UNIVERSITY OF RWANDA

ADDRESSING THE SNPs ASSOCIATED WITH MALARIA TREATMENT FAILURE OF KING FAISAL HOSPITAL PATIENTS.

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ADDRESSING THE SNPs ASSOCIATED WITH MALARIA TREATMENT FAILURE OF KING FAISAL HOSPITAL PATIENTS.

By

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Dr. Edgar KALIMBA

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Declaration of the independent work

I, MUKANDAYISENGA Esther, hereby declare that this research project submitted to the University of Rwanda, for the degree Master of Science in Biotechnology is my own original work and has not been submitted before to any institution by myself or any other person in fulfilment of the requirements to the award of any degree or any other qualification.

Signature:  **Date:** 18/09/2025

Student name: MUKANDAYISENGA Esther

Dedication

First, I thank Almighty God for the gift of life and for sustaining me throughout this journey.

I dedicate this work to:

My beloved husband for granting me the opportunity to pursue my master's studies, for his constant encouragement, his help made my journey possible.

My beloved children for patiently stay at home and took care of it in my absence.

My lecturers who shared knowledge and worked closely with me throughout my academic journey.

Many Thanks to my supervisors for guidance and support, especially in providing the necessary resources to complete the study.

I am sincerely thankful.

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May God bless you.

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Pfcrt: Plasmodium falciparum chloroquine resistance transporter

Pfmdr1: Plasmodium falciparum multidrug resistance 1

BC: Before Jesus Christ

ACTs: Artemisinin-based combination therapies

RBC: Rwanda Biomedical Centre

(CQ): chloroquine

PCR: Polymerase chain reaction

SNPs: Single Nucleotide Polymorphisms

PfK13: *Plasmodium falciparum* Kelch13

KFH: King Faisal Hospital

ONT: Oxford nanopore technology

THF: tetrahydrofolate

DNA: Deoxyribonucleic Acid

DHA-PPQ: Dihydroartemisinin-Piperaquine

Abstract

Malaria is a major health burden nationally and regionally, especially in sub-Saharan Africa which experiencing more than 94% of cases and 95% of deaths attributed to malaria, and *Plasmodium falciparum* being the most lethal parasite species. Rwanda, continues to have a high transmission of malaria despite the national commitment and malaria control programs. The major cause of this failure to malaria control strategies includes the Resistance of *Plasmodium falciparum* to antimalarial drugs, affecting more treatment programs globally including the use of Artemisinin-based combination therapies (ACTs). There is therefore an urgent need to improve the control strategy.

This study focused on addressing Single Nucleotide Polymorphisms (SNPs) associated with malaria treatment failure. *P. falciparum*, the deadliest malaria parasite species, and the gene mutation associated with it, specifically in the *Plasmodium falciparum Kelch 13* (*PfK13*), *Plasmodium falciparum* chloroquine resistance transporter *(Pfcrt*) and *Plasmodium falciparum* multidrug resistance 1(*Pfmdr1*) genes were studied. These genetic markers are widely implicated in resistance to drugs like chloroquine, lumefantrine, and amodiaquine. In Rwanda, as many other countries adopted ACTs as the first line therapy but the presence of mutation associated with *PfK13* and *pfmdr1* caused reduced treatment efficacy of ACTs. In this study, validation of oxford nanopore technology (ONT) findings by sanger sequencing were performed. In total 64 samples (collected in 2024) were sequenced by ONT and validated by sanger sequencing. Results obtained revealed the concordance of results to be 92.05 % and Cohen's Kappa k=0.479, chi-square test (x^2 = 25.000), $p < 0.001$ confirms concordance and the Pearson Chi-Square test also confirms a strong association of the ONT and Sanger sequencing and finally the Likelihood Ratio Test confirms the concordance (x^2 = 8.397, p = 0.015).

Next, another cohort of 43 samples (collected in 2025) were analyzed for prevalence, 36/43(83.7%) of samples presented SNPs associated with malaria treatment failure, *PfK13*-R561H (51.2%) and *PfMDR1*-Y184F (60.5%) were the most prevalence SNPs identified**.** These mutations compromise rapid malaria parasites clearance thus causing ACT failure and plays a role in multidrug resistance and reduce the efficacy of Artemether-Lumefantrine (Coartem).

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Chapter 1. Introduction

1.1 . Background

Malaria, the vector borne infectious disease of human and other primates, caused by mosquito originating from the protozoan of the genus plasmodium. The disease causes symptoms that typically include fever, chills and headache; in severe cases can progress to coma or death. Five *plasmodium* species cause clinical malaria in human (*P. falciparum, P. vivax, P. Ovale, P. malariae, and P. Knowless*). *P.falciparum* infection causes the greatest threat due to factors such as its severity, high mortality rate, resistance to treatment and elevated transmission rate(Alruwaili *et al*., 2025a). In1880 Charles Louis Alphonse Laveran first identifies the malaria parasite; he is awarded the 1907 Nobel Prize for the discovery. In 1898 Sir Ronald Ross demonstrates that mosquitoes transmit malaria, he wins the 1902 Nobel Prize for this work. In1934 Hans Andersag in Germany discovers the anti- malarial drug chloroquine, which is not widely used until after World War II, he won the Nobel Prize for this work in 1948. In1952 malaria was eliminated in the United States.1955 World Health Organization (WHO) launches Global Malaria Eradication Campaign, which excludes sub-Saharan Africa and is eventually abandoned.1957 First documented case of resistance to chloroquine was reported. 1976 William Trager and JB. Jensen grow malaria parasites in culture for the first time, opening the way for drug discovery and vaccine researches.1989 The United States. Food and Drug Administration approves the use of the anti-malaria drug mefloquine, hydrochloride, registered as Lariam® by Hoffman-LaRoche (Nosten *et al.*, 2022).

1.2. Malaria epidemiology.

Despite the control measures, malaria continues to be a critical global health concern, especially in tropical and subtropical regions (Yutura *et al*., 2024). According to the World Health Organization (WHO) World malaria report 2023, In 2022 malaria estimated on 249 million malaria cases worldwide, an increase from 247 million in 2021. The global malaria death toll stood at approximately 608 deaths, with a majority of these fatalities occurring in sub-Saharan Africa. Figure 1, Highlight, 70% of the global malaria burden is concentrated in 11 countries: India, Burkina Faso, Cameroon, Democratic Republic of the Congo, Ghana, Mali, Mozambique, Niger, Nigeria. Africa region bears the highest burden of malaria, accounting for 94% (233 million) of all cases, more than 50% of all deaths occurred in just four countries; Nigeria (31%), the Democratic Republic of the Congo (12%), Niger (6%), and Tanzania (4%) (Li *et al*., 2024) and 95% (58000) of malaria deaths of which 80% of this

death occur in children under the age of five years (WHO, 2023; Li *et al*., 2024; Nchang *et al.*, 2023).

1.2.1. Worldwide *plasmodium falciparum* incidence

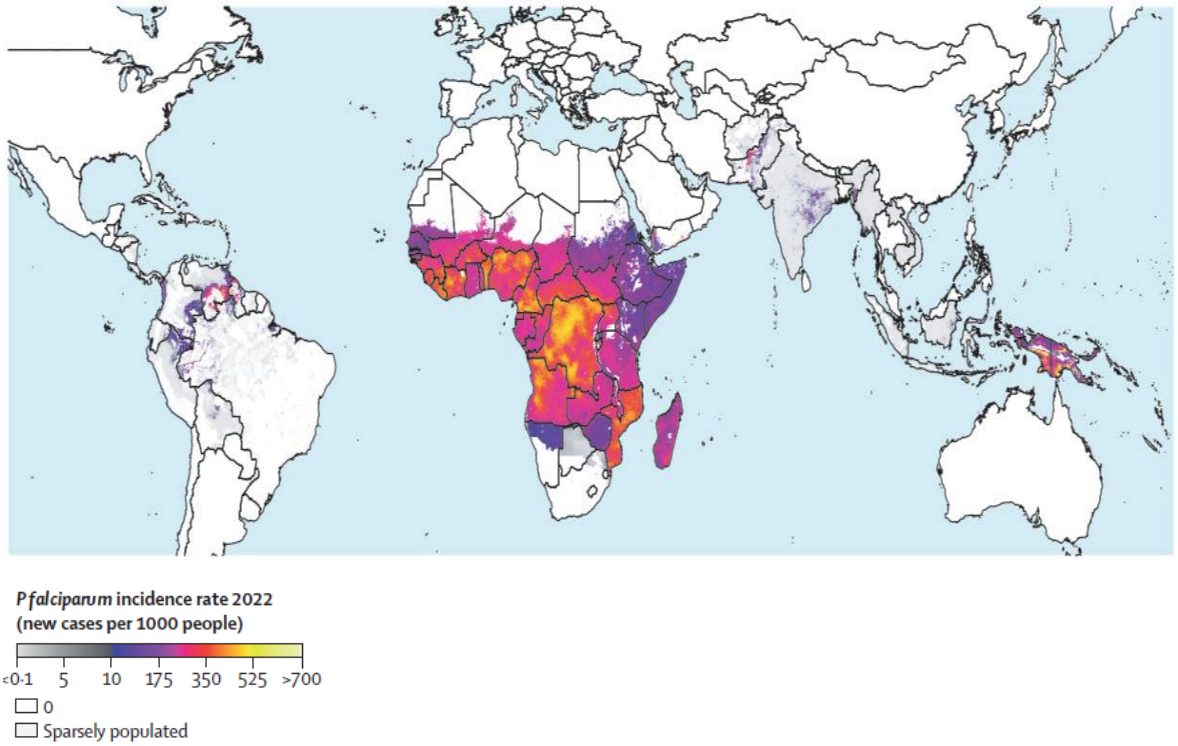

Figure 1. *Plasmodium falciparum* incidence (Tripathi *et al.*, 2023).

Several challenges were reported to hinder malaria response efforts, including "limited health-care access, conflicts and emergencies, the continuing effects of COVID-19 on service delivery, inadequate funding, and uneven implementation of core malaria interventions." Of the 88 malaria-endemic countries that provided data, 78 have noted vector resistance to at least one insecticide class. Climate change and extreme weather events such as flooding and heatwaves are linked to malaria outbreaks and increased transmission *was* highlighted as a substantial risk to progress against malaria (Venkatesan, 2024).

Unless Rwanda showed a decline in malaria incidence, 345 cases per 1000 persons reported in 2018, decreased in 255 in 2019,165 in 2020,97in 2021 ,58 in 2022 and the incidence become 40 in 2023. The positive progression represented on 88% of reduction even though malaria cases more increased in eastern and southern province of Rwanda followed by Kigali city where persistent is reported (Umugwaneza *et al*., 2025).

1.2.2 Malaria burden in the region

In Rwanda, malaria remains a leading cause of illness and death and affect all kind of people, despite control measures putted in place and supported by Presidents Malaria Initiative, WHO and Global Fund to fight against malaria which include indoor residual spraying (IRS), home-based malaria management by community health workers (CHWs), insecticide-treated nets (ITNs) and introduction of Artemisinin-based Combination Therapies (ACT)(Umugwaneza *et al*., 2025**),** still drug resistance is a major challenge for malaria control. *Plasmodium falciparum* is the most predominant species in Rwanda, account for 97% of all malaria cases and being the most severe and potentially fatal if not promptly treated (Sato, 2021)**.** The resistance of *p. falciparum* to many drugs such as chloroquine (CQ), sulfadoxine, and pyrimethamine (SP) are associated with Single nucleotide polymorphisms in *P. falciparum* genes. Key polymorphisms include *pfcrt*- K76T for chloroquine resistance, *pfkelch 13*-R561H mutations for artemesinin, *dhfr* mutations for sulfadoxine- pyrimethamine resistance, *pfatp6* for quinine resistance and *pfmdr1* mutations for resistance to both mefroquine and lumefantrine especially in a combination with arthemeter (Jalei *et al.*, 2023). *Pfcrt* -H97Y, F145I, M343L, or G353V genes mutations confer resistance to Piperaquine (PPQ) (Baina *et a*l., 2024).

Malaria infection was associated with socio-economic status and low altitude(Umugwaneza *et al*., 2025). The emergence and spread of parasite resistance against available antimalarial constitutes a major threat towards the efforts of the elimination program, artemisinin resistance was first identified in Cambodia and spread quickly at different foci in countries of Greater Mekong sub region (Laos, Myanmar, Thailand, and Vietnam) causing high rates of ACT treatment failure (Uwimana *et al.*, 2020).

