PREVALENCE OF PLASMODIUM FALCIPARUM ANTIMALARIAL DRUGS RESISTANCE GENETIC MARKERS IN SELECTED LAKE VICTORIA ISLANDS, WESTERN KENYA

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Prevalence of *Plasmodium falciparum* Antimalarial Drugs Resistance Genetic Markers in Selected Lake Victoria Islands, Western Kenya.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature………………………………………. Date…………………………….

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

This thesis is dedicated to my entire family of Mr. Pithon Kihuga and Tabitha Waweru and friends who inspired me to complete this work.

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ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin-based Combination Therapies
AMOVA	Analysis of Molecular Variance
ART	Artemisinin
BAM	Binary Alignment/Map Format
BQSR	Base Quality Score Recalibration
BWA	Burrows-Wheeler Alignment tool
CQ	Chloroquine
DNA	Deoxyribonucleic Acid
GATK	Genome Analysis Tool kit
HF	Halofantrine
IPTp	Intermittent Preventive Treatment of malaria during pregnacy
MEGA	Molecular Evolutionary Genetics Analysis
MQ	Mefloquine
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* Chloroquine Resistance Transporter
Pfdhfr	*Plasmodium falciparum* Dihydrofolate Reductase
Pfdhps	*Plasmodium falciparum* Dihydropteroate Synthase

Pfmdr1	*Plasmodium falciparum* Multidrug Resistant-1
QN	Quinine
SAM	Sequence Alignment/Map format
SEA	Southeast Asia
SNP	Single Nucleotide Polymorphisms
SP	Sulfadoxine Pyrimethamine
VCF	Variant Calling Format
WGS	Whole Genome Sequencing
WHO	World Health Organization

ABSTRACT

Plasmodium falciparum multidrug resistance has been linked to Single Nucleotide Polymorphisms (SNPs) on chloroquine resistance transporter (*Pfcrt*), multidrug resistant-1 (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*), and dihydropteroate synthase (*Pfdhps*) genes. Delayed parasite clearance against Artemisinin-based Combination Therapies (ACTs) has been linked to the Kelch-13 (*Pf-k13*) gene SNPs. The global health burden due to this resistance translates to approximately 229 million cases and over 400,000 deaths annually, with significant morbidity, economic impact This cross-sectional study analyzed isolates from Lake Victoria islands, Kenya, a malaria-endemic region, providing baseline information on the prevalence of SNPs in the five genes associated with drug-resistant parasites. Fifty-nine whole blood samples were collected from symptomatic malaria patients visiting dispensaries in five selected islands on Lake Victoria, Kenya between 2014 and 2016. DNA was extracted and quality checks performed.Whole-genome sequencing of the isolates was then performed on the Illumina platform. Reads were analyzed for antimalarial resistance-associated SNPs via a pipeline based on the Genome Analysis Tool kit (GATK) practices and data compared with isolates from West Africa and East Africa parasite populations derived from MalariaGEN repository. Out of twenty-seven isolates that passed quality checks, it was observed that all isolates harbored the *Pf-k13* wildtypes for all resistance validated markers. K76T mutation of *Pfcrt* was observed at 18.51%, n=5, *Pfmdr1 Y*184F (48.15%,n=13), D1246Y (7.40%, n=2), *Pfdhps* K540E (88.89%,n=24), *Pfdhfr* N51I (96.30%,n=26), C59R (77.78%,n=21) and S108N (96.30%, n=26). *Pfcrt* haplotype CVMNK was observed in 81.48%(n=22) of isolates, *Pfdhfr* haplotype IRN (70.37%, n=19), and *Pfdhps* SAEAA (74.01%, n=20). This study provides baseline data for subsequent surveillance studies on the prevalence of antimalarial resistance markers. This study revealed a low circulation of chloroquine (CQ) and artemisinin-resistant associated alleles, and a high prevalence of polymorphisms linked to SP resistance. Similar trends of prevalence were also observed in West Africa and East Africa parasite populations. Surveillance studies are recommended to monitor resistance in the region as well as temporal trends of these polymorphisms.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria remains a significant global cause of morbidity and mortality, with countries in Sub-Saharan Africa accounting for 90% of all cases and deaths(WHO, 2021). Despite interventions and strategies by the World Health Organization (WHO) to combat this scourge, in the absence of an effective vaccine, the biggest challenge remains the emergence of resistance against antimalarial drugs(Usoro *et al.*, 2024; WHO, 2023). Over the last decades, several antimalarial drugs have been used as the first-line treatment for malaria. However, emergence of antimalarial drugs resistant *P. falciparum* strains has rendered majority of these drugs ineffective in treating malaria(Humphreys *et al*., 2007). For instance, chloroquine, previously used to treat uncomplicated malaria, was discontinued in most African countries due to increased resistance. Over the last decade, antimalarial drugs such as amodiaquine (AQ), mefloquine (MQ), quinine (QN), and halofantrine (HF) have been reported to have reduced efficacy in treating *P. falciparum* malaria and in some cases, complete resistance has been recorded(Baraka *et al*., 2018; Moyeh *et al*., 2018; Tyagi *et al*., 2018). The combination of Sulfadoxine and Pyrimethamine (SP) has continued to be used as Intermittent preventative treatment (IPT) of *P. falciparum* malaria in pregnant women and infants less than five years (WHO, 2023) because it is well tolerated and has minimal side effects. However, there are concerns regarding its effectiveness in preventing malaria in pregnancy due to the emergence of SP-resistant *P. falciparum* parasite strains, leading to its substitution with other drugs, such as Artemisinin-based Combination Therapy (ACTs). ACTs are recommended as the first line treatment drugs in managing uncomplicated malaria (Kamau *et al*., 2015; WHO, 2016). However, there have been reports of the emergence and spread of *P. falciparum* parasite populations resistant to artemisinin (ART) from Southeast Asia (SEA) and Africa(Hassett & Roepe, 2019), jeopardizing the effectiveness of ACTs for the treatment of uncomplicated malaria.

Resistance toward these antimalarial drugs is attributed to *P. falciparum* genetic mutations. Polymorphisms correlating to resistance are Single Nucleotide Polymorphisms (SNPs)s, Copy Number Variations, and haplotypes of specific genes (Lucchi *et al*., 2015). SNPs at codon K76 of *P. falciparum* Chloroquine-resistant transporter gene (*Pfcrt*) on chromosome 7 mediates resistance against chloroquine. The substitution of lysine (K) with threonine (T) results in a mutant protein which is believed to act as an exporter protein, transporting CQ out of the parasitophorous vacuole compromising its mechanism of action (Mwai *et al*., 2009; Sidhu *et al*., 2005). *In vitro*, studies have also shown that *Pfcrt* mutations also influence *P. falciparum* susceptibility to quinine, halofantrine, and artemisinin (Humphreys *et al*., 2007)

The *P. falciparum* multidrug resistance-1 gene (*Pfmdr*-1) mutations are attributed to resistance toward multiple antimalarial drugs. The precise mechanisms by which *Pfmdr*1 contributes to multidrug resistance have not yet been fully elucidated(Shafik *et al.*, 2022). However, it is understood that polymorphisms in *Pfmdr*-1 and *Pfcrt* that cause chloroquine resistance simultaneously increase the parasite's susceptibility to lumefantrine and mefloquine a phenomenon known as collateral drug sensitivity(Shafik *et al.*, 2022). Polymorphisms at codons N86Y, Y184F, and D1246Y are linked to reduced parasite clearance and susceptibility to Chloroquine (CQ), mefloquine (MQ), Quinine (QN), and artemisinin(Baraka *et al*., 2018; Moyeh *et al*., 2018; Tyagi *et al*., 2018), while studies on variations in *Pfmdr1* copy number have demonstrated treatment failure of artesunate and mefloquine (Lim *et al*., 2009).

Polymorphisms in the *P. falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes confer resistance against Sulphadoxine Pyrimethamine (SP). Mutations at codons *dhfr*-51, *dhfr*-59, *dhfr*-108, *dhps*-437, and *dhps*-540 have been associated with SP treatment failure in pregnant women and infants (Alker *et al*., 2008; Tahita *et al*., 2015). Studies have found these polymorphisms in various regions of Africa, including Nigeria and Tanzania. In Nigeria, 80.8% of parasites exhibited dhfr gene polymorphisms, with the dhfr triple mutant I51R59N108 being the most common(Kayode *et al*., 2021). In Tanzania, nearly all samples carried the wild-type N86 in the P. falciparum multidrug

resistance 1 (*Pfmdr*1) gene, which is associated with reduced susceptibility to lumefantrine(Bakari *et al.*, 2024). However, increases in Y184F mutations and NFD haplotype were observed between 2016 and 2021. The quintuple and sextuple mutated genotypes are highly prevalent in East Africa(Tuedom *et al.*, 2021).

Pf-k13 gene encodes a putative kelch protein associated with delayed clearance of the *P. falciparum* parasite and reduced susceptibility to ACTs. Studies in SEA have associated non-synonymous mutations on the C-terminus end of K-13 protein at codons Y493H, R539H, I543T, and C580Y with higher ring-stage parasite survival rates as compared with the wildtype (Ariey *et al.*, 2014). In Africa, a mutation at codon A578S and K189T has been recorded but not yet linked to artemisinin resistance(Kamau *et al.*, 2015). Additionally, several other mutations in the *Pf-k13* gene have been identified. Specifically, five validated *Pf-k13* partial resistance markers have been found: R561H in Rwanda and Tanzania, M476I in Tanzania, F446I in Mali, C580Y in Ghana, and P553L in an Angolan isolate(Owoloye *et al.*, 2021). However, these mutations are present at low frequencies and there have been no reports of clinical treatment failure, except for Rwanda(Ndwiga *et al.*, 2021a).

Monitoring molecular markers for antimalarial resistance is essential in tracking the emergence and spread of resistant genes in malaria-endemic areas. Therefore, this study explored the prevalence of antimalarial drugs resistance markers in *Pfcrt*, *Pfmdr1*, *Pfdhfr*, *Pfdhps,* and *Pf-k13* genes in isolates collected from four islands on Lake Victoria (Mfangano, Takawiri, Kibuogi, and Ngodhe) a coastal mainland (Ungoye) to provide baseline data for antimalarial resistance surveillance studies in the region. Further, the observed marker genes from Lake Victoria were compared against isolates from East Africa and West Africa parasite populations.

1.2 Problem Statement

Malaria continues to be a leading cause of mortality in developing nations, as reported by the WHO in 2023(WHO, 2024). In the absence of a potent vaccine, the primary strategy for managing and treating malaria relies on the use of antimalarial drugs. However, the effectiveness of these drugs is increasingly being undermined by

the emergence of drug-resistant strains of the parasite. Resistance to antimalarial drugs has been attributed to mutations in various genes. *Pfcrt* gene mutations have been linked to Chloroquine resistance, polymorphisms in *Pfmdr1* are linked to multidrug resistance, combined point mutations of *Pfdhfrs* and *Pfdhps* genes are linked to SP resistance while most recently mutations on *Pfk*-13 gene have been associated with ACTs resistance.

After Chloroquine resistance was established in 1999, Sulphadoxine-Pyrimethamine (SP) was adopted as the first choice for treating malaria until 2003, when cases of resistance were reported. This led to the introduction of ACTs as the first line of treatment for malaria. (Amin, 2007). However, evidence from various studies has demonstrated that the *P. falciparum* parasite population on the Thai Cambodian border, a historical epicenter for multidrug resistance spread spanning over 50 years, has developed resistance against ACTs and other artemether combinations. Delayed clearance is associated with a heightened risk of gametocytaemia, presumably with a potential for increased transmissibility of drug-resistant parasites(Sowunmi *et al*., 2010). Recent studies in sub-Saharan Africa, including Kenya, identified two key non-synonymous SNPs (A578S and V561I) on the *Pf-k13* gene associated with ACTs resistance(Jeangn *et al*., 2024; Maniga *et al*., 2023). These two SNPs are significant due to their proximity to the C580Y mutation, a key SNP determinant for artemisinin resistance in Southeast Asia. The A578S prevalence currently is at 2.7% in Western Kenya (Kamau, Campino, Amenga-Etego, Drury, Ishengoma, Johnson, Mumba, Kekre, Yavo, Kwiatkowski, *et al*., 2015)*et alet al*A study conducted in Msabweni, coastal Kenya, revealed a diminished prevalence of *Pfcrt* 76T resistance marker 13 years after CQ withdrawal(Kiarie *et al*., 2015). Different studies show that the *Pfap2mu* variant alleles are more prevalent following AL post-treatment and could be a novel candidate gene for tracking artemisinin resistance in Africa(Henriques *et al*., 2014). Therefore, it is critical to study the prevalence of these resistance markers for surveillance and monitoring of resistance patterns of *Plasmodium falciparum.*

1.3 Justification

The management, treatment and control of malaria is anchored on the wide-scale deployment of malaria control interventions laid out by WHO which includes treatment with the available antimalarial drugs(WHO, 2024). However, the emerging resistance towards these drugs threatens the emergence of endemic-resistant malaria if surveillance and monitoring are not done promptly.

Molecular markers are invaluable in tracking antimalarial drug resistance, offering a precise and swift means to pinpoint genetic changes linked to resistance. These markers, typically specific genes or single nucleotide polymorphisms (SNPs), are instrumental in identifying resistance to a range of antimalarial medications, including chloroquine, sulfadoxine-pyrimethamine, and artemisinin combinations. Through the surveillance of these markers' prevalence and patterns in areas plagued by malaria, health officials can gauge the rise and dissemination of resistant strains of the parasite, thus facilitating prompt modifications to therapeutic approaches. Moreover, these markers are crucial for distinguishing actual treatment failures from subsequent infections, ensuring that measures to counteract resistance are effectively implemented. The tangible outcomes of such studies include enhanced treatment protocols, reduced incidence of drug-resistant malaria cases, and improved patient outcomes in regions affected by malaria.Thus, by determining the prevalence of molecular markers associated with *P. falciparum* resistance in different regions, the information can be used to monitor resistance spread and help develop policies and strategies to combat it(Gadalla *et al*., 2015; Musyoka *et al*., 2020).

1.4 Objectives

1.4.1 General Objective

To determine the prevalence of *Plasmodium falciparum* genetic polymorphisms associated with antimalarial resistance on selected malaria endemic islands in Lake Victoria, western Kenya.

1.4.2 Specific Objectives

1. To determine the prevalence of antimalarial drug-associated polymorphisms in *Plasmodium falciparum*, *Pfcrt*, *Pfmdr1*, *Pfdhfr, Pfdhps,* and *Pf-k13* genes.

2. To compare the prevalence the identified anti-malarial drug associated polymorphisms using parasite populations in other African malaria-endemic regions.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Malaria remains one of the most severe life-threatening protozoan diseases, classically identified by paroxysm, fever, and flu-like symptoms recurring in 48-h or 72-h cycles . It is caused by parasites of the Plasmodium genus transmitted by the Anopheles mosquito vector. The predominant species of this genus that caused malaria in humans are *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium falciparum*, *Plasmodium malariae,* and the simian *Plasmodium knowleski.* The most lethal and thus vastly studied species is *Plasmodium falciparum,* which is responsible for most malaria deaths globally(WHO, 2023).

2.2 Geographical Distribution of Malaria

Globally, malaria's geographical distribution is influenced by several factors. The most affected areas are typically warm and wet climates, which are conducive to the transmission of malaria through the bites of infected mosquitoes during their blood meals. This transmission is further affected by the behavior and ecology of mosquitoes, which in turn are influenced by human activities, rainfall patterns, soil types, and population distribution(Bejon *et al*., 2014).

