UNIVERSITY OF RWANDA

PREVALENCE OF *PLASMODIUM FALCIPARUM* K13 MUTATIONS AND ITS ASSOCIATION WITH PARASITEMIA LEVELS IN GISAGARA DISTRICT, RWANDA

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Jeanne BATAMURIZA

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By
Jeanne BATAMURIZA Reg.Number:223027893

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Supervisor: Dr. Jean Paul SINUMVAYO
Co-Supervisor: Dr Masceline Jenipher MUTSAKA-MAKUVAZA
Co-Supervisor: Dr Jules NDOLI MINEGA

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Declaration of independent work

I, Jeanne BATAMURIZA 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.

Jeanne Batamuriza

Signature: 

Supervisors 'approval

This is to certify that this dissertation was carried out under our guidance and is being submitted with our full approval as supervisors.

Supervisor 1:

Dr. Jean Paul SINUMVAYO

Chair, Department of Microbiology and parasitology

University of Rwanda

Signature: 

Date: 8/August/2025

Supervisor 2:

Dr. Masceline Jenipher MUTSAKA-MAKUVAZA

Senior Lecturer

University of Rwanda

Signature: 

Date:8/August/2025

Supervisor 3:

Dr. Jules NDOLI MINEGA

Head of Clinical Education and Research Division

CHUB

Signature…  ……..……..

Date:8/August/2025

Dedication

I humbly dedicate this work to God, whose blessings and strength have carried me through every challenge.

To my family, whose love and encouragement have been my greatest motivation.

To my lecturers, for their wisdom and dedication in nurturing my learning.

And to my supervisors, for their guidance, support, and invaluable advice throughout this journey.

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Acronyms

ACT: Artemisinin based combination therapy

ART-R: Artemisinin partial resistance

ARMEA: Artemisinin Resistance Monitoring in East Africa

CHUB: University Teaching Hospital of Butare

CHW: community health worker

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9

DHA: Dihydroartemisinic

DNA: Deoxyribonucleic acid

EDTEA: Ethylenediaminetetraacetic acid

IBM SPSS: International Business Machines Statistical Package for the Social Sciences Software

K13: Kelch13

NRL: National Reference Laboratory

PCR: Polymerase Chain Reaction

RNEC: Rwanda National Ethics Committee

RSA: Ring Stage Survival Assay

RBC: Red Blood Cell

RDT: Rapid Diagnostic Test

ROS: Reactive Oxygen Species

WHO: World Health Organisation

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Abstract

Malaria remains one of the most significant public health challenges globally, particularly in sub-Saharan Africa, where *Plasmodium falciparum* accounts for the majority of malaria-related morbidity and mortality. In Rwanda, malaria remains endemic. Despite the widespread implementation and success of artemisinin-based combination therapies (ACTs), the emergence of artemisinin resistance primarily associated with mutations in the *P. falciparum* Kelch 13 (K13) gene poses a serious threat to current treatment efficacy. K13 mutations have been increasingly reported in Rwanda, warranting continuous molecular surveillance. This study aimed to investigate the prevalence of *P. falciparum* K13 mutations and their association with parasitemia levels in Gisagara District.

A cross-sectional study was conducted using 215 Rapid diagnostic test (RDT)-positive samples which were collected under the Artemisinin Resistance Monitoring in East Africa (ARMEA) project from November to December 2024. Malaria-positive participants were initially identified using the Bioline^{TM} Malaria Ag *P. f*/Pan, Abbott Diagnostics Korea Inc RDTs and subsequently referred to health centers for venous blood collection. Samples were transported to the University Teaching Hospital of Butare (CHUB) for further laboratory analysis.

Thick and thin smears were used for parasitemia identification and quantification, while polymerases chain reaction assay (PCR) and capillary-based Sanger sequencing were employed to detect K13 mutations. Of the 215 samples, 195 were confirmed positive by microscopy. Among these 190 were identified as *P. falciparum.* The majority of *P. falciparum* cases were trophozoites, while 17 samples presented with gametocytes. Additionally, five samples were identified as *P. malariae,* with 3 containing schizonts. The 195 microscopy-positive samples were classified into 3 categories: 31 exhibited low parasitemia,73 fell into the moderate range and 91 shower high parasitemia levels

PCR amplification was successful in 203 samples, and sequencing revealed K13 mutations in 2.8% of cases, with R561H being the most frequently observed mutation. Notably, mutants samples exhibited moderate to high parasitemia, with no cases in the low parasitemia category.

These findings highlight the significance of continued molecular surveillance for early detection of K13 mutations to combat outbreaks due to artemisinin resistance.

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CHAPTER I. INTRODUCTION

1.1. Background

Malaria is a parasitic disease which remains as one of the greatest challenges for global health. In 2023, around 263 million cases and 597 000 deaths were reported globally with 94% of cases and 95% of deaths emanating from the World Health Organisation (WHO) African region(WHO, 2024). Most malaria related deaths in Africa occur in children under 5 years of age, with *Plasmodium falciparum* responsible for 99% of malaria cases on the continent (Naß & Efferth, 2019).

Despite global efforts to control malaria, the emergence of drug resistance poses a serious challenge to treatment efficacy and disease elimination. Artemisinin-base combination therapies (ACTs) have been the global standard for treating uncomplicated *P. falciparum* infection. The combination therapies include a rapidly acting artemisinin derivative with a longer-lasting partner drug to ensure the complete elimination of remaining parasites(Eastman & Fidock, 2009; P. J. Rosenthal et al., 2024). However, resistance to artemisinin has been reported in several regions, firstly in Southeast Asia, and is now increasingly observed in Africa(F. Ariey et al., 2014; Uwimana et al., 2021).

Artemisinin partial resistance (ART-R) is primarily associated with specific non-synonymous mutations in the propeller domain of the *P. falciparum* kelch13 (K13) gene (*PF*3D7_1343700). The K13 mutation has been been linked to delayed parasite clearance and reduced the drug's effectiveness in eliminating *P. falciparum*(F. Ariey et al., 2014; Dondorp et al., 2009; C. Schreidah et al., 2024). Studies show that these mutations can lead to a delay in parasite clearance which can complicate treatment monitoring("Association of mutations in the Plasmodium falciparum Kelch13 gene (Pf3D7_1343700) with parasite clearance rates after artemisinin-based treatments-a WWARN individual patient data meta-analysis," 2019; Asua et al., 2021; Huang et al., 2015; Straimer et al., 2022). Recent studies in Rwanda have indicated a concerning shift towards an increase in K13 mutations prevalence. Precisely, research conducted in Huye district, Southern province has documented a substantial rise in the prevalence of these mutations, escalating from 9.1% in 2019 to 17.5% in 2023(van Loon et al., 2024).

A critical consequence of K13 mutations is their association with lower pre-treatment parasite densities in infected individuals(van Loon et al., 2024). This phenomenon has significant implications for malaria diagnostics, particularly for rapid diagnostic tests (RDTs) which are widely used at the community level and in primary healthcare settings in Rwanda due to their ease of use and rapid results(Nsengimana et al., 2023). RDTs

typically rely on detecting parasite antigens in the blood, and lower parasite densities can fall below the detection threshold of some RDTs, leading to false-negative results. These findings underscore the need for further surveillance and research to better understand the prevalence and association of K13 mutations with parasitemia (Bergmann et al., 2021). Parasitemia, the concentration of *P. falciparum* parasites in an infected individual's bloodstream, is a key indicator of malaria severity, reflects parasite replication efficiency and host immune response(Su et al., 2025). K13 mutations have the ability to influence the level of parasitemia by affecting critical biological processes within malaria parasite

The process of haemoglobin endocytosis is an essential process for parasite's acquisition of nutrients required for its growth and survival inside red blood cells, could be affected by K13 mutations. This process is crucial for the parasite to obtain amino acids from host red blood cells. Alterations in *K13* may disrupt the formation or function of cytostomes specialized structures used for haemoglobin uptake leading to slower parasite metabolism and reduced drug activation, particularly of artemisinin. This delayed haemoglobin digestion is thought to contribute to parasite survival during early ring stages under drug pressure (Birnbaum et al., 2020; Tutor et al., 2023).

