

2026-08

Prevalence, Diagnostic Performance, and Associated Factors of Plasmodium Infections among Individuals with Presumptive Malaria in Northwest Ethiopia

Desale, Sisay

<http://ir.bdu.edu.et/handle/123456789/17477>

Downloaded from DSpace Repository, DSpace Institution's institutional repository



BAHIR DAR UNIVERSITY
COLLEGE OF MEDICINE AND HEALTH SCIENCES
SCHOOL OF MEDICAL LABORATORY SCIENCES EDUCATION AND
SERVICE
DEPARTMENT OF MEDICAL PARASITOLOGY AND ENTOMOLOGY
EDUCATION AND SERVICE

**PREVALENCE, DIAGNOSTIC PERFORMANCE, AND ASSOCIATED
FACTORS OF *PLASMODIUM* INFECTIONS AMONG INDIVIDUALS
WITH PRESUMPTIVE MALARIA IN NORTHWEST ETHIOPIA**

BY: SISAY DESALE (MSc candidate)



August 2026

Bahir Dar, Ethiopia

BAHIR DAR UNIVERSITY
College of Medicine and Health Sciences
School of Medical Laboratory Sciences Education and Service
Department of Medical Parasitology and Entomology Education and Service

**Prevalence, Diagnostic Performance, and Associated Factors of
Plasmodium Infections among Individuals with Presumptive
Malaria in Northwest Ethiopia**

BY: Sisay Desale (MSc Candidate)

Advisors:

Tadesse Hailu (PhD, Associate Professor)

Animen Ayehu (MSc, Assistant Professor)

A thesis submitted to the Department of Medical Parasitology and Entomology Education and Service, School of Medical Laboratory Sciences, College of Medicine and Health Sciences, Bahir Dar University, in partial fulfillment of the requirements for the degree of Master of Science in Medical Parasitology and Vector Control.

BAHIR DAR UNIVERSITY
College of Medicine and Health Sciences
School of Medical Laboratory Sciences Education and Service
Department of Medical Parasitology and Entomology Education and Service

Advisors' approval for thesis defense

We hereby certify that this thesis titled “**Prevalence, Diagnostic Performance, and Associated Factors of *Plasmodium* Infections among Individuals with Presumptive Malaria in Northwest Ethiopia,**” submitted by Sisay Desale for the master’s degree in medical Parasitology and Vector Control, was carried out under our supervision, and the thesis has not been previously submitted in part or in full for any degree or diploma of this or any other university.

Advisors' name	Signature	Date
1. _____	_____	_____
2. _____	_____	_____

DECLARATION

I, the undersigned Medical Parasitology and Vector Control student, declare that this thesis is my original work and has not been presented for a degree at any other university, and all sources of material used for this thesis have been properly acknowledged.

Name: Sisay Desale

Signature_____

Date_____

Place of submission:Department of Medical Parasitology and Entomology, School of Medical Laboratory Science Education and Service, College of Medicine and Health Sciences, Bahir Dar University.

Date of submission: _____

Examiner(s)

	Name	Signature	Date
1.	_____	_____	
2.	_____	_____	_____

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors, Dr. Tadesse Hailu and Mr. Animen Ayehu. Their guidance, constructive feedback, and support throughout this study were invaluable. I am also grateful to Dr. Mulat Yimer and Dr. Banchamlak Tegegn for their support during the laboratory work and logistical aspects of my MSc research. My sincere appreciation also goes to the Department of Medical Parasitology and Entomology Education and Service, School of Medical Laboratory Sciences Education and Service, College of Medicine and Health Sciences, Bahir Dar University for providing me with the opportunity and academic environment to undertake this research granted me ethical approval. I also equally thank Wollo University for sponsoring me.

I am also deeply grateful to the Amhara Public Health Institute for providing a letter of support, laboratory facilities, reagents, extraction kits, and technical support throughout the laboratory work. In addition, I extend my deepest gratitude to the Armauer Hansen Research Institute (AHRI) for providing laboratory facilities, materials, and reagents essential to this research. My sincere thanks also go to the malaria and NTD laboratory staff at AHRI for their technical assistance, expertise, and willingness to support me, particularly during nPCR, qPCR, and gel electrophoresis procedures.

Finally, I would like to express my sincere appreciation to the district health offices and the laboratory professionals of the selected health institutions for their cooperation and support during data and specimen collection. I am also indebted to all study participants for their willingness to participate and provide the information and specimens required for this study.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	II
TABLE OF CONTENTS.....	III
LIST OF TABLES.....	V
LIST OF FIGURES	VI
ACRONYMS/ ABBREVIATIONS	VII
ABSTRACT.....	VIII
1. INTRODUCTION.....	1
1.1 BACKGROUND	1
1.2 STATEMENT OF THE PROBLEM	3
1.3 SIGNIFICANCE OF THE STUDY	5
2. LITERATURE REVIEW	6
3. OBJECTIVES OF THE STUDY	13
3.1 GENERAL OBJECTIVE	13
3.2 SPECIFIC OBJECTIVES	13
4. MATERIALS AND METHODS	14
4.1 STUDY AREA.....	14
4.2 STUDY DESIGN AND PERIOD	15
4.3. POPULATION	15
4.3.1 SOURCE POPULATION.....	15
4.3.2 STUDY POPULATION.....	15
4.3.3 STUDY PARTICIPANTS	16
4.4 INCLUSION AND EXCLUSION CRITERIA	16
4.4.1 INCLUSION CRITERIA	16
4.5 SAMPLE SIZE DETERMINATION AND SAMPLING TECHNIQUES	16
4.5.1 SAMPLE SIZE DETERMINATION	16
4.5.2 SAMPLING TECHNIQUE AND PROCEDURE.....	19
4.6 VARIABLES OF STUDY	20
4.6.1 DEPENDENT VARIABLES	20

4.6.2 INDEPENDENT VARIABLES	20
4.6.3 OPERATIONAL DEFINITIONS	21
4.7 DATA COLLECTION AND LABORATORY PROCEDURES	21
4.7.1 SOCIODEMOGRAPHIC, BEHAVIORAL, AND ENVIRONMENTAL DATA	21
4.7.2 BLOOD SAMPLE COLLECTION	21
4.7.3 THICK AND THIN BLOOD FILM PREPARATION AND MICROSCOPY	22
4.7.4 RAPID DIAGNOSTIC TEST BIOCREDIT MALARIA AG PF/PV (PLDH/PLDH)	22
4.7.5 DNA EXTRACTION FROM DBS.....	23
4.7.6 DNA AMPLIFICATION AND DETECTION	23
4.8 QUALITY ASSURANCE	26
4.9 DATA MANAGEMENT AND ANALYSIS.....	27
4.10 ETHICAL CONSIDERATIONS.....	27
5. RESULTS.....	28
6. DISCUSSION.....	39
7. CONCLUSION AND RECOMMENDATIONS	45
7.1 CONCLUSION	45
7.2 RECOMMENDATIONS	46
8. REFERENCES	48
9. ANNEXES.....	60

LIST OF TABLES

Table 1: Proportional allocation of sample size across selected health facilities in Northwest Ethiopia, 2026	19
Table 2: Primers and probes used for the molecular detection and speciation of Plasmodium species	25
Table 3: Sociodemographic Characteristics of Malaria-Suspected Individuals (N=410) in Northwest Ethiopia, 2026.	28
Table 4: Concordant and Discordant Diagnostic Outcomes among RDT, Microscopy, and PCR in the PCR-Tested Subsample (n = 220), Northwest Ethiopia, 2026.	34
Table 5: Diagnostic Performance and Agreement Between RDT, Microscopy, and PCR	35
Table 6: Sensitivity of RDT and Microscopy across qPCR-Determined Parasite Burden Categories for <i>P. falciparum</i> and <i>P. vivax</i> , Northwest Ethiopia, 2026.	35
Table 7: Factors Associated with Plasmodium species infection Based on the Composite Reference (RDT, Microscope and PCR) Standard (N = 410), Northwest Ethiopia, 2026.	37

LIST OF FIGURES

Figure 1: Location of the study area map in Northwest Ethiopia.....	15
Figure 2: Malaria species distribution by RDT, Light Microscope, and PCR among malaria-suspected individuals in Northwest Ethiopia, 2026.	30
Figure 3: Distribution of <i>Plasmodium</i> species and total prevalence in each district across the Study Sites, northwest Ethiopia, 2026.	31
Figure 4: Correlation Between Microscopy-Derived Parasite Density and qPCR-Derived Parasite Burden for <i>P. falciparum</i> and <i>P. vivax</i> infections, Northwest Ethiopia, 2026.	33

ACRONYMS/ ABBREVIATIONS

- ✓ ACTArtemisinin-Based Combination Therapy
- ✓ AHRIArmauer Hansen Research Institute
- ✓ AOR Adjusted Odds Ratio
- ✓ APHI Amhara Public Health Institute
- ✓ CI Confidence Interval
- ✓ COR Crude Odds Ratio
- ✓ DBS Dried Blood Spot
- ✓ DNA Deoxyribonucleic Acid
- ✓ EPHI Ethiopian Public Health Institute
- ✓ FAM 6-Carboxyfluorescein
- ✓ IQR Interquartile Range
- ✓ IRB Institutional Review Board
- ✓ IRS Indoor Residual Spraying
- ✓ ITN Insecticide-Treated Net
- ✓ PCR Polymerase Chain Reaction
- ✓ pLDH*Plasmodium*lactate dehydrogenase
- ✓ qPCR Quantitative Polymerase Chain Reaction
- ✓ RDT Rapid Diagnostic Test
- ✓ RPM Revolutions Per Minute
- ✓ rRNA Ribosomal Ribonucleic Acid
- ✓ SD Standard Deviation
- ✓ SOP Standard Operating Procedure
- ✓ SPSS Statistical Package for the Social Sciences
- ✓ TAE Tris-Acetate-EDTA
- ✓ WHO World Health Organization

ABSTRACT

Background: Malaria remains a major public health problem in Ethiopia, including the Amhara Region. Although *Plasmodium falciparum* and *Plasmodium vivax* predominate, shifts in their relative distribution and recent reports of *Plasmodium ovale* indicate changing epidemiology. However, updated data on prevalence, species distribution, diagnostic performance, and associated factors remain limited. Routine diagnostics often miss low-density and mixed infections due to their low sensitivity. As well, the application of PCR is limited in the study area.

Objective: The main aim of this study was to assess the prevalence, species distribution, diagnostic performance, and factors associated with *Plasmodium* infections among malaria-suspected individuals in Northwest Ethiopia.

Methods: A health facility-based cross-sectional study was conducted from February to April 2026 in four study sites in Northwest Ethiopia. A total of 410 malaria-suspected individuals were enrolled using systematic random sampling. Sociodemographic, behavioral, and environmental data were collected using a structured questionnaire. A capillary blood sample was collected for *Plasmodium* detection and preparation of dried blood spots. Detection of *Plasmodium* was conducted using microscopy, BioCredit Malaria Pf/Pv pLDH rapid diagnostic test (RDT), and Polymerase Chain Reaction (PCR). Molecular detection and species identification were performed using multiplex real-time and nested PCR targeting the *Plasmodium* 18S rRNA gene. Data were entered using the statistical package for social science version 27 and analyzed in R version 4.5.1. Prevalence and species distribution were computed using descriptive statistics. Agreement between diagnostic methods was assessed using Cohen's kappa (κ). Logistic regression was also used to identify factors associated with *Plasmodium* infection. P -value < 0.05 in the multivariate regression was taken as statistically significant.

Results: Among 410 malaria-suspected participants, the overall prevalence of *Plasmodium* species was 36.1% (148/410); positivity by RDT and by light microscopy was 23.2% (95/410) and 25.4% (104/410), respectively. Among 220 study participants, tested by PCR 52.7% (116/220) tested positive for *Plasmodium* species. Among RDT and microscopy-positive participants, PCR identified *P. falciparum* in 32/123 (26.0%), *P. vivax* in 24/123 (19.5%), mixed *P. falciparum*/*P. vivax* infections in 33/123 (26.8%), and *P. malariae* infection in 1/123

(0.8%). PCR detected a higher proportion of *P. falciparum* and mixed *P. falciparum/P. vivax* infections than RDT and microscopy. The prevalence of *P. malariae* among 220 study participants was 1.7% (2/116). Most microscopy-positive infections were of low parasite density (60.6%; <1,000 parasites/ μ L), with median parasitemia of 2,505.0 parasites/ μ L for *P. falciparum* and 104.4 parasites/ μ L for *P. vivax*. RDT and microscopy showed substantial agreement ($\kappa = 0.688$). Sensitivity and specificity of RDT compared with microscopy were 73.1% and 93.8%, respectively. Unimproved housing wall material, proximity to surface or stagnant water, nocturnal outdoor activity, and recent travel were independently associated with increased odds of *Plasmodium* infection.

Conclusion and recommendations: Conventional diagnostic methods showed reduced sensitivity for low-density infections, while molecular methods revealed an additional *Plasmodium* species profile, including *P. malariae*, missed by conventional methods. Infection risk was associated with household, environmental, and behavioral factors. Strengthening diagnostic quality, targeted molecular surveillance, housing improvement, drying stagnant water, and site-specific vector-control interventions are recommended to support malaria control in Northwest Ethiopia.

Keywords: Diagnostic performance, Northwest Ethiopia, *Plasmodium* infections, *Plasmodium* species

1. INTRODUCTION

1.1 BACKGROUND

Malaria is a life-threatening infectious disease caused by protozoan parasites of the genus *Plasmodium*, transmitted by the bites of infected female *Anopheles* mosquitoes (WHO, 2024a). *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* are the five species that cause malaria in humans (WHO, 2025b). Of these, *P. falciparum* causes severe malaria (Alharbi and Ahmed, 2026). *P. vivax* and *P. ovale* can form dormant liver stages called hypnozoites, responsible for relapses (Huber *et al.*, 2021).

Globally, malaria remains a major public health problem, with an estimated 282 million cases and 610,000 deaths in 2024. According to the World Health Organization (WHO), the African Region accounted for 94% of global malaria cases and 95% of malaria deaths (WHO, 2025d). Persistent transmission in the region is due to climatic suitability, widespread *P. falciparum*, and efficient vectors such as *Anopheles gambiae* (Villena *et al.*, 2022). Ethiopia is also responsible for approximately 4.4% of global cases and 3.7% of global deaths (WHO, 2025d). *Plasmodium falciparum* accounted for 65–70% of infections, and *P. vivax* contributed 30–35% (WHO, 2024b). Nearly 75% of the Ethiopian landmass is considered malaria-endemic, and transmission is seasonal (Berhe *et al.*, 2019, Adugna *et al.*, 2022, WHO, 2024b).

The *Plasmodium* species undergoes a complex life cycle involving both human and mosquito hosts. Transmission begins when an infected *Anopheles* mosquito injects *Plasmodium* sporozoites into the bloodstream; these travel to the liver, multiply, and release merozoites that invade red blood cells, causing the clinical symptoms of malaria. Some parasite stages differentiate into gametocytes, which a mosquito ingests during a blood meal and develops into new sporozoites, completing the cycle when the mosquito bites another person (van der Watt *et al.*, 2022).

Malaria often shows flu-like symptoms, such as high fever, chills, and headache, but its complexity extends far beyond these symptoms. A hallmark feature is malarial paroxysm. Each cycle of *Plasmodium* invasion and red cell rupture releases parasites and inflammatory

mediators, resulting in episodes. Repeated cycles progressively lead to hemolysis and anemia(Obeagu, 2024, Sanyaolu *et al.*, 2025).

In *P. falciparum*, PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein-1)-mediated cytoadherence of infected erythrocytes to the vascular endothelium leads to sequestration, microvascular obstruction, and impaired tissue perfusion. This leads not only to cerebral malaria but also to severe complications, including severe anemia, metabolic acidosis, acute respiratory distress syndrome (ARDS), acute kidney injury, and placental malaria with adverse pregnancy outcomes(White, 2022, Meena *et al.*, 2026). *Plasmodium malariae* and *P. ovale* spp, can cause significant morbidity, including nephrotic syndrome, progressive renal failure, anemia, and, more recently, severe malaria(Yman *et al.*, 2019).

The Federal Ministry of Health in Ethiopia (FMOH) recommends parasitological confirmation of all suspected malaria cases before treatment (FMOH Ethiopia., 2020, WHO, 2025c). Diagnosis primarily relies on light microscopy and rapid diagnostic tests (RDTs). Microscopy is a reference standard for species identification and parasite quantification. Although it depends on skilled personnel and quality laboratory infrastructure, RDTs are widely used in peripheral health facilities due to their simplicity and rapid turnaround. Variability in sensitivity, particularly in non-falciparum species, remains a concern (WHO, 2025c). Molecular methods, such as Polymerase Chain Reaction (PCR), have higher sensitivity and can detect low-density and mixed-species infections (Opoku Afriyie *et al.*, 2023b). However, their routine use is constrained by cost, applicability, and limited availability. Molecular diagnostics in routine surveillance, particularly in endemic areas, can improve malaria control and play a role in Ethiopia's malaria reduction and elimination goals (Martín-Díaz *et al.*, 2018).

Malaria treatment is primarily artemisinin-based combination therapy (ACT) for *P. falciparum* and chloroquine for *P. vivax*. Additionally, primaquine is used for radical cure to eliminate hypnozoites(WHO, 2025c). Key prevention strategies in Ethiopia are the use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS). Furthermore, Ethiopia has officially launched the R21/Matrix-M malaria vaccine, which is the 23rd African country to introduce a malaria vaccine. Fifty-eight (58) districts across nine regions have been included in this program (WHO, 2025a).

1.2 STATEMENT OF THE PROBLEM

Malaria remains a major public health challenge in Ethiopia, particularly in the Amhara Region, which contributes substantially to the national burden. In 2024, Ethiopia reported 7.3 million malaria cases between January and October, compared with 4.1 million cases in 2023, with the Amhara Region accounting for approximately 18% of the national burden (WHO, 2024b, Lake *et al.*, 2025). An 11-year surveillance analysis (2014–2024) reported a 12.6% annual increase in malaria incidence since 2018, with resurgence occurring in 83% of districts in the region. During this period, the proportion of *P. vivax* infections increased from 24.9% to 43%, although *P. falciparum* remained the predominant species (Lake *et al.*, 2025). These changes highlight the need for accurate identification of the *Plasmodium* species circulating in the region.

Despite this changing epidemiology, the true distribution of *Plasmodium* species in Northwest Ethiopia remains incompletely understood. In addition to *P. falciparum* and *P. vivax*, molecular studies have identified *P. ovale curtisi* and *P. ovale wallikeri*, while *P. malariae* and *P. ovale* spp. remain relatively under-investigated (Alemu *et al.*, 2013). Routine microscopy and RDTs may fail to detect low-density, mixed, or less common infections, potentially leading to underestimation of the true malaria burden and incorrect species identification.

Evidence from Ethiopia demonstrates important limitations in routine diagnosis. Microscopy detected only 8.3% of mixed *P. falciparum*/*P. vivax* infections compared with PCR in Arba Minch (Yigezu *et al.*, 2023), while sensitivity compared with qPCR was 73.8% in peripheral blood and 62.2% in placental blood in Northwest Ethiopia (Tamir *et al.*, 2025). In North Gondar, microscopy missed 16.3% of PCR-confirmed infections, including all *P. ovale* and mixed infections (Alemu *et al.*, 2013). Microscopy similarly misdiagnosed non-falciparum infections in pediatric patients regionally (Getie *et al.*, 2026). Similarly, the BIOCREDIT Pf/Pv RDT showed only 55.6% sensitivity for *P. falciparum* compared with PCR and cannot reliably distinguish *P. ovale* from *P. malariae* (Tegegne *et al.*, 2025). However, the comparative performance and agreement of microscopy, RDT, and PCR remain insufficiently evaluated in this setting.

In addition, the factors influencing *Plasmodium* infection are not well characterized. Although age, residence, insecticide-treated net use, outdoor nighttime activities, and family history of malaria have been associated with infection (Woldesenbet *et al.*, 2024), the combined effects of

socioeconomic, personal, behavioral, and environmental factors remain poorly understood. This limits the ability to identify individuals and communities at increased risk.

Therefore, there is a need for locally generated evidence on the prevalence and species distribution of *Plasmodium* infections, the performance and agreement of commonly used diagnostic methods, and the factors associated with infection among malaria-suspected individuals in Northwest Ethiopia. Such evidence is essential for improving malaria diagnosis, surveillance, and control strategies in the region.

1.3 SIGNIFICANCE OF THE STUDY

This study will generate locally relevant evidence on the prevalence, species distribution, sensitive diagnostic methods, and associated factors of *Plasmodium* infections for patients and the community, clinicians, policymakers, and researchers.

For patients and the community, the study may contribute to better recognition of malaria infections that are difficult to detect by routine diagnostic methods, particularly low-density, mixed, and less commonly reported species such as *P. ovale* and *P. malariae*. Identifying factors associated with infection may also improve understanding of individual- and community-level risks and support more appropriate malaria prevention practices. Through this, the findings will provide evidence on the comparative performance of routine diagnostics center. This may improve awareness of possible discrepancies between clinical presentation and routine diagnostic results, particularly when patients have persistent or atypical symptoms despite negative or inconclusive routine test results.

For policymakers and malaria control programs, the study may provide evidence on the current *Plasmodium* species distribution and the diagnostic challenges in Northwest Ethiopia. The findings may help inform malaria surveillance strategies, interpretation of routine diagnostic data, and decisions regarding the need for improved or complementary diagnostic approaches. Evidence on socioeconomic, personal, behavioral, and environmental factors may also help identify populations and circumstances requiring greater attention in malaria prevention and control.

For researchers and academic institutions, the study will provide baseline data on *Plasmodium* species distribution, diagnostic performance, and associated factors in the study area. The findings may serve as a reference for future molecular surveillance, epidemiological studies, diagnostic evaluations, and investigations of less commonly detected and mixed *Plasmodium* infections. The study may also identify important knowledge gaps that can guide further research in Northwest Ethiopia and other malaria-endemic areas of Ethiopia.

Overall, the study may contribute to a better understanding of the local epidemiology and diagnosis of malaria and provide evidence that can support improved surveillance, and malaria control efforts in Northwest Ethiopia.

2. LITERATURE REVIEW

2.1 Prevalence of *Plasmodium* spp with each diagnostic method (PCR, Microscopy, RDT)

In India, a survey was conducted from 2017 to 2019 with $n = 17,405$. Nested PCR detected malaria in 436 individuals (2.50%), compared with 196 (1.13%) by RDT and 118 (0.68%) by microscopy. Of the PCR-positive infections, 263 (60.4%) were detected only by PCR and were submicroscopic infections. *P. falciparum* accounted for many PCR-detected cases, with *P. vivax* also present (Singh *et al.*, 2022). In contrast, a study among 422 clinically suspected malaria patients in Odisha, India, reported malaria detection rates of 76.5% by PCR, 64.4% by microscopy, and 63.0% by RDT. Although microscopy and RDT showed relatively high sensitivity for *P. falciparum* compared with PCR, their performance was substantially lower for *P. vivax* (57.0%) and mixed *P. falciparum*–*P. vivax* infections (18.0%). These findings indicate that the diagnostic performance of conventional methods may vary considerably according to *Plasmodium* species and infection type, with molecular testing providing greater detection of infections missed by microscopy and RDT (Ahmad *et al.*, 2021).