The highest rates of transmission are found in tropical regions of sub-Saharan Africa, India, and Southeast Asia **(Figure 2.1).** These areas are marked by a high moisture index and elevated temperatures, which shorten the breeding and incubation period of the parasite and increase the reproduction rate of the mosquito vector(Kar *et al*., 2014). Notable regions in Kenya that fall into this category include the Lake Victoria basin, Western Kenya, and the South Coast.

The spatial heterogeneity of malaria transmission makes controlling the disease challenging, yet it also presents an opportunity to focus control efforts on

transmission hotspots. Targeting these specific areas has been predicted to be highly effective in combating malaria(Baidjoe *et al*., 2016; G. Zhou *et al*., 2024).

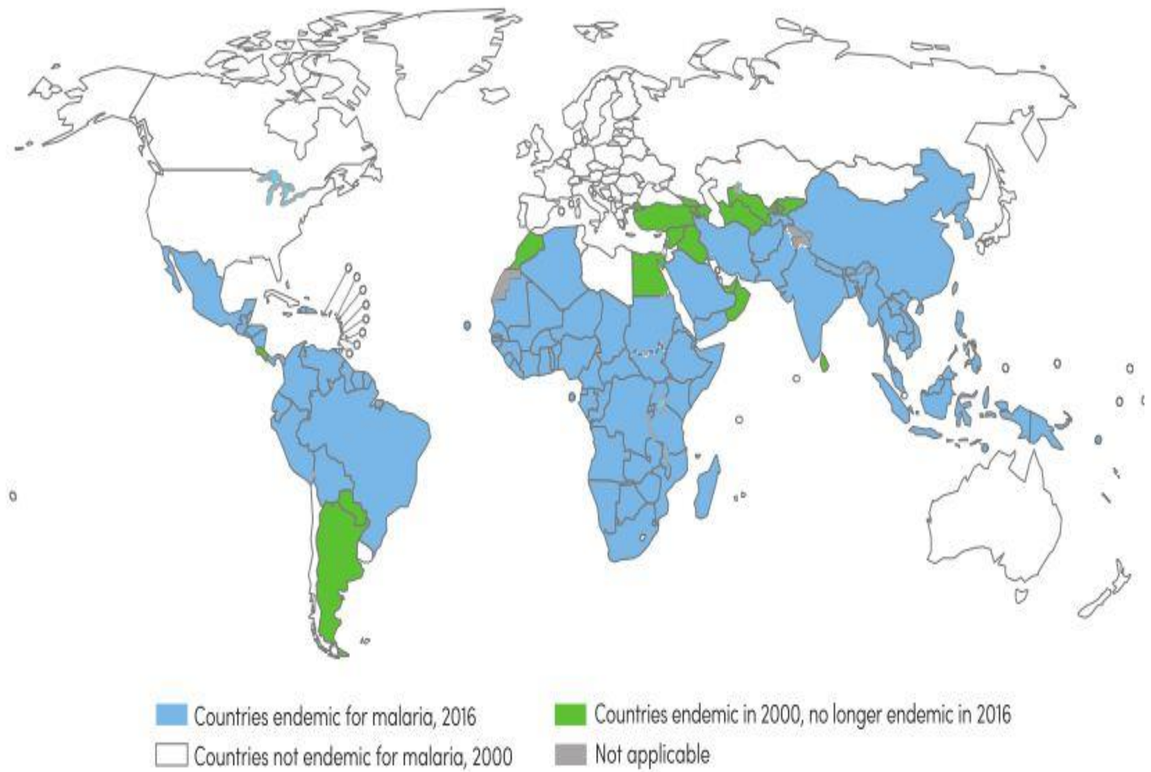

Figure 2.1:The geographical distribution of malaria (WHO, 2016)

2.3 Parasite Lifecycle

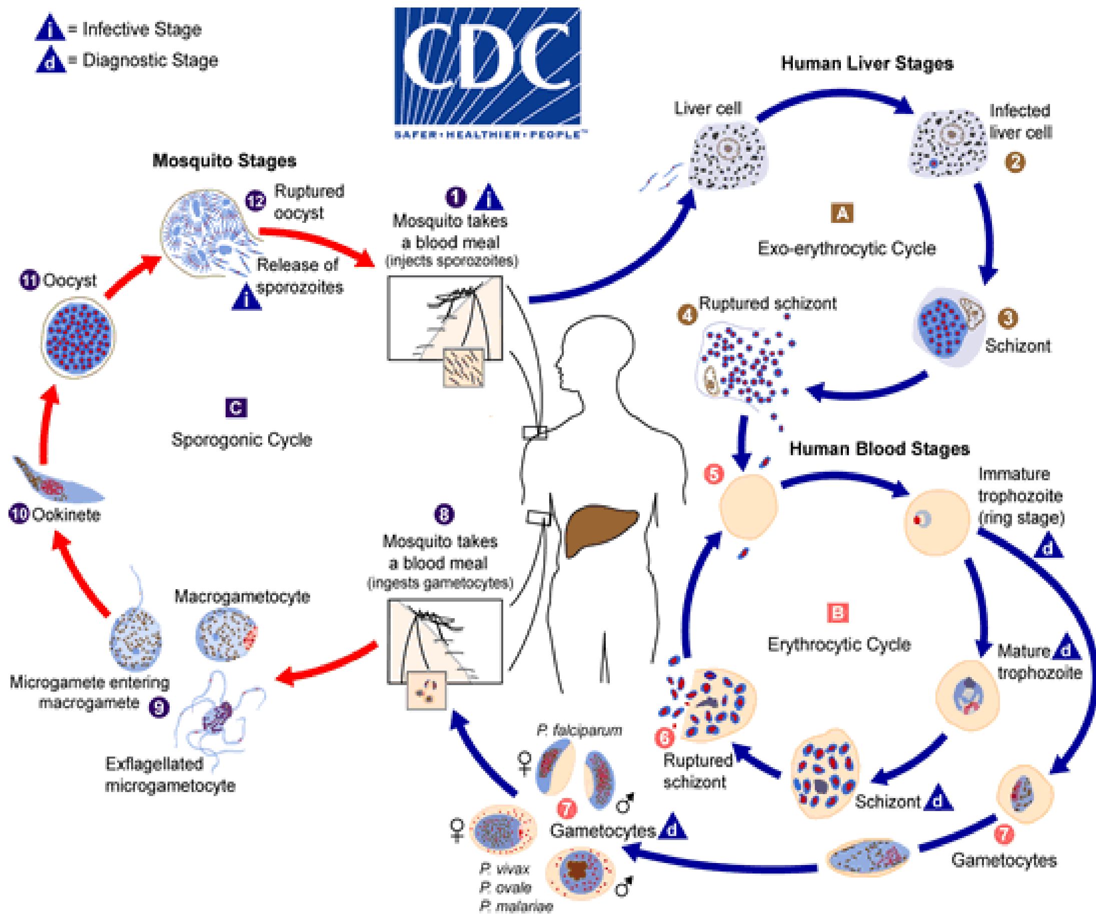

Figure 2.2: The Complete Lifecycle of the *P. Falciparum* Parasite (CDC, 2020)

The life cycle of *P. falciparum* is usually classified into three stages: (a) Exo-erythrocytic Cycle, (b) Erythrocytic Cycle, and (c) Sporogonic Cycle **(Figure 2.2).** It has a complex lifecycle that involves two hosts to complete. Sexual replication occurs in the human host erythrocytes, while asexual reproduction occurs in the mosquito vector (Kathleen et al., 2015) Human malaria transmissions are facilitated by the female mosquito of the genus *Anopheles* which occurs during the mosquito blood meal. Four species cause human malaria: *P. falciparum*, *P. vivax*, *P. ovale,* and *P. malariae,* while *P. knowlesi* causes the simian malaria*.* *P. falciparum* is the most lethal species due to its ability to inhabit blood vessels and evade the immune system (Kathleen et al., 2015).

The malaria parasite is introduced into the human host via the bite of an infected Anopheles mosquito while taking a blood meal, injecting a small number of sporozoites into the skin. Through the bloodstream, the sporozoites travel to the liver, where they invade the hepatocytes and mature into schizonts. The schizonts replicate asexually, where after about seven days, each infected hepatocyte ruptures releasing merozoites into the bloodstream. Once released into the bloodstream, the merozoites quickly recognize and invade the erythrocytes, initiating a repeated asexual replication cycle. The parasite progresses through the "ring stages" and trophozoites stages before replicating into daughter merozoites at the schizont stage. (schizogony) (CDC, 2020).

At this stage, the red blood cells rupture, releasing merozoites into circulation and initiating another round of asexual replication. The mature asexual stages exhibiting trophozoites and schizonts bind to the vascular system in various organs, allowing them to avoid splenic clearance(Nilsson *et al*., 2015).After several cycles of asexual replication, a small subset of the parasite deviates into sexual progeny that differentiates into gametocytes. A further smaller subset of gametocytes exits the external vascular system and penetrate the bone marrow extracellular space, where they undergo a maturation stage known as gametocytogenesis. At the final stage, infection-competent gametocytes re-enter the circulation(Talman *et al*., 2004).

During a blood meal, the mosquito ingests male and female gametocytes, which later mature into gametes via the gametogenesis process. Within the mosquitos midgut, fertilization occurs where a microgamete (male ex-flagellated gametocyte) fuses with a macrogamete(female ex-flagellated gametocyte) to form a zygote and matures into an invasive ookinete through meiosis, that can penetrate the mosquito gut wall. The ookinete forms an oocyst where the *P.falciparum* asexually replicates into thousands of sporozoites (Liu *et al*., 2011). The oocyst ruptures after a while, releasing sporozoites, which migrate to the salivary glands and can be transmitted back to a human host via a blood meal. If the parasites transmitted are mutants, the resistance characteristic is also passed on to the infected human host. This way, resistance to antimalarial is passed on via new hosts. (Kathleen *et al.,* 2015).

2.4 Pathogenicity

Clinical manifestations of Plasmodium falciparum infection are triggered by the invasion of merozoites into red blood cells (RBCs). The pathogenicity of the disease is attributed to alterations in RBCs induced by the parasite and abnormalities in microcirculation, which are accompanied by both systemic and local immune responses, leading to varying clinical severity(Buffet *et al.*, 2011). Trophozoites, considered the primary agents of clinical manifestations, alter the adhesion and deformability of RBCs, promoting their sequestration in small vessels and preventing their circulation in the peripheral bloodstream. This sequestration is facilitated by the adherence of RBCs to endothelial cells, other blood cells, platelets, and uninfected RBCs, mediated by a variant *P. falciparum* adhesin, *P. falciparum* Erythrocyte Membrane Protein 1 (P*f*EMP1), encoded by the var multigene family(Buffet *et al.*, 2011). The vascular obstruction caused by various endothelial ligands, receptors, and the inflammatory response triggered by the parasite are key factors in *P. falciparum* pathogenesis. Events such as damage to RBCs, hepatocytes, endothelial cells, loss of endothelial integrity, initiation of cell destruction, and activation of cell death promoters contribute to organ damage, particularly in the brain and lungs, and the general acidotic state observed in malaria(Buffet *et al.*, 2011).Pregnant women are particularly susceptible to severe malaria infection and its related complications due to the physiological alterations that occur during pregnancy and changes in immune responses. The pathogenicity of malaria in pregnant individuals is driven by the placental sequestration of infected erythrocytes, immune dysregulation, and the subsequent release of inflammatory mediators(Rijken *et al.*, 2012). The placenta, during pregnancy, becomes a site for the sequestration of infected red blood cells (iRBCs), which plays a significant role in the pathogenesis of placental malaria. This sequestration process is facilitated by specific parasite adhesion molecules, such as VAR2CSA, which bind to receptors present on the placental tissue. This leads to inflammation, compromised placental function, and ultimately results in adverse pregnancy outcomes(Sharma and Shukla, 2017).Immune dysregulation is another crucial aspect of malaria pathogenicity in pregnancy. A study by Kwenti *et al*. (2021) highlighted that alterations in the immune response during pregnancy, including

changes in cytokine profiles and immune cell populations, can impact the susceptibility to malaria infection and disease severity(Kwenti *et al.*, 2017). Pregnant women exhibit a shift towards a Th2-biased immune response, which can dampen the pro-inflammatory immune response needed for parasite clearance, thereby increasing the risk of severe malaria(Feeney, 2020; Sánchez and Spencer, 2022).

Moreover, host genetic factors play a role in malaria pathogenicity during pregnancy. Polymorphisms in genes encoding immune-related molecules, such as human leukocyte antigen (HLA) genes, have been associated with variations in susceptibility to malaria infection and the severity of placental malaria(Tukwasibwe *et al.*, 2020). A study by Opi *et al.* (2021) demonstrated that certain HLA alleles are associated with an increased risk of placental malaria, indicating a genetic influence on the pathogenicity of malaria in pregnancy(Opi *et al.*, 2021).

2.5 Prevention and Treatment of Malaria

The WHO has recommended various strategies to prevent and reduce the risk of malaria infection in endemic and non-endemic regions. These strategies can be categorized into malaria prevention strategies and malaria treatment strategies.

2.5.1 Malaria Prevention

2.5.1.1 Vector Control

Vector control, a pivotal strategy in malaria prevention, targets the Anopheles mosquito population, which is responsible for the transmission of malaria parasites(WHO, 2024). This strategy encompasses interventions such as the use of insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), and larviciding. ITNs, in particular, are recognized as an exceptionally effective tool for vector control. Numerous studies have attested to their effectiveness in curtailing malaria transmission and safeguarding individuals from mosquito bites. A systematic review conducted by Pryce (2018) revealed that ITNs significantly diminished malaria infection rates and child mortality(Pryce *et al.*, 2018). Furthermore, a study by Bhatt *et al.* (2015) estimated that ITNs were instrumental in achieving a 68% reduction in

malaria cases in sub-Saharan Africa between 2000 and 2015(Bhatt *et al*., 2016). However, to optimize the impact of ITNs, it is crucial to address challenges such as insecticide resistance, low adherence to net usage, and human behavior concerning net usage.IRS involves spraying insecticides on the walls and surfaces of houses, targeting mosquitoes that rest indoors. Several studies have demonstrated the effectiveness of IRS in reducing malaria transmission. A review by Pluess *et al*. (2010) reported a significant reduction in malaria transmission and clinical cases with IRS implementation(Choi *et al.*, 2021). Similarly, a study in Zambia by Chanda *et al*. (2015) showed a substantial decline in malaria cases after the introduction of IRS. However, the emergence of insecticide resistance poses a challenge to the long-term effectiveness of this intervention, emphasizing the need for rotational use of insecticides and monitoring of resistance patterns.

Larviciding involves the application of larvicides to mosquito breeding sites, targeting mosquito larvae. While larviciding is less commonly implemented than ITNs and IRS, it has shown potential in reducing malaria transmission. A study by Fillinger *et al*. (2009) demonstrated that larviciding in urban areas effectively reduced malaria vector populations(Fillinger *et al*., 2009). Larviciding can be particularly valuable in areas with extensive breeding sites or in combination with other vector control interventions. However, operational challenges, cost-effectiveness, and sustainability must be considered when implementing larviciding programs.

