Thesis Ref.No___

**ADDIS ABABA UNIVERSITY**

**COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE**

**DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PUBLIC HEALTH**

# DIAGNOSTIC PERFORMANCE OF PCR AND MICROSCOPY AND MOLECULAR SURVEILLANCE OF CHLOROQUINE RESISTANCE -MARKERS IN *PLASMODIUM FALCIPARUM* CLINICAL ISOLATES IN HAWASSA, SIDAMA ETHIOPIA

**MSC THESIS**

**BY**

**DIBORA HAILU**

**JUNE, 2026**

**BISHOFTU, ETHIOPIA**

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Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa. University in partial fulfillment of the requirements for the degree of Master of Science in Tropical Infectious Disease

**By**

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As MSc in tropical infectious disease research advisors, we hereby certify that we have read and evaluated this thesis prepared under our guidance by Dibora Hailu, entitled" *Diagnostic Performance of PCR and Microscopy and Molecular Surveillance of Chloroquine Resistance-Markers in Plasmodium falciparum Clinical isolates in Hawassa Sidama Ethiopia.* "We recommend that it be submitted as fulfilling the MSc thesis requirement for the Degree of Master of Science in Tropical Infectious Disease.

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**DEDICATION**

I dedicate this work to my family for their unwavering support, encouragement, and for always being there for me throughout this journey until its successful completion.

**STATEMENT OF AUTHOR**

I, Dibora Hailu, solemnly declare that the thesis entitled ― *Diagnostic Performance of PCR and Microscopy and Molecular Surveillance of Chloroquine Resistance-Markers in Plasmodium falciparum Clinical isolates in Hawassa Sidama Ethiopia*" is my original work and has not been previously submitted in whole or in part for the award of any degree, diploma, scholarship, or other academic title at any university or institution. All sources of information, including data, literature, and intellectual contributions used or cited in this work, have been duly acknowledged with proper citations and references. Any assistance received in the preparation of this thesis has been appropriately credited. This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Tropical Infectious Disease, at the College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia.

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**ACKNOWLEDGMENTS**

I would like to sincerely acknowledge Professor Lemu Golassa for his willingness to involve me in this project and for his continuous support, guidance, and encouragement from the beginning to the completion of this work.

I would also like to express my heartfelt gratitude to Dr. Samson Leta for his valuable support, constructive comments, and overall contribution to this study.

My special thanks also go to Dr. Aynalem Mandefro, Yonas Alemu, and Sena Dejene for their incredible support during and after my laboratory work. Their assistance and guidance will never go unnoticed. They kindly taught me molecular laboratory techniques, shared their knowledge and experience generously, and supported me throughout the entire process until the completion of this work.

## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ACT | Artemisinin-based Combination Therapy |
| AL | Artemether–Lumefantrine |
| ALIHR | Aklilu lemma institute of health research |
| CI | Confidence Interval |
| CQ | Chloroquine |
| DBS | Dried Blood Spot |
| DNA | Deoxyribonucleic Acid |
| EtBr | Ethidium Bromide |
| NPV | Negative Predictive Value |
| OR | Odds Ratio |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| *Pf* | Plasmodium falciparum |
| *Pfcrt* | Plasmodium falciparum Chloroquine Resistance Transporter Gene |
| *Pfmdr1* | Plasmodium falciparum Multidrug Resistance Gene 1 |
| *Pv* | Plasmodium vivax |
| PPV | Positive Predictive Value |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic Acid |
| SNP | Single Nucleotide Polymorphism |
| SP | Sulfadoxine–Pyrimethamine |
| TBE | Tris–Borate–EDTA Buffer |
| UV | Ultraviolet |
| WHO | World Health Organization |

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**ABSTRACT**

Malaria is still a major public health problem in Ethiopia, particularly in areas with seasonal transmission, such as Hawassa, southern Ethiopia. Accurate diagnosis and continuous monitoring of antimalarial drug resistance are essential for effective control and elimination efforts. This study aimed to evaluate the diagnostic performance of PCR and microscopy and conduct molecular surveillance of CQ resistance markers in *Plasmodium falciparum* clinical isolates. A cross-sectional study was performed from September 2025 to May 2026 in selected health centers found in Hawassa, southern Ethiopia. A total of 1428 dried blood samples were collected from malaria-suspected individuals. Nested PCR targeting the 18S rRNA gene was used for species confirmation, while genotyping was conducted by restriction fragment length polymorphism (RFLP) to detect mutations in the *pfcrt* (K76T) and *pfmdr1* (N86Y) genes. Statistical analysis was performed using R software version 4.3.2. Logistic regression was used to assess the association between infection status and participants' characteristics, and Cohen's kappa was used to assess agreement between microscopy and PCR. Out of 1428 samples, 546 (38.2%) were PCR positive for *Plasmodium* species, of which 214 were confirmed as *P. falciparum*. Sex was significantly associated with PCR positivity, with male participants exhibiting higher odds of infection compared to females (OR = 1.33, 95% CI: 1.04–1.69, $p = 0.021$). Age (as a continuous variable) showed no statistically significant association with malaria infection overall or by species (Pf and Pv), as the odds ratios were close to unity and the confidence intervals included 1 (Overall PCR: OR = 0.99, 95% CI: 0.98–1.00, $P = 0.40$; Pf: OR = 0.99, 95% CI: 0.98–1.00, $P = 0.211$; Pv: OR = 0.99, 95% CI: 0.97–1.00, $P = 0.30$). But age was significantly associated with microscopy positivity, (OR = 0.98, 95% CI: 0.97–0.99, $p < 0.001$). In conclusion, PCR showed superior diagnostic performance compared to microscopy, particularly for malaria species identification. Microscopy demonstrated moderate agreement for *P. falciparum* ($\kappa = 0.426$) and fair agreement for *P. vivax* ($\kappa = 0.289$), while overall agreement was substantial ($\kappa = 0.611$; accuracy = 82.56%). Molecular analysis revealed fixation of the *pfcrt* K76T mutation (100%) and absence of the *pfmdr1* N86Y mutant allele, indicating persistent chloroquine resistance markers despite drug withdrawal. These findings highlight the need for continued molecular surveillance and strengthened malaria diagnosis and treatment monitoring in Ethiopia.

**Keywords**: *Chloroquine resistance, Malaria, Microscopy, PCR, pfcrt, Pfmdr1*

# 1. INTRODUCTION

## 1.1.Background

Malaria persists as a major global health challenge, with reported cases showing an increasing trend. In 2024, approximately 282 million cases and 610,000 deaths were recorded, a 9 million case increase compared to 2023 (WHO, 2025). Sub-Saharan Africa bears the highest share of this burden, accounting for 95% of malaria cases and deaths globally (WHO, 2025).

Malaria is endemic in Ethiopia, with approximately 68% of the population living in areas at risk, where *P. vivax* and *P.falciparum* coexist (FMoH, 2020; Negatu, Abebe, and Yalew, 2022). The disease is characterized by unstable and seasonal transmission patterns, often leading to periodic outbreaks (Degefa *et al*., 2015). The two predominant malaria species in Ethiopia are *P. falciparum* and *P. vivax*, which together account for nearly all malaria infections in the country (Girum *et al*., 2019). Despite substantial progress in malaria control through vector management, improved diagnostics, and effective treatment strategies, Ethiopia has experienced a marked resurgence of malaria since 2022, reporting its highest burden in 2024 and contributing substantially to the global malaria burden (WHO, 2024; WHO, 2025).

Accurate and timely diagnosis and proper case management are a cornerstone of effective malaria control and elimination, to ensure proper case management (WHO, 2016). Conventional diagnostic methods include light microscopy and rapid diagnostic tests (RDTs), both of which are widely used in endemic settings, including in Ethiopia. Microscopy, in particular, has long been considered the gold standard due to its ability to identify parasite species and estimate parasite density (Oyegoke *et al*., 2022).

However, microscopic diagnosis faces several limitations in these regions, including a shortage of skilled personnel, its labor-intensive and time-consuming nature, and poor quality control. These constraints often lead to poor diagnostic performance, including false negative results and decreased effectiveness in detecting low-level parasitism, especially in asymptomatic individuals, mixed infections, and early parasite stages (Tegegn *et al*., 2024). In contrast, Polymerase Chain Reaction (PCR) offers high sensitivity and specificity, and is

capable of detecting very low parasite levels with consistent repeatability and reproducibility. Despite this advantage its very expensive and not applicable in field settings (Adiatmaja *et al*., 2020).

In addition to diagnostic challenges, the development and dissemination of resistance to antimalarial drugs continue to threaten malaria control efforts. *P. falciparum* has developed resistance to several commonly used antimalarial drugs, including Chlorquine, Sulfadoxin phyramitimien, and, more recently, artemisinin derivatives and their partner drugs. Antimalarial drug resistance has become a major challenge to malaria control, undermining the effectiveness of timely treatment with appropriate medications (WHO, 2025). The World Health Organization recommends Artimissinin Combination Theraphy as the first-line treatment for uncomplicated *P. falciparum* malaria. However, the rise and spread of *P. falciparum* strains resistant to artemisinins and their partner drugs pose a serious threat to global malaria control efforts (WHO, 2024). Notably, in the early 1990s, a CQ–resistant *P. falciparum* strain emerged as a key challenge to malaria prevention and control in Ethiopia (Ayalew *et al*., 2017).

Artemisinin resistance was initially identified in western Cambodia in Southeast Asia during 2006-2007 and has since spread to various regions worldwide, including Eastern Africa. Validated markers of artemisinin resistance (ART-R ), such as C580Y, R539T, Y493H, I543T, N458Y, P574L, F446I, P553L, have been confirmed in countries such as Ethiopia (Fola *et al*., 2023), Rwanda (Uwimana *et al*., 2021), Uganda (Balikagala *et al*., 2021), Tanzania (Ishengoma *et al*., 2024), and the Democratic Republic of the Congo (DRC) (van Loon *et al*., 2024). This resistance likely contributes to the accelerated development of resistance to partner drugs, resulting in treatment failures, as observed in Southeast Asia (Fola *et al*., 2025).

*P. falciparum* parasites have developed resistance to CQ. After its introduction in the 1950s, CQ quickly became a treatment choice for treating *P. falciparum* infections because of its proven efficacy, favorable safety, cost-effectiveness, and ability to reduce fever (WHO, 2010). Research has identified that mutations in the *pfcrt* (K76T) and *Pfmdr1* (N86Y) genes are linked to resistance against CQ and other antimalarial drugs. (Kritsiriwuthinan and Ngrenngarmlert, 2015).

CQ resistance in *P. falciparum* is linked to mutations in the *pfcrt* gene, which encodes the PfCRT protein located on the membrane of the parasite's digestive vacuole (DV). This protein is responsible for transporting 4-aminoquinoline drugs, such as CQ, out of the vacuole. Resistant mutant alleles of *pfcrt* hinder Chloroquine from accumulating within the acidic Digestive vacuole (DV), thereby preventing the drug's toxic effects. Specifically, mutations in codons 72 to 76 of the *pfcrt* gene are associated with the CQ-resistant phenotype (Fontecha *et al*., 2021). The *pfmdr1* gene, although less prominently than *pfcrt*, has also been implicated in CQ resistance, particularly when the 86Y mutation occurs alongside the 76T mutation in *pfcrt* (Wurtz *et al*., 2014).

In southern Ethiopia, the exceptionally high prevalence of P.falciparum resistance markers strongly highlights that the infectivity of older antimalarial drugs, such as SP and CQ. This situation shows the urgent need of expended molecular monitoring across the country. The limited detection of certain resistance markers in Ethiopia, particularly in southern areas such as Hawassa, is essential to gain a good understanding of resistance patterns and distributions (Schunk et al., 2006).

### 1.2.Statement of Problem

Microscopic examination has long been the standard diagnostic method for detecting malaria parasites due to its effectiveness and lower cost compared to other techniques. However, this method has limitations, including the potential for species misidentification, especially when slides are poorly prepared, parasite densities are low, or mixed infections occur. Additionally, microscopy demands a high level of expertise. To improve diagnostic accuracy, molecular approaches such as PCR are increasingly used as complementary tools (Feleke *et al*., 2021).

The limitations of microscopy present a major obstacle to malaria elimination, as misidentification of low parasite densities and mixed-species infections contributes to 20–50% of human-to-mosquito transmission. Research in Ethiopia has shown that while microscopy specificity exceeds 90%, its sensitivity ranges widely from 44% to 96% (Tegegn *et al*., 2024)

For many years, CQ has been widely used as the primary treatment for uncomplicated malaria in Africa due to its affordability, effectiveness, and safety. However, the rapid development and spread of CQ-resistant parasites have significantly reduced its usefulness, creating an urgent need for alternative treatments (Sibley and Ringwald, 2006).

CQ was introduced in 1947 and became the best treatment for *P. falciparum* worldwide due to its efficacy and cost-effectiveness (Wellems and Plowe, 2001). However, resistance to this drug emerged during the 1950s across Southeast Asia and South America and spread to Africa by the 1980s, leading to treatment failure (Talisuna *et al*., 2004). In Ethiopia, CQs were used for decades since this resistance emerged; the drug policy was changed in the 2000s, and replaced with ACTs (FMOH, 2004; Talisuna *et al*., 2004). Despite this, CQ is still used for *P. vivax* in this country (Abreha *et al*., 2017). This withdrawal results in drug selection pressure and the emergence of drug-sensitive parasites (Kublin *et al*., 2003). At the molecular level, resistance to CQ is primarily associated with mutation in the *pfcrt* gene, particularly the K76T mutation, and to a lesser extent, *pfmdr1* polymorphism (Djimdé et al., 2001). After CQ discontinuation, studies show a significant reduction in mutant allele and show withdrawal (Kublin *et al*., 2003; Laufer *et al*., 2006).

In response, Ethiopia replaced CQ with Sulfadoxin Pyramithimene as an antimalarial treatment policy, and subsequently adopted Arthemether Lumefantrine in 2004 as the first-line therapy for uncomplicated *P.falciparum* malaria. Monitoring the prevalence of CQ resistance-associated markers more than two decades after the drug's withdrawal is critical for understanding the ongoing selection pressures on key genetic targets (FMOH, 2004).

