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EXPLORING GENETIC VARIABILITY OF *Plasmodium falciparum*: INSIGHTS FROM *PFCSP* AND *PFK13* GENES AND THEIR IMPLICATIONS FOR MALARIA CONTROL IN RWANDA

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ARLENE UWITUZE

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By

ARLENE UWITUZE, Ref. Number 223027873

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Supervisor: **Dr. Peter RWIBASIRA**
Co-Supervisors: **Assoc. Prof. Dieudonne MUTANGANA**
Dr. Diane UMUHOZA
Dr. Jean Pierre MUSABYIMANA

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DECLARATION

I, UWITUZE Arlene 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.

Arlene UWITUZE

CERTIFICATION

This is to certify that this dissertation has been conducted in collaboration with the University of Rwanda and Rwanda Biomedical Centre. It is of the original work carried out by Arlene Uwituze for the master of Science in Biotechnology at University of Rwanda, College of Science and Technology (UR-CST) during the 2024-2025 academic year.

1. Dr. Peter RWIBASIRA 

2. Assoc. Prof. Dieudonne MUTANGANA 

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LIST OF ABBREVIATIONS, SYMBOLS, OR NOMENCLATURE

PfCSP – *Plasmodium falciparum* Circumsporozoite Protein

PfK13 – *Plasmodium falciparum* Kelch 13 gene

P. falciparum – *Plasmodium falciparum*

SNP – Single Nucleotide Polymorphism

LD – Linkage Disequilibrium

PCA – Principal Component Analysis

RTS, S/AS01 – RTS, S malaria Vaccine Formulated with AS01 Adjuvant

WHO – World Health Organization

***pfhrp2/3* gene**- *Plasmodium falciparum* Histidine-Rich protein 2/3

RDTs- Rapid Diagnostic Tests

ACT- Artemisinin based Combination Therapy

Th2R-T-helper 2 Receptor

Th3R- T- helper 3 Receptor

CD8+ Cluster of Differentiation 8 Positive

EDTA- Ethylenediaminetetraacetic Acid

PCR- Polymerase Chain Reaction

ONT-Oxford Nanopore Technology

SAM- Sequence Alignment Map

BAM- Binary Alignment Map

VCF: Variant Call Format

RBC-Rwanda Biomedical Centre

ARMEA- Artemisinin Resistance Monitoring in East Africa

ABSTRACT

Purpose and Scope: the purpose of this study was to characterize the genetic variability of plasmodium falciparum in Rwanda, focusing on two critical genes: the *circumsporozoite protein gene* (*PfCSP*), a target for malaria vaccine development, and *kelch13 gene* (*Pfk13*), which serves as a marker for artemisinin resistance. The research aims to assess the implications of genetic variability in these genes for malaria control strategies.

Methods: A quantitative, cross-sectional molecular design was employed, analyzing 27 *P. falciparum* isolates. The study was conducted on samples from Huye and Kirehe districts, as malaria transmission areas. Blood samples were treated with QIAamp DNA Mini Kit to extract DNA, and PCR amplification of *PfCSP* and *PfK13* genes. Sequencing was performed using Oxford Nanopore Technology, and bioinformatics tools (e.g., Minimap, Samtools, and Python packages) were used for variant calling, population genetic analysis and polygenetic tree construction. Statistical tests (Tajima's D, Fu and Li's D), and Principal component analysis (PCA) were used to assess population structure, selection pressures and genetic diversity.

Principal Findings: the study identified significant single nucleotide polymorphisms (SNPs) in PfCSP, such as A98G, D199N and A361E observed at high frequencies (up to 69%). In Pfk13, the R561H mutation, a validated maker of artemisinin resistance was detected in 11 samples. Tajima's D analysis indicated balancing selection in *PfCSP* (D=0.77), suggesting immune driven evolutionary pressure. Weak Linkage disequilibrium (LD) among SNPs suggested high genetic variability, while PCA revealed distinct clustering patterns, explaining 53% of genetic variation. Phylogenetic tree analysis showed genetic divergence between samples from Huye and Kirehe, likely influenced by regional transmission dynamics.

Significance of Findings: The results demonstrate the genetic complexity of *P. falciparum* in Rwanda, which has consequences for the development of a vaccine against malaria and the tracking of resistance. The presence of R561H underscores the need for surveillance to mitigate artemisinin resistance. Balancing selection in *PfCSP* suggests ongoing immune evasion, which might affect how well the RTS, S/AS01, and R21 vaccinations work. This study provides the first district-level genetic analysis in Rwanda, offering important data to inform national malaria control programs and contributing to regional and global efforts in malaria elimination.

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CHAPTER 1: INTRODUCTION

1.1. Background

Malaria is an infectious disease caused by plasmodium species, remaining one of the world's biggest health problems, with over 263 million cases in 2023 and approximately 597,000 deaths reported in 2023, mostly affecting sub-Saharan Africa, especially children under five and pregnant women (WHO, 2023). Sub-Saharan Africa accounted for almost 95% of the cases and fatalities, with Plasmodium *falciparum (P. falciparum)* being the primary cause of mortality (Li et al., 2024).

In Rwanda, malaria incidence declined from 345 cases per 1,000 population in 2018 to 40 cases per 1,000 in 2023, where the highest burden was initially recorded in the eastern province (Umugwaneza et al., 2025). However, most districts had recorded substantial improvements by 2023; where Nyamagabe and Gisagara in the southern province reported incidence rates above the national average. This was largely attributed to irrigation-related mosquito breeding, cross-border movements, and inadequate housing conditions. In contrast, the Northern province consistently showed the lowest malaria burden due to its higher altitudes and environmental conditions for mosquito breeding.

Improved malaria control in Rwanda has been driven by several key interventions, including community-based malaria management, improved access to diagnosis and treatment, indoor residual spraying (IRS) and the use of insecticide-treated nets (Umugwaneza et al., 2025). Despite these successes, there are still issues, especially with new medications, pesticide resistance, and the existence of *pfhrp2/3* gene deletion and gaps in sustainable funding. To maintain and build on this progress, experts recommend strengthening surveillance systems by integrating advanced molecular tools, such as sequencing technologies, to enhance detection and support timely public health responses (Umugwaneza et al., 2025).

The complexity of genetic diversity in *Plasmodium falciparum*, particularly within genes like *PfK13* linked with artemisinin resistance and *PfCSP* genes linked with malaria vaccine, necessitates the integration of advanced molecular surveillance techniques to track mutations that may impact antimalarial drugs and the efficacy of vaccines. This surveillance is crucial for monitoring emerging drug-resistant strains and understanding how genetic variability influences parasite behavior, immune evasion, and treatment response. (Harrott et al., 2025)

Plasmodium falciparum (Pf) exhibits genetic variability, with approximately 5000 genes contributing to its ability to express various proteins, enabling it to invade multiple types of host cells. This genetic variability, particularly antigenic variation, makes it challenging for *Pf* to be targeted with vaccines as the parasite continually evolves to evade immune responses (Sallam et al., 2025).

The primary surface protein of the sporozoite stage is encoded by the *PfCSP gene* found in the circumsporozoite protein *(*PfCSP*),* which is the basis for the RTS, S/AS01, and R21/matrix vaccines. However, PfCSP protein contains high levels of polymorphism, particularly in its central repeat and C-terminal regions that affect malaria vaccine efficacy (Kojom Foko et al., 2024; Laurens, 2020; Maina et al., 2024a; Zavala & Zavala, 2022). In Kenya, genetic diversity of *PfCSP* gene was evaluated in a study carried out with sophisticated molecular techniques on samples gathered in the western area. The N-terminal of *PfCSP* was found to be moderately conserved with an A98G mutation present in all isolates, indicating diversity in P*fCSP* haplotypes. The need for *PfCSP* genetic variability research to guide the creation of a malaria vaccine is highlighted by this work (Maina et al., 2024b).

In January 2016, two malaria vaccines, RTS, S/AS01, and R21/matrix vaccines, that target *Plasmodium falciparum* sporozoites, were approved by the World Health Organization (WHO). The RTS, S/AS01 phase III trial was carried out in a number of sub-Saharan African nations between 2009 and 2014. These test locations reflected low, medium, and high malaria transmission scenarios. The findings demonstrated the vaccine's safety and 58% effectiveness against severe malaria. (Ngulube, 2023). Both vaccines target the circumsporozoite protein (PfCSP), an antigen implicated in liver stage invasion by sporozoites.

The challenge of artemisinin resistance is also a rising concern. Mutations in the PfK13 propeller domain, namely the C580Y allele, were initially discovered in Southeast Asia and have been connected to treatment failure and delayed parasite clearance (Rutering et al., 2014; Uwimana et al., 2021a). Although the prevalence of PfK13 resistance-associated alleles remains low in Africa, a study conducted in Southern province showed that 17.5% of malaria patients had PfK13 markers linked to artemisinin resistance (Loon et al., 2023). This shows evidence that continuous genomic surveillance is needed to monitor and evaluate local mutations across Africa (Amato et al., 2016; Andreína et al., 2019). Region-specific molecular epidemiological data to inform national treatment guidelines are needed.

Ongoing efforts to eliminate malaria by 2030 can benefit from a comprehensive approach that includes vector control and chemotherapeutic strategies and targeted vaccine development informed by genetic insights. By focusing on the genetic variation of key malaria genes, particularly *PfCSP* and *PfK13*, Rwanda can better anticipate challenges to treatment and vaccine deployment, allowing for the development of more effective and region-specific interventions. The analysis to understand the PfK*13* genetic diversity and its linkage patterns within Rwandan parasite populations will help identify emerging challenges and guide antimalarial policymakers (Pacheco et al., 2019).

This study could support the artemisinin resistance monitoring, adapting antimalaria policies to combat resistance in real time. Therefore, continued research into *P. falciparum* genetic diversity remains vital for Rwanda's effort to eradicate malaria.

This study focused on characterizing the genetic variations and evolutionary patterns of *PfCSP* and *PfK13 genes* in p. *falciparum* samples collected from Huye (South province) and Kirehe (East province) districts in Rwanda. These regions are epidemiologically significant and provide a picture of parasite diversity in both high and low transmission regions. Mutations in *PfCSP* and *PfK13* may reveal the strategies to eliminate malaria in Rwanda. (Umugwaneza et al., 2025)

One of the most promising strategies to address malaria's treatment resistance is through the development of effective vaccines. Evaluating the potential impact of malaria vaccines in the context of drug resistance is essential for assessing their long-term efficacy once deployed as a permanent solution. However, high polymorphism in parasite genes, such as *PfCSP*, complicates the development of universally effective vaccines, as these variants can evade immune responses triggered by the vaccine (Sallam et al., 2025).

Through this study, we expect to generate crucial data that will enhance our understanding of the local *P. falciparum* evolutionary background. Our findings will inform the national malaria control program in Rwanda by refining genomic surveillance strategies, guiding treatment decisions, and supporting the design of next-generation vaccines that include circulating polymorphic variants.

