EVALUATION OF NOVEL BIOCREDIT RAPID DIAGNOSTIC TESTS FOR DETECTION OF *PLASMODIUM* SPECIES, AND PREVALENCE OF PfHRP2/3 GENE DELETIONS AMONG FEBRILE PATIENTS AT MAKSEGNIT HEALTH CENTER, NORTHWEST ETHIOPIA

BY: ALAYU BOGALE (MSc CANDIDATE)

A RESEARCH THESIS SUBMITTED TO SCHOOL OF MEDICAL LABORATORY SCIENCES, FACULTY OF HEALTH SCIENCES, INSTITUTE OF HEALTH, JIMMA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR MASTER OF SCIENCE DEGREE IN MEDICAL PARASITOLOGY

JANUARY, 2022

JIMMA, ETHIOPIA

JIMMA UNIVERSITY

INSTITUTE OF HEALTH

FACULTY OF HEALTH SCIENCES

SCHOOL OF MEDICAL LABORATORY SCIENCES

EVALUATION OF NOVEL BIOCREDIT RAPID DIAGNOSTIC TESTS FOR DETECTION OF *PLASMODIUM* SPECIES, AND PREVALENCE OF PfHRP2/3 GENE DELETIONS AMONG FEBRILE PATIENTS AT MAKSEGNIT HEALTH CENTER, NORTHWEST ETHIOPIA

BY: ALAYU BOGALE (MSc CANDIDATE)

ADVISORS:

- Dr. TEFERI ESHETU (PhD)
- Dr. TESHOME DEGEFA (PhD)
- Dr. FITSUM GIRMA (PhD)
- Dr. CRISTIAN KOEPFLI (PhD)
- Dr. MIGBARU KEFFALE (DVM, PhD CANDIDATE)

ABSTRACT

Background: Rapid diagnostic test (RDT) plays an essential role for prompt diagnosis of malaria in settings where using microscopy is not feasible. However, there is an increasing concern that *Plasmodium falciparum* histidine rich protein (PfHRP) gene deletions could impede the performance of the commonly used RDTs, resulting in a false negative diagnosis. This suggests the need to develop and evaluate new RDT kits to overcome such challenges.

Objective: To evaluate the diagnostic performance of Biocredit RDTs Pf(pLDH/HRP2) and Pf/Pv(pLDH/pLDH) for detection of *Plasmodium* species and determine the prevalence of PfHRP 2/3 gene deletions among febrile patients at Maksegnit Health Center (MHC), northwest Ethiopia.

Methods: A health facility-based cross-sectional study was conducted from September to December 2021 in 384 malaria suspected febrile study subjects at MHC. Finger-prick blood samples were collected for malaria diagnosis using microscopy, RDTs and Quantitative Polymerase Chain Reaction (qPCR). Sensitivity, specificity, positive and negative predictive values of the RDTs were determined by comparing with the gold standard microscopy and qPCR Digital Polymerase Chain Reaction (dPCR) was used to detect PfHRP 2/3 gene deletion.

Results: The prevalence of malaria among febrile patients was 69.3%, 67.2%, 42.2% and 71.4% by microscopy, Biocredit RDT Pf/Pv (pLDH/pLDH), SD Bioline RDT and qPCR, respectively. By taking microscopy as a reference, the Biocredit Pf/Pv (pLDH/pLDH) RDT had sensitivity and specificity of 97.9% and 97.4% for *P. falciparum* and 94.5% and 97.5% for *P. vivax,* respectively. The Biocredit Pf (pLDH/HRP2) RDT had sensitivity and specificity of 97.4% and 97.5%. In contrast, SD Bioline RDT had sensitivity and specificity of 51.3% and 93.2% for *P. falciparum*, and 86.3% and 96.5% for *P. vivax*, respectively. By taking qPCR as a reference, the sensitivity and specificity of Biocredit Pf/Pv (pLDH/pLDH) were 95.5% and 96.4% for *P. falciparum,* and 90.8% and 99.1% for *P. vivax,* respectively. The Biocredit Pf (pLDH/pHRP2) RDT had sensitivity and specificity of 94.9% and 97.4%, respectively, whereas the SD Bioline RDT had sensitivity and specificity of 50.0% and 96.5% for *P. falciparum*, and 83.0% and 96.5% for *P. vivax*, respectively. Out of 99 SD Bioline RDT negative samples, Pfhrp2 and Pfhrp3 exon 2 gene deletions were observed in 23.2% (46/198) and 27.7% (55/198) of the PCR-positive samples,

respectively. Double deletions in pfhrp2 and pfhrp3 were detected in 13.1% (26/198) of the PCR positive samples.

Conclusion: The sensitivity and specificity of Biochredit RDTs kits documented in this study comply with the WHO limit of detection for routine diagnosis of clinical malaria, with more reliable diagnostic performance compared to the conventional (SD Bioline) RDT. This study confirms the presence of 13.1% of pfhrp2/3 gene deletions So, we should consider alternative diagnostic tool like Pf-pLDH in the study area. Further nationwide survey on the prevalence of hrp 2/3 gene deletion is crucial.

Keywords: *Plasmodium falciparum*, *Plasmodium vivax,* RDTs, *hrp* 2/3, Ethiopia

ACKNOWLEDGMENTS

First of all, I would like to express my sincere gratitude to my advisors Dr. Teferi Eshetu, Dr. Teshome Degefa, Dr. Fitsum Girma, Dr. Cristian Koepfli and Dr. Migbaru Keffale for all their intellectual guidance, support, and leadership throughout this thesis work. Secondly, I would like to thank the data collectors for their invaluable effort in collecting the data. My deep gratitude also extends to the study participants for their voluntary participation and all staffs of Malaria and Neglected Tropical Diseases at Armauer Hansen Research Institute and laboratory staffs of Maksegnet Health Center for their cooperation. Additionally, I would like to thank all my friends who have in one way or another contributed to the completion of this research.

My gratitude also goes to Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences, and Medical Parasitology Unit for giving this chance to conduct this study. Finally, I am also thankful to my sponsor organization, Dilla University and Armauer Hansen Research Institute.

.

TABLE OF CONTENTS

Contents pages

ABSTRACT I
ACKNOWLEDGMENTS III
TABLE OF CONTENTS IV
LIST OF TABLES VII
LIST OF FIGURES VIII
ABBREVIATIONS AND ACRONYMS IX
CHAPTER ONE 1
1. INTRODUCTION 1
1.1. Background 1
1.2. Statement of the problem 3
1.3. Significance of the study 6
CHAPTER TWO 7
2. LITERATURE REVIEW 7
2.1. Malaria epidemiology in Ethiopia 7
2.2. Life cycle of the malaria parasites 8
2.3. Pathogenesis and clinical presentation of malaria 9
2.4. Diagnosis of malaria 10
CHAPTER THREE 18
3. OBJECTIVE 18
3.1. General Objective 18
3.2. Specific Objectives 18
CHAPTER FOUR 19
4. MATERIALS AND METHODS 19
4.1. Study Area 19
4.2. Study Design and period 20
4.3. Population 20
4.3.1. Source population 20
4.3.2. Study population 20
4.4. Sample size and sampling technique 21
4.4.1. Sample Size 21

4.4.2. Sampling Technique ... 21
4.5. Inclusion and Exclusion Criteria ... 22
4.5.1. Inclusion Criteria ... 22
4.5.2. Exclusion Criteria ... 22
4.6. Study Variable ... 22
4.6.1. Dependent Variables ... 22
4.6.2. Independent Variables ... 22
4.7. Data Collection and laboratory methods ... 23
4.7.1. Socio-demographic and clinical data ... 24
4.7.2. Blood sample collection and processing ... 24
4.7.3. Malaria microscopy ... 24
4.7.4. Rapid Diagnostic Tests ... 25
4.7.5. Molecular Diagnosis ... 26
4.8. Operational definition ... 33
4.9. Data Quality Assurance and Management ... 34
4.10. Statistical Analysis ... 34
4.11. Ethical Consideration ... 36
4.12. Dissemination of the Result ... 36
CHAPTER FIVE ... 37
5. RESULTS ... 37
5.1. Socio-demographic data ... 37
5.2. Malaria prevalence by Microscopy, RDTs and qPCR ... 37
5.3. Sensitivity and specificity of Biocredit and SD Bioline RDTs ... 38
5.4. Socio-demographic factors and parasite density ... 40
5.5. HRP 2/3 gene deletion typing by dPCR ... 43
CHAPTER SIX ... 46
6. DISCUSSION ... 46
CHAPTER SEVEN ... 50
7. CONCLUSION AND RECOMMENDATION ... 50
7.1. Conclusion ... 50
7.2. Recommendation ... 51
REFERENCES ... 52
ANNEX ... 58

Annex- I: Participant Information Sheet English/Amharic Version 58
Annex- II: Certificate of consent English/Amharic Version (>18 age of years) 62
Annex- III: Certificate of Assent English/Amharic Version (<18 age of years) 65
Annex- IV: Questionnaire English/Amharic Version 71
Annex-V: Malaria Blood film preparation and examination 74
Annex-VI: DNA extraction using NucleoMag® Blood Extraction Method 78
Annex-VII: Quantitative polymerase chain reaction for species detection 81
Annex-VIII: Digital polymerase chain reaction for typing *hrp 2/3* deletion 85
Annex-IX: Declaration 87

LIST OF TABLES

Table 1: Summary of literatures which is related to this study and shows the main findings...... 14
Table 2: Primers and probes concentration in reaction volume for multiplex qPCR 28
Table 3: Primers and probes sequence for *P. falciparum* and *P. vivax* 18s qPCR. 28
Table 4: Primers and probes concentration in reaction volume for hrp-2/hrp 3 by dPCR 31
Table 5: Primers and probes sequence for hrp-2, tRNA and hrp-3 31
Table 6: Contingency table (2×2 table) to calculate diagnostic performance measurement........ 34
Table 7: Prevalence of malaria by different diagnostic tests among febrile patients in Maksegnet health center, Northwest Ethiopia, 2022 (n=384). 38
Table 8: Performance of different RDTs kits compared to microscopic examination (n=384) in Maksegnet health center, Northwest Ethiopia, 2022. 39
Table 9: Performance of different RDTs kits compared to qPCR examination (n=384) in Maksegnet health center, Northwest Ethiopia, 2022. 40
Table 10: Distribution of parasite density by gender, age-group, and living area in Maksegnet health center, Northwest Ethiopia, 2022 41
Table 11: The hrp2 and hrp3 deletions status in Maksegnet health center, North West Ethiopia, 2022.. 43

LIST OF FIGURES

Figure 1: Immunological reaction on a positive RDT strip for *P. vivax* infection. 12
Figure 2: Map of the study area (Maksegnit, Gonder zuria woreda). 20
Figure 3: Study flowchart for malaria rapid diagnostic tests (RDTs), microscopy, and molecular assay 23
Figure 4: Procedure of SD Bioline RDT performance to investigate malaria 25
Figure 5: Manufacturer instruction for one step malaria antigen Pf/Pv (pLDH/pLDH) Rapid test. 26
Figure 6: NucleoMag® Blood 200µL Kit DNA extraction in kingfisher flex machine. 27
Figure 7: CFX96 Touch Real-Time PCR Detection System 29
Figure 8: Amplification and standard curves of qPCR results for samples collected from 29
Figure 9: Work flow of QIAcuity dPCR 30
Figure 10: QIAcuity Digital PCR System 33
Figure 11: Gender and residence of study participants in Maksegnet health center, Northwest Ethiopia, 2022. 37
Figure 12: Comparison of sensitivity and specificity of SD Bioline and Biocredit RDTs in Maksegnet health center, Northwest Ethiopia, 2022. 39
Figure 13: Geometric mean of parasite densities among age groups of study participants in Maksegnet health center, Northwest Ethiopia, 2022. 42
Figure 14: Geometric mean of parasite densities among gender of study participants in Maksegnet health center, Northwest Ethiopia, 2022. 42
Figure 15: Sample positive and negative partition for hrp 2 deletion 1D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022. 44
Figure 16: Sample positive and negative partition for tRNA 1D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022. 44
Figure 17: Over all examples of hrp2 exon 2 deletion typing by dPCR 2D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022. 45

ABBREVIATIONS AND ACRONYMS

ACT:	Artemisinin based Combination Therapy
AHRI:	Armauer Hansen Research Institute
ALERT:	All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre
CI:	Confidence intervals
Ct:	Cyclic threshold
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleotide Triphosphates
dPCR:	Digital Polymerase Chain Reaction
EDTA:	Ethylene Di-amino Tetra Acetic acid
FMoH:	Federal Ministry of Health
HMIS:	Health management information system
HRP:	Histidine-rich protein
MHC:	Maksegnet health center
NPV:	Negative predictive value
NTC:	Non template control
PCR:	Polymerase Chain Reaction
pLDH:	*Plasmodium* Lactate Dehydrogenase
PPV:	Positive predictive value
qPCR:	Quantitative Polymerase Chain Reaction
RBC:	Red blood cell
RDTs:	Rapid diagnostic tests
rRNA	Ribosomal Ribonucleic acid
SOPs:	Standard operating procedures
tRNA:	Transfer Ribonucleic acid
WBC:	White blood cell
WHO:	World Health Organization

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Malaria is one of the most serious vector-borne infectious diseases, and it continues to cause significant morbidity and mortality in the world. *Plasmodium falciparum* and *P. vivax* are the two most common *Plasmodium* species in Ethiopia, accounting for 65% and 35% of all *Plasmodium* infections, respectively (1). Both species are found in all malaria-endemic areas, albeit their relative frequency varies depending on the time and location within a given geographical range (2). The human malaria parasite *P. falciparum* is the most lethal, and highly polymorphic, and individual hosts are frequently infected by numerous parasite clones at the same time (3).

The management and control of malaria necessitates quick and effective diagnostic methods. The front line methods for identifying malaria in the field are microscopy and rapid diagnostic test (RDT) (4). The microscopic tests involve staining and direct microscopic observation of the parasite. In the majority of contexts, from the clinical laboratory to field surveys, the direct microscopic observation of the parasite on the thick and/or thin blood smears has been the standard approach for diagnosing malaria for more than a century (5). As of right now, the "gold standard" for diagnosing malaria is the thorough analysis of a well-prepared and well-stained blood film. The peripheral smear examination is one of the most frequently utilized microscopic tests (6).

RDT use immune-chromatographic technique to detect parasite proteins in the blood (7). The histidine rich protein 2 (hrp2) and the structural homologous protein, the histidine rich protein 3 (hrp3) are detected by more than 90% of RDTs used to identify *P. falciparum* (8). Hrp2 is an appropriate target for diagnostics since it is highly produced by the parasite and released into the plasma (9). Parasite lactate dehydrogenase (pLDH) antigen, on the other hand, is consistently expressed in all *Plasmodium* species. *P. falciparum* and *P. vivax* have diverse pLDH amino acid sequences, allowing for the development of species-specific pLDH for the identification of various malaria parasite species (10,11). For instance, the *P. vivax* pLDH (Pv-pLDH) RDT is used to identify *P. vivax* malaria infection while the *P. falciparum* pLDH (Pf-pLDH) RDT is utilized to diagnose *P. falciparum* malaria infection (12).

Deletions of the genes encoding the histidine-rich proteins 2 and 3 (pfhrp2 and pfhrp3) impair the effectiveness of RDT based on the hrp 2, making it more difficult to correctly detect the *P. falciparum* (13). The aim of this study is to compare the performance of new Biocredit RDTs to gold standard microscopic examination and Quantitative Polymerase Chain Reaction (qPCR), as well as to determine the prevalence of hrp 2/3 gene deletion among febrile patients visiting Maksegnit Health Center in Northwest Ethiopia.

1.2. Statement of the problem

The predicted number of malaria cases and deaths globally in 2020 was 241 million cases and 627, 000 deaths, respectively (14). This represents about 14 million more cases in 2020 compared to 2019, and 69 000 more deaths (14,15). Around 95% of cases from the global coverage were covered by the WHO African Region, with an estimated 228 million cases in 2020. The region of Sub-Saharan Africa was the most affected, accounting for the majority of malaria morbidity and mortality (14).

In Ethiopia with a population of about 102.8 million in 2020, 5.4 million estimated and 904,495 confirmed malaria cases were recorded in 2019(16). Malaria was responsible for 30% of all disability-adjusted life years (DALY) lost in Ethiopia , making it a major stumbling block to social and economic growth (17). Malaria discourages investments and tourism, affects land use patterns and crop selection resulting in sub-optimal agricultural production, reduces labor productivity, and impairs learning (18,19).

Ethiopia has shown a significant reduction in malaria cases over the last two decades attributing to the widespread use of long-lasting insecticide-treated nets (LLINs) (20), Indoor residual spray (IRS), RDTs, and Artemisinin-based combination therapy (ACT), as well as the deployment of more than 30,000 community health extension workers at the millennium's start (21,22). However, the disease remains a major public health concern in the country, with an increase in malaria cases recently reported in some parts of the country.

For effective case management in malaria control and elimination, improved access to diagnostic tests and rapid treatment are critical. The gold standard for diagnosis is still microscopy, which is affordable, sensitive to 50–100 parasites/µl, and allows for the identification of parasite species and density (4). However, lack of competent microscopists, poor quality control, and the potential for misdiagnosis due to low parasitemia or mixed infections are challenging the use of microscopic diagnosis in many malaria-endemic areas (5).

In situations with low parasite rates or mixed infections, PCR was more sensitive and specific tha n evaluation of thick or thin blood smears (4).The WHO suggests that nucleic acid amplification assays for malaria molecular detection only be used for epidemiological studies and survey mapping sub-microscopic infections (23). The use of molecular techniques as diagnostic tools in sub-Saharan Africa is challenging because of the equipment needed, the upkeep of reagents, and the well experienced professionals (4,23,24). PCR is the most sensitive method, and detect parasitemia at levels as low as 2–5 parasites/µl. However, due to the fact that it is an expensive and complicated approach, it cannot be used in the field and routine diagnosis of malaria (25).

RDTs serve as the major approach for diagnosing clinical cases in recent decades (26). RDTs offer results in 10–30 minutes, are cost–effective, and have a detection limit of more than 100 parasites/µl (27). They also require minimum training for staff members of health centers. Over 300 million RDTs are used annually throughout the world, and their introduction has greatly improved case management, leading to a notable decrease in the burden of malaria (28).

Furthermore, RDTs are designed to detect clinically relevant parasite and antigen levels, although it's unclear how many parasite infections (antigen positive) they miss (29). RDTs based on hrp 2 and pLDH are now the most sensitive on the market, including new "ultrasensitive" RDTs which are capable to detect <100 parasites/µl.

However, RDT performance has been harmed by a variety of factors, including high temperatures and humidity, which cause antibody denaturation, hrp-2 gene polymorphism and deletions, operational issues, parasite level throughout the test and pro-zone effect (30,31).The pro-zone effect, an absent or faint test line due to an overabundance of antigens obstructing the binding sites of both detection and capture antibodies, preventing binding of the antigen-detection antibody-conjugate complex to the capture antibody, resulting in pink line generation failure (32).

Moreover, the performance of the Pfhrp2 RDT is influenced by a number of circumstances. The absence of HRP2 antigen synthesis in *P. falciparum* parasites is caused by a deletion of sections or all of the pfhrp2 gene, among other things(33). Surprisingly, partial or complete deletion of the pfhrp2 and pfhrp3 genes in *P. falciparum* populations could lead to false-negative RDT-based malaria diagnoses, delaying urgent anti-malarial drug prescriptions and allowing the fast spread of

genotypes from untreated patients into communities (34). Accurately identifying the distribution and prevalence of *P. falciparum* parasites lacking hrp2/3 would be a critical first step in determining which RDTs targeting other antigens, or microscopy, should be used in these areas (33,34).

The false negativity of SD Bioline RDTs might be due to the deletion of the hrp2/3 genes and their flanking regions, as well as a partial coding region between exon 1 and exon 2 (intron 1) (8,35), minor mutations on amino acid repeat units recognized by commercial antibodies (36), and the development of a new *P. falciparum* parasite strain with a wider genetic diversity of parasite antigens (37,38). Generally various gene deletion rates have been reported in Ethiopia, ranging from 100% for both Pf hrp 2 and Pf hrp 3 (39) in Adama to 2.1% for Pf hrp 2 and 74.5% for Pfhrp 3 (40) in Jimma. If the prevalence of hrp2 deletion is greater than 5%, the WHO suggests switching to non-hrp2 based RDTs or other diagnostic methods (31). The widespread distribution of parasites lacking the hrp2 gene poses a significant new challenge for malaria control and elimination efforts.

RDT performance evaluations for a specific country will aid in lowering the financial impact of RDT failure in malaria control and elimination initiatives. We used Biocredit RDTs (Pf pLDH/HRP2 and Pf/Pv pLDH/pLDH) for this study which is in WHO pre -qualification pipeline which has already passed laboratory evaluation. Ethiopia plans to eliminate malaria by 2030 (1) So, the country needs effective diagnostic tool for appropriate case management. In order to guarantee patient test findings and recommend appropriate treatment, there are a limited number of studies conducted globally in general on the diagnostic performance of Biocredit RDTs and hrp 2/3 gene deletion by digital polymerase chain reaction(dPCR). Hence, the aim of this study was to compare the performance of novel Biocredit RDTs with the gold standard microscopic examination and qPCR, as well as to determine the prevalence of PfHRP2/3 gene deletions among febrile patients visiting at Maksegnit Health Center in Northwest Ethiopia.

.

1.3. Significance of the study

This study generated data about Biocredit RDTs and hrp 2/3 gene deletion status that will be used by local, regional, and other stakeholders to choose appropriate diagnostic techniques and contribute to malaria control. Laboratory evaluation of RDTs gives a comparable, standardized measure of RDT performance for distinguishing between well-performing and poorly-performing tests, which can be used by malaria control organizations to make purchasing decisions. In addition to this, based on the prevalence of hrp 2/3 gene deletion, hrp 2 based RDTs may be replaced with non-hrp-2 RDTs. The study also serves as a starting point for future researchers interested in learning more about the diagnostic evaluation of various RDTs for malaria and hrp 2/3 gene deletion which is a driving force for false negative result of rapid diagnostic test.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Malaria epidemiology in Ethiopia

The majority of outpatient visits in Ethiopia are due to the burden of malaria, which continues to generate a significant amount of morbidity and mortality (41). One of the biggest reasons for hospitalization and fatalities in the nation has been malaria. The majority of the malaria-risky areas, which are located below 2000 meters above sea level, are home to about 60% of the population (41,42). However, locations above this altitude have a number of pockets where micro-epidemiological circumstances encourage malaria transmission. The two predominant parasites that cause the bulk of malaria cases in Ethiopia are *P. falciparum* and *P. vivax*. Other *Plasmodium* species , *P. ovale and P. malariae*, have a very small impact in the country (43).

