UNIVERSITY OF RWANDA

COMPARATIVE STUDY OF MICROSCOPY AND CONVENTIONAL PCR FOR *PLASMODIUM FALCIPARUM* IDENTIFICATION ON PATIENT SAMPLES FROM GISAGARA AND HUYE DISTRICTS, SOUTHERN PROVINCE, RWANDA

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DECLARATION

I, **Janvier NDINKABANDI**, hereby declare that this Research project submitted to the University of Rwanda, Rwanda for the degree Master of Science in Biotechnology is my 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.

Janvier Ndinkabandi

DEDICATION

This thesis is dedicated:

To the Almighty God,

To my beloved family, whose unwavering love, encouragement, and sacrifices have guided me every step of the way. Their belief in me has been my greatest motivation.

ACKNOWLEDGEMENT

My acknowledgements go to Enabel-EU project Kwigira, BK foundation, Ministry of Health and University of Rwanda for making possible this program success,

Also, to my supervisors, **Dr TOLESSA MULETA DABA**, **Dr NDOLI MINEGA Jules**, and **Mr. HABARUGIRA Felix**, for the invaluable guidance and support provided throughout the research process. Your mentorship has been instrumental in shaping the quality of this work.

Special thanks to the University Teaching Hospital of Butare, for providing the Laboratory research environment and resources necessary for this work.

Finally, my heartfelt thanks to my family for their patience, encouragement, and unwavering belief in me.

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LIST OFABBREVIATIONS

ACT: Artemisinin Combination Therapy

API: Annual Parasite Incidence

AUC: Area Under the Curve

bp: Base Pair

CDC: Center for Disease Control

cDNA: Complementary DNA

CHUB: Centre Hospitalier Universitaire de Butare

CHWs: Community Health Workers

CI: Confidence interval

dPCR: Digital PCR

EDTA: Ethylenediaminetetraacetic acid

HC: Health Center K13: Kelch 13

iRBC: Infected Red Blood Cells

LAMP: Loop-mediated Isothermal Amplification

LOD: Limit of Detection

MgCl2: Magnesium chloride

P.f: *Plasmodium falciparum*

PCR: Polymerase Chain Reaction

pfHRP2-RDT: *P. falciparum* histidine-rich protein based rapid diagnostic test

qPCR: quantitative PCR

RDT: Rapid Diagnostic Test

ROC: Receiver Operating Characteristic

RT-PCR: Reverse Transcription PCR

SPSS: Statistical Package for the Social Sciences

Std-error: standard error

WHO: World Health Organization

μL: Microlite

ABSTRACT

Malaria is a critical global public health concern. Sub-Saharan African nations including Rwanda are known for high malaria cases and transmission. In Rwanda, particularly the districts of Gisagara and Huye are known for the endemic and seasonal transmission. To tackle high transmission and to apply early treatment, effective malaria management which depends on accurate and timely diagnosis is very imperative. Microscopy has been serving as the gold standard diagnostic technique due to its affordability, ability to detect and quantify Plasmodium species. However, factors such as technician skills, equipment quality and parasite density limit its effectiveness. Its low sensitivity, especially in detecting low-sensitive infections or lower ability in distinguishing between closely related species is also among its limitation. In contrary, conventional PCR, a molecular technique, offers superior sensitivity and specificity, especially for low parasitemia and mixed infections that microscopy often misses. Conventional PCR overcomes many of microscopy limitations through high sensitivity, targeting parasite DNA and enabling precise species identification even at low concentrations. This study is therefore, focused on comparison of microscopy with conventional PCR for successful identification of *Plasmodium falciparum*. It sought to determine prevalence of *Plasmodium falciparum*; to assess the diagnostic sensitivity; specificity of the two methods and evaluate their concordance. The study population includes RDT-positive patients identified by community health workers, then referred to health centers for screening to be recruited in ARMEA project. A sample size was determined using statistical calculations with Daniel's formula. Ethical clearance was secured before data collection process begun. Blood samples undergone both microscopy (with thick and thin smear), and detection using conventional PCR. The raw data was recorded using Microsoft Excel and analyzed using SPSS Version 25. This study revealed that high prevalence rates were detected with both methods, though PCR detected slightly more cases. Microscopy demonstrated strong diagnostic performance with high sensitivity (95.26%) and positive predictive value (98.96%), but a lower negative predictive value (54.76%), reflecting its limitations in ruling out infection. Despite PCR's superior sensitivity, microscopy showed excellent diagnostic accuracy (AUC = 0.902) and substantial agreement with PCR (Cohen's Kappa = 0.639), supporting its continued use in resource- limited, high-transmission settings. In conclusion, the study supported integrating microscopy with molecular tools like PCR for malaria diagnosis, as microscopy remains valuable due to its high sensitivity and accuracy.

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CHAPTER ONE: INTRODUCTION

1.1 Background

The Mesopotamians and Egyptian tablets remain confirms the malaria presence as early as 3200 years before Christ in history. The disease likely spread from Africa to the Mediterranean, Greece and eventually Rome, where it may have contributed to the Empire's decline (Nosten *et al*., 2022). Over centuries, it thrived in clouded European settlements and semi-tropical regions of India and China (Nosten *et al*., 2022). In the America, malaria arrived with European colonists and Africa slaves, who carried both the parasite and partial genetic resistance (Nosten et al., 2022). In the United State (U.S), malaria was widespread in the early 20th century (Nosten *et al*., 2022).

Malaria remains a world health threat, especially in low- and middle-income nations, with Africa bearing the heaviest burden. In 2023, the WHO reported an estimated 263 million malaria cases and 597,000 fatalities globally (Report, 2024). Africa encountered for about 95% of these cases and 96% of the deaths with the top five concentrated at Nigeria (26%), the Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%) (World Malaria Report, 2024). East Africa, including countries like Uganda, Tanzania, Kenya and Rwanda, continue to be a high transmission zone, with malaria being endemic and peaking during rainy seasons (Nosten *et al*., 2022).

According to WHO, the African region remained the most hit, accounting for 94 % of global cases. The Eastern Mediterranean region also saw a sharp 57% increase in incidence since 2021. Global malaria deaths in 2023 were estimated at 597,000, with a mortality rate of 13.7 per 100,000 showing a gradual decline from 2020. The African region also bore 95% of global malaria deaths (World Malaria Report, 2024).

In Rwanda also, malaria remains a significant public health issue, particularly in the Southern Province districts of Gisagara and Huye. These areas are endemic to malaria, with seasonal outbreaks that heavily burden the local health system. Malaria transmission remains moderate to high, especially in swampy, lowland areas that support mosquito breeding. Accurate and timely diagnosis of Plasmodium infections is critical for effective case management and the reduction of malaria-related morbidity and mortality (Umugwaneza *et al.,* 2025).

Different diagnostic tests including microscopy have been in use to diagnosis malaria. Microscopic analysis remains the "gold standard" for laboratory confirmation of malaria. These tests need to be performed by an experienced microscopist immediately when ordered by a healthcare provider. A Rapid Diagnostic Test (RDT) which is an alternative way of quickly establishing the diagnosis of malaria infection by detecting specific malaria antigens in a person's blood. Molecular detection in which parasite nucleic acids are detected using polymerase chain reaction (PCR) (CDC, 2024).

Microscopy has long been the gold standard for malaria diagnosis in many parts of the world, including Rwanda. It is widely used due to its cost-effectiveness, ability to quantify parasitemia, and its applicability in identifying different *Plasmodium* species, primarily *Plasmodium falciparum* and *Plasmodium vivax*, with occasional detection of *Plasmodium ovale* and *Plasmodium malariae* (Lin *et al*., 2022; Wangai *et al*., 2011).

1.2 Statements of the Problem

The Southern Province of Rwanda is experiencing shifting malaria transmission dynamics due to climatic, ecological, and human factors. This evolution underscores the need to assess the reliability of microscopy compared to molecular methods like Conventional PCR in order to improve malaria diagnosis. Research across different endemic regions has highlighted significant discrepancies between microscopy and molecular diagnostic methods such as PCR (Opoku Afriyie *et al*., 2023). However, microscopy is heavily dependent on the skills of the technician (expert dependent), quality of the equipment, and the density of parasites in the blood sample. In case of low parasitemia, microscopy may fail to detect infection.

1.3 Significance of the Study

This study seeks to evaluate and compare the diagnostic accuracy of microscopy and Conventional PCR in the identification of *Plasmodium* species among patients in Gisagara and Huye districts. This comparative evaluation of microscopy and conventional PCR can provide valuable insights into their relative effectiveness and inform National diagnostic guidelines and malaria control strategies.

1.4 Research Question

i. What is the prevalence of *Plasmodium falciparum* in the study area detected by both used methods?

ii. How is sensitivity and specificity of conventional PCR compared to microscopy for malaria parasite detection?

iii. What is the level of agreement between conventional PCR and microscopy results?

1.5 Objectives

1.5.1 General Objective

The main objective of this study was to determine the comparative diagnostic accuracy of microscopy and conventional PCR in detecting *Plasmodium falciparum* among patient samples from Gisagara and Huye districts.

1.5.2 Specific Objectives

The specific objectives of this study were:

i. To investigate the prevalence of *Plasmodium falciparum* using microscopy and conventional PCR among malaria patient samples from Gisagara and Huye districts.

ii. To assess the sensitivity and specificity of microscopy in comparison to conventional PCR as a reference standard.

iii. To evaluate the concordance between microscopy and conventional PCR results for *P. falciparum* detection.

