UNIVERSITY OF RWANDA

COMPARATIVE EVALUATION OF MALARIA PREVALENCE USING LIGHT MICROSCOPY AND QUANTITATIVE PCR IN SELECTED HEALTH CENTERS OF SOUTHERN PROVINCE, RWANDA.

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SAMANTHA IKIREZI

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By: SAMANTHA IKIREZI Ref.Number:216095689

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Supervisor: Prof. Jeanne Primitive UYISENGA

Co-Supervisors: Prof. Jean Claude TWIZERE

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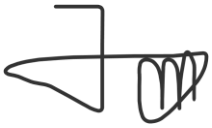
DECLARATION

I**, Samantha Ikirezi,** hereby declare that this research project submitted to the University of Rwanda, Rwanda for the degree Master of Science in Biotechnology is my own original work and has not been submitted before to any Institution by myself or any other person in fulfilment of the requirements to the award of any degree or any other qualification.

Samantha Ikirezi, 216095605



Date: July,2025

DEDICATION

This work is sincerely dedicated to my beloved family, whose constant support, prayers, and encouragement have sustained me throughout this academic journey. I am deeply grateful to God for the strength, wisdom, and grace that guided me through every step of this work. To my mentors and supervisors, your guidance and belief in my potential have made this achievement possible. And to every student or researcher working quietly behind the scenes to contribute to science and technology in Africa, this is for you.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACRONYM	FULL MEANING
AL	Lysis Buffer (Buffer AL)
AW1, AW2	Wash Buffers 1 and 2
CDC	Centers for Disease Control and Prevention
CHW	Community Health Workers
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
IC	Internal Control
IRS	Indoor Residual Spraying
ITN	Insecticide-Treated Net
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RDT	Rapid Diagnostic Test

RT-PCR	**Reverse Transcription Polymerase Chain Reaction**
SPSS	**Statistical Package for the Social Sciences**
WHO	**World Health Organization**
CHUB	**Centre Hospitalier Universitaire de Butare**
CRF	**Case Report Form**
LM	**Light Microscopy**
LLINs	**Long-Lasting Insecticidal Nets**
PBO	**Piperonyl Butoxide**
RBC	**Rwanda Biomedical Centre**
SD	**Standard Deviation**

ABSTRACT

Background: Rwanda is one of the sub-Saharan African countries where malaria remains endemic. Over the past decade, the country experienced a sharp increase in malaria incidence, from 48 cases per 1,000 population in 2012 to 403 cases per 1,000 in 2018. Despite this trend, there is limited literature on the distribution of *Plasmodium* species in Rwanda. This study assessed the prevalence of *Plasmodium* species among malaria-positive patients in two health centers, Byimana and Rwaniro, in Southern Rwanda.

Methodology: A total of 508 participants were screened using light microscopy and q RT-PCR using the RealStar® Malaria Screen & Type PCR Kit 1.0. The detection rates and species distribution were compared across both diagnostic methods.

Results: Of the 508 samples analyzed, q RT PCR detected malaria in 114 participants (22%), while light microscopy identified 94 positive cases (19%). PCR-based species identification revealed *P. falciparum* in 97 cases (85.09%), followed by *P. malariae* in 15 cases (13.16%), *P. vivax* in 7 cases (6.14%), and *P. ovale* in 6 cases (5.26%). Light microscopy identified *P. falciparum* in 91 of the 94 positive cases (96.8%) and *P. malariae* in 4 cases (4.26%). No cases of *P. vivax* or *P. ovale* were detected by microscopy.

Conclusion: This study provides updated insights into the distribution of *Plasmodium* species in Southern Rwanda. *P. falciparum* remains the predominant species across both diagnostic methods. However, the higher detection rate and broader species coverage by q RT PCR highlight the limitations of microscopy alone and support the integration of molecular tools for more accurate malaria diagnosis and surveillance.

Keywords: Malaria, Prevalence, q RT- PCR, Plasmodium falciparum dominance, Southern Rwanda.

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CHAPTER 1: INTRODUCTION

1.1 Background of Study

Malaria remains a paramount global public health challenge. In 2022, an estimated 249 million cases occurred across 85 endemic countries, with the World Health Organization (WHO) African Region accounting for an overwhelming 94% of this burden (WHO, 2023). This landscape underscores the urgent need for robust and adaptive control strategies, particularly in sub-Saharan Africa, the epicenter of global malaria transmission (Kubana et al., 2023).

Rwanda, a nation within the highly endemic region, has made significant strides in terms of fighting against malaria. However, the country's journey has been marked by dramatic fluctuations in disease incidence. After a period of successful control, malaria incidence increased more than eight-fold, from 48 cases per 1,000 population in 2012 to a peak of 403 cases per 1,000 in 2018. While recent intensified efforts contributed to a reduction to approximately 43 cases per 1,000 in the 2022-2023 period, this resurgence highlights the fragility of control gains (Karema et al., 2020) (Rubuga et al., 2024).

