UNIVERSITY OF RWANDA

GENOMIC CHARACTERIZATION OF ANTIMALARIAL DRUG RESISTANCE MARKERS IN *PLASMODIUM FALCIPARUM* USING SELECTIVE WHOLE GENOME AMPLIFICATION AND THE NOMAD NANOPORE SEQUENCING IN HUYE, GISAGARA, AND KIREHE DISTRICTS.

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By

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Kigali, Rwanda 2025

DECLARATION OF INDEPENDENT WORK

I, Laetitia IRANKUNDA, hereby declare that this research project, submitted to the University of Rwanda for the degree of Master of Science in Biotechnology, is my original work. It has not been submitted previously to any institution by myself or any other person in fulfillment of the requirements for the award of any degree or other qualification.

Laetitia IRANKUNDA| 223027923

Signature:

Dates: 21st September 2025

DEDICATION

First and foremost, I offer my deepest gratitude to Almighty God, the eternal source of wisdom and strength, whose infinite grace and guidance have faithfully sustained me throughout this academic journey. It is by His divine providence that I have been able to overcome challenges and reach this significant milestone.

I dedicate this work to the Government of Rwanda, the Enabel-EU Kwigira Project, and the BK Foundation for their generous, continuous, and invaluable support that enabled the successful completion of my master's studies. I am profoundly grateful to the Rwanda Biomedical Centre (RBC) and the ARMEA project for their essential contributions and steadfast backing throughout this research.

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Finally, I wholeheartedly dedicate this achievement to my beloved family and friends, whose unwavering love, patience, and motivation have been an enduring source of inspiration and strength throughout this endeavor.

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LIST OF SYMBOLS AND ACCRONYMS

ACT: Artemisinin-based Combination Therapy

ARMEA: Antimalarial Resistance Monitoring in East Africa

CSP: Circumsporozoite Protein

Dhfr: Dihydrofolate Reductase gene

Dhps: Dihydropteroate Synthase gene

DNA: Deoxyribonucleic Acid

K13: Kelch 13 gene (pfkelch13)

MDR1: Multidrug Resistance Protein 1 gene

MFT: Multiple First-Line Therapies

NOMAD: Nanopore Optimization and Molecular Analysis Device

PCR: Polymerase Chain Reaction

Pf: Plasmodium falciparum

Pfcrt: Plasmodium falciparum chloroquine resistance transporter gene

sWGA: Selective Whole Genome Amplification

TES: Therapeutic Efficacy Studies

WGS: Whole Genome Sequencing

WHO: World Health Organization

ABSTRACT

Malaria remains a major public health challenge, with increasing cases and emerging drug resistance threatening global control efforts. This study evaluates and compares two DNA amplification strategies selective whole genome amplification (sWGA) and MVP within the nomadic sequencing (NOMAD) nanopore sequencing protocol to enhance genomic surveillance of Plasmodium falciparum in Rwanda.

Our findings demonstrate that sWGA produces significantly higher sequencing reads compared to MVP, although it does not markedly improve genome coverage or depth. The NOMAD protocol delivers robust performance across multiple key resistance and vaccine target genes, maintaining high sequencing depth and coverage even with varying DNA input levels from clinical field samples. Molecular surveillance identified mutations in crucial resistance markers including pfkelch13, pfcrt, pfmdr1, pfdhfr, and pfdhps, providing actionable insights into regional parasite populations and drug resistance dynamics.

This work underscores the practical utility of rapid, field-deployable nanopore sequencing combined with selective amplification methods to support real-time malaria control efforts. Strategic use of sWGA should balance increased throughput benefits against higher costs and longer processing times, while MVP may suit rapid, low-bias applications. Continued optimization and nationwide scale-up of this protocol, alongside sustained molecular surveillance and functional validation of emerging mutations, will strengthen Rwanda's capacity for adaptive malaria control and elimination.

Keywords: **Malaria, *P. falciparum*, Antimalaria drug resistance, Molecular surveillance, NOMAD protocol, Nanopore sequencing**

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CHAPTER 1. INTRODUCTION

Malaria remains a global public health concern, with an estimated 263 million new cases and 597,000 deaths reported worldwide in 2023. Approximately 95% of malaria cases and fatalities are reported in the World Health Organization (WHO) African Region, reflecting major disparities in healthcare access and disease prevention (Organization, 2024). Despite significant progress since 2000, when around 2.2 billion cases and 12.7 million deaths were averted through intensified control efforts, the global malaria estimated cases increased since 2020, with a notable increase of 11 million cases from 2022 to 2023 (WHO, 2023) . Children under five, pregnant women, indigenous peoples, migrants, and people living in remote areas mostly affected, underscoring the need for more equitable access to life-saving tools and targeted interventions (WHO, 2023)(WHO, 2024a). Malaria cases in Africa is primarily driven by *Plasmodium falciparum*, the most lethal parasite species linked to severe illness and mortality (WHO, 2024c).

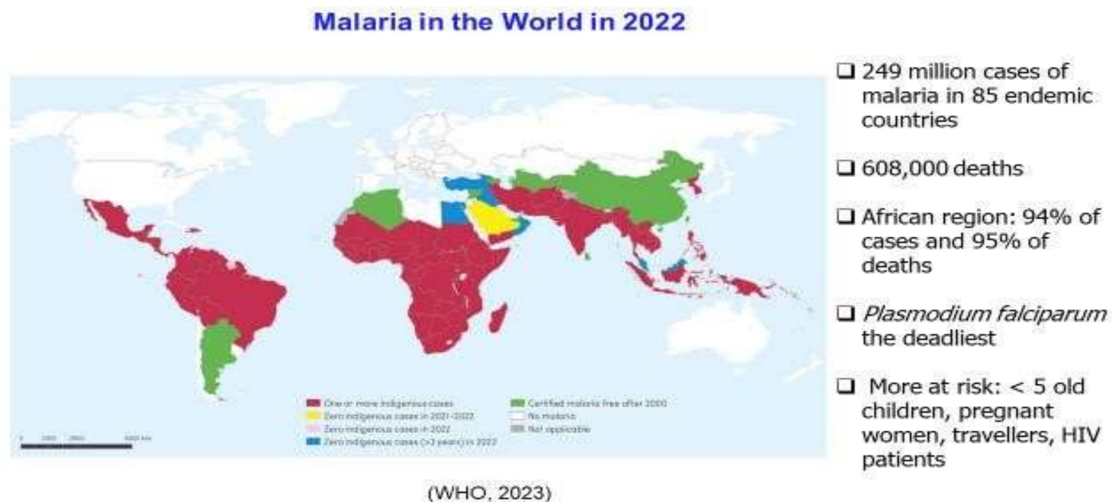

Figure 1: Prevalence of malaria across the world in 2022 (WHO, 2023)

Rwanda witnessed a substantial reduction in malaria cases, declining from approximately 4.8 million in 2016/2017 to around 620,000 in 2023/2024 an overall decrease of about 88% (Ministry of Health, 2018). However, in 2024, over 800,000 uncomplicated malaria cases were reported nationwide, marking a significant increase. Despite this progress, 2024 saw a resurgence, with over 800,000 cases of uncomplicated malaria reported nationwide. Between

January and October 2024 alone, 630,000 cases were recorded, compared to 432,000 during the same period in 2023. Notably, more than 80% of these cases were concentrated in 10 districts, including Gisagara and Kirehe, which border Burundi and Tanzania respectively, as well as Huye District, which borders Gisagara (Ministry of Health, 2018) (Agency, 2024).

Multiple factors contribute to the global resurgence of malaria, undermining the effectiveness of established prevention and control measures. These factors include limited access to quality healthcare, the effects of climate change, political instability, the emergence of insecticide resistance in mosquito populations, shifts in mosquito biting behavior, and the growing resistance to antimalarial drugs especially Artemisinin resistance (WHO, 2024d). The emergence of antimalarial drug resistance, particularly artemisinin resistance, warrants urgent attention as it directly undermines the effectiveness of malaria treatment and control efforts. Rwanda was the first African country to report *P. falciparum* mutations specifically in the *Kelch 13 (K13)* gene validated as molecular markers of partial artemisinin resistance. Since then, additional reports have emerged from several predominantly East African countries identifying either validated or candidate resistance markers. The spread of these mutations poses a serious threat to the efficacy of artemisinin-based combination therapies (ACTs), leading to prolonged infections, increased transmission, and a growing malaria burden (Uwimana et al., 2020).

Mutations in the K13 gene are associated with partial resistance to artemisinin, while mutations in genes such as *crt, mdr1, dhps,* and *dhfr* are linked to reduced susceptibility to ACT partner drugs (Siddiqui et al., 2021). In response, the World Health Organization (WHO) recommends routine monitoring of validated molecular resistance markers to complement Therapeutic Efficacy Studies (TES) (Nsanzabana, 2021). Molecular surveillance provides timely, region specific data to guide treatment policy and supports WHO's Multiple First-Line Therapies (MFT) strategy, which involves deploying two or more effective ACTs concurrently to mitigate the risk and spread of resistance (WHO, 2024b). Rwanda has already introduced key interventions to contain the spread of antimalarial resistance, including MFT and active case finding (RBC/MOH, 2024). The country also recognizes the critical role of molecular surveillance and started planning its implementation countrywide (Rwanda Ministry of Health., 2020).