Moreover, these mutations influence how the parasite responds to stress particularly when exposed to antimalarial drug like ACTs. By modifying these stress response pathways, the parasite may be able to tolerate drug pressure, potentially contributing to the presence of parasite in the bloodstream after treatment(Angwe et al., 2024). Understanding how these mutations affect parasitemia at a mechanistic level is essential to fully grasp their impact on diagnosis and treatment outcomes. This persistence, even after treatment, may act as silent reservoirs, and increase the risk of spreading of artemisinin resistance consequently undermining malaria control and elimination efforts. This underscores the importance of strengthening molecular surveillance, particularly in regions with heterogeneous malaria transmission where localized resistant strains could result in broader outbreaks.

Gisagara District, situated in Rwanda's Southern Province, is characterized by high malaria transmission and remains a malaria endemic area (Rudasingwa & Cho, 2020; van Loon et al., 2024). Although this study was limited to Gisagara, evidence of K13 mutations genetic indicators linked to artemisinin resistance has been documented in Huye District, which borders Gisagara and has lower but ongoing malaria transmission(A. Umugwaneza et al., 2025). The geographical proximity of the two districts, along with their differing transmission intensities, highlights the relevance of investigating the prevalence and implications of K13 mutations in Gisagara's high-transmission context. To the best of our

knowledge, no comprehensive study has been conducted to assess K13 mutations and or their association with parasitemia in Gisagara Districts. This information could offer valuable insights into the dynamics of artemisinin resistance and guide future malaria control strategies.

1.2. Problem Statement

Although ACTs remain the cornerstone of malaria treatment in Africa, the increasing emergence of *P. falciparum* resistance threatens to reverse the gains in malaria control and elimination. Central to this resistance is the presence of mutations in the K13 gene, which have been associated with delayed parasite clearance following ACT administration. Rwanda is among the few African countries where K13 mutations particularly the R561H allele have been confirmed at significant frequencies, indicating possible in-country artemisinin partial resistance (Straimer et al., 2022)

Despite national level reports of these mutations, there is a significant gap in the literature concerning their prevalence in the whole country and biological impact particularly within Gisagara district. This district exhibits higher endemicity which may influence parasite biology and clinical outcomes. However, there is no available data on K13 mutations and its correlate with pretreatment parasitemia levels, despite emerging evidence suggesting that K13 mutant parasites may present with altered fitness and parasitemia profiles (Stokes et al., 2021).

The absence of localized data in Gisagara result in delayed detection of emergence of resistance, allowing parasites to spread silently in the local populations. The absence of such information also means that the district will rely on generalized data, missing opportunities for targeted invention, thus, resulting in suboptimal treatment policies, weakening progress towards elimination and consequently leading to vulnerability in malaria resurgence. Without detailed knowledge of the presence of K13 mutations and how they influence parasitemia levels, it becomes challenging to design regionally tailored surveillance and treatment strategies. This knowledge gap restricts the ability of malaria control programs to detect early signs of treatment failure, adapt diagnostic approaches, and target interventions effectively. It also raises the risk of importation and unmonitored spread of the resistant strains to other settings

Therefore, investigating the prevalence of K13 mutations and their association with pretreatment parasitemia in Southern Rwanda (Gisagara) is critical to understand local dynamics of artemisinin resistance and support evidence-based policymaking for more effective malaria control and elimination strategies.

1.3. Research question

What is the prevalence of *P. falciparum* K13 mutations in Gisagara district of Southern Rwanda, and how are these mutations associated with pretreatment parasitemia?

1.4. Rational of the study

Globally, ACTs are the cornerstone of malaria treatment. However, the emergence and spread of *P. falciparum* stains with K13 mutations pose a threaten malaria control and elimination efforts. Such mutations have been reported in Rwanda, particularly in Huye in the Southern province. However, such data is lacking in many districts in Rwanda including Gisagara, a high transmission district adjacent to Huye. Given the proximity of Gisagara to Huye district, and the contrasting transmission intensities between the two districts, lack of data on the molecular characteristics of K13 in Gisagara represents a critical gap in Rwanda's national malaria surveillance data. This research was particularly relevant in the context of Rwanda's national malaria control strategy, which emphasizes the need for continuous surveillance of resistance markers to guide treatment policies. Understanding the dynamics of K13 mutations guided early warning systems, treatment policies and consequently prevents the establishment and silent spread of malaria resistant strains. Additionally, the findings will contribute to the broader understanding of malaria resistance trends in Africa and support global efforts to combat drug-resistant of *P. falciparum* strains.

1.5. Objectives

1.5.1. Main objective

To investigate *P. falciparum* K13 mutation prevalence, and its relationship with parasitemia levels in Gisagara district.

1.5.2. Specific Objectives

- To assess parasitemia levels in RDT positive blood samples collected from Gisagara district.
- To determine the prevalence of K13 mutations in *P. falciparum* isolates Gisagara district.
- To investigate the association between K13 mutation prevalence and parasitemia levels.
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CHAPTER II. LITERATURE REVIEW

2.1.Global malaria epidemiology and significance of *Plasmodium falciparum*

Malaria remains a major global health concern, particularly affecting population in low resource settings. Despite extensive control strategies over the years, the disease continues to pose a significant burden. It is endemic in 83 countries, with highest transmission rates reported in sub-Saharan Africa, Southeast Asia, and parts of Latin America. Sub-Saharan Africa is disproportionately affected, contributing to approximately 94% of all malaria cases and 95% of associated deaths worldwide. Alarmingly, young children under the age of five account for about 76% of these fatalities (WHO, 2024).

The genus *Plasmodium* is comprised of over 200 species but only five are responsible for malaria in human. These include *P. vivax, P. ovale, P. malariae, P. knowlesi* and *P. falciparum* (Wang et al., 2022).

The most widespread and deadly malaria causing specie is *P. falciparum*, particularly in sub-Saharan Africa, where it is responsible for the majority of infections and severe clinical outcomes. Its high pathogenicity is linked to several biological traits, including rapid replication, the ability to reach high parasite densities in the blood, and its unique capacity to sequester infected red blood cells in the microvasculature of vital organs such as the brain, lungs and placenta. This sequestration allows the parasite to evade splenic clearance and contributes to life-threatening complications, most notably cerebral malaria(Cowman et al., 2016).

2.2.History and use of Artemisinin Based combination therapies

Artemisinin, derived from Artemisia annua by researchers at Chinese Academy of traditional Chinese medicine, emerged as a timely breakthrough and represents natural compound identified for malaria treatment(Ma et al., 2020). Artemisinin was discovered during the time when malaria parasites were rapidly developing resistance to chloroquine and sulfadoxine-pyrimethamine, which were the existing treatments Artemisinin-based combination therapies were introduced as the first-line treatment for uncomplicated *P. falciparum* infection in the early 2000s. Their use revolutionized malaria treatment globally, primarily due to artemisinin' potent antimalarial properties and its ability to rapidly clear *P. falciparum* parasites from bloodstream(Ogbonna & Uneke, 2008). Artemisinin-based combination therapies have become a foundation in the fight against malaria, with their extensive use leading to a considerable decline in malaria related morbidity and mortality. This is especially notable is sub-Saharan Africa which has the highest malaria burden globally. According to WHO, the introduction and widespread

adoption of ACTs contributed to achieving a 60% reduction in deaths caused by malaria worldwide since 2000(WHO, 2020).