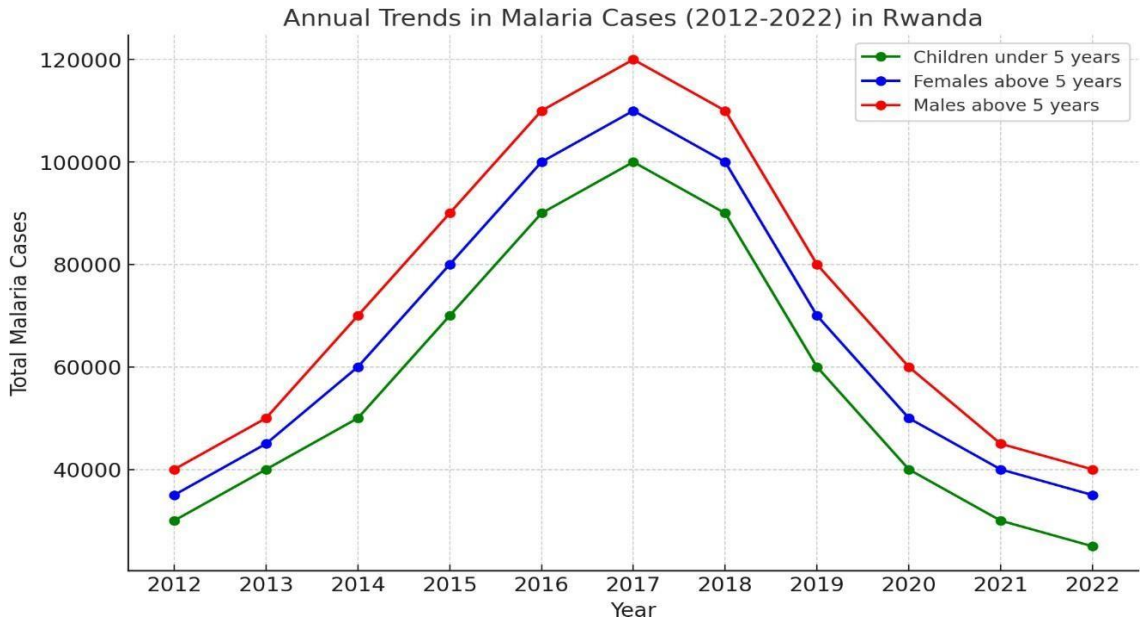

Figure 1: Annual trends in reported malaria cases in Rwanda from 2012 to 2022, stratified by three demographic groups.

reflects the national trend in malaria burden, with case counts peaking between 2015 and 2017 across all demographic groups. This aligns with Rwanda's reported rise in incidence from 48 to 403 cases per 1,000 population between 2012 and 2018. Following intensified control efforts, both incidence and case numbers declined significantly, reaching their lowest levels by 2022. The graph visually highlights this pattern and emphasizes the impact and vulnerability of malaria control progress (Rubuga et al., 2024).

Malaria transmission in Rwanda is highly heterogeneous and seasonal, with peaks typically occurring in April-May and November-December. Geographically, the lower-altitude Eastern and Southern Provinces are the most affected. Notably, districts in the Southern Province, including Huye, Gisagara, Nyanza, and Muhanga, have persistently represented a significant portion of the national malaria burden (Kubana et al., 2023) (Gaither et al., 2024).

In 2014, this province accounted for 38% of all cases, and as recently as 2022, districts such as Muhanga, Nyaruguru, Gisagara and Nyamagabe recorded high incidence rates of 73,91,100 and 111 cases per 1,000 individuals, respectively, far exceeding the national average (Kubana et al., 2023) (Umugwaneza et al., 2025). The communities selected for this study, Rwaniro and Byimana, are situated within this high-burden context.

The primary vector of malaria in Rwanda is Anopheles gambiae sensu, and the secondary vectors are Anopheles funestus and Anopheles arabiensis, with P. falciparum being the most common malaria species in Rwanda (Kubana et al., 2023). Although data on non-falciparum malaria in Rwanda are limited, one study conducted in highland southern Rwanda found that Plasmodium falciparum accounted for 90.7 % of infections among 259 patients, Plasmodium vivax (P.vivax) was seen in 8.1% of patients, Plasmodium malariae (P. malariae) in 11.6%, and Plasmodium ovale (P. ovale) in 5.0% (van Loon et al., 2023). Another study that used real-time PCR to detect four Plasmodium species among 4595 mainly adult participants in the 2014–2015 Demographic and Health Survey found an overall prevalence of 23.6 %, with P. falciparum and non-falciparum infections at 17.6 % and 8.3 %, respectively (Gaither et al., 2024).

While malaria remains a significant global health burden, particularly in sub-Saharan Africa, recent data on the prevalence and species distribution of *Plasmodium* in Rwanda reveal a complex and evolving landscape of malaria transmission. Studies conducted across different regions of the country have consistently highlighted *Plasmodium falciparum* as the most prevalent species, in line with regional trends observed throughout sub-Saharan Africa. Despite considerable progress in malaria control through interventions such as insecticide-treated nets and indoor residual spraying, the resurgence of malaria in specific hot spots, including the Southern Province, continues to present challenges. Previous research has pointed to the limitations of conventional diagnostic methods, such as light microscopy, which cannot detect submicroscopic and non-falciparum infections that may sustain undetected transmission.

This study aims to address these diagnostic gaps by comparing the performance of light microscopy and quantitative PCR (qPCR) in detecting malaria infections in the Southern Province of Rwanda, with a focus on both microscopic and submicroscopic infections. By directly

comparing these diagnostic techniques, this research contributes to the growing body of knowledge on malaria detection in Rwanda. The findings will provide valuable evidence regarding the sensitivity and accuracy of molecular diagnostics, which is essential for informing future surveillance strategies and refining control interventions in endemic regions. This study is particularly relevant for regions where malaria prevalence remains a significant public health concern.

1.2 Problem Statement

The national malaria control strategy in Rwanda relies heavily on light microscopy (LM) and Rapid Diagnostic Tests (RDTs) for surveillance and case management. However, these methods are constrained by a detection limit of 50-100 parasites/μL, leading to two critical diagnostic gaps:

1.2.1 The Submicroscopic Reservoir: A significant proportion of infections, particularly in asymptomatic individuals in endemic settings, exist at densities below the detection threshold of microscopy. These low-density, submicroscopic infections constitute a silent, infectious reservoir that sustains transmission and undermines control efforts (van Eijk et al., 2023).

1.2.2 Under-detection of Species Diversity: The accurate identification of non-falciparum species is challenging with microscopy, especially in mixed infections where P. falciparum may predominate. This lack of speciation data hinders the deployment of appropriate treatment regimens and provides an incomplete picture of malaria epidemiology.

Therefore, the true prevalence of malaria, including the contribution of both submicroscopic infections and non-falciparum species in hotspots in Rwanda, remains poorly quantified. This lack of high-resolution data poses a significant obstacle to design targeted interventions necessary for achieving pre-elimination malaria. The highly sensitive method for malaria detection is required for timely defective management of the disease.

1.3 Justification of the Study

This research is designed to address the diagnostic gaps and is justified by its potential public health and scientific impact. By employing a highly sensitive molecular method (qPCR) alongside

Conventional microscopy, this study will provide a more accurate and comprehensive assessment of malaria prevalence and species distribution in the high-burden settings of Rwaniro and Byimana. The findings will furnish the Rwanda Biomedical Centre (RBC) and local health partners with crucial evidence to:

- Quantify the true parasite reservoir, including the hidden burden of submicroscopic infections.
- Understand the local prevalence of non-falciparum species to guide diagnostic and treatment policies.
- Refine surveillance strategies and better target resources to interrupt residual transmission.