2.5.1.2 Chemoprophylaxis

C Chemoprophylaxis, the use of antimalarial drugs to prevent infection, is a fundamental approach in malaria prevention. Its effectiveness in averting malaria in at-risk individuals has been demonstrated in various studies. A systematic review by Schlagenhauf *et al*. (2017) assessed the efficacy of different antimalarial drugs, reporting a notable decrease in malaria incidence among travelers and residents in malaria-endemic regions who took prophylactic medications(Sturrock, Novotny, *et al.*, 2013). The selection of antimalarial drugs for chemoprophylaxis hinges on multiple factors, including geographic location, patterns of drug resistance, and

individual characteristics such as age, pregnancy status, and medical history. Artemisinin-based combination therapies (ACTs) are typically recommended as the first-line chemoprophylaxis in areas with multidrug-resistant Plasmodium falciparum. Jauréguiberry *et al.* (2013) demonstrated the effectiveness of atovaquone-proguanil, doxycycline, and mefloquine as chemoprophylactic options in various malaria-endemic regions. For pregnant women in high-transmission areas, Sulphadoxine pyrimethamine (SP) is recommended for Intermittent Preventive Treatment (IPT), which aids in protecting against malaria and reducing the risk of pregnancy complications. While chemoprophylaxis is a potent malaria prevention strategy, its implementation faces several challenges. Adherence to the drug regimen is vital for its effectiveness, yet suboptimal adherence rates have been observed, particularly among travelers(Nayyar *et al.*, 2012). Additionally, the emergence of antimalarial drug resistance presents a significant challenge to chemoprophylaxis.

2.5.1.3 Malaria Elimination Strategies

Various malaria elimination strategies have been employed to achieve sustained and effective prevention of malaria transmission in malaria-endemic regions. These include Active Case Detection (ACD) and Mass Drug Administration (MDA). ACD involves actively identifying and treating individuals infected with malaria, including asymptomatic carriers, to interrupt transmission chains. Several studies have demonstrated the effectiveness of ACD in reducing malaria transmission. A systematic review by Sturrock *et al.* (2013) found that ACD combined with prompt treatment significantly reduced malaria prevalence in low-transmission settings(Sturrock, Novotny, *et al.*, 2013). Furthermore, this study showed that ACD, when integrated with other interventions, led to a substantial decline in malaria cases, supporting the importance of proactive case management.

MDA involves administering antimalarial drugs to an entire population, regardless of infection status, to clear existing infections and reduce the parasite reservoir. While MDA has successfully reduced malaria transmission in some settings, its effectiveness can vary depending on transmission intensity, drug efficacy, and population coverage. A study in Zanzibar by Stuck *et al.* (2020) reported a

significant reduction in malaria transmission after three rounds of MDA, highlighting the potential of this approach in specific contexts(Stuck *et al*., 2020). However, challenges such as drug resistance, logistical constraints, and community acceptance must be carefully addressed for successful implementation.

2.5.2 Malaria Treatment

2.5.2.1 Antimalarial Drugs

Effective treatment of malaria is crucial for reducing mortality and morbidity associated with the disease. Artemisinin-based combination therapies (ACTs) are the recommended first-line treatment for uncomplicated *Plasmodium falciparum* malaria. ACTs combine an artemisinin derivative with a partner drug to provide rapid symptom relief and reduce the risk of developing drug resistance. Numerous studies have demonstrated the high efficacy of ACTs in clearing malaria parasites and improving clinical outcomes. *et al*Meta-analysis studies have showed that ACTs achieved high cure rates and rapid parasite clearance in patients with uncomplicated falciparum malaria across multiple endemic regions(Maiga *et al*., 2021). However, the emergence and spread of artemisinin resistance in known malaria endemic regionspose a significant challenge to the long-term effectiveness of these drugs as the first line treatment strategy for uncomplicated malaria.

Quinoline-based antimalarials, such as chloroquine and hydroxychloroquine, were once widely used as first-line treatment for uncomplicated malaria. However, widespread resistance to these drugs, particularly in *Plasmodium falciparum*, led to their decreased effectiveness in many endemic regions. Despite the reduced efficacy, these drugs continue to play a role in treating non-falciparum malaria and as a component of combination therapies in areas with low levels of drug resistance(Foley and Tilley, 1997; van Schalkwyk *et al*., 2019).

Various antimalarial drugs treat malaria, either as monotherapy or in combination with other drugs. These include sulfadoxine-pyrimethamine, mefloquine, atovaquone-proguanil, and lumefantrine. Their effectiveness varies depending on the specific malaria parasite species and the local drug resistance patterns. For instance,

sulfadoxine-pyrimethamine has been used for intermittent preventive treatment in pregnancy and seasonal malaria chemoprevention programs. Studies have shown its efficacy in reducing the burden of malaria in pregnant women and young children in certain settings(Figueroa-romero and Pons-duran, 2022).

Despite the availability of effective antimalarial drugs, several challenges are associated with their use. Drug resistance is a major concern, particularly with artemisinin-based therapies, and efforts to monitor and contain resistance are essential. Adherence to treatment regimens and access to quality-assured medications remain crucial for successful malaria treatment outcomes(Banek *et al.*, 2021). Additionally, special considerations are needed for specific populations, such as pregnant women and children, who may require alternative or adjusted dosing regimens.

2.5.2.2 Diagnosis and Monitoring

Accurate diagnosis and effective monitoring of malaria play a critical role in successfully treating and managing the disease. Microscopy and RDTs are the primary diagnostic tools for malaria detection in resource-limited settings. Microscopy involves the examination of blood smears under a microscope to identify and quantify malaria parasites. RDTs are rapid, point-of-care tests that detect specific malaria antigens in the blood. Several studies have compared the accuracy and performance of microscopy and RDTs, with RDTs generally showing high sensitivity and specificity comparable to microscopy. A systematic review by Wongsrichanalai *et al*. (2007) demonstrated the overall high diagnostic accuracy of RDTs, making them a valuable tool for malaria diagnosis, especially in areas with limited laboratory infrastructure(Wongsrichanalai *et al.*, 2007). However, microscopy requires well-trained personnel, quality assurance, and infrastructure, which may be lacking in resource-constrained settings. RDTs, while more accessible, have limitations, including the potential for false-positive or false-negative results and shorter shelf life (WHO, 2023).

Monitoring treatment response is crucial for assessing the effectiveness of antimalarial drugs and identifying treatment failures. Various parameters, such as clinical symptoms, parasite clearance rates, and molecular markers of drug resistance, are used to monitor treatment response. The World Health Organization (WHO) recommends using molecular markers, such as genotyping of *Plasmodium falciparum* parasites, to differentiate treatment failure due to recrudescence (persistent infection) from new infections. A study by Ashley *et al*. (2014) demonstrated the utility of molecular markers in distinguishing between treatment failures and new infections, providing valuable information for refining treatment policies and combating drug resistance (Ashley *et al*.,, 2015).

2.6 Parasite Drug Resistance

Drug resistance in parasites can be characterized as the ability of the parasite to withstand increasing concentrations of a drug over time, eventually leading to a lack of response to the drug at therapeutic doses(Usoro *et al*., 2024). Specifically, resistance to antimalarial drugs in Plasmodium falciparum is defined as the capacity of the parasite to survive and replicate despite the administration of an antimalarial drug. This resistance often manifests as a resurgence of the parasite days after an apparent reduction in parasitemia in patients (Sutherland *et al*., 2017).

Given that malaria treatment relies heavily on chemoprophylaxis, the emergence of drug resistance in the parasite poses a significant threat to future malaria control and management efforts. Chloroquine (CQ), an antimalarial drug that was widely used for many years, was discontinued in 1998 due to the emergence of resistance. Recent studies have also reported instances of resistance to Artemisinin-based Combination Therapies (ACTs)(Caroline *et al*., 2023; Fola *et al*., 2023; Grossman *et al*., 2023).

Resistance to ACTs and other artemether combination drugs is particularly concerning for malaria control efforts, as ACTs are currently the first-line treatment for malaria worldwide. The resistance to CQ has been attributed to mutations in the *Pfcrt*-codon K76T and *Pfmdr*1- codons N86Y, N1042D, and D1246Y, which result

in the efflux of CQ from the parasite's food vacuole, thereby significantly reducing the effectiveness of CQ.

2.7 Factors Contributing to Antimalarial Drug Resistance.

2.7.1 Genetics

Mutations in the *Plasmodium falciparum* genome have been the major causative of antimalarial resistance over the past decades. *P. falciparum* genome has undergone several mutations that have enabled its survival under inhibitory concentrations of administered drugs. Mutations in the *Pfcrt*, *Pfmdr*1, and Kelch-13 propeller genes have been associated with resistance towards Chloroquine and Artemisinin-based drugs(Achieng *et al*., 2015).In *Plasmodium*, drug resistance is mediated by two processes: (a) the rate at which de novo mutations conferring resistance are selected and (b) the spread of those resistant alleles.

The emergence of drug resistance in Plasmodium is primarily driven by the selection of spontaneous mutations that confer a survival advantage in the presence of antimalarial drugs. De novo mutations can occur naturally in the parasite's genome, leading to alterations in drug targets or metabolic pathways that render the parasite less susceptible to the effects of the drug. Various factors, including the intrinsic mutation rate of the parasite, the intensity and duration of drug exposure, and the genetic diversity of the parasite population, influence the rate at which these mutations occur(Id *et al*., 2021).

Once a resistant Plasmodium strain emerges within the parasite population, its subsequent spread can occur through various mechanisms. One important factor is the transmission dynamics of the parasite, which involves the transfer of resistant parasites from human hosts to mosquito vectors and subsequent transmission to other individuals. The spread of resistant alleles can also be influenced by factors such as the geographic distribution of drug use, population movement, and the intensity of malaria control measures(Talisuna *et al*., 2007).

Understanding the mechanisms underlying the spread of drug resistance is crucial for effective malaria control. Mathematical models and population genetics studies have provided insights into the dynamics of resistance spread. For instance, mathematical models have shown that the rate of spread is influenced by factors such as the fitness cost of resistance, the level of drug pressure, and the migration patterns of infected individuals. Population genetics studies have investigated the genetic diversity and population structure of Plasmodium parasites, shedding light on the patterns and pathways of resistance dissemination(Mulenge *et al*., 2016; Neafsey, 2021).

2.7.2 Pharmacological Factors

The incomplete understanding of pharmacokinetics elements may precis the useful life of malaria treatment drugs and may increase the spread of resistance. In the early years of drug trials, the drug levels were rarely calibrated, so all clinical trials and treatment failures were attributed to inherent parasite resistance(White, 2013). Failure to meet therapeutic drug levels prompted clinical outcomes of inaccurate drug efficacy and parasite susceptibility deduction. The doses selected are the minimum lethal and therapeutic during dose selection studies. As the resistance spreads, low drug levels permit the circulation of resistant strains because the therapeutic levels required to kill mildly resistant parasites are higher than those required to eliminate susceptible ones. A pharmacokinetic model suggests that mefloquine resistance would have emerged slower had mefloquine been administered at 24mg/kg rather than 15mg/kg (White et al., 2009). Thus, some investigators have suggested the inclusion of an assessment of drug levels at day seven during drug efficacy trials to determine whether therapeutic concentrations have been achieved in the blood.

2.7.3 Counterfeit Medication

Counterfeiting medication refers to producing and distributing substandard or falsified drugs that do not meet quality standards or contain incorrect ingredients. Falsified medication poses a major threat to public health and contributes to the emergence and spread of antimalarial drug resistance in the context of antimalarial

drugs(Bui *et al*., 2022; Jackson *et al*., 2020). Substandard antimalarial medication usually lead to the inadequate treatment of malaria infections, promoting the survival and proliferation of drug-resistant parasites. These drugs may contain insufficient or incorrect active ingredients, rendering them ineffective against the malaria parasite(CDC, 2020). This partial or ineffective treatment allows resistant parasites to survive and multiply, increasing the likelihood of drug resistance development. Several studies have demonstrated the association between counterfeit antimalarial drugs and drug resistance. *et al*Studies conducted in Southeast Asia have demonstrated that patients treated with counterfeit antimalarials had higher treatment failure rates and were more likely to harbor drug-resistant parasites(Nayyar *et al*., 2012). Similarly, a study in Nigeria *et al*revealed a correlation between the prevalence of counterfeit antimalarial drugs and the spread of antimalarial drug resistance(Beargie *et al*., 2019). Factors such as weak regulatory systems, inadequate enforcement of intellectual property rights, lack of quality control infrastructure, and limited access to genuine medications have been attributed to the proliferation of counterfeit antimalarial drugs(Newton *et al*., 2010).

2.7.4 The Human Host

The human host plays a significant role in developing and spreading. Individuals' adherence to antimalarial treatment regimens is crucial for drug therapy's effectiveness and drug resistance prevention. Poor adherence, including incomplete treatment courses, inadequate dosing, or self-medication, can result in subtherapeutic drug levels, allowing resistant parasites to survive and multiply. Various factors, including access to healthcare, patient education, socioeconomic status, cultural beliefs, and drug-related side effects, can influence treatment adherence. Several studies have shown the association between treatment adherence and the development of antimalarial drug resistance. For instance, a study by Gesase *et al*. (2009) in Tanzania demonstrated that poor adherence to artemether-lumefantrine treatment contributed to the emergence of resistance in *Plasmodium falciparum*(Gesase *et al*., 2009). Through education, monitoring, and supportive interventions, improved treatment adherence is essential to prevent the spread of drug resistance.

The genetic makeup of the human host plays a role in determining the response to antimalarial drugs and susceptibility to drug resistance. Genetic factors can influence drug metabolism, targets, and the immune response against the malaria parasite. Polymorphisms in genes encoding drug transporters, drug targets, or enzymes involved in drug metabolism can affect the efficacy of antimalarial drugs and contribute to the development of resistance. Genetic variations in human genes, such as cytochrome P450 enzymes, have been linked to variations in drug metabolism and treatment outcomes(Zhao *et al*., 2021).

Additionally, asymptomatic individuals infected with malaria parasites can serve as a hidden reservoir, contributing to the persistence and spread of antimalarial drug resistance. These individuals serve as a reservoir for ongoing transmission, as mosquitoes can acquire the parasites during blood feeding. The persistence of parasites in asymptomatic hosts creates an environment where drug-resistant strains can survive and potentially spread to others. Several studies have shown a higher prevalence of drug-resistant malaria parasites in asymptomatic individuals than in those with symptomatic infections. A study by Sondo *et al*. (2020) in Burkina Faso demonstrated a higher proportion of drug-resistant parasites in asymptomatic individuals, indicating their potential role in maintaining and disseminating drug resistance(Sondo *et al*., 2020). Detecting asymptomatic individuals is challenging because they do not exhibit typical malaria symptoms, leading to underestimation of the reservoir of infections. Traditional diagnostic methods, such as microscopy and rapid diagnostic tests (RDTs), have limited sensitivity in detecting low-level parasitemia in asymptomatic individuals. Treating asymptomatic individuals poses additional challenges as they are less likely to seek healthcare and receive appropriate treatment because they do not exhibit symptoms. The use of ineffective or suboptimal antimalarial drugs in this population can promote the survival and spread of drug-resistant parasites. Targeted interventions, such as active case detection and mass drug administration, have been employed to identify and treat asymptomatic infections, thereby reducing the reservoir of drug-resistant parasites(Sturrock, Hsiang, *et al*., 2013).

The presence of asymptomatic human hosts infected with drug-resistant malaria parasites has significant implications for malaria control strategies. These individuals can contribute to the ongoing transmission of drug-resistant strains and undermine the effectiveness of antimalarial interventions. The persistence of drug-resistant parasites in asymptomatic hosts can serve as a source for re-establishing transmission after control efforts(Bushman *et al*., 2018).