In 2006, Rwanda revised its national malaria treatment policy to introduce artemisinin-based combination therapies, specifically artemether-lumefantrine which was adopted as the first line treatment for uncomplicated *P. falciparum* malaria. This policy shift was driven by increasing resistance to previously used antimalarials, including chloroquine and later the combination of amodiaquine with sulfadoxine-pyrimethamine(Karema et al., 2012).

2.3.Challenges associated with Artemisinin-based combination therapies

Artemisinin-based combination therapies have remained the keystone of malaria treatment globally since their authorization by WHO in the early 2000s(WHO, 2015). Their rapid parasite clearance and ability to reduce disease severity have significantly contributed to declines in malaria-related morbidity and mortality(Dondorp et al., 2010; WHO, 2020). Despite their success, ACTs face a range of challenges that threaten their long-term effectiveness, especially in malaria-endemic regions.

A major concern is the circulation of substandard and falsified antimalarials. These medicines often contain incorrect doses of active ingredients or lack them completely, resulting in reduced therapeutic efficacy. Such products are most prevalent in sub-Saharan Africa and Southeast Asia. World health organisation estimates that approximately 10% of medical products in low and middle-income countries are substandard or counterfeit, with antimalarials being among the most commonly affected(Who, 2017).

Inadequate patient adherence to ACTs regimes further undermines treatment success. The established three-day treatment protocol is often not completed, extremely in areas with low health literacy or limited access to health facilities. Some patients stop treatment prematurely due to the rapid resolution of symptoms or because of side effects, while others may lack understanding of the importance of completing the full dose(Banek et al., 2014; Yakasai et al., 2015). Incomplete treatment increases the likelihood that some parasites will survive and be exposed to sub-therapeutic drug levels, setting stage for resistance development.

Diagnostic challenges are also a threat to ACTs effectiveness. In many settings malaria diagnosis is based on clinical symptoms alone which can be nonspecific and lead to overuse of ACTs(Leslie et al., 2012; Manguin et al., 2017).

2.4.Emergence of K13 mutation and Artemisinin Resistance

Artemisinin resistance which was first detected in western Cambodia, Southeast Asia, has caused alarm across the globe(Noedl et al., 2008). It is now used as key indicator of resistance in therapeutic efficacy(Noedl et al., 2008). Artemisinin partial resistance is associated with mutation in *P. falciparum kelch* 13 (K13) gene encoding a protein of 727 amino acid involved in parasite cytoprotective and antioxidant responses that play an important role in the survival of *P. falciparum* under drug pressure, which result in delayed parasite clearance and threaten the efficacy of ACTs(F. Ariey et al., 2014; Menard & Dondorp, 2017). Although initially restricted to southeast Asia *K13* mutations linked to artemisinin resistance have now been identified in multiple African countries, including Rwanda raising concerns about the potential spread and impact of resistance on the continent(Ashley et al., 2014; Balikagala et al., 2021; A. Uwimana et al., 2020).

Recent studies indicate that Eastern Africa has become a hotspot for the emergence of ART-R with multiple validated K13 mutations including 469Y and 675V in Uganda, 561H in Rwanda and Tanzania, 622l in Eritrea and Ethiopia arising independently and spreading to neighbouring countries(Juliano et al., 2023; P. J. Rosenthal et al., 2024; C. Schreidah et al., 2024). The K13 R561H mutation was first reported in *P. falciparum* isolates collected from Masaka, Rwanda during the 2014-2015 surveillance period, where it was detected at a frequency of 7.4%. Interestingly, during the same time frame, this mutation was not observed in samples collected from Rukara a geographically nearby location. Yet, subsequent surveillance in 2018 revealed a notable increase in the prevalence of K13 561H mutation in both areas. Specifically, the mutation was found in 19.6% of isolates from Masaka and in 22% of isolates from Rukara(Cecile Schreidah et al., 2024). Furthermore, recent studies highlighted the emergence and spread of specific *P. falciparum* K13 gene mutations namely C469F, R561H, and A675V in southern Rwanda associated with delayed parasite clearance (Balikagala et al., 2021). In 2019, a prevalence of 9.1% of these mutations was recorded in Huye district, indicating a concerning rise in resistance markers. Specifically, the R561H mutation, a validated marker for artemisinin resistance, was identified in patient isolates from the region(van Loon et al., 2022).Further research in 2023 revealed that the prevalence of these K13 mutations had nearly doubled to 17.5% in the same area (van Loon et al., 2024).

In addition, other nonsynonymous mutations such as but not limited to P574L have also been reported at low frequencies in different location (Masaka, Ruhuha, Huye) (Tacoli et al., 2016). However, these mutations have not been directly associated with delayed

parasite clearance in vivo as evidence by the absence of persistent day 3 parasitaemia but have demonstrated increased survival rates in vitro using ring stage survival assay (RSA) suggesting reduced susceptibility to artemisinin (A. Uwimana et al., 2020; Uwimana et al., 2021). These findings highlight the critical importance of continuous and systematic molecular surveillance of K13 mutations to track the emergence and distribution of resistance for informing and adapting malaria control and treatment strategies in a timely and effective manner (C. Schreidah et al., 2024; A. Uwimana et al., 2020) and to combat the spread of drug-resistant malaria in the region

Genomic analysis revealed that the 561H mutation found in Rwanda had different surrounding microsatellite patterns compared to the 561H mutations previously seen in Southeast Asia. This strongly suggests that the mutation developed independently in Africa (Tacoli et al., 2016; A. Uwimana et al., 2020). However, the available data remain limited, particularly regarding its distribution and clinical impact in other parts of the country.

2.4.1. Parasitemia and its clinical importance

Parasitemia refers to the presence and concentration of malaria parasites in an individual's peripheral blood and is a key metric in diagnosing and managing infection. Accurate measurement of parasitemia is essential for evaluating the severity of infection, guiding treatment decisions, and evaluating transmission risk in endemic regions. *Plasmodium falciparum,* the most virulent of the human malaria species, is responsible for the majority of severe cases and malaria related deaths worldwide (Genton et al., 2008; Satpathy et al., 2004). The level of parasitemia is measured either as the number of parasites per microliter (μL) of blood, commonly assessed using thick blood smears or as the percentage of red blood cells infected with parasites, which is often determined through thin blood smear analysis(Moody, 2002).

The severity of malaria is closely correlated with the level of parasitemia, mostly in cases of *P. falciparum*, which is known for its rapid replication and potential to cause life-threatening complications. Elevated parasite loads are frequently linked to critical conditions such as severe anemia, cerebral malaria, low blood sugar and failure of multiple organs remarkably, in high-risk groups like young children and pregnant women(Genton et al., 2008; Taylor et al., 2004). Conversely, people who have acquired partial immunity through repeated exposure to malaria in endemic settings often exhibit low levels of parasitemia, which may lead to mild or asymptomatic infections (Bousema et al., 2014). Despite their low density, these infections can still play a significant role in sustaining

transmission, particularly when gametocytes persist and are not effectively eliminated through treatment (Ashley et al., 2014).

2.5.Association Between K13 Mutations and Parasitemia Levels

The direct impact of K13 mutations on pretreatment parasitemia levels is not well-defined. Some studies suggest that infections with K13 mutant parasites may present with lower baseline parasitemia. For example, research in Huye southern of Rwanda indicated that patients infected with *P. falciparum* carrying artemisinin resistance markers had lower pretreatment parasite densities compared to those with wild-type parasites (van Loon et al., 2024). However, other studies have not found significant differences in baseline parasitemia between mutant and wild-type infections, suggesting that factors such as host immunity and parasite fitness may influence parasitemia levels.

