UNIVERSITY OF RWANDA

MOLECULAR SURVEILLANCE OF ACT DRUG RESISTANCE MARKERS AND GENETIC DIVERSITY IN PLASMODIUM FALCIPARUM IN GASABO DISTRICT

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By

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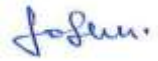
DECLARATION

I, Jean de Dieu NIYOYITA, 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.

Jean de Dieu NIYOYITA

Signature: 

Date: 31/August/2025

DEDICATION

This master dissertation is dedicated to:

- Almighty God
- My family
- University of Rwanda, College of Science and Technology
- My mentor Prof. Jean Claude TWIZERE, Université de Liège
- My supervisors: Prof. Jacob Souopgui**,** Prof. Antoine NSABIMANA and Dr. Edgar Kalimba
- European Union and ENABEL, through the KWIGIRA project which funded the master of Science in Biotechnology through University of Rwanda, College of Science and Technology.

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May God bless you all.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACT: Artemisinin-based Combination Therapy

AL: Artemether Lumefantrine

ART: Artemisinin

ASPY: Artesunate Pyronaridine

BP: Base Pair

CHWs: Community Health Workers

DHA-PPQ: Dihydroartemisinin-Piperaquine

HC: Health Center

KFHR: King Faisal Hospital, Rwanda

MoH: Ministry of Health

MSP2: Merozoites Surface Protein2

NC: Negative Control

ONT: Oxford Nanopore Technologies

PCR: Polymerase Chain Reaction

PfCRT: Plamodium Falciparum Chloroquine Resistance Transporter

PfK13: Plamodium Falciparum kelch 13

PfMDR1: Plamodium Falciparum Multi-Drug Resistance 1

RDT: Rapid Diagnostic Test

RM: Remera Health Center

RSC: Research System Controller

SNPs: Single Nucleotide Polymorphisms

WHO: World Health Organization

ABSTRACT

The emergence of *Plasmodium falciparum* resistance to artemisinin-based combination therapies (ACTs) threatens malaria control in sub-Saharan Africa, which accounts for 94% of global malaria cases and over 95% of malaria-related deaths(WHO, 2023). This study assessed ACT drug resistance markers and the genetic diversity of *P. falciparum* in Gasabo District, Kigali, Rwanda. A cross-sectional molecular epidemiological study was conducted from April to July 2025, during which 392 venous blood samples were collected. Of these, 290 samples were successfully amplified for target genes, and 54 samples were sequenced using Oxford Nanopore Technologies. The study targeted four genes: pfk13, pfmdr1, and pfcrt, which are involved in ACT metabolism, and msp2, used to assess parasite genetic diversity. Bioinformatics processing and statistical analysis using SPSS were performed to identify patterns of drug resistance and parasite diversity.

Molecular analysis revealed that 70% (n = 38) of sequenced isolates harbored single nucleotide polymorphisms (SNPs) in pfk13, pfmdr1, or pfcrt**.** The WHO-validated resistance marker R561H in pfk13 was detected in 39% (n = 21) of isolates, the pfmdr1 Y184F mutation occurred in 54% (n = 29), and pfcrt K76T, historically associated with chloroquine resistance, was observed in 7% (n = 4). Structural modeling of the novel A569V mutation in pfk13 suggested a destabilizing effect on the protein. Co-occurrence of resistance markers, particularly between pfk13 and pfmdr1, was observed in 31% of isolates, indicating a possible reduction in the efficacy of Coartem, the first-line treatment used in Rwanda. MSP2 genotyping revealed limited allelic diversity, with predominant 3D7 strains. Only a subset of the amplified samples (54 of 250) was sequenced due to academic constraints, which limited the available time.

These findings highlight ongoing transmission and emerging drug resistance, emphasizing the need to integrate molecular surveillance into national malaria control strategies to support evidence-based policy decisions and preserve ACT efficacy.

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I. INTRODUCTION

I.1 Background

The *Plasmodium* genus consists of protozoan parasites that are the causative agents of malaria in humans. There are five species known to infect humans: *Plasmodium falciparum*, *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi* (Li et al., 2024). Malaria, primarily caused by *P. falciparum*, continues to pose a significant public health threat in sub-Saharan Africa, especially affecting young children and pregnant women (Sarfo et al., 2023). Efforts to control and prevent the disease have included vector control, the use of artemisinin-based combination therapies (ACTs), and the initiation of RTS,S and R21 vaccine delivery in some countires, all of which have played an important role in lowering malaria cases (Nguyen et al., 2023). Despite these advancements, malaria persists as a major health issue, with a concerning resurgence noted in recent years.

In 2023 alone, 263 million malaria cases and 597,000 deaths were reported globally, with the African region accounting for over 94% of cases and more than 95% of deaths, which represents about 11 millions more cases reported in 2022(WHO, 2023). Clearly highlighting the region's heavy malaria burden, as shown in the map below.

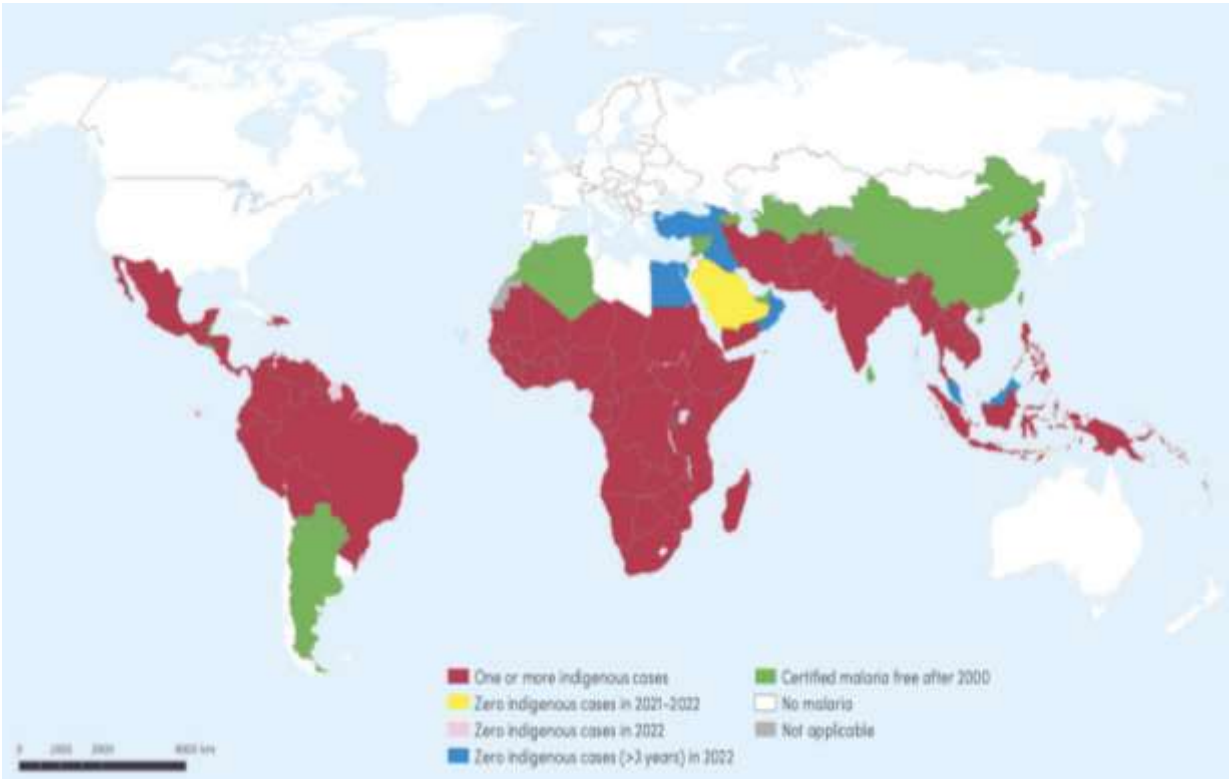

Figure 1 Word malaria map showing the global distribution (WHO,2023).

I.1 Plasmodium falciparum life cycle

Plasmodium falciparum is responsible for causing malaria. The disease is transmitted when a female mosquito bites a human seeking blood meal. During this bite, the mosquito injects sporozoites into the human bloodstream. These sporozoites go to the liver, where they invade liver cells(hepatocytes) and undergo the exoerythrocytic①. Inside the hepatocytes sporozoites develop into schizonts, which mature and rupture, then release merozoites into the blood stream. The merozoites infect red blood cells, initiating the erytrocytic cycle②**.** Within the red blood cells, merozoites develop into trophozoites ring stage and then sexual forms called gametocytes or schizonts. These schizonts rupture the RBCs, releasing more merozoites that invade new red blood cells, continuing the cycle and causes fever and chills. When another female mosquito bites an infected person and ingests gametocytes, the sexual stage of *plasmodium falciparum* life cycle begins in the *anopheles* mosquito's midgut③. The gametocytes (males and females) fuse to form a zygote which undergoes several developmental stages to become an oocyst. This oocyst matures and ruptures and release much sporozoites that migrate to the *anopheles* mosquito's salivary gland and ready to infect a new human host during the next blood meal.

