Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys

Endashaw Esayas Boda

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

February, 2024

Adama, Ethiopia

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Major Advisor: Prof. Hunduma Dinka (Ph.D.)

Co-advisor: Dr. Endalamaw Gadissa (Ph.D.)

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DECLARATION

I hereby declare that this Master Thesis entitled **"Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys"** is my original work. Thus, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through appropriate citations.

Endashaw Esayas ---------- ----------

Name of student Signature Date

RECOMMENDATION

We, the advisors of this thesis, hereby certify that, we have read the revised version of the thesis entitled **"Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys"** prepared under our guidance by **Endashaw Esayas** submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology. Therefore, we recommend the submission of a revised version of the thesis to the department following the applicable procedures.

Prof. Hunduma Dinka (Ph.D.) ---------- ----------
Major Advisor Signature Date

Dr. Endalamaw Gadissa (Ph.D.) ---------- ----------
Co-advisor Signature Date

APPROVAL SHEET

We, the advisors of the thesis entitled **"Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys"** and developed by Endashaw Esayas, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor: Prof. Hunduma Dinka (Ph.D.) Signature_____ Date _____

Co-advisor: Dr. Endalamaw Gadissa (Ph.D.) Signature _____Date _____

We, the undersigned, members of the Board of Examiners of the thesis by Endashaw Esayas have read and evaluated the thesis entitled "Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Dr. Dejene Getachew (Ph.D.) Internal Examiner	_____ Signature	_____ Date
Dr. Jifar Hassen (Ph.D.) External Examiner	_____ Signature	_____ Date

Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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Table of Contents

List of Annexes

Contents **Page**

List of Tables

Contents **Page**

List of Figures

Contents **Page**

LIST OF ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based combination therapy
AHRI	Armauer Hansen Research Institute
bpn	bites per person per night
CDC-LT	Centers for Disease Control and Prevention Light Trap
CHW	Community health workers
Cox1	Cytochrome C oxidase subunit 1
CSA	Central Statistical Agency
DBS	Dried blood spots
ELISA	Enzyme-linked Immuno-sorbent assays
EPHI	Ethiopian Public Health Institute
FMOH	Federal Ministry of Health
HBO	Human behavior observation
HBR	Human-biting rate
HRP	High risk population
IRB	Institutional review board
IRS	Indoor residual spraying
ITS2	Internal transcribed spacer region 2
LAMP	Loop mediated isothermal amplification
LLIN	Long-lasting insecticide treated net
LSM	Larval source management
NATs	Nucleic acid amplification-based tests
NMEP	National malaria elimination program
OPD	Outpatient department
qPCR	Quantitative polymerase chain reaction
RDT	Rapid diagnostic test
SOP	Standard operating procedures
WHO	World health organization

ABSTRACT

Seasonal migrant workers, who move between areas with low and high malaria risks for agricultural work, are potential high-risk groups for infection and can fuel transmission dynamics. Hence, the aim of this study was to investigate malaria transmission among seasonal migrant agricultural workers in the Metema district, Northwestern Ethiopia. A targeted parasite survey and entomological assessments were conducted in major (September to December, 2022) and minor (March to May 2023) malaria transmission seasons. A total of 1597 individuals were enrolled and tested to estimate the prevalence of malaria cases using rapid diagnostic tests (RDT) and quantitative polymerase chain reaction (qPCR). Dried blood spots (DBS) were collected, and DNA was extracted using chelex to detect malaria infection using 18S ribosomal RNA gene-based qPCR. Hourly centers for disease control (CDC) light trap collections coupled with human behavior observations (HBO) were conducted around the farm areas where targeted parasite survey was taking place. Morphologically identified 266 anopheline specimens underwent DNA sequencing targeting internal transcribed spacer region 2 (ITS2) and cytochrome oxidase subunit 1(COX1) genes for species confirmation. The finding of this study showed that the prevalence of malaria infection among seasonal migrant agricultural workers was 23.2% (371/1597) and 38.3% (612/1597) by RDT and qPCR, respectively. Among positive cases identified, P. falciparum was the most prevalent species (21.3% by RDT and 34.4% by qPCRs), followed by P. vivax (1.3% by RDT and 2.3% by qPCRs). Only 8.02% of seasonal migrant workers possessed LLINs. A total of 532 mosquitoes belonging to 10 species were molecularly confirmed, with Anopheles arabiensis (76.69%) as the primary malaria vector. Approximately 71% of human exposure to mosquito bites happened outdoors (awake or asleep without bed nets). Agricultural Migrant workers were exposed to storms for malaria transmission: low LLIN use, high prevalence of malaria, and high levels of outdoor exposure to mosquito bites. Thus, preventing malaria in mobile populations requires multi-pronged action. These could include boost LLINs, shield outdoor exposure (like mosquito repellents), and vectors to protect migrant workers and their communities from malaria's ongoing threat.