The growing prevalence of K13 mutations in Rwanda highlights the need for ongoing surveillance and research to monitor drug resistance patterns. While the 561H mutation has been detected at increasing frequencies, the clinical significance of newer mutations such as 675V remains unclear. Investigating the association between *Plasmodium falciparum* K13 mutations and pretreatment parasitemia levels is critical for deepening our understanding of malaria pathogenesis and its clinical progression. Such insights can inform whether artemisinin-resistant parasites exhibit altered replication rates or growth dynamics, which in turn could influence disease severity, diagnostic accuracy, and therapeutic response. Establishing this relationship may also help predict treatment outcomes and guide the development of more effective intervention strategies, especially in regions where K13 mutations are increasingly prevalent.

CHAPTER III. METHODOLOGY

3.1.Study Design

This study was a cross-sectional investigation assessing the prevalence of *P. falciparum* Kelch13 mutations and their association with parasitemia levels in Gisagara district, Rwanda. The study utilized samples collected in the Artemisinin Resistance Monitoring in East Africa (ARMEA) project conducted in November and December 2024. In this ARMEA project, malaria cases were identified by community health workers (CHWs) at the community level and at health centers using RDTs, Parasitemia identification and quantification by microscopy with molecular analysis was conducted at University Teaching Hospital of Butare (CHUB) and National Reference Laboratory (NRL).

3.2.Brief description of the ARMEA Project

Artemisinin Resistance Monitoring in East Africa (ARMEA) project is a multicounty, observational study aiming to rapidly generate molecular data *on P. falciparum* K13 mutations linked to emerging artemisinin resistance in Rwanda, Uganda, and the Democratic Republic of Congo. In Rwanda, the study will enroll 1,200 malaria patients from multiple districts (Huye, Gisagara and Kirehe), to assess the spread of resistance-associated mutations and potential risk factors. The project also emphasizes capacity building through training of local scientists and strengthening laboratory infrastructure, with the goal of supporting evidence-based malaria control and treatment policies across the region.

3.3.Study area and population

The study was conducted in Gisagara district, which is located in Southern province of Rwanda. This district was selected due to its higher malaria transmission intensity, demographic structure and accessible to healthcare making appropriate for investigating the relationship between *P. falciparum* K13 mutations and parasitemia levels.

3.3.1. Geographical and demographic characteristics

According to Rwanda population and housing census, Gisagara district has a population of approximately 397 051residents is predominantly rural with scattered settlements and limited infrastructure development.

The populations rely on subsistence agriculture, growing crops such as beans, maize, cassava, sweet potatoes and rice cultivation is also prominent, especially in the marshlands, playing a vital role in both food security and income generation. Livestock farming is widespread alongside small-scale trade in local markets.

3.3.2. Malaria burden and transmission

According to Rwanda biomedical Centre in 2023, Gisagara district experiences higher malaria burden with incidence rate of around 100 cases per 1 000 people Although malaria-related mortality has declined nationally as a result of widespread use of insecticide-treated nets and improved treatment protocol, Gisagara continues to report more severe malaria cases. This may reflect proximity to breeding sites for *Anopheles* mosquitoes, variations in access to timely treatment and the higher number of rural households with limited healthcare access.

3.3.3. Climate and seasonality

Gisagara district experience a bimodal rainfall pattern, with the long rainy season occurring from march to May and the short season from middle October to December. Annual rainfall averages between 1 200 mm and 1 600 mm and temperatures typically range from 16^{o}c to 28^{o}c. These conditions support perennial malaria transmission. Although peaks occur during the rainy seasons. Gisagara lies at a lower altitude and has slightly warmer conditions that reason its malaria transmission season tends to be longer and more intense.

3.3.4. Healthcare accessibility

Gisagara district has at least one health center per administrative sector but residents in remote areas may face long distances and transportation challenges to access the services. Community health workers play an essential role by providing frontline malaria testing and referring patients for further care.

3.4.Inclusion and exclusion criteria

3.4.1. Inclusion criteria

- Participant must be one year old or above
- Residence in study area for at least 6 months
- A positive result for malaria

3.4.2. Exclusion criteria

- Age below one year of age
- Health condition not allowing for study participation such as severe illiness due to diseases other than malaria

3.5.Sampling type and sample size determination

In this study, a proportion-based sampling approach was employed to estimate the prevalence of K13 mutations in *P. falciparum* isolates from Gisagara District. This method was chosen because the primary objective is to determine the proportion of samples

carrying specific mutations associated with antimalarial resistance. Based on existing data, it was assumed that the prevalence of K13 mutations in the target population is approximately 5%. To ensure a reliable estimate, the sample size was calculated using the formula for estimating a single proportion:

$$n = (Z^2 \cdot p \cdot (1 - p)) / E^2$$

Where:

n=required sample size

Z=Z-value corresponding to the desired confidence level (1.96 for 95%)

P= estimated proportion of the population 5%

1-p=complement of the proportion (95%)

E=margin of error (3.6%)

The resulting minimum sample size calculated was 141. However, to enhance statistical power and account for potential data loss, a total of 215 samples were collected from Gisagara district. This sample size allows for a 95% confidence interval ranging from 2.4% to 9.0%, ensuring accurate estimation of the mutation prevalence within the study population from the region.

3.6.Sample collection and laboratory procedures

Malaria positive participants were recruited through a community-based screening program conducted by trained CHWs as part of their routine activities for individuals with malaria-like symptoms. Individuals who met the study's inclusion and exclusion criteria and tested positive for *P. falciparum* using RDTs (Bioline Malaria Ag *P.f*/Pan, Abbott Diagnostics Korea Inc) at the community level were transferred to health centers for enrolment. At the health centers, written informed consent was obtained from adult participants, while parental or guardian consent and child assent were obtained for minors prior to any study procedures. Following consent, venous blood samples (5mL) were collected in EDTA tubes. By recruiting participants across multiple health centers, the study aimed to enhance sample representativeness and improve the generalizability of findings to the broader population of malaria cases in Gisagara

The blood samples were transported under triple packaging conditions to CHUB for malaria confirmation and parasitemia quantification, thick and thin blood smears were prepared and stained with 10% Giemsa stain. These smears were examined under microscopy by two qualified independent microscopists who identified and quantified parasitemia by counting the number of parasites per microliter of blood cells and

calculating parasite density based on the total WBC count following the World Health Organisation criteria(Ba et al., 2015). *P. falciparum* was distinguished from other malaria species, by examining specific morphological features, including small ring forms within red blood cells (RBCs), applique forms and distinctive crescent-shaped gametocytes. For molecular analysis, DNA was extracted from the remaining blood sample using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) following the manufacturer's protocol.

The K13 gene was amplified using polymerase chain reaction (PCR) with specific existing primers after being validated in CHUB research laboratory (forward primer: 5'CGGAGTGACCAAATCTGGGA3'and reverse primer:5'GCCTTGTTGAAAGAA CAGA3') targeting the most mutation-prone region of the K13 gene, specifically the propeller domain where mutations associated with artemisinin resistance have been observed previously (Frédéric Ariey et al., 2014).The PCR products size were 883bp. PCR amplification was performed under the following cycling conditions: an initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 94 °C for 30 sec, 40 cycles of annealing at 60 °C for 60 sec, and elongation at 72 °C for 60 sec; followed by one cycle of final elongation at 72 °C for 10 min. Amplicons were visualized by gel electrophoresis to confirm successful amplification.then analyzed by gel electrophoresis to confirm successful amplification.

These amplicons underwent capillary electrophoresis-base Sanger sequencing with BigDye™ Terminator.

3.7.Data Analysis

The obtained sequences chromatogram were inspected for quality, and low-quality bases at the ends were clipped. Sequence alignment, assembly and mutation calling were carried out using CodonCode Aligner version 12.0.1 against *P. falciparum* 3D7 reference sequence (PF3D7_1343700) obtained from PlasmoDB Single nucleotide polymorphisms (SNPs) were identified by comparing aligned sequences with the reference. Mutations were considered valid if they were present in both forward and reverse reads and supported by high-quality base calls (≥80% confidence).