Scientifically, this study will contribute valuable, context-specific data on the comparative performance of microscopy and molecular diagnostics in a region of moderate-to-high endemicity, enriching the evidence base for malaria control in sub-Saharan Africa.

1.4 Research Questions

1. What is the prevalence and species distribution of Plasmodium infections in Rwaniro and Byimana as detected by light microscopy?
2. What is the prevalence and species distribution of Plasmodium infections in the same communities as detected by the highly sensitive quantitative Polymerase Chain Reaction (qPCR) assay?
3. What is the magnitude of submicroscopic and non-falciparum infections missed by routine microscopy in these settings?

1.5 Objectives

1.5.1 Main Objective

To assess the prevalence of plasmodium species in malaria-positive patients in two health centers (Byimana and Rwaniro) in Southern Rwanda using light microscopy (LM) and q RT-PCR

1.5.2 Specific Objectives

1) To determine the prevalence and species distribution of *Plasmodium* infections in malaria-positive patients from Byimana and Rwaniro health centers using light microscopy (LM).
2) To determine the prevalence and species distribution of *Plasmodium* infections in the same study population using the qRT-PCR.
3) To compare the diagnostic accuracy of light microscopy and qRT-PCR in detecting *Plasmodium* species.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Malaria in human is caused by Protozoan parasites of the genus Plasmodium. Infected female Anopheles mosquitoes transmit the parasite exclusively through their bite (Fikadu & Ashenafi, 2023). The parasite's complex lifecycle requires both the mosquito for sexual reproduction and a human host for asexual replication (Nureye & Assefa, 2020).

The cycle begins when an infected mosquito injects sporozoites into the human bloodstream. These sporozoites travel to the liver and initiate clinically silent exo-erythrocytic (liver) stage, where they multiply into thousands of merozoites (Milner, 2018). When the liver cells rupture, they release merozoites into the bloodstream. This event initiates the symptomatic erythrocytic (blood) stage. Merozoites invade red blood cells, multiply, and rupture them in a cycle that causes fever and anemia. After several cycles, some parasites differentiate into male and female gametocytes (Real & Mancio-Silva, 2022). The transmission cycle concludes when a non-infected mosquito ingests these gametocytes during a blood meal, eventually becoming infectious to another human (Nightingale et al., 2018).

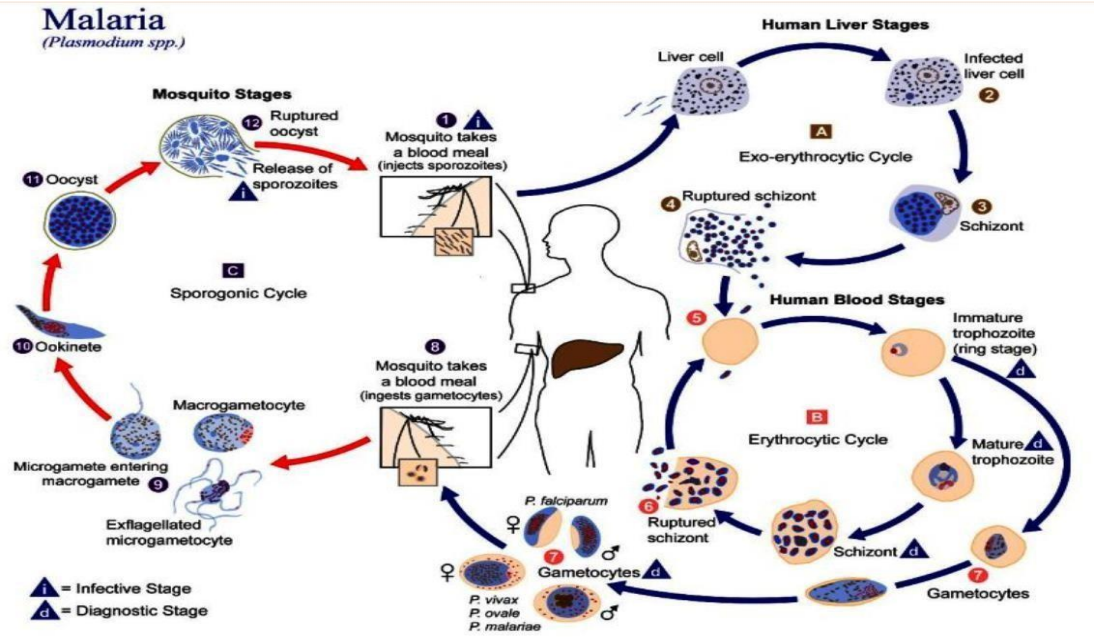

Figure 2: The Lifecycle and Transmission Cycle of the Plasmodium Parasite. The diagram illustrates the key human organs (liver and blood) and mosquito stages, clearly showing the points of transmission between the two hosts (Makler & Gibbins, 1991)**.**

2.2 The Global and Regional Burden of Malaria

Malaria remains one of the most significant infectious diseases of our time. The World Health Organization 2023 World Malaria Report documented a persistent and substantial global burden, detailing an estimated 249 million cases and 608,000 deaths in 2022. The WHO African Region continues to be the epicenter of this crisis. It shoulders a staggering 94% of cases and 95% of deaths. Four countries within this region, Nigeria, the Democratic Republic of Congo, Uganda, and Mozambique, accounted for nearly half of all malaria deaths globally (WHO, 2023) .

This immense burden persists despite decades of investment and the widespread deployment of effective control measures (Code, n.d.). A confluence of biological and environmental threats further complicates progress. These threats include the spread of insecticide resistance in mosquito vectors and the emergence of parasite strains with HRP2/3 gene deletions that evade detection by common rapid diagnostic tests (RDTs) (Fitri et al., 2022). This complex global landscape necessitates a move away from one-size-fits-all strategies towards data-driven, locally adapted

control programs.

2.3 Malaria in Rwanda: A Tale of Progress and Persistent Challenges

Rwanda's national malaria control program serves as a public health success story. By leveraging strong political will and an integrated health system, the country has made remarkable strides against the disease.