According to the current national malaria diagnosis and treatment guidelines, Arthemether Lumefantrine is the first-line treatment for uncomplicated *P. falciparum* infections, while CQ remains the first-line treatment for *P. vivax* infections. The observed trend toward reversal of resistance may be influenced by a country's drug policies; however, data on the impact of CQ withdrawal on the circulating parasite population in Ethiopia remain limited and insufficiently studied(Golassa *et al.*, 2014).

In southern Ethiopia, particularly in Hawassa, there is limited and inconsistent data on the current status of CQ resistance markers and the diagnostic performance of microscopy compared to molecular methods. This lack of up-to-date evidence hinders effective

monitoring of resistance trends and limits the ability to optimize diagnostic and treatment strategies.

## 1.3. Objectives

### *1.3.1. General objective*

- To evaluate the diagnostic performance of PCR and microscopy and to determine molecular markers associated with chloroquine resistance in *P. falciparum* isolates from Hawassa, Southern Ethiopia.

### *1.3.2. Specific objectives*

- To assess sensitivity of microscopy agnainst PCR and the level of agreement between microscopy and PCR in the detection of *P. falciparum* and *P. vivax*
- To evaluate factors associated with malaria positivity detected by PCR and microscopy.
- To detect the presence of the *pfmdr1* N86Yand *pfcrt* K76T mutations in *P. falciparum* isolates

## 2. LITERATURE REVIEW

### 2.1.Overview of Malaria and Its Life Cycle

Malaria is a disease transmitted by the bite of infected female *Anopheles mosquitoes* and is caused by five species of *Plasmodium* parasites. In Ethiopia, the two most prevalent species responsible for malaria cases are *P.falciparum* and *P. vivax*, which account for approximately 60% and 40% of infections, respectively(Assemie *et al*., 2022). Globally, malaria continues to be a major cause of illness and death in low-income countries, affecting 263 million people and resulting in 597,000 deaths worldwide, according to the World Health Organization's 2023 World Malaria Report (Minwuyelet *et al*., 2025).

The life cycle of malaria is complex; it relies on the expression of specialized proteins essential for survival in both vertebrate and invertebrate hosts. These proteins facilitate the parasite's survival both inside and outside the cells, enable them to invade various cell types and to evade the host's immune defenses. When an *anopheles mosquito* bites a person, an infective sporozoite is released from the salivary gland along with an anticoagulant to maintain the smooth blood flow. Once in the bloodstream, the parasite travels to the liver, invades hepatocytes, and remains there for 9 to 16 days. During this period, the parasites undergo asexual replication called exo-erythrocytic schizogony. Each sporozoite produces tens to thousands of merozoites within the hepatocyte, and upon release, each merozoite can infect a red blood cell(RBC) (Tuteja *et al.*, 2007).

Once inside the red blood cell, the parasite begins asexual replication and progresses through several developmental stages. The early stage, which is called trophozoite or "ring form," is named for its distinctive shape. When the tropozoite grows, it exhibits active metabolism, including glycolysis of large amounts of glucose, ingestion of host cytoplasm, and breakdown of hemoglobin into amino acids. Since the parasite cannot degrade the toxic heme by-product of hemoglobin digestion, they convert it to a crystalline substance called hemozoin (malaria pigment), which is stored in food vacuoles. When a mosquito feeds on the infected person, it can ingest sexual-stage parasites called gametocytes. Inside the mosquito midgut, macrogametes develop into macrogametes, and microgametocytes undergo exflagellation to produce microgametes. These gametes fuse during fertilization to form a zygote, then

transform to ookonate. Then develops into an oocyst, then continues the parasite's life cycle within the mosquito(Tuteja *et al.*, 2007).

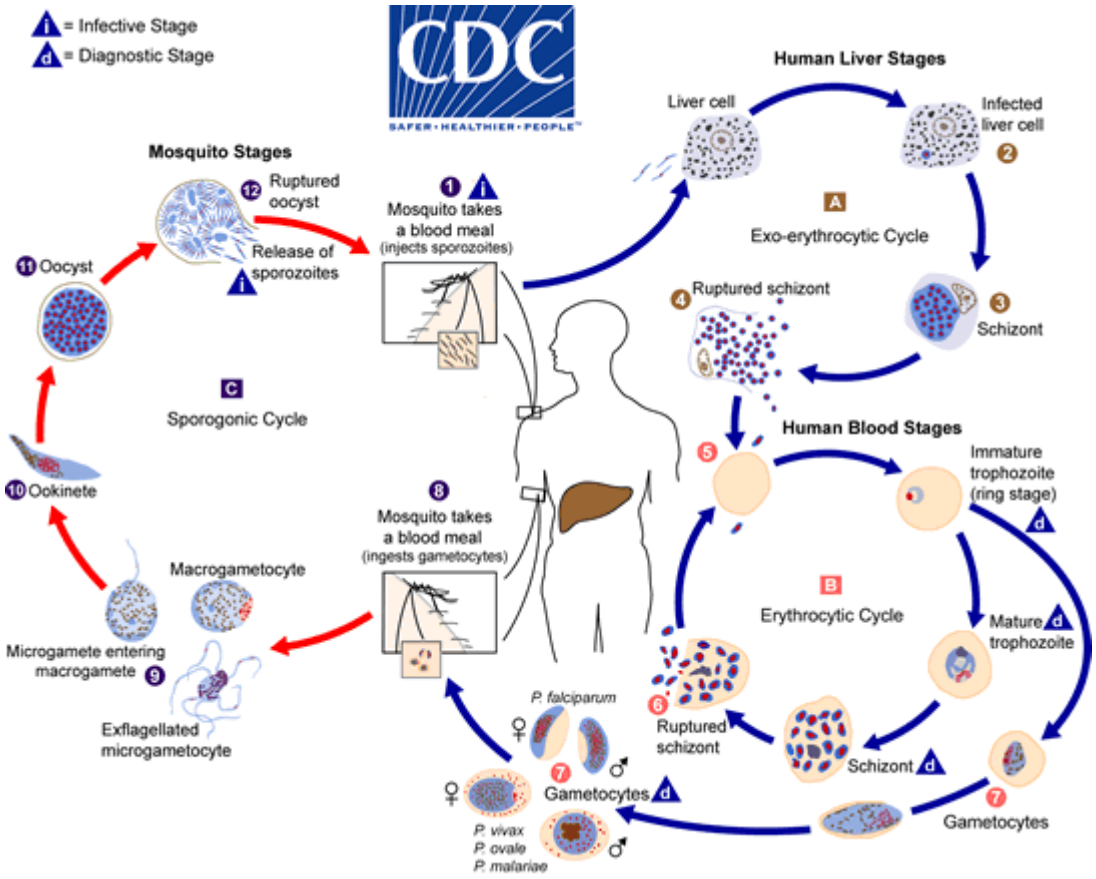


**Figure 1:** Life cycle description for malaria (Centers for Disease Control and Prevention,2024)

## 2.2. Diagnosis of malaria(Microscopy and PCR methods)

Light microscopy is capable of detecting malaria parasites, identifying infecting species, and estimating parasite load. However, it requires skilled personnel and a good power supply, and this limits its use in resource-limited settings. Microscopy could not always detect parasite load or treatment follow-up. An alternative diagnostic method is nested PCR, which includes two rounds of amplification that target the 18S ribosomal RNA gene using a thermocycler. However, nested PCR cannot quantify parasite load (Moyeh *et al*., 2019).

Microscopy identifies about 75% of malaria infections in high-transmission areas but can fail to detect up to 88% in low-transmission settings due to parasite densities below the detection limit. It also has limited sensitivity for mixed *Plasmodium* infections, especially when the parasite levels are too low(Mekonnen *et al*., 2014). Additionally, from malaria diagnostic methods, rapid diagnostic tests, which can detect the parasite antigen within short minutes, but has it, have inherent limitations that affect their reliability. Especially with deletions in the histidine-rich protein 2/3 (HRP2/3) genes, it can result in false-negative results in *P. falciparum*–specific tests even if the parasites are present (Moyeh *et al*., 2019).

Generally, nested PCR is more sensitive and can detect mixed infection well, and is widely used for diagnosis, epidemiological studies, drug efficacy assessment, and to measure the accuracy of microscopy (Feleke *et al*., 2021).

## 2.3.Antimalarial Drug Resistance: Global Overview

The WHO defines antimalarial drug resistance as the ability of the parasite strain to survive despite the administration of drug doses that are equal to or exceeding the standard or recommended amounts, where the drug should reach the target, in the right amount, without making the patient sick. This is due to a mutation in the parasite gene, which reduces their susceptibility to the drug, associated with alteration in structure, function, or abundance of specific proteins, resulting in genetic changes such as single–nucleotide polymorphism(SNP) or gene amplification(Shibeshi, Kifle, and Atnafie, 2020)

Poor adherence to malaria treatment guidelines, the drug's strength, the parasite mutation rate, improper dosing, and poor pharmacokinetic properties result in insufficient drug exposure on parasites, and low quality of antimalarial drugs may also contribute to the development of resistance to currently available antimalarial medications(Shibeshi, Kifle, and Atnafie, 2020).

The development of this resistance to antimalarial drugs shows a significant challenge to effective malaria control and treatment programs. To track this, molecular markers are a very important tool to assess the ongoing effectiveness of the current antimalarial drug and inform the design of new treatment strategies. Antimalarial drugs act to eliminate the erythrocytic stages of the parasites that are responsible for human illness. The drug regimens for the treatment of *Plasmodium* species are different for *P. falciparum* and *p.vivax*. ACT is now recommended for uncomplicated malaria in nearly all areas, due to resistance to the former drugs (Abebe *et al*., 2025).

### 2.4.Antimalarial Drug Use and Resistance in Ethiopia

In Ethiopia, *P. falciparum* and *p.vivax* co-exist across areas with varying levels of malaria endemicity. For more than two decades, AL has been the first-line treatment for uncomplicated malaria caused by *P. falciparum*, while CQ is still used for *p.vivax* infections. However, the implication of this high level of co-endemicity and continued CQ use for *p. vivax* remains unclear. In particular, it is not well understood whether the replacement of CQ with AL for *P. falciparum* has influenced AL efficacy or contributed to the reversion of mutations associated with CQ resistance(Hailemeskel et al., 2019). AL primarily targets the asexual stage of the malaria parasite, but it also has gametocidal activity. This activity interrupts the cycle that is between the mosquito vector and the human host (Tesfaye *et al*., 2024).

The emergence and spread of resistance to both artemisinin and partner drugs affects the effectiveness of ACT to treat and eradicate the parasite before getting worse. Their assessment of national treatment policies is needed to guarantee real-time evidence and to determine the therapeutic efficacy of first-line ACT. Few investigations on AL have been conducted since ACT was introduced in Ethiopia in 2004(Abamecha *et al*., 2020).

As a result of ACT and vector control programs, the global burden of malaria has decreased. However, malaria remains uncontrollable due to factors such as pesticide-resistant mosquito vectors, the emergence of drug resistance parasite, and the lack of an efficient malaria vaccine. Due to this, management of *P. falciparum* largely depended on chemoprophylaxis and chemotherapy. Additionally, *P. falciparum* develops resistance to all existing drugs that are currently available, which poses a significant challenge to control malaria worldwide (Woldesenbet *et al*., 2025).

### 2.5.Molecular Basis of Drug Resistance in *P. falciparum*

Potential genetic determinants of CQ resistance include the *pfmdr1*, which encodes pgh1, a homologue of p-glycoprotein, and the *pfcrt*, which encodes the CQ resistance transporter protein. Point mutations in *pfmdr1* at nucleotide positions 754, 1049, 3598, 362, and 4234 result in amino acid substitutions at codon 86, 184, 1034, 1042, and 1246, respectively. These amino acid changes have been associated with CQ resistance. Among them, the mutation at codon 86(Asparagine to tyrosine; N86Y) is considered particularly important, as it may alter the substrate specificity of the encoded p-glycoprotein homolog and potentially modify its transport function(Shrivastava *et al.*, 2014).

A mutation in the *pfcrt* gene is a key determinant of CQ resistance. The K76T substitution is considered the principal marker of CQ resistance and sensitivity. This mutation occurs in the first trans membrane domain of the PFCRT protein, where the positively charged lysine residue is replaced by a neutral threonine at position 76. This alteration facilitates the efflux of deprotonated CQ from the parasite's digestive vacuole through active transport mechanisms. Variations in this protein also influence susceptibility to other antimalarial drugs, including AQ, pipraquine, quinine, and luefantrine(Antony *et al*., 2016).

The *pfcrt* 76T mutation is characterized by the ACA codon encoding threonine, confirming the presence of a single amino acid substitution at this position. CQ resistance resulting from a single mutation can be further intensified by additional associated mutations. Specifically, the level of resistance linked to the *pfcrt* K76T substitution is higher when occurres along side other point mutations within the transmembrane regions of the CQ transporter protein(Chaijaroenkul *et al*., 2011). Consequently, mutations affecting parasite membrane

proteins can alter membrane permeability to CQ, thereby promoting selection and dominance of the CQ-resistant parasite. This mutation remains one of the principal determinants of CQ resistance.

The discovery of *pfmdr1* initially suggested its involvement in CQ resistance, but it soon became evident that polymorphisms in this gene were not the main cause. Further genetic analysis of offspring from a cross between CQ-sensitive and resistant strains identified the *Pfcrt* gene. This gene encodes a protein located in the food vacuole membrane and is believed to be part of the drug/metabolite transporter superfamily, playing a key role in CQ resistance(Cui *et al.*, 2015).