In Ethiopia, a health facility-based study shows the overall frequency of malaria among adults was 13.61%. According to subgroup analysis based on the different categories of malaria cases, the prevalence of malaria in symptomatic and asymptomatic individuals was 15.34% and 11.99%, respectively. Similar to this, a regional subgroup study revealed that the Southern Nations, Nationalities, and Peoples' Region (SNNPR) had the greatest malaria prevalence (16.17%), followed by Oromia Regional State (13.11%) and Amhara Regional State (12.41%) (44). In the Gonder Zuria district, the overall malaria count for 2019 was 5893, compared to 31, 550 for 2020 and 33, 248 for 2021. Incidence of malaria per 1000 individuals was 2.39 $\pm$ 5.4 on average per month in 2019, 10.64 $\pm$16.99 in 2020, and 11.19 $\pm$16.59 in 2021. Malaria elimination programs in the area or the nation as a whole are in grave risk as the annual incidence increased from 24 cases/1000 people in 2019 to 139.08 cases/1000 people in 2021(45).

With the exception of the low ground along the southwest international border, where transmission occurs all year round, the levels of malaria risk and transmission intensity, however, exhibit significant seasonal, inter-annual, and geographic variations (22). The main transmission season, which follows the main rainy season from June to September, lasts from September to December in the majority of the country. In some areas, the short rainy season is followed by a brief

transmission season from April to May. The main vector are *Anopheles arabiensis* with minor transmission roles played by *An. pharoensis, An. funestus*, *An. Stephensi* and *An. Nili*(22,46).

Generally, the diverse ecology of the country supports a wide range of transmission intensities ranging from low-seasonal to high-perennial transmission. For planning purposes and targeting of intervention strategies, the Federal Ministry of Health (FMoH) of Ethiopia has stratified the country's malaria transmission burden using 'woreda' (district)-level transmission intensity according to annual parasite incidence per 1000 population (API) and elevation. Accordingly, four broad strata were identified by the mixed criteria of the FMoH and WHO — malaria free, low, moderate, and high transmission (16,22).

2.2. Life cycle of the malaria parasites

Plasmodia goes through a number of stages and has two host life cycles. The parasite undergoes sexual reproduction in female *Anopheles* mosquitoes, and asexual reproduction in human. In the midgut of vector female *Anopheles* mosquitoes, the parasite's sporozoites stage is released during sexual reproduction and travels to the salivary gland.When a female *Anopheles* mosquito feeds on human blood, sporozoites are injected into tiny blood arteries (47,48).

After 30 minutes following injection, sporozoites infiltrate liver parenchymal cells. The parasite grows inside the liver cell to become a spherical, multinucleate liver stage known as a schizont, which contains thousands of merozoites (49). Exoerythrocytic schizogony is the term used to describe the massive proliferation of the *Plasmodium* parasites. Depending on whether species of *Plasmodium* is involved, the exoerythrocytic or hepatic phase of the illness typically lasts 5 to 21 days. The maturation of liver-stage schizonts, however, may be postponed for 1–2 years in infections with *P vivax* and *P. ovale*. Hypnozoites are the name for these dormant liver-phase parasites (50).

Regardless of how long it takes for development to take place, mature schizonts finally burst, discharging tens of thousands of uninucleate merozoites into the circulation. Every merozoite is capable of attacking a red blood cell. By means of erythrocytic; schizogony, the merozoite matures to either a spherical or banana shaped, uninucleate gametocyte inside the red cell or an erythrocytic

stage (blood stage) schizont (51,52). When the schizont matures and ruptures, 8 to 36 merozoites, are discharged into the bloodstream. Another generation of blood's erythrocytes is infected as a result of the released merozoites. The length of time needed for erythrocytic schizogony varies depending on the type of *Plasmodium* and dictates the space between the releases of subsequent generations of merozoites, which accounts for the traditional periodicity of fever in malaria (53).

When mosquitoes take a blood meal, the gametocyte, which is the sexual stage, infects them. Gametocytes within the mosquito mature into female and male gametes (known as macrogamete s and microgametes, respectively), which are fertilized and then expand over the course of two to three weeks into sporozoites that infect people(54).Female mosquitoes need to survive at least 2 to 3 weeks before they can transmit malaria because there is a gap between becoming infected an d the maturation of sporozoites (55).

2.3. Pathogenesis and clinical presentation of malaria

The development of severe malaria may result from mixed reactions of parasite-specific factors. Such factors include adherence and sequestration in the vasculature and the release of active biomolecules, together with host inflammatory immune reactions (24). Which include cytokine and chemokine production and cellular infiltrates. Cerebral malaria is clinically presented by diffuses symmetrically to encephalopathy with high temperature and absent or few central neurological signs. Patients may recover full consciousness after a seizure; thus, transient postictal coma must be excluded. Multiple attacks are frequent, and up to 50% of unconscious children have subclinical convulsions or status epilepticus (56). Other clinical manifestation includes; Ocular funduscopic findings which includes vessel color change, macular and white-centered retinal bleeding. The median time to coma recovery is roughly 24 hours in children and 48 hours in adults. There is the ability of retinal abnormalities to resolve with no residual visual deficit. Post malaria neurological syndrome is self-limiting; however, long exposure to neurological symptoms, including cognitive deficits and epilepsy, are reported among children. The quite a number of malaria patients are exposed to risk factors for developing acute kidney injury (AKI), which includes; volume depletion, hypoalbuminemia, concomitant bacterial sepsis, black water fever (BWF) co- morbidity, such as diabetes (57).

2.4. Diagnosis of malaria

Quality demonstration of malaria parasite is critical to proper malaria case management. The worldwide effect of malaria disease has propelled interest in manufacturing successful testing strategies for resource-limited areas where malaria is a major public health concern in the community and developing countries, where malaria diagnostic expertise and equipment is often inadequate (58). Malaria disease is a potential medical emergency and should to be treated urgently. Delays in diagnosis and treatment are the leading causes of death in many countries .Such delays can be due to many factors such as diagnosis can be confusing where malaria is no longer endemic for healthcare providers, unfamiliar with the disease and clinicians may forget to consider malaria disease among some patients (59,60). Lack experience and technical skills from the laboratory personnel which can lead to failure to detect parasites when examining blood smears under a microscope. Malaria can be diagnosed in the laboratory using various techniques, including microscopic diagnosis by use of stained thin and thick peripheral blood films, concentration techniques, RDTs and molecular diagnostic methods (14).

Microscopy

Microscopic examination of stained blood films using Giemsa stains is the gold standard for diagnosis of malaria. Microscopy is important for identification and detection of *Plasmodium* species when using thick films, whereas, thin blood films is useful for species' confirmation. Although the expert microscopist can detect up to 5parasites/µl, the average microscopist detects only 50-100 parasites/µl. The low parasitemia level probably underestimated malaria percentage rate of infection, especially in low parasitemia individuals and asymptomatic malaria cases.

The challenge associated with implementing and sustaining a level of skilled microscopy appropriate for clinical diagnosis, particularly in the field setting, has prompted the development of various malaria RDTs devices. Microscopy is the most widely diagnostic tool used to demonstrate malaria at health facilities. In capable hands it is very sensitive for parasitemia >50parasites/µL it gives species identification, parasitic stages and parasitemia level. However, quality of microscopy is difficult to implement and maintain. It is labor intensive and requires highly skilled personnel and constant regular quality assurance measures (6,61).

Rapid diagnostic tests (RDTs)

Malaria rapid diagnostic tests are immune-chromatographic tests, which are used to demonstrate the presence of malaria parasites in suspected malaria cases by detecting the presence of one or a combination of the following *Plasmodium* antigens. *Plasmodium* Histidine-rich protein (HRP) 2 for *P. falciparum* or a 'pan-specific' aldolase or *Plasmodium* lactate dehydrogenase (pLDH) to detect other species such as *P. vivax*. These are antibodies based on the ability to capture circulating antigens from *Plasmodium* species, making them fast and reliable. There is numerous malaria RDTs commercially available, all of which detect malaria antigens or antibodies in the blood. Most RDTs that detect *P. falciparum* are histidine rich protein-2(HRP2) based. Other tests detect the presence of parasite enzyme Lactate Dehydrogenase (pLDH), using either monoclonal antibodies that react with LDH of all species including *P. falciparum* (Pan or pLDH) or antibodies specific for *P. falciparum* LDH. A blood sample from a patient need to be obtained using a lancet and standard sample collection devise is used to collect the blood sample and put in a sample well. Drop the reagent buffer solution on a test cassette, and interprets the results within 20 minutes (25,62).

Though there are variations among the more than 200 malaria RDT products on the market, the principles of the tests are similar. Immunochromatographic relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat anti-mouse antibody capture ensures that the system is controlled for migration (25,29).

How RDTs work

RDTs are lateral-flow immunoassays that allow visualization of specific antigen–antibody recognition events. In routine use, a specified amount of finger stick blood is transferred to one end of the RDT, the sample pad, which is loaded with reagents that lyse the blood cells to release any malaria antigens present and allow binding of monoclonal antibodies labelled with colloidal gold or another visible colorimetric indicator. Addition of a liquid buffer helps the blood wick up through the nitrocellulose membrane towards an absorbent pad. On the way, it crosses one or more test lines on the strip, where immobilized monoclonal antibodies can bind to exposed epitopes on *Plasmodium* proteins (*P. vivax* in Fig.1). In addition to test lines, which darken when malarial proteins are bound and detected, there is also a control line, which ensures that the sample pad reagents were liquefied and wicked appropriately up the RDT membrane. As each test may have a slightly different configuration and require different handling (e.g. amount of buffer to be added, time until results), the instructions accompanying the tests must be followed closely (63).

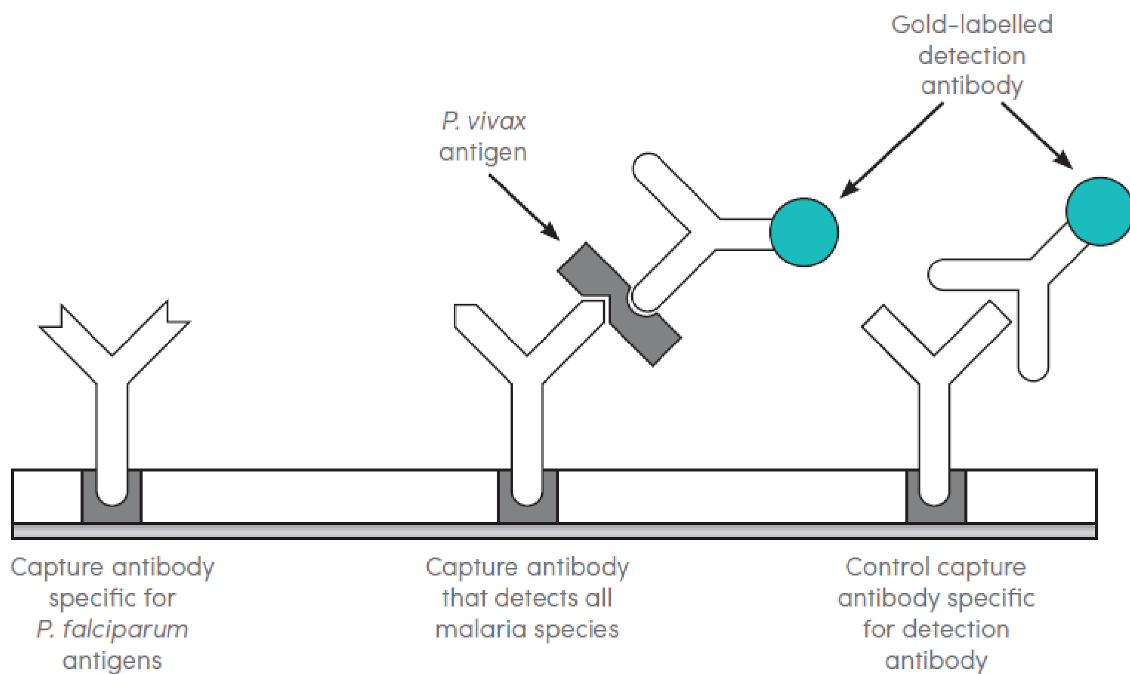


Figure 1: Immunological reaction on a positive RDT strip for *P. vivax* infection.

RDTs give high-quality results, reliable and are cost-effective. The RDTs procedure is simple and do not require high levels of technical expertise enhance the ability to be used by a wide range of personnel, including community health volunteers, which may constantly reduce the miss use of antimalarial drugs. However, performance characteristics decreases as parasitemia level decrease to less than 200 parasites/µl. HRP2 RDTs have disadvantages as their performance has been shown to be affected by product quality and parasite-related factors such as pfhrp2/3gene deletion, non-*P. falciparum* species and prozone effects that leads to false negative RDTs (25).

RDTs have proven to be a valuable tool in implementing the WHO-recommended parasite-based diagnosis in areas where expert microscopy is unavailable. When adopting RDTs, one of the problems that must be answered is whether these tests are relevant in a certain endemic location as well as which RDT to apply. Data on the sensitivity and specificity of RDT test kits is crucial for national malaria control programs to make informed decisions about which tests to use. *P. falciparum* parasites lacking the pfhrp2 and pfhrp3 genes pose a danger to malaria diagnostic tests in malaria endemic country.

Table 1: Summary of literatures which is related to this study and shows the main findings.

S.no	Title of the research	Study design	Sample size	Study population	Study period	Result	Reference
1	Diagnostic Performance of Three Rapid Diagnostic Test Kits for Malaria Parasite *P. falciparum in korea.*	Cross-sectional	1129	Febrile patients	-------	• Sensitivity and specificity of Biocredit Malaria Ag Pf(pLDH/pHRP 2)- 99.0% and 100%, • PPV, NPV and Accuracy=100, 99.8 and 99.8 respectively. • Sensitivity and specificity of Biocredit Malaria Ag Pv(pLDH)- 95.8% and 100%, • PPV, NPV and Accuracy =100,100 and 100	(64)
2	Comparative Assessment of Diagnostic Performances of	Cross-sectional	229	Febrile patients	---------	• The detection sensitivities and specificities for *P. falciparum and P. vivax* of Biocredit Malaria Ag *Pf/Pv* (*p*LDH/*p*LDH) were 87.83% and 100% respectively	(65)

	Two Commercial Rapid Diagnostic Test Kits for Detection of *Plasmodium* spp. in Ugandan Patients with Malaria						
3	*P. falciparum hrp2* and *hrp3* gene deletion status in Africa and South America by highly sensitive and specific digital PCR	Cross-sectional	47	Febrile patients	June to November 2016	• The hrp 2 exon 2 deletion was 2.1% • The hrp 3 deletion was 74.5% • The hrp 2 + hrp 3 gene deletion was 2.1%	(40)
4	Identification of *P. falciparum* isolates lacking histidine-rich protein 2 and 3 in Eritrea by nested PCR	Cross-sectional	135	Febrile patients	November 2013 and November 2014	• In the analyzed samples, 56% (81/144) of isolates were pfhrp2/pfhrp3 positive, while 9.7% (14/144) showed deletion of exon 2 of pfhrp2 gene and • 43% (62/144) of isolates lacked the pfhrp3 gene.	(66)
5	Major Threat to Malaria Control Programs by *P.*	Cross-sectional	50	Febrile patients	-------	• A study was showed that 80.8% (21/26) of patients at Ghindae Hospital and 41.7% (10/24)	(67)

	falciparum Lacking Histidine-Rich Protein 2, Eritrea nested PCR					at Massawa Hospital were infected with pfhrp2-negative parasites and • 92.3% (24/26) of patients at Ghindae Hospital and 70.8% (17/24) at Massawa Hospital were infected with pfhrp3-negative parasites.	
6	*P. falciparum* parasites with *pfhrp2* and *pfhrp3* gene deletions in two endemic regions of Kenya by nested PCR	Cross-sectional	274	Asymptomatic children	--------	• In samples with evidence of deletion, the result shows failed to amplify pfhrp2from 25 of 131 (19.1%) PCR-confirmed samples. • Of these, only 8 (10%) samples were microscopic positive and were classified as pfhrp2-deleted. • Eight microscopically-confirmed pfhrp2-deleted samples with intact pfhrp3locus were positive by HRP2-based RDT. • No sample harbored parasites lacking both genes.	(68)
7	High prevalence and extended deletions in *P. falciparum hrp2/3* genomic loci in	Cross-sectional	189	Febrile patients		• Pfhrp 2 and Pfhrp 3gene deletion was 100%	(39)

	Ethiopia by nested PCR						
8	Detection of high prevalence of *P. falciparum* histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: implication for malaria diagnosis by nested PCR	Cross-sectional	218	Febrile patients		• Pfhrp2 deletion-17.9% whereas pfhrp3 gene deletion -9.2% • pfhrp2exon1-2 (50%) was also detected while the deletions of the pfhrp3exon1-2 gene were 4.1%	(33)

The Biocredit RDTs is novel and under pre-qualification study in WHO. There are a few numbers of studies on diagnostic performance of this novel RDTs in the world particularly in Korea, Uganda and Burundi. In Ethiopia, there is no studies which were conducted on this novel RDTs and there also varying hrp 2/3 gene deletion were reported from different regions and communities especially through nested PCR. In our case the hrp 2/3 gene deletion was typing through digital PCR. The diagnostic performance and hrp 2/3 gene deletion report is vary based on location due to various factors. Conventional *hrp2* deletion typing relies on nested PCR followed by gel electrophoresis whereby a missing band is interpreted as *hrp2* deletion. The method requires up to a dozen individual PCRs. Recently we used highly sensitive assay for *hrp2/3* deletion diagnosis based on digital PCR (dPCR). The assay quantifies *hrp2*, *hrp3*, and a control gene with very high accuracy.

CHAPTER THREE

3. OBJECTIVE

3.1. General Objective

To evaluate the diagnostic performance of novel Biocredit RDTs (pLDH/ pHRP2 and pLDH /pLDH) for detection of *P. falciparum* and *P. vivax* and determine the prevalence of *P. falciparum* histidine rich protein 2/3 gene deletions among febrile patients at Maksegnit Health Center, Northwest Ethiopia

3.2. Specific Objectives

1. To evaluate the diagnostic performance of Biocredit Pf pLDH/ pHRP2 based rapid diagnostic test for the diagnosis of *P. falciparum* malaria in febrile patients.
2. To evaluate the diagnostic performance of Biocredit Pf/Pv pLDH/pLDH based rapid diagnostic test for the diagnosis of *P. falciparum* and *P. vivax* malaria in febrile patients.
3. To determine the prevalence of *P. falciparum* histidine rich protein 2/3 gene deletions in the study area

CHAPTER FOUR

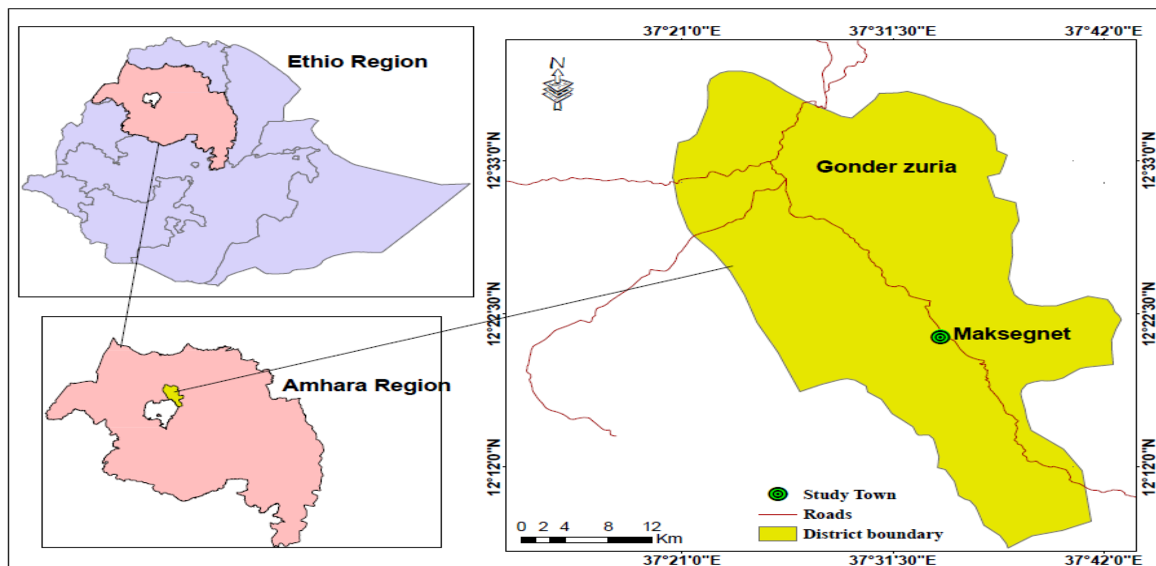
4. MATERIALS AND METHODS

4.1. Study Area

The study was conducted in Gondar zuria district which is located in central Gonder zone, Northwest Ethiopia. This district is located 45 km from Gondar town and 729 km from Addis Ababa, the capital of Ethiopia. Gondar zuria has a latitude and longitude of 12° 39' 59.99" N and 37° 19' 60.00" E with an elevation of 2133 meters above sea level. Gondar zuria district has an estimated total population of 208,889, of whom 108,388 were men and 100,501 women; 37,600 or 18.60% were urban inhabitants in an area of 1,108.53 square kilometers. Gondar zuria district is bordered on the south by the Debub Gondar zone, on the southwest by Lake Tana, on the west by Dembiya, on the north by Lay Armachiho, on the northeast by Wegera, and on the southeast by Mirab Belessa. The lowest and highest average temperature is 9.8°C and 29.7°C respectively and 1151mm average annual rainfall. The majority of its population depends on subsistence farming. Gondar zuria district is serving as a landing zone for seasonal migrants during the agricultural off season, which sees a high number of clinical cases despite its high altitude of above 2000m. According to the zonal health department report, the district is malarious; the streams and irrigation water serve as permanent *Anopheles* breeding sites during dry season. Gondar zuria district has seven health centers and 44 health posts providing services for people in and around the district. Maksegnit health center was selected due to high intensity of transmission according to the annual parasite incidence report (16) and also there is a significant number of seasonal migrants on the Northwest border of our country.

Maksegnit health center is one of the health centers found in Gonder zuria district. The health center was established in 1974. It is providing health care services for Gonder zuria administrative areas and the residents around from the boarder of the Gonder city. The health center provides medical services for 55,900 clients in outpatient settings. It serves for the catchment population of more than 93,456. The health center has a laboratory service mainly in outpatient laboratory. Among the services that is given in the health center laboratory include, stool examination, blood film examination, variety of serological tests, urine examination and blood group test. (Health Management Information System of Maksegnit Health Center) The location of study site was taken

by handheld Global positioning system and the map generated using ArcGIS version 10.7 software (Figure 2).



The map was generated using ArcGIS version 10.7 software

Figure 2: Map of the study area (Maksegnit, Gonder zuria woreda).

4.2. Study Design and period

A health facility based cross–sectional study was conducted from September 2021 to December 2021.

4.3. Population

4.3.1. Source population

All febrile individuals who came up to the health center for malaria diagnosis during the study period.