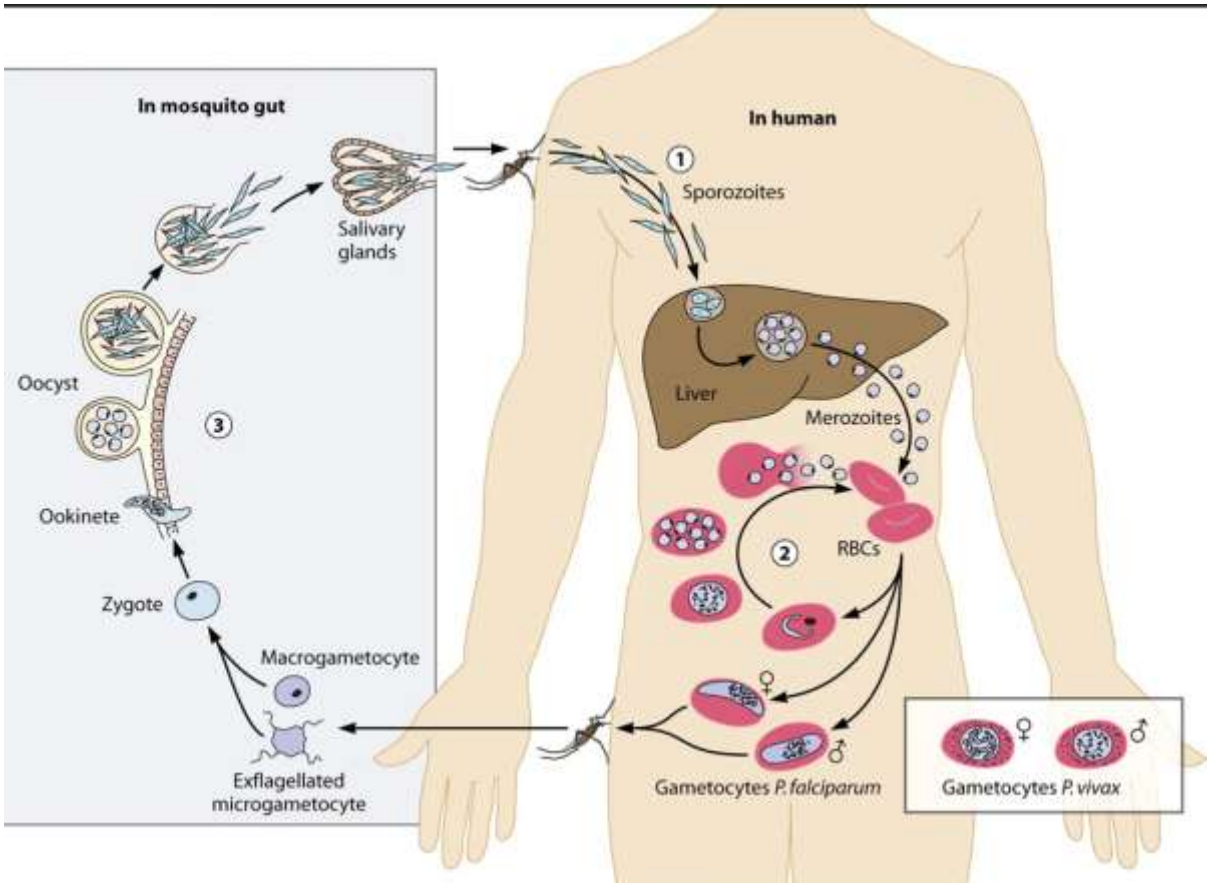

Figure 2 Plasmodium falciparum life cycle (source: https://doi.org/10.1128/cmr.00051-10)

During the sexual stage of *plasmodium falciparum* life cycle, within the mosquito, genetic recombination may occur, contributing to the genetic diversity in the parasite. The msp2 gene of *plasmodium falciparum* is widely used as molecular marker to assess this diversity(Rosenthal et al., 2024). It comprises two major allelic family 3D7 and FC27 which are commonly used for strain identification. Both allelic types contain a central variable region characterized by distinct repeat motifs, and each may further exhibit sub-alleles giving rise to sub-strains(Uwimana et al., 2020). In Africa, the 3D7 sub-alleles has been extensively reported as more prevalent over FC27. In Rwanda, the diversity within the 3D7 families was found to contribute to immune evasion, which can increase the disease burden(Mwesigwa, Ocan, et al., 2024). In addition to genetic diversity, the continued persistence of malaria may be driven by the increase of parasite resistance to antimalarial drugs. This resistance is largely attributed to single nucleotide polymorphisms(SNPs) in specific genes involved in drug action.

The first SNP associated with antimalarial drug(artemisinin) resistance was identified in the *Pf k13* gene propeller domain of clinical isolates from Southeast Asia, and later reported in Kenya, eventually spreading across Africa(Meier-Scherling et al., 2025). In addition to artemisinin resistance, *Plasmodium falciparum* parasites have developed resistance to partner drugs, driven by SNPs in genes such as *pfcrt* and *pfmdr1*, depending on the specific drug class. To date, the World Health Organization(WHO) has validated around 20SNPs as markers of partial resistance to ACTs including; R561H, P553L,K76T,K189T,Y184F and C580Y(Millogo et al., 2024). In response to this challenge, WHO through its member states has implemented a range of control measures, including improved clinical case management, vector control and introduction of antimalarial vaccines. In this framework, Rwanda prioritized community awareness, use of insecticide treated bed nets(ITNs), implementation of indoor residual spraying(IRS), improved diagnostic tools supported by community health workers (CHWs), and the introduction of artemisinin-based combination therapies(Umugwaneza et al., 2025).

These interventions have collectively contributed to a significant decrease in malaria incidence and mortality in Rwanda. However, the country has recently experienced a notable resurgence in malaria cases with 802428 cases treated in 2024, an increase from 600,000 in 2023(RBC report, World malaria day, April2025), this suggests a potential link to parasite genetic diversity and antimalarial drug resistance. To assess antimalarial drugs resistance, various genetic markers associated with artemisinin-based combination therapies have been investigated both globally and within the country, as outlined in the literature review below.

II. LITERATURE REVIEW

Up to date, artemisinin-based combination therapies(ACTs) remain the cornerstone of *Plasmodium falciparum* treatment, particularly in sub-Saharan Africa, and have demonstrated high efficacy across many endemic regions(Schreidah et al., 2024). However, the emergence and spread of *plasmodium falciparum* strains with reduced susceptibility to ACTs pose a significant problem to malaria control efforts. Molecular surveillance has identified gene specific key markers in *plasmodium falciparum* which are strongly associated with artemisinin resistance and decreased efficacy of partner drugs(Loon et al., 2023). Studies conducted in Africa have reported various genetic markers in the *Pfk13* gene linked to artemisinin resistance and *Pfcrt* and *Pfmdr1*genes for partner drugs with prevalence varying across the region.

II.1 *Plasmodium falciparum* kelch 13(Pf13) gene markers causing artemisinin resistance

The *plasmodium falciparum* kelch13 gene, located on chromosome 13 , encodes a protein involved in the artemisinin mechanism of action, a key factor in parasite clearance during artemisinin(ART) treatment(Wicht et al., 2021). Mutations in this gene, specifically single nucleotide polymorphisms(SNPs) can impair the drug efficacy, leading to delayed parasite clearance. Since 2014, East Africa has seen the emergence of locally derived *plasmodium falciparum* mutations/SNPs associated with artemisinin drug resistance following treatment with ACTs(Rosenthal et al., 2024). Reports from Rwanda, Uganda, Eritrea, and Ethiopia have documented the presence of some WHO-validated *pfk13* gene markers associated with ART drug resistance including; C469Y(cysteine to tyrosine), R561H(arginine to histidine), A622I(alanine to isoleucine), and P675V(proline to valine)(Conrad et al., 2023).

Specifically, in Rwanda *PfK13* gene markers such as R561H, A675V, C469F, and P574L have been documented, with R561H being the most prevalent and strongly associated with resistance to artemisinin or its derivatives. This molecular marker has been reported across multiple endemic regions over the years(Schallenberg et al., 2025a). Reported prevalence of the R561H mutation includes: 2.47% in Ngoma(2014-2015), 5.47% in Kirehe(2014-2015), 7.4% in Masaka(2015), 19.6% in Masaka(2018) and22% in Rukara(2018) and 4.5% in Huye district (2019)(Schreidah et al., 2024). Despite the emergence of resistance to artemisinin or its derivatives driven by mutations in *Pfk13* gene, reduced efficacy of partner drugs have also been reported, primarily due to the mutations in *pfmdr1* and *pfcrt* genes, further contributing to

ACTs resistance. The figure below shows the distribution of drug-resistant associated with *pf k13* mutations previous reported studies from selected regions.

