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# Prevalence of Placental Malaria, Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene Deletions and Kelch-13 Gene Mutations among Pregnant Women in Selected Health Facilities of Ethiopia

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BAHIR DAR UNIVERSITY

COLLEGE OF SCIENCE

DEPARTMENT OF BIOLOGY

# PREVALENCE OF PLACENTAL MALARIA, *PLASMODIUM FALCIPARUM'S HISTIDINE RICH PROTEIN 2/3 GENE* DELETIONS AND *KELCH-13 GENE* MUTATIONS AMONG PREGNANT WOMEN IN SELECTED HEALTH FACILITIES OF ETHIOPIA

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A dissertation submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences.

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BAHIR DAR

## DECLARATION

This is to certify that the thesis entitled “Prevalence of Placental Malaria, *Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene* Deletions and *Kelch-13 Gene* Mutations among Pregnant Women in Selected Health Facilities of Ethiopia”, submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences of College of Sciences, Department of Biology, Bahir Dar University, is a record of original work carried out by me and has never been submitted to this or any other institution to get any other degree or certificates. The assistance and help I received during the course of this investigation have been duly acknowledged.

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## Advisors' approval of dissertation for defense

I hereby certify that I have supervised, read and evaluated this dissertation

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## Examiners' approval of dissertation for defense result

As members of the board of examiners, we examined this dissertation/thesis entitled "Prevalence of Placental Malaria, *Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene* Deletions and *Kelch-13 Gene* Mutations among Pregnant Women in Selected Health Facilities of Ethiopia" by Banchamlak Tegegne. We hereby certify that the thesis is accepted for fulfilling the requirements for the award of the degree of PhD in Biomedical Sciences.

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## Abstract

**Background:** Placental malaria (PM) represents a significant public health burden for both pregnant women and their fetus. The diagnosis of malaria in Ethiopia primarily relies on blood film microscopy using stained thick and thin blood films and Rapid Diagnostic Tests (RDTs). However, RDTs *pfhrp2* and *pfhrp3* gene deletions result in misdiagnosis of *Plasmodium falciparum* infection. According to WHO recommendation, artemisinin-based combination therapies (ACTs) were used as a first line anti-malarial drug in the country. On the contrary, a mutation in the *PfKelch13 gene* was found to be associated with artemisinin resistance in endemic countries including Ethiopia.Therefore, this study aimed to determine the prevalence of placental malaria, *pfk13 gene* mutation, *pfhrp2/3 gene* deletion and performance evaluation of lactate dehydrogenase based RDTs among symptomatic and asymptomatic pregnant women in selected health facilities (Andasa Health Center, Chis Abay Health Center, Hamusit Health Center, Kurgen Health Center, Uffa Health Center, Gambella General Hospital, Gambella Primary Hospital, and Gebretsadik Shewa Hospital) of Ethiopia. **Methods:** A facility based cross-sectional study was conducted from July to November 2023 at selected eight health facilities of Ethiopia. Nine hundred twenty-two pregnant women were enrolled for placental malaria prevalence study, and their placenta samples were examined for *Plasmodium* infection using smear microscopy and Loop-mediated isothermal amplification method. For LDH based RDT kit performance evaluation, 302 pregnant women suspected of malaria were recruited and samples were examined using blood film microscopy, CareStart RDT, Boicredit RDT and PET-PCR for the detection of *Plasmodium* species. For *hrp2/3 gene* deletion and ACT drug resistance molecular marker detection, 275 pregnant women with *P. falciparum* infection were purposively recruited. For *hrp2/3 gene* deletion detection, Bio-Rad QX200 ddPCR machine was used to look at 3 targets (*HRP2 exon 1, HRP 2 exon 2* and *HRP 3*) and their concentration in relation to tRNA ligase. Genomic DNA from *P. falciparum* laboratory reference strains was included in each ddPCR assay as experimental controls. The *P. falciparum k13* propeller domain was amplified from genomic DNA samples by nested PCR and its products were run on 2% agarose gel and bidirectional Sanger sequencing was performed. Sociodemographic characteristics of the participants and necessary clinical data were collected using Redcap software in the anti-natal clinic and labor ward. **Results:** Of the total recruited pregnant women, thirteen samples (1.4%) were positive with LAMP methods of which eight (61.5%) of them had

*P. falciparum* infections and five of them (38.5%) had *P. vivax* infections. From the total analyzed samples with ddPCR, 15% (31/207) of parasites had fully deleted *hrp2/3 genes*. A separate analysis of *hrp2* and *hrp3 gene* deletion showed that 17.9% (37/207) and 41.5% (86/207) of the samples carried *hrp2* and *hrp3 gene* deleted parasites respectively. The sensitivity and negative predictive value of BIOCREDIT Pf/Pv (pLDH/pLDH) were 55.6% (95%CI: 46.1-64.7) and 77.9% (95% CI: 74.2-81.2) against a PET-PCR reference test respectively which was higher as compared to *CareStart*™ Pf/Pv (HRP2/pLDH). From the total 227 sequenced samples, 18.9 % (43/227) had *pfk13 gene* mutations with a total of 13 SNPs (point mutations) including ten non-synonymous mutations and three synonymous mutations. The G596E, H588Y, I406R and T587I are novel non-synonymous mutations which have never been described elsewhere. Three WHO approved k13 resistance markers (R622I, A675V and R539T) were detected. **Conclusion:** Both *P. falciparum* and *P. vivax*, including asymptomatic infections, contribute to placental malaria. High *pfhrp2/3* deletion rates and the presence of *pfk13* resistance mutations pose major diagnostic and treatment concerns in Ethiopia. Continued molecular surveillance using ddPCR and whole genome sequencing is essential.

**Key words:** Placental malaria, K-13 mutation, Histadine rich protein 2, Ethiopia

Table of contents

**Acknowledgements** ..... IV

**Abstract** ..... VI

**Table of contents** ..... VIII

**List of tables** ..... XII

**List of figures** ..... XIII

**List of abbreviations and acronyms** ..... XIV

**CHAPTER ONE INTRODUCTION** ..... 1

**1.1. Background** ..... 1

**1.2. Statement of the problem** ..... 3

**1.3. Objectives of the Study** ..... 6

1.3.1. General Objective ..... 6

1. 3.2. Specific Objectives ..... 6

**1.4. Research questions** ..... 5

**1.5. Significance of the study** ..... 7

**1.6. Scope of the work** ..... 8

**1.7. Operational definition** ..... 9

**CHAPTER TWO: LITERATURE REVIEW** ..... 10

**2.1. Life cycles of *Plasmodium* species infection** ..... 10

**2.2. Epidemiology of placental malaria** ..... 11

2.2.2. Placental malaria in West Africa ..... 11

2.2.3. Placental malaria in North Africa ..... 12

2.2.4. Placental malaria in East Africa ..... 12

**2.3. Pathophysiology of malaria in pregnancy** ..... 13

**2.4. Diagnosis of malaria** ..... 14

2.4.1. Microscopic examination of malaria ..... 15

2.4.2. Malaria diagnosis using RDTs ..... 16
2.4.3. Molecular diagnosis of malaria ..... 19
**2.5. Treatment of malaria** ..... 19
2.5.1. Factors contributing to anti-malarial treatment failure ..... 20
2.5.2. Function and impact of the *P.falciparum kelch 13* mutations on antimalaria drugs ..... 21
2.5.3. WHO validated and candidate pfk13 mutation ACT resistance markers ..... 21
**2.6. Prevention and control of malaria** ..... 25
2.6.1. Long Lasting Insecticidal Nets (LLINs) ..... 26
2.6.2. Indoor Residual Spraying (IRS) ..... 26
2.6.3. Larval Source Management ..... 27
**CHAPTER THREE: MATERIALS AND METHODS ..... 28**
**3.1. Study Design, Period and Area** ..... 28
**3.2. Source population** ..... 29
**3.3. Study population** ..... 29
**3.4. Study Participants** ..... 30
**3.5. Inclusion and Exclusion Criteria** ..... 30
3.5.1. Inclusion Criteria ..... 30
3.5.2. Exclusion Criteria ..... 30
**3.6. Sample Size determination and sampling technique** ..... 31
3.6.1. Sample size determination ..... 31
3.6.2. Sampling technique ..... 32
**3.7. Data collection and Laboratory Diagnosis methods** ..... 35
3.7.1. Socio-demographic and clinical data collection ..... 35
3.7.2. Sample Collection, Processing and Laboratory investigations ..... 35
**3.8. Study Variables** ..... 40
**3.9. Data quality control** ..... 40

**3.10. Data processing and analysis** ..... 40

**3.11. Ethical considerations** ..... 41

**CHAPTER FOUR RESULTS** ..... **42**

**4.1 Placental malaria prevalence** ..... 42

4.1.1 Socio-demographic characteristics of pregnant women recruited for PM ..... 42

4.1.2. Placental malaria prevalence ..... 42

4.1.3. Regional distribution of placental malaria ..... 43

**4.2. HRP2/3 gene deletion** ..... 44

4.2.1. Detection of *Pfhrp2* and *Pfhrp3* genes using ddPCR ..... 44

4.2.2. *Pfhrp2* and *Pfhrp3* gene deletion status by region ..... 45

4.2.3. *Pfhrp2* and *Pfhrp3* gene deletion status by health facility ..... 46

4.2.4. *Pfhrp2* and *Pfhrp3* gene deletion among pregnant women with symptomatic and asymptomatic *Plasmodium* infection ..... 46

**4.3. Evaluation of PLDH based RDTs** ..... 48

4.3.1 Evaluation of Rapigen/Biocredit PFPV/PLDH mRDT kit ..... 48

**4.4. Kelch 13 mutation** ..... 53

4.4.1. *Plasmodium falciparum* k13 gene mutation ..... 53

4.4.2. Regional variation of *pfk13 gene* mutation ..... 55

4.4.3. Co-dominance of *hrp2/3 gene* deletion and *k13 gene* mutation ..... 56

**5.1. Placental malaria prevalence** ..... 57

**5.2. Deletion of *pfhrp2* and *pfhrp3* genes** ..... 58

**5.3. Evaluation of the diagnostic performance Pf/Pv/PLDH based RDT** ..... 61

**5.4. *Plasmodium falciparum* keltch 13 gene mutation** ..... 63

5.4.1. Distribution of pfk13 mutation ..... 64

5.4.2. Co-dominance of *pfhrp2/3 gene* deletion and *pfk13 gene* mutation ..... 66

**CHAPTER SIX CONCLUSIONS** ..... **67**

**CHAPTER SEVEN RECOMMENDATION** ..... **69**

**CHAPTER EIGHT REFERENCES** ..... **70**

**CHAPTER NINE ANNEXES** ..... **96**

**Annex I. Participant information sheet** ..... 96

**Annex II. Consent form** ..... 98

**Annex III. Questionnaire** ..... 98

**Annex IV. Standard operating procedures** ..... 102

**Annex V. Details of WHO approved and candidate pfk13 mutation** ..... 117

**Annex VI. Details of pfk13 mutations detected from samples collected from 2021-2023 at selected regions of Ethiopia** ..... 118

List of tables

Table 1. PfKelch13 markers of artemisinin partial resistance (WHO,2025) ..... 21
Table 2 : Socio-demographic characteristics of study participants from 2021-2023 in selected areas of Ethiopia ..... 42
Table 3: Placental malaria prevalence status by study site in selected areas of Ethiopia from 2021-2023 ..... 44
Table 4: Summary of hrp2/3 gene deletion status by region from 2021-2023 in selected areas of Ethiopia ..... 45
Table 5: Summary of pfhrp2/3 gene deletion in symptomatic and asymptomatic *Plasmodium* infections from 2021-2023 ..... 47
Table 6: Associated risk factors with pfhrp2/3 gene deletion from 2021 to 2023 at selected areas of Ethiopia ..... 47
Table 7: Prevalence of *Plasmodium* infection by different diagnostic techniques (N=302) ..... 48
Table 8: Comparison of BIOCREDIT Pf/Pv and CareStart™ Pf/Pv against microscopy and PET-PCR ..... 50
Table 9: Performance of BIOCREDIT Pf/Pv (pLDH/pLDH) and CareStart™ Pf/Pv (HRP2/pLDH) in detecting *Plasmodium* infection ..... 52
Table 10: Details of samples analyzed for k13 gene sequencing by region and study site in selected areas of Ethiopia from 2021-2023 ..... 53
Table 11: K13 gene mutation status by study site in selected areas of Ethiopia from 2021-2023 55

## List of figures

Figure 1. Life cycles of Plasmodium parasite species in human (https://www.immunopaedia.org.za/immunology/special-focus-area/4-immunity-to-malaria/).. 10

Figure 2. Binding of infected erythrocytes to microvasculature endothelium versus placenta (Smith and Deitsch, 2004) ..... 14

Figure 3. Map of selected study sites ..... 29

Figure 4. Flow chart showing selection of study sites ..... 33

Figure 5. Flow chart for selection of specimens for genotyping and placental malaria with results……35

Figure 6. Placental and peripheral blood malaria positivity rate in selected areas of Ethiopia from 2021-2023 ..... 43

Figure 7. A Venn diagram showing summary of the overall pfhrp2/3 gene deletion status in selected areas of Ethiopia from 2021-2023 ..... 45

Figure 8. Pfhrp2/3 gene deletion status by study site in selected areas of Ethiopia from 2021-2023..... 46

Figure 9. Venn diagram showing detection of P. falciparum (A), P. vivax (B) and Mixed (C) infections..... 49

## List of abbreviations and acronyms

ACTs……...Artesunate Combination Treatments

AL……Arthemether Lumefantrine

ANC……Antenatal care

API……...Annual parasite incidence

APHI……..Amhara Public Health Institute

BLAST…… Basic Local Alignment Search Tool

CD36……...Cluster of differentiation 36

CSA……...Central Statistical Agency

DBS……Dried Blood Spot

DNA……..Deoxyribose Nucleic Acid

RNA…….….Ribose Nucleic Acid

EDTA……. Ethylene Diamine Tetraacetic Acid

GAP……..Genome Assembly Program

HRP2/3……. Histidine Rich Protein -2/3

IES……Infected Erythrocytes

K-13……. Kelch-13

KD……...kilodalton

KLHLS……Kelch-like gene family members

LAMP……Loop-Mediated Isothermal Amplification

LBW……...Low Birth Weight

LLINS……..Long Lasting Insecticide Treated Nets

LSM……..Larval Source Management

MOH……. Ministry of Health

ND…….....Nanodrop

nPCR……Nested Polymerase Chain Reaction

PET-PCR……. Photo-Induced Electron Transfer PCR

*pfcrt* ……*P. falciparum* chloroquine resistance transporter

*pfdhfr*……..*P. falciparum* dihydrofolate reductase

*pfdhps* ……*P. falciparum* dihydropteroate synthase

PfEMP1/2……...*Plasmodium falciparum* Erythrocyte Membrane Protein 1/2

*pfmdr1* ……...*P. falciparum* multi-drug resistance gene1

RDT……. Rapid Diagnostic Test

SNP…….....Single Nucleotide Polymorphism

SPSS……..Statistical Package for the Social Sciences

UV……..... Ultra Violet

VAR2CSA…… Variant two Chondroitin Sulfate A

WBC……. White Blood Cell

WHO……. World Health Organization

# CHAPTER ONE INTRODUCTION

## 1.1. Background

Malaria is a disease caused by intracellular protozoan parasites of the genus *Plasmodium*, transmitted via female *Anopheles* mosquitoes. Five *Plasmodium* species: namely *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*) and *Plasmodium knowlesi* (*P. knowlesi*) are known to cause malaria in humans (WHO, 2024).

According to 2024 world malaria report, the global tally of malaria cases reached 263 million and 597,000 deaths; of which 94% of the total cases are from WHO African region. *P. falciparum* causes 96% of deaths globally and resulted in deaths of all age groups (WHO,2024).

According to this report, from the total 36 million pregnant women in moderate and high transmission countries in African region, 34% (12.4 million) pregnant women were exposed to malaria of which 551 000 neonates were with low birth weight.

In Ethiopia, malaria continues to be a major cause of morbidity and mortality. About 75 % of the country's landmass is favorable for malaria transmission primarily associated with altitude and rainfall (MOH, 2022). Yet, the disease primarily occurs up to 2000-meter (m) elevation but can also occasionally affect areas up to 2300m elevation putting approximately 52% of the Ethiopian population at risk (MoH, 2022).*P. falciparum* and *P. vivax* are the two predominant species causing malaria in Ethiopia, with relative prevalence of 70% and 30%, respectively (MoH,2018; MoH,2022). *Anopheles arabiensis* is the main malaria vector*;* while *An. pharoensis, An. funestus* and *An. nili* play a role as secondary vectors. Recently, an invasive vector, *An. stephensi* is detected mainly along the eastern, northeastern and southeastern corridors of the country (MoH, 2022).

In 2024, Ethiopia reported a total of 5,677,912 malaria cases and 766 malaria deaths, of which 96.9% (5,500,835) were laboratory-confirmed through microscopy or rapid diagnostic tests (RDTs) and 67.1% of the cases were due to *P. falciparum* (unpublished annual report from ministry of health). According to the above data, incidence of malaria for three regions (Amhara, Southwest Ethiopia and Gambella), where our eight study sites exist was; incidence per 1000

population at risk was 85.4, 264 and 308.7 for Amhara, Southwest Ethiopia and Gambella regions, respectively.

In pregnant women, *P. falciparum* infected red blood cells in the placenta express unique variant surface antigens (VSA), predominantly the Variant Surface Antigen 2 Chondroitin Sulfate A (VAR2CSA) protein, and lack of immunity to these pregnancy-specific variant surface antigens explains some of the pregnancy-associated malaria susceptibility (Stephen *et al*.,2007). Altered immunity during pregnancy and ability of *P. falciparum* infected erythrocytes to sequester to placenta are responsible for severe malaria especially in first pregnancy (Mackintosh *et al*., 2004; Stephen *et al*.,2007). Malaria in pregnancy (MiP) poses a dangerous health risk to both mothers and their fetuses, causing severe outcomes such as preterm delivery, intrauterine growth restriction, miscarriage, stillbirth, and neonatal and maternal death (Berhe, *et al*.,2023).

As a result, early laboratory diagnosis using microscopy and malaria rapid tests (RDTs) and treatment have decreased such consequences and transmission at the community level. However, RDTs *pfhrp2* and *pfhrp3* gene deletions have deleterious effect on early and prompt malaria diagnosis and treatment as reported by different African scholars (Gamboa *et al*., 2010; Koita *et al*., 2012; Wurtz *et al*., 2013; Amoah *et al*.,2016; Golassa *et al*.,2020; Feleke *et al*.,2021; Alemayehu *et al*.,2021; Fola *et al*.,2023; Rogier *et al*.,2024; Mekonen *et al*.,2024; Kamaliddin *et al*.,2024;Adamu *et al*.,2025). Based on the above evidence, the Ethiopian Ministry of Health switched from HRP2 based RDTs to lactate dehydrogenase (pLDH) based RDTs targeting the plasmodial lactate dehydrogenase (pLDH) specific for *P. falciparum* and *P. vivax* (RapiGEN BIOCREDIT Malaria Ag Pf/Pv pLDH/ pLDH) in the beginning of the year 2024 (unpublished policy brief from ministry of health).

On top of this, according to WHO recommendation, artemisinin-based combination therapies (ACTs) were used as a first line anti-malarial drug in 2001 in the world and in 2004/2005 in Ethiopia (MoH, 2018). On the contrary, mutations in the PfKelch13 (K13) gene were identified to be definitively associated with artemisinin resistance which is dominant in Southeast Asian countries and minor presence in Africa including Ethiopia (Ariey *et al*., 2014; Ménard *et al*.,2016; Bayih *et el*.,2016; Kamaliddin *et al*.,2024; Jeang *et al*.,2024; Juliano *et al*.,2024). This also has a negative impact on the treatment of malaria during pregnancy and causes maternal, fetal and newborn deaths and increased the transmission of malaria among the population.

## 1.2. Statement of the problem

Malaria prevalence in pregnancy can reach 36% globally and 60% in sub-Saharan Africa with placental malaria affecting up to 28% of cases (Minwuyelet *et al*.,2025). There are also reports in the study areas that showed high prevalence of pregnancy malaria (Almaw *et al*.,2022; Alemayehu *et al.*,2024; Almaw *et al*.,2024). Different authors reported that placental malaria has been the main problem that can cause complications both on the mother and her fetus in African countries (Minwuyelet *et al*.,2025). On the contrary, there is scarcity of data in malaria endemic areas of Ethiopia except few studies in Gambella, South and Northwest Ethiopia (Solomon *et al.,* 2020; Tamir *et al*.,2023; Alemayehu *et al.,*2024) on the prevalence of placental malaria among asymptomatic and symptomatic pregnant women. As a result, the prevalence of placental malaria among asymptomatic and symptomatic pregnant women in selected eight facilities of Ethiopia needs further assessment.

Placental malaria, *hrp2/3* gene deletions, and *pfk13* mutations collectively undermine malaria control in pregnancy by obscuring parasite detection with sequestered placental infections, causing false-negative rapid diagnostic tests, and reducing treatment efficacy while complicating surveillance of emerging drug resistance.

In malaria endemic areas, majority of pregnant women may remain asymptomatic or subclinical but still associated with complications on the mothers and their fetuses, serve as reservoirs, and act as potential transmitters of infection (Tilahun *et al.*, 2020; Alemayehu *et al.,* 2024). For early diagnosis and treatment, microscopy and *pfhrp2/3* based RDTs are being used and are mandatory. However, reports showed that parasites could harbor deleted *hrp2/3 genes* in African countries including Ethiopia that can hamper its detection ability for *P. falciparum* infection (Rogier *et al*., 2024; Mekonen *et al.*,2024; Kamaliddin *et al*.,2024; Adamu *et al*.,2025).

According to WHO response plan for *pfhrp2* deletion, countries that are using a best-in-class HRP2 based RDT will need to switch before the *pfhrp2* deletion prevalence in the parasite population reaches 25% (WHO,2024).Therefore, to know the actual burden of *pfhrp2* deletion in Ethiopia, more studies in different study sites should be conducted using the most accurate method like ddPCR and study findings needed to be used for the decision makers. In addition, there is limited evidence on the burden of *hrp2/3* deletions specifically among pregnant women.

Therefore, the currently *pfhrp2* and *pfhrp3* gene deletions status of parasites among the symptomatic and asymptomatic pregnant women in selected health facilities needed to be investigated. In addition, pLDH-based RDTs have not been field-evaluated**.** Therefore**,** it is very important to evaluate the performance of the alternative LDH based malaria RDT kits (pf/pv LDH). For this, the results from symptomatic patients better represent a clinical setting, where the majority of RDTs are used.

Nowadays, efficacy of the current anti-malaria drug, artemisinin and its combinations are threatened by emergence and spread of resistance due to mutations in Southeast Asia and Africa including Ethiopia (Kamaliddin *et al*.,2024; Jeang *et al.*,2024; Juliano *et al*.,2024). Therefore, further studies across different geographical regions are necessary to map the spread of drug-resistant malaria parasites, monitoring and managing drug resistance, revising treatment guidelines, informing the development of new antimalarial drugs and this will help to provide valuable input for malaria control and elimination programs. (WHO,2020; WHO,2022; Chaves *et al.*,2024).

To effectively detect and monitor the spread of ACTs resistance and to address future related challenges, regular monitoring of drug resistance molecular markers is essential in various endemic areas (WHO,2022; Xu *et al*.,2023). In addition, this will be the first study focusing on this specific population segment, pregnant women who represent a high-risk group for malaria.

## 1.3. Research questions

- ✓ What is the prevalence of placental malaria in selected areas of Ethiopia?
- ✓ What is the prevalence and geographic distribution of *pfhrp2* and *pfhrp3 gene* deletions among *P. falciparum* isolates from infected pregnant women?
- ✓ How does the diagnostic performance of LDH-based malaria RDTs compared to HRP2-based RDTs in symptomatic pregnant women?
- ✓ What is the prevalence and spectrum of *pfk13* propeller gene mutations among *P. falciparum* isolates from infected pregnant women?

## 1.4. Objectives of the Study

### 1.4.1. General Objective

To assess Prevalence of Placental Malaria, *Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene* Deletions and *Kelch-13 Gene* Mutations among Pregnant Women in Selected Health Facilities (Andasa health center, Chis Abay health center, Hamusit health center, Kurgen health center, Uffa health center, Gambella General Hospital, Gambella Primary Hospital, and Gebretsadik Shewa Hospital) of Ethiopia.