Advancements in molecular technologies have significantly improved the speed and robustness of generating genetic data, making routine molecular surveillance more feasible (Nsanzabana, 2021)(Nsanzabana et al., 2018).

While traditional methods like Sanger sequencing remain the gold standard for accurately detecting known antimalarial resistance mutations, they are time-consuming and less sensitive in low parasite density samples. To overcome these limitations, innovative tools such as the Nomad protocol a portable, rapid, and cost-effective nanopore sequencing method and selective whole genome amplification (sWGA) have been introduced. These technologies enable comprehensive and timely detection of genetic markers not only related to drug resistance (*pfk13, pfcrt, pfmdr1, pfdhfr, pfdhps*), but also those associated with vaccine targets (*pfama1, pfcsp*) and diagnostic markers (*hrp2/3*) (Oyola et al., 2016) (de Cesare et al., 2024). These molecular approaches enhance the capacity of national malaria programs to monitor resistance evolution and refine malaria management strategies in real time.

Despite the growing recognition of molecular surveillance in guiding treatment policy and supporting the implementation of MFT, Rwanda is yet to integrate it routinely into its malaria control program. Furthermore, the geographic distribution of validated antimalarial resistance markers remains poorly characterized at regional and district levels.

This study, therefore, aimed to optimize and validate the Nomad nanopore sequencing protocol combined with selective whole genome amplification for effective molecular surveillance of *P. falciparum* resistance markers. It also mapped the distribution of *pfk13, pfcrt, pfmdr1, pfdhfr,* and *pfdhps* in clinical samples collected from Gisagara, Kirehe, and Huye districts.

General Objectives

To optimize and validate the NOMAD nanopore sequencing protocol combined with Selective Whole Genome Amplification for effective molecular surveillance of *Plasmodium falciparum* resistance markers.

Specifics Objectives

1- Evaluate the performance of the NOMAD nanopore sequencing protocol across clinical samples with Amplification results and depth coverage and by comparing the SWGA and MVP based on Concentration and depth coverage.

2- Determine the prevalence and geographic distribution of key *Plasmodium falciparum* antimalarial drug resistance markers in clinical samples collected from Gisagara Kirehe, and Huye districts, using the NOMAD sequencing protocol.

3- Leveraging artificial intelligence (AI) to compare K13-Propeller domain and corresponding mutant of interest.

CHAPTER 2. LITERATURE REVIEW

2.1. Malaria Burden and Antimalarial Drug Resistance in Rwanda

Plasmodium falciparum is a unicellular protozoan parasite and the deadliest species causing malaria in humans. It is transmitted through the bite of an infected female Anopheles mosquito. The parasite has a complex life cycle involving liver and red blood cell stages in humans, where it multiplies and causes the clinical symptoms of malaria. P. falciparum is known for its ability to modify the surface of infected red blood cells, promoting adhesion to blood vessel walls, which can lead to severe complications like cerebral malaria. This parasite is responsible for the majority of malaria-related deaths, particularly in sub-Saharan Africa, making it a major target for disease control and elimination efforts (Das et al., 2022) (Kale et al., 2024).

Malaria cases have increased in Rwanda since 2024, with a high burden in districts including Huye, Gisagara, and Kirehe. This resurgence is driven by environmental challenges, changes in mosquito behavior, and the spread of Plasmodium falciparum resistance to artemisinin-based combination therapies (ACTs). Addressing these complex factors is essential for designing effective and sustainable malaria control interventions (WHO, 2024d).

Antimalarial drug resistance remains a critical challenge in malaria control efforts, particularly in Rwanda and the broader East African region. The molecular basis of resistance involves several key genes that influence the parasite's susceptibility to commonly used drugs. Among these, the *pfcrt* gene, encoding a transporter protein in the parasite's digestive vacuole membrane, harbors mutations such as K76T that disrupt drug accumulation to confer chloroquine resistance (Silva et al., 2022) (Schallenberg et al., 2025).
The pfmdr1 gene, encoding a P-glycoprotein homolog, contains mutations (e.g: N86Y and Y184F) and gene copy variations that modulate resistance to multiple antimalarials, including components of artemisinin-based combination therapies (ACTs) (Li et al., 2014). The *pfdhfr* and *pfdhps* genes code for enzymes targeted by sulfadoxine-pyrimethamine (SP), and mutations in these loci (such as N51I and S108N in *pfdhfr*, and A437G and K540E in *pfdhps*) reduce drug binding efficacy, undermining SP treatment and prevention strategies (Kateera et al., 2016). Particular significance is the *pfk13* gene, which encodes the *Kelch13* protein

containing BTB and Kelch-repeat propeller (KREP) domains involved in ubiquitin-dependent protein degradation and parasite hemoglobin uptake through the cytostome. Mutations within the *K13* propeller domain, such as the prevalent C580Y and notably the R561H mutation first identified in Rwanda, impair hemoglobin endocytosis, limiting heme availability required to activate artemisinin inside the parasite and thereby conferring artemisinin resistance characterized by delayed parasite clearance and treatment failure (Yan et al., 2022) (Tutor et al., 2023). The R561H mutation, initially detected around 2014 in the Masaka area, has increased in prevalence and is linked to delayed clearance of parasites following ACT treatment, representing the earliest confirmed marker of artemisinin partial resistance in Africa with a local origin in Rwanda (Uwimana et al., 2021a).

Additionally, increasing prevalence of *pfmdr1* alleles linked to reduced sensitivity against ACT partner drugs like lumefantrine poses further threats to treatment efficacy (Silva et al., 2022). Local studies stress the importance of these molecular markers for predicting therapeutic outcomes and optimizing malaria control strategies. Therefore, a comprehensive understanding of these genetic resistance mechanisms, especially the functional implications of *Kelch13* mutations such as R561H, is essential for tailoring effective malaria treatment policies and sustaining progress in malaria control in the region.

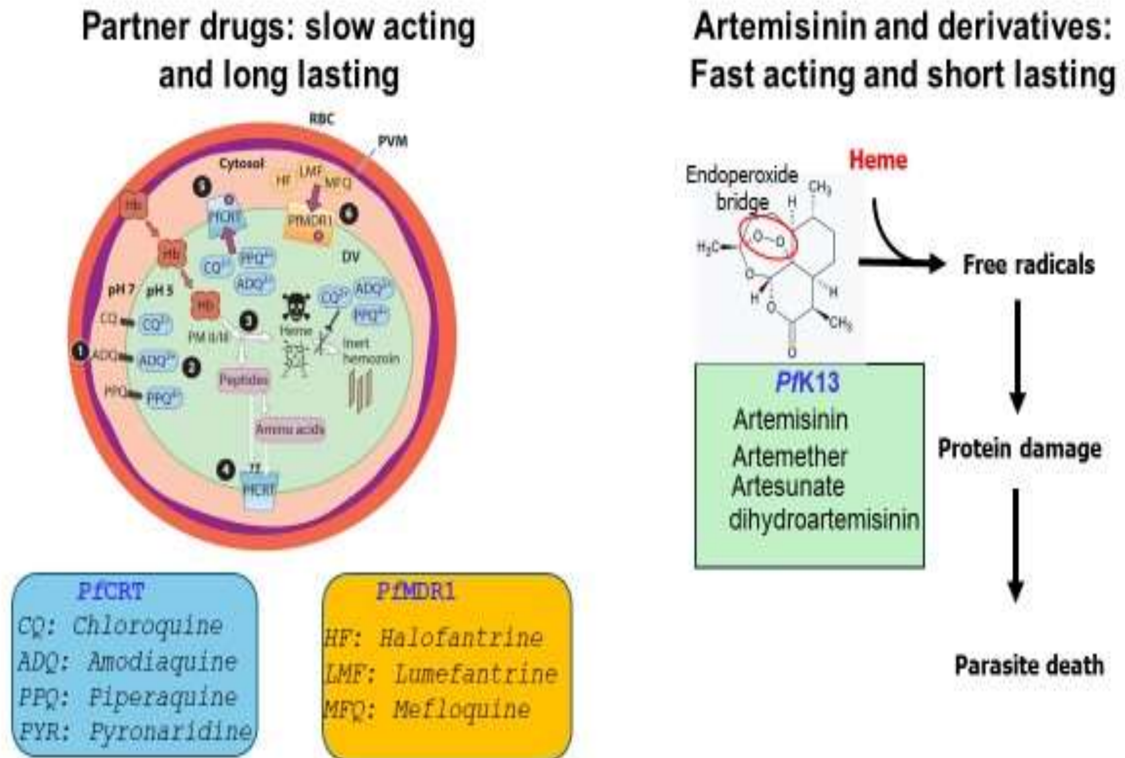

Figure 2: Mechanisms of action and resistance to ACT drugs

2.2. Genomic Surveillance of Plasmodium Falciparum Drug Resistance

2.2.1. Importance of Genetic and Genomic Methods for Monitoring Resistance

Genomic surveillance plays a pivotal role in malaria control and elimination efforts by enabling the early detection, tracking, and characterization of drug-resistant *Plasmodium falciparum* populations. Molecular detection of resistance markers informs treatment policies, helps avert widespread therapeutic failures, and guides public health interventions to contain or prevent the spread of resistant strains (Tang et al., 2021). Genetic monitoring supports understanding parasite population structure, transmission dynamics, and adaptive evolution under drug pressure, crucial for anticipating resistance emergence and dissemination.