1.2. **Research aims and objectives**

The aim was to investigate *Plasmodium falciparum* genetic variability by focusing on two key genes: the *Circumsporozoite protein (PfCSP*) and the *kelch13* (*PfK13*) *genes*. These genes are critical in the context of malaria control, with *PfCSP* being a primary target in malaria vaccine development, and *PfK13* playing a central role in artemisinin resistance monitoring. By analyzing isolates collected from Huye and Kirehe districts in Rwanda, the goal of the study was to advance knowledge of the genetic landscape of these genes and their implications for public health interventions.

1.2.1. **Main Objective**

To characterize the genetic diversity of *Plasmodium falciparum Circumsporozoite protein* (*PfCSP*) and *kelch13* (*K13*) genes in isolates collected from Huye and Kirehe and assess the implications of this diversity for vaccine development and artemisinin resistance monitoring.

1.2.2. Specific Objectives:

1. To identify and characterize single-nucleotide polymorphisms (SNPs) in the *PfCSP* gene
2. To assess the genetic variations and selection pressure acting on the *PfCSP* gene using Tajima's D and Fu and Li'sD.
3. To evaluate patterns of linkage disequilibrium (LD) within the PfCSP gene.
4. To explore genetic relatedness and clustering patterns using principal component analysis (PCA).
5. To explore the genetic variability and evolutionary patterns of the *Plasmodium falciparum keck13*(PfK13) gene and evaluate their relevance for malaria resistance monitoring.

1.3. Research questions

1. Are there any variations at Single Nucleotide Polymorphism (SNP) for PfCSP and PfK13 in P. falciparum isolates from Huye and Kirehe districts?

2. What are the implications of these genetic variations for current and future malaria control strategies?

3. If the *PfCSP* and *Pfk13* genes show polymorphic variations, might there be an influence of any pressure on these SNPs, and what do these reveal about the adaptive landscape of P. falciparum relevant to disease control?

1.4. Significance of the study

P. falciparum is responsible for more than 95% of all malaria cases and fatalities in Rwanda, making malaria a significant public health concern. Despite this burden, Rwanda contributes very limited genomic data to global malaria databases compared to neighboring countries such as Tanzania, Uganda, Kenya. The latest MalariaGEN Pf dataset, encompassing over 33,000 *plasmodium falciparum* genomes from 122 locations, with no data from Rwanda (MalariaGEN, 2025). This limited representation creates a critical gap for understanding local parasite evolution and tailoring malaria control strategies to Rwanda epidemiological context.

This study focuses to address critical knowledge gaps regarding genetic diversity of two key parasite genes: *PfCSP*, essential for malaria vaccine development, and *PfK13*, a validated marker for artemisinin resistance. This research focused on two districts (Huye and Kirehe) where genomic changes may significantly influence malaria control outcomes.

Variability in the *PfCSP* gene compromises the efficacy of RTS, S/AS01, and R21 vaccines by allowing immune evasion, while efficacy of frontline artemisinin-based combination treatments is threatened by mutations in *PfK13* gene. Achieving Rwanda's goal of malaria elimination depends on timely and targeted genomic surveillance to detect and mitigate these emerging biological threats.

This study will generate the first district-level analysis of PfCSP and PfK13 polymorphisms, including data on selection pressures from Huye and Kirehe districts. The findings will be crucial in several domains. For vaccine strategy, identifying circulating PfCSP haplotypes will guide

evaluation of regional vaccine suitability and inform the design of next-generation vaccines. For treatment policy, detecting resistance mutations will support real-time decisions on antimalarial drug use. From a national policy perspective, the results will provide the Rwanda Malaria Control Program with actionable data to enhance genomic surveillance, vector control efforts, and resource allocation.

Integrating the study of *PfCSP* and *PfK13* gene will provide a comprehensive understanding of the genetic landscape of *P. falciparum* in Rwanda. This approach will enhance genomic surveillance, inform treatment strategies, and guide vaccine deployment tailored to local parasite populations, thereby supporting Rwanda's goal of malaria elimination by 2030.

Beyond Rwanda, the study will contribute to regional and global surveillance efforts by contributing to malaria databases needed for tracking parasite evolution. By uncovering the genetic drivers behind vaccine evasion and drug resistance, this study will directly support Rwanda's malaria elimination strategy and serve as a framework for precision malaria control in other endemic settings around the world.

CHAPTER 2: LITERATURE REVIEW

Plasmodium falciparum (Pf) is the most virulent and lethal species of malaria, contributing to morbidity and mortality, especially in sub-Saharan Africa, which includes Rwanda. Malaria is still a major global public health concern. *Pf* was the main causal agent in 2022, accounting for a projected 249 million cases and 608,000 deaths, with the biggest burden occurring in sub-Saharan Africa (Young et al., 2024). The prevalence of malaria has been rising again, which highlights the necessity of sustained innovations in malaria control and treatment.

Various interventions against malaria in Rwanda were conducted, including the use of insecticides, chemotherapy and other preventive measures. Despite these efforts, malaria incidence and mortality rates have significantly reduced over the past two decades (van Loon et al., 2024a). However, the persistence of malaria, along with increasing drug resistance, continues to challenge eradication efforts. In response to a recent malaria outbreak, the Rwanda government has updated its national malaria treatment guidelines to include dihydroartemisinin-piperaquine (DHAP) and artesunate pyronaridine as second-line treatments for uncomplicated malaria. This initiative aims to stop the growing resistance to existing antimalaria drugs (RBC, 2020).

In all suspected cases of malaria, WHO advises parasitological confirmation prior to treatment, with a focus on the use of light microscopy and rapid diagnostic tests (RDTs) as foundational tools in malaria case management. (WHO Guidelines for Malaria, 30 November 2024).

In alignment with this recommendation, Rwanda has implemented a mandatory parasitological diagnosis before administering Artemisinin-based Combination therapy (ACTs). Light microscopy is regarded as the gold standard method due to its ability to identify Plasmodium species, quantify parasitemia, and detect gametocytes. Although it is labor-intensive and reliant on skilled technicians, microscopy is highly valued for its accuracy in cases with high parasite density and is widely used in health facilities. (Umugwaneza et al., 2025)

Different districts in Rwanda show varying levels of malaria transmission as identified according to the Rwanda Malaria strategic plan 2020-2024 (RBC, 2020). Kirehe and Huye, due to their geographic locations along borders with neighboring countries and the presence of vast marshlands, play a significant role in the transmission of malaria. These factors make them critical focus regions for malaria control and surveillance efforts (Umugwaneza et al., 2025).

A validated mutation linked to resistance to artemisinin-based treatments in the *Plasmodium kelch 13 gene* (R561H), was observed in different districts of Rwanda (Uwimana et al., 2021b). This highlights the growing concern of drug resistance in the region. Following recommendations from the WHO, the Rwandan government has introduced dihydroartemisinin-piperaquine (DHAP) in Kirehe as a new treatment strategy. This intervention underscores the importance of closely

monitoring these districts for resistance markers and ensuring the effectiveness of the new treatment option in controlling malaria transmission (RBC, 2020)

Plasmodium falciparum cycle

Malaria infection results from a complex interaction between humans, mosquitoes, and the Plasmodium parasite. Only female mosquitoes of the *Anopheles* genus can spread malaria to humans. When the mosquitoes experience a shortage of essential nutrients such as protein and plant sugar needed for egg maturation, they alter their egg-laying behavior and seek human blood as an alternative source. During a successful blood meal, the mosquito can transmit malaria-causing *Plasmodium* into the human bloodstream infection (Savi, 2023).

Plasmodium Falciparum undergoes a complex life cycle that includes two main phases: sexual and asexual. The human host experiences the asexual period, whereas the mosquito vector experiences the sexual phase. The parasite's survival and spread depend on this dual host life cycle. Infection begins when sporozoites enter the bloodstream via mosquito bite, travel to the liver, where they undergo exoerythrocytic schizogony (Sato, 2021).
They proliferate in the liver, and after seven to ten days, the sporozoites turn into merozoites, which are then released into the circulation to infect red blood cells. From there, they change into trophozoites and, eventually, schizonts, which contain a large number of merozoites. The cycle is continued when the schizonts rupture, producing merozoites that infect further red blood cells. This stage is called erythrocytic schizogony, which is the focus of most malaria treatments (Ngulube, 2023).
Certain merozoites undergo differentiation into gametocytes, both male and female, which mosquitoes subsequently consume during a blood meal. Gametocytes are fertilized inside the mosquito's gut to create zygotes, which mature into ookinetes and ultimately oocysts. The sexual part of the parasite's life cycle is completed when these oocysts release sporozoites, which move to the mosquito's salivary gland and are prepared to infect a new human host. (Sato, 2021)

By targeting particular proteins on the parasite, such as thrombospondin-related adhesion protein (TRAP) and circumsporozoite protein (CSP), malaria vaccines seek to elicit immune responses. CSP is still the key contender for vaccines that target the pre-erythrocytic stage of the malaria cycle since it is essential for sporozoite invasion of liver cells (Ngulube, 2023).

Importance of *PfCSP* and *PfK13* in Malaria Control

The Circumsporozoite protein (*PfCSP*) and *Kelch13 (PfK13) genes* are critical targets in malaria interventions. The RTS, S/AS01 malaria vaccine targets PfCSP, which encodes the primary sporozoite stage surface protein. This vaccine offers some protection against *P. falciparum* malaria (Caspers et al., 1989; Pringle et al., 2018). On the other hand, *PfK13* mutations are known molecular indicators of artemisinin resistance, endangering the effectiveness of first-line antimalarial therapy (Ariey et al., 2013; Amato et al., 2016).

Genetic Diversity and Polymorphism in *PfCSP*: Implications for Vaccine Development

Genetic diversity in *PfCSP gene* is a major challenge for malaria vaccine efficacy, as the antigenic polymorphism can affect immune recognition. Numerous studies have found high levels of polymorphism, particularly in the central repeat and C-terminal regions of PfCSP, which harbor immunodominant epitopes (Caspers et al., 1989; Zeeshan et al., 2012; Pradel Kojom Foko et al., 2024). The central NANP repeat region varies in length and repeat number, influencing antibody binding and vaccine effectiveness (Maina et al., 2024).

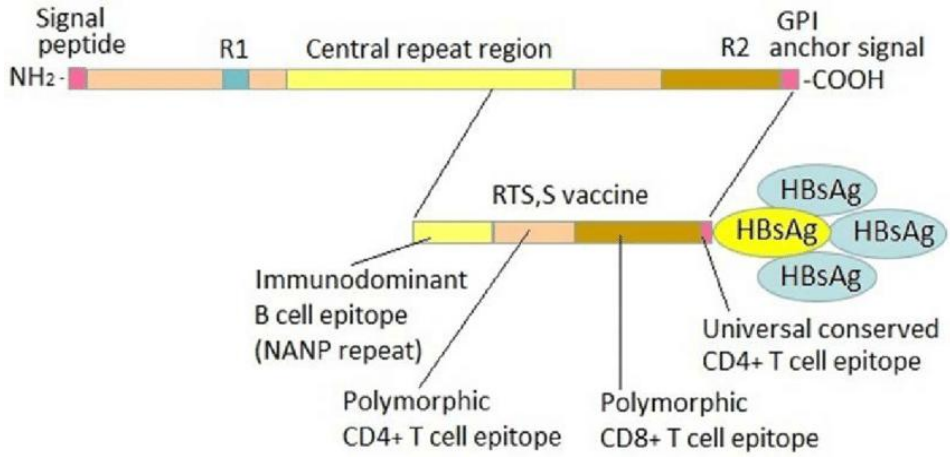


Figure 1: Parts of CSP protein (Adapted from Ngulube, 2023)

Studies also document geographic variation, where African isolates tend to be more polymorphic than Asian ones (Caspers et al., 1989; Tanabe et al., 2013). In Rwanda, research has been limited, but countries like Cameroon showed distinct haplotypes and evidence of balancing selection in PfCSP ([Pradel Kojom Foko et al., 2024]). The limited polymorphism observed in the N-terminal region suggests conserved vaccine targets, while immunity is compromised by a significant degree of variation in the C-terminal Th2R and Th3R epitopes that CD4+ AND CD8+ T cells detect (Mohamed et al., 2021).