In southwestern Saudi Arabia, Madkhali *et al.* (2022) evaluated malaria diagnostic methods among 400 febrile patients in the low-transmission Jazan region using light microscopy, an HRP2/pLDH-based RDT, and nested PCR. Malaria was detected in 10.5% (42/400) of samples by microscopy, 12.0% (48/400) by RDT, and 14.3% (57/400) by PCR. When PCR was used as the reference method, RDT demonstrated higher sensitivity than microscopy (79.0% vs. 71.9%), while both methods showed high specificity (99.1% and 99.7%, respectively). Importantly, false-negative results by both RDT and microscopy were associated with low parasite density, and PCR identified additional infections missed by conventional methods. These findings demonstrate the limitations of microscopy and RDTs in detecting low-density *P. falciparum* infections among febrile patients and support the complementary role of molecular methods in malaria diagnosis (Madkhali *et al.*, 2022).

In a Ghanaian cross-sectional study of 500 asymptomatic children ≤ 5 years old in July 2015, the prevalence of malaria was 36.8% by nested PCR (184/500), 31.2% by *P. falciparum*HRP-2 RDT (156/500), and 23.2% by microscopy (116/500). The study showed that microscopy and *P. falciparum*-specific RDTs can miss non-falciparum infections in asymptomatic children (Okyere et al., 2020). In contrast, in a cross-sectional study of 200 febrile children in Nigeria (April 2023–February 2024), the malaria prevalence was 35.0% by nested PCR (70/200), 56.0% by microscopy (112/200), and 30.0% by *P. falciparum*-specific RDT (60/200) (Osun et al., 2024).

In Adama Woreda, East Shoa Zone, Ethiopia (Dec 2016–Feb 2017), a facility- and community-based cross-sectional study enrolled 330 participants, consisting of 202 symptomatic febrile patients. Overall malaria prevalence was 24.55% by PCR (81/330), 10.91% by microscopy (36/330), and 9.4% by CareStart RDT (31/330). Among PCR-positive cases, *P. vivax* predominated (69.1%, 56/81), followed by *P. falciparum* (23.5%, 19/81), with mixed infections comprising 7.4% (6/81) of cases. Microscopy revealed that *P. vivax* accounted for 72.2% (26/36), *P. falciparum* for 22.2% (8/36), and mixed infections for 5.6% (2/36), whereas the RDT detected 77.4% *P. vivax* (24/31), 15.6% *P. falciparum* (5/31), and 6.45% mixed infections (2/31) among positives (Tegegn et al., 2024a). In contrast, a facility-based cross-sectional study in Ziway Town, Adami Tulu District, Oromia (June–July 2019) enrolled 899 consecutive malaria-suspected febrile outpatients from Batu and Dembel health centers during the low-transmission season. Malaria prevalence was 7.5% by PCR (67/899), comprising 5.8% *P. falciparum* (55/899) and 1.7% *P. vivax* (12/718). Microscopy identified 4.9% (44/899), and *P. falciparum*HRP2-based RDT detected 4.5% (40/899). All *P. vivax* infections were asymptomatic and PCR-positive (Carlier et al., 2022).

Studies conducted in different malaria-endemic settings of northwest Ethiopia have reported substantial variation in malaria prevalence among febrile and malaria-suspected patients. In Jawi District, a facility-based cross-sectional study among febrile patients reported a 41.2% malaria prevalence by microscopy, with *P. falciparum* predominating, followed by *P. vivax* and mixed infections (Dagneu et al., 2025). Similarly, an earlier study in Jawi District involving febrile patients reported malaria prevalence estimates of 26.4% by expert microscopy, while *P. falciparum* HRP2- and pLDH-based RDTs detected 30.3% and 24.1%, respectively (Alemayehu

et al., 2020). At Andassa Health Center, a study of 303 malaria-suspected febrile outpatients using multiplex PCR detected malaria in 61.4% of participants, comprising *P. falciparum* (42.6%), *P. vivax* (12.9%), and mixed infections (5.9%) (Zelege *et al.*, 2023). These findings demonstrate considerable heterogeneity in malaria burden across northwest Ethiopia and highlight the influence of geographical setting, transmission intensity, study population, season, and diagnostic approach on the observed prevalence and species distribution.

A facility-based cross-sectional study conducted at Shewa Robit and Metehara health centres in northeastern and eastern Ethiopia, respectively, enrolled 271 malaria-suspected febrile outpatients. Multiplex real-time PCR detected malaria in 32.5% (88/271) of participants, including *P. falciparum* (25.5%), *P. vivax* (5.9%), and mixed *P. falciparum*–*P. vivax* infections (1.1%) (Belachew *et al.*, 2022). In contrast, study in southwestern Ethiopia, a facility-based cross-sectional study among 297 malaria-suspected febrile individuals in Bonga compared light microscopy with quantitative PCR (qPCR). Microscopy detected malaria in 7.0% of participants, whereas qPCR identified 17.4%, including *P. vivax*, *P. falciparum*, and mixed infections. The study demonstrated that a substantial proportion of infections were missed by microscopy, particularly low-density and submicroscopic infections, highlighting the value of molecular methods for improving malaria detection and species identification in symptomatic individuals (Abagero *et al.*, 2022).

2.2 Performance of diagnostic methods

Studies in Ethiopia have consistently shown that conventional diagnostics (microscopy and RDTs) have only moderate sensitivity and often miss low-density infections that molecular tests can detect (Alemu *et al.*, 2014, Cuadros *et al.*, 2015). The diagnostic performance of RDTs, light microscopy, and PCR for malaria has been studied across Ethiopia and sub-Saharan Africa (Felege *et al.*, 2021, Ojeniyi *et al.*, 2025).

Meta-analysis of 29 Ethiopian studies (2001–2020) established the foundational pattern: RDTs versus microscopy showed pooled sensitivities of 79–100% and specificities of 80–100%. The sensitivity fell when PCR was used as the reference, suggesting that both conventional tools systematically underestimate the true infection. The authors concluded that microscopy and

RDTs are sufficient for diagnosing febrile, high-density cases, but that PCR-based methods are indispensable for detecting submicroscopic infections (Feleke *et al.*, 2021).

The clinical context of testing determines RDT performance. In symptomatic, febrile outpatients with relatively high parasite densities, pLDH-based RDTs can approach near-perfect accuracy. A 2025 cross-sectional study in Northwest Ethiopia (Dembia and North Achefer districts, n=460) found that a pan-pLDH RDT achieved 99.6% sensitivity and 100% specificity versus expert microscopy, with near-perfect agreement ($\kappa = 0.97$), confirming that under optimal quality-assurance conditions, pLDH-based RDTs can meet WHO performance targets in febrile populations (Demass *et al.*, 2025b). However, this performance does not generalize to lower-density infections. A cross-sectional facility study at Adama, East Shoa (n=330) by Tegegn *et al.* (2024) showed that among symptomatic patients, both RDT and microscopy detected only approximately 50% of PCR-confirmed cases (specificity 100%).

Studies of asymptomatic individuals, RDT sensitivity collapsed to 15.4% (specificity 98.0%) and microscopy to 19.2%; PCR identified 26 infections versus only 8 by microscopy and 5 by RDT (Tegegn *et al.*, 2024b). A facility-based survey in Bahir Dar Zuria district (n=310 febrile outpatients) by Zeleke *et al.* (2023) reinforced this finding: a PfHRP2/pLDH CareStart RDT achieved only 81.0% sensitivity versus microscopy and 75.8% versus PCR, missing 19% and 24.2% of confirmed cases, respectively, which is below the WHO-mandated 95% sensitivity threshold for *P. falciparum* (Zeleke *et al.*, 2023).

A 2025 multiplex qPCR study in Jawi District, Amhara (n=835 pregnant women) by Tamir *et al.* demonstrated that qPCR achieved 100% sensitivity (specificity 94.8%), peripheral blood microscopy sensitivity was only 73.8% and RDT sensitivity 67.6%; on placental blood, both microscopy and RDT sensitivity fell to 62.2%, with RDT-PCR agreement substantially lower than microscopy-PCR agreement ($\kappa = 0.684$ vs 0.823) (Tamir *et al.*, 2025). Parallel findings emerged from two linked studies in Majang Zone, Gambella: a cross-sectional survey among 425 pregnant women at ANC clinics reported a qPCR-based prevalence of 17.6% versus 11.3% by RDT and 11.8% by microscopy (Alemayehu *et al.*, 2024a), while a companion study of 640 pregnant and 180 parturient women evaluating peripheral, placental, and cord blood found peripheral-blood RDT sensitivity of 63.5% and microscopy 73.1% against qPCR, declining further on placental samples (RDT 56.3%, microscopy 81.3%). Both investigations independently concluded that conventional diagnostics are suboptimal in pregnancy and that

molecular methods are required for accurate detection in this high-risk group (Alemayehu *et al.*, 2025).

Recent evidence from Ethiopia has demonstrated the potential value of pLDH-based RDTs, including BIOCREREDIT assays, for malaria diagnosis. In Bahir Dar, a 2025 study among 302 antenatal care attendees evaluated the BIOCREREDIT Pf/Pv pLDH/pLDH RDT against CareStart HRP2/pLDH RDT, microscopy, and PCR. BIOCREREDIT showed higher sensitivity for *P. falciparum* than CareStart when microscopy was used as the reference method (75.4% vs. 39%), although its sensitivity decreased to 55.6% when PCR was used as the reference. For *P. vivax*, both RDTs demonstrated relatively high and comparable sensitivity (84–87%)(Tegegne *et al.*, 2025).

Similarly, a prospective field evaluation among 708 febrile patients in southern Ethiopia compared four malaria RDTs, including two BIOCREREDIT pLDH-based combinations, with expert microscopy and qPCR. At parasite densities ≥ 20 parasites/ μ L, the evaluated RDTs achieved sensitivities exceeding 96% for *P. falciparum* and 92% for *P. vivax*, compared with lower sensitivities reported for routine health-center microscopy (63% and 72%, respectively). The study also detected only limited failure of pLDH-based tests among samples with *pfhrp2/3* deletions, supporting their potential utility where HRP2-based diagnosis may be affected by parasite genetic variation(Oduma *et al.*, 2025).

The relevance of pLDH-based RDTs is further supported by evidence of *pfhrp2/3* gene deletions in *P. falciparum* populations in Ethiopia. Such deletions can compromise the performance of HRP2-based diagnostic tests because these assays depend on the detection of the HRP2 antigen. Consequently, pLDH-based platforms such as BIOCREREDIT PfLDH/PvLDH may provide an important complementary diagnostic approach, particularly in settings where *pfhrp2/3* deletions occur(Golassa *et al.*, 2020).

2.3Factors Associated with Malaria Infection

Sociodemographic characteristics, particularly sex, age, and place of residence, are among the most consistently identified independent predictors of *Plasmodium* infection across analytical studies in sub-Saharan Africa,including Ethiopia(Obasohan *et al.*, 2021, Warkaw *et al.*, 2022, Anjorin *et al.*, 2023). A ten-year retrospective analysis at Bichena Primary Hospital in the Amhara Region, Northwest Ethiopia, using bivariable and multivariable regression with Pearson chi-square tests on 24,107 blood film examinations found that males were 1.28 times more likely

to be infected than females, and individuals in the 15-24 age group had 1.27 times higher odds of malaria compared to other age groups (Minwuyelet *et al.*, 2025).

A case-control study conducted in Dembia District, Northwest Ethiopia, identified being male, being aged 15 years or above, and rural residence as independent predictors of malaria infection, with the heightened risk in males attributed to occupational exposure to outdoor vector-biting environments during farming and night-time activities (Agegnehu *et al.*, 2018).

A multilevel analysis of malaria indicator survey data from 13 sub-Saharan African countries confirmed these demographic patterns among children: children aged 48–59 months had significantly higher odds of malaria, compared to younger infants, rural residents faced more than twice the risk of urban counterparts, and children from the richest household (Obasohan *et al.*, 2021)s had markedly lower odds of infection, compared to the poorest, underscoring the role of socioeconomic status alongside demographic vulnerability (Chilot *et al.*, 2023). Similarly, a bivariable and multivariable logistic regression study at Kidist Hana Health Centre, Fogera District, Northwest Ethiopia, found that children aged ≤ 5 years and 6–15 years had significantly higher odds of malaria than adults ≥ 36 years, and that low household income and widowed marital status were also independently associated with confirmed *Plasmodium* infection (Alebele *et al.*, 2026).

Behavioral and environmental factors related to vector exposure represent the most analytically robust modifiable determinants of *Plasmodium* infection, with insecticide-treated net (ITN) non-use, outdoor sleeping, proximity to stagnant water, and lack of indoor residual spraying (IRS) consistently emerging as significant predictors in multivariable logistic regression models.

A facility-based cross-sectional study in Wogera District, Northwest Ethiopia, using multivariable logistic regression among 585 under-five children found that regular sleeping under long-lasting insecticide-treated nets (LLINs) was strongly protective against malaria, while the presence of stagnant water near the household and outdoor sleeping at night were independently associated with significantly higher odds of infection (Tsegaye *et al.*, 2021). These findings are mirrored in a multivariable logistic regression study at Mizan Tepi University Teaching Hospital in Southwest Ethiopia, where proximity to stagnant water was the strongest predictor of malaria infection, followed by lack of IRS coverage (Duguma *et al.*, 2022).

A study among febrile children in Northwest Ethiopia using multivariable logistic regression further reported that shorter distance from stagnant water, outdoor sleeping, irregular bed net use, late bedtime, and larger family size were all statistically significant independent determinants of *Plasmodium* infection incidence (Hailu *et al.*, 2018). A meta-analysis and review of travel as a malaria risk factor in sub-Saharan Africa using a random-effects model on 22 studies calculated a pooled odds ratio for travel to endemic areas, with particularly high odds in urban residents travelling to rural or high-transmission areas, establishing travel history as another major independently confirmed risk factor for malaria infection (Ahmed *et al.*, 2020).

Previous malaria infection history also independently determines susceptibility to subsequent *Plasmodium* infection: a prospective follow-up study in Northwest Ethiopia using binary logistic regression found that having no prior malaria attack nearly 10-fold increased the risk of unsuccessful treatment outcome, and that being infected with *P. vivax* was associated with approximately ninefold higher odds of treatment failure compared to *P. falciparum*, with implications for understanding how prior exposure, acquired partial immunity, and species identity interact as factors shaping infection outcomes in Northwest Ethiopia (Demass *et al.*, 2025a).

Generally, existing evidence shows that malaria transmission in Ethiopia is heterogeneous. *P. falciparum* and *P. vivax* co-circulate and contribute to complex infections, but the true prevalence of *P. ovale* and *P. malariae* is not explicitly known. Studies have consistently shown that routine diagnostics underestimate the true malaria prevalence. Low-density, mixed, and submicroscopic infections are detectable mainly by molecular techniques. Molecular epidemiology is crucial for revealing parasite diversity and hidden reservoirs. Furthermore, malaria is influenced by a combination of demographic, behavioral, and environmental factors. However, studies integrated molecular diagnostics with associated factors within a single study are limited. Therefore, comprehensive comparative diagnostic and molecular epidemiological investigations are important to characterize the true malaria prevalence, species distribution, and to inform targeted control and elimination strategies in Northwest Ethiopia.

3. OBJECTIVES OF THE STUDY

3.1 GENERAL OBJECTIVE

To assess the prevalence, diagnostic performance of BIOCREREDIT Malaria Ag Pf/Pv (pLDH/pLDH), and associated factors of *Plasmodium* infections among malaria-suspected individuals in Northwest Ethiopia.

3.2 SPECIFIC OBJECTIVES

- To determine the prevalence and of *Plasmodium* infections among individuals suspected of having malaria using microscopy, RDT, and PCR.
- To compare the diagnostic performance and level of agreement of the diagnostic methods for *Plasmodium* infections
- To identify the factors associated with *Plasmodium* infections among malaria-suspected individuals

4. MATERIALS AND METHODS

4.1 STUDY AREA

The study was conducted in the Amhara Region of Northwest Ethiopia, encompassing Bahir Dar city, Ayikel, East Dembiya (Kola Diba), and Metema (Figure 1). Bahir Dar is the regional capital and a major urban center on the southern shore of Lake Tana, located at the outlet of the Abbay at approximately 1,820 m above sea level. The climate is tropical savanna (borderline highland), with warm temperatures and a pronounced rainy season year-round. The large rural districts of North Gondar lie to the west and north (Alemayehu *et al.*, 2024b).

The Chilga district, whose district capital is Ayikel town, lies to the west of Bahir Dar near the Sudanese border. Chilga is a mid-altitude area, 1,000-1,500 m, in the Maekelawi Gondar Zone. It is bounded by Dembiya to the east and Metema to the west (Asfaw *et al.*, 2022). South of Chilga is the Dembiya district, which fronts Lake Tana and ranges from 1,500 to 2,600 m in elevation. Dembiya lies in North Gondar Zone, about 750 km north of Addis Ababa. Its population is 91% rural and predominantly engaged in mixed farming (Tarekegn *et al.*, 2021). Metema District, which is on Chilga's western flank, is a lowland border district (500–1,000 m, average 750 m) set in hot, arid environs. Metema is a major agricultural investment area with vast irrigated farms and large seasonal labor migration; it borders Sudan and lies at the southern terminus of a corridor to the Sudanese town of Gallabat (Esayas *et al.*, 2025).

All four sites lie in Ethiopia's "malarious belt," where unstable, seasonal malaria transmission prevails. In Ethiopia, peak transmission follows the main rains (June–August), with a secondary transmission period after short rains (April–May). Bahir Dar's altitude and climate sustain malaria seasonally despite temperatures seldom dropping below 22°C, and outbreaks can occur after the rains. Chilga and Dembiya have the typical highland-to-midland ecology (woyna dega), with bimodal rains (Mar–May and Jun–Sep) (Tarekegn *et al.*, 2021). Metema's lowland, irrigated environment allows year-round transmission, with pronounced peaks during agricultural harvest seasons (Esayas *et al.*, 2025).

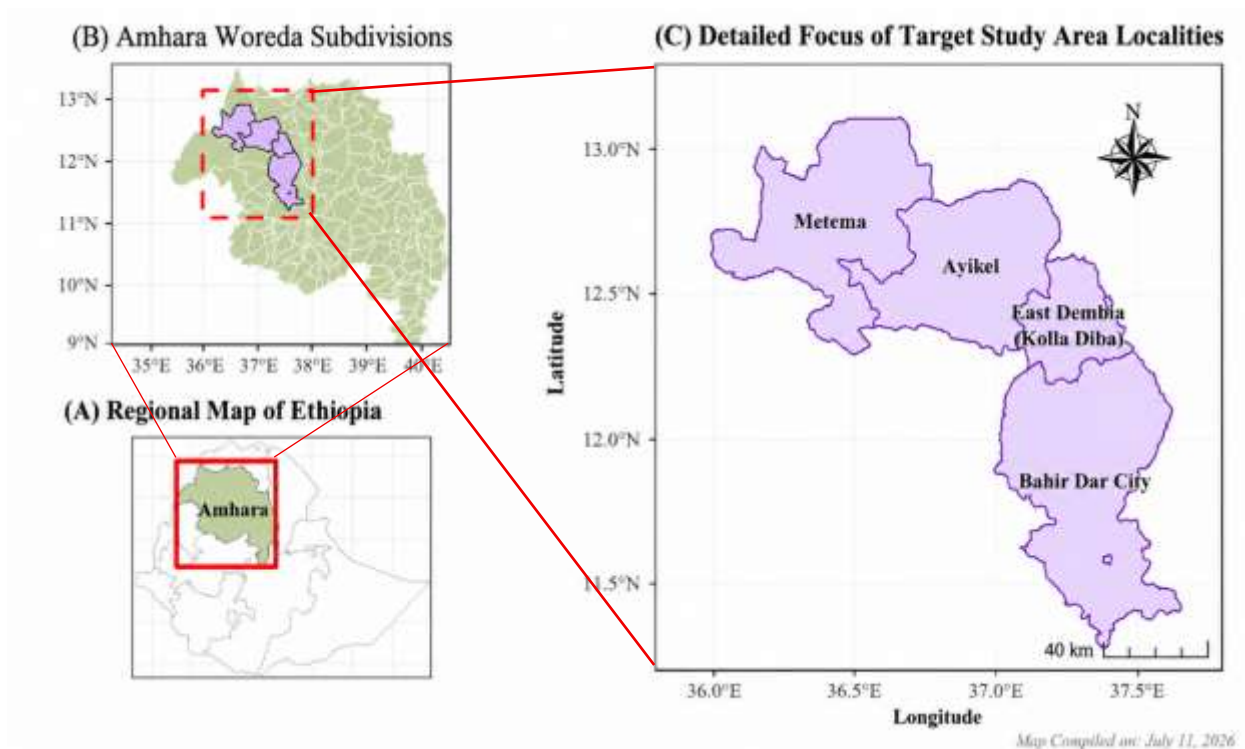


Figure 1: Location of the study area map in Northwest Ethiopia.

4.2 STUDY DESIGN AND PERIOD

A health-facility-based cross-sectional study was conducted from February to April, 2026.

4.3. POPULATION

4.3.1 SOURCE POPULATION

The source population consists of all individuals who live in the catchment areas of the selected public health facilities at Bahir Dar City, Ayikel, Koladiba, and Metema districts.

4.3.2 STUDY POPULATION

The study population included all malaria-suspected individuals who visited the inpatient, outpatient, and emergency departments of the selected facilities during the study period.

4.3.3 STUDY PARTICIPANTS

The study participants were those from the study population who volunteered to participate in the study and provided informed consent (or assent).

4.4 INCLUSION AND EXCLUSION CRITERIA

4.4.1 INCLUSION CRITERIA

Febrile malaria-suspected patients who attended the selected health facilities during the study period provided written informed consent and/or assent and were included in the study.

4.4.2 EXCLUSION CRITERIA

Malaria-suspected patients who received antimalarial treatment within the two weeks before enrollment and were critically ill during data collection time were excluded.

4.5 SAMPLE SIZE DETERMINATION AND SAMPLING TECHNIQUES

4.5.1 SAMPLE SIZE DETERMINATION

The sample size for this study was determined by calculating the minimum number of participants required to achieve adequate statistical power for each of the three specific research objectives.