Traditional molecular surveillance methods for monitoring drug resistance in *Plasmodium falciparum* primarily involve polymerase chain reaction (PCR)-based assays targeting specific known resistance mutations and Sanger sequencing, which allows targeted genotyping of candidate gene loci such as *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps*, and *pfk13*. These approaches are highly sensitive and cost-effective for detecting known single nucleotide polymorphisms (SNPs) and small insertions or deletions within pre-identified resistance genes (Tang et al., 2021). However, they have notable limitations. The methods are inherently limited to a targeted scope, meaning they only assay predefined mutations and therefore cannot detect novel or rare variants outside the known loci. Additionally, these conventional techniques have low multiplexing capacity, typically analyzing only a few loci per run, which restricts throughput. PCR-based methods are also sensitive to parasite density, often failing to amplify DNA successfully when parasitemia is low, as seen in asymptomatic or post-treatment cases. Furthermore, they provide limited phylogenetic and population genomic insights, as their focus on specific loci yields minimal data on genome wide variation or evolutionary selection dynamics. This restricts their utility in understanding the broader genomic landscape of resistance and hampers comprehensive surveillance efforts (Park et al., 2012).

Next-generation sequencing (NGS) technologies have revolutionized malaria research by enabling rapid, cost-effective, and high-throughput analysis of parasite genomes, facilitating detailed insights into genetic diversity and drug resistance mechanisms. Among these, whole genome sequencing (WGS) provides an unbiased, high-resolution approach to interrogate the entire *Plasmodium falciparum* genome, delivering comprehensive information on known and novel resistance mutations, copy number variations, structural variants, and genome-wide signatures of selection and adaptation (Park et al., 2012) (Cowell et al., 2018).

WGS plays a crucial role in surveillance by elucidating the evolutionary trajectories of resistance genes and identifying genetic backgrounds that influence drug sensitivity. Its advantages include genome-wide coverage that allows simultaneous interrogation of all genomic loci for mutations and haplotypes, discovery potential to detect emerging resistance markers before clinical treatment failures are observed, and the ability to provide population

genomic insights into parasite gene flow, population structure, and transmission bottlenecks. Furthermore, WGS enables multiplex pathogen detection, allowing differentiation of mixed infections and coinfections with other Plasmodium species. However, sequencing parasite DNA from low-density or clinical samples presents significant challenges (Kunasol et al., 2022). Low parasite DNA abundance, especially in asymptomatic or partially treated patients, results in insufficient material for standard WGS library preparation. High levels of host (human) DNA contamination in blood samples complicate parasite genome enrichment and increase sequencing noise and costs (Auburn et al., 2012).

Additionally, sample quality and storage conditions affect sequencing success rates, particularly in resource-limited endemic settings (Schwartz et al., 2015). The high cost and bioinformatics requirements further limit widespread access to WGS technology (de Cesare et al., 2024). Complexity arising from mixed-strain infections complicates data interpretation and allele phasing (Zhu et al., 2019). To mitigate these obstacles, enrichment methods such as selective whole genome amplification (sWGA), targeted genome capture, and optimized sample processing protocols are employed to enhance parasite DNA yield and sequencing coverage from clinical specimens (de Cesare et al., 2024). Continuous improvements in sequencing platforms, informatics pipelines, and cost reductions are progressively increasing the feasibility of integrating WGS into routine malaria resistance surveillance programs.

2.3. Selective Whole Genome Amplification and Nanopore Sequencing Technologies

Selective Whole Genome Amplification (SWGA) is a targeted molecular method that enriches *Plasmodium falciparum* DNA from samples with high human DNA contamination, such as dried blood spots with low parasite levels. It uses custom primers that preferentially bind the parasite's highly AT-rich genome over the human genome, enabling exponential amplification of parasite DNA by phi29 polymerase while minimizing host DNA. This process can boost the proportion of parasite DNA reads from under 1% to over 70% in sequencing libraries, even from limited or low-parasite samples. SWGA bypasses the need for large blood volumes or leukocyte depletion, making whole genome sequencing feasible for many clinical samples that would otherwise fail due to overwhelming host contamination

or low parasite density. This technique has significantly advanced genomic surveillance capacity in malaria research (Oyola et al., 2016) (Coonahan et al., 2023).

Nanopore sequencing technology, developed by Oxford Nanopore Technologies, represents a transformative advancement in DNA sequencing characterized by its portability, real-time data acquisition, and scalability. The technology employs protein nanopores embedded in a membrane through which native DNA strands are passed electrically. Changes in ionic current as the DNA translocates through the pore are interpreted to read the nucleotide sequence. The NOMAD platform builds on this technology by providing a user-friendly, portable sequencing setup that integrates field-friendly sample preparation, rapid sequencing, and real-time bioinformatics analysis, all in a compact device. This allows malaria researchers and public health workers to perform genomic surveillance, including sequencing of *P. falciparum* DNA, directly in endemic field settings without requiring extensive laboratory infrastructure. Key benefits include rapid turnaround times facilitating near-immediate decision making, elimination of cold chain dependence, and the potential for large-scale deployment in remote or resource-limited regions (de Cesare et al., 2024)(Holzschuh et al., 2024).

The combination of Selective Whole Genome Amplification (SWGA) and Nanopore sequencing effectively overcomes key challenges in malaria genomic surveillance by enriching *Plasmodium falciparum* DNA from low-density clinical samples and enabling rapid, portable sequencing in field settings. SWGA reduces human DNA contamination and boosts parasite DNA yield, while Nanopore technology provides real-time, flexible sequencing without the need for extensive laboratory infrastructure. This integrated approach allows for scalable, sensitive detection of drug resistance mutations and supports timely epidemiological responses to emerging resistance in real-world conditions (de Cesare et al., 2024)(De Meulenaere et al., 2024).

Examples of successful applications of Selective Whole Genome Amplification (SWGA) combined with Nanopore sequencing in malaria research include studies in endemic regions such as Ghana and Mozambique. These studies utilized SWGA to enrich *Plasmodium falciparum* DNA from dried blood spots, followed by Nanopore sequencing to profile drug

resistance markers and population structure effectively. Adaptive sampling on Nanopore devices has further enhanced the proportion of parasite reads from clinical samples with human DNA contamination, achieving 3- to 6-fold enrichment. This integration enables robust reconstruction of parasite genomes at sufficient coverage, demonstrating reproducibility, practical feasibility, and significant epidemiological value for malaria genomic surveillance in field and clinical settings (Coonahan et al., 2023) (Holzschuh et al., 2024)(De Meulenaere et al., 2024).

There is a notable gap in detailed genomic surveillance data on *Plasmodium falciparum* drug resistance in the Huye, Gisagara, and Kirehe districts of Rwanda, limiting the ability of local malaria control programs to effectively track evolving resistance mutations and parasite population dynamics. While national efforts monitor antimalarial resistance broadly, these districts each with distinct transmission intensities lack fine-scale genomic data necessary to understand localized resistance patterns and genetic diversity. Generating high-resolution, district-level genomic profiles is critical for guiding appropriate treatment policies, anticipating the spread of resistant strains, and implementing targeted interventions to improve malaria control outcomes in these regions (Schallenberg et al., 2025) .

The use of Selective Whole Genome Amplification (SWGA) combined with the portable NOMAD Nanopore sequencing platform is particularly justified for these settings, where low parasite densities and high human DNA contamination in clinical samples pose technical challenges to standard sequencing methods. SWGA selectively enriches *P. falciparum* DNA from contaminated samples, while NOMAD enables rapid, real-time whole genome sequencing in decentralized, resource-limited environments without requiring advanced laboratory infrastructure (Oyola et al., 2016).

This integrated approach can significantly enhance the sensitivity and scalability of genomic surveillance, providing timely and actionable data on both known and novel resistance mutations.

Consequently, this study’s effective application of Selective Whole Genome Amplification (SWGA) combined with NOMAD Nanopore sequencing is well-positioned to enhance the precision of drug resistance monitoring across Rwanda.

CHAPTER 3. METHODOLOGY

3.1. Study Design

This was a cross-sectional molecular surveillance study. The NOMAD nanopore sequencing protocol combined with selective whole genome amplification were optimized and validated for characterizing *Plasmodium falciparum* antimalarial resistance markers. Samples were stratified by parasitemia level to compare NOMAD nanopore sequencing performance across different parasite densities. The study also assessed the prevalence and distribution of antimalarial resistance markers in these regions.