Another study done in Ethiopia found that the SNPs, such as A98G, were observed in the central region repeats. At least one substitution was seen in the polymorphism of the Th2R and Th3R epitopes in the C-terminal region. The study's use of phylogenetic tree and haplotype network analyses highlighted the significant genetic diversity of PfCSP by revealing similarities between isolates (Mandefro et al., 2025a).

RTS, S Malaria vaccine functions

The RTS, S malaria vaccine is designed to trigger both cell-mediated and humoral immune responses, generating a strong immune defense against *Plasmodium falciparum*. Key elements of the vaccine are reflected in the name itself: S stands for the hepatitis B surface antigen (HBsAg), T for the T cell epitopes inside CSP, and R for the central repeat region of CSP. The N-terminal part of the hepatitis B surface antigen, which is joined to the C-terminus of CSP, and 19 NANP repeats from the middle region of CSP make up the structure of the vaccine antigen. While other

QS-21 Saponin uses an adjuvant containing monophosphoryl lipid A and Quillaja Saponaria Molina in oil-water emulsion to boost innate and adaptive immunity together, thereby increasing the vaccine efficacy against *P. Falciparum*, the vaccine formulation combines the RTS, S antigen with ASO1 adjuvant to enhance immune response. In an effort to prevent the parasite from developing during the pre-erythrocytic stage, before its invasion into liver cells, the vaccine specifically targets CSP (Sallam et al., 2025).

R21/matrix-M uses the adjuvant (Matrix M) to offer enhanced protection compared to RTS, S/AS01. At the onset of infection, innate immune cells such as dendritic cells recognize *Plasmodium falciparum* via receptor recognition, which initiates immune signaling and DC maturation (Laurenson & Laurens, 2024). As the infection progresses, adaptive immunity takes over. T and B cells are activated, leading to antibody production. Sporozoites are deposited into the skin by mosquitoes during the pre-erythrocytic stage. The circumsporozoite proteins (CSP) on the sporozoite surface are targets for neutralizing antibodies and T-cell responses. Vaccines are designed to mimic CSP to stimulate both antibody and T cell response, aiming to block the parasites before they reach the hepatocytes (Sallam et al., 2025).

Pressures Acting on *PfCSP gene*

PfCSP gene exhibits signs of balancing selection and positive selection driven by host immune pressures, as shown in different populations using statistical tests such as Tajima's D and Fu and Li's D (Naung et al., 2022; Lê et al., 2018). The recurrent polymorphisms in the immunodominant repeat region arise due to immune evasion strategies, which complicate vaccine design. Linkage disequilibrium analysis shows recombination events break down associations among polymorphisms, further increasing genetic diversity (Caspers et al., 1989).

***PfK13* Genetic Diversity and Artemisinin Resistance Surveillance**

A few *PfK13* propeller domain mutations have been confirmed to be a molecular indicator of artemisinin resistance, first described in Southeast Asia (Ariey et al., 2013). One of the Resistance-associated mutations in this domain is C580 which has been observed to emerge rapidly.

The combination of artesunate and mefloquine was one of the first artemisinin-based combination therapies (ACTs). It was safe, well-tolerated, and very effective in treating uncomplicated multidrug-resistant *P. falciparum* malaria, with high efficacy rates achieved within a 3-day regimen, even though the drugs' pharmacokinetic properties (artemisinin) or parasite resistance (mefloquine) prevented them from being fully effective on their own.

During the first three days of treatment, the extremely powerful but quickly removed artemisinin component rapidly reduces the overall parasite load. The less strong but longer-lasting companion medicine subsequently eliminates the remaining parasites (van der Pluijm et al., 2021). When it comes to treating simple malaria brought on by drug-sensitive parasites, ACTs are remarkably

effective. Concern has been raised by the advent of ACT resistance, which is characterized by delayed parasite clearance, despite its known effectiveness. Mutations in the *Kelch (K13)* of *P. falciparum* are linked to this resistance. The first reports of partial resistance, which is characterized by extended parasite clearance, were made in Cambodia, despite the World Health Organization's (WHO) recommendation to use artemisinin-based combination therapy (Young et al., 2024).

Artemisinin (ART) effectiveness relies on the activation by heme (molecules containing iron released when the parasite in the blood breaks down hemoglobin). When ART encounters heme, the endoperoxide bond is cleaved, generating highly toxic radicals that kill parasites. (Rahman et al., 2024a) However, mutations in the *Plasmodium falciparum kelch 13*, such as C580Y and R539T, reduce the protein's ability to bind heme, leading to lower artemisinin activation and contributing to drug resistance, which has now been reported in East Africa. (Rahman et al., 2024b).

A delayed removal of *Plasmodium falciparum* parasites from the bloodstream after treatment with ACTs is known as artemisinin partial resistance (ART-R). Mutations in the propeller domain of the P. *falciparum kelch 13 (k13*) gene are the main cause of the resistance. ACT partner medications resistance is frequently seen in conjunction with this, which was first noticed in Southeast Asia. Clinical treatment failures and parasite recrudescence following ACT delivery have been associated with these combination mutations (Schreidah et al., 2024).

***Plasmodium* Molecular marker genes**

Identifying drug-resistance genes helps elucidate the molecular mechanisms of resistance and develop molecular markers for surveillance, offering a potential solution to the problem of drug resistance. (Iwanaga et al., 2022). These genes include *pfk13, pfcrt, pfmdr1, dhfr, dhps, and cytb;* they are key to understanding how the parasite adapts under drug pressure, and each is implicated in conferring resistance to specific treatments.

***P. falciparum* multidrug resistance protein 1 (PfMDR1)** is a homolog of the human MDR1 P-glycoprotein, an ATP-binding cassette (ABC) transporter found on chromosome 5 (PF3D7_0523000, Pfmdr-1 gene). A decrease in mefloquine, lumefantrine, and piperaquine, among other ACT partner medicine susceptibility, is linked to resistance mechanisms. By regulating the intracellular accumulation of these medications in the parasite, these genetic changes reduce the therapeutic efficacy of ACT (Bell, 2017).

Decreased resistance to quinine, mefloquine, chloroquine, and artemisinin derivatives has also been linked to point mutations and single-nucleotide polymorphisms (SNPs) of this gene's pfmrp1 (Gupta et al., 2014).

The *MSP2* gene, which encodes Merozoite Surface Protein 2, is essential for the invasion of red blood cells by P. *falciparum* (Adamu et al., 2020). Although *MSP2* is not directly associated with drug resistance, it is vital to understand the parasite's biology and pathogenicity. This highly polymorphic gene displays significant genetic diversity, which helps the parasite evade the host's immune response. This variability can complicate efforts to control parasite through immune-based interventions (Adamu et al., 2020). Regarding resistance to ACTs, studying *MSP2* can provide information about the genetic makeup of P. *falciparum* populations.

Understanding the interaction between *MSP2* variability and other genetic markers, such as *K13* gene mutations linked to artemisinin resistance, can help researchers better monitor and interpret resistance patterns (Jamil et al., 2021). Comprehensive genomic surveillance, including *MSP2,* is essential for tracking the spread and evolution of drug-resistant strains, ultimately guiding the development of effective treatment strategies and containment measures (Marwa et al., 2022).

Sulfadoxine, a key partner medication in several ACTs, is known to target the Plasmodium *falciparum dihydropteroate synthase (PfDHPS)* gene, which is essential to the parasite's folate production process. Mutations in the *PfDHPS* gene confer resistance to sulfadoxine, reducing the efficacy of ACTs that include sulfadoxine-pyrimethamine (SP) as the partner drug.
These mutations decrease sulfadoxine's binding affinity to the *DHPS* enzyme, allowing the parasite to continue folate synthesis and survive despite the drug's presence (Issa et al., 2022). Common *PfDHPS* mutations include A437G, K540E, A581G, and A613S/T, with the A437G single mutant being predominant in many regions. *PfDHPS* resistance significantly challenges the effectiveness of ACTs, necessitating continuous genetic surveillance and potential adjustments in treatment strategies (Guémas et al., 2023).

The *PfDHFR* gene (Plasmodium *falciparum dihydrofolate reductase*) is crucial for the malaria parasite's resistance to certain drugs, particularly in combination therapies. *PfDHFR* mutations are primarily associated with resistance to antifolate drugs such as pyrimethamine and sulfadoxine, but they can also affect the efficacy of ACTs. In ACTs, an artemisinin derivative is combined with a partner drug, often an antifolate. *PfDHFR* gene mutations can lessen the parasite's vulnerability to the ACT's antifolate component, compromising the overall effectiveness of the combination therapy (Yan et al., 2021).

The 3.1 kb gene *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) has 13 exons, with codons 72–76 found in exon 2 (Chatterjee et al., 2016). Resistance to ACTs is closely linked to mutations in the *PfCRT* gene. Drug resistance is mostly dependent on the PfCRT protein, which is found on the parasite's digesting vacuole membrane. Mutations like *K76T* in the *PfCRT* genes are key factors in chloroquine resistance, as they alter the protein's structure, reducing the drug's accumulation in the digestive vacuole and thus its effectiveness.

Besides chloroquine, *PfCRT* mutations are linked to resistance against other antimalarials used in ACTs, such as amodiaquine. The presence of specific *PfCRT* mutations can affect the parasite's susceptibility to various partner drugs in ACTs, impacting the overall efficacy of the combination therapy (Wicht et al., 2020).

Circumsporozoite protein (CSP), the main surface protein of sporozoites, is a multifunctional protein necessary for sporozoite formation and probably mediates a number of these processes (Coppi et al., 2011). Hepatocyte invasion and sporozoite function depend on the *Plasmodium falciparum* circumsporozoite protein (CSP). A phase III human CSP malaria vaccine experiment is now underway due to the importance of the disease. Three sections make up the CSP: a four-amino acid repeat region (NANP), a thrombospondin-like type I repeat (TSR) domain at the C-terminus, and an N-terminus that binds heparin sulfate proteoglycans (Plassmeyer et al., 2009).

Plasmodium falciparum Kelch 13(pfk13) is a 1-exon gene that codes for a putative kelch protein and has three domains: a C-terminal six-blade propeller, a BTB/POZ, and a plasmodium-specific domain. Mutations in the propeller region have been connected to resistance. Across Plasmodium species, *Pfk-13* is highly conserved and promotes protein-protein interactions. (Mishra et al., 2015). Artemisinin resistance is associated with mutations in the Plasmodium *falciparum* Kelch 13 (PfK13) protein.

