^{-9}$|
| 95    | 283        | E [G]     | K [A]  | Trans| N   | 44.4 (18) | 55.6 (18)| $1 \times 10^{-9}$|
| 75    | 225        | S [T]     | S [C]  | Trans| S   | 44.4 (18) | 55.6 (18)| $7.9 \times 10^{-9}$|
| 71    | 213        | K [G]     | K [A]  | Trans| S   | 44.4 (18) | 44.4 (18)| $2 \times 10^{-9}$|
| 59    | 175        | E [G]     | K [A]  | Trans| N   | 44.4 (18) | 55.6 (18)| $1 \times 10^{-9}$|
| 57    | 170        | R [G]     | K [A]  | Trans| N   | 44.4 (18) | 55.6 (18)| $1.6 \times 10^{-11}$|
| 47    | 139        | V [G]     | F [T]  | Transv| N  | 22.2 (18) | 44.4 (18)| $2.6 \times 10^{-8}$|
| 50    | 149        | G [G]     | E [A]  | Trans| N   | 0 (18)    | 11.1 (18)|
| 81    | 243        | S [A]     | S [T]  | Transv| S  | 0 (18)    | 11.1 (18)|
| 68    | 203        | I [T]     | K [A]  | Transv| N  | 0 (18)    | 11.1 (18)|
| 61    | 182        | Q [A]     | P [C]  | Transv| N  | 0 (18)    | 11.1 (18)|
| 60    | 180        | N [T]     | N [C]  | Transv| S  | 0 (18)    | 11.1 (18)|
| 149   | 447        | S [T]     | S [G]  | Transv| S  | 50 (12)   | 16.7 (12)|
| 179   | 537        | G [T]     | G [A]  | Transv| S  | 50 (4)    | 0 (4)  |
| 156   | 466        | E [G]     | K [A]  | Trans| N   | 50 (4)    | 0 (4)  |
| 158   | 474        | E [G]     | E [A]  | Trans| S   | 0 (4)     | 50 (4) |
| 183   | 547        | S [A]*    | G [G]  | Trans| N   | 50 (4)    | 50 (4) |
| 168   | 504        | K [G]     | K [A]  | Trans| S   | 50 (4)    | 50 (4) |
| 161   | 481        | A [G]     | P [C]  | Transv| N  | 50 (4)    | 50 (4) |
| 159   | 477        | K [G]     | K [A]  | Trans| S   | 50 (4)    | 50 (4) |

A.A: amino acids. Trans: transition. Transv: transversion. N: non-synonymous. S: synonymous. * Blue: Artemisinin reduced susceptibility marker. * Represents mutation previously annotated in PlasmoDB. White: synonymous mutations. SNPs observed in more than one isolate are assigned with a $P$-value. The non-common single point mutations are not assigned with a $P$-value. The frequency (%) represents the percentage of reads that harboured a variant over the total number ($n$) of reads that had data for the given variant’s locus.
Table 3. *P. falciparum* single nucleotide polymorphisms (SNPs) markers.