Plasmodium falciparum artemisinin partial resistance in the African region is of great concern, as are increasing reports of anopheles species resistance to pyrethroid insecticides(Venkatesan, 2024**)**. The *pfk13* artemisinin partial resistance mutations continue to increase in prevalence for East African countries with the overall level of mutant infections reaching 32% in Rwanda highlighting the urgent need for ongoing surveillance and intervention measures to prevent the spread and impact of these resistant strains (Schreidah *et al*., 2024).

1.3. Malaria transmission

Management of malaria transmission remains complicated due to multiple interaction of factors. The mosquito capacity to adapt to the environment like temperature and humidity is facilitated by their sensitivity to such conditions. The study conducted in Kenya confirms that mosquito feeding behavior and transmission can shift over time. The Figure 2 illustrates how malaria infection starts when an infected female mosquito bites a human, injecting plasmodium parasites through its saliva into bloodstream (Savi, 2023).

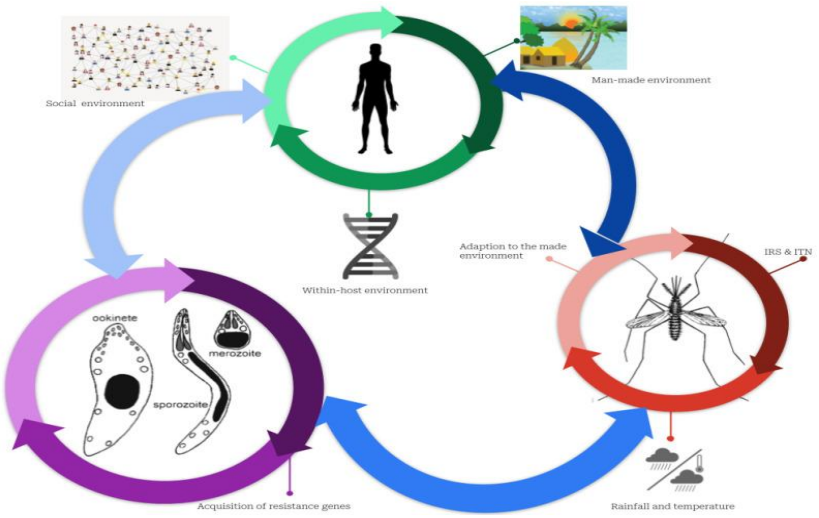

Figure 2.Malaria transmission cycle and interaction of three subsystems (Savi, 2023).

Malaria transmission can vary by how the hosts physiology or behavior attract mosquito vectors. Malaria transmission is sustained by interaction between three subsystems, humans, mosquitoes and plasmodium parasites. Human subsystem is determined not only by biological factors like genetic factors, housing, social behaviors and health care behavior, where the mosquito subsystems determined by the respond to intervention put in place to eliminate them such as Insecticide treated nets(ITNs) and indoor spraying(IRS) and by adaptation to environment. The parasite subsystem have ability to protect its self through the development of resistance to antimalarial drugs (Savi, 2023)and this support the adaptability of *p. falciparum* to various transmission settings. *Anopheles Gambiae* is the primary anopheles that is known to transmit malaria (Alruwaili *et al.*, 2025b)**.**

1.4. Malaria life cycle

Malaria parasite life cycle is a complex cycle divided into different cycles, sporogonic cycle (in Mosquito also called the sex cycle) and in Human (exoerythrocytic and erythrocytic cycles-the asexual cycle). The cycle is continuous, sporogonic cycle starts when the anopheles' mosquito bites an infected human and ingests gametocytes of *Plasmodium* in mosquito's gut, the male and female gametocytes fuse together to give a zygote which develops into ookinete and this form the oocyst in the gut of mosquito. The oocyst produces sporozoites and they migrate to the mosquito salivary gland. In human, the infected mosquito bites a human and injects sporozoites into blood stream, then they travel to the liver invading liver cells and multiply into schizonts, these burst and release merozoites which causes symptoms like fever and anemia. Some merozoites develop into gametocytes which are taken again by mosquito and make the continuous cycle (Figure 3)(Dejen Nueye, 2021);(Tripathi *et al*., 2023).

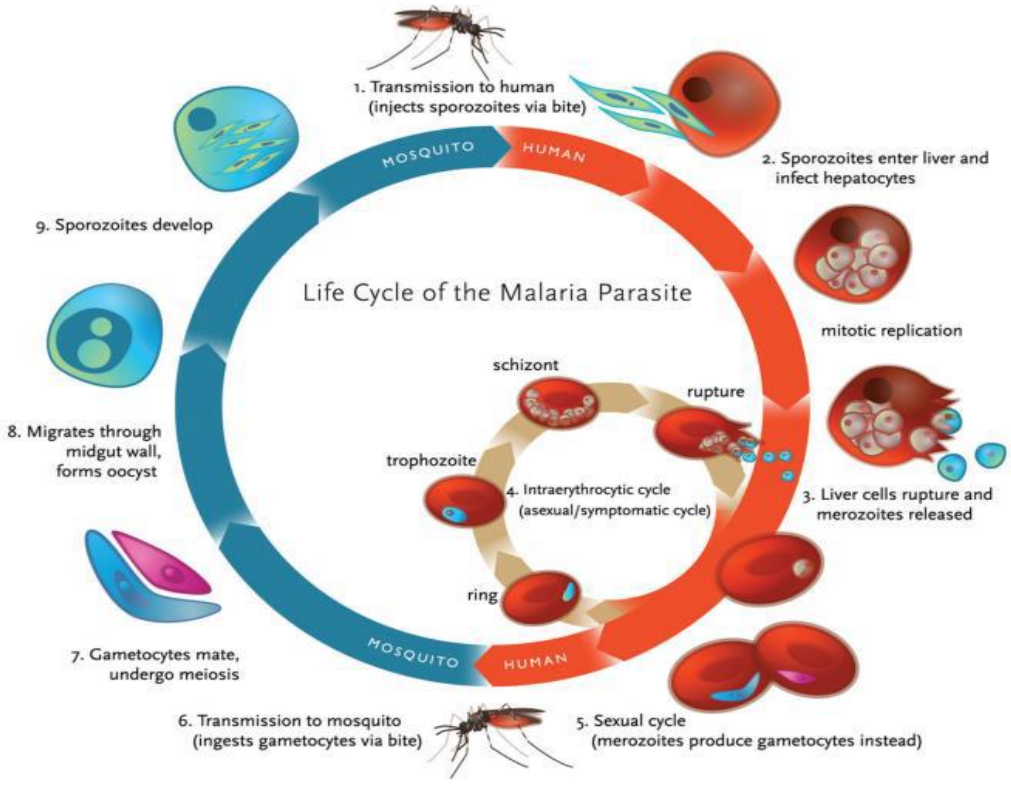

Figure 3.Malaria life cycle (Dejen Nueye, 2021).

1.5. Antimalarial treatment and drug resistance mechanisms

1.5.1. Current anti malaria drug in Rwanda

Artemesinin based combination therapy (ACT) is a treatment for malaria that combines an artemisinin and its derivatives (such as artesunate, artemether, or dihydroartemisinin) with one or more other antimalarial drugs called partner drugs (lumefantrine, mefloquine, amodiaquine, and piperaquine). WHO recommends Artemesinin bases combination therapy as the successful treatment in controlling malaria. Artemesinin is extracted in Chinese sweet worm wood Artemesia annua with its derivatives artemether,artesunate and dihydroartemisinin acts rapidly and contain unique endoperoxide (Wicht *et al*., 2020a).
They are the most effective antimalarial currently available, effective in rapidly reducing the number of Plasmodium parasites in the blood. Artemisinin is administered in combination with partner drugs (lumefantrine, piperaquine, mefloquine,amodiaquine and pyronaridine) due to its short half-life. A partner drug refers to the non-artemisinin component of Artemisinin Combination Therapy (ACT). The figure 4 illustrates ACT is combined with an artemisinin derivative to enhance treatment efficacy and reduce the risk of resistance development. It works synergistically with the artemisinin derivative to clear remaining parasites. After the artemisinin derivative rapidly reduces the parasite load, the partner drug helps to eliminate any residual parasites preventing resistance (Geneva: World Health Organization, 2022).

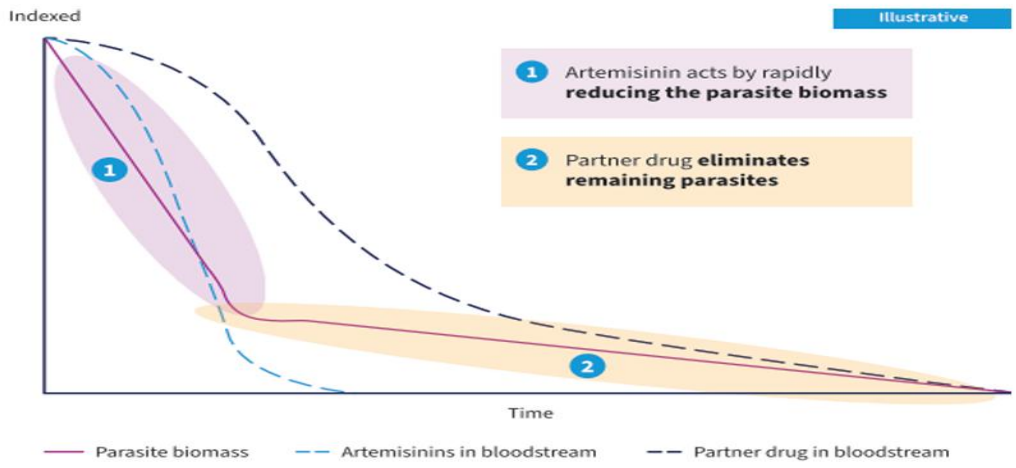

Figure 4. Evolution of parasite biomass in the body following ACT administration (Geneva: World Health Organization, 2022).

Artemether-lumefantrine, often known as Coartem, is still the first-line treatment for uncomplicated malaria caused by *Plasmodium falciparum,* according to the most recent

malaria treatment guidelines published by the Rwanda Biomedical Center (RBC) and the Ministry of Health incorporated new treatment include dihydroartemisinin–piperaquine (DHAP) and artesunate–pyronaridine (AS-PY) into the national treatment policy. Since their official introduction into public healthcare settings in april 2025, these medications have increased treatment alternatives and expected to decrease selective drug pressure, which might hasten the development of resistance (America, 2025;WHO, 2023)**.**

1.5.2. *Plasmodium falciparum* genes and molecular mechanisms resistance

The Resistance mechanism associated with ACT is caused by certain single nucleotide polymorphisms in the *pfk13* gene, and it was first identified in Southest Asia. Mutations conferring resistance and/or delay in parasite clearance have been observed in East Africa, including Rwanda, since 2010**.** *Pfk13*-R561H,pfk13-C469Y, and *pfk13* -A675V genotypes were observed in Rwanda and said to be associated with slow parasitemia in vivo and reduced vulnerability in Vitro (Alruwaili *et al*., 2025a).

Drug-resistant genes in malaria parasites are genetic mutations that confer resistance to multiple antimalarial drugs. These genes can reduce the effectiveness of treatment and complicate efforts to control and eliminate malaria. Some key multidrug-resistant genes in *Plasmodium falciparum,* the deadliest malaria parasite, include: *Pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter): Mutations encoding amino acid substitutions in the chloroquine resistance transporter *PfCRT* (PF3D7_0709000) at positions 72, 74, 75, 76,97, 220, 271, 326, 356 and 371 have been associated with increased resistance to chloroquine (CQ), with the K76T substitution being the primary mediator of resistance. Evidence suggests that mutated *PfCRT,* particular K76T mutation confers chloroquine resistance by allowing enhanced efflux of chloroquine from the parasite`s digestive vacuole, thereby reducing the concentration of drug available to bind target heme (Amambua-Ngwa *et al.*, 2023).

The resistance to treatment arise due to specific point mutations in *pf* genes and interfere with treatment action through inhibition of transport, changed metabolism and treatments targets change (Wicht *et al*., 2020a)**.**

Pfcrt, pfmdr1, pfk13 play many roles in mediation of resistance. The mechanisms of resistance include digestive vacuole drug efflux, digestion of hemoglobin cause impaired treatment activation and enhance parasite survival (Wicht *et al*., 2020b). The table 1 summarize *pf* genes and mechanisms of resistance to malaria treatment.