3.2. Study Area

This study included three *Plasmodium falciparum*-positive clinical samples collected between October 2024 and May 2025 from three Rwandan districts: Gisagara, Huye, and Kirehe. Gisagara and Huye are located in the Southern Province of Rwanda, with Gisagara bordering Burundi, while Kirehe lies in the Eastern Province, sharing a border with Tanzania. These districts were selected based on their high malaria burden and differing antimalarial treatment regimens.

3.3. Study Population

The study population consisted of individuals aged over 1 year from Gisagara, Huye, and Kirehe districts who had confirmed malaria diagnoses by microscopy at local health centers. Eligible participants were randomly selected, and only those who provided informed consent for participation and agreed to the use of their samples for future research were included in the study.

3.4. Inclusion criteria

Participants were included if 1) their age is above 1, 2) residing in in Gisagara, Huye, and Kirehe districts during the study period, 3) Confirmed *Plasmodium falciparum* infection by microscopy at a local health facility, 4) Diagnosis of uncomplicated malaria, including cases

with low parasite density, 5) provided written informed consent (or assent with parental/guardian consent, and 6) Willingness to comply with study procedures and attend follow-up visits if required.

3.5. Exclusion criteria

Samples were excluded from analysis if individuals had not provided consent for their use in research. Additionally, samples with insufficient volume or that were visibly hemolysed were excluded from further molecular analysis.

3.6. Sample size

Based on the prevalence of K13 mutations in Plasmodium falciparum in southern province, the sample size was calculated using the formula with an expected prevalence (p) of 13%, a 95% confidence level (Z = 1.96), and a 5% margin of error (d = 0.05). This yielded a minimum sample size of 174, ensuring sufficient precision for molecular characterization of resistance markers.

$$n = \frac{Z^2 \times p \times (1 - p)}{d^2}$$

3.7. Sampling Strategy and Data Collection Procedures

A multistage cluster sampling approach was implemented in Gisagara, Huye, and Kirehe districts by selecting health facilities or communities as clusters. Within each cluster, malaria-positive individuals confirmed by rapid diagnostic tests (RDTs) were randomly sampled, with sample sizes allocated proportionally to the malaria burden. Both whole blood and dried blood spot (DBS) samples were collected from participants at the selected health facilities; however, this study utilized only whole blood samples for molecular analysis, while DBS samples were reserved as backup and stored for potential future experiments. Whole blood was obtained via venipuncture, stored at 2–8°C, and transported under cold chain conditions (2–8°C) to maintain sample quality. Samples collected in Gisagara and Huye were sent to the Centre Hospitalier Universitaire de Butare (CHUB) for DNA extraction, after which DNA

extracts were transferred and stored at –20°C at the National Reference Laboratory. This sampling and processing strategy ensured high-quality DNA suitable for comprehensive molecular characterization of *Plasmodium falciparum* resistance genes using selective whole genome amplification, providing a representative resistance profile for the study areas.

3.8. Laboratory analysis

3.8.1. DNA Extraction

The DNA extraction from whole blood was performed using QIAamp DNA Mini Kit. It involves lysing blood cells with protease and lysis buffer, binding the genomic DNA to a silica spin column membrane, washing to remove contaminants, and eluting purified DNA ready for downstream molecular applications. Starting with 200 µL of whole blood, protease and AL lysis buffer are added and incubated at 56°C to release DNA. Ethanol is then added to facilitate DNA binding to the silica membrane during centrifugation. Subsequent wash steps with AW1 and AW2 buffers remove impurities, and DNA is finally eluted in AE buffer at room temperature after incubation and centrifugation. The protocol requires standard molecular biology equipment including a thermomixer, centrifuge, and pipettes, and special care is taken to avoid contamination by cleaning workspaces and equipment with DNA-away solutions prior to processing. Extracted DNA was stored at –20°C. This method provides high-quality genomic DNA efficiently and reliably from whole blood samples for molecular characterization of pathogens.

3.8.2 Targeted PCR and sWGA Amplification

Selective whole genome amplification (sWGA) was performed on a subset of samples to enrich *Plasmodium falciparum* DNA prior to targeted molecular analysis. The sWGA primers preferentially amplify *P. falciparum* genomic sequences through incubation at 45°C for 60 minutes, followed by dilution of the amplified products. Targeted PCR was subsequently carried out using MVP primers at a working concentration of 10 µM, which amplify key resistance and diagnostic genes including *pfama1*, *pfcrt*, *pfcsp*, *pfdhfr*, *pfdhps*, *hrp2/3*, *pfk13*, and *pfmdr1*. PCR reactions were performed under established conditions using Kapa Readymix with either sWGA enriched DNA or directly extracted DNA. Amplification

success was verified by agarose gel electrophoresis using a 2% agarose gel prepared in 1x TAE buffer with SYBR Safe stain. Negative controls (water only) were included in each PCR run to monitor contamination, and DNA bands were visualized under UV light.

3.8.3 Library Preparation and Sequencing

The post-PCR clean-up process uses AMPure XP beads to purify PCR products by selectively binding DNA and removing contaminants such as primers and enzymes. After bringing the beads to room temperature and thoroughly mixing, a 0.5X bead-to-sample ratio of AMPure XP beads is added to the PCR product and incubated at room temperature. The bead-DNA complexes are then immobilized on a magnetic rack, the supernatant is carefully removed while retaining a small volume, and the beads are washed twice with freshly prepared 80% ethanol to eliminate impurities. Following thorough removal of ethanol and air drying, DNA is eluted from the beads using nuclease-free water. The purified DNA is quantified using the Qubit assay according to standard protocols. For multiplex sequencing, DNA fragments were end-repaired and ligated to sequencing adapters using a ligation kit. After adapter ligation, the samples were pooled and underwent another AMPure XP bead clean-up [17]. The pooled library was eluted, quantified again, and then loaded onto the flow cell for sequencing.

3.9. Data analysis

Raw sequencing data in the form of FAST5 files were generated using the Oxford Nanopore GridION platform. These raw files were basecalled to produce FASTQ sequence files for downstream analysis. Quality control was performed to filter low-quality reads and ensure sequence accuracy. The reads were aligned to the *Plasmodium falciparum* reference genome, and mutations within the eight target genes (*pfama1*, *pfcrt*, *pfcsp*, *pfdhfr*, *pfdhps*, hrp2/3, *pfk13*, and *pfmdr1*) were identified. Data organization, cleaning, and statistical analyses exploring mutation patterns and associations with drug resistance were conducted using R. To address the impact of SNPs of interest on the structural feature and function of K13 AlphaFold 3 server was employed.

3.10. Ethical consideration

We obtained ethical clearance under the ARMEA project, which mandates conducting research on antimalarial resistance. This clearance covered this study activity as part of the broader ARMEA initiative focused on monitoring and understanding artemisinin resistance in *Plasmodium falciparum* across East Africa. This study exclusively genotyped *Plasmodium falciparum* for the purpose of molecular surveillance; no human genomic analysis was performed on participant samples.

CHAPTER IV. RESULTS

A total of 175 clinical samples were collected from Gisagara, Huye, and Kirehe districts. DNA extraction and NOMAD sequencing were performed, however, only 34 samples have been fully processed and are available for inclusion in the current results. The remaining samples are still undergoing bioinformatics analysis. Of the 34 samples analyzed in this report, 24 were collected from Kirehe District and 10 from Huye District. This study focuses on evaluating the performance of NOMAD nanopore sequencing protocol and assessing the frequencies of key *Plasmodium falciparum* resistance markers in *pfkelch13 (K13), pfmdr1, pfcrt, pfdhfr,* and *pfdhps* genes.

4.1. Gel Electrophoresis

The PCR amplification of the target DNA was followed by gel electrophoresis to separate and visualize the resulting products. The image shown depicts the gel from our lab work, where the presence of multiple bands indicates that multiple primers targeted different regions of the DNA. Gel electrophoresis was essential for confirming the success of the amplification, estimating fragment sizes, and verifying the specificity and purity of the PCR products.