2.8 Molecular Markers of Antimalarial Drug Resistance

The emergence of mutations in the genome of Plasmodium falciparum forms the genetic underpinning of antimalarial drug resistance. A multitude of studies have reported the genetic elements involved in this process. Specific mutations in certain parasite genes have been pinpointed as crucial factors in resistance to a range of antimalarial drugs. Insights into the genomic diversity and genetic signatures related to drug resistance have been gleaned from whole-genome sequencing studies. These investigations have identified new potential genes and genetic markers that could play a role in drug resistance(Akoniyon *et al*., 2022).2.8.1 *Plasmodium falciparum* chloroquine resistance transporter

The *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene has been extensively studied as a key genetic determinant of antimalarial drug resistance, particularly in the case of chloroquine resistance. The gene is located on chromosome 7 of the *P. falciparum* genome and encodes a transmembrane protein localized to the parasite's digestive vacuole membrane(Bray *et al*., 2005). This protein has 10 predicted transmembrane domains (TMDs) and was termed *Pfcrt* for ‘Plasmodium falciparum Chloroquine Resistance Transporter’ in recognition of the ‘transporter-like’ nature of its predicted secondary structure **(Figure 2.3).** Recent research, including studies from 2018 onwards, has shed light on the role of *Pfcrt* mutations in conferring resistance to chloroquine and other antimalarial drugs(Gomez *et al*., 2023; Ibraheem *et al*., 2014). Several specific mutations in the *Pfcrt* gene have been associated with chloroquine resistance, the most well-known being the K76T mutation. This mutation is highly prevalent in chloroquine-resistant strains of *P. falciparum* and has been widely studied for its impact on drug efficacy.

A study *et al*conducted in pakistan investigated the association between *Pfcrt* mutations and chloroquine resistance in isolates from different regions of Pakistan. The study found the presence of high frequency of resistant allele 76T remained despite the removal of CQ, supporting its role in chloroquine resistance in the local population(Nadeem *et al*., 2023).

Furthermore, the *Pfcrt* gene has also been implicated in resistance to other antimalarial drugs, such as amodiaquine and piperaquine. Mutations in *Pfcrt*, including the K76T mutation, have been associated with decreased susceptibility to these drugs. A study conducted in 2019*et al* explored the genetic diversity of *Pfcrt* in isolates from different regions of Angola and found a range of mutations associated with resistance to multiple antimalarials, highlighting the complex interplay between *Pfcrt* polymorphisms and drug resistance(Zhou *et al*., 2019).

In Africa, several *Pfcrt* haplotypes have been identified and linked to antimalarial drug resistance. The most extensively studied haplotype is the SVMNT haplotype, associated with chloroquine resistance. A study conducted in Tanzania demonstrated a strong correlation between the presence of the SVMNT haplotype and chloroquine resistance in *P. falciparum* isolates from various regions of Africa (Gadalla *et al*., 2015). Studies have also shown that the CVIET haplotype is prevalent in chloroquine-resistant parasites in different African countries.*et al*.

Furthermore, the impact of *Pfcrt* haplotypes on treatment outcomes with other antimalarial drugs has also been investigated. Venkatesan *et al*. (2014) studied the association between *Pfcrt* haplotypes and treatment efficacy in malaria patients from multiple African countries. The study revealed that specific haplotypes, such as CVIET and SVMNT, were associated with decreased artemether-lumefantrine and artesunate-amodiaquine treatment efficacy(Venkatesan *et al*., 2014).

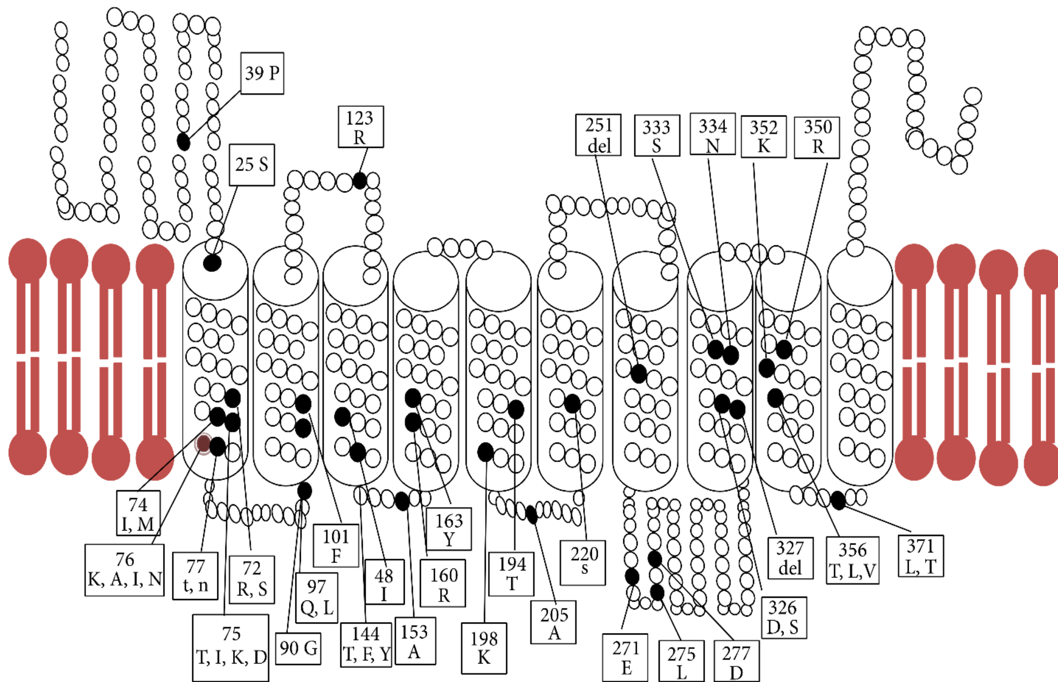

Figure 2.3: Detailed structure of *Pfcrt* protein. It is made up of 422 amino acids distributed over 10 transmembrane domains. Inside the structure are 32 candidate codons for point mutations that confer for changing *Pfcrt* function (Ibraheem *et al*., 2014).

2.8.1 *Pfmdr1*

The *Pfmdr*1 (*Plasmodium falciparum* multidrug resistance 1) gene is essential to genetic antimalarial multi-drug resistance. This gene encodes a membrane transporter protein that plays a crucial role in the efflux of various antimalarial drugs, leading to reduced drug accumulation within the parasite and subsequent resistance. *Pfmdr*1 contains two transmembrane domains (TMDs) and two conserved nucleotide-binding domains (NBDs), following a typical TMD-NBD-TMD-NBD arrangement of ABC transporter **(Figure 2.4).** Numerous studies have investigated the association between *Pfmdr*1 polymorphisms and drug resistance, highlighting its significance as a genetic marker. One key polymorphism in *Pfmdr*1 that has been extensively studied is the N86Y mutation. This mutation is associated with reduced susceptibility to multiple antimalarial drugs, including chloroquine and amodiaquine. Sidhu *et al*. (2002) demonstrated that the N86Y mutation in *Pfmdr*1 was associated

with decreased sensitivity to chloroquine in *P. falciparum* isolates from Asian, African, or South American malaria-endemic regions. Price *et al*. (2004) demonstrated that the N86Y mutation in *Pfmdr*1 was associated with decreased susceptibility to amodiaquine in African isolates(Price *et al*., 2015). In addition to the N86Y mutation, other polymorphisms in *Pfmdr*1 have been identified and linked to antimalarial drug resistance. The Y184F mutation, for instance, has been associated with decreased susceptibility to artemisinin derivatives. A Takala-Harrison *et al*. (2013) study reported that parasites carrying the Y184F mutation exhibited delayed parasite clearance rates following artemisinin-based combination therapy(Takala-Harrison *et al*., 2013). Furthermore, polymorphisms such as S1034C, N1042D, and D1246Y have also been implicated in altering parasite susceptibility to multiple antimalarial drugs(Pirahmadi *et al*., 2013; She *et al*., 2020).

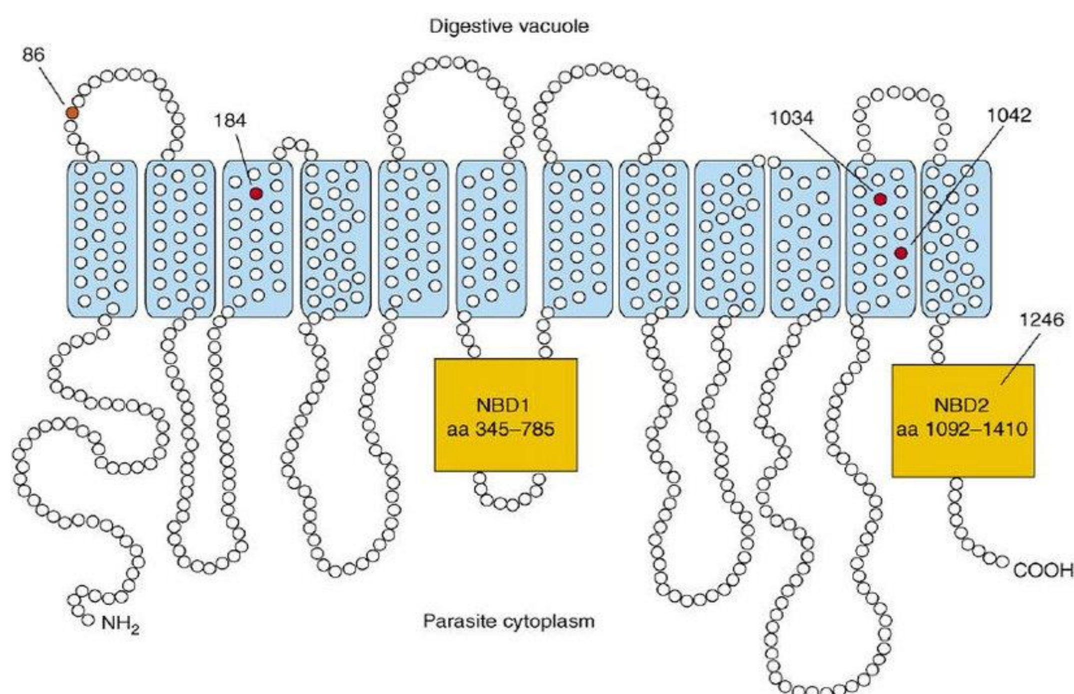

Figure 2.4: The predicted 2-dimensional structure of Pfmdr1 protein (Valderramos & Fidock, 2006)**. The red dots indicated mutations associated with high CQ resistance.**

2.8.2 Pfdhfr and Pfdhps

The *Pfdhps* (*Plasmodium falciparum* dihydropteroate synthetase) and *Pfdhfr* (*Plasmodium falciparum* dihydrofolate reductase) genes play a crucial role in genetic antimalarial drug resistance in *Plasmodium falciparum*. *Pfdhps* is 323 amino acids long and folds into a triosephosphate isomerase (TIM) barrel single-domain protein **(Figure 2.5).** The protein features a well-structured eight-stranded core of parallel β-sheets surrounded by peripheral α-helices. The active site of the protein is a highly flexible tunnel formed by the core β-sheets, flanked by loops. The dihydrofolate reductase domain (*Pfdhfr*) of Plasmodium falciparum bifunctional dihydrofolate reductase–thymidylate synthase (*Pfdhfr*-TS) is a well defined target of antifolate antimalarial drugs such as pyrimethamine (Pyr) and cycloguanil (Cyc)(Chusacultanachai *et al*., 2002).These genes encode enzymes in the folate biosynthesis pathway, targeted by antimalarial drugs such as sulfadoxine and pyrimethamine. Mutations in these genes can alter the encoded enzymes' structure and function, reducing the drug's binding affinity and decreasing drug susceptibility. One well-established mutation in the *Pfdhfr* gene is the N51I/C59R/S108N triple mutation. This mutation is associated with resistance to pyrimethamine. A study by Plowe *et al*. (1997) demonstrated that the N51I/C59R/S108N mutation in *Pfdhfr* was strongly associated with pyrimethamine resistance in *P. falciparum* isolates from four countries with increasing levels of pyrimethamine-sulfadoxine resistance: Mali, Kenya, Malawi and Bolivia(Sirawaraporn *et al*., 1997). A study by Nzila *et al*. (2000) found that parasites carrying this triple mutation displayed significantly reduced susceptibility to pyrimethamine(Nzila *et al*., 2000).

Similarly, the *Pfdhps* gene has mutations associated with resistance to sulfadoxine. The A437G and K540E mutations in *Pfdhps* have been identified as key contributors to sulfadoxine resistance. A study by Roper *et al*. (2004) showed that these mutations were prevalent in sulfadoxine-resistant parasites in African isolates from southeast Asia and strongly associated with decreased drug susceptibility(Roper *et al*., 2004).

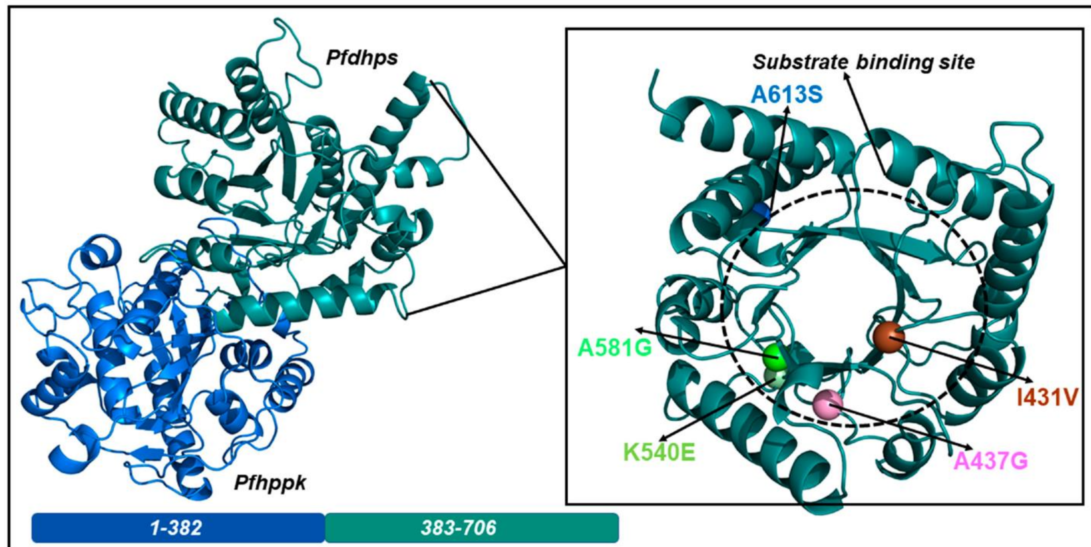

Figure 2.5: The Structural Components of Pfdhps. The Amino Acid Ranges for Pfdhps Segment is Highlighted in Deep Teal , Mutations Occurring iIn Pfdhps Are Indicated by Spheres in the Right Panel (Boateng et al., 2023)**.**

2.8.3 Pf-k13

The *Pf-k13* (*Plasmodium falciparum* kelch 13) gene has emerged as a crucial genetic marker for antimalarial drug resistance, particularly artemisinin resistance. *Pf-k13* encodes a 726-amino acid protein (*Pf-k13*) consisting of a poorly conserved Apicomplexa-specific N-terminal region and three annotated, highly conserved domains9 : a coiled-coil-containing (CCC; amino acids 212–341), a BTB (Broad-complex, tramtrack and bric-à-brac; also known as BTB/POZ; amino acids 350–437) and a C-terminal Kelch-repeat propeller (KREP; amino acids 443–726) which harbors nearly all *Pf-k13* alleles associated with ART-R **(Figure 2.6).** Mutations in the *Pf-k13* gene have been associated with reduced parasite susceptibility to ACTs, which are frontline treatments for malaria. A seminal study by Ariey *et al*. (2014) identified the *Pf-k13* gene as a key mediator of artemisinin resistance(Ariey *et al*., 2014a). The study demonstrated a strong association between specific *Pf-k13* mutations, such as C580Y, and delayed parasite clearance rates following artemisinin treatment in Southeast Asia. Subsequent studies conducted in various malaria-

endemic regions further confirmed the role of *Pf-k13* mutations in artemisinin resistance(Ndwiga *et al*., 2021b). For instance, a study by Ménard *et al*. (2016) examined the distribution and prevalence of *Pf-k13* mutations in Africa. The study revealed the presence of multiple non-synonymous mutations in *Pf-k13*, including the C580Y mutation, in *P. falciparum* isolates from several African countries. These mutations were associated with delayed parasite clearance and reduced artemisinin susceptibility, highlighting the emerging threat of artemisinin resistance in Africa(Ménard *et al*., 2016). A similar study by Takala-Harrison *et al*. (2018) investigated the genetic diversity of *Pf-k13* across different malaria-endemic regions. The study identified numerous *Pf-k13* mutations associated with artemisinin resistance, including validated and candidate resistance-conferring mutations(Takala-Harrison *et al*., 2013). The findings emphasized the need for continued surveillance and monitoring of *Pf-k13* mutations to track the spread of artemisinin resistance globally. The *Pf-k13* gene has become a critical marker for artemisinin resistance in *P. falciparum;* thus, monitoring the presence and distribution of *Pf-k13* mutations is vital for detecting and responding to emerging artemisinin resistance, informing treatment guidelines, and developing effective strategies to combat drug resistance in malaria-endemic regions.