4.3.2. Study population

All individuals who came up to the health center with fever and fulfill the inclusion criteria

4.4. Sample size and sampling technique

4.4.1. Sample Size

We used the sample size calculation methods of Buderer *et al* 1996(69). In this method, we first calculated the True positive (TP) plus false negative (FN) for sensitivity and the true negative (TN) plus false positive (FP) for specificity through the following equation:

$TP+FN= Z^2 \times se\ (1-se)\ /W^2$ and

$TN+FP= Z^2 \times sp\ (1-sp)/W^2$

Where Z, the normal distribution value, is set to 1.96 as corresponding with the 95% confidence interval (CI), W, the maximum acceptable width of the 95% CI, is set to 5%, and the expected sensitivity of 96% and specificity of 93% are defined based on the estimates from previous studies done in Assosa, Ethiopia(70). The next step was to calculate the sample size (N) required for sensitivity for specificity through the following equations:

$N\ (se) = TP+FN/P$ and $N\ (sp) = TN+FP/1-P$

Where P is the prevalence of malaria. Taking the prevalence of symptomatic malaria by Pf-hrp 2 based RDT of 30.3% in 2020 in the northwest part of Ethiopia(70), a minimum of 195 for sensitivity and 144 samples for specificity will be required.

The required sample size (n) is calculated by using a single population proportion formula for which is $n = Z\alpha/2\ p\ (1-p)/d^2$, where n = the sample size, z = 1.96 at 95% confidence interval (CI), d = margin of error (5%), P = investigation of reported pfhrp2 exon1-2 gene deletion results in Assosa, Ethiopia (50%) (33). Accordingly, a total of 384 febrile study participants were enrolled. So, the sample size for sensitivity, specificity and hrp 2/3 gene deletion was 195, 144 and 384. The maximum sample size is **384** was selected based on statical significance.

4.4.2. Sampling Technique

Convenient sampling technique was used to select those febrile individuals until the desired sample size were obtained

4.5. Inclusion and Exclusion Criteria

4.5.1. Inclusion Criteria

Self-presenting febrile individuals ($\geq$37.5°C) suspected of malaria and ordered for blood film examination at the health center and willing to participate in the study were included.

4.5.2. Exclusion Criteria

- Critically ill patients who are unable to give blood samples
- Those who have taken antimalarial drugs before four weeks during study period

4.6. Study Variable

4.6.1. Dependent Variables

- Diagnostic performance of Biocredit Pf pLDH/ pHRP2 rapid malaria test
- Diagnostic performance of Biocredit Pf/Pv pLDH/pLDH rapid malaria test
- Histidine-rich protein 2 and 3 genes deletion

4.6.2. Independent Variables

- Age,
- Sex and
- Residence

4.7. Data Collection and laboratory methods

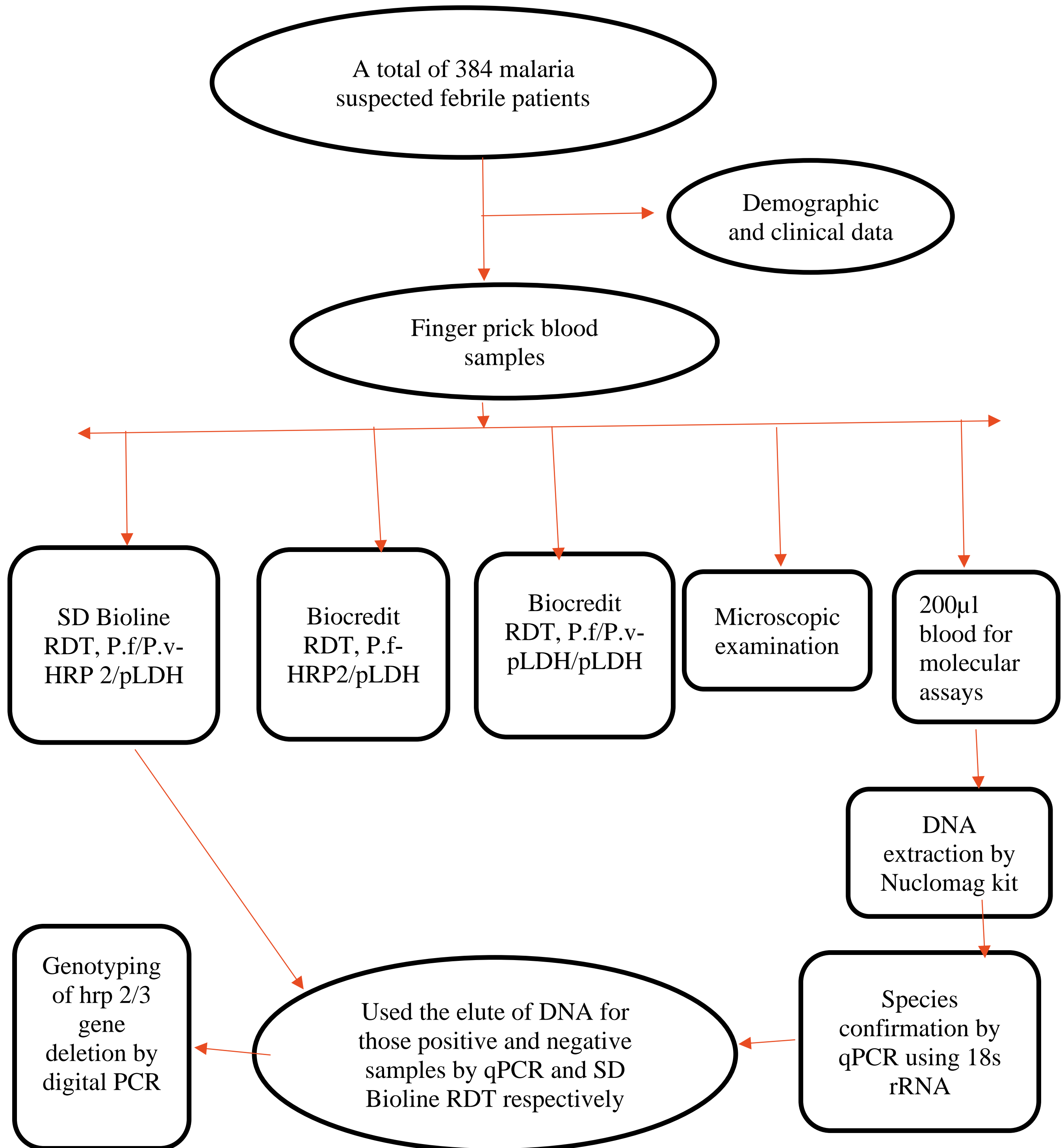


Figure 3: Study flowchart for malaria rapid diagnostic tests, microscopy, and molecular assay

4.7.1. Socio-demographic and clinical data

Information related to socio-demographic and clinical data were collected using structured questionnaire (Annex-IV). Face-to-face interviews were employed to collect socio-demographic and clinical data from the study participants. Each study participant was clinically examined for fever by nurse for assessment of auxiliary body temperature measured by thermometer, for adults >37.5^{o} C and for children> 37.2^{o}C were considered as febrile (71). Before data collection, data collectors selected patients who fulfilled the inclusion criteria and taken consent or assent form. If the study participants were children, consent and assent was taken from their parent/guardian and respective children respectively (Annex-II and III).

4.7.2. Blood sample collection and processing

Blood was drawn in accordance with standard operating procedures (72). The patient's finger was cleansed with an alcohol wipe and let to dry. The alcohol must dry in order for the region to be cleansed and for the person to experience less discomfort. A single-use safety lancet was used to take blood samples. The phlebotomist punctures the skin in the fleshy area of the finger pad, around 1 cm from the tip of the finger between the midline and side of the finger, to get a blood sample. With cotton, the initial blood sample was wiped away. A blood sample was taken after that for microscopic examination, RDT, and 200µl blood in EDTA microtainer tube for molecular analysis. Cotton is put on the fingertip when the blood draw was finished, and study participant was interviewed (73).

4.7.3. Malaria microscopy

Thick and thin smears were prepared on a single slide for each patient from capillary blood by finger prick using a sterile lancet. Each blood smear was air-dried and the thin film was fixed with methanol, then stained with Giemsa and was examined under the oil immersion microscope objective. A hundred fields were examined before reporting a negative result. The thick smear was used to detect *Plasmodium* infection and parasite quantification. A thin smear was used to identify the type of *Plasmodium* species (Annex-V). The number of parasites per microliter (*u*l) of blood were calculated by counting the number of asexual parasites in a set number of white blood cells (WBCs) (200) with a hand tally counter (74).

The microscopist was blinded to the results of the RDT. All blood films were re-read a second time by an experienced microscopist at AHRI who was also blinded to initial microscopy and RDT results. Quality control check was instituted by ensuring that both positive and negative slides were cross-checked by another trained microscopist in MHC and AHRI. Microscopy results and parasite counts were corrected according to the third reading, in cases where the results were discordant. All the microscopist involved in the study had pre-study training and were qualified.

4.7.4. Rapid Diagnostic Tests

A three band SD Bioline Malaria Ag P.f/P.v (HRP2/pLDH) RDT (Lot.No.05FK80) is a qualitative and differential test for the detection of HRP-2 and pLDH of *P. falciparum and P. vivax in* human whole blood respectively. The RDT contains a membrane strip, which is pre-coated with mouse monoclonal antibodies specific to the HRP-2 and the lactate dehydrogenase of *P. falciparum and P. vivax on* test band region (75).

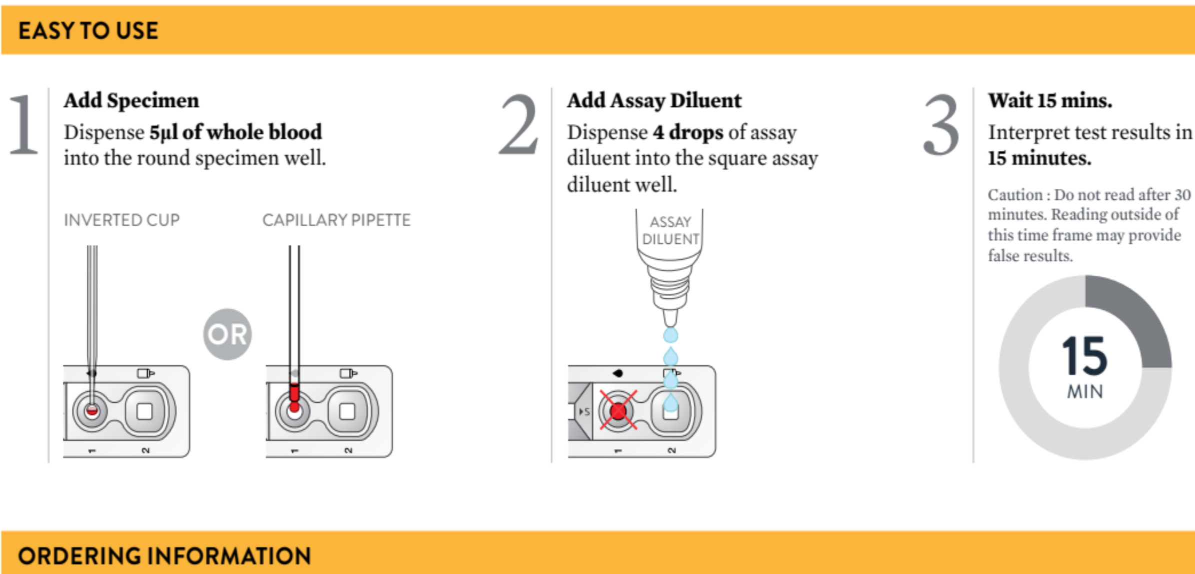


Figure 4: Procedure of SD Bioline RDT performance to investigate malaria

In this study we also used two novel malaria RDT products developed by RapiGEN Inc.(Gunpo, Korea), - Biocredit Malaria Ag Pf/Pv (pLDH/pLDH) (Lot. No. HO16A001DA) and Biocredit Malaria Ag Pf (pLDH/HRP2) (Lot. No. HO52A0010B). The Biocredit Malaria Ag Pf/Pv (pLDH/pLDH) is a combo test that detects *P. falciparum* and *P. vivax* on a single device. Biocredit Malaria Ag Pf (pLDH/HRP2) targets both Pf-pLDH and HRP2 antigens in *P. falciparum.* This

novel RDTs have a detection ability of <100 parasites/µl. Each RDT comprised a membrane strip pre-coated with antibodies specific to each target protein and was tested according to the manufacturer's instructions. Each sample was blinded against the results obtained from the other diagnostic tests.

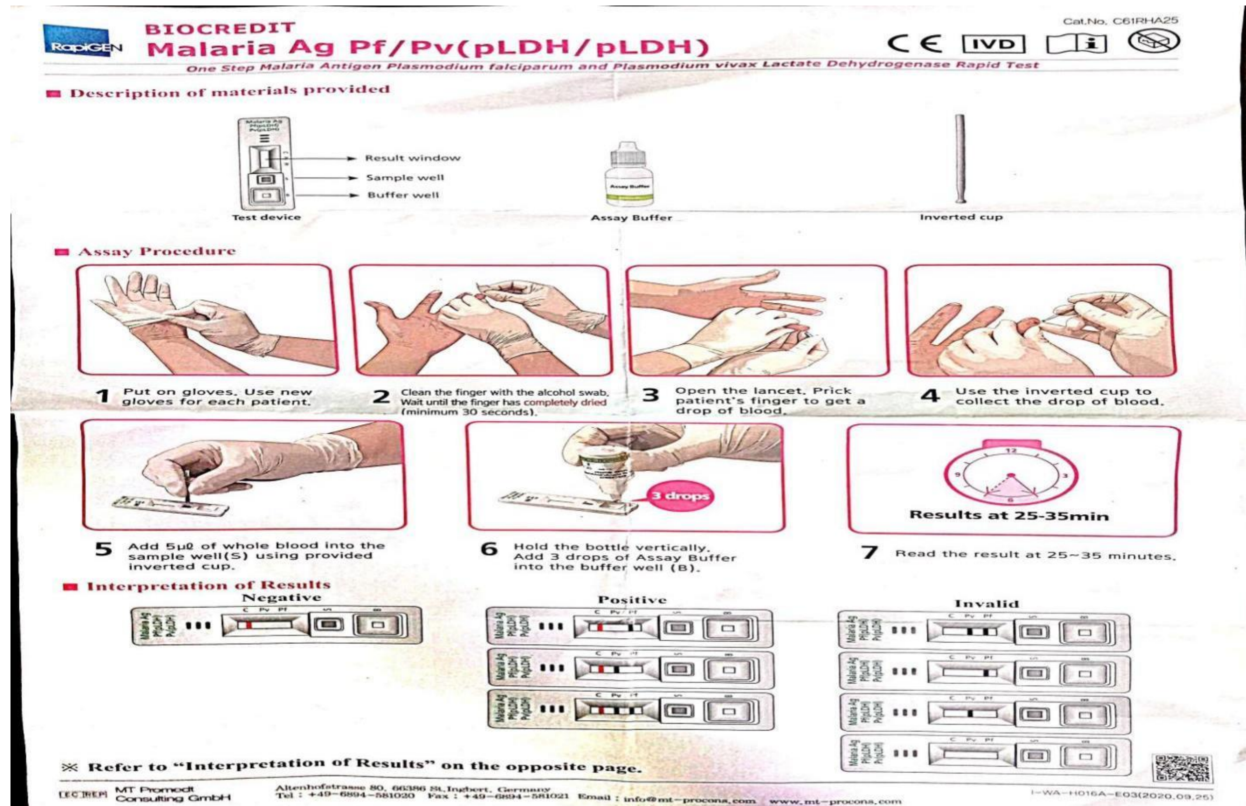


Figure 5: Manufacturer instruction for one step malaria antigen Pf/Pv (pLDH/pLDH) Rapid test.

4.7.5. Molecular Diagnosis

4.7.5.1. DNA extraction

Genomic DNA was extracted from blood using NucleoMag® Blood 200µL Kit method. The NucleoMag purification kit is one of the advanced methods of nucleic acid extraction technologies that use NucleoMag magnetic bead-based nucleic acid isolation technology to produce high yields of purified DNA that are free of PCR inhibitors from a variety of sample types. The magnetic beads not only have a large available binding surface but they can also be fully dispersed in solution, allowing thorough nucleic acid binding, washing, and elution (Annex-VI). (SOPs of NucleoMag® Blood 200µL Kit for DNA extraction in AHRI laboratory)

(Source: AHRI laboratory)

Figure 6: NucleoMag® Blood 200µL Kit DNA extraction in kingfisher flex machine**.**

4.7.5.2. Multiplex Quantitative PCR

Plasmodium falciparum and *P. vivax* identification was confirmed by TaqMan probe based quantitative PCR (qPCR) assay by targeting the 18S rRNA gene using species-specific primers (Annex-VII)(76). We used FAM and Texas Red as a reporter molecule for *P. falciparum* and *P. vivax* respectively. We used NF-54 and plasmids for *P. falciparum* and *P. vivax* as positive control respectively and negative control also involved. Serially diluted known quantity of parasite DNA amplified to generate a standard curve. In brief, qPCR amplification was carried out in a total reaction volume of 20 µL containing 2.57 µL of nuclease free water, 10 µL of TaqMan master mix, 0.6 µL each of the forward and reverse primers, and 5 µL of extracted DNA under the following PCR cycling conditions: initial denaturation at 95°C for 3 minutes, followed by 45 cycles of amplification at 94°C for 15 seconds, and 60°C for 1 minute each (Annex-VII). (SOPs of qPCR in AHRI laboratory)

Table 2: Primers and probes concentration in reaction volume for multiplex qPCR

18s qPCR mix	(Stock) µM	(Final) µM	Volume final 1x	100x
TaqMan fast advanced mix 2x	10	2	10	1000
Pf 18s FW	10	0.3	0.6	60
Pf 18s REV	10	0.3	0.6	60
Pf 18s Probe	10	0.11	0.22	22
Pv18s FW	10	0.2	0.4	40
Pv 18s REV	10	0.2	0.4	40
Pv 18s Probe	10	0.105	0.21	21
dH2O			2.57	257
Total			15	1500
			15 + 5µl DNA input=20	

Table 3: Primers and probes sequence for *P. falciparum* and *P. vivax* 18s qPCR.

Pf18S rRNA Forward primer	5'-GTAATTGGAATGATAGGAATTTACAAGGT-3'
Pf18S rRNA Reverse primer	5'-TCAACTACGAACGTTTTAACTGCAAC-3'
Pf18S rRNA probe	6FAM-AACAATTGGAGGGCAAG–MGBNFQ
Pv18SrRNA Forward primer	5'ACGCTTCTAGATTAATCCACATAACT3'
Pv18S rRNA Reverse primer	5'ATTTACTCAAAGTAACAAGGACTTCCAAGC3'
Pv18S rRNA Probe	HEX-5'TTCGTATCGACTTTGTGCGCATTTTGC3'

(Source: AHRI laboratory)

Figure 7: CFX96 Touch Real-Time PCR Detection System

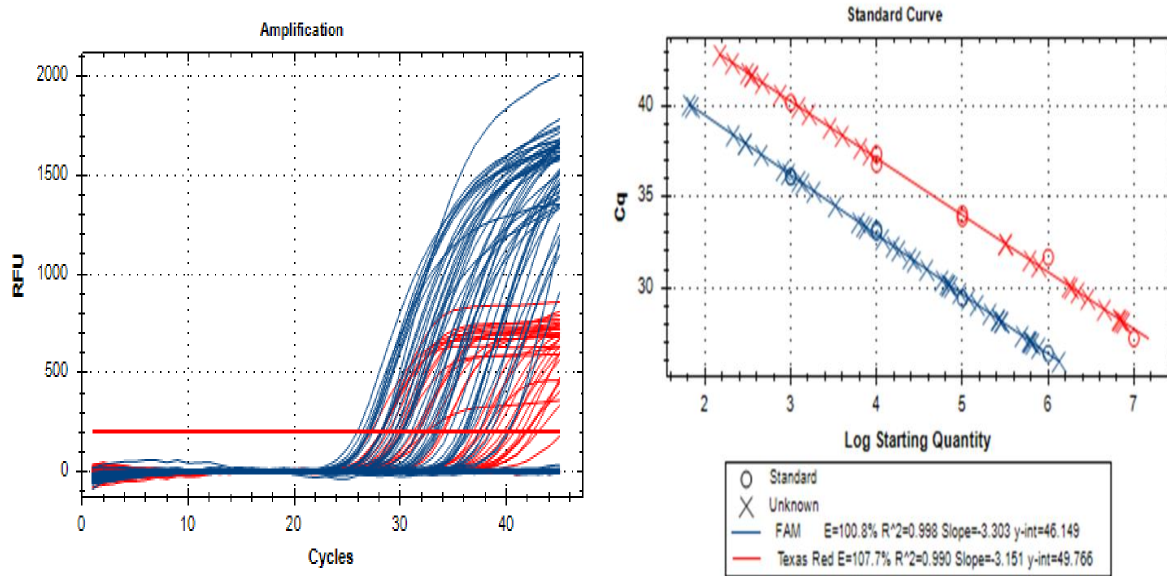


Figure 8: Amplification and standard curves of qPCR results for samples collected from study participants in Maksegnet health center, Northwest Ethiopia, 2022.

4.7.5.3. Genotyping of hrp2/hrp3 deletion by dPCR

Just like in qPCR experiments, sample preparation includes the transfer of master mix, probes and primers to a 96 well nano plate, followed by the addition of samples. In brief, dPCR amplification was carried out in a total reaction volume of 12 µL containing 2 µL of nuclease free water, 3 µL of probe mix, 0.96 µL each of the forward and reverse primers for HRP 2 exon 2, 0.48µl probe of HRP 2 exon 2, 0.24 µL each of the forward and reverse primers for tRNA, 0.12 µl probe of tRNA and 4 µL of extracted DNA under the following PCR cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 50 cycles of amplification at 94°C for 15 seconds each, 56°C for 1 minute each and 72°C for 15 seconds each. The reaction volume followed by cycling condition for hrp 3 also similar with HRP 2. The system integrates partitioning, thermocycling and imaging into a single fully automated instrument that takes users from the sample to result in under 2 hours. One can perform analysis on the Software Suite, providing the concentration in copies per microliter of your target sequence as well as for quality control such as positive samples or NTC. This analysis can also be extended to remote computers within the same local area network (LAN). The QIAcuity Nanoplate dPCR workflow involves three basic steps: pipetting and loading, running the experiment and analyzing results (Annex-VIII).