***Keywords**: Anopheles, Human behavior, Malaria transmission, Metema, Plasmodium, Seasonal migrant workers.*

CHAPTER 1

1. INTRODUCTION

1.1. Background

Malaria is a widely spread human parasitic disease, particularly in tropical and subtropical parts of the globe (World Health Organization (WHO), 2023). In 2022, malaria caused an estimated 249 million clinical episodes, and 608,000 deaths were recorded worldwide. The disease comprises protozoan parasite belonging to the genus *Plasmodium* (the causative agent of malaria), human hosts and female *Anopheles* mosquitoes (spread the disease from person to person through the bites) (Snow *et al.,* 2005). Five species of *Plasmodium* parasites are responsible for human malaria: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* (Mendis *et al.,* 2001; Snow *et al.,* 2005) and *P. knowlesi* (zoonotic *Plasmodium* infection which is prevalent in Southeast Asia) (Lee *et al.,* 2022). *Plasmodium falciparum* is the dominant and is responsible for majority of malaria related deaths in the sub-Saharan African countries whereas *P. vivax* is the predominant in most nations outside of sub-Saharan Africa (WHO, 2023).

In Ethiopia, malaria remains a significant public health issue which causes a substantial amount of morbidity (Federal Ministry of Health (FMOH), 2020). Transmission of malaria in Ethiopia is generally unstable and heterogeneous in space and time due to diverse eco-topography and climate. The highest malaria transmission is concentrated in lowlands, highland fringe areas, and in the western part of the country along the border with Sudan and South Sudan (Ethiopian Public Health Institute (EPHI), 2016). Previously, malaria was known to occur up to 2,000 meters above sea level (m a.s.l.) however, several pockets with micro-epidemiological conditions supporting malaria transmission occur in areas above this altitude up to 2,300 meters under weather condition anomalies (Tesfaye *et al.,* 2011; Daygena *et al.,* 2017).

In most parts of Ethiopia, transmission has a bimodal pattern. The major season occurs from September to December, following June to September rainy season and from March to May, during and after the February to March rainy season. However, in parts of South and Southwest lowlands, the main rainy season is from February to March resulting in malaria major season to be in April and May (EPHI, 2016). Except for the occurrence of small-scale outbreaks and seasonal case build-ups, countrywide malaria epidemics have not been observed in Ethiopia after

implementing wide-scale control strategies and introduction of artemether-lumefantrine as first-line antimalarial drug for uncompilicated *P. falciparum* since 2004 (FMOH, 2017). A report by Ethiopian health management information system indicated a significant drop of confirmed malaria cases from 2016 to 2019 by 47% (FMOH, 2020), and the country was fore-mentioned by World Health Organization (WHO) for applying malaria prevention strategies effectively to achieve a reduction by 40% in 2020 (WHO, 2023).