### 1. 4.2. Specific Objectives

- ✓ To determine the prevalence of placental malaria among symptomatic and asymptomatic pregnant women
- ✓ To determine prevalence of *Pfhrp2/3 gene* deletion among symptomatic and asymptomatic *P. falciparum* infected pregnant women
- ✓ To assess diagnostic performance of LDH based malaria RDT against PCR among symptomatic pregnant women
- ✓ To determine prevalence of *pfKelch-13 Gene* mutations among symptomatic and asymptomatic *P. falciparum* infected pregnant women

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## 1.5. Significance of the study

Placental malaria causes a great health risk to mother and her fetus and results in abortion, still birth, intra uterine growth retardation, and low birth weight. Apart from these, according to the National malaria guideline of the Ethiopian Ministry of Health, early malaria diagnosis and treatment is a corner stone for the control and prevention of malaria (MoH, 2020). For early diagnosis, RDTs are the routine diagnostic tools at the health post level and in other health facilities when microscopy service is not available. However, nowadays, they are threatened with *Pfhrp2/3 gene* deletions. The spread of drug resistant *P.falciparum* parasites remains one of the major challenges for malaria control and elimination in sub-Saharan Africa. Monitoring of molecular markers conferring resistance to ACTs is important to track the spread of resistant parasites and to optimize the therapeutic lifespan of current drugs.

It is also obvious that both treatment and diagnostic failures in asymptomatic and symptomatic malaria could potentially hamper the malaria elimination program set by the Ethiopian Ministry of Health by 2030 and hence, this study has a paramount importance, as it will provide information:

- ✓ On additional knowledge about placental malaria during pregnancy in Ethiopia and will design different control and prevention intervention strategy since there is scarcity of data in Ethiopia (except study at Wolkite, Jawi and Gambella).
- ✓ On the prevalence of *pfhrp2/3 gene*-deleted parasites and this justifies switching malaria rapid diagnostic tests to other alternative diagnostic options in Ethiopia especially for high-risk groups (pregnant women).
- ✓ On field performance of LDH based RDTs to know the malaria detection performance and to plan further switching if it had lower sensitivity/specificity.
- ✓ On profiling *Kelch-13 Gene* mutations that could be used to track the possible emergence of ACTs drug resistance due to treatment failure or delay in parasite clearance.

## 1.6. Scope of the work

- ✓ Study areas are eight health facilities (Andasa health center, Chis Abay health center, Hamusit health center, Kurgen health center, Uffa health center, Gambella General Hospital, Gambella Primary Hospital, and Gebretsadik Shewa Hospital) selected from the Amhara, Gambella and Southwest regions of Ethiopia.
- ✓ Study participants are pregnant women that are symptomatic and asymptomatic for malaria.
- ✓ Study participants included in the study if they are willing to give data, placental and venous blood samples and after signing written concsent.They recruited from 2021 to 2024.
- ✓ Samples analyzed to check weither they are infected with *plasmodium* parasites, carried *pfhrp2/3* gene deleted and *pfkl3* mutated strains.

## 1.7. Operational definition

- **Asymptomatic malaria:** Women with absence of fever (≥37.5°C) in the last 48 hours . headache, nausea, vomiting within 24 hours but were positive for *Plasmodium* infection by any of the diagnostic methods.
- **Symptomatic malaria** : Women who presented with fever (axillary temperature ≥37.5°C) or history of fever in the last 48 hours, along with other symptoms such as headache, chills, or vomiting, and were positive for *Plasmodium* infection by any diagnostic method.
- **Submicroscopic malaria**: Any women diagnosed positive for malaria by molecular methods LAMP/PCR but negative by microscopy and/or RDT.
- **Malaria species identified:** *P. falciparum, P. vivax* and mixed (*Pf* & *Pv*) infections (three subgroups).

# CHAPTER TWO: LITERATURE REVIEW

## 2.1. Life cycles of *Plasmodium* species infection

Malaria is a disease transmitted to humans by the bite of female *Anopheles* mosquitoes. *Anopheles* mosquitoes feed on blood for survival and production of eggs. During feeding, infected female *Anopheles* mosquito inoculates the infective sporozoites from its salivary gland into human skin. After inoculation, parasites circulate with blood and those reaching to the liver undergo one cycle development. Then, parasites infect and multiply inside red blood cells to bring the characteristic signs and symptoms (MoH,2020).

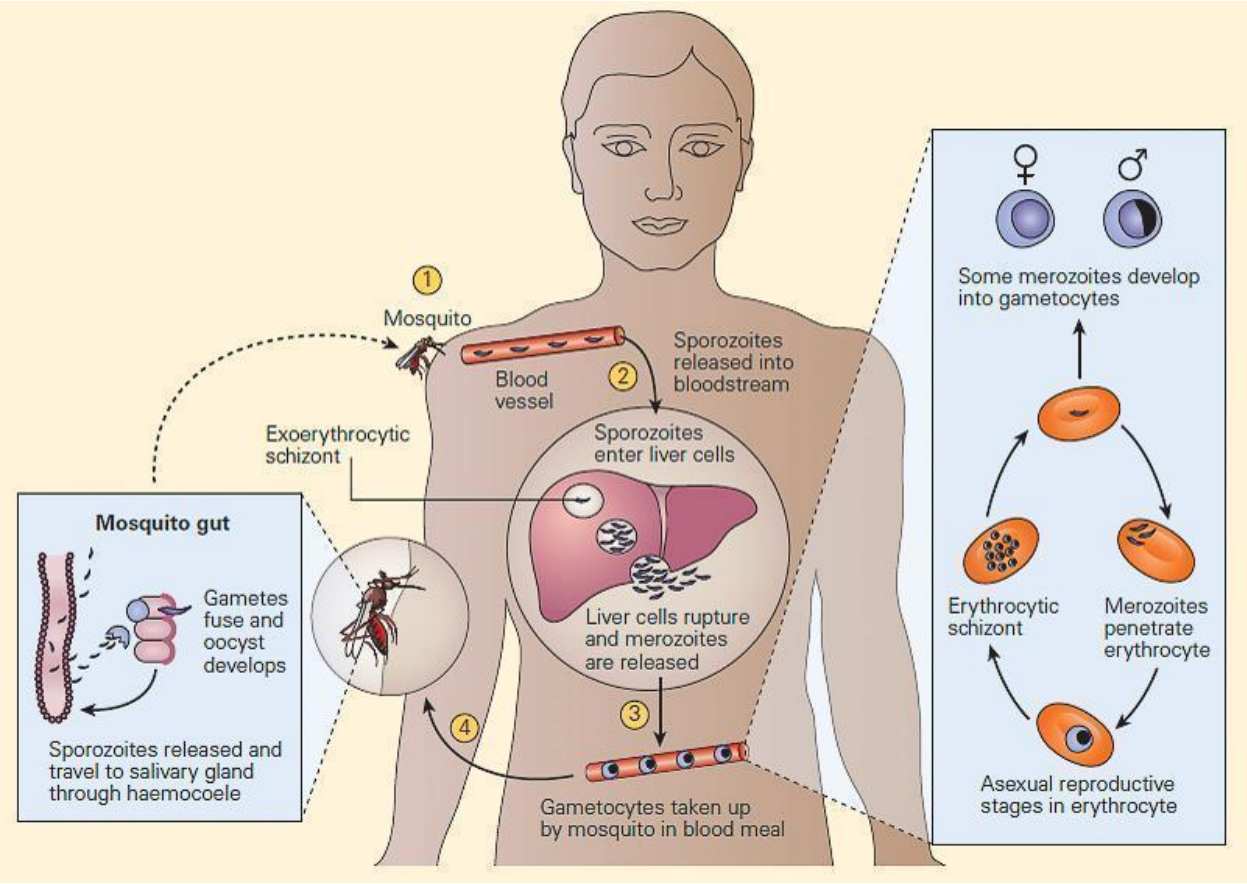



Figure 1. Life cycles of *Plasmodium* parasite species in human (https://www.immunopaedia.org.za/immunology/special-focus-area/4-immunity-to-malaria/)

## 2.2. Epidemiology of placental malaria

In areas endemic for malaria, pregnant women frequently present with a placenta that has been commonly parasitized by *P. falciparum*. This is due to parasite's variant surface antigen (VAR2CSA) mediates infected red blood cells to bind to chondroitin sulfate A (CSA) in the placental intervillous space, evading the spleen's clearance mechanism.

### 2.2.1. Placental malaria in South America

In South America particularly in Colombia, reports showed that from diagnosed 602 placental blood samples of women with spontaneous and vaginal delivery (with Microscopy and nPCR), prevalence of PM was 27.7% with 92% (155/167) of them are submicroscopic that Microscope detected 13 positives (9 *pv* and 4 *pf*) and qPCR detected 167 positive cases (69 *pv*,74 *pf* and 24 mixed)(Arias *et al*.,2022). Again, a recent study conducted with the same researcher at the same place showed that, from diagnosed 431 pregnant women, 24.8% of them had placental malaria (Arias *et al*., 2024).

### 2.2.2. Placental malaria in West Africa

In Cote d'Ivoire, from 300 women who delivered and enrolled in the study, 7.3% (22/300) of them had positive placental malaria by microscopy (Toure *et al*., 2020).

The study conducted in Ebolowa, Southern Cameroon showed that from the total collected 197 placental samples during delivery time, 42 samples were positive for *P. falciparum* diagnosed microscopically of which 31 were primigravidae (Hesran *et al*.,1997).

In 2012, a study in Ghana showed that from the total 107 placental samples collected and diagnosed microscopically, 56/107(52%) had malaria positive results. Again in 2019, from 50 placental samples tested for *Plasmodium*, 6/50(12%) were infected with the parasite by both rapid diagnostic test and microscopy (Spronsen *et al*.,2012; Ahenkorah *et al*.,2019).In Mali there was report of 71% placental malaria from histopathological diagnosed 249 delivered mothers (Vincenz *et al*.,2022*).*

A study in Guinea showed that, from 1451 placenta samples examined by histology, placental malaria was detected in 269 (18.5%) pregnant women; of which 55 (3.8%) were chronic infection (Lufele *et al*.,2017).

Study conducted in Nigeria showed that, from 365 placentas examined, 254 (69.6%) showed histological evidence of *Plasmodium* infection. Again in 2017, from 331 placental blood sample tested microscopically, 160(48.3%) samples were positive for *plasmodium*. Another study from the same area showed that, from the total 252 placental histology samples, 162(64%) had *plasmodium* infections (Ezebialu *et al*.,2012; Okonkwo *et al*.,2017; Eki-udoko *et al*.,2021).

Again, a study in Nigeria showed that, placenta blood samples were collected from 207 delivering mothers, and resulted in 21.3% placental malaria prevalence (Okoro *et al*., 2023). A recent study from the same area showed that a histopathological analysis from 357 delivered mothers had 38.4% placental malaria prevalence (Oranuka *et al*.,2024).

### 2.2.3. Placental malaria in North Africa

A study in Sudan showed that from the total 1149 mothers recruited, 59.3% of the placental films were infected with *P. falciparum*. Again in 2021 the same author and his friends showed that, from the total 185 placental blood samples collected and examined *plasmodium* parasites were detected in 97 samples (Omer *et al*.,2017; Omer *et al*.,2021). Again in 2023, study from central Sudan showed that, from microscopically screened 2384 delivered mothers, 734 (30.8%) women had placental malaria (Ahmed *et al*.,2023).

A study in Sub-Saharan Africa showed that, from examined 491 placenta smears and histology *P. falciparum* was detected in 11.4% and 10.8% of total samples, respectively (Ouédraogo *et al*.,2019).

### 2.2.4. Placental malaria in East Africa

In 2015, a study in Tanzania showed that from 350 pregnant women studied, 28 (11.5%) had *plasmodium* positive placental blood samples. Again in 2000, from the total examined 1207 placental blood samples, 415 were microscopically positive for *P. falciparum* infection. A recent study finding from the same study area showed, from the total 1115 women who had histopathology and DNA PCR examination, 93 (8%) had histopathology placental infection, and 136 (12%) had the sub-microscopic placental infection (Ndeserua *et al*., 2015; Menendez *et al*.,2000; Kalinjuma *et al*., 2020).

A study in Ethiopia showed that, from the total 230 placental samples examined *plasmodium* was detected in 3.9% (9/230). Of which, *P. falciparum* was detected in 1.7% and *P. vivax* was detected in 2.2% of placental blood films (Solomon *et al.,* 2020).

Two studies in northwest Ethiopia showed that, from the 218 delivered mothers, microscopic prevalence of placental was 2.3% (5/218) in Kuch district and from 526 pregnant women, molecular prevalence of placental malaria was 10.9% (95% CI 8.2–14.1 in Jawi district (Limenih *et a*l.,2021; Tamir *et al*.,2023).

Recent study in Ethiopia, Gambella region showed that from 180 parturient women attending delivery ward prevalence of placental malaria were 26.7% by qPCR (Alemayehu *et al*.2024*).*

## 2.3. Pathophysiology of malaria in pregnancy

Malaria in pregnancy can lead to the development of placental malaria, where *P. falciparum*-infected erythrocytes adhere to placental receptors, triggering placental inflammation and subsequent damage, causing harm to both mother and her infant (Chua *et al*., 2021; Berhe *et al*.,2023). Pregnant women, especially first-time pregnant mothers, are susceptible to massive sequestration of infected erythrocytes in the placenta. Placental isolates are antigenically and functionally distinct from those found systemically and do not bind the primary microvasculature receptor CD36, but instead bind to CSA (Smith and Deitsch, 2004). The parasite is able to exploit a gap in malaria immunity to establish high-density infections in the placenta. During pregnancy, women develop antibodies to the infected erythrocyte surface that may either block infected erythrocyte binding or coat them for destruction by monocytes, thereby reducing disease severity in subsequent pregnancies (Smith and Deitsch, 2004; Rogerson *et al*.,2007; Tomlinson *et al*., 2021).

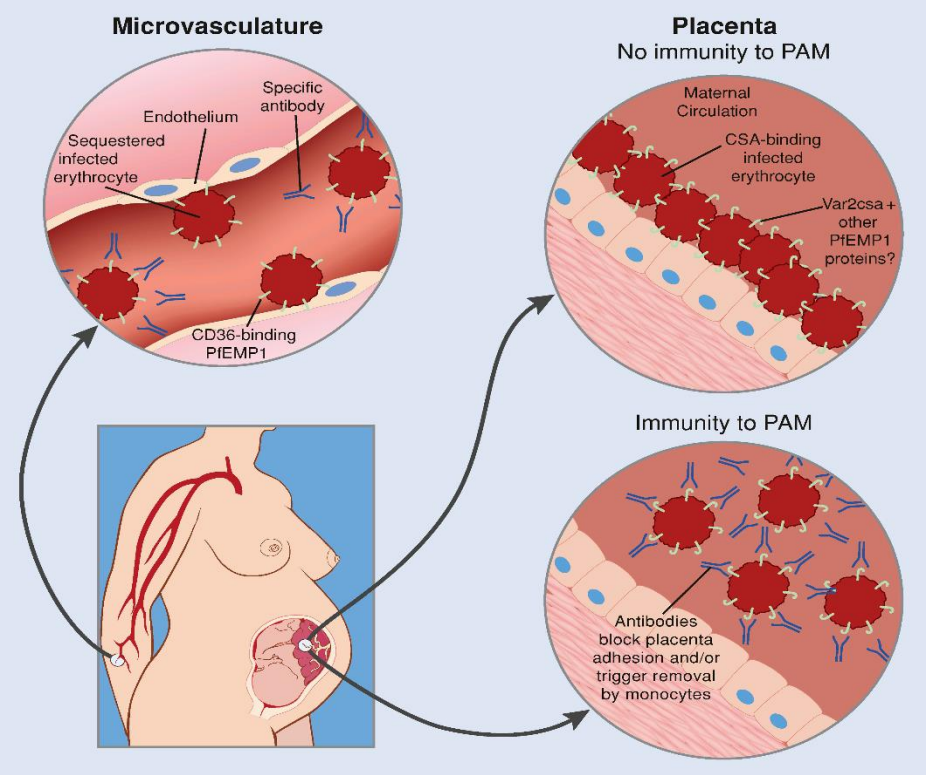



Figure 2. Binding of infected erythrocytes to microvasculature endothelium versus placenta (Smith and Deitsch, 2004)

## 2.4. Diagnosis of malaria

Malaria can be diagnosed using clinical and laboratory diagnosis. Clinical diagnosis has made based on when a patient from malaria endemic area has fever or history of fever in the last 48 hours. Or if a patient from non-malaria endemic area has fever or history of fever in the last 48 hours and has a history of travel to malaria-endemic areas within the last 30 days and spending at least one night in a malaria endemic area (MoH, 2020; MoH, 2022). In general malaria is suspected clinically primarily on the basis of fever or a history of fever. Due to this, clinical diagnosis can be mostly similar with other commonly reported diseases, such as visceral leishmaniasis, tuberculosis etc. Hence, suspicion during physical examination is resolved using laboratory diagnosis and making the diagnosis of malaria on clinical features alone is not acceptable (MoH,2022; WHO,2022).

In the laboratory, malaria is diagnosed using different techniques, e.g. conventional microscopic diagnosis by staining thin and thick peripheral blood smears, Concentration techniques, like quantitative buffy coat (QBC) method, Rapid diagnostic tests, Serological tests like ELISA, and

molecular diagnostic methods, such as polymerase chain reaction (PCR) and Loop-mediated isothermal amplification (LAMP). Some advantages and shortcomings of these methods are related to sensitivity, specificity, accuracy, precision, time consumed, cost-effectiveness, labor intensiveness, the need for skilled microscopists, and the problem of less experienced technicians (Fitri *et al*., 2022; Oyegoke *et al*.,2022).

The most widely accepted and existing laboratory diagnostic methods for malaria are parasitological, serological and molecular methods (MoH, 2020).

The two methods used routinely for parasitological diagnosis of malaria are light microscopy and immuno-chromatographic RDTs. Detection of antibodies to parasites and nucleic acid-based amplification techniques have no role in the clinical management of malaria or in routine surveillance systems (MoH, 2020; WHO,2022).

### 2.4.1. Microscopic examination of malaria

Light microscopy using thick blood films not only provides a highly sensitive and specific diagnosis of malaria but it also allows quantification of malaria parasites and identification of the infecting species when performed well. The accuracy of diagnosis is strongly dependent on the competence of the microscopists. (Tegegne *et al*.,2020; WHO,2016).

Light microscopy is a ‘gold standard test’ for malaria examination having the following advantages: low direct costs, if laboratory infrastructure to maintain the service is available, high sensitivity, if the performance of microscopy is high, differentiation of *Plasmodia* species, determination of parasite densities, detection of parasite stages including gametocytaemia, allows monitoring of responses to therapy (Siahaan *et al*.,2018; Berzosa *et al*.,2018).

Disadvantages include: Accuracy depends heavily on the skill and experience of the microscopist, it requires well-maintained equipment and a consistent supply of quality staining reagents, it can be time-consuming, and in areas with limited resources, it may not be readily available and it can be less sensitive than other methods, like PCR and LAMP, especially when parasite levels are low (Tegegne *et al*.,2020; Fitri *et al*., 2022).

### 2.4.2. Malaria diagnosis using RDTs

Rapid diagnostic tests (RDTs) are immuno-chromatographic tests for detecting parasite-specific antigens in a blood sample. Some tests allow detection of only one species (*P. falciparum*); others allow detection of one or more of the other species of human malaria parasites (*P. vivax, P. malariae* and *P. ovale*) (WHO,2003; WHO, 2004). They are available commercially in various formats, e.g. dipsticks, cassettes and cards. Cassettes and cards are easier to use in difficult conditions outside health facilities. RDTs are relatively simple to perform and to interpret, and they do not require electricity or special equipment.

Rapid diagnostic tests are based on the detection of histidine-rich protein 2 (HRP2), which is specific for *P. falciparum*, pan-specific or species-specific *Plasmodium* lactate dehydrogenase (PLDH) or pan-specific aldolase (WHO, 2020; Alemayehu *et al.*, 2020).

These tests had many potential advantages, including rapid provision of results and extension of diagnostic services to the lowest-level health facilities and communities, fewer requirements for training and skilled personnel (for instance, a general health worker can be trained in 1 day), and reinforcement of patient confidence in the diagnosis and in the health service in general. (Makanjuola *et al*.,2020; Alemayehu *et al.*, 2020).

They also have potential disadvantages, including inability to distinguish new infections from recently and effectively treated infections due to the persistence of *pf*hrp2 in the blood for 1–5 weeks after effective treatment, the heterogeneous quality of commercially available products and the existence of lot-to-lot variation (WHO,2020).

Besides the above-mentioned limitations of malaria RDT diagnosis, parasites having deleted *hrp2/3 genes* in *P.falciparum* parasites in different malaria endemic countries can hamper its detection ability and make HRP2-based tests not suitable in that region (WHO,2017). This will be reviewed in detail as follows:

A study conducted in Vietnam showed that the prevalence of false-negative RDT results among 270 symptomatic cases was 15 (5.6%) despite the absence of *pfhrp2* and *pfhrp3* deletion (Thang *et al*., 2022). A study conducted by Bharti *et al*., (2016) in eight highly endemic states of India showed that of the 1521 positive samples collected and examined, 50 of them were negative by

*pfhrp2* based RDT test. Of those negatives, the frequency of *pfrhp2* deletions varied between sites ranged from 0-25% and for dual deletions ranged from 0-8% by PCR.

According to the global systematic review conducted by Molina- de la Fuente *et al*., (2021), higher prevalence of *pfhrp3* deletion was reported 43% in South Central America, followed by 3 % in Africa and 1% in Asia compared to *pfhrp2 gene* deletion accounted for 18% in South Central America, 4% in Africa and 3% in Asia. Moreover, in this review, the prevalence of deletions among the false negatives ranged from 0 to 100% in Africa. However, 90% to 48% in Asia and South-Central America.

A study conducted by Kaaya *et al*., (2022) in Northeastern Tanzania showed that of the 122 samples examined by nPCR, there were 2 (1.6%) and 61 (50%) deletions in *pfhrp2* and *pfhrp3 genes* respectively.

A recent study in Tanzania showed that, from the 140 samples analized for *pfhrp2/3*, 89.2% (125/140) were *pfhrp2*+/*pfhrp3*+, nine were *pfhrp2* deleted and six were *pfhrp3* deteted patasites. No dual *pfhrp2/3* deleted parasites were detected (Rogier *et al.*,2024).

Another global systematic review conducted by Zeleke *et al*., (2022) showed that pooled prevalence of *pfhrp2* and *pfhrp3* gene deletions were 21.3% and 34.5%, respectively. This review also reported that among the false negatives, the prevalence of *pfhrp2 gene* deletion was 41.1%.

According to the African systematic review conducted by Agaba *et al*., (2019), the level of *pfhrp2/3* gene deletion across Africa ranged from the highest 62% to the lowest 0.4%. Another study conducted by Thomson *et al*., (2019) depicted that of the 911 samples collected in Ghana, Tanzania and Uganda, *pfhrp gene* deletions were reported from Tanzania (3 *pfhrp2* and 2 *pfhrp3*) and Uganda (7 *pfhrp2* and 2 *pfhrp3*) by PCR. However, *pfhrp2/3 gene* deletions were not identified in Ghana.

A study conducted by Mihreteab *et al*., (2021) in Eritrea showed that molecular analyses of 715 samples revealed the overall prevalence of *hrp2*-, *hrp3*-, and dual *hrp2/3*-deleted parasites as 9.4%, 41.7% and 7.6%, respectively.

A study in Adama, Ethiopia showed, amplifications of 50 samples done by semi-nested PCR to detect *pfhrp*2/3 genes resulted all (100%) deletions for *pfhrp*2 and *pfhrp*3 genes (Golassa *et al*.,2020).

According to a study from Benishangul Gumuz Regional State, Northwest Ethiopia, of 218 *P. falciparum* positive samples, exon 2 deletions were observed in 17.9% of *pfhrp*2 gene and in 9.2% of *pfhrp*3 gene. A high proportion of deletions in short segments of *pfhrp2* exon1-2 (50%) was also detected while the deletions of the *pfhrp3* exon1-2 gene were 4.1% (Alemayehu *et al*.,2021).

Another study conducted by Molina-de la Fuente *et al*., (2022) in Amhara Region Ethiopia, showed that among the 354 symptomatic malaria patients, deletion frequency in exon 1-2 and exon 2 were 22 and 4.6% for *pfhrp2* and 68 and 48% for *pfhrp3*, respectively.

A study in Pawe, Ethiopia investigated *pfhrp2/3 dual* genes deletions in three samples from 20 samples collected for Therapeutic Efficacy Study in 2017 (Rogier *et al*.,2022).

From 233 samples collected and analyzed during Ethiopian national survey, 51(22%) samples harbored complete *hrp2/3* deletions by ddPCR. Also, this report showed from the total 31 samples carried R622I SNPs, 90.3% of samples (28 of 31) also had an *hrp3* deletion (Kamaliddin *et al*.,2024).

A recent study in Northwest Ethiopia showed that from 33 samples analyzed by nested PCR 16 (48.5%) tested negative for *Pfhrp*3, *pfhrp2* and flanking genes (Getie *et al*.,2024). Again recent study in Southern Ethiopia showed that, among the 249 *msp-2* positive samples, dual deletion of *hrp2* and *hrp3* genes was observed in 33 (13.3%) of the isolate (Mekonen *et al*.,2024).