Antifolate resistance has been associated with point mutations in the dihydrofolate reductase

(*Pfdhfr)* gene and dihydropteroate synthetase(pfdhps) gene. *Pfdhfr* catalyzes the reaction of dihydrofolate (DHF) to tetrahydrofolate (THF), essential for deoxyribonucleic acid (DNA) synthesis and replication in the parasites. Antimalarial like pyrimethamine and proguanil inhibit *Pfdhfr* by blocking the production of THF, leading to the parasite`s death. Point mutations at the codons 16,51,59,108, and 164 of *Pfdhfr* alter the enzyme`s active site, decreasing the efficacy of pyrimethamine while mutation at 436,437,540,580 and 613 of *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps)* reduce the substrate binding capacity and confer resistance to Sulphadoxine (Patel *et al*., 2017). Mutation in *Plasmodium falciparum Kelch 13* (*Pfkelch13*) gene of *Plasmodium falciparum* are associated with artemisinin resistance, a critical challenge in malaria treatment. These mutations enhance the parasite's ability to manage oxidative stress caused by artemisinin, reducing the drug's effectiveness. Common mutations like C580Y, R539T, and Y493H alter the K13 protein's function, enabling the parasite to withstand oxidative damage. This results in delayed parasite clearance, increased treatment failures, and the need for alternative antimalarial therapies (Kale *et al.*, 2024).

Plasmodium falciparum multi-drug resistance 1 (*pfmdr1***)** gene on chromosome 5 encodes a digestive vacuole transmembrane glycoprotein (Pgh1 protein), an ATP-binding cassette transporter involved in transporting substances across cellular membranes. This protein is primarily localized on the membrane of the digestive vacuole in the *Plasmodium falciparum* parasite, which is crucial for hemoglobin degradation, a process essential for the parasite's survival within red blood cells. digestive vacuole transmembrane glycoprotein (Pgh1) is involved in the efflux (pumping out) of antimalarial drugs from the digestive vacuole, which can decrease the concentration of these drugs at their site of action, thereby diminishing their effectiveness. This efflux mechanism is a significant contributor to the parasite's resistance to various antimalarial drugs (Silva *et al*., 2022).

Table 1.Drug resistance genes, chromosomes, position and mutation association

Genes	Chromosome and Position	Mutation association
Pfkelch13 (PF3D7_1343700):	Chromosome 13, positions around 1727920 to 1731024	Artemisinin resistance.
Pfcrt (PF3D7_0709000):	Chromosome 7, positions around 403440 to 405984	Resistance to chloroquine and amodiaquine
Pfmdr1 (PF3D7_0523000):	Chromosome 5, positions around 957640 to 961290	Resistance to multiple drugs, including mefloquine, lumefantrine, and artemisinin.
Pfdhfr (PF3D7_0417200):	Chromosome 4, positions around 795044 to 796616	Resistance to antifolate drugs like sulfadoxine and pyrimethamine.
Pfdhps (PF3D7_0810800):	Chromosome 8, positions around 549737 to 550817	Resistance to antifolate drugs like sulfadoxine and pyrimethamine.

Genes associated with invasion of the host's red blood cells (RBCs) and liver

Merozoites Surface Proteins (MSP2): is expressed on the surface of *plasmodium* merozoites and plays a role in the recognition and invasion of red blood cells during erythrocytic stage of malaria. Circumsporozoite protein (*CSP):* expressed on the surface of *plasmodium* sporozoites and facilitate the parasite entry into hepatocytes during the initial infection phase (Chandley *et al.*, 2023).

1.6. Malaria resistance and evidence of molecular surveillance in the region-Rwanda

The development and spread of resistance to antimalarial drugs especially in Eastern Africa has presented challenges for malaria control. In Cameroon the study was done to investigate the evolution of plasmodium falciparum antimalarial drug resistance markers and it reveals a high prevalence of *pfmdr1*- 184F mutant allele during post-ACT adoption. *Pfcrt* -76T and *pfmdr*186Y mutant alleles have declined between 2004 and 2020 with $p < 0.0001$ (Niba *et al*., 2023).

The study in Congo analyzed the polymorphisms in *Pfcrt, Pfmdr1* and *Pfk13* genes' markers

of resistance to artemisinin-based combination therapy in *plasmodium falciparum*, reported the prevalence of K76, N86Y, Y184F mutations to be 26.0,6.8% and 27.7% respectively. No mutation associated with *K13* was identified (Baina *et al*., 2024).

The figure 4 highlight that in Rwanda both in rural and urban region they have been done studies and reported significant resistance associated mutations including *pfkelch13* R561H,A675 and C469F and these are linked to delayed parasites clearance and reduce treatment efficacy (Uwimana *et al.*, 2020).

The study was done in southern province of Rwanda to investigate the prevalence of mutations in *plasmodium falciparum* in children under five years, 104 *p. falciparum* isolates were genotyped for SNPs identification and *pfmdr1* N87,184F and D1246 genotypes have been reported, the frequencies of these alleles were 98% 97/99; 44.4% 44/99; 86% 85/99 respectively. The *pfmdr1*-N86 increased from 60.6% in 2010 to 98% in 2023 (Alruwaili *et al.*, 2025b)**.**

Between 2010 and 2023, Schallenberg *et al*., (2025) conducted a study to look *p. falciparum pfcrt* -K76T mutation, the prevalence of *Pfcrt* -K76T were decreased overtime period, from 75.8% to 25.7% and the study showed a decline of *pfcrt*- K76T mutation in Huye district, southern province of Rwanda and the mutation frequency appeared to stabilize around 25% (Schallenberg *et al*., 2025)**.** Despite the reported prevalence of *Pfcrt* -K76T and *Pfmdr1* -N86Y mutations in different studies done in different region of the world, the prevalence varies according to the region, in southern province of Rwanda the reported prevalence was significant whereas research in Brazzaville reported a slightly lower frequency (Baina et al., 2024); Schallenberg *et al*., 2025). This difference may vary according to local transmission dynamics, variations in drug pressure and region climate (Kubana *et al*., 2023).Therefore surveillance in King Faisal hospital is necessary to know the local resistance landscape.

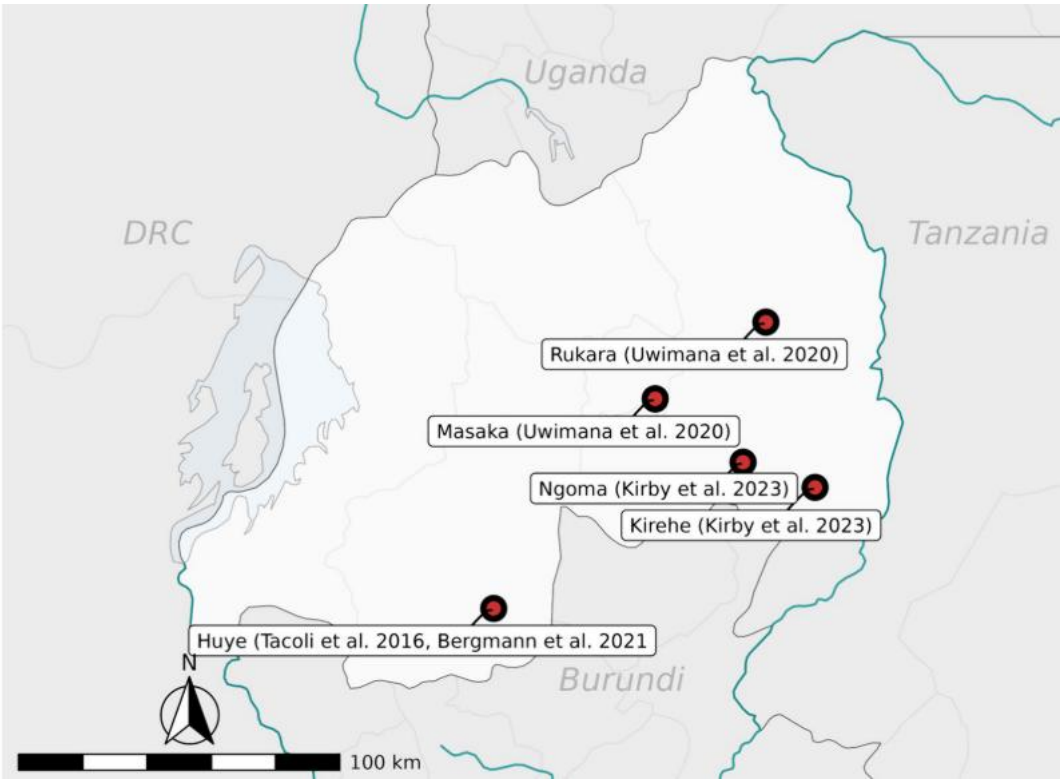

Figure 5.Malaria resistance in Rwanda (Schreidah *et al*., 2024)

1.7. Rationale for the study at King Faisal Hospital

The emergence of resistance to artemesinin and its partner drugs compromises current anti-malaria treatment protocols. Resistance to ACTs is primarily attributed to certain single nucleotides polymorphisms in the propeller domain of *pfk13* gene and it was initially identified in Mekong -southeast Asia and also published in other sub-Saharan African countries including Tanzania, Kenya, Uganda ,Ertitrea, Rwanda and from Kagera region (Ngasala *et al.*, 2024).

In Africa it have been reported that if treatment policies are not changed,antimalarial drug resistance will reach 36% by 2060,resulting in approximately 53 million treatment failure annually, there is an urgent in surveillance of resistance-associated mutations and timely policy intervention to prevent large-scale treatment failure (Watson *et al*., 2024).

A study in Rwanda confirmed local emergency of artemisinin partial resistance, on the figure 6, *pf kelch 13* -R561H mutation was observed on prevalence of 13 % in 2018, increased in 2019, it indicates a significant rise in the R561H mutation across important regions of Rwanda including Masaka as the catchment area surrounding King Faisal Hospital, this implies the active emergence and dissemination of partial resistance to artemisinin (Uwimana *et al.*, 2021 ;Alruwaili *et al*., 2025b). This is an indication of local emergence and

spread of partial resistance.

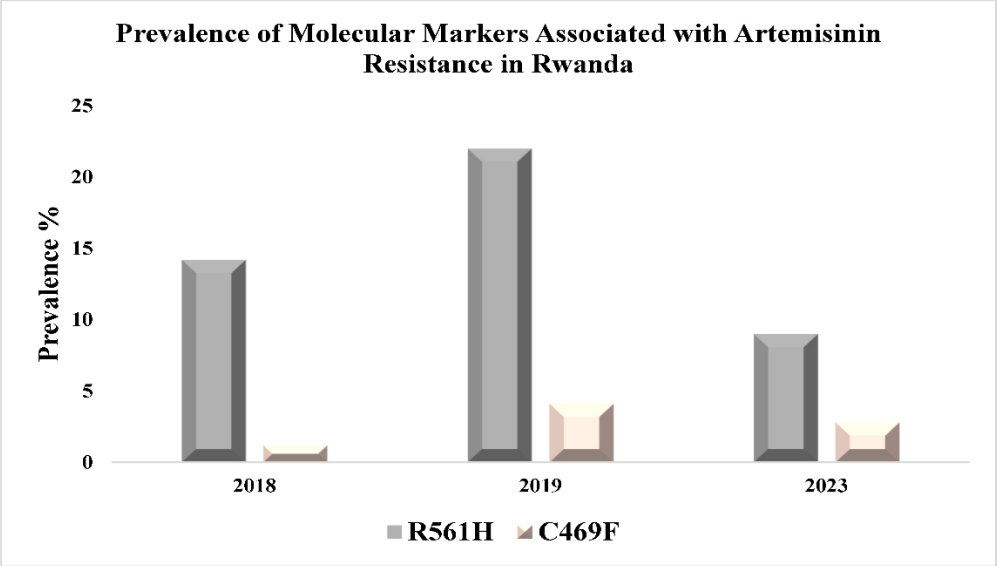

Figure 6.Prevalence of molecular marker associated with artemesin resistance in Rwanda (Alruwaili *et al*., 2025b).