2.3.1 The Era of Successful Control (2005-2012)

Between 2005 and 2012, Rwanda achieved one of the most dramatic reductions in malaria burden ever recorded in Africa. The aggressive, universal scale-up of core interventions drove this success. Mass campaigns for long-lasting insecticidal nets (LLINs) reached near-universal coverage, and the program implemented robust indoor residual spraying (IRS) campaigns in high-burden districts (Fullman et al., 2013). Concurrently, the health system strengthened through the introduction of community health workers (CHWs) equipped to test and treat uncomplicated malaria. This approach drastically reduced delays in malaria treatment (Cross, 2010). These integrated efforts resulted in a reduction of over 85% in malaria incidence and mortality, demonstrating the profound impact of well-executed control programs (Tambo et al., 2012).

2.3.2 The Post-2012 Resurgence and Epidemiological Shift

A concerning resurgence of malaria (Bizimana & Nduwayezu, 2021) marked the period following this success, with incidence rising more than eight-fold between 2012 and 2018 (Hakizimana et al., 2016). This reversal highlighted the fragility of the gains and the complex epidemiology of residual malaria. Researchers have implicated several factors in this resurgence. These include the waning of population immunity following years of low exposure and potential shifts in vector behavior. Critically, studies in Rwanda have confirmed the emergence of high levels of pyrethroid resistance in the primary vector, Anopheles gambiae, a development that undermines the efficacy of standard ITNs (Hakizimana et al., 2016). Significant spatial heterogeneity continues to characterize transmission, with the warmer, lower-altitude Eastern and Southern Provinces serving as persistent hotspots (Dao et al., 2023). The study sites for this thesis, Rwaniro and Byimana, are in this Southern Province, a region that continues to challenge the national goal of pre-elimination.

2.4 Core Malaria Prevention and Control Strategies

The global strategy to combat malaria integrates prevention, diagnosis, and treatment.

2.4.1 Vector Control in Depth: ITNs, IRS, and the Challenge of Resistance

Vector control is the primary method of malaria prevention. It aims to reduce human-mosquito contact. The two main interventions are Insecticide-Treated Nets (ITNs) and Indoor Residual Spraying (IRS) (Fullman et al., 2013). The effectiveness of these tools, however, is threatened by widespread insecticide resistance. Pyrethroids, the class of insecticide historically used on all ITNs, are now less effective against many vector populations (Sougoufara et al., 2020). This challenge has driven the development and deployment of next-generation nets. These include nets co-treated with the synergist piperonyl butoxide (PBO) or with novel classes of insecticides. Countries like Rwanda are now scaling up these new tools to combat resistance (Rulisa et al., 2023).

2.4.2 Chemoprevention and the New Era of Vaccination

Chemoprevention involves administering antimalarial drugs to prevent infection in vulnerable groups (Runge et al., 2023). The recent rollout of the world's first malaria vaccines, RTS, S/AS01 and R21/Matrix- M, marks a historic milestone (Nambatya et al., 2025). These vaccines primarily target the circumsporozoite protein on the surface of the sporozoite, aiming to prevent the parasite from establishing an infection in the liver (Littmann et al., 2024). The WHO recommends their use in children living in moderate-to-high transmission areas (Greenwood et al., 2021). These vaccines are a powerful new tool to be used in conjunction with existing interventions (Kwambai et al., 2020).

2.5 Diagnostic Methods for Malaria: The Foundation of Control

Accurate diagnosis is the bedrock of malaria control. It influences individual patient care, clinical outcomes, public health surveillance, and the strategic deployment of resources (Oyegoke et al., 2022). The two principal methods this thesis evaluates, light microscopy and qPCR, represent two distinct eras of diagnostic technology. Each has a unique profile of strengths and operational limitations (Gitta & Kilian, 2020).

2.5.1 Light Microscopy: The Enduring, but Imperfect, Gold Standard

For over a century, the microscopic examination of a Giemsa-stained blood smear has served as the operational gold standard. Its enduring utility in resource-limited settings is due to its low consumable cost and its rich diagnostic output (Members, 2021). A skilled microscopist using a well-prepared slide can perform two crucial functions: species identification, which is critical for guiding treatment, and quantification of parasitemia, which is essential for assessing disease severity (Mpina et al., 2022). The methodology relies on two types of smears. The thick smear concentrates parasites for sensitive screening, while the thin smear preserves cell morphology for definitive species identification (Jan et al., 2018).

Despite these advantages, microscopy's performance is critically dependent on the skill of the technician, and quality assurance is a major operational challenge. Its most significant limitation is its analytical sensitivity (Das et al., 2021). Under ideal laboratory conditions, its detection limit is approximately 50 parasites/µL of blood. In routine field settings, this can be closer to 100 parasites/µL or higher. This sensitivity threshold means that microscopy systematically misses low-density, submicroscopic infections, creating a major gap in disease surveillance (Maturana et al., 2022).

2.5.2 Molecular Diagnostics: Unveiling the Hidden Reservoir with qPCR

The advent of real-time quantitative PCR (qPCR) has revolutionized malaria epidemiology and now serves as the reference standard for research studies. Unlike microscopy, which detects whole parasites, PCR-based methods detect and amplify specific sequences of parasite DNA (Hofmann et al., 2018).

The RealStar® Malaria PCR kit, like many modern qPCR assays, targets a highly conserved region of the parasite's 18S ribosomal RNA (rRNA) gene (Batista-Dos-Santos et al., 2018). This gene is an ideal target because it exists in multiple copies within the parasite genome. This feature provides a naturally amplified signal that significantly enhances the assay's sensitivity. This molecular approach offers unparalleled analytical sensitivity, achieving a limit of detection often between 0.1 and 5 parasites/µL. This represents a 100-fold or greater improvement over expert microscopy.

The quantitative aspect of qPCR provides another layer of valuable data. The instrument measures fluorescence in real-time as it amplifies the DNA. The resulting cycle threshold (Ct) value is inversely proportional to the amount of starting target DNA (Ola S. H. Almusaddar & Cyuzuzo Callixte,

2021). A low Ct value therefore indicates a high parasite density, while a high Ct value indicates a low density. This allows for an objective, semi-quantitative estimation of parasitemia. The primary disadvantages of qPCR, including its cost and complexity, have historically limited its use to research and reference laboratories (Britton et al., 2016).