The *pfmdr1* gene is located on chromosome 5 and comprises a single exon encoding the p-glycoprotein homolog 1(pgh1). The encoded protein consists of 1419 amino acids with an approximate molecular weight of 162.25kDa. PFMDR1 is a transmembrane protein containing two homologous domains, each with six transmembrane helices, as well as a nucleotide-binding fold that functions as an ATP –binding site. It is localized in the parasite's digestive vacuole and belongs to the ATP-binding cassette (ABC) transporter family, similar to PFCRT. Mutations in *pfmdr1*, particularly N86Y and N1042D, have been associated with AQ resistance. In addition, substitution at codons 86 and 184 has been implicated in altered responses to AL, although evidence across studies is not fully consistent. In the case of CQ, PFMDR1 is believed to play a modulatory role in resistance but is not considered a primary determinant of CQ resistance(Heuchert *et al.*, 2015)

Mutations K76T and A220S in the *pfcrt* gene, together with the N86Y substitution in the *pfmdr1* gene, have been strongly associated with high levels of CQ resistance in field isolates of *P. falciparum*. In addition, increased copy number of the *pfmdr1* gene has been linked to reduced susceptibility to several antimalarial drugs, including quinine, mefloquine, halofantrine, lumefantrine, and artemisinin(Antony *et al*., 2016).

## 2.6. Factors Influencing Development of Resistance

Despite extensive and ongoing efforts, developing effective antimalarial drugs remains a significant challenge for malaria control. This challenge is underscored by the emergence of resistance to widely used antimalarial medications, which has exerted substantial selective pressure on *P. falciparum*. As a result, resistance to conventional antimalarial drugs has become widespread in many malaria-endemic regions, leading to increased morbidity and mortality from malaria(Mita and Tanabe, 2012).

The emergence of antimalarial drug resistance is more likely in low-transmission settings, mainly due to reduced host immunity and limited within-host competition among different parasite genotypes. The spread of parasites resistant to ACT partner drugs is strongly influenced by the periods of sub-therapeutic drug exposure, referred to as the selection window. Evidence suggests that resistance-associated mutations are more readily established and maintained in such low-transmission environments. When resistance to partner drugs is confirmed, control strategies should prioritize molecular surveillance and monitoring of therapeutic efficacy. In these situations, transitioning to an ACT regimen with an effective partner drug is recommended to sustain treatment success(Masserey *et al.*, 2022).

Almost all antimalarial drugs that target *P. falciparum* are metabolized by cytochrome P450 enzymes in the human liver, which makes the drug efficacy dependent on human genetic factors. Additionally, population movement, such as refugee displacement, travel, and transhumance, facilitates the spread of drug resistance. When individuals move to regions that are resistant to a particular antimalarial drug, they can spread resistance to new locations. This mechanism has been a key factor in the widespread dissemination of CQ resistance (Nikiema *et al*., 2025).

Chloroquine efficacy is primarily attributed to its ability to disrupt haematin detoxification in *P. falciparum* parasites during their growth inside host blood cells. CQ targets the trophozoite and schizont stages of the parasite. As the parasite consumes and digests hemoglobin within its digestive food vacuole, large amounts of haematin are released. Normally, haematin is detoxified by polymerizing into harmless crystals called haemozoin. CQ interferes with this process by forming a drug-haematin complex, preventing proper detoxification. This

disruption of haematin detoxification by quinolone drugs leads to toxic effects that are lethal to the parasites(Mulenga *et al.*, 2021).

### 2.7. Evidence from Ethiopia (and Southern Ethiopia specifically)

In 1998, widespread resistance of *P. falciparum* to CQ in Ethiopia led to a shift in first-line treatment to SP. However, recent data indicate a high SP treatment failure rate of up to 72% in some regions. As a result, a transition to AL was recommended in 2004. Despite this, AL availability remains limited, and with 85% of the population living in rural areas with restricted healthcare access, presumptive treatment with available drugs like CQ or SP continues to be common. Although CQ has been officially withdrawn as the first-line treatment, continued use of CQ may be contributing to the persistence of resistant parasite strains in the population(Schunk *et al.*, 2006).

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In Ethiopia, CQ was replaced with SP as first-line treatment for uncomplicated *P. falciparum* in 1998. This was later followed by a policy revision in 2004, in which SP was replaced by ACT. In Kenya, ACT was widely introduced in 2006, with AL becoming available in health facilities. In contrast, Ethiopia began nationwide implementation of AL in 2005(Jeang *et al.*, 2024).

In Ethiopia, there are significant interregional variations in malaria endemicity and transmission, coupled with a notable lack of comprehensive data on antimalarial drug effectiveness. In 1999, the CQ treatment failure rate within the first two weeks was as high as 88% in central regions of the country. By 2005, SP therapeutic failure rates ranged between 36% and 72% within two to four weeks of follow-up. In contrast, treatment with AL in the same year demonstrated a high efficacy, achieving an appropriate clinical and parasitological response rate of 99%(Mula *et al.*, 2011).

**Table 1:**Comparsion of Previous studies on CQ associated resistance markers

| First author | Data collection year | DNA extraction method | Genotyping method | *pfcrt*d, (No) | *pfcrt*-76K (No, %) | *pfcrt*-76T (No, %) | *Pfmdr*1 genotyped (No) | *Pfmdr*1-86N (No, %) | *Pfmdr*1-86Y (No, %) |
|---|---|---|---|---|---|---|---|---|---|
| (Golassa *et al.*, 2015) | 2014 | chelex extraction | nRFLP | 152 | 6(3.9) | 146(96) | 103 | 89(86.4) | 14(13.5) |
| (Hassen *et al.*, 2022) | 2019-2021 | Chelex extraction | nRFLP | 83 | 7(8.4) | 76(91.5) | 80 | 80(100) | 0 |
| (Hailemeskel *et al.*, 2019) | 2016-2017 | Chelex extraction | nRFLP | 169 | 40(23.7) | 129(76.3) | 168 | 158(94) | 10(6) |
| Mekonnen *etal.*, 2014) | 2009 | Qiagen | Sequencing | 195 | 164(84.1%) | 31(15.9%) | 195 | 166(85.1) | 29(14.9) |
| (Schunk *et al.*, 2006) | 2004 | Qiagen | nRFLP | 69 | 0 | 69 (100%) | 69 | 24 (34.7) | 45 (65.2) |
| (Mula *et al.*, 2011) | 2007-2009 | Speed tools kit | nRFLP | 76 | 0 | 76(100) | 76 | 51( | 25(32.9) |
| Tadele *et al.*, 2023) | 2020 | Chelex | HRM | 215 | 115(53.4) | 100(46.5) | 208 | 166(79.8) | 42(20.2) |
| (Golassa *et al.*, 2014) | 2012 | Chelex | nRFLP | 99 | 0 | 99(100) | NR | NR | NR |
| (Heuchert *et al.*, 2015) | 2013 | Chelex | nRFLP | 159 | 7(4.4) | 152(95.6) | 163 | 161(88.8) | 2(1.2) |

# 3. MATERIALS AND METHODS

## 3.1. Description of the Study Area

This study was conducted in Hawassa City, the capital of Sidama regional state in Ethiopia. Hawassa City is the capital of Sidama Regional State. Located approximately 275 Kilometers south of Addis Ababa, at 7°03'43.38"North and 38° 28' 34.86" E longitude approximately, a distance of 275 kilometers south of Addis Ababa (Atara, Tolossa, and Denu, 2019). The city lies within an area of seasonal malaria transmission and has local environmental features that support vector breeding, notably the extensive lake shoreline, swampy and marshy areas, and peri-urban water containers, which sustain *Anopheles* breeding habitats and seasonal epidemics. Community surveys and surveillance in Hawassa report ongoing malaria risk and variable prevention practices among households, and recent entomological studies have documented the presence of multiple efficient vectors (including the invasive *Anopheles stephensi*), with elevated *Plasmodium* sporozoite infection rates in primary/secondary vectors in the area (Hawaria *et al*., 2025). These ecological and entomological features, together with the local temperature and rainfall patterns that favor vector survival and parasite development, make Hawassa an appropriate site for fieldwork on malaria prevalence and vector risk (Hawaria *et al*., 2023; Hawaria *et al*., 2025).

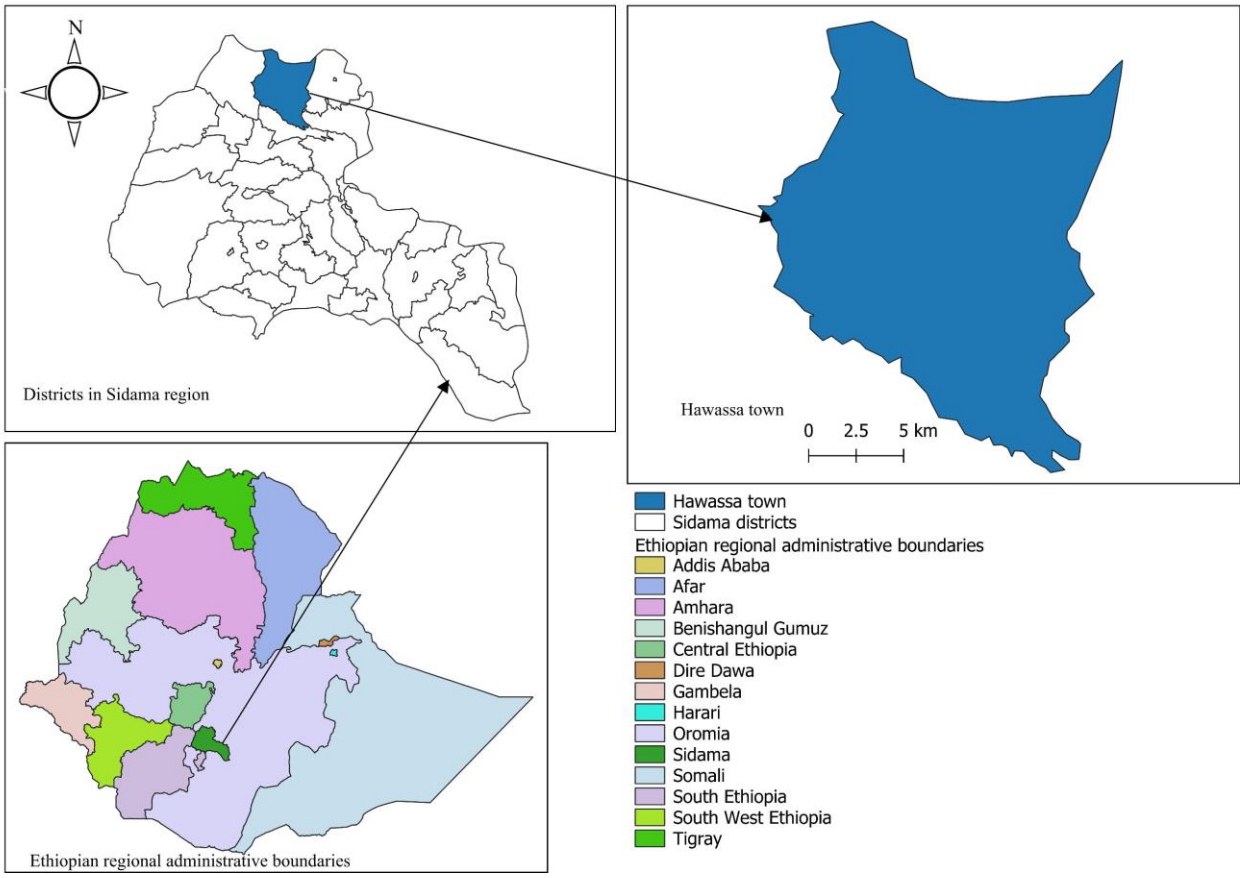


**Figure 2:** Map of the study area

**3.2.** Study Design and Population

This study employed a cross-sectional design and was conducted during (September 2025 to May 2026). The sample collection was carried out at three selected health centers in Hawassa, Ethiopia, where samples were obtained from outpatient attendees.

Inclusion and Exclusion Criteria

- Inclusion-criteria

All individuals aged six months and above who were clinically suspected of malaria according to the Ethiopian national malaria case definition (fever or history of fever with no obvious alternative cause), attended the selected health centers during the study period, and provided informed consent were included in the study.

- Exclusion criteria:

Pregnant women and severely ill individuals who were unable to provide blood samples or consent were excluded from the study.

**3.3.** Data Collection Methods

*3.3.1. Sample collection and handling*

Finger-prick blood samples were collected by trained health professionals from patients at the three health centers. All collected blood samples were used to prepare thin and thick blood films and were also spotted onto Whatman 3MM filter paper to prepare dried blood spots. The samples were then air-dried and stored in self-sealing plastic bags for subsequent molecular analysis (Golassa *et al.*, 2015). Over all 1428 blood samples were obtained from all study sites: Telte health center (n=482, Gameto health center (n=456 and Gararikeṭa health center (n=491). The blood spots were air-dried horizontally, kept at room temperature for at least 4 hours, away from dust, insects, and direct sunlight. The samples were then placed in a sealed container and transported to the Malaria Molecular Laboratory at Addis Ababa University, ALIHR, for molecular analysis. Upon arrival, the samples were stored at −20 °C until DNA extraction.

### 3.4. Laboratory Procedure

*3.4.1. Microscopic diagnosis*

For confirmatory identification of *Plasmodium* species, blood smears were stained using 10%Giemsa solution for 10 minutes. Microscopic examinations were performed by trained laboratory personnel following WHO guidelines(WHO,2016). Then, each slide was independently read by two blinded microscopists, with examination conducted across 200 high-power fields and parasite density estimated assuming a mean leukocyte count of 8,000/ µl. In cases of discordant results, a third independent blinded microscopist re-evaluated the slides to reach a final result.

*3.4.2. DNA extraction*

DNA was extracted from dried blood spot (DBS) using a Chelex-based method as previously described (Simon *et al.,* 2020). With minor modifications. Two 6mm discs were punched from each blood spot using a sterile manual puncher and put in micro centrifuge tubes. Then 1ml of PBS and Tween 20 was added, and then kept overnight at $4^0$ °C with intermittent mixing. The next day after centrifugation, the supernatant was removed, and the discs were washed with PBS and further incubated at $4^0$ °C. Following additional centrifugation and removal of wash buffer, 6% Chelex solution was added to each tube. DNA was then released by heating at $95^0$c for 12 minutes with intermittent vortexing. The samples were centrifuged again, and the resulting supernatant was carefully transferred to new tubes. The final extract was stored at-$20^0$ °C until molecular analysis.