| Codon | Nucleotide | Wild type | Mutant | Type | N/S | 2016/2017 | 2018 | P-value |
|-------|------------|-----------|--------|------|-----|-----------|------|---------|
| 62    | 185        | S [G]     | N [A]* | Trans | N   | 42.8 (28) | 50 (14) | 1 \times 10^{-34} |
| 65    | 193        | K [A]     | Q [C]* | Trans | N   | 28.5 (28) | 57.1 (28) | 2.5 \times 10^{-26} |
| 67    | 200        | E [A]     | G [G]* | Sub   | N   | 35.7 (28) | 57.1 (28) | 7 \times 10^{-40} |
| 67    | 201        | E [A]     | G [T]* | Sub   | N   | 35.7 (28) | 50 (28)  | 7 \times 10^{-40} |
| 120   | 359        | S [G]     | I [T]* | Trans | N   | 23 (26)   | 23 (26)  | 1 \times 10^{-10} |
| 130   | 390        | E [G]     | D [C]* | Trans | N   | 7.8 (26)  | 15.38 (26) | 3.3 \times 10^{-9} |
| 146   | 437        | R [G]     | K [A]  | Trans | N   | 45.4 (22) | 36.3 (22) | 2 \times 10^{-13} |
| 150   | 448        | D [G]     | N [A]  | Trans | N   | 45.4 (22) | 54.5 (22) | 1.6 \times 10^{-20} |
| 155   | 463        | T [A]     | P [C]  | Trans | N   | 45.4 (22) | 54.5 (22) | 6.3 \times 10^{-14} |
| 160   | 479        | R [G]     | K [A]  | Trans | N   | 45.4 (22) | 54.5 (22) | 4 \times 10^{-16}  |
| 163   | 487        | D [G]     | N [A]  | Trans | N   | 45.4 (22) | 54.5 (22) | 3.2 \times 10^{-17} |
| 164   | 492        | K [G]     | K [A]  | Trans | S   | 45.4 (22) | 54.5 (22) | 4 \times 10^{-16}  |
| 179   | 536        | R [G]     | K [A]  | Trans | N   | 45.4 (22) | 45.4 (22) | 10^{-14} |
| 188   | 562        | E [G]     | K [A]  | Trans | N   | 36.3 (22) | 45.4 (22) | 4 \times 10^{-16}  |
| 194   | 582        | A [A]     | A [C]  | Trans | S   | 27.2 (22) | 45.4 (22) | 5.7 \times 10^{-9}  |
| 195   | 584        | R [G]     | Q [A]  | Trans | N   | 45.4 (22) | 54.5 (22) | 2.5 \times 10^{-7}  |
| 238   | 714        | K [G]     | K [A]  | Trans | S   | 50 (20)   | 30 (20)  | 6.8 \times 10^{-12} |
| 271   | 811        | D [G]     | N [A]  | Trans | N   | 33.3 (18) | 33.3 (18) | 5 \times 10^{-9}  |
| 148   | 443        | Y [A]     | F [T]  | Trans | N   | 18.18 (22) | 18.18 (22) |          |
| 203   | 609        | N [T]     | K [G]  | Trans | N   | 9 (22)    | 9 (22)   |          |
| 229   | 687        | T [A]     | T [C]  | Trans | S   | 0 (20)    | 10 (20)  |          |
| 279   | 835        | D [G]     | H [C]  | Trans | N   | 11.11 (16) | 0 (16)   |          |
| 305   | 914        | Q [A]     | L [T]  | Trans | N   | 20 (10)   | 0 (10)   |          |
| 306   | 916        | D [G]     | N [A]  | Trans | N   | 20 (10)   | 20 (10)  |          |

A.A: amino acids. Trans: transition. Transv: transversion. N: non-synonymous. S: synonymous. *Represents SNPs annotated in PlasmoDB. Blue: Lumefantrine selection marker. White: synonymous mutations. SNPs observed in more than one isolate are assigned with a P-value. The non-common single point mutations are not assigned with a P-value. The frequency (%) represents the percentage of reads that harboured a variant over the total number (n) of reads that had data for the given variant’s locus.

occurred in the parasite genome during only one month, then we defined 2016/17 as a one-time point. Based on the defined sample collection time point, the Chi-square test for trend was performed to find out significant trend of mutants over time. Pearson correlation coefficient was calculated to check the correlation mutant’s data between time points. Statistical results were considered significant if P < 0.05. All the graphics were constructed using OriginPro, v9.6 software. We further checked proteins sequences of the three genes tracking the point mutations in proteins domains and motifs, using InterPro database, SMART tool, and NCBI conserved domain database (CDD).
Table 4. *Pfpm2* single nucleotide variations (SNVs).

| Codon Nucleotide Wild type | Mutant Nucleotide | Mutant Type | N/S | 2016/2017 (n) | 2018 (n) |
|----------------------------|-------------------|-------------|-----|---------------|----------|
| 79                         | H [C]             | N [A]       | Transv | 11.11 (18)   | 33.33 (18) |
| 188                        | R [G]             | T [C]       | Transv | 7.7 (26)      | 0 (26)   |
| 260                        | V [G]             | L [T]       | Transv | 0 (20)        | 10 (20)  |
| 261                        | D [G]             | H [C]       | Transv | 10 (20)       | 0 (20)   |
| 309                        | E [G]             | L [C]       | Transv | 0 (10)        | 20 (10)  |
| 322                        | D [T]             | E [A]       | Transv | 0 (10)        | 20 (10)  |

A.A: amino acids. Transv: transversion. N: non-synonymous. The frequency (%) represents the percentage of reads that harboured a variant over the total number (n) of reads that had data for the given variant’s locus.