In Ethiopia, since 2000 there has been a substantial increase in investment to support malaria interventions. Due to this commitment over the past two decades, a significant reduction of malaria has been registered (FMOH, 2020). This improvement could be attributed to improved coverage of key malaria control interventions, including, distribution of long-lasting insecticide treated net (LLINs) through mass campaigns targeting the entire population at risk, indoor residual spraying (IRS) in designated epidemic-prone areas, and expanded diagnostic testing and effective antimalarial treatment to people at risk (Abeku *et al.,* 2015; FMOH, 2017). Encouraged by recent progress in malaria control, the country has set an ambitious goal: nationwide malaria elimination by 2030. To achieve this, FMOH are starting with sub-national elimination in districts with low malaria transmission (FMOH, 2020). In these lower endemic areas where malaria transmission is the most heterogeneous, there will be a probability of importing parasites and transmission can be restarted (Churcher *et al.,* 2014) due to peoples' seasonal travel patterns (Schicker *et al.,* 2015; Haile *et al.,* 2017) and efficacy failure of commonly used antimalarial drugs (White, 2004), diagnostic failure (Murray *et al.,* 2014) and vector resistances (Ranson and Lissenden 2016).

Population movement is considered as one of the key factors fueling and maintaining malaria transmission in low transmission areas of Ethiopia (Graves *et al.,* 2009; Schicker *et al.,* 2015). Seasonal migrant workers account for most of the population movement in regions like Amhara and Tigray and are considered a key high-risk population (HRP) as they move annually from the highlands (relatively low malaria endemicity) to the lowlands (higher malaria endemicity) to seek work at agricultural farms (Dugassa *et al.,* 2021). Driven by the growth of large-scale agriculture, especially in western Ethiopia, seasonal labor participation has grown significantly (FMOH, 2021).

Seasonal migrant workers are susceptible to malaria infections during their travel and/or stay at farm camps due to low coverage and use of malaria prevention and control measures (LLINs, IRS) coupled with high levels of indoors and outdoors exposure to mosquito bites (Argaw *et al.,* 2013; Dugassa *et al.,* 2021). Furthermore, seasonal migrant workers risk returning to their home community with untreated malaria infections potentially contributing to malaria transmission in receptive areas (Wimberly *et al.,* 2012; Alemu *et al.,* 2014).

The national malaria elimination strategic plan (2021-2025) identified 68 high-risk districts nationally. Of these, 36 are in four regions stretching from North to South along the western borders with Sudan and South Sudan (FMOH, 2020). Studies conducted in the Amhara Region provided some initial data on migrant workers and the movement patterns (Schicker *et al.,* 2015; Bansil *et al.,* 2018). Malaria outpatient department (OPD) data from 2014-2017 in 138 midland and highland health posts in Ethiopia revealed a high prevalence of male patients (75%) among 8,163 rapid diagnostic tests (RDT) positive cases. This trend may be attributed to the migration of young men for agricultural work in large areas, as evidenced by the higher incidence in older children and adults (18%). Fifty-nine percent of the malaria cases who reported travel history were diagnosed between September and December. Case documentation and investigation with reactive focal test and treat was conducted from 2014 until 2017 in 41 health post catchment areas. Of the 436 malaria index cases, 37% (159/436) reported travel history, compared to 0.7% (49/6706) of the household members of neighbors who tested negative (Bansil *et al.,* 2018).

1.2. Statements of the problem

Ethiopia has made significant progress in controlling malaria over the past decade. The national malaria burden has declined by over 70% since 2005, due to a major scale-up and the implementation of various malaria control interventions (Aregawi *et al.,* 2014; Taffese *et al.,* 2018). However, despite this progress, malaria transmission remains heterogeneous in Ethiopia. Stubborn pockets of high transmission persist, mainly in the country's lowlands and western regions. In areas with low or very low transmission, the disease often becomes hyper-localized, clustering within specific high-risk groups like seasonal migrant workers (Haile *et al.,* 2017; Dugassa *et al.,* 2021).