CHAPTER TWO: LITERATURE REVIEW

2.0 Introduction

This literature aims to critically access and compare microscopy and conventional PCR in the context of *Plasmodium falciparum* identification. By analyzing a range of studies, the review highlights the diagnostic performance, advantages, limitations and practical considerations associated with each method.

2.1 Theoretical Review

2.1.1 Overview on Malaria

Five different Plasmodium species: *Plasmodium falciparum* (*P. falciparum), Plasmodium vivax(P.vivax), Plasmodium malariae(P.malariae), Plasmodium ovale(P.ovale) and Plasmodium knowlesi(P.knowlesi)*, can infect humans and cause malaria which is still a major health concern worldwide. *P.knowlesi* is still regarded as a zoonotic malaria, however three of the four species are recognized as genuine human parasites (Fitri et al., 2022). The two most common species among them are *P. falciparum and P. vivax*, with infection caused by *P. falciparum* being especially linked to serious malaria consequence. Malaria continue to be a significant source of illness and mortality in tropical and subtropical regions, especially in developing states (Muyidi et al., 2025). In 2023, there were an estimated 263 million new malaria cases in 83 countries worldwide, up from 252 million in 2022 and 226 million in 2015 (World Report, 2024).

Malaria is a major cause of illness and death in Rwanda, affecting many people, though pregnant women, children under five, and refugees are especially vulnerable (Umugwaneza *et al.*, 2025). According to the Annual Parasite Incidence, there are four zones where malaria is endemic: High (> 450 API per 1000), Moderate (250-450 API per 1000), Low (100-250 API per 1000), and very low (<100 API per 1000). About 70% of Rwanda's malaria cases happen in 19 districts across the country's eastern and southern provinces (Umugwaneza *et al.*, 2025). Throughout the year, transmission peaks during the rainy seasons (May to June and November to December). *Plasmodium falciparum* is the most common species, while Anopheles arabiensis is the main vector. Parasitological diagnosis before administering Artemisinin-based combination therapies (ACT) became necessary when light microscopy was used in clinics and community health workers used pfHRP2-RDT at the community

level (Umugwaneza *et al*., 2025).

To date, three malaria parasites species have been identified in Rwanda: *Plasmodium falciparum*, *Plasmodium ovale* and *Plasmodium malariae*. The most prevalent parasite, *Plasmodium falciparum*, accounts for 97 - 99% of the total population. With 0.5 - 2%, *P. ovale*, is the second most prevalent species, while *P. malariae*, which is mono-infecting, comes in third with 0.5 -1. *P. vivax* has not been detected to date in Rwanda (RMOH, 2015). To provide information on malaria management techniques to lower morbidity and mortality, epidemiological screening and surveillance have long struggled to provide a suitable and reliable diagnostic for the identification of malaria parasites (Fitri *et al*., 2022). There are currently several methods for diagnosing malaria, ranging from the first traditional method that used microscopy to the new way that uses PCR (Fitri *et al*., 2022). Numerous studies have demonstrated the limitations of microscopy in malaria diagnosis, especially in detecting low parasitemia and mixed infections (Opoku Afriyie *et al*., 2023; Tamir *et al*., 2025). Research across different endemic regions has highlighted significant discrepancies between microscopy and molecular diagnostic methods such as conventional PCR (Opoku Afriyie *et al*., 2023).

2.1.2 Pathogenesis of Malaria

The pathogenesis of the *Plasmodium* genus are protozoan parasites that cause malaria. Five species can infect people*: P.falciparum, P.vivax*, *P.malariae*, *P.ovale* and *P.knowlsi* (Milner, 2018). The disease is primarily transmitted through the bite of an infected female Anopheles mosquito. Once bitten, the parasite's sporozoites enter the bloodstream and migrate to the liver, where they invade hepatocytes and undergo asexual reproduction (Milner, 2018). This liver stage is asymptomatic but critical, as it produces thousands of merozoites that are later released into the bloodstream. These merozoites invade red blood cells, initiating the symptomatic blood stage. Within red blood cells, the parasites multiply further, leading to cell rupture and the release of more merozoites, causing cycles of fever, chills and anemia (Agent & Cycle, 2015; CDC, 2017). The typical clinical presentation includes periodic fevers, headaches, vomiting and muscle aches. In case of *P. falciparum*, the disease can progress to severe forms, such as cerebral malaria, acute renal failure, respiratory distress, metabolic acidosis and death. Some species, such as *P.vivax* and *P. ovale*, can remain dormant in the liver as hypnozoites, causing relapses weeks or months after the infection (Milner, 2018).

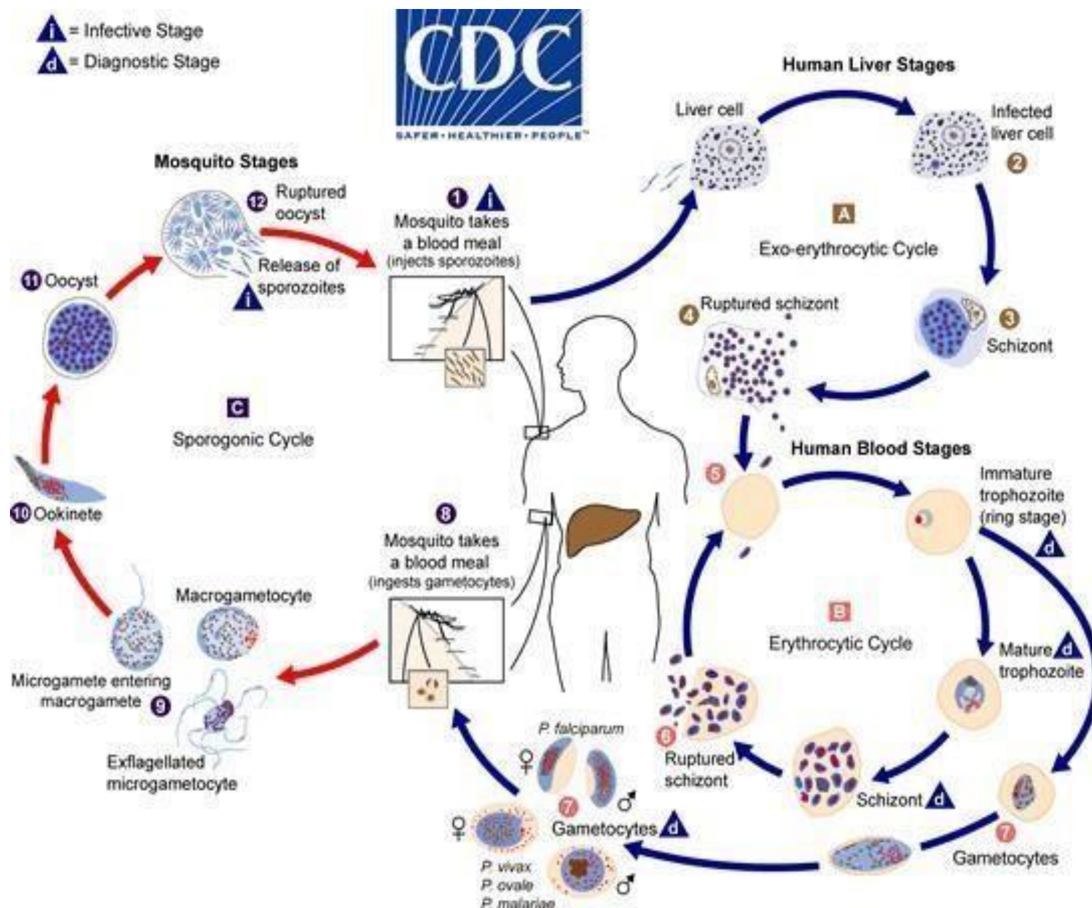

Figure 1: Life cycle of *Plasmodium* Source: (CDC, 2017)

2.1.3 Burden of Malaria

Malaria remains a global health threat, particularly in low- and middle-income countries, with Africa bearing the heaviest burden. In 2023, the World Health Organization reported an estimated 263 million malaria cases and 597,000 deaths worldwide. Africa encountered for about 95% of these cases and 96% of the deaths with the top five concentrated at Nigeria (26%), the Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%) (World Malaria Report, 2024). East Africa, including countries like Uganda, Tanzania, Kenya and Rwanda, continue to be a high transmission zone, with malaria being endemic and peaking during rainy seasons. The WHO African region remained the most affected, accounting for 94% of global cases. The WHO Eastern Mediterranean region also saw a sharp 57% increase in incidence since 2021. Global malaria deaths in 2023 were estimated at 597,000, with a mortality rate of 13.7 per 100,000 showing a gradual decline from 2020. The African region also bore 95% of global malaria deaths (World Malaria Report, 2024).

In Rwanda, malaria also remains a significant public health issue, particularly in the Southern Province districts of Gisagara and Huye. These areas are endemic to malaria, with seasonal outbreaks that heavily burden the local health system. Malaria transmission remains moderate to high, especially in swampy, lowland areas that support mosquito breeding. Accurate and timely diagnosis of *Plasmodium* infections is critical for effective case management and the reduction of malaria-related morbidity and mortality (Umugwaneza *et al.*, 2025).

Different diagnostic tests include microscopy where microscopic examination remains the "gold standard" for laboratory confirmation of malaria. These tests should be performed by an experienced microscopist immediately when ordered by a healthcare provider. A Rapid Diagnostic Test (RDT) which is an alternate way of quickly establishing the diagnosis of malaria infection by detecting specific malaria antigens in a person's blood. Molecular detection in which parasite nucleic acids are detected using polymerase chain reaction (PCR) (CDC, 2024).