*3.4.3. DNA Amplification by Nested PCR*

A nested PCR was employed for molecular confirmation of *Plasmodium* infection through two amplification stages. The first round (N1) targeted the *Plasmodium* genus to detect parasite DNA, while the second round (N2) was used for species-level identification, particularly distinguishing *P.falciparum* and *P.vivax*. In the primary PCR, genus-specific primers (rPLU5 and rPLU6) were used to amplify target DNA, which subsequently served as the template for the second amplification. Species identification in the secondary reaction was performed using primers specific for *P.falciparum*(rFAL1/rFAL2) and *P. vivax* (rVIV1/rVIV2), as previously described by Snounou *et al*. (2002). The first round amplification involved an initial denaturation at $95^0$ °C for 10 minutes, followed by 35cycles consisting of denaturation at $95^0$ °C for 60 seconds, annealing at $58^0$ °C for 60seconds, and extension at $72^0$c minutes. The second-round PCR followed a similar thermal profile, except that it included 30 cycles and ended with a final extension at $70^0$c for 10 minutes. PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under a UV trans illuminator after running at 120V for approximately one hour. Fragment sizes were estimated using a 100-bp DNA ladder (Biochain). Each run included a positive control (*P.falciparum* 3D7 strain DNA) and a negative control containing nuclease-free water to validate assay performance.

*3.4.4. Genotyping of Pfcrt K76T and Pfmdr1 N86Y codons by RFLP of PCR products*

Following amplification of the target codons using the primer sets listed in Table 3 and the PCR conditions provided in Annex 2, genotyping of *pfcrt* K76T and *pfmdr1* N86Y polymorphisms was carried out using PCR-restriction fragment length polymorphisms(PCR-RFLP) analysis with the ApoI resistance enzyme(New England Biolabs). Enzymatic digestion was performed according to the manufacturer's instructions in a total reaction volume of 20 µL containing nuclease-free water, NEBuffer r3.1, ApoI enzyme, and 5 µL of nested PCR product. Reference control strains, including Pf3D7 (wild type) and Dd2(mutant type), together with undigested DNA controls, were included in each assay run. The ApoI enzyme selectively cleaves the wild *pfcrt* 76K and *pfmdr1* N86 alleles, whereas the mutant *pfcrt* 76T and *pfmdr1* 86Y alleles remain undigested. Digested products were separated by electrophoresis using 2.0% agarose gel for *pfcrt* and 2.5% agarose gel for *pfmdr1,* followed by visualization under a UV transiluminator (Hassen *et al*., 2022). Mutant alleles were

identified by the presence of undigested fragments corresponding to the expected product sizes of 145bp for *pfcrt* 76T and 481bp for *pfmdr1*86Y.

**Table 2**:Primer name and sequence used for confirmation of *p.falciparum* and *P.vivax* infection targeting the 18S ribosomal RNA gene

| | Primers name | Sequence (5'-3') | Size |
|---|---|---|---|
| Primary PCR(N1) | rPlu5 | CCT GTT GTT GCC TTA AAC TTC | 1200bp |
| | rPlu6 | TTA AAA TTG TTG CAG TTAAAACG | |
| Nested PCR | rFal1 | TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT | 205bp |
| | Rfal2 | ACA CAA TAG ACT CAA TCA TGA CTA CCC GTC | |
| | rViv1 | CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC | 120bp |
| | Rviv2 | ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA | |

**Table 3:** Primer name and sequnce used to genotype the *pfcrt* K76T and *pfmdr1* N86Y genes

| Codon | Primer | Sequence5'-3' | Amplicon size(bp) |
|---|---|---|---|
| *pfcrt*, K76T | CRT-1F | CCGTTAATAATAAATACACGCAG | 537 |
| | CRT-1R | CGGATGTTACAAAACTATAGTTACC | Djimde *et al.*, (2001) |
| | CRT-2F | TGTGCT CAT GTG TTT AAA CTT | 145 |
| | CRT-2R | CAAAACTATAGTTACCAATTTTG | |
| *Pfmdr1*,N86Y | MDR1-1F | TTAAATGTTTACCTGCACAACATAGAAAATT | 612 Fontecha *et al.* (2021) |
| | MDR1-1R | CTCCACAATAACTTGCAACAGTTCTTA | |
| | MDR1-2F | TGTATGTGCTGTATTATCAGGA | 526 |
| | MDR1-2R | CTCTTCTATAATGGACATGGTA | |

### 3.5. Quality Control

To minimize contamination, all laboratory procedures were conducted under strict sterile conditions. Laboratory workflow will follow a unidirectional layout, with physically separated areas designated for DNA extraction, master mix preparation, and template addition. During the punching of (DBS) filter papers, special care was taken to avoid cross-contamination by sterilizing punchers using flame and 70% ethanol between samples. Different laboratory coats and gloves were worn in each work area to maintain cleanliness. In addition, positive and negative controls were included at every critical step, including DNA extraction and PCR amplification, to monitor for contamination and to validate assay performance. As positive and negative controls, *P.falciparum* strain 3D7(CQ sensitive) and Dd2( CQ resistant) were used during amplification before genotyping. Then all genotyped products were evaluated before being digested by restriction enzymes(Ocan *et al.*, 2016).

### 3.6. Data Management and Statistical Analysis

All collected data, including participants' age, sex, and microscopy result, were entered into an Excel spreadsheet, and then summaries were performed using pivot tables. The cleaned data were then imported into the R software 4.3.2 (R Core Team, 2023) for statistical analysis. A *p*-value <0.005 was considered statistically significant, and the odds ratio and confidence interval 95% and corresponding p-values were calculated.sensitivity and specifity with positive predictive value and negative predictive value were analyzed using caret package (Kuhn *et al.*, 2008). Agreement between microscopy and PCR was assessed using Cohen's kappa coefficient (Landis *et al*., 2011) with the IRR package (Gamer *et al*., 2019). For antimalarial drug resistance markers (*pfcrt* and *pfmdr1*), all isolates were classified as either mutant or wild-type (100% characterization). These results were summarized descriptively using charts and narrative statements.

### 3.7. Ethical Approval and Consideration

Ethical approval for the study was obtained from the Institutional Review Board (IRB) of the Aklilu Lemma Institute of Health Research, Addis Ababa University (Ref. No. ALIHR IRERC/198/2018/25(Annex4). Written informed consent was obtained from all adult participants, while assent was appropriately obtained from participants under 18 years of age. All procedures were conducted in accordance with established ethical guidelines.

# 4. RESULTS

## 4.1. Characteristics of the study participants

The study includes both male and female participants aged between 6months and 85 years. A total of 1428 individual were enrolled, comprising 708(49.6%) males and 720(50.4%) females. Although some microscopy data were missing, PCR amplification was successfully performed for all participants, including PCR positivity and associated participant factors.

## 4.2. PCR positivity and associated risk factor

Among the 1,428 participants included in the analysis, 546(38.2%) were PCR positive for *Plasmodium* species. Sex was statistically significantly associated with PCR positivity, with male participants exhibiting higher odds of infection compared to females (OR = 1.33, 95% CI: 1.04–1.69, $p = 0.021$).

Sex was not significantly associated with PCR positivity for *P. falciparum*, with males having similar odds of infection compared to females (OR = 1.14, 95% CI: 0.85–1.53, $p = 0.384$), and Age showed no significant association with PCR positivity (OR = 0.99, 95% CI: 0.98–1.00, $p = 0.211$). In contrast, for sex were significantly associated with PCR positivity for *P. vivax*, male participants had higher odds of infection compared to females (OR = 1.33, 95% CI: 1.04–1.68, $p = 0.021$). Age (as a continuous variable) showed no statistically significant association with malaria infection overall or by species (Pf and Pv), as the odds ratios were close to unity and the confidence intervals included 1 (Overall PCR: OR = 0.99, 95% CI: 0.98–1.00, $P = 0.40$; Pf: OR = 0.99, 95% CI: 0.98–1.00, $P = 0.211$; Pv: OR = 0.99, 95% CI: 0.97–1.00, $P = 0.30$). (Table 4).

**Table 4**:PCR positivity and Associated Risk factors

| Variable | Category | Sample Size | PCR Overall (n) | OR (95% CI) | P-value | PCR Pf (n) | OR (95% CI) | P-value | PCR Pv (n) | OR (95% CI) | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | Female | 720 | 251 | Ref | 0.0214 | 102 | Ref | 0.384 | 162 | Ref | 0.021 |
| | Male | 708 | 295 | 1.33(1.04–1.69) | | 112 | 1.14(0.85–1.53) | | 197 | 1.33 (1.04–1.68) | |
| Age | Continuous | 1428 | 546 | 0.99 (0.98–1.00) | 0.40 | 214 | 0.99(0.98–1.00) | 0.211 | 359 | 0.99 (0.97–1.00) | 0.30 |

### 4.3. Microscopy Detection of *Plasmodium* Species and Association with Participant Characteristics

The association between participant characteristics and microscopy positivity was evaluated. Both sex and age were significantly associated with microscopy positivity. Male participants had higher odds of microscopy positivity compared to females (OR = 1.53, 95% CI: 1.20–1.95, $p < 0.001$). Age was also significantly associated with microscopy positivity, with increasing age associated with a decrease in the odds of microscopy positivity (OR = 0.98, 95% CI: 0.97–1.00, $p < 0.001$).

The microscopic *P. falciparum* positivity was associated with participant age and sex. Sex showed a statistically significant association with *P. falciparum* positivity by microscopy, with male participants having higher odds of microscopy positivity compared to females (OR = 1.49, 95% CI: 1.13–1.97, $p = 0.005$). Similarly, age was also significantly associated with microscopy positivity, with increasing age associated with a decrease in the odds of microscopy positivity (OR = 0.98, 95% CI: 0.97–0.99, $p < 0.001$).

On the other hand, *P. vivax* microscopy positivity was not significantly associated with sex, with male participants showing comparable odds to females (OR = 1.30, 95% CI: 0.90–1.89;

$p$ = 0.166). However, age remained significantly associated, with increasing age associated with a decrease in the odds of microscopy positivity (OR = 0.98, 95% CI: 0.97–0.99; $p$ = 0.009). (Table 5).

**Table 5**:Microscopy positivity and Associated Risk factors

| Variable | Category | N | Overall (n) | OR (95% CI) | P | Pf (n) | OR (95% CI) | P | Pv (n) | OR (95% CI) | P |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sex** | Female | 684 | 159 | Ref | 0.000 | 105 | Ref | 0.005 | 55 | Ref | 0.166 |
| | Male | 672 | 212 | 1.53 (1.20–1.95) | | 143 | 1.49 (1.13–1.97) | | 69 | 1.30 (0.89–1.89) | |
| **Age** | Continuous | 1356 | 372 | 0.98 (0.97–0.99) | 0.000 | 248 | 0.98 (0.97–0.99) | 0.000 | 124 | 0.98 (0.97–0.99) | 0.009 |

### *4.4.* Diagnostic performance of microscopy against PCR for *P.falciparum* and *P.vivax*

The diagnostic performance of microscopy was evaluated against PCR as the reference standard for overall samples as well as separately for *Plasmodium falciparum* and *Plasmodium vivax*. Overall, microscopy demonstrated moderate diagnostic accuracy, correctly identifying a substantial proportion of PCR-negative samples; however, both false-positive and false-negative results were observed. This indicates that while microscopy performs reasonably well in routine screening, it is not fully reliable when used as a standalone diagnostic tool.

For *P. falciparum*, microscopy showed comparatively better performance, with a relatively higher number of true positives and true negatives. Although some false-negative results were still observed, the overall pattern suggests that microscopy is more effective in detecting *P. falciparum* infections than *P. vivax,* with a relatively balanced sensitivity and specificity.
In contrast, for *P. vivax*, microscopy exhibited reduced sensitivity, as indicated by a higher proportion of false-negative results compared to true positives. This suggests that a

considerable number of *P. vivax* infections confirmed by PCR were missed by microscopy. However, false positives were relatively few, indicating good specificity for ruling out non-infected cases. Overall, these findings indicate that microscopy has acceptable but variable diagnostic performance, with better detection accuracy for *P. falciparum* compared to *P. vivax*, and a general tendency to underestimate true malaria prevalence when compared with PCR.