2.6 The Challenge of Submicroscopic Malaria

Submicroscopic malaria presents a substantial challenge to malaria control and elimination efforts, primarily due to its asymptomatic nature and the limitations of conventional diagnostic methods. Traditional techniques, such as microscopy and rapid diagnostic tests (RDTs), are ineffective at detecting low-density infections, which are common in areas with low or declining transmission (Whittaker et al., 2021). These infections, although asymptomatic, can sustain malaria transmission by serving as a reservoir for gametocytes, thus contributing to onward transmission. The inability to identify such infections underlines a critical gap in current diagnostic capabilities (Nyenke, 2024). Furthermore, submicroscopic infections are often undetected in regions with declining transmission, hindering accurate assessments of transmission intensity. Consequently, the failure to address these hidden reservoirs may undermine malaria control programs, particularly in settings aiming for disease elimination (Telfils et al., 2024).

The prevalence of submicroscopic malaria varies geographically and seasonally, adding complexity to efforts to monitor and control its spread. In low-transmission areas, submicroscopic infections are more common and contribute significantly to the infectious reservoir, despite being asymptomatic (van Eijk et al., 2023). These variations in prevalence are influenced by factors such as historical transmission intensity, local malaria control strategies, and diagnostic limitations. Effective malaria elimination strategies must account for these dynamics by integrating sensitive molecular diagnostic methods and robust surveillance systems to detect and monitor submicroscopic infections (Bahati et al., 2020). Moreover, understanding the role of submicroscopic infections in transmission dynamics is essential for refining control interventions. Addressing these challenges requires tailored diagnostic tools, improved surveillance frameworks, and targeted intervention strategies to effectively reduce the burden of malaria in both endemic andeliminationcontext

CHAPTER 3: METHODOLOGY

3.1 Study Design and Area

This study employed a cross-sectional diagnostic accuracy design, aimed at comparing the performance of light microscopy and the q RT PCR in detecting Plasmodium species among febrile patients. The research was conducted at two health centers (Byimana and Rwaniro) located in the malaria-endemic Southern Province of Rwanda. Both facilities serve rural communities with a high malaria burden and provide routine diagnostic services, including microscopy.

3.2 Study Population

The study population consists of patients consulting at Byimana and Rwaniro Health Center and presenting with clinical symptoms suggestive of malaria, such as fever, chills, fatigue, and nausea. Individuals were recruited consecutively from outpatient consultations at the two selected health centers.

Inclusion Criteria:

Participants were eligible if they met the following inclusion criteria:

- Aged 5 years or older
- Presenting with signs and symptoms indicative of malaria
- Willing to provide informed consent (or assent for minors with guardian consent)

Exclusion Criteria:

Patients exhibiting signs of severe illness or symptoms consistent with central nervous system infections, as defined by WHO guidelines, were excluded to ensure participant safety and compliance with study procedures (Hatz, 2001).

3.3 Sample Size Determination

Our sample size was determined using the standard formula for diagnostic accuracy studies

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

, with a base calculation of 227 participants needed for 18% prevalence and 5% precision. We then applied a design effect of 1.5 to account for clustering at two Health centers and added 20% to account for potential sample failures or incomplete testing.

This resulted in approximately 500 participants, which we rounded to 508 to ensure equal distribution between sites (256 and 252). This sample size provides us with an expected 90+ malaria-positive cases, giving us adequate power to estimate sensitivity within ±10% and specificity within ±5%, while also being logistically feasible for the study sites.

3.4 Participant Recruitment and Data Collection

Potential participants were identified by healthcare personnel during their routine clinical visits. After providing a full explanation of the study, participants who agreed to take part signed informed consent documents. A structured case report form (CRF) was used to collect demographic data (such as age and sex), symptom history, recent medication use, and prior malaria diagnosis. These CRFs were also used to document diagnostic test results.

3.5 Sample Collection and Handling

Two types of blood specimens were collected from each participant:

- Capillary Blood: Approximately 50 µL was obtained via finger prick. This sample was used to prepare thick and thin smears on glass slides for light microscopy. The slides were stained using 10% Giemsa solution and air-dried before microscopic evaluation.
- Venous Blood: A 4 mL sample was collected into EDTA tubes through venipuncture. Tubes were stored in cooler boxes containing ice packs and temperature monitors, ensuring transport at 2 to 8°C to the central laboratory at CHUB for molecular testing.

3.6 Diagnostic Testing Procedures

3.6.1 Laboratory workflow

Table 1: Microscopic Diagnosis of Malaria Parasites

Step	Procedure	Description
1	Slide Preparation (Thick/Thin)	I prepared both thick and thin blood smears on clean glass slides.
2	Air Drying	The smears were air-dried in a horizontal position.

3	Fixation	I fixed the thin smear using absolute methanol for 30 seconds.
4	Staining	Both smears were stained using 10% Giemsa solution for 10 minutes.
5	Washing and drying	After staining, the slides were gently rinsed with buffered water and air-dried.
6	Microscopic Examination	I examined the smears under oil immersion at 100× magnification. Parasite species and stages were identified morphologically.

Table 2: DNA Extraction Using QIAamp® DNA Blood Mini Kit

Step	Procedure	Description
1	Sample Volume	I used 200 µL of whole blood collected in EDTA tubes for each extraction.
2	Lysis and Digestion	20 µL proteinase K and 200 µL Buffer AL were added to each sample. The mixture was incubated at 56°C for 10 minutes.
3	Precipitation	I added 200 µL absolute ethanol and mixed by pulse vortexing.
4	Column Binding	The lysate was transferred to QIAamp spin columns and centrifuged.
5	Washing Steps	I performed two washes using Buffer AW1 and AW2 respectively, with centrifugation after each wash.
6	Final Spin	A high-speed spin at 14,000 rpm was done to remove residual ethanol.