C580Y, R539T, I543, and Y493Y are common mutations; the Greater Mekong subregion is where C580Y is most common. *PfK13* is located in the cytostome, where the parasite ingests hemoglobin, a process critical for artemisinin activity. Resistance may be conferred by mutations in *PfK13* that decrease artemisinin activation and hemoglobin endocytosis. (Si et al., 2023) PfK13 alterations do not, however, cause all artemisinin resistance; certain parasites that do not have these changes nevertheless exhibit resistance. *PfK13*-mutant parasites have less hemoglobin endocytosis and require less iron and hemoglobin. Another theory for resistance is that artemisinin kills parasites by interfering with their use of iron and hemoglobin. It is essential to comprehend how PfK13 mutations impact malaria parasites (Si et al., 2023).

The *Plasmodium falciparum kelch 13* gene *(P.fk13*) has 124 non-synonymous mutations worldwide, 46 in Southeast Asia, 62 in sub-Saharan Africa, and 16 shared in both regions. Based on in vitro and in vivo data, WHO has confirmed that nine of these mutations, F446I, N458Y, M476I, Y493H, P533L, R539T, I543T, R561H, and C580 are linked to decreased susceptibility and delayed parasite clearance. In addition, 11other mutations, including P441L, F63I, and others, are classified as associated or suspected to cause delayed parasite clearance and reduce susceptibility to artemisinin but require more data for confirmation. Several other variants have been reported but not statistically significant due to the low number of documented cases. (Ikegbunam et al., 2021; Dafalla et al., 2020).

Point mutations in the beta-propeller domain of the gene encoding the Kelch protein 13-Pfk13, which are likely to cause decreased susceptibility to ART and its derivatives, provide an explanation for the fundamental mechanism of ART resistance. (Arya et al., 2021)
Pf13, is a critical protein involved in the parasite's cellular processes, believed to participate in the ubiquitin dependent protein degradation pathway through its BTB and kelch repeat propeller domains (KRPD). The variants like F446I and C580Y were found to be closely linked to artemisinin resistance. The variation in the amino acids in the KRPD region through hydrogen bonds reveal how the protein's structure may impact drug resistance (Figure 3) (Yan et al., 2022).

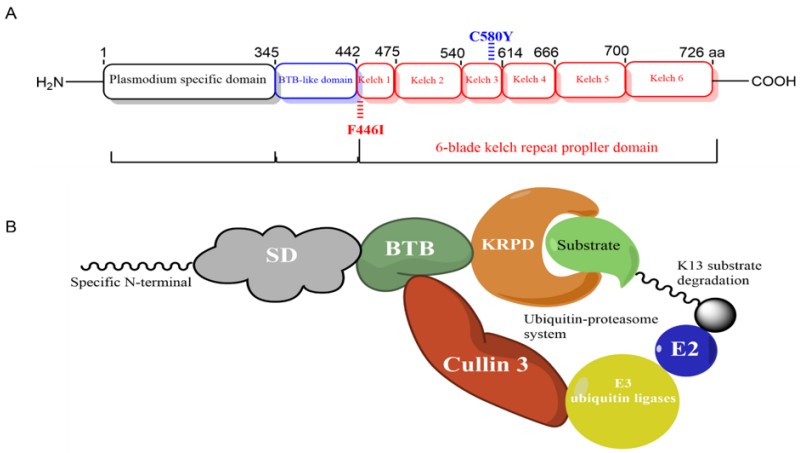


Figure 2: Structure (A) and function (B) of pfkelck 13 protein (Adapted from Yan et al., 2022).

Panel A: domain architecture of the pfk13 protein, which consists of a plasmodium-specific domain with 1-345 amino acids, a BTB domain with 346 to 442 amino acids and a six-bladed kelch repeat propeller domain from 443 to 726 amino acids; each kelch (1to 6) is labeled with red color. The mutations associated with artemisinin resistance, such as F446I and C580Y, are located within kelch 1 and kelch3, respectively. These mutations have been implicated in altering protein function and reducing susceptibility to treatment.

Panel B: demonstrates a proposed role of pfk13 in the ubiquitin proteasome system, where pfk13 is suggested to function as a substrate adapter, where its BTB domain binds to cullin 3, forming a complex with E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. The kelch propeller domain is shown to interact with specific substrate proteins, targeting them for ubiquitination and subsequent degradation. This pathway is critical for regulating protein and cellular stress responses, especially under antimalarial drug pressure (Yan et al., 2022).

Emerging resistance to ACTs highlights the urgent need for advanced diagnostic approaches such as sequencing technologies, including whole genome sequencing (WGS), and amplicon deep sequencing. Sequencing is a high-throughput technology that provides valuable insights into the malaria parasite genome, enhancing our understanding of its biology, evolution, and drug resistance. Such technologies are highly recommended where malaria burden remains high (Akoniyon et al., 2022).

Geographic isolation and epidemiological factors strongly influence the population genetic structure of P. *falciparum*. Studies show isolation-by-distance patterns with genetic diversity decreasing with distance from Central Sub-Saharan Africa, highlighting regional differentiation of vaccine candidate antigen alleles (Tanabe et al., 2013). Rwanda, situated in East Africa, likely harbors unique parasite populations distinct from those in West or Central Africa, reinforcing the need for local genetic data to guide interventions. Despite intensified malaria control measures like insecticide-treated nets, high genetic diversity in PfCSP and other antigens persists in African settings, indicating ongoing transmission and evolutionary adaptation (Sallam et al., 2025).

Gaps and Rationale for the Current Study

While some data exist on *PfCSP* and *PfK13* diversity in multiple global and African settings, there is limited genetic information from Rwanda, particularly regarding isolates from the Huye and Kirehe districts. Given the heterogeneity in genetic variants and local selective pressures, it is critical to characterize:

- The SNP landscape, evolutionary dynamics, and population structure of *PfCSP* and *PfK13* in Rwanda.
- The implications of observed genetic diversity on RTS, S/AS01 vaccine efficacy and artemisinin resistance surveillance.
- The impact of genetic variability on adaptive capacity and resistance emergence to guide public health strategies.

Understanding genetic diversity and evolutionary forces shaping the *PfCSP* and *PfK13* genes is essential for enhancing malaria vaccine design and resistance monitoring in Rwanda. Previous research underscores the presence of extensive polymorphisms affecting vaccine efficacy, genetic diversity, emerging mutations in resistance markers, and complex population structures that influence regional malaria intervention outcomes. Building on this foundation, the proposed study addresses critical local knowledge gaps that can inform precision malaria control and contribute to global elimination goals.

This study was conducted within the Artemisinin Resistance Monitoring in East Africa (ARMEA project. with a focus on genotyping the kelch13 gene in *Plasmodium falciparum* isolates for identifying mutations associated with artemisinin resistance. This study is under Rwanda Biomedical Centre (RBC) in collaboration with Charité universitätsmedizin Berlin.

CHAPTER 3: METHODOLOGY

3.1.**Research design**

This is quantitative molecular study with cross-sectional design. The primary focus is on understanding the genetic diversity of the P. *falciparum* parasite, specifically the *Kelch 13 gene* (*PfK13*) and the circumsporozoite protein (*PfCSP*) gene. The study investigates the isolates of *P. falciparum* were gathered from two Rwandan districts Huye and Kirehe. The goal is to assess the genetic variability of these genes and explore their implications for malaria resistance monitoring and the potential deployment of malaria vaccines in Rwanda.

The study involved patients with uncomplicated malaria who tested positive for *P. falciparum* parasites with rapid diagnostic testing (RDT) and had lived in the study area for a minimum of six months. These patients were seeking care at health facilities within Huye and Kirehe districts. This criterion ensures that participants are representative of the local malaria transmission dynamics, and the informed consent of all eligible participants was acquired in accordance with ethical guidelines to safeguard their well-being and rights.

3.2.**Training of study personnel**

Comprehensive training was conducted for study personnel, starting from community health workers, nurses, and laboratory personnel involved in participant recruitment, clinical assessment, sample collection, and data documentation. This training ensured rigorous adherence to the standard protocol and consistency across all study sites. The topics covered included malaria case identification, informed consent procedures, blood collection techniques, sample transport, and the accurate use of data, emphasizing confidentiality and ethical research practices.

3.3.**Sample size Determination**

Based on the previously estimated frequency of *PfK13* resistance markers (van Loon et al., 2024a), the study's sample size was established, and the following parameters were used for the calculation:

Confidence level: 95%(z=1.96%), which is a standard value to represent the high level of confidence that the sample estimate will reflect the true population value.

Frequency of resistance marker: 17.5%

Margin of error (d): 5% used in public health studies and genetic studies was also used in SNPs association studies (Politi et al., 2023).

The sample size formula used is based on fundamental statistical principles as outlined by Barlett, and others who designed the formula as:

$n = \left| Z^2 x \frac{Px(1-P}{d^2} \right|$ (Bartlett, J. E., Kotrlik, J. W., & Higgins, C. C., 2001)

Where n: minimum sample size

Z: Z-score corresponds to a confidence level of 1.96 for 95% confidence

P: estimated proportion

D: margin of error

The minimum sample size to achieve 95% confidence interval and 5% margin of error is calculated
as:

$$n = \left|1.96^2 x \frac{0.175x(1-0.175)}{0.05^2}\right| = 222 \text{ samples}$$

Therefore, the minimum sample size was 222 participants to accurately estimate the frequency of *PfK13* gene and *PfCSP* gene resistance markers.

The sample size calculation for this study was based on estimating the frequency of *Pfk13* resistance markers with a target of 222 participants; however, the study is still ongoing due to time frame and logistical constraints. As of now, 168 samples have been sequenced, with 27 samples currently being analyzed from Huye and Kirehe districts for this phase of the study. While the analysis is based on these 27 samples, the additional informational data for *Pfk13* and *PfCSP* genetic analysis will be provided in the future phases enabling a comprehensive understanding of gene diversity in those regions.

3.4.**Procedural flow**

Figure 4 outlines the comprehensive methodology used to analyze Plasmodium *falciparum* isolates in this study, beginning with rapid diagnostic tests (RDTs) to identify malaria-positive cases. Blood samples were collected from confirmed cases, followed by microscopy examination to quantify parasite density and validate infections. These samples were subjected to DNA extraction using the QIAamp DNA Mini Kit, and target genes (*PfCSP* and *PfK13*) were amplified via PCR with gene-specific primers. Successful amplification was confirmed through gel electrophoresis before sequencing using Oxford Nanopore Technology (MVP protocol). Finally, bioinformatics tools processed the sequencing data to identify SNPs, assess genetic diversity, and analyze population structure, enabling insights into vaccine efficacy and drug resistance markers. This end-to-end workflow ensured robust genomic characterization of *P. falciparum* in Rwanda, supporting the study's objectives of informing malaria control strategies.