Sample size determination for objective one

To determine the prevalence and species distribution of *Plasmodium* infections among malaria-suspected patients, the required sample size was calculated using the single population proportion formula:

$$n = \frac{Z_{\alpha}^2 P(1 - P)}{d^2}$$

Where:

- **n** = required sample size
- **Z** = standard normal value at 95% confidence level (1.96)

- **P** = expected malaria prevalence
- **d** = margin of error (0.05)

Recent studies conducted in northwest Ethiopia have reported varying malaria prevalence among febrile patients. Dagnew *et al.* (2025) reported a malaria prevalence of 41.2% among febrile patients in Jawi District. Therefore, the higher recent prevalence estimate of 41.2% was used as the prevalence (*p*) for calculating the sample size of the current study(Dagnew *et al.*, 2025).

$$n = \frac{(1.96)^2 \times 0.412(1 - 0.412)}{(0.05)^2}$$

$$n = \frac{3.8416 \times 0.2423}{0.0025}$$

$$n = 372$$

Therefore, the minimum sample size required for estimating malaria prevalence and species distribution was 372.

Sample size determination for objective two

To evaluate the diagnostic performance of routine light microscopy and the BIOCREREDIT Malaria Ag Pf/Pv (pLDH/pLDH) RDT using nested PCR as the reference standard, the sample size was calculated using Buderer's formula for diagnostic accuracy studies (Buderer, 1996):

$$n = \frac{Z^2 \times Se(1 - Se)}{L^2 \times P}$$

Where:

- ✓ **n** = required sample size
- ✓ **Z** = 1.96 (95% confidence level)
- ✓ **Se** = anticipated sensitivity of the index test
- ✓ **L** = desired precision (0.05)
- ✓ **P** = expected disease prevalence

An expected malaria prevalence of 41.2% was obtained from Dagne *et al.* (2025) (Dagne *et al.*, 2025). The anticipated sensitivity of the BIOCREREDIT Malaria Ag Pf/Pv (pLDH/pLDH) rapid diagnostic test was 91.2%, according to a recent systematic review and meta-analysis. Evaluating the diagnostic performance of the BIOCREREDIT species-specific lactate dehydrogenase-based immunochromatographic assay, which reported a pooled sensitivity of approximately 91.2% (Gebremeskele *et al.*, 2026), for *P. falciparum* detection compared with molecular reference methods.

Substituting these values:

$$n = \frac{(1.96)^2 \times 0.912(1 - 0.912)}{(0.05)^2 \times 0.412}$$

$$n = \frac{0.3083}{0.00103}$$

$$n = 300$$

Therefore, a minimum sample size of 300 participants was required to evaluate diagnostic sensitivity and agreement between the diagnostic methods.

Sample size determination for objective three

To identify factors associated with malaria infection, the sample size was calculated using the formula for comparing two population proportions. Educational status (formal education versus no formal education) was selected as the primary exposure variable because it was identified as a significant predictor of malaria infection in northwest Ethiopia by Dagne *et al.* (2025) (Dagne *et al.*, 2025). Assuming a 95% confidence level, 80% statistical power, a 1:1 ratio of exposed to unexposed participants, and a malaria prevalence of 49% among individuals without formal education and 33% among those with formal education. The required sample size was calculated to be 294 participants.

The sample size requirements for the three study objectives were compared. The prevalence objective required the largest sample size (372 participants), compared with 300 participants for diagnostic accuracy assessment and 294 participants for identifying associated factors. Lastly, the largest calculated sample size (372) was selected as the baseline sample size.

Considering possible non-response, a 10% contingency was added, and the final size was 410.

4.5.2 SAMPLING TECHNIQUE AND PROCEDURE

Initially, study sites were purposively selected based on malaria burden and patient flow. In Bahir Dar, four health centers, Shimbit Health Center, Han Health Center, Abay Health Center, and Shum Abo Health Center, were included due to their high malaria caseload. Similarly, in other districts, facilities serving large numbers of febrile patients were selected, including Metema Primary Hospital, Ayikel Health Center, and Kola Diba Primary Hospital. Then, a systematic random sampling technique was used to recruit malaria-suspected patients attending selected health facilities during the study period.

Then, the total sample size ($n = 410$) was allocated proportionally across study sites by considering the 2017 E.C annual parasite incidence report from the Amhara Regional State Health Bureau. The number of study participants was 40, 82, 140, and 148 for Chilga, Kola Diba, Bahir Dar city, and Metema, respectively

Within each selected facility, the sampling interval (K) was determined by dividing the estimated number of malaria-suspected patients during the same period in the previous year (2017 E.C) by the allocated sample size for that facility. The calculated sampling intervals were as follows: Shimbit Health Center ($K=6$), Han Health Center ($K=6$), Abay Health Center ($K=6$), Shum Abo Health Center ($K=6$), Metema Primary Hospital ($K=3$), Ayikel Health Center ($K=5$), and Kola Diba Primary Hospital ($K=4$) (Table 1).

The first participant was selected randomly from the first K eligible patients. After that, every K^{th} malaria-suspected patient presenting to the outpatient department was recruited.

Table 1: Proportional allocation of sample size across selected health facilities in Northwest Ethiopia, 2026

Districts	Allocated sample	Health center (n)	Average number of eligible patients during the same period in the previous year (2017 E.C)
Bahir Dar	140	Shimbit HC (44)	289
		Han HC (36)	238
		Abay HC (26)	167
		Shum Abo HC (36)	234
Metema	148	Metema Hospital (148)	543
Chilga	40	Ayikel HC (40)	232

Kola Diba	82	Kola Diba Primary Hospital (82)	360
-----------	----	------------------------------------	-----

4.6 VARIABLES OF STUDY

4.6.1 DEPENDENT VARIABLES

 *Plasmodium* infection status

4.6.2 INDEPENDENT VARIABLES

Sociodemographic variables

- Age
- Sex
- Residence
- Marital status
- Educational level
- Occupation
- Family size

Behavioral factors

- Bed-net ownership and utilization
- Indoor residual spraying (IRS)
- Travel history within the previous 14 days
- Sleeping/working outdoors at night
- Use of protective clothing
- Use of insect repellents

Environmental factors

- Presence and distance of surface water
- House wall material
- Cracks/holes in walls
- Window availability and screening
- Windows/doors kept open at night
- Gaps between doors and floor/walls

4.6.3 OPERATIONAL DEFINITIONS

Critically ill: A malaria-suspected patient with an acute, life-threatening clinical condition requiring immediate resuscitation, continuous monitoring, or urgent specialized medical management, as determined by the attending healthcare professional at the time of enrollment. This included patients with altered consciousness, respiratory distress, shock, repeated convulsions, or other signs of severe systemic illness requiring emergency intervention.

4.7 DATA COLLECTION AND LABORATORY PROCEDURES

4.7.1 SOCIODEMOGRAPHIC, BEHAVIORAL, AND ENVIRONMENTAL DATA

Data on sociodemographic characteristics, behavioral factors, and environmental variables were collected from each study participant using a structured and pre-tested interviewer-administered questionnaire. The questionnaire was prepared in English and translated into Amharic. The questionnaire captured information on age, sex, residence, marital status, educational level, occupation, and household size. In addition, data on behavioral and environmental risk factors including bed net ownership and usage habits, indoor residual spraying (IRS) history within the preceding 12 months, presence of surface water within 500 meters of the household, house construction materials, presence of cracks or holes in walls, window and door status at night, history of travel outside the residence within the previous 14 days, and the use of personal protective measures such as protective clothing and insect repellents were also collected.

4.7.2 BLOOD SAMPLE COLLECTION

Capillary blood was collected from each study participant by finger prick. A sterile, single-use lancet was used following standard operating procedures at [Annex X](#). The first drop of blood was wiped away, and subsequent blood drops were collected and used for blood film preparation and RDT testing.

Blood samples were also collected from the capillary for molecular analysis using DBS (Dry Blood Spot). The card was a Whatman 903 Protein Saver filter paper card (Whatman Inc., UK) in the form of DBS. Each of the pre-printed circles on the filter paper card had a diameter of 12–13 mm. It was filled completely by gently touching the fingertip to the center of the circle. It was allowed to absorb the blood uniformly and spontaneously. Each filled circle contains approximately 75–80 µL of whole blood.

Following blood spotting, the filter paper cards were laid flat. Allowed to air-dry completely at room temperature for a minimum of four hours. DBS were individually packaged in sealed, zipper bags containing silica gel desiccant. Each card was labeled with a unique participant identification number, date of collection, and study site. Cards were stored at room temperature until transport to the molecular laboratory at Amhara Public Health Institute, where they were kept at -20°C until DNA extraction.

In addition to DBS preparation for molecular analysis, separate drops of capillary blood were collected at the same time for preparation of thick and thin blood films for light and RDT.

4.7.3 THICK AND THIN BLOOD FILM PREPARATION AND MICROSCOPY

For each participant, thick and thin blood films were prepared on clean, grease-free glass slides at the time of blood collection in accordance with [Annex XI](#). *Plasmodium* species identification and parasite density were determined from the thin and thick blood films, respectively. A blood film was considered negative for malaria when no *Plasmodium* parasites, including asexual forms and gametocytes, were detected after careful examination of at least 100 microscopic fields. Parasite density was calculated as the number of asexual parasites counted per 200 white blood cells (WBCs), assuming a standard WBC count of 8,000 WBCs/μL. Each blood film was independently examined by two trained microscopists, who recorded the presence or absence of *Plasmodium* parasites and identified the infecting species. In cases of discordant results between the two primary readers, the blood film was re-examined by a third senior WHO-certified microscopist, whose assessment was accepted as the final microscopy result (WHO and UNICEF, 2015).

4.7.4 RAPID DIAGNOSTIC TEST BIOCREDIT MALARIA AG PF/PV (pLDH/pLDH)

Malaria rapid diagnostic testing was performed using the BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH) test kit (RapiGEN Inc., Republic of Korea). It is a one-step immunochromatographic assay that qualitatively detects *P. falciparum*-specific lactate dehydrogenase (PfLDH) and *P. vivax*-specific lactate dehydrogenase (PvLDH) antigens in human whole blood. The test allows simultaneous differentiation of *P. falciparum* and *P. vivax* infections on a single device (Tegegne *et al.*, 2025). The test was performed strictly according to the manufacturer's instructions; see [Annex XII](#).

4.7.5 DNA EXTRACTION FROM DBS

DNA from dried blood spot (DBS) samples was extracted using the QIAamp DNA Mini Kit (QIAGEN, Germany)(QIAGEN., 2024). The DBS spot (3 mm diameter) was punched and added into an Eppendorf tube and lysed with 200 μ L of ATL buffer following vortexing. After that, the sample was incubated at 85°C for 10 minutes, after which 20 μ L of proteinase K was added and incubated at 56°C for 1 hour with brief centrifugation. After enzymatic digestion, 200 μ L of AL buffer and 200 μ L of absolute ethanol were consecutively added to facilitate nucleic acid precipitation. The mixture was applied to a spin column and centrifuged at 8,000 RPM for 1 minute. The column was washed with 500 μ L each of AW1 and AW2 buffers, centrifuged at 8,000 RPM and 14,000 RPM, respectively. It was followed by a final dry centrifugation at 14,000 RPM in 3 minutes. DNA was eluted with 50 μ L of nuclease-free water (NFW) following a 3-minute incubation at room temperature, centrifuged at 8,000 RPM for 1 minute, and stored at –20°C for downstream analyses, as it is shown in [Annex XIII](#).

4.7.6 DNA AMPLIFICATION AND DETECTION

Due to financial and laboratory resource limitations, molecular analyses were done on a subset of 220 samples. This subset comprised all composite-positive samples, microscopy and/or RDT (n = 123), and 97 randomly selected samples from participants who were negative by both methods. Therefore, molecular findings represent the molecularly tested subset rather than the entire study population and were used primarily for molecular species identification, parasite quantification, and evaluation of agreement and diagnostic performance with conventional diagnostic methods.

Plasmodium infection was initially assessed at the genus level using the first round of nested PCR (N1). Samples positive by N1 were subsequently subjected to species-specific molecular testing. *Plasmodium ovale* and *P. malariae* were identified using species-specific nested PCR (N2), while *P. falciparum* and *P. vivax* were identified and quantified using multiplex quantitative real-time PCR (qPCR).

First-Round (Genus-Specific) Nested PCR (N1)

Primary, genus-level amplification of the *Plasmodium* 18S ribosomal RNA gene was done using the nested PCR method of Snounou *et al.* (1993)(Snounou *et al.*, 1993). Each 25 μ L reaction

contained 5 µL of extracted genomic DNA and a master mix prepared with GoTaq Flexi DNA Polymerase (Promega, Cat. No. M7808), comprising 1× GoTaq Flexi PCR Buffer, 2.0 mM MgCl₂, 0.25 mM of each dNTP, 1.0 U of polymerase, and 0.25 µM each of the outer genus-specific primers rPLU6 and rPLU5 (Table 2). Cycling conditions consisted of an initial denaturation at 95°C for 10 min; 35 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 90 s; a final extension at 72°C for 10 min; and a 4°C hold. The expected N1 amplicon size was approximately 1200 bp.

Second-Round Nested PCR for *Plasmodium ovale* and *Plasmodium malariae* (N2)

All N1-positive samples undergo a second-round, species-specific nested PCR to identify *P. ovale* and *P. malariae*. Each 25 µL reaction used 2 µL of N1 amplicon as template, with a master mix of the same as N1 (1× GoTaq Flexi PCR Buffer, 2.0 mM MgCl₂, 0.25 mM of each dNTP, 1.0 U GoTaq Flexi DNA Polymerase) with 0.25 µM of the appropriate species-specific primer pair: rOVA1/rOVA2 for *P. ovale* (749 bp) or rMAL1/rMAL2 for *P. malariae* (144 bp) (Table 2). Conditions comprised an initial denaturation at 95°C for 10 min; 30 cycles of 95°C for 60 s, 54°C for 60 s, and 72°C for 60 s; a final extension at 72°C for 10 min; and a 4°C hold.

Gel Electrophoresis

N1 and N2 amplicons were resolved on 2.0% agarose gels stained with SYBR Safe DNA Gel Stain (0.5 µg/mL) in 1× TAE buffer, alongside a 100 bp DNA ladder, and visualised under UV illumination (254 nm) on a gel documentation system.

Multiplex Real-Time PCR for *Plasmodium falciparum* and *Plasmodium vivax*

P. falciparum and *P. vivax* were detected and quantified in all N1-positive corresponding samples by dual multiplex TaqMan real-time PCR targeting the 18S rRNA gene, using TaqMan Fast Advanced Master Mix (2×, Applied Biosystems, Cat. No. 4444964). Each 12 µL reaction contained 3 µL of DNA template. Target-specific primers and probes (Table 2) were used at final concentrations of 300 nM (Pf18S primers) and 200 nM (Pv18S primers). The FAM- and Texas Red-labelled probes at 110 nM and 105 nM, respectively. Cycling conditions were 50°C hold for 2 min, enzyme activation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s, with fluorescence acquired in the FAM (Pf) and Texas Red (Pv) channels at the end of each

extension step. Parasite densities were estimated from 10-fold serial standard curves run in parallel on each plate: cultured *P. falciparum* NF54 strain genomic DNA (10^6 – 10^2 parasites/mL) and a plasmid construct containing the *P. vivax* 18S rRNA gene insert (10^7 – 10^3 copies/mL). The lowest standards corresponded to Ct values of 35.815 for *P. falciparum* at 10^2 parasites/mL and 37.48 for *P. vivax* at 10^3 copies/mL. These lowest standards were used as the lower limits of the respective standard curves for quantitative assessment [Annex XIV](#).

Table 2: Primers and probes used for the molecular detection and speciation of *Plasmodium* species

Assay	Target	Primer or probe	Sequence (5'→3')	Amplicon size
N1	<i>Plasmodium</i> spp.	rPLU6 (F)	TTAAAATTGTTGCAGTTAAAACG	1200 bp
		rPLU5 (R)	CCTGTTGTTGCCTTAAACTTC	
N2 N2	<i>P. ovale</i>	rOVA1 (F)	ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA	749 bp
		rOVA2 (R)	GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG	
	<i>P. malariae</i>	rMAL1 (F)	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	144 bp
		rMAL2 (R)	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA	
qPCR	<i>P. falciparum</i>	Pf18SFW	GTAATTGGAATGATAGGAATTTACAAGGT	
		Pf18SRV	TCAACTACGAACGTTTTAACTGCAAC	
		Pf18Sprobe	FAM-AACAATTGGAGGGCAAG-MGBNFQ	

qPCR	<i>P. vivax</i>	Pv18SFW	AGCAGCCGCGGTAATTCCA	
		Pv18SRV	ATGCGCACAAAGTCGATACGAAG	
		Pv18Sprobe	TEXASRED- AGCAACGCTTCTAGCTTAATCCACAT-BHQ2	

4.8 QUALITY ASSURANCE

Quality assurance procedures were implemented at all stages of the study. The questionnaire was developed in English and translated into Amharic. Before data collection, the questionnaire was pretested with 5% of the sample in a health facility outside the study sites. Data collectors received training on study objectives, ethical issues, interview techniques, sample collection, and documentation procedures.

Laboratory quality assurance was kept in accordance with standard operating procedures (SOPs). Blood sample collection, handling, transportation, and storage were performed in accordance with the WHO-recommended protocols for malaria diagnostics. Microscopy quality was maintained through proper smear preparation, standardised Giemsa staining, and double-blind slide reading. A third malaria microscopist (WHO-licensed) resolved discordant results.

RDT was done by trained personnel according to the manufacturer's instructions at the health center. The application of blood and buffer and the interpretation of results within the specified reading time were ensured. Each test was checked to ensure the control line appeared correctly. Tests without a valid control line were considered invalid and repeated using a new test device. RDT results were documented throughout the study.

DNA extraction was conducted at the Amhara Public Health Institute (APHI), Bahir Dar City, Ethiopia. Similarly, PCR amplification was performed at the Armauer Hansen Research Institute (AHRI) in Addis Ababa, Ethiopia. Each PCR run included positive controls, negative controls, and extraction controls (blanks). Equipment calibration, reagent checks, and adherence to molecular SOPs were maintained throughout the process. PCR results were accepted only when

all control reactions produced valid outcomes, and all laboratory results were documented immediately, cross-checked, and stored.

4.9 DATA MANAGEMENT AND ANALYSIS

All data taken from questionnaire responses, RDT readings, microscopy examinations, and PCR detection were reviewed for completeness and accuracy at the time of collection and detection. Each participant had a unique identification code. Data were entered into SPSS (version 27) for data cleaning and exported to R version 4.5.1 in RStudio version 2026.6.0+241 for analysis.

Descriptive statistics were used to summarize socio-demographic characteristics, *Plasmodium* infection, species distribution, and sensitivity of diagnostic methods. Microscopy and RDT results were compared to PCR results to assess agreement using Cohen's kappa (κ) statistic. The κ value was interpreted as slight agreement ($\kappa = 0.01$ – 0.20), fair agreement ($\kappa = 0.21$ – 0.40), moderate agreement ($\kappa = 0.41$ – 0.60), substantial agreement ($\kappa = 0.61$ – 0.80), and almost perfect agreement (0.81 – 1.00) (Tamir *et al.*, 2025). Sensitivity, specificity, and predictive values were calculated. Bivariate analyses were conducted to assess associations between malaria infection and key independent variables. Variables demonstrating statistically significant associations ($p < 0.25$) in bivariate testing were included in a multivariable logistic regression model. Adjusted odds ratios with corresponding 95% confidence intervals were reported with statistical significance of $p < 0.05$.

4.10 ETHICAL CONSIDERATIONS

Ethical approval was obtained from the Institutional Review Board (IRB) of the College of Medicine and Health Sciences, Bahir Dar University (Protocol number: 6039/2026), and supportive letters and permission were obtained from the APHI and district health office before data collection. The purpose, expected benefits, and potential risks of the study were explained. Written informed consent and/or assent were obtained from each study participant before any data collection. Personal identifiers were not recorded, and all information was used only for research purposes. Participants who tested positive by RDT and microscope for malaria were linked to the doctor/physician for treatment.

5. RESULTS

5.1 Socio-demographic Characteristics of Study Participants

In this study, a total of 410 malaria-suspected participants were enrolled. Of these, 212 (51.7%) were male. The mean and median age of the study participants were 29.8 ± 17.8 years and 28 years (IQR: 17–41), respectively. Most participants (78.8%) were aged ≥ 15 years. Rural participants accounted for 52.7%, and 29% of the malaria-suspected patients were farmers. More than half had a primary school education or below (58%) (Table 3).

Table 3: Sociodemographic Characteristics of Malaria-Suspected Individuals (N=410) in Northwest Ethiopia, 2026.

Variables	Characteristics	Frequency	Percent (%)
Study Site	Metema	148	36.1
	Kolla Diba	82	20.0
	Ayikel	40	9.8
	Bahir Dar	140	34.1
Age Group (Years)	<15	87	21.2
	≥ 15	323	78.8
Sex	Male	212	51.7
	Female	198	48.3
Residence	Urban	194	47.3
	Rural	216	52.7
Marital Status	Single	177	43.2
	Married	177	43.2
	Divorced	43	10.5
	Widowed	13	3.2
Educational Status	Unable to read and write	107	26.1
	Primary school	133	32.4
	Secondary school	82	20.0
	College and above	88	21.5

Occupational Status	Farmer	111	27.1
	Student	107	26.1
	Employed	45	11.0
	Merchant	49	12.0
	Unemployed	34	8.3
	Housewife	34	8.3
Family Size	<5	324	79.0
	≥5	86	21.0

5.2 Malaria Prevalence and *Plasmodium* Species Distribution

Among the 410 malaria-suspected individuals, the prevalence of *Plasmodium* infection using a combination of three diagnostic methods (RDT, Microscopy, and PCR) was 36.1% (148/410). The prevalence of *Plasmodium* infection was 23.2% (95/410) by RDT and 25.4% (104/410) by blood-film microscopy.

5.2.1 PCR-based Detection of *Plasmodium* species

Molecular analysis was conducted on 220 study participants, comprising 123 who were positive by the RDT and/or microscopy composite reference standard and 97 who were composite-negative. Among the 123 conventional-test-positive participants, 95/123 (77.2%) were positive by RDT, comprising 43 *P. falciparum*, 48 *P. vivax*, and 4 mixed infections, while 104/123 (84.6%) were positive by microscopy, comprising 46 *P. falciparum*, 51 *P. vivax*, and 7 mixed infections. Seventy-six/123 (61.8%) participants were positive by both RDT and microscopy. Among the 123 conventional test-positive participants, PCR identified 32/123 (26.0%) *P. falciparum*, 24/123 (19.5%) *P. vivax*, 33/123 (26.8%) mixed *P. falciparum/P. vivax*, and 1/123 (0.8%) *P. malariae* infection, while 32/123 (26.0%) were PCR-negative. Among all 220 PCR-tested participants, 116/220 (52.7%) were PCR-positive, including 114/220 (51.8%) detected by qPCR and 2/220 (0.9%) by nPCR. Of the 123 blood samples that were positive by either RDT or microscopy, 91 (74.0%) were PCR-positive, while 32 (26.0%) were PCR-negative. Among the 97 samples that were negative by both RDT and microscopy, 25 (25.8%) were PCR-positive, whereas 72 (74.2%) were PCR-negative.