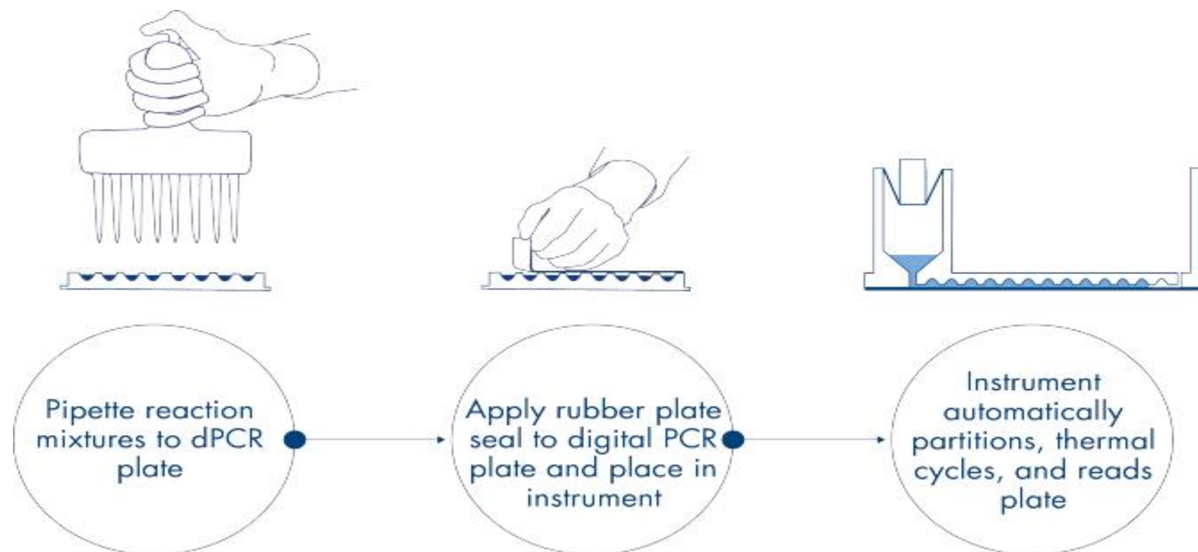


Figure 9: Work flow of QIAcuity dPCR

Table 4: Primers and probes concentration in reaction volume for hrp-2/hrp 3 by dPCR

Master Mix:	[Final] μM	Volume Final 1X	98X
Probe Mix 4X		3	294
hrp2_Exon 2-FW/hrp 3 forward	0.8	0.96	94.1
hrp2_Exon 2-RV/hrp 3 reverse	0.8	0.96	94.1
hrp2_Exon 2-Probe/ hrp 3 probe	0.4	0.48	47
tRNA_FW	0.2	0.24	23.5
tRNA_RV	0.2	0.24	23.5
tRNA_Probe	0.1	0.12	11.8
ddH2O		2	196
Total		8	784

Table 5: Primers and probes sequence for hrp-2, tRNA and hrp-3

hrp2 exon 2 forward	CATTTTTAAATGCTTTTTATTTTTATATAG
hrp2 exon 2 reverse	CTTGAGTTTCGTGTAATAATCTC
hrp2 exon 2 probes	FAM-CGCATTTAATAATAACTTGTGTAGCAAAAATGC- BHQ-1
hrp3 forward	ATGCTAATCACGGATTTCATTTTA
hrp3 reverse	ATCGTCATGGTGAGAATCATC
hrp3 probe	FAM-CCTTCACGATAACAATTCCCATACTTTAC- BHQ-1
tRNA forward	CATCAAATGAAGATTTAACAAGAG
tRNA reverse	CTTTTTGATTCTATAGTTTCATCTTTATG
tRNA probe	HEX-CTACCTCAGAACAACCATTATGTGCT- BHQ-1

We used three brand-new dPCR assays. One test, located next to the histidine-rich repeats, is focused on the first 120 conserved base pairs of hrp2 exon 2. There have been numerous breakpoints for the deletion of hrp2. The new primers for exon 2 are in a region that is deleted in all known deletion variations, hence hrp2 deletion will be discovered regardless of the precise breakpoint. The second test is aimed at hrp3. The assay amplifies a 101-bp region in exon 2's middle. A test that screened for serine-tRNA (Transfer Ribonucleic acid) ligase was multiplexed with each test. The conserved, single-copy gene tRNA is commonly used as a reference for gene expression testing. TRNA and hrp2 or hrp3 copy numbers are same in wild type infections without a deletion.

The reaction volume in a dPCR experiment is divided into around 8,500 partitions, which are subsequently subjected to endpoint PCR. If the partition includes template DNA, amplification will take place, and each partition acts as a separate PCR reaction. DPCR is superior to qPCR for quantification in many ways. Utilizing Poisson statistics, the quantity of positive and negative partition can be used to directly calculate the concentration of template DNA. External benchmarks are not necessary. Two targets, such as a control gene (tRNA) and a target gene, can be measured in a single reaction well using two separate probes (hrp 2 and tRNA or hrp 3 and tRNA). In comparison to gel-based assays, the innovative assay significantly decreases the number of reactions that must be done, has good sensitivity and precision, and can identify the deletion in polyclonal infections. The assay was extensively validated using culture strains, and field samples.

The QIAcuity is designed as a walk-away instrument that integrates and automates all plate processing steps. Only the plate preparation must be done manually before starting the run. This includes the pipetting of the target, reagents, and master mix in the plate’s input wells and the closing of the wells with the Nanoplate Seal. Once the preparation is done and the experiment is set up, the plate is placed in a free plate slot of the instrument tray. By reading the barcode of the plate, the instrument links the plate to the experiment previously defined in the software. After pressing the play button, all further steps are performed fully automated by the instrument(77).

Figure 10: QIAcuity Digital PCR System

4.8. Operational definition

Acceptable diagnostic performance of RDT by WHO based on microscopy as standard(78)

- Sensitivity > 95%
- Specificity >90%

How to interpret Kappa(79)

- Poor agreement = Less than 0.20
- Fair agreement = 0.20 to 0.40
- Moderate agreement = 0.40 to 0.60
- Good agreement = 0.60 to 0.80
- Excellent agreement = 0.80 to 1.00

Threshold of hrp 2/3 gene deletion by WHO(80)

- hrp 2/3 gene deletion > 5%

4.9. Data Quality Assurance and Management

To ensure the quality of the data, pre-analytical, analytical and post-analytical phases were maintained during sample processing by carefully following standard operating procedures. The English version of the questionnaire was translated into local language (Amharic), and re-translated to the English version for its accuracy and consistency (Annex-IV). Again, the questionnaire was pretested in 5% of the sample size number in one public health facility (Enfranz Health Center) before actual data collection and training was given for the data collectors to reduce technical and observer bias. Quality control for working equipment and reagents were ensured using standard controls. The result of each test was properly recorded. Finally, the questionnaires were checked by the investigator for their consistency and completeness.

4.10. Statistical Analysis

Data were checked for completeness and entered into Redcap (Research Electronic Data Capture) software and exported to Statistical Package for Social Sciences (SPSS) version 25 software (IBM Corporation, USA) for analysis. Diagnostic test evaluation calculator - MedCalc Software (Version 20.114) (free software available under the Clarified Artistic License) was used to calculate the sensitivity, specificity, positive and negative predictive values and accuracy with 95% confidence intervals (CI) for each RDTs using microscopy and qPCR as the gold standard. Cohen Kappa was used to assess the agreement between these malaria diagnostic methods. Calculations were based on cross-tabulation (contingency table) as shown below.

Table 6: Contingency table (2×2 table) to calculate diagnostic performance measurement.

<table>
<tr><td rowspan="5">RDTs</td><td></td><td colspan="3">Microscopic examination and qPCR (Gold standard)</td></tr>
<tr><td></td><td>Positive</td><td>Negative</td><td>Total</td></tr>
<tr><td>Positive</td><td>TP</td><td>FP</td><td>TP+FP</td></tr>
<tr><td>Negative</td><td>FN</td><td>TN</td><td>FN+TN</td></tr>
<tr><td>Total</td><td>TP+FN</td><td>FP+TN</td><td>TP+FN+FP+TN</td></tr>
</table>

Digital PCR relies on Poisson statistics to determine the absolute target quantity following an end-point amplification. As the target molecule is distributed randomly across all available partitions, Poisson distribution estimates the average number of molecules per partition (zero, one or more) and calculates the copies of the target molecule per positive partition. Poisson statistical analysis of the number of positive and negative reactions yields precise, absolute quantitation of the target sequence. Poisson statistics is applied to the probability of a given number of events in a fixed period if the events occur at a known constant rate and are independent of the occurrence of the previous event.

$$\lambda = -\ln\left(\frac{Number\ of\ valid\ partition - Number\ of\ postive\ partition}{Number\ of\ postive\ partition}\right)$$

The total amount of target DNA in all partitions of a well is calculated by multiplying the amount of average target DNA per partition with the number of valid partitions. Calculation of target concentration is determined by referring to the volume in all analyzable partitions, that is, partitions which were filled with reaction mix. The total number of filled partitions is identified by a fluorescent dye present in the reaction mix itself. Absolute quantification by dPCR eliminates the need for standard curves to determine amounts of target DNA in a given sample.

The result of wild type target and mutant target in a sample throughout the selected wells is represented in the diagram with one point showing the concentration in copies/µl value together with the confidence interval. The Concentration diagram tab shows a diagram that displays the distribution of concentration values in the wells together with their confidence intervals. A concentration diagram has two axes. The x-axis shows the analyzed wells and samples, and the y-axis represents the concentration values. P-values < 0.05 will be interpreted as statistically significant (77).

The prevalence is calculated as the number of pfhrp2/3deletion mutants causing false-negative RDTs divided by the total number of cases of *P. falciparum* malaria. Proportion of *P. falciparum* cases with suspected hrp 2/3 deletion (81)=

$$\frac{\#\ No\ of\ Pf\ case\ with\ discordant\ result\ by\ RDT\ and\ Microscopy}{\#\ Confirmed\ Pf\ cases\ by\ Microscopy\ or\ qPCR\ or\ Pf - pLDH\ RDT}$$

4.11. Ethical Consideration

Ethical clearance was obtained from the AHRI/ALERT Research and Ethical Review Committee and Institutional Review Board (IRB) of Jimma University, Institute of Health. A support letter from the post graduate program directorate of Jimma University was submitted to AHRI and MHC. A permission letter was also taken from health office of Gonder zuria where the data was collected. The data collectors informed the objective of the study to the study participants by reading or giving to read the information sheet which is prepared in the participants' language (Annex-I). Besides, written informed consent/assent were obtained from the study participants before data collection (Annex-II and III). Participation in the study was voluntary and refusal at any time is possible. To ensure confidentiality of data, the study subject was identified using codes instead of individual identifier and the unauthorized person is not able to access the collected data. The results were notified to the physicians at the outpatient department for proper treatment and management based on national guideline.

4.12. Dissemination of the Result

The finding of this study will be submitted to Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Science, MHC and AHRI. The findings of this study will be also published in peer-reviewed scientific journals. It will also be presented on different scientific forums.

CHAPTER FIVE

5. RESULTS

5.1. Socio-demographic data

Among a total of 384 malaria-suspected self-presenting febrile patients, 64.6% (248/384) were males and the remaining 35.4% (136/384) were females. The mean age of the study participants with standard deviation was 19.76 ± 13.5 years. Sixty-two percent 61.7% (237/384) of the study participants were from rural areas, whereas 38.3% (147/384) were from semi-urban areas (figure 11).

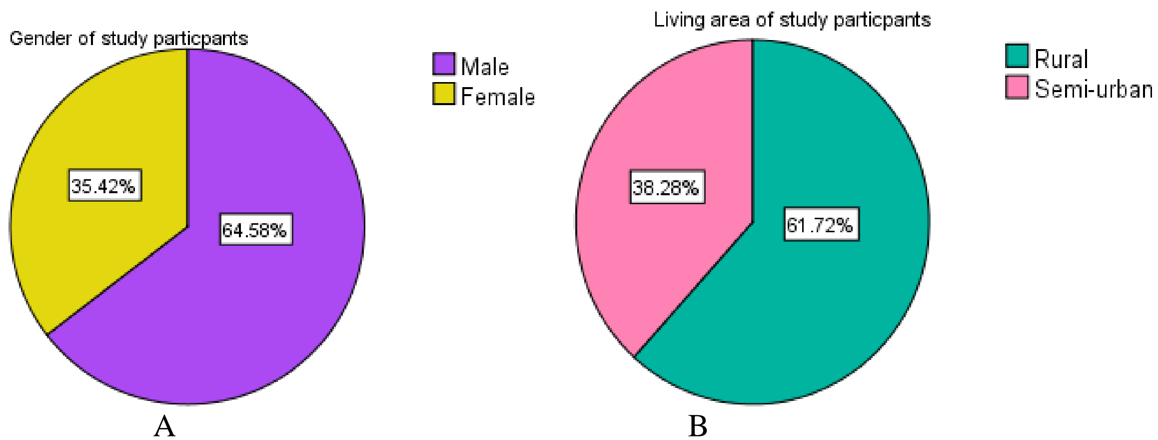


Figure 11: Gender and residence of study participants in Maksegnet health center, Northwest Ethiopia, 2022.

5.2. Malaria prevalence by Microscopy, RDTs and qPCR

Microscopy had a *P. falciparum* positivity rate of 50.3% (193/384) compared to Biocredit Pf-HRP2 and Pf-pLDH RDT's based positivity rates of 29.4% (113/384) and 48.9% (188/384) respectively. By using qPCR, the prevalence of *P. falciparum* was 51.6 % (198/384), almost no more difference with the microscopy or the Biocredit Pf-pLDH RDT. Microscopy had a *P. vivax* positivity rate of 19% (73/384), compared to Pv-pLDH RDT positivity rate of 17.9% (69/384). The prevalence of *P. vivax* was found to be 19.8% (76/384) using qPCR. The prevalence of *P. falciparum* and *P. vivax* by using SD Bioline RDT were25.7% (99/384) and 16.4% (63/384)

respectively (Table 7). With kappa values of 0.97, there was excellent agreement between the qPCR and microscopy techniques for both *P. falciparum* and *P. vivax* species. There was no discordance between field and AHRI reading of blood film species identification.

Table 7: Prevalence of malaria by different diagnostic tests among febrile patients in Maksegnet health center, Northwest Ethiopia, 2022 (n=384).

Species	Microscopy	Biocredit RDT Pf (pLDH/pHRP2)	Biocredit RDT Pf/Pv (pLDH/pLDH)	SD Bioline RDT	qPCR
P. falciparum (%)	193 (50.3)	188(48.9)	189 (49.2)	99(25.8)	198 (51.6)
P. vivax (%)	73 (19.0)	-	69 (17.9)	63(16.4)	76 (19.8)
Total (%)	266(69.3)	189(49.2)	258(67.2)	162(42.2)	274(71.4)

5.3. Sensitivity and specificity of Biocredit and SD Bioline RDTs

The sensitivity and specificity of Biocredit Pf-HRP2/pLDH RDT was 97.4% and 97.5% respectively, using microscopy as the gold standard. With kappa values of 0.943, there was a perfect amount of agreement between the Biocredit Pf-HRP2/pLDH RDT and reference microscopy technique. The sensitivity of Biocredit Pf-pLDH and Pv-pLDH RDT was 97.9% and 94.5% respectively, using microscopy as the gold standard. The respective specificity rates were 97.4% and 97.5% (Table 8). Excellent agreement was found between the Biocredit Pf-pLDH and Pv-pLDH RDT and reference microscopy method with kappa values about 0.945.

The SD-Bioline RDT have low diagnostic sensitivity as compared to the Biocredit RDTs (Table 8, figure 12). The PfHRP2 component of SD Bioline and Biocredit RDT detect 99 and 113 out of 193 microscopically confirmed *P. falciparum* species respectively. The novel Biocredit HRP2 based RDT showed improved sensitivity for the diagnosis of *P. falciparum* than the conventional SD Bioline HRP2 based RDT by considering both microscopy and qPCR as a reference.

Table 8: Performance of different RDTs kits compared to microscopic examination (n=384) in Maksegnet health center, Northwest Ethiopia, 2022.

Parameters	Biocredit Pf (pLDH/pHRP2)	Biocredit Pf/Pv (pLDH/pLDH)		SD Bioline RDT	
		Pf (pLDH)	Pv (pLDH)	Pf-HRP2	Pv-LDH
Sensitivity	97.4(94.1 to 99.2)	97.9(94.8 to 99.4)	94.5 (86.6 to 98.5)	51.3(44.0 to58.5)	86.3(76.3 to 93.2)
Specificity	97.5(92.8 to 99.5)	97.4 (92.7 to 99.5)	97.5 (92.8 to 99.5)	93.2(87.1 to 97.0)	96.5(91.3 to 99.0)
PPV	98.4(95.4 to 99.5)	98.4(95.4 to 99.5)	95.8(88.3 to 98.6)	92.5(86.2 to 96.1)	94.0(85.7 to 97.6)
NPV	95.8 (90.6 to 98.2)	96.6 (91.5 to 98.7)	96.6 (91.7 to 98.7)	53.9(50.1 to 57.7)	91.7(86.1 to 95.1)
Accuracy	97.4 (94.9 to 98.8)	97.7 (95.4 to 99.1)	96.4(92.6 to 98.5)	67.2(61.7 to 72.4)	92.5(87.8 to95.9)

Pf-HRP2 = *Plasmodium falciparum* histidine-rich protein 2; Pf-LDH = *Plasmodium falciparum* lactate dehydrogenase; Pv-LDH = *Plasmodium vivax* lactate dehydrogenase, RDT = rapid diagnostic test.

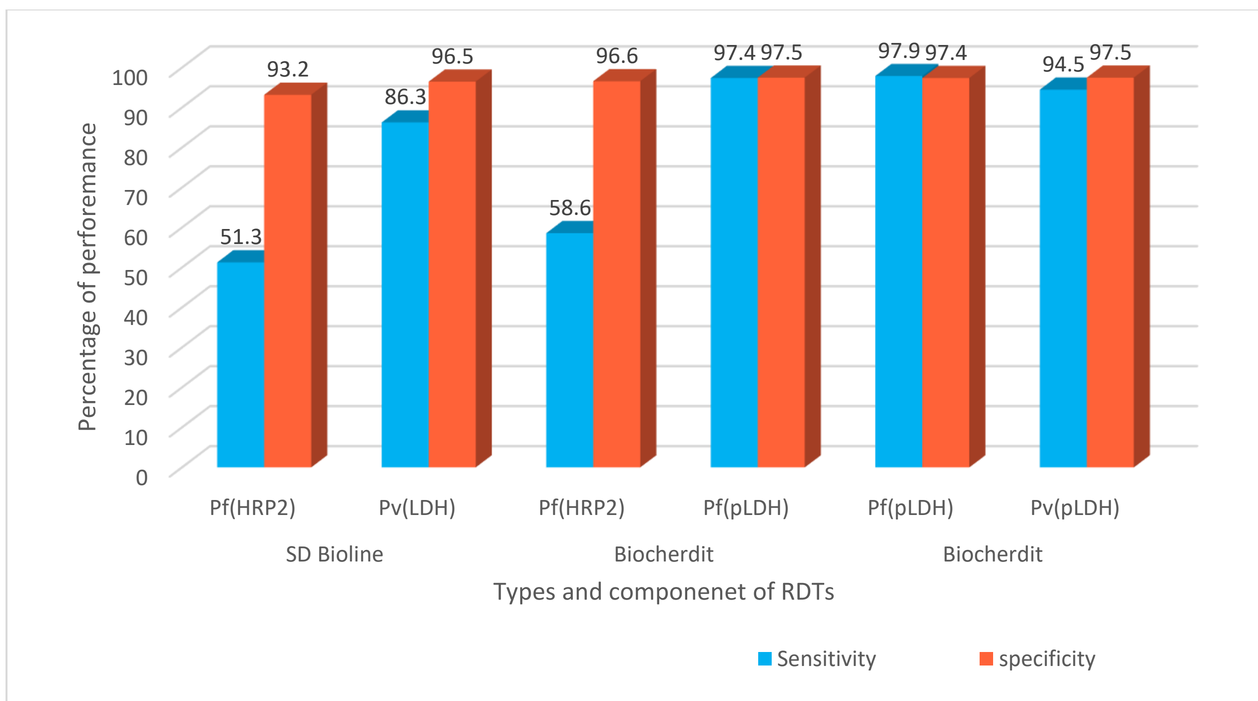


Figure 12: Comparison of sensitivity and specificity of SD Bioline and Biocredit RDTs in Maksegnet health center, Northwest Ethiopia, 2022.

When qPCR was used as a reference method, Biocredit Pf-HRP2/pLDH had a sensitivity and specificity of 94.9% and 97.4 % respectively. With a kappa value of 0.939, the Biocredit RDT (Pf-HRP2/pLDH) and qPCR likewise demonstrated a perfect degree of agreement. The sensitivity of Biocredit Pf-pLDH and Pv-pLDH RDT had 95.5 % and 90.8 % when qPCR was employed as a reference test. Additionally, the specificity of Biocredit RDT Pf-pLDH had 96.4 % and Pv-pLDH had 99.1 % (Table 9). With a kappa value of 0.941, the Biocredit RDT (Pf-pLDH and Pv-pLDH) and qPCR likewise shown excellent measure of agreement.

Table 9: Performance of different RDTs kits compared to qPCR examination (n=384) in Maksegnet health center, Northwest Ethiopia, 2022.

Parameters	**Biochredit Pf (pLDH/pHRP2)**	**Biochredit Pf/Pv (pLDH/pLDH)**		**SD Bioline RDT**	
		Pf (pLDH)	Pv (pLDH)	Pf-HRP2	PV-LDH
Sensitivity	94.9 (90.9 to 97.6)	95.5 (91.6 to 97.9)	90.8(81.9 to 96.2)	50(42.8 to 57.8)	83(72.5 to 90.6)
Specificity	97.4 (92.4 to 99.5)	96.4 (90.9 to 99.0)	99.1 (95.0 to 99.9)	96.5(91.3 to 99)	96.5(91.3 to 99)
PPV	98.4 (95.5 to 99.5)	97.9 (94.8to 99.2)	98.6 (90.7 to 99.8)	96.1(90 to 98.5)	94(85.7 to 97.7)
NPV	91.7 (85.7 to 95.3)	92.2(86.2 to 95.7)	93.9 (88.5 to 96.9)	52.6(49 to 56.2)	89.4(83.7 to 93.3)
Accuracy	95.8 (92.9 to 97.8)	95.8 (92.9 to 97.7)	95.7 (91.7 to 98.1)	67(61.5 to 72.2)	91(86 .0 to 94.7)

Pf-HRP2 = *Plasmodium falciparum* histidine-rich protein 2; Pf-LDH = *Plasmodium falciparum* lactate dehydrogenase; Pv-LDH = *Plasmodium vivax* lactate dehydrogenase qPCR = quantitative PCR; RDT = rapid diagnostic test

5.4. Socio-demographic factors and parasite density

Microscopic parasite density ranged from 53 to 89,043 parasite/µl. With respect to the study participants' gender and age categories, parasitemia levels differed. Among the study participant who have a parasite density most of them were within 5-14 age groups (Table 11). As parasite density distributions were skewed, geometric mean densities are given whenever densities are reported. The geometric parasite density of the study participant was 4598.75. Age groups 55-64 have highest geometric parasite density (figure 13) when compare to others. When we see the

geometric mean of parasite density among gender of participant in bar graph the female had high mean of parasite densities than male (figure 14).

Table 10: Distribution of parasite density by gender, age-group, and living area in Maksegnet health center, Northwest Ethiopia, 2022

		Study participant, No (%)							
Parasite density	Number	Gender		Age groups				Living area	
		Male	Female	5-14	15-24	25-34	>35	Rural	Semi-urban
50-200	7	6(85.7)	1(14.3)	3(42.8)	2(28.6)	1(14.3)	1(14.3)	3(42.8)	4(57.1)
201-500	23	20(86.9)	3(13)	10(43.5)	9(39.1)	3(13)	1(4.3)	18(78.2)	5(21.7)
501-2000	48	35(72.9)	13(27.1)	20(41.7)	13(27.1)	6(12.5)	9(18.8)	30(62.5)	18(37.5)
2001-10,000	92	57(61.9)	35(38)	36(39.1)	32(34.8)	14(15.2)	10(10.9)	58(63)	34(36.9)
>10,000	96	62(64.5)	34(35.4)	50(52.1)	3(32.3)	4(4.17)	11(11.5)	67(69.8)	29(30.2)
P-value		<0.119		<0.599				< 0.348	

There was no statistically significant association of the living area ($P < 0.348$), gender ($P < 0.119$), and age-group ($P < 0.599$) of the study participants with microscopic parasite density.