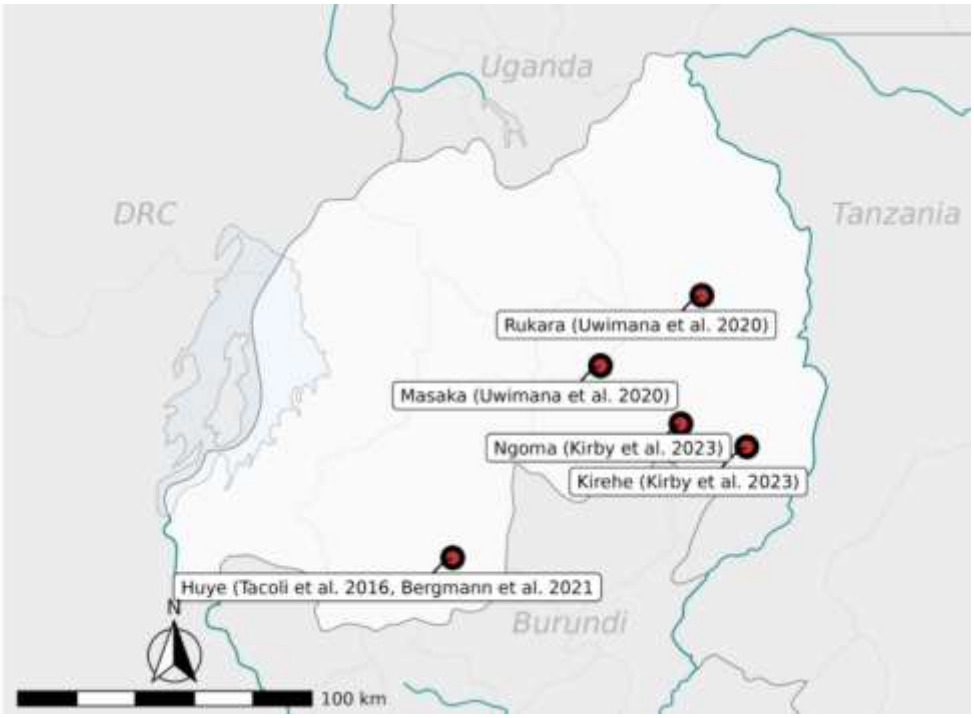

Figure 3 The map illustrating the geographic distribution of previous reported K13 mutations(Schreidah et al., 2024)

II.2 *Plasmodium falciparum* multidrug resistance1(pfmdr1) gene markers to ART partner drugs resistance.

Pfmdr1 gene is located on chromosome 5 of *plasmodium falciparum* and encodes a membrane-bound ATP binding cassette (ABC) transporter protein that helps regulate the movement of substances across the parasite digestive vacuole (DV) membrane. In the wild type parasite, ABC does not actively expel significant amounts of antimalarial drugs from the DV, allowing effective drug accumulation within the vacuole, which contributes to parasite killing(Van Loon et al., 2021). However, in mutant forms, *pfmdr1* proteins(ABC) more efficiently pump drugs out of the DV, reducing their intracellular concentration and thereby compromising drug efficacy, which can lead to treatment failure(Ross & Fidock, 2019). SNPs in *pfmdr1* protein, particularly at amino acids N86Y**,** Y184F**,** and D1246Y, have been consistently documented across multiple studies in Rwanda to alter the efficacy of ACT partner drugs (lumefantrine and mefloquine), where Y184F variant has been reported at prevalence of 53.8%(Loon et al., 2021).

II.3 *Plasmodium falciparum* chloroquine resistance transporter gene (*Pfcrt*) mutations

The *pfcrt* gene located on chromosome 7 of plasmodium falciparum encodes transmembrane transporter protein on the digestive vacuole. In wild type, this protein does not affect antimalarial drug activity. However, when mutations occur, the structure and function of the protein are altered enabling the efflux of drugs thereby reducing their efficacy and leading to treatment failure. The *pfcrt* gene is critically associated with ART-partner drug resistance (piperaquine and pyronaridine). The K76T mutation is a well characterized marker of chloroquine drug resistance and remains highly prevalent in Rwanda(Schallenberg et al., 2025b). Beyond K67T, recent studies in Southeast Asia have identified additional *pfcrt* mutations such as F145I, M343L, and G353V that are associated with reduced susceptibility to piperaquine and pyronardine(Wicht et al., 2022). However, these mutations are not yet documented in Rwanda. For all molecular markers discussed above, their interaction with parasite genetic diversity further complicates malaria control efforts. This genetic diversity is commonly assessed through msp2 genotyping, which helps in identifying circulating *plasmodium* strains and estimating the multiplicity of infection(Kiwuwa, et al., 2024).

II.4 *Plasmodium falciparum* msp2 genetic diversity and multiplicity of infection(MOI)

Merozoite Surface Protein 2 (msp2) gene located in chromosome2 encode for antigenic protein and is a highly polymorphic marker expressed during the merozoite stage of *plasmodium falciparum*, a factor that promote immune evasion and complicate effective disease management(Mwesigwa, Kiwuwa, et al., 2024). A systematic review of 52 studies from 23 sub-Saharan Africa countries reported 3D7and FC27 msp2 allelic families occurring at 60 and 55%respectively and an overall MOI prevalence of 63%(Mwesigwa, Ocan, et al., 2024). The current study, conducted in Gasabo district, similarly demonstrated high *msp2* polymorphism, with a predominance of 3D7 alleles(Kalimba et al., 2025). Despite the availability of molecular data, the Rwanda Ministry of Health(MoH) continues to prioritize malaria control through microscopy-based diagnosis at health facilities and rapid diagnostic tests in the community through CHWs, alongside the use of ACTs and other control interventions. However, molecular surveillance remains a limited component of the national malaria control strategy.

II.5 Rationale of the study

Artemisinin-based combination therapies have played a pivotal role in reducing malaria morbidity and mortality globally, particularly in sub-Saharan Africa, where *plasmodium falciparum* remains the predominant malaria parasite(Schreidah et al., 2024). In Rwanda, the ACTs are the cornerstone of malaria treatment, supported by extensive vector control measures(Schallenberg et al., 2025b). However, the persistence and resurgence of *P. falciparum* malaria cases in recent years have raised concerns about the emergence and spread of antimalarial drug resistance. Previous molecular surveillance has identified specific genetic markers in the *pfk13,pfcrt* and *pfmdr1* genes that are associated with reduced susceptibility to ACT drugs (Schallenberg et al., 2025a). In response, the Rwandan ministry of health has strengthened community-based malaria case management and introduced second-line treatments in selected areas alongside other malaria control measures.

Despite these efforts, continued reports of rising malaria cases and treatment failures coupled with limited current molecular data, underscore the urgent need for sustained molecular monitoring to inform timely policy adjustments. Given the introduction of dihydroartemisinin-piperaquine and artesunate pyronaridine as a second line therapies in selected regions, including Kigali city, and the increasing reports of malaria incidence and treatment failures across health facilities in Gasabo district, it is imperative to monitor *pfcrt* mutations associated resistance to the second line therapies, alongside other key molecular markers associated with ACT drug resistance. A comprehensive assessment of *pfk13, pfcrt* and *pfmdr1* polymorphisms combined with *msp2*-based genotyping provide critical insights into both drug resistance patterns, the genetic diversity and multiplicity of infection of circulating *P. falciparum* strains. Through the following study objectives, this research aims to address the current knowledge gap and support evidence-based malaria control strategies.

II.5.1 Objectives of the study

II.5.1.1 Main objectives of the study

Based on the above rationale the overall objective of this study was to evaluate ACT-drug resistance markers and genetic diversity in *plasmodium falciparum* in Gasabo district, Kigali city. To meet this overall objective, the following specific objective were implemented.

II.5.1.2 Specific objectives

1. To determine the prevalence of Single Nucleotides Polymorphisms in the *Pfk13, Pfmdr1and Pfcrt* genes associated with ACTs drug resistance in *P. falciparum* isolates. Artemether lumefantrine combination(AL) commonly known as Coartem(1st line), Pyronaridine and piperaquine-based (2nd line) are treatments for malaria in Rwanda. Resistance to artemisinin combination therapies is primarily linked to single nucleotides polymorphisms(SNPs)in the *PfKelch13* gene for artemisinin or its derivatives, and in the *Pfmdr1(*lumefantrine*)* and *Pfcrt (*piperaquine and pyronaridine*)* genes for partner drugs.