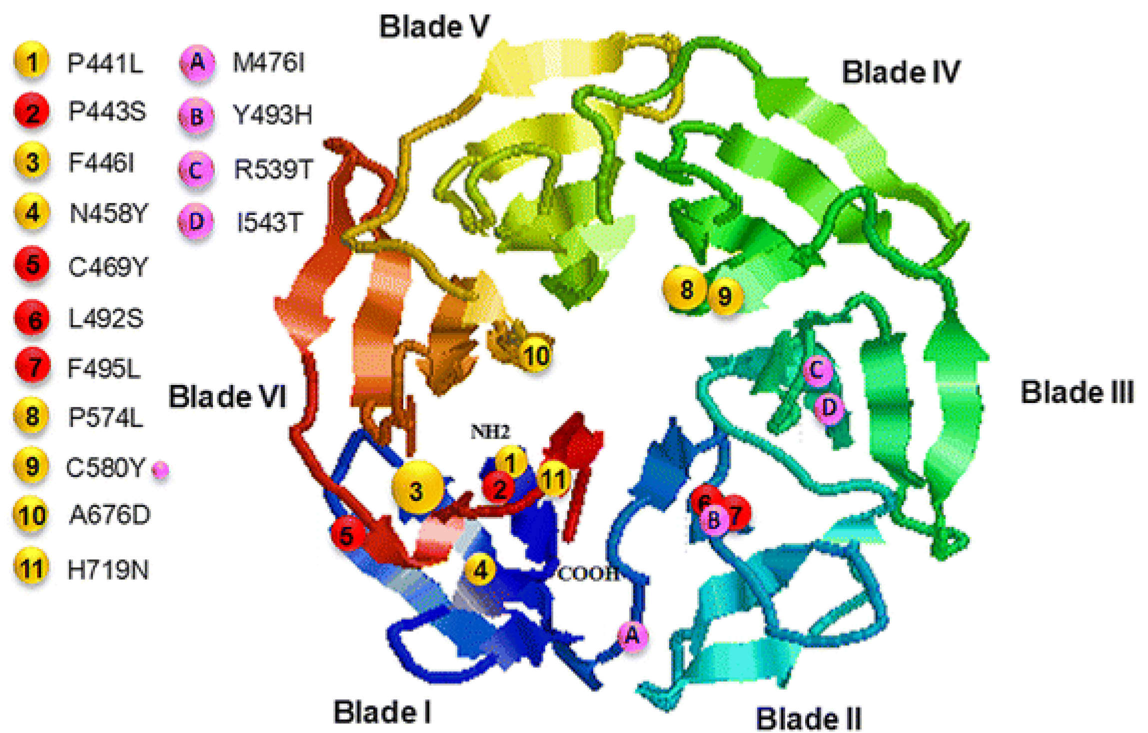

Figure 2.6: Predicted 3-Dimensional Model of Pfk-13 Gene and Mutations Locations on its Six Propeller Blades(Wang *et al*., 2015).

2.9 Global Prevalence of Drugs Resistance Markers

Plasmodium falciparum resistance has spread from Southeast Asia (SEA) on various occasions. The plummeting prevalence of antifolate and chloroquine resistance has rendered these drugs ineffective in most malaria-endemic regions(Srimuang *et al*., 2016).The historic path of this resistance is well known, where SEA is considered the epicentre of malaria resistance, which then spreads to India via Myanmar and then to Africa **(Figure 2.7).**

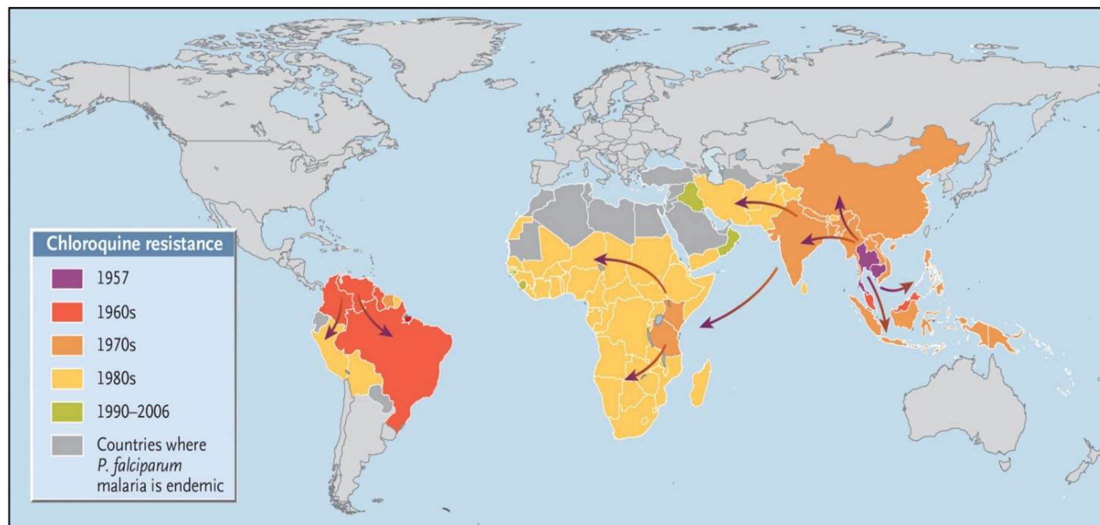

Figure 2.7: Spread of Chloroquine Resistance. The Map Shows the Spread of Chloroquine Resistance Over the Last Decades Across the World From Southeast Asia(Medical, 2014).

Monitoring this resistance has been enhanced by surveillance of the molecular markers' causative of resistance. A study by (Srimuang et al., 2016) showed the prevalence of *Pfmdr1* at MaeSot on Thailand–Myanmar border at an estimated 59%. The Y184F mutation was most prevalent in and around Cambodia, and the F1226Y mutation was prevalent in 50% of samples in Mae Sot. The K76T mutation on *Pfcrt* prevalence was at 98.2% of isolates. The CVIET haplotype associated with CQ resistance comprised 95% of the western SEA parasite population.

In comparison, the CVIDT haplotype was common between 30% & 40% in north and north-eastern Cambodia, southern Laos, and southern Vietnam. A cross-sectional study by (Kyaw *et al*. 2015) showed the prevalence of *Pfk*-13 propeller marker at 39% of all samples collected at malaria treatment centres at 55 sites in ten administrative regions in Myanmar, and in relevant border regions in Thailand and Bangladesh, between January 2013, and September 2014 where twenty-six different alleles, including nine mutations not previously observed in Southeast Asia, were recorded. In Homalin, 47% of 45 parasite samples harbored K13-propeller mutations(Tun *et al*., 2015).

In a report by (Kamau *et al.* 2015) to show the prevalence of K-13 propeller mutations in sub-Saharan Africa, 22 unique mutations were detected, of which seven were non-synonymous. Ghana recorded the highest SNPs, approximately 3% for the non-synonymous V566I and synonymous C469C. The prevalence of the A578S mutant allele was highest in parasites from Kenya, at 2.7%. (Kamau et al., 2014). A study in Msambweni, Kenya, between 2008 and 2014 showed a significant decline in CQR parasites(Maraka *et al.*, 2020). The 76T codon prevalence for CQR was recorded at 41% from 63%, five years after a retrospective study was conducted in the same area. This decline may be attributed to the reduced drug pressure years after CQ was discontinued.

2.10 Monitoring of Drug Resistance

2.10.1 *In vivo* Tests

In vivo test entails treating symptomatic and infected people with calibrated drug doses and subsequent surveillance and monitoring of the parasitological and clinical response over time. By studying drug responses in vivo, researchers can gain insights into the effectiveness of different antimalarial drugs, the prevalence of resistance, and the factors contributing to resistance development. In vivo tests allow for monitoring parasite clearance and recrudescence rates and assessing the therapeutic efficacy of antimalarials(Cui *et al.*, 2015).

However, it is essential to consider the challenges associated with in vivo tests for monitoring drug resistance in *Plasmodium falciparum*. Ethical considerations, including the use of animal models or human subjects, should be carefully addressed and justified. Additionally, the complexities of the parasite's lifecycle and the genetic diversity of *Plasmodium falciparum* strains may impact the interpretation of in vivo results(Njue *et al.*, 2018). Therefore, it is crucial to carefully select appropriate study populations, use standardized protocols, and employ molecular techniques to characterize genetic markers associated with drug resistance,

2.10.2 *In vitro* Tests

In vitro tests entail obtaining a blood sample via pricking of the finger, then culturing the parasites, exposing them to known dosages of various drugs, and monitoring the inhibition of maturation into schizonts. In vitro tests avoid many confounding factors that influence in vivo tests by removing parasites from the host and placing them into a controlled experimental environment. In addition, various tests can be conducted on multiple parasites and with various drugs. Experimental drugs can also be tested without risking the health of host volunteers. However, factors like the link between in vitro response and clinical response in the host are not clear or consistent, and the link seems to depend on the level of acquired immunity within the population being tested(La Rosa and Diamond, 2012). Some pro-drugs, such as proguanil which require host conversion into active metabolites, can't be evaluated, including drugs that require synergism with the host's immune system(Bloland and Bloland, 2001).

2.10.3 Animal Models Tests

Animal model tests are carried out in animals with nearly identical physiology as humans and, thus, are affected by many similar extrinsic factors as in vivo tests. The impact of the host immunity is diminished by using animals reared in the laboratory or animal-parasite amalgamations unlikely to exist in nature. However, other host factors remain (Bloland, 2001). These tests allow for the testing of parasites that cannot be adapted to in vitro environments, provided a suitable animal host is available and the testing of experimental drugs not yet approved for use in humans. One major disadvantage is that only parasites that can grow and be adapted to primates and mice models can be investigated.

2.10.4 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) can be used for genotyping mutations associated with resistance against various antimalarial drugs. In theory, the percentage prevalence of specific gene alleles within a sample of parasites collected from infected patients from a specific area could indicate the prevalence of drug resistance compared to data acquired from in vitro techniques. Mutations are recorded in

population surveys in the target regions, and when phenotypes are available in numbers enough to achieve statistical power, the most prevalent mutations are associated with delayed parasite clearance after antimalarial treatment and reduced in-vitro responses; resistance(Tun *et al.*, 2015). CQR and AL resistance markers have been documented and are used to monitor resistance by studying their prevalence in target regions.

2.10.5 Next Generation Sequencing

Next-generation sequencing (NGS) techniques have revolutionized genomics in the last decade and demonstrated outstanding potential for malaria drug resistance monitoring. NGS allows for high-throughput sequencing of the malaria parasite's genome, providing detailed insights into genetic variations associated with drug resistance (Akoniyon *et al.*, 2022). Several studies have utilized NGS to identify known drug resistance markers and discover novel genetic mutations associated with drug resistance, enabling early detection, and tracking of emerging resistance patterns(Manske *et al.*, 2012; Miotto *et al.*, 2015).

A key advantage of NGS in malaria drug resistance monitoring lies in its ability to detect low-frequency drug resistance mutations that may escape detection using conventional methods such as polymerase chain reaction (PCR) or Sanger sequencing(Talundzic *et al.*, 2018). NGS allows for a comprehensive assessment of the genetic diversity within a parasite population, enabling the identification of rare drug-resistant clones that can contribute to treatment failure or the spread of resistance. This capacity to monitor low-frequency mutations empowers early warning systems for emerging drug resistance and facilitates targeted interventions to prevent its dissemination (Taylor *et al.*, 2017). Furthermore, NGS techniques provide valuable information on the population structure and dynamics of drug-resistant parasites, shedding light on transmission patterns and aiding the development of effective malaria control strategies.

Despite the vast potential of NGS in malaria drug resistance monitoring, its widespread implementation encounters specific challenges. The high costs associated

with sequencing and data analysis, as well as the requirement for specialized infrastructure and expertise, limit the accessibility of NGS technologies, particularly in resource-limited settings where the malaria burden is most pronounced (Ghansah *et al*., 2019; Grandjean Lapierre *et al*., 2023). However, ongoing efforts are underway to develop cost-effective and field-friendly NGS platforms that can be deployed in malaria-endemic regions(Aydemir *et al*., 2018). Additionally, integrating NGS data with other surveillance tools, such as molecular epidemiology and clinical data, can enhance the interpretation of drug resistance patterns and inform evidence-based policy decisions (Amato *et al*., 2020). Continued research collaboration is essential to overcome these challenges and fully leverage the potential of NGS in malaria drug resistance monitoring, thereby supporting effective malaria control and elimination efforts strategies.(Kamau *et al*., 2015)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

This study was conducted on four selected islands (Mfangano, Takawiri, Kibuogi, and Ngodhe) situated on Lake Victoria, along with a coastal mainland site (Ungoye) **(Figure 3.1)** Malaria transmission in these islands is perennial, with high intensity and prevalence varying from 7% to 29%, with peak periods corresponding to the rainy season(Olanga *et al*., 2015). The main economic activity on these islands is fishing which exposes the residents to malaria transmission. Also, due to the continuous use of antimalarial drugs in the population, it is expected that malaria parasite resistance will occur due to drug pressure. The study enrolled malaria-symptomatic patients with fever and parasitemia ≥2000 parasites/ul of blood who visited government health centres between July 2014 and July 2016. Informed consent was obtained from participants or legal guardians. Experimental procedures of this study were carried out at the Centre for Research in Infectious Diseases, Mount Kenya University.