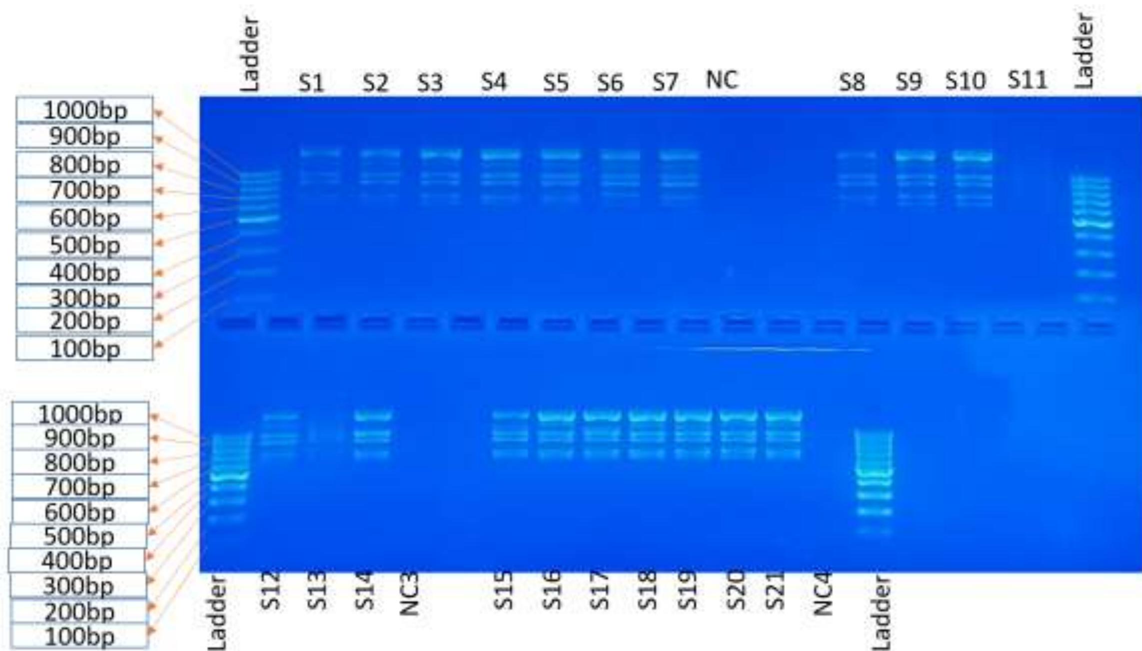

Figure 3: Results PCR Gel Electrophoresis

4.2. Comparison of Nomadic Sequencing Performance by DNA amplification strategy

We evaluated the performance of the nomadic sequencing protocol using two DNA amplification strategies: sWGA and MVP. Key metrics read count, mean sequencing depth, and genome coverage were compared across matched samples (figure 5). sWGA yielded significantly more sequencing reads than MVP (mean difference = 1,018.2; 95% CI: 27.8–2,008.7; $t(9) = 2.33$; $p = 0.045$), suggesting higher output. However, no significant difference was observed in mean sequencing depth (mean difference = 266.8; 95% CI: -113.5–647.1; $t(9) = 1.59$; $p = 0.147$). Similarly, genome coverage did not differ significantly between protocols ($V = 45$; $p = 0.084$), although a non-significant trend favored sWGA.

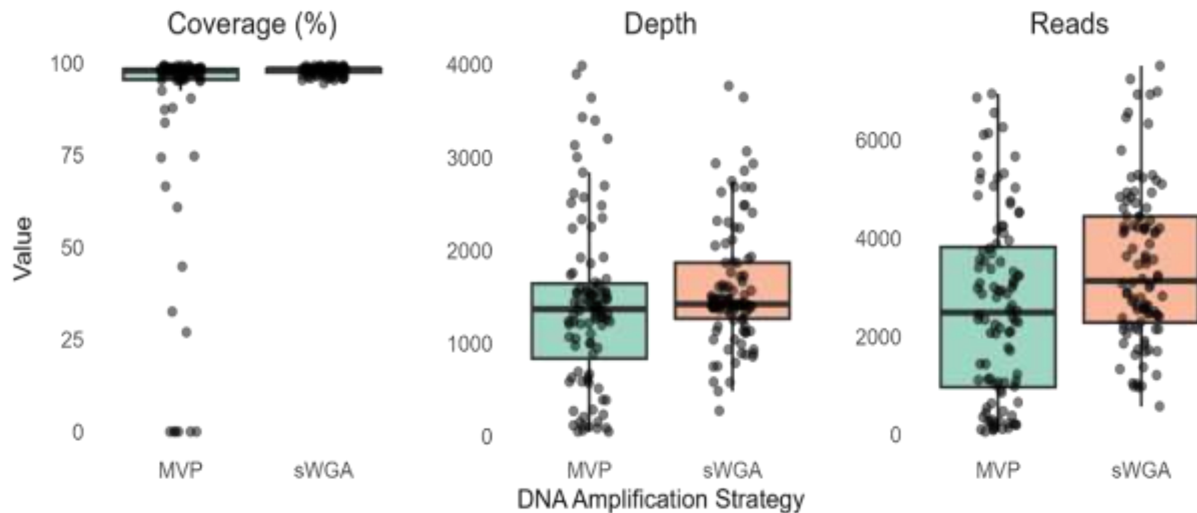

Figure 4: Distribution of coverage, reads, and depth by DNA Amplification Strategy

4.3. Sequencing Depth Distribution Across Genes

Sequencing depth, also called read depth, is the average number of times each nucleotide in the genome is sequenced, reflecting how many reads cover a given base. It ensures greater accuracy in identifying genetic variants by providing redundant observations of the same genomic position. We first assessed the sequencing depth distribution per gene using boxplots

(Figure 5). Most genes have a median depth above 2,000, indicating strong overall coverage. CSP (NANP Repeats) shows the most consistent (least variable) depth, while MDR1 (10K–12.6K) and PK13 (C-terminal) display the most variation and several high-depth outliers, suggesting inconsistent coverage. Overall, the sequencing appears robust but with variability in a few gene regions that might benefit from further optimization.

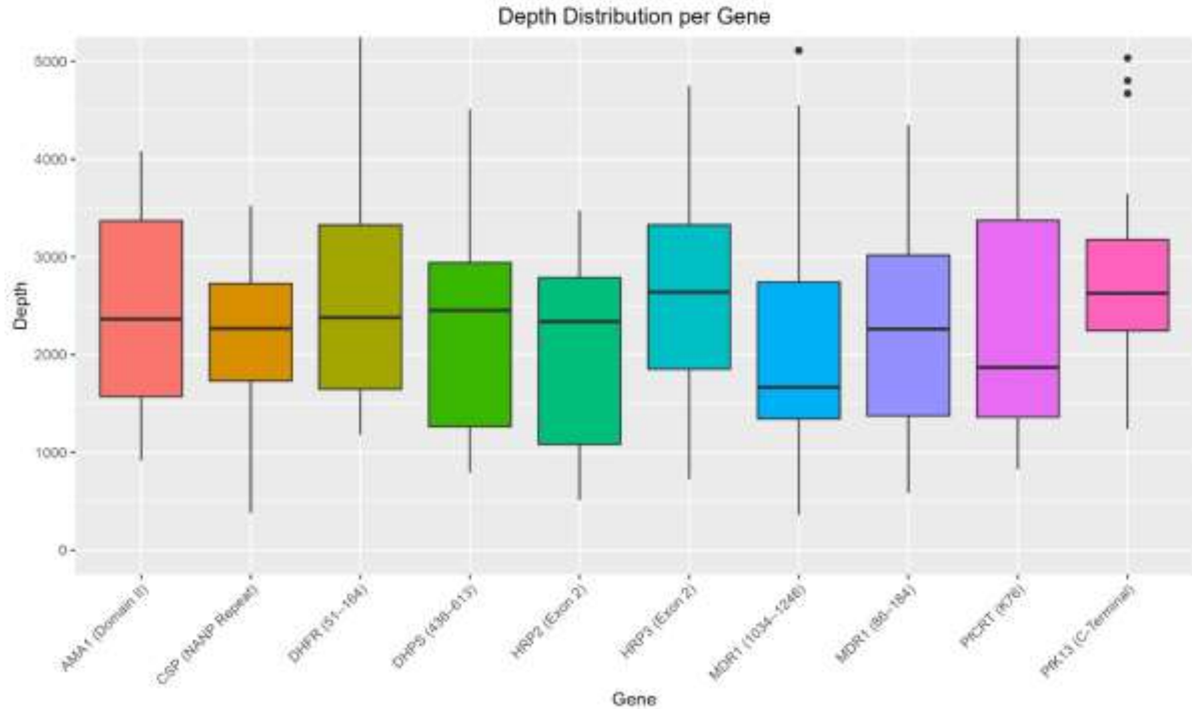

Figure 5: Boxplots showing the distribution of sequencing depth for each target gene across clinical samples.

4.4. Sequencing Depth Profiles Across Genes and DNA Input Levels

Variation in sequencing depth across genes and DNA concentrations showed generally robust performance, with most genes maintaining average depths above 1,000 reads regardless of input concentration. Higher DNA concentrations were associated with increased depth, particularly for genes such as *kelch13* (C-terminal), *dhps*, and *hrp2*, which consistently exceeded 3,000 reads. In contrast, genes like *hrp3*, *dhfr*, and *mdr1* exhibited greater variability, with markedly reduced depths at lower concentrations, occasionally nearing zero or resulting in missing data (figure 6).