4. Results

4.1. SNPs Markers Detected in *Pfcoronin* Gene

Twenty-two SNPs codons (Table 2), were detected in *Pfcoronin* gene target region, in which six codons (R100K, D96N, E95K, E59K, R57K, and V47F) contained non-synonymous mutations, the rest three codons (K125K, S75S, and K71K) harboured synonymous mutants alleles. The frequency of SNPs mutants’ alleles is shown in (Figure 1). Artemisinin reduced susceptibility mutation, 100K ($5 \times 10^{-16}$) [26], was detected, at increased frequencies throughout the time, 44.4% in 2016/17 and 55.6% in 2018. The highest strong signal was observed in allele 125K ($6.3 \times 10^{-20}$), and both, 125K and 71K ($2 \times 10^{-9}$) were found at consistent frequency through all time points (50% and 44.4%) respectively. 96N, 95K, and 59K had the same signal ($1 \times 10^{-9}$), and their frequencies evolved similarly (44.4%) in 2016/17, reaching (55.6%) in 2018. Similar frequency was made in 75S ($7.9 \times 10^{-9}$) and 57 K ($1.6 \times 10^{-11}$) alleles, which frequencies were stable at 44.4% in 2016/17, attaining 55.6% in the 2018 period. Transversion allele 47F doubled frequency from 22.2% in 2016/17 to 44.4% in 2018. The mutant’s alleles showed significant temporal trend in both time points ($X^2 = 20.23, P < 0.0001$) in 2016/17 and ($X^2 = 36.64, P < 0.0001$) in 2018. There high positive correlation between mutants from both periods of time ($r = 0.8855, P < 0.0001$). The following five codons (G50E, S81S, I68K, Q61P, and N60N) showed no variation in 2016/17 whereas mutants were observed at frequencies of 11.1% in the 2018 time point. The allele 50E in codon G50E has been reported to reduce artemisinin susceptibility [26]. Mutants’ alleles in codons (S183G, K168K, A161P, and K159K) had similar stable frequencies over all the time (50%). The allele 183G was found annotated in PlasmoDB database (https://plasmodb.org/plasmo/app). S149S, G179G, and E156K mutants showed similar frequencies (50%) in 2016/17 while a remarkable decline (16.7%) in 149S frequency was noted in 2018, and mutants 179G and 156K were not observed. Mutation E150E was only observed in 2018 at frequency reaching 50%. The frequency of wild type and mutants’ alleles in the *Pfcoronin* gene is gathered in (Figure S1).
Figure 1. Frequency of mutants’ alleles in the Pf.coronin gene and in the Pf.cysteine desulfurase gene from 2016/17 and 2018 time point. (a) = frequency of mutants’ alleles in the Pf.coronin gene. (b) = frequency of mutants alleles in the Pf.cysteine desulfurase gene. The X-axes represent the polymorphic codons that carry the mutations.