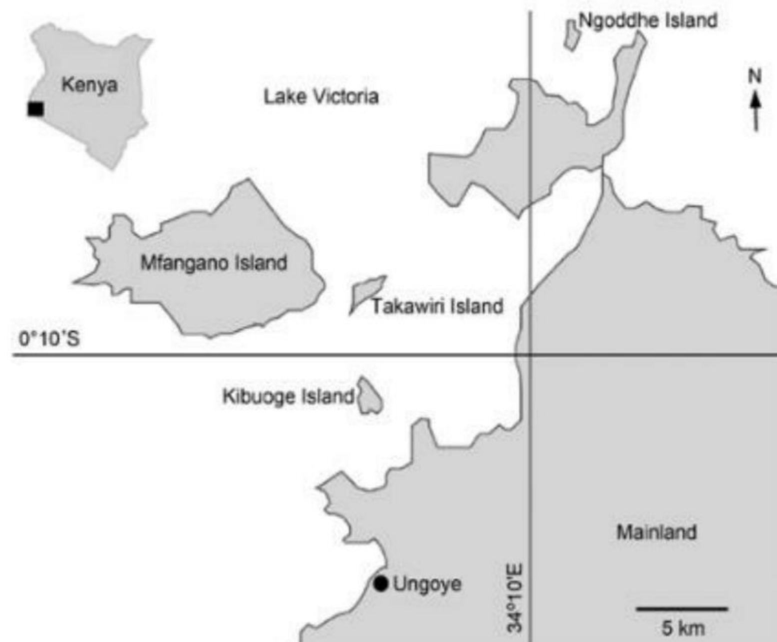

Figure 3.1: Shows the Study Sites Selected to Investigate the Prevalence of Antimalarial Drug Resistance Markers on Lake Victoria Islands.2015-2016. Inset Shows the Study Area in Kenya. This Map Was Generated Using DIVA-GIS Software Version 7.5.0 http://www.diva-gis.org/

3.2 Ethical Considerations

This study's approval was obtained from the Kenyatta National Hospital, the University of Nairobi (KNH-UON) ethical review committee (P609/10/2014), and the Mount Kenya University Ethics Review Committee (MKU-ERC) approval number 038/2014.

3.3 Sampling Procedure and Sample Collection

3.3.1 Sampling Design

The study employed a cross-sectional design to investigate the prevalence of antimalarial associated polymorphisms in selected islands in Lake Victoria, Kenya. The study is part of a main project conducting surveillance for artemether-

lumefantrine clinical response, lumefantrine in vitro sensitivity and molecular markers of tolerance in currently circulating *Plasmodium falciparum* isolates in Kenya.

3.3.2 Sample Size Determination

The sample size was determined using Fisher's formula. The estimated prevalence of malaria in selected Lake Victoria islands is between 7% during low transmission season to 29% during high transmission season. The total population in the selected islands varies with fishing patterns. The samples were collected during high transmission season where a the estimated proportion of population with target mutations was estimated to be 96(Musyoka *et al.*, 2020).

$$n = \frac{Z^2 * p*q}{d^2}$$

Z = Standard normal deviation at required confidence interval (95%),

p = proportion of the target mutations within the population, which is 96%, q = 1-p

d = level of statistical significance at 95% confidence interval.

$n = 1.96^2 * 0.96 * 0.04/0.05^2 = 59$ samples

3.3.3 Sample Collection

Fifty- nine blood samples (3 ml) were obtained from patients confirmed positive for malarial infection using microscopy. As previously described, *P. falciparum* parasites were adapted for in-vitro culture (Poindexter, 1976); briefly, in RPMI-1640 medium containing 10% heat-inactivated pooled type AB+ human serum, 200 mM hypoxanthine, 20 µg/mL gentamicin and O+ human RBC at 2% hematocrit.

3.4 Genomic DNA Extraction

After a short period of culture (≤15 days, median 7 days), genomic DNA was extracted from the short-term cultures using the QIAamp DNA mini kit (Qiagen, Valencia, CA). Briefly, 200 µl of blood sample was introduced into a 1.5 ml microcentrifuge tube, followed by 200 µl lysis buffer (Buffer AL). Protein digestion was achieved by adding 20 µl of proteinase K. The resulting mixture was subjected to pulse-vortexing for 15 seconds and incubated at 56 ^{o}C for 10 minutes to ensure effective lysis of the Plasmodium pellet. Purification of DNA was accomplished using 200 µl of 96% ethanol, followed by micro-centrifugation at 8000 rpm for 1 minute. The resulting filtrate was discarded, and the spin column was transferred to a sterile 2ml collection tube. Subsequent DNA washing steps involved the addition of 500 µl of wash buffer, followed by centrifugation at 8000 rpm for 1 minute, and removing the filtrate. 500 µl of buffer AW2 was added to the spin column and centrifuged at 14,000 rpm for 3 minutes. DNA elution from the spin column was achieved by adding 100 ul of elution buffer and centrifuging at 8,000 rpm for 1 minute. The purified genomic DNA was stored at -20 ^{o}C until further use. Extracted DNA was quantified using the dsDNA high-sensitivity method in a Qubit Fluorometer (Invitrogen, Marsiling, Singapore) per the manufacturer's instructions.

3.5 Whole Genome Sequencing

3.5.1 Library Preparation and Sequencing

Paired-end sequencing libraries were prepared using Nextera XT DNA library preparation Kit according to the manufacturer's recommended protocol (https://www. Illumina .com). Briefly, 2ng genomic DNA was fragmented using Nextera XT transposome to a mean fragment distribution of 300-500bp, and sequencing adapters were ligated to each fragment. The tagmented DNA fragments were purified from the transposome before subsequent downstream processes. Amplification of the purified tagmented DNA fragments was performed using Index 1 (i7) and Index 2 (i5) primers. A 5-cycle PCR was performed using the following protocol: Initial denaturation at 98 ^{o}C followed by 10 cycles at 98 ^{o}C for 10 seconds, 56 ^{o}C for 30

seconds, 72 °C for 30 seconds and a final elongation step at 72 °C for 5 minutes. The amplified libraries were purified using AMPure XP beads and size selected to remove short library fragments. The libraries were further normalized in Nextera Dilution Plate and pooled in the Nextera Pooled Plate to contain enriched DNA.

As previously described, whole genome sequencing was performed on the Illumina platform (Manske *et al.*, 2012b). The pooled libraries were used for paired-end sequencing of 150-bp reads using the Illumina MiSeq reagent kit v.2 with 500 cycles. The Illumina Analysis Pipeline was utilized for image analysis, base calling, and error estimation.

3.6 Bioinformatics Data Analysis

3.6.1 Quality Control

As part of quality control checks on the generated reads, adapter sequences and low-quality bases were filtered using Trimmomatic v0.36 (USADELLAB, USA) (Bolger, Lohse, and Usadel 2014), with the following parameters: adapter trimming; LEADING-cutting bases at the start of a read if below a threshold quality of 20; TRAILING- cutting bases at the end of a read if below threshold quality of 25; SLIDING WINDOW- scanning the read with a 4-base sliding window and cutting when the average quality per base drop below 15; MINLENGTH- dropping a read below 35 base pairs long. Further quality control on the Fastq files was performed using the FASTQC (Babraham Insitute, UK) toolkit version 0.11.5.

3.6.2 Mapping

After quality control checks, the reads that passed the threshold were aligned against the *P. falciparum* 3D7 reference genome (https://plasmodb.org/common/downloads/release-59/Pfalciparum3D7/fasta/data/) version 9.3 using Burrows-Wheeler Alignment tool (BWA) (Li & Durbin, 2009) (http://bio-bwa.sourceforge.net) with default parameters. The resulting SAM files were then converted to BAM format using Samtools(Li *et al.*, 2009).

3.6.3 BAM Files Preprocessing

After mapping each sample to the 3D7 reference genome, PCR and sequencing duplicates which may originate from the library preparation stage or genome amplification bias, were marked using Picard SortSam and the MarkDuplicates function of the Genome Analysis Toolkit's (GATK) (McKenna *et al*., 2010) pipeline. Realignment around InDels (insertion and deletion sequence variants) was done using GATK's RealignerTargetCreator/IndelRealigner to improve the accuracy of variant calling and alignment of sequencing reads. Base quality score recalibration was done with GATK's BaseRecalibrator to correct systematic errors, account for platform-specific biases, and improve variant calling.

The following *P. falciparum* genetic crosses 1.0 databases were used for the base quality
recalibration:ftp://ngs.sanger.ac.uk/production/malaria/pfcrosses/1.0/7g8_gb4.combined.final.vcf.gz; ftp://ngs.sanger.ac.uk/production/malaria/pf-crosses/1.0/hb3_dd2.combined.final.vcf.gz and ftp://ngs.sanger.ac.uk/production/malaria/pf-crosses/1.0/37_hb3.combined.final.vcf.gz.

3.6.4 Variant Calling and Filtering.

The recalibrated BAM files were used as input for this step. Genome Analysis Toolkit (GATK) Haplotype caller was used to call variants using the following parameters: genotyping mode DISCOVERY, --output mode EMIT_VARIANTS_ONLY, --stand_emit_conf 10 and –stand_call_conf 30. Each sample's resulting individual Variant Calling Format (VCF) files were merged using VCF tools(Danecek *et al*., 2011). Further, we used the GATK SelectVariants tool to select SNPs from the merged VCF file. Initial hard filtering was performed using quality parameters provided by GATK developers(McKenna *et al*., 2010) as follows; minimum Quality by depth (QD) of 2.0, P-value of Fisher exact test of detecting strand bias <0.001, Minimum Mapping quality phred score (MQ) of 40 (mapping quality is the probability that the read is incorrectly aligned), Minimum depth of coverage at every SNP position of 10.0, Z-score of rank sum test for mapping

qualities <-12.5 (mapping quality of all reads with alternate versus reference allele), and Z-score for rank sum test for relative positioning of reference versus alternative alleles within the reads of 8.0. Phred uses a logarithmic scale to assign quality scores (-10 * log10(P_error)), where P_error represents the probability of an incorrect base call (Zhang *et al*., 2017).

Further quality hard filtering was performed to filter out SNPs with the following features: SNPs with very poor sequencing depth (<10 reads in 1 sample) and coverage across samples; SNPs that had more than 2 alleles(non-biallelic) ; and SNPs located in the highly polymorphic *var*, *rifin*, and *stevor* regions.

Finally, we produced functional annotation with snpEFF tool v.5.1(Cingolani *et al*., 2012) using Ensembl functional annotation of the *Plasmodium falciparum* 3D7 (ASM276v2) as input. The SNPs were inspected and analyzed in Artemis(Rutherford *et al*., 2000). The DNA and predicted amino acid sequences from each locus were analyzed by alignment in MEGA 11(Tamura *et al*., 2021) against a 3D7 reference sequence.

3.7 *Pfcrt* Haplotype Determination

Th*e Pfcrt* haplotype was genotyped using a method previously described by Srimuang *et al*. (2016). Briefly, Samtools was used to extract two invariant sequences flanking the core *Pfcrt* haplotype, and the reads aligned against the 3D7 reference genome after discarding low-quality reads. The core haplotype was read at amino acid positions 72 - 76 from the resulting alignment for each sample(Srimuang *et al*., 2016).

3.8 *Pfdhps* and *Pfdhfr* Haplotype Determination

Genotyping of *Pfdhps* and *Pfdhfr* genes haplotypes was performed using a method previously described by Jiang *et al*. (Jiang *et al*., 2019). Briefly, each haplotype reconstitution was performed by observing 3 and 5 distinct genotypes in *Pfdhfr* and *Pfdhps*, respectively. The codons observed were at positions 51, 59,108, and 436, 437, 540, 581, 613 of *Pfdhfr* and *Pfdhps* genes, respectively.

3.9 Comparison and Validation of Drug Resistance Markers Frequencies in Lake Victoria With other Malaria-Endemic Regions in Africa

We accessed the catalogue of drug resistance markers in *P. falciparum* in the global MalariaGEN database v6.0 for comparing and validating the SNPs identified from the Lake Victoria sample population. This dataset comprised genomic variation records of 7,113 *P. falciparum* samples from 28 malaria-endemic countries. The method used to retrieve the data was previously described by(Amato *et al*., 2017). The dplyr v1.0.9 package(Wickham *et al*., 2021) in R v4.2.1 was used to filter out the *Pfcrt, Pfmdr1, Pfdhps, Pfdhfr,* and *Pfk*-13 genes using their PlasmoDB unique identifiers. The SNPs identified from Lake Victoria parasite population were further screened against the ones identified from MalariaGEN dataset for missed variants due to differences in analysis methodologies. The observation of similar mutations in both populations was used to validate the SNPs observed in Lake Victoria samples.

3.10 Analysis of Parasite Isolates With Mixed Gene Mutations.

Further, we analyzed *P. falciparum* isolates from Lake Victoria region with multiple drug-resistance gene mutations cutting across *Pfcrt*, *Pfmdr1*, *Pfdhps,* and *Pfdhfr* genes. *Pfcrt* mutations were inspected at codons spanning position 72 to 76, *Pfmdr* at codons 86, 184 and 1246 while mutations on the *Pfdhps* and *Pfdhfr* genes were inspected at codons 51, 59, 108 and 540 respectivly. Isolates habouring mutations on both the *Pfcrt* and *Pfmdr1* gene were classified as double mutants while those habouring mutations at all codons in *Pfdhps* and *Pfdhfr* genes classified as quadruple mutants(Ikegbunam *et al*., 2019; Jiang *et al*., 2019).

CHAPTER FOUR

RESULTS

4.1 Parasite Culture and Genomic DNA Extraction

In the present study, fifty-nine whole blood samples were collected from patients exhibiting symptoms of malaria, including fever and parasitemia levels of 2000 parasites/µl or higher. Following short-term parasite culture, fifteen of these samples were excluded due to insufficient growth. Subsequently, an additional nine samples were removed from the analysis due to suboptimal DNA quality. Thus, the final analysis was conducted with a reduced number of samples.

4.2 Whole Genome Sequencing

4.2.1 Sequencing Analysis

The whole-genome sequencing generated 4.6 to 13.8 million paired-end reads per sample with 150bp mean read depth and average genome coverage of 62X from a total of twenty seven of the thirty five samples sequenced. Mapping statistics indicated an average alignment rate of 92.1% (**Table 4.1**).