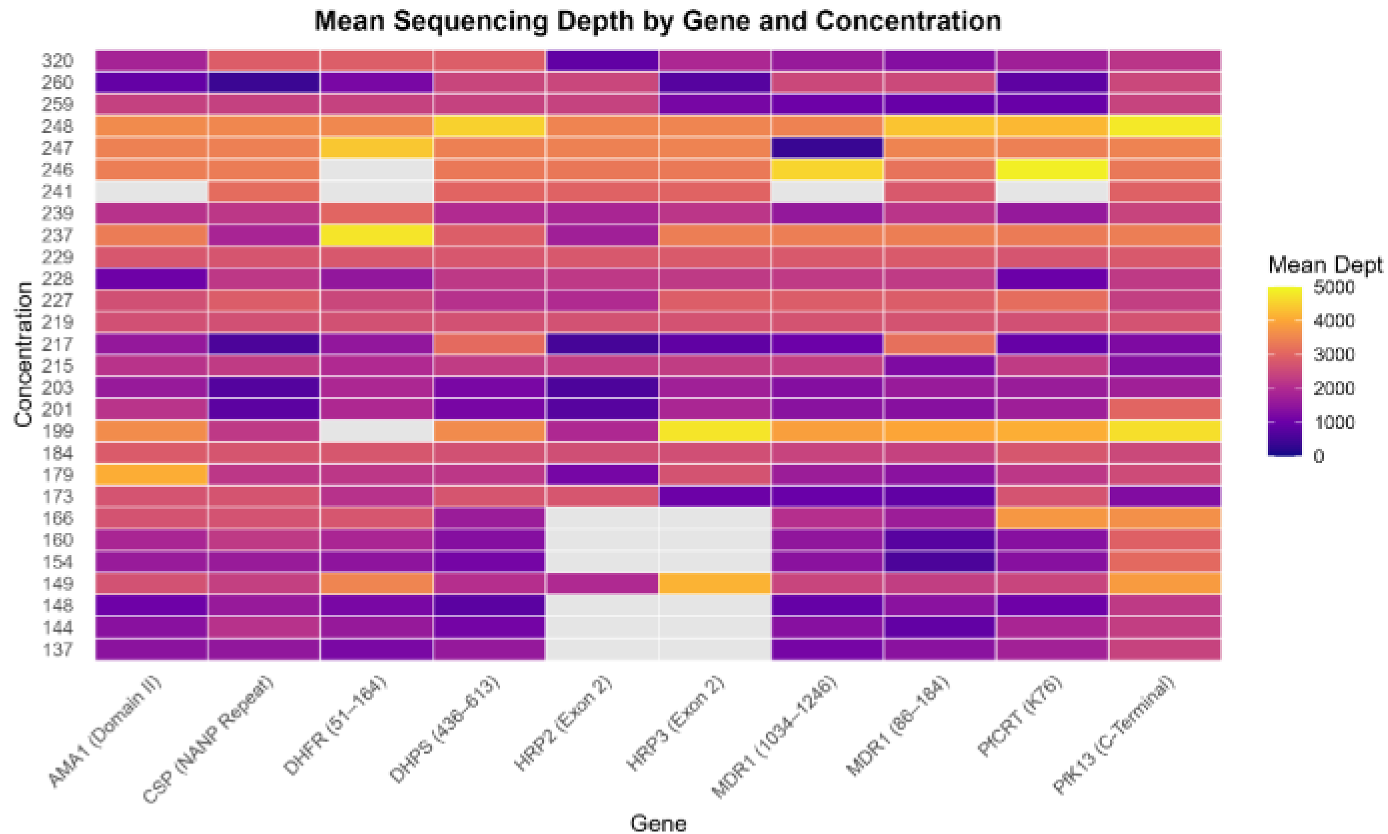

Figure 6: Heatmap showing the average sequencing depth across studied gene regions (x-axis) for different concentrations (y-axis).

The color gradient indicates the mean depth, ranging from 0 (dark purple) to 5000 (bright yellow).

4.5. Mean Coverage Per Gene

Analysis of the mean sequencing coverage revealed consistently high coverage across all ten target genes, with average values nearing 100% (figure 7). Notably, each gene exceeded the established quality threshold of 90%, indicating robust and comprehensive coverage suitable for downstream analyses.

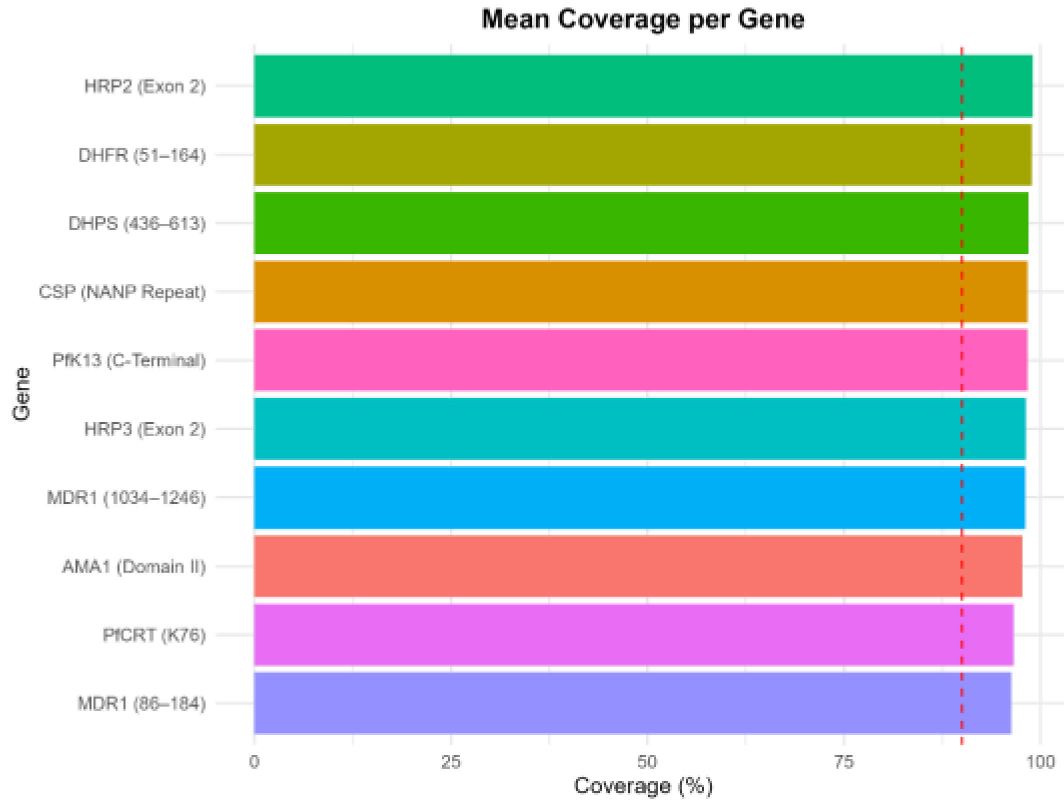

Figure 7: Horizontal bar plot represents the mean percentage coverage achieved for each gene across samples.

4.6. Artemisinin Resistance

The R561H mutation within the *kelch13* propeller domain was detected in 4.1% of the samples from the Kirehe district but was absent in all samples from Huye. Additionally, the WHO candidate mutation G449A was observed in 10% of samples from Kirehe. The N490T mutation was detected in 8.3% of samples from Kirehe, marking its first reported occurrence and unprecedentedly reported in the country (table 1).

4.6.1. Structural and functional implications of the N490T mutation in the *K13* propeller domain

The N490T mutation emerged in our study as an emerging single nucleotide polymorphism (SNP) unprecedentedly reported in Rwanda. Although N490T has not yet been formally linked to artemisinin resistance, its prevalence of about 8% prompted us to investigate its potential structural impact on *K13* function. We used the AlphaFold 3 server to predict the

three dimensional structures of the *K13* propeller domain for both the wild-type and mutant variants. For this purpose, the PD sequence started on amino acid in position 438 (K) of the full protein.

No significant alterations in the overall tertiary structure were observed between the two models (Figure 8).