A recent study conducted in the Amhara region by Adamu *et al*. (2025) analyzed 179 samples and reported that 92 samples (51.4%) had dual *pfhrp2/3* deletions, 4 samples (2.2%) had *pfhrp2* deletions alone, and 62 samples (34.6%) had *pfhrp3* deletions.

Finally, according to WHO recommendation to change HRP2 based RDT to LDH based RDT, data is needed to confirm the *hrp2 gene* deletion status in each area whether it is > 5% or not. Therefore, our study aimed to show its prevalence in eight selected facilities (Andasa health center, Chisabay health center, Hamusit health center, Kurgen health center, Uffa health center, Gambella General Hospital, Gambella Primary Hospital, and Gebretsadik Shewa Hospital) of

Ethiopia using the most accurate and recent method ddPCR on the *P. falciparum* infected pregnant women, hence, study and review is not done on this segment of population. Based on the above limited evidence, the Ethiopian Ministry of Health switched from HRP2-based RDTs to lactate dehydrogenase (pLDH)-based RDTs targeting the plasmodial lactate dehydrogenase (pLDH) specific for *P. falciparum* and *P. vivax* (RapiGEN BIOCREDIT Malaria Ag Pf/Pv pLDH/ pLDH) at the beginning of the year 2024. But performance of the new LDH based RDT in clinical samples is not evaluated yet in Ethiopia. Therefore, we evaluated the diagnostic performance of alternative LDH based RDT among pregnant women with suspected malaria in Northwest Ethiopia.

### 2.4.3. Molecular diagnosis of malaria

Molecular diagnosis of malaria refers to advanced laboratory techniques that detect genetic material (DNA or RNA) of malaria parasites in a patient's blood. Molecular diagnostic techniques have become increasingly important in malaria detection due to their high sensitivity and specificity, especially in detecting low parasitemia or asymptomatic infections that are often missed by microscopy and rapid diagnostic tests (RDTs) (Lucchi *et al*.,2018). Polymerase chain reaction (PCR) and its variants such as nested PCR, real-time PCR (qPCR), and loop-mediated isothermal amplification (LAMP) allow for accurate identification of *Plasmodium* species and detection of mixed infections (Snounou *et al*., 1993; Rougemont *et al*., 2004). These methods are also crucial for identifying *P. falciparum* strains with deletions in the *pfhrp2* and *pfhrp3* genes, which undermine the efficacy of HRP2-based RDTs (Gatton *et al*., 2020). While molecular techniques offer superior diagnostic performance, their use is often limited in endemic regions due to higher costs, the need for technical expertise, and laboratory infrastructure (Alemayehu *et al*.,2023). Nonetheless, they play a vital role in surveillance, confirmatory testing, and research applications.

## 2.5. Treatment of malaria

Early and effective treatment of malaria is very important and plays a role in malaria prevention and control. According to the Ethiopian National Malaria Treatment Guideline, treatment of malaria should be based upon a parastiologically confirmed diagnosis. For instances, if the RDT or microscopy test indicates a *P. falciparum* infection, then the patient should be treated with appropriate doses of Arthemether –Lumefantrine (AL) and single dose of primaquine (PQ) with

the first dose of AL. If the RDT or microscopy reveals a *P. vivax* infection only, then chloroquine (CQ) treatment should be dispensed (MoH, 2018; MoH, 2020). In addition, to prevent relapse of *P. vivax* malaria, treat children and adults (except pregnant women, infants less than 6 months of age and lactating women), with a 14-day course of primaquine in all transmission settings at all levels of the health system. Mixed infection confirmed by microscopy should be treated with AL and 14 days course of primaquine. Pregnant women in any of trimester should be treated with AL when uncomplicated *P. falciparum* infection is present (MoH, 2018; MoH, 2020).

### 2.5.1. Factors contributing to anti-malarial treatment failure

Treatment failure in malaria remains a major challenge and obstacles in the fight against malaria. One significant cause of treatment failure is true drug resistance, which is considered after ruling out other contributing factors such as poor patient compliance and substandard drug quality (WHO,2010). Additional causes include re-infection with a new strain of the parasite during the treatment period and, specifically in the case of *Plasmodium vivax*, the relapse caused by the activation of dormant liver-stage parasites known as hypnozoites (Nath *et al.*,2021; Vestergaard and Ringwald,2007).

Antimalarial treatment failure is a complex issue influenced by multiple factors related to the parasite, host, and treatment regimen. One of the primary causes is drug resistance, particularly mutations in parasite genes such as *pfcrt*, *pfmdr1*, *k13*, and others, which are associated with resistance to chloroquine, mefloquine, and artemisinin derivatives (WHO, 2023; Dondorp *et al*., 2009). Inadequate drug absorption, due to vomiting or malnutrition, and substandard or counterfeit medications can also reduce therapeutic efficacy. Incorrect dosing, poor adherence to treatment regimens, and monotherapy rather than combination therapies increase the risk of recrudescence and selection of resistant strains (White, 2004). Additionally, host immunity, especially in non-immune individuals or young children, plays a significant role in treatment outcomes, as a weaker immune response may be insufficient to clear residual parasites. Delayed parasite clearance times are now recognized as a key marker of emerging resistance, particularly to artemisinin-based combination therapies (ACTs) (Ashley *et al*., 2014).

Artemisinin-combination therapies (ACTs) are highly effective against *P. falciparum* malaria but, anti-malarial drug resistance including ACTs are a major threat to global malaria control and

elimination efforts (WHO,2020).The fight against malaria is constantly challenged by the parasite's ability to evolve resistance, and by the problems of getting high quality medications to the people that need them. Resistance arises from genetic mutations in the parasite, which happened when the parasite is exposed to the drug. It is reviewed in detail as follows:

### 2.5.2. Function and impact of the *P.falciparum kelch 13* mutations on antimalaria drugs

The *P.falciparum Kelch 13* (PfK13) protein is expressed in all stages of the intraerythrocytic cycle and partially localized in the endoplasmic reticulum of *P. falciparum*. The *kelch13-proplle*r *gene,* which is found on chromosome 13, is associated with single point mutations in the PfK13 protein and is associated with artemisinin resistance. However, PfK13 mediates artemisinin resistance is not understood completely. Other scholars claimed that PfK13 is essential for asexual erythrocytic development, but its function is not yet known (Siddiqui *et al*., 2020; Ndwiga *et al*., 2021).

### 2.5.3. WHO validated and candidate pfk13 mutation ACT resistance markers

The list of validated and candidate resistance markers is continually being updated. The current list is provided below.

Table 1. PfKelch13 markers of artemisinin partial resistance (WHO,2025)

| Sr.No | Validated markers | Candidate or associated markers |
|---|---|---|
| 1 | F446I | P441L |
| 2 | N458Y | G449A |
| 3 | C469Y | C469F |
| 4 | M476I | A481V |
| 5 | Y493H | R515K |
| 6 | R539T | P527H |
| 7 | I543T | N537I/D |

| | | |
|---|---|---|
| 8 | P553L | G538V |
| 9 | R561H | V568G |
| 10 | P574L | |
| 11 | C580Y | |
| 12 | R622I | |
| 13 | A675V | |

*Footnote*: F446I meaning, Change of amino acid phenylalanine to Isoleucine at position 446 in the K13 protein. (See detailed descriptions of all WHO approved and Candidate pfk13 mutation on annex V)

The discovery that mutations in portions of a *P. falciparum* gene encoding kelch (K13) propeller domains are the major determinant of resistance has provided opportunities for monitoring such resistance on a global scale.

K13-propeller sequence polymorphism was analyzed from 14,037 samples collected in 59 malaria endemic countries. Of those, 108 nonsynonymous K13 mutations were identified and showed marked geographic disparity in their frequency and distribution. For instances, in Asia, 36.5% of the K13 mutations were distributed within two area: One in Cambodia, Vietnam, and Laos and the other in western Thailand, Myanmar, and China with no overlap. However, in Africa, we observed a broad array of rare nonsynonymous mutations that were not associated with delayed parasite clearance (Menard *et al*., 2016).

A study conducted by Mohon *et al*., (2014) in Bangladish showed that, from sequenced 253 *P. falciparum* positive samples one non-synonymous mutation (A578S) was reported. On the contrary, a study by Patel *et al*., (2017) from Chhattisgarh, central India showed that no k 13gene mutation was found.

A study by Chidimatembue *et al*., (2021) in Mozambique showed that from Sanger sequenced 109 *falciparum* positive samples no *pfk13* mutations associated with artemisinin partial resistance were observed.

A study by Matrevi *et al*., (2022) in Ghana showed that from 977 sanger sequenced samples, The novel SNPs include R404G, P413H, N458D/H/I, C473W/S, R529I, M579T/Y, C580R/V, D584L, N585H/I, Q661G/L were detected.

A study in Cameroon by Niba *et al* .,(2023) revealed nine pfk13 mutations, including G453V, M472I, C510W, V520I, P527H, I590V, A676S, D680N, and H697N.

A study by Xu *et al*., (2023) showed that from 237 *P. falciparum* infections imported from central Africa to Zhejiang Province, China, seven samples (3.1%, 7/232) presented pfk13 mutations, including two (0.9%, 2/232) non-synonymous mutations (D464E and K503E) and five (2.1%, 5/232) synonymous mutations (F442F, R471R, T535T, V589V, and G690G).

A study in Cotdevore in 2023 showed that from Sanger sequenced 186 samples, prevalence of pfk13 mutation was His-493 (1.26%), Thr-539 (3.52%) and Ile-476 (1.29%) (Oléfongo *et al*.,2023).

A study in South Sudan showed that from analyzed 468 samples from symptomatic malaria patient they found 15 mutations: S623G, Q654Q, Y519H, R561L, E605K, A486S, N694N, D516D, F673F, A578S, E567E, E602K, G496G, P615S and G449G (8 non-synonymous and 7 synonymous). But none were validated or candidate markers of artemisinin resistance (Fuente *et al*.,2023).

A study with 186 patients conducted in Mali, Gabon, Ghana, Uganda, and Rwanda showed an allele frequency of 22% for R561H and 2 with parasites that carried novel mutations Q661E and P667S (Straimer *et al*.,2022).

According to a systematic review in Nigeria, the overall prevalence of non-synonymous SNPs mutations of pfk13 gene was 2.3% and there was no confirmed (WHO-validated) K13 mutation. There were two non-synonymous mutations, K189T and Q613H (Iwuafor AA *et al*.,2023).

A study in Niger showed that from 159 samples sequenced ten different mutations were observed: Seven of these mutations were non-synonymous (C469R, T508S, R515T, A578S, I465V, I437V, and F506L) while three were synonymous (P443P, P715P and L514L) (Arzika *et al.*, 2023).

A study in Djibouti showed that from targeted sequencing of 79 *P. falciparum* clinical isolates using the Miseq Illumina, only the "African type" PfK13 substitution, R622I, was found in a single isolate (1.4%) for the first time in the country (Mze *et al*.,2024).

Again, recent study by Laminou *et al*., (2024) in Niger showed that from sequenced 255 samples, ten mutations (SNP) were found, including 7 synonyms (K248K, G690G, E691E, E612E, C469C, G496G, P718P) and 3 non-synonyms (N594K, R255K, V714S).

According to a study from Western Kenya, from 226 *P. falciparum* infected and Sanger sequenced DNA samples, they found validated ART-R K13 mutation C469Y in three samples (Makau *et al*.,2024). Reports from sequenced 176 samples from Northwestern Tanzania showed Arg561His mutation in 18 samples (Ishengoma *et al*.,2024).

Again, a report from a recent study in Tanzania showed that from genotyped 6855 samples, sequencing across the k13 gene identified three WHO validated ART-R mutations: Arg561His (54 isolates), Arg622Ile (one isolate), and Ala675Val (two isolates) (Juliano *et al*.,2024).

A recent study in Democratic Republic of Congo showed that from analyzed 1065 samples, two of the non-synonymous mutations occurred more than once are Q613E and V637I and the candidate P441L mutant found in one sample from Rushuru, and the validated R561H, from Kabondo (Kahunu *et al*.,2024. Report from republic of Congo showed that no *Pfk13* mutations associated with artemisinin-based resistance were detected (Baina *et al*., 2024).

A recent study in Nigeria by Jada *et al*., (2024) revealed that, from genotyped 163 samples, no validated mutations linked to artemisinin resistance were found, but the A578S mutation was detected in 15.09% of the analyzed samples.

A recent study in Uganda showed that from 238 samples sequenced, three *pfk13* mutations were identified; C469Y in 32/238 (13.5%) samples, A675V in 14/238 (5.9%) and S522C in (1/238 (0.42%) samples across the four surveyed regions (Agaba *et al*.,2024).

A recent study in Southeastern Sudan showed among 257 *P. falciparum infected* samples, 22% harbored the *pfkelch13* R622I mutation and 10.7% showed *hrp2/3* gene deletions (L'Episcopia *et al.*,2025).

Five different non-synonymous mutations: Glu605Lys, Arg622Ile, Asn657Lys, Lys658Glu, and Ser679Leu were detected in Eritrea from sequenced 587 samples. The most prevalent mutation (11.9%) was *pfk13* Arg622Ile (Mihreteab *et al*.,2024).

A study by Jeang *et al*., (2024) in Kenya and Ethiopia reported two WHO validated mutations from analyzed 1102 samples: A675V in three isolates from Kenya and R622I in four isolates from Ethiopia.

A study in Northwest Ethiopia revealed that of the 125 k13 genes amplified and sequenced, a unique k13 gene mutation (R6221) was identified in 3 (2.4%) of the samples (Bayih *et al*., (2016). In contrast to this, another study from central Ethiopia by Lo *et al*., (2017) showed no k 13 gene mutation was reported.

From deep sequenced key drug-resistance mutations and 1,832 SNPs in the parasite genomes of 609 malaria cases collected during a diagnostic-resistance surveillance study in Ethiopia, 8.0% of malaria cases were caused by *P. falciparum* carrying *kelch13* (*K13*) 622I mutation of which 8.2% of *K13* 622I parasites also carried the *pfhrp2/3* deletions (Fola *et al*.,2023).

A more recent report in Ethiopia by Brhane *et al.,* (2025) reported 15.7% R622I mutation and showed distribution in the Amhara, Somali, Gambella, and Oromia regions. In addition, A675V *pfk13* mutation detected in six isolates in the Gambella region.

Finally, from all reviewed papers, almost all authors recommend continuous surveillance and research to know the sensitivity of the parasites to artemisinin treatments and to maintain successes and address any future challenges posed by drug resistance. Additionally, there is absence of data on drug resistance molecular markers on *P. falciparum* infected pregnant women and hence no review of literature has been done in this segment of population.

## 2.6. Prevention and control of malaria

Besides to early diagnosis and treatment, prevention and control of malaria depends on, vector control using long lasting insecticide nets (LLINs) and indoor residual spraying (IRS) to avoid mosquito bites and the use of chemoprophylaxis. Moreover, larval source management (LSM) such as environmental management by community mobilization and Larvicide. are used for prevention of malaria in Ethiopia (MoH, 2018; MoH, 2020).

### 2.6.1. Long Lasting Insecticidal Nets (LLINs)

LLINs are effective tools to significantly reduce morbidity and mortality due to malaria; especially when coverage rates are high and human biting by vectors takes place after people have gone to sleep, LLINs also can have an impact on vector populations. LLINs have three main functions: i) When mosquitoes are in contact with the net, it has a knock-down effect, temporarily incapacitating or even killing mosquitoes; ii) It has a repellent effect; and, iii) It reduces contact between the person sleeping under the net and mosquitoes by acting as a physical barrier (MoH,2022).

### 2.6.2. Indoor Residual Spraying (IRS)

IRS is the application of long-lasting residual insecticides to potential malaria vector resting surfaces such as internal walls, eaves, and ceilings of all houses or structures (including domestic animal shelters) where such malaria vectors might come into contact with the insecticide. IRS is one of the most common vector control interventions for reducing and interrupting malaria transmission, and one of the most effective methods for obtaining rapid large-scale impact on reduction of both vector populations and malaria morbidity/mortality. The effectiveness of IRS as a malaria control intervention arises from the fact that many important malaria vectors are endophilic; searching for blood meals they rest on the walls, ceilings and other interior surfaces before and/or after feeding on inhabitants (WHO, 2022).

The objectives of IRS towards curtailing malaria transmission are: a) Reducing the lifespan of vector mosquitoes to less than the time it takes for the malaria sporozoites to develop; b) Reducing the density of vector mosquitoes by immediate killing; and c) Reducing human-vector contact through repellent effect, thereby reducing the number of mosquitoes that enter sprayed rooms (Nsekuye *et al*.,2024; WHO,2015).

IRS can be effective in areas with unstable malaria transmission; it will prevent seasonal increases in transmission, will prevent and control epidemics, and can eliminate local transmission of malaria. In areas with stable malaria, IRS will prevent seasonal increases in malaria transmission, prevalence, morbidity and mortality (WHO, 2022).

### 2.6.3. Larval Source Management

Larval source management (LSM) is an additional strategy for malaria control. Unlike (LLINs) and IRS, which target the adult mosquito vector, LSM targets the immature, aquatic stages of the mosquito (larvae and pupae), thereby reducing the abundance of adult vectors. To ensure the prevention and control of malaria, it is important that all temporary or permanent breeding sites, which are not needed, are identified and drained through active participation of communities (MoH, 2022).

# CHAPTER THREE: MATERIALS AND METHODS

## 3.1. Study Design, Period and Area

A facility based cross-sectional study was conducted from 01 July to 30 November 2023 at selected health facilities of Ethiopia. The geographical study areas were three health facilities from Amhara Region (Andasa, Chis Abay and Hamusit Health centers), three health facilities from Gambella Region (Gambella General Hospital, Gambella Primary Hospital and Kurgen Health Center) and two health facilities from Southwest Ethiopia Peoples' Region (Gebretsadik Shewa Hospital and Uffa Health Center).The facilities were selected based on prevalence of malaria Annual Parasite Incidence (API) ≥ 50.

Based on 2013 Central Statistical Agency (CSA) of Ethiopia, with population projection for 2023, the Amhara Region has an estimated total population of 80,075,952 consisting of 40,103,964 male and 39,971,988 female. 84.4% of the population is estimated to be rural inhabitants, while 15.6% are urban dwellers. Bahir Dar has a tropical savannah climate with one distinct rainy period. The average elevation of the town is around 1,840 meters above sea level. Temperatures are warm year-round, with the hot season from February to May and the cool season from July to September. The average humidity is around 58% (NMA, 2013). Gambella Region has an estimated total population of 1,584,000 consisting of 828,000 males and 756,000 females with 68.7% of the population is estimated to be rural inhabitants. Gambella town, located in the Gambella Region of Ethiopia, experiences a hot, humid climate with an average elevation of 526 meters (1,726 feet). The mean annual temperature is around 26.7°C, and the average rainfall is approximately 1200mm per year. Relative humidity also tends to be high, reflecting the town's lowland location and proximity to the Baro River (Alemayehu *et al*.,2020). According to CSA report, there is no data on the total populations of Southwest Ethiopia Peoples' Region. Due to various and diverse geographical areas in the regions, malaria vector breading sites are commonly there. Bonga town, located in southwestern Ethiopia, has a humid, warm climate with an elevation around 1,700 meters (5,600 feet) above sea level. The average temperature ranges from 15°C to 25°C (59°F to 77°F). Bonga experiences a wet season with overcast conditions and a dry season with partly cloudy skies. The town is known for its lush greenery and high rainfall, especially during the wet season from April to September (Unpublished data from the Southwest region health bureau).

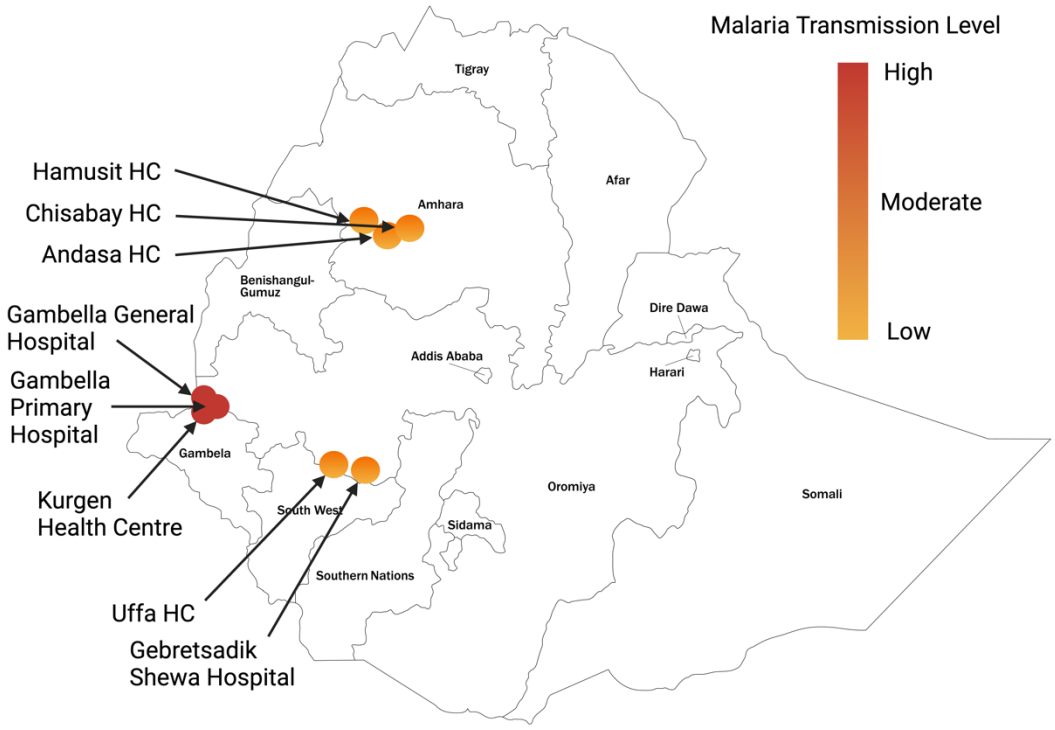



Figure 3. Map of selected study sites

## 3.2. Source population

All pregnant women who lived in selected districts of Amhara Regional State, Southwest Ethiopia Region and Gambella Regional State are source population.

## 3.3. Study population

For placental malaria prevalence:

All pregnant women who presented at the labor unit of the selected health facilities for delivery

For *HRP2/3 gene* deletion and k13 mutation:

All pregnant women who came for their regular scheduled and unscheduled ANC visit and had confirmed *P. falciparum* infection

For LDH based RDT evaluation:

All pregnant women had sign symptoms of malaria and came to selected health facilities for diagnosis and treatment

### 3.4. Study Participants

For placental malaria prevalence:

All pregnant women who presented at the labor unit of the selected health facilities for delivery and willing to give written consent, placental blood sample and data.

For *pfhrp2/3 gene* deletion and *pfk13 gene* mutation:

All pregnant women who came for their regular scheduled and unscheduled ANC visit and had confirmed *P. falciparum* infection and willing to give written consent, venous blood sample and data.

For LDH based RDT evaluation:

All pregnant women had sign symptoms of malaria and came to selected health facilities for diagnosis and treatment and willing to give written consent, venous blood sample and data.

## 3.5. Inclusion and Exclusion Criteria

### 3.5.1. Inclusion Criteria

- Being a permanent resident in the study area
- All pregnant women who delivered at the selected health facilities (for PM study)
- All pregnant women who came for ANC follow up or any clinical complains and *P. falciparum* positive by microscopy/LAMP and able to give consent (for *HRP2/3 gene* deletion and drug resistance molecular markers detection).
- All pregnant women with malaria sign symptoms and who came for blood film examination (for LDH based RDT evaluation)

### 3.5.2. Exclusion Criteria

- Pregnant women who were severely sick and unable to respond to research questions
- Pregnant women negative for *P. falciparum* infection either by microscopy or LAMP (for *pfhrp2/3 gene* deletion and *pfk13 gene* mutation detection)

## 3.6. Sample Size determination and sampling technique

### 3.6.1. Sample size determination

‘’LAMPREG Trial’’ a pragmatic randomized diagnostic outcomes trial conducted in three different Ethiopian regions from 2021 to 2023. This study aimed to evaluate the impact of enhanced case detection using molecular testing called loop-mediated isothermal amplification (LAMP) on birth outcomes in a prospective study design. In total 2425 women were enrolled in this pragmatic randomized trial. Pregnant women enrolled and randomized in their first or second trimester based on ultrasound dating to either standard of care (SOC) passive malaria case detection using microscopy, rapid diagnostic tests, or active case detection using LAMP technology (Loopamp$^{TM}$, Human Diagnostica). The women were treated with Artemether-Lumefantrine (Co-artem$^{TM}$) or Chloroquine if positive for *plasmodium* infection. The primary outcome of the study was the proportion of newborns with low birth weight (LBW) (<2500 g) at day 0, at day 28, and the weight gain between day 0 and day 28. Secondary outcomes included maternal and newborn anemia, pregnancy loss and prematurity (Gebresenbet *et al.*,2022; Teferi *et al.*,2025).