Table 4.1: Whole Genome Sequencing, Mapping and Genome Wide Coverage Statistics

Sample ID	Total No. of raw sequences	Total mapped sequences	Genome-wide coverage
Pf001	14,306,978	13,884,962 (97.05%)	86X
Pf002	6,440,125	6,357,072 (98.71%)	37X
Pf003	8,839,814	8,439,200 (97%)	40X
Pf004	13,343,786	13,150,225 (98%)	85X
Pf005	4,685,318	4,621,017(98%)	30X
Pf006	9,358,688	9,186,255(98.16%)	40X
Pf008	13,839,352	13,643,675 (98.65%)	85X
Pf009	7,947,592	7,798,919 (98.22%)	50X
Pf010	7,359,666	7,252,365 (98.61%)	45X
Pf011	9,139,494	8,990,190 (98.43%)	56X
Pf012	10,033,062	9,815,506 (97.83%)	61X
Pf013	6,200,124	6,035,112 (97.34%)	39X
Pf015	10,224,152	9,994,626 (97.85%)	62X
Pf016	13,042,210	12,705,879 (97.54%)	79X
Pf017	12,110,680	11,625,228 (97.14%)	76X
Pf018	7,794,526	7,597,518 (97.58%)	47X
Pf019	11,617,020	11,267,793 (96.99%)	70X
Pf020	10,387,722	10,044,699 (96.84%)	62X
Pf021	11,803,462	11,526,081 (97.65%)	74X
Pf022	13,257,921	12,785,939 (96.44%)	40X
Pf023	9,114,332	8,890,143(97.65%)	55X
Pf024	20,285,560	18,365,207 (90.53%)	57X
Pf025	13,385,168	12,983,510 (97.14%)	81X
Pf026	10,164,980	9,892,649 (97.32%)	62X
Pf027	10,672,875	10,502,109 (98.40%)	68x
Pf028	10,559,674	10,241,521 (97%)	64X
Pf029	12,178,546	11,825,118 (97.24%)	74X

4.2.2 Quality Control

Quality controls checks were performed using FastQC software. Quality scores were measured on the phred scale (a logarithmic function representing base-calling error probabilities), and their corresponding positions in the read sequence. Across all samples, the phred scores consistently exceeded 30, indicating an error rate of less than 1 in 1,000 bases. The results signified that the base calls obtained from the samples exhibited high quality, thereby confirming their suitability for subsequent downstream analysis. (**Figure 4.1).**

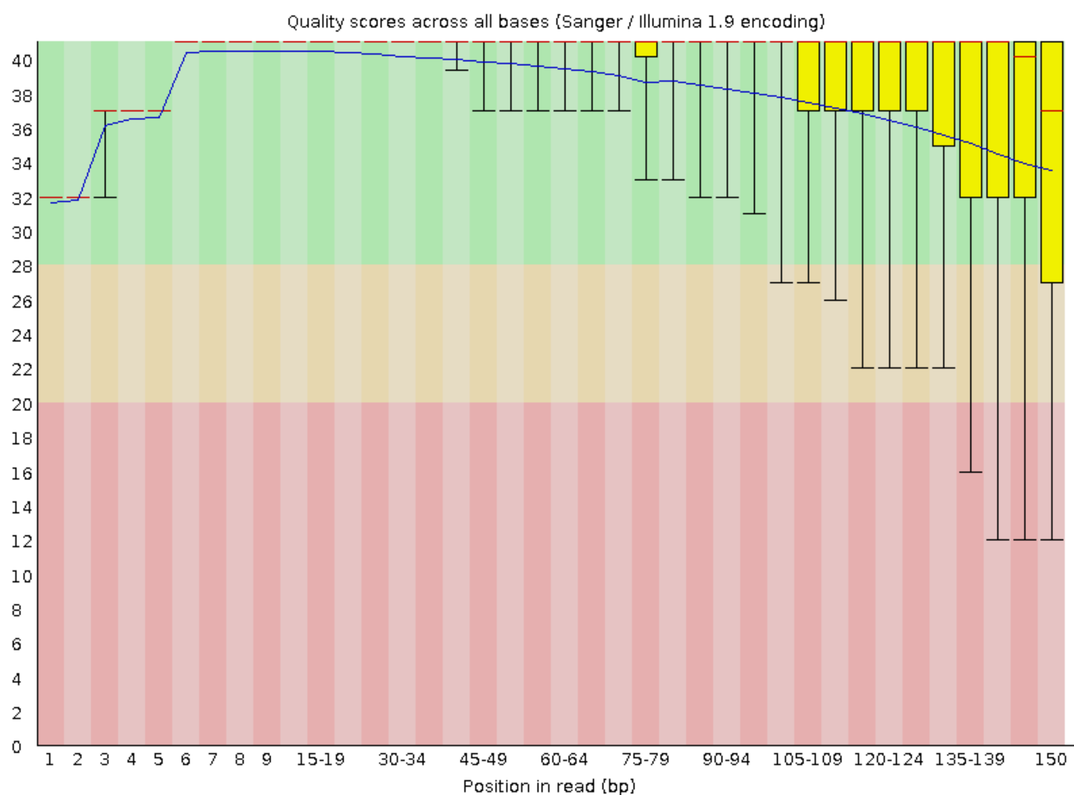

Figure 4.1: Quality Scores Across All Bases Results as Analyzed With Fastqc Software.

Additionally, average quality per read, it was observed that the phred scores reached approximately 39 (**Figure 4.2**). This value implies that the likelihood of an erroneous base call was less than 1 in 1,000 bases, indicating a high level of accuracy in the base calls, surpassing 99.9%

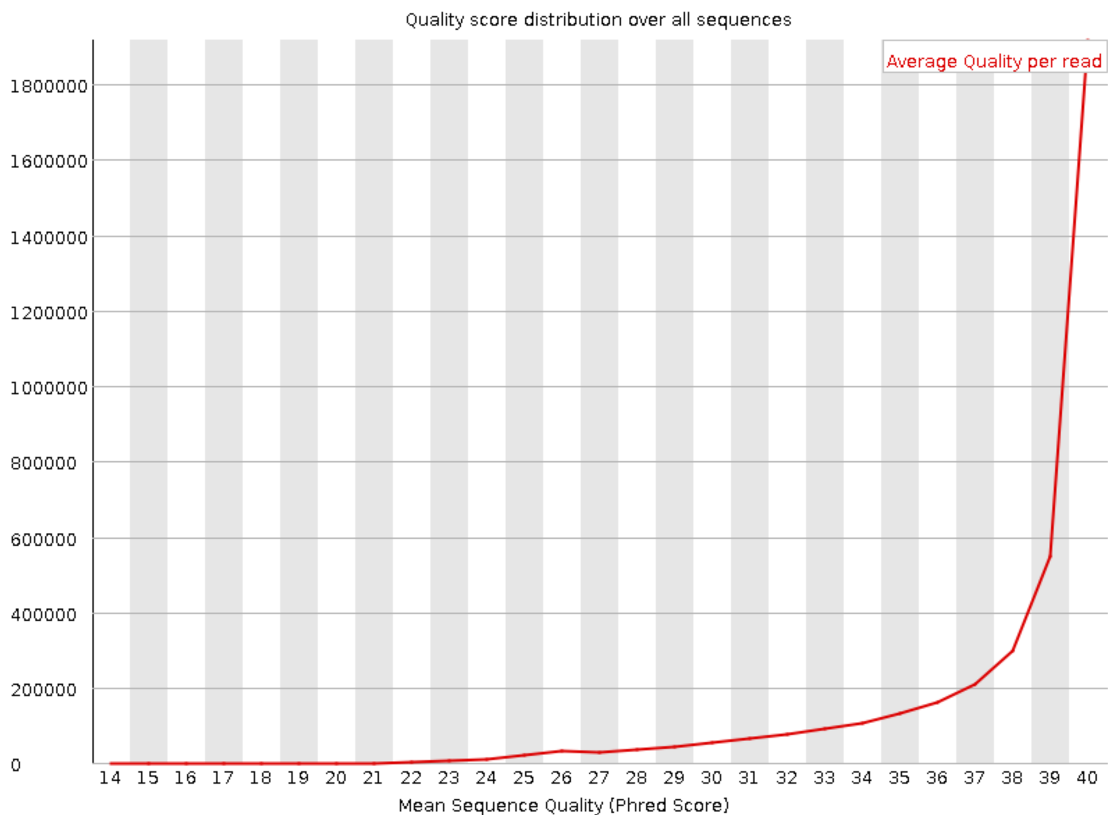

Figure 4.2: Quality Score Distribution Over All Sequences Results as Analyzed with Fastqc Software.

4.3 Prevalence of *P. falciparum* Antimalarial Resistance Polymorphisms

4.3.1 *Pfcrt*

The *Pfcrt* gene K76**T** marker for CQ resistance was observed in 18.52% of the Lake Victoria isolates, 16.67% of East Africa isolates, and 13.21% of West Africa isolates. Three additional mutations, A220**S**, Q271**E,** and R371**I,** were observed in low frequencies in Lake Victoria isolates, 14.81%, 11.11%, and 3.70%, respectively (**Table 4.1**): East Africa isolates, 15.35%, 16.23%,17.54%, and in West Africa isolates, 12.58%, 12.58%, and 11.95% **(Figure 4.3).** The wildtype haplotype CVMNK corresponding to codons 72-76 of *Pfcrt* was observed in high frequencies of 81.48 % in the Lake Victoria isolates **(Table 4.1).** None of the Lake Victoria samples analyzed harbored the SVMNK, SVIET, and CVTNT haplotypes.

Table 4.2: Single Nucleotide Polymorphisms of *Pfcrt, Pfmdr1* and *Pf-k13* Genes

Gene	Codon position	Reference Amino acid sequence	Mutant Amino acid sequence	Total number of samples %
Pfcrt	76	K	T	5/27 (18.52)
	220	A	S	4/27 14.81)
	244	Q	E	3/27 (11.11)
	271	R	I	3/27 (11.11)
	371	D	Y	1/27 (3.70)
Pfmdr1	184	Y	F	13/27 (48.15)
	255	N	K	1/27 (3.70)
	651	D	N	2/27 (7.41)
	1246	D	Y	2/27 (7.41)
Pf-k13	189	K	T	7/27 (25.93)
	578	A	S	1/27 (3.70)

4.3.2 *Pfdhps &Pfdhfr*

Pfdhfr gene mutations associated with SP resistance were observed at varying frequency levels for each allele. The N51**I** mutation was observed at high frequencies of 96.3% (n = 26) in Lake Victoria isolates **(Table 4.2)** and other studied regionsat 93.42% and 92.45% in East Africa and West Africa isolates, respectively. The C59**R** mutation was observed at high frequencies 77.8%, 86.84%, and 93.08% in Lake Victoria, East Africa, and West Africa isolates, respectively. The S108**N** mutation was observed at low frequencies in Lake Victoria isolates, 7.41% compared to 99.56% of East Africa and 93.08% of West Africa isolates. Mutation I164**L** was observed in low frequencies (Lake Victoria = 7.41% and East Africa = 2.19%), while no isolates were observed in the West Africa population **(Figure 4.3)**. The triple mutant haplotype IRN of *Pfdhfr* gene was observed at high frequency, 70.37% compared to the double mutant ICN haplotype and the triple mutant haplotypes IRS

and NRN observed at low frequencies, 22.22%,3.70%, and 3.70%, respectively. None of the isolates carried the wildtype haplotype NCS **(Table 4.4).**

The K540**E** mutation on *Pfdhps* linked to sulfadoxine resistance was observed at low frequencies in Lake Victoria and West Africa populations, 14.81% and 29.56%, respectively, compared to the high frequency observed in the East Africa population, 85.53%.

Pfdhps mutant haplotypes SAEAA, HAEAA, and SAEAA, were analyzed in the Lake Victoria isolates. The double mutant *Pfdhps* haplotypes SAEAA and HAEAA were observed at frequencies of 74.01% and 7.41%, respectively. A single mutant haplotype SAEAA was observed in 7.41% of the isolates **(Table 4.3).**

Table 4.3: Single Nucleotide Polymorphisms of *Pfdhps* and *Pfdhfr* Genes

Gene	**Codon position**	**Reference amino acid sequence**	**Mutant amino acid sequence**	**Total number of samples %**
Pfdhps	436	S	H	2/27 (7.41)
	540	K	E	24/27 (88.89)
	581	A	G	1/27 (3.70)
Pfdhfr	51	N	I	26/27 (96.30)
	59	C	R	21/27 (77.78)
	108	S	N	26/27 (96.30)

Table 4.4: Haplotype Frequencies for *Pfcrt*, *Pfdhps*, and *Pfdhfr* Genes

Gene	Haplotype	Type	Haplotype frequency, n (%) (n=27)	Chi-square score, p-value
Pfcrt	CVMNK	Wildtype	22(81.5)	21.41, $p < 0.00001$
	CVMNT	Single mutant	5(18.5)	
Pfdhps	SAEAA	Double mutant	20(74)	38.37, $p < 0.00001$
	HAEAA	Double mutant	2(7.4)	
	SAAEA	Single mutant	2(7.4)	
Pfdhfr	IRN	Triple mutant	19(70.4)	42.81, $p < 0.00001$
	ICN	Double mutant	6(22.2)	
	IRS	Triple mutant	1(3.7)	
	NRN	Triple mutant	1(3.7)	
	NCS	Wild type	0(0)	

4.3.3 *Pf-k13*

Mutations on *Pf-k13* associated with artemisinin delayed clearance were not recorded for all populations. However, we observed three key mutations previously reported across Africa, K189**T**, R255**K,** and A578**S**. The K189**T** mutation was observed in 25.93% of Lake Victoria isolates (**Table 4.2**), 11.84% of East Africa isolates, and 34.59% of West Africa isolates. In contrast, mutation R255**K** was observed at low frequencies, 7.41%, 2.63%, and 6.29% in Lake Victoria, East Africa, and West

Africa isolates. The A578S mutation was not observed in West Africa isolates but in very low frequencies of 3.70% and 1.32% in Lake Victoria and East Africa isolates **(Figure 4.3).**

4.3.4 *Pfmdr1*

Mutation D1246**Y** of *Pfmdr1* gene associated with chloroquine resistance and lumefantrine delayed clearance(Humphreys *et al.*, 2007) was observed in low frequencies (Lake Victoria = 7.41%) **(Table 4.2)** (East Africa = 11.40%) and (West Africa = 31.45%). The N86**Y** and Y184**F** associated with altered invitro parasite drug sensitivity to AQ and AL were observed at varying frequencies. The N86**Y** mutation was not observed in Lake Victoria isolates, while East Africa and West Africa populations occurred at 12.72% and 24.53%, respectively. Mutation Y184**F** was observed at relatively high frequencies in Lake Victoria isolates, 44.44% compared to 37.28% and 15.72% in East and West Africa isolates **(Figure 4.3).**

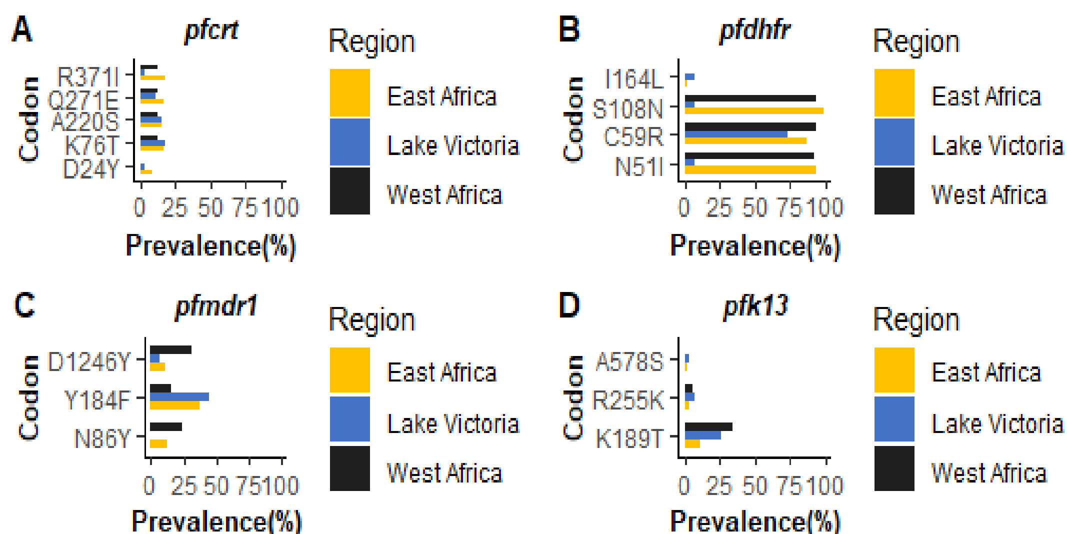

Figure 4.3: Mutation Frequencies of P. Falciparum Genes Associated With Drug Resistance in Lake Victoria (n=27), East Africa (except Kenya) (n=228) , and West Africa (159) Isolates.

4.4 Mixed Gene Mutations

Analysis of Lake Victoria isolates with completely defined haplotypes for mixed gene mutations revealed that some *P. falciparum* parasites carried quadruple gene mutations across *Pfcrt*, *Pfmdr1*, *Pfdhps,* and *Pfdhfr* genes. These were characterized by *Pfcrt* haplotypes CVMNK/T for codons 72 to 76, *Pfmdr1* NFY, NYY, NYD, and NFD for codons 86, 184, and 1246, *Pfdhps* and *Pfdhfr* haplotypes IRNE, ICNE, IRSE, and NRNE for codons 51,59, 108 and 540 respectively**(Table 4.5)**

Table 4.5: Occurrence of *Plasmodium Falciparum* Isolates Carrying Multiple Mutations Across Four Genes Associated With Drug Resistance.