5.2.2 *Plasmodium* species Distribution across Diagnostic Methods

Plasmodium species distribution varied according to the diagnostic method used. Using the three-method composite, 148/410 (36.1%, 95% CI: 31.6%–40.9%) participants were positive, including *P. falciparum* 55/410 (13.4%, 95% CI: 10.5%–17.1%), *P. vivax* 51/410 (12.4%, 95% CI: 9.6%–16.0%), *P. malariae* 2/410 (0.5%, 95% CI: 0.1%–1.8%), and mixed infections 40/410 (9.8%, 95% CI: 7.2%–13.0%). Among the 410 participants tested by RDT, 95 (23.2%, 95% CI: 19.3%–27.5%) were positive, with *P. vivax* predominating 48/95 (50.5%, 95% CI: 40.6%–60.4%). Light microscopy identified 104/410 (25.4%, 95% CI: 21.4%–29.8%) positive study participants, with *P. vivax* again predominating 51/104 (49.0%, 95% CI: 39.6%–58.5%). Among the 220 participants tested by PCR, 116/220 (52.7%, 95% CI: 46.1%–59.2%) were positive, comprising *P. falciparum* 43/116 (37.1%, 95% CI: 28.8%–46.1%), mixed *P. falciparum*/*P. vivax* infections 40/116 (34.5%, 95% CI: 26.5%–43.5%), *P. vivax* (31/116, 26.7%, 95% CI: 19.5%–35.4%), and *P. malariae* 2/116 (1.7%, 95% CI: 0.5%–6.1%). Notably, *P. malariae* was detected only by PCR, and no *P. ovale* was detected (Figure 2).

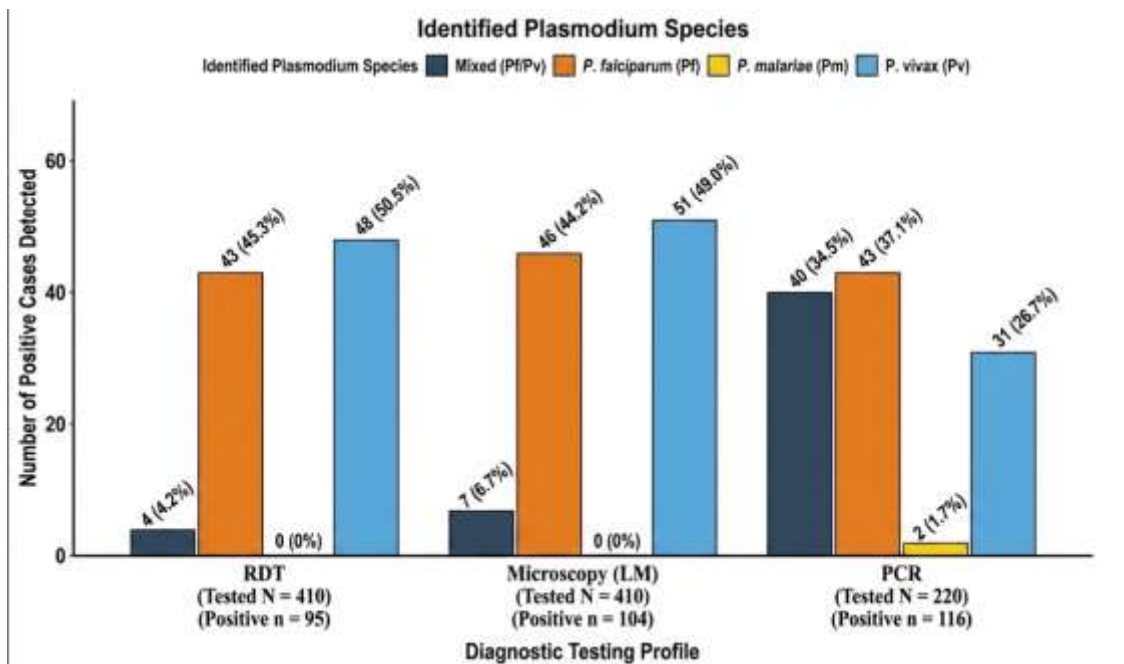


Figure 2: Malaria species distribution by RDT, Light Microscope, and PCR among malaria-suspected individuals in Northwest Ethiopia, 2026.

5.2.3 PCR-based Prevalence and *Plasmodium* Species Distribution across the Study Area

Among the 220 PCR-tested samples, 116/220 (52.7%) were PCR-positive and 104/220 (47.3%) were PCR-negative. The number of PCR-tested samples varied across the study sites according to the predetermined sample allocation, with 74 samples from Metema, 64 from Bahir Dar, 50 from Kolla Diba, and 32 from Ayikel. Molecular prevalence was highest in Kolla Diba 36/50 (72.0%), followed by Bahir Dar 38/64 (59.4%), Metema 31/74 (41.9%), and Ayikel 11/32 (34.4%). At the species level, *P. falciparum* was predominant in Kolla Diba 20/50 (40.0%) and Metema 13/74 (17.6%), while *P. vivax* was predominant in Bahir Dar, 16/64 (25.0%). In Ayikel, mixed infections were most frequent, 5/32 (15.6%), followed by *P. vivax* 4/32 (12.5%) and *P. falciparum* 2/32 (6.3%). Mixed infections were also common in Bahir Dar 14/64 (21.9%) and Kolla Diba 13/50 (26.0%). *P. malariae* was detected exclusively in Metema 2/74 (2.7%) (Figure 3).

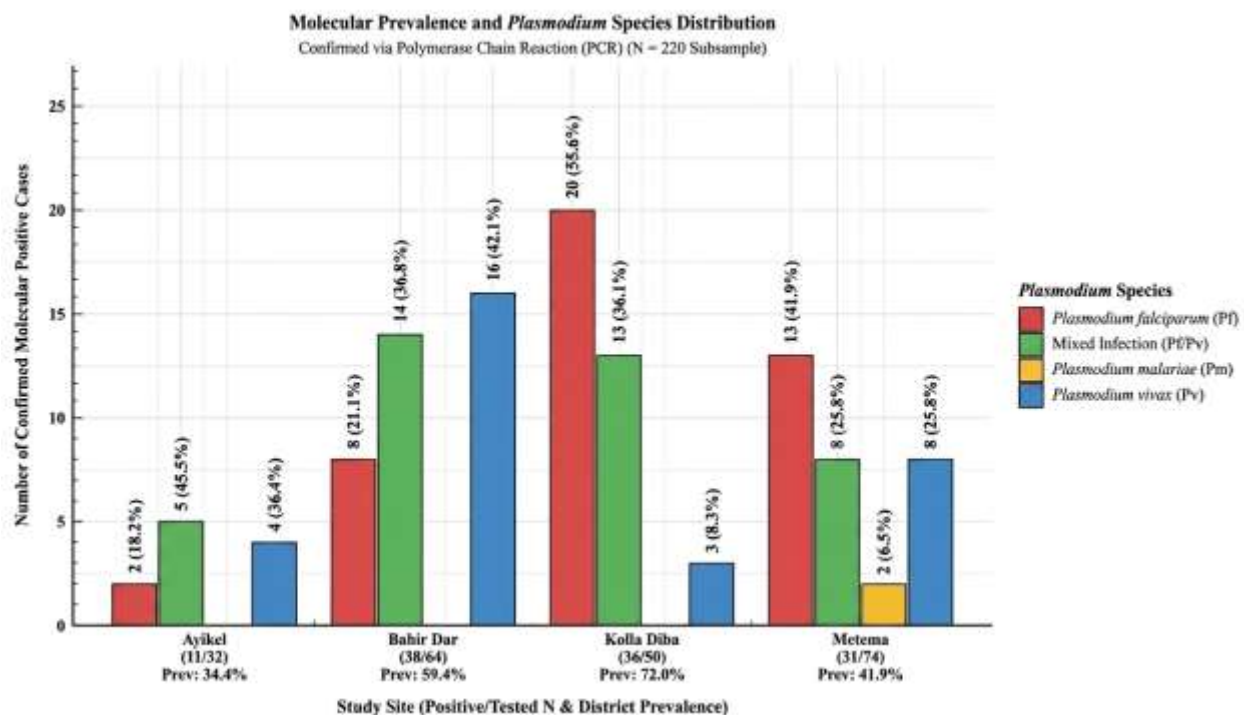


Figure 3: Distribution of *Plasmodium* species and total prevalence in each district across the Study Sites, northwest Ethiopia, 2026.

5.2.4 Quantitative Parasite Density Assessment for *P. falciparum* and *P. vivax*

Among the 104 microscopy-positive participants, 63 (60.6%) had low parasite density (<1,000 parasites/ μ L), 21 (20.2%) had moderate (1,000–4,999 parasites/ μ L), and 20 (19.2%) had high (5,000–99,999 parasites/ μ L); no hyperparasitemia ($\geq$ 100,000 parasites/ μ L) was observed. Median parasitemia was 2,505.0 parasites/ μ L (IQR: 69.6–42,354.6) for *P. falciparum* and 104.4 parasites/ μ L (IQR: 30.3–4,939.4) for *P. vivax*.

Multiplex-qPCR quantification used species-specific standard curves calibrated differently: *P. falciparum* against the culturable NF54 reference strain (parasites/ μ L), and *P. vivax* against a plasmid standard of known copy number (gene copies/ μ L), as no continuous *P. vivax* culture system exists. The two outputs are therefore not directly comparable and are reported separately. Median qPCR values were 114.7 parasites/ μ L (IQR: 29.4–4,520) for *P. falciparum* and 3,910 gene copies/ μ L (IQR: 112–10,027.5) for *P. vivax*; one *P. falciparum* sample showed hyperparasitemia, and none among *P. vivax* samples.

5.2.5 Correlation Between Microscopy and qPCR Parasite Burden

A strong positive correlation was observed between microscopy-derived parasite density and qPCR output for both species (Figure 4), indicating that the molecular assay tracked microscopy-based parasitemia closely within each species. For *P. falciparum*, microscopy parasite density was strongly correlated with qPCR-derived parasite density (Spearman's $\rho = 0.836$, $p < 0.001$). For *P. vivax*, microscopy parasite density was strongly correlated with qPCR-derived gene copy number (Spearman's $\rho = 0.980$, $p < 0.001$). As the *P. vivax* qPCR output is a copy-number rather than a parasite-density measure, this correlation should be interpreted as evidence that the assay

reliably tracks relative *P. vivax* parasitemia, not as a direct parasite-density validation.

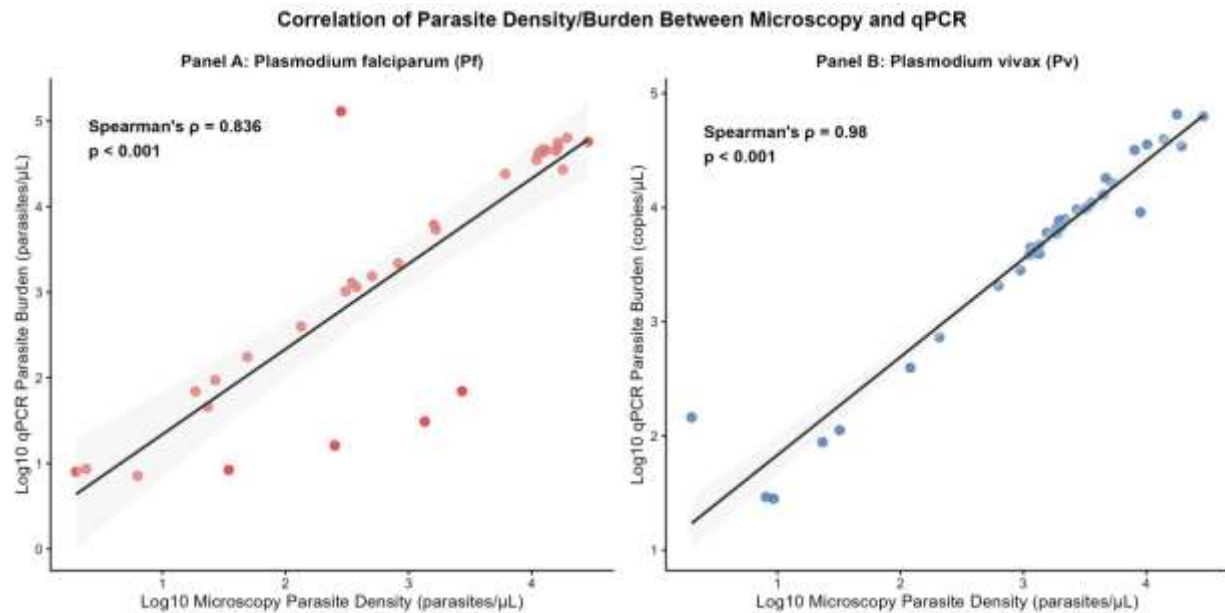


Figure 4: Correlation Between Microscopy-Derived Parasite Density and qPCR-Derived Parasite Burden for *P. falciparum* and *P. vivax* infections, Northwest Ethiopia, 2026.

5.3 Comparative Diagnostic Performance of Malaria Diagnostic Methods

5.3.1 Frequency and Distribution of Discordant Results

Among the 220 participants evaluated by all three diagnostic methods, 72 (32.7%) were negative by all three methods, while 62 (28.2%) were positive by all three methods. Discordant diagnostic results were observed in 86 (39.1%) participants. PCR alone detected 25 infections (11.4%) that were missed by both RDT and microscopy. Additionally, 16 cases (7.3%) were positive by microscopy and PCR but negative by RDT, while 14 cases (6.4%) were positive by both RDT and microscopy but negative by PCR. Exclusive positivity was observed in 11 cases (5.0%) for microscopy and 7 cases (3.2%) for RDT (Table 4).

Table 4: Concordant and Discordant Diagnostic Outcomes among RDT, Microscopy, and PCR in the PCR-Tested Subsample (n = 220), Northwest Ethiopia, 2026.

Diagnostic Outcome Combination Profile	Frequency (n)	Distribution n (%)
All Three Methods Negative (Concordant Negative)	72	72 (32.7%)
All Three Positive Methods (Concordant Positive)	62	62 (28.2%)
Microscopy (+) and PCR (+), but RDT (–)	16	16 (7.3%)
Microscopy Only Positive (+), others (–)	11	11 (5%)
PCR Only Positive (+), others (–)	25	25 (11.4%)
RDT (+) and Microscopy (+), but PCR (–)	14	14 (6.4%)
RDT (+) and PCR (+), but Microscopy (–)	13	13 (5.9%)
RDT Only Positive (+), others (–)	7	7 (3.2%)

5.3.2 Comparative Diagnostic Performance and Agreement Between RDT, Light Microscopy, and PCR

Inter-test agreement was assessed using Cohen's kappa. Among all 410 participants, RDT showed substantial agreement with light microscopy ($\kappa = 0.688$, 95% CI: 0.541–0.835; $p < 0.001$), with a sensitivity of 73.1% (95% CI: 63.5%–81.3%) and a specificity of 93.8% (95% CI: 90.5%–96.2%) relative to microscopy. Among the subset of 220 participants tested molecularly, agreement with PCR was moderate for both light microscopy ($\kappa = 0.411$, 95% CI: 0.273–0.550; $p < 0.001$) and RDT ($\kappa = 0.450$, 95% CI: 0.307–0.592; $p < 0.001$). Using PCR as the reference standard, microscopy achieved a numerically higher sensitivity than RDT (67.2%, 95% CI: 57.9%–75.7% vs. 64.7%, 95% CI: 55.2%–73.3%) and a slightly higher negative predictive value (67.5%, 95% CI: 58.2%–75.9% vs. 66.9%, 95% CI: 57.9%–75.1%), with overlapping confidence intervals between the two tests; conversely, RDT outperformed microscopy on specificity

(79.8%, 95% CI: 70.8%–87.0% vs. 76.0%, 95% CI: 66.6%–83.8%) and positive predictive value (78.1%, 95% CI: 68.5%–85.9% vs. 75.7%, 95% CI: 66.3%–83.6%) (Table 5).

Table 5: Diagnostic Performance and Agreement Between RDT, Microscopy, and PCR

Primary Comparison	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Kappa (κ)	95% CI (κ)	P-Value	Agreement Level
RDT vs. Microscopy (LM: reference)	410	73.1% (63.5–81.3%)	93.8% (90.5–96.2%)	80.0% (70.5–87.5%)	91.1% (87.4–94%)	0.688	0.541 to 0.835	<0.001	Substantial
Microscopy vs. PCR	220	67.2% (57.9–75.7%)	76.0% (66.6–83.8%)	75.7% (66.3–83.6%)	67.5% (58.2–75.9%)	0.411	0.273 to 0.550	<0.001	Moderate
RDT vs. PCR	220	64.7% (55.2–73.3%)	79.8% (70.8–87%)	78.1% (68.5–85.9%)	66.9% (57.9–75.1%)	0.450	0.307 to 0.592	<0.001	Moderate

5.3.3 Effect of Parasite Density on Diagnostic Performance

Detection by RDT and microscopy varied with molecular parasite burden. For *P. falciparum*, RDT and microscopy detected 61.8% and 65.5% of low-burden infections, compared with 65.0% and 75.0% of high-burden infections, respectively. For *P. vivax*, detection increased from 53.8% for low-burden infections to 72.4% by RDT and 82.8% by microscopy for high-burden infections (Table 6).

Table 6: Sensitivity of RDT and Microscopy across qPCR-Determined Parasite Burden Categories for *P. falciparum* and *P. vivax*, Northwest Ethiopia, 2026.

qPCR Parasite Burden	PCR-Positive (N)	RDT Sensitivity (95% CI)	Microscopy Sensitivity (95% CI)
A: Effect of <i>P. falciparum</i> Molecular Burden (parasites/μL)			
Low parasite load (<1,000 parasites/μL)	55	61.8% [49.0%–74.7%]	65.5% [52.9%–78.0%]
Moderate (1,000–4,999 parasites/μL)	7	28.6% [0.0%–62.0%]	42.9% [6.2%–79.5%]

qPCR Parasite Burden	PCR-Positive (N)	RDT Sensitivity (95% CI)	Microscopy Sensitivity (95% CI)
High (5,000–99,999 parasites/μL)	20	65.0% [44.1%–85.9%]	75.0% [56.0%–94.0%]
Hyperparasitemia ($\geq 100,000$ parasites/μL)	1	100.0% [100.0%– 100.0%]	100.0% [100.0%– 100.0%]

B: Effect of *P. vivax* Molecular Burden (copies/μL)

Low parasite load (<1,000 copies/μL)	26	53.8% [34.7%–73.0%]	53.8% [34.7%–73.0%]
Moderate (1,000–4,999 copies/μL)	15	73.3% [51.0%–95.7%]	60.0% [35.2%–84.8%]
High (5,000–99,999 copies/μL)	29	72.4% [56.1%–88.7%]	82.8% [69.0%–96.5%]

5.4 Factors Associated with *Plasmodium* Infection Based on the combination of diagnostic methods (RDT, Microscopy, and PCR)

Based on the composite reference standard, multivariable analysis identified unimproved housing wall material (AOR = 1.71, 95% CI: 1.04–2.81; $p = 0.034$), proximity to surface or stagnant water (AOR = 1.68, 95% CI: 1.02–2.76; $p = 0.041$), nocturnal outdoor sleeping or working (AOR = 2.51, 95% CI: 1.63–3.87; $p < 0.001$), and recent travel history (AOR = 2.00, 95% CI: 1.24–3.22; $p = 0.004$) as significant factors associated with *Plasmodium* infection. Other factors were not significantly associated with infection after adjustment (Table 7).

Table 7: Factors Associated with *Plasmodium* species infection Based on the Composite Reference (RDT, Microscope and PCR) Standard (N = 410), Northwest Ethiopia, 2026.

Variables	Positive (n)	Negative (n)	COR (95% CI)	P-value	AOR (95% CI)	P-value
Residence						
Urban	58	136	1	–	1	–
Rural	90	126	1.67 (1.11–2.52)	0.014*	1.53 (0.96–2.45)	0.077
Age Category						
>=15 years	116	207	1	–	1	–
<15 years	32	55	1.04 (0.64–1.7)	0.881	1.19 (0.68–2.1)	0.547
Sex						
Male	83	129	1	–	1	–
Female	65	133	0.76 (0.51–1.14)	0.183	0.81 (0.53–1.25)	0.346
Educational Background						
Formal education	107	196	1	–	1	–
Unable to read/write	41	66	1.14 (0.72–1.79)	0.578	0.99 (0.59–1.67)	0.976
Housing Wall Material						
Improved (Cement/Brick)	37	108	1	–	1	–
Unimproved (Wood/Mud)	111	154	2.10 (1.33–3.32)	0.001*	1.71 (1.04–2.81)	0.034*
Insecticide-Treated Net Ownership						
Yes	95	193	1	–	1	–
No	53	69	1.56 (1.01–2.41)	0.045*	1.43 (0.9–2.28)	0.126

Variables	Positive (n)	Negative (n)	COR (95% CI)	P-value	AOR (95% CI)	P-value
Proximity to Surface/Stagnant Water						
No	76	180	1	—	1	—
Yes	72	82	2.08 (1.35–3.20)	0.001*	1.68 (1.02–2.76)	0.041*
Nocturnal Outdoor Sleeping/Working						
No	60	164	1	—	1	—
Yes	88	98	2.45 (1.62–3.71)	0*	2.51 (1.63–3.87)	0*
Functional Window Screening						
Yes	74	127	1	—	1	—
No	74	135	0.94 (0.63–1.41)	0.766	0.82 (0.53–1.28)	0.383
Recent Travel History Profiles						
No	97	201	1	—	1	—
Yes	51	61	1.73 (1.11–2.7)	0.015*	2 (1.24–3.22)	0.004*

Where, *Statistically significant; 1=reference

6. DISCUSSION

In this study, 410 malaria-suspected individuals were enrolled, of whom 51.7% were male and 78.8% were aged ≥ 15 years. Overall malaria prevalence was 36.1% (148/410) using a composite of RDT, microscopy, and PCR, with individual test positivity of 23.2% by RDT and 25.4% by microscopy. *P. falciparum* (13.4%) and *P. vivax* (12.4%) were the predominant species, followed by mixed infections (9.8%) and *P. malariae* (0.5%), the latter detected exclusively by PCR. Among the 220 participants tested molecularly, 52.7% were PCR-positive. Relative to PCR, microscopy showed slightly higher sensitivity than RDT (67.2% vs. 64.7%), while RDT showed higher specificity (79.8% vs. 76.0%); RDT demonstrated substantial agreement with microscopy ($\kappa = 0.688$) and moderate agreement with PCR ($\kappa = 0.450$). Multivariable analysis identified unimproved housing wall material, proximity to stagnant water, nocturnal outdoor sleeping or working, and recent travel history as independent risk factors for *Plasmodium* infection. These findings are discussed in relation to existing literature below.