King Faisal hospital uses several ACTs to treat patients, including artemeter –lumefantrine (Coartem), dihydroartemisinin -piperaquine and artesinate-pyronardine. Even though the study done by Ngasala *et al*. (2024), reported that AL is still efficacy for malaria treatment as the cure rate was observed to be ≥97%(Ngasala *et al*., 2024), the SNPs with in *Pfcrt*, *pfk13* and *pfmdr1*genes may compromise future treatment efficacy.

Addressing resistance markers associated with treatment failure can help identify whether resistance to a drug is present in a population and how it evolves over time, allowing for a better understanding of treatment failures to provide valuable information for rational drug policy formulation, managing malaria cases and the development of effective methods to alleviate the impact of drug resistance (Alruwaili *et al*., 2025a)**.**

Assessing the SNPs in patients treated at King Faisal Hospital is essential to detect resistance markers associated with ACTs, informing local treatment guideline , national malaria policies and preventing large scale treatment failure to support malaria elimination goals and support malaria case management in Rwanda.

1.8.Problem statement

In Rwanda, there is a high transmission of malaria despite control strategies available in Rwanda, malaria still persists. Among the major cause of this failure in control strategies put in place to control and eliminate malaria is *P. falciparum* strains resistance to drug like artemisisin-based combination therapy (ACTs)(Moser *et al.*, 2022).

In the study done by Uwimana *et al*. (2021) titled "Association of *Plasmodium falciparum kelch13*-R561H genotypes with delayed parasite clearance in Rwanda*"*, they reported a high prevalence of *Pfmdr1*, for 240 samples assessed 217 (90%)had *Pfmdr1*haplotypes.

Significant genetic changes in and around *pfcrt* and *pfmdr1* have been highly correlated with the decreased susceptibility to antimalarial drugs including chloroquine and partner drugs as ACT. However in Rwanda, only two studies have been done to assess these genetic markers (Alruwaili *et al.*, 2025b)**.**

Considering these mutations reported in Rwanda and other regions of the world, this study addressed the SNPs associated with malaria treatment failure of king Faisal hospital patients thus determining *Plasmodium falciparum* drug resistance-associated mutations in *pfk13*, *pfcrt* and *pfmdr1* genes to support early detection of drug resistance, guide update to treatment policies in Rwanda and support malaria control and elimination strategies.

1.9. Clinical significance of the study

Plasmodium falciparum drug resistance poses a major challenge to malaria control and treatment efforts; mutations in *pfk13*, *pfcrt* and *pfmdr1* genes are linked with reduced sensitivity to commonly used antimalarial drugs particularly those in Artemesinin-based combination therapy (ACTs) which are the common standard first line treatment in Rwanda and globally. As the resistance to drug increase, thus increase the risk of treatment failure leading to prolonged infections, increased transmission and thus cause higher morbidity and mortality.

The existence of mutations is not only indicator of treatment failure but it is also suggesting population dynamics changes in parasite populations due to drug selective pleasure and therefore addressing SNPs associated with treatment failure is necessary to guide treatment decision and management of cases in Rwanda and worldwide.

Understanding the prevalence and SNPs associated with malaria treatment failure and determining *Plasmodium falciparum* genes *pfk13*, *pfcrt* and *pfmdr1* genes mutation could

support timely updates to treatment guidelines, improving patient's outcomes and help in malaria control and elimination strategies in Rwanda and other malaria endemic-regions.

1.10. Research question

What is the concordance of oxford nanopore technology (ONT)results comparing to sanger sequencing results?

What is the prevalence of SNPs in *pfk13, pfcrt* and *pfmdr1*genes associated with malaria treatment failure at King Faisal hospital?

1.11. Objectives

1.11.1. General objective.

To address the SNPs associated with malaria treatment failure for king Faisal hospital patients thus determining drug resistance-associated mutations in *pfk13, pfcrt* and *pfmdr1* genes.

1.11.2 . Specific objectives

1. To validate oxford nanopore technology (ONT)results in determination of SNPs of *Plasmodium falciparum* genes by Sanger sequencing.

Justification of ONT results validation by sanger sequencing: ONT, a third generation sequencing method that provide long reads even if it has an increased number of errors ranked on 5 to 15% comparing to sanger sequencing which has error rate of 0.001% (Lin *et al*., 2021). Validating the ONT results with the golden standard technique sanger sequencing demonstrated how ONT results are accurate in detecting SNPS in *plasmodium* genes and strengthened confidence in precision of ONT generated data which support its use in molecular surveillance and research on malaria.

2. To determine the prevalence of SNPs in *pfk13, pfcrt* and *pfmdr1*genes associated with malaria treatment failure at King Faisal hospital.

Justification of assessing the prevalence of SNPs: the prevalence of SNPs in *pfk13, pfmdr, pfcrt* genes for KFH patients, the national referral and tertiary hospital that serve patients with malaria that often resist to treatment, severe malaria and recurrent malaria is highly needed as described in Figure 5, studies were done in Rwanda published mutation in Huye, Masaka and rukara , *pfk13* -R561H published on over 15 % (Uwimana *et al*., 2021) and no data is available from a high level clinical settings like KFH, furthermore review highlight that AL treatment has not yet reach the national threshold for failure, however an increase in *pfmdr1* and *pfcrt* -SNPs could impact the future treatment efficacy. Without molecular surveillance of SNPs, there would be uncontrolled spread of resistance and failure of malaria control by relying on treatments that are already failing in tertiary hospital.

Chapter 2. Methodology

2.1. Research design

This study was a cross-sectional study aimed at addressing the SNPs associated with malaria treatment failure of KFH patients, genetic mutations associated with *Plasmodium falciparum* genes *Pfmdr, Pfk13* and *Pfcrt* genes isolates were sequenced and analysed. Blood samples were collected at single point in time and analyzed using the molecular techniques (DNA extraction, PCR, gel electrophoresis and sequencing by oxford nanopore technology and sanger sequencing to detect mutations in the targeted genes (*Pf k13, Pfmdr1 and Pfcr*t) known to be involved in treatment failure for drug currently used in king Faisal hospital.

2.2. Study area

King Faisal hospital, located in Gasabo district, Kigali city and it is the largest, national referral and tertiary hospital in Rwanda.

2.3.Study population

Individually microscopically diagnosed with *plasmodium* in King Faisal hospital Rwanda.

2.4.Inclusion criteria

Microscopically confirmed malaria cases

2.5.Exclusion criteria

All malaria negative samples were excluded.

2.6. Sample size

The total number of 107 positive samples were collected and sequenced for SNPs analysis.

2.7.Sampling strategy:

Convenience sampling was used to collect all *plasmodium*- positive blood samples from patients attending King Faisal hospital. For all patients of all ages who tested positive for malaria by microscopy, blood samples were collected in EDTA tube and conserved in fridge 2-8^{0}c prior to laboratory processing.

2.8.Laboratory procedures

2.8.1 Blood sample collection

Blood samples were collected at king Faisal hospital for patients who tested positive for malaria by microscopy. EDTA tubes were used in samples collection and kept in fridge 2-80 c prior to extraction**.**

2.8.2. DNA extraction

DNAs were extracted from *plasmodium* infected blood samples: using Zymo quick DNA kit. To extract DNA, samples were thawed at room temperature and add 20µl of proteinase K Solution and 200µl of sample to micro centrifuge tubes, then 200µl of biofluid&cell buffer was added and the mixture vortexed for 10 seconds, and incubated at 55°C for 30 minutes. Addition of 1 volume of genomic binding buffer to the digested buffer and vortexed for 10 sec. Transfer of mixture in zymo-spin TM II-XLR Column in a collection and centrifuge for 1 min at 12,000xg ,then 400 µl of DNA pre wash buffer were added to the spin column in a new collection tube then 700 ml of g-DNA and centrifuge at 12,000xg for 1 min, then 200 ml of g-DNA after, elution was done using 70ml of elution buffer and centrifuge at maximum speed 21,000x g for 1 min after 15 min of incubation.

2.8.3.DNA extracts quantification

DNA extraction results were Quantified and Qualified by using both spectrophotometry, nanoDrop and fluorometry method, Qubit to accurately assess DNA concentration and quality. For nanodrop any debris and contaminants such as proteins, phenol, and salts absorb at different wavelengths and can affect the A260/A280 and A260/A230 ratios, providing misleading information about DNA purity. Fluorometry methods using Qubit was more specific for DNA and less affected by contaminants and providing a more accurate measurement of DNA concentration. For quantificatin 1µl of each sample and 200µl QuantiFluor® ONE dsDNA Dye, were mixes and incubate for 5 minutes, then measure fluorescence using the Quantus™ Fluorometer(Qubit).

2.8.4. Amplification

Conventional PCR was done to amplify specific genetic markers for analysis. The use of PCR enabled the identification and quantification of these genetic sequences, critical for understanding genetic variations and potential drug resistance. Specific primers were used to amplify *pfcrt, pfmdr1*and *pfk13* genes.

Additionally, a multiplex PCR approach was employed to simultaneously amplify multiple drug resistance genes (*pfcrt, pfmdr1*and *pfk13*), allowing for comprehensive genetic analysis. The use of PCR enabled the identification and quantification of these genetic sequences, critical for understanding genetic variations and potential drug resistance.

2.8.5. Gel electrophoresis

In this step, gel electrophoresis was done to check if DNAs as target are present in our samples prior to continue for next step.

2.8.6. Normalization and amplicon pooling

To ensure libraries have the same concentration before pooling, I made sure that the final volume calculated does not exceed 15µl.The normalization volume was 11.5µl, for volumes that exceed 15µl but are close to 15µl, adjustments were made by reducing the amount of DNA in the amplicon product.

2.8.7. Oxford nanopore library preparation

Oxford nanopore library preparation for DNA Repair and End-Prep were done, reagents included 11.5µL of amplicon DNA, 1µL per sample of diluted DNA Control, 1.75µL of Ultra II End- Prep reaction buffer, and 0.75µL of Ultra II End-Prep Enzyme Mix, totaling 15µL per sample across all samples. The protocol involved mixing, spinning down, and incubating at 20°C for 5 minutes followed by 65°C for 5 minutes. **Native Barcode Ligation** followed, where 7.5µL of End-Prepped DNA, 3.5µL of Native Barcode, and 10µL of TA Ligase Master Mix were combined. After mixing and incubating at 20°C, the reaction was stopped with 4µL of EDTA. Samples are then pooled, treated with AMPure Beads, washed with 80% ethanol, and suspended in 35µL nuclease-free water before quantification with Qubit. To perform **adapter ligation and clean-up**, mix 30 µl of pooled barcoded sample, 5 µl of Native Adapter (NA), 10 µl of NEBNext Quick Ligation Reaction Buffer (5X), and 5 µl of Quick T4 DNA ligase in a 1.5 ml Eppendorf LoBind tube. After incubating the reaction for 20 minutes at room temperature, add 50 µl of suspended AMPure XP Beads (AXP), wash the beads twice with 125 µl Short Fragment Buffer (SFB), and elute the DNA library in 15 µl of Elution Buffer (EB). Finally, **Priming and Loading the SpotON Flow Cell** required thawing and mixing of necessary buffers(Flow cell Flush 1,170µl,Bovin serum Albumin 5µl and Flow Cell Tether 30µl), priming the flow cell, loading 0.3µl of the library into the SpotON sample pot, and sequencing run initiated on **MinION**, an oxford nanopore technology used to determine the SNPs associated with malaria treatment failure for malaria-positive samples of King Faisal hospital patients and Validation of SNPs were done Using Sanger sequencing.

2.8.8. Oxford Nanopore Technology

ONT was used to determine molecular markers of resistance associated with artemisinin and its partner drugs in isolates from malaria- positive clinical samples, collected at KFH. The sequenced results were analyzed using Bioinformatic.

Oxford Nanopore Technology developed third generation sequencers that are portable, able to sequence DNA in remote locations and produce ultra-long reads. It uses a barrel-shaped protein called α- hemolysin, naturally found as a 'pore' in a cell membrane, regulating which molecules can enter or leave a cell. This is called a nanopore. α-hemolysin has a diameter of 1 nanometer, just big enough to allow a single strand of DNA through. In ONT, the α-hemolysin nanopore is embedded into an artificial membrane inside a sequencing chamber. When a current is applied to the membrane, the DNA travels through the nanopore. As the DNA travels through the nanopore, it obstructs the current flowing across the membrane. The four bases of the DNA (A, T, C and G) are of different shape and size, so cause variations in the current. These variations are measured by an electronic chip. An algorithm converts the data into a sequence which can then be read (Wang *et al.*, 2021).