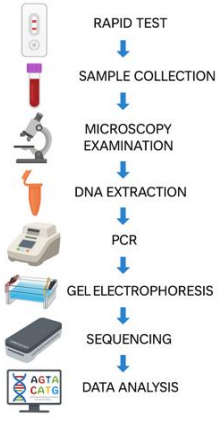


Figure 3: Procedural Flow for Molecular and Bioinformatic Analysis of P. falciparum Isolates

3.5.**Sample collection**

Community Health Workers conducted rapid diagnostic tests (RTDs) to screen individuals for malaria infection. Those who tested positive were referred to the nearest health facility for further clinical evaluation and Blood sample collection. At the health centers within Kirehe and Huye districts, prior to administering any malaria treatment, trained laboratory technicians will collect venous blood into EDTA tubes, and thick and thin blood smears were prepared for microscopic identification of *Plasmodium* parasites. The EDTA blood samples collected were sent to the National Reference Laboratory (NRL) for molecular analysis. The samples were transported to NRL within 24 hours to ensure sample integrity.

3.6.**DNA Extraction**

Samples received at the National Reference Laboratory and underwent DNA extraction. Using the QIAamp DNA Mini kit and the manufacturer's instructions, DNA was extracted from the entire sample (200µL). A 200µL aliquot of whole blood was put into a 1.5 mL microcentrifuge tube that had a special sample identification label on it. 200µL of AL buffer was added after 20µL of proteinase K for cell lysis. On a thermomixer, the samples were combined by vortexing and then incubated for ten minutes at 85°C. Following incubation, each sample received 200µL of 100% concentrated ethanol, and the mixture was once more vortexed to induce precipitation.

After transferring the lysate-ethanol mixture to a spin column, it was centrifuged for one minute at 800 revolutions per minute (rpm) to allow the DNA to attach to the silica membrane and discard the rest. After putting the spin column in a fresh collection tube and adding 500µL of AW1 buffer, it was centrifuged for one minute at 800 rpm. To get rid of any impurities, the column was centrifuged for three minutes at 14 rpm after the washing process was repeated with 500µL of AW2 buffer. The spin column was filled with 200µL of AE buffer, allowed to sit at room temperature for five minutes, and then centrifuged for one minute at 800 rpm in order to elute the DNA. The eluate containing extracted DNA was transferred to a microcentrifuge tube well labeled with sample ID and the DNA was stored at -20˚C for further processing.

3.7.**Polymerase chain reaction (PCR) amplification of *P. falciparum* kelch 13 propeller domains.**

The kelch-13 propeller domain of *Plasmodium falciparum*, a genomic locus linked to artemisinin resistance, was amplified in this work using PCR (a molecular method for selectively amplifying particular DNA sections), with primers K13 Forward out: 5'-CGGAGTGACCAAATCTGGGA-3' and 5'-GCCTTGTTGAAAGAAGCAGA-3' as *K13* reverse primer from the Solis Biodyne kit. (Ariey et al., 2014)

The PCR reagents were prepared according to the concentrations outlined in the table below, and amplification was carried out using a Bio-Rad thermal cycle, programmed with specific conditions optimized for kelch 13 propeller domain.

Table 1: PCR Master Mix preparation

Reagent	**Final concentration**	**Volume (µL)**
Nuclease-free water	-	13.5
B1PCR Buffer (solis Biodyne,10x)	1x	2
Mgcl2 (25mM)	2.5mM	2
dNTPs (10nM)	0.2mM	0.4
Hot Firepol Taq polymerase (5U/ul)	0.5U	0.1
Primer K13 Fw out (10uM)	0.25µM	0.5
Primer K13 Rv in (10uM)	0.25µM	0.5
DNA template	-	**1**

Table 2: PCR cycling conditions

Step	**Temperature**	**Time**	**cycle**
Initial denaturation	95°C	10 min	
Denaturation	94°C	1 min	
Annealing	60°C	45 sec	
Extension	72°C	1 min	40
Final elongation	72°C	10 min	1
Hold	15°C	∞	

3.8.**Gel electrophoresis**

SYBR Green dye was used to resolve the PCR results on a 1% agarose gel. A 100 bp DNA ladder was added to each well as a molecular weight marker, along with 5 ul of the PCR product combined with 1 ul of loading dye. Electrophoresis was carried out at 100-150 V for approximately one hour to visualize the expected amplicon (~883 bp). Upon confirmation of successful amplification, DNA extracts were used for downstream sequencing using Nanopore Oxford Technology with NOMADS protocol. The remaining PCR products were stored at -20°C for future use.

3.9.**Sequencing of Plasmodium falciparum using NOMADS protocol**

The sequencing of *P. falciparum* was performed using the MVP primers, followed by PCR amplification, amplicon clean up, library preparation with barcoding, pooling, rapid adapter addition, flow cell checks and sequencing. This method focuses on sequencing specific amplicons from the parasite's genome, providing valuable insights into the genetic factors that contribute to malaria transmission, resistance and the potential for targeted therapeutic strategies.

3.10. **The MVP protocol for Plasmodium falciparum sequencing**

The Malaria variant protocol (MVP) was employed to selectively amplify and sequence DNA from Plasmodium falciparum DNA, targeting genes of interest involved in malaria drug resistance and vaccine development. The protocol is based on the amplification of specific genetic markers within the *plasmodium falciparum* genome using gene specific primer pairs. The genes investigated included *pfama1*, *pfcrt*, *pfcsp*, *pfdhfr*, *pfdhps*, *pfhrp2*, *pfhrp3*, *pfk13*, and *pfmdr1*.
The primers were lyophilized and resuspended to a 100µM concentration with a TE buffer. To prepare the primer pool, 10µl of each primer was combined, bringing the total to 100µl. The primer pool was diluted to 10µM by adding 900 ul of TE buffer. Proper procedures were followed to avoid contamination, including the use of aliquots and storage at -20˚C for future use.

A master mix containing Kapa ReadMix 2X was used, with MVP primer pool (10µM). The prepared master mix of each sample was 17µl and sample added was 8µl in each well. This reaction was performed in a thermocycler with the following conditions: Preparation of master mix on ice was recommended as follows:

Table 3: MVP Master Mix preparation

Reagent	**Volume per sample(µL)**
Kapa Readymix 2x	**15.5**
MVP primer pool (10 uM)	**1.5**
Total	**17**

Table 4: Set up for MVP PCR

Step	**Temperature (µL)**	**Time**	**No of cycles**
Prepare block	95	Forever	1
Initial denaturation	95	3min	1
Denaturation	98	20 sec	35
Extension	60	3 min	
Final extension	60	10 min	1
Hold	8	forever	1

After amplification, DNA concentration was measured using Qubit DNA assay to ensure an optimal amount of DNA for sequencing. A rapid barcoding kit 96 v14 and sequencing protocol

involves preparing samples, barcoding, library preparation and loading onto the flow cell was used. 200ng of each sample was transferred into a new plate, ensuring a total of 10µL per sample by adding nuclease-free water, ensuring that each sample is well represented and contributes equally to the final sequencing data output. 1 µL of rapid barcode was added to each sample. The plate was mixed, spun, and incubated in the thermal cycler with a specific program.

Table 5: Incubation thermocycler set up condition

Step	**Temperature (°C)**	**Time**
Prepare block	30	Forever
Tagmentation	30	2 min
Inactivation	80	2 min
Hold	8	Forever

After barcoding, the samples are pooled, and 5 µL from each barcoded sample is transferred into a clean 1.5mL. The pool underwent SPRI bead clean up with 0.5x bead to sample ratio, followed by ethanol washes to remove contaminants. After eluting the DNA in 15 µL of the elution buffer, it is allowed to sit at room temperature for 10 minutes. The qubit assay is used to quantify the eluate, ensuring the recommended 800 ng as final concentration to be loaded on the R10 flow cell. The rapid adapter (RA) should be diluted in a new 1.5 mL Eppendorf DNA ligation tube by adding 1.5 µL of RA and 3.5 µL of adapter buffer (ADB), then pipetting the mixture together. To make sure the contents are at the bottom, add 1 ul of diluted rapid adapter to the barcoded DNA sample and gently mix by flicking the tube and spinning down. To enable adapter ligation, incubate for five minutes at room temperature.

Sequencing was performed using Oxford Nanopore Technology GridION platform, a compact benchtop device with integrated computer. GridION generates up to 240 Gb of data per run. Library sequencing was carried out on R10.4 flow cells using chemistry 14 kits. Each flow cell was checked for pore health (number of pores available) and Primed using Priming kits to avail the nanopores from the storage buffer before loading. The library was mixed with sequencing buffer and Library beads to ensure proper density on the flow cell membrane over pores while sequencing. After loading the library, sequencing was initiated using MinKNOW Software and the resulting data were processed and analyzed using different Bioinformatics tools.

3.11. **Sequencing of the PfCSP gene**

To examine the *Plasmodium falciparum circumsporozoite protein (PfCSP)* gene's genetic diversity, primers were designed to amplify N-terminal, central repeat and C-terminal regions of the gene. Forward primer sequence: TCGCAAACGTAATTAAATATTCACAAA, and reverse primer sequence as GCACTGGTATTCCTGGAATAAGG The amplicon size was 1274 base pairs, which covers central repeat and regions. This approach ensured to capture the immunologic regions relevant for malaria vaccine including the RTS, S/AS01 vaccine.

3.12. **Variant calling and population genetics analysis method**

This analysis of *Falciparum* sequences involved a stepwise bioinformatics pipeline from raw Fastq reads to phylogenetic tree. The workflow was designed to ensure high confidence variant detection, consensus generation, and genetic diversity analysis using different tools.

Raw FASTQ files obtained from amplicon sequencing were first analyzed for quality check using Nanoplot (De Coster, 2018). This tool provided quality score and read length distributions, helping to assess overall sequencing run quality. FastP (Chen, Zhou, Chen, & Gu, 2018) was then employed to perform additional quality checks and evaluate the overall read quality and read distributions, ensuring that the data met the required quality standards for further analysis. Porechop (Wick, 2017) was used to trim low-quality bases and remove adapter sequences. Low-quality reads with Q <20 and adapter sequences were trimmed using Porechop.

After trimming, Minimap (Li, 2018) was used to align the cleaned reads to the Plasmodium *falciparum* 3D7 reference genome. This tool accurately mapped the sequencing data to the reference genome, facilitating downstream analysis of genetic variations. The resulting alignment files were in SAM format, which were processed using Sam tools (Danecek, et al., 2021). The SAM files were compressed to the BAM format using BAM tools (Barnett, 2011) to ensure compatibility with computational tools as Binary data. BAM files were further used to inspect read depth coverage across target gene regions.

Medaka (Technologies, 2025) was applied for variant calling, a 50% of allele frequency threshold was set of 50% for variant calling to ensure high confidence quality. The output VCF (Variant Calling Format) and FASTA files were generated. Python scripts (Foundation, 2023) were used to generate VCF files (Variant Calling files). From these files consensus sequences were generated using Medaka, with a threshold set to call a nucleotide only when it had a 50% frequency at the specified depth, ensuring accurate variant calls.

MaFT pipeline (Katoh & Standley, 2013) was used for generating Multiple Sequence Alignment (MSA), the resulting MSA files were visualized with AliView software, which facilitates the identification of regions with high genetic variability, focusing on high complexity regions characterized by diverse nucleotides. Low complexity regions where sequence ambiguity might introduce errors were removed to ensure the accuracy of the variant calls.