In the present study, the overall malaria prevalence was 36.1% (95% CI: 31.4%–40.7%), which is comparable to that reported in a health-facility-based study from Southern Ethiopia, where malaria prevalence was 36.4% by microscopy, 33.2% by RDT, and 40.8% by qPCR among febrile patients (Bogale *et al.*, 2026). It is also consistent with findings from Fogera District, Northwest Ethiopia, where prevalence was 40.5% by microscopy and 38.9% by RDT (Alebele *et al.*, 2026). Similarly, a study in Tanzania reported an RDT-based prevalence of 37.3%, although the microscopy-based prevalence (14.3%) was considerably lower (Hayuma *et al.*, 2021). This similarity might be attributed to comparable levels of malaria endemicity, transmission intensity, and health-facility catchment characteristics (febrile, health-facility-based sampling) between the settings.

Based on the composite reference standard combining RDT, microscopy and PCR, among 410 malaria-suspected individuals, *P. falciparum* accounted for 13.4% (55/410; 95% CI: 10.1%–16.7%), *P. vivax* for 12.4% (51/410; 95% CI: 9.2%–15.6%), mixed *P. falciparum/P. vivax* infections for 9.8% (40/410; 95% CI: 6.9%–12.6%), and *P. malariae* for only 0.5% (2/410; 95% CI: 0.1%–1.8%). Among the 220 PCR-tested participants, *P. falciparum* (37.1%) and mixed infections (34.5%) were more frequent than pure *P. vivax* (26.7%), while *P. malariae* remained

rare (1.7%), and no *P. ovale* was detected. This pattern of *Plasmodium* co-dominance is broadly consistent with findings from a comparative diagnostic study in Northwest Ethiopia, which similarly identified *P. falciparum* and *P. vivax* as the two most prevalent species (Minwuyelet *et al.*, 2026).

In the present study, *P. malariae* was identified only among PCR-tested participants (2/220, 1.7% of PCR-positive samples; 2/74, 2.7% in Metema specifically) and was missed by both microscopy and RDT. This finding shows the well-documented insensitivity of conventional diagnostic tools for this species and is consistent with a study from western Kenya, where 71% (32/45) of PCR-confirmed *P. malariae* infections were undetected by microscopy, characteristically low parasitaemia of this species (Lo *et al.*, 2017), and a Ghanaian PCR survey in which up to 98.7% of *P. malariae* infections confirmed molecularly were missed by microscopy (Amoah *et al.*, 2022). Taken together, this systematic underestimation of *P. malariae* by antigen-based and microscopy-based methods indicates a need to incorporate molecular diagnostics into malaria surveillance to avoid underdiagnosis of non-falciparum species, with direct implications for chloroquine-based radical treatment policy and malaria elimination planning.

A parallel treatment-policy implication applies to *P. vivax*, which, together with mixed infections, accounted for over a fifth of all infections in this study. Undiagnosed or delayed-diagnosed *P. vivax* infections risk being missed for primaquine-based radical cure, with consequent risk of relapse; this reinforces the need for molecular confirmation, particularly for mixed infections, ahead of radical treatment decisions in this setting.

For the diagnostic performance, the sensitivity of both RDT and microscopy fell considerably at low parasite densities, particularly for *P. vivax*, for which detection increased from only 53.8% at low burden to 72.4% (RDT) and 82.8% (microscopy) at high burden. This pattern is consistent with reports from Ethiopia's low-endemic settings, where microscopy detected only 0.7% of infections that qPCR identified in 12.7% of the same population, because parasite densities were low (median 24.4 parasites/ μ L for *P. falciparum* and 16.4 parasites/ μ L for *P. vivax*) (Tadesse *et al.*, 2017). Similarly, in Nigeria, microscopy achieved only 77% sensitivity against qPCR, compared with 95% for RDTs (Ikegbunam *et al.*, 2024), a sensitivity gap wider than microscopy (67.2%) and RDT (64.7%) in this study.

RDT showed substantial agreement with light microscopy ($\kappa = 0.688$, 95% CI: 0.541–0.835), with a sensitivity of 73.1%, specificity of 93.8%, PPV of 80.0%, and NPV of 91.1%. This distinction is operationally important: RDT–microscopy concordance reflects agreement between two conventional field tools, whereas the more moderate agreement with PCR indicates that a proportion of molecularly confirmed infections are missed by both routine methods, with direct implications for surveillance and case detection during the low-transmission or elimination phase, when submicroscopic infections predominate.

The sensitivity observed in our study (73.1%) was comparable to the 81.0% sensitivity and 96.8% specificity against microscopy (Zelege *et al.*, 2023), 78.8% against microscopy, though with lower specificity at 92.7% (Opoku Afriyie *et al.*, 2023a). In contrast, higher sensitivity was reported in Tanzania, with 99.8% sensitivity but lower specificity (87.6%) (Bwire *et al.*, 2019), Cameroon, 93.4% sensitivity with specificity (68.5%) (Missoup *et al.*, 2025) and Brazilian, sensitivity was 83.9% with specificity 99–100% (de Melo *et al.*, 2021). Our sensitivity was considerably higher than that reported in Adama Woreda, Ethiopia (50.0%) (Tegegn *et al.*, 2024a). These differences may be due to transmission intensity affecting parasite density distributions, varying antigen expression levels of *Plasmodium* species, and the quality and standardization of microscopy procedures, which cause the observed differences. Study population characteristics: age, immunity status, and clinical presentation further influence RDT performance.

Among the 220 participants evaluated by all three diagnostic methods in the current study, concordant results across all three tests were observed in 60.9% of cases (32.7% concordant-negative and 28.2% concordant-positive), while discordant results were observed in 39.1% of participants, most notably 25 cases (11.4%) that were positive by PCR alone. This overall level of discordance was considerably higher than the low discordance implied in Djibouti, 8.1% and 5.9% of *P. falciparum*-positive samples (Abdi Moussa *et al.*, 2024). It was, however, consistent with sensitivity (55.6%, a false-negative rate of approximately 44% against PCR) (Tegegne *et al.*, 2025), similar to the microscopy-and-PCR-positive-but-RDT-negative category (7.3%) observed in the present study. The discordance to false-negative RDT results in this study was comparable to the false-negative proportion (approximately 48%, derived from a 52.2% sensitivity against qPCR) reported in Ghana, where most missed infections were of low parasite density (Kayode *et*

al., 2024), and lower than the specificity, 82.4–84.4% versus a 93.4–96.2% HRP2-based comparator described in Burundi (Niyukuri *et al.*, 2022). Likewise, the PCR-positive infections missed by both microscopy and RDT in the present study (11.4%) were lower than 22.2% of nested-PCR-confirmed infections in Colombia (Vásquez *et al.*, 2018).

The relatively high level of discordance in this study, particularly the fraction detected by PCR alone, is due to low-density or submicroscopic parasitaemia, which was below the detection thresholds of RDT and microscopy, given that low parasite densities (<1,000 parasites/μL) accounted for 60.6% of microscopy-positive participants in this study. Other likely contributing factors include inter-reader variability in blood film, the relatively high proportion of *P. vivax* and mixed infections, which the pLDH-based BIOCREDIT platform detects via a lower-abundance, higher-limit-of-detection antigen than HRP2, and sample handling or storage conditions that can affect pLDH antigen stability, consistent with the antigen-concentration-driven sensitivity limitations described for this same RDT platform in Djibouti, Bahir Dar, Burundi, and Ghana.

Collectively, these findings indicate that a submicroscopic parasite reservoir, undetected by both RDT and microscopy, persists among malaria-suspected individuals in the study area. This has direct implications for onward transmission and for the reliability of routine RDT- and microscopy-based surveillance data used to guide malaria control and elimination planning in this setting.

In the present study, multivariable analysis identified unimproved housing wall material (AOR = 1.71, 95% CI: 1.04–2.81), proximity to surface or stagnant water (AOR = 1.68, 95% CI: 1.02–2.76), nocturnal outdoor sleeping or working (AOR = 2.51, 95% CI: 1.63–3.87), and recent travel history (AOR = 2.00, 95% CI: 1.24–3.22) as independent risk factors for *Plasmodium* infection. The association between proximity to stagnant water and malaria infection in the present study (AOR = 1.56; 95% CI: 1.00–2.43) is consistent with findings from Ethiopia. Studies in Tigray and Kola Diba reported significantly increased odds of malaria among individuals living near stagnant water (AOR = 6.00 and AOR = 2.06, respectively) (Mesele *et al.*, 2025, Nuru *et al.*, 2025). Similar associations have been reported outside Ethiopia: living close to stagnant water was associated with malaria infection in Ghana (AOR = 1.34) and among

pregnant women in Pru East Municipality (AOR = 4.96) (2024; 2025) (Opoku Afriyie *et al.*, 2024). This association is biologically plausible because stagnant water provides suitable larval habitats for *Anopheles* mosquitoes, potentially increasing local vector abundance and human-vector contact. In Fogera district, northwest Ethiopia, outdoor sleeping and proximity to mosquito breeding sites were highly prevalent (68.5% and 69.2%, respectively) but did not remain statistically significant after adjustment, whereas ITN availability and household ITN ownership emerged as the dominant predictors of infection (Alebele *et al.*, 2026).

Nocturnal outdoor sleeping or working was significantly associated with malaria infection in the present study (AOR = 2.51; 95% CI: 1.63–3.87). This finding is consistent with evidence from Northwest Ethiopia, where outdoor sleeping at night was independently associated with increased odds of malaria infection among under-five children in Wogera District (Tsegaye *et al.*, 2021). Similarly, a case-control study in Mudzi District, Zimbabwe, reported night-time outdoor social and religious activities as an independent risk factor for malaria infection (AOR = 8.13; 95% CI: 1.74–37.90) (Fambirai *et al.*, 2025). The increased risk may be explained by greater exposure to *Anopheles* mosquitoes during peak nocturnal biting hours when individuals sleep or work outdoors.

Recent travel history was associated with malaria infection in the present study (AOR = 2.00, 95% CI: 1.24–3.22), consistent with findings from South Ethiopia, where travel to malaria-endemic lowland areas during the preceding 30 days increased the odds of *P. falciparum* infection (AOR = 2.60, 95% CI: 1.27–5.33) (Assefa *et al.*, 2024), and from Malawi, where travel outside city was associated with increased odds of malaria infection (AOR = 2.36, 95% CI: 1.31–4.20) (Ahmed *et al.*, 2020). Similarly, a case-control study in Kampala, Uganda, found that recent overnight travel was strongly associated with malaria infection (AOR = 58.9; 95% CI: 23.1–150.1) (Arinaitwe *et al.*, 2020). Evidence from Southern Province, Zambia, also demonstrated that recent travel was independently associated with malaria infection (AOR = 2.55; 95% CI: 2.28–2.85) (Porter *et al.*, 2020).

Housing quality and proximity to potential vector habitats in the present study are consistent with findings from Edo-North, Nigeria, where malaria was more common among residents of mud houses and among those living near bushes, streams, and rivers (Braimah *et al.*, 2024). In

Uganda, modern houses characterized by brick walls, metal roofs and closed eaves had a 53% lower human biting rate than traditional houses (aIRR = 0.47, 95% CI: 0.32–0.67, $p < 0.001$), while closed eaves and metal roofs were independently associated with lower mosquito biting rates (IRR = 0.40, 95% CI: 0.25–0.65 and IRR = 0.40, 95% CI: 0.24–0.68, respectively) (Musiime *et al.*, 2022), show the role of structural housing improvements in reducing indoor vector exposure. In Cambodia, Chen *et al.* reported that 29% of participants lived in partially open primary structures and 39% in partially open secondary structures, with important gaps in mosquito-bite protection during outdoor and nighttime activities among forest-exposed populations (Chen *et al.*, 2025).

Limitations of the Study

The main limitation of this study was the inability to perform PCR testing on all 410 study participants because of resource and laboratory capacity constraints. PCR was performed on all samples that were positive by the routine diagnostic methods and on a subset of samples that were negative. Consequently, not all participants had the same opportunity to undergo the reference molecular test. In addition to this, health facilities were purposefully selected, and behavioral and environmental factors data were based on participant reporting.

7. CONCLUSION AND RECOMMENDATIONS

7.1 CONCLUSION

This study demonstrated that *Plasmodium* infection remains an important public health concern in the study area. Molecular investigation identified a broader diversity of *Plasmodium* infections, including *P. falciparum*, *P. vivax*, *P. malariae*, and mixed-species infections that were missed by microscopy and/or RDT. A comparison of the two routine diagnostic methods showed that RDT was comparable to light microscopy for detecting *Plasmodium* infection in the study population. Several household, environmental, socioeconomic, and behavioral factors were independently associated with *Plasmodium* infection.

7.2 RECOMMENDATIONS

1. Recommendations to the Community

- Community members should consistently use insecticide-treated bed nets, particularly in malaria-endemic areas and during peak transmission periods.
- Communities should reduce exposure to mosquito bites by avoiding outdoor activities during peak mosquito-biting hours where possible and using appropriate personal protective measures.
- Households should improve environmental sanitation and reduce potential mosquito breeding sites around homes.

2. Recommendations to Health-Care Workers

- Community health education should emphasize that malaria may occur with mild or atypical symptoms
- Microscopy should be strengthened through regular training, competency assessment, quality assurance, and supervision, particularly for accurate species identification and detection of low-density and mixed infections.
- Less commonly detected species, including *P. malariae*, should be considered in endemic sites.

3. Recommendations to Policymakers and Malaria Control Programs

- Regional and national malaria programs should strengthen routine surveillance of *Plasmodium* species, including monitoring of non-dominant and mixed infections.
- Diagnostic quality-assurance programs should be strengthened to improve the sensitivity and species identification capacity of routine microscopy and RDTs.
- Malaria prevention strategies should consider the clinical, household, behavioral, socioeconomic, and environmental factors identified as associated with infection in the study.

4. Recommendations to Researchers

- Future studies should be performed using PCR on all study participants, regardless of microscopy or RDT results, to obtain more accurate estimates of species distribution and diagnostic performance.
- Larger, multicenter studies should be conducted across different districts and transmission settings of Northwest Ethiopia to establish the broader epidemiology of *P. malariae*, *P. ovale species*, and mixed infections.
- Further research should evaluate the clinical significance of low-density, mixed, *P. malariae*, and other non-dominant infections.

8. REFERENCES

- ABAGERO, B. R., KEPPLER, D., PESTANA, K., WITHERSPOON, L., BIRUKSEW, A., ADANEW, A., BAHARU, F., HANSEL, S., LOPEZ, K., JANIES, D. A., LO, E. & YEWHALAW, D. 2022. Low density Plasmodium infections and G6PD deficiency among malaria suspected febrile individuals in Ethiopia. *Frontiers in Tropical Diseases*, Volume 3 - 2022.
- ABDI MOUSSA, R., PAPA MZE, N., YONIS ARREH, H., ABDILLAH HAMOUD, A., MOHAMED ALALEH, K., MOHAMED ADEN, F., YONIS OMAR, A. R., OSMAN ABDI, W., KAYAD GUELLEH, S., AHMED ABDI, A. I., BASCO, L. K., ABDI KHAIREH, B. & BOGREAU, H. 2024. Assessment of the Performance of Lactate Dehydrogenase-Based Rapid Diagnostic Test for Malaria in Djibouti in 2022-2023. *Diagnostics (Basel)*, 14.
- ADUGNA, F., WALE, M. & NIBRET, E. 2022. Prevalence of malaria and its risk factors in Lake Tana and surrounding areas, northwest Ethiopia. *Malaria Journal*, 21, 313.
- AGEGNEHU, F., SHIMEKA, A., BERIHUN, F. & TAMIR, M. 2018. Determinants of malaria infection in Dembia district, Northwest Ethiopia: a case-control study. *BMC Public Health*, 18, 480.
- AHMAD, A., SONI, P., KUMAR, L., SINGH, M. P., VERMA, A. K., SHARMA, A., DAS, A. & BHARTI, P. K. 2021. Comparison of polymerase chain reaction, microscopy, and rapid diagnostic test in malaria detection in a high burden state (Odisha) of India. *Pathog Glob Health*, 115, 267-272.
- AHMED, S., REITHINGER, R., KAPTOGE, S. K. & NGONDI, J. M. 2020. Travel Is a Key Risk Factor for Malaria Transmission in Pre-Elimination Settings in Sub-Saharan Africa: A Review of the Literature and Meta-Analysis. *Am J Trop Med Hyg*, 103, 1380-1387.
- ALEBELE, S., ABATE, A., SISAY, M., ASNAKE, T., ALEHEGN, M., ADISU, D. & MUNSHEA, A. 2026. Prevalence and associated risk factors of malaria among febrile patients at Kidist Hana Health Center Fogera District, Amhara Regional State, Northwest Ethiopia. *Parasite Epidemiol Control*, 33, e00488.
- ALEMAYEHU, A., ABOSSIE, A., ZEYNUDIN, A., BEYENE, J. & YEWHALAW, D. 2024a. Asymptomatic malaria in pregnancy and associated risk factors in Majang Zone,

- Gambella Region, Southwest Ethiopia: a hard-to-reach malaria hotspot. *Malaria Journal*, 23, 210.
- ALEMAYEHU, A., ZEYNUDIN, A., BEYENE, J. & YEWHALAW, D. 2025. Performance of malaria rapid diagnostic test, microscopy, polymerase chain reaction, and histopathology to diagnose malaria among pregnant and parturient women using peripheral, placental, and cord blood, and placental biopsy in Majang Zone of Gambella Region, Southwest Ethiopia. *Malar J*, 24, 175.
- ALEMAYEHU, G. S., LOPEZ, K., DIENG, C. C., LO, E., JANIES, D. & GOLASSA, L. 2020. Evaluation of PfHRP2 and PfLDH Malaria Rapid Diagnostic Test Performance in Assosa Zone, Ethiopia. *Am J Trop Med Hyg*, 103, 1902-1909.
- ALEMAYEHU, K. D., MITIKU, B. A., ALEMU, Y. F. & NIBRET, T. B. 2024b. Knowledge, attitude, and prevention practices towards common zoonotic diseases in and around Bahir Dar city, north western Ethiopia. *Transboundary and Emerging Diseases*, 2024, 6642766.
- ALEMU, A., FUEHRER, H.-P., GETNET, G., KASSU, A., GETIE, S. & NOEDL, H. 2014. Comparison of Giemsa microscopy with nested PCR for the diagnosis of malaria in North Gondar, north-west Ethiopia. *Malaria Journal*, 13, 174.
- ALEMU, A., FUEHRER, H.-P., GETNET, G., TESSEMA, B. & NOEDL, H. 2013. Plasmodium ovale curtisi and Plasmodium ovale wallikeri in North-West Ethiopia. *Malaria journal*, 12, 346.
- ALHARBI, B. F. & AHMED, M. A. 2026. Malaria in the 21st Century: Global Disease Burden, Epidemiological Insights, and Strategic Control Approaches. *Biology*, 15, 575.
- AMOAHA, L. E., ASARE, K. K., DICKSON, D., ANANG, S. F., BUSAYO, A., BREDU, D., ASUMAH, G., PEPRAH, N., ASAMOAHA, A., ABUAKU, B. & MALM, K. L. 2022. Nationwide molecular surveillance of three Plasmodium species harboured by symptomatic malaria patients living in Ghana. *Parasit Vectors*, 15, 40.
- ANJORIN, S., OKOLIE, E. & YAYA, S. 2023. Malaria profile and socioeconomic predictors among under-five children: an analysis of 11 sub-Saharan African countries. *Malar J*, 22, 55.
- ARINAITWE, E., MPIMBAZA, A., NANKABIRWA, J. I., KAMYA, V., ASIIMWE, A., KUULE, J. K., KAMYA, M. R., DRAKELEY, C., DORSEY, G., ROSENTHAL, P. J. & STAEDKE, S. G. 2020. Malaria Diagnosed in an Urban Setting Strongly Associated with

- Recent Overnight Travel: A Case-Control Study from Kampala, Uganda. *Am J Trop Med Hyg*, 103, 1517-1524.
- ASFAW, D. M., SHIFAW, S. M., BELETE, A. A. & AYCHILUHM, S. B. 2022. The Impact of Community-Based Health Insurance on Household's Welfare in Chilga District, Amhara Regional State, Ethiopia. *Front Public Health*, 10, 868274.
- ASSEFA, M., MASSEBO, F., ASHINE, T. & WEGAYEHU, T. 2024. Population travel increases the risk of Plasmodium falciparum infection in the highland population of Gardula Zone, South Ethiopia: A longitudinal study. *PLOS ONE*, 19, e0315900.
- BELACHEW, M., WOLDE, M., NEGA, D., GIDEY, B., NEGASH, L., ASSEFA, A., TASEW, G., WOYESSA, A. & ABERA, A. 2022. Evaluating performance of multiplex real time PCR for the diagnosis of malaria at elimination targeted low transmission settings of Ethiopia. *Malar J*, 21, 9.
- BERHE, B., MARDU, F., LEGESE, H. & NEGASH, H. 2019. Seasonal distribution and seven year trend of malaria in North West Tigray: 2012–2018, Ethiopia; 2019. *Tropical Diseases, Travel Medicine and Vaccines*, 5, 15.
- BOGALE, A., MITIKU, A., TEFERA, S., KASSIE, A., ABREHAM, A., BAYIH, A., DUBE, S. J., TEKLESELASSIE, T., ABEBE, A. A., TEREFE, H. & DEGEFA, T. 2026. Comparative evaluation of a multi-antigen malaria rapid diagnostic test against qPCR and microscopy in a co-endemic area of Southern Ethiopia. *Sci Rep*.
- BRAIMAH, J. O., EDIKE, N., OKHAIOMOJE, A. I. & CORREA, F. M. 2024. The fight against malaria in Edo-North, Edo State, Nigeria: identifying risk factors for effective control. *PeerJ*, 12, e18301.
- BUDERER, N. M. F. 1996. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. *Academic Emergency Medicine*, 3, 895-900.
- BWIRE, G. M., NGASALA, B., KILONZI, M., MIKOMANGWA, W. P., FELICIAN, F. F. & KAMUHABWA, A. A. 2019. Diagnostic performance of CareStart™ malaria HRP2/pLDH test in comparison with standard microscopy for detection of uncomplicated malaria infection among symptomatic patients, Eastern Coast of Tanzania. *Malaria journal*, 18, 354.