4.2. SNPs Markers Detected in Pfifs Gene

A total of 24 polymorphism codons were recovered within the target region of interest (Table 3). The polymorphism codon K65Q which is lumefantrine selection marker [16] was identified. The strongest signal reported was the mutant’s allele 65Q \( (P = 2.5 \times 10^{-76}) \) and the allele was recovered to increase frequencies over the time points from 28.5% in 2016/17 to 57.1% in 2018 while the wild type K65 which is responsible for lumefantrine selection [16], was detected at 14.2% in 2016/17, but not observed in 2018 time point. The 62N mutant allele \( (P = 1 \times 10^{-14}) \) in codon S62N evolved at the same frequency (42.8%) as the mutant 65Q, and its frequency reached 50% in 2018. The most polymorphic codon recovered...
was E67G, in which two contiguous nucleotides were substituted (Wt. AA vs Mt. GT) and were found to evolve together in mutant data at the same frequency (35.7%) in 2016/17 and the mutant 67G-(G) ($P = 7 \times 10^{-40}$) reached 57.1% in 2018 while the 67G-(T) ($P = 7 \times 10^{-40}$) was found at 50% at the same time point. A transversion was found to occur in codons S120I and E130D with frequencies that ranged from 7.8% to 15.3% for 130D ($P = 3 \times 10^{-9}$) from 2016/17 to 2018 respectively. The allele 120I ($P = 1 \times 10^{-10}$) had stable frequency (23%) over time. A transition was recorded in codon 188, with mutant 188K ($P = 4 \times 10^{-16}$) frequency at 36.3% in 2016/17 reaching 45.4% in 2018. The six mutants aforementioned have been reported in Kilifi isolates (Coastal Kenya) at high frequencies in the 2017/18 period (65Q (80%), 62N (20%), 67G (80%), 120I (32%), 130D (22%), and 188K (5.9%)) in a study conducted [16]. Interestingly, the author found a temporal trend in codon K65Q Post-ACT introduction, with decline in the frequency of wild type allele K65. This correlates with our results. We further stretched the research by screening PlasmoDB database to check whether the SNPs recorded have been annotated, and we found that the following five mutant mutants (65Q, 62N, 67G, 120I, and 130D) were reported in the database, strengthening the evidence of our findings. In addition, other SNPs codons were also recovered, although not previously reported, they were found with strong $P$-values, among which mutants in the following codons (D150N, T155P, R160K, D163N, R195Q) showed similar frequencies (Figure 1), ranging from 45.4% in 2016/17 to 54.5% in 2018. Mutant R146K, and R179K, had frequencies of 45.4%, in 2016/17, and 179K was stable across the 2018 time point (45.4%) while a decrease was observed in 146K frequencies (36.3%) in 2018. The 146K-allele has not been reported in previous studies, therefore we cannot describe it as resistant mutation. However, the oscillation (decrease/increase) in alleles’ frequency naturally occurs and this results from genetic drift. Genetic drift is a non-directional change in allele frequency that occurs by chance between generations by decreasing or increasing a certain allele’s frequency, but it does not result from the accommodation of individuals to the environment since the affected allele may be beneficial or harmful allele [44]. Therefore the decline observed in 145K-allele in the 2018 period as well the oscillation observed in all the other alleles that were not previously reported to be associated with drug resistance may be a result of genetic drift. D271N showed no change in mutant allele frequency (33.3%) across all time points. Three synonymous polymorphisms occurred at codons K164K, A194A and K238K with frequencies (45.4%, 27.2%, and 50%) respectively in 2016/17 whereas 164K, and 194A increased (54.5%, 45.4%) respectively in 2018. A decline in 238k frequency was observed, 30% in 2018 time point. There was significant trend in SNPs mutant’s alleles in both time points ($X^2 = 50.32, P < 0.0001$) in 2016/17, and ($X^2 = 61.90, P < 0.0001$) in 2018. The correlation between mutants in both periods was highly positive and statistically significant ($r = 0.8652, P < 0.0001$). Transversion was found to occur in the following five codons (Y148F, N203K, T229T, D279H,
and Q305L). Variants 148F, 203K, and 306N (codon D306N) showed stable frequencies in all-time points (18.1%, 9%, 20%) respectively. Mutation 229T was absent in 2016/17 time point and observed at frequency of 10% in 2018 while 279H and 305L had frequencies (11.1% and 20%) respectively in 2016/17 but not observed in the 2018 period (Table 3). The frequency of wild type and mutants’ alleles in the \textit{Pfcysteine desulfurase} gene is shown in (Figure S2).