**Table 6**:Confusion matrix of Microscopy based prediction for all compared with PCR combined samples(pf and pv)

| | **Reference 0 (PCR−)** | **Reference 1 (PCR+)** |
|---|---|---|
| Microscopy negative (Prediction 0) | TN = 788 | FN = 194 |
| Microscopy positive (Prediction 1) | FP = 42 | TP = 329 |

**Table 7**:Confusion matrix of alternatitive model performance for *P.falciparum* compared with PCR refrence standared

| | **Reference 0 (PCR−)** | **Reference 1 (PCR+)** |
|---|---|---|
| Microscopy negative( Prediction 0) | TN = 1021 | FN = 87 |
| Microscopy positive (Prediction 1) | FP = 130 | TP = 118 |

**Table 8**:Confusion matrix for microscopy based prediction for *p.vivax* compared with PCR refrence standared

| | **Reference 0 (PCR−)** | **Reference 1 (PCR+)** |
|---|---|---|
| Microscopy negative (Prediction 0) | TN = 978 | FN = 254 |
| Microscopy positive (Prediction 1) | FP = 34 | TP = 90 |

### 4.5. Diagnostic Performance and Agreement of Microscopy Compared with PCR for Malaria Detection

Microscopy showed substantial overall performance compared with PCR, with a sensitivity of 62.91% and high specificity of 94.94%, indicating that it correctly identified most negative cases but failed to detect a substantial proportion of PCR-confirmed positive cases (false negatives). The overall agreement between microscopy and PCR was substantial ($\kappa = 0.611$), with an accuracy of 82.56% (95% CI: 80.43–84.54). For *P. falciparum*, microscopy demonstrated lower sensitivity (57.56%) but high specificity (88.71%), with moderate agreement ($\kappa = 0.426$) and accuracy of 84.00%, indicating better performance in ruling out infection than detecting all true cases. In contrarst For *P. vivax*, microscopy performance was poor in sensitivity (26.16%) despite very high specificity (96.64%), with weak agreement ($\kappa = 0.289$) and lower accuracy (78.76%), showing that many infections were missed by microscopy. Overall, microscopy performed well in confirming negative cases but had limited sensitivity, particularly for *P. vivax***,** compared to PCR**.**

**Table 9**:Agreement and diagnostic accuracy of microscopy compared to PCR indetection of malaria among study participants

| Diagnostic Comparison | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Kappa | Accuracy (%) | 95% CI for Accuracy |
|---|---|---|---|---|---|---|---|
| Microscopy vs PCR (Overall) | 62.91 | 94.94 | 88.68 | 80.24 | 0.611 | 82.56 | 80.43–84.54 |
| *P. falciparum* Microscopy vs PCR | 57.56 | 88.71 | 47.58 | 92.15 | 0.426 | 84.00 | 81.94–85.91 |
| *P. vivax* Microscopy vs PCR | 26.16 | 96.64 | 72.58 | 79.38 | 0.289 | 78.76 | 76.49–80.91 |

### 4.6. Molecular Marker Profiles of chloroquine resistance-associated markers in *P. falciparum*: *pfcrt* K76T and *pfmdr1* N86Y

Among the 546 PCR-positive samples, species-specific analysis showed 214 *P. falciparum* infections and 359 *P. vivax* infections, with mixed infections included in both categories, resulting in overlapping counts. Since the study specifically focused on chloroquine resistance in *P. falciparum*, all *P. vivax* samples were excluded from further analysis. Consequently, genotyping of *pfcrt* and *pfmdr1* was performed only on the *P. falciparum* isolates. Out of 214 *P. falciparum* samples, 84 were excluded due to resource limitations, and the remaining 130 samples were subjected to genotyping of both genes. During laboratory processing, a subset of samples failed to produce amplification bands, likely due to DNA quality issues or low parasite density, resulting in reduced numbers for downstream analysis. Accordingly, 123 samples were successfully analyzed for *pfcrt* K76T following ApoI digestion, while 115 samples were successfully analyzed for *pfmdr1* N86Y. Analysis of *P. falciparum* isolates revealed distinct patterns of chloroquine resistance–associated markers. The *pfcrt* gene showed fixation of the mutant allele (100%) among successfully analyzed samples, with no wild-type allele detected in the study population. In contrast, all analyzed isolates carried the wild-type allele for *pfmdr1* N86Y, and no mixed or mutant genotypes were observed. These findings indicate a homogeneous genetic profile for these key drug-resistance markers in the study area.

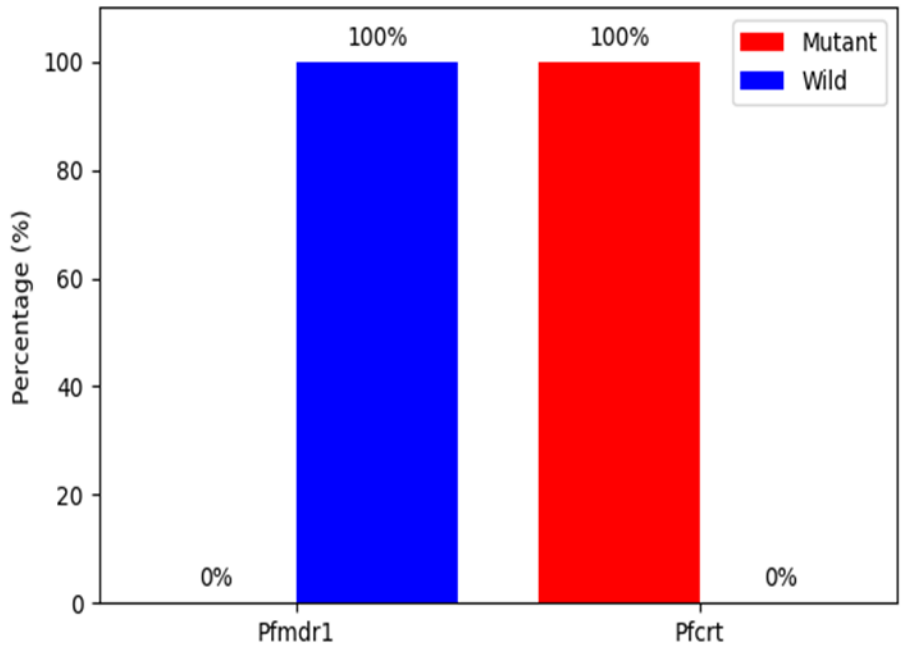


**Figure 3:** Fixation of *pfcrt* 76T, and wild-type *pfmdr1* N86

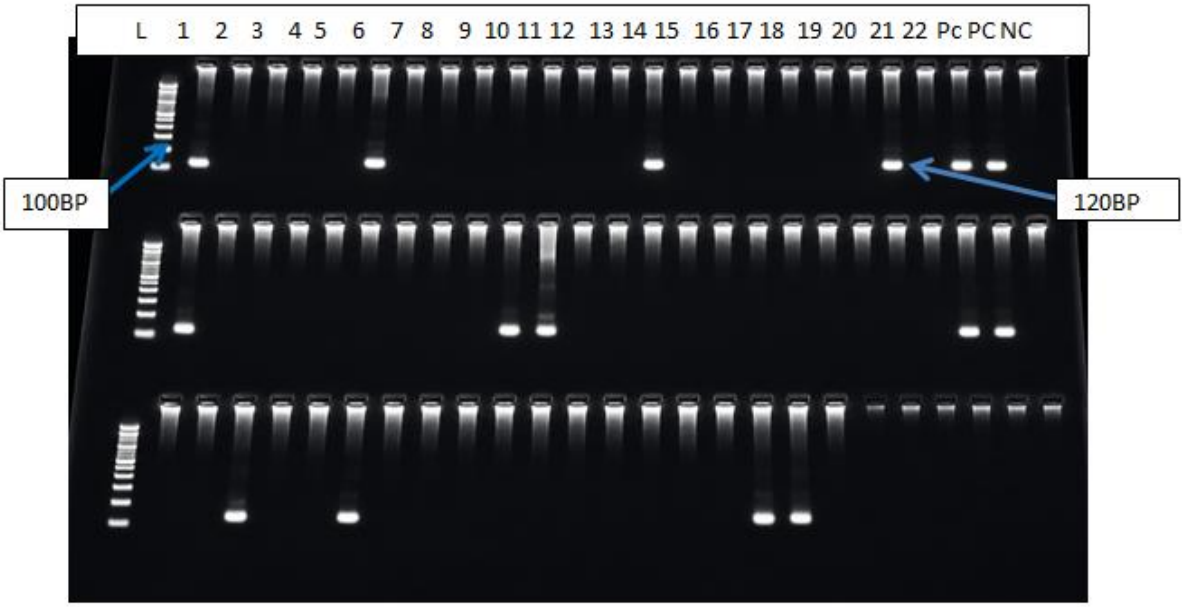


**Figure 4:**Gel electrophoresis image for species confirmation of *p.vivax* L=100bp ladder;pc=positive control(known sample), NC=Negative control(nuclease-free water). Well sample 2,6,14,21,23,32,33,47, and 50 were positive for *p.vivax*; the remaining were negative

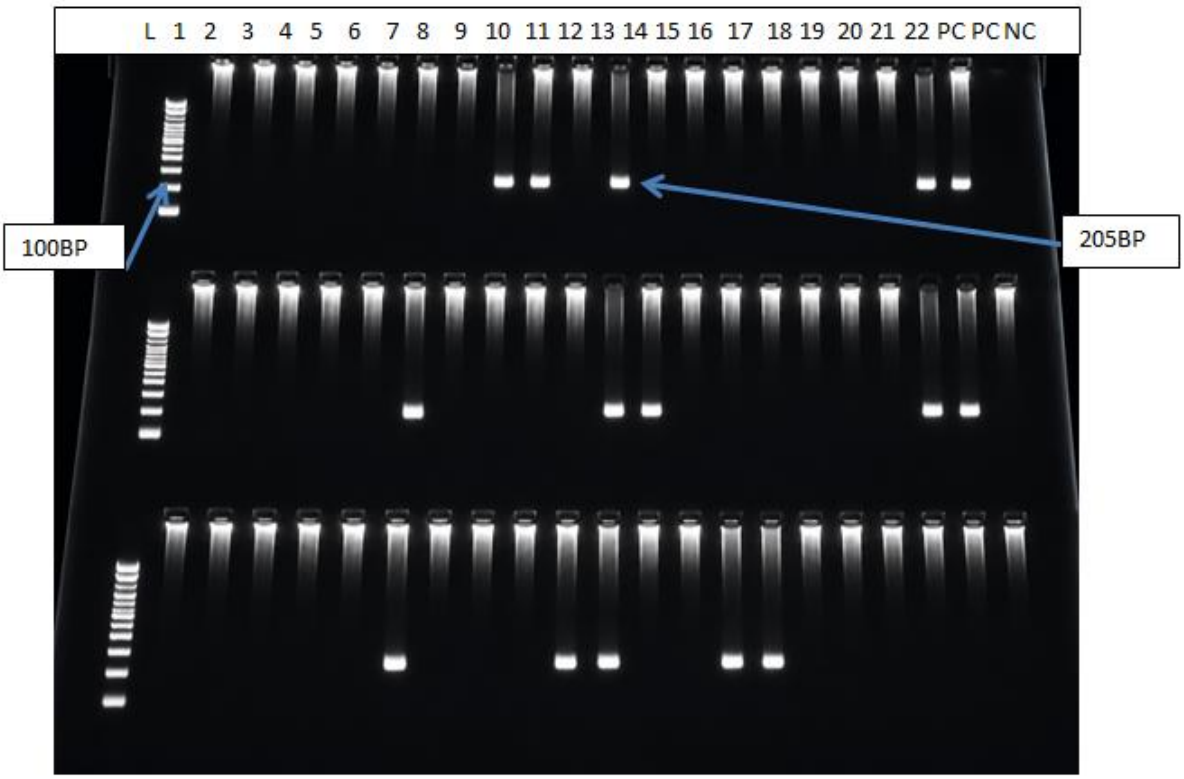


**Figure 5:**Gel image for species identification of *P.falciparum* L=100bp ladder; pc=positive control, NC= negative control(nucleus-free water). Well sample 10, 11,14,30,36,37,52,56 and 58 were positive at 205bp, the remaining were negative.


**Figure 6:** Gel electrophoresis image for genotyping of *pfcrt* (K76T), gene showing a band at 145bp, L=100bp ladder, pc=3D7(Drug sensitive strain), NC=Dd2(Drug resistant strain)

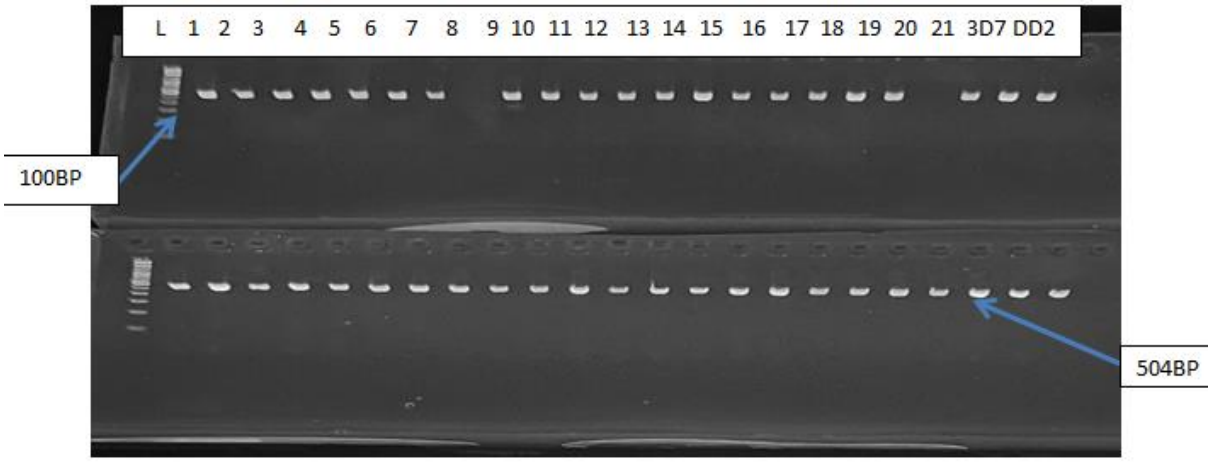


**Figure 7:** Gel electrophoresis image for genotyping of *pfmdr1* (N86Y) gene, L=100bp, pc(3D7), NC(Dd2), well in the first lane, samples 8 and 20 did not show a band, the remain shows a band at 504 bp

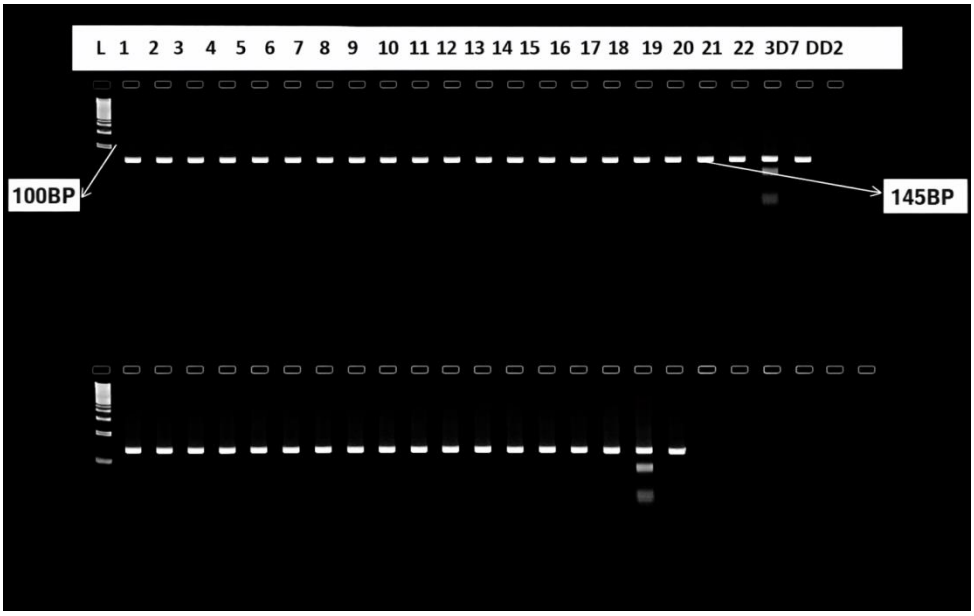


**Figure 8:**Gel electrophoresis image for *pfcrt*(K76T), after digested by ApoI enzyme showing that all samples were mutant at 145bp, L= 100 bp , pc(3D7),NC(Dd2)