		Number of isolates with:				
Pfcrt **Haplotypes**	***Pfmdr1*** **haplotypes**	**Double gene mutation (*Pfcrt*+ *Pfmdr1* only)**	**Quadruple gene mutation (*Pfdhfr*+*Pfdhps*)**			
			IRNE	ICNE	IRSE	NRNE
CVMNK	NFY	1(3.70%)	1(3.70%)	0(0%)	0(%0)	0(%0)
	NYY	1(3.70%)	1(3.70%)	0(0%)	0(%0)	0(%0)
	NYD	10(37.04%)	6(22.22)	1(3.7)	0(%0)	0(%0)
	NFD	10(37.04%)	9(33.33)	1(3.7)	0(%0)	0(%0)
CVMNT	NFY	2(7.41%)	0(0%)	1(3.7)	0(%0)	0(%0)
	NYY	0(0%)	0(0%)	0(0%)	0(%0)	0(%0)
	NYD	3(11.11%)	1(3.70%)	2(7.4)	0(%0)	0(%0)
	NFD	2(7.41%)	1(3.70%)	0(0%)	0(%0)	1(3.70%)

* All allele mutations are underlined. The *Pfcrt* haplotypes CVMNK and CVMNT correspond to codons 72 to 76, while *Pfmdr*1 NFY, NYY, NYD, and NFD haplotypes correspond to codons 86, 184, and 1246. The combined *Pfdhfr* and *Pfdhps* haplotype IRNE, ICNE, IRSE, and NRNE correspond to codons 51, 59, 108, and 540, respectively.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Despite efforts by WHO to eradicate malaria, the emergence and widespread of *P. falciparum* resistance to antimalarial drugs have drastically hindered the control, prevention, and total elimination of this scourge (Ljolje *et al*., 2018; WHO, 2016). Over the decades, several antimalarial drugs have been withdrawn as the first line of treatment due to parasite acquiring resistance. Recent cases of delayed parasite clearance after treatment with ACTs have been reported in Southeast Asia and Sub-Saharan Africa. (Noedl *et al*., 2008; Suresh and Haldar, 2018; Uwimana *et al*., 2021). The emergence of parasites tolerant to artemisinin and ACTs indicates the field's spread and filtering of ACT-resistant strains. Studies have provided evidence of multidrug-resistant parasites lineage spread from Southeast Asia across Sub-Saharan Africa by carrying known markers in *Pfcrt, Pfmdr1, Pfdhfr, Pfdhps Pf-k13* genes (Achieng *et al*., 2015; Lim *et al*., 2009; Noedl *et al*., 2008). Surveillance with these molecular markers allows early detection of antimalarial drugs resistance, susceptibility, and temporal trends in the spread of malaria resistance. This information provides fundamental data for drug policy and treatment strategies of *P. falciparum* malaria infections (Vestergaard and Ringwald, 2007).

Since the withdrawal of chloroquine as a treatment for uncomplicated malaria two decades ago, the prevalence of *P. falciparum* resistance alleles has declined moderately in most malaria-endemic countries (Lu *et al*., 2017). This decline has been attributed to low drug pressure on the parasite over the years (Mwai *et al*., 2009; Ocan *et al*., 2019). This study revealed the persistence of the K76T CQ resistance marker in 18.51% of Lake Victoria isolates and East Africa populations at 16.67%. These findings add to the body of evidence for a fitness cost in CQ-resistant *P. falciparum* in several African countries that have seen the return of widespread CQ-sensitive *P.falciparum* strains(Frosch *et al*., 2014; Konaté *et al*., 2018; Mulenga *et al*., 2021). It is anticipated that chloroquine could be re-introduced for malaria

treatment across Africa when the drug pressure on the parasite is eradicated. However, the slow diminishing rates of the CQ resistance markers suggest continued illegal sale and use of chloroquine for malaria treatment or otherwise suggest parasite's low fitness cost associated with maintaining this mutation.

Previous studies indicated that lumefantrine selects wildtype alleles in codon K76 and N86, D1246 of *Pfcrt* and *Pfmdr1,* respectively (Achieng *et al*., 2015; Malmberg *et al*., 2013) Invivo studies have also shown drug pressure-induced directional selection of *Pfmdr1* 86Y, Y184, and 1246Y for amodiaquine and N86, 184F, D1246 for AL. The current study revealed a high prevalence of N86 wildtype allele (100%) as well as mutations Y184F (44.44%) and D1246Y (7.40%). The findings can be attributed to the continued use of artemether lumefantrine as the first line of treatment for uncomplicated malaria in these regions.

Sulfadoxine pyrimethamine is widely used for intermittent preventive treatment (IPTp) due to the lack of drugs available for safe use in pregnant women and infants under five years of age (Iriemenam *et al*., 2012). This study revealed polymorphisms on *Pfdhfr*; N51I (7.40%), C59R (74.07%), S108N (7.40%), and *Pfdhps*; K540E (14.81%) in Lake Victoria isolates. However, these markers' were also observed in East Africa isolates, and West Africa isolates, suggesting that the continued use of SP as an IPTp does not come at a high fitness cost to *P.falciparum* parasite and the high drug pressure to maintain these polymorphisms(Conrad *et al*., 2017).

Resistance to artemisinin and ACTs manifest as reduced susceptibility and delayed parasite clearance after treatment with the recommended dose of the drug (Ljolje *et al*., 2018; Suresh and Haldar, 2018). This mechanism is attributed to the mutation of the *Pf-k13* gene on the kelch 13 propeller protein domain on chromosome 13 of *P. falciparum.* Over the last decade, there has been the emergence and spread of ART resistance in Cambodia, India, and Myanmar. This emergence has been attributed to the decline of *Pf-k13* wildtype alleles; thus, it is purported that mutations on the kelch gene could prove as valuable markers for tracking ACTs resistance (Ariey *et al*., 2014b). Studies have suggested that C580Y, Y493H, and R539T mutant alleles could be the markers for ACT resistance(Achieng *et al*., 2015; Ariey *et al*., 2014b;

Ljolje *et al*., 2018). This study reports two mutations: K189T (25.92%) and A578S 3.70% on the *Pf-k13* gene. The A578S and K189T allele are of interest due to their prevalence in countries spanning from east and central Africa to western Africa and could be potential resistance markers (Kamau *et al*., 2015). The findings from this study concur with previous studies conducted in Kenya (Osborne *et al*., 2021), that predict the slow return of chloroquine sensitive parasites in Malaria endemic regions of western Kenya as well as the emergence of SP resistant parasites. This is attributed to the withdrawal of chloroquine in the Kenyan market as well as the continued use of SP for IPTp treatment. The presence of IRN-SEAA haplotype previously associated with SP resistance(Lin *et al*., 2020), highlights the importance of monitoring parasite populations in these regions for the increase of existing and novel resistance polymorphisms that could compromise the effiacy of pregnancy malaria treatment in Kenya and Africa at large.(Osborne *et al*., 2021)

The data obtained from this study, a high number of whole-genome sequenced samples and analysis with next-generation sequencing tools would increase the robustness of our results.

5.2 Conclusion

The study provides baseline data on antimalarial resistance markers in the selected regions on Lake Victoria, Kenya. Analysis of resistance markers in other malaria endemic regions in East and West Africa revealed a similar pattern of resiatnce markers prevalence suggesting a balancing selection across these regions. While our findings suggest a favorable susceptibility profile to ACTs, caution is warranted due to the limitations of the study, including its cross-sectional design and the dynamic nature of parasite populations. Continued surveillance efforts are essential for monitoring changes in resistance patterns and informing malaria control strategies in the region.

5.3 Recommendations

1. This study revealed a low proportion of CQ resistant parasites strains. It is however unclear whether this is as a result low CQ drug pressure of whether

the low resistance could be attributed the use of other antimalarial drugs. This study recommends further investigation into the factors contributing to this effect.

2. There is need for investigation into the temporal trends of antimalarial drugs resistance markers in the selected islands in Lake Victoria, Kenya and other malaria endemic regions in East and West Africa.

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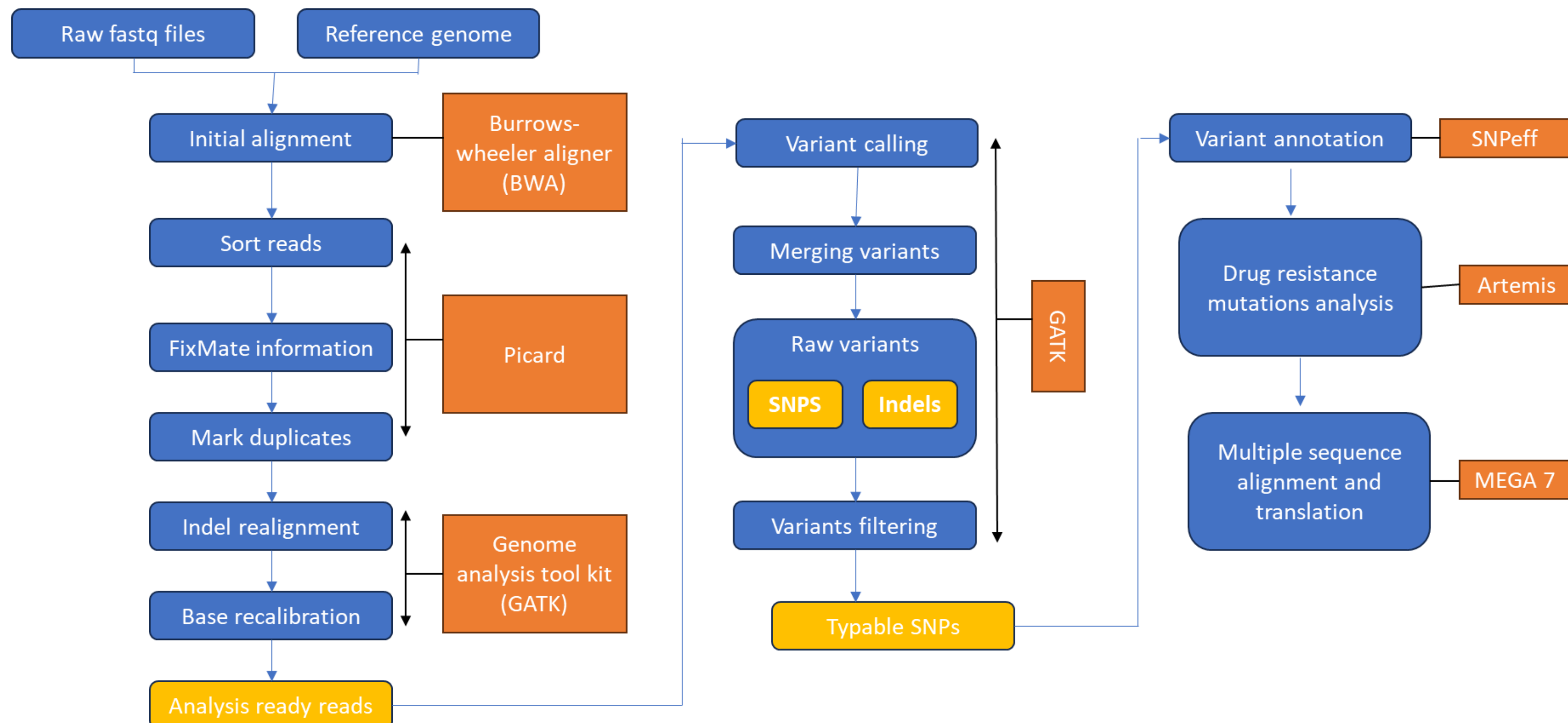
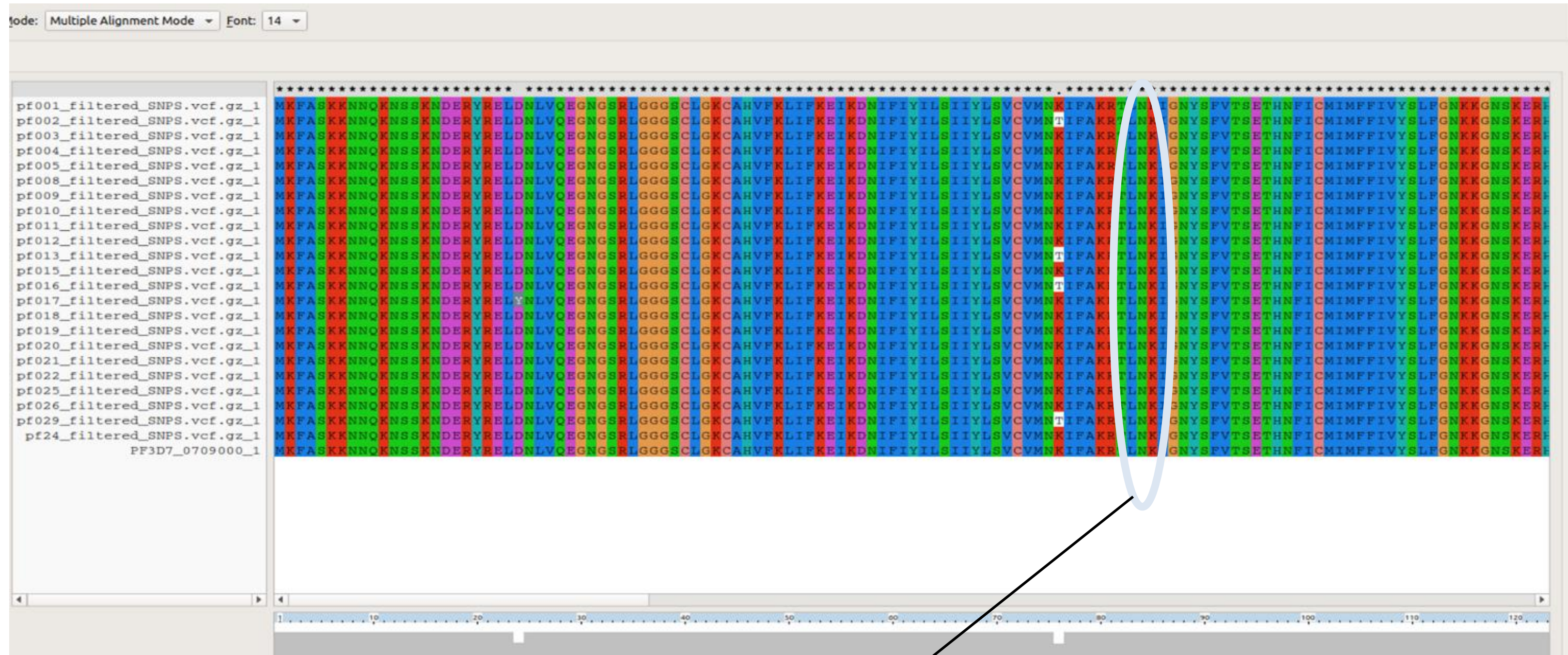
APPENDICES

Appendix I: Variant Calling Pipeline

Diagram showing the analysis of raw reads to generate typable variants. The blue boxes indicate the analysis steps, yellow boxes indicate the output, and the orange boxes indicate the software and tools used for analysis.



Appendix II: Multiple Sequence Alignment Showing Mutations on Pfcrt gene.



K76T mutation

Appendix III: Publication

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Limited genetic variations of the Rh5-CyRPA-Ripr invasion complex in *Plasmodium falciparum* parasite population in selected malaria-endemic regions, Kenya

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