- CARLIER, L., BAKER, S. C., HUWE, T., YEWHALAW, D., HAILESELASSIE, W. & KOEPFLI, C. 2022. qPCR in a suitcase for rapid *Plasmodium falciparum* and *Plasmodium vivax* surveillance in Ethiopia. *PLOS Global Public Health*, 2, e0000454.
- CHEN, I., DOUM, D., MCIVER, D. J., KEO, V., VONG, P., PECH, S., METH, V., BUN, S., PEN, K. & CHEA, S. 2025. Identifying malaria risks amongst forest going populations in Mondulkiri province and Kampong Speu province, Cambodia: a large cross-sectional survey. *Malaria Journal*, 24, 59.
- CHILOT, D., MONDELAERS, A., ALEM, A. Z., ASRES, M. S., YIMER, M. A., TONI, A. T. & AYELE, T. A. 2023. Pooled prevalence and risk factors of malaria among children aged 6–59 months in 13 sub-Saharan African countries: A multilevel analysis using recent malaria indicator surveys. *PLOS ONE*, 18, e0285265.
- CUADROS, J., PÉREZ-TANOIRA, R., PRIETO-PÉREZ, L., MARTIN-MARTIN, I., BERZOSA, P., GONZÁLEZ, V., TISIANO, G., BALCHA, S., RAMOS, J. M. & GÓRGOLAS, M. 2015. Field Evaluation of Malaria Microscopy, Rapid Malaria Tests and Loop-Mediated Isothermal Amplification in a Rural Hospital in South Western Ethiopia. *PLOS ONE*, 10, e0142842.
- DAGNEW, A., YESHANEW, S., NIBRET, E., ATENAFU, G., KENDIE, F. A., ASSEFA, M. & HABTAMU, A. 2025. Prevalence of malaria and associated factors among febrile patients in selected healthcare facilities of Jawi District, northwest Ethiopia. *Malaria Journal*, 24, 327.
- DE MELO, G. C., NETTO, R. L. A., MWANGI, V. I., SALAZAR, Y. E. A. R., DE SOUZA SAMPAIO, V., MONTEIRO, W. M., DE ALMEIDA E VAL, F. F., ROCHELEAU, A., THOTA, P. & LACERDA, M. V. G. 2021. Performance of a sensitive haemozoin- based malaria diagnostic test validated for vivax malaria diagnosis in Brazilian Amazon. *Malaria Journal*, 20, 146.
- DEMASS, T. B., BESHAW, M. A., BANTIE, G. M., BEYENE, B. B., TADEGE, M., ALEHEGN, A. A., BERNEH, A. A., YIMER, M., MELESE, A. A. & DEGU, W. A. 2025a. Treatment outcomes of patients with uncomplicated malaria and associated factors in Northwest Ethiopia: a prospective follow-up study, 2024. *BMC Infectious Diseases*, 25, 387.

- DEMASS, T. B., BEYENE, B. B., BANTIE, G. M., TADEGE, M., ALEHEGN, A. A., BERNEH, A. A., JEGNIE, M., BESHAW, M. A., MELESE, A. A. & DEGU, W. A. 2025b. Comparing the diagnostic accuracy of the newly introduced and existing malaria tests in Northwest Ethiopia. *PLOS ONE*, 20, e0322366.
- DUGUMA, T., TEKALIGN, E., MULETA, D. & SIMIENEH, A. 2022. Malaria prevalence and risk factors among patients visiting Mizan Tepi University Teaching Hospital, Southwest Ethiopia. *PLOS ONE*, 17, e0271771.
- ESAYAS, E., LOUIE, W., ROUTLEDGE, I., MURPHY, M., DEMEKE, N. N., DE AMARAL, F., ARANDA-DÍAZ, A., GREENHOUSE, B., KASSA, F. A. & NIGUSSE, T. 2025. High connectivity and low differentiation of *Plasmodium falciparum* parasite populations in a setting with high seasonal migration. *Scientific Reports*.
- FAMBIRAI, T., CHIMBARI, M. J. & NDARUKWA, P. 2025. Risk factors for contracting malaria in six wards of Mudzi District, Zimbabwe: A case control-study. *PLoS One*, 20, e0329093.
- FELEKE, D. G., ALEMU, Y. & YEMANEHIRHANE, N. 2021. Performance of rapid diagnostic tests, microscopy, loop-mediated isothermal amplification (LAMP) and PCR for malaria diagnosis in Ethiopia: a systematic review and meta-analysis. *Malaria Journal*, 20, 384.
- FMOH ETHIOPIA. 2020. *ETHIOPIA MALARIA ELIMINATION STRATEGIC PLAN: 2021-2025* [Online]. Available: [https://www.moh.gov.et/National Malaria Elimination Program](https://www.moh.gov.et/National_Malaria_Elimination_Program) [Accessed 14 April 2026].
- GEBREMESKELE, B. T., GETU, E., DEMEKE, A. D., BOGALE, A. & CHURISO, G. 2026. Diagnostic accuracy of novel Biocredit species-specific LDH-based immunochromatographic assay for *Plasmodium falciparum* and *Plasmodium vivax* detection: a systematic review and meta-analysis. *BMC Infectious Diseases*, 26, 721.
- GETIE, S., MEKONNEN, G. G., WORKU, L., BIRHANIE, M., MEDA, A. L., ABERE, A. & NOEDL, H. 2026. Unmasking Malaria: Microscopy and nPCR Reveal the Hidden Misdiagnosis of *Plasmodium* Infections Among Febrile Pediatric in Northwest Ethiopia. *Interdisciplinary Perspectives on Infectious Diseases*, 2026, 9983415.

- GOLASSA, L., MESSELE, A., AMAMBUA-NGWA, A. & SWEDBERG, G. 2020. High prevalence and extended deletions in *Plasmodium falciparum* hrp2/3 genomic loci in Ethiopia. *PLOS ONE*, 15, e0241807.
- HAILU, T., ALEMU, M., MULU, W. & ABERA, B. 2018. Incidence of *Plasmodium* infections and determinant factors among febrile children in a district of Northwest Ethiopia; a cross-sectional study. *Tropical Diseases, Travel Medicine and Vaccines*, 4, 8.
- HAYUMA, P. M., WANG, C. W., LIHELUKA, E., BARAKA, V., MADEBE, R. A., MINJA, D. T. R., MISINZO, G., ALIFRANGIS, M. & LUSINGU, J. P. A. 2021. Prevalence of asymptomatic malaria, submicroscopic parasitaemia and anaemia in Korogwe District, north-eastern Tanzania. *Malar J*, 20, 424.
- HUBER, J. H., KOEPFLI, C., ESPAÑA, G., NEKKAB, N., WHITE, M. T. & ALEX PERKINS, T. 2021. How radical is radical cure? Site-specific biases in clinical trials underestimate the effect of radical cure on *Plasmodium vivax* hypnozoites. *Malaria Journal*, 20, 479.
- IKEGBUNAM, M., MAURER, M., ABONE, H., EZEAGWUNA, D., SANDRI, T. L., ESIMONE, C., OJURONGBE, O., WOLDEAREGAI, T. G., KREIDENWEISS, A., HELD, J. & FENDEL, R. 2024. Evaluating Malaria Rapid Diagnostic Tests and Microscopy for Detecting *Plasmodium* Infection and Status of *Plasmodium falciparum* Histidine-Rich Protein 2/3 Gene Deletions in Southeastern Nigeria. *Am J Trop Med Hyg*, 110, 902-909.
- KAYODE, T. A., ADDO, A. K., ADDISON, T. K., TWENEBOAH, A., AFRIYIE, S. O., ABBAS, D. A., SETH, A., BADU-TAWIAH, A. K., BADU, K. & KOEPFLI, C. 2024. Comparison of three rapid diagnostic tests for *Plasmodium falciparum* diagnosis in Ghana. *Malar J*, 23, 265.
- LAKE, M. W., ASEMAHAGN, M. A., NIGUSSIE, T. Z., GELAYE, K. A., MUCHIE, K. F., ENGEDAW, H. A. & YENESEW, M. A. 2025. Resurgence of malaria in the Amhara Region, Ethiopia (2014-2024): trends, spatial expansion, and control challenges. *Malar J*, 24, 425.
- LO, E., NGUYEN, K., NGUYEN, J., HEMMING-SCHROEDER, E., XU, J., ETEMESI, H., GITHEKO, A. & YAN, G. 2017. *Plasmodium malariae* prevalence and csp gene diversity, Kenya, 2014 and 2015. *Emerging infectious diseases*, 23, 601.

- MADKHALI, A. M., GHZWANI, A. H. & AL-MEKHLAFI, H. M. 2022. Comparison of Rapid Diagnostic Test, Microscopy, and Polymerase Chain Reaction for the Detection of *Plasmodium falciparum* Malaria in a Low-Transmission Area, Jazan Region, Southwestern Saudi Arabia. *Diagnostics (Basel)*, 12.
- MARTÍN-DÍAZ, A., RUBIO, J. M., HERRERO-MARTÍNEZ, J. M., LIZASOAIN, M., RUIZ-GIARDIN, J. M., JAQUETI, J., CUADROS, J., ROJO-MARCOS, G., MARTÍN-RABADÁN, P., CALDERÓN, M., CAMPELO, C., VELASCO, M. & PÉREZ-AYALA, A. 2018. Study of the diagnostic accuracy of microbiological techniques in the diagnosis of malaria in the immigrant population in Madrid. *Malaria Journal*, 17, 314.
- MEENA, P., SINGLA, V., DESHPANDE, A., KASTURI, U., KHAN, E. F., KUMAR, V. R. K. & KALYESUBULA, R. 2026. Clinical characteristics and outcome of malaria-associated acute kidney injury in adult patients: a systematic review and meta analysis. *Malar J*, 25.
- MESELE, F. B., BELAY, D. S., GEBRESLASSIE, K. B., ABRHA, M. G., TESFAY, B. G. & GEBREANENIA, F. A. 2025. Prevalence of malaria and its associated factors among adults living in Samre Woreda, Tigray, Ethiopia, 2023/24: a community-based cross-sectional study. *BMC Public Health*, 25, 2162.
- MINWUYELET, A., YEWHALAW, D. & ATENAFU, G. 2025. Retrospective analysis of malaria prevalence over ten years (2015–2024) at Bichena Primary Hospital, Amhara Region, Ethiopia. *PLOS ONE*, 20, e0322570.
- MINWUYELET, A., YEWHALAW, D. & ATENAFU, G. 2026. Diagnostic challenges in malaria detection: A comparative diagnostic performance of HRP2-based rapid diagnostic tests, microscopy, and PCR at Bichena primary hospital, Northwest Ethiopia. *Parasite Epidemiology and Control*, e00485.
- MISSOUP, V. O., SUH, P. F., NDJEBAKAL, C. N., TAGNE, D., POUMACHU, Y., JOKO, S., KOUAMENDJOUO DJILLA, A., NJIOKOU, F., MBACHAM, W. & WONDJI, C. 2025. Malaria infection prevalence and diagnostic performance of the Abbott Bioline Malaria Ag Pf/Pan rapid test in rural populations of Central Cameroon. *Scientific Reports*, 15, 27930.
- MUSIIME, A. K., KREZANOSKI, P. J., SMITH, D. L., KILAMA, M., CONRAD, M. D., OTTO, G., KYAGAMBA, P., ASIIMWE, J., REK, J., NANKABIRWA, J. I., ARINAITWE, E., AKOL, A. M., KAMYA, M. R., STAEDKE, S. G., DRAKELEY, C.,

- BOUSEMA, T., LINDSAY, S. W., DORSEY, G. & TUSTING, L. S. 2022. House design and risk of malaria, acute respiratory infection and gastrointestinal illness in Uganda: A cohort study. *PLOS Glob Public Health*, 2, e0000063.
- NIYUKURI, D., SINZINKAYO, D., TROTH, E. V., ODUMA, C. O., BARENGAYABO, M., NDEREYIMANA, M., HOLZSCHUH, A., VERA-ARIAS, C. A., GEBRE, Y., BADU, K., NYANDWI, J., BAZA, D., JUMA, E. & KOEPFLI, C. 2022. Performance of highly sensitive and conventional rapid diagnostic tests for clinical and subclinical *Plasmodium falciparum* infections, and hrp2/3 deletion status in Burundi. *PLOS Glob Public Health*, 2, e0000828.
- NURU, H., TEKESTE, Z. & ALELIGN, A. 2025. Epidemiology of malaria and utilization of insecticide-treated bed nets in Kola Diba, northwest Ethiopia. *Scientific African*, 28, e02720.
- OBASOHAN, P. E., WALTERS, S. J., JACQUES, R. & KHATAB, K. 2021. A Scoping Review of Selected Studies on Predictor Variables Associated with the Malaria Status among Children under Five Years in Sub-Saharan Africa. *Int J Environ Res Public Health*, 18.
- OBEAGU, E. I. 2024. Role of cytokines in immunomodulation during malaria clearance. *Annals of Medicine and Surgery*, 86.
- ODUMA, C. O., LULU, B., EWNETU, Y., LOWN, L. A., KAYODE, T. A., HAWARIA, D. & KOEPFLI, C. 2025. First field evaluation of novel LDH- and HRP2-based rapid tests for *Plasmodium vivax* and *Plasmodium falciparum* malaria diagnosis. *PLOS Neglected Tropical Diseases*, 19, e0013307.
- OJENIYI, F. D., AYOOLA, A. O., IBITOYE, O., OPALEYE, O. O., OLOWE, O. A., EHIGIE, L. O., THOMAS, B. N. & OJURONGBE, O. 2025. Performance and challenges of malaria rapid diagnostic tests in endemic regions of Africa. *Scientific Reports*, 15, 43663.
- OKYERE, B., OWUSU-OFORI, A., ANSONG, D., BUXTON, R., BENSON, S., OSEI-AKOTO, A., OWIREDU, E.-W., ADJEI, C., XORSE AMUZU, E., MARFO BOAHENG, J. & DICKERSON, T. 2020. Point prevalence of asymptomatic *Plasmodium* infection and the comparison of microscopy, rapid diagnostic test and nested PCR for the diagnosis of asymptomatic malaria among children under 5 years in Ghana. *PLOS ONE*, 15, e0232874.

- OPOKU AFRIYIE, S., ADDISON, T. K., GEBRE, Y., MUTALA, A.-H., ANTWI, K. B., ABBAS, D. A., ADDO, K. A., TWENEBOAH, A., AYISI-BOATENG, N. K. & KOEPFLI, C. 2023a. Accuracy of diagnosis among clinical malaria patients: comparing microscopy, RDT and a highly sensitive quantitative PCR looking at the implications for submicroscopic infections. *Malaria Journal*, 22, 76.
- OPOKU AFRIYIE, S., ADDISON, T. K., GEBRE, Y., MUTALA, A.-H., ANTWI, K. B., ABBAS, D. A., ADDO, K. A., TWENEBOAH, A., AYISI-BOATENG, N. K., KOEPFLI, C. & BADU, K. 2023b. Accuracy of diagnosis among clinical malaria patients: comparing microscopy, RDT and a highly sensitive quantitative PCR looking at the implications for submicroscopic infections. *Malaria Journal*, 22, 76.
- OPOKU AFRIYIE, S., ANTWI, K. B., MUTALA, A.-H., ABBAS, D. A., ADDO, K. A., TWENEBOAH, A., ADDISON, T. K., OSEI, E., KOEPFLI, C. & BADU, K. 2024. Socio-demographic factors, housing characteristics, and clinical symptoms associated with falciparum malaria in two rapidly urbanizing areas in the Ashanti region of Ghana. *Malaria Journal*, 23, 354.
- OSUN, O. G., AHMED, A. S., SULIMAN, S. A., OLORUNFEMI, A. B., THOMAS, B. N., OJURONGBE, O., OSUN, O. & OLORUNFEMI, A. 2024. Comparative evaluation of microscopy, rapid diagnostic tests, and polymerase chain reaction (pcr) for malaria diagnosis in Nigerian children. *Cureus*, 16.
- PORTER, T. R., FINN, T. P., SILUMBE, K., CHALWE, V., HAMAINZA, B., KOOMA, E., MOONGA, H., BENNETT, A., YUKICH, J. O., STEKETEE, R. W., KEATING, J., MILLER, J. M. & EISELE, T. P. 2020. Recent Travel History and Plasmodium falciparum Malaria Infection in a Region of Heterogenous Transmission in Southern Province, Zambia. *Am J Trop Med Hyg*, 103, 74-81.
- QIAGEN. 2024. QIAamp DNA Mini and Blood Mini Handbook. 5th Edition ed.
- SANYAOLU, A., MARINKOVIC, A., PRAKASH, S., BALENDRA, V., SHAZLEY, O., GARDELLINI, T., JAN, A., YOUNIS, K., OKORIE, C. & IZURIETA, R. 2025. Emerging Molecular Mechanisms in Malaria Pathogenesis and Novel Therapeutic Approaches: A Focus on P. falciparum Malaria. *Biomolecules*, 15, 1038.
- SINGH, A., SINGH, M. P., BHANDARI, S., RAJVANSHI, H., NISAR, S., TELASEY, V., JAYSWAR, H., MISHRA, A. K., DAS, A., KAUR, H., LAL, A. A. & BHARTI, P. K.

2022. Significance of nested PCR testing for the detection of low-density malaria infection amongst febrile patients from the Malaria Elimination Demonstration Project in Mandla, Madhya Pradesh, India. *Malar J*, 21, 341.
- SNOUNOU, G., VIRIYAKOSOL, S., JARRA, W., THAITHONG, S. & BROWN, K. N. 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and biochemical parasitology*, 58, 283-292.
- TADESSE, F. G., VAN DEN HOOGEN, L., LANKE, K., SCHILDKRAUT, J., TETTEH, K., ASEFFA, A., MAMO, H., SAUERWEIN, R., FELGER, I., DRAKELEY, C., GADISSA, E. & BOUSEMA, T. 2017. The shape of the iceberg: quantification of submicroscopic *Plasmodium falciparum* and *Plasmodium vivax* parasitaemia and gametocytaemia in five low endemic settings in Ethiopia. *Malar J*, 16, 99.
- TAMIR, Z., ANIMUT, A., DUGASSA, S., GEBRESILASSIE, A., BELACHEW, M., ABERA, A. & ERKO, B. 2025. Comparative performance of microscopy, rapid diagnostic tests, and multiplex real-time PCR for detection of malaria parasites among pregnant women in northwest Ethiopia. *Malaria Journal*, 24, 19.
- TAREKEGN, M., TEKIE, H., DUGASSA, S. & WOLDE-HAWARIAT, Y. 2021. Malaria prevalence and associated risk factors in Dembiya district, North-western Ethiopia. *Malaria Journal*, 20, 372.
- TEGEGN, G., GNANASEKAREN, N., GADISA, E., GETIE, M., MOLLA, A., MEHARIE, T., DEBASH, H., BERHAN, A., ALMAW, A. & HUSSIEN, A. 2024a. Comparative assessment of microscopy, malaria rapid diagnostic test and polymerase chain reaction as malaria diagnostic tools in Adama Woreda, East shoa zone of Ethiopia: a cross-sectional study. *BMC Infectious Diseases*, 24, 1363.
- TEGEGN, G., GNANASEKAREN, N., GADISA, E., GETIE, M., MOLLA, A., MEHARIE, T., DEBASH, H., BERHAN, A., ALMAW, A., HUSSIEN, A. & ABEJE, G. 2024b. Comparative assessment of microscopy, malaria rapid diagnostic test and polymerase chain reaction as malaria diagnostic tools in Adama Woreda, East shoa zone of Ethiopia: a cross-sectional study. *BMC Infect Dis*, 24, 1363.
- TEGEGNE, B., NIBRET, E., MUNSHEA, A., TEFERI, M., YIMER, M., ALEMU, G., YEWHALAW, D. & PILLAI, D. R. 2025. Performance of BIOCREDIT Pf/Pv lactate

- dehydrogenase-based malaria rapid diagnostic test among pregnant women with suspected malaria infection in Bahir Dar City Administration, northwest Ethiopia. *PLoS One*, 20, e0322362.
- TSEGAYE, A. T., AYELE, A. & BIRHANU, S. 2021. Prevalence and associated factors of malaria in children under the age of five years in Wogera district, northwest Ethiopia: A cross-sectional study. *PLOS ONE*, 16, e0257944.
- VAN DER WATT, M. E., READER, J. & BIRKHOLTZ, L. M. 2022. Adapt or Die: Targeting Unique Transmission-Stage Biology for Malaria Elimination. *Front Cell Infect Microbiol*, 12, 901971.
- VÁSQUEZ, A. M., ZULUAGA, L., TOBÓN, A., POSADA, M., VÉLEZ, G., GONZÁLEZ, I. J., CAMPILLO, A. & DING, X. 2018. Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for screening malaria in peripheral and placental blood samples from pregnant women in Colombia. *Malar J*, 17, 262.
- VILLENA, O. C., RYAN, S. J., MURDOCK, C. C. & JOHNSON, L. R. 2022. Temperature impacts the environmental suitability for malaria transmission by *Anopheles gambiae* and *Anopheles stephensi*. *Ecology*, 103, e3685.
- WARKAW, Y. M., MITKU, A. A., ZERU, M. A. & AYELE, M. 2022. Spatial pattern and predictors of malaria in Ethiopia: Application of auto logistics regression. *PLOS ONE*, 17, e0268186.
- WHITE, N. J. 2022. Severe malaria. *Malaria Journal*, 21, 284.
- WHO. 2024a. *Malaria* [Online]. WHO. Available: <https://www.who.int/news-room/fact-sheets/detail/malaria> [Accessed 17 November 2025].
- WHO. 2024b. *Malaria - Ethiopia* [Online]. WHO. Available: <https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON542#:~:text=Approximately%203,has%20been%20a%20shift%20in> [Accessed 16 November 2025].
- WHO. 2025a. *Ethiopia introduces malaria vaccine to protect children and strengthen health systems* [Online]. WHO. Available: <https://www.afro.who.int/countries/ethiopia/news/ethiopia-introduces-malaria-vaccine-protect-children-and-strengthen-health-systems> [Accessed 14 April 2026].

- WHO. 2025b. *Malaria* [Online]. World Health Organization. Available: <https://www.afro.who.int/health-topics/malaria#:~:text=%2A%20In%202022%2C%2094,in%20the%20WHO%20African%20Region> [Accessed 25 August 2026].
- WHO. 2025c. *WHO guidelines for malaria, 13 august 2025* [Online]. WHO. Available: <https://iris.who.int/items/62e89e1d-c8f7-4965-940c-8789af5a1b19> [Accessed 25 August 2026].
- WHO. 2025d. *World malaria report 2025* [Online]. WHO. Available: <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2025> [Accessed 25 August 2026].
- WHO & UNICEF 2015. Microscopy for the detection, identification and quantification of malaria parasites on stained thick and thin blood films in research settings (version 1.0): procedure: methods manual.
- WOLDESENBET, D., TEGEGNE, Y., SEMAW, M., ABEBE, W., BARASA, S., WUBETIE, M., TAMENE, E., ANTENEH, M., YIMER, A. & WOLDE, D. 2024. Malaria Prevalence and Risk Factors in Outpatients at Teda Health Center, Northwest Ethiopia: A Cross- Sectional Study. *Journal of Parasitology Research*, 2024, 8919098.
- YIGEZU, E., WONDALE, B., ABEBE, D., TAMIRU, G., ELIGO, N., LINDTJØRN, B., GADISA, E., TADESSE, F. G. & MASSEBO, F. 2023. Malaria misdiagnosis in the routine health system in Arba Minch area district in southwest Ethiopia: an implication for malaria control and elimination. *Malar J*, 22, 273.
- YMAN, V., WANDELL, G., MUTEMI, D. D., MIGLAR, A., ASGHAR, M., HAMMAR, U., KARLSSON, M., LIND, I., NORDFJELL, C., ROTH, I., NGASALA, B., HOMANN, M. V. & FÄRNERT, A. 2019. Persistent transmission of *Plasmodium malariae* and *Plasmodium ovale* species in an area of declining *Plasmodium falciparum* transmission in eastern Tanzania. *PLOS Neglected Tropical Diseases*, 13, e0007414.
- ZELEKE, M. T., GELAYE, K. A., HIRPA, A. A., TESHOME, M. B., GUMA, G. T., ABATE, B. T. & YENESEW, M. A. 2023. Diagnostic performance of pf HRP2/pLDH malaria rapid diagnostic tests in elimination setting, Northwest Ethiopia. *PLOS Global Public Health*, 3, e0001879.