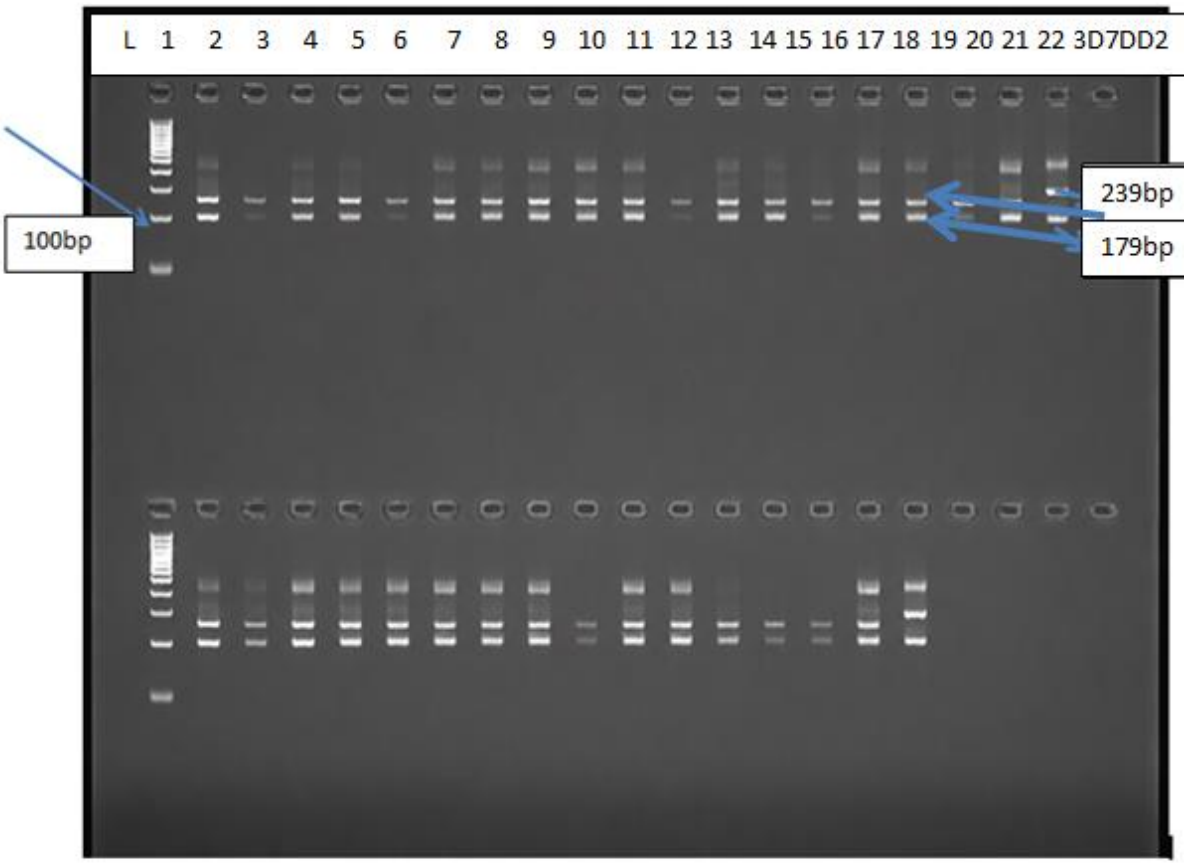


**Figure 9:** Gel electrophoresis image for *pfmdr1* (N86Y) gene after digested by ApoI enzyme showing a band at 179 and 239, which is wild type, L=100bp ladder, PC(3d7), NC(Dd2)

## 5. DISCUSSION

Molecular surveillance is key to knowing the persistence of mutations or genetic changes in the population of parasites that are exposed to drug pressure. These findings provide important insight into the current resistance landscape and diagnostic performance of PCR and microscopy and molecular surveillance of CQ associated resistance profile in Hawassa, Sidama Ethiopia. As the demographic characteristics indicate, the study includes both male and female participants, with males 708(49.6%) and females 720(50.4%) within the age range between 6 months and 85 years. All samples were successfully amplified using nested PCR. The main findings were complete fixation of the *pfcrt* K76T mutation, absence of the *pfmdr1* N86Y mutation, and substantial overall agreement of PCR and microscopy, and species-specific discrepancies between these two diagnostic methods.

Recent research indicated that *P. vivax* outnumbered *P.falciparum*, as in Butajira (62.5%) (Woyessa *et al.,* 2012), Hallaba (70.41%) (Tefera G *et al.,* 2014), Adim Tullu (84.6%) (Gari T *et al*., 2016), and Wolkite districts (69.7%) (Solomon *et al.,* 2020). This variation may be influenced by differences in transmission intensity, parasite biology, and diagnostic limitations. In particular, low parasite densities and mixed infections can lead to misclassification by microscopy, especially when performed by less experienced personnel, resulting in underestimation of true species distribution. Molecular methods such as PCR have been shown to improve species detection and reduce such misclassification (Golassa *et al*., 2020; Moyeh *et al*., 2019).

Of the 546 microscopy-positive samples, PCR confirmed 359 *P. vivax* infections and additionally identified mixed-species infections that were not consistently recognized by microscopy. These discrepancies likely reflect the challenges of species identification using microscopy, particularly in cases of low parasite density and morphological overlap between Plasmodium species. Accurate species identification is essential because treatment recommendations differ among species, and misclassification may result in inappropriate case management. Technical factors such as staining quality and microscopist expertise may further affect diagnostic performance. Similar observations have been reported in studies from Ethiopia, Cameroon, and Indonesia, where molecular methods detected infections that were either missed or incorrectly classified by microscopy (Heuchert *et al*., 2015; Moyeh *et al*., 2019). The high sensitivity and specificity of nested PCR make it particularly valuable for

detecting mixed-species and low-density infections (Mekonnen *et al.,* 2014b; Gatti *et al.,* 2007).

Demographic analysis showed significant associations between sex, and malaria infection. Increasing age was consistently associated with lower odds of infection detected by both microscopy and PCR. Specifically, age was not significantly associated with overall PCR-confirmed infection (OR=0.99, 95%CI: (0.98–1.00, *P*=0.40),but for microscopy-detected infection(OR = 0.98, 95% CI: 0.97–0.99, $p < 0.001$), and species-specific outcomes, including *P. falciparum* and *P. vivax*. This trend likely reflects the gradual development of immunity with increasing age due to repeated exposure. Consequently, adults are more likely to harbor asymptomatic or low-density infections, which are more readily detected by molecular methods (Kayiba *et al.,* 2024; Mulugeta *et al.,* 2022).

Male sex were associated with higher odds of infection, particularly for microscopy detected infections(OR = 1.53, 95% CI: 1.20–1.95, $p < 0.001$) and PCR confirmed *p.vivax* infection(OR = 1.33, 95% CI: 1.04–1.68, $p = 0.021$). This is likely due to outdoor and occupational exposure, and this aligns with previous studies(Mulugeta et al., 2022). These highlight how demographic, behavioral, and combined partial immunity shape malaria epidemiology. But for *P.falciparum* it is not significant (OR = 1.14, 95% CI: 0.85–1.52, $p = 0.38$), (OR = 0.99, 95% CI: 0.98–1.00, $p = 0.211$). This may be due to the widespread and more homogeneous transmission of *P.falciparum* in the study area, where exposure occurs across all demographic groups, reducing detectable differences between them. In addition, PCR detects both high and low density infections, including submicroscopic infections that are more evenly distributed across populations, thereby attenuating observable associations with the host factor(WHO,2024; Belay *et al.*,2025; Demash *et al.*,2024).

The present study demonstrated that microscopy had high specificity and substantial overall agreement with PCR, indicating its usefulness as a routine diagnostic tool for malaria detection. However, its moderate sensitivity and particularly low sensitivity for *P.vivax* indicate that a considerable number of infections may be missed. These findings are consistent with the systematic review and meta-analysis conducted in Ethiopia by Feleke *et al.* (2021), which reported that microscopy showed good diagnostic accuracy but lower sensitivity when PCR was used as the reference standard. The superior sensitivity of PCR enables the detection of low-density and submicroscopic infections that may not be identified

by conventional microscopy. Therefore, although microscopy remains appropriate for routine clinical diagnosis in resource-limited settings because of its affordability and availability, PCR should be considered the reference method for confirmatory diagnosis, surveillance, and epidemiological studies.

Cohen's kappa analysis showed substantial overall agreement between microscopy and PCR ($\kappa = 0.611$), but lower agreement at the species level, with moderate agreement for *P. falciparum* ($\kappa = 0.426$) and fair agreement for *P. vivax* ($\kappa = 0.289$). These findings indicate that although microscopy is useful for general malaria diagnosis, PCR provides more accurate species identification, which is critical for appropriate treatment and avoiding unnecessary drug exposure (Jaleta *et al.*, 2020). The lower agreement, particularly for *P. vivax*, may be attributed to low parasite densities and difficulties in species differentiation, whereas the relatively higher agreement for *P. falciparum* may reflect its higher parasite densities and more distinct morphological characteristics. Similar findings have been reported by Ehtesham *et al.* (2015), who observed only moderate concordance between microscopy and PCR for species identification and detection of mixed infections.

Regarding molecular resistance, all successfully amplifies *P.falciparum* isolates show fixation of mutant alleles for *pfcrt* K76T, with no wild-type haplotype detected. This result contrasts with reports from western Ethiopia, which showed partial re-emergence of CQ-sensitive strains after withdrawal of this drug (Tadele *et al*., 2023). The persistence of this fixation can be due to continued selective pressure from informal use of CQ for *p.vivax*, which contributes to higher transmission intensity, which maintains resistant alleles despite the withdrawal. Similar persistence was documented elsewhere in Ethiopia (Golassa *et al.,* 2014), whereas other studies reported partial recovery(Mekonnen *et al.,* 2014). The persistence of this 76T allele in Ethiopia is likely due to the incomplete withdrawal of CQ for *P. vivax,* which accounts 40% prevalence in Ethiopia. Additionally, *P. vivax* can relapse during the dry season, which may also have an impact on exposing *P. falciparum* to CQ pressure (Hassen *et al.,* 2022).

These discrepancies highlight geographical and temporal heterogeneity in resistance dynamics, which may be influenced by population immunity, local drug use, transmission intensity, and parasite population structure, all of which shape how resistant or sensitive alleles rise or decline (Masserey *et al*., 2022). Persistent use of CQ for p.vivax may influence

the maintenance of *P.falciparum* strains. A study from West Bengal, India, reported sustained prevalence of *pfcrt* K76T despite reduced *P.falciparum* incidence, which was attributed to the continued use of CQ for *p.vivax*. This finding highlights how indirect drug pressure can affect the persistence and potential re-emergence of CQ-sensitive or resistant parasite populations (Chatterjee et al., 2016).

Regarding the *pfmdr1* gene, no mutations were detected; all isolates carried the wild-type N86Y allele. This pattern may reflect low selection pressure for *pfmdr1* mutations in areas where AL is used as first-line therapy, which tends to favor the wild-type allele(Ramirez *et al.,* 2025). previous studies have reported variable prevalence of this mutation( Mula *et al.,* 2011),and systematic review indicating regional heterogeneity(Mitiku *et al.,* 2025). Other study such as conducted in Qatar reported the predominance of wild type *pfmdr1* alongside persistent *pfcrt* mutations, which aligns with this study(Okell *et al.,* 2018), other similar studies reported low prevalence of *pfmdr1* copy number amplification(1%) from Democratic Republic of Congo and other African countries, in contrast to studies in southeast Asia, were amplification is more common due to mefloquine pressure(Mvumbi *et al.,* 2017). Although the copy number was not assessed in this study, the absence of the N86Y mutation supports limited selective pressure on the *pfmdr1* setting.

Other similar studies from Angola using PCR-RFLP reported high prevalence of *pfcrt* K76T(93.9%); however, unlike our findings, the mutant *pfmdr1* N86Y allele was detected in 61.3% of isolates(Figueiredo *et al*., 2008). The absence of the *pfmdr1* N86Y mutation in oursetting may be due to selective pressures and regional heterogeneity across sub-Saharan Africa (Figueiredo *et al*., 2008).In contrast to this study from Botswana, which reported a high prevalence of *pfmdr1* N86Y(95.8%) and wild-type *pfcrt* K76T, this can be due to resistance patterns, while supporting the continued efficacy of AL were N86Y predominates (Tawe *et al*., 2018).

Globally, *pfcrt* mutations show notable regional variability. Studies from Yemen showed high prevalence of *pfcrt* K76T mutations(97.7%) across multiple districts, with other genes varying frequencies, which reflects the concept that CQ resistance patterns are shaped by local transmission intensity, parasite population, and historical drug pressure. This emphasizes the importance of ongoing molecular surveillance(Atroosh *et al.,* 2016). In contrast, a study from Rwanda reported re-emergence of CQ –sensitive parasites, 50.8% of

isolates carrying the wild-type pfcrt K76T allele. This showed regional differences in the dynamics of CQ resistance recovery across Africa. Similarly, this study reported a high prevalence of pfmdr1 N86Y allele, supporting continued ACT efficacy (Kateera et al., 2023).