Importance of ONT for malaria diagnosis and drug resistance monitoring

Firstly, an ultra-long read sequence generated by nanopore sequencing, where the top exceeds, 4 Mb in length, promotes genomic assembly, as well as analysis of copy number and presence/absence variants, and repetitive regions. Nanopore sequencing is also the only sequencing technology that offers real-time analysis (for rapid insights), in fully scalable formats, can analyze native DNA or RNA, and can sequence any length of a fragment to achieve short to ultra-long read lengths. The portability of Nanopore sequencers promotes real-time analysis of data on the field, as well as projecting monitoring power to environments where otherwise genomic profiling may not be possible. The different designs of flow cells used in Nanopore sequencers also allow for greater scalability, due to the flexibility of this method (Wang *et al.,* 2021).

2.8.9. Sanger sequencing

Nucleic acid sequencing is a method for determining the exact order of nucleotides present in a given DNA or RNA molecule, developed by Frederick Sanger in 1977. Sanger sequencing also called chain terminator involves the use of ddNTPs during DNA replication. These are incorporated to terminate the replication. ddNTPs lacks 3'-OH group required to

form phosphodiester bond with next nucleotide, its incorporation it prevent the addition of other nucleotides thus terminating the DNA chain and the resulting in fragments with different lengths which are separated by size using capillary electrophoresis. Single stranded DNA is used as template to be sequenced, primer to bind template to initiate synthesis is added, the mixture of DNA polymerase, normal dNTPs and small proportion of labelled ddNTPs are used for reaction extension. Then during the synthesis chain terminator(ddNTPs) are incorporated results in chain termination. Capillary gel electrophoresis separates by size different nucleotides length produced. Detection of nucleotides is done by laser which detect fluorescent labels attached to the terminating ddNTPs, each labels produce its own color and the computer finally assembles the data into a readable DNA (Guo & Pyle, 2023).

2.8.10. ONT and sanger sequencing results analysis

ONT sequenced results were analyzed using bioinformatics tools google colab; nanoplot for quality control, porechop for barcode and adapter trimming, minimap2 for alignment, samtools for alignment filtration and statistics, clair3 for variants calling, bcf tools for variants filtration, snpEff for variants annotation, plasmoDB for SNPs identification and (IGV) for visualization and explore genomic data. Analysis of Sanger sequencing results were done using DNASTAR/Lasergene.

2.9. Data analysis

Data analyzed were done using SPSS version 25, microsoft excel and R studio version 4.4.1

2.10. Ethical consideration

Ethical clearance was obtained from NEC prior to sample collection (see Appendix 2).

Chapter 3. Results presentation

3.1. Molecular diagnostic tests for detection of SNPs

3.1.1. Sample extraction results presentation

A total number of 107 samples were extracted for the purpose of assessing the SNPs associated with malaria treatment failure. Extraction was performed following the procedure as described in section 2.8.2. The Table 2 is describing the selected DNA extracts quantification done using nanodrop to assess concentration and purity.

Table 2. DNA Extracts measurement by Nanodrop

Sample ID	Concentration ng/uL	A260	A260/A280	A260/A230
Blank	0	0	0	0
KFD021	112.39	2.25	1.79	0.75
KFD022	67.9	1.36	1.64	0.47
KFD023	49.58	0.99	1.66	1.66
KFD024	34.02	0.68	1.84	0.23
KFD025	29.85	0.6	1.66	0.97
KFD031	45.93	0.92	1.63	0.71
KFD032	86.82	1.74	1.64	0.36
KFD033	55.23	1.1	1.75	0.12
KFD051	42.59	0.85	11.38	0.5
KFD052	48.55	0.97	1.62	0.92
KFD063	59.69	1.19	1.67	1.15
KFD064	48.79	0.98	1.62	1
KFD065	45	0.9	1.53	0.24
KFD066	82.76	1.66	1.62	1.08
KFD067	60.62	1.21	1.67	0.55
KFD068	75.02	1.5	1.74	1.03
KFD069	35.12	0.7	1.51	0.21
KFD070	40.97	0.82	1.63	0.24

DNA concentration for selected extracted samples, measurement done using nanodrop spectrophotometer to assess concentration and purity for downstream molecular analysis. The measurement ranged from 29.85ng/ul to112.39ng/ul, most samples fallen within acceptable range for PCR Amplification and sequencing. The A260/280 which indicate protein contamination were in good range of 1.6 to 1.8, DNA extracts had high purity.

3.1.2. PCR Amplification Results

Polymerase Chain Reaction (PCR) was used in this study to amplify *pfk13, pfmdr1* and *pfcrt* genes to generate copies from a small sample (see Appendix 1 for detailed amplification steps). The technique of PCR ensured selective amplification of my targeted regions and reduce sample complexity and enhancing sequencing efficiency. After amplification 107 samples were successful amplified.

3.1.3. Gel electrophoresis

Agarose gel electrophoresis was performed to verify the success and specificity of PCR amplification by visualizing DNA bands at expected sizes. It helps assess the quality and integrity of the amplified DNA, ensuring it is suitable for downstream applications like sequencing.

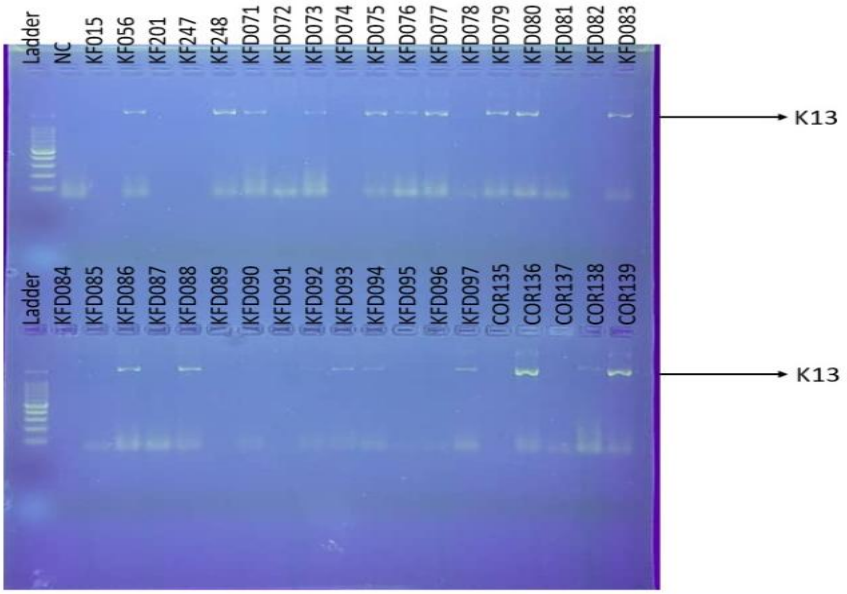

Figure 7. Gel electrophoresis results

Successful amplified samples were confirmed using Gel electrophoresis. The figure 7. Shows *Pfk13* gene. All amplicons were run on gel for amplification verification. A total of 107 samples successful amplified and were suitable for subsequent sequencing analysis.

3.1.4. Bioinformatics

The Nanoplot-report.html analysis showed in Table 3 provides a comprehensive visual and statistical summary of long- read sequencing data and highlight key metrics such as read length distributions and quality scores facilitating data interpretation and decision making.

Table 3.Nanoplot report table.

Mean read length	**620.5**
Mean read quality	10.0
Median read length	597.0
Median read quality	10.8
Number of reads	118,592.0
Read length N50	616.0
STDEV read length	652.0
Total bases	73,582,737.0

The nanoplot report for this selected sample sequenced showed a mean read length of 620.5 bases and a mean read quality of 10.0, with a total of 118,592 reads contributing to 73,582,737 total bases. The median read length is 597.0 bases, indicating most reads are relatively short, and the read length N50 of 616.0 bases reflects a distribution skewed towards shorter reads, with a standard deviation of 652.0 bases indicating significant variability in read lengths.

Figure 8.Read quality for true variants and sequencing error differentiation.

Read quality for this selected sample sequenced is indicating the relationship between read lengths and average read quality for my sequenced sample using ONT. Most reads are shorter for this sample with reads below 2000bases, some reads are beyond 5000 bases. On Read quality the average quality reads are higher or tends to be higher for shorter reads and most of reads have a quality score of 10 to 15.

3.1.5. Identified SNPs genes.

Identification of SNPs was done using Bioinformatics tool, Porechop used for barcode and adapter trimming, Minimap2 for alignment, Samtools for alignment filtration and statistics, clair3 for variants calling, bcf tools for variants filtration, SnpEff for variants annotation and PlasmoDB for SNPs identification. The table 5. Illustrate the distribution of SNPs and their frequency per sample in all 43 samples of king Faisal hospital patients sequenced and review confirmed their association with malaria treatment failure. Analysis of Sanger sequencing results were done using DNASTAR/Lasergene.

3.2. Validation of ONT results in determination of SNPs of *Plasmodium falciparum* genes by Sanger sequencing.

3.2.1. Validation of ONT delivered *pfk13*- R561H mutation by sanger sequencing

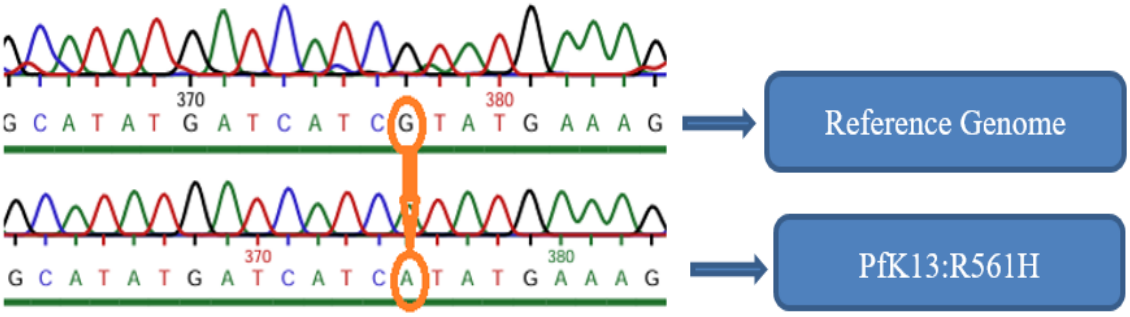

Figure 9.Validation of *PfK13*-R561H mutation in sample EF73789273 by sanger sequencing. Comparing the Results to the normal reference Genome using DNASTAR/Lasergene.

To validate ONT results SNPs in the *plasmodium falciparum k13* gene on codon 561, R561H, 24 samples were sequenced to determine the reliability of ONT. The figure 10 shows 22 samples (91.7%) confirmed the same results by presenting *pfk13*-R561H mutations and 2(8.3%) samples did not show the mutation. The results indicated a strong concordance. 8.3% indicate false positive in ONT results.

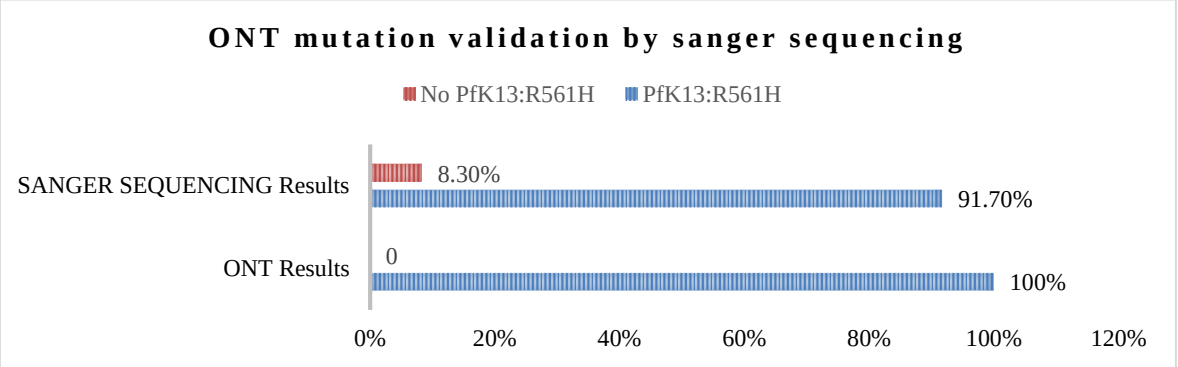

Figure 10.ONT results and Sanger sequencing results

3.2.1.a. Cohen's kappa determined for assessing agreement between ONT and Sanger sequencing

Table 4 and 5 illustrate Cohen's kappa coefficient used and assess the ONT and sanger sequencing agreement in detecting mutation associated with *pfk13*-R561H for 24 samples tested. It gives a moderate agreement of k=0.479 which is statistically significant Chi – square =25.000, the *p* value ($p < 0.001$). These two molecular tests are in the same line in detecting mutations even though ONT is likely to give mutations in wild type samples.