Microscopy has long been the gold standard for malaria diagnosis in many parts of the world, including Rwanda. It is widely used due to its cost-effectiveness, ability to quantify parasitemia, and its applicability in identifying different *Plasmodium* species, primarily *Plasmodium falciparum* and *Plasmodium Vivax*, with occasional detection of *Plasmodium Ovale* and *Plasmodium malariae* (Lin *et al.*, 2022; Wangai *et al.*, 2011).

2.1.4 Laboratory Diagnosis of Malaria

Microscopy

This method remains the gold standard that has been in use to quantify and stage malaria parasites (Fitri *et al.*, 2022). This technique is based on the collection of peripheral blood from patients with suspected malaria infection for the preparation of the slides (Gimenez *et al.*, 2021). The testing is made by observing thick and thin blood films. Frequently, the thick smear is the preferred technique for diagnosing malaria because it increases the likelihood of discovering infected red blood cells(iRBC) by allowing for the observation of a large number of blood cells in a comparatively small area (Gimenez *et al.*, 2021). Giemsa solution is used to stain the slides, and an optical microscope with a 100x oil immersion objective is used form examination. This approach is both qualitative and quantitative since it allows for species- level parasite separation and offers a parasite count by field. A more detailed

parasite's morphology and characteristic changes in the parasitized erythrocytes is made possible by the thin film (Gimenez *et al*., 2021; Prairie, 2012).

Through microscopic analysis, up to 5-10 parasites per µl of blood can be found. This limitation, however, is based on the microscopist's level of experience and prior training in test interpretation. This method's limit of detection (LOD) in the field is roughly 50-200 parasites/µl. Many biases are involved, even though microscopy is still the most common method for diagnosing malaria. These include the technical skills required to prepare the slide, the lysis of red blood cells and the resulting changes in parasite morphology, which can lead to errors in species identification, the quality of optical image and the illumination of microscope, the competence and care of microscopist, and, ultimately, the degree of parasitemia (Gimenez *et al*., 2021). The disadvantage is that those with low parasitemic (submicroscopic) infections- most of whom are asymptomatic would go undiagnosed and untreated because of the LOD which will let the transmission cycle continue in the community (Gimenez *et al*., 2021).

Sadly, there are many restrictions with the microscopic approach as well. The morphology of mature trophozoites, schizonts, and gametocytes between *P. knowlesi* and *P. malariae*, as well as the early trophozoite stage between *P. knowlesi* and *P. falciparum*, could not be differentiated by microscopic analysis (Fitri *et al*., 2022). These restrictions frequently result in misdiagnosis. The problem of this method is the inability to identify a tiny number of parasites below the microscopic threshold of 50 parasites/µl (Fitri *et al*., 2022).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) has been used as a more sensitive diagnostic tool for both detection and quantification of parasite DNA (Mixson-Hayden *et al*., 2010). Polymerase Chain Reaction (PCR) is a widely used technique in molecular biology that allows scientists to amplify specific DNA sequences (Kombo, 2025). This method was first introduced by Kary Mullis in 1983 and has since become a crucial tool in various fields, including medical diagnostics, forensic science, and genetic research. PCR is based on the natural process of DNA replication and consists of three main steps: denaturation, annealing, and extension (Kombo, 2025). Denaturation involves heating the double-stranded DNA to divide it into two single strands. During the annealing stage, small synthetic DNA sequences called primers attach to the target regions on each strand. Finally, during the extension phase, DNA

polymerase synthesizes new DNA strands by adding nucleotides to the primers, resulting in amplification of the target sequence. This procedure is done several times to produce millions of copies of the DNA section of interest (Kombo, 2025).

These include conventional PCR, real-time PCR, digital PCR (dPCR), multiplex PCR, quantitative PCR (qPCR), reverse transcription PCR (RT-PCR), nested PCR, touchdown PCR, hot start PCR, colony PCR, and in situ PCR. Conventional PCR is the standard form of PCR that involves thermal cycling to amplify DNA. It requires gel electrophoresis for the visualization of amplified products (Kombo, 2025).

Real-Time PCR (qPCR), also known as quantitative PCR, enables researchers to measure the amount of DNA contained in a sample during the amplification process. Digital PCR (dPCR) is a highly sensitive technique that partitions a DNA sample into thousands of individual reactions, allowing for absolute quantification of target sequences.

Multiplex PCR is designed to amplify multiple DNA targets simultaneously using multiple primer sets. Reverse Transcription PCR (RT-PCR) prior to amplification, RNA is converted into complementary DNA.

Nested PCR is a modification of standard PCR that involves two consecutive amplification reactions to improve specificity and reduce non-specific amplification.

Hot Start PCR involves the use of a DNA polymerase that remains inactive at low temperatures, preventing non-specific amplification (Fitri *et al*., 2022; Kombo, 2025; Thermo Fisher Scientific, 2018).

Each type of Polymerase Chain Reaction (PCR) requires specific reaction components and conditions to achieve optimal amplification. The fundamental components include Taq polymerase, $MgCl_2$, buffer, dNTPs, primers, DNA sample, and nuclease-free water. Conventional PCR, the standard form of PCR, requires Taq polymerase to facilitate DNA amplification, $MgCl_2$ as a cofactor for enzymatic activity, and a buffer solution to maintain an optimal reaction environment, dNTPs serve as building blocks for new DNA strands, while primers ensure specificity in target sequence amplification. The DNA sample acts as a template, and nuclease- free distilled water is used to maintain the desired reaction volume (Kombo, 2025).

PCR is very effective tool in diagnosing and estimating current malaria infections. However, the availability of such method in resource limited areas is lacking, and the requirement for a trained technician, quality control, and equipment maintenance might be 'far-reached' in endemic populations. The challenges might affect the effectiveness of this method and my expose the process to contamination, which can lead to false-positive results. The knowledge of design and selection of primers also have impact on PCR specificity and the reliance of PCR on precise thermal cycling conditions and poses another challenge (Hawkes and Kain, 2007; Krampa *et al*., 2017).

2.1.5 Use of PCR in Disease Detection

Polymerase Chain Reaction (PCR) techniques are now extensively employed in both life sciences and clinical settings for the efficient amplification of specific DNA fragments. In the context of infectious diseases, PCR is utilized for targeted or broad-spectrum pathogen detection, monitoring emerging infections, identifying biological threat agents early, and analyzing antimicrobial resistance (Wang et al., 2024). For genetic disorders, PCR enables prenatal diagnostics and the screening of neonatal conditions like Down syndrome and thalassemia. In cancer research, PCR supports the detection of genes associated with tumors, including oncogenes, tumor suppressor genes, metastasis-related genes, tumor-associated viruses, and genes linked to resistance or mutations affecting anticancer therapies (Wang et al., 2024).

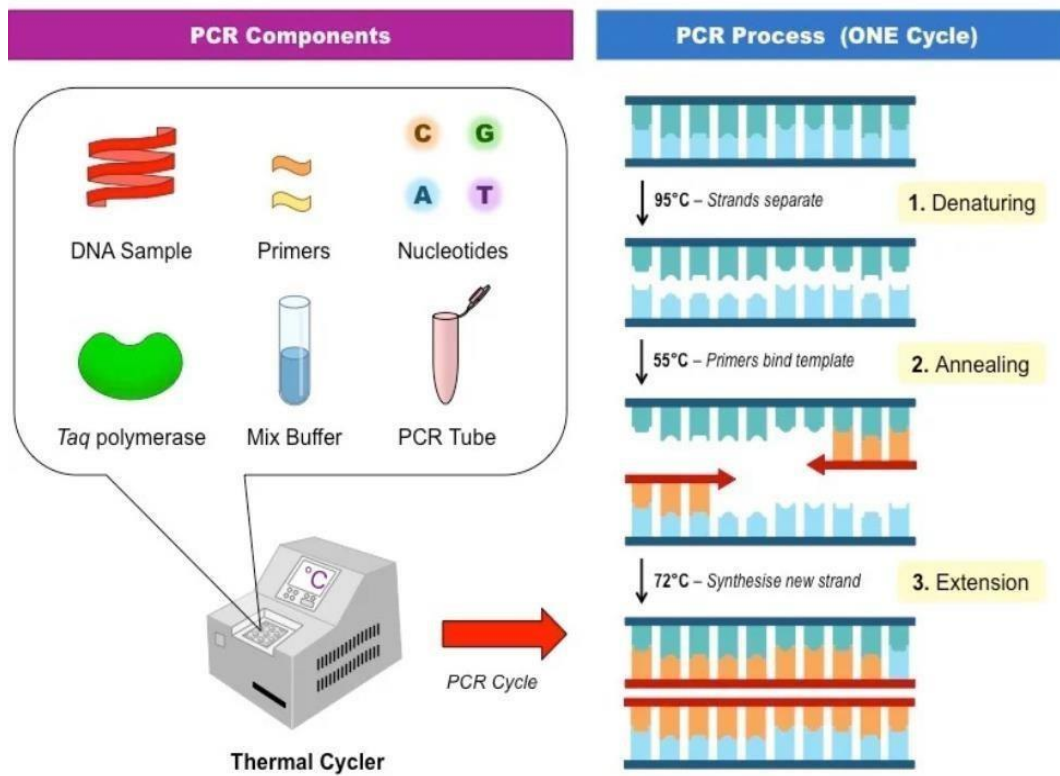

Figure 2: Illustration of PCR in General Source: (Reaction, 2019).