7	DNA Elution	Purified DNA was eluted in 100 µL of pre-warmed Buffer AE and stored at −20°C.

Table 3: Real-Time PCR Detection of Plasmodium spp. Using RealStar® Kit1.0

Step	Procedure	Description
1	Internal Control Addition	1 µL of internal control was added to each sample before extraction to monitor inhibition.
2	Master Mix Preparation	two master mixes were prepared: o **Pf/Pv Mix:** for *P. falciparum* and *P. vivax* o • **Pk/Pm/Po Mix:** for *P. knowlesi*, *P. malariae*, and *P. ovale*
3	Reaction Setup	Each 30 µL reaction contained 20 µL master mix and 10 µL extracted DNA. Positive and negative controls were included.
4	Thermal Cycling	PCR was run on a Bio-Rad CFX96™ using the kit's protocol: 95°C for 10 min; 45 cycles of 95°C for 15 sec, and 60°C for 60 sec.
5	Primers and Targets	The assay used species-specific primers targeting the conserved **18S rRNA gene**. Primer sequences were proprietary to the manufacturer.

6	Fluorescence Detection Channels	The amplification was monitored using the following channels: ○ **FAM**: *P. falciparum*, *P. malariae* ○ **Cy5**: *P. vivax*, *P. ovale* ○ **ROX**: *P. knowlesi* ○ • **JOE/VIC**: Internal Control
7	Result Interpretation	The sample was considered positive based on Ct ≤ 40 . Mixed infections were confirmed by simultaneous amplification in multiple fluorophore channels.

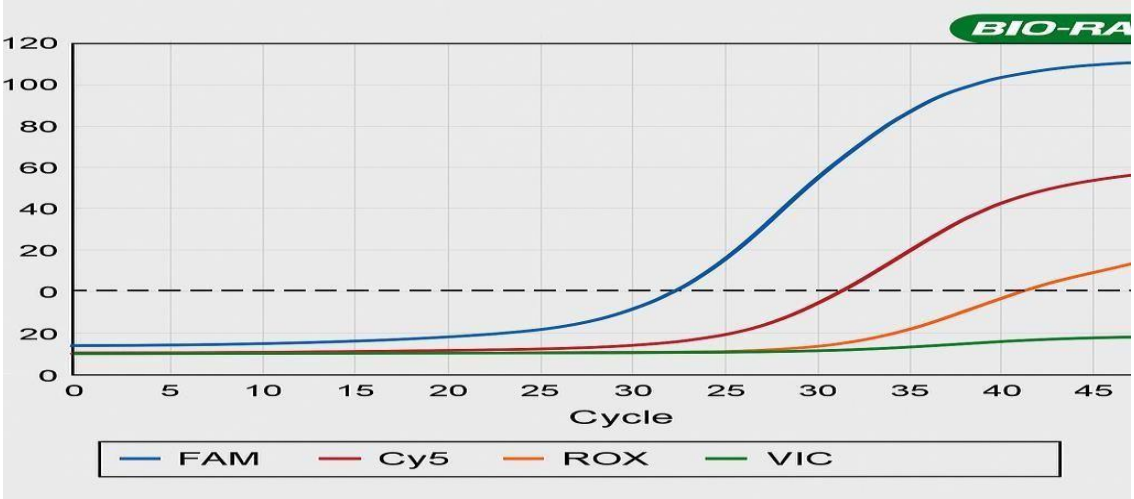

Figure 3: Amplification Plot of Study Samples Using Bio-Rad CFX96 Real-Time PCR System

This amplification plot represents results from the current study, showing positive detection of Plasmodium falciparum and P. vivax in selected blood samples. The fluorescence curves reflect target-specific amplification using the RealStar® Malaria PCR Kit. Internal control amplification was successful in all samples, confirming the absence of PCR inhibition.

The graph displays amplification curves for different fluorescent dye channels used in qPCR: FAM (blue) for detecting the target sequence, Cy5 (red) for another target, ROX (orange) as a passive reference dye for normalization, and VIC (green) for an additional target. The dashed line represents the threshold for detectable fluorescence, marking the start of the exponential amplification phase.

3.7 Statistical Analysis

All data were entered into OpenClinica, a secure electronic data capture platform. Before entry, datasets were carefully checked for accuracy and completeness. Descriptive statistics, including means, standard deviations, frequencies, and proportions, were used to summarize participant sociodemographic and clinical characteristics. Diagnostic performance of light microscopy was evaluated against qRT-PCR, which served as the reference standard. Since the dataset did not include PCR-negative samples, only sensitivity was calculated for each *Plasmodium* species.Sensitivity was calculated with 95% confidence intervals using Wilson's score method. All analyses were performed using SPSS.

3.8 Ethical Considerations

Ethical approval for this study was obtained from the Rwanda National Ethics Committee (Reference No. 683/RNEC/2022) and the Rwanda Food and Drugs Authority (Ref No. 023/CTAC/FDA/2023). Written informed consent was obtained from all adult participants, and assent was secured for minors along with guardian consent. A research identification was given to each sample to assure the confidentiality of participants and used solely for research purposes. All procedures were conducted in accordance with the Declaration of Helsinki and Rwandan regulations on research on human subjects.

CHAPTER 4: RESULTS

4.1 Clinical characteristics of study participants

A total of 508 participants were enrolled in the study, with 325 (64%) females and 183 (36%) males. The participants' ages ranged from 5 to 93 years, with a mean age of 32 (SD = 15), with most (97%) being adults aged 18 years and above. Nearly all participants (99%) reported a history of malaria, with an average of 26 months since their most recent diagnosis. Recent anti-malarial drug use was rare, with only one participant reporting intake in the four weeks preceding the study. Similarly, only 3% took other medications or vitamins during that period.

The average body temperature recorded was 38°C (SD = 0.72), consistent with febrile illness, the most reported symptoms were headache and chills (97%), followed by fatigue (96%), fever (93%) and nausea (82%). Less common symptoms included abdominal pain, conjunctival redness and cough, while vomiting, dysuria, diarrhea and rash were reported in fewer than 25% of participants.