Among the 2425 sample size, the number of participants enrolled for prevalence of placental malaria, *hrp2/3 gene deletion* and *k13 gene* mutation was calculated as follows:

The sample size estimated for placental malaria prevalence was 922. Pregnant women who delivered during the study period enrolled for the study using 50% prevalence, a design effect of two and 20% non-response rate. Therefore, a confidence interval of 95 % which corresponds to 1.96 standard values, and a margin of error of five and 20 % non-response rate, the sample size was calculated as follows,

$$n = \frac{(Z_{\alpha/2})^2 P (1-P)}{d^2}$$ (Naing *et al.*,2006)

$$n = \frac{(1.96)^2 (0.5) (1-0.5)^2}{(0.05)2} = 384$$

Where;

n = number of sample size.

P= the proportion (p = 0.5) taken as 50% prevalence

d= marginal error between the sample and population (0.05).

Z= critical value at 95% certainty (1.96).

In addition, 20% non-response rate was used (384 x .2= 76.8). Finally, the sample size was 384 + 76.8 = 461 x 2= 922.

For *pfhrp2/3 gene* deletion and *pfk13 gene* mutation detection, all pregnant women with *P. falciparum* infection were purposively recruited from the total participants of the large-scale study.

For LDH based RDT kit performance evaluation study, 317 participants were recruited as follows:

Sample size was calculated using Buderer's formula for diagnostic test studies (Buderer, 1996).

Study participant**s:** $n = \underline{(Z^2 S_N (1- S_N))/ d^2}$

Where n = sample size

z = 95% statistic for level of confidence (Z =1.96)

$S_N$ = Sensitivity = 75% from WHO recommendation of minimum sensitivity for any malaria RDT (WHO, 2019).

d = margin of error tolerated (d = 0.07)

$n = \underline{((1.96)^2 \times 0.75(1 - 0.75))/ (0.05)^2} = 288$

After adding 10% (29) to compensate for non-respondents, a total of 317 pregnant women with suspected malaria were recruited for the study.

### 3.6.2. Sampling technique

From 13 regions of Ethiopia, five top malaria endemic regions were selected based on annual incidence report from MoH, 2021 (unpublished data). From those selected top five regions, three regions were selected by lottery method. From the three selected regions, all health facilities having API≥50 framed from DHIS2 data source were given a unique code in the computer program; then those health facilities were generated with their number. Finally, the eight health facilities were taken, and the codes were recorded. Those selected study health facilities were encountered from Amhara Regional State the three health facilities (Andasa, Chis Abay and Hamusit Health centers), three health facilities from Gambella Region (Gambella General Hospital, Gambella Primary Hospital and Kurgen Health Center) and two health facilities from

Southwest Ethiopia Peoples' Region (Gebretsadik Shewa Hospital and Uffa Health Center) as shown below in fig 4.

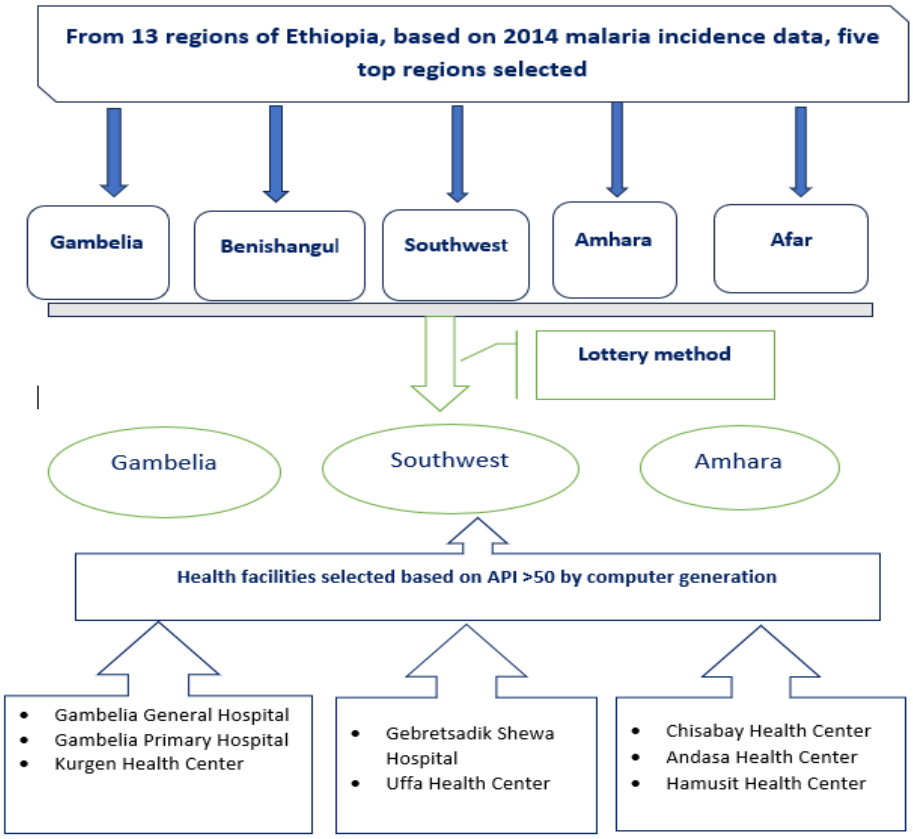



Figure 4. Flow chart showing selection of study sites

Then for placental malaria prevalence, by using convenient sampling technique, pregnant women who came to the selected health facilities were recruited until the required sample size fulfilled with in specified period. Proportional allocation of the number of cases to participate in the study from each health facility was considered by using the previous one-year ANC follow up data. In the 2022 fiscal year, number of pregnant women who visited the selected health facilities at Southwest Ethiopian people's region were 1301 and 1205 in Gebretsadik Shewa Hospital and Uffa Health Center, respectively. From Gamella Region, 2530, 1901 and 1106 pregnant women visited Gambella General Hospital, Gambella Primary Hospital and Kurgen Health Center respectively. From Amhara Region, 1104, 1296, and 1956 pregnant women visited Chis abay, Andasa and Hamusit Health Centers, respectively.

Therefore, 96,90,188,142,83,83,96, and 144 numbers of pregnant women were allocated for Gebretsadik Shewa Hospital, Uffa Health Center, Gambella General Hospital, Gambella Primary

Hospital, Kurgen Health Center, Chis abay health center, Andasa health center and Hamusit health center respectively. Finally, 922 pregnant women coming for delivery service to the selected health facilities according to their enrollment order (beginning from the first client) were recruited until the required sample size fulfilled with in specified period.

Again, for *hrp2/3 gene* deletion and *pf* drug resistance markers detection, all *P. falciparum* infected mothers diagnosed either by microscopy or LAMP or both were recruited purposively from all study sites within specified period.

For LDH based RDT evaluation, the two health institutions (Addis Alem Hospital and Bahir Dar Health Center) were selected purposively for data collection based on the malaria case flow and proximity to Amhara Public Health Institute, where the laboratory tests (RDT, microscopy and polymerase chain reaction (PCR)) were performed. A sample size of 168 and 134 were proportionally allocated to Addis Alem Hospital and Bahir Dar Health Center, respectively based on the case flow. After estimating (from previous year's data) the total number of suspected pregnant mothers expected to visit the data collection sites, a sampling interval (k) was calculated, and a systematic random sampling technique was used to select study participants. The first participant was selected by lottery method among the first k pregnant women with suspected malaria, and then every $k^{th}$ woman was enrolled in the study. Participants were traced at the antenatal care clinic in both health facilities.

Therefore, the total sample size consisted of 1,499 study participants, categorized as follows: 922 participants were recruited to assess the prevalence of placental malaria; 275 *P. falciparum*-positive participants were included for the investigation of *pfhrp2/pfhrp3 gene* deletions and molecular markers of drug resistance (*pfk13* mutations); and 302 symptomatic pregnant women were enrolled to evaluate the performance of LDH-based rapid diagnostic tests (RDTs).

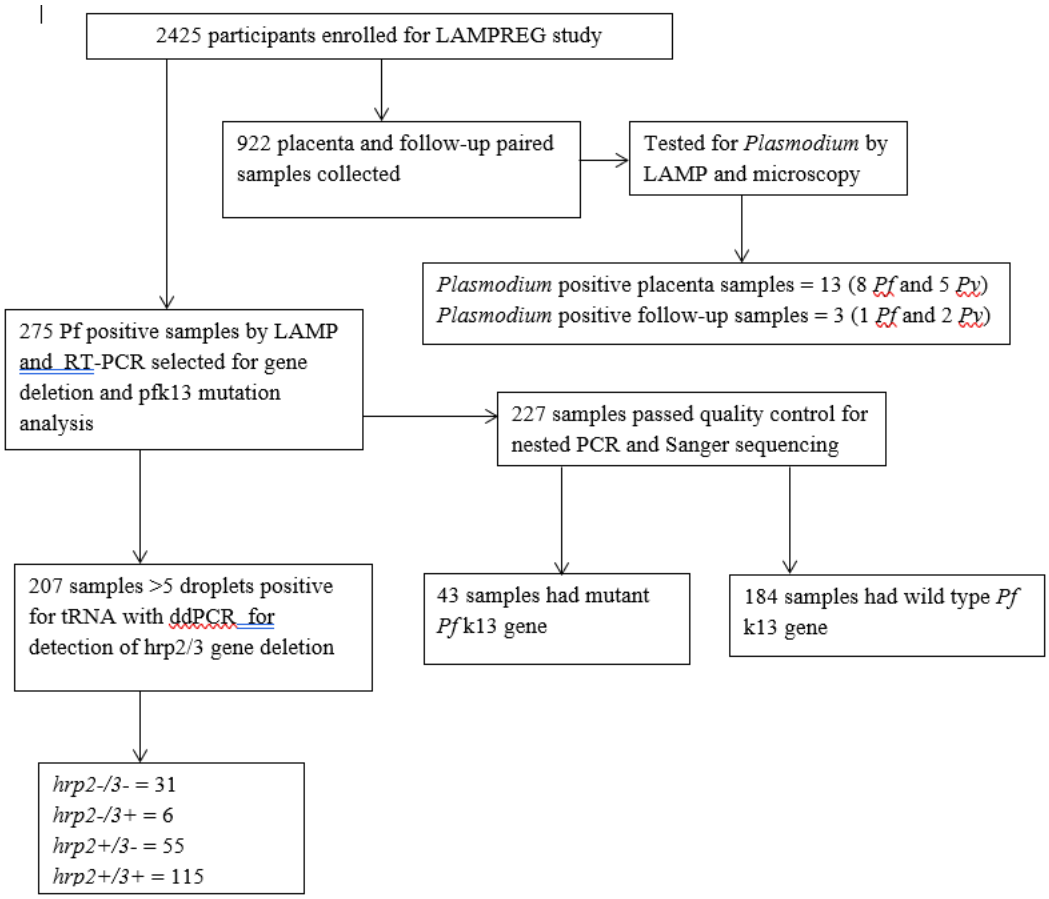



Figure 5. Flow chart for selection of specimens for genotyping and placental malaria with results

## 3.7. Data collection and Laboratory Diagnosis methods

### 3.7.1. Socio-demographic and clinical data collection

After obtaining written informed consent, Sociodemographic characteristics of the participants and necessary clinical data were collected using Redcap software in the ANC and labor ward. An enrollment form containing demographic data and past medical and obstetric history were completed for every participant by trained midwives.

### 3.7.2. Sample Collection, Processing and Laboratory investigations

#### 3.7.2.1. Placental blood sample collection for placental *Plasmodium* infection detection

From eligible and consented participants placental blood was collected immediately after delivery of the placenta. The delivered placenta was placed in a wide kidney dish with the maternal side facing upwards. A sterile soaked gauze with savlon was used to clean the maternal side of the placenta. An incision of about 1.5 cm deep was made in the mother's part of the

placenta through the intervillous space with a scalpel blade and a 5cc syringe was used to collect 2 ml placental blood from the pool of blood into EDTA anticoagulated tube.

3.7.2.2. Venous blood sample collection for peripheral *Plasmodium* detection, *HRP2/3 gene* deletion and drug resistance molecular markers detection

Two milliliter of venous blood sample were collected from pregnant women coming for their unscheduled/scheduled ANC visit and 30 µl sample was used for LAMP assay, Dried blood Spot (DBS) samples prepared using Whatman 903 filter paper (Cytiva, United Kingdom) were dried, wrapped and sealed in plastic bags according to the protocol, stored at the study site and then sent to APHI in a weekly basis and stored under -20 $^{O}$C until shipped to UCalgary for further laboratory analysis.

3.7.2.3. Sample processing and Laboratory investigation

3.7.2.3.1. DNA extraction

Genomic DNA was extracted from DBS samples of known *P. falciparum* infection (examined using LAMP) by using QIAamp® DNA Blood Mini kit (Qiagen, Hilden, Germany), as recommended by the manufacturer. DNA concentrations were measured with Qubit Fluorometer, using qubit dsDNA HS (High Sensitivity) Assay Kit (manufactured by Thermo Fisher Scientific around the world) (Using manufacturer's instruction, Qubit $^{TM}$ 4.0 Fluorometer instructions).The Qubit Fluorometer kit is designed to be accurate for initial DNA sample concentrations of 0.005 to 120 ng/µL, providing a detection range of 0.1-120 ng.

DNA samples stored at −20°C before use for ddPCR and nPCR. Again, DNA was extracted using the same extraction kits from whole blood samples for PET-PCR assay. A detailed procedure is shown in annex IV.

3.7.2.3.2. Microscopic Examination

Both thick and thin smears were prepared from placental blood samples and then they were labeled properly based on the established protocol. After proper labeling and air-drying, thin films were fixed with absolute methanol for 5 s while both thin and thick films were stained with freshly prepared 10% Giemsa working solution diluted with buffered water (PH 7.1–7.2) for 10 min and examined under 100X magnification. Thin smears were used to identify *Plasmodium* parasites species while the thick smears were examined to detect and measure the density of the

parasite against 200 white blood cells (WBC), assuming mean WBC count was 8000/µl (Parasites/µl = no. of asexual parasites x 8000/no. of WBC counted) as per WHO recommendations (WHO,2015). The slides were re-read by trained laboratory technologists under light microscope and positive results for malaria parasites were recorded in terms of parasite density (counts) against WBC on thick film. For discrepant results between two readers, a third reading by an independent and certified microscopist was taken as the final result. A detailed procedure is shown in annex IV.

3.7.2.3.3. Malaria rapid diagnostic tests (RDTs)

It is well known that malaria RDTs diagnose malaria antigens from the blood of infected patients. In this study, *CareStart*™ Pf/Pv (HRP2/pLDH) Ag Combo RDT (USA, RMVM-02591, 2025/02/20) specifically targets PfHRP2 expressed by *P. falciparum* and lactate dehydrogenase specific to *P. vivax* in human whole blood and BIOCREDIT Pf/Pv (pLDH/pLDH) RDT (Republic of Korea, H016D022D, 2026/03/27) targeting the plasmodial lactate dehydrogenase (pLDH) specific for *P. falciparum* and *P. vivax* were used. Manufacturers' instructions and quality control measures were strictly followed. Briefly, for *CareStart*™ Pf/Pv Malaria RDT, five microliters of blood samples were added using a micropipette to the sample well and two drops (60 µl) of buffer solution were added to the buffer well and results were read just after 20 minutes. For BIOCREDIT Pf/Pv (pLDH/pLDH) RDT, five microliters of blood samples were added using micropipette to the sample well and three drops (90 µl) of buffer solution were added to the buffer well and results were read just after 25 minutes. Negative readings at 25 minutes were read again at 35 minutes.

3.7.2.3.4. Loop-Mediated Isothermal Amplification (LAMP) assay

For this study Loop-Mediated isothermal amplification (LAMP) method was used to diagnose *Plasmodium* infection in placental and peripheral blood samples. LAMP malaria diagnosis emerging as a recent alternative to PCR and it can be employed directly on whole blood samples (with minimum processing) and purified DNA or RNA (Notomi *et al.*, 2000; Mori *et al*.,2009; Chander *et al*.,2014). LAMP targets 18S rRNA for the species and for the *genus plasmodium* detection. This technique is based on auto cycling and high DNA strand displacement activity mediated by *Bst* polymerase from *geobacillus stearothermophilus,* under isothermal conditions. It uses four to six primers: the two outer primers (F3 and B3) and the two inner primers (FIP and

BIP), which are the core set required for amplification, along with two optional loop primers (LF and LB) to accelerate the reaction (Notomi *et al.*,2000; Yang *et al*., 2024). The reaction consists of two steps: an initial step and a combination of a cycling amplification step with an elongation/recycling step. The isothermal amplification was carried out at 60-65$^{o}$C, the optimum temperature for *Bst* polymerase activity (Yang *et al*.,2024). Here for our study, both maternal peripheral blood and placental blood was examined using LAMP by Humaloopamp machine (Human Gesellschaft fur Biochemica and Diagnosticambh, Wiesbaden, Germany) which has heating unit, amplification unit and detection unit. The work flow consists of four main steps; sample transfer and lysis step, Loopamp PURE DNA extraction step, Loop-mediated isothermal amplification step and result reading steps (https://www.youtube.com/watch?v=IUKLNxXvzOI). A detailed procedure is shown in annex IV.

3.7.2.3.5. PET-PCR diagnosis

Photo-induced electron transfer PCR (PET-PCR) was used as a gold standard method for LDH-based malaria RDT kit performance evaluation. The assay was run using Agilent Technologies strata gene Mx3005p PCR machine following the protocol described elsewhere (Tilahun *et al*.,2020; Tilahun *et al*.,2024). The following genus and species level primers were used: Original Genus 18sFor, 5'-GGC CTA ACA TGG CTA TGA CG-3'

Original Genus FAM 18s Rev, 5'-AGG CGC ATA GCG CCT GGC TGC CTT CCT TAG ATG TGG TAG CT-3'*P. falciparum* For, 5'-ACC CCT CGC CTG GTG TTT TT-3' *P. falciparum* Rev, HEX-5'-AGG CGG ATA CCG CCT GGT CGG GCC CCA AAA ATA GGA A-3'

*P. vivax* For, 5'-GTA GCC TAA GAA GGC CGT GT-3' *P. vivax* Rev, HEX-5'- AGG CGC ATA GCG CCT GGC CTG GGG GAT GAA TAT CTC TAC AGC ACT GT-3'

Amplification of the *Plasmodium* 18S rRNA gene specific to *Plasmodium* genus, *P. falciparum* or *P. vivax* was performed in a 20 µl reaction following the protocol explained elsewhere (Tilahun *et al*.,2024). Samples with a CT value of 40 or below were considered positive (Lucchi *et al.*,2013). The genus and *P. falciparum* multiplex assays were performed for all samples. *P. vivax* singleplex assay was performed for genus-positive samples regardless of the result for *P. falciparum.* A detailed procedure is shown in annex IV.

3.7.2.3.6. Droplet Digital PCR (ddPCR) assays for *HRP2/3 gene* deletion

For *HRP2/3 gene* deletion analysis, Bio-Rad QX200 ddPCR machine was used to look at 3 targets (HRP2 exon 1, HRP 2 exon 2 and HRP 3) and tRNA ligase. The analysis was done by comparing the concentration of the hrp 2 (exon 1 / 2) or hrp 3 gene to the house keeping gene (tRNA ligase) (Vera-Arias.*et al*.,2022; Kamaliddin *et al*.,2024).

Only droplet counts >10, 000 per reaction were retained for downstream analysis and samples were included if ≥5 *tRNA ligase* droplets were present to show that sufficient parasitic DNA material was present for analysis as described on the previous study protocol (Kamaliddin *et al*.,2024).

The result calculation and interpretation were as follows: ratios of *hrp2/3* to *tRNA ligase* that were >0.75 were reported as no deletion present, ratios between 0.75 and 0.25 were reported as mixed infections, and ratios <0.25 were reported as full deletion as reported elsewhere (Kamaliddin *et al*., 2024). A detailed procedure is shown in annex IV.

3.7.2.3.7. Amplification of pfk13 and targeted sequencing

Nested PCR and Sanger sequencing were used to amplify and detect *pfk13 gene* mutations. The *P. falciparum k13* propeller domain was amplified from genomic DNA samples by nested PCR, as described elsewhere (Ariey *et al*., 2014; Mohon *et al*.,2014; Kamaliddin *et al*., 2024). PCR products were run on 2% agarose gel with a 1-kb ladder (New England Biolabs), and the bands at 849 base pairs were purified using the QI Quick Gel Extraction Kit (Qiagen, Germany). Bidirectional Sanger sequencing of the purified PCR products was performed using BigDye Terminator chemistry with an Applied Biosystems 3730XL instrument at the Centre for Health Genomics and Informatics (University of Calgary). Sequencing results were assessed individually by analyzing both strands with an MAFFT local pairwise alignment tool on a Benchling cloud-based platform (MAFFT version 7) against the *P. falciparum* 3D7 *k13* reference sequence (PlasmoDB: PF3D7_1343700) (Kamaliddin *et al*., 2024). A detailed procedure is shown in annex IV.

### 3.8. Study Variables

- *Placental Plasmodium* infection
- *Plasmodium falciparum hrp2/3* gene deletion
- *LDH based RDT performance evaluation*
- *Plasmodium falciparum drug resistance molecular markers*
- Symptoms of malaria (fever, head ache…)
- History of malaria
- Socio-demographic factors (age, educational status)

## 3.9. Data quality control

Data were collected using Redcap software by trained midwife and laboratory professionals hired at each study site. Venous blood and placental blood were collected by trained and experienced laboratory professionals and mid wives respectively. The blood film slides were read by two trained laboratory technologists. For discrepant results between two readers, a third reading by an independent and certified microscopist was taken as the final result. Manufacturers instruction for mRDT diagnosis strictly followed. Every laboratory test was done using positive and negative controls against each test. Moreover, the expiry date and storage conditions of reagents and kits were checked before starting the laboratory work.

## 3.10. Data processing and analysis

The collected data was exported to excel and SPSS version 21 (SPSS, Chicago, IL, USA). The results were organized in terms of frequencies, percentage and were presented with descriptive measures, frequency tables and graphs. The association and strength between variables were assessed by Chi-square analysis. P-value < 0.05 was considered as statistically significant. For the *hrp2/3* gene deletion, all assays were run in triplicate, i.e., HRP2 exon 1, HRP 2 exon 2 and HRP 3; analysis done by comparing the concentration of the hrp 2 (exon 1 / 2) or hrp 3 gene to the house keeping gene (tRNA ligase). For drug resistance molecular markers, nested PCR was used on the samples and then purified and sent for Sanger sequencing. Sequencing results were assessed individually by analyzing both strands with an MAFFT local pairwise alignment tool on a Benchling cloud-based platform (MAFFT version 7) against the *P. falciparum* 3D7 *k13* reference sequence (PlasmoDB: PF3D7_1343700) (Kamaliddin *et al*., 2024).

## 3.11. Ethical considerations

Ethical clearance was obtained from Bahir Dar University, College of Sciences Research Ethics Committee with a reference number PRCSVD/8142023 and permission letter was obtained from Amhara Public Health Institute, Gambella Regional Health Bureau and Southwest Ethiopia Regional Health Bureau. Both verbal and written consent were obtained from each study participant. Ethical approval was obtained from Ethiopia Ministry of Science and Higher Education National Research Ethics Review Committee (approval SRA/11.7/7115/20), to ship for those samples that could not be done in Ethiopia and done at Calgary University, Canada. All *plasmodium* infected pregnant women were treated with anti-malarial drugs based on the national malaria treatment protocol.

# CHAPTER FOUR RESULTS

## 4.1 Placental malaria prevalence

### 4.1.1 Socio-demographic characteristics of pregnant women recruited for PM

A total of 922 pregnant women were recruited for placental malaria study and placental blood samples were collected from all these delivering mothers. Parity status of recruited pregnant women showed that 30.8% (284/922) were primigravidae. The age range of participants were between 18 and 40 years and 47.6% accounted under the age range of ≤24 years, and 3.8% were at the age range of ≥35 years. Based on the level of education of participants, 18.4% and 23.9% of them were Unable to read/write and post-secondary respectively (Table 2).

Table 2 : Socio-demographic characteristics of study participants from 2021-2023 in selected health facilities of Ethiopia

| **Variable** | **Category** | **Frequency** | **Percentage** |
|---|---|---|---|
| Age(year) | ≤24 | 439 | 47.6% |
| | 25-29 | 337 | 36.6% |
| | 30-34 | 111 | 12% |
| | ≥35 | 35 | 3.8% |
| Educational status | Unable to read/write | 170 | 18.4% |
| | Read/write | 77 | 8.4% |
| | Primary | 248 | 26.9% |
| | Secondary | 207 | 22.4% |
| | Post secondary | 220 | 23.9% |

### 4.1.2. Placental malaria prevalence

Placental blood samples were collected from all 922 participants according to the established protocol. All the placental samples were diagnosed with smear microscopy and LAMP methods for the presence of *Plasmodium* infections but none of them were diagnosed positive with smear microscopy whereas thirteen samples (1.4%) were positive with LAMP methods. The peripheral bloods of the participants were checked for the presence of *Plasmodium* parasite using LAMP during their follow up visits. Of which three of them resulted in *Plasmodium* positive (one *P.*

*falciparum* and two *P. vivax* infections) which was same result as their placental blood diagnosis (figure 6). The positive placental malaria by educational background showed that 15.4%, 69.2%, 7.7% and 7.7% were among participants who were unable to read and write, primary, secondary and post-secondary, respectively. Among those 13 positive samples, eight (61.5%) of them had *P. falciparum* infections whereas five of them (38.5%) had *P.vivax* infections. Again, from those positive samples, ten (76.9%) were multigravida and three (23.1%) were primigravida participants. The prevalence of placental malaria was higher (ten (76.9%)) among asymptomatic participants than symptomatic (three (23.1%)) participants.