To address the above objective, the following work packages were implemented:

1.Venous blood sample collection from patients with confirmed *Plasmodium falciparum* infection at Remera health center(RM).
2. Genomic DNA extraction from collected samples.
3. Targeted gene amplification and purification (*Pfk13, Pfmdr, pfmsp2 and Pfcrt*).
4. Oxford Nanopore Technology (ONT) library preparation and sequencing using MinION.
5. Bioinformatics and data analysis.

2. Studying *Pfmsp2* genetic diversity and multiplicity of infection in Gasabo district

Parasite diversity remain a major obstacle in malaria control efforts. The genetic variability of the merozoites surface protein 2(msp2) gene makes it a useful marker for studying this diversity. Its polymorphic nature also allows for the identification of infections caused by a single parasite strain or multiple different strains within the same host. To achieve this second objective, the following work packages were done: Amplification of the msp2 gene using DNA extracted in the previous work package (related to objective1), followed by target gene purification, sequencing using ONT and subsequent analysis using bioinformatics tools.

3.To investigate the co-occurrence of genetic markers in *Plasmodium falciparum* isolates to assess potential for multidrug resistance to ACT.

Artemisinin resistance is primarily associated with SNPs in *pfk13*, while resistance to partner drugs is linked to SNPs in *pfmdr1* and/or *pfcrt*. When SNPs occur across these genes within the same parasite strain, resistance may develop against both artemisinin (or its derivatives) and partner drugs, potentially resulting in multidrug resistance. Based on the the previous work package (Objective 1), an Excel dataset was generated to visualize the co-occurrence of the genetic markers within individual parasite strains and to present their frequencies using chart representations.

4.Molecular impact of key mutations on the protein structural features and function

For a mutation to impact drug efficacy, it must alter the structure of the associated protein in a way that affects its biological function. Structural changes in proteins are well known to disrupt their normal activity, and this principle applies to drug–target interactions. In the case of *Plasmodium falciparum* proteins involved in the mechanism of action of ACTs, SNP-induced alterations may impair drug binding or activity, and lead to drug resistance. To investigate this, protein structure prediction and analysis were performed using the AlphaFold3 server

III. METHODOLOGY

III.1 Study Plan

A cross-sectional molecular epidemiological study was conducted between April to July 2025 to provide a snapshot of the genetic polymorphism of *Plasmodium falciparum* in the study area.

III.2 Study area

This study was conducted at Remera Health Center (RM-HC) for participants' recruitment and venous blood sample collection. All subsequent laboratory procedures were performed at the molecular biology research laboratory of King Faisal Hospital (KFH). Remera HC is a public healthcare facility located in Gasabo district, Kigali City, serving population from Remera and neighboring sectors. King Faisal Hospital is a teaching hospital at tertiary level and a multidisciplinary referral institution known for its excellence in specialized clinical care, diagnostics, and research.

III.3 Study population, inclusion and exclusion criteria

The target population consisted of patients attending Remera Health Center who were confirmed, through microscopy, to be infected with *Plasmodium falciparum*. All individuals who tested positive for *P. falciparum* were eligible to participate in the study, whereas those infected with other *Plasmodium* species were excluded.

III.4 Sampling technique

A convenience sampling technique was used to recruit participants meeting the study criteria. 400 Venous blood samples were collected from individuals who provided informed consent. To meet the study objectives, laboratory procedures were performed for downstream processing, beginning with genomic DNA extraction.

III.5 Genomic DNA extraction

Genomic DNA was extracted from 400 EDTA whole blood samples following the manufacturers' protocols using two approaches: a manual method with the Quick-DNA Miniprep Plus Kit (Zymo Research, D4069) and a semi-automated method utilizing the Maxwell® 48 RSC instrument with the Maxwell® RSC Blood DNA Kit (Promega, AS1400). The manual extraction involved lysing cells by mixing blood with lysis buffer and Proteinase K to break down cell membranes and digest proteins. A genomic binding buffer was then added to facilitate DNA binding to the silica membrane of a spin column. The column underwent

sequential washes to remove proteins, salts, and other impurities, and purified DNA was finally eluted using nuclease-free water. In the semi-automated method, the Maxwell® 48 RSC instrument was used to streamline DNA extraction by automating the lysis, binding, washing, and elution steps. Whole blood samples were processed according to the kit protocol, with magnetic beads capturing the DNA during automated washing cycles. The end product from both methods was high-quality DNA extracts suitable for downstream applications such as PCR amplification.

III.6 Target gene amplification using PCR

Uniplex, multiplex, and nested PCR methods were employed to amplify 380 samples for specific Plasmodium *falciparum* genes (*kelch13*, *pfmdr1*, *pfcrt*, and *msp-2)*. Initial amplifications were carried out using GoTaq® green master mix (Promega, M712). For samples that failed to amplify with this master mix, a second attempt was made using GoTaq® endure qPCR master mix (Promega, A6220) to improve amplification efficiency. All PCR procedures were conducted on a Bio-Rad thermal cycler. Following initial screening, samples identified with *pfk13* mutations were re-amplified using primers targeting the *pfk13* gene's propeller domain to ensure specificity. Similarly, samples harboring mutations in *pfmdr1* and *pfcrt* were also re-amplified to prepare them for mutation validation through Sanger sequencing. This approach ensured accurate detection and confirmation of genetic polymorphisms in the target genes.

Table1: Primes used in amplification of *Plasmodium falciparum* target genes

Oligonucleotides	**Sequence (5′-3′)**	**Gene size(bp)**
PFK13 Forward	GATGCAGCAAATCTTATAAATGATGATTCTGG	1612
PFK13 Reverse	GCCAAGCTGCCATTCATTTG	
PFCRT Forward	TGTCTTGGTAAATGTGCTCA	177
PFCRT Reverse	AGTTGTGAGTTTCGGATGTT	
PFMDR1 Forward	TGTGTTTGGTGTAATATTAAAGAACA	363
PFMDR1 Reverse	ACATAAAGTCAAACGTGCATTT	
PFMSP2 Forward	ATGAAGGTAATTAAAACATTGTCTATTATA	811
PFMSP2 Reverse	ATATGGCAAAAGATAAAACAAGTGTTGCTG	

III.7 Agarose gel electrophoresis

To confirm that the sequence amplified corresponded to the intended target genes, the PCR products were subjected to agarose gel electrophoresis, which separates DNA fragments based on size. The resulting DNA bands were then compared to a DNA ladder/marker containing fragments of known sizes. To do that, the gel was prepared by dissolving 4 g of agarose powder in 200 mL of 0.5X TAE (Tris-acetate-EDTA) buffer, followed by melting the mixture in a microwave. After cooling slightly, 2ul of SYBR Safe DNA stain was added. The molten gel was poured into casting trays fitted with combs to form wells and allowed to solidify prior to sample loading. Subsequently, 4 µL of each PCR product was loaded into individual wells of a 2% agarose gel and electrophoresed at 100 V for 30 minutes. The PCR amplicons were then visualized under UV illumination. DNA fragment was estimated using the BenchTop 100 bp DNA Ladder (Promega, G8291), which contains 11 fragments ranging from 100bp to 1500bp.

III.8 PCR products purification

Following electrophoresis, PCR products corresponding to the target genes were pooled to reconstruct the complete gene set for each sample. The pooled amplicons were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, A9282) to remove residual primers, nucleotides, and other PCR components, yielding pure DNA amplicons for 300 samples.

III.9 PCR products quantification

Purified DNA quality and quantity were evaluated using two complementary approaches. First, the EzDrop 1000 spectrophotometer was used for an initial concentration check targeting quality. Second, DNA purity and accurate quantification were determined using the QuantiFluor® ONE dsDNA System (Promega), which selectively binds double-stranded DNA for fluorescence-based measurement. Based on the QuantiFluor results, DNA concentrations were normalized to meet library preparation requirements: 250 ng for high-yield amplicons and 150 ng for lower-yield amplicons. Only samples meeting these criteria were selected for Oxford Nanopore Technologies (ONT) library preparation.

III.10 Library preparation for ONT sequencing

ONT library preparation is a process of converting amplified PCR products into a format that can be recognized and sequenced by the oxford nanopore platform. It involves a series of enzymatic reactions and clean-up steps that ensure the DNA is intact, has the correct chemical

ends, and carries the necessary sequencing adapters. The workflow typically follows four main stages and library was prepared for 54 samples.

III.10.1 DNA repair and End-preparation

Normalized DNA amplicons were enzymatically treated to correct chemical or structural imperfections that could interfere with sequencing, ensuring compatibility with downstream ligation steps. PCR products intended for sequencing may contain nicks, damaged bases, or irregular ends, and were therefore processed using the NEBNext Ultra II End Repair/dA-Tailing Module. This treatment repaired DNA ends and added a single adenine (A) overhang to the 3′ termini, enabling precise pairing with thymine (T) overhangs on the sequencing adapters, thereby enhancing ligation efficiency. A small DNA control sample was included to monitor workflow quality. The reaction was carried out in a thermocycler at 20 °C for 5 minutes, followed by 65 °C for 5 minutes, to maximize enzyme performance, yielding double stranded DNA with repaired bases, standardized ends, and optimal chemical features for the next stage barcode ligation.