The data from the study was initially entered and cleaned in Microsoft Excel. After ensuring data accuracy and completeness, the cleaned dataset was imported into a system designed in programming language "Python" for analysis and visualization using the Streamlit library. Descriptive statistics were generated to summarize the prevalence of

K13 mutations and parasitemia levels. Parasitemia levels were quantified from microscopy-positive samples and categorized as low (less than 1,000 parasitemia), moderate (between 1,000 and 9,999), or high (10,000 and above).

Cross-tabulations were performed to evaluate parasitemia distribution based on malaria species, developmental stage, age group, sex. Age was categorized into six groups (<5, 5–15, 15–25, 25–35, 35–45, >45) to assess differences in parasitemia across age categories. These categories were used in the analysis and visualized through interactive graphs in Streamlit to highlight age-related trends.

Associations between K13 mutations and parasitemia were assessed using Chi-square tests at a 95% confidence level, implemented via Python's streamlit library. Comparisons of parasitemia levels between groups with and without K13 mutations were conducted using the Mann–Whitney U test, depending on data distribution.

3.8.Ethical considerations

The ARMEA study was approved by Rwanda National Ethics Committee RNEC/548/2024 This sub-study requested approval for data use from CHUB Ethics Committee REC/CHUB/051/2025. Informed consent was obtained from all participants or their guardians before sample collection. Participants were informed that participation in the study was voluntary and they were free to withdraw from the study at any time. Confidentiality was maintained by assigning unique identification codes to each sample, and all data were securely stored. Access to the data is limited to the research team only.

3.9. Outcomes

The study provided insights into the prevalence of K13 mutations in Gisagara district and their potential impact on malaria parasitemia levels. The findings contribute to national malaria control efforts by informing treatment guidelines and resistance monitoring strategies.

CHAPTER4. PRESENTATION OF FINDINGS

This chapter outlines the key findings of a study according to its objectives. The findings are organized into four main sections; study population characteristics, parasitemia levels and plasmodium species distribution by age and sex, detection of *P. falciparum* K13 mutations and the association between K13 mutation status and parasite density.

4.1. Study population characteristics

A total of 215 individuals who tested positive for malaria using RDTs were enrolled in the study. Among them 120 were female and 95 were male. Participant ages ranged from 1 year to 75 years old, with the majority falling within 15-25 years category. Parasitemia levels and K13 mutation patterns were analysed by stratifying the data according to Age and sex to assess variations across these categories.

4.2. Parasitemia levels and plasmodium species distribution

Microscopy results for the 215 RDT-positive participants are summarized in Figure 1, showing species distribution and parasite stages observed in positive samples. Of the 215 participants, 195 (90.7%) were confirmed positive by microscopy. *P. falciparum* was the predominant species, accounting for 97.4% of infections, with trophozoites being the most frequently observed stage. Seventeen *P. falciparum* samples contained gametocytes, indicating ongoing transmission. Additionally, five samples were identified as *P. malariae,* three of which contained schizonts.

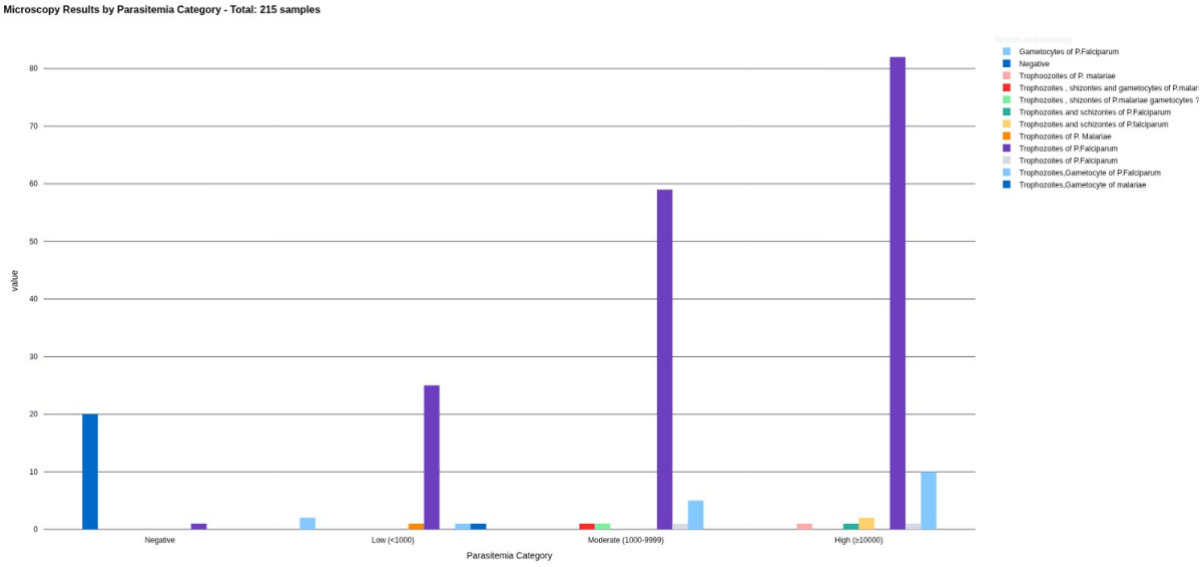

Figure 1.Microscopy results by species and parasite stage distribution. Among RDT-positive participants (n=215). The x-axis represents malaria species and parasite stages (trophozoites, gametocytes, schizonts) and their distribution in parasitemia categories , while the y-axis represents the number of samples observed. P. falciparum and P. malariae

were the species detected. Trophozoites were the most common stage observed, while gametocytes and schizonts were less frequent.

Parasitemia levels among study participants varied substantially, showing a right-skewed distribution, indicating that a small proportion of individuals harbored high parasite burdens. Figure 2 presents the distribution of parasitemia levels across age groups, highlighting higher densities among participants aged 15–25 years. Overall, 91 (46.7%) participants exhibited high parasitemia (≥10,000 parasites/µL), 73 (37.4%) had moderate parasitemia (1,000–9,999 parasites/µL), and 31 (15.9%) had low parasitemia (<1,000 parasites/µL).

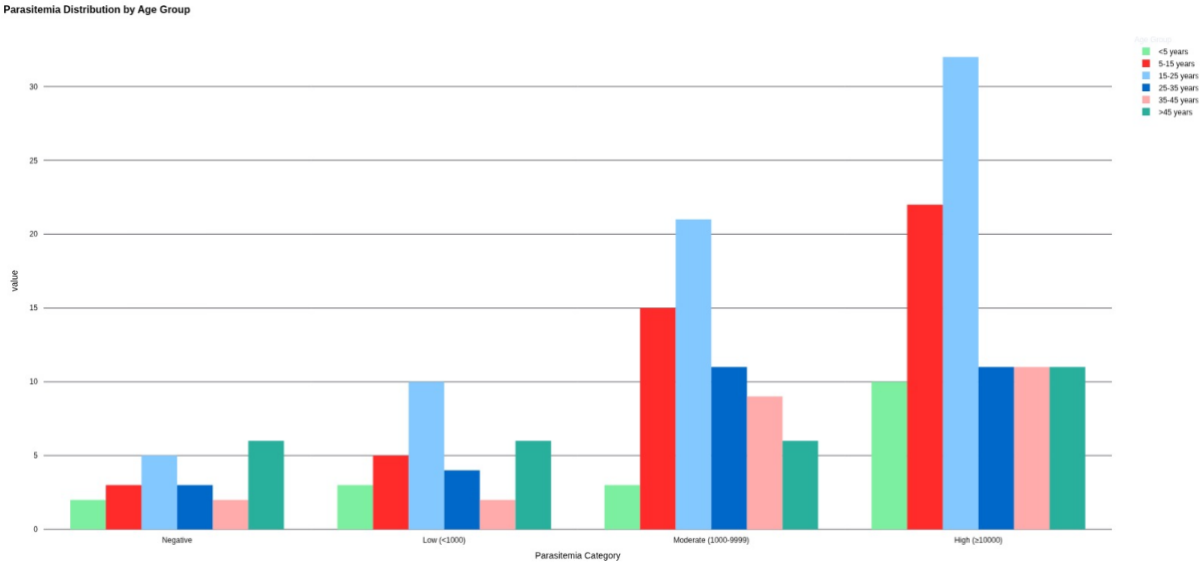

Figure 2.Distribution of parasitemia Level in age (Years) The x-axis represents both age groups (<5 years, 5-15 years,15-25 years, 25-35 years, 35-45 years, > 45 years) and parasitemia categories (negative, low <1,000 parasites/µL, moderate 1,000–9,999, high ≥10,000), and the y-axis represents the number of participants in each category