2.1.6 Use of PCR for Malaria Diagnosis

Molecular techniques have significantly improved the accuracy and sensitivity of malaria diagnosis by enabling the amplification and detection of nucleic acids. These methods facilitate the identification and characterization of *Plasmodium* parasites with high precision (Tests, 2024).

PCR-based assays typically target conserved regions of *Plasmodium* DNA, such as the 18S rRNA gene. Its high sensitivity and specificity make PCR particularly effective for detecting low-level parasitemia and distinguishing among different *Plasmodium* species (Tests, 2024).

Advancements in PCR technology such as conventional PCR, nested PCR, multiplex PCR, and real-time PCR have further improved malaria detection. Conventional PCR is the standard form of PCR that involves thermal cycling to amplify DNA. It requires gel electrophoresis for the visualization of amplified products (Kombo, 2025). Nested PCR involves two rounds of amplification, enhancing sensitivity and specificity by focusing more precisely on target sequences (Tests, 2024). Multiplex PCR allows simultaneous detection

of multiple *Plasmodium* species within a single reaction, facilitating rapid and accurate species identification. Real-time PCR, or quantitative PCR (qPCR), enables precise quantification of parasite DNA, offering insights into parasite burden and treatment response (Tests, 2024).

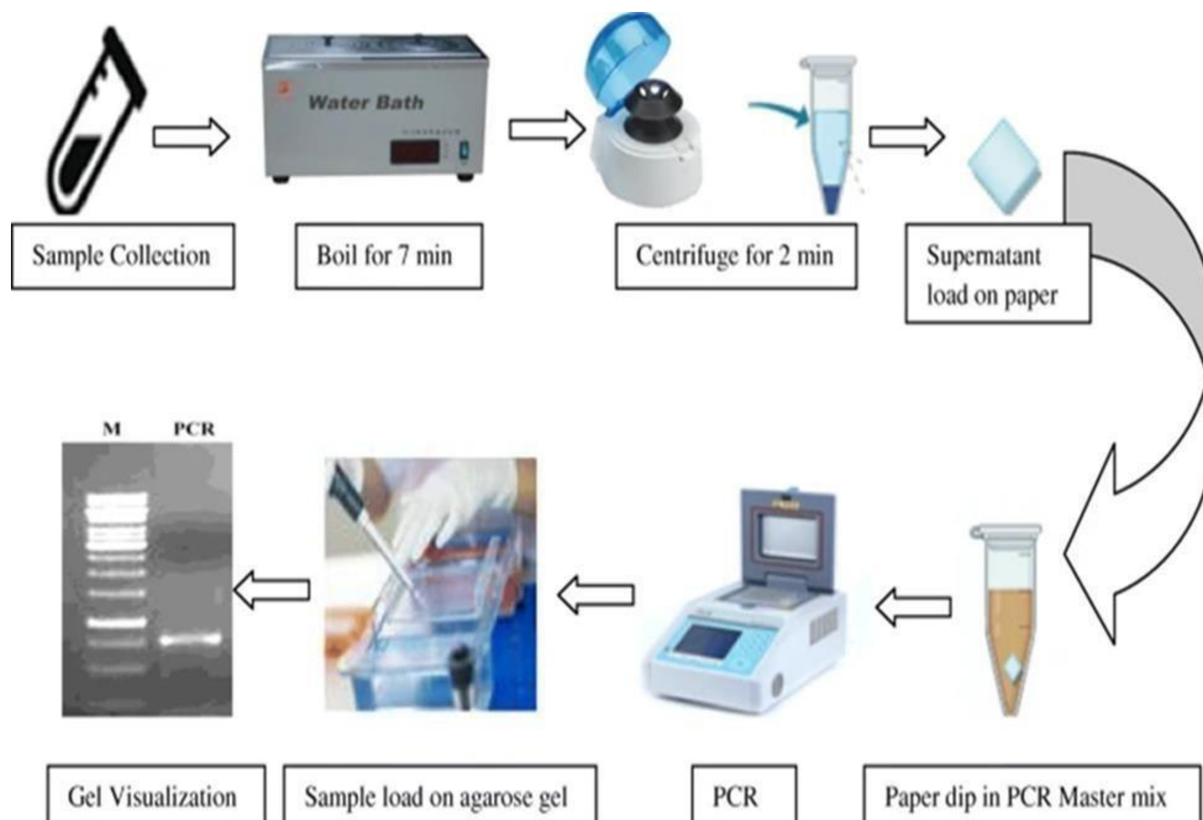

Figure 3: Overall Procedure of conventional PCR

Source:(Collins et al., 2021)

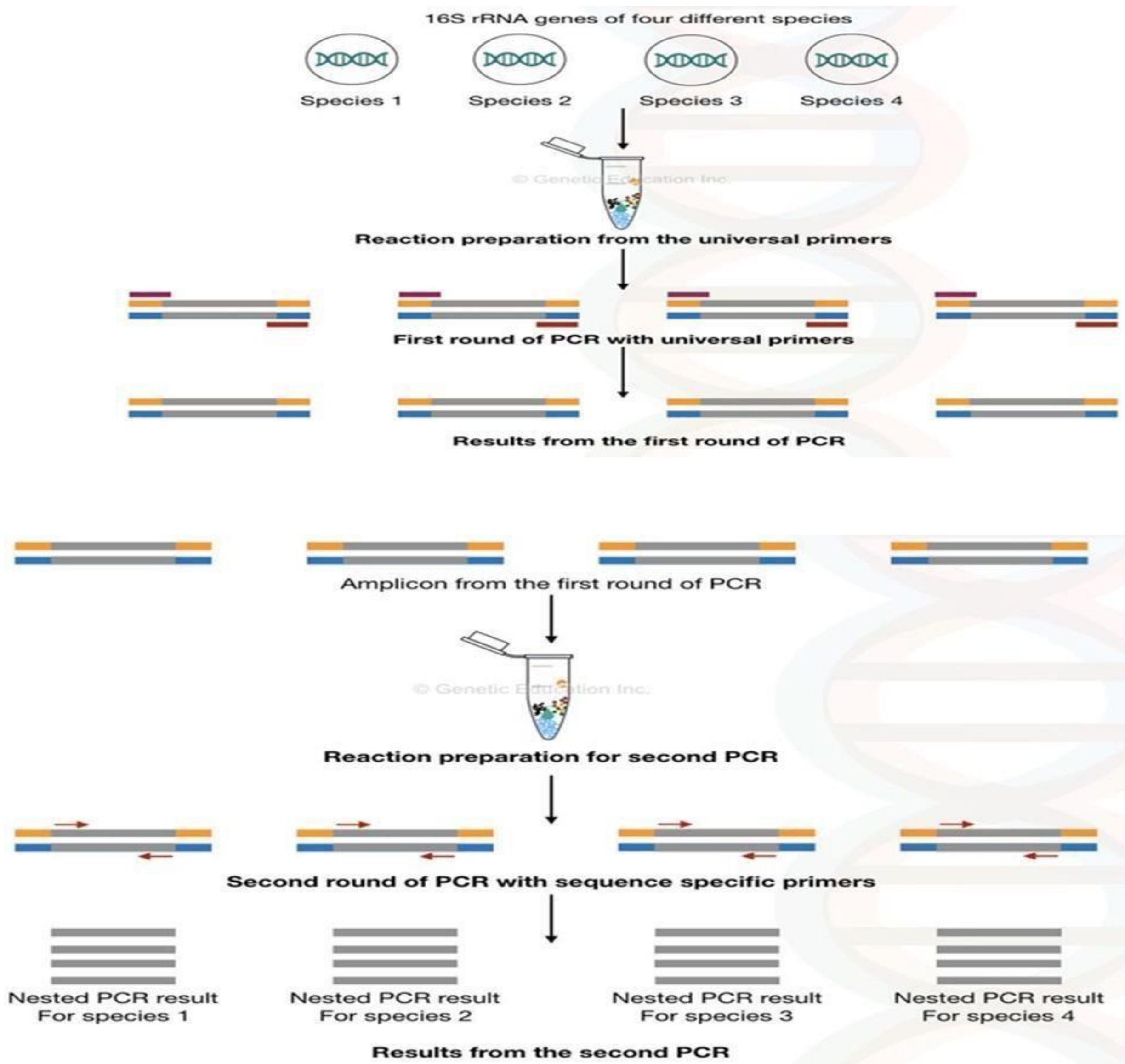

Figure 4: Procedure of Nested PCR

Source:(Chauhan, 2019)