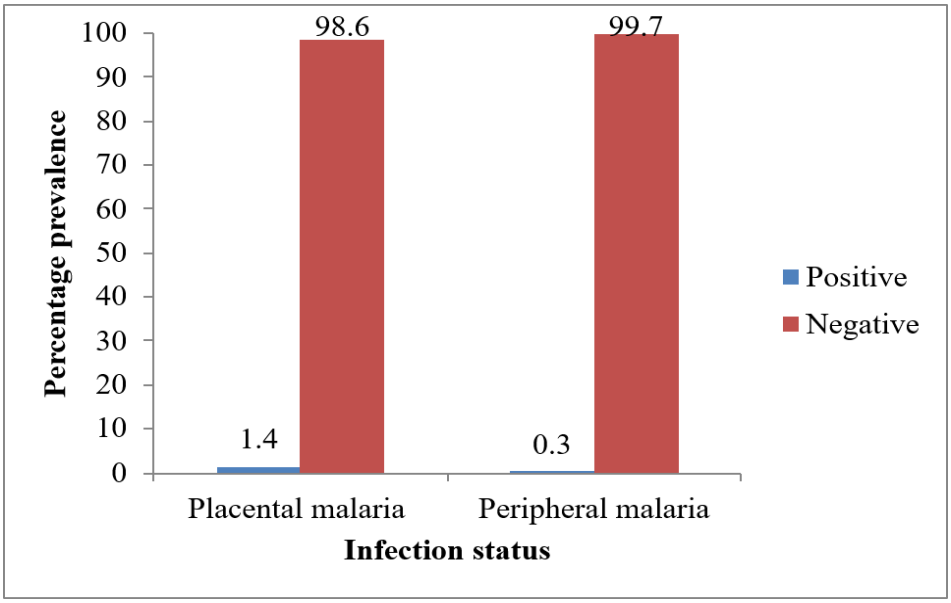



Figure 6. Placental and peripheral blood malaria positivity rate in selected health facilities of Ethiopia from 2021-2023

### 4.1.3. Regional distribution of placental malaria

Distribution of PM by region showed that 30.8% (4/13) were from Amhara Regional State, 61.5% (8/13) were from Gambella Regional State, and 7.7% (1/13) were from the Southwest Regional State. Regional distribution of placental malaria by species level showed that, two *P. falciparum* infections were from Amhara region, five *P.falciparum* infections were from Gambella Regional State and one *P.falciparum* infection was from Southwest Ethiopia region. From the total five *P. vivax* infections, two were from Andasa Health Center, Amhara Regional State and three of them were from Gambella General Hospital, Gambella Regional State (Table 3).

Table 3: Placental malaria prevalence status by study site in selected health facilities of Ethiopia from 2021-2023

| **Region** | **Plasmodium species identified** | | | | |
|---|---|---|---|---|---|
| | ***Pf n* (%)** | ***Pv n* (%)** | **Mixed n (%)** | **Neg n (%)** | **Total** |
| **Amhara** | | | | | |
| Andassa Health Center (AHC) | 2 (2.1) | 2 (2.1) | 0 | 92 (95.8) | 96 |
| Chis Health Center (CHC) | 0 | 0 | 0 | 83 (100) | 83 |
| Hamusit Health Center (HHC) | 0 | 0 | 0 | 144 (100) | 144 |
| **Sub total** | **2 (0.6)** | **2 (0.6)** | **0** | **319 (98.8)** | **323** |
| **Gambella** | | | | | |
| Gambella Primary Hospital (GPH) | 3 (2.1) | 0 | 0 | 139 (97.9) | 142 |
| Gambella General Hospital (GGH) | 2 (1.1) | 3 (1.6) | 0 | 183 (97.3) | 188 |
| Kurgen Health Center (KHC) | 0 | 0 | 0 | 83 (100) | 83 |
| **Sub total** | **5 (1.2)** | **3 (0.7)** | **0** | **405 (98.1)** | **413** |
| **Southwest** | | | | | |
| Gebretsadik Shewa Hospital (GSH) | 1 (1) | 0 | 0 | 95 (99) | 96 |
| Uffa Health Center (UHC) | 0 | 0 | 0 | 90 (100) | 90 |
| **Sub total** | **1 (0.5)** | **0** | **0** | **185 (99.5)** | **186** |
| **Total** | **8 (0.9)** | **5 (0.5)** | **0** | **909 (98.6)** | **922** |

*Pf (P. falciparum), Pv (P. vivax*), mixed (pf &pv)

## 4.2. HRP2/3 gene deletion

### 4.2.1. Detection of *Pfhrp2* and *Pfhrp3* genes using ddPCR

Out of the 275 selected samples, 207 met the ≥5 tRNA ligase droplet threshold required for ddPCR analysis. Among these 207 samples, wild type *pfhrp2/3* genes were detected in 55.6% (115/207), fully deleted *hrp2/hrp3* genes were found in 15% (31/207) of the samples, while 26.5% (55/207) showed mixed deletions (*hrp2+*/*hrp3-*), and 2.9% (6/207) exhibited partial deletions (*hrp2-*/*hrp3+*). When hrp2 deletions and hrp3 deletions analyzed separately, it was

found that 17.9% (37/207) and 41.5% (86/207) of samples contained parasites with hrp2- and hrp3- deletions, respectively (Figure 7). The overall prevalence of *pfhrp2/3 gene* deletion was 44.4% (92/207) which is deletion of at least by one of the two *hrp2/3* genes.

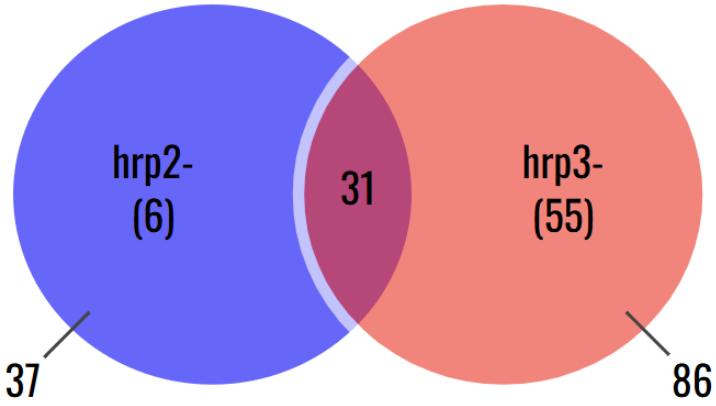



Figure 7. A Venn diagram showing summary of the overall *pfhrp2/3 gene* deletion status in selected health facilities of Ethiopia from 2021-2023

### 4.2.2. *Pfhrp2* and *Pfhrp3* gene deletion status by region

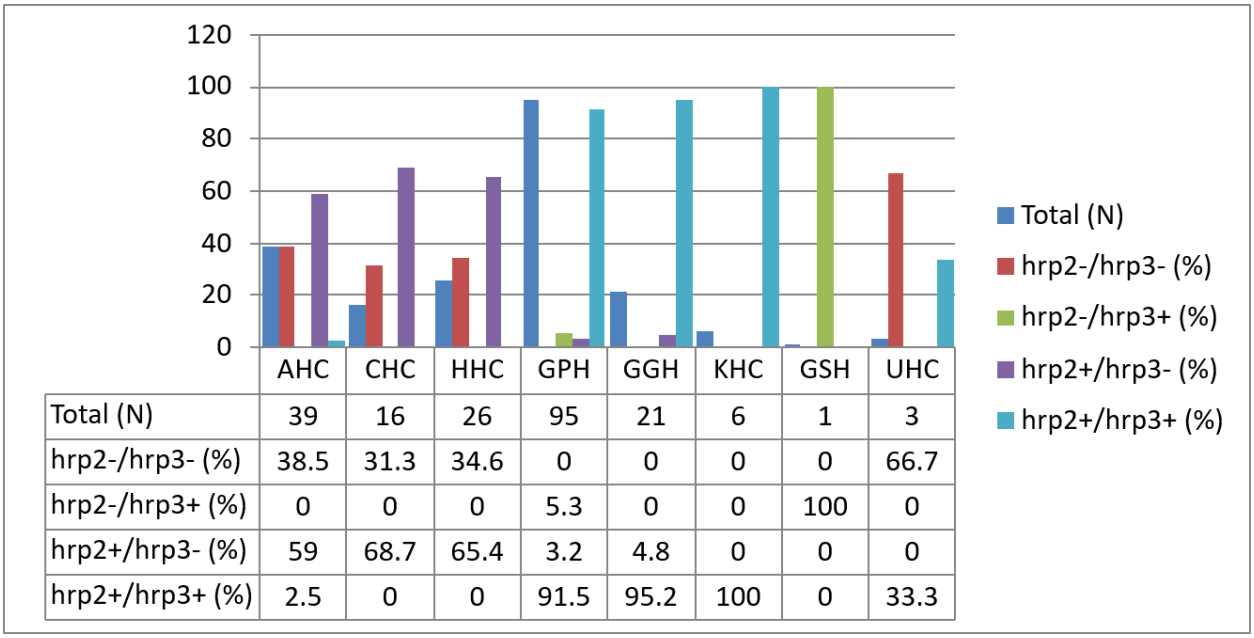
From the total 31 samples with fully deleted *pfhrp2/3* genes, 29 (93.5%) of them were from Amhara Regional State. Five partially deleted parasites were from Gambella Regional State and 51 mixed infected samples (*pfhrp2+/pfhrp3-*) were from Amhara Regional State (Table 4).

Table 4: Summary of *hrp2/3 gene* deletion status by region from 2021-2023 in selected health facilities of Ethiopia

| **Region** | **hrp2-/hrp3-(full deletion) n (%)** | **hrp2-/hrp3+ (partial deletion) n (%)** | **hrp2+/hrp3- (mixed deletion) n (%)** | **hrp2+/hrp3+ (Wild type) n (%)** |
|---|---|---|---|---|
| **Amhara** | 29 (14%) | 0 | 51 (24.6%) | 1 (0.5%) |
| **Gambella** | 0 | 5 (2.4%) | 4 (2%) | 113 (54.5%) |
| **Southwest** | 2 (1%) | 1 (0.5%) | 0 | 1 (0.5%) |
| **Total** | **31 (15%)** | **6 (2.9%)** | **55 (26.5%)** | **115 (55.6)** |

### 4.2.3. *Pfhrp2* and *Pfhrp3* gene deletion status by health facility

An analysis of full deletion of *hrp2/3 gene* by each health facility revealed that 38.5% (15/39) were from AHC, 31% (5/16) were from CHC, and 34.6% (9/26) were from HHC. There were 59 % (23/39) and 68.8% (11/16) mixed *hrp2/3 gene* deletions from AHC and CHC respectively. There were also partially deleted *hrp2/3 genes* from GPH in five samples (5.3 %) (Figure 8).




| | AHC | CHC | HHC | GPH | GGH | KHC | GSH | UHC |
|---|---|---|---|---|---|---|---|---|
| Total (N) | 39 | 16 | 26 | 95 | 21 | 6 | 1 | 3 |
| hrp2-/hrp3- (%) | 38.5 | 31.3 | 34.6 | 0 | 0 | 0 | 0 | 66.7 |
| hrp2-/hrp3+ (%) | 0 | 0 | 0 | 5.3 | 0 | 0 | 100 | 0 |
| hrp2+/hrp3- (%) | 59 | 68.7 | 65.4 | 3.2 | 4.8 | 0 | 0 | 0 |
| hrp2+/hrp3+ (%) | 2.5 | 0 | 0 | 91.5 | 95.2 | 100 | 0 | 33.3 |

Figure 8. *Pfhrp2/3 gene* deletion status by study site in selected health facilities of Ethiopia from 2021-2023

AHC: Andasa Health Center, CHC: Chisabay Health Center, HHC: Hamusit Health Center, GPH: Gambella Primary Hospital, GGH: Gambella General Hospital, KHC: Kurgen Health center, GSH: Gebretsadik Shewa Hospital, UHC: Uffa Health Center

### 4.2.4. *Pfhrp2* and *Pfhrp3* gene deletion among pregnant women with symptomatic and asymptomatic *Plasmodium* infection

Of the total participants, 62.8% (130/207) were asymptomatic for malaria and carried 23.1%, 0.8% and 36.1% of full deletion, partial deletion and mixed infections of *hrp2/3 genes* respectively. Our data showed that from the total samples with wild types of *hrp2/3 genes*, 45% of them were asymptomatic infections (Table 5).

Table 5: Summary of *pfhrp2/3 gene* deletion in symptomatic and asymptomatic *Plasmodium* infections from 2021-2023

| **Gene deletion status** | **Total examined** | **hrp2-/hrp3- n (%)** | **hrp2-/hrp3+ n (%)** | **hrp2+/hrp3- n (%)** | **hrp2+/hrp3+ n (%)** |
|---|---|---|---|---|---|
| Asymptomatic | 130 | 30 (23.1) | 1 (0.8) | 47 (36.1) | 52 (40) |
| Symptomatic | 77 | 1 (1.3) | 5 (6.5) | 8 (10.4) | 63 (81.8) |
| Total | 207 | 31 (15%) | 6 (2.9) | 55 (26.5) | 115 (55.6%) |

The deletion of *hrp2, hrp3,* and *hrp2/3* dual genes is significantly associated with malaria symptoms and transmission settings, but not with previous malaria history. Asymptomatic individuals in moderate transmission areas are more likely to harbor parasites with *hrp2/3* gene deletions (Table 6).

Table 6: Associated risk factors with *pfhrp2/3 gene* deletion from 2021 to 2023 in selected health facilities of Ethiopia

| | Variables | | Number participated n (%) | Gene deletion *n* (%) | $\chi^2$ | *p-value* |
|---|---|---|---|---|---|---|
| *hrp2* gene deletion | Malaria symptom | Symptomatic | 77 (37.2) | 6 (7.8) | 8.49 | 0.004 |
| | | Asymptomatic | 130 (62.8) | 31 (23.8) | | |
| | Transmission setting | Low | 4 (1.9) | 3 (75.0) | 42.40 | <0.001 |
| | | Moderate | 81 (39.1) | 29 (35.8) | | |
| | | High | 122 (59.0) | 5 (4.1) | | |
| | Previous malaria history | Yes | 168 (81.2) | 28 (16.7) | 0.886 | 0.347 |
| | | No | 39 (18.8) | 9 (23.1) | | |
| *hrp3* gene deletion | Malaria symptom | Symptomatic | 77 (37.2) | 9 (11.7) | 45.01 | <0.001 |
| | | Asymptomatic | 130 (62.8) | 77 (59.2) | | |
| | Transmission setting | Low | 4 (1.9) | 2 (50.0) | 182.89 | <0.001 |
| | | Moderate | 81 (39.1) | 80 (98.8) | | |
| | | High | 122 (59.0) | 4 (3.3) | | |
| | Previous malaria history | Yes | 168 (81.2) | 67 (39.9) | 1.02 | 0.313 |
| | | No | 39 (18.8) | 19 (48.7) | | |
| *hrp2/3* dual | Malaria symptom | Symptomatic | 77 (37.2) | 1 (1.3) | 18.01 | <0.001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| deletion | | Asymptomatic | 130 (62.8) | 30 (23.1) | | |
| | Transmission setting | Low | 4 (1.9) | 2 (50.0) | 52.94 | <0.001 |
| | | Moderate | 81 (39.1) | 29 (35.8) | | |
| | | High | 122 (59.0) | 0 (0) | | |
| | Previous malaria history | Yes | 168 (81.2) | 22 (13.1) | 2.48 | 0.116 |
| | | No | 39 (18.8) | 9 (23.1) | | |

## 4.3. Evaluation of PLDH based RDTs

### 4.3.1 Evaluation of Rapigen/Biocredit PFPV/PLDH mRDT kit

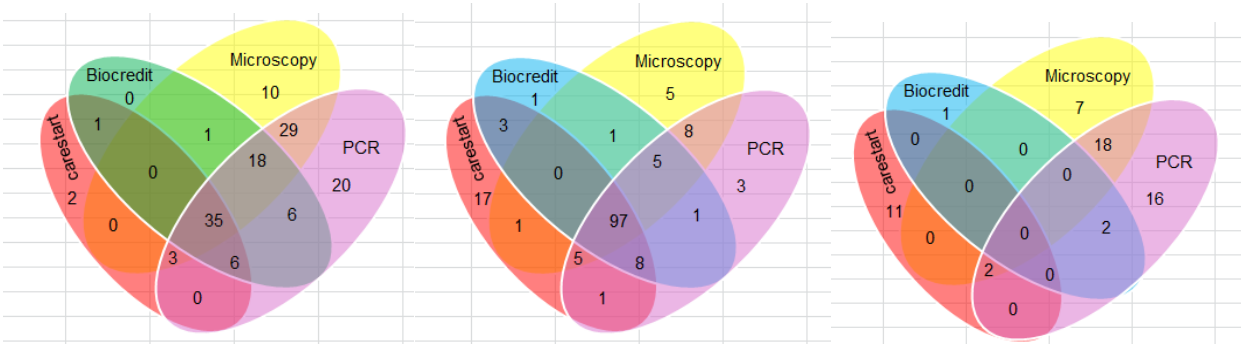
Of the 317 participants initially recruited, samples collected from 302 participants were appropriate for all diagnostic tests and hence included in the data analysis. Of 302 samples examined, BIOCREDIT Pf/Pv (pLDH/pLDH) and CareStart™ Pf/Pv (HRP2/pLDH) proved positive in 180 (59.6%) and 166 (55.0%) samples, respectively. Two hundred seven (68.5%) samples tested positive by PET-PCR, whereas 191 (63.2%) samples were positive by microscopy. Results of species-level analysis showed that a higher number of samples tested positive for *P. falciparum* using PET-PCR (117, 38.7%) and the lower using *CareStart*™ Pf/Pv (HRP2/pLDH) (47, 15.6%). Likewise, *P. vivax* was detected in 132 (43.7%) samples with *CareStart*™, followed by PET-PCR (128, 42.4%) and microscopy (122, 40.4%) (Table 7).

Table 7: Prevalence of *Plasmodium* infection by different diagnostic techniques (N=302)

| *Plasmodium* infection | BIOCREDITPf/Pv (pLDH/pLDH) n (%) | *CareStart*™Pf/Pv (HRP2/pLDH) n (%) | Microscopy n (%) | PET-PCR n (%) |
|---|---|---|---|---|
| *P. falciparum* | 67 (22.2) | 47 (15.6) | 96 (31.8) | 117 (38.7) |
| *P. vivax* | 116 (38.4) | 132 (43.7) | 122 (40.4) | 128 (42.4) |
| Mixed (Pf/Pv)* | 3 (1.0) | 13 (4.3) | 27 (8.9) | 38 (12.6) |
| Total positive | 180 (59.6) | 166 (55.0) | 191 (63.2) | 207 (68.5) |
| Negative | 122 (40.4) | 136 (45.0) | 111 (36.8) | 95 (31.5) |

*Mixed infections are also included while calculating *P.falciparum* and *P.vivax* prevalence

A total of 131 (43.4%) and 156 (51.7%) participants were tested positive for *P. falciparum* and *P. vivax* infections, respectively at least by one of the diagnostic tests. Of these, 58 (19.2%) were mixed infections (Figure 9).




A B C

Figure 9. Venn diagram showing detection of *P. falciparum* (A), *P. vivax* (B) and Mixed (C) infections

### 4.3.2. Comparison of RDTs with microscopy and PET-PCR

In comparison to PET-PCR and microscopy, BIOCREDIT Pf/Pv RDT misdiagnosed *P. falciparum* in 32 (10.6%) and 54 (17.9%) of the 302 samples, when PET-PCR and microscopy were used as a reference test, respectively. Similarly, BIOCREDIT Pf/Pv RDT misdiagnosed *P. vivax* in 22 (7.3%) and 32 (10.6%) samples, when evaluated against PET-PCR and microscopy reference tests, respectively. When PET-PCR was used as a reference test, *CareStart*$^{TM}$ *Pf/Pv* (*pfhrp2*/pLDH) misdiagnosed *P. falciparum* in 76 (25.2%) and *P. vivax* in 38 (12.6%) samples, respectively (Table 8).

Table 8: Comparison of BIOCREDIT *Pf/Pv* and CareStart™ *Pf/Pv* against microscopy and PET -PCR

| Spp | Results | Biocredit Pf/Pv (pLDH/pLDH) | | | *CareStart*™ Pf/Pv (HRP2/pLDH) | |
|---|---|---|---|---|---|---|
| | | CareStart as a reference test n (%) | Microscopy as a reference test n (%) | PCR as a reference test n (%) | Microscopy as a reference test n (%) | PCR as a reference test n (%) |
| *Plasmodium falciparum* | TP | 42 (13.9) | 52 (17.2) | 65 (21.5) | 38 (12.6) | 44 (14.6) |
| | FP | 25 (8.3) | 15 (5.0) | 2 (0.7) | 9 (3.0) | 3 (1.0) |
| | TN | 230 (76.2) | 218 (72.2) | 183 (60.6) | 197 (65.2) | 182 (60.3) |
| | FN | 5 (1.7) | 17 (5.6) | 52 (17.2) | 58 (19.2) | 73 (24.2) |
| *Plasmodium vivax* | TP | 108 (35.8) | 103 (34.1) | 111 (36.8) | 103 (34.1) | 111 (36.8) |
| | FP | 8 (2.6) | 13 (4.3) | 5 (1.7) | 29 (9.6) | 21 (7.0) |
| | TN | 162 (53.6) | 167 (55.3) | 169 (59.0) | 151 (50.0) | 153 (50.7) |
| | FN | 24 (7.9) | 19 (6.3) | 17 (5.6) | 19 (6.3) | 17 (5.6) |

*Key: TP = true positive; FP = false positive; TN = true negative; FN = false negative; n = number; % = percentage*

### 4.3.3. Performance of BIOCREDIT Pf/Pv and *CareStart*™ Pf/Pv in detecting *Plasmodium* infection

The sensitivity and NPV of BIOCREDIT *Pf/Pv* (pLDH/pLDH) in detecting *P. falciparum* infection was 75.4% (95%CI: 63.5-85.0) and 92.8% (95%CI: 89.5-95.1), respectively using microscopy as a reference. The sensitivity and NPV were further reduced to 55.6% (95%CI: 46.1-64.7) and 77.9% (95% CI: 74.2-81.2) against a PET-PCR reference. However, BIOCREDIT Pf/Pv (pLDH/pLDH) was more sensitive in detecting *P. falciparum* as compared to *CareStart*™ Pf/Pv (HRP2/pLDH), which had a sensitivity of 39.6% (95%CI: 29.8-50.1) and 37.6% (95%CI: 28.8-47.0) using microscopy and PET-PCR as reference tests, respectively. Hence, BIOCREDIT *Pf/Pv* was more accurate than *CareStart*™ *Pf/Pv* (HRP2/pLDH) when evaluated against microscopy (89.4% vs. 77.8%) and PET-PCR (82.1% vs. 74.8%). The sensitivity of BIOCREDIT *Pf/Pv* and *CareStart*™ Pf/Pv was comparable in the diagnosis of *P. vivax* compared to both microscopy (84.3% vs. 84.4%) and PET-PCR (86.7% vs. 86.7%) reference tests (Table 9).