III.10.2 Native Barcode Ligation

Oxford Nanopore Technologies (ONT) enables simultaneous sequencing of multiple samples on a single flow cell by assigning each sample a unique molecular identifier called a barcode. Each repaired DNA fragment was enzymatically ligated to a unique barcode adapter. These barcode adapters have complementary thymine (T) overhangs that specifically pair with the adenine (A) overhangs previously added to the 3′ ends of DNA fragments during the dA-tailing step, ensuring efficient and accurate barcode attachment to each molecule. After barcoding, the ligation reaction was stopped by adding EDTA to stop enzymatic activity, and all barcoded samples were pooled together. The combined DNA pool was purified using AMPure XP magnetic beads, which selectively bind DNA to remove excess enzymes, unligated adapters, and small unwanted fragments. The purified DNA was then eluted in nuclease-free water, quantified, and prepared for the next critical step of adapter ligation.

III.10. 3 Adapter ligation and clean-up

After barcoding, the DNA molecules were ligated to ONT sequencing adapters, which are specially designed to include motor proteins and tethering elements essential for controlling

DNA movement through the nanopores during sequencing. The motor proteins regulate the speed of DNA translocation, allowing accurate base detection, while the tethers help stabilize the DNA within the flow cell. Following ligation, a magnetic bead cleanup was performed to remove unligated adapters, enzymes, and small unwanted DNA, ensuring a pure DNA library. The purified, adapter-ligated library was quantified to determine its concentration, ensuring the correct amount was prepared for loading onto the sequencing flow cell.

III.10.4 Priming and Loading the Flow Cell

Before sequencing begins, the nanopore flow cell was primed to ensure efficient nanopore function throughout the run. A buffer solution containing tethering proteins and bovine serum albumin (BSA) was used to stabilize the environment and prepare the nanopores for optimal performance. The prepared DNA library was then mixed with sequencing buffer and library beads, which help evenly distribute DNA molecules across the flow cell. Prior to sequencing, the quality of each flow cell was checked by evaluating the number of active pores. Finally, this mixture was gently loaded dropwise onto the SpotON port of the flow cell, making it ready for sequencing on the ONT device. Sequencing was carried out using the MinION Mk1B device from Oxford Nanopore Technologies (ONT) FLO-MIN114 (R10.4.1), operated via MinKNOW software (v24.06.16, ONT, UK) which convert electrical signals data into FASTQ reads for downstream analysis.

III.11 Bioinformatics Pipeline and Variant Analysis

After sequencing, the raw DNA data from the Oxford Nanopore MinION device was collected in the form of FASTQ files, which contain the sequences of DNA reads along with quality scores. The first step was to evaluate the quality of these reads using specialized software tools like pycoQC and NanoPlot. These tools provide visual summaries and statistics about the length of the reads and their overall quality, helping to identify any problems early. The raw sequences data often include leftover adapter sequences from the library preparation, as well as low-quality bases at the ends of reads, which can interfere with accurate analysis. To clean these up, a program called Porechop was used to trim away adapters and remove poor quality ends. After this trimming, the data was checked again with NanoPlot to confirm that all unwanted sequences and low-quality reads (those with a quality score below 10) have been removed. The cleaned, high-quality reads are then aligned to the well-studied *Plasmodium falciparum* 3D7 genome. This alignment was performed by a software called Minimap2**,** which

efficiently finds where each read best matches on the reference. The alignment results are then processed with a set of tools called samtools to sort, filter, and index the data, preparing it for detailed analysis.

Additional quality checks on the alignments were done using Qualimap, and the data was visually inspected with the integrative genomics viewer (IGV) to spot any anomalies or errors. Once the data was aligned and verified, the next step was to identify genetic variations, focusing on single nucleotide polymorphisms (SNPs) changes in the DNA sequence that may affect parasite and lead to drug resistance. SNPs were called using a program called Clair3**,** which analyzes the aligned reads to detect and report these variations in a format known as gVCF. These variant calls were then filtered to keep only high confidence SNPs based on quality scores, sequencing depth, and minor allele frequency, which helps exclude sequencing errors or extremely rare variants unlikely to be biologically relevant. The filtered variant files were merged and converted into a standard format (VCF) suitable for further analysis. Using a program called SnpEff**,** these variants were annotated, meaning they were linked to specific genes (*pfmsp2*, *pfkelch13*, *pfcrt*, and *pfmdr1*). To understand the significance of detected mutations, the results were cross-referenced with the PlasmoDB database, which catalogs known mutations in *Plasmodium falciparum*. The obtained results were analyzed using Excel and the Stata statistical package to calculate proportions and generate graphs.

IV. THE STUDY RESULTS

The overall objective of the study was successfully achieved. Target genes were amplified using PCR, and successful amplification was confirmed by agarose gel electrophoresis, with DNA bands visualized under UV illumination, as shown in the figures below.

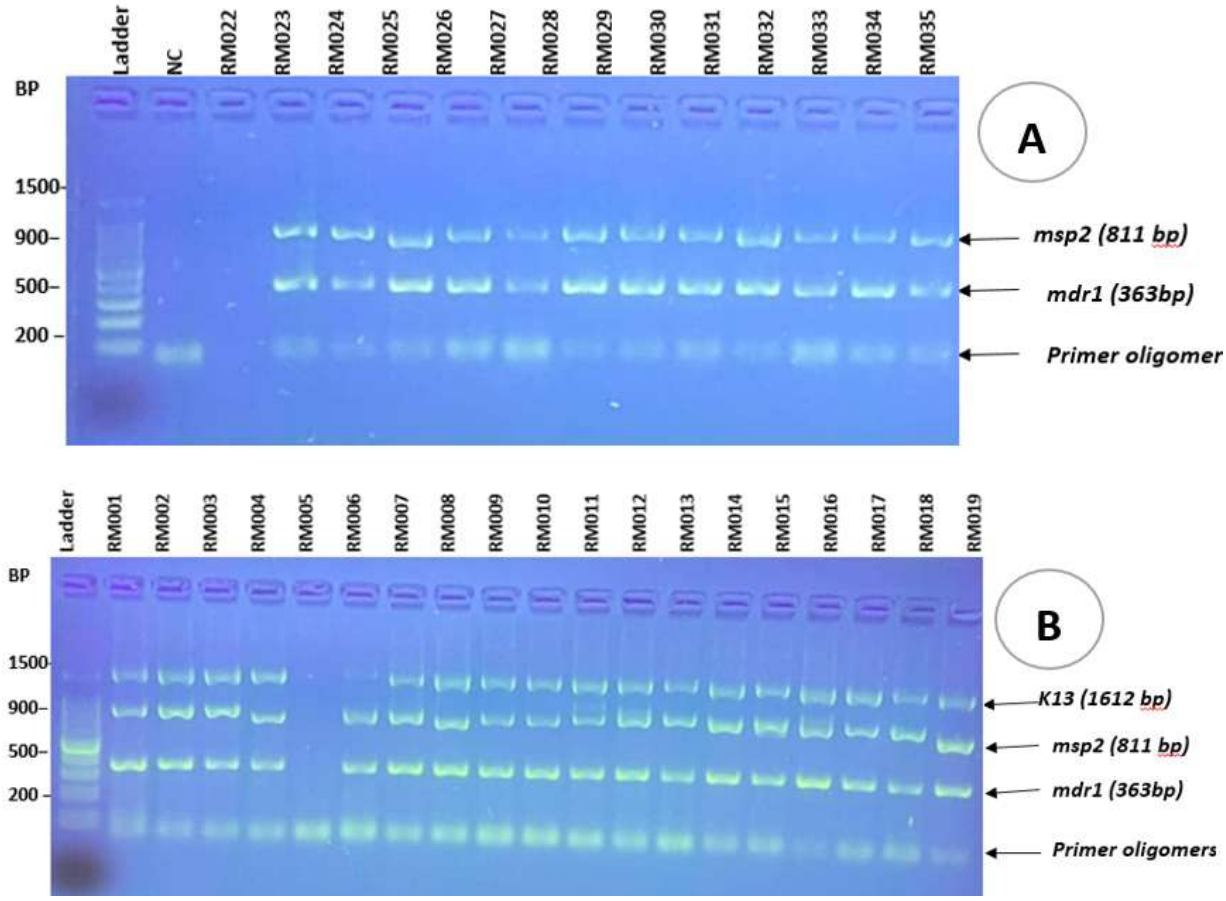

Figure 4*:* Gel electrophoresis image showing PCR products visualized under UV illumination

The above figure represented by images A and B, display the multiplex PCR products for a subset of the target genes. Specifically, in image A, detectable bands indicate successful amplification *pfmsp2* and *pfmdr1* genes in samples RM023 to RM035. In contrast, sample RM022 showed no detectable band, indicating the lack of amplification for these genes. The smallest bands migrating beyond the lowest marker band are likely primer oligomers. The negative control(NC) exhibited no amplification, confirming the absence of contamination. The DNA marker(ladder) displayed distinct bands, serving as a reference for estimating the sizes of the amplified gene fragments. Additional target gene not shown in previous image is presented in the figure below.