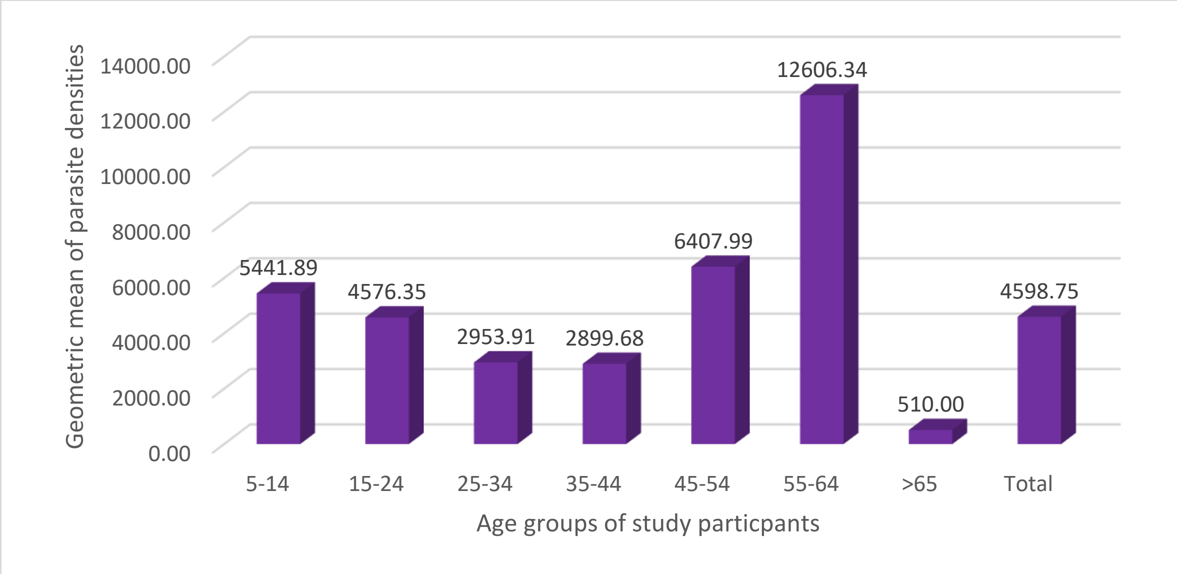


Figure 13: Geometric mean of parasite densities among age groups of study participants in Maksegnet health center, Northwest Ethiopia, 2022.

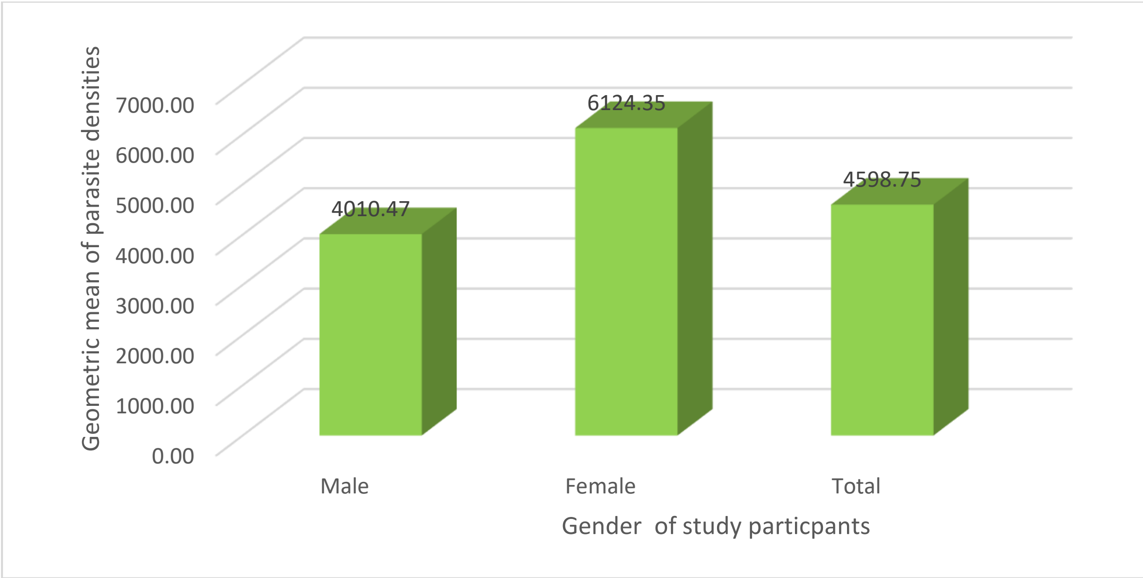


Figure 14: Geometric mean of parasite densities among gender of study participants in Maksegnet health center, Northwest Ethiopia, 2022

5.5. HRP 2/3 gene deletion typing by dPCR

According to the qPCR and SD Bioline field and laboratory data, there are a round 99 samples out of the 198 *P. falciparum* qPCR positive samples that are negative for the SD Bioline HRP 2 which are a target for hrp 2/3 gene deletion by dPCR.

A total of 99 *P. falciparum* positive samples by qPCR were included based on inclusion criteria for molecular analysis of pfhrp2 and pfhrp3 gene deletion. Of the study participants positive for *P. falciparum* by qPCR, 67.7% (67/99) and 45.5% (45/99) of the positive cases were male and in the youngest age group (5–14 years), respectively. *P. falciparum* positive samples with cyclic threshold (Ct) value ranged with 18.5-35.6 were included for pfhrp2/3molecular analysis by dPCR.

During optimization those sample with Ct value 18 to 21 needs dilution, in our case there are eight samples that being diluted in order to prevent over concentration of the parasites in the partition. Samples ranging from 2.3 to 352589.2 parasites/µl in qPCR were successfully typed, with samples at densities of >43,550 parasites/µl diluted in elution buffer. In wild-type samples, a very similar quantification of hrp2, hrp 3 and tRNA was expected.

Out of 99 SD Bioline Pf HRP2 RDT negative samples 23.2% (46/198) have hrp 2 exon 2 deletions, 27.7% (55/198) have hrp 3 deletion and 13.1% (26/198) have both hrp 2 exon 2 and hrp 3 deletion.

Table 11: The hrp2 and hrp3 deletions status in Maksegnet health center, North West Ethiopia, 2022.

Study site	Sample positive by qPCR	SD-Bioline Pfhrp2 RDT negative	*hrp2* exon 2	*hrp3*	*hrp2+hrp3*
MHC	198	99	46/198=23.2%	55/198=27.7%	26/198=13.1%

When we see the positive and negative partition of hrp 2 and tRNA, for those samples which have hrp 2 deletion there is no positive partition but they contain tRNA partition. Look at the figure 15

and 16 (example well F1 for hrp 2 deletion, F3 no hrp 2 deletion and figure 16 tRNA concentration on 1D scatter).

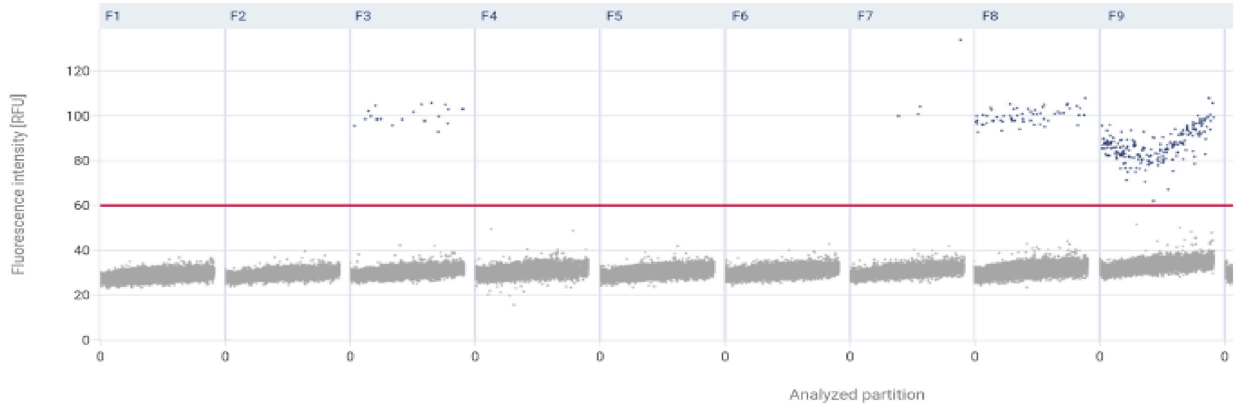


Figure 15: Sample positive and negative partition for hrp 2 deletion 1D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022**.**

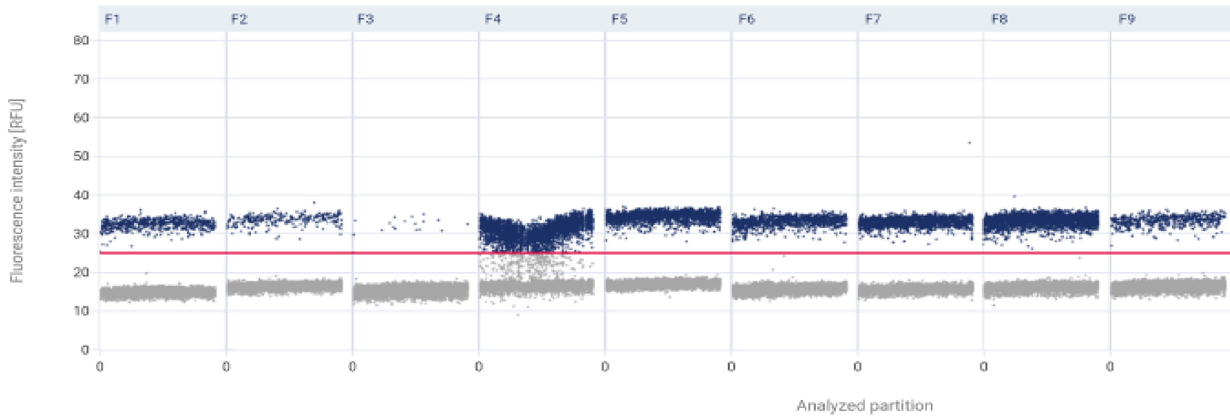


Figure 16: Sample positive and negative partition for tRNA 1D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022.

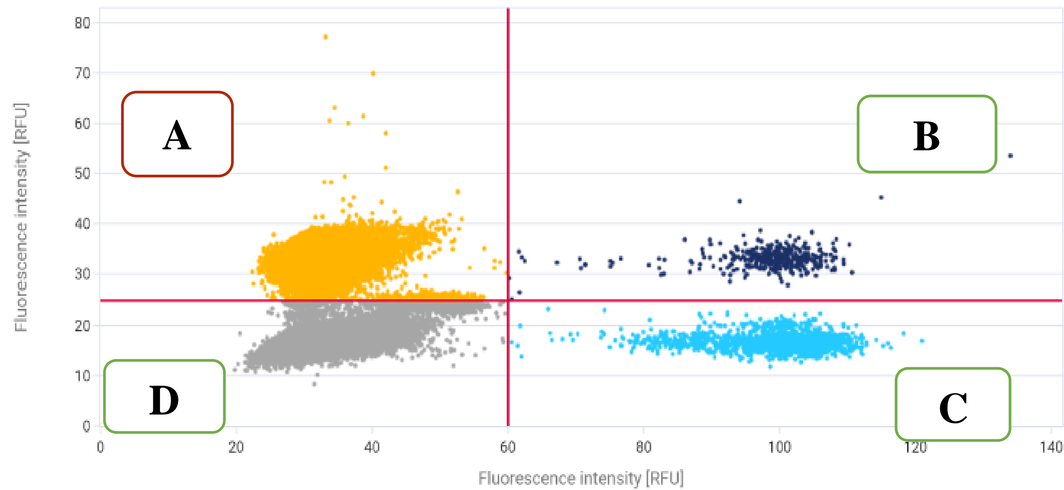


Figure 17: Over all examples of hrp2 exon 2 deletion typing by dPCR 2D scatter plot status in Maksegnet health center, Northwest Ethiopia, 2022.

A, Partition positive for *tRNA* are shown in yellow (top left).

B, Partition positive for *hrp2* are shown in blue black (top right).

C, Partition positive for *hrp2* and *tRNA* are shown in blue (bottom right).

D, Negative Partition (for both *hrp2* and *tRNA*) are shown in gray (bottom left).

CHAPTER SIX

6. DISCUSSION

A precise diagnosis is necessary for effective malaria case management, whether the test is based on microscopy or an RDT. Quality-assured RDT and microscopy have been considered the diagnostic methods of choice for the confirmation and management of suspected clinical malaria even in areas of low transmission due to their high diagnostic performance capabilities in detecting clinical malaria, their relative affordability, and their availability. To support the global effort of the control and elimination campaign, the efficacy of malaria RDTs in places where the disease is endemic must be periodically assessed. In order for RDTs to be effective and valuable diagnostic tools, the WHO recommends that they have higher than 95% sensitivity and 90% specificity (78).

In the current study, the sensitivity of the Biocredit RDT kits was 97.41% and specificity was 97.46% when using Biocredit Pf (pLDH/pHRP 2) RDT kit respectively. This result was almost in agreement with a study which was conducted in Korea (64) as well which is acceptable based on WHO recommendation. However, the sensitivity of HRP 2 in Biocredit Pf (pLDH/pHRP 2) RDT in this finding which is 58.6% (figure 12) was low when compared with a study which was conducted in Korea (64). When qPCR was used as a reference method, Biocredit Pf-HRP2/pLDH had high diagnostic performance when we compared with the study which was conducted in Burundi (26).

This might be due to hrp 2/3 gene deletion, genetic variability and sequence variation without deletion of the hrp2gene, prozone effect and low parasitemia level. However, the Biochredit Pf HRP2/pLDH based *P. falciparum* RDT was important as a diagnostic tool whether there is false negative result in HRP 2 component was present or not because there is a backup of ultrasensitive Biochredit Pf-pLDH which is an alternative for *P. falciparum* detection in single test device. This RDT was in perfect agreement with the microscopic examinations and the 18s ribosomal RNA-based qPCR results.

Interestingly, the sensitivity of Biocredit Pf/Pv(pLDH/pLDH) RDT was 97.93% and 94.52% for both *P. falciparum* and *P. vivax* malaria and with corresponding specificity was 97.44% and 97.46% for *P. falciparum* and *P. vivax* respectively which is fulfill the criteria of WHO recommendation. This finding was in line with the result which were done in Korea (64). In contrast this result was higher when we compared with a study which was conducted in Uganda (82).This might be due to parasitemia level and persistence of the antigen. The Biocredit RDT (Pf-pLDH and Pv-pLDH) and microscopy and qPCR also showed excellent measure of agreement with a kappa value of more than 0.9.

The geographical difference that could account for the high prevalence of *P. falciparum* in this study area is that during the agricultural off-season, a sizable number of seasonal migrants cross the country's northwestern border from the region where malaria could be imported and exported between Sudan, Eritrea, and Ethiopia. Additionally, when compared to semi-urban areas, the incidence of *Plasmodium* was higher in rural areas. This may be a result of the extensive and widespread outdoor agricultural operations taking place in the rural area.

Using the Biocredit Pf-pLDH RDT, this study found that males had a greater infection rate of *P. falciparum* (51.2%, 127/248) than females (44.9%, 61/136). This outcome was in line with a previous investigation carried out in the northwest Ethiopian district of Assosa (70). Males are more likely than females to spend more time outdoors working in agriculture and mining, which exposes them to more disease-carrying mosquitoes. Gender, age, and residency (in rural and semi-urban areas) did not substantially correlate with the parasite density as measured by microscopy. This outcome was consistent with earlier studies carried out by Cameron and Assosa (70,83). It does not, however, agree with a study which was conducted in Uganda (84). This could be as a result of the different age groups, immune status, geographical location and difference in clinically suspected cases of malaria among the study participants.

The Biocredit Pf-pLDH RDT was used in this investigation to identify the malaria, which had a high negative predictive value (NPV) (95.83%) and positive predictive value (PPV) (98.43%) using microscopy as the gold standard which is in line with a study which was conducted in Korea (64). By using the Biocredit Pf-pLDH RDT, these high NPV and PPV correspondingly demonstrated minimal false-negative and false-positive rates. These results showed that the

Biocredit Pf-pLDH RDT performs pretty well for detecting malaria-free patients as true negatives and malaria-positive people as true positives in peripheral health facilities.

In comparison to estimates of those previously used commercial RDTs, the Biocredit RDT Pf/Pv (pLDH/pLDH) and Pf (pLDH/pHRP 2) evaluated in this experiment had superior sensitivity and specificity. In a field context, these Biocredit kits produced positive blood test results for *P. falciparum* and *P.vivax*, resulting in precise diagnosis. Given their effectiveness, we advise utilizing these Biocredit RDT kits for *P. falciparum* and *P. vivax* screening and confirmation at healthcare facilities with limited staffing and equipment availability.

Out of a total of 50% (99/198) SD Bioline Pf-HRP2 RDT negative samples in this study, a high proportion of dPCR confirmed deletions were observed in pfhrp2 exon2 and pfhrp3compared to WHO pfhrp2/3 deletion threshold level. Approximately twenty-three percent of 23.2% (46/198) SD Bioline Pf-HRP2 RDT negative samples lacked exon 2 of pfhrp2, the main amino acid coding region of the pfhrp2gene. Moreover, high prevalence of deletion was detected for pfhrp3 which accounts 27.7% (55/198). dPCR confirmed the absence of both pfhrp2/3genes to be 13.1% (26/198) of the *P. falciparum* strains. Double deletions of the pfhrp2/3 genes were found in this study, which could imply a difficulty in SD Bioline Pf-HRP2 RDT-based malaria detection in the study area. The diagnostic performance of SD Bioline Pf-HRP2 RDT would surely be impacted by the complete deletions of the pfhrp2/3 genes found in this investigation.

This investigation verified that clinical isolates of *P. falciparum* in the Gonder zuria district had a significant proportion of pfhrp2/3 gene deletions. These numerous false-negative individuals run the risk of acting as malaria reservoirs, hastening the spread of malaria in the community, if they are left undiagnosed and untreated. According to this study, the deletion of the pfhrp2/3 gene in *P. falciparum* populations should be the cause of the high percentage of SD Bioline Pf-HRP2 RDT false-negative results.

The prevalence of pfhrp2 exon2 deletion in this study 23.2% (46/198) varied from the findings reported by previous studies. It is lower compared to the findings of studies conducted in Eritrea (67), Sudan (85), Djibouti (86) and Adama, Ethiopia (39)and higher than in a study that was done in Ethiopia (87–89). The prevalence of pfhrp3deletion in this study 27.7% (55/198) varied from

the findings and showed higher result in a study which was conducted in Assosa, Ethiopia (89) and lower results in Eritrea (67), Djibouti (90) and Adama and Jimma in Ethiopia (39,87). Double deletions of the pfhrp2/3 genes were found in 13.1% (26/198), this finding was higher when compared to studies which conducted in Nigeria, Sudan and South Sudan (91) and Jimma, Ethiopia (92) and lower compared with a study conducted in Eritrea (67), Djibouti (86) and Adama, Ethiopia (39). The possible explanation for these differences might be due to variation in transmission intensity, host immunogenic response, drug used, geographical locations, sample size, and laboratory methods used to analyze pfhrp2/3 genes deletions.

According to our extensive literature review, in our country there has been no comprehensive study evaluating this novel Biocredit RDTs by considering microscopy and qPCR as a standard test and typing of hrp 2/3 gene deletion by dPCR which is addressed by the current study. Despite its strength, our study has some limitations. As a limitation the effectiveness of the Biocredit Pf-HRP2, Biocredit Pf-pLDH and Biocredit Pv-pLDH RDTs among asymptomatic people and variable malaria transmission seasons were not taken into account.

CHAPTER SEVEN

7. CONCLUSION AND RECOMMENDATION

7.1. Conclusion

In general, the sensitivity and specificity of both Biocredit RDTs in this study comply with the WHO limit of detection for routine diagnosis of clinical malaria. There was a perfect agreement and moderate agreement between Biocredit RDTs and SD Bioline RDT with the reference microscopy and qPCR for the diagnosis of clinical malaria in this study respectively. Hence, this currently in pre-qualification, Biocredit RDTs based on WHO could be used as an alternative diagnostic tool in the absence of microscopy. Biocredit kits were shown to be reliable diagnostic kits to detect *P. falciparum* and *P. vivax* malaria infections and can contribute to malaria control efforts as a possible replacement for microscopic examination in front-line diagnosis. This study confirms the presence of 13.1% of pfhrp2/3 gene deletions in *P. falciparum* isolates in the study area. Therefore, based on the prevalence of hrp 2/3 gene deletion there should be consider alternative diagnostic tool like Biocredit Pf-pLDH based RDT at study area. The high proportion of SD Bioline Pf-HRP2 RDT false-negative results due to the deletion of pfhrp2/3 gene could affect malaria control and elimination efforts in the country. Besides further nationwide survey on the prevalence of hrp 2/3 gene deletion is crucial.

7.2. Recommendation

To National Malaria Control Program:

After further country-wide attention to evaluation of this novel Biocredit RDTs, guidelines for the management of malaria should consider to incorporating this novel and ultrasensitive Biocredit RDTs as screening and confirming of *Plasmodium* for early detection and treatment of infected individuals and hence improve the overall care of patients with malaria. Moreover, the importance of molecular surveillance and mapping of pfhrp2/3 deletions in other place in Ethiopia to guarantee appropriate patient care, control and ultimately contribute to achieving malaria elimination

To Maksegnet health center:

The prevalence of Pfhrp2/3 gene deletion in Maksegnit, Gonder zuria woreda exceeds the 5% threshold, thus making the HRP2 based rapid diagnostic tests not completely reliable for malaria diagnosis in the study area so, it is recommended that Pf-pLDH-based RDTs will be used.

To the researchers:

A number of studies should be conducted using a large sample size. Also, endemicity, travel history, season of transmission, clinical classifications of the participants including asymptomatic individuals, and risk area for polymorphism and hrp 2 deletion should be considered. It might be very important to have other studies across the country for patients to provide rational care.