### 4.3. Point Mutations Detected in \textit{Pfpm2} Gene

\textit{Pmp2} was the less mutational gene with only six point mutations (H79N, R188T, V260L, D261H, E309L, and D322E) identified in the target region (Table 4). The variants 260L, 309L, and 322E were absent in the 2016/17 period, and observed at 20% (309L, and 322E) in 2018, while 260L had frequency at 10%. Both alleles 188T and 261H were found at frequencies 7.7% and 10% respectively in 2016/17 but not observed in the 2018 period. Mutant 79N was found to increase frequency, 11.1% in 2016/17 and 33.3% in 2018. No significant temporal trend was observed in 2016/17 ($X^2 = 0.12$, $P = 0.7186$), while trend was significant in 2018 ($X^2 = 4.96$, $P = 0.0258$). Markedly low and negligible positive correlation was observed between mutants ($r = 0.00$, $P = 0.999$). The mutants’ alleles’ frequency is reported in (Figure 2). The wild-type and mutants’ alleles’ frequencies are reported in (Figure S3).

### 5. Discussion

\textit{Pf.coronin} is one of the members of the WD40-propeller residues protein family [26] [45]. It is an actin filament-binding protein, sharing structural features, (the \textit{\beta}-propeller domain) with \textit{PfKelch13} protein [26] [46]. \textit{Pf.coronin} protein is conserved, and expressed in late schizogony, further localized to the merozoite’s periphery during the invasion [46]. The protein is known to be involved in multiple functions including the F-actin binding and its organization, cytoskeletal motility and, vesicular transport [26] [45] [46] [47]. Damas et al., have discovered a novel function \textit{Pfcoronin} which therefore involved coronin protein in drug resistance making it a putative gene for tracking resistant mutations [26]. The author reported that the artemisinin resistance mutation (R100K and G50E) appeared in \textit{Pfcoronin gene} at 400 nM concentration of dihydroartemisinin (DHA) after pulsing intermittently the parasite with DHA (the active metabolite artemisinin) over 4 years period. The long-term use of antimalarial drugs exerts pressure on the parasite population. \textit{Plasmodium falciparum} developed the ability to stand drug pressure which allows this parasite to survive under treatment. Adoption of resistance fitness mostly results from the resistant mutations due to drug pressure, for instance, mutation C580Y has been reported to be associated with artemisinin resistance in Cambodia [21]. In Kenya, malaria parasites are exposed to artemether-lumefantrine and dihydroartemisinin-piperaquine pressure. Mutation 100K is associated with reduced artemisinin susceptibility. Although a decline in the frequency of drug selection alleles may be observed in...
some cases, however, the frequency of drug selection alleles increases under drug pressure over time and decreases after withdrawal or discontinuation of the drug responsible for the pressure \[48\], therefore, the temporal increase in the frequency of artemisinin selection allele 100k may be attributed to the continuous use of artemether-lumefantrine and dihydroartemisinin-piperaquine regimen. We also noticed an increase in the frequencies of mutants 96N, 95K, 59K, 57K, and 47F suggesting that these alleles may be under ACT drug pressure. Importantly, care must be taken in the surveillance of alleles 100k, and 50E, two of the alleles recovered by Damas et al. \[26\] to confer reduced artemisinin susceptibility. No variants were recovered in codon I68K, Q61P, and G50E, in 2017 time point, while mutants were observed in 2018, indicating that, pressure in these alleles has started in 2018. For further analysis, we used InterPro database, SMART tool, and NCBI CD-Search, and we found in a conserved domain (WD40 repeats), five successive predicted WD40-repeats motifs covering a region of 199aa, in which the following mutations (E95K, D96N, R100K, S149S, K71K, S75S, S81S, K125K, K159K, E158E, A161P, K168K, G179G, and S183G) were found to occur in WD40 motifs (Figure S4). Interestingly, these findings correlate with the results of the selection mutations reported by Dams, et al., \[26\] which were also discovered in WD40 motifs. G50E, N60N, E59K, R57K, Q61P, E156K, and I68K were localized outside WD repeats motifs, but took part of the WD40 domain. Only V47F was found outside the domain. Interestingly, WD40 repeats (repetitive tandem residues, containing tryptophan (W)-aspartic acid (D) at the C-terminus while having glycine-(G) and histidine-(H) at the N terminus), are conserved domains that contain a β-propeller structure providing a scaffold for protein-protein and protein-DNA interaction and are known to involve in multiples functions viz, signal transduction, regulation of transcription.
Mutations in these motifs are of crucial importance as they may impair drugs effect pathways. Variants, 125K, 75S, 71K, 168K, 81S, 60N, 149S, 179G, 150E, and 159K, are synonymous mutants. Although synonymous mutations are thought to be less harmful, they can affect the stability of the stem-loops, helices