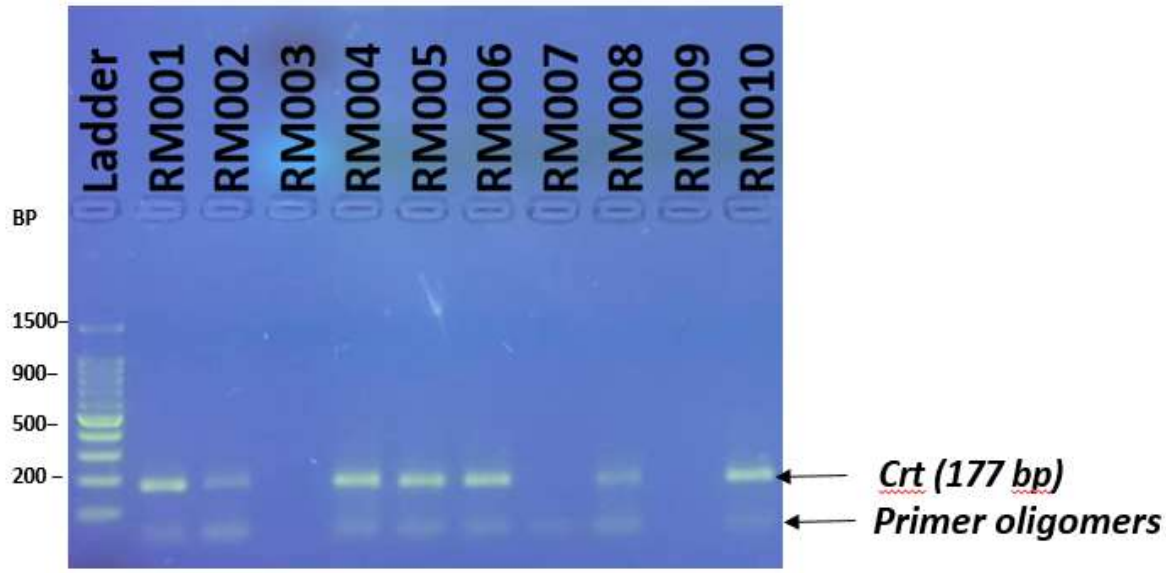

Figure 5: Gel electrophoresis image showing uniplexed PCR products

This figure shows uniplex PCR products for *pfcrt* gene amplification. Seven samples exhibit bands at approximately 200 bp**,** indicating successful amplification. In contrast, three samples (RM003**,** RM007, and RM009) showed no visible bands, suggesting the absence or failed amplification of the *pfcrt* gene target.

The successfully amplified genes underwent subsequent processing, including sequencing using Oxford Nanopore Technology(ONT). The resulting data were analyzed using bioinformatics tools and the prevalence of genetic markers in target genes was calculated. Among 54 samples successfully sequenced, 70%(n=38) exhibited mutations(SNPs) in at least one of the target genes (*pfk13, pfmdr1* and *pfcrt*). Specifically, mutations(SNPs) in *pfk13* were identified in 56%(n=30) of these samples, *pfmdr1* mutations(SNPs) were present in 59%(n=30) and *pfcrt* mutations were observed in 7%(n=4) as shown in the below figure.

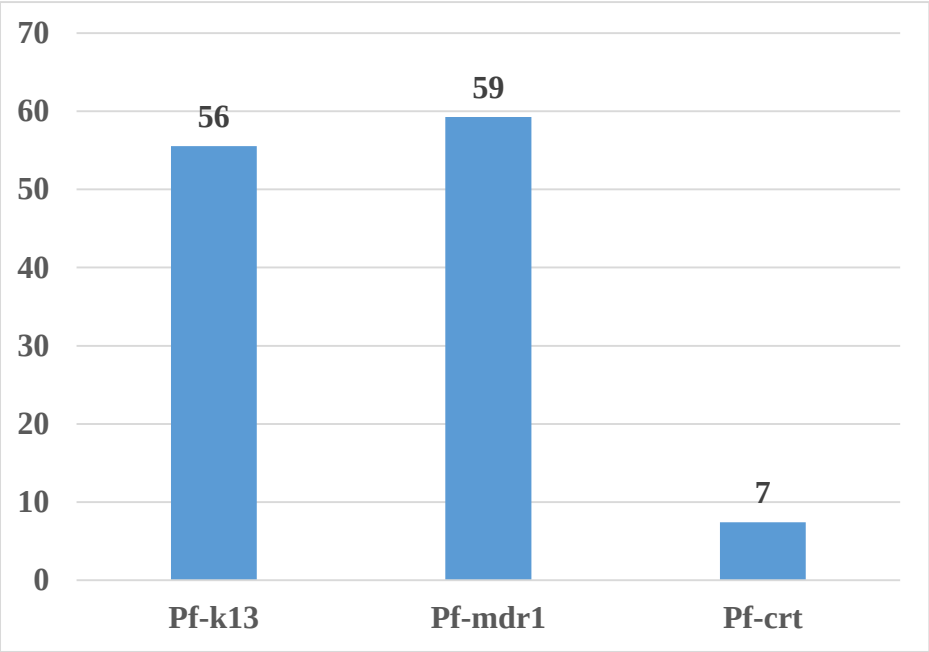

Figure 6: Prevalence of *pfk13, pfmdr1* and *pfcrt genes mutations* (% n=54)

IV.1 Prevalence of Single Nucleotides Polymorphism(SNPs) in the *pfk13, pfmdr1* and *pfcrt* genes associated with ACT-resistance in *P. falciparum* isolates.

The prevalence of individual single nucleotide polymorphisms in the target genes was assessed to identify the markers associated with resistance to artemisinin-based combination therapies(ACTs). Initially, six distinct SNPs were identified in ***pfk13***: K189T, R561H, A569V, P441L, T207K, and A578S with different proportion. Among these, K189T (41%) and R561H (39%) are WHO-validated markers associated with artemisinin resistance. In *pfmdr1*, the Y184F (54%) mutation associated with reduced susceptibility to lumefantrine and mefloquine partner drugs in ACT, was found at high frequency. For ***pfcrt***, three mutations were identified: K76T (7%), a well-known marker of resistance to the former antimalarial drug chloroquine, and two frameshift mutations, M74fs and N75fs, which currently have no established association with resistance to ACT partner drugs. The below figure summarize prevalence of all SNPs identified.

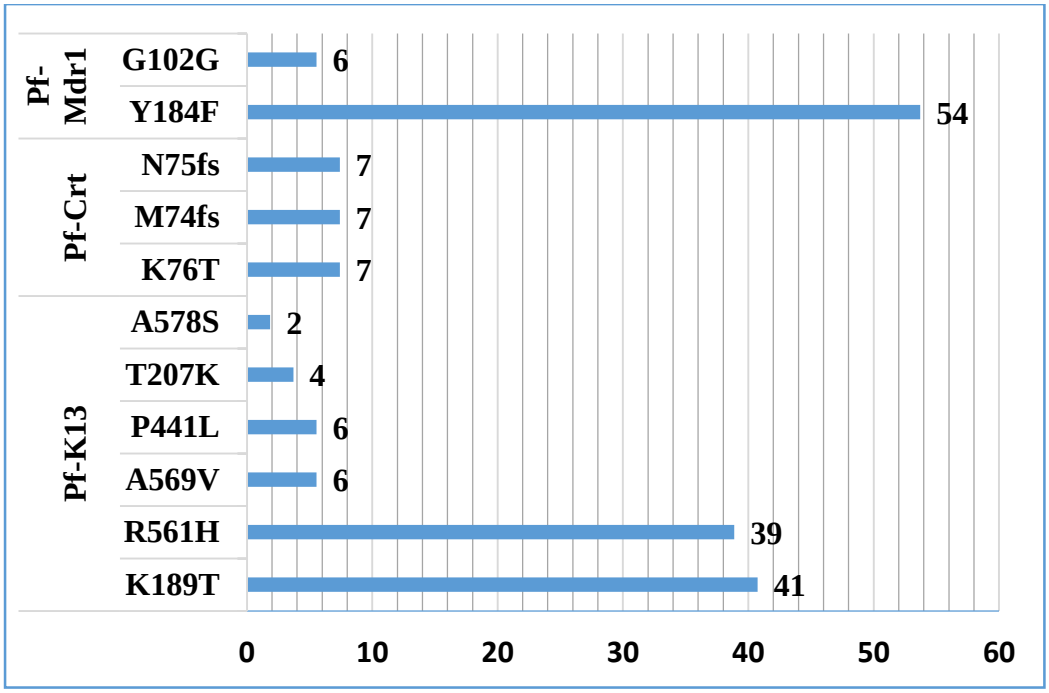

Figure 7: Prevalence of genetic SNPs emerging in the study site (%, n=54).