Mutations associated with *pfmdr1* modulate parasite susceptibility to ACT partner drugs but are not associated with artemisinin resistance. While codons N86Y, Y184F, and D1246Y influence sensitivity to amodiaquine and lumefantrine in Sub-Saharan Africa, the absence of N86Y suggests continued efficacy of AL (Adamu *et al.*, 2020; WHO, 2022). The persistence of wild-type *pfmdr1* N86, and fixation of *pfcrt* 76T reflects a complex resistance landscape shaped by CQ exposure and ongoing ACT use, which means CQ remains unsuitable for *P.falciparum*, while ACTs are likely still effective. The near-complete reversion to the wild-type allele observed in this setting may be attributed to ACT driven tratement pressure. Therefore, we recommend concurrent surveillance of molecular markers to assess the impact of deployed antimalarial drugs in areas where *p.vivax* and *P. falciparum* are co-endemic(Ariey *et al.*, 2014).

If the particular resistant allele shows sensitivity to CQ in Ethiopia, we can use it as part of combination therapy with other short-acting drugs, which provides a cost-effective, safe, and easily administered antimalarial option (Mekonnen *et al.*, 2014). Overall, this study contributes updated molecular evidence on CQ resistance in Hawassa, southern Ethiopia, emphasizing the need for continuous monitoring to inform treatment policy and resistance containment strategies

The present study has several limitations that should be considered. First, the cross-sectional study design did not allow assessment of temporal trends in resistance markers or evaluation of clinical treatment outcomes. Second, the study was conducted in Hawassa, Southern Ethiopia; therefore, the findings may not be generalizable to other regions of Ethiopia due to geographical variations in malaria transmission and antimalarial drug pressure. Third, DNA sequencing was not performed to confirm the detected mutations because of resource and time constraints. Despite these limitations, the study provides important data on the diagnostic performance of microscopy and PCR and contributes valuable molecular surveillance evidence on CQ resistance markers in *P. falciparum* isolates from Southern Ethiopia.

## 6. CONCLUSION AND RECOMMENDATIONS

This present study investigated the diagnostic performance of PCR and microscopy and to determine molecular markers associated with chloroquine resistance in *P. falciparum* isolates from Hawassa, Southern Ethiopia. as the result indicated that increasing age was associated with decreased odds of infection. Likely reflecting the development of acquired immunity, repeated exposure. Regarding sex, male were more frequently affected, which might be due to their occupational or outdoor exposure. Overall, microscopy demonstrated high specificity and substantial agreement with PCR; however, its moderate sensitivity particularly low for *P. vivax* indicates that a considerable number of infections may be missed, especially in cases of mixed infections and low parasitaemia, highlighting its limitations in accurate species-level diagnosis compared to PCR. Molecular analysis showed complete fixation of *pfcrt* K76T alleles, while no mutations were detected in the *pfmdr1* N86Y locus among the analyzed isolates. The persistence of this *pfcrt* mutant allele may be linked to the continued use of CQ for *p.vivax* in Ethiopia, which may maintain drug selection pressure in co-endemic settings. This finding provides essential baseline molecular evidence for Hawassa and underscores the need for strengthened molecular surveillance, improved diagnostic accuracy, and careful monitoring of drug policy implications to safeguard the efficacy of first-line therapies and support malaria elimination efforts in Ethiopia. Based on the findings of this study, the following recommendations are forwarded:

- Regular molecular surveillance should be strengthened in Hawassa and other endemic areas.
- Molecular diagnostic methods should be integrated into the reference laboratory level to increase and improve species identification accuracy.
- Future studies should include resistance markers such as *pfkelch13* (*P.falciparum kelch 13* gene), which is the recent in our country to incorporate therapeutic efficacy assessments, and longitudinal and therapeutic efficacy studies should be conducted to correlate molecular markers with clinical treatment outcomes.
- The Ministry of Health should review malaria treatment policies based on updated molecular evidence, and training on molecular techniques should be enhanced to support local research and surveillance systems.
- Since DNA sequencing gives extensive information on the resistance markers and the amino acid sequence, and it’s the most reliable, it should be conducted for the future.

## 7. REFRENCE


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## 8. APPENDIES

### Annex 1 : Informed Consent and Assent format

Participant Information Sheet

Project Title: Diagnostic Performance of PCR and Microscopy and Molecular Surveillance of Chloroquine Resistance-Markers in *Plasmodium falciparum* Clinical isolates from Southern Ethiopia

Principal investigator:

Advisor(s):

Institution: Addis Ababa University, College of Veterinary Medicine and Agriculture

Introduction

My name is Dibora Hailu, I am an MSC student and currently conducting research entitled "Diagnostic Performance of PCR and Microscopy and Molecular Surveillance of Chloroquine Resistance-Markers in *Plasmodium falciparum* Clinical isolates from Southern Ethiopia." You are being invited to take part in this study. Before you decide, you need to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

Purpose of the study

The goal of the study is to compare the diagnostic performance of microscopy and PCR, and to detect drug resistance markers for chloroquine in Hawassa, southern Ethiopia. We are using dried blood spot (DBS), which is a simple way to collect and store blood for testing.

Procedure

If you agree to participate

1. A trained health professional will clean your finger with an alcohol swab.
2. A small prick will be made on your fingertip using a sterile, disposable lancet.
3. A few drops of blood, approximately (3-5 drops), will be dropped onto a special filter paper card.
4. The procedure takes about 5-10 minutes in total

   Risks and discomforts

There is minimal risk involved. You may feel a slight sting when your finger is pricked, and there is a small chance of minor bruising or slight bleeding, which will be managed immediately by the collector.

Benefit:

There may be no direct clinical benefit to you today. However, the information gained from this study will help to improve malaria drug resistance markers (chloroquine) detection and public health strategies in the future.

Confidentiality:

All information collected will be kept strictly confidential. Your sample will be assigned a code number. Your name will not appear on the sample or in any published reports. Only the research team will have access to the data.

Volunteer participation and withdrawal:

Your participation is entirely voluntary. You can choose to not participate or to withdraw at any time without any penalty or loss of medical care at this facility.

Informed consent form

Statement of the participants

I have read (have had read to me) the information provided above. I have had the opportunity to ask questions, and any question I asked has been answered to my satisfaction. I understand that my participation is voluntary and that I can withdraw at any time.

I agree to take part in this study and allow the collection of a DBS sample for the detection of chloroquine resistance markers.

Participant name: _____

Signature/Thumb print: _____ date: _____

Statement of the researcher:

I confirm that I have explained the nature and effect of the research to the participant, and I believe that the participant understands the implications of the study.

Researcher name: _____

Signature _____ date: _____

Contact information: _____

Principal investigator: _____

Assent form

For this research, you will give us a blood sample from a finger-prick. We will keep your entire blood sample laboratory result private; only the principal researcher from Addis Ababa University working on the study will see it.

We do not think that any big problem will happen to you as you give a blood sample for this study; there will be no financial or other direct benefit to you. But during each follow-up period of 8 days, your parents/guardians will take 100 birr for transportation and lose their jobs. More than this, your assent for us to give a blood sample to test chloroquine-resistant markers will help us to know the efficacy of the antimalarial drugs.

You should know that:

You do not have to be in this study if you do not want to. You will not get into trouble if you say no.
You may stop being in this study at any time.
Your parent(s)/guardian(s) will be asked if it is ok for you to be in this study. Even if they say it's ok, it's still your choice whether or not to take part
You can ask any questions you have now or later.
Sign this form only if you:

Have understood what you will be doing for this study
Have all your questions answered
Have talked to your parent(s)/guardian(s)

Agree to take part in this research

Name(participant) signature _____date: _____

Name (investigator) signature _____date_____

**Annex 2: Laboratory Procedures**

All laboratory procedures were conducted under Biosafety Level 2 (BSL-2) conditions due to the handling of human blood samples potentially containing infectious agents such as *Plasmodium* species and other blood-borne pathogens.

Standard laboratory safety precautions were strictly followed, including the use of:

Laboratory coats
Disposable gloves
Face masks
Protective eyewear when necessary

- All biological samples were treated as potentially infectious. Sample handling, DNA extraction, and PCR preparation were performed in designated laboratory areas to prevent contamination.

- Work surfaces were disinfected before and after procedures using 70% ethanol or appropriate laboratory disinfectants. Pipette tips and consumables were disposed of in biohazard waste containers.

- Sharps (if any were used) were discarded in puncture-resistant safety boxes. All infectious waste materials were autoclaved before final disposal according to institutional biosafety guidelines.

- In case of spills or accidental exposure, immediate decontamination procedures were followed using approved disinfectants, and incidents were reported to laboratory supervisors.

- PCR setup was carried out in a contamination-free area using separate pipettes and consumables to avoid cross-contamination of DNA samples.

- Malaria parasite DNA is extracted from dried blood spots (DBS) by heating samples in A Chelex suspension (5%-30%). The samples are heated in a water bath at 96°C in an alkaline solution, which disrupts cell membranes and breaks down proteins, including Heat-sensitive

enzymes. The Chelex solution, acting as an ion chelator, helps protect the DNA by inactivating nucleases and binding heavy metals that could otherwise damage parasite DNA.

Equipment and Materials

- Puncher /scissor - Permanent marker
- Forceps - PBS-container
- 1.5 ml micro centrifuge tubes - Chelex- container
- 0.5 ml micro centrifuge tubes - Refrigerator
- Micropipette (20µl, 100-200 µl, 1000 µl) - Bunsen burner (optional)
- Micropipette Tip ( 20 µl, 200 µl, 1000 µl) - Plate sealer
- Digital balance - Shaker (optional)
- Water bath or dry bath - Tissue paper
- Micro Centrifuge - Aspirator (Optional)

Required chemicals and reagents

- PBS solution
- 0.5% Saponin/PBS
- 6% Chelex

Remark: Condition to be considered before starting the procedure

- Clean the workbench with disinfectant ( 1:10 diluted bleach) and 70% alcohol
- Collect the necessary material
- Prepare required working reagent if not available (NB. Chelex reagent should be made fresh each day based on the need)
- Cover the workbench with M-tork (Tissue paper)
- Arrange the DBS/sample, disinfectant, and/ or Bunsen burner in the appropriate place

Procedures [Day 1]

1. Enter sample information in Excel (Extraction list) or logbook. Specify the date of extraction

2. Prepare the work area (Disinfect the area and cover the bench with tissue paper)

3. Label 1.5 ml micro centrifuge tubes or the well of a 96-well microliter plate with the filter paper sample ID (label both the lid and the side of the tube)

4. Disinfect the puncher/scissor and forceps before and after punching by dipping in 70 % Alcohol and passing through a Bunsen flame

5. Cut 2-3 pieces of 3 mm x 3 mm or punch a 3 mm-5mm disk (holds approx. 3-5µl of Dried blood) from the filter paper. Cut into small strips before putting into a 1.5ml tube.

6. Put the small pieces of filter paper into the corresponding 1.5 Micro centrifuge tube or well of a 96-well microliters plate using forceps

7. Add 1ml of PBS containing 0.5 % Saponin (950µl PBS + 50µl 10% Saponin) into the Corresponding 1.5ml micro centrifuge tube or 96-well microliters plate

NB. Ensure filter papers are soaked in buffer (PBS)

8. Mix well and make sure filter papers are completely immersed in buffer (PBS)

9. Incubate micro centrifuge tube or 96-well microliter plate at 4 o C for 1- 2 nights , depending on the colour change

Procedures [Day 2]

1. Vortex and then centrifuge the mixture at 13,000 rpm for 10 minutes at room temperature

2. Discard the liquid content using the P1000 pipettor

3. Vortex and add 1ml of PBS and leave at 4 °C for 30 mins

4. Centrifuge at 13,000 rpm for 5 mins

5. Discard as much liquid as possible using P1000 pipette tips

6. Add 300µl 6 % Chelex in TE buffer

7. Vortex and incubate at 95 °C for 12 mins. Open the cap to release pressure after 2 min and vortex every 3 mins

8. Centrifuge at 13,000 rpm for 8 mins

9. Transfer supernatant into clean, labelled 1.5 ml centrifuge tubes

10. Centrifuge again at 13,000 rpm for 1 min

11. Transfer liquid to clean labelled 0.5 ml centrifuge tubes. Avoid the chelex particles that reside at the bottom. Volume should be ~250ul for each tube

12. Put finished tubes in a white cardboard box. Good quality DNA should be of a clear colour, maybe light yellowish sometimes

13. Label the box with a consistent labelling system and then store the box at -20 o C

- PBS preparation

PBS Tablet……one Tablet

Distilled water……200ml

Procedure for preparation of PBS solution

1. Measure 200 mL of distilled water in a measuring cylinder

2. Transfer 200 mL of distilled water into the flask and add one tablet of PBS

3. Dissolve the tablet using an electronic stirrer

4. Autoclave dissolved PBS solution

- 0.5% saponin /PBS solution

Saponin powder……..0.5gm

PBS solution……..100ml

- 6% Chelex

1. Chelex 100 resin……6gm

2. DNase/ RNase free water ……. .100ml

**Table 10:** Mastermix formulation for species confirmation primary PCR (N1)

| PCR ingredient | Volume per reaction (microliters) | Total amount |
|---|---|---|
| Nuclease-free water | 6.25 | |
| Primer forward (PLU5) | 0.625 | |
| Primer Reverse (PLU6) | 0.625 | |
| Hot start master mix(2x) | 12.5 | |
| Aliquate per well | 20 | |
| DNA Template | 5 | |
| Total volume | 25 | |

**Table 11**:Primary PCR(N1) program for species confirmation using nested PCR

| Steps /cycle | PCR program Time | Temperature (0c) | Purpose | |
|---|---|---|---|---|
| 1st step | 10min | 95 | Initial denaturing | |
| 2nd step | 60sec | 95 | Denaturing | |
| 3rd step | 60sec | 58 | Annealing | 35 cycles |
| 4th step | 90sec | 72 | Extending | |
| 5th step | 10 min | 72 | Extending | |
| | ∞ | 10 | Hold | |

**Table 12**:Mastermix formulation for species confirmation PCR(N2)

| **PCR ingredient** | **Volume per reaction (microliters)** | **Total amount** |
|---|---|---|
| Nuclease-free water | 9.25 | |
| Primer forward(fal1/viv1) | 0.625 | |
| Primer reverse (fal2/viv2) | 0.625 | |
| Hot start master mix(2x) | 12.5 | |
| Aliquate per well | 23 | |
| DNA template | 2 | |
| Total volume | 25 | |

**Table 13**:Primary(N1) PCR program for species confirmation using nested PCR

| **Steps/ cycle** | **PCR program Time** | **Temperature (0c)** | **Purpose** | |
|---|---|---|---|---|
| 1st step | 10min | 95 | Initial denaturation | |
| 2nd step | 60sec | 95 | Denaturing | 30 cycles |
| 3rd step | 60sec | 58 | Annealing | |
| 4th step | 90sec | 72 | Extending | |
| 5th step | 10min | 72 | Extending | |
| | ∞ | 10 | Hold | |

Principle

- This protocol is based on the amplification of specific regions of the *Plasmodium* falciparum *pfcrt* and *pfmdr1* genes using conventional nested PCR, followed by Restriction Fragment Polymorphism(RFLP) analysis to differentiate wild-type and mutant alleles.
- PCR generates sufficient copies of the target DNA segments, which are then digested with allele-specific restriction enzymes that recognize or fail to recognize sequence changes caused by the K76T and N86Y mutations.
- The resulting DNA fragments are separated by agarose gel electrophoresis, and the banding patterns are interpreted to determine whether each sample contains wild-type, mutant, or mixed genotypes.