Table 4.Cohen's kappa for ONT and Sanger sequencing agreement

Symmetric Measures

		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	kappa	.479	.301	4.056	.000
N of Valid Cases		24			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Table 5.Chi-Square Tests for ONT validation by Sanger sequencing

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	25.000[a]	2	.000
Likelihood Ratio	8.397	2	.015
N of Valid Cases	24		

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is .04.

3.2.2 Validation of ONT delivered *pfmdr1* -Y184F mutations by Sanger sequencing

A total number of 40 king Faisal hospital patients were sequenced to validate *Pfmdr1*-Y184F mutations delivered ONT by sanger sequencing. The mutation involves the change of codon tyrosine(Y) to phenylalanine(F) at postion184. The Figure 11shows samples analyzed using DNASTAR/Lasergene18 and aligned to reference genome, all samples were analyzed to codon 184, the wild type codon TAT or TAC which encode tyrosine; in 37 (92.5%)samples the wild type codon was replaced by mutant codon TTT or TTC which encodes phenylalanine. Only 3(7.5%) shows wild type codon.

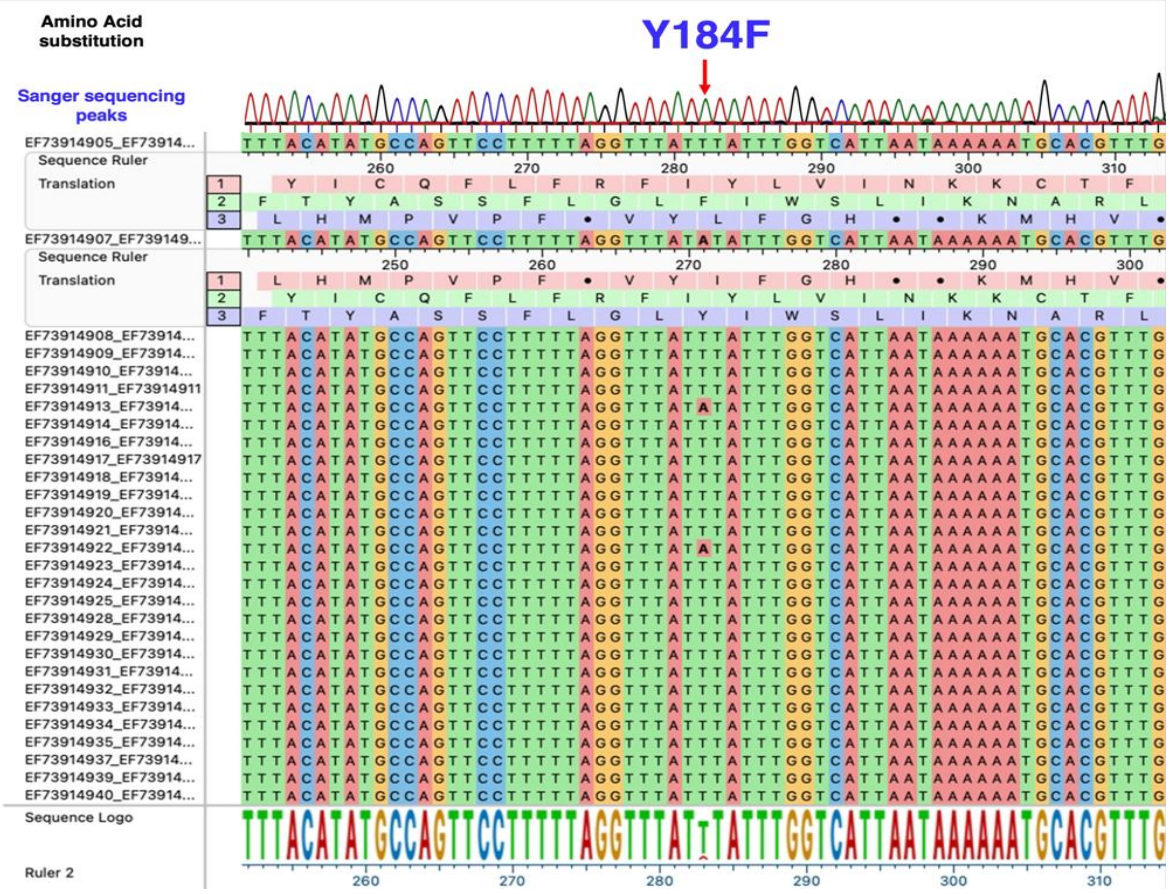

Figure 11.Sanger sequencing alignment shows mutants codon in 40 samples which results in *pfmdr1* -Y184F and 3 wild type codon.

***Pfmdr1*- Y184F Mutation by sanger sequencing (%)**

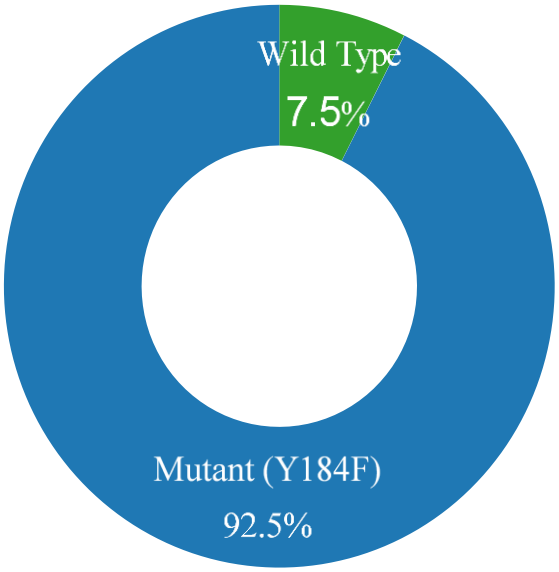

Figure 12.Validation of *Pfmdr*1 -Y184F ONT results by Sanger sequencing

37/40 (92.5%) of PfMDR1 are confirmed by Sanger sequencing and 3/40 (7.5%) not confirmed by Sanger

3.3.The prevalence of SNPs in *pfk13, pfcrt* and *pfmdr1*genes associated with malaria treatment failure at King Faisal hospital.

3.3.1. Mutation frequency distribution among sequenced samples

In total of 43 samples were sequenced and 36 (83.7%)samples presented SNPs, 2 of samples presented an increased number of SNPs, 6 different SNPs identified in one Sample.

Table 6.Distribution of SNPs identified and their frequency per sample

SN	Samples ID	Frequency	Percent	Cumulative Percent
1	KFD001	2	1.8	1.8
2	KFD002	4	3.6	5.4
3	KFD006	4	3.6	9.0
4	KFD008	3	2.7	11.7
5	KFD010	3	2.7	14.4
6	KFD011	2	1.8	16.2
7	KFD012	4	3.6	19.8
8	KFD013	3	2.7	22.5
9	KFD014	3	2.7	25.2
10	KFD015	2	1.8	27.0
11	KFD021	3	2.7	29.7
12	KFD022	4	3.6	33.3
13	KFD025	2	1.8	35.1
14	KFD031	2	1.8	36.9
15	KFD034	3	2.7	39.6
16	KFD035	3	2.7	42.3
17	KFD036	3	2.7	45.0
18	KFD038	2	1.8	46.8
19	KFD044	3	2.7	49.5
20	KFD048	2	1.8	51.4
21	KFD050	6	5.4	56.8
22	KFD054	6	5.4	62.2
23	KFD060	2	1.8	64.0
24	KFD063	2	1.8	65.8
25	KFD066	2	1.8	67.6
26	KFD070	6	5.4	73.0
27	KFD072	4	3.6	76.6
28	KFD075	5	4.5	81.1
29	KFD080	3	2.7	83.8
30	KFD083	1	0.9	84.7
31	KFD086	2	1.8	86.5
32	KFD088	2	1.8	88.3
33	KFD092	2	1.8	90.1
34	KFD093	1	0.9	91.0
35	KFD094	5	4.5	95.5
36	KFD097	5	4.5	100.0
	Total	**111**	**100.0**	

ONT sequencing of 43 samples were done to determine mutation associated with malaria treatment failure, Table 6 is showing 36 samples presented mutation and the frequency of mutation and ranging from 1 to 6 per sample. The most frequencies were observed in samples KFD050, KFD054 and KFD070 showing frequency of 6.

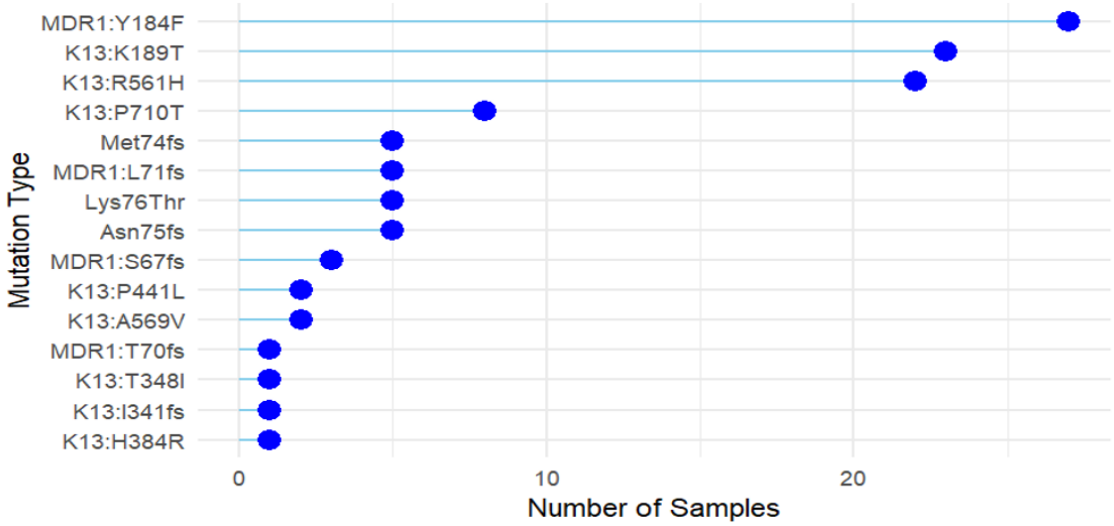

Figure 13.Types of SNPS Identified in 43 samples

Most of *Pfmdr1*-Y185F is the first with samples having the mutation, secondary *pfk13*-K189T.

3.3.2 The prevalence of SNPs in *pfk13, pfcrt* and *pfmdr1* genes

Table 7.Prevalence of SNPs associated with malaria treatment failure of KFH patients

PF GENES	SNPs	Frequency	Total sample sequenced	Prevalence(%)
PfMDR1 **mutation**	L71fs	5	43	11.6
	S67fs	3	43	7.0
	T70fs	1	43	2.3
	Y184F	26	43	60.5
PfCRT **mutation**	Asn75fs	5	43	11.6
	Lys76Thr	5	43	11.6
	Met74fs	5	43	11.6
PfK13 **mutation**	A569V	2	43	4.7
	H384R	1	43	2.3
	I341fs	1	43	2.3
	K189T	23	43	53.5
	P441L	2	43	4.7
	P710T	8	43	18.6
	R561H	22	43	51.2
	T348I	1	43	2.3

In total 43 samples sequenced for addressing mutation or SNPs in *PfK13*, *Pfmdr1* and *PfCRT* genes, the anti-malaria resistance associated genes. The results of ONT sequencing showed different mutation with different prevalence. The *PfMDR1* -Y184T as in Figure 15 is the most prevalent with 60.5% suggesting high persistence of Y184T mutation in King Faisal patients and this is associated with treatment containing lumefantrine as artemether-lumefantrine, other frameshift mutation in *mdr1* gene were also identified such as L71fs, S67fs and T70fs with prevalence of 11.6%,7% and 2.3% respectively. Figure 14 presents *PfCRT* genes, three SNPs were identified Asn75fs, Lys76Thr and Met74fs, with 11.6 % for each of the gene. The Lys76Thr is associated with reduced piperaquine sensitivity in Southeast Asia and in Rwanda. The use of DHA-PPQ (Dihydroartemisinin-Piperaquine) requires continuous monitoring, which is of great importance as Lys76Thr can compromise the treatment efficacy.