9. ANNEXES

Annex I: Participant information sheet [English version]

Principal Investigator: Sisay Desale, Bahir Dar University

Title of the study: Prevalence, Diagnostic Performance, and Associated Factors of *Plasmodium* Infections among Individuals with Presumptive Malaria in Northwest Ethiopia

Purpose of the Study

The purpose of this study is to determine the Prevalence, Species Distribution, Diagnostic Performance, and Associated Factors of *Plasmodium* Infections among Malaria-Suspected Individuals in Northwest Ethiopia. The results will help improve malaria diagnosis and control strategies in the area.

Procedures to be carried out.

You are invited to participate in this study after giving your consent. A small amount of blood will be collected during your routine malaria test (finger-prick or venous blood). No extra blood will be taken beyond what is normally required for diagnosis. You may also be asked a few short questions related to your health and malaria history.

Risks Associated with Study

There is no major risk involved in this study. The only possible discomfort is mild pain during blood collection, which is like routine clinical procedures.

Benefits of the Study

There is no financial benefit to you. However, the findings of this study will help improve malaria diagnostic accuracy and may support better patient management and public health planning.

Confidentiality of Your Information

Your information will remain confidential. Laboratory results and questionnaire data will not include your name. Only the researchers and responsible medical personnel will have access to the coded data. No personal identifiers will be associated with your results.

Termination or Withdrawal from the Study

Your participation is entirely voluntary. You may refuse to take part or withdraw from study at any time without affecting the medical care you receive. Your decision will be respected. You may also request your test results if you wish.

Agree to participate? ☐ Yes ☐ No

Annex II: Participant information sheet [Amharicversion]

የጥናቱ ርዕስ፡- በሰሜንምዕራብ ኢትዮጵያ የወጣ ልጅ ባለባቸው ግለሰቦች ላይ የሚታየው የወጣዝርያዎች በሞላ ኪዳን ላይ (PCR) እና ተያያዥ መንስኤዎች ጥናት።

ጥናቱን የሚያጠናው፡- ሲሳይደሳለ፣ በባህር ዳር ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅ እጩተ መራቂ።

የጥናቱ ዓላማ፡-

በሰሜንምዕራብ ኢትዮጵያ በሚገኙ በተመረጡ የመንግስት ጤና ተቋማት የወጣተ ጠርጣሪ ሆነው ለምርመራ ከሚመጡ ግለሰቦች የሚወሰዱ የደምና ሙሽን እጅግ ዘመናዊ በሆነ የሞላ ኪዳን (Real-Time PCR) በመመርመር፡-

- ያሉትን የወጣዝርያዎች በትክክል መለየት።
- የበሽታውን ስርጭት መጠን ማወቅ።
- ለበሽታው የሚያጋልጡ መንስኤዎችን በመለየት ለወደፊት መከላከያና መቆጣጠሪያ ዘዴዎች ግብዓት የሚሆን መፍትሄ ማቅረብ።

ለጥናቱ ተሳታፊዎች ያለው ጥቅም፡- ለተሳታፊዎች የሚከፈል የገንዘብ ክፍያ የለም። ነገር ግን ከጥናቱ የሚገኘው የላቦራቶሪ ውጤት የእርስዎን የጤና ሁኔታ በትክክል ለመረዳትና ለህክምናዎ ተጨማሪ መረጃ ለመስጠት ይረዳል።

በተሳታፊዎች ላይ የሚደርስ ጉዳት፡- ናሙና በሚወሰድበት ወቅት ከሚሰማ መጠነኛ የመርፌ መውጋት ስሜት ውጭ ምንም አይነት ዘላቂ ጉዳት አይኖርብዎትም። በተጨማሪም ከወትሮው የተለየ የሚባከን ሰዓት የለም።

የመረጃ ሚስጥራዊነት፡- የሚሰጡት መረጃ ሙሉ በሙሉ በሚስጥር ይያዛል። መረጃው የሚመዘገበው በመለያ ቁጥር (Code) እንጂ በስምዎ አይደለም። በጥናቱ ላይ ለመሳተፍ ሙሉ መብት ያለዎት ሲሆን፣ በማንኛውም ሰዓት ያለምንም ቅጣት ራስዎን ማግለል ይችላሉ።

እኔም የጥናቱ ተሳታፊ ይሆን በመገንዘብ ጥናቱ ላይ ለመሳተፍ ተስማምቼ ያለሁ።

ፊርማ _____

መረጃውን የሰበሰበው ግለሰብ ስም _____ ፊርማ _____

Annex III: Informed consent form [English version]

I, the undersigned individual, am informed about the objective of the study. I have been told that all my information will be kept confidential and used only for this research.

Your signature below indicates that you have read or listened to, and understood, the information provided to you about the study.

Before you sign, please make sure you understand the purpose of the study, the procedures, possible risks and benefits of participation, your right to refuse or withdraw at any time, confidentiality and privacy protections, and who to contact if you have any questions.

I have read or listened to the description of the study, and I understand what procedures will be performed and what will happen to me during the study.

Based on the above information, I voluntarily agree to participate in this research.

Participant's Signature: _____ Date: _____

Name of Data Collector: _____ Signature: _____

If you have any questions, you may ask the principal investigator at any time.

Principal Investigator:

Sisay Desale(BSc, MSc Candidate)

Mobile: +251928363976

Email: sisaydesale64@gmail.com

Annex IV: Informed consent [Amharicversion]

በዚህጥናት ላይ ሚና ተጫውቶ የሚገኙትን ሰዎች ለመግለጽ ሚና ተጫውቶ የሚገኙትን ሰዎች መጠየቅ ይቻላል

እኔ ፊርማዬ ከዚህ በታች የተቀመጠው ግለሰብ ከእኔ የሚወሰደውና ሙና ለጥናቱ አላማ ብቻ እንደሚወልድ ተረድቻለሁ፡፡

ሁሉም መረጃዎች እና የጥናቱ ውጤቶችም ለሰው ጤና ምንም ስጦታ ሊሰጡም ሆኑ ንጉሥ ገንዘብ አለሁ፡፡

በጥናቱ ላይ በመሳተፌ ምንም የገንዘብ ክፍያ እንደማላገኝ ተረድቻለሁ፡፡

ከምርመራ መሳተፍ ወይም አለመሳተፍ መብቴ የተጠበቀ መሆኑን እና ላለመሳተፍ በወስን በላቦራቶሪው በሚደረግ በልሻ ምርመራ ላይም ንምትፅዕኖ እንደማይኖረኝ ተረድቻለሁ፡፡

ስለዚህ የጥናቱን ጠቃሚነት አምኜ በትየስምምነት ቃሌ ንየሰጠሁት በፍፁም ፈቃደኝነት ነው፡፡ ስለዚህ ጥናት ማንኛውም ጥያቄ ካለዎት በማንኛውም ጊዜ ከዚህ በታች በተጠቀሱት አድራሻዎች መጠየቅ ይቻላል፡፡

እኔም የጥናቱ ተሳታፊ ሆኖ በመገንዘብ ጥናቱ ላይ ለመሳተፍ ተስማምቼ ያለሁ፡፡

የጥናቱ ተሳታፊ ፊርማ _____ ቀን _____

መረጃውን የሰበሰበው ግለሰብ ስም _____ ፊርማ _____

ሲሳይደሳለ

ባህር ዳርቱ ቨርሲቲ፣ የጤና ሳይንስ ኮሌጅ፣ የህክምና ፓራሳይቶሎጂ እና ኢንቶሞሎጂ ፓርትመንት እጩተ መራቂ

ባህር ዳር

ኢትዮጵያ

ኢ-ሜይል፣ sisaydesale64@gmail.com

ስ.ቁ +251-928-363976

Annex V. Parental/Guardian Consent Form [English Version]

I, the undersigned, have been told about this research. My child has the right to choose if they want to be in the study. I have been informed that other people will not know my child's results because the information will be coded with a number instead of a name.

I understand that there may be no benefit to my child personally, apart from the clinical service they receive from the test results. I have been encouraged to ask questions, and my questions have been answered.

I have been told that participation in this study is voluntary, and I may refuse to let my child be in the study. I also understand that my child's agreement (assent) will be required. By signing below, I agree to allow my child to participate in this research study.

Parent/Guardian Signature: _____ Date: _____

Name of Data Collector: _____ Signature: _____

If you have any questions, you can ask the principal investigator.

Principal Investigator:

Sisay Desale (BSc, MSc Candidate)

Mobile: +251 928 363 976

Email: sisaydesale64@gmail.com

Annex VI. Parental/Guardian Consent Form [Amharic Version]

የወላጅ/የአሳዳጊ/ የሞግዚት የስምምነት መጠየቂያ ቅጽ

እኔ ፊርማዬ ከዚህ በታች የተቀመጠው—የታማሚው ወላጅ/አሳዳጊ/ ሞግዚት ስምን የዚህን ጥናት አላማ በወልተረድቻለሁ፡፡
በጥናቱ ወቅት ምታማሚው መረጃዎች በሚሰጥ ስለሚያዝበሉ ላለው ዘንድ እንደማይታወቅ ተረድቻለሁ፡፡
በውጤቱ ከሚገኘው የህክምና አገልግሎት በቀር ሌላ ታማሚው በግሉ የሚያገኘው ጥቅም እንደሌለ ተረድቻለሁ፡፡
ጥያቄ እንደጤዎታል ተሰጥቶ ሻለ ጥያቄዎቼም በቁምላሽ አግኝቻለሁ፡፡
የልጄ በጥናቱ መሳተፍ በእኔ ፍላጎት ብቻ እንደሆነ እና በጥናቱም አለመሳተፍ ምንም አይነት ተፅዕኖ ታማሚው ላይ እንደማያሰከትል ተረድቻለሁ፡፡ በከዚህ በላይ ገርታማሚው በጥናቱ ውስጥ ለመካተት የእኔ ወላጅ/አሳዳጊ/ሞግዚት ፈቃድ እንደሚያስፈልግ ተረድቻለሁ፡፡
በእኔ ፍቃድ ነት ታማሚው በጥናቱ እንደሚሳተፍ ከዚህ በታች በፊርማዬ አረጋግጣለሁ፡፡

የጥናቱ ተሳታፊ ወላጅ/አሳዳጊ/ሞግዚት ፊርማ _____

መረጃውን የሰበሰበው ግለሰብ ስም _____ ፊርማ _____

ሲሳይደሳለ

ባህርዳር ዩኒቨርሲቲ፣ የጤና ሳይንስ ኮሌጅ፣ የህክምና ፓራሳይቶሎጂ እና ኢንቶሞሎጂ ፓርትመንት እጩተመራቂ

ባህርዳር

ኢትዮጵያ

ኢ-ሜይል፡ sisaydesale64@gmail.com

ስ.ቁ +251-928-363976

Annex VII: Questionnaires [English version]

Instruction: For the following questions, please tick (✓) the appropriate box.

Section	Question	Response
Sociodemographic Variables	Age (years)	
	Sex	☐ Male ☐ Female ☐ other
	Residence	☐ Urban ☐ Rural
	Marital status	☐ Single ☐ Married ☐ Divorced ☐ Widowed
	Educational level	☐ Unable to write and read ☐ Primary ☐ Secondary ☐ College/Above
	Occupation	☐ Farmer ☐ Student ☐ employed ☐ Merchant ☐ Unemployed ☐ Housewife
	Household size	_____
Behavioral & Environmental Factors	Do you have a bed net?	☐ Yes ☐ No
	Habit of Bed-net use	☐ Always ☐ Sometimes ☐ Never
	Has your house been sprayed for malaria in the last 12 months (IRS)?	☐ Yes ☐ No
	Is there surface water within 500m from home?	☐ Yes ☐ No
	Distance of surface water from home (meter)	☐ 1–100 ☐ 101–500
	What is the main material of the house wall?	☐ Mud ☐ Wood ☐ Stone ☐ Cement/Brick
	Are there cracks or holes in the walls?	☐ Yes ☐ No
	Does the house have windows?	☐ Yes ☐ No
	Are the windows screened with mesh/net?	☐ Yes ☐ No
	Do you usually keep windows open at night?	☐ Yes ☐ No

	Habit of sleeping or working outside at night		☐ Yes	☐ No
	Are there gaps between the door and the floor/wall?		☐ Yes	☐ No
	Is the door usually kept open at night?		☐ Yes	☐ No
	Have you traveled outside your residence in the last 14 days?		☐ Yes	☐ No
	If yes:	Destination	_____	
		Was the area malaria-endemic?	☐ Yes	☐ No
	Protective clothing at night		☐ Yes	☐ No
Ointment/repellent use at night		☐ Yes	☐ No	

AnnexVIII: Questionnaire [Amharic]

መመሪያ፡ለቀጣዩጥያቂዎችእባክዎትከከለኛውንሳጥን (✓) ይምረጡ።

የጥያቄክፍል	ጥያቄ	መልስ
	እድሜ (በዓመት)	
	ፆታ	☐ ወንድ ☐ ሴት ☐ ሌላ
	መኖሪያቦታ	☐ ከተማ ☐ ገጠር
	የጋብቻሁኔታ	☐ ያላገባ/ች ☐ ያገባ/ች ☐ የፈታ/ች ☐ መበለት/ት
ማህበራዊናስነት-ህዝብመረጃ	የትምህርትደረጃ	☐ ማንበብናመጻፍየማይችል/ች / ☐ አንደኛደረጃ ☐ ሁለተኛደረጃ ☐ ኮሌጅእናከዚያበላይ
	ሥራ	☐ ገበሬ ☐ ተማሪ ☐ ሰራተኛ ☐ ነጋዴ ☐ ሰራአጥ ☐ የቤት እመቤት
	የቤተሰብመጠን	
ባህሪያዊናአካባቢያዊአጋላጭምከንዎቶች	የወባመከላከያአጎበርአለዎት?	☐ አዎ ☐ አይ
	የወባመከላከያአጎበርየመጠቀምልምድ?	☐ ሁልጊዜ ☐ አንዳንዴ ☐ አልጠቀምም
	ቤትዎበአለፉት 12 ወራትየወባመከላከያመዳኒት (IRS) ተረጭቷል?	☐ አዎ ☐ አይ
	ከቤትዎ እስከ 500 ሜትርርቀትላይየገጸምድርውሃአለ?	☐ አዎ ☐ አይ
	የገጸምድርውሃከቤትርቀት (ሜትር) ምንያህልነው?	☐ 1-100 ሜትር ☐ 101-500 ሜትር
	የቤቱግድግዳዋናቀሳቀሰምንድንነው?	☐ ጭቃ ☐ እንጨት ☐ ድንጋይ ☐ ሲሚንቶ/ጡብ
	በግድግዳላይስንጥቆችወይምቀዳዳዎችአሉ?	☐ አዎ ☐ አይ
	ቤቱመስኮቶችአሉት?	☐ አዎ ☐ አይ
	መስኮቶቹበመጋረጃ/መረብ (mesh/net) ተሸፍነዋል?	☐ አዎ ☐ አይ
	ብዙጊዜበሌሊትመስኮቶችንከፍትትተዋላችሁ?	☐ አዎ ☐ አይ
	በሌሊትውጭየመተኛትወይምየመስራትልምድ?	☐ አዎ ☐ አይ

የጥያቄክፍል	ጥያቄ	መልስ
	በበሩእናወለሉ/ግድግዳመካከልክፍተቶችአሉ?	☐ አዎ ☐ አይ
	በሌሊትብዙጊዜበሩክፍትይቀራል?	☐ አዎ ☐ አይ
	ባለፉት 14 ቀናትከመኖሪያቦታዎውጭተጉዘዋል?	☐ አዎ ☐ አይ
	የጉዞመዳረሻ	
	ያንድካባቢወባየተለመደቦታነው?	☐ አዎ ☐ አይ
	ማታማታሰውነትንየሚሸፍንልብስይሉብሳሉ?	☐ አዎ ☐ አይ
	ማታማታየወባመከላከያቅባት /ስፕሬይ/ ይጠቀማሉ?	☐ አዎ ☐ አይ

የተሞላበትቀን/የጤናተቋም/ ID..... Parasite (if any)

AnnexIX: Laboratory data collection form.

Study Title: Prevalence, Species Distribution, Diagnostic Performance, and Associated Factors of *Plasmodium* Infections among Malaria-Suspected Individuals in Northwest Ethiopia

Participant code: _____

Date _____ of _____ sample _____ collection: _____

Data collector name: _____

Table 3: Laboratory Results

Test	Result	Remark
Microscopy (Thick Smear)	☐ Positive ☐ Negative	Parasite density (/µL): _____
Microscopy (Thin Smear)	☐ Positive ☐ Negative	Species: ☐ <i>P. falciparum</i> ☐ <i>P. vivax</i> ☐ Po ☐ Pm ☐ Mixed ☐ Other: _____
Molecular Analysis (qPCR)	☐ Positive ☐ Negative	Species detected: ☐ <i>P. falciparum</i> ☐ <i>P. vivax</i> ☐ Po ☐ Pm ☐ Mixed ☐ Other: _____
RDT	☐ Positive ☐ Negative	☐ <i>P. falciparum</i> ☐ <i>P. vivax</i> ☐ Mixed

Table 4: Laboratory Processing Details

Step	Date	Responsible Initials	Remark
Blood sample received	____ / ____ / ____	_____	_____
DNA extraction completed	____ / ____ / ____	_____	_____
qPCR performed	____ / ____ / ____	_____	_____
Data entry/validation	____ / ____ / ____	_____	_____

Comments.....

.....

Annex X

SOP1: Blood Sample Collection and Dried Blood Spot (DBS) Preparation

1. Purpose

To standardize capillary blood collection by finger prick and preparation of dried blood spots on Whatman 903 filter paper for downstream molecular analysis, alongside thick/thin blood film slides and RDT.

2. Scope

Applies to all participants at point-of-care collection sites. Covers fingertip capillary blood collection, DBS preparation, slide preparation, and RDT sample allocation.

3. Responsibilities

- ✓ Trained laboratory technician: Perform finger prick and allocate blood samples.
- ✓ Laboratory supervisor: Ensure consumables are adequately stocked and SOPs are followed.

4. Materials & Equipment

- ✓ Sterile single-use lancets
- ✓ 70% ethanol swabs
- ✓ Whatman 903 Protein Saver filter paper cards
- ✓ Clean, grease-free glass slides
- ✓ BIOCREDIT RDT kit (with capillary tubes)
- ✓ Zip-lock plastic bags with silica gel desiccant
- ✓ Permanent marker/participant ID labels
- ✓ Disposable gloves (nitrile)
- ✓ Sharps disposal container
- ✓ Cool box with ice packs (for transport)

5. Procedure

Preparation

1. Wear gloves before each participant.
2. Label a Whatman 903 filter paper card and clean glass slides with the participant's unique ID number, date of collection, and site code.

3. Prepare the RDT kit: remove one device, lay it on a flat surface, label with participant ID number like that on the questionnaire and DBS.

Fingertip Preparation and Puncture

4. Select the ring or middle fingertip of the non-dominant hand.
5. Clean the fingertip with 70% ethanol and allow it to air-dry completely.
6. Using a sterile single-use lancet, puncture the fingertip with one firm motion.
7. Wipe away the first drop of blood with a clean, dry swab.

Sample Allocation

8. Thick film: Touch the slide to the fingertip and spread approximately 5 μ L of blood into a circular area of 1–1.5 cm diameter.
9. Thin film: Spread approximately 2 μ L of blood using the standard push technique.
10. RDT: Collect 5 μ L of whole blood using the INVERTED CUP provided in the kit and transfer to the sample well.
11. DBS: Touch the fingertip gently to the center of each pre-printed circle on the Whatman 903 card. Allow blood to absorb uniformly and fill the entire circle. Do not press hard or layer blood.

DBS Drying, Packaging, and Storage

12. Lay filter paper cards flat and allow them to dry completely at room temperature for a minimum of 4 hours. Do not stack wet cards.
13. Once fully dried, place each card individually in a labelled zipper bag containing fresh silica gel desiccant.
14. Seal the bag and store at room temperature until transport to the Amhara Public Health Institute (APHI), Bahir Dar.
15. Upon arrival at APHI, store DBS cards at -20°C until DNA extraction.

Contamination Prevention

16. Use a new sterile lancet for every participant. Never reuse lancets.
17. Use a clean, individually packaged filter paper card for each participant.
18. Dispose of all sharps immediately into a designated sharps container.

AnnexXI

SOP2:Thick and Thin Blood Film Preparation, Giemsa Staining, and Microscopy

1. Purpose

To standardize preparation, staining with 10% Giemsa, and microscopical examination of thick and thin blood films for *Plasmodium* species identification and parasite density calculation, per WHO SOP.

2. Scope

Applies to all malaria blood film preparation and reading at study sites and the central laboratory.

3. Responsibilities

- ✓ Laboratory technician: Prepare and stain blood films.
- ✓ Two independent experienced microscopists (blinded): Read and record results.
- ✓ Senior WHO–licensed microscopist (tiebreaker): Resolve discordant results.
- ✓ Laboratory supervisor: Ensure quality of staining reagents and microscopes.

4. Materials and equipment

- ✓ Clean, grease–free glass slides
- ✓ Absolute methanol (for thin film fixation)
- ✓ 10% Giemsa stain solution (pH 7.2)
- ✓ Phosphate–buffered water (pH 7.2)
- ✓ OilImmersion
- ✓ Binocular light microscope with 10×, 40×, and 100× (oil immersion) objectives
- ✓ Slide staining rack
- ✓ Timer

5. Procedure

Film preparation

1. Thick film: spread approximately 5 µL of capillary blood in a circular area of 1 cm in diameter.
2. Thin film: spread approximately 2 µL using the standard push technique to achieve a monolayer.
3. Air–dry slides at room temperature on a clean dust–free surface.

Fixation and staining

4. Fix the thin film only by dropping absolute methanol (2–3 dips); do NOT fix the thick film.
5. Prepare fresh 10% Giemsa stain: dilute 1 mL Giemsa stock in 9 mL phosphate–buffered water (pH 7.2).
6. Place slides on the staining rack and flood with 10% Giemsa.
7. Allow air–drying in a vertical position for 10–15 minutes.