Table 9: Performance of BIOCREDIT Pf/Pv (pLDH/pLDH) and CareStart™ Pf/Pv (HRP2/pLDH) in detecting *Plasmodium* infection

| *Plasmodium* infection | Diagnostic tool | Sensitivity (95%CI) | Specificity (95%CI) | PPV (95%CI) | NPV (95%CI) | Accuracy (95%CI) | Kappa value |
|---|---|---|---|---|---|---|---|
| *Plasmodium falciparum* | Biocredit vs *CareStar* | 89.4 (76.9-96.5) | 90.2 (85.9-93.6) | 62.7 (53.3-71.2) | 97.9 (95.3-99.1) | 90.1 (86.1-93.2) | 0.678 |
| | Biocredit vs microscopy | 75.4 (63.5-85.0) | 93.6 (89.6-96.4) | 77.6 (67.6-85.2) | 92.8 (89.5-95.1) | 89.4 (85.4-92.6 | 0.696 |
| | *CareStart*™ vs microscopy | 39.6 (29.8-50.1) | 95.6 (91.9-98.0) | 80.9 (68.0-89.3) | 77.3 (74.2-80.0) | 77.8 (72.7-82.2) | 0.408 |
| | Biocredit vs PCR | 55.6 (46.1-64.7) | 98.9 (96.2-99.9) | 97.0 (89.0-99.2) | 77.9 (74.2-81.2) | 82.1 (77.3-86.3) | 0.591 |
| | *CareStart*™ vs PCR | 37.6 (28.8-47.0) | 98.4 (95.3-99.7) | 93.6 (82.3-97.9) | 71.4 (68.4-74.2) | 74.8 (69.5-79.6) | 0.404 |
| *Plasmodium vivax* | Biocredit vs *CareStar* | 81.8 (74.2-88.0) | 95.2 (90.9-98.0) | 93.1 (87.2-96.4) | 87.1 (82.4-90.7) | 89.4 (85.4-92.6) | 0.782 |
| | Biocredit vs microscopy | 84.3 (76.8-90.4) | 92.8 (88.0-96.1) | 88.8 (82.4-93.1) | 89.8 (85.3-93.0) | 89.4 (85.4-92.6) | 0.778 |
| | *CareStart*™ vs microscopy | 84.4 (76.8-90.4) | 83.9 (77.7-88.9) | 78.0 (71.6-83.3) | 88.8 (84.0-92.4) | 84.1 (79.5-88.0) | 0.674 |
| | Biocredit vs PCR | 86.7 (79.6-92.1 | 97.1 (93.4-99.1) | 95.7 (90.3-98.1) | 90.9 (86.5-93.9) | 92.7 (89.2-95.4) | 0.849 |
| | *CareStart*™ vs PCR | 86.7 (79.6-92.1) | 87.9 (82.1-92.4) | 84.1 (77.9-88.8) | 90.0 (85.2-93.4) | 87.4 (83.1-90.9) | 0.743 |

Key: *PPV = positive predictive value; NPV = negative predictive value; PCR = polymerase chain reaction; CI = confidence interval*

## 4.4. Kelch 13 mutation

### 4.4.1. *Plasmodium falciparum* k13 gene mutation

A total of 275 *P. falciparum* positive samples were processed to be sequenced for identification of mutations in the k13 gene associated with artemisinin resistance. Of these, 227 samples passed quality control and successfully sequenced and analyzed for resistance markers detection. Among the samples analyzed, 18.9% (43/227) carried mutations in the *pfk13 gene* (Table 10).

Table 10: Details of samples analyzed for k13 gene sequencing by region and study site in selected health facilities of Ethiopia from 2021-2023

| **Region** | **Study site** | **No of samples analyzed (n)** | **No of samples passed controls** | **Wild type (n)** | **Mutant (n)** |
|---|---|---|---|---|---|
| Amhara | AHC | 45 | 37 | 22 | 15 |
| | CHC | 19 | 16 | 14 | 2 |
| | HHC | 47 | 38 | 27 | 11 |
| | Sub total | 111 | 91 | 63 | 28 |
| Gambella | GGH | 42 | 35 | 32 | 3 |
| | GPH | 104 | 86 | 76 | 10 |
| | KHC | 10 | 8 | 8 | 0 |
| | Sub-total | 156 | 129 | 116 | 13 |
| Southwest | GSH | 4 | 4 | 2 | 2 |
| | UHC | 4 | 3 | 3 | 0 |
| | Sub total | 8 | 7 | 5 | 2 |
| Total | | 275 | 227 | 184 | 43 |

In total, 13 single nucleotide polymorphisms (SNPs) were identified, comprising 10 non-synonymous mutations A675V, G596E, H588Y, I265S, I402R, I406R, R539T, R622I, T587I, and E606K and three synonymous mutations D584D, I587I, and N585N. Notably, three of the non-synonymous mutations R622I, A675V, and R539T are recognized by the World Health Organization (WHO) as validated *k13* markers of artemisinin resistance. The Pfk13 R622I mutation was detected in 25 out of 227 sequenced samples (11.0%) collected across three regions

of Ethiopia. Of these 25 cases, 14 (56%) were from Andasa Health Center, one (4%) from Chis Abay Health Center, and seven (28%) from Hamusit Health Center, all located in the Amhara region. Additionally, one case (4%) was from Gambella General Hospital in the Gambella region, and two cases (8%) were from Gebretsadik Shewa Hospital in the Southwest region. The Pfk13 A675V mutation was identified in two samples (0.9%), both from Gambella General Hospital. The Pfk13 R539T mutation was detected in one sample (0.44%) from Gambella Primary Hospital, also in the Gambella region.

The remaining seven non-synonymous Pfk13 mutations were detected at low frequencies across the study sites. G596E was identified in one out of 227 samples (0.44%), while T587I and H588Y were detected in two (0.88%) and three (1.32%) samples, respectively all from Gambella Primary Hospital in the Gambella region.

In the Amhara region, I265S and I402R were each found in two samples (0.88%) from Hamusit Health Center. I406R was detected in one sample (0.44%) from Chis Abay Health Center, and E606K was identified in one sample (0.44%) from Andasa Health Center.

The three synonymous Pfk13 mutations D584D, I587I, and N585N were each detected in one out of 227 samples (0.44%), all originating from Gambella Primary Hospital in the Gambella region. The prevalence of the wild-type (non-mutated) Pfk13 allele across all analyzed samples was 81.1%.

Importantly, four non-synonymous mutations G596E, H588Y, I406R, and T587I are novel and have not been previously reported in the literature, suggesting potential emerging variants that warrant further investigation (Table 11).

Table 11: K13 gene mutation status by study site in selected health facilities of Ethiopia from 2021-2023

| Sr. No | pfk13 gene mutation detected | Region | | | | | | | | Total | Synonymous/Nonsynonymous |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Amhara | | | Gambella | | | Southwest Ethiopia | | | |
| | | AHC | CHC | HHC | GPH | GGH | KHC | GSH | UHC | | |
| 1 | A675V | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | NSY* |
| 2 | D584D | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | SYN** |
| 3 | G596E* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | NSY |
| 4 | H588Y | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | NSY |
| 5 | I265S | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | NSY |
| 6 | I402R | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | NSY |
| 7 | I406R | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | NSY |
| 8 | I587I | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | SYN |
| 9 | T587I | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | NSY |
| 10 | N585N | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | SYN |
| 11 | R539T | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | NSY |
| 12 | R622I | 14 | 1 | 7 | 0 | 1 | 0 | 2 | 0 | 25 | NSY |
| 13 | E606K | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | NSY |
| Total | 13 | 15 | 2 | 11 | 10 | 3 | 0 | 2 | 0 | 43 | |

NSY* (Non-Synonymous), **SYN (Synonymous),

The details of amino acids representing each SNPs are described in annex VI.

### 4.4.2. Regional variation of *pfk13 gene* mutation

In the Amhara Regional State, 30.8% (28/91) of the sequenced samples carried non-synonymous Pfk13 mutations, including I265S, I402R, I406R, R622I, and E606K.Again, 65% (28/43) of all mutated samples were from Amhara region. Among the study sites in this region, Andasa Health Center accounted for the highest proportion, contributing 53.6% (15/28) of all detected mutations. Notably, 22 out of the 25 total R622I mutated samples (88.0%) originated from the

Amhara region. Within this group, Andasa Health Center was the primary source, contributing 14 of the 22 R622I cases (63.6%).Among the four novel non-synonymous mutations, I406R was detected in one sample from Chis Abay health center, Amhara Regional State.

In the Gambella Regional State, pfk13 mutations were found in 10.1% (13/129) of the analyzed samples, with 76.9% (10/13) of these originating from GPH. Notably, the two WHO-approved *k13* resistance markers, A675V and R539T, were also identified in samples from Gambella region. Additionally, three of the four novel non-synonymous mutations G596E, H588Y, and T587I were detected in this region. In the Southwest Ethiopia Regional State, pfk13 mutations were detected in 28.6% (2/7) of the analyzed samples, with both carrying the R622I mutation. Notably, R622I is the only WHO-approved *k13* resistance marker identified across all three regions Amhara, Gambella, and Southwest. The Amhara region accounted for the majority (88%) of the R622I mutations, while the Southwest region showed the lowest prevalence, representing 8% (2/25) of the total R622I cases.

### 4.4.3. Co-dominance of *hrp2/3 gene* deletion and *k13 gene* mutation

Our study found that among the 31 samples carrying double-deleted *pfhrp2/3* genes, 29% (9/31) harbored the WHO-approved *k13* resistance marker R622I, while 6.45% (2/31) and 3.2% (1/31) carried the novel SNPs I402R and I406R, respectively. Notably, all these diagnostic and treatment resistant *P. falciparum* parasites were identified in samples from the Amhara region of Ethiopia. Among the 55 samples infected with mixed gene-deleted parasites (*pfhrp2+/pfhrp3-*), 14.5% (8/55) carried the *pfk13* R622I mutation, and 1.8% (1/55) harbored the E606K mutation. Except for one sample, all these mixed infections were detected in the Amhara region, northwest Ethiopia.

Nine individuals including one from CHC, three from HHC, and five from AHC in Northwest Ethiopia were found to be infected with *P. falciparum* strains harboring both *hrp2/hrp3 dual gene* deletions and the R622I mutation. Similarly, eight other participants two from HHC, five from AHC, and one from GGH were infected with parasites showing a mixed *hrp2+/hrp3-* deletion in combination with the R622I mutation.

# CHAPTER FIVE DISCUSSION

## 5.1. Placental malaria prevalence

The majority of the study participants (47.6%) were aged 24 years or younger, indicating a predominantly younger age group. Additionally, 30.8% of the participants were primigravidae. Our study found a placental malaria prevalence of 1.4% (13 out of 922) using the loop-mediated isothermal amplification (LAMP) diagnostic method, while placental blood smear microscopy detected no cases (0%) among the 922 pregnant women who delivered in selected areas of Ethiopia. Of the participants who tested positive for placental malaria, 69.2% were multigravidae. This finding contrasts with the commonly observed pattern in the literature, where primigravidae are typically more susceptible to malaria. This increased susceptibility in primigravidae is attributed to the lack of acquired immunity against placental malaria, particularly the absence of antibodies targeting VAR2CSA, a protein that enables malaria parasites to adhere to the placenta (Kassie *et al*., 2024; Berhe *et al*.,2023; Fried & Duffy., 2017). In our study, however, the higher number of infected multigravidae may be explained by the overall distribution of participants: 69.2% of the study participants were multigravidae. The other possible reasons might be due to infection with a genetically different strain of *P. falciparum*, incomplete immunity which may decline over time especially if they have not had repeated malaria exposure between pregnancies and immune suppression due to co-existing health issues. Even though they are generally more protected than primigravidae, the risk is not eliminated. On the other hand, the absence of positive cases by microscopy, despite detection by LAMP, may be attributed to the lower sensitivity and specificity of microscopic diagnosis compared to the more sensitive LAMP method. Peripheral blood diagnosis in our study revealed that only 0.3% (3 out of 922) of participants had detectable *Plasmodium* infections. The occurrence of placental infection in the absence of peripheral parasitemia may be explained by the expression of the VAR2CSA protein on infected red blood cells. This protein facilitates the binding of infected erythrocytes to chondroitin sulfate A (CSA) in the placenta, enabling sequestration and evasion from peripheral circulation. Additionally, the absence of peripheral parasitemia might result from prior clearance of peripheral parasites due to the host immune response or effective antimalarial treatment, which may have cleared circulating parasites while leaving placental sequestration intact (Berhe *et al.,*2023). The placental malaria prevalence observed in our study (1.4% using

LAMP) is considerably lower than figures reported in other countries. For instance, Cardona-Arias *et al*. reported a prevalence of 27.7% using thick blood smear and 13.2% using qPCR in northwestern Colombia (Cardona-Arias *et al*., 2022; 2024). Similarly, higher prevalence rates were documented in several African countries. In Nigeria, Okoro *et al*. reported a prevalence of 21.3% using microscopy, while Oranuka *et al*. found a prevalence of 38.4% using histopathological analysis. In Ghana, Mwin *et al*. reported a 7% prevalence using microscopy (Okoro *et al*., 2023; Oranuka *et al*., 2024; Mwin *et al*., 2021). When we compare our findings with previous studies conducted in Ethiopia, our prevalence report is lower than that reported by Solomon *et al*. (3.9% using microscopy), Limenih *et al*. (2.3% using microscopy), and Tamir *et al*. (10.9%) (Solomon *et al*., 2020; Limenih *et al*., 2021; Tamir *et al*., 2023). One possible reason for this lower prevalence could be that our study included both symptomatic and asymptomatic participants that might reduce the overall burden of infection. Among participants who tested positive for placental malaria, 61.5% were from the Gambella region. This may be attributed to the fact that Gambella experiences stable or year-round malaria transmission, which increases the risk of exposure (Assefa *et al*.,2025). Regarding the species distribution among *Plasmodium*-positive cases, out of the thirteen positive samples, eight were *P. falciparum* infections, while five were *P. vivax* infections. Although *P. falciparum* is the well-established cause of placental malaria due to its sequestration characteristics, we detected *P. vivax* in 38.5% (5/13) of the positive placental blood samples. This finding in lined with studies conducted by Tamir *et al*. and Solomon *et al*. in Ethiopia, which reported *P. vivax* prevalences of 12% (6/50) and 2.2% (5/9), in placental blood samples respectively (Solomon *et al*., 2020; Tamir *et al.,* 2023). Similarly, a study by Cardona-Arias *et al*. in Colombia reported a *P. vivax* prevalence of 43.9% (25/57) in placental blood samples (Cardona-Arias *et al*., 2024). These findings suggest that *P. vivax* may be an emerging contributor to severe malaria, capable of causing anemia, acute respiratory distress, multiple organ failure, and other pathological effects. In contrast to our findings and the aforementioned studies, a study by Limenih *et al*. reported only *P. falciparum* infections in placental blood samples (Limenih *et al.*, 2021).

## 5.2. Deletion of *pfhrp2* and *pfhrp3* genes

The primary impact of *pfhrp2/3* gene deletions is the generation of false-negative results by HRP2-based RDTs hence these genes encode proteins (HRP2 and HRP3) that are targeted by the

most rapid diagnostic tests (RDTs) for *P. falciparum* ( Golassa *et al*.,2020; Grignard *et al*.,2020; Kong *et al*.,2021; McCaffery *et al*.,2021; Rogier *et al*.,2022; Fola *et al*.,2023).

For this experiment, we used the Bio-Rad QX200 droplet digital PCR (ddPCR) system, which offers enhanced sensitivity and specificity for detecting low-density infections compared to nested PCR (nPCR). We targeted three genomic regions hrp2 exon 1, hrp2 exon 2, and hrp3 and quantified their relative concentrations using the *tRNA ligase* gene as a reference following the methodology described by Kamaliddin *et al.* (2024).

Among 207 samples analyzed, we found 15% (31/207) full gene deletions (*hrp2-/hrp3-*), 26.5% (55/207) mixed infections (*hrp2+/hrp3-*), 2.9% (6/207) partial deletions (*hrp2-/hrp3+*) and 55.6% (115/207) wild type (*hrp2+/hrp3+*) infections. The overall prevalence of *pfhrp2/3 gene* deletions was 44.4% (92/207), indicating deletion of at least one of the two genes, *hrp2* or *hrp3*. These results highlight both the prevalence and the diversity of *hrp2/hrp3* deletion patterns within the studied population.

Our findings indicate a lower prevalence of *Pfhrp2* and *Pfhrp3 gene* deletions compared to recent studies conducted in Ethiopia. For example, Getie et al. (2024) reported *Pfhrp2* deletions in 63.6% (21/33) of samples, *Pfhrp3* deletions in 84.9% (28/33), and *dual hrp2/hrp3* deletions in 60.6% (20/33). Similarly, Mekonen *et al.* (2024) reported *Pfhrp2* deletions in 27.3%, *Pfhrp3* deletions in 30.5%, and dual deletions in 13.2% of samples. Again, recent study conducted in the Amhara region by Adamu *et al*. (2025) reported 51.4% dual *pfhrp2/3* deletions, 2.2% *pfhrp2* deletions and 34.6% *pfhrp3* deletions.

Our finding have higher *hrp2/3 gene* deletion prevalence than some previous studies conducted in Eritrea by Mihreteab *et al*., (2021) which had overall prevalence of *hrp2-*, *hrp3-*, and dual *hrp2/3*-deleted parasites 9.4%, 41.7% and 7.6%, respectively, in Tanzania (3 *pfhrp2* and 2 *pfhrp3*) (Thomson *et al*., 2019) and ( 9 *pfhrp2,* 6 *pfhrp3 and no* dual *pfhrp2/3* deleted) parasites were detected (Rogier *et al*.,2024).Again in Uganda (7 *pfhrp2* and 2 *pfhrp3*) were detected (Thomson *et al*., 2019).In Ethiopia study by Rogier *et al*.,2022 reported only three dual *pfhrp2/3* deleted parasites, and Mekonen *et al*.,2024 reported 33 (13.3%) dual deletion of *hrp2* and *hrp3* genes.

The observed differences in gene deletion prevalence across studies may be attributed to host immune responses, methodological variations, and geographical locations. Notably, our study

participants were symptomatic and asymptomatic pregnant women who possibly had suppressed immune systems, and our study areas are in high, middle and low transmission set ups, and we used ddPCR for analyzing samples which offers higher sensitivity and specificity, particularly for detecting low-density infections and distinguishing between mixed and monoclonal infections. All the above listed studies employed conventional nested PCR (nPCR) or quantitative PCR (qPCR) assays to detect *pfhrp2* and *pfhrp3* deletions. These classical methods have inherent limitations in accurately characterizing gene deletions that the absence of an amplification band is interpreted as a gene deletion, false-negative results can occur due to suboptimal PCR conditions or low parasite densities, where stochastic amplification failure is more likely. Additionally, in polyclonal infections involving both wild-type and deletion-carrying parasites, the presence of the wild-type clone can mask the deletion, leading to misclassification (Vera-Arias *et al.*, 2022).

Another contributing factor could be differences in sample size. The studies conducted in northwest Ethiopia by Getie *et al*. (2024) included relatively small sample numbers (33) which may not be representative and could lead to overestimation and underestimation of gene deletion prevalence.

A previous study conducted in southwestern Ethiopia by Vera-Arias et al. (2022) using ddPCR identified single *hrp2* deletions among 47 analyzed samples. In contrast, our study detected substantially higher levels of gene deletions. This discrepancy might be attributed to differences in sample size, host immune responses, and geographical differences of study sites. Notably, our participants were pregnant women recruited from areas with varying malaria transmission intensities high, medium, and low.

A recent ddPCR-based study by Kamaliddin et al. (2024) reported *pfhrp2/pfhrp3* dual deletions in 17.16% of samples, *pfhrp2* deletions in 30.47%, and *pfhrp3* deletions in 69.1% of the analyzed parasites which was higher than our report. These differences might be attributed to variations in study populations, geographical regions, transmission seasons, and differences in parasite population.

Despite these variations, both our study and previous ddPCR-based investigations consistently reveal a concerning prevalence of *pfhrp2* deletions in Ethiopia, underscoring the critical need for ongoing molecular surveillance to guide malaria diagnostics and control efforts.

The World Health Organization recommends switching to a non-HRP2 RDT for *P. falciparum* clinical case diagnosis when the prevalence of *pfhrp2* deletions ≥5% to return false-negative results (WHO, 2016). Therefore, all the above evidence and findings from this report, which indicate high rates of *pfhrp2*/*pfhrp3* gene deletions, support the continued use of LDH-based RDT kits for malaria diagnosis.

The impact of *hrp2/3 gene* deletion on infected pregnant women includes: false-negative malaria diagnosis which resulted undiagnosed malaria; this means malaria could not diagnosed and treated timely and allowed the infection to progress, increasing the risk of severe complications for both the mother and the fetus (Berhe *et al.*,2023; Takem and Alessandro,2013) like maternal anemia, placental malaria, low birth weight (LBW) and preterm birth , stillbirth and miscarriage (Mlugu *et al*.,2020 ; Umbers *et al.*,2011; CDC,2024). Finally, it challenges the surveillance and control programs making difficult to accurately assess disease prevalence and target interventions (Kamalidinn *et al.*,2024) and need for alternative diagnostic strategies in areas with a high prevalence of *hrp2/3 gene* deletions.

## 5.3. Evaluation of the diagnostic performance Pf/Pv/PLDH based RDT

To deliver healthcare services at the community level, Ethiopia launched a health extension program in 2004 (Banteyerga, 2011). One of the program's main goals has been the prevention and treatment of malaria.

The prevalence of both *P. falciparum* and *P. vivax* in the present study was high because; (i) data were collected from participants who fulfilled the clinical case definition (i.e. febrile in the last 48 hours and coming from malaria endemic area) (FMOH, 2022), (ii) data were collected during the major malaria transmission season (September to December), (iii) there was malaria epidemics in the study area at the time of data collection, and (iv) we used sensitive molecular diagnostic test (PET-PCR), in addition to microscopy and RDTs. In Ethiopia *P. falciparum* is known to account for 70% of malarias, while the remaining 30% are due to *P. vivax*, but this figure may vary by season and locality (FMOH, 2022). Hence, the prevalence of *P. vivax* was higher than that of *P. falciparum* in the present study (Table 7). This is supported by recent literature reporting a 7-fold national increase in *P. vivax* malaria cases from 100,000 in 2018 to 730,000 in 2022, unlike the steady increase in *P. falciparum* cases (Zhou *et al*.,2024). Similarly, a higher prevalence of *P. vivax* than *P. falciparum* was reported in a previous study in Ethiopia

(Woldesenbet *et al*., 2024). The shift in the proportion of the two species is multifactorial, with climate change and relapse might being primary contributors (Tesfaye *et al*.,2011). A recent report suggested the expansion of *P. vivax* populations that could infect both Duffy-positive and Duffy-negative individuals in sub-Saharan Africa, including Ethiopia, contributing to the increasing proportion of *P. vivax* compared to *P. falciparum* (Twohig *et al.*, 2019). However, to provide a definitive justification, large-scale studies are recommended to assess the role of vectors, human factors, the environment and climate-related drivers contributing to the distribution of each *Plasmodium* species.

The present study revealed that, compared to *CareStart*™, the BIOCREDIT Pf/Pv RDT showed good performance in detecting *P. falciparum*, with only 5 false-negative (FN) results compared to 25 false-positive (FP) results. This was inconsistent with a prior study in Djibouti (*Abdi et al*.,2024). The higher FP rate might be due to *hrp2/3 gene* deletions missed by the *CareStart*™ but detected by the BIOCREDIT RDT (Alemayehu *et al*., 2021; Feleke *et al*., 2021, Molina-de la *et al*.,2022; Mekonen *et al*.,2024). The BIOCREDIT RDT was also more sensitive than the previously used *CareStart*™ in detecting *P. falciparum* when evaluated against microscopy (75.4% vs. 39.6%) and PCR (55.6% vs. 37.6%) reference tests, which was in line with a previous study from Burundi (Niyukuri *et al*.,2022). However, BIOCREDIT RDT's sensitivity (55.6%) against PCR was less than the WHO's minimal cut-off value (≥75%) (WHO,2019). Additionally, it fell short of sensitivity rates observed in other studies, such as 79.9% in Burundi (Niyukuri *et al*.,2022) , 88.2% in Djibouti (Abdi Moussa *et al*.,2024), 87.8% in Uganda (Bahk *et al*.,2018), 69% (Feleke *et al*.,2022) and 89% in Ethiopia (Alemayehu *et al*.,2020). These discrepancies may be due to factors such as parasite densities, RDT manufacturers, storage and transportation conditions, and operator skills (Coldiron *et al*.,2019; Ali *et al*.,2021; Tetteh *et al*.,2021; Albertini et al.,2012). Although we did not quantify parasite density in this study, we anticipated that most individuals had minimal parasitemia because the study area was under a malaria elimination program. Hence, 5.6% FN results were reported compared to PCR (Table 8). This might have occurred due to the higher detection limit of malaria RDTs (>100 parasites/µl) compared to that of the reference PCR test (<5 parasites/µl) (Rubio *et al*.,1999). The impact of operator skill on RDT performance was demonstrated in Djibouti, where retraining and close supervision increased the sensitivity to identify *P. falciparum* from 69.8% to 88.2% ($p<0.01$) (Abdi *et al*.,2024).

The high specificity (98.9%) and PPV (97.0%) were substantiated by the fact that, when compared to PCR, only two samples had FP results with BIOCREDIT. This was consistent with earlier findings (Abdi *et al*.,2024; Bahk *et al*.,2018; Park *et al*.,2020). When compared to microscopy, the FP was higher (5.0%), which could be because of the limited sensitivity of microscopy itself (5-20 parasites/µl compared to a detection limit of <5 parasites/µl by PCR) (Rubio et al.,1999; Bruce-Chwatt LJ et al.,1984). Moreover, *P. falciparum*-infected red blood cells are sequestered in the placenta. So, some *P. falciparum* infections might have been overlooked by microscopy but detected by the BIOCREDIT RDT. It is also crucial to remember that individuals with rheumatoid factor (Grobusch *et al*.,1999) or those with persistent pLDH following treatment (Tahar *et al*.,2013) could have experienced BIOCREDIT false positive results.