IV.2 The co-occurrence of genetic markers to assess potential for multidrug resistance.

Some *P. falciparum* isolates exhibited single nucleotide polymorphisms (SNPs) in more than one gene. Co-occurrence was more frequent between *pfk13* and *pfmdr1* markers (31%) than between *pfk13* and *pfcrt* markers (2%). The *pfk13–pfmdr1* association may indicate a substantial reduction in susceptibility to artemether–lumefantrine (AL), the current first-line treatment in Rwanda. However, the very low *pfk13–pfcrt* co-occurrence suggests a minimal likelihood of multidrug resistance. The chart below illustrates the patterns of co-occurring *pf* gene markers among the identified SNP.

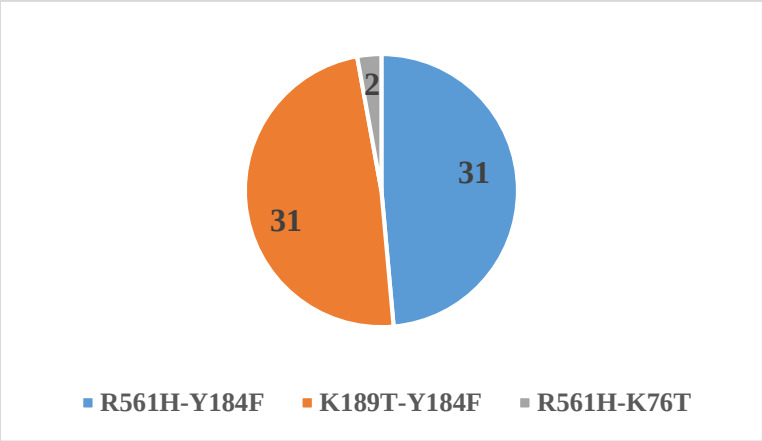

Figure 8: Prevalence of co-occurrence of ACTs resistant markers

IV.3 Proportion of *plasmodium falciparum* msp*2* genetic diversity in Gasabo district

Analysis of *msp2* gene diversity revealed three circulating *Plasmodium falciparum* strains, all belonging to the 3D7 allelic family but present at different proportions: Pf3D7_05_v3(59%), Pf3D7_07_v3(7%)**,** and Pf3D7_13_v3(56%)**.** To assess the potential of multiplicity of infection, the co-occurrence of sub-alleles was examined. Notably, Pf3D7_05_v3 co-occurred with Pf3D7_13_v3 in 46% of the samples, suggesting the presence of multiple infections within individuals. The below graph summarizes the prevalence of genetic diversity.

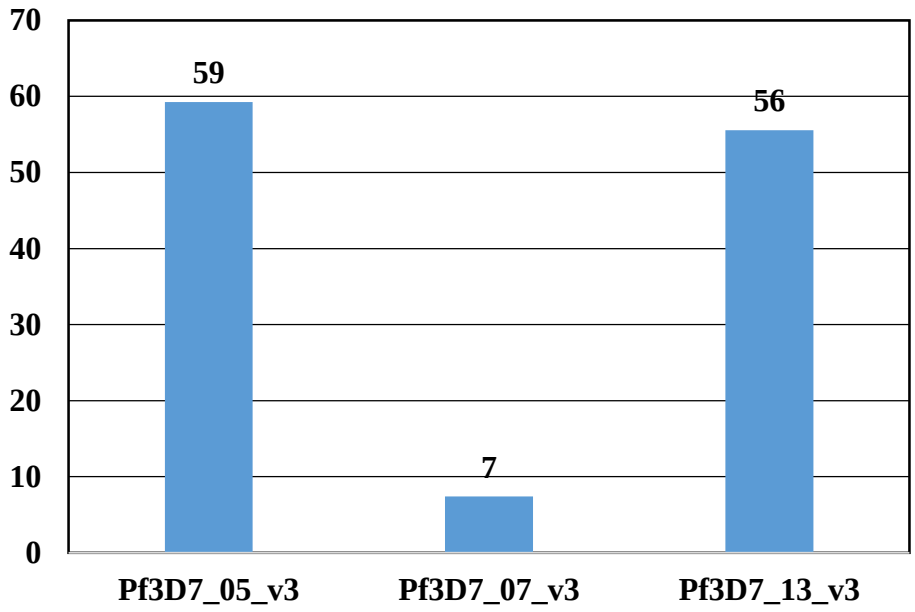

Figure 9: Proportion of Pfmsp2 genetic diversity as marker of parasite strains

IV.4 Molecular impact of key mutations on the protein structural features and function

To assess the potential structural impact of the mutations, structural modeling of the *Pfk13* protein was performed using the AlphaFold AI server. The pfk13 A569V mutation was found to disrupt hydrogen bond interactions within the K13 gene propeller domain. Specifically, as illustrated in the below image, this mutation led to the loss of stabilizing hydrogen bonds between amino acid residues, which may alter the protein conformation, impair its normal function, and potentially interfere with drug binding.

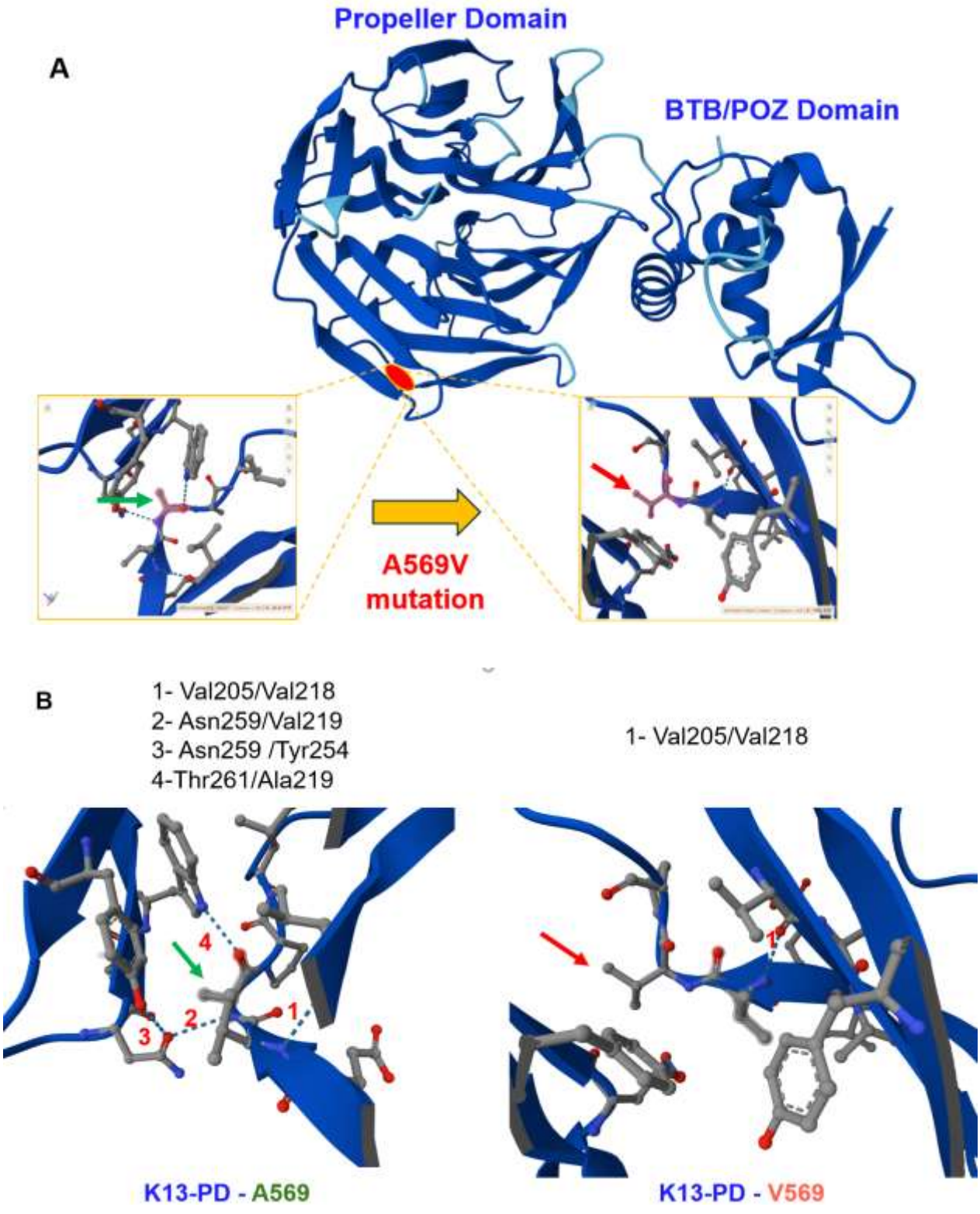

Figure 10(A&B): AlphaFold predicted protein structural change induced by A569V mutation

V. DISCUSSION

The overall objectives of the study were successfully achieved, markers of *plasmodium falciparum* associated with resistance to ACTs were thoroughly evaluated. Among 54 samples successfully sequenced isolates from Gasabo district 70%(n=38) harbored SNPs in at least one of the three key resistance associated genes: *pfk13*, *pfmdr1* and *pfcrt*. Specifically, for *pfk13*, six SNPs were identified: K189T, R561H, A569V, P441L, T207K and A578S. Notably R561H one of WHO-validated marker of artemisinin resistance was detected at high prevalence (39%). The finding aligns with previous national reports documenting the spread of R561H across the country, reported prevalence includes: 19.6% in Masaka(2018) and 22% in Rukara(2018). Some other mutations such as P441L have previously reported but their phenotypic relevance remains unclear. To explore the potential structural implications of the detected mutations, protein modeling was conducted using the AlphaFold AI server.