Table 4: clinical characteristics of study participants

Variable	Number of Participants	Percentage (%) / Mean
Total Participants	508	
Sex		
Male	183	36%
Female	325	64%
Age (Categorical)		

Mean Age		Mean = 32, SD = 15
<12	8	2%
12-17	1	<1%
≥18	499	97%
Previous Malaria Diagnosis		
Yes	504	99%
No	4	1%
Months Since Latest Diagnosis	504	Mean = 26, SD = 32
Anti-malarial Drugs in the Preceding 4-week Period		
Yes	1	<1%
No	507	>99%
Other Medicines or Vitamins		
Yes	17	3%
No	491	97%
Temperature	508	Mean = 38°C, SD = 0.72
Symptoms		
Abdominal Pain	235	46%
Sweats	364	72%
Dizziness	373	73%

Chills	494	97%
Nausea	415	82%
Stomach-ache	95	19%
Fatigue	488	96%
Fever	470	93%
Cough	147	29%
Dysuria	34	7%
Headache	494	97%
Diarrhea	25	5%
Vomiting	129	25%
Rash	12	2%
Conjunctival Redness	228	45%

4.2 Diagnostic results

The Quantitative RT-PCR test detected 114 positive cases while LM detected 94 cases. Thus, q RT-PCR detected 20 more positive malaria cases than light microscopy, corresponding to a 4% absolute increase in detection rate. The chi-square test revealed a statistically significant difference ($p = 0.03$), indicating that the PCR assay has a higher sensitivity in detecting malaria infections compared to expert light microscopy. This supports the use of molecular diagnostics for improved surveillance and accurate case identification in endemic settings like Rwanda.

Table 5: Comparison of Malaria Detection by Light Microscopy and q RT- PCR.

Test Method	Positive Cases (n)	% of Total Cases (N = 508)	Difference in Detection	p-value
Light Microscopy (LM)	94	18.5%	—	—
Quantitative RT-PCR	114	22.4%	+4% (n = 20)	0.03

4.3 Species identification by using Light Microscopy

Out of 94 malaria-positive cases detected by light microscope, the major prevalent species was *P. falciparum* (91 cases, 96.8%). Four cases (4.26%) contained *P. Malariae*, and there were no cases of *P. vivax* and *P. ovale* identified on the Light Microscopy.

Table 6: Light microscopy positive results by species

Species	Positive cases (n=94)	Percent of Positive Cases (n = 94)
P. falciparum	91	96.8%
P. malariae	4	4.26%
P. vivax	0	-
P. ovale	0	-

4.4 Identification of Species using Quantitative RT-PCR

Among the 114 malaria-positive cases identified with the RealStar® Malaria Screen & Type PCR Kit 1.0, *P. falciparum* was the most prevalent species, detected in 97 cases (85.1 %), followed by P. malariae (15 cases, 13.16 %), P. vivax (7 cases, 6.14 %), and P. ovale (6 cases, 5.26 %).

Table 7: Quantitative RT-Positive Results by Species

Species	Absolute Number	Percent of Positive Cases (n = 114)
P. falciparum	97	85.09%
P. malariae	15	13.16%
P. vivax	7	6.14%
P. ovale	6	5.26%

4.5 Diagnostic Performance of Light Microscopy Compared to qRT-PCR for Plasmodium Detection

The diagnostic performance of light microscopy was evaluated against quantitative real-time PCR (qRT-PCR), which served as the reference standard in this study. Sensitivity was assessed as the primary accuracy metric because only PCR-positive cases were available in the dataset. The absence of PCR-negative samples limited the ability to compute specificity, predictive values, or Cohen's kappa (κ) coefficient, which typically require both true positives and true negatives.

Microscopy demonstrated high sensitivity in detecting *Plasmodium falciparum*, identifying 91 out of 97 qRT-PCR-confirmed cases (93.8%). However, its performance was significantly lower for non-falciparum species. Only 4 out of 15 *P. malariae* cases were detected microscopically (26.7%), and none of the *P. vivax* (n=7) or *P. ovale* (n=6) infections were identified by microscopy. Overall,

microscopy detected 82% of all PCR-confirmed cases but exhibited a strong bias toward *P. falciparum* detection. These findings reinforce the limitations of microscopy in areas where mixed or non-falciparum infections may occur and underscore the importance of incorporating molecular diagnostic tools in routine malaria surveillance.

Table 8: Sensitivity of Light Microscopy for Plasmodium Species Detection Compared to qRT-PCR

Plasmodium Species	**PCR Positive (n)**	**Microscopy Positive (n)**	**Sensitivity (%)**
P. falciparum	**97**	**91**	**93.8%**
P. malariae	**15**	**4**	**26.7%**
P. vivax	**7**	**0**	**0.0%**
P. ovale	**6**	**0**	**0.0%**

CHAPTER 5: DISCUSSION

5.1 Prevalence and Species Distribution by Light Microscopy

The first objective of this study was to determine the prevalence and species distribution of *Plasmodium* infections using the conventional method of light microscopy. Our results showed an overall malaria prevalence of 18.5% in the study population. The species analysis revealed a near monolithic presence of Plasmodium falciparum, which was identified in 91 of the 94 positive cases (96.8%). A minor fraction of infections were identified as P. malariae (4 cases, 4.26%), while no cases of *P. vivax* or *P. ovale* were detected.

This overwhelming dominance of *P. falciparum* is consistent with the well-established epidemiology of malaria across sub-Saharan Africa, where it accounts for the most infections. This trend is driven by a combination of factors, including the region's tropical climate which supports the year-round breeding of the *Anopheles* vector, and the intrinsic biological advantages of *P. falciparum*, such as its rapid replication and ability to evade the host immune system (Snow et al., 2017). Our findings align strongly with regional studies, such as research in Mali that reported *P. falciparum* in 96.77% of symptomatic cases, and broader reviews from East Africa confirming its prevalence at over 90%. This consistency validates our microscopy results and situates them firmly within the known malaria landscape of the continent.

5.2 Prevalence and Species Distribution by qPCR

The second objective sought to determine prevalence and species distribution using a more sensitive molecular method, qPCR. This assay revealed a higher malaria prevalence of 22.4% (114 positive cases) and painted a more complex epidemiological picture. While P. falciparum was still the most common species, found in 97 cases (85.1%), qPCR also uncovered a significant and previously hidden burden of non-falciparum infections namely. P. malariae in 15 cases (13.16%), P. vivax in 7 cases (6.14%), and P. ovale in 6 cases (5.26%).

The lower relative percentage of *P. falciparum* found by qPCR (85.1% vs. 96.8% by LM) does not imply lower diagnostic capability. On the contrary, it demonstrates the superior ability of qPCR to

identify co-circulating non-falciparum species and mixed infections that are invisible to microscopy, especially at low parasite densities. The detection of these other species is of great public health significance. for instance, *P. malariae* can cause chronic, long-lasting infections, while the presence of *P. vivax* and *P. ovale* is particularly critical, as these species form dormant liver-stage hypnozoites that can lead to relapsing malaria weeks or months after an initial infection. These relapses contribute to ongoing morbidity and transmission, undermining elimination efforts if not properly diagnosed and treated.