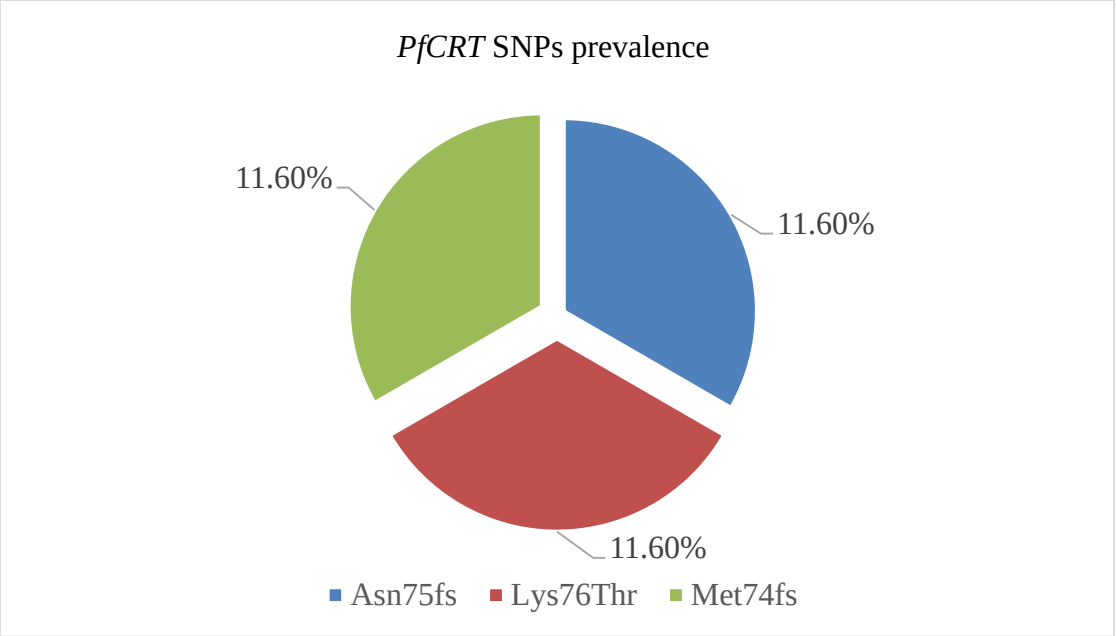

Figure 14. *Pfcrt* genes SNPs prevalence.

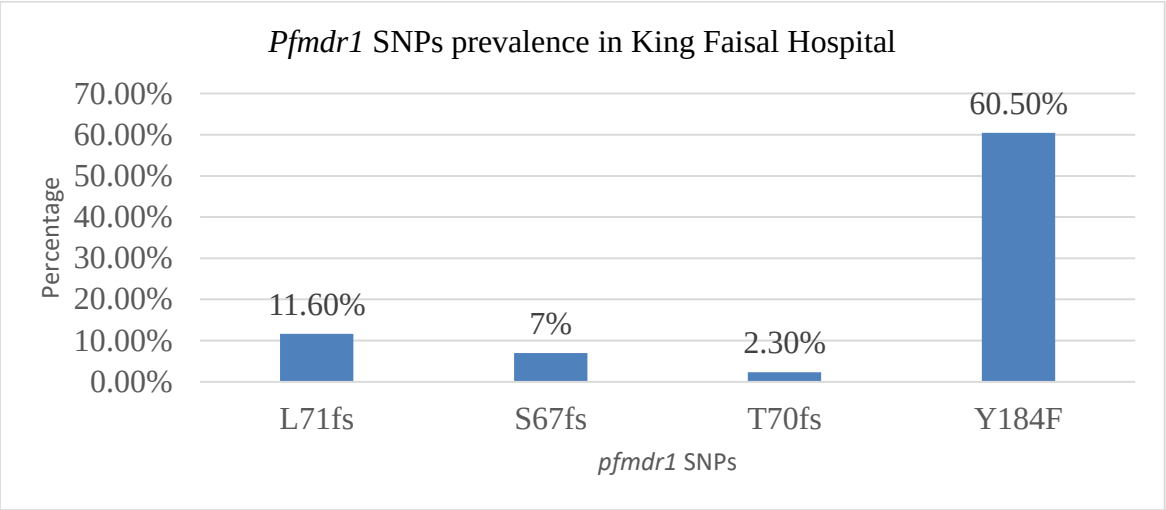

Figure 15.*Pfmdr1* -SNPs prevalence. *Pfmdr1*- Y184T is the most prevalent with 60.5%

Figure16 presents mutation in *PfK13* gene K189T (53.5%) and R561H (51.2%) are the most prevalent and R561H is a marker of artemisinin resistance. P710T, A569V, H348R, P441L, I341fs and T348 were detected low in prevalence of 19%,4.7,2.3%,4.7%,2.3%,2.3% respectively.

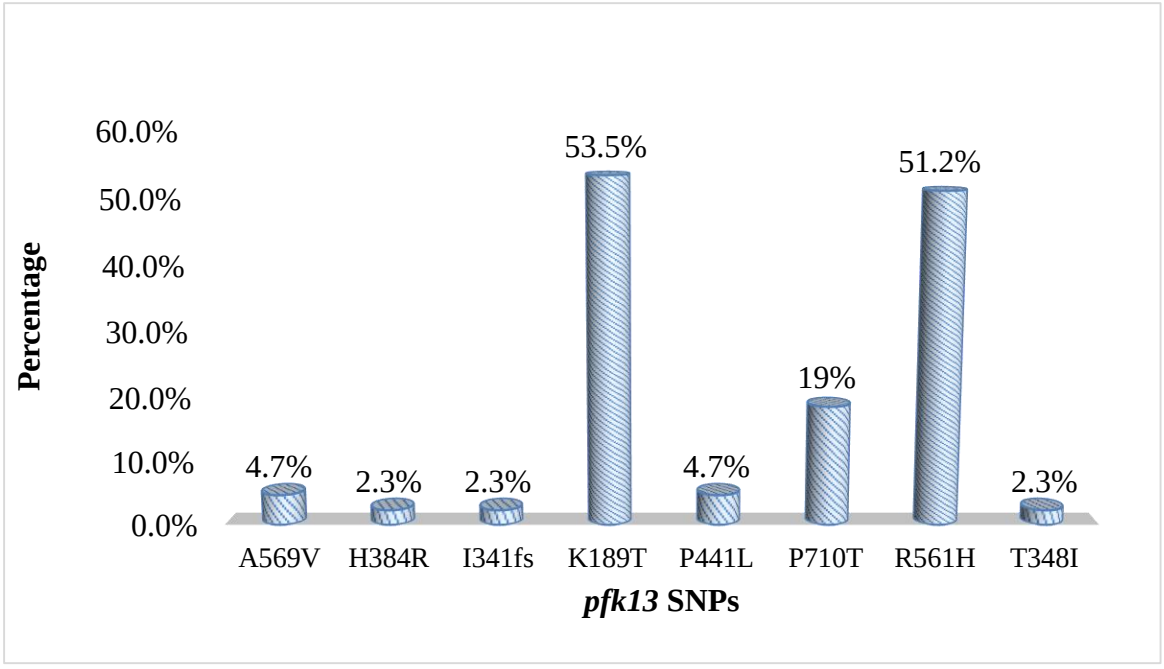

Figure 16.*PfK13* prevalence in King Faisal Hospital.

In *PfK13* genes the most first SNPs identified is R561H (51.2%)

Gene ID identified in results of ONT sequencing are highlighted in table 8. The majority of Gene ID identified are PF3D7_13437000 (54.1 %) namely *PfK13* gene is the most mutated

gene, PF3D7_0523000, PfMDR1genes (32.4%) is the secondly mutated and lastly PF3D7_0709000.1, *PfCRT* has the lowest percentage of 13.5%, it is less common mutation but clinical significant to cause treatment failure.

Table 8.Summary of gene ID and associated mutations frequency

Gene ID	Frequency	Percent	Cumulative Percent
PF3D7_0523000: *Pfmdr1*	36	32.4	32.4
PF3D7_13437000: *Pfk13*	60	54.1	86.5
PF3D7_0709000.1: *Pfcrt*	15	13.5	100.0
Total	111	100.0	

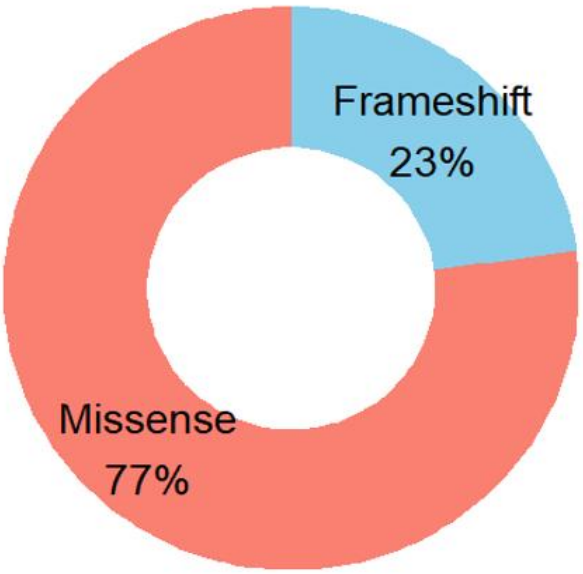

Figure 17. Frame shift and missense variant distribution among the samples presented mutation.

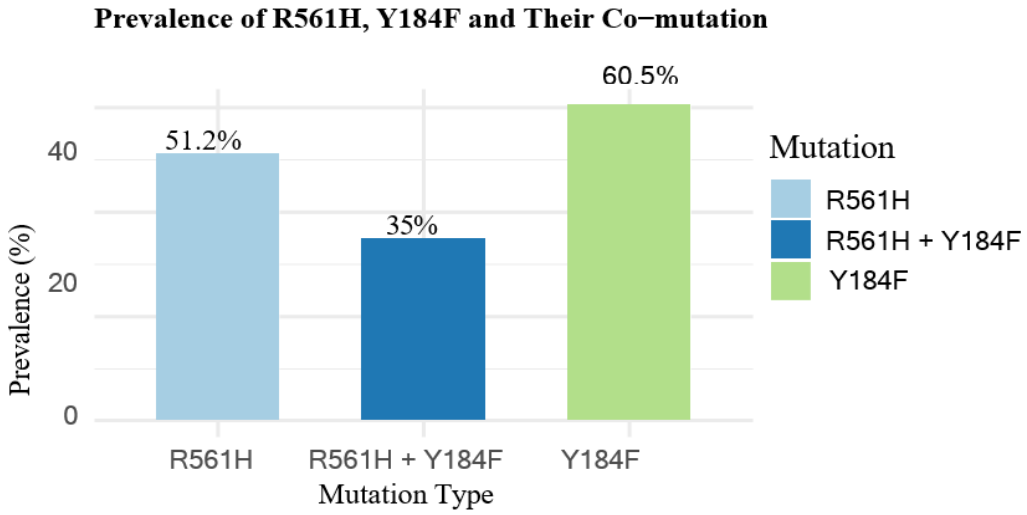

Figure 18.Mutation combination. *PfK13*- R651H and *Pf MDR1*-Y184F

presented on 35%. Even though mutation can alone have impact on treatment, combination with another mutation could widen ACT treatment efficacy.

Chapter 4. Discussion

4.1. Validation of ONT delivered *pfk13*-R561H and *pfmdr1*- R561 Mutation by Sanger sequencing

Validation of ONT, a portable, cost effective methods which speed up the results by another molecular technique like sanger sequencing, the golden standard technique is very important as it has reported to have an errors rates. The validation ensured that the SNPS produced by ONT is acceptable and no incorrect treatment decision is taken due to false positive or negative results.

My study validated a total of 64 samples, 40 samples previously tested in year 2024 and gave positive SNPs of *PfMDR1*- Y184F for all and 24 samples with *PfK13*- R561H. These SNPs are associated with malaria treatment failure. This study shows the concordance of results to be 92.05 % for *Pfmdr1* -Y184F; this concordance highlight that ONT is an alternative test that should be used for SNPs identification in low and middle income countries. It is in line with other studies done in Zambia reported over than 95% and Ghana reported a high concordance especially when parasitemia was ≥0.1% (de Cesare *et al*., 2024).