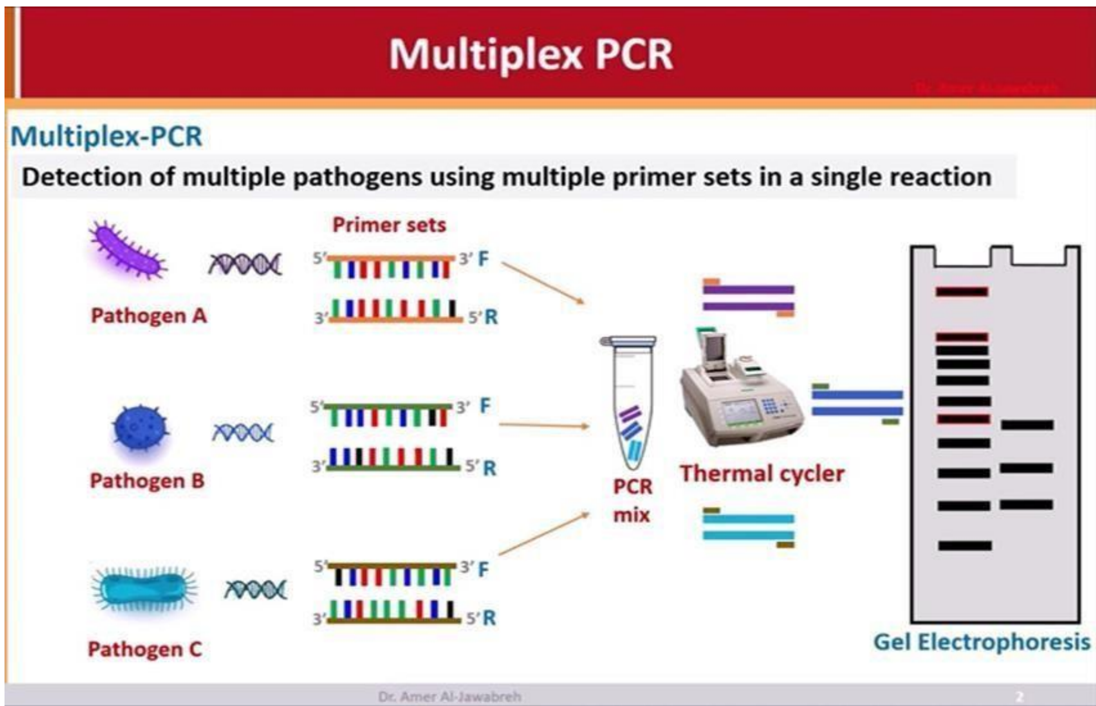

Figure 5: Multiple Pathogen Detection using multiplex PCR

Source: (Datta, 2023)

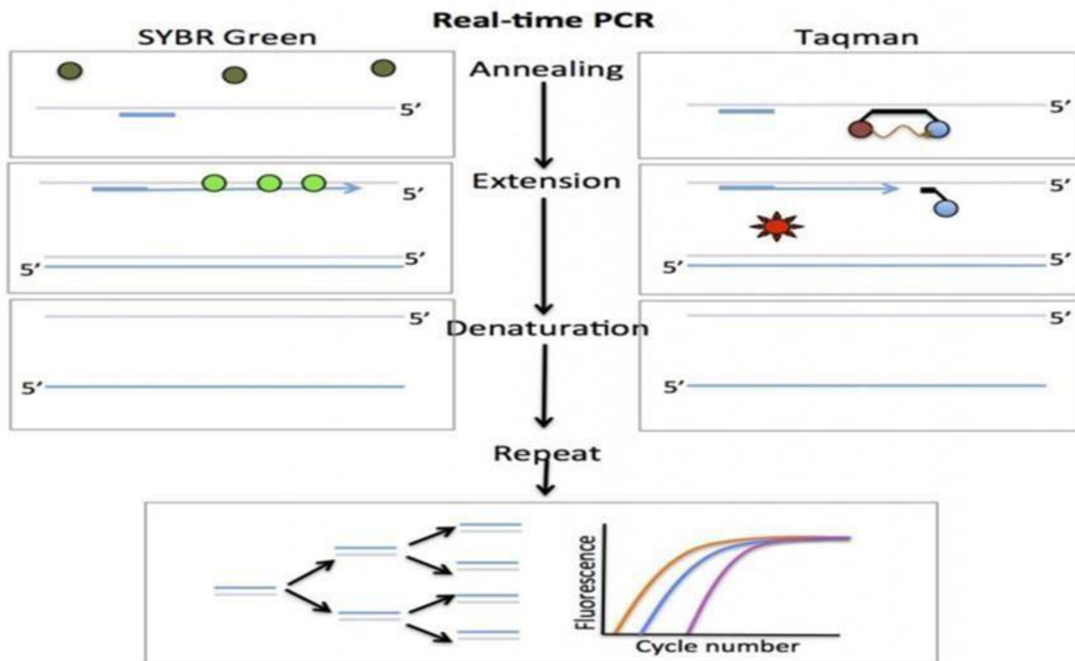

Figure 6: Stepwise process of real time PCR Source: (Datta, 2023)

2.1.7 Types of Malaria Specimen Samples Appropriate for PCR Detection

The following malaria specimen are appropriate for detection using PCR.

Whole Blood: Blood collected via venipuncture in tubes with anticoagulants EDTA can be used for PCR malaria detection. This type of sample has advantages of High parasite DNA yield and it is most reliable for detection and quantification. Whoever it requires to collect high amount sample(2-3ml), refrigeration or cold chain. It is best for clinical diagnostics, research labs, high-sensitivity detection (Li *et al*., 2014).

Dried Blood Spots (DBS): Capillary blood from a finger prick is spotted onto Whatman filter paper and dried. This type of sample is easy to collect and store, no refrigeration needed, field friendly and cost-effective. However, it can give slightly lower DNA yield than fresh blood and more PCR inhibitors possible. It is best for field surveys, remote settings, large-scale screening (Li *et al*., 2014).

Plasma or Serum: The liquid component of blood collected in tube after centrifugation. It can be used if whole blood is not available and easier to handle in some lab settings. However, it has much lower DNA content and less sensitive than whole blood or DBS. It is best for experimental or supplementary use (Li *et al*., 2014).

Buffy Coat: The white blood cell layer obtained after centrifuging whole blood. This type can concentrate parasite DNA. However, it requires lab processing and not widely used in routine diagnostics. It is best for research applications where parasite enrichment is needed

Tissue Samples: Biopsies or autopsy tissue in rare or post-mortem cases. It is useful in unusual presentations or severe malaria. However, it is highly invasive and rarely used outside of research or forensic settings. It is best for research or histopathological studies (Li *et al*., 2014).

2.1.8 Discrimination Ability for Identifying Malaria Species

Accurate identification of *Plasmodium* species is essential for appropriate treatment and for malaria surveillance programs. PCR-based methods offer superior sensitivity and specificity compared to microscopy and rapid diagnostic tests (RDTs), particularly in detecting Low-density infections, mixed-species infections and morphologically similar species (e.g., *P. knowlesi* versus *P. malariae*)

Conventional PCR: Uses genus-specific primers in the first round, followed by species-specific primers in a second (nested) round. It is good for high sensitivity in differentiating all five human malaria species, however it requires post-PCR handling like agarose gel electrophoresis and there is an increasing contamination risk (Snounou *et al*., 1993).

Nested PCR: Involves two consecutive amplification reactions to improve specificity and reduce non-specific amplification with species-specific primers in the second round. This discriminates mixed infections accurately and detects species missed by microscopy or RDTs (Singh, *et al*., 1999).

Real-Time PCR (qPCR): Uses fluorescent probes or SYBR Green to detect DNA in real-time. It can be quantitative (estimates parasite quantity. It uses species-specific primers/probes allow precise identification and it is fast, sensitive, and high-throughput, but requires more sophisticated equipment (Rougemont, 2004).

Multiplex PCR: Simultaneously amplifies multiple targets in one reaction. Allows detection of several species in a single test and it is efficient for epidemiological surveys or screening mixed infections (Morris *et al*., 2013).

LAMP (Loop-mediated Isothermal Amplification): While not strictly PCR, LAMP is used in species detection. It uses species-specific primers enable identification with minimal lab infrastructure. It is field-deployable and faster than conventional PCR (Collins *et al*., 2021).

CHAPTER THREE: METHODOLOGY

3.1 Study Design

The study was a cross-sectional comparative study aimed at evaluating microscopy and conventional PCR for malaria parasite identification in research patient samples from Gisagara and Huye districts, Southern Province, Rwanda.

3.2 Study Area

The samples were collected from 5 selected health centers in Gisagara of the wetland zone where the prevalence of malaria is high and 8 health centers in Huye districts but accessible for easy sample transportation to the laboratory of study.

3.3 Study Population

Patients from one and above years case history with uncomplicated malaria tested from community health workers were considered from those selected health centers.

3.4 Sample Size Determination

Sample size was calculated based on expected malaria prevalence using Daniel's formula which is:

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

Where: n is the sample size, p is the predictive local malaria prevalence, d is the precision error, and Z= statistic for level of confidence.

In this study: p=5%, d=5% and z =1.96

Accordingly, a minimum of 428 patients was required for the study (Naing *et al*., 2006).

Inclusion Criteria

Study participants were a consecutive sample of patients who presented to selected health facilities in Gisagara and Huye districts and who tested positive by malaria rapid diagnostic

test (pfHRP2-RDT) by community health workers, then referred to health center and recruited to participate in the Artemisinin resistance monitoring in East Africa (ARMEA) project. All patients with one or more than one year, not having signs of complicated malaria were selected.

Exclusion Criteria

All patients below one-year cases and those presenting signs of complicated malaria were excluded from the study candidacy.

3.5 Sample Collection Strategy

The 3-4 ml venous blood sample from participants fulfilling inclusion criteria was drawn into four milliliters (4ml) EDTA tube at the health center and then transported at the study site laboratory for both blood smear and Conventional PCR examination.

3.6 Test Procedures

Microscopy: Thick and thin smear was prepared from peripheral blood, stained with Giemsa and examined under microscope for malaria parasite identification and quantification by two experienced microscopists.