Microscopy examination

8. Examine slides under oil immersion ($\times 100$ objective).
9. Reader 1 and Reader 2 examine independently and are blinded to each other's results and to RDT results.
10. Identify *Plasmodium* species and assess parasite density from the thick blood film.
11. Declare a slide negative only after examining at least 100 microscopic fields with no asexual parasites.

Parasite density calculation

12. Count the number of asexual parasites per 200 white blood cells (WBCs).
13. Calculate parasite density using $\text{parasites}/\mu\text{L} = (\text{parasites counted} / 200 \text{ WBCs}) \times 8,000 \text{ WBCs}/\mu\text{L}$.

Discordant results

14. If Reader 1 and Reader 2 results are discordant (species or positivity), submit the slide to a third senior WHO–licensed microscopist (tiebreaker).
15. Accept the majority result (2 of 3 readings) as the final microscopy result.

AnnexXII

SOP3:Malaria Rapid Diagnostic Test (RDT) BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)

1. Purpose

To standardize performance and interpretation of the BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH) immunochromatographic assay (RapiGEN Inc., Republic of Korea) for qualitative detection and differentiation of *P. falciparum* and *P. vivax* in whole blood.

2. Scope

Applies to all RDT testing performed at point-of-care study sites for enrolled malaria-suspected participants.

3. Responsibilities

- ✓ Laboratory technician: Perform the RDT according to the manufacturer's instructions.

4. Materials and equipment

- ✓ BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH) test kits (Cat. No. C61RHG25, RapiGEN Inc.)
- ✓ Capillary tube (provided in kit)
- ✓ Assay diluent buffer (provided in kit)
- ✓ Capillary blood sample (5 µL)
- ✓ Timer
- ✓ Result recording sheet

5. Procedure

Test preparation

1. Allow the RDT kit components to equilibrate to room temperature (15–30°C) before use if stored refrigerated.

2. Label the test device with the participant's unique ID number.
3. Remove the test device from the foil pouch and place it on a flat, horizontal surface.

Sample Application

4. Collect exactly 5 µL of capillary whole blood using the inverted cup provided.
5. Transfer the 5 µL blood sample to the sample well (S) of the test device.
6. Add 3 drops of assay diluent buffer to the buffer well (B) immediately after blood application.

Result reading

7. Start the timer immediately after adding the buffer. Read results in exactly 20–30 minutes.
8. Do not read results before 20 minutes or after 30 minutes.

Result interpretation

9. **NEGATIVE:** Control line (C) only appears no malaria detected.
10. ***P. falciparum* POSITIVE:** Control line (C) and Pf test line appear.
11. ***P. vivax* POSITIVE:** Control line (C) and Pv test line appear.
12. **MIXED INFECTION:** All three lines (C, Pf, and Pv) appear.
13. **INVALID:** No control line appears. Discard device, repeat test with a new kit.

Documentation

14. Record the result immediately on the participant's result sheet and in the study questionnaire
15. Photograph the test device, if possible, for quality assurance purposes.

AnnexXIII

SOP 4: DNA Extraction from Dried Blood Spot (DBS) Samples

1. Purpose

This SOP describes the standardized procedure for extracting high-quality genomic DNA from Dried Blood Spot (DBS) samples for use in downstream molecular analyses, PCR amplification, and detection.

2. Scope

This procedure applies to all laboratory personnel engaged in molecular biology research involving DBS-derived samples. It must be followed precisely to ensure reproducible, high-quality DNA yields and to reduce contamination risk.

3. Health and Safety Precautions

- ✓ All procedures were performed in the Amhara Public Health Institute
- ✓ Personal Protective Equipment (PPE): gloves and a laboratory coat must always be worn.
- ✓ DBS samples are derived from human blood and should be treated as potentially infectious. Handle in accordance with institutional biosafety guidelines.
- ✓ Ethanol is flammable. Keep away from open flames and heated surfaces.
- ✓ Proteinase K is an irritant. Avoid skin and eye contact.
- ✓ All liquid waste must be decontaminated (10% bleach) before disposal.
- ✓ Solid biohazardous waste (tips, tubes) must be disposed of in appropriate biohazard bags.

4. Equipment and Reagents

Reagent or Equipment	Volume Specification	or	Purpose
ATL Buffer	200 µL		Tissue lysis buffer for initial sample digestion
Proteinase K	20 µL		Enzymatic digestion of proteins at 56°C

AL Buffer	200 µL	Lysis optimization buffer
Absolute Ethanol (99%)	200 µL	DNA precipitation
AW1 Wash Buffer	500 µL	First column wash
AW2 Wash Buffer	500 µL	Second column wash removes salts
Elution Buffer (NFW)	50 µL	Nuclease-free water for DNA elution
DBS Sample Discs	1 spot (3 mm punch)	Dried Blood Spot source material
Eppendorf Tube (1.5 mL)	×2	Lysis tube and labeled collection tube
Spin Column	×1	Silica membrane column for DNA binding
Heat Block	Set to 70°C / 85°C	Incubation of sample at required temperatures
Water bath	56 °C	Incubation of sample at required temperatures
Centrifuge	Up to 14,000 RPM	Column centrifugation steps
DBS Puncher		To punch the DBS spot
Vortex Mixer		Sample mixing
Freezer (–20°C)		Storage of extracted DNA

5. Step-by-Step Procedure

5.1 Pre-Procedure Preparation

- ✓ Switch on the heat block and allow it to equilibrate to 70°C (for the 85°C incubation step, the block thermometer may read 70°C).
- ✓ Switch the water bath and allow it to equilibrate to 56°C
- ✓ Label all tubes clearly with sample ID, date, and operator initials before use.

- ✓ Prepare all buffers and reagents at room temperature. Vortex buffer bottles gently before use.
- ✓ Ensure centrifuge is balanced and set to appropriate RPM before loading columns.

5.2 Extraction Steps

The following procedure was performed for each sample. All steps were carried out at room temperature unless otherwise specified. Sterile technique was maintained throughout, and a new set of gloves was worn for each extraction run to minimize the risk of cross-contamination.

A. Sample Preparation

1. Using a sterile 5 mm puncher, three DBS discs were punched from the sample card and transferred into a labelled 1.5 mL Eppendorf tube.
2. 200 μ L of ATL buffer was added to the tube containing the DBS discs, followed by brief vortexing to ensure the discs were fully submerged.
3. The tube was incubated at 85°C for 10 minutes using a heat block (pre-set to 70°C). This step facilitates initial elution of blood from the disc matrix.

B. Protein Digestion

4. 20 μ L of proteinase K was added to the sample, followed by vigorous vortexing.
5. The tube was incubated at 56°C for 1 hour to allow complete enzymatic digestion of proteins, then briefly centrifuged to collect the sample at the bottom of the tube.

C. Lysis and Precipitation

6. 200 μ L of AL buffer was added to the digested sample and mixed thoroughly by vortexing.
7. The mixture was incubated at 70°C for 10 minutes to complete cell lysis and deactivate proteinase K.
8. 200 μ L of absolute ethanol (96–100%) was added to the sample and mixed immediately by vortexing, followed by a brief centrifuge spin. This step precipitates the DNA and conditions the lysate for binding to the silica membrane.

D. Column Binding

9. The entire sample mixture (including any precipitate) was carefully applied to a QIAamp Mini spin column placed in a 2 mL collection tube, avoiding wetting the rim of the column.
10. The column was centrifuged at 8,000 RPM for 1 minute. The filtrate tube was discarded.

E. Washing

11. The spin column was carefully reopened and 500 µL of AW1 wash buffer was added. The column was centrifuged at 8,000 RPM for 1 minute to remove residual contaminants.
12. 500 µL of AW2 wash buffer was added to the column, followed by centrifugation at 14,000 RPM for 3 minutes to remove residual salts and ethanol.
13. A final dry-run centrifugation was performed at 14,000 RPM for 3 minutes to completely remove any residual wash buffer from the silica membrane. This step is critical to prevent carryover of inhibitors into the eluate.

F. Elution and Storage

14. A new 1.5 mL Eppendorf tube was labelled with the sample identifier. The spin column was transferred to this labelled tube.
15. 50 µL of elution buffer (nuclease-free water, NFW) was applied directly to the center of the silica membrane and incubated at room temperature for 3 minutes to maximize DNA yield.
16. The column was centrifuged at 8,000 RPM for 1 minute. The eluate containing purified genomic DNA was collected in the labelled tube.
17. Extracted DNA was immediately placed in a -20°C freezer for long-term storage until used in downstream molecular analyses.

6. Quality Control

- ✓ Include at least one negative extraction control (blank DBS disc, no blood) per extraction batch.

Annex XIV

SOP 5: Nested PCR for Genus Detection and Species Identification (*P. ovale* and *P. malariae*)

1. Purpose

This Standard Operating Procedure (SOP) describes the method for genus-level detection of *Plasmodium* spp. by first-round nested PCR (N1), followed by second-round nested PCR (N2) for species-level identification of *Plasmodium ovale* (Po) and *Plasmodium malariae* (Pm), based on amplification of the 18S small subunit ribosomal RNA (18S rRNA) gene.

2. Scope

This procedure applies to all extracted genomic DNA samples from malaria-suspected individuals processed within the study, for the purpose of confirming *Plasmodium* genus positivity and identifying *P. ovale* and *P. malariae* infections not reliably distinguished by microscopy or rapid diagnostic tests (RDTs).

3. Responsible body

- ✓ Laboratory Supervisor: Responsible for overall quality assurance of the nested PCR procedure, approval of results, and ensuring compliance with this SOP.
- ✓ Principal Investigator: Responsible for performing the nested PCR amplification and gel electrophoresis according to this SOP, maintaining equipment, and documenting all procedures.

4. Principle

The assay follows the nested PCR protocol originally described by Snounou *et al.* (1993), with optimized reaction conditions. The first round (N1) amplifies a conserved region of the 18S rRNA gene common to all human-infecting *Plasmodium* species using the genus-specific primer pair rPLU6/rPLU5, producing an amplicon of approximately 1200 bp. Amplicons generated in the N1 reaction are used as template in a second-round (N2) reaction employing species-specific

primer pairs (rOVA1/rOVA2 for *P. ovale*; rMAL1/rMAL2 for *P. malariae*), increasing both sensitivity and specificity.

5. Reagents, Consumables, and Equipment

- GoTaq Flexi DNA Polymerase kit (Promega, Cat. No. M7808), including 5× GoTaq Flexi Buffer and 25 mM MgCl₂
- dNTP mix (25 mM stock)
- Oligonucleotide primers: rPLU6, rPLU5, rOVA1, rOVA2, rMAL1, rMAL2 (see Section 6)
- Nuclease-free water
- Genomic DNA template extracted using the Qiagen DNA extraction kit
- Known positive genomic DNA controls (*Plasmodium* spp., *P. ovale*, *P. malariae*) and non-template control
- Thermal cycler
- Nuclease-free PCR tubes/plates, filter tips, PCR workstation with UV hood
- 2.0% agarose, SYBR Safe DNA Gel Stain, 1× TAE buffer, 100 bp DNA ladder, horizontal electrophoresis unit, UVITEC Gel Documentation system

6. Procedure for First-Round PCR (N1)

1. Prepare the N1 master mix on ice in a dedicated pre-PCR workstation, sufficient for the required number of samples plus positive and non-template controls (see Section 6.1 for the per-reaction composition).
2. Dispense 20 µL of master mix into each PCR tube/well.
3. Add 5 µL of extracted genomic DNA template to give a total reaction volume of 25 µL. For the non-template control, add 5 µL of nuclease-free water instead of DNA.

4. Cap/seal reactions and load the plate into the thermal cycler.
5. Run the N1 thermal cycling program described in Section 6.2.
6. Store N1 amplicons at 4°C for immediate use as N2 template, or at –20°C for later use.

7. Reaction Composition and Thermal Cycling for N1

7.1 N1 Master Mix (per 25 µL reaction)

Component	Final concentration / amount	Volume per reaction
GoTaq Flexi PCR Buffer (5×)	1×	5.0 µL
MgCl ₂ (25 mM)	2.0 mM	2.0 µL
dNTP mix (25 mM)	0.25 mM each	0.25 µL
Primer rPLU6 (Forward)	0.25 µM	included in mix
Primer rPLU5 (Reverse)	0.25 µM	included in mix
GoTaq Flexi DNA Polymerase	1.0 U	0.2 µL
Nuclease-free water		11.925 µL
Master mix subtotal		20 µL
Genomic DNA template		5 µL
Total reaction volume		25 µL

7.2 N1 Thermal Cycling Conditions

Step	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	60 sec	
Annealing	58°C	60 sec	35
Extension	72°C	90 sec	
Final extension	72°C	10 min	1
Hold	4°C	∞	

8. Procedure Second–Round PCR (N2): *P. ovale* and *P. malariae*

7. Perform N2 amplification only on samples that tested positive in the N1 (genus–level) reaction.
8. Prepare separate N2 master mixes for the *P. ovale* primer pair (rOVA1/rOVA2) and the *P. malariae* primer pair (rMAL1/rMAL2), each on ice in the pre–PCR workstation (composition in Section 8.1).
9. Dispense 23 µL of the relevant species–specific master mix into each reaction well.
10. Add 2 µL of the N1 amplicon as template DNA, giving a total reaction volume of 25 µL. Include a known positive control and a non–template control for each species–specific run.
11. Run the N2 thermal cycling program described in Section 8.2.
12. Proceed to gel electrophoresis (Section 9) to visualize and interpret results.

9. Reaction Composition and Thermal Cycling for N2

9.1 N2 Master Mix (per 25 μ L reaction)

Component	Final concentration / amount	Volume per reaction
GoTaq Flexi PCR Buffer (5 $\times$)	1 $\times$	5.0 μ L
MgCl ₂ (25 mM)	2.0 mM	2.0 μ L
dNTP mix (25 mM)	0.25 mM each	0.25 μ L
Species-specific forward primer (rOVA1 or rMAL1)	0.25 μ M	included in mix
Species-specific reverse primer (rOVA2 or rMAL2)	0.25 μ M	included in mix
GoTaq Flexi DNA Polymerase	1.0 U	0.2 μ L
Nuclease-free water		14.925 μ L
Master mix subtotal		23 μ L
N1 amplicon (template)		2 μ L
Total reaction volume		25 μ L

9.2 N2 Thermal Cycling Conditions

Step	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	60 sec	
Annealing	54°C	60 sec	30
Extension	72°C	60 sec	
Final extension	72°C	10 min	1
Hold	4°C	∞	

10. Primer Sequences (N1 and N2)

Assay	Target	Primer	Sequence (5'→3')
N1	<i>Plasmodium</i> spp. (genus)	rPLU6 (F)	TTAAAATTGTTGCAGTTAAAACG
		rPLU5 (R)	CCTGTTGTTGCCTTAAACTTC
N2	<i>P. ovale</i>	rOVA1 (F)	ATCTCTTTTGCTATTTTTTAGTATTGGAGA
		rOVA2 (R)	GGAAAAGGACACATTAATTGTATCCTAGTG
N2	<i>P. malariae</i>	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC

Assay	Target	Primer	Sequence (5'→3')
		(F)	
		rMAL2 (R)	AAAATTCCCATGCATAAAAAATTATACAAA

Expected amplicon sizes: N1 (genus) =1200 bp; N2 *P. ovale* = 749 bp; N2 *P. malariae* = 144 bp.

11. Gel Electrophoresis and Result Interpretation

11.1 Gel Preparation

1. Prepare a 2% agarose gel by dissolving 2 g of agarose powder in 100 mL of 1× TAE buffer; heat to fully dissolve.
2. Add 4 µL of Ethidium Bromide to the molten agarose, mix gently, and cast the gel with an appropriate comb.
3. Allow the gel to set fully before use, then place it in the electrophoresis tank and submerge in 1× TAE running buffer.

10.2 Loading and Run Conditions

4. Load 4 µL of the 100 bp DNA ladder in the first well of each gel.
5. Load 8 µL of each N1 or N2 (PCR product) sample, including positive and non-template controls, into the remaining wells.
6. Run the gel at 120 V and 400 mA for 60 minutes.

10.3 Visualization and Interpretation

7. Visualize bands under UV illumination using a UVITEC Gel Documentation system.

8. A sample is scored positive for a given target when a band of the expected size (Section 9) is present and absent in the corresponding non-template control.

9. Document and archive gel images with sample identifiers, run date, and operator initials.

11. Quality Control

- Include one known positive genomic DNA control per target species and one non-template control in every N1 and N2 run.
- Repeat any run in which the positive control fails to amplify or the non-template control shows amplification.
- Discard reagents beyond their validated storage period or upon visible contamination.

12. Safety Precautions

- Wear appropriate personal protective equipment (gloves, lab coat) always.
- Perform pre- and post-PCR steps in physically separated workstations to prevent amplicon contamination.

SOP 6: Multiplex Real–Time Quantitative PCR (qPCR) for Detection and Quantification of *P. falciparum* and *P. vivax*

1. Purpose

This SOP describes the dual multiplex real–time quantitative PCR (qPCR) assay used to detect and quantify *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv) in N1–positive samples, targeting the 18S rRNA gene using species–specific TaqMan hydrolysis probes.

2. Scope

This procedure applies to all samples confirmed positive for *Plasmodium* spp. by first–round nested PCR (N1) and requiring species–level detection and parasite density quantification for *P. falciparum* and *P. vivax*.

3. Principle

The assay uses TaqMan Fast Advanced Master Mix in a dual–channel multiplex format. Species–specific forward and reverse primers and hydrolysis probes labeled with FAM (*P. falciparum*) and Texas Red (*P. vivax*) allow simultaneous detection and differentiation of both species within a single reaction. Fluorescence signal accumulation is measured in real time and compared against 10–fold serial dilution standard curves to determine parasite density.

4. Responsible Body

- ✓ Laboratory Director/Supervisor: Responsible for overall quality assurance of the nested PCR procedure, approval of results, and ensuring compliance with this SOP.
- ✓ Principal Investigator: Responsible for performing the nested PCR amplification and gel electrophoresis according to this SOP, maintaining equipment, and documenting all procedures.

5. Reagents, Consumables, and Equipment

- TaqMan Fast Advanced Master Mix (2×, Applied Biosystems, Cat. No. 4444964)

- Primers and probes: Pf18S_FW/RV/probe (FAM-labeled); Pv18S_FW/RV/probe (Texas Red-labeled) (see Section 6)
- Nuclease-free water
- N1-positive genomic DNA template
- *P. falciparum* standard: cultured NF54 strain genomic DNA (10^6 – 10^2 parasites/mL)
- *P. vivax* standard: plasmid DNA construct containing the Pv 18S rRNA gene insert (10^7 – 10^3 copies/mL)
- Non-template control
- Real-time PCR detection system with FAM and Texas Red detection channels
- Optical 96-well plates/tubes, optical adhesive covers, cold block/ice

6. Procedure

13. Thaw TaqMan Fast Advanced Master Mix, primers, probes, and DNA templates on ice, protecting probes from light.
14. Prepare the multiplex reaction master mix containing both Pf and Pv primer/probe sets at the concentrations given in Section 6.1, sufficient for all samples plus standards and controls.
15. Dispense 9 μ L of multiplex master mix into each well of an optical reaction plate.
16. Add 3 μ L of DNA template (sample, standard dilution, or nuclease-free water for the non-template control) to give a total reaction volume of 12 μ L.
17. Seal the plate with an optical adhesive cover, briefly centrifuge to collect contents at the bottom of the wells, and load into the real-time PCR instrument.
18. Run the qPCR program described in Section 6.2, acquiring fluorescence in the FAM (Pf) and Texas Red (Pv) channels at the end of each extension step.

19. Include the *P. falciparum* and *P. vivax* standard curves (10-fold serial dilutions) in every run to enable parasite density quantification.
20. Export amplification curves, Ct values, and standard curve parameters (slope, R², efficiency) for analysis.

7. Reaction Composition and Cycling Conditions

7.1 Multiplex qPCR Master Mix Primer/Probe Final Concentrations (per 12 µL reaction)

Target	Oligonucleotide	Final concentration	Label
<i>P. falciparum</i> (Pf18S)	Pf18S_FW (Forward)	300 nM	
	Pf18S_RV (Reverse)	300 nM	
	Pf18S_probe	110 nM	FAM–MGBNFQ
<i>P. vivax</i> (Pv18S)	Pv18S_FW (Forward)	200 nM	
	Pv18S_RV (Reverse)	200 nM	
	Pv18S_probe	105 nM	Texas Red–BHQ2

Reaction volume: 9 µL multiplex master mix (TaqMan Fast Advanced Master Mix, 2×, plus primers/probes at the above concentrations, made up with nuclease-free water) + 3 µL DNA template = 12 µL total.

7.2 Thermal Cycling Conditions

Step	Temperature	Time	Cycles
Pre-wash incubation (UNG step)	50°C	2 min	1
Enzyme activation	95°C	10 min	1
Denaturation	95°C	15 sec	45
Combined annealing/extension (data acquisition)	60°C	60 sec	45

8. Primer and Probe Sequences

Target	Oligonucleotide	Sequence (5'→3')
<i>P. falciparum</i>	Pf18S_FW	GTAATTGGAATGATAGGAATTTACAAGGT
	Pf18S_RV	TCAACTACGAACGTTTTAACTGCAAC
	Pf18S_probe	FAM-AACAATTGGAGGGCAAG-MGBNFQ
<i>P. vivax</i>	Pv18S_FW	AGCAGCCGCGGTAATTCCA
	Pv18S_RV	ATGCGCACAAAGTCGATACGAAG
	Pv18S_probe	TEXASRED- AGCAACGCTTCTAGCTTAATCCACAT-BHQ2

9. Standard Curves and Quantification

- *P. falciparum* standard: cultured NF54 strain genomic DNA, 10-fold serial dilutions from 10^6 to 10^2 parasites/mL.
- *P. vivax* standard: plasmid DNA construct containing the Pv 18S rRNA gene insert, 10-fold serial dilutions from 10^7 to 10^3 copies/mL.
- Run both standard curves in every plate; accept the run only if standard curve efficiency, slope, and R^2 fall within the laboratory's validated acceptance criteria.
- Determine parasite density/copy number for each sample by interpolating its Ct value against the corresponding standard curve.

10. Quality Control

- Include a known positive genomic DNA control for each target species and a non-template control in every qPCR run.
- Repeat any run in which a positive control fails to amplify within the expected Ct range or the non-template control shows amplification.
- Monitor standard curve performance (efficiency 90–110%, $R^2 \geq 0.98$) as an ongoing assay performance check.

11. Safety Precautions

- Wear appropriate personal protective equipment (gloves, lab coat) always.
- Protect fluorescently labelled probes from light exposure during preparation and storage.
- Handle plasmid standards and cultured parasite DNA according to institutional biosafety guidelines.