Lumefantrine selection marker, K65 was previously reported in Kilifi isolates together with its neighbour’s mutants 62N, and 67G [16]. Mutants, 65Q, 62N, and 67G in addition to 120I and 130D were identified to increase frequencies in Kilifi (65Q (80%), 62N (20%), 67G (80% - 80%), 120I (32%), 130D (22%), and 188K (5.9%)) in 2017/18 period [16]. Consistently with our findings, an increase in the frequency of these alleles is observed in the present study although slightly lower in mutants 65Q (57.1%), 67G (57.1% - 50%), 120I (23%), 130D (15.3%), compared to the findings in Kilifi field. The author also reported a decline in the frequency of wild type K65 (from 38% to 20%) in Kilifi isolates [16], this supports our results, as we found K65 at low frequency (14.2%) in Mbita isolates. Conversely, mutant 62N (50%), and 188K (45.4%) frequencies are higher than found in Coastal Kenya. This irregularity may be due to the differences in drug pressure in both settings. The increase in frequencies observed in mutant’s alleles may be a result of Arthemetr-lumefantrine drug pressure as observed in Pícronin mutants. Conserved domain search using NCBI-CDD and InterPro revealed a conserved sequence (Aminotran_5 domain) in Pfnfs protein, composed of 367aa (Figure S5), localized within ATT-I region, a region of 387aa. Excluding the mutations (R146K, S62N, K65Q, E67G, S120I, E130D), all the rest of the variants codons were found in the Aminotran_5 domain. 120I was found in a predicted disordered region made up of 23aa, the function of which includes protein modification, molecular assembly, molecular recognition, and serve as entropic chains [53].

By using SMART database, the predicted disorder region was found to span a low complexity motif of 20aa, and another low complexity motif was predicted downstream (14aa), in which occurred mutation N203K. These motifs play adhesive and structural roles, they transduce molecular movement, interact with phospholipid bilayers, modulate protein translation and act as frame-shift checkpoints [54]. From 2016/17 to 2018, the mutant 150N, 155P, 160K, 163N, 195Q, and 164K increased frequencies (9.1%), while 194A gained (18.2%). These alleles in addition to 238k, and 146K which decreased in frequencies (20%) and (9.1%) and also (179K, 271N, 148F, 203K, 306N) were not previously reported. Therefore we could not confirm their selection, though, the evolution their frequencies need to be monitored. 279H, 305L, were not observed in the 2018 time point indicating the restoration of the wild-types. All these mutants were localized in protein conserved features except the ones aforementioned. Together, knowing the functions of Pf.cysteine desulfurase IscS (Iron-sulfur cluster System), mutants reported in this study may have an impact on parasites and this may result in acquiring selective fitness. Cysteine desulfurase IscS is a member of
aspartate aminotransferase superfamily fold type I (AAT_I) and belongs to the class-V pyridoxal-phosphate-dependent aminotransferase family. The protein IscS is involved in the biosynthesis and delivery of iron-sulfur clusters to diverse metabolic pathways, such as Fe-S complex synthesis [31] [55]. IscS pathway is crucial for erythrocytic stage parasite development and is an inherent drug target [56]. Iron homeostasis is capital for erythrocytic stage parasite growth and is involved in quinolones drug mechanisms [31], lumefantrine drug family. In apicomplexans, proteins containing Fe-S cluster are modulated during parasites’ resistance, stress conditions and cell development [31] [56] [57]. They occupied important roles in gene expression and are also antimalarial drugs targets [58].