Conventional PCR: DNA of all samples collected above was extracted from a 200µl of whole blood using the QIAamp DNA Blood Mini kit (Qiagen, Germany), eluted in a 200µl final volume and stored at -20^{0}C until use.

PCR protocol for *plasmodium falciparum* kelch-13 propeller domain, with Solis Biodyne reagents was used (Ariey *et al*, 2014). Positive and Negative controls for all runs, were used respectively, then the kelch-13 specific gene for *Plasmodium falciparum* detection was done by gel electrophoresis. PCR assay QC and contamination prevention were based on inclusion of extraction blanks to monitor contamination, positive controls to confirm assay sensitivity, negative controls to check for false positives, adherence to contamination prevention practices like separate work areas, sterile consumables, barrier tips, and routine decontamination. The *Plasmodium falciparum* kelch-13 (K13) propeller domain was amplified using an inner PCR approach to ensure both species-specific detection and reliable identification of molecular markers of Artemisinin resistance. The K13 locus is the only validated genetic marker of resistance, and nested PCR enhances sensitivity for detecting

low-density infections that are common in endemic settings such as Gisagara and Huye. This method provides high specificity for *P. falciparum* while enabling surveillance of emerging resistance mutations, making it a practical and standardized tool for monitoring treatment efficacy in East Africa (Ariey et al., 2014). Targeting the *P. falciparum* kelch-13 (K13) propeller domain with inner PCR allowed us to confirm parasite species while simultaneously screening for mutations linked to Artemisinin resistance, providing critical surveillance data for Gisagara and Huye.

In this protocol, the PCR amplification procedure was followed (Adapted by Welmoed van Loon, 2023). The specific primers, short sequences of DNA that match the target *P. falciparum* DNA, are used. These primers flank a region of the parasite's DNA that is unique to *P. falciparum*. If *P. falciparum* DNA is present, the specific DNA sequence was amplified. The detection and analysis of amplicons (PCR products) are sorted by size using gel electrophoresis. The presence of a band of the expected size of 849bp for a common *P. falciparum* primer set that confirms the presence of *P. falciparum*. The intensity of the band can also give an indication of the parasite load. Simple gel-based detection is sufficient for the *P. falciparum* kelch-13 (K13) inner PCR because the amplified fragment has a well-defined and expected size (~849 bp), allowing straightforward confirmation of successful amplification and parasite detection without the need for more complex assays. Sequencing or other downstream analyses can then be applied only to PCR-positive products to identify resistance-associated mutations (Ariey et al., 2014; WHO, 2019) as described in protocol of ARMEA project.

Alternative molecular assays such as nested 18S PCR or qPCR were considered but not employed in this study due to resource limitations and cost constraints. In contrast, it could help to investigate the discordance results between microscopy and PCR.

3.7 Data Analysis

After experimental work, the raw data were retrieved in Microsoft excel version 2021. Data was cleaned and coded and analyzed using the Statistical package for Social Sciences (SPSS) version 25 statistical software (IBM Corporation, 2017). Counts and percentages in graphs and tables were used to present categorical data. Sensitivity and specificity were calculated. The level of agreement between microscopy and conventional PCR were evaluated using Cohen’s Kappa statistics.

3.8 Ethical Consideration

Recruitment of participants was voluntary and selected among patients fulfilling inclusion criteria then informed consent were obtained from all study participants before sample collection, and data confidentiality was maintained throughout the study. Ethical approval for this study was obtained from CHUB Ethics committee as an institutional of the study site.

CHAPTER FOUR: RESULTS

4.0 Introduction

This chapter represents the results of comparison between microscopy and conventional PCR for *Plasmodium falciparum* identification on patient samples from Gisagara and Huye districts, Southern province, Rwanda. The general objective of this study was to determine the comparative diagnostic accuracy of microscopy and conventional PCR in detecting *Plasmodium falciparum* among patient samples from Gisagara and Huye districts. This study was guided by following specific objectives: i. to investigate the prevalence of *Plasmodium falciparum* using microscopy and conventional PCR among malaria patient samples from Gisagara and Huye districts. ii. to assess the sensitivity and specificity of microscopy in comparison to conventional PCR as a reference standard. iii.to evaluate the concordance between microscopy and conventional PCR results for *P. falciparum* detection.

4.1 Data analysis and presentation

4.1.1 Prevalence of *Plasmodium falciparum* using microscopy and conventional PCR.

Table 1: Prevalence of *Plasmodium falciparum* using Microscopy

	Microscopy			
Districts	**Negative**		**Positive**	
	n	%	n	%
Huye	22	10.3	192	89.7
Gisagara	20	9.3	194	90.7
Total	42	9.8	386	90.2

Table 1 above shows the prevalence of *Plasmodium falciparum* using microscopy. The prevalence of *Plasmodium falciparum* was 89.7% and 90.7% in Huye and Gisagara districts, respectively.

Table 2: Prevalence of Plasmodium falciparum using Conventional PCR

Districts	Conventional PCR			
	Negative		Positive	
	n	%	n	%
Huye	16	7.5	198	92.5
Gisagara	11	5.1	203	94.9
Total	27	6.3%	401	93.7

Table 2 above shows the prevalence of *Plasmodium falciparum* using conventional PCR. The prevalence of *P. falciparum* is 92.5% in Huye district while it is 94.9 in Gisagara district.

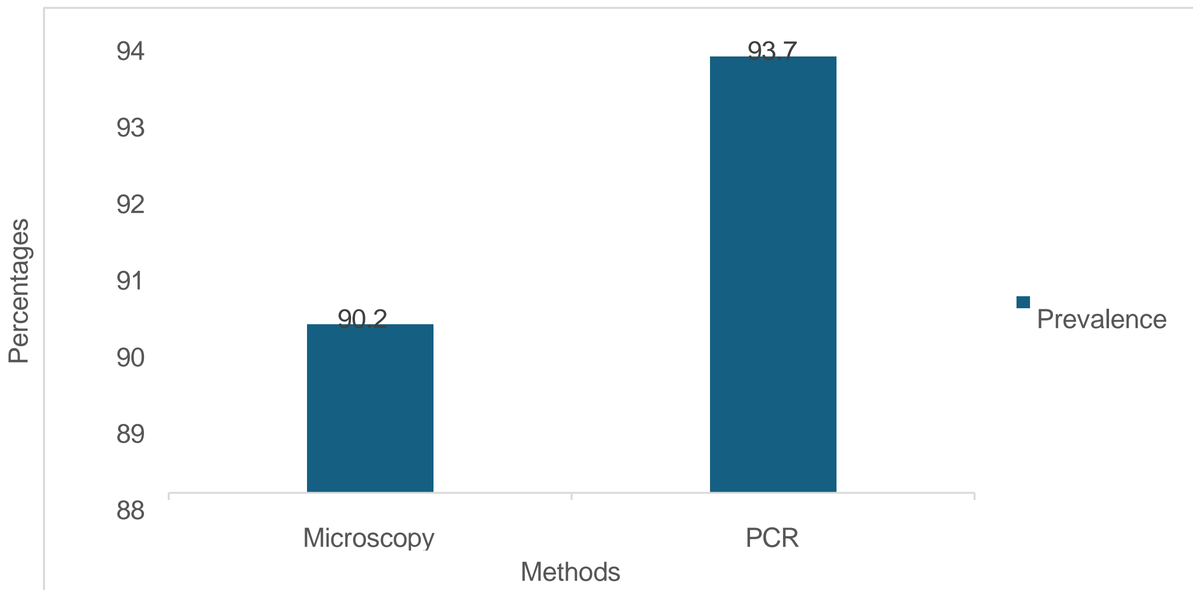

Figure 7: Overall *Plasmodium falciparum* prevalence comparison between Microscopy and Conventional PCR

The figure 7 above shows the overall prevalence of *P. falciparum* comparison between Microscopy and conventional PCR. Conventional PCR showed the overall prevalence of 93.7% while microscopy detection shows 90.2% prevalence of *Plasmodium falciparum.*

4.1.2 Sensitivity and Specificity of Microscopy compared to PCR as a reference method

Table 3: Diagnostic evaluation of microscopy against conventional PCR as reference method

Test characteristics	Microscopy evaluation with PCR as reference method	
	Value	95% CI
True Negative	23 (54.76%)	39.95% – 68.78%
False Negative	4 (1.04%)	0.40% – 2.63%
True positive	382 (98.96%)	[97.37 – 99.60]
False Positive	19 (45.24%)	31.22% – 60.05%
Sensitivity	95.26%	[92.70 - 97.12]
Specificity	85.19%	[66.27 - 95.81]
Positive likelihood ratio	6.43	[2.60 - 15.89]
Negative likelihood ratio	0.06	[0.03 - 0.09]
Positive predictive value	98.96%	[97.48 - 99.58]
Negative predictive value	54.76%	[43.16 - 65.86]
Accuracy	94.63%	[92.05 - 96.56]

Table 3 above present the diagnostic characteristics of microscopy with conventional PCR as method of reference. According to the findings, true negative results were 23 (54.76%), 19 (45.24%) false positive, 382 (98.96%) true positive, and 4 (1.04%) false negative. The findings showed the sensitivity of 95.26% [92.70% - 97.12%] and specificity of 85.19 [66.27% - 95.81%]. The positive likelihood ratio was 6.43 [2.60 - 15.89] while negative likelihood ratio was 0.06 [0.03 - 0.09]. The positive predictive value was 98.96 [97.48% - 99.58%] with Negative predictive value of 54.76% [43.16% - 65.86%]. The accuracy was found to be 94.63% [92.05% - 96.56%].