Python packages were utilized to extract SNPs from VCF files and convert them into matrix format (samples and SNP positions) to help visualize population analysis. Pandas and numpy in python packages were used to convert the extracted data into SNP matrix (sample and SNP positions), with each cell representing the allele observed at a given position. This matrix served as the basis for downstream statistical analysis.

Linkage Disequilibrium (LD) analysis was performed using scikit-allel's python package. The squared correlation coefficient (r^2) between pairs of SNPs was calculated to measure the non-random association alleles. The resulting LD matrix was visualized as a heatmap using the seaborn library (Waskom, 2021), allowing identification of linkage across the different loci, which is essential for understanding the genetic relationship between SNPs within the population.

Tajima's D was calculated in sliding window format using scikit allel package in python, which allows genetic metrics to be computed helping determine whether the population is evolving neutrally or under selection pressure.

PCA was performed using scikit-learn and visualize with matplotlib to assess genetic clustering, finally, a pairwise genetic matrix was computed from SNPs matrix using scikit bio and a phylogenetic tree was constructed with bio. phylo module from Biopython to visualize the evolutionary relationships between sequences. The analysis of this genetic divergence enabled us to track mutations and better understand the factors influencing the spread and evolution of the parasite

3.13. **Ethical considerations**

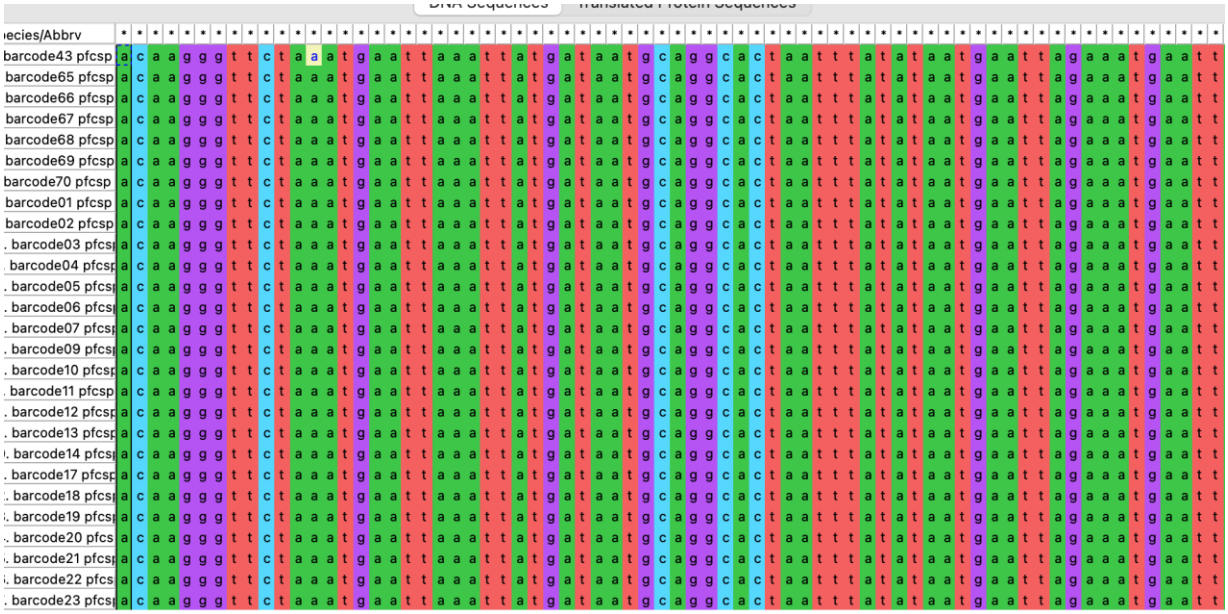
The Rwanda Biomedical Centre (RBC) created biosafety and ethical protocols that were followed during the investigation. The Rwanda National Ethics Committee (RNEC) granted ethical clearance for this study, guaranteeing compliance with the standards necessary for this kind of research. Participants gave their written informed consent, and participation was entirely voluntary. The confidentiality was taken into consideration, and the sample was anonymized with unique identifiers in all data handling and analysis. This measure was implemented to protect the participant's privacy throughout the study. All biological samples were bio-banked in protected freezers, and all data information was documented and stored with strict adherence to confidentiality, and data integrity.

CHAPTER 4: RESULTS

This chapter presents the findings related to the genetic diversity and evolutionary dynamics of the *PfCSP* and *PfK13* genes in *plasmodium falciparum* isolated and collected from Huye and Kirehe districts in Rwanda.

4.1. Single Nucleotide Polymorphisms in *PfCSP* gene

A visualization with AliView after multiple alignment showing a nucleotide at each position. SNP was presented where C Substitutes G to a particular position at high frequency in different samples.



Visualization of Polymorphic Site

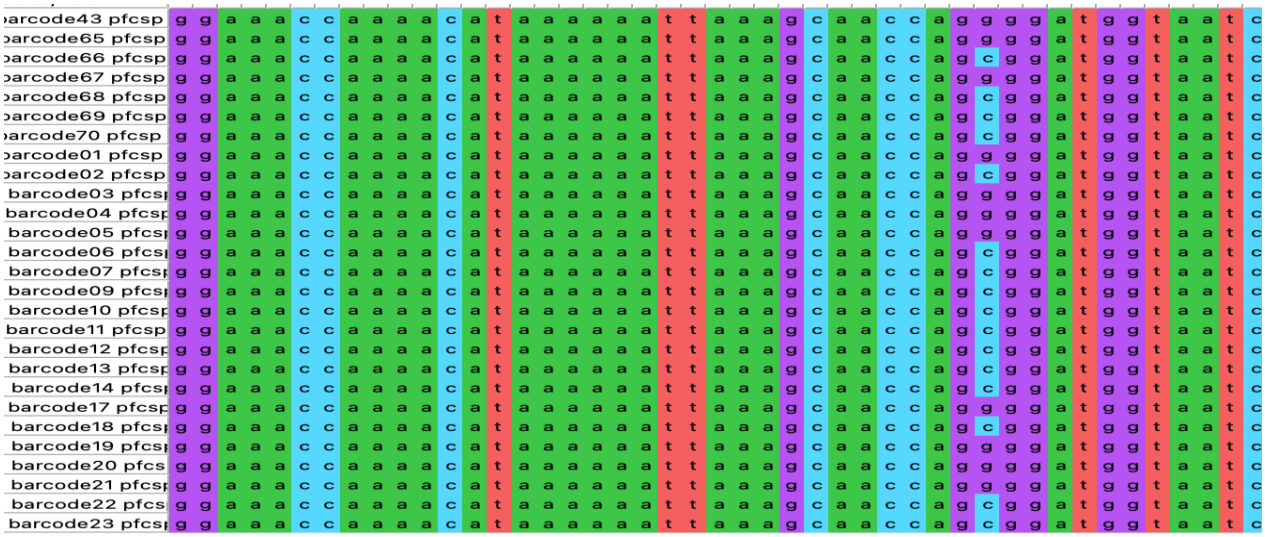


Figure 4: Single Nucleotide Polymorphisms in PfCSP gene

Visualization using AliView after multiple alignment sequences shows nucleotides at each position. The upper panel depicts non-polymorphic sites; the lower panel highlights polymorphic sites where a Cytosine (c) is frequently substituted by guanine (G) across different samples

4.2.**PfCSP Gene analysis**

4.2.1. **SNP identification and frequency**

The *PfCSP* gene was analyzed in samples from Huye and Kirehe districts.

Table 6: SNPs in the PfCSP gene from Huye district

Mutation type	**CSP_aa change_HUYE**	**Frequency(count)**	**Frequency (%)**
Missense	A361E	19	65.5
Missense	A98G	20	69.0
Missense	D199N	20	69.0
Missense	D294N	20	69.0
Missense	D356N	20	69.0
Missense	D359N	20	69.0
Missense	E357Q	20	69.0
Missense	K314Q	20	69.0
Missense	K317E	20	69.0
Missense	K317T	20	69.0
Missense	K322E	20	69.0
Missense	K322T	20	69.0
Missense	L327I	20	69.0
Missense	N298K	20	69.0
Missense	N321K	20	69.0
Missense	N352G	20	69.0
Missense	Q324K	20	69.0
Missense	S301N	20	69.0
Missense	V198A	20	69.0

Missense mutations were observed in nearly all samples. Most mutations (A98G, D199N, D294N) were present in 20 samples (69%) while A361E was observed in 19 (65.5%) of the total samples.

Table 7: SNPs in the PfCSP gene from Kirehe district

Mutation type	Frequency(count)	Frequency (%)
A361T	7	24.14
A98G	7	24.14
D199N	7	24.14
D294N	7	24.14
D356N	7	24.14
D359N	7	24.14
E295K	7	24.14
E357Q	7	24.14
K314Q	7	24.14
K317E	7	24.14
K322E	7	24.14
L327I	8	27.59
N321K	7	24.14
N321Q	8	27.59
P354S	7	24.14
Q324K	7	24.14
S301N	7	24.14
V198A	8	27.59
N298K	6	20.69
N352D	6	20.69
P200P	7	24.14
V377V	6	20.69

Frequencies of missense mutations were generally lower. Most mutations were observed in 7 samples (24.14%), with L327L, N321Q, and V198A appeared in 8 samples (27.6%), while N298K, N352D, and V377V were detected in 6 samples, accounting for 20.69% of the total.

Table 8: Homozygous mutations

Mutation type	CSP- SNP aa-change	Frequency count	Frequency (%)	Origin
Missense	D199N	11	41	Huye
Missense	V198A	11	41	Huye
Missense	D359N	4	15	Huye
Missense	E357Q	4	15	Huye
Missense	L327I	2	7	Huye
Missense	Q324K	2	7	Huye

Missense	K322T	2	7	Huye
Missense	N321K	3	11	Huye
Missense	N298K	2	7	Huye
Missense	D356N	2	7	Kirehe
Missense	P354S	2	7	Kirehe
Missense	N352D	3	11	Kirehe
Missense	K322E	1	4	Kirehe
Missense	K317E	3	11	Kirehe
Missense	D294N	1	4	Kirehe
Missense	A98G	2	7	Kirehe
Missense	N321Q	2	7	Kirehe
Missense	K314Q	1	4	Kirehe

(Table 8) illustrates the frequencies of missense mutations in *PfCSP* gene with distribution of homozygous mutant genotypes (1/1) based on high genotype quality (GQ) and depth of coverage (DP). D199N and V198A mutations show the highest frequency (41%), while other SNPs like D359N and E357Q ranged between 4-15% across Kirehe and Huye districts.