Reagents and chemicals

- ✓ DNA template
- ✓ 2x Go Taq hot start green master mix(Promega)
- ✓ Nuclease-free water
- ✓ Primers
- ✓ Agarose powder and TBE buffer(10x)
- ✓ DNA ladder(100bp, 50bp,1kb)
- ✓ Apo I-HF( Restriction enzymes ) and corresponding 10x restriction buffer
- ✓ ETH bromide or Syber Safe or Red Gel
- ✓ Disinfectant( Bleach and Ethanol)

**Table 14**:Mastermix formulation for primary PCR amplification *pfcrt* K76T(N1)

| **PCR ingredient** | **Volume per single reaction(µl)** | **Total Volume Example (N=5)** |
|---|---|---|
| 1. Nuclease-free water | 4 | 10X4=40 |
| 2.Primer Forward | 1 | |
| 3.Primer Reverse | 1 | |
| 4. 2x MM | 10 | |
| Aliquot MM | 16 | |
| 5. DNA Template | 4 | |
| Total volume of the PCR reaction | 20 | |

**Table 15**:PCR program for primary reaction(N1) *pfcrt* K76T

| | **PCR program (N1)** | | | |
|---|---|---|---|---|
| | **Time** | **Temperature($^{o}$C)** | **Purpose** | |
| **Step/cycle** | | | | |
| 1$^{st}$ step | 3min | 94 | Initial denaturing | |
| 2$^{nd}$ step | 30sec | 94 | Denaturing | **x30** |
| 3$^{rd}$ step | 30sec | 56 | Annealing | |
| 4$^{th}$ step | 1min | 65 | Extending | |
| 5$^{th}$ step | 3min | 65 | Extending | |
| | ∞ | 10 | To hold the N1 product | |

**Table 16**:Mastermix formulation formulation for secondary nested PCR amplification for *pfcrt* K76T

| **PCR ingredient** | **Volume per Single reaction(µl)** | ***Total Volume** |
|---|---|---|
| 1. Nuclease-free water | 7 | |
| 2.Primer Forward | 1 | |
| 3.Primer Reverse | 1 | |
| 4. 2x Hot start master mix | 10 | |
| Aliquot MM | 19 | |
| N1 DNA Template | 1 | |
| Total volume of the PCR reaction | 20 | |

**Table 17**:PCR program for nested (N2) for *pfcrt* K76T

| | **PCR program (N1)** | | | |
|---|---|---|---|---|
| **Step/cycle** | **Time** | **Temperature ($^{o}$C)** | **Purpose** | |
| 1$^{st}$ step | 5min | 95 | Initial denaturing | |
| 2$^{nd}$ step | 30sec | 92 | Denaturing | **X40** |
| 3$^{rd}$ step | 30sec | 52 | Annealing | |
| 4$^{th}$ step | 30sec | 65 | Extending | |
| 5$^{th}$ step | 3min | 65 | Extending | |
| | ∞ | 10 | To hold the N1 product | |

**Table 18**:Mastermix formulation for primary amplification (N1) *pfmdr1* N86Y

| **PCR ingredient** | **Volume per single reaction(µl)** | **Total Volume** |
|---|---|---|
| 1. Nuclease-free water | 6 | |
| 2.Primer Forward | 1 | |
| 3.Primer Reverse | 1 | |
| 4. 2x MM | 9 | |
| Aliquot MM | 17 | |
| 5. DNA Template | 1 | |
| Total volume of the PCR reaction | 18 | |

**Table 19**:PCR program for primary reaction(N1) for *pfmdr1* N86Y

| | **PCR program (N1)** | | | |
|---|---|---|---|---|
| **Step/cycle** | **Time** | **Temperature($^{o}$C)** | **Purpose** | |
| 1$^{st}$ step | 3min | 95 | Initial denaturing | |
| 2$^{nd}$ step | 30sec | 95 | Denaturing | **X20** |
| 3$^{rd}$ step | 30sec | 52 | Annealing | |
| 4$^{th}$ step | 1min | 72 | Extending | |
| 5$^{th}$ step | 5min | 60 | Extending | |
| | ∞ | 10 | To hold the N1 product | |

**Table 20**:Mastermix formulation for nested (N2) *pfmdr1* N86Y

| PCR ingredient | Volume per single reaction(µl) | Total Volume |
|---|---|---|
| 1. Nuclease-free water | 6 | |
| 2.Primer Forward | 1 | |
| 3.Primer Reverse | 1 | |
| 4. 2x MM | 9 | |
| Aliquot MM | 17 | |
| 5. DNA Template | 1 | |
| Total volume of the PCR reaction | 18 | |

**Table 21**:PCR program for nested (N2) for *pfmdr1* N86Y

| | **PCR program (N1)** | | | |
|---|---|---|---|---|
| **Step/cycle** | **Time** | **Temperature($^{o}$C)** | **Purpose** | |
| 1st step | 3min | 95 | Initial denaturing | |
| 2nd step | 30sec | 95 | Denaturing | **X25** |
| 3rd step | 30sec | 54 | Annealing | |
| 4th step | 1min | 72 | Extending | |
| 5th step | 5min | 72 | Extending | |
| | ∞ | 10 | To hold the N1 product | |

**Procedure for Agarose Gel Electrophoresis and Storage of Nested PCR Products**

- Prepare a 2.0% agarose gel for *pfcrt* K76T PCR products.
- Prepare a 2.5% agarose gel for *Pfmdr1* N86Y PCR products.
- Stain gels with ethidium bromide (EtBr**)**.
- Load 5 µl of nested PCR products onto the respective gels.

- Add a 100 bp DNA ladder to estimate band sizes.
- Run electrophoresis under the following conditions:
    - Voltage: 120 V
    - Duration: 45 minutes
    - Buffer: 0.5X TBE
- Visualize DNA bands under a UV trans-illuminator
- Store the remaining nested PCR products at 2–8 °C

**Table 22**: Restriction Enzyme digestion incubation steps(APO1)

| **Ingredient** | **Volume per reaction** | **Total amount** |
|---|---|---|
| PCR amplicon | 5 | |
| 10xApoI buffer | 2 | |
| Apo I enzyme | 5 | |
| Nuclease-free water | 8 | |
| Total volume | 20 | |

Remark: aliquot 15ml + 5ml of amplicon

Always refer to the manufacturer's instructions before preparing the reaction.

Mix gently by pipetting and spin down briefly

Keep the reaction tube on ice until incubation starts

1. Incubation and heat inactivation
    - ✓ Incubate the reaction at $37^0$ °C for 1 hour
    - ✓ Heat –inactivate the enzyme at $80^0$c for 20min
2. Store or load on gel
    - ✓ Digested products are loaded immediately on the 2.5% gel
    - ✓ Or store at $-20^0$ °C for a short period of time

Agarose gel preparation

Material and reagent

| | |
|---|---|
| Agarose powder | ethidium bromide |
| 1xTBE buffer | weighing balance |

Microwave or heating plate, measuring cylinder or beaker, PPE

Gel casting tray and comb

2.5% Agarose Gel preparation (100ml)

Agarose powder:2.5g

1xTBE buffer: 100ml

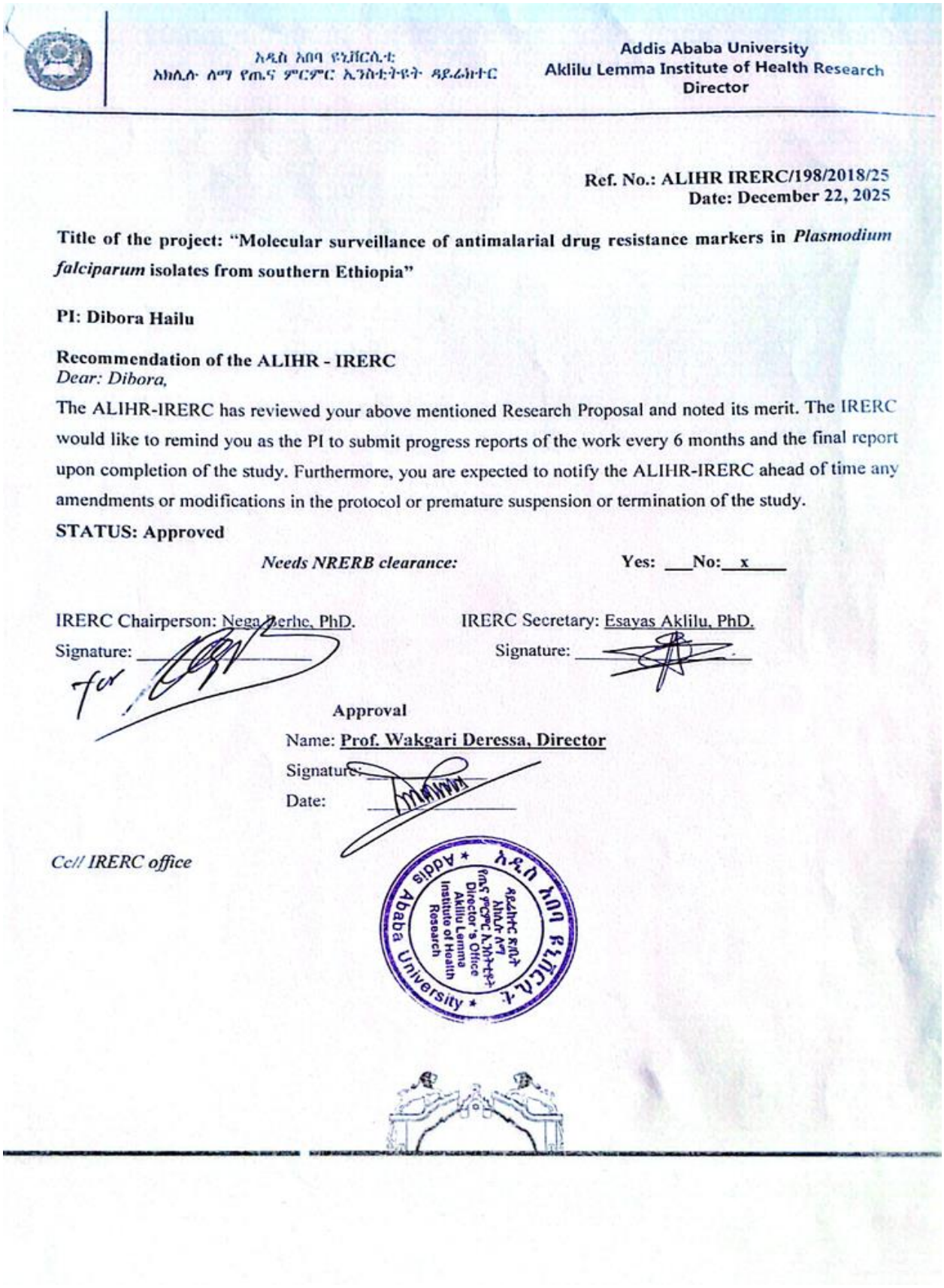
Steps:

1. Weigh 2.5g of agarose
2. Add it to a flask or beaker with 100ml of 1xTBE
3. Microwave( or heat) until fully dissolved( clear solution)
4. Cool to ~$60^0$ °C

**Annex 3: Pictures taken during laboratory work**


**Figure 10:** Laboratory work flow for malaria molecular diagnostic, showing PCR amplification using a thermal cycler, master mix preparation, DNA extraction, and Template addition

## Annex 4:Ethical Clearance format



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Addis Ababa University
Aklilu Lemma Institute of Health Research
Director

**Ref. No.: ALIHR IRERC/198/2018/25**
**Date: December 22, 2025**

**Title of the project: "Molecular surveillance of antimalarial drug resistance markers in *Plasmodium falciparum* isolates from southern Ethiopia"**

**PI: Dibora Hailu**

**Recommendation of the ALIHR - IRERC**
*Dear: Dibora,*

The ALIHR-IRERC has reviewed your above mentioned Research Proposal and noted its merit. The IRERC would like to remind you as the PI to submit progress reports of the work every 6 months and the final report upon completion of the study. Furthermore, you are expected to notify the ALIHR-IRERC ahead of time any amendments or modifications in the protocol or premature suspension or termination of the study.

**STATUS: Approved**

***Needs NRERB clearance:*** **Yes: ___No:__x____**

IRERC Chairperson: Nega Berhe, PhD.
Signature: _____

IRERC Secretary: Esayas Aklilu, PhD.
Signature: _____

**Approval**
Name: **Prof. Wakgari Deressa, Director**
Signature: _____
Date: _____

*Cc// IRERC office*

Addis Ababa University
Director's Office
Aklilu Lemma Institute of Health Research
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