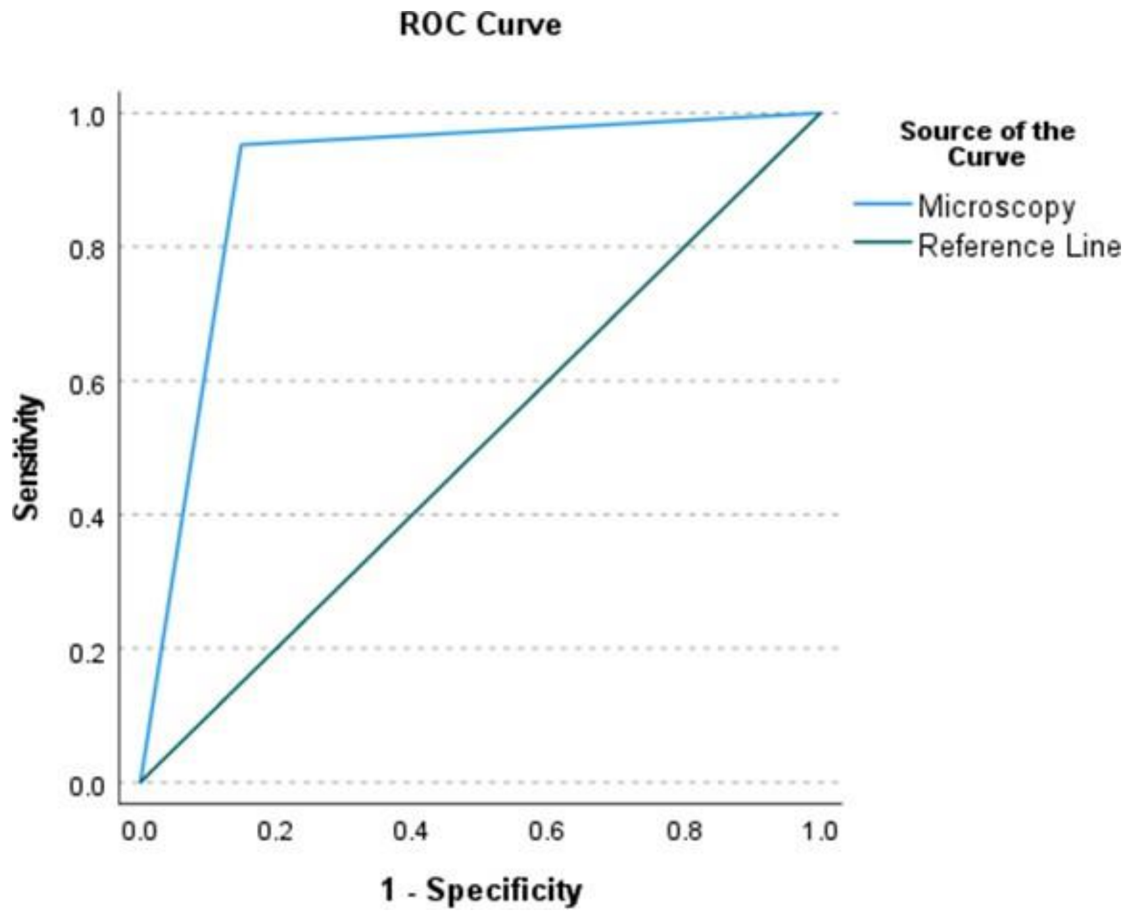

Figure 8: Receiver operating characteristics (ROC) for Malaria microscopy versus PCR as reference methods

Table 4: Area Under the ROC Curve (AUC) for Microscopy in detecting *Plasmodium falciparum* using PCR as the reference method

Test variable	Area under the curve	Std. Error	CI 95%	P value
Microscopy	0.902	0.040	0.823 – 0.981	**<0.001**
PCR	0.769	0.049	0.672 – 0.865	**<0.001**

The ROC analysis showed that Microscopy had an (area under the curve) AUC of 0.902 (95% CI: 0.823–0.981), indicating excellent diagnostic accuracy in detecting *P. falciparum* compared to PCR. The result was statistically significant ($p < 0.001$).

The ROC analysis of PCR showed a moderate to good diagnostic performance, with an AUC of 0.769. This suggests that the test correctly classifies cases about 76.9% of the results confirmed with microscopy. The confidence interval (0.672–0.865) is wider and includes values in the "fair" range, indicating more variability in performance. However, the result statistically significant, as shown by the p-value (<0.001).

4.1.3 Concordance between microscopy and conventional PCR results for *P. falciparum*

Table 5: Cohen's Kappa statistic showing agreement between Microscopy and PCR results for *Plasmodium falciparum* detection.

Measure	Value	Std. Error	Approx. T	CI 95%	P value
Kappa	0.639	0.069	13.601	0.504 – 0.774	<0.001

The Cohen's Kappa statistic was 0.639, indicating a substantial level of agreement between Microscopy and PCR results for the detection of *Plasmodium falciparum*. The 95% confidence interval ranged from 0.504 to 0.774, suggesting that the true agreement lies between moderate and substantial. The result was statistically significant ($p < 0.001$), confirming that the observed agreement is unlikely to be due to chance. This supports the reliability of Microscopy as a diagnostic tool, although PCR remains the reference standard.

CHAPTER FIVE: DISCUSSION

The comparative study of microscopy and conventional PCR for the detection *of Plasmodium falciparum* revealed a consistently high prevalence of infection in both Huye and Gisagara districts. Microscopy detected a prevalence of 89.7% in Huye and 90.7% in Gisagara, with an overall prevalence of 90.2%. Conventional PCR, which is a more sensitive molecular method, showed slightly higher prevalence rates of 92.5% in Huye and 94.9% in Gisagara, resulting in an overall prevalence of 93.7%. The higher detection rates by PCR indicate its superior ability to identify low-density parasitemia that might be missed by microscopy, reinforcing its role as the reference standard.

When microscopy was evaluated against PCR, the diagnostic performance was strong. Microscopy achieved a sensitivity of 95.26%, indicating that it was able to detect most of the true positive cases. Its specificity was 85.19%, showing moderate accuracy in identifying true negatives, though with some false positives. The positive predictive value was very high at 98.96%, suggesting that nearly all microscopy-positive cases were confirmed by PCR. However, the negative predictive value was relatively low at 54.76%, meaning a negative microscopy result did not reliably rule out infection. The overall diagnostic accuracy of microscopy was 94.63%, confirming its usefulness in field settings, particularly in areas of high malaria transmission In line with other research, this study demonstrated that microscopy performance may be influenced by microscopist expertise and the parasite density when PCR is used as a reference method (Muyidi *et al.*, 2025).

Further analysis using ROC curve demonstrated that microscopy had an area under the curve (AUC) of 0.902, with a 95% confidence interval of 0.823 to 0.981 and a p-value of less than

0.001. This indicates excellent diagnostic ability in distinguishing between infected and uninfected individuals when PCR is used as the gold standard. The high AUC supports microscopy's role as a practical and effective diagnostic approach, especially in resource-constrained environments but also depended on expertise of microscopists. The concordance between microscopy and PCR was assessed using Cohen's Kappa statistic, which yielded a value of 0.639. This corresponds to substantial agreement between the two diagnostic methods. The 95% confidence interval ranged from 0.504 to 0.774, suggesting that agreement could range from moderate to substantial. The statistically significant p-value (<0.001) confirms that this agreement is unlikely to be due to chance.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

This study indicated that PCR diagnosis showed superior sensitivity and accuracy compared to microscopy despite the fact that microscopy showed relatively low negative predictive value and presence of false negatives. However, because of its easy use and affordability, the continued use of microscopy in malaria diagnosis with supplemental diagnostic methods like PCR is highly fruitful.

In addition, continuous training of microscopists in endemic areas, especially in settings where detecting all cases, including submicroscopic infections, is critical. In nutshell, the findings of this study support a diagnostic strategy that integrates microscopy with PCR to enhance malaria detection and control efforts in endemic regions.