Table 1. ***Distribution of parasitemia level among study participants by Sex.*** Parasitemia categories are defined as high (≥10,000 parasites/µL), moderate (1,000–9,999 parasites/µL), and low (<1,000 parasites/µL). Slightly higher proportion of females (47.7%) than males (45.3%) fell into the high parasitemia category. A detailed breakdown of parasitemia levels by sex is presented in below Table 1.

Sex	Parasitemia			Total
	<1,000 n (%)	1,000–9,999 n (%)	≥10,000 n (%)	
Female	16(14.7)	41(37.6)	52(47.7)	109(55.9)
Male	15(17.4)	32(37.2)	39(45.3)	86(44.1)
Total	**31(15.9)**	**73(37.4)**	**91(46.7)**	**195(100)**

The mean parasite density observed was 18,777 parasites/µL, the median was 7,420 parasites/µL, reflecting the variability in parasite density across the study population. The data suggest a wide variation in parasite burden among individuals, with 46.7% of cases falling within the high parasitemia range.

4.3. Prevalence of K13 Mutations

Given the role of K13 mutations in mediating artemisinin resistance in *P. falciparum*, this section presents the prevalence of K13 gene mutations identified in the study samples.

Twelve samples failed to yield PCR amplification, 9 of them were from the samples that were negative on microscopy and 3 were likely due to very low parasite density then were excluded from sequencing. Out of 203 samples that were successfully amplified by PCR and sequenced for the K13 gene, Sequence quality was assessed using CodonCode Aligner**,** where chromatograms were visually inspected for clear, non-overlapping peaks and base calling confidence. Sequences with extensive background noise, mixed peaks, or stretches of ambiguous bases (Phred score <20) were classified as poor quality and excluded from further analysis (n=25). Re-sequencing of poor-quality samples was not possible due to budgetary constraints. As a result, only high-quality sequences were retained for mutation prevalence estimates (n=178) . The details were presentanted in below figure 3.

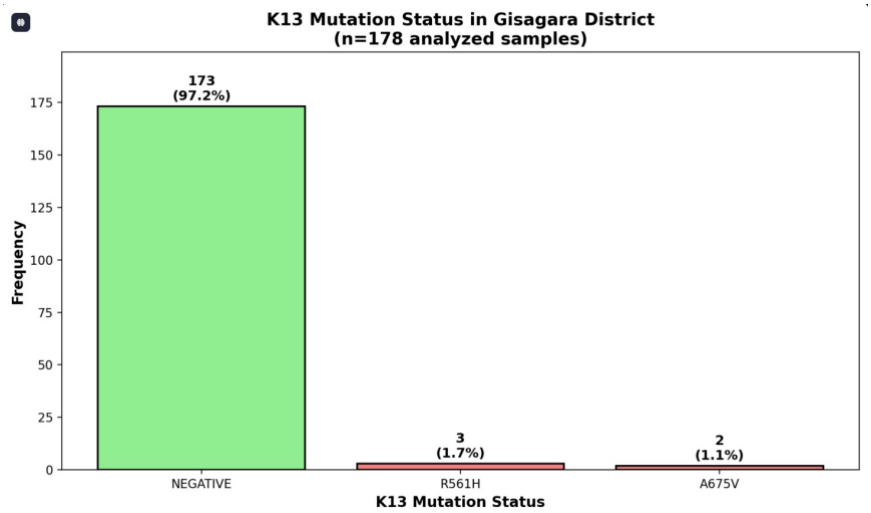

Figure 3. K13 Mutation status in Gisagara district. The overall prevalence of K13 mutations among 178 sequences analysed were 2.8% (5 mutants/178 analysed sequence). The R561H mutation was the most frequently observed at 1.7% followed by A675V at 1.1%. R561H.

To assess how specific K13 mutations relate to parasite density, parasitemia levels were compared between samples carrying the R561H and A675V alleles. The R561H mutation showed a more compact parasitemia distribution, whereas A675V exhibited a broader and more variable range of parasitemia values more details are presented below in figure 4.

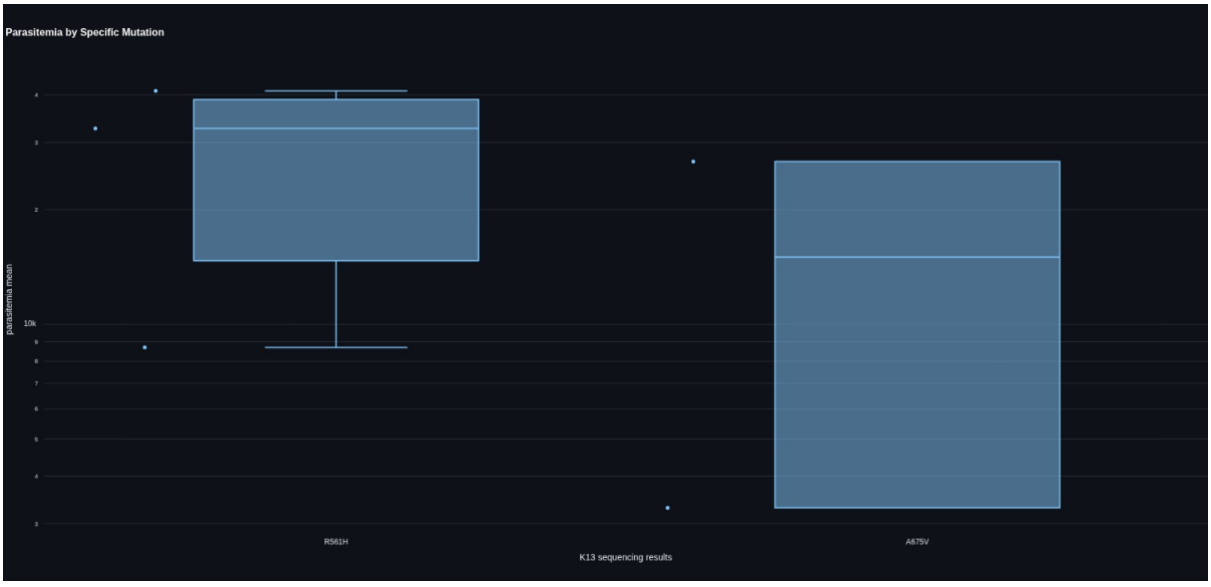

Figure 4. Distribution of parasitemia level by K13 mutation types. This figure shows parasitemia values for samples carrying the *R561H* and *A675V* mutations, with *R561H* exhibiting a compact parasitemia distribution around the median and *A675V* showing a wider spread indicating greater variability.

4.4. Association Between K13 Mutations and Parasitemia Levels

The relationship between *P. falciparum* K13 mutations and parasitemia levels was assessed. Although the number of mutant samples was small, K13 mutations were detected in samples with moderate to high parasitemia. Statistical analysis indicated that the difference in parasitemia levels between K13 mutant and wild-type isolates was not significant (p = 0.081).