REFERENCES

1. Federal Minstry of health(FMoH) E. Ethiopia malaria elimination strategic plan. Vol. 1. 2021. 10–12 p.
2. Howes RE, Battle KE, Mendis KN, Smith DL, Cibulskis RE, Baird JK, et al. Global epidemiology of Plasmodium vivax. Am J Trop Med Hyg. 2016;95(6):15–34.
3. Sondo P, Derra K, Lefevre T, Diallo-Nakanabo S, Tarnagda Z, Zampa O, et al. Genetically diverse Plasmodium falciparum infections, within-host competition and symptomatic malaria in humans. Sci Rep. 2019;9(1):1–9.
4. Berzosa P, De Lucio A, Romay-Barja M, Herrador Z, González V, García L, et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea 11 Medical and Health Sciences 1108 Medical Microbiology. Malar J [Internet]. 2018;17(1):1–12. Available from: https://doi.org/10.1186/s12936-018-2481-4
5. Azikiwe CCA, Ifezulike CC, Siminialayi IM, Amazu LU, Enye JC, Nwakwunite OE. A comparative laboratory diagnosis of malaria: Microscopy versus rapid diagnostic test kits. Asian Pac J Trop Biomed. 2012;2(4):307–10.
6. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria diagnosis: A brief review. Korean J Parasitol. 2009;47(2):93–102.
7. Ho MF, Baker J, Lee N, Luchavez J, Ariey F, Nhem S, et al. Circulating antibodies against Plasmodium falciparum histidine-rich proteins 2 interfere with antigen detection by rapid diagnostic tests. Malar J. 2014;13(1):1–12.
8. Mcdonald WM. Plasmodium falciparum Histidine-Rich Protein 2 and 3 Gene Deletions and Their Implications in Malaria Control. Br Med J. 1937;1(3987):1–19.
9. Uyoga S, Wanjiku P, Rop JC, Makale J, Macharia AW, Nyutu GM, et al. Plasma Plasmodium falciparum Histidine-rich Protein 2 Concentrations in Children With Malaria Infections of Differing Severity in Kilifi, Kenya. Clin Infect Dis. 2021;73(7):2415–23.
10. Mehlotra RK, Howes RE, Cramer EY, Tedrow RE, Rakotomanga TA, Ramboarina S, et al. Plasmodium falciparum Parasitemia and Band Sensitivity of the SD Bioline Malaria Ag P.f/Pan Rapid Diagnostic Test in Madagascar. Am J Trop Med Hyg. 2019;100(5):1196–201.
11. Lee J, Kim TI, Lê HG, Yoo WG, Kang JM, Ahn SK, et al. Genetic diversity of Plasmodium vivax and Plasmodium falciparum lactate dehydrogenases in Myanmar isolates. Malar J. 2020;19(1):1–15.
12. Jain P, Chakma B, Patra S, Goswami P. Potential biomarkers and their applications for rapid and reliable detection of malaria. Biomed Res Int. 2014;2(1):1–21.
13. Góes L, Chamma-Siqueira N, Peres JM, Nascimento JM, Valle S, Arcanjo AR, et al. Evaluation of histidine-rich proteins 2 and 3 gene deletions in plasmodium falciparum in endemic areas of the Brazilian Amazon. Int J Environ Res Public Health. 2021;18(1):1–9.
14. World Health Organization (WHO). Word Malaria Report 2021. Word Malaria report. 2021. 2013–2015 p.
15. World Health Organization(WHO). World Malaria Report [Internet]. World Health Organization. 2020. 2–15 p. Available from: https://www.who.int/publications/i/item/9789240015791
16. Federal Minstry of health(FMoH) E. Ethiopia malaria elimination strategic plan: 2021-2025. 2021. 11–12 p.

17. Moges B, Amare B, Belyhun Y, Tekeste Z, Gizachew M, Workineh M, et al. Comparison of CareStart HRP2/pLDH COMBO rapid malaria test with light microscopy in north-west Ethiopia. Malar J [Internet]. 2012;11(1):1. Available from: ???
18. Jeffrey S, Malaney P. The economic and social burden of malaria. Nature. 2002;415(2):680–5.
19. Tefera DR, Sinkie SO, Daka DW. Economic burden of malaria and associated factors among rural households in Chewaka district, Western Ethiopia. Clin Outcomes Res. 2020;12(1):141–52.
20. Wubishet MK, Berhe G, Adissu A, Tafa MS. Effectiveness of long-lasting insecticidal nets in prevention of malaria among individuals visiting health centres in Ziway-Dugda District, Ethiopia: matched case–control study. Malar J [Internet]. 2021;20(1):1–15. Available from: https://doi.org/10.1186/s12936-021-03833-9
21. President's Malaria Initiative. President ' S Malaria Initiative Technical. Vol. 1, CDC. 2016. 9–11 p.
22. Taffese HS, Hemming-Schroeder E, Koepfli C, Tesfaye G, Lee MC, Kazura J, et al. Malaria epidemiology and interventions in Ethiopia from 2001 to 2016. Infect Dis Poverty. 2018;7(1):1–9.
23. World Health Organization (WHO). Policy brief on malaria diagnostics in low-transmission settings September 2014. Program Glob Malar. 2014;3(1):1–12.
24. Cunningham JA, Thomson RM, Murphy SC, De La Paz Ade M, Ding XC, Incardona S, et al. WHO malaria nucleic acid amplification test external quality assessment scheme. Malar J [Internet]. 2020;19(1):1–12. Available from: https://doi.org/10.1186/s12936-020-03200-0
25. Bisoffi Z, Gobbi F, Van Den Ende J. Rapid diagnostic tests for malaria. Am socieity Microbiol. 2014;348(1):66–78.
26. Niyukuri D, Sinzinkayo D, Troth E V., Oduma CO, Barengayabo M, Ndereyimana M, et al. Performance of highly sensitive and conventional rapid diagnostic tests for clinical and subclinical Plasmodium falciparum infections, and hrp2/3 deletion status in Burundi. PLOS Glob Public Heal [Internet]. 2022;2(7):e0000828. Available from: http://dx.doi.org/10.1371/journal.pgph.0000828
27. Ansah EK, Epokor M, Whitty CJM, Yeung S, Hansen KS. Cost-effectiveness analysis of introducing RDTs for malaria diagnosis as compared to microscopy and presumptive diagnosis in central and peripheral public health facilities in Ghana. Am J Trop Med Hyg. 2013;89(4):724–36.
28. Feleke DG, Alemu Y, Yemanebirhane N. Performance of rapid diagnostic tests, microscopy, loop-mediated isothermal amplification (LAMP) and PCR for malaria diagnosis in Ethiopia: a systematic review and meta-analysis. Malar J [Internet]. 2021;20(1):1–11. Available from: https://doi.org/10.1186/s12936-021-03923-8
29. Murray CK, Gasser RA, Magill AJ, Miller RS. Update on rapid diagnostic testing for malaria. Clin Microbiol Rev. 2008;21(1):97–110.
30. Marti X, Skjefte M, Sikka R, Gupta H. Factors Affecting the Performance of HRP2-Based Malaria Rapid Diagnostic Tests. Trop Med Infect Dis. 2022;7(265):1–22.
31. World Health Organization(WHO). Response plan to pfhrp2 gene. 2019. 1–33 p.
32. Gillet P, Mori M, Van Esbroeck M, Van Den Ende J, Jacobs J. Assessment of the prozone effect in malaria rapid diagnostic tests. Malar J. 2009;8(1):1–7.
33. Alemayehu GS, Blackburn K, Lopez K, Cambel Dieng C, Lo E, Janies D, et al. Detection

of high prevalence of Plasmodium falciparum histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: implication for malaria diagnosis. Malar J [Internet]. 2021;20(1):1–11. Available from: https://doi.org/10.1186/s12936-021-03629-x

34. Houzé S, Hubert V, Le Pessec G, Le Bras J, Clain J. Combined deletions of pfhrp2 and pfhrp3 genes result in plasmodium falciparum malaria false-negative rapid diagnostic test. J Clin Microbiol. 2011;49(7):2694–6.
35. Fontecha G, Mejía RE, Banegas E, Ade MP, Mendoza L, Ortiz B, et al. Deletions of pfhrp2 and pfhrp3 genes of Plasmodium falciparum from Honduras, Guatemala and Nicaragua. Malar J [Internet]. 2018;17(1):1–10. Available from: https://doi.org/10.1186/s12936-018-2470-7
36. Baker J, Ho MF, Pelecanos A, Gatton M, Chen N, Abdullah S, et al. Global sequence variation in the histidine-rich proteins 2 and 3 of Plasmodium falciparum: Implications for the performance of malaria rapid diagnostic tests. Malar J. 2010;9(1):1–12.
37. Dorado EJ, Okoth SA, Montenegro LM, Diaz G, Barnwell JW, Udhayakumar V, et al. Genetic characterisation of plasmodium falciparum isolates with deletion of the pfhrp2 and/or pfhrp3 genes in Colombia: The amazon region, a challenge for malaria diagnosis and control. PLoS One. 2016;11(9):1–17.
38. Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, et al. Effect of sequence variation in Plasmodium falciparum histidine-rich protein 2 on binding of specific monoclonal antibodies: Implications for rapid diagnostic tests for malaria. J Clin Microbiol. 2006;44(8):2773–8.
39. Golassa L, Messele A, Amambua-Ngwa A, Swedberg G. High prevalence and extended deletions in Plasmodium falciparum hrp2/3 genomic loci in Ethiopia. PLoS One [Internet]. 2020;15(11):1–11. Available from: http://dx.doi.org/10.1371/journal.pone.0241807
40. Vera-Arias CA, Holzschuh A, Oduma CO, Badu K, Abdul-Hakim M, Yukich J, et al. Plasmodium falciparum hrp2 and hrp3 gene deletion status in Africa and South America by highly sensitive and specific digital PCR. medRxiv [Internet]. 2021;1(1):1–35. Available from: https://www.medrxiv.org/content/10.1101/2021.06.01.21258117v1
41. Tegegne Y, Worede A, Derso A, Ambachew S. The Prevalence of Malaria among Children in Ethiopia: A Systematic Review and Meta-Analysis. J Parasitol Res. 2021;2(1):1–6.
42. Esayas E, Tufa A, Massebo F, Ahemed A, Ibrahim I, Dillu D, et al. Malaria epidemiology and stratification of incidence in the malaria elimination setting in Harari Region, Eastern Ethiopia. Infect Dis Poverty [Internet]. 2020;9(1):1–12. Available from: https://doi.org/10.1186/s40249-020-00773-5
43. Deress T, Girma M. Plasmodium falciparum and Plasmodium vivax Prevalence in Ethiopia: A Systematic Review and Meta-Analysis. Malar Res Treat. 2019;2(1):1–12.
44. Tegegne Y, Worede A, Derso A, Ambachew S. The Prevalence of Malaria among Children in Ethiopia: A Systematic Review and Meta-Analysis. J Parasitol Res. 2021;1(1):1–9.
45. Ewnetu Y, Lemma W. Highland Malaria Transmission Dynamics in Space and Time Before Pre-elimination Era, Northwest Ethiopia. J Epidemiol Glob Health [Internet]. 2022;12(3):362–71. Available from: https://doi.org/10.1007/s44197-022-00034-8
46. Alkadir S, Gelana T, Gebresilassie A. A five year trend analysis of malaria prevalence in Guba district, Benishangul-Gumuz regional state, western Ethiopia: A retrospective study. Trop Dis Travel Med Vaccines. 2020;6(1):1–7.
47. Venugopal K, Hentzschel F, Valkiūnas G, Marti M. Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. Nat Rev Microbiol. 2020;18(3):177–

89.
48. Donahoe. Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host. Mol Cell Biochem. 2012;23(1):1–7.
49. Walker M, Kublin JG, Zunt JR. Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. Cell Res. 2009;42(1):115–25.
50. Vaughan AM, Kappe SHI. Malaria Parasite Liver Infection and Exoerythrocytic Biology. Cold Spring Harb Lab Press. 2017;1(2):1–22.
51. Counihan NA, Chisholm SA, Bullen HE, Srivastava A, Sanders PR, Jonsdottir TK, et al. Plasmodium falciparum parasites deploy RhopH2 into the host erythrocyte to obtain nutrients, grow and replicate. Elife. 2017;6(1):1–31.
52. Perrin AJ, Bisson C, Faull PA, Renshaw MJ, Lees RA, Fleck RA, et al. Malaria parasite schizont egress antigen-1 plays an essential role in nuclear segregation during schizogony. Am socieity Microbiol. 2021;12(2):1–16.
53. Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJI, Richards JS. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. FEMS Microbiol Rev. 2016;40(3):343–72.
54. Lakhani CM, Benjamin M. Davis, Glen F. Rall MJS. Gametocytogenesis in malaria parasite: commitment, development and regulation Zhenyu. Physiol Behav. 2017;176(3):139–48.
55. Bennink S, Kiesow MJ, Pradel G. The development of malaria parasites in the mosquito midgut. Cell Microbiol. 2016;18(7):905–18.
56. Chaudhary A, Kataria P, Surela N, Das J. Pathophysiology of Cerebral Malaria: Implications of MSCs as A Regenerative Medicinal Tool. Bioengineering. 2022;9(6):1–12.
57. Idro R, Marsh K, John CC, Newton CRJ. Cerebral malaria: Mechanisms of brain injury and strategies for improved neurocognitive outcome. Pediatr Res. 2010;68(4):267–74.
58. Dufera M, Dabsu R, Tiruneh G. Assessment of malaria as a public health problem in and around Arjo Didhessa sugar cane plantation area , Western Ethiopia. BMC Public Health. 2020;2(1):1–10.
59. Morris AM, Epi SM, Liles WC. Practice Delay in diagnosis : malaria in a returning traveller. Can Med Assoc J. 2009;180(11):1129–31.
60. Plucinski MM, Ferreira M, Ferreira CM, Burns J, Gaparayi P, João L, et al. Evaluating malaria case management at public health facilities in two provinces in Angola. Malar J. 2017;16(186):1–10.
61. World Health Organization (WHO). GIEMSA STAINING OF MALARIA BLOOD FILMS. 2016. 1–6 p.
62. Oyegoke OO, Maharaj L, Akoniyon OP, Kwoji I, Roux AT, Adewumi TS, et al. Malaria diagnostic methods with the elimination goal in view. Parasitol Res [Internet]. 2022;121(7):1867–85. Available from: https://doi.org/10.1007/s00436-022-07512-9
63. World Health Organization (WHO). Response plan to pfhrp2 gene. WHO. 2019. 1–33 p.
64. Park SH, Jegal S, Ahn SK, Jung H, Lee J, Na BK, et al. Diagnostic performance of three rapid diagnostic test kits for malaria parasite Plasmodium falciparum. Korean J Parasitol. 2020;58(2):147–52.
65. Bahk YY, Park SH, Lee W, Jin K, Ahn SK, Na BK, et al. Comparative assessment of diagnostic performances of two commercial rapid diagnostic test kits for detection of plasmodium spp. in Ugandan patients with malaria. Korean J Parasitol. 2018;56(5):447–52.
66. Menegon M, Episcopia ML, Nurahmed AM, Talha AA, Nour BYM, Severini C.

Identification of Plasmodium falciparum isolates lacking histidine-rich protein 2 and 3 in Eritrea. Infect Genet Evol [Internet]. 2017;55(1):131–4. Available from: http://dx.doi.org/10.1016/j.meegid.2017.09.004

67. Berhane A, Anderson K, Mihreteab S, Gresty K, Rogier E, Mohamed S, et al. Major threat to malaria control programs by plasmodium falciparum lacking histidine-rich protein 2, Eritrea. Emerg Infect Dis. 2018;24(3):462–70.
68. Beshir KB, Sepúlveda N, Bharmal J, Robinson A, Mwanguzi J, Busula AO, et al. Plasmodium falciparum parasites with histidine-rich protein 2 (pfhrp2) and pfhrp3 gene deletions in two endemic regions of Kenya. Sci Rep. 2017;7(1):1–10.
69. Buderer NM. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. Acad Emerg Med [Internet]. 1996;3(9):895–900. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8870764%5Cnhttp://onlinelibrary.wiley.com/doi/10.1111/j.1553-2712.1996.tb03538.x/abstract?systemMessage=Wiley+Online+Library+will+be+disrupted+on+the+18th+October+from+10:00+BST+(05:00+EDT)+for+essential+maintenance
70. Alemayehu GS, Lopez K, Dieng CC, Lo E, Janies D, Golassa L. Evaluation of PfHRP2 and PfLDH malaria rapid diagnostic test performance in assosa zone, Ethiopia. Am J Trop Med Hyg. 2020;103(5):1902–9.
71. Crump JA, Newton PN, Baird SJ, Lubell Y. Febrile Illness in Adolescents and Adults. Dis Control Priorities, Third Ed Major Infect Dis. 2017;6(1):365–85.
72. Safety O, Act P. STANDARD OPERATING PROCEDURE (SOP) Phlebotomy Stations Outside of Clinical Care Areas. Univ Pittsburgh [Internet]. 2009;7194(412):4–6. Available from: file:///D:/SkyDrive/Etica/Investigacion/SOP_PhlebotomyStation.pdf
73. Monica Cheesbrough. WHO guidelines on drawing blood : best practices in phlebotomy. World Heal Organ. 2010;1(1):1–105.
74. Dr. Vladimir VF. Standard operating procedures for malaria microscopy. Gastron ecuatoriana y Tur local. 2017;1(69):5–24.
75. Kilauzi AL, Mulumba JGT, Magafu MGMD, Matchaba-Hove R, Tapera R, Magafu NS, et al. SD Bioline malaria antigen Pf (HRP-2/pLHD) for assessing efficacy of Artemisinin combination therapy against plasmodium falciparum in pediatric patients in the democratic republic of the Congo. Pan Afr Med J. 2015;22(1):1–7.
76. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, et al. Development of a Real-Time PCR Assay for Detection of Plasmodium for Routine Clinical Diagnosis. J Clin Microbiol. 2004;42(3):1214–9.
77. GmbH Q. QIAcuity User Manual March 2021. 2021. 165–171 p.
78. WHO. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 8 (2016–2018). Geneva: World Health Organization; 2018 [Internet]. Vol. 3, WHO Publications. 2018. 154 p. Available from: http://www.who.int/malaria/publications/atoz/9789241510035/en/
79. Viera AJ, Garrett JM. Understanding Interobserver Agreement : The Kappa Statistic. 2005;(May):360–3.
80. Policy M, Committee A. Protocol for estimating the prevalence of pfhrp2 / pfhrp3 gene deletions among symptomatic falciparum patients with false-negative RDT results. 2017;(October).
81. WHO. Master protocol for surveillance of pfhrp2/3 deletions and biobanking to support

future research. 2020. 1–37 p.
82. Bahk YY, Park SH, Lee W, Jin K, Ahn SK, Na B, et al. Comparative Assessment of Diagnostic Performances of Two Commercial Rapid Diagnostic Test Kits for Detection of Plasmodium spp . in Ugandan Patients with Malaria. Parasitology. 2018;56(5):447–52.
83. Kwenti TE, Njunda LA, Tsamul B, Nsagha SD, Assob NJC, Tufon KA, et al. Comparative evaluation of a rapid diagnostic test, an antibody ELISA, and a pLDH ELISA in detecting asymptomatic malaria parasitaemia in blood donors in Buea, Cameroon. Infect Dis Poverty. 2017;6(1):1–9.
84. Nankabirwa JI, Yeka A, Arinaitwe E, Kigozi R, Drakeley C, Kamya MR, et al. Estimating malaria parasite prevalence from community surveys in Uganda: A comparison of microscopy, rapid diagnostic tests and polymerase chain reaction. Malar J. 2015;14(1):1–11.
85. Hamid MA, Awad-Elgeid M, Nasr A. Gene Variation and Suspected Plasmodium Falciparum Histidine-Rich Protein 2 Gene Deletion and Its Impact on Sensitivity of Malaria Rapid Diagnostic Tests in Sudan . BMJ Glob Heal. 2017;2(Suppl 2):A21.2-A21.
86. Iriart X, Menard S, Chauvin P, Mohamed HS, Charpentier E, Mohamed MA, et al. Misdiagnosis of imported falciparum malaria from African areas due to an increased prevalence of pfhrp2/pfhrp3 gene deletion: the Djibouti case. Emerg Microbes Infect. 2020;9(1):1984–7.
87. Vera-Arias CA, Holzschuh A, Oduma CO, Badu K, Abdul-Hakim M, Yukich J, et al. Plasmodium falciparum hrp2 and hrp3 gene deletion status in Africa and South America by highly sensitive and specific digital PCR. medRxiv [Internet]. 2021;1(1):5–35.
88. Feleke SM, Reichert EN, Mohammed H, Brhane BG, Mekete K, Mamo H, et al. Plasmodium falciparum is evolving to escape malaria rapid diagnostic tests in Ethiopia. Nat Microbiol [Internet]. 2021;6(10):1289–99.
89. Alemayehu GS, Blackburn K, Lopez K, Cambel Dieng C, Lo E, Janies D, et al. Detection of high prevalence of Plasmodium falciparum histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: implication for malaria diagnosis. Malar J [Internet]. 2021;20(1):1–12.
90. Iriart X, Menard S, Chauvin P, Mohamed HS, Charpentier E, Mohamed MA, et al. Misdiagnosis of imported falciparum malaria from African areas due to an increased prevalence of pfhrp2/pfhrp3 gene deletion: the Djibouti case. Emerg Microbes Infect [Internet]. 2020;9(1):1984–7. Available from: https://doi.org/10.1080/22221751.2020.1815590
91. Prosser C, Gresty K, Ellis J, Meyer W, Anderson K, Lee R, et al. Plasmodium falciparum histidine-rich protein 2 and 3 gene deletions in strains from Nigeria, Sudan, and South Sudan. Emerg Infect Dis. 2021;27(2):471–9.
92. Vera-Arias CA, Holzschuh A, Oduma CO, Badu K, Abdul-Hakim M, Yukich J, et al. High-throughput Plasmodium falciparum hrp2 and hrp3 gene deletion typing by digital PCR to monitor malaria rapid diagnostic test efficacy. Elife. 2022;11(1):1–16.

ANNEX

Annex- I: Participant Information Sheet English/Amharic Version

Title of the Research: Evaluation of performance of novel Biocredit RDTs for detection of *Plasmodium* species. and histidine rich protein 2/3 gene deletions in febrile patients in Maksegnet health center.

Name of Principal Investigator: Alayu Bogale

Advisors: Dr. Teferi Eshetu, Dr. Teshome Degefa, Dr. Fitsum Girma, Dr. Cristian Koepfli and Dr. Migbaru Keffale

Name of the Organization: Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences.

Introduction: This information sheet is prepared for the aim of explaining the research that you are asked to join as a research participant. This information sheet describes the research.

Aim of the study: This study aims to evaluate the performance of novel Biochredit RDTs (pLDH/ pHRP2 and pLDH) for detection of *P. falciparum and P. vivax,* and histidine-rich protein 2/3 gene deletions in febrile patients in Maksegnet health center.

Procedure: If the patient is agreed to take part in the study, the clinical nurse were give verbal and/or written information about the study and the patient was signed on the consent form. Patients are kindly requested to give the correct information about themselves and the necessary measurements were performed by the assigned nurse. When we prick your finger to do the routine malaria rapid diagnostic test, we were also taking a few extra drops to do an additional malaria test today and put onto 500µl on EDTA tube for further testing to see whether the malaria parasites have changed in ways that make them hard to find. These tests were carried out in Armauer Hansen Research Institute, Addis Ababa.

Risk and discomfort: Participating in this research doesn’t cause more discomfort than is required you could go through for routine examination. There may be pain during blood sample collection.

The amount of blood taken from each volunteer throughout the study period is very small which doesn't affect your health.