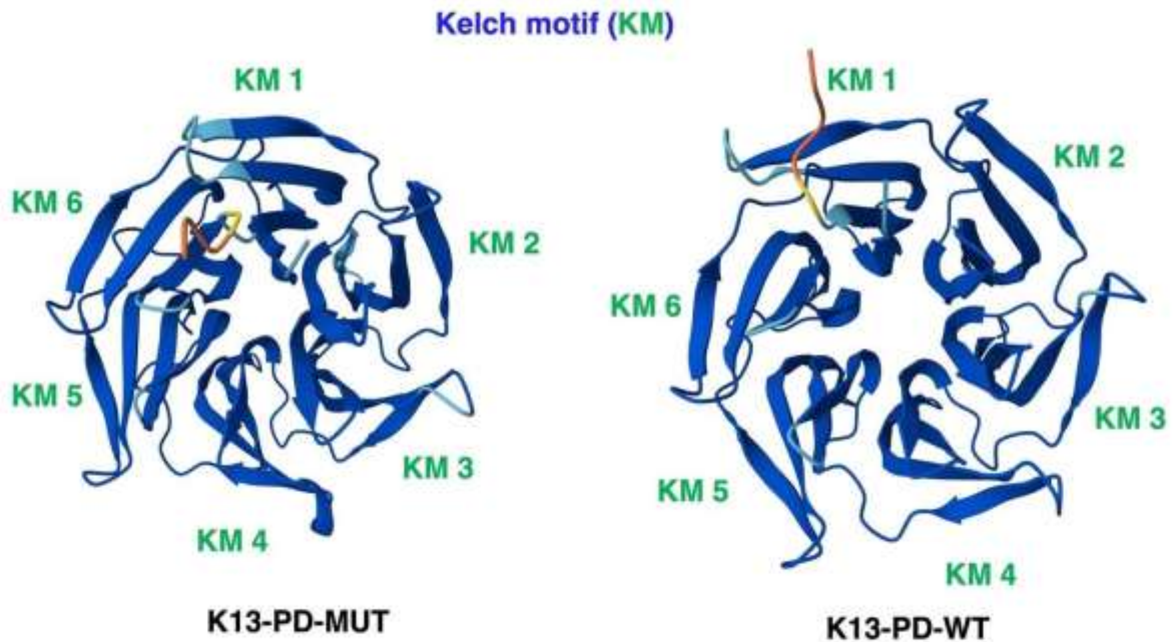

Figure 8: Structural and functional implications of the N490T mutation in the K13 propeller domain

However, detailed structural analysis at higher resolution revealed subtle yet potentially meaningful local changes (Figure 9). The substitution of asparagine (N) with threonine (T) which does not notably affect the local electrostatic environment. Nonetheless, the mutation led to a marked reduction in the number of hydrogen bonds, from eight in the wild-type structure to five hydrogen bonds in the mutant (Figure 9). Particularly, the hydrogen bond formed by N53 (corresponding to the position 490 of the full sequence) and N288 (corresponding to the position 725 of the full sequence) was missing in the mutant. Also, hydrogen bonds linking distant amino acids such Asn53/Asn288 and Ile289/Arg76 were replaced by hydrogen bounds linking proximal amino acids.

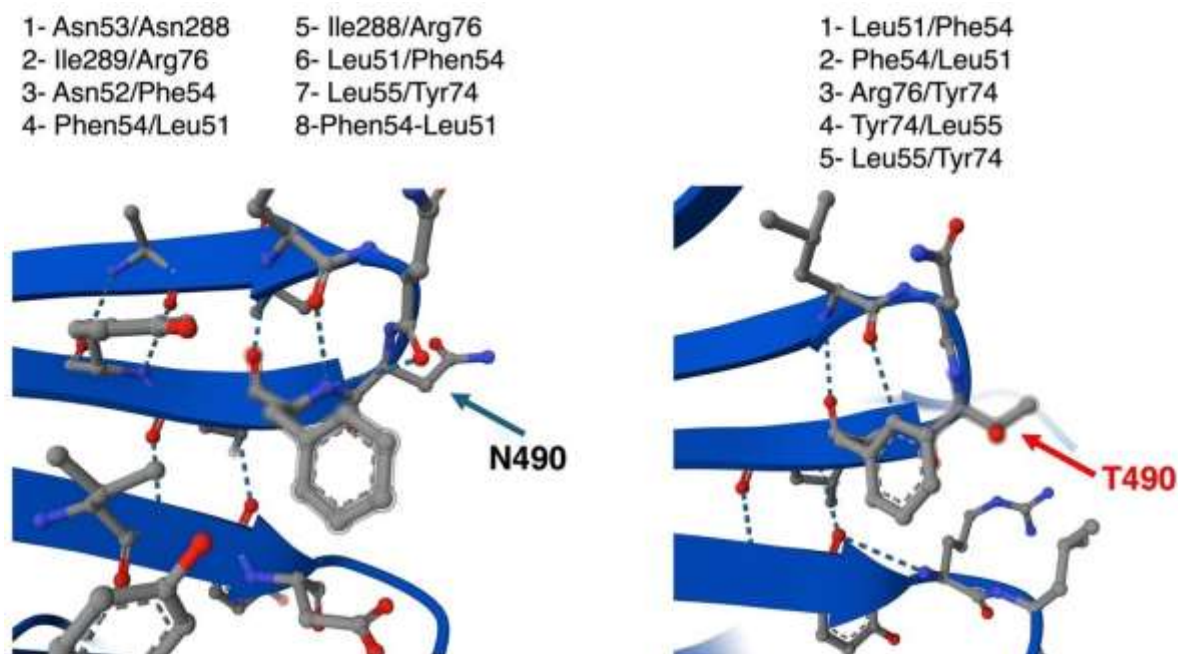

Figure 9: Compared high magnification of N490 and T490 local structures

4.7. Sulfadoxine-pyrimethamine (SP) Resistance

Molecular variants in the *pfdhfr* and *pfdhps* genes, associated with sulfadoxine-pyrimethamine (SP) resistance were highly prevalent. The *pfdhfr* mutation *N51I* was detected at 95.8% in Kirehe and 100% in Huye samples. The *C59R* mutation occurred in 79.1% of Kirehe and 80% of Huye samples, while *S108N* was fixed at 100% frequency in both districts. In the *pfdhps* gene, the *A437G* mutation was observed in 87.5% of Kirehe and 80% of Huye samples, and *K540E* was present at 83.4% in Kirehe and 80% in Huye. The *A581G* mutation showed a lower prevalence, detected only in 16.7% of Kirehe samples and absent in Huye (table1).

4.8. Resistance to ART partner drugs and Chloroquine

Our study observed mutations in *pfcrt* and *pfmdr1* genes. The *pfcrt* D24Y mutation was detected in 29.1% samples from Kirehe and 10% in Huye, in *pfcrt* K76T locus, 100% of samples exhibited the wild type allele. In the *pfmdr1* gene, the Y184F mutation was observed in 41.7% of samples Kirehe and 20% in Huye (table 1).

Table 1: Frequencies of molecular markers associated with antimalarial Resistance in Kirehe and Huye Districts

			Kirehe(n=24)	**Huye(n=10)**
Gene	**Mutation**	**Mutation status**	**n (%)**	
k13	G449A	Wild type	0(0)	9(90)
		Mutant	0(0)	1(10)
	N490T	Wild type	22(91.7)	0(0)
		Mutant	2(8.3)	0(0)
	R561H	Wild type	19(79.2)	0(0)
		Mutant	1(4.1)	0(0)
		Mixed	4(16.7)	0(0)
	P667S	Wild type	22(91.6)	0(0)
		Mutant	1(4.2)	0(0)
		Mixed	1(4.2)	0(0)
	F699C	Wild type	22(91.7)	0(0)
		Mutant	2(8.3)	0(0)
dhfr	N51I	Wild type	1(4.1)	0(0)
		Mutant	23(95.8)	10(100)
	C59R	Wild type	3(12.5)	1(10)
		Mutant	19(79.1)	8(80)
		Mixed	1(4.1)	1(10)
	S108N	Mutant	24(100)	10(100)
	I164L	Wild type	0	9(90)

		Mutant	0	1(10)
dhps	A437G	Wild type	2(8.3)	2(20)
		Mutant	21(87.5)	8(80)
		Mixed	1(4.2)	0(0)
	A581G	Wild type	14(58.3)	0(0)
		Mutant	4(16.7)	0(0)
		Mixed	6(25)	0(0)
	K540E	Wild type	2(8.3)	2(20)
		Mutant	20(83.4)	8(80)
		Mixed	2(8.3)	0(0)
crt	D24Y	Wild type	13(54.1)	9(90)
		Mutant	7(29.1)	1(10)
		Mixed	4(16.7)	0(0)
	K76T	Wild type	24(100)	0(0)
		Mutant	0(0)	0(0)
		Mixed	0(0)	0(0)
mdr1	Y184F	Wild type	9(37.5)	7(70)
		Mutant	10(41.7)	2(20)
		Mixed	5(20.8)	1(10)
	Y1197N	Wild type	21(87.5)	0(0)
		Mixed	3(12.5)	0(0)

CHAPTER 5. DISCUSSION

In this study, we compared sWGA and MVP amplification strategies within the nomadic sequencing protocol, finding that sWGA yields significantly more reads but no significant improvement in sequencing depth or genome coverage. The choice to use sWGA should consider its potential benefits in throughput against possible amplification bias and longer preparation times. Careful evaluation in the context of specific experimental goals and resource availability is recommended to determine the most appropriate use of each method.

Our study demonstrated that the NOMAD nanopore sequencing protocol delivers robust performance by generating high sequencing depth across multiple target genes, with median depths exceeding 2,000 reads and consistent mean coverage above 90% across all loci. The protocol showed reliable and uniform coverage, particularly in genes such as CSP (NANP repeats), though some variability was noted in *MDR1* and *PK13* regions, which may require further optimization. Performance remained consistently strong across a range of DNA input levels, maintaining average depths above 1,000 reads even with reduced inputs, illustrating its suitability for diverse and often low-yield sample types common in field surveillance. Notably, critical resistance genes like kelch13, dhps, and hrp2 consistently exhibited high read depths above 3,000 reads, supporting the protocol's sensitivity for detecting important mutations. Although some gene-specific input-dependent variability was observed (e.g., in *hrp3, dhfr,* and *mdr1*), overall coverage quality and consistency fulfilled the requirements for precise variant calling and molecular surveillance, establishing NOMAD as a practical, scalable approach for genomic monitoring of malaria parasites. While the current dataset included a limited number of samples, the consistently high sequencing depth and coverage underscore the robustness and reliability of the NOMAD protocol in accurately capturing genomic variation, supporting its potential for expanded use in malaria surveillance (de Cesare et al., 2024)(Girgis et al., 2022).