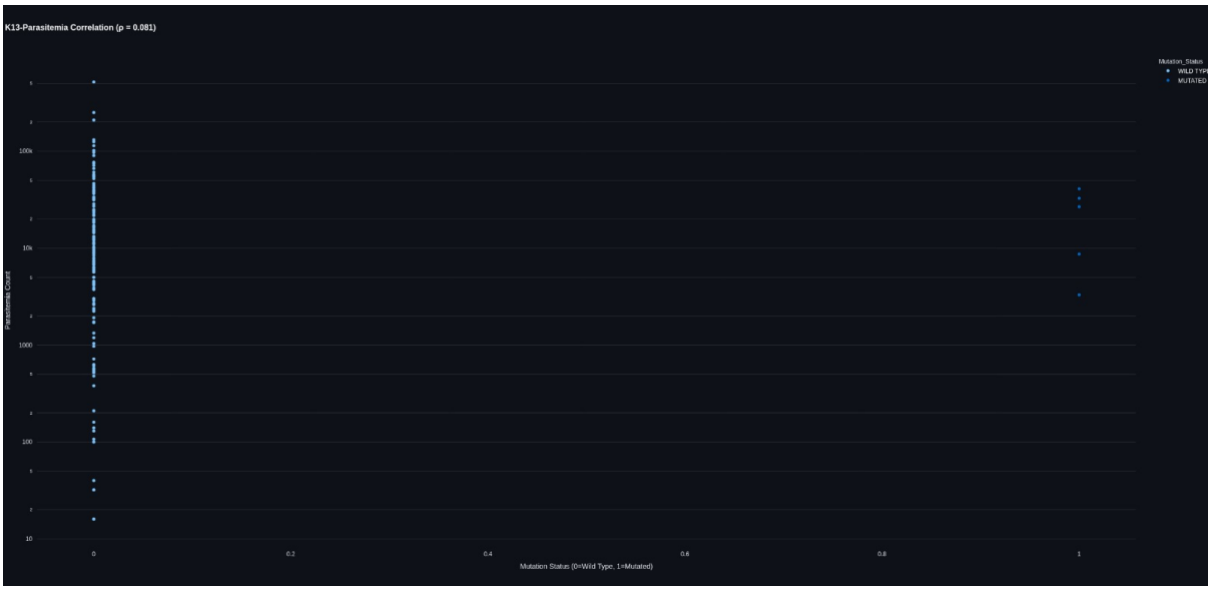

Figure 5.Association of K13 mutations and parasitemia levels. Mutant isolates were found in moderate to high parasitemia samples, while wild-type isolates were distributed across a wider range of parasitemia values. No significant difference was observed between the two groups (p = 0.081).

4.5. Summary of Key Findings

A total of 215 RDT-positive samples were collected, of which 195 were confirmed positive by microscopy. *Plasmodium falciparum* was the predominant species, identified in 97.4% of cases. Trophozoites were the most frequently observed stage (89.5%), while 8.9% of the samples presented with gametocytes and 1.6% with schizonts. Most participants had moderate to high parasitemia. Additionally, five samples were identified as *P. malariae*, and 3 of these showed schizonts. PCR amplification was successful in 94.4% samples, and sequencing revealed K13 mutations in 2.8% of the cases, with R561H being the most frequent mutation observed. Statistical analysis showed no significant association between mutation status and parasitemia level.

CHAPTER.5 DISCUSSION

This study investigated the prevalence of *P. falciparum* K13 mutations and their association with parasitemia levels among RDT-positive individuals in Gisagara District, Out of 215 RDT-positive samples 195 (90.7%) were confirmed positive by microscopy. with *P. falciparum* being the predominant species and trophozoites the most frequently observed stage. Seventeen samples were identified at the gametocyte stage, indicating ongoing transmission potential within the district(Chawla et al., 2021).Additionally, five were identified as *P. malariae*. Parasitemia levels varied, with the majority of samples falling within the high category (≥10,000 parasites/μL) consistent with the known high malaria transmission intensity of Gisagara(Kubana et al., 2023). The mean parasite density was 18,777/μL, female represented 55.9% and male 44.1%. A total of 178 samples were successfully analyzed. The overall prevalence of K13 mutations was (5 mutats /178 high-quality) 2.8%, with R561H and A675V as the identified variants, while 97.2% of samples carried wild-type alleles.

In this study, K13 mutations were detected in a small proportion of *P. falciparum* isolates, with R561H being the most frequent. These mutations have been associated with artemisinin resistance in other regions. However, no phenotypic assessments, such as Day-3 parasitemia following ACT treatment or in-vitro RSA, were performed. Therefore, the presence of these mutations should be interpreted cautiously, as their actual impact on resistance in this population is unknown. Future studies should integrate genotyping with phenotypic assays or therapeutic efficacy studies to confirm the clinical relevance of these mutations. Although samples with K13 mutations tended to have higher parasitemia, the association between mutation status and parasite density was not statistically significant (p=0.081).

Most participants showed high parasite densities (≥10,000 parasites/μL), consistent with the intense malaria transmission in Gisagara District. The distribution of parasitemia observed in this study was very common seen in malaria datasets. The mean parasite density (18,777/μL) far exceeds the median (7,420/μL), indicating a right-skewed distribution with a few individuals carrying very higher parasite burdens(Mischlinger et al., 2018; Popkin-Hall et al., 2024). Similar patterns have been observed in other high-endemic regions, where mean parasite densities exceed 10,000 parasites/μL(Andolina et al., 2021).

The presence of 17 *P. falciparum* gametocytes cases indicated the presence of sexual stages of the parasite responsible for transmission, as gametocytes are the parasite stage responsible for infecting mosquitoes This supports the persistence of active transmission in

the region(Oyibo et al., 2023).Additionally, the detection of *P. malariae* in a few cases suggests that while *P. falciparum* dominates, there is mixed infections with other species continuing to circulate at low levels. Female participants accounted for a 59.9 % slightly higher proportion of infections than male. This pattern is comparable to findings in Uganda, where women showed a higher malaria incidence, this may be related to greater exposure during evening chores activities or because women tend to seek health care more frequently leading to higher detection of case(Okiring et al., 2022). The high parasitemia in the 15-25 age group may be due to increased exposure from working in marshland and rice plantations, which provide abundant mosquito breeding habitats. Similar findings have been reported in other malaria-endemic regions, where young adults involved in agricultural activities, particularly in irrigated or swampy areas, face higher malaria risk due to prolonged outdoor exposure and limited protective measures during peak mosquito biting hours(Dolo et al., 2004).The detection of K13 mutations in *P. falciparum* isolates from Gisagara District confirms the circulation of artemisinin resistance-associated alleles even in high-transmission areas. The most commonly observed mutation was R561H which has been reported in various regions across Africa.These findings are consistent with recent reports of K13 mutations in Rwanda, particularly in high-transmission districts such as Masaka, and moderate transmission districts such as Rukara, and Huye. However, the presence of such mutations in Gisagara with relatively high malaria burden suggests that resistance markers are no longer confined to low-transmission settings (Philip J. Rosenthal et al., 2024; Talisuna et al., 2012; Arlette Umugwaneza et al., 2025). Therefore, the presence of K13 variants in Gisagara could reflect both ongoing drug pressure and potential local adaptation. The most frequently detected K13 mutation in this study, was R561H has also been reported in other African settings. In Rwanda, R561H is linked to delayed parasite clearance and has been found in Masaka and Rukara. By contrast, mutations A675V, recently reported in neighboring Huye District, have not yet been associated with resistance(Kirby et al., 2023).The detection of 2.8% in Gisagara high-transmission and previously unsampled area highlights the need for ongoing molecular surveillance to assess its clinical relevance and track potential spread.