There is an agreement of concordance between ONT and sanger sequencing as illustrated in Table 4 and 5. The Cohen's kappa assessed this and the results showed a moderate agreement of k=0.479 which is statistically significant, chi-square test revealed (x^2= 25.000), with $p <$ 0.001 proving both techniques to be consistent in SNPs identification.

Even if ONT provided some positive results in samples that sanger classified in wild type, the overall agreement supports the two test to be reliable and ONT can be used as alternative of sanger sequencing but due to slow difference shown ONT have to be cautiously interpreted. The Pearson chi-square test confirms a strong association of the ONT results validated by Sanger sequencing and finally the likelihood ratio test confirms the concordance (x^2 = 8.397, p = 0.015).

4.2. The prevalence of SNPs in *pfk13, pfcrt* and *pfmdr1* genes

Plasmodium falciparum isolates of 43 patients were analyzed in 2025 using ONT, and a numbers of Single Nucleotide Polymorphisms were detected and some of them are associated with treatment failure for current drugs used at King Faisal hospital. Among the SNPs identified *PfMDR1* -Y184F are the most prevalent with 60.05%, other frame shift

mutation identified are outlined in Figure 11. In *PfK13* artemesinin resistance is presented on 51.2% (22/43) of *PfK13* -R561H, and *PfK13*- K189T, P441L and P710T were also presented in Figure16 at lowest level. *PfCRT* frameshift mutation also reported in approximately 26% of samples. The results highlight the predominance of *PfMDR1*- Y184T followed by *PfK13*-R651H, which are key markers in emerging artemisinin resistance.

PfK13- R561H SNPs are increasing, it was reported 9% in 2020, over 15% in 2023 and now reported on 51.2% at King Faisal Hospital (Uwimana *et al*., 2021).

This study conducted at KFH in addition revealed a higher prevalence of Y184T (60.5%), the rising frequency of SNPs indicates a localized selective pressure likely associated with particular antimalarial drug use patterns. Regional *PfK13*-R651H prevalence increased in Uganda from about 20%(2018), to 23%(2022). In Senegal the *pfmdr1* -Y184F was reported in 56%of isolate (2015). The findings are in line with King Faisal Hospital study revealed data.

Comparing mutation to the study done in Chine reporting *pfk1*3 propeller SNPs in 3.6% of isolates and *pfmdr1* in 24.7%, KFH- SNPs are highly increased showing mutation burden for king Faisal hospital patients especially in *PfK13*- R651H (51.2%) and *pfmdr1*- Y184F (60.5%).

The increase SNPs in *pfmdr1*- Y184F is showing with evidence the adaptation of malaria parasites to lumefantrine and other ACT, leading to treatment failure due to treatment efficacy reduction. *Pfk13* -R651H suggest the emergence surveillance of artemesinin resistance in Rwanda as the SNPs delay parasite clearance. The rise in prevalence of SNPs associated with Malaria treatment failure at King Faisal Hospital, Rwanda is well illustrated in the Figure 17 comparing *Pfk13*- R651H and *PfMDR1* -Y184F to other studies done in Rwanda which published lower prevalence. Studies conducted in year 2015 in Masaka and 2018 in Rukara showed *PfK13* -R651H (1.34%and 22% respectively), the study in Huye observed *pfmdr1*-Y184F(59%)(Alruwaili *et al.*, 2025b;Schallenberg *et al*., 2025;Karema *et al*., 2020). The increased frequency of *PfK13*-R561H (51.2%) and *PfMDR1* -Y184F (60.5%) raises concern with treatment failure and burden of mutation in Kigali, in 43 clinical samples molecular characterized to detect SNPs 36(83.7%) had different mutations as described in Table 7. Without Quick intervention and precaution in Rwanda, the increase in the mutation will results in increased malaria death cases in Rwanda due to treatment failure as even the newly introduced DHAPQ and pyronardine will be inefficacy due to mutations in *Pfcrt* observed.

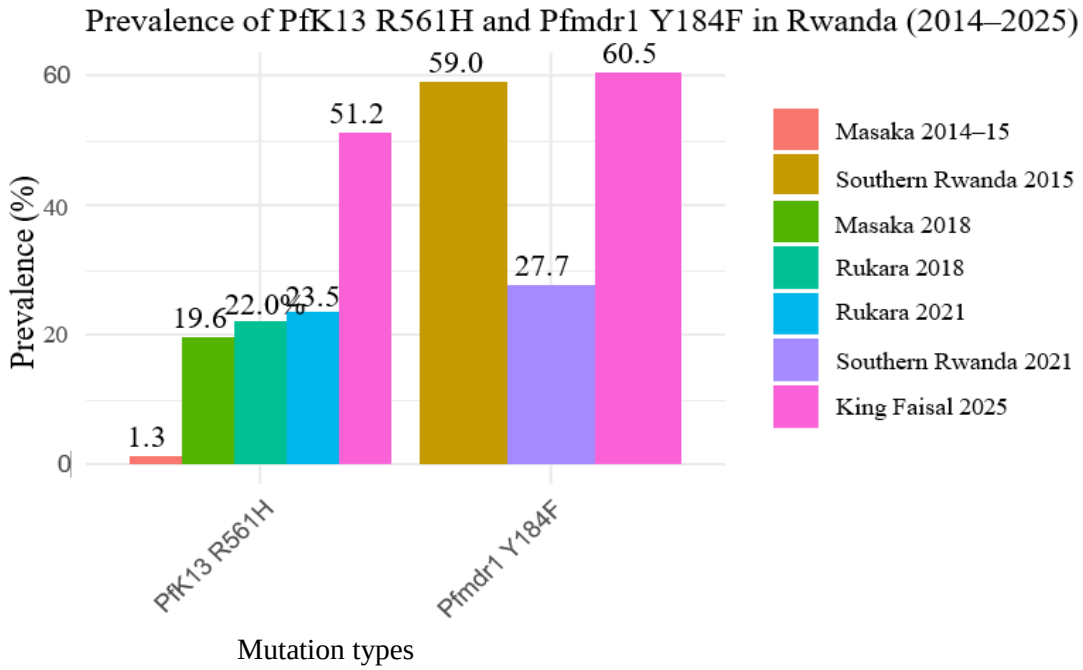

Figure 19.Comparison and progress in *Pf K13*-R561H and *Pfmdr1*- Y184F SNPS**.** KFH SNPs are increased compared to other places in Rwanda

Chapter 5. Conclusion and recommendations

5.1. Conclusion

Singles Nucleotide polymorphisms in *Plasmodium falciparum* is becoming a big challenge for malaria control and elimination worldwide and in Rwanda. SNPs in Lys76Thr in *pfcrt* gene associated with chloroquine resistance is present in Rwanda and has historically caused treatment failure. The *pfk13*-R561H SNPs is of critical mutation associated with artemesinin resistance and has been confirmed by WHO. The mutation Compromises rapid malaria parasites clearance thus causing ACT failure. *Pfmdr1*-Y184F gene Plays a role in multidrug resistance and reduce the efficacy of ACT partner drug like lumefantrine and amodiaquine. The combination of the two mutation at high prevalence of 35% as presented on Figure 16 shows a serious problem and suggest an Urgent control measure and continuous molecular surveillance.

5.2. Perspective and recommendations

The study finding highlighted increased number of mutations, it underscores the growing concern of malaria treatment failure in Rwanda. Mutations in *Pfmdr1*-Y184F, *Pfk13*-R561H and *Pfcrt*-K76T among king Faisal Hospital patients were observed to be high comparing to other region of Rwanda and it highlight the adaptation of malaria parasites to currently used anti-malaria drug artemisinin based combination therapy, artemether-lumefantrine(Coartem). The persistence of malaria despite control measures that we have in Rwanda suggest routine molecular surveillance, periodic screening of mutations, updating treatment guidelines is necessary if mutation continue to increase, enhance laboratory capacity to test SNPs associated with malaria in laboratory dairy activities, enhance surveillance of malaria control guideline implementation and vector control measures in Rwanda and other malaria endemic region. Other studies are needed to monitor the spread of drug resistance associated mutations in *plasmodium falciparum* and the study to look clinical impact of *pfk13*-R561H and *pfmdr1*-Y184F on treatment outcome for KFH.

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APPENDIX 1. Steps for amplification (adapted from promega, GoTaq® Endure qPCR Master Mix protocol)

Promega

Assembling the GoTaq® Endure qPCR Master Mix Amplification Mix (continued)

1. Prepare the amplification mix (minus the DNA template) by combining the GoTaq® Endure Master Mix, 2X, PCR primers, hydrolysis probe and Nuclease-Free Water as described below. The DNA template is added in Step 5. Vortex briefly to mix.

Component	Volume	Final Concentration
GoTaq® Endure Master Mix, 2X	10µl	1X
forward primer (20X)	1µl	200nM–1µM
reverse primer (20X)	1µl	200nM–1µM
hydrolysis probe (20X)	1µl	100–300nM
template DNA	2–5µl	≤250ng
Nuclease-Free Water to a final volume of	**20µl**	—

Note: Optimize the concentrations of primers and hydrolysis probe for each primer combination.

2. Add the appropriate volume of amplification mix (without the DNA template) to each PCR tube or well of an optical-grade PCR plate.

3. Add the DNA template or Nuclease-Free Water for no-template control (NTC) reactions, to the appropriate wells of
the reaction plate.

4. Seal the tubes or optical plate. Centrifuge plates briefly at 300 × *g* to collect contents at the bottom of the wells. Protect from extended light exposure and elevated temperatures before cycling. The samples are now ready for thermal cycling.

Note: Assembled reaction plates can be stored protected from light at ambient temperature for up to 4 hours.

APPENDIX 2. Ethical clearance

REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA

NATIONAL ETHICS COMMITTEE / COMITE NATIONAL D'ETHIQUE

E-mail: info@rnecrwanda.org
Web site: www.rnecrwanda.org

Ministry of Health
P.O. Box. 84
Kigali, Rwanda.
FWA Assurance No. 00001973
IRB 00001497 of IORG0001100

21 November 2024.

Principal Investigator: Prof Jacob Souopgui

ANNUAL RENEWAL APPROVAL NOTICE: RNEC597/2024

Protocol Title: "Addressing both naturally occurring and ACT induced Plasmodium reservoirs using Artemisia infusions and develop a family registry for malaria to accelerate malaria elimination and eradication in Rwanda: A proof of concept Randomized Controlled Trial."

After review of the protocol, progress report consent forms requested during the RNEC meeting of 09th November 2024 where quorum was met , **the requested annual renewal was approved.**

Please note that approval of the protocol and consent form both English and Kinyarwanda version is valid for **12 months**.

You are responsible for fulfilling the following requirements:

1. Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.
2. Only approved consent forms are to be used in the enrollment of participant
3. All consent forms signed by subjects should be retained on file. The RNEC may conduct audits of all study records, and consent documentation may be part of such audits.

RWANDA NATIONAL ETHICS COMMITTEE R.N.E.C P.O BOX:84 KIGALI

4. A continuing review application must be submitted to the RNEC in a timely fashion and before expiry of this approval.
5. Failure to submit a continuing review application will result in termination of the study.
6. Notify the Rwanda National Ethics committee once the study is completed.

Sincerely,

Date of Approval: 09 November 2024
Expiration date: 08 November 2025

Dr. Vedaste NDAHINDWA
Chairperson, Rwanda National Research Ethics Committee.

RWANDA NATIONAL ETHICS COMMITTEE
R.N.E.C
P.O BOX:84 KIGALI

C.C.
- Hon. Minister of Health.
- The Permanent Secretary, Ministry of Health

Approval

This is to certify that this dissertation has been developed under our supervision and that it is being submitted with supervisor's approval.

Supervisor 1:

Prof. JACOB SOUOPGUI

Professor Université Libre de Bruxelles

Brussels, Belgium

Signature…… Date… **18 / 09 / 2025**……...

Supervisor 2:

Dr. Edgar Kalimba

Deputy CEO in Charge of Medical Services.

King Faisal Hospital, Rwanda

Signature…… …….Date…**18 / 09/ 2025**……..……

Academic Supervisor:

Prof. Antoine NSABIMANA

Signature… ..…Date…**18 / 09/ 2025**……..…