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APPENDICES

PCR protocol for *Plasmodium falciparum* Kelch-13 propeller domain with <u>Solis Biodyne</u> reagents. Adapted by Welmoed van Loon, 2023

Primers:

Primer name	Outer/Inner PCR	Sequence (5' – 3')
K13_F_in	Inner PCR	GCCAAGCTGCCATTCATTTG
K13_R_in		GCCTTGTTGAAAGAAGCAGA

Inner PCR Mastermix:

Reagent	Stock conc.	Final conc.	Vol.x1	Vol.x*N*
Nuclease-free ddH2O	-	-	13.4 µL	
B1 PCR Buffer (Solis BioDyne)	10X	1X	2.0 µL	
MgCl2	25 mM	2.5 mM	2.0 µL	
dNTP (each)	10 mM	0.2 mM	0.4 µL	
HotStart FirePOL (Solis BioDyne)	5 U/µL	1 U	0.2 µL	
Primer – K13_F_in	10 µM	0.25 µM	0.5 µL	
Primer – K13_R_in	10 µM	0.25 µM	0.5 µL	
Total			**19.0 µL**	
DNA template (=outer PCR product)			1.0 µl	

Inner PCR cycling conditions:

Cycle	Temp (°C)	Time	No. of cycles
Initial Denaturation	95	10 min	1
Denaturation	94	30 sec	
Annealing	59	90 sec	
Extension	72	45 sec	40
Final extension	72	10 min	1
Cooling	15	Unlimited	1

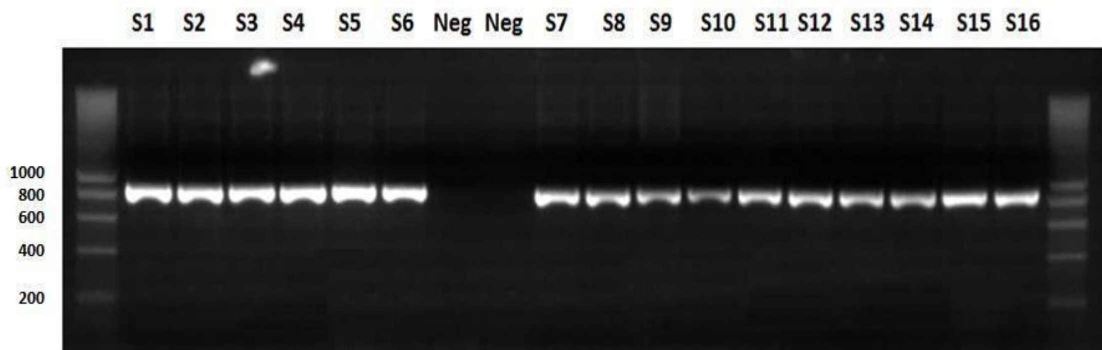
Product size inner PCR: 849 bp

Gel electrophoresis, sequencing, storage:

Load 5 µL (+1 µL loading dye) on 1% agarose gel, include a 100 bp ladder, run for approx. 1 hour at 100-150 V.

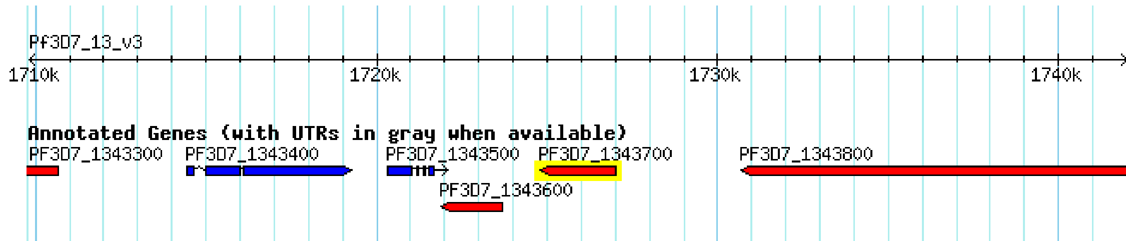
Ensure successful PCR amplification

Store at 4°C up to 1 week, or at -20°C to -80°C for long-term storage.



3D7 sequence of PF3D7_1343700 Kelch-13 protein propeller domain

P. falciparum 3D7 protein coding gene on Pf3D7_13_v3 from 1,724,817 to 1,726,997 (Chromosome: 13)



Below: 3D7 complete genome sequences flanking K13 candidate marker SNPs, outer PCR primers in yellow, inner PCR primers in green. >gi|124513603|ref|XM_001350122.1| *P. falciparum* 3D7 kelch protein, putative (PF13_0238) mRNA, complete cds

ATGGAAGGAGAAAAAGTAAAAACAAAAGCAAATAGTATCTCGAATTTTTCTATGACGTATGAT
AGGGAATCTGGTGGTAACAGCAATAGTGATGATAAAAGCGGAAGTAGTAGCGAGAATGATTCT
AATTCATTTATGAATCTAACTAGTGATAAAAATGAGAAAACGGAAAATAATAGTTTCCTTTTAA
ATAATAGTAGTTATGGAAATGTTAAAGATAGCCTATTAGAATCCATTGATATGAGTGTATTAGA
TTCGAACTTTGATAGTAAAAAAGATTTTTTACCAAGTAATTTATCAAGAACATTTAATAATATGT
CTAAAGATAATATAGGAAATAAATATTTAAATAAATTGTTAAATAAAAAAAAAGATACTATTA
CAAATGAAAATAATAATATTATCATAATAATAATAATAATAATCTGACAGCAAATAATATAACT
AATAATCTTATTAATAATAATATGAATTCTCCATCAATTATGAATACCAACAAAAAAGAGAATT
TTTTAGATGCAGCAAATCTTATAAATGATGATTCTGGATTAAACAATTTAAAAAAATTTTCAACT
GTAAATAATGTAAATGATACTTATGAAAAGAAAATTATTGAAACGGAATTAAGTGATGCTAGTGA
TTTTGAAAATATGGTAGGTGATTTAAGAATTACATTTATTAATTGGTTAAAAAAGACACAAATG
AATTTTATTCGAGAAAAAGATAAATTATTTAAAGATAAGAAAGAACTAGAAATGGAAAGAGTA
CGATTGTACAAAGAATTAGAAAACCGTAAAAATATTGAAGAACAGAAATTACATGATGAAAGA
AAGAAATTAGATATTGATATATCTAATGGTTATAAACAAATAAAAAAAGAAAAAGAAGAACAT
AGGAAACGATTTGATGAAGAAAGATTAAGATTTTTACAAGAAATCGATAAAATTAAATTAGTA
TTATATTTAGAAAAAGAAAAATATTATCAAGAATATAAAAATTTTGAGAATGATAAAAAAAAA
ATTGTTGATGCAAATATTGCTACTGAAACTATGATTGATATTAATGTTGGTGGAGCTATTTTTGA
AACATCTAGACATACCTTAACACAACAAAAAGATTCATTTATAGAGAAATTATTAAGTGGAAG
ACATCATGTAACCAGAGATAAACAAGGAAGAATATTCTTAGATAGGGATAGTGAGTTATTTAG
AATTATACTTAACTTCTTAAGAAATCCGTTAACTATACCCATACCAAAAGATTTAAGTGAAAGT
GAAGCCTTGTTGAAAGAAGCAGAATTTTATGGTATTAAATTTTTACCATTCCCATTAGTATTTTG
TATAGGTGGATTTGATGGTGTAGAATATTTAAATTCGATGGAATTATTAGATATTAGTCAACAA
TGCTGGCGTATGTGTACACCTATGTCTACCAAAAAAGCTTATTTTGGAAGTGCTGTATTGAATA

ATTTCTTATACGTTTTTGGTGGTAATAACTATGATTATAAGGCTTTATTTGAAACTGAGGTGTAT
GATCGTTTAAGAGATGTATGGTATGTTTCAAGTAATTTAAATATACCTAGAAGAAATAATTGTG
GTGTTACGTCAAATGGTAGAATTTATTGTATTGGGGGATATGATGGCTCTTCTATTATACCGAAT
GTAGAAGCATATGATCATCGTATGAAAGCATGGGTAGAGGTGGCACCTTTGAATACCCCTAGAT
CATCAGCTATGTGTGTTGCTTTTGATAATAAAATTTATGTCATTGGTGGAACTAATGGTGAGAG
ATTAAATTCTATTGAAGTATATGAAGAAAAAATGAATAAATGGGAACAATTTCCATATGCCTTA
TTAGAAGCTAGAAGTTCAGGAGCAGCTTTTAATTACCTTAATCAAATATATGTTGTTGGAGGTA
TTGATAATGAACATAACATATTAGATTCCGTTGAACAATATCAACCATTTAATAAAAGATGGCA
ATTTCTAAATGGTGTACCAGAGAAAAAAATGAATTTTGGAGCTGCCACATTGTCAGATTCTTAT
ATAATTACAGGAGGAGAAAATGGCGAAGTTCTAAATTCATGTCATTTCTTTTCACCAGATACAA
ATGAATGGCAGCTTGGCCCATCTTTATTAGTTCCCAGATTTGGTCACTCCGTTTTAATAG
CAAATATAT

CLINICAL EDUCATION AND RESEARCH DIVISION
DIRECTORATE: RESEARCH -ETHICS COMMITTEE **Huye,21st, July,2025**

RESEARCH

Approval Notice: No: REC/CHUB/091/2025

Janvier NDINKABANDI

Email:ndinj12@gmail.com

Reference is made to your letter requesting ethical clearance for **"Comparative study of microscopy and conventional PCR for plasmodium falciparum identification in patient samples from Gisagara and Huye, Southern province"** Having reviewed your application, project proposal, been satisfied with existing ARMEA project protocol and it's approval number: RNEC/548/2024, 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 for fulfilling the following requirements:

- Changes, amendments and addenda to the protocol or consent form must be submitted to the committee for review and approval before 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

COLLEGE OF SCIENCE AND TECHNOLOGY

SCHOOL OF SCIENCE

Date: 15th July 2025.

Dear Sir/Madam,

TO WHOM IT MAY CONCERN

I the Undersigned, Prof, Antoine NSABIMANA hereby confirm that the research proposal entitled "Comparative study of microscopy and conventional PCR for *plasmodium falciparum* identification in patient samples from Gisagara and Huye, Southern province, RWANDA for Mr. Janvier NDINKABANDI (Reg.No 219015180) has been approved by the Department of biology.

The study involves data collection from participants and/or departments within CHUB, Additionally, we request your support in **facilitating the data collection process** once ethical clearance is granted and therefore, He will submit his protocol to your committee for ethical review and approval at the institutional level.

Sincerely,

Prof Antoine NSABIMANA(PhD)

Program coordinator of MSc in Biotechnology (CMHS, CAFF, CVAS and CST)