Given that this is the first study to explore K13 prevalence in Gisagara District and its association with parasitemia, the findings offer important baseline data for future drug efficacy monitoring and resistance mapping efforts in Rwanda.

Among the mutant group, samples exhibited moderate to high parasitemia levels, with no cases falling into the low parasitemia category. In contrast, the wild-type group showed a

broader distribution, including more samples with low and moderate parasitemia and fewer with high parasite densities. However, statistical analysis revealed no significant association between mutation status and parasitemia level. These findings are consistent with previous studies from Uganda and Rwanda have identified R561H mutation in infections with moderate to high parasitemia, suggesting that some K13 variants may maintain sufficient fitness in high-transmission settings(Balikagala et al., 2021; Aline Uwimana et al., 2020).

In Corroborating this study, in Cambodia a high prevalence of K13 mutations with no strong correlation between mutation status and parasite burden was reported(Kheang et al., 2017). The relationship between mutation and parasitemia may vary depending on transmission setting and host immunity factors in high transmission areas, repeated exposure often leads to partial immunity, allowing individuals to control parasite levels. Therefore, differences in parasitemia between mutated and wild-type infections may reflect both genetic resistance and host immune response.(Amaratunga et al., 2019; Uwimana et al., 2021). In our study, conducted in the high-transmission setting of Gisagara, the observed pattern warrants further investigation to determine whether these mutations confer a selective growth advantage or reflect delayed clearance.

5.4. Strength and limitations of the study

5.4.1. Strength

The study focused on Gisagara District, a high-transmission area in Rwanda where K13 mutation data are limited. It combined both microscopy and molecular techniques (PCR and Sanger sequencing) for accurate detection of malaria and K13 mutations. Parasitemia levels were categorized, allowing exploration of their relationship with mutation status. The study also provided baseline information useful for future resistance monitoring and public health planning in Gisagara and other highly burdened settings.

5.4.2. Limitations

Parasitemia was assessed at a single time point, which limited the ability to understand dynamic parasite behavior during treatment. In addition, some sequences exhibited low-quality chromatograms, which may have slightly underestimated the true prevalence of K13 mutations. The cross-sectional design also restricted the study from making causal inferences or capturing temporal trends in the emergence of mutations.

CHAPTER 6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The current study findings provide baseline data on the prevalence of K13 mutations and their association with parasitemia in Gisagara, a district with high transmission of malaria in the Southern province of Rwanda. The detection of K13 mutations associated with artemisinin resistance underlines the importance of continuous molecular surveillance in the district. While there was no statistical significance between K13 mutations and parasitemia, the presence of moderate to high parasitemia requires vigilant monitoring to combat the spread of resistant strains.

Future research should include longitudinal follow-up, treatment outcome data, and expanded molecular analysis to further characterize the clinical and epidemiological implications of K13 mutations in high endemic areas like Gisagara. Overall, the study findings underscore the need for integrating molecular surveillance of resistant species into the national malaria control programme.

6.2. Recommendation

Based on the study outcomes, continuous molecular surveillance of *P. falciparum* should be strengthened in Gisagara District and other high-transmission areas of Rwanda. Monitoring parasitemia levels, including Day-3 parasitemia after ACT treatment, alongside K13 mutations, should be incorporated into resistance surveillance to better understand their clinical significance. Future research should adopt longitudinal designs, include treatment outcome monitoring, and integrate genotyping with phenotypic assays such as in-vitro RSA or therapeutic efficacy studies. Finally, community education and effective case management are essential to reduce malaria transmission and limit the spread of resistant strains.

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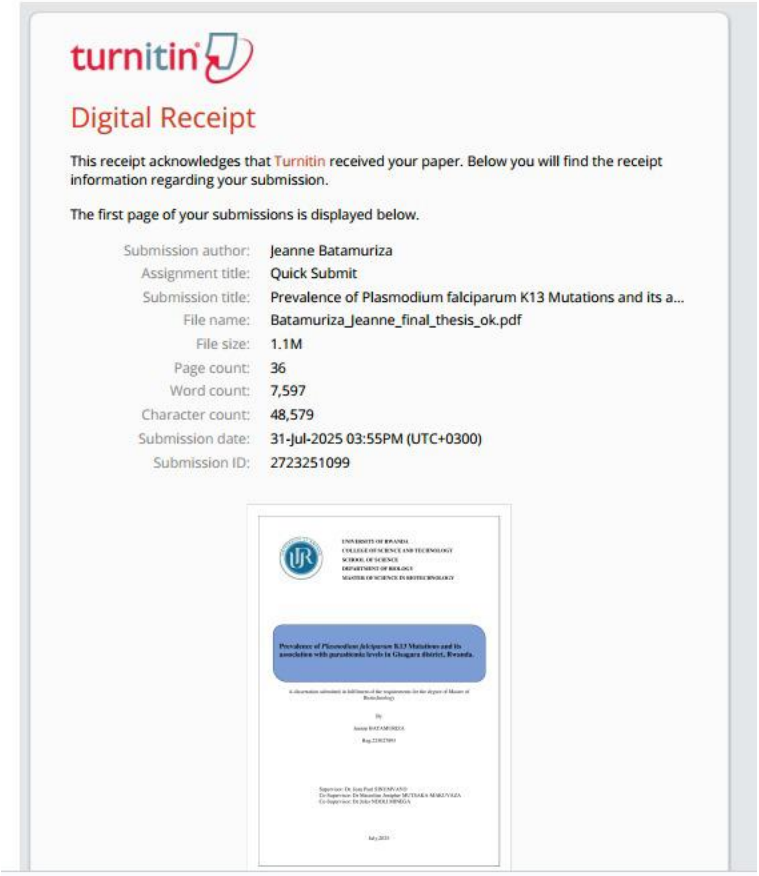
APPENDICES



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UNIVERSITY OF RWANDA
COLLEGE OF SCIENCE AND TECHNOLOGY
SCHOOL OF SCIENCE
DEPARTMENT OF BIOLOGY
MASTER OF SCIENCE IN BIOTECHNOLOGY

Prevalence of Plasmodium falciparum K13 Mutations and its association with parasitemia levels in Gisagara district, Rwanda.

Jeanne Batamuriza	Prevalence of Plasmodium falciparum K13 ...	14%		2723251099	31-Jul-2025

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CLINICAL EDUCATION AND RESEARCH DIVISION **RESEARCH**
DIRECTORATE: RESEARCH -ETHICS COMMITTEE **Huye,4th, June,2025**

Approval Notice: No: REC/CHUB/051/2025

Jeanne BATAMURIZA

Email: batamurizajeanneo4@gmail.com

Reference is made to your letter requesting for ethical clearance **"Prevalence of *Plasmodium falciparum* K13 Mutations and their Association with Parasitemia Levels in Gisagara and Huye Districts, Rwanda"** Having reviewed your application and been satisfied with your protocol and previous ethical approval related to this protocol, your study is hereby granted ethical clearance and should be conducted within University Teaching Hospital of Butare. Please note that approval of the protocol and consent form is valid for one year starting on the issue date and shall be renewed on request. You are responsible to fulfilling the following requirements:

- 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
- Only approved consent forms are to be used in the enrollment of participants
- All consent forms signed by subjects should be retained on file.
- The committee may conduct audits of all study records. Consent documentation may be part of such audits
- A continuing review application must be submitted to the committee in a timely fashion and before expiry of this approval
- Failure to submit continuing review application result in termination of study
- Notify the committee once the study is finished
- Identification of participants must be kept confidential for the duration of the study

Sincerely

Dr. HABIMANA Emmanuel
Chairperson of Ethics Committee/CHUB
Cc: - Director General
- Head of Clinical Education and Research Division
- Head of Clinical Service Division
- Director of DTS
- Head Pathology Department