Benefits: If you are participating in this study, there may not be a direct benefit to you, but your participation is likely to help us an important input on evaluation of novel RDTs for detection of *P. falciparum* and *P. vivax* and histidine-rich protein 2/3 in the parasite isolates obtained from patients visiting Maksegnet Health Center.

Incentives and payment for participating in the study: You don't be provided with any direct incentives for your participation in this study.

Confidentiality: All information about the patients were kept confidential. The name of the participant was coded. The information sheet that links the coded number to a patient name was locked inside a computer and it doesn't reveal to anyone except your physician and the principal investigator.

Right to refuse or withdraw: You have the full right to withdraw from participating in the study at any time before and after consent without explaining the reason and not respond to some or all the questions. Your decision doesn't affect your right to get the health service you are supposed to get otherwise.

Contact Address

If you have any questions or concerns, you can contact Alayu Bogale at any time using the following address:

Alayu Bogale, Medical Parasitology MSc student at Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences.

Tel: 09-36-68-01-41

Email: bgialex11@gmail.com

Jimma, Ethiopia

Thank you very much!

ስለጥናቱ መረጃ በአማርኛ (Participant information sheet in Amharic version)

የጥናቱርዕስ፡ በጎንደር ዙሪያ በ ማክሰኝት ከተማ አካባቢ በሚኖሩ ሰዎች አዲስ የወባ መመርመሪያ መሳርያ አቅምን መፍተሻ አና የወባን ዘረ መልን የመመርመር አቅምን መዳሰስ

የተማራማሪ ስም፡- አላዩ በጋለ፤የድርጅቱ ስም -ጅማ ዩኒቨርስቲ /የህክምና ላብራቶሪ ሳይንስ ት/ቤት

የአማካሪዎች ስም፡- ዶ/ር ተፈሪ እሸቱ ዶ/ር ተሾመ ደገፋ ዶ/ር ፍጹም ግርማ ዶ/ር ክርስቲያን ኮኦፊል እና ዶ/ር ምግባሩ ከፋለ

መግቢያ፡-ይህ የተዘጋጀው በአጭሩ ስለሚካሂደው ጥናት ለጥናቱ ተሳታፊዋች በጥናቱ ጉዳይ ሃሳብ ለማካፈል ነዉ።ሰለሆነም የዚህ ጥናት ዋና አላማ አዲስ የወባ መመርመሪያ መሳርያ አቅምን መፍተሻ አና የወባን ዘረ መልን የመመርመር አቅምን መዳሰስ የበሽታዉን ማህበራዊ እና ኢኮኖሚያዊ ጉዳት ለመቀነስ አና ከመከላከል አንጻር የዚህ ጥናት ውጤት የበኩሉን ሚና ይጫዎታል።

የጥናቱ ሂደት ዝርዝር

በጥናቱ ለመሳተፍ ከተሰማሙ የሚከተሉትን መረጃዋች እና ናሙና እንወሰዳለን

- ✓ የ 5 ደቂቃ ቃለ-መጠይቅ ይደረግላቸዋል
- ✓ ከልጆዎ ወይንም ከእርሶ የደም ናሙና ተወሰዶ ምርመራ ይደረጋል
- ✓ የተሰበሰበው ናሙና በአርመዋር ሀንሰን የምርምር ተቋም የወባ በሽታ ዘረ -መል ምርመራ ይደረጋል

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሰውን ናሙና በ ሚወስድበት ጊዜ በ ናሙና መውሰጃ መረፊ አነስተኛ የሆነ ጊዜያዊ ህመም ወጪ ሌላ ምንም ችግር አንደማያጋጥም የተረጋገጠ ነዉ። ሊያስገኛቸው የሚችሉት ጥቅሞች በዚህ ጥናት ውስጥ ልጅዎ ውይም እርሶ በመሳተፉ በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም። የጥናቱ ተሳታፊዎች በምርመራ ዉጤት ላይ በመመርኮዝ በሽታው በልጅው ወይም በርሶ ከተገኘ ለበለጠ ህክምና እና እንክብካቤ በማክሰኝትጤናጣቢያመድሃኒትእንድሰጣቸዉይደረጋል።

የጥናቱ ምስጢራዊነት

ማንኛዉም በጥናቱ የተገኙ መረጃዎች ምስጢራዊነቱ የተጠበቀ ነዉ:: የጥናቱ መረጃዎች በሙሉ የተቀመጡት ለጥናቱ ተብሎ በሚሰጠው ስውር ቁጥር ሲሆን ጥናቱን ከሚያስከሄዱት ባለሙያዎች በስተቀር ማንም ሊያውቅ አይችልም። የጥናቱ ተሳታፊ ማንነት በሚገልጥ መልኩ የተዘጋጀውን መረጃ የጥናቱ ተሳታፊ በፊርማዉ የተረጋገጠ ፍቃድ ሳይሰጥ ይፋ አይደርግም፡፡ ይህ ጥናት ሳይንሳዊ መረጃ እንደመሆኑ መጠን በወረቀት ታትሞ ቢወጣ ወይንም በሚድያ ቢነገር የጥናቱ ተሳታፊ ስም በምንም መልኩ አይጠቀስም፡፡

ያለመቀበል ወይም ጥሎ የመውጣት መብት

በዚህ ጥናት ዉስጥ የሚኖርዎት ተሳትፎ ሙሉበሙሉ ፈቃደኝነት ላይ የተመሰረተ ሲሆን በማንኛውም ጊዜ ይህንን ጥናት የማቋረጥ መብታቸዉ ሙሉበሙሉ የተጠበቀነዉ። በጥናቱ ባለመሳተፋቸዉ ወይም ከጥናት በመገለላቸዉ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታ ላይ ተፅዕኖ አይኖርም።

የተመራማሪ መገኛ

በጥናቱ ላይ ጥያቄ ወይም አሰተያየት ካሎት የጥናቱን ባለቢት አላዩ ቦጋለን በዚህ አድራሻ ማግኘት ይችላሉ።

አላዩ ቦጋለ ፤የድርጅቱ ስም -ጅማ ዩኒቨርስቲ /የህክምና ላብራቶሪ ሳይንስ ት/ቤት ተማሪ

ስልክ ቁጥር ፡ 09-36-68-01-41

ኢሚይል፡bgialex11@gmail.com

ጅማ፣ ኢትዮጵያ

በጥናቱ ስለተሳተፉ ከልብ አመሰግናለሁ::

Annex- II: Certificate of consent English/Amharic Version (>18 age of years)

Survey ID: __________

I have been invited to participate in a study that aims to better understand the diagnostic evaluation of novel RDTs and hrp 2/3 gene deletion. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study.

Name of participant: _____________________Signature: _______Date: ______________ (dd/mm/yyyy)

<table>
<tr><td rowspan="2"><u>Long-term storage and future studies:</u>
I agree to allow the study team to store my blood sample for future studies on malaria. I understand that I can change my mind to not have my blood sample stored and used for future research.</td><td colspan="2">If you agree, circle “YES,” if you Do not agree, circle ‘NO’.</td></tr>
<tr><td>YES</td><td>NO</td></tr>
<tr><td>Name: __________________________</td><td>Signature</td><td>Date
_______/______/______</td></tr>
</table>

<u>Witness’ signature</u>: *A witness’ signature and the patient’s thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.*

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumbprint of participant

Name of witness: _____________________Signature: _______Date: _______________(dd/mmm/yyyy)

<u>Investigator’s signature</u>:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:___________________ Signature: ________ Date: ______________(dd/mm/yyyy)

<u>የፈቃድ አና የምስክር ፊርማ ቅፅ</u>

የዳሰሳ ጥናት መታወቂያ:______________________

አዲስ የወባ መመርመሪያ መሳርያ አቅምን መፍተሻ አና የወባን ዘረ መልን የመመርመር አቅምን ለመዳሰስበደንብለመረዳትበሚያስችልጥናትላይእንድሳተፍተጋበዣለው፡፡ከላይየተጠቀሱትንመረጃዎችአን ብቤአለሁወይምተነበልኛል፡፡ጥያቄዎችንየማቅረብእድልአግኝቻለሁ፣እናየጠየኳቸውጥያቄዎችሁሉበበቂሁኔ ታመልስአግኝተዋል፡፡በዚህጥናትውስጥለመሳተፍፈቃደኛመሆኔንበፊርማዬአረጋግጣለሁ፡፡

የተሳታፊውስም:___________________ ፊርማ:________ቀን:__________(ቀን /ወር /ዓ.ም)

<u>ለወደፊትጥናቶችየረጅምጊዜማከማቻስምምነትቅፅ፡</u>	ከተስማሙ፣ "አዎ" ያክብቡ፣ ካልተስማሙ፣ "አይ" ያክብቡ፡፡	
ለወደፊቱ በወባላይለሚደረጉጥናቶችየጥናትቡድኑየእኔን የደምናሙናእንዲያከማችለመፍቀድእስማማለሁ፡፡የደምናሙናእንዳይከ ማችእናለወደፊቱምርምርጥቅምላይእንዳይውልለመወሰንሀሳቤንመለወ ጥእንደምችልተረድቻለሁ፡፡	አዎ	አይ
	ፊርማ	ቀን ________/____/_____

<u>የምስክርፊርማ</u>

የምስክር ፊርማ እና የታካሚ ውጣት አሻራ የሚፈለገው ታካሚው ማንበብ የማይችል ከሆነ ብቻ ነው፡፡በዚህ ጊዜ ማንበብና መጻፍ የሚችል ታካሚ፣ፊርማ መፈረም አለበት፡፡ከተቻለ ይህ ሰው በተሳታፊው መመረጥ አለበት እና ከጥናቱ ቡድን ጋር ምንም ዓይነት ግንኙነት ሊኖረውአይገባም፡፡) ተሳታፊው/ዋ ጥያቄዎችን የመጠየቅ እድል እንዳገኘ/እንዳገኘች እና የስምምነት ቅጹንበትክክል እንደተረዳ/እንደተረዳችተመልክቻለሁ፡፡ ተሳታፊው/ዋፈቃዱንበነፃነትመስጠቱንአረጋግጣለሁ፡፡

የተሳታፊየጣትአሻራ [ ]

የምስክርስም: ________________________ ፊርማ: ______ቀን: ________________(ቀን/ወር/ዓ.ም)

<u>የተመራማሪውፊርማ</u>

ተሳታፊውየስምምነትቅጹንበትክክልአንደተነበበለትእናአንደተረዳጥያቄዎችንምለመጠየቅዕድልእድልእንዳገ
ኝአይቻለሁ፡፡ተሳታፊውፈቃዱንበነፃነትመስጠቱንበፊርማዬአረጋግጣለሁ፡፡

የተመራማሪ ስም: ____________________ፊርማ: __________ቀን:___________________(ቀን/ወር/ዓ.ም)

Annex- III: Certificate of Assent English/Amharic Version (<18 age of years)

Survey ID: __________

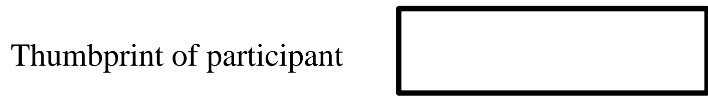
I have been invited to participate in a study that aims to better understand the diagnostic evaluation of novel RDTs and hrp 2/3 gene deletion. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study.

Name of participant: ______________________Signature: _______Date: _______________ (dd/mm/yyyy)

<table>
<tr><td rowspan="2"><u>Long-term storage and future studies:</u>
I agree to allow the study team to store my blood sample for future studies on malaria. I understand that I can change my mind to not have my blood sample stored and used for future research.</td><td colspan="2">If you agree, circle “YES,” if you Do not agree, circle ‘NO’.</td></tr>
<tr><td>YES</td><td>NO</td></tr>
<tr><td>Name: ____________________________</td><td>Signature</td><td>Date
______/_____/______</td></tr>
</table>

Witness' signature: *A witness' signature and the patient's thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.*

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.



Thumbprint of participant

Name of witness:_______________________Signature: _______Date: _______(dd/mmm/yyyy)

Investigator's signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:____________________ Signature: ________ Date: _______________(dd/mm/yyyy)

Certificate of Assent

I understand this research is to understand the diagnostic evaluation of novel RDTs and hrp 2/3 gene deletion. I understand that I will get a finger prick for two malaria tests today and the a few drops on paper to use for other malaria research in the future.

I have read this information (or had the information read to me) I have had my questions answered and know that I can ask questions later if I have them. I agree to take part in the research.

Only if child assents:

Name of child ____________________Signature of child: _________Date: _________________

Long – term storage:

<table>
<tr><td rowspan="2"><u>Long-term storage and future studies:</u>

I agree to allow the study team to store my blood sample for future studies on malaria. I understand that I can change my mind to not have my blood sample stored and used for future research.</td><td colspan="2">If you agree, circle “YES,” if you do not agree, circle ‘NO’.</td></tr>
<tr><td>YES</td><td>NO</td></tr>
</table>

<u>Only if child assents:</u>

Name of child ____________________Signature of child: ______Date: _________________

<u>If illiterate:</u> A literate witness must sign (if possible, this person should be selected by the participant, not be a parent, and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

<u>Witness' signature:</u> I have witnessed the accurate reading of the assent form to the child, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Thumbprint of participant

Witness name: _______________________ Signature: _______ Date: _______________

<u>Investigator's signature</u>:

I have accurately read or witnessed the accurate reading of the assent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given assent freely.

Name of Investigator:____________________ Signature: ________ Date: _______________(dd/mm/yyyy)

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the child understands that the following will be done: (1). finger prick for malaria test and blood spot on filter paper (2). Long term storage of the filter paper for future research

I confirm that the child was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of person taking the assent________________________ Signature: _______ Date: _______________

Parent/Guardian has signed an informed consent___Yes___No___(Name of researcher/assistant)

የፈቃድ እና የምስክር ፊርማ ቅፅ

የዳሰሳጥናትመታወቂያ፡_________________________

አዲስ የወባ መመርመሪያ መሳርያ አቅምን መፍተሻ እና የወባን ዘረ መልን የመመርመር አቅምን ለመዳሰስበደንብለመረዳትበሚያስችልጥናትላይእንድሳተፍተጋበዣለው፡፡ከላይየተጠቀሱትንመረጃዎችአንብቤአለሁ ወይምተነበልኛል፡፡ጥያቄዎችንየማቅረብእድልአግኝቻለሁ፣እናየጠየኳቸውጥያቄዎችሁሉበበቂሁኔታመልስአግኝተዋል፡፡ በዚህጥናትውስጥለመሳተፍፈቃደኛመሆኔንበፊርማዬአረጋግጣለሁ፡፡

የተሳታፊውስም፡______________________ ፊርማ፡_________ቀን፡___________(ቀን /ወር /ዓ.ም)

ለወደፊትጥናቶችየረጅምጊዜማከማቻስምምነትቅፅ፡	ከተስማሙ፣ “አዎ” ያክብቡ፣ ካልተስማሙ፣ “አይ” ያክብቡ፡፡	
ለወደፊቱበወባላይለሚደረጉጥናቶችየጥናትቡድኑየእኔን	አዎ	አይ

የደምናሙናእንዲያከማችለመፍቀድእስማማለሁ።የደምናሙናእንዳይከማችእና ለወደፊቱምርምርጥቅምላይእንዳይውልለመወሰንሀሳቤንመለወጥእንደምችልተ ረድቻለሁ።	ፊርማ	ቀን _________/_____/______

<u>የምስክርፊርማ</u>

የምስክር ፊርማ እና የታካሚ ውጣት አሻራ የሚፈለገው ታካሚው ማንበብ የማይችል ከሆነ ብቻ ነው።በዚህ ጊዜ ማንበብ ና መጻፍ የሚችል ታካሚ፣ ፊርማ መፈረም አለበት። ከተቻለ ይህ ሰው በተሳታፊው መመረጥ አለበት እና ከጥናቱ ቡድን ጋር ምንም ዓይነት ግንኙነት ሊኖረው አይገበም።) ተሳታፊው/ዋ ጥያቄዎችን የመጠየቅ እድል አንዳገኘ/እንዳገኘች እና የስምምነት ቅጹን በትክክል አንደተረዳ/አንደተረዳች ተመልክቻለሁ። ተሳታፊው/ዋፈቃዱንበነፃነትመስጠቱንአረጋግጣለሁ።

የተሳታፊየጣትአሻራ

የምስክርስም፡ ____________________________ ፊርማ፡ ________ቀን፡ __________________(ቀን/ወር/ዓ.ም)

<u>የተመራማሪውፊርማ</u>

ተሳታፊውየስምምነትቅጹንበትክክልአንደተነበበለትእናአንደተረዳጥያቄዎችንምለመጠየቅዕድልእድልእንዳገኘአይቻለ ሁ።ተሳታፊውፈቃዱንበነፃነትመስጠቱንበፊርማዬአረጋግጣለሁ።

የተመራማሪስም: _____________________ፊርማ: ___________ቀን:____________________(ቀን/ወር/ዓ.ም)

<u>የፈቃድአናየምስክርፊርማቅፅ</u>

ይህምርምርአዲስየወባመመርመሪያመሳርያአቅምንመፍተሻአናየወባንዘረመልንየመመርመርአቅምንለመ ዳሰስበደንብለመረዳትእንደሆነተረድቻለሁ፡፡ለአንድጊዜየወባምርመራዎችከጣቴላይጠብታዎችእንደሚወሰ ድተረድቻለሁ።የሚቀረውየደምጠብታለወደፊቱለሌላየወባምርምርእንደሚጠቀምይገባኛል፡፡ይህንንመረጃ አንብቤያለሁ(ወይምመረጃውተነቦልኛል)።ለጥያቄዎቼመልስተሰጥቶኛል።ከዚህበኋላምጥያቄዎችንመጠየቅ እንደምችልአውቃለሁ፡፡በምርምርውውስጥለመሳተፍእስማማለሁ፡፡

<u>ልጅፈቃደኝነቱንካረጋገጠብቻ :</u>

የልጁስም ____________________የልጁፊርማ ___________ቀን ________________ (ቀን / ወር / ዓመት)

<u>ለወደፊትጥናቶችየረጅምጊዜማከማቻስምምነትቅፅ</u>፡

ለወደፊቱበወባላይለሚደረጉጥናቶችየጥናትቡድኑየእኔን

የደምናሙናእንዲያከማችለመፍቀድእስማማለሁ፡፡የደምናሙናእንዳይከማችእናለወደፊቱምርምርጥቅምላይእንዳይውልለ

<u>ልጅፈቃደኝነቱንካረጋገጠብቻ :</u>

የልጁስም ____________________ የልጁፊርማ ____________ቀን ________________ (ቀን / ወር / ዓመት)

<u>ልጁ/ልጅቷማንበብየማይችል/የማትችልከሆነ</u>

ማንበብናመጻፍየሚችልሰውየምስክርነትወረቀትመፈረምአለበት (ከተቻለይህሰውበተሳታፊውመመረጥአለበት፣ወላጅመሆንየለበትም፣እናከምርምርቡድኑጋርምንምዓይነት ግንኙነትሊኖረውአይገባም)፡፡ማንበብናመጻፍየማይችሉተሳታፊዎችምየአውራጣትአሻራቸውንማካተት አለባቸው፡፡

<u>የምስክርፊርማ፡</u>ተሳታፊውጥያቄዎችንየመጠየቅእድልአንዳገኘላለውተሳታፊየስምምነትቅጹንበትክክልአን ደተረዳተመልክቻለሁ፡፡ተሳታፊውፈቃዱንበነፃነትመስጠቱንአረጋግጣለሁ፡፡

የተሳታፊየአወራጣትአሻራ:

የምስክርስም(ወላጅአይደለም): ____________________________የምስክርፊርማ:____________

ቀን: ___________________________________ (ቀን/ወር/ዓ.ም)

<u>የተመራማሪውፊርማ፡</u>የስምምነትቅጹንለተሳታፊዉበትክክልአንደተነበበለትእናአንደተረዳጥያቄዎችንምለ መጠየቅዕድልእንዳገኘአይቻለሁ፡፡ተሳታፊውፈቃዱንበነፃነትመስጠቱንበፊርማዬአረጋግጣለሁ።

የተመራማሪስም:____________________ፊርማ:___________ቀን:_________________ (ቀን/ወር/ዓ.ም):

የመረጃውበሙሉለተሳታፊውበትክክልአንብቤያለሁ፣እናበሚችለውሁሉህፃኑየሚከተለውእንደሚከናወንመ ገንዘቡንአረጋግጫለሁ።

1. በወባበሽታምርመራእናበማጣሪያወረቀትላይየደምጠብታከጣትበመውጋትአንደሚወሰድ

2. የተወሰነየደምጠብታለወደፊቱምርምርየማጣሪያወረቀትላይእናበቲዩብረጅምጊዜማከማቸት

ልጁስለጥናቱጥያቄዎችንየመጠየቅእድልመሰጠቱንአረጋግጣለሁ፣እናእሱ / እሷየጠየቋቸውጥያቄዎችሁሉበትክክልእናበቻልኩትአቅምሁሉእንደተመለሱአረጋግጣለሁ።ግለሰቡናሙናእ ንዲሰጥያልተገደደመሆኑንእናበነፃፈቃደኝነትመሰጠቱንአረጋግጣለሁ።

ስምምነቱንየሚወስድየተመራማሪስም፡ ______________________ፊርማ፡ ___________ቀን፡________________(ቀወዓ)

❖ ወላጅ / አሳዳጊበመረጃላይየተመሠረተስምምነትተፈራረሟል፡ - **አዎ /አይደለም**

የዋና / ረዳትተመራማሪስም፡ ____

Annex- IV: Questionnaire English/Amharic Version

Socio-Demographic Information and Survey Case Report Form

A. Participant Information

1. Patient ID ________ 2. Health Centre ________ 3. Date of visit __ __/__ __/__ __ __ __
4. Age (years) ______ 5. Sex ☐ Male ☐ Female 6. Kebele __________________
7. Is febrile ☐ Yes ☐ No 8. Axillary T^0 ____________ ^{0}C

B. RDT Results

9. SD Pf-HRP2/ Pv-LDH based RDT

   ☐ Pf ☐ Pv ☐ Mixed ☐ Negative ☐ Not done
10. BIOCREDIT PfHRP2/LDH based RDT

   ☐ PfHRP2 ☐ PfLDH ☐ Both ☐ Negative ☐ Not done
11. BIOCREDIT Pf-LDH/Pv-LDH based RDT

   ☐ Pf-LDH ☐ Pv-LDH ☐ Mixed ☐ Negative ☐ Not done

C. Microscopy Examination

Field health facility

12. Status ☐ Positive ☐ Negative ☐ Not done
13. Species ☐ *P. falciparum* ☐*P. vivax* ☐ Mixed species
14. Parasite count (parasites per microliter) _________ 15. Name of microscopist_____________

Confirmation at AHRI

16. Status ☐ Positive ☐ Negative ☐ Not done
17. Species ☐ P. falciparum ☐ P. vivax ☐ Mixed species
18. Parasite count (parasites per microliter) _________ 19. Name of microscopist_____________

If positive to questions 9, 10, 11 or 12 above, provide treatment.