When compared to PCR, the BIOCREDIT RDT's sensitivity (86.7%) and NPV (90.9%) for detecting *P. vivax* were above the minimum sensitivity limit of RDTs (WHO,2019), but lower than findings of earlier studies (Abdi *et al*.,2024; Park *et al*.,2020). The sensitivity was similar to that of the *CareStart*™ (86.7%), implying that either RDT could be recommended for the diagnosis of *P. vivax* infections in Ethiopia. However, the BIOCREDIT RDT is more appropriate as both *P. falciparum* and *P. vivax* are co-endemic in most parts of the country (FMOH,2022).
In the present study, *CareStart*™ (13 samples) detected higher number of mixed infection compared to BIOCREDIT (3 samples). Differences in cross-reactivity, and hence specificity between the two RDTs might bring this variation. Biocredit is 100% specific in detecting *P. vivax* than *Carestart* having a specificity of 97.5% (source: manufacturers' leaflets), possibly contributing for the higher report of *P. vivax* mono-infection as well as mixed infection by *Carestart*.

## 5.4. *Plasmodium falciparum* keltch 13 gene mutation

Effective antimalarial drugs are essential for malaria control and elimination. Continuous monitoring of their efficacy is needed to update treatment policies in malaria-endemic countries, and to ensure early detection of, and response to, drug resistance (WHO, 2025).
We thought to determine if any SNPs in the K13 gene that were associated with artemisinin resistance (ART-R) were present in 275 extracted genomic DNA samples collected from eight study sites in three regions of Ethiopia. Identification of molecular markers of partial artemisinin

resistance (*pfk13* mutations) have been used as early pointers of partial artemisinin resistance to inform early response and containment efforts to mitigate the inevitable reduction in effectiveness of these antimalarial drugs.

### 5.4.1. Distribution of pfk13 mutation

In this study, bidirectional Sanger sequencing was performed on purified PCR products from 227 genomic DNA samples. Among these, 81.1% (184/227) were identified as wild type for the *pfk13* gene, while 18.9% (43/227) harbored *pfk13* mutations. A total of 13 distinct mutations were detected: 10 (76.9%) were non-synonymous (A675V, G596E, H588Y, I265S, I402R, I406R, R539T, R622I, T587I, and E606K), and 3 (23.1%) were synonymous (D584D, I587I, and N585N). Notably, three mutations A675V, R622I, and R539T are recognized by the WHO as validated markers of artemisinin resistance. Of the thirteen *pfk13* gene mutations identified, four were novel (G596E, H588Y, I406R, and T587I), and were detected exclusively in the Gambella and Amhara regions of Ethiopia. Among the WHO-approved artemisinin resistance markers, the East African *pfk13* mutation R622I was the most prevalent, accounting for 58.1% (25/43) of the mutated samples. The *pfk13* R539T mutation, also WHO-approved and reported here for the first time in Ethiopia and Africa, was found in 2.3% (1/43) of the mutated samples. The third WHO-recognized resistance marker, A675V, was detected in 4.6% (2/43) of the mutated samples.

To assess previous reports of WHO-approved *pfk13* resistance markers identified in this study, the R622I mutation has previously been detected at low frequency in neighboring countries. It was identified in a single sample (1.4%) in Djibouti (Papa *et al*., 2024) and has also been reported in Tanzania (one sample) and Eritrea (11.9%) (Mihreteab *et al*., 2024; Juliano *et al*., 2024). In Ethiopia, *pfk13* R622I was first reported by Bayih *et al*. (2016) (2.4% mutations) in Northwest Ethiopia. Since then, its presence has been confirmed in the Amhara, Tigray, and Gambella regions (8% mutations) (Fola *et al*., 2023), with further evidence from Amhara and Tigray (31 samples) (Kamaliddin *et al*., 2024), and Eastern Ethiopia (four samples) (Jeang *et al*., 2024). A more recent report by Brhane *et al*. (2025) has expanded this distribution (15.7% mutation) to include the Amhara, Somali, Gambella, and Oromia regions.

Another WHO-approved *pfk13* resistance marker, R539T strongly associated with artemisinin resistance and delayed parasite clearance was detected for the first time in Ethiopia in this study.

Similarly, the A675V mutation was also identified for the first time in Ethiopia, following a recent report by Brhane *et al*. (2025), marking the second confirmed detection of this mutation in the country.

The G596E, H588Y, I406R, and T587I mutations identified in this study represent novel *pfk13* variants, detected exclusively in the Gambella and Amhara regions of Ethiopia. These single nucleotide polymorphisms (SNPs) have not yet been functionally characterized to assess their potential role in artemisinin (ART) resistance. However, documenting their presence is important, given the possibility that such mutations could be selected under increasing drug pressure and potentially evolve into markers of ART resistance in Africa. Previous studies have suggested that *pfk13* mutations found in Africa may have an independent origin from those observed in Southeast Asia, forming distinct genetic clusters. This supports the expectation that additional resistance-associated mutations could emerge independently within African parasite populations (Pacheco *et al*., 2019).

The presence of *pfk13* mutations in *P. falciparum* has been associated with delayed parasite clearance and, in some cases, treatment failure in infected individuals, including pregnant women (Straimer *et al.*, 2021). Although delayed clearance does not always result in outright treatment failure, it allows the parasite to persist longer in the maternal bloodstream. This prolonged parasitemia can increase the risk of recrudescence, extended illness, and a higher likelihood of severe malaria, all of which contribute to adverse pregnancy outcomes (Berhe *et al.*,2023).

If artemisinin-based combination therapies (ACTs) become less effective due to the emergence of *pfk13* mutations, reliance on alternative interventions or the development of new treatment strategies becomes increasingly critical particularly for vulnerable populations such as pregnant women, who require consistent and effective malaria protection (WHO, 2023). The detection of *K13* mutations reinforces the urgent need for continuous molecular surveillance to monitor the emergence and spread of artemisinin resistance, enabling timely public health responses (Molina-de la Fuente *et al*., 2023).

### 5.4.2. Co-dominance of *pfhrp2/3 gene* deletion and *pfk13 gene* mutation

Our study revealed that from the total 31 samples which carried double deleted *pfhrp2/3 genes*, 29% (9/31), 6.45% (2/31) and 3.2% (1/31) of them carry WHO approved k13 resistance marker R622I, new SNPs I402R and I406R respectively. This finding is in line with the recent study from Southeastern Sudan that showed from 22% *pfkelch13* R622I mutated samples 10.7% of them were *hrp2/3* gene deleted (L'Episcopia *et al.*,2025), same country Ethiopia that showed 8.2% of *K13* 622I parasites also carried the *pfhrp2/3* deletions (Fola *et al*.,2023), and 90.3% of samples (28 of 31) containing an R622I SNP also had an *hrp3* deletion (Kamaliddin *et al*.,2024).

In addition to this, our study showed that from the total 55 samples infected with mixed *(hrp2+/hrp3-) gene* deleted parasites, 14.5% (8/55) carried R622I pfk13 mutations and 1.8% (1/55) sample carried E606K pfk13 mutations. Almost all these diagnostic and treatment resistant parasites are from the Amhara regions of Northwest Ethiopia.

The most important information in this study is that, nine participants one from CHC, three from HHC, and five from AHC all from Northwest Ethiopia, were infected with *P. falciparum* parasites carrying both *hrp2/hrp3* double deletions and the R622I mutation. Additionally, eight participants, two from HHC, five from AHC, and one from GGH were infected with parasites exhibiting a mixed *hrp2+/hrp3*- deletion along with the R622I mutation. This co-dominance may be due to the long-term, widespread implementation of both RDT-based diagnosis and ACTs in the country and the finding will strengthen the evidence that both *hrp2/3* deletions and *k13* mutations may arguably have similar roles in providing *P. falciparum* resistance to ACT. However, the exact genetic and evolutionary mechanism of this coexistence remains to be clarified (Nair *et al*.,2022; Kamaliddin *et al*.,2024).

*Limitations of the study:*

Correlation of the RDT results with the *hrp2/3 gene* deletion results was not done due to some missed data on RDT examination of participants. As therapeutic efficacy was not evaluated in this study, it is not possible to determine the impact of the new pfk13 mutations reported on parasite clearance half-life.

# CHAPTER SIX CONCLUSIONS

All placental smear samples in this study were negative by microscopic examination which showed that all the cases were submicroscopic. Therefore, molecular diagnosis is important for the detection of placental malaria to know the actual burden of malaria in pregnancy and to intervene accordingly. In this study, *P.vivax* and asymptomatic infections showed significant contribution for placental malaria that could be a big challenge for the malaria control/elimination program, and this should be considered for each intervention taken against malaria.

An overall high number of *pfhrp2/3* gene deletions were detected in this study. Therefore, the continued use of the current LDH-based RDT kits by the Ministry of Health is recommended for malaria diagnosis in the country, especially in the Northwest Amhara region, where nearly all cases with double gene deletions were identified.

The BIOCREDIT Pf/Pv RDT demonstrated superior performance in diagnosing *P. falciparum* compared to the previously in-use *CareStart*™ Pf/Pv (HRP2/pLDH) and for *P. vivax*, its performance was comparable to that of *CareStart*™. Continuous nationwide monitoring of the performance of LDH based RDT kit is important to support malaria control and elimination program in Ethiopia.

WHO approved pfk13 resistance markers R622I, A675V and R539T detected in this study are a major concern in Ethiopia, as they are associated with delayed artemisinin-based combination therapies clearance. R622I mutations, detected from samples in three regions (Amhara, Gambella and Southwest) indicate a broader geographic spread of this resistance mutation within the country. The co-dominance of diagnostic and treatment resistance genes; Parasites with both K13 R622I mutation and *pfhrp2/3 gene* deletion specially in Northwest Amhara region of Ethiopia are big challenges for malaria control and elimination program as these gene-deleted and mutated parasites may have a selective advantage over their wild-type counterparts, or potentially be imported to other regions. Additionally, the novel mutations reported in this study suggest that new resistance markers may be emerging in the country. These markers need to be closely monitored to understand their specific functional roles in parasite resistance to ACTs, as well as to track their frequency over time in Ethiopia.

Finally, continuous and rigorous genomic surveillance of *pfhrp2/3* deletions and *pfk13* mutations is crucial for monitoring the emergence and spread of resistance, and for supporting malaria control and elimination efforts in Ethiopia.

In summary, both *K13* mutations and *hrp2/3* gene deletions pose significant threats to the health of infected pregnant women. *K13* mutations compromise the efficacy of frontline antimalarial treatments, potentially resulting in more severe and prolonged infections. Meanwhile, *hrp2/3* deletions undermine the effectiveness of rapid diagnostic tests, leading to missed diagnoses and delayed treatment both of which can have serious consequences for maternal and fetal health. Continuous genomic surveillance, timely adaptation of treatment protocols, and the development and deployment of alternative diagnostic tools are essential to mitigate these risks.

# CHAPTER SEVEN RECOMMENDATION

For Ministry of Health/Regional Health Bureaus

- ✓ Molecular diagnosis should be considered for the detection of placental malaria to save lives of mothers and newborns
- ✓ Asymptomatic and *P.vivax* infections should be emphasized for each intervention activities on malaria control and elimination program
- ✓ Health facilities should promote using LDH-based RDTs for malaria diagnosis
- ✓ Continuous monitoring of LDH based RDT performance is needed

For researchers,

- ✓ Nationwide study of *hrp2/3 gene* deletion and *pfk13* mutations using ddPCR and whole genome sequencing, respectively
- ✓ Impact of novel and candidate mutations on parasite survival and fitness in vitro should be studied

For health facilities,

- ✓ All health posts and those health centers that used RDT before should use PLDH based RDTs
- ✓ Diagnose pregnant women for *plasmodium* infection at each ANC visit

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# CHAPTER NINE ANNEXES

## Annex I. Participant information sheet

**Study Title:** "Prevalence of Placental Malaria, *Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene* Deletions and *Kelch-13 Gene* Mutations among Pregnant Women in Selected Health Facilities of Ethiopia".

**Name of Investigator:** Banchamlak Tegegne

**Name of Organization**: Bahir Dar University

**Introduction:**

My name is Banchamlak Tegegne; PhD student from Bahir Dar University and I am ready to do my research for partial fulfillment of my PhD dissertation on prevalence of placental malaria, hrp2/3 *gene* deletion and *pfk13 gene* mutations in pregnant women.

**Purpose**

I have planned to conduct a study with the objective to assess Prevalence of Placental Malaria, *Plasmodium Falciparum's Histidine Rich Protein 2/3 Gene* Deletions and *Kelch-13 Gene* Mutations among Pregnant Women in Selected Health Facilities of Ethiopia. The knowledge gained from this work is believed to help malaria program to reduce the morbidity and mortality associated with pregnancy malaria.

**Procedure** For this study placental blood and venous blood samples will be collected and examined for the presence plasmodium species. You are selected as one of the study participants. If you are willing to participate you are kindly requested to give requested samples to the sample collectors.

**Risk and /or Discomfort;** by participating in this research project, you may fill discomfort due to wasting your time about 10-15 minute for sample collection and for provision of information. No risk in participating in this study project.

**Benefits**

If you participate in this research and if you are found to have malaria parasitic infection, you may be able receive appropriate treatment more quickly. We will facilitate diagnosis and

medication in the health facilities. More over your participation helps as to assess prevalence of placental malaria, *hrp2/3 gene* deletion and *Kelch-13 Gene* Mutations whuch will finally used for designed malaria control and elimination program to reduce the morbidity and mortality associated with pregnancy malaria.

**Compensation for participation:** There is no any compensation/money/ given for your participation.

**Confidentiality:** The information collected from you will be kept confidential. It will be stored in a file using codes without your name. And it will be used only for this particular research not revealed to anyone except the principal investigator.

**Voluntary to participation (right to refusal)**

You have full right to refuse from participating in this research you can refuse to give specimen.

**Person to Contacts**

This research will be reviewed and approved by the Bahir Dar University, College of Science Research ethics committee. If you want to know more information, you can contact the following individual and you may ask what you want.

**Investigator:** Banchamlak Tegegne

Mobile: +2510931708006

E-mail Banteg92@gmail.com

Address: Bahir Dar University

## Annex II. Consent form

This agreement form is for participation in the assessment of prevalence of placental malaria, *pfhrp2/3 gene* deletion and drug resistance molecular markers; so, if you are volunteer to participate you have to put your signature below after reading it.

1. I knew the purpose of the study and I knew that I can get the researcher at any time.

2. I knew that collected sample will be for research purpose only.

3. I knew that any information provided for research and any results will be kept confidentially.

4. I knew that there is no compensation will be given for participation.

5. I knew I have the right to refusal.

6. I agree to participate on the study

Code no. of participant ----------

Signature of participant----------

Date of agreement ----------

## Annex III. Questionnaire

**Questionnaire on Socio-demographic and clinical characteristics of the respondent**

This questionnaire contains two parts that Socio-demographic and clinical characteristics. For each question, please give the answer carefully with patience. Your name is not included in the questionnaire and your answer will not be exposed to anyone.

Questionnaire no._____ Name of interviewer _____Date_____

1. **Socio-demographic characteristics**
   1.1.Code no. of participant _____.
   1.2.Age _____
   1.3.Address, Kebele _____

**2. Clinical characteristics of the patient**

2.1.Duration of your pregnancy in week/month? _____

2.2.Is your pregnancy for the first time? _____

2.3.How many children you have? _____

2.4.Have you had laboratory confirmed malaria in the previous six months? A. Yes B. No

When? Please specify_____

2.5.Do you travel to other malaria endemic areas in the last 30 days A. Yes B. No

2.6.Do you have fever that repeats with 48 hours? A. Yes B. No

2.7.Did you have shivering and chills? A. Yes B. No

2.8.Do you have severe headaches? A. Yes B. No

2.9.Do you have vomiting? A. Yes B. No

3. Laboratory diagnosis results

3.1.Microscopic/LAMP results of peripheral blood----------

3.2.Microscopic results of Placental blood----------

3.3.LAMP results of placental blood----------

**Amharic Version of patient information**

***መግቢያ***

እኔ ባንቻምላክ ተገኘ የተባልኩ በባህር ዳር ዩኒቨርሲቲ፤ ሳይንስ ኮሌጅ የፒኤችዲ ተማሪ ስሆን የመመረቂያ ጥናቴን የእንግዴ ልጅ ዎባ ምርመራ እንዲሁም "*pfhrp2/3 gene* deletion and *pfk13 gene* mutations" በነፍሰጡር እናቶች ላይ ለመስራት ተዘጋጅቻለሁ።።

ስለሆነም እርስዎ በዚህ የመመረቂያ ጥናት ላይ እንዲሳተፉ ተጋብዘዋል።።እባኮዎ በዚህ ጥናት ላይ ከመስማማቶ በፊት ከዚህ ቀጥሎ የሚገኘዉን ንባብ በጥሞና ያንብቡ/ይመልሱ ግልå ያልሆነዉን ማንኛዉንም ሃሳብ ይጠይቁ።።እርሶ በዚህ ጥናት ላይ የሚኖርዎት ተሳትፎ ሙሉ ለሙሉ በበጎ ፈቃደኝነት ላይ የተመሠረተ ነዉ።።በዚህ ጥ ናት ዉስጥ ላለመሳተፍ ከወሰኑ በዚህ ጤና ተቃም ዉስጥ የሚሰጥዎት አገልግሎት እንደነበረው ይቀጥላል።።አሁነ ተስማምተው ኋላ ሀሳባቸውን ቢቀይሩ በትናቱ አለመሳተፍ ይችላሉ፣አገልግሎቱ ግን እንዳለ ይቀጥላል።።ለመሳተፍ የሚስማሙ ከሆነ የስምምነት ቅጹ ላይ በጹሑፍ ወይም በጣት ፊርማዎ ማስቀመጥ ይጠበቅቦታል።።።

**የጥና ተሳታፊ በመሆንዎ የሚጠበቅብዎት ነገር**

በዚህ ጥናት ለመሳተፍ ሚስማሙ ከሆነ ከሁለት ሚሊሊትር ያልበለጠ የደምና የፕላሲንታ ሙና እንደሚወሰድ እና ለጥናቱ እንዲዉል መስማማት ይጠበቅቦታል።።በተጨማሪም ለጥናቱ አስፈላጊ የሆኑ መረጃዎች ለማግኘት ስለእርሶ አንዳንድ ጥያቄዎች ሲጠየቁ መልስ መስጠት ይጠበቅበዎታል።።

**በዚህ ጥናት በመሳተፍዎ የሚፈጅብዎት ጊዜ**

የተዘጋጀዉን መጠይቅ ለመሙላት የስምምነት ቅጹ ላይ ለመፈረምና የደም ናሙና ለመስጠት 10-15 ደቂቃ ያስፈልጋል፡

**በዚህ ጥናት በመሳተፍዎ ሊያጋጥሙዎት የሚችሉ ችግሮች**

ናሙና ሲወሰድብዎ ትንሽ ፍርሃትና የህመም ስሚት ሊሰማዎት ይችላል፡፡ነገር ግን የህመም ስሚቱ ከተወሰኑ ሰዐታት በኋላ ይጠፋል፡፡

**የሕክምና መረጃ ሚስጥርን በተመለከተ**

ስለራሶ የሰጡት ማንኛዉም መረጃ ከተወሰደዉ ናሙና ላይ የተገኘዉ የላቦረቶሪ ዉጠቶች የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ፡፡የተወሰደዉም የደም ናሙና ከዚህ ጥናት ውጭ ለሊላ አላማ አይዉልም፡፡በጥናቱ ላይ የተሳታፊው ማንነት ለማንም አይገለፅም፡፡ስለእርሶ የሰበሰብናቸው መረጃዎች ሁሉ ሚስጢራዊና በስምዎ ሳይሆን በቁጥር የተመዘገቡ ናቸው፡፡የጥናቱ አባላት ብቻ ስለርስዎ የመዝገብ ቁጥርና መረጃ እንዲያዉቁ ይደረጋል፡፡

**በዚህ ጥናት በመሳተፍዎ የሚያስገኛቸዉ ጥቅሞች**

ይህ ጥናት የፔኢችዲ ዲግሪ መመረቂያ እንደመሆኑ መጠን ለተሳታፊዎች ገንዘብ አይሰጥም ነገር ግን የእርስዎ ተሳትፎ በትክክል የዎባ በሽታ በንፍሰ ጡር እናቶች ላይ እያስከተለ ያልውን ጉዳት እንድናውቅ ይረዳል፡፡

**በዚህ ጥናት ተሳታፍ በመሆንዎ ያሉዎት መብቶቼ**

በጥናቱ ዉስጥ ያሎትን ተሳትፎበ ማንኛዉም ጊዜ የማቋረጥ ሙሉ መብትዎ የተጠበቀ ከመሆኑም በላይ ራሶን ከጥናቱ በማግለሎ ምክንያት የሚቀርቦት ምንም ዓይነት የሆስፒታል አገልግሎት አይኖርም ከዚህም በተጨማሪ ጥናቱን በተመለከተ ማንኛዉንም ዓይነት ጥያቄ የመጠየቅና ገለፃ የማግነት መብት አሎት:: የላቦራቶሪ ምርመራ ዉጤቱንም በነፃ ማግኘት ይቻላል::

**ጥያቄ ካለዎ ወይም ችግር ቢያጋጥምዎት**

ይህን ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥዉ ድንገተኛ ችግሮች ወይም ጥያቄ ካሎት በሚከተለዉ አድራሻ ይጠቀሙ፡፡

ጥናቱን የሚያጠናው ስም፡ባንቻምላክ ተገኘ

ስልክቁጥር፡2510931708006

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## Amharic version of Consent form

ይህ የጥናት ተሳታፊዎች ስምምነት ቅጽ የእንግዴ ልጅ ዎባ ምርመራ እንዲሁም “placental malaria, *pfhrp2/3 gene* deletion and *pfk13 gene* mutations” በነፍሰጡር እናቶች ላይ ለሚሰራው ጥናት ተሳታፊዎች የሚሞላ ነው፡፡ በጥናቱ ተሳታፊ ለመሆነ ከዎሰኑ ፊርማዎትን አንብበው ከጭረሱ በሁዋላ መፈረም ይጠበቅበዎታል፡፡

1.የጥናቱን አላማ አውቃለሁ፤ተመራማሪዎችንም በፈለግሁት ጊዚ እንደማገኛቸው አውቃለሁ፡፡

2.የተሰበሰበው ናሙና ለዚህ ጥናት ብቻ እንደሚውል ተረድቻለሁ፡፡

3.ለጥናቱ የሰጥሁት መረጃ ሚስጥሩ የተጥበቀ እንደሚሆን አውቃለሁ፡፡

4.በዚህ ጥናት በመሳትፊ ምንም አይነት ልዩ ጥቅም እንደማላገኝ አውቃለሁ::

5.በዚህ ጥናት ያለመሳተፍ መብት እንዳለኝ አውቃለሁ::

6.በዚህ ጥናት ለመሳተፍ ፈቃደኝ ነኝ ::

የተሳታፊ መለያ ቁጥር----------

የተሳታፊ ፊርማ----------

ስምምነት የተወሰደበት ቀን ----------

**Amharic version of Questionnaire**

**ስለ ራስዎና ስለ ህመም ስሜትዎ መረጃ ለመሰብሰብ የተዘጋጀ መጠይቅ ቅጽ**

መጠይቁ ሁለት ክፍሎች ይኖሩታል፡የመጀመሪያው ስለራስዎ ምላሽ የሚሰጡበት ሲሆን ሁለተኛው ደግሞ ስለህመም ምልክትና ስሜትዎ የሚገልጹበት ነዉ::ለእያንዳንዱ ጥያቄ በጥንቃቄና በታማኝነት ያመኑበትን ይመልሱ::የእርስዎ ማንነት በመጠይቁ ውስጥ አይካተትም::የሰጡት አስትያየትም የእርስዎ ስለመሆኑ በምንም ሁኔት አይገለጽም::

መጠይቅ ቁጥር ----------

መጠይቁን የሚያቀርበዉ ሰዉ ስም----------

ቀን----------

**<u>ክፍል አንድ፡</u>**እርስዎን በተመለከተ ጥያቄዎች

የመለያ ቁጥር----------

ዕድሜ----------

አድራሻ:ቀበሌ_____

**ክፍል ሁለት፡**ስለ ህመም ምልክትና ስሜትዎ በተመለከተ ጥያቄዎች

እርግዝናው ስንት ጊዜው ነው?_____

እርግዝናው ለመጀመሪያ ጊዜ ነው_____

ካሁን በፊት ስንት ልጅ አለሽ?_____

ባለፈዉ ስድስት ወራት ዉስጥ ወባ በላቦራቶሪ ተመርምረዉ ተገኝቶቦታል ሀ. አዎ ለ. የለም

ባለፈዉ ወር ዉስጥ ወባ ወዳለበት ቦታ ተንቀሳቅሰዋል ሀ. አዎ ለ. የለም

በየሁለት ቀኑ የሚመላለስ የሰዉነት መተኮሰ አለብዎት ሀ. አዎ ለ. የለም

አንዳንዴ የሰዉነት ማንቀጥቀጥና ቅዝቃዜ ይሰማወታል ሀ. አዎ ለ. የለም

ከፍተኛ የሆነ የራስ ምታት ይሰማዎታል ሀ. አዎ ለ. የለም

አንዳንዴ ያስታዉከዎታል ሀ. አዎ ለ. የለም

## Annex IV. Standard operating procedures

### 1. Procedure for Microscopic examination of Blood Film

- On a single slide, prepare both thick and thin blood films (use 6 µl blood for thick film and 2 µl blood for thin film)
- Fix the thin blood films fixed with methanol for 2-3 second
- After air-drying slides stained with a 10% Giemsa solution for 10 minutes
- Wash the slides and air dry
- Examine thick and thin film under 100X microscopic objective
- Report as negative if no *Plasmodium* parasites seen after examining 100 fields per 100 high power objectives.If positive report species, stage and parasite density against WBC count. (WHO,2016)

### 2. Procedure for Loop-mediated isothermal amplification procedure (Humaloop amp)

The work flow consists of four main steps;

- sample transfer and lysis step,
- Loopamp PURE DNA extraction step,
- Loop-mediated isothermal amplification step and
- result reading steps.