The PfK13 A569V SNP was of particular interest, as modeling indicated disruption of hydrogen bond interactions within the propeller domain of the K13 protein. This disruption could lead to the loss of stabilizing interactions between amino acid residues, potentially altering protein conformation, impairing normal function, and affecting drug-binding capacity. While these structural insights suggest that A569V may play a role in modulating artemisinin susceptibility, functional predictions could not be confirmed, as protein–ligand docking analyses were not performed due to time constraints. Therefore, complementary approaches, including protein–ligand docking as well as in vivo and in vitro studies, are needed to validate the functional impact of this mutation and determine whether it can contribute to ACT drug resistance.

The mutations in *pfmdr1* were identified in 59%(n=32) of the sequenced samples, with Y184F being the most frequent (54%) and a marker of ACTs drug resistance specifically linked to reduced sensitivity to lumefantrine and mefloquine, and its continued presence mirrors trends observed in Rwanda and neighboring countries. This raises concern over diminishing efficacy of artemether lumefantrine(AL), the current 1st line treatment in Rwanda. Further to this, the known chloroquine resistance associated mutation K76T was observed at *pfcrt* gene with prevalence of 7%. The findings are relevant with the reports in southern Rwanda in 2023 with the prevalence of 25%, underscoring the ongoing circulation of resistant parasite strains. Fortunately, no *pfcrt* mutations associated with reduced efficacy of second -line therapies

(DHA-PPQ and ASPY) were detected in this study, but continued monitoring remains essential.

The co-occurrence of resistance markers, particularly between *pfk13(R561H)* and *pfmdr1(Y184F)* was observed in multiple isolates at 31%. This pattern suggests a potential reduction in the efficacy of both artemether and its partner drug lumefantrine. The absence of *pfk13* (R561H/K189T)–*pfcrt* co-occurrence suggests no likelihood of multidrug resistance. These findings support the consideration of piperaquine- or pyronaridine-based ACTs as viable alternative therapies. However, sustained molecular surveillance is needed to ensure *pfcrt* does not acquire mutations conferring resistance to these partner drugs. In addition, the *msp2* genotyping revealed a narrow allelic distribution, with three 3D7 family alleles Pf3D7_05_v3, Pf3D7_07_v3 and Pf3D7_13_v3 circulating in the study population. However, due to academic constraints, full *msp2* genotyping could not be completed, limiting insight into the comprehensive diversity of circulating strains. Nevertheless, the preliminary results are consistent with recent studies conducted at King Faisal Hospital and across Rwanda, which also reported a predominance of the 3D7 allelic family. The observed co-occurrence of multiple *msp2* subtypes, particularly between Pf3D7_05_v3 and Pf3D7_13_v3 in 46% of samples, suggests multiple infections and repeated exposure to genetically distinct parasites.

This study utilized both ONT for SNPs identification and Sanger sequencing for SNPs validation, with all reported SNPs confirmed by Sanger. Some mutations initially identified by ONT were later revealed to be contamination, emphasizing the importance of cross-platform validation. ONT offers notable advantages, including real-time sequencing, long read capability, and portability for field-based applications. However, it also presents limitations such as complex data analysis and a higher likelihood of base-calling errors. In contrast, Sanger sequencing provides highly accurate base calls and straightforward data interpretation but is restricted to short reads and requires specialized laboratory infrastructure. Although Sanger is more expensive per sample for large-scale studies due to its low throughput, ONT proves more cost effective for processing multiple samples at once, despite its higher initial setup costs. By combining both platforms, ONT for broad detection and Sanger for precise validation the study ensured greater accuracy, reliability, and confidence in the genetic findings.

The general limitation of the study is based on limited samples size due to academic time constraints. While the findings are consistent with regional studies, the small sample size limit the generalizability of the results across the entire Gasabo district. Moreover, although preliminary msp2 genotyping revealed three 3D7 family alleles and indicated multiple infections, the full msp2 dataset is still under analysis. A larger set of around 100 samples is currently being processed, which is expected to provide a more comprehensive understanding of *plasmodium falciparum* genetic diversity. The current dataset is insufficient for constructing a robust phylogenetic tree to distinguish between major strain clusters such as 3D7 and FC27. As sample processing continues, the complete data will enable a more detailed characterization of the genetic structure of circulating plasmodium falciparum strains and help inform targeted malaria control strategies in Kigali city.

VI. CONCLUSION

This study successfully addressed all its objectives: the prevalence of SNPs in the *pfk13*, *pfmdr1*, and *pfcrt* genes associated with ACTs drug resistance; assessment of *pfmsp2* genetic diversity; investigating the co-occurrence of ACTs drug resistance markers; and evaluating the structural impact of key mutations. The findings revealed a high prevalence of resistance associated SNPs, particularly *pfk13* R561H (39%), *pfmdr1* Y184F (54%), and *pfcrt* K76T (75%), which raise significant concerns about the continued effectiveness of ACTs. The co-occurrence of SNPs markers R561H in *pfk13* and Y184F of *pfmdr1* in 46% of isolates suggests the high risk for reduced efficacy of artemether-lumefantrine(AL). Although no markers of piperaquine/pyronaridine resistance were detected, the long-term efficacy of second-line treatments remains uncertain without continuous monitoring. Additionally, *msp2* genotyping revealed multiple infections and allelic diversity, further pointing to sustained transmission within the study area.

These findings highlight the urgent need for ongoing molecular studies to inform national malaria treatment policies and strengthen control strategies. However, a key limitation of the study was the restricted sample size due to academic time constraints, which may have limited the statistical power to generalize findings to the broader district. Despite this, the consistency of the results reinforces the reliability and relevance of the conclusions drawn.

VI.1 PERSPECTIVES

The findings of this study have inspired me to pursue advanced research focused on tackling the escalating challenges in malaria control. My research interests center on understanding how drug resistance–associated mutations impact protein structure and function, parasite-host interactions, and the mechanisms of antimalarial drugs. Given the increasing prevalence of drug resistance mutations in the region, there is an urgent need to explore alternatives beyond chemotherapy, including vaccine development. Although genetic diversity particularly in the *msp2* gene complicates malaria control efforts, vaccines targeting conserved parasite stages such as the circumsporozoite protein (CSP) or gametocytes present promising strategies. These approaches, combined with novel biologics, hold potential to disrupt transmission and accelerate malaria elimination. Consequently, I am committed to pursuing advanced studies aimed at developing innovative, molecularly informed solutions that directly address the critical global health challenges posed by malaria and other infectious diseases.

VI.2 RECOMMENDATIONS

To strengthen malaria control efforts in Rwanda, decision-makers, particularly MoH need to prioritize the integration of molecular surveillance into routine malaria monitoring programs. Regular tracking of drug resistance markers for *pfk13*, *pfmdr1*, and *pfcrt* genes mutations and msp2 genotyping, will enable timely detection of emerging resistant strains and support evidence-based treatment policies. Expanding diagnostic capacity to include genetic analysis beyond traditional microscopy and rapid tests can significantly enhance the accuracy of treatment decisions and help limit the spread of resistant parasites. Investment in research and capacity building in molecular biology and bioinformatics through universities is also essential to empower local people to conduct sustained genomic surveillance.

Given the genetic diversity of circulating parasites, further efforts should support vaccine research and the development of transmission-blocking strategies, such as targeting gametocytes. In parallel, it is critical to strengthen community awareness, ensure consistent availability of ACTs and maintain robust vector control measures, including the distribution of insecticide-treated nets and indoor residual spraying. A coordinated multisector, and evidence-driven approach linking policy, research, health facilities and public health action will be vital to achieving long-term malaria control and eventual elimination in Rwanda.

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Appendix 1 ETHICAL CLEARENCE

REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA

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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.

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