D. Sample Types Collected

20. Is the EDTA sample collected? ☐ Yes ☐ No
21. Is DBS prepared? ☐ Yes ☐ No

E. Previous malaria infection

22. In the past 4 weeks, have you had a test for malaria? ☐ Yes ☐ No
23. If yes, what was the result of the test? ☐ Positive ☐ Negative ☐ Don't know

24. In the past 4 weeks, have you taken any medicine for malaria? ☐ Yes ☐ No

25. If yes, what was the antimalarial medicine taken?

☐ ACT ☐ AL ☐ Artesunate ☐ Fansidar (SP) ☐ Quinine

☐ Panadol (available antipyretic) ☐ Other ________________ ☐ Unknown

26. Data collector name and signature

: ______________________________

ቃለ-መጠይቅ

የማህበራዊ-አካባቢያዊ እና የዳሰሳ ጥናት መረጃ

A.የተሳታፊዎች መረጃ

1. መለያቁጥር:________ 2. የጤናጣቢያው ስም: ____________ 3. የመጡበት ቀን __ __/__ __/__

4. ዕድሜ (ዓመት) ______ 5. ፆታ ☐ወንድ ☐ሴት 6. ቀበሌ :-

7. ሙቀተ አለው አዎ የለዉም 8. የሙቀት መጠን--------------

B. ፈጣንየወባመመርመሪያ

9.የኢስዲ ባዮላይን ፈጣንየወባመመርመሪያ ወጤት

☐*ፕ.ፋልሲፓረም* ☐*ፕ.ቫየቫክስ* ☐ሁለቱም ☐ነገቲፍ ☐አልተሰራአም

10. የባዮክሪዲት ፈጣንየወባመመርመሪያ ወጤት

☐ሒስቲዲን ሪች ፕሮቲን ☐ላክቲት ዲሀይድሮጅን ☐ሁለቱም ☐ነገቲፍ ☐አልተሰራአም

11. የባዮክሪዲት ፈጣንየወባመመርመሪያ ወጤት

☐*ፕ.ፋልሲፓረም*-ላክቲት ዲሀይድሮጅን ☐*ፕ.ቫየቫክስ* ላክቲት ዲሀይድሮጅን

☐ሁለቱም ☐ነገቲፍ ☐አልተሰራአም

C. የማይክርስኮፒ ወጤት

የመስክ ጤና ጣቢያ

12. የዉጤት ሁኔታ ☐ፐዘቲፍ ☐ነገቲፍ ☐አልተሰራአም

13. ዘርያ ☐ *ፕ.ፋልሲፓረም* ☐ *ፕ.ቫየቫክስ* ☐ሁለቱም

14. የተህዋስ ቁጥር በµሊትር ደም ውስጥ _________ 15. የጤናባለሞያውስም______________

አሃሪ ሲረጋገጥ

16. የዉጤትሁኔታ ☐ፐዘቲፍ ☐ነገቲፍ ☐አልተሰራአም

17. ዘርያ ☐ፕ.ፋልሲፓረም ☐ፕ.ቫየቫክስ ☐ሁለቱም

18. የተህዋስ ቁጥር በሚሊትር ደም ውስጥ ________ 19. የጤናባለሞያውስም____________

D. የተሰነበሰበዉ የናሙና አይነት

20. ኢዲቲኤባለውቲዩብየደምናሙናተሰብስቧል? ☐ . አዎ ☐አይ
21. ዲቢኢስተዘጋጅ~ል? ☐ . አዎ ☐አይ

E. ከአሁን በፊት የወባ በሽታ ሁኔታ

22. ባለፉት ሁለት ሳምንት የወባ መርመራ አድርገዋል? ☐አዎ ☐አይ
23. ወጤቱ ምን ነበር? ☐ፖዘቲፈ ☐ኒጊቲፈ ☐አላዉቀዉም
24. ባለፉት 4 ሳምንት የወባ መድሀኒት ወስደዋል? ☐አዎ ☐አይ
25. ከወሰዱ መድሀኒቱ ምን ነበር

☐አርቲሚሲን ኮንባይኒሽ ቲራፒ ☐አርቲሚተር-ሉሚፋንትሪን ☐አርቱስኒት ☐ፈአንሲዳር ☐ኪዩኒን

☐ፓናዶል ☐ሊላ ________________ ☐አይታወቅም

26. የመረጃ ሰብሳቢ ስም እና ፈረማ : ____________________________

Annex-V: Malaria Blood film preparation and examination

Specimen Collection and Processing

Kinds of blood film: In malaria microscopy, two kinds of blood film are used: thick and thin.

The thick film: A thick film is always used to search for or detect malaria parasites. The film consists of many layers of red and white blood cells. During staining, the hemoglobin in the red cells dissolves, So that large amounts of blood can be examined quickly and easily. Malaria parasites, when present, are more concentrated than in a thin film and are easier to see and identify.

The thin film: The thin film is used to confirm the malaria parasite species when this cannot be done in the thick film. It is used to search for parasites only in exceptional situations. A well prepared thin film consists of a single layer of red and white blood cells spread over less than half the slide. The frosted end of the slide is used for labeling.

Required equipment's

•***Sterile lancet***—Retractable type is preferred; tip less than 2.4 mm.

•***Alcohol wipes***—Wipes containing 70% isopropyl alcohol will be used.

•***Sterile gauze pads***—for removal of a first free-flowing drop of blood and for pressure application after collection.

•***Gloves***—made of latex, rubber, vinyl, etc.; worn to protect the patient and the collector

•***Sharps disposal unit***—Lancets were placed in a proper disposal unit after use.

•***Frosted end slides*****__**Cleaned, wrapped frosted end slides; frosted ends used for labeling

•***Absorbent cotton wool*****__**to clean the finger & wipe off the blood

•***A slide box or tray*** for drying slides horizontally and protecting them from flies and dust;

•***Record forms or a register***; to record the field & laboratory data

•***Ballpoint ink-pen*** for the record forms or register; and

•***A lead pencil*** to give code or write on the frosted end of slides film and small sharpener.

•***Absolute Methanol*****__**to fix the thin blood films

•***Giemsa stain*****__**to stain the blood films

•***Slide rack*****__**to put the slides on for staining

•***Light microscope and immersion oil*****__**to examine the blood films

Procedure for finger pricking and blood film preparation

1. The retractable lancet is used most often for safety reasons. The retractable lancet is spring-loaded and the lancet retracts into the body of the device after skin puncture.
2. The recommended depth of puncture is 2.5 mm for adults and 2.0 mm for children All lancets will be sterile and for one-time use only.
3. The finger was massaged to increase the blood flow. This may be done by ***gently*** squeezing the finger from hand to fingertip 5 or 6 times. This maneuver is not overused as it may cause erroneous results due to the concentration of tissue fluids.
4. Fingertip cleansed with 70% isopropyl alcohol. It was then wiped, dried with a clean, dry piece of gauze or cotton. The finger should be thoroughly dry, as blood will not well up and form a drop at the puncture site of a moist finger.
5. The lancets will be removed from its package and grasped between the thumb and forefinger. The finger puncture devices will be used referring to the instructions for the device we will be using it.
6. Using a sterile lancet, a skin puncture will be made just off the center of the finger pad. The puncture should be made perpendicular to the ridges of the fingerprint so that the drop of blood does not run down the ridges. The first drop of blood, which tends to contain excess tissue fluid will be wiped away.
7. The drops of blood will be collected into the collection device by gently massaging the finger. Excessive pressure will be avoided because it may squeeze tissue fluid into the drop of blood.
8. Through wiping off the first drop of blood, thick and thin films will be made on the same slide from the next blood drops. For thick films, ~12 μl of blood was spread over a diameter of 15mm, while ~2 μl of blood will be used for thin films.

Staining of malaria blood smears

1. The staining technique and blood film examination will be conducted employing WHO guidelines.
2. We used a 10% Giemsa working solution at PH of 7.2 to stain for 10 minutes. One hand of commercially available Giemsa liquid stock solution and 9 hands of distilled water will be diluted, checked for PH and used immediately for staining. Every time of

staining, new working solutions will be used for a new batch, discarding the remaining solution.

3. After the thin slides had been fixed with 100% methanol for 30 sec, the stain will be gently poured onto the slides (or a pipette will be used to drop the stain onto the slide).
4. After 10 minutes of staining, the stain gently flushed off the slide by adding drops of clean water. The stain never poured off the slides, otherwise, the surface scum will stick to the filmand spoil it for microscopic examination.
5. The slide will be placed in the drying rack, film side downwards, to drain and dry. The thick film will be placed carefully as not to touch the edge of the rack.

Microscopic examination of the films

1. Microscopic examination of thick films, using high power magnification for the presence of parasites and parasite species identification using thin films under a 100× Oil immersion objective will be carried out by an experienced laboratory technicians/technologist. The parasitic load will be also counted following WHO guidelines**.**
2. Parasite density will be determined from thick smears at 100x oil immersion objective by counting the number of asexual parasites per 200 leukocytes (or per 500 leukocytes when the number of parasites was below 10) and expressed as parasites/µl, assuming a standard white blood cell count of 8,000leukocytes/µl.

$$\text{Parasitemia} = \frac{Number\ of\ parasites\ counted}{Number\ of\ leukocyte\ counted} \times (8000\ \text{leukocyte}/ul)$$

Depending on the number of parasites observed, stop counting after you have examined 200 or 500 white cells. If you have counted ≥ 100 parasites in 200 white cells, stop counting, and record the results as the number of parasites per 200 white cells and If you have counted ≤ 99 parasites in 500 white cells, stop counting, and record the results as the number of parasites per 500 white cells

3. Each slide was read by two microscopist blinded to the other’s readings. Slides with discrepant results between the first and second readings were settled by a third microscopist.

Criteria for a second microscopy reading was: slides positive for Plasmodium spp. at first microscopy reading; individuals with discrepant microscopy results read by third one.

4. The discrepant microscopy result was resolved by calculating the percentage discrepancy.

$$\% \text{ Discrepancy} = \frac{count\ 1 - count\ 2}{Mean\ count\ of\ 1\ and\ 2} * 100\%$$

5. For parasite counts with percentage discrepancy less than 20%, the count was accepted and the mean parasite count was taken as the parasite density.
6. For parasite counts with % discrepancy ≥ of 20%, the films were examined by Reader 3.
7. The count by Reader 3 and the closest from either Reader 1 or Reader 2 were used to calculate the % discrepancy, and their mean count was taken as parasite density providing the% discrepancy is < 20%.
8. A definitive diagnosis of malaria will be made when a reddish chromatin dot with a purple orblue cytoplasm of the malaria parasites have seen together.
9. The microscopist spent an average of 15 min to 1 hr on each thick and thin film, respectively. A slide was reported negative after 100 high power fields have been examined using ×100 oil immersion objective lens and no parasite seen

Annex-VI: DNA extraction using NucleoMag® Blood Extraction Method

SOPs for NucleoMag® Blood Extraction Method

Principle:

The NucleoMag® Blood procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. Whole blood is lysed with Lysis Buffer MBL1 and Proteinase K. Following lysis incubation, magnetic beads are added and binding conditions under which the DNA binds to the magnetic beads are adjusted by the addition of Binding Buffer MBL2. After magnetic separation and removal of the supernatant, the paramagnetic beads are washed three times to remove contaminants and salt. There is no need for a drying step as ethanol from previous wash steps is removed by Wash Buffer MBL4. Finally, highly purified DNA is eluted with low-salt Elution Buffer MBL5 and can directly be used for downstream applications. The NucleoMag® Blood kit can be used either manually or automated on standard liquid handling instruments or automated magnetic separators.

Reagents and Supplies

Reagents
NucleoMag® B-Beads - 12 mL
Lysis Buffer MBL1 - 45 mL
Binding Buffer MBL2 - 160 mL
Wash Buffer MBL3 - 900 mL
Wash Buffer MBL4 - 500 mL
Elution Buffer MBL5* - 125 mL
Proteinase K, lyophilized**, and Proteinase Buffer PB - 4 x 50 mg and 15 mL

Supplies and equipment
- Kingfisher Flex magnetic particle processor with 96 deep wellhead and 96 well heat blocks. - Kingfisher Flex 96 Deep well plate - Kingfisher Flex 96 Tip comb and plate - Adjustable micropipettes - Micro-plate shaker - Incubator

- Vortex mixer
- Adhesive film sealer
- M-tork

Procedure

- ✓ **Before first use of the kit**

Before proceeding to the extraction prepare the PK buffer

- Was added 2.5ml PK buffer to each of the 4 PK vials then store at -20°c

- ✓ **Prepare Bead and MBL2 mix**

- Was added 25ul bead to the 300ul MBL2 (binding buffer) for a single sample. Calculate the mix according to your sample

Components	Volume per well
B-beads	25ul
MBL2(binding buffer)	300ul
Total Mix	325ul

Note: *Vortex the bead before and after mix*

- While adding into sample avoid settling of the beads

Note: Don't store the premix of the bead& MBL2 longer than 12hrs

- ✓ **Preparing the sample**

A. Was added 20ul of PK mix to each tube
B. Was added 200ul blood
C. Was added 80µl MBL1 (lysis buffer). Mix 3-5 times pipetting up and down
D. Shaked for 8min at 700rpm at room temperature
E. Was added 325ul bead mix (bead + MBL2) to each sample.

- ✓ **Process samples on the instrument**

A. Label the Deep well as 'wash 1' and added 800µl MBL3 to each well

B. Label the second Deep well as ‘Wash 2’ and added 800µl MBL3 to each well

C. Label the third Deep well as ‘wash 3’ and added 900ul 80% Ethanol to each well

D. Was added 75ul MBL5(elution buffer) to each well

E. After finishing labeling and adding the reagents turn on the kingfisher flex instrument

F. On the user section was select the DNA

G. Select the protocol ‘A2595’and press start

H. Put the deep wells as the machine suggested and run the sample

I. When prompted by Kingfisher flex machine added 75ul of MBL5

Note: *Do not allow the purified samples to sit uncovered at room temperature for more than 10 minutes, to prevent evaporation and contamination.*

The purified samples are ready for immediate use. Alternatively, store the covered Elution Plate:

- At 2–8°C for up to 24 hours.
- At –20°C or –80°C for long-term storage.

Annex-VII: Quantitative polymerase chain reaction for species detection

Principle of Multiplex qPCR

Real-Time Polymerase Chain Reaction (qPCR) is a technique that monitors the progress of PCR reaction in real-time. It is based on the detection of the fluorescence produced by a reporter molecule. There are many different fluorescence markers (fluorophore-containing DNA probes) used in qPCR but one of the most common is TaqMan probe. It is a hydrolysis probe which bear a reporter dye, often fluorescein (FAM) and Texas Red at its 5' end and a quencher tetramethylrhodamine[(TAMRA), attached to the 3' end of the oligonucleotide. As the taq-polymerase start to synthesize new DNA strand in the extension stage, it causes degradation of the probe by 5' end nuclease activity and the fluorescein is separated from the quencher as a result of which fluorescence signal is generated. As this procedure continues, in each cycle the number of signal molecule increases, causing the increase in fluorescence which is positively related with the amplification of the target.

In general, Bio-Rad's CFX Dx real-time PCR amplification systems allow PCR quantification with standard curve.

Reagents and Supplies:

Reagents
70% alcohol
DNA away solution
PCR master mix reagents (TaqMan MM 2x buffer, 18S forward primers, 18S reverse primers and probes)
PCR water
Pf18S and Pv18S serial dilutions (plasmids)

Supplies and materials
✓ M-Tork ✓ Fine tip markers ✓ Gloves ✓ Pipette tips (10µL, 20µL, 100µL, 200µL, 1000/1250µL)

- ✓ PCR plate
- ✓ PCR tubes
- ✓ Plate sealer (Microseal® 'B' PCR Plate Sealing Film, Bio-Rad)
- ✓ 2 mL Eppendorf tubes

Equipment:

- Rack for holding the Eppendorf and PCR tubes
- Box (Bowel) to remove used tips
- Dispenser
- Pipettes (10µL, 20µL, 100µL, 200µL, 1000µL)
- Biosafety cabinet
- PCR plate spinner
- Tube spinner
- Ice maker machine
- CFX96 Touch™ Real-Time PCR Detection System

Procedure:

Step	Action
1.	Take the master mix box to the master mix room.
2.	Turn on the master mix safety hood and also put on the light.
3.	Clean the master mix cabinet by using 70% ethanol followed by wiping away with M-tork.
4.	Also clean all pipettes, tip boxes and rack by 70% ethanol. Use "DNA away" to clean pipettes in the MM room if we use them in other rooms.
5.	Take out the master mix reagents from the fridge except the probes, then leave them and put on the rack to thaw for some time. **Note:** Withdraw the probes from the fridge after the other components are mixed.
6.	In between MM preparation activities, take out the extracted samples to be tested and serial dilutions/plasmids from -20ºC and put them on the sample room to thaw.
7.	Ready a 2 mL Eppendorf tube to mix all master mix components.

8.	According to their order, add TaqMan MM 2x (buffer), species-specific Primer FW and Primer RV (*P. falciparum/vivax* FW and RV primers), nuclease free water (PCR-water, or MQ, or ddH2O) into the labelled tube. **Note:** While adding the components, mix them using the pipette by sucking up and down manner. Also, care has to be taken to not create bubbles. Switch off the light when we use the probe as it’s light sensitive.
9.	Return all the primer and probe aliquots to the fridge.
10.	Dispense the prepared MM (15µL in each well) into the PCR plate wells considering the duplicate plasmids, negative controls (MQ/NTCs) and number of target samples (unknown).
11.	Switch off the light in the sample room as our mix contains probe that is light sensitive.
12.	Dispense 5µL of the duplicate plasmids (standards), samples and MQs into the dispensed master-mix as of the plate layout.
13.	After pipetting our PCR samples align the adhesive film (the Bio-Rad microseal® ‘B’ seals) to the plate so that all wells are covered tightly.
14.	Spin the plate in the PCR spinner to remove all bubbles.
15.	Turn on the computer and then turn on the BIO-RAD by the switch at the back of the machine.
16.	On the desktop of the computer, double click on the icon “Bio-Rad CFX Maestro” software.
17.	Click on the “User-defined” run type button in the main software toolbar.
18.	From the window that appears click on “Select Existing”, on Local Disc D then select “18S TaqMan”.
19.	Check /adjust the real time PCR machine thermal cycler conditions on the Protocol window as follows:

Thermocycler conditions		
Temp	**Time**	**Cycle**
50°C	2:00	1
95°C	10:00	1
95°C	0:15	45X
60°C	1:00	

20.	Click on "Next" and in the window that appears click on "Edit selected". Insert all the information and indicate which wells are containing the standards, non-templates and unknown samples followed by selecting the fluorophores,
21.	Click on the "Open lid" button located on the software's "Start Run" tab to open the motorized lid.
22.	Load samples in the "block" of the CFX96 Deep Well system, then click on "Close lid" to close the motorized lid.
23.	Select our target gene as "18S".
24.	Start the reaction by clicking on "Start Run", after filling all the required information for the reaction.
25.	Once cycling is completed, check our results by adjusting the baseline thresholds for each species of *Plasmodia*.
26.	Export the result to excel and save the excel workbook.
27.	Remove the samples from the block and close the system.
28.	Press the power switch on the back of the C1000 thermal cycler to power it down.

Result Reporting and Interpretation:

A successful real-time PCR experiment will have the following characteristics:

- Curves are all S-shaped
- Dilution series has expected spacing
- Replicates are tightly clustered
- Baselines are relatively flat
- Plateau height doesn't matter
- Curves are smooth
- Melt curve has one peak per product
- No signal for NTC (non-template control).

In general, slopes between -3.1 and -3.6 giving PCR efficiencies between 90 and 110% are typically acceptable

Annex-VIII: Digital polymerase chain reaction for typing *hrp 2/3* deletion

Principle of dPCR

Just like in qPCR experiments, sample preparation includes the transfer of master mix, probes and primers to a 96- or 24-well nano plate, followed by the addition of samples. The system integrates partitioning, thermocycling and imaging into a single fully automated instrument that takes users from the sample to result in under 2 hours. One can perform analysis on the Software Suite, providing the concentration in copies per microliter of your target sequence as well as for quality control such as positive samples or NTC. This analysis can also be extended to remote computers within the same local area network (LAN).

Procedures

Step 1: Prepare and load

As a first step, it is important to get your samples ready. Depending on your application, the sample can also be tested in different dilutions. Next, create an experiment in the QIAcuity Software Suite by defining the dPCR parameters – priming, cycling and imaging profiles, reaction mixes, samples & controls and plate layout. Once the plate has been defined, it is ready for a run. Prepare the reaction mix in a pre-plate by combining the QIAcuity PCR Master Mix with the sample, primers, RNase-free water and optionally restriction enzyme. The final reaction volume depends on the QIAcuity Nanoplate used. The prepared reaction mix can then be pipetted into the nanoplate. To prevent evaporation and contamination the nanoplate should be sealed properly. A roller is used to fix the foil on the plate macrostructure. The proper fixing of the plate seal by manual rolling is important for a good filling result. It is critical to hold the plate at the side edges or by the tray and transport it to the QIAcuity smoothly without shaking or turning to ensure that the reaction mix is at the bottom of the input well. When the instrument and suite are connected, all modules are ready for use and plates can be loaded and started.

Step 2: Run setup and amplify

Once the plate is loaded, the instrument will highlight the slot in blue and scan the barcode. The preferred experiment previously set up in the Control Software can be linked to the plate, the priming, cycling and imaging profile defined and run initiated.

First, the plate is processed in the priming/rolling module. At this step, the reaction mix of each well is partitioned into a thousand little individual reactions. Then PCR is performed in a thermocycler. The suitable template material within one or the other partition will lead to a positive fluorescence signal which is detected during imaging. The images are sent to the QIAcuity Software Suite for image processing.

Step 3: Analyze results

To analyze, select mutation detection. The list view shows the concentration, confidence interval as well as valid, positive and negative partitions. A heat map view shows the target channel alongside the reference channel. Use either histogram, 1D scatterplot or 2D scatterplot to change threshold settings. Click “recalculate” to get results based on the changed threshold.

Annex-IX: Declaration

I the undersigned, declare that this thesis is my own work and has never been presented for any degree or other purposes at Jimma University or any other institution of higher learning. I also declare that, when other people work has been used, it has been carefully acknowledged and referenced following the requirements. Therefore, I agree to accept responsibility for the scientific, ethical and technical conduct of the research project and for the provision of required progress reports as per the terms and conditions of the Institute of Health in effect at the time of grant is forwarded as the result of this application.

Name of the student: Alayu Bogale

Signature: ______________________ Date: _____/_______/__________

This thesis has been approved by the supervision of university advisors:

1. Name of 1st advisor: Dr. Teferi Eshetu (PhD)

Signature: ____________________ Date: ______/______/________

2. Name of 2nd advisor: Dr. Teshome Degefa (PhD)

Signature: ____________________ Date: ______/______/________

3. External Examiner: Professor Daniel Yilma (Prof)

Signature: ___________________ Date: ______/______/________

4. Internal Examiner: Dr. Abdulhakim Abamecha (PhD)

Signature: ____________________ Date: ______/______/________

5. Name of School Head: Dr. Teshome Degefa (PhD)

Signature: ____________________ Date: ______/______/________