➢ To get started, PURE DNA extraction kits, Human pan/pf/pv detection kits and general laboratory materials like micropipette and gloves, are needed. Before the start of LAMP

examination, work area will be cleaned with bleaching solution and distilled water as written in the protocol.

- PURE DNA extraction kit contains heating tube containing lysis solution, adsorbent tube containing powder, injection cap and injection nozzle.

1. Take out the aluminum bag with the heating tube and transfer 30 ul normal saline in to the heating tube,
2. After mixing the blood/placenta sample several times and transfer 30 ul of sample in the respective heating tube and mix the samples by shaking the heating tubes.
3. Check temperature of the Human lop amp machines (heat blockers) which should be 75 $^{O}$C, put the tubes in to the heating block and start the sample lysis, the remaining time is shown in the machine.
4. To start the DNA extraction process, take out the adsorbent tubes and prepare appropriate number of tubes for the sample number we need to extract DNA,
5. Loosen the powder in the adsorbent tube by taping it to the bottom,
6. After completing the lysis process, let the tubes cool down for 2 minutes,
7. Remove the caps of the adsorbent tube but not discard it, screw the heating tube into the adsorbent tube, shake the tubes thoroughly until the extraction solution of the heating tube is fully mixed with the white powder to form milky emulsion.
8. Take the appropriate number of reaction tubes with the respective species(*pan,pf,pv*) including for negative and positive controls with the help of the scissor, reseal the bag immediately to avoid contamination.
9. Prepare appropriate number of injection caps and put the cap of the adsorbent tube onto the nozzle of the injection cap and screw them on to adsorbent tube,
10. Squeeze the tubes and inject DNA directly into reaction tubes, close the lids of reaction tubes immediately and label,

11. Take out positive control from the kit and transfer 30 ul of it in to the respective reaction tubes.

12. To start the LAMP step, since the reagents are in the lid turn the tubes upside down to reconstitute LAMP reagent for 2 minutes,

13. Mix reaction tubes (up and down rotating the tubes) at least five times and make sure the liquid is at the bottom,

14. Put the tubes in the amplification unit of HumaLoop amp with the temperature of 65 $^{O}$C and start isothermal amplification which takes 40 minute and subsequent inactivation for 5 minutes.

15. To read the results, after completing LAMP reaction, put the tubes in to the fluorescence detection unit and turn on UV light to read the results.

16. Positive samples will fluoresce green will be double check with the positive and negative controls. Record the results from each run.

(https://www.youtube.com/watch?v=IUKLNxXvzOI)

## 3. SOP for DNA extraction using Qiagen extraction kit (from DBS sample)

1. Puch DBS sample (3 spots with 5 mm puncher) to the Eppendorf tube
2. Add 200ul of ATL buffer to the tube containing DBS sample ⟹ Vortex
3. Incubate tube at 85 $^{o}$C for 10 minutes (set heat block to 70 $^{o}$C)
4. Add 20ul proteinase k ⟹ Vortex!
5. Incubate at 56$^{o}$C for 1hr ⟹ brief centrifuge!
6. Add 200 AL buffer to the sample.
7. Incubate at 70$^{o}$C for 10 minutes.
8. Add 200ul absolute Ethanol to sample and vortex ⟹ brief centrifuge!
9. Apply mixture to spin column (without wetting the rim) and centrifuge at 8000 RPM for 1 minute ⟹ discard filtrate tube!
10. Carefully open spin column ⟹ add 500ul AW1 buffer & centrifuge at 8000 RPM for 1 minute.

11. Add 500ul AW2 buffer ⟹ centrifuge at 14,000 RPM for 3 minutes!
12. Centrifuge at 14,000 RPM for 3 minutes (dry run)
13. Label 1.5 ml Eppendorf
14. Place spin column to the labeled tube and add 50ul elution buffer (NFW)
15. Incubate at room temperature for 3 minutes ⟹ centrifuge at 8000 RPM for 1 minute!
16. Place extracted DNA in freezer.

## 4. SOP for PET-PCR diagnosis

DNA was isolated using the QIAamp® DNA Blood Mini kit (Qiagen, Hilden, Germany), as described in the above. The PET-PCR assay was run using Agilent Technologies strata gene Mx3005p PCR machine.

*Photo -induced electron transfer PCR (PET-PCR) Primer sequence*

Original Genus 18sFor, 5'-GGC CTA ACA TGG CTA TGA CG-3'.

Original Genus FAM 18sRev, 5'-agg cgc ata gcg cct ggC TGC CTT CCT TAG ATG TGG TAG CT-3'.

Falciparum For, 5'-ACC CCT CGC CTG GTG TTT TT-3'.

P. vivax For, 5'-GTA GCC TAA GAA GGC CGT GT-3'.

P. vivax Rev, HEX-5'- agg cgc ata gcg cct ggC CTG GGG GAT GAA TAT CTC TAC AGC ACT GT-3'.

**Procedure**

- All working stock primers should be prepared at 10 μM concentration.
- DNA samples should be stored at 4 $^{o}$C until testing or -20 $^{o}$C for long term storage.
- Store all primer stocks at -20 $^{o}$C for up to 1 year.
- Unopened tubes of ABI TaqMan Environmental Buffer should be stored at -20 $^{o}$C for a maximum of six months. Once thawed, store at 4 $^{o}$C for up to six months. The reagent must be used within the expiration date provided by the manufacturer.
- All samples should be tested in duplicate

**Master-mix preparation**

- ✓ The PET-PCR reaction mix is prepared by mixing the TaqMan Environmental Master Mix buffer, primers, and water as shown below.
- ✓ Determine the number of reactions to run and multiply the total number of samples to test (including positive and negative controls) by two because every sample will be tested in duplicate. For example, if there are 10 samples to tests then multiply this by two to get 20.
- ✓ Add two to four extra reactions to account for loss of solution during pipetting. This gives a total number of reactions of 22.
- ✓ Multiply this number with the volumes below for each component to get the total master-mix volume required for the experiment.

**Multiplex reaction**

**Genus and P. falciparum master mix preparation**

- In a 1.5mL tube, prepare your master-mix by multiplying the volumes shown below with the total number of reactions you need to run.

| | |
|---|---|
| H2O | 3.25µl |
| 2X ABI TaqMan buffer | 10.0µl (1x) |
| Genus For primer | 0.5 µl (250nM) |
| FAM-genus Rev primer | 0.5 µl (250nM) |
| *P. falciparum* For primer | 0.5 µl (250nM) |
| HEX-*P. falciparum* Rev primer | 0.25 µl (125nM) |

**Total Volume 15.00µl**

**Singleplex reactions**

In a 1.5mL tube, prepare your master-mix by multiplying the volumes shown below with the total number of reactions you need to run (e.g. 22 as described above).

**Genus master mix preparation**

| | |
|---|---|
| H2O | 4.0µl |
| 2X ABI TaqMan buffer | 10.0µl (1x) |
| Genus For primer | 0.5 µl (250nM) |
| FAM-genus Rev primer | 0.5 µl (250nM) |

**Total Volume 15.00µl**

***P. falciparum* master mix preparation:**

| | |
|---|---|
| H2O | 4.25µl |
| 2X ABI TaqMan buffer | 10.0µl |
| *P. fal* For primer | 0.5 µl (250nM) |
| HEX-*P. fal* Rev primer | 0.25 µl (125nM) |
| **Total Volume** | 15.00µl |

***P. vivax* master mix preparation:**

| | |
|---|---|
| H2O | 4.0µl |
| 2X ABI TaqMan buffer | 10.0µl |
| *P. vivax* For primer | 0.5 µl (250nM) |
| HEX-*P. vivax* Rev primer | 0.5 µl (250nM) |
| **Total Volume** | 15.00µl |

**Addition of master mix**

- ✓ Mix the prepared master mix well by vortexing briefly.
- ✓ Centrifuge the master mix briefly to remove any solution trapped in the tube cap.
- ✓ Arrange the optically clear PCR tubes on a PCR-tube rack following the PCR plate layout. Add 15 µl of the PET-PCR master mix prepared above to each PCR tube.
- ✓ 5 µl nuclease-free water to the wells designated as the no template control (NTC).
- ✓ Loosely put the lids on the tubes filled with master mix solution.
- ✓ Return all reagents to the freezer and refrigerator before proceeding to the next step.
- ✓ Take the assembled plate containing the tubes with PCR master mix solution to the PCR template area.

**Addition of DNA samples**

- Add 5 µl of the unknown DNA samples to the tubes with the master mix according to the plate layout. Cap the tubes tightly after adding the samples. The total volume of PCR reaction is 20.0 µl after addition of the template.

- Add positive control DNA to each positive control tube with master-mix. Cap the tubes after each positive control is added.
- Add 5.0 µl of DNase-free H2O to the tubes designated as the no-template control (NTC) and close that tube tightly.
- Make sure each sample has been added to the correct tube and that all tubes are tightly capped.
- Briefly centrifuge the tubes to remove any solution trapped in the tube walls.
- Make sure there are no bubbles in the tubes.

**PCR Cycling conditions for the Genus and *P. falciparum* multiplex**

1) 95°C 15 mins

2) 95°C 20secs

3) 60°C 40secs x 45 cycles

**Cycling conditions for the *P. vivax* and *P. falciparum* singleplex**

1) 95°C 15 mins

2) 95°C 20secs

3) 60°C 40secs x 45 cycles

4) 72°C 30sec

**Running the PCR assay**

1. Take the ready-to-run PCR tubes to the thermocycler room. Place the tubes on the heat block in the real-time thermocycler. Be sure to follow the pattern designed in the plate layout.

2. Close the lid over the tubes and shut the door to the thermocycler. Start the run.

**Results Interpretation**

**Cut off for positive and negative:**

- **Positive PCR:** A positive sample produces a fluorescence signal above the threshold/noise level. Positive samples are designated a Ct value 40.0 (≤40).
- **Negative PCR**: No fluorescence signal above the threshold/noise level. Negative samples have no Ct or have a Ct value above 40.0.

The test should be repeated if:

- The NTC control has a positive Ct value.
- The positive control yields no positive results

## 5. Procedure for Molecular identification of *pfhrp2/3 gene* using ddPCR

- Genomic DNA will be extracted from dry blood spots collected by using QIAamp mini-DNA kits (QIAGEN, https://www.qiagen.com) according to the manufacturer's directions.

Reagents required (Thaw Enzymes ON ICE before use)

- Reagent mastermix and reagents appropriate for your reaction (probe-based; if SYBR based, new ddPCR droplet oil must be purchased and installed)
- Primers and Probes
- NFW
- Samples
- Controls
- 2x deep well BioRad 96-well plates for each plate to be run
- 1 cartridge cassette for every 4 columns to be run
- 2 columns of tips for every column (8 samples) to be run

**Droplet Generation**

Extract samples if required – be sure to include appropriate positive and negative extraction controls during extraction

If running an assay for the first time, to test performance, set up a temperature gradient and run the same sample in all wells of a column

If designing an assay, FAM/HEX can be paired, and FAM/VIC can be paired. FAM and HEX and VIC can be run alone. Can not run VIC/HEX, or any other fluorophores

1) Turn droplet reader on, wait 3 min, then turn software on.
2) Ensure droplet reader is ready
    a. Connect to software, or see if orange blinking light on machine – if oil is low, replace by changing empty oil bottle over and putting waste sticker on it to make it the new waste bottle, removing waste bottle, and adding bleach to waste bottle [template contamination will be a risk, be careful!], and installing new waste

bottle). Ensure stickers are facing the outside of the machine upon installation. Run a prime then a flush in the tools area of the software.

b. If machine has not been run in 7 days (check calendar by computer), run a prime in the tools area of the software.

c. If machine has not been run in 7 days, check to ensure that pump tubing is installed in pump behind metal panel (unscrew top two screws, remove panel, and flip up black pump cover on top left).

3) Make reaction mastermix for x+1 samples for every 24 samples run. Make sure to thoroughly vortex Supermix/reagent mastermix before loading.

4) Load tip boxes, cartridges, and 1 PCR plate on the BioRad cold block (freezer A) into the ddPCR AutoDG [warning => lifting a box or cartridge cassette will cause the machine to reset and think it is new. For tips, it will cycle through until it finds tips before attempting to load. For cartridges, used cartridges will be registered as clean and reused, which is bad. Also, not loading a PCR plate into the cold block in the AutoDG will result in sample being loaded directly into the cold block]

5) Ensure droplet oil level is OK + proper oil for your application (default is probe based oil that we use in the lab)

6) Load 1 BioRad deep well PCR plate onto a cold block for sample/mastermix loading

7) Turn plate sealer on, hit OK to allow it to start to preheat.

8) Turn PCR machine on. Ensure your melt-anneal-extension steps have a ramp rate of 2*C/s (can be set under options for that step).

9) Thoroughly vortex reaction mastermix (15 seconds minimum) before loading into PCR plate on cold block external to the ddPCR AutoDG. Do not load samples into plate in the AutoDG.

10) Load samples into PCR plate (no need to pipette to mix, just put on side of well), loading NTC and negative control first and positive controls last

11) Place ONE foil seal (red stripe should be visible) on plate with samples. Place into metal block (should always be stored outside of head sealer), and into the plate sealer. Press seal once heat sealer reaches 180*C. Ensure only 1 foil is on the plate.

12) Remove sealed plate with metal block and close heat sealer, remove plate, vortex at all four corners and middle of plate for 15-30s each. Replace plate onto the cold block.
13) Load plate into centrifuge and spin 1 minute
14) Load plate into AutoDG. Be careful not to bump it and disturb spun down liquid.
15) Select “configure plate”, select columns to be loaded, and press OK
16) Select “start droplet generation”
17) Once droplets generated, carefully move plate from cold block in AutoDG (right) to heat sealer. Place metal film (red stripe up), and seal with one foil.
18) Carefully remove plate and heat block from heat sealer. Load plate into PCR machine, close and tighten lid, and run protocol. Put heat block next to heat sealer and turn off. Remove used cartridges and waste tips/plate from AutoDG and dispose of in autoclave waste. If liquid is in white waste tip bin, please wipe down with DNAzap before putting back in AutoDG.

**Droplet Reading**

1) Allow plate to sit at 4 degrees for at least 30 min, up to ½ day before measuring
2) Carefully load plate into droplet reader by opening green door, lifting tabs on metal cover to lift metal cover off, placing plate into black metal holder, and replacing the metal cover on the top of the plate (leave the foil on).
3) Use top left button of software (looks like a plate) to add plate, and then click configure plate
4) Name plate, select mastermix used, and add wells to be read. You can also add in information about wells, though this can be done during analysis
5) Start run in order to measure droplets

NOTE: IF machine is not to be run for 7 subsequent days, remove pump tubing from pump to prevent solidification. (See step 2c).

**Master mix preparation for ddPCR**

| Hrp2 Mastermix set up | | | |
|---|---|---|---|
| Reagent | Concentration | Vol/rxn | |
| ddPCR Multiplex super mix | 1x | 5.5 | 198 |
| Ddtt 300 nM | 3.7 nM | 0.27 | 9.72 |
| CK3 F 100 uM | 900 nM | 0.198 | 7.128 |
| CK3 R 100 uM | 900 nM | 0.198 | 7.128 |
| CK4 F 100 uM | 900 nM | 0.198 | 7.128 |
| CK4 R 100 uM | 900 nM | 0.198 | 7.128 |
| CK6 F 100 uM | 900 nM | 0.198 | 7.128 |
| CK6 R 100 uM | 900 nM | 0.198 | 7.128 |
| CK3 probe 10 uM | 250 nM | 0.55 | 19.8 |
| CK4 Probe1 10 uM | 125 nM | 0.275 | 9.9 |
| CK4 Probe2 10uM | 125 nM | 0.275 | 9.9 |
| CK6 Probe 10 uM | 250 nM | 0.55 | 19.8 |
| NFW | N/A | 3.392 | 122.112 |
| Sample | N/A | 10 | 160 |
| MM total | N/A | 12 | 192 |
| MM + Sample total | N/A | 22 | 594 |

| Hrp3 mastermix set up | | |
|---|---|---|
| Reagent | Volume/Rxn | |
| BioRad ddPCR Multiplex Supermix (4x) | 5.5 | 148.5 |
| Dtt | 0.27 | 7.29 |

| | | |
|---|---|---|
| CK5 Forward | 0.198 | 5.346 |
| CK5 Reverse | 0.198 | 5.346 |
| CK5 Probe | 0.275 | 7.425 |
| CK6 Forward | 0.198 | 5.346 |
| CK6 Reverse | 0.198 | 5.346 |
| CK6 Probe | 0.55 | 14.85 |
| NFW | 4.62 | 124.74 |
| Master Mix total | 12.007 | |
| Template | 10 | |
| Reaction total | 22 | |

**PCR Conditions**

| Temperature | Duration | | |
|---|---|---|---|
| 95$^{o}$C | 10:00 | 39 cycles | Step 1 |
| 94 $^{o}$C | 0:30 | | |
| 58 $^{o}$C | 1:00 | | |
| 98 $^{o}$C | 10:00 | 1 cycle | Step 2 |
| 4 $^{o}$C | ∞ | | |

**ddPCR data analysis**

1.Calculate average values of NTC, Trna ligase and HRP2 from the control

2.Subtract the above average value from the total concentration

3.Calculate ratio of hrp2 exon1 and exon2 to Trna ligase (hrp/Trna lig)

4.Identify failed Trna ligase<0.1, identify gene deletion status from hrp2 exon1 and 2

5.Report the status as no deletion(0.75<gene<1), Composite (0.25<gene<0.74) and full deletion(0<gene<0.24).

## 6. SOP for Single nucleotide polymorphisms (SNPs) of antimalarial resistance in *pfk13* genes

Procedure involves sample collection, DNA extraction, gene amplification, sequencing, and data analysis:

- Dried Blood Spots (DBS), a small volume of blood, spotted onto specialized filter paper and allowed to air dry used
- DNA extracted using commercially available DNA extraction kits (QIAamp DNA Blood Mini Kit)
- To obtain a sufficient amount of the *pfk13* gene for sequencing, a polymerase chain reaction (PCR) is performed. Due to the low parasite densities often found in clinical samples, a nested PCR used.

  Here, Outer PCR: The first round of PCR uses a set of primers to amplify a larger fragment of the *pfk13* gene. Nested PCR: The product from the outer PCR is then used as a template for a second round of PCR with a different set of primers that bind within the initial amplicon. This specifically amplifies the propeller domain of the *pfk13* gene, which is the region of interest for resistance mutations.

  **Reaction Mix (25 µL total volume):**

| **Primary PCR Component** | **Volume per reaction** |
|---|---|
| Invitrogen 10X PCR Buffer (-MgCl2) | 2.50 µL |
| Invitrogen 50 mM MgCl2 | 1.50 µL |
| NEB deoxynucleotide Solution Mix, 10 mM | 0.50 µL |
| *pfk13* primary forward primer | 0.40 µL |
| *pfk13* primary reverse primer | 0.40 µL |
| Invitrogen platinum *Taq* DNA polymerase | 0.40 µL |
| NFW | 18.3 µL |
| Template | 1 µL |
| Total | 25 µL |

| **Nested PCR Component** | **Volume per reaction** |
|---|---|
| Invitrogen 10X PCR Buffer (-MgCl2) | 2.50 µL |
| Invitrogen 50 mM MgCl2 | 1.25 µL |
| NEB deoxynucleotide Solution Mix, 10 mM | 0.50 µL |
| *pfk13* primary forward primer | 0.40 µL |
| *pfk13* primary reverse primer | 0.40 µL |
| Invitrogen platinum *Taq* DNA polymerase | 0.40 µL |
| NFW | 17.55 µL |
| Template | 2.00 µL |
| Total | 25.00 µL |

## PCR condition

| **Step** | **Temperature (celcius)** | **Time (min)** | **Number of cycles** |
|---|---|---|---|
| initial denaturation | 94 C | 5:00 | 1 |
| denaturation | 94 C | 0:30 | 40 |
| Annealing | 65.5 C | 0:45 | 40 |
| Extension | 72 C | 1:30 | 40 |
| Final Extension | 72 C | 10:00 | 1 |
| Hold | 4°C | ∞ | |

- ❖ For quality check, run 5 µL of nested PCR product on a 1.5% agarose gel. Expected band size: ~850 bp.
  - Once the *pfk13* gene fragment has been amplified, the next step was Sanger sequencing to determine its exact DNA sequence.
  - The amplified PCR products were purified and then sequenced using a capillary electrophoresis-based sequencer.
  - To analyze and interpret the data, the bioinformatics workflow typically includes the following steps: 1, **Sequence Alignment:** The obtained sequences were aligned to a reference *pfk13* gene sequence (3D7 strain) to identify any variations. 2, **SNP Calling:** Specialized software was used to call the SNPs, which were the single base differences between the sample sequence and the reference sequence. 3, **Interpretation:** The identified SNPs were then compared against a database of known *pfk13* mutations associated with artemisinin resistance.

## Annex V. Details of WHO approved and candidate *pfk13 gene* mutations

1. A481V means that at position 481 alanine is replaced by valine.
2. A675V refers to Alanine at position 675 is replaced by Valine
3. C469F means Cysteine at position 469 is replaced by Phenylalanine
4. C469Y refers to Cysteine (C) at position 469 is replaced by Tyrosine (Y)
5. C580Y refers to Cysteine at position 580 is replaced by Tyrosine.
6. F446I mutation refers to a change from Phenylalanine (F) to Isoleucine (I) at amino acid position 446.
7. G449A refers to Glycine at position 449 is replaced by Alanine.
8. G538V is a mutation where Glycine changes to Valine at position 538
9. I543T means that at position 543 in the protein, Isoleucine has been replaced by Threonine.
10. M476I refers to Methionine (M) at position476 replaced by Isoleucine (I)
11. N458Y refers to Asparagine (N) at position 458 is replaced by Tyrosine (Y) in the Kelch 13 (K13) protein.
12. N537I/D means that the Asparagine at position 537 can be replaced by either Isoleucine or Aspartic acid in different parasite variants
13. P441L refers to Proline at position 441 is replaced by Leucine.
14. P527H is a substitution of Proline with Histidine at position 527 in the pfk13 protein.
15. P553L means that Proline at position 553 is replaced by Leucine in a specific protein
16. P574L refers to Proline at position 574 is replaced by Leucine
17. R515K means Arginine is replaced by Lysine at position 515 in the pfk13 protein
18. R539T refers to Arginine at position 539 replaced by Threonine
19. R561H refers to Arginine at position 561 is replaced by Histidine
20. R622I refers to arginine at position 622 is replaced by isoleucine
21. V568G is a mutation where Valine is replaced by Glycine at position 568
22. Y493H refers to Tyrosine (Y) at amino acid position 493 changed to Histidine (H)

## Annex VI. Details of *pfk13 gene* mutations detected from samples collected from 2021-2023 at selected health facilities of Ethiopia

1. A675V corresponds to amino acid Alanine (A) at position 675 is replaced by valine (V)
2. D584D, is a synonymous (silent) mutation, meaning the amino acid at position 584 is still Aspartic acid
3. E606K means glutamic acid at position 606 has been replaced by lysine
4. G596E corresponds to change of glycine (G) to glutamic acid (E) at position 596
5. H588Y means that histidine at position 588 has been replaced by tyrosine
6. I265S indicates that isoleucine at position 265 has been replaced by serine.
7. I402R means isoleucine at position 402 has been replaced by arginine
8. I406R indicates that isoleucine at position 406 has been replaced by arginine.
9. I587I is a synonymous (silent) mutation, meaning the amino acid at position 587 is still isoleucine
10. N585N, is a synonymous (silent) mutation, meaning the amino acid at position 585 is still asparagine
11. R539T means arginine at position 539 is replaced by threonine
12. R622I means arginine at position 622 has been replaced by isoleucine
13. T587I means threonine at position 587 has been replaced by isoleucine.