In Rwanda, expanding the use of the NOMAD protocol is crucial to strengthen genomic surveillance through scalable and precise sequencing of *Plasmodium falciparum*, enabling early detection of resistance markers and effective monitoring of parasite populations.

Empowering local laboratories and health programs with this advanced technology will improve intervention strategies and accelerate progress toward malaria elimination. Continued efforts are needed to optimize amplification methods, define DNA input standards, integrate the latest nanopore sequencing advancements, and build local capacity to fully realize the benefits of this approach.

Building on our previous findings, our study identified the validated *pfkelch13* R561H mutation in 4.1% of samples from Kirehe District, while this mutation was not detected in any of the samples analyzed from Huye District. The 4.1% prevalence observed in Kirehe is substantially lower than previously reported rates within the same Eastern Province in the southeast of Kigali city [38]. Notably, although previous studies documented R561H in 4.5% of samples from Huye, our failure to detect this mutation in the Huye may be attributable to our smaller sample size, which may limit the sensitivity to detect low-frequency variants (Straimer et al., 2022). Significantly, we also observed the candidate G449A mutation in 10% of Huye's samples. Additional non-synonymous mutations in the *pfkelch13* propeller domain (P667S and F699C) were detected at low frequencies. Our results highlight pronounced geographic variability both in the presence and frequency of resistance-associated and candidate mutations underlining the importance of continuous and expanded surveillance efforts (Uwimana et al., 2021b).

Among these *pfkelch13* propeller domain, the N490T mutation observed in our study warrants particular attention due to its potential functional implications. This mutation is expected to modulate the stability and weaken local structural integrity, thereby interfering with proper folding or substrate binding within the propeller domain. Such disruption could impair the K13 protein's ability to interact with its physiological targets, which are likely involved in key processes such as hemoglobin uptake and proteasome-mediated degradation. Consequently, compromised K13 function may reduce the susceptibility of *Plasmodium falciparum* to artemisinin and its derivatives. Given these potential effects, further in vivo validation and longitudinal clinical studies are essential to clarify the functional significance of the N490T mutation and its role in mediating or enhancing artemisinin resistance.

Our study observed high frequencies of mutations in *pfdhfr* and *pfdhps* genes associated with sulfadoxine-pyrimethamine (SP). Specifically, near fixation of the *pfdhfr* N51I, C59R, and S108N mutations, alongside substantial prevalence of the *pfdhps* A437G and K540E mutations, was observed in samples from both districts. These findings align closely with resistance profiles that have been widely reported across sub-Saharan Africa (Wang et al., 2022)(Bohissou et al., 2024). Interestingly, persistence of such high frequencies occurs despite SP no longer being endorsed for treatment or intermittent preventive therapy in Rwanda. This paradox may be explained by additional selective pressures maintaining the resistant parasite populations, including possible cross-resistance with other antifolate compounds, the prolonged half-life of SP leading to residual drug pressure, or potential fitness advantages conferred by these resistance mutations (Nana et al., 2023). Further research combining longitudinal molecular surveillance, clinical efficacy data, and detailed assessment of local preventive drug use is needed to elucidate these mechanisms and assess implications for malaria control strategies relying on SP.

Polymorphisms in the *pfcrt* gene, historically associated with chloroquine resistance, were not detected in our study population. This observation aligns with previous reports documenting a decline in *pfcrt* mutations following the cessation of chloroquine use, likely due to the reduction in drug pressure (Rana et al., 2022) (Ocan et al., 2019). Such findings suggest the potential for reconsidering chloroquine as part of future antimalarial treatment strategies. Additionally, the *pfmdr*1 Y184F variant, which has been linked to altered susceptibility to artemisinin-based combination therapy (ACT) partner drugs such as lumefantrine, was detected at a decreased frequency of 41.7% and 20% in Kirehe and Huye, respectively compared to previous reports. disparities likely reflect adaptive parasite responses to local drug use patterns, particularly the widespread use of artemether-lumefantrine as first-line treatment in Rwanda (Van Loon et al., 2021)(Wicht et al., 2020). The spatial heterogeneity of these mutations between the two regions underscores the complexity and localized nature of antimalarial drug resistance evolution. Collectively, these findings emphasize the critical need for continuous and expanded molecular surveillance integrated with clinical data to effectively guide malaria treatment policies and containment efforts.

CHAPTER 6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

This study successfully optimized and validated the NOMAD nanopore sequencing protocol as a practical, scalable tool for molecular surveillance of *Plasmodium falciparum* antimalarial resistance markers in Rwanda. While sWGA amplification increased sequencing throughput by generating significantly more reads than MVP, both strategies produced comparable depth and coverage, suggesting the choice of method should be tailored to experimental goals and resource considerations. The protocol consistently delivered high-quality, uniform coverage across key resistance and vaccine target genes. Detection of important resistance mutations such as *pfkelch13* R561H, the established N490T mutation within the *pfkelch13* propeller domain, and persistent sulfadoxine-pyrimethamine resistance markers underscores the value of this approach for ongoing genomic monitoring. Geographic variability in mutation prevalence highlights the need for expanded and continuous surveillance to guide tailored intervention strategies. Empowering local laboratories with this technology and optimizing amplification and sequencing workflows will strengthen Rwanda's capacity for timely, data-driven malaria control and elimination efforts.

6.2. Perspective

This study underscores the practical utility and promises of the NOMAD nanopore sequencing protocol paired with established amplification strategies like selective whole genome amplification (sWGA) and MVP for real-time molecular surveillance of *Plasmodium falciparum* drug resistance. The consistently high read depth and genome coverage across critical resistance and vaccine target genes demonstrate the robustness and adaptability of the approach, even when faced with variable sample quality and DNA input. Differences observed between sWGA and MVP highlight the need for tailored amplification strategies that balance sequencing throughput, potential biases, cost, and processing time according to specific program goals.

Detection of key resistance markers such as *pfkelch13* R561H with geographically variable prevalence, as well as persistent sulfadoxine-pyrimethamine resistance mutations and diverse

molecular marker patterns, reflect the complex, evolving antimalarial resistance landscape in Rwanda. These findings emphasize the necessity of continuous, localized genomic surveillance to inform adaptive malaria control and elimination strategies. The identification of mutations like pfkelch13 N490T further calls for focused functional and longitudinal studies to anticipate emerging challenges to artemisinin efficacy.

Empowering local laboratories through integration of portable, cost-effective technologies like NOMAD within routine malaria control programs offers transformative potential for rapid resistance mutation detection, timely policy support, and targeted intervention deployment. Nonetheless, sustainable implementation demands ongoing capacity building, infrastructure enhancement, and alignment with existing surveillance systems to ensure long-term success.

6.3. Recommendation

Selective Application of Amplification Methods: Selective whole genome amplification (sWGA) is valuable for detecting new gene mutations, especially in cases of clinical failure or low parasitemia. However, due to its higher cost and longer processing time, sWGA should be used strategically based on project goals. It is best suited for projects needing maximum sequencing throughput, while MVP may be preferred for faster processing with less bias. Further efforts to optimize sWGA to reduce bias and preparation time will improve its practical use.

Expansion and Standardization of NOMAD Protocol Implementation: Scale up the NOMAD nanopore sequencing protocol nationwide to enable broad, cost-effective genomic surveillance of malaria. Develop standardized guidelines for DNA input, amplification, and bioinformatics to ensure consistent data quality and comparability across all sites.

Functional Validation of Emerging Mutations: To determine the clinical and public health significance of new mutations such as N490T in *pfkelch13*, it is crucial to conduct focused laboratory and clinical studies. These should involve *in vitro* testing of parasite drug susceptibility and in vivo monitoring of treatment outcomes in affected patients. Combining

molecular surveillance with phenotypic data will clarify the mutation's role in artemisinin resistance and support informed malaria control strategies.

Capacity Building and Resource Allocation: Investment in laboratory infrastructure, training of personnel in molecular techniques and bioinformatics, and integration of genomic data into national malaria information systems are necessary for sustainable surveillance.

Policy Adaptation and Responsive Control Strategies: Surveillance data should guide malaria control programs and policies, including treatment and elimination efforts. The absence of *pfcrt* mutations suggests chloroquine could be reconsidered under monitoring. Regional differences in pfmdr1 and other markers should inform ACT selection. The rising prevalence of *dhfr* and *dhps* resistance mutations requires close monitoring to understand selective pressures and implement preventive treatment. Continuous molecular surveillance is essential for timely, effective strategy adaptation and sustained malaria control.

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