4.2.2 **CSP SNPS distribution across districts**

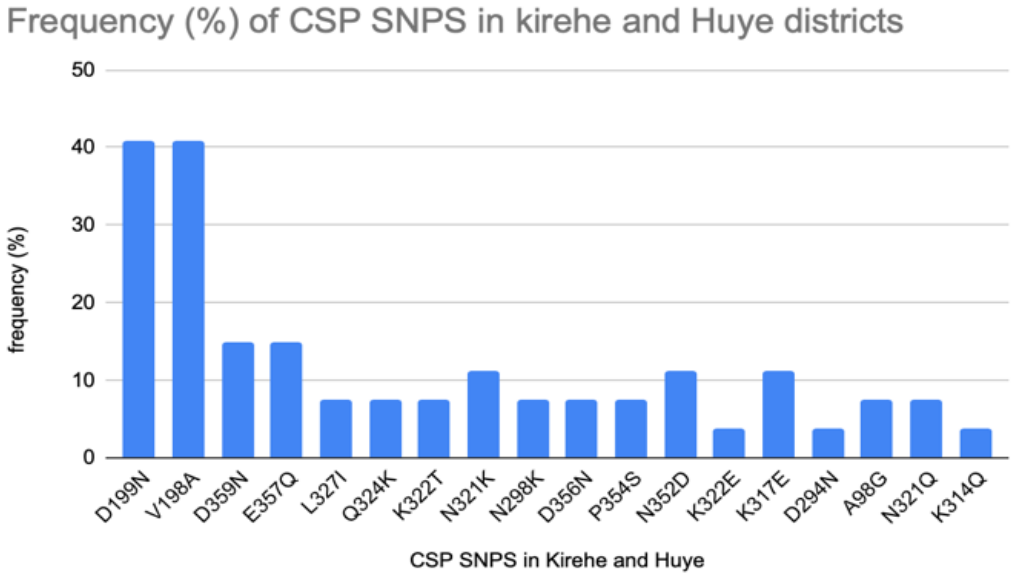


Figure 5: Visualizes the predominance of D199N and V198A mutations in both districts.

4.2.3. **Genetic Diversity and Selection**

Table 9: Tajima's neutrality test for PfCSP gene

Metrics	**M**	**S**	***Ps***	**Θ**	**Π**	**D**
Value	27	53	0.044	0.01	0.01	0.77

D: Tajima's D is a statistical test used to detect selection pressures. Which is 0.77
M: number of sequences analyzed, which is 27 coding nucleotide sequences.
S: number of segregating sites which is 53 segregating sites was observed across the sequences
Ps: Polymorphic sites refer to positions where mutations or variations occur within sequences which was 0.04 relatively low level of polymorphism across analyzed alleles.
Θ: Is Watterson's theta which is 0.01
□: Nucleotide diversity which is also 0.01

Tajima's D = 0.77, suggesting balancing selection. Other metrics: M = 27, S = 53, Ps = 0.044, Θ = 0.01, π = 0.01.
The *PfCSP* gene showed a positive Tajima's D (0.77), suggesting that the *PfCSP* gene is under balancing selection.

Tajima's D was computed using a Python package and analyzed using a sliding window approach to assess genetic diversity and selection pressures across the *PfCSP* gene. This method provides insights into the evolutionary forces shaping the genetic structure of the population.
Three key regions showed different selection patterns in the below figure 8: 200 bp (selective sweep, D ≈ -3.5), 400–600 bp (purifying selection, D ≈ -3), 800–1000 bp (slight balancing selection, D ≈ -2.5)

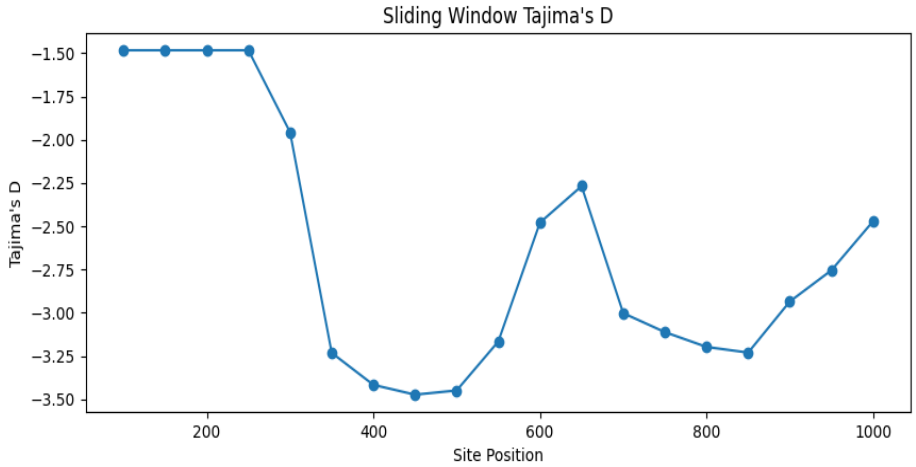


Figure 6: Sliding window Tajima's D analysis of PfCSP gene

4.2.4. **Linkage Disequilibrium (LD) Heatmap**

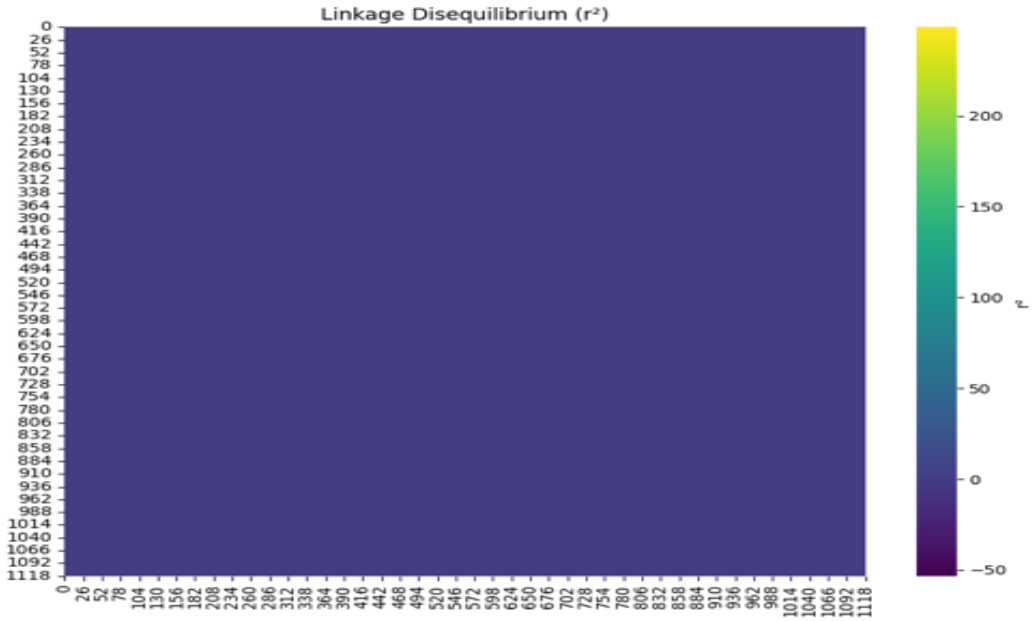


Figure 7: Linkage Disequilibrium (LD) assessment Heatmap

The heat map shows weak LD, suggesting that SNPs are not strongly linked. The uniform purple color across the heatmap indicates weak LD among SNPs.

X and Y axes represent the position of different SNPs in the sequence, represented by the color's differences. Strong linkage disequilibrium is shown as yellow to green, a weak linkage disequilibrium (WL), meaning those SNPs are inherited independently.

4.2.5. **Population structure**

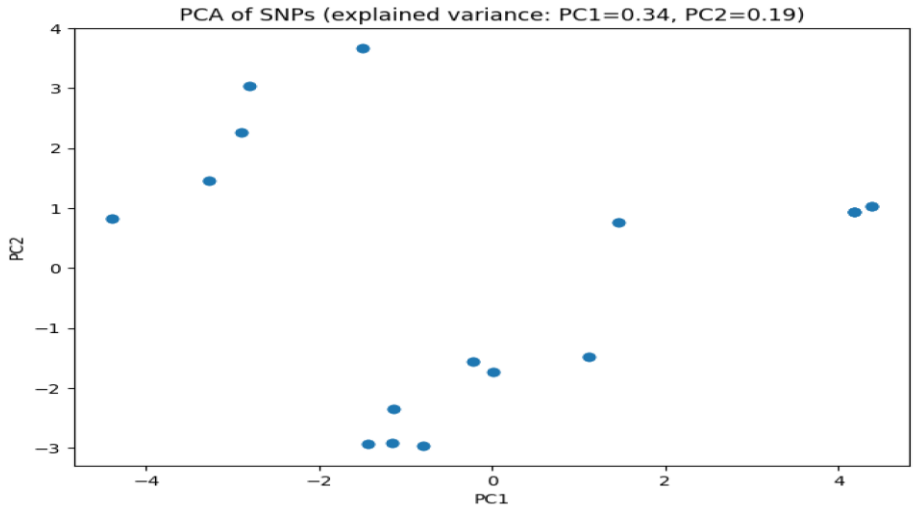


Figure 8: Principal Component Analysis (PCA) of PfCSP SNPs.

PC1 and PC2 explain 53% of the total variance. The distribution of points shows diversity within the population.

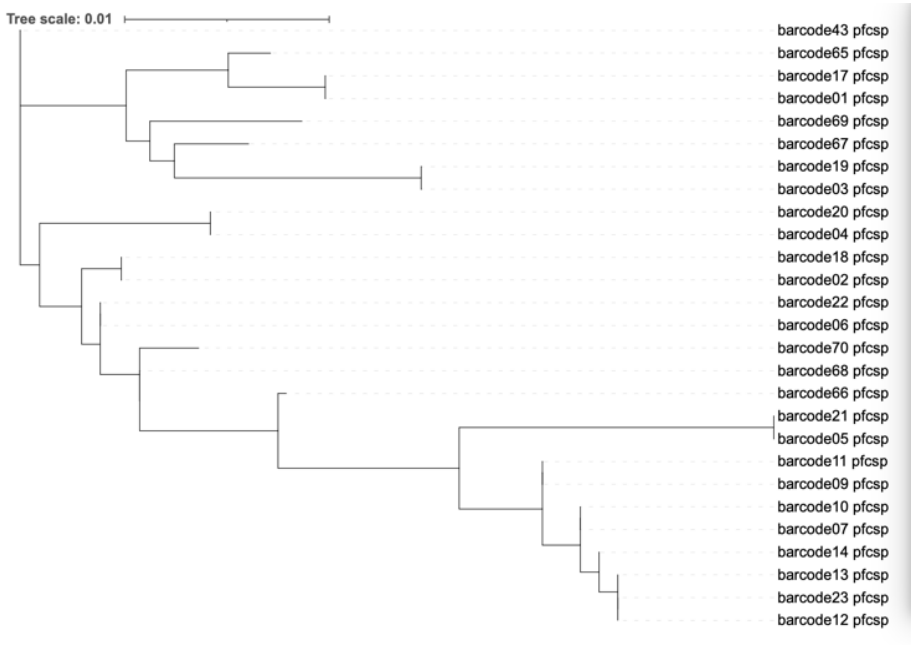


Figure 9: Phylogenetic tree of PfCSP variability

Samples are grouped based on SNP similarity. Barcodes indicate sample identification, closely clustered barcodes suggest shared evolutionary traits, while distant barcodes indicate genetic divergence.