The remarkable observation we made in Pfpm2 point mutations is that all the variants discovered were transversion mutations, among which five mutants (188T, 260L, 261H, 309L, and 322E) were localized in an active site (aspartic-peptidase active site), predicted within peptidase A1 domain, a region makes up of 308aa, (Figure S6), found in the amino acid sequence of Pfpm2 protein. Only mutation H79N was localized outside the peptidase A1 domain. Aspartic-peptidase active site is involved in catalysis, and binding of substrates for cleavage [59] [60]. Transversions mutations largely impact gene expression and impair the amino acid sequence of proteins [61]. Pfpm 2 is a digestive vacuole enzyme of aspartic proteases family A1 and is a potential antimalarial drug target [62] [63] [64], expressed in the erythrocytic parasite stage as integral membrane protein. The enzyme is implicated in the digestion and host haemoglobin degradation, processing of cytoskeletal protein, host cell remodelling, and effector export [64] [65] and the amplification of its copy number has been to be associated with piperaquine resistance. Mutations found in the active site may alter the binding affinity of substrates, protein structure, functions, therefore may impart to parasites reduced sensitivity fitness to the drugs. Taking together, mutations found in the three genes are localized in critical regions of proteins in which they were identified, and the trend analysis was found to be significant in both Pfcoronin and Pfcysteine markers’ data with a positive correlation between time points. This gives importance to our findings, and the slight increase in mutant frequencies over time may be the result of ACT drugs pressure used in the study area. We detected several SNPs at increased frequencies that were not previously reported, this call for further study to lighten their role as selection markers or resistant point mutations.

6. Conclusion

The present study has identified two point mutations in Pfcoronin previously reported to be associated with reduced artemisinin susceptibility and also a marker of selection for lumefantrine in Pfcysteine desulfurase. The results call attention to continuous surveillance of P.falciparum molecular markers associated with drug resistance in the study setting known for high transmission of malaria. To our knowledge, artemether-lumefantrine, as well as dihydroartemisinin-piperaquine,
are still efficacious in western Kenya, and no clinical failures have yet been reported. Despite this, parasite’s genetic monitoring will be important in informing about prospective resistance. We have also identified several other SNPs markers that were first reported in this study, but more remains to be known about their impacts. Close study of these mutants and the corresponding genes through gene expression analysis, linkage disequilibrium and the mechanism by which the three genes might confer resistance will be useful in tracking resistance.

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Authors’ Contributions

V.A.M., J.K.H., J.K. supervised the work; H.D., E.E.M., V.A.M. P.O.O. designed the experiments; H.D., E.E.M. performed the experiments; J.K.H. provided samples; H.D., E.E.M., R.M.I. V.A.M. were involved in the data analysis; H.D. wrote the manuscript; H.D., V.A.M., J.K.H., J.K., E.E.M. and P.O.O. read, edited, reviewed and interpreted the final manuscript.

Accessibility of Data and Material

Data are available upon reasonable request. Correspondence, H.D.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental Data File

**Figure S1.** Frequency of wild type and mutant alleles in the *P*.coronin from 2016/17 and 2018 time point. The X-axis represents the polymorphic codons that carry the mutations.

**Figure S2.** Frequency of wild type and mutant alleles in *P*.cysteine desulfurase gene from 2016/17 to 2018 time point. The X-axis represents the polymorphic codons that carry the mutations.

**Figure S3.** Frequency of wild type and mutant alleles in *P*.plasmepsin 2 gene from 2016/17 to 2018 time point. The X-axis represents the polymorphic codons that carry the mutations.
Figure S4. The protein sequence of *Pfcoronin* (ID: XP_001350896.1) with point mutations indicated. WD40 domain starts from 50 to 249, underlined (Gray). This domain covers WD40-1 motif (Red dash), WD40-2 motif (Short dot), and WD40-4 motif (Turquoise). Yellow = SNPs mutations. Pink = SNVs mutations.

Figure S5. Protein sequence of *Pf cytelines* (ID: XP_001349169.1) with point mutations indicated. Aminotran_5 domain starts from 148 to 535, underlined (Gray), and localized within AAT-I region (from 148 to 535). The domain spans a disordered sequence (Turquoise). Green = low complexity motif. Yellow = SNPs mutations. Pink = SNVs mutations.
**Figure S6.** Protein sequence of *Pf plasmepsin 2* (ID: XP_001348250.1) with point mutations indicated. **Gray** = Peptidase A1 domain (140 - 448), **Green** = Aspartic-peptidase active site (154 - 345), **Pink** = SNVs mutations.