5.3 Comparative Diagnostic Performance of Light Microscopy and qPCR

The third objective was to directly compare the diagnostic performance of light microscopy against qPCR, using the latter as the reference standard. The results provide clear, quantitative evidence of the limitations of microscopy in this setting. Overall, qPCR detected 20 more positive cases than microscopy, an absolute increase of 4% in the detection rate that was statistically significant ($p = 0.03$).

A deeper analysis of sensitivity revealed a stark diagnostic bias. Microscopy performed well for P. falciparum, detecting 91 of the 97 PCR-confirmed cases, yielding a sensitivity of 93.8%. However, its performance collapsed for non-falciparum species. The sensitivity for P. malariae was a mere 26.7%, and it completely failed to identify any infections of P. vivax or P. ovale.

The results of our research showed the diagnostic gap due to the relying on microscopy alone. While it remains a useful tool for detecting high-density *P. falciparum* infections, it is profoundly unreliable for comprehensive malaria surveillance in areas where other species co-circulate. This limitation results in a significant under-reporting of the true malaria burden and a mischaracterization of its species diversity. As acknowledged in our study design, the absence of a true-negative cohort prevented the calculation of specificity or predictive values. However, the sensitivity data alone is compelling enough to advocate for the integration of molecular diagnostics into routine surveillance to ensure that all malaria infections, regardless of species or density, are detected.

5.4 Contextualizing Findings and Public Health Implications for Rwanda

While the 19–22% malaria prevalence found in our study seems high, it must be interpreted within the context of Rwanda's extraordinary national success against the disease. Rwanda achieved a remarkable 86% reduction in malaria incidence between 2016 and 2023, a testament to its robust control program built on widespread LLIN distribution, targeted IRS, and a strong community health worker network.

Our findings, therefore, do not contradict this national success but rather highlight a critical challenge in the final push toward elimination: the existence of residual transmission hotspots. The high prevalence in the Southern Province suggests that while national strategies have been effective, localized areas persist where transmission remains intense. Addressing these hotspots requires a more nuanced, data-driven approach.

The results of this study provide a clear path forward. To achieve its 2030 malaria elimination goal, Rwanda must complement its existing strategies with enhanced surveillance capabilities. This study provides strong evidence that integrating highly sensitive molecular tools like qPCR is essential to uncover the hidden burden of submicroscopic and non-falciparum infections that fuel transmission in these hotspots. By strengthening diagnostic capacity, particularly in high-burden zones, Rwanda can move from broad control to targeted, precision-based public health interventions, ensuring that no infection is left behind.

CHAPTER 6: RECOMMENDATION & CONCLUSION

Malaria remains a persistent and complex public health challenge in Rwanda, particularly in the Southern Province. This study sets out to assess the prevalence of Plasmodium species among malaria-positive patients at BYIMANA and RWANIRO health centers, and to compare the diagnostic performance of light microscopy with quantitative PCR (qPCR).

The findings reveal that while Plasmodium falciparum is the most prevalent species, accounting for 96% of cases detected by microscopy, this traditional method falls short in identifying non-falciparum and low-density infections. qPCR, on the other hand, uncovered a broader spectrum of Plasmodium species, including P. malariae, P. vivax, and P. ovale, as well as mixed and submicroscopic infections that microscopy often misses. These results highlight the limitations of relying solely on microscopy and underscore the value of integrating molecular diagnostics into routine malaria surveillance.

By providing a more accurate and comprehensive picture of malaria transmission, this research contributes important evidence to guide Rwanda's malaria control strategies. The study demonstrates that advancing diagnostic methods is essential for effective disease management and for achieving the national goal of malaria elimination.

6.2 RECOMMENDATIONS

Based on the study's findings, the following recommendations are proposed:

1. Integrate Molecular Diagnostics: The Ministry of Health should prioritize the adoption of qPCR and other molecular tools in routine malaria surveillance to improve detection of all Plasmodium species, especially those missed by microscopy.
2. Enhance Training and Capacity Building: Continuous training for healthcare workers, particularly in rural and high-burden areas, is crucial to ensure accurate diagnosis and effective use of advanced diagnostic technologies.
3. Strengthen Targeted Interventions: Focus malaria control efforts in identified hotspots like the Southern Province, expanding vector control measures and supporting community health workers in early detection and treatment.
4. Monitor All Malaria Species: Establish surveillance systems that track non-falciparum species, as these can influence treatment protocols and prevention strategies.
5. Empower Community Health Workers: Equip community health workers with improved diagnostic tools and ongoing support to enhance malaria control at the grassroots level.

6.3 Limitations

While this study provides valuable insights, several limitations should be acknowledged:

1. **Sample Size and Scope:** The research was limited to two health centers in the Southern Province, which may not fully represent malaria trends across Rwanda. A larger, multi-site study would provide a more comprehensive national perspective.

2. **Diagnostic Specificity:** The study could not assess the specificity of microscopy due to the absence of PCR-negative samples. Future research should include both positive and negative cases to evaluate diagnostic accuracy more thoroughly.
3. **Variability in Microscopy:** The accuracy of light microscopy depends on the skill of the technician and the quality of blood smears, which can lead to underreporting of infections, especially at low parasite densities.
4. **Complexity of Mixed Infections:** Although qPCR improved detection of mixed infections, accurately identifying and quantifying these cases remains challenging and warrants further methodological development.

6.4 Final Thoughts

This study underscores the urgent need for innovation in malaria diagnosis and control in Rwanda. By comparing light microscopy and qPCR, it becomes clear that embracing molecular diagnostics is not just an option, but a necessity for capturing the true burden of malaria. Investing in advanced diagnostics, building local capacity, and empowering communities will be key to closing the detection gap and accelerating progress toward malaria elimination.

As Rwanda moves forward, a commitment to evidence-based strategies and continuous improvement will be essential. With the right tools and targeted interventions, the vision of a malaria-free Rwanda by 2030 is within reach. This research stands as a call to action for policymakers, health professionals, and communities to work together for a healthier, malaria-free future.

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