4.3. *PfK13* gene analysis

4.3.3. PfK13 SNP identification and frequency

PfK13 gene was analyzed to assess artemisinin resistance marker

Table 10: SNPs frequencies in the Pfk13 gene

Mutation type	K13_aa change	Frequency(count)	frequency (%)	origin
Missense	G449A	2	7.4	Huye
Missense	R561H	3	11.1	Kirehe
Missense	N490T	1	3.7	kirehe

Missense mutations G449A (Huye), R561H(Kirehe)and N490T(Kirehe) were observed at 7.4%, 11% and 3.7%, respectively, with R561, a validated artemisinin resistance marker, being the most frequent.

4.3.4. Distribution across districts.

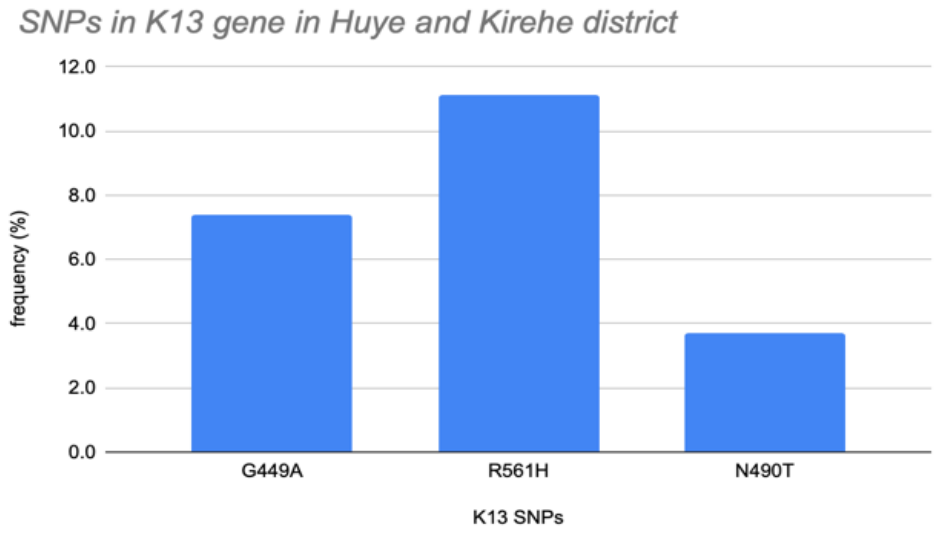


Figure 10: Visualization of mutation frequencies highlights the predominance of R561H in the population.

CHAPTER 5: DISCUSSION

This study investigated the genetic variability of *Plasmodium falciparum* by focusing on two key genes: The Circumsporozoite protein (*PfCSP*) and the *kelch13* (*PfK13*) *genes*. The findings provide insights into parasite population dynamics that are critical for guiding vaccine development strategies and malaria resistance monitoring programs in Rwanda.

The *PfCSP* gene showed a predominance of non-polymorphic sites characterized by an abundance of adenine (A) and Thymine (T) nucleotides consistent with other East African *P. falciparum* populations particularly in the central repeat region. This is consistent with other *Plasmodium falciparum* studies that reported that the central repeat of *PfCSP* is rich in A-T bases, aligning with research (Mandefro et al., 2025a). The nucleotide substitution of C to G at high frequency in different samples confirms the SNPs occurrence.

SNP analysis revealed several high frequency variants in both districts. In Huye, A361E appealed in 65%, indicating the overall observation of SNPS. D199N and V198A were the most frequent SNPs, with 41%, while N321K, D359N, and E357Q show moderate frequencies of 15% in samples collected from Huye. N352D and K317E are highly observed in the Kirehe district. Similar SNPS in the central repeat and C-terminal region of *PfCSP* gene are of particular interest because they could influence the binding of vaccine-induced antibodies and potentially reduce the efficacy of vaccines like RTS, S/AS01 and R21 which target PfCSP epitopes. These SNPs have been reported in Sudan, Kenya, and Cameroon. D199N was documented at moderate frequencies in East African isolates (Maina et al., 2024b; Mohamed et al., 2021b). A98G was found to be linked to the mutation in the central region with NANP and NVDP repeats per haplotype in the study conducted in Ethiopia (Mandefro et al., 2025b).

Despite reports of PfCSP SNPs in other East African countries, there is limited data on PfCSP polymorphisms in Rwanda. Notably, Rwanda has not yet implemented the RTS, S/AS01 malaria vaccine, which targets PfCSP and is recommended by the WHO for regions with moderate to high malaria transmission (Kenya, 2023). Understanding the local genetic diversity of PfCSP is therefore critical for anticipating how these SNPs might influence vaccine efficacy once introduced.

By employing statistical tests such as Tajima's D, Fu and Li's D, principal component analysis (PCA) and Phylogenetic tree, we assessed PfCSP genetic structure, identify potential selection pressures and explore the relationship between CSP SNPs. This approach is necessary for understanding the selection pressures to guide the policy making and development of effective vaccines. (Lyimo et al., 2024)

A positive 0.77, Tajima's D value was observed during this study, suggesting that the *PfCSP* gene is under balancing selection and shows no strong signs of recent expansion. In addition, Fu and Li's D was found to be 0.1329, supporting the absence of recent population expansion. The

balancing selection further indicates that multiple alleles are maintained, likely reflecting adaptive responses to host immune pressure. This pattern aligns with the findings from Muheza and Muleba districts of Tanzania which also reported slightly positive Tajima's D (Lyimo et al., 2024).

The observed balancing selection may reflect evolutionary pressures even in the regions without current vaccine deployment, highlighting the adaptive potential of local parasite populations. Regional vaccination efforts in neighboring countries, genetic drift, human migration and cross-border movement, contributing to SNPs presence in *CSP gene* (Sané et al., 2025). Studies have shown that the gene flow between populations can introduce new alleles and increase genetic diversity (Mwesigwa et al., 2025). Introducing malaria vaccines in neighboring countries could influence genetic variation observed in the *CSP gene*, which reduces parasite transmission and alters selection pressures, potentially leading to changes in allele frequencies (Mwesigwa et al., 2025).

Sliding window analysis showed variation in Tajima's D value across the *CSP* gene: a sharp decrease around position 200 suggests a beneficial allele increased rapidly in frequency, reducing genetic diversity at this site, at 400 to 600 position, possible directional selection which ends to the 800-1000 position, suggesting balancing selection. This observation was also observed in other *Plasmodium falciparum* genes such as AMA1, which is also a candidate to develop a Malaria vaccine. (Zhu et al., 2016)

Linkage Disequilibrium revealed weak associations among SNPs in the *PfCSP* gene, suggesting independent inheritance of alleles and high genetic variability. When alleles at several loci are not distributed randomly, this is referred to as linkage disequilibrium (LD). This shows that particular alleles are inherited in tandem, suggesting that the loci are either close to each other or there has been some recent development. The weak LD was observed across SNPs pairs in the *PfCSP gene*, suggesting a high degree of genetic variability. LD is consistent with other plasmodium species studies reflecting the frequent recombination and the dynamic evolutionary landscape of the parasite. (Feingold, 2001). This high variability may challenge the effectiveness of vaccines targeting conserved regions of PfCSP gene.

Principal component analysis (PCA) showed that PC1 and PC2 explain 63% of the total SNPs variability (PC1:34%, PC2:19%) and highlighted clustering patterns of genetically related isolates. (Oriero et al., 2022). These clusters likely reflect local transmission dynamics and genetic drift. Phylogenetic analysis further confirmed relationships among samples: Barcodes 43, 65, and 15 are closely, suggesting they may share a common evolutionary trait. In contrast, Barcode 12 and Barcode 43 are positioned far apart on the tree, indicating greater genetic divergence between these samples, suggesting they might have evolved separately due to geographic separation, genetic drift or other evolutionary pressures, which showed that 12 and 43 barcodes are from Huye and Kirehe samples, respectively. This divergence might result from regional differences in malaria transmission, influencing local *Plasmodium falciparum* populations. Similar study was done using 172 sequences, a phylogenetic tree was constructed from north central and south of

Ghana, showing the high relatedness among *PfK13* mutations in all clades, such patterns are important for understanding parasite adaptation and anticipating vaccine escape (Dieng et al., 2023).

The *PfK13* gene analysis revealed several nonsynonymous mutations with R561H being the most common, followed by G449 and N490T. The predominance of R561H is particularly significant as it is a validated molecular marker of artemisinin resistance. Previous studies in Rwanda and other East African countries have consistently associated this mutation with delayed parasite clearance and treatment failures (Uwimana et al., 2021c; van Loon et al., 2024b). Given its prevalence, the continued monitoring of *PfK13* mutations in Rwanda is necessary for evaluating effectiveness of current malaria treatment and guiding future public health interventions. The detection of G449A and N490T, although less common, is also noteworthy, as these variants have been reported at moderate frequencies in some East African populations and could represent emerging alleles under drug selection pressure (Molina-de la Fuente et al., 2023).

Artemisinin based combination therapies (ACTs) remain the first line treatment for uncomplicated malaria in Rwanda. However, the rise of resistance could compromise the clinical efficacy of ACTs, particularly if partner drug resistance evolves simultaneously like lumefantrine or piperaquine. This concern is reinforced by WHO reports that highlight the regional spread of k13 mutations in East Africa, indicating that artemisinin resistance is no longer confined to Southeast Asia but has become a growing challenge for African malaria control (WHO, 2023).

The detection of R561H and other PfK13 variants underscores the urgent of continuous molecular surveillance to safeguard the effectiveness of ACTS. This study provides a preliminary district-level genetic analysis of *plasmodium falciparum* based of PfCSP and PfK13 sequences, offering early insights into the local genetic landscape. However, given the limited size largely due to logistical and time constraints, as the study is part of an ongoing project, the findings reveal meaningful patterns worth further exploration.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

This study investigated the genetic diversity of *plasmodium falciparum* in Rwanda by focusing on the *PfCSP* and *K13* genes, which are the key targets for malaria vaccines and drug resistance monitoring. Single Nucleotide Polymorphism (SNP) analysis revealed mutations of potential biological and clinical significance, including A361E and D199N in pfCSP, and R561H in *Pfk13*. These findings provide the first district level molecular data from Huye, and Kirehe offering preliminary insights into parasite dynamics in Rwanda.

This population genetic analysis demonstrated that the *PfCSP* gene is under balancing selection, suggesting maintenance of multiple alleles likely driven by immune pressure. Principal Component analysis (PCA) and phylogenetic tree analysis further indicated genetic divergence across regions, reflecting both localized selective pressures and the possible influence of human migration and cross-border parasite gene flow.
Importantly, these genetic variations have potential implications for malaria vaccine efficacy as polymorphisms in PfCSP epitopes could alter vaccine induced antibody recognition and for antimalaria treatment effectiveness, given the presence of Pfk13 mutations associated with artemisinin resistance. These results emphasize the need for continuous genetic surveillance to inform public health strategies in Rwanda, particularly in anticipation of future vaccine rollout in the context of ongoing ACT base therapies.

This study acts as a proof of concept for further research on Rwanda's genetic diversity in malaria, highlighting the critical need for sustained genomic surveillance to inform public health decisions and strategies. However, it was limited by relatively small sample size, future research remains highly needed with a larger sample size and expanding to include more settings across the country. Integrating clinical and epidemiological data will provide deeper insights into how host factors influence genetic variation.

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