Northwestern parts of Ethiopia witness a significant annual migration of people, estimated at 1.5 million, for seasonal work in agricultural investment areas (FMOH, 2021). Seasonal migrant workers constitute a critical workforce for Ethiopia's agricultural sector. While vital to Ethiopia's food security, the influx of seasonal workers into areas with varying malaria risks adds a layer of difficulty to controlling malaria. Studies suggest their infection rates double the national average, highlighting their vulnerability (Haile *et al.,* 2017; Dugassa *et al.,* 2021). Given the crowded living conditions, limited healthcare access, and direct exposure to infective mosquito bite while working create a perfect storm for transmission. Their migration not only exposes them to high risks but also fuels transmission, potentially reigniting outbreaks back home and jeopardizing malaria elimination efforts (Dugassa *et al.,* 2021).

Studies on seasonal migrant work forces related to malaria infection rates, transmission dynamics, and risk factors are scarce. This knowledge gap hinders the development of targeted interventions to protect this vulnerable group and prevent onward transmission. Therefore, moving towards malaria elimination necessitates characterizing seasonal migrant workers and implementing tailored interventions. Generating evidence on high-risk migrant worker environments' transmission dynamics, disease burden, entomological drivers, and human behavior is crucial to identify factors influencing mosquito exposure and prevention measure utilization. By prioritizing these actions, this study attempts to bridge the malaria control gap for migrant workers by characterizing the malaria transmission dynamics and its burden among seasonal migrant workers in Northwestern Ethiopia. Addressing this knowledge gap can support the malaria elimination agenda via development of targeted interventions to protect this vulnerable group and prevent further expansion of the disease to new localities.

1.3. Significance of the Study

The information obtained from this study illuminates the malaria transmission dynamics among seasonal migrant workers. This study's findings provide valuable insights for the National Malaria Control and Elimination Program (NMEP) to tailor interventions for seasonal migrant workers and their communities. It informs strategic resource allocation and the development of targeted prevention, testing, and treatment plans, thereby contributing to Ethiopia's malaria elimination efforts.

1.4. Research questions

- What is the prevalence of malaria cases among seasonal migrant agricultural workers in the Metema district?
- What are the species compositions, abundance, and biting behaviors of *Anopheles* mosquitoes around seasonal migrant agricultural workers in the Metema district?
- What are the human behavioral drivers of malaria transmission among seasonal migrant agricultural workers in the Metema district?

1.5. Objectives of the Study

1.5.1. General Objective

The general objective of this study is to investigate malaria transmission among seasonal migrant agricultural workers in the Metema district, Northwestern Ethiopia.

1.5.2. Specific Objectives

- To estimate the prevalence of malaria cases among seasonal migrant agricultural workers in the Metema district.
- To characterize *Anopheles* mosquito's species compositions, abundance, and bionomics in the seasonal migrant agricultural workers in Metema district.
- To determine human behavioral drivers of malaria transmission among seasonal migrant agricultural workers in the Metema district.

1.6. Delimitations and limitations of the study

In this study while polymerase chain reaction (PCR) was successfully used to detect malaria infections among seasonal migrant workers, time and resource constraints limited the ability to investigate deeper into the parasite's potential for resistance. This means the study couldn't explore the diversity and genetic relatedness of parasite strains through genotyping, nor assess for drug resistance mutations in identified cases. Moreover, the current study unable to investigation of *Anopheles* mosquitos' infection status and potential role in malaria transmission within the studied population.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Burden of Malaria

Malaria claims millions of lives globally, particularly in tropical and subtropical parts of the globe. Nearly half the world's population lives in areas at risk of malaria transmission in 85 countries and territories (WHO, 2023). The burden of the disease continues to cause a considerable number of morbidity and mortality in the sub-Saharan African and cause severe economic losses in endemic countries (Murray *et al.,* 2014). An estimated 82% of malaria cases and 94% of deaths in 2022 was from sub-Saharan African countries. Worldwide, millions of people die annually from malaria, which is also known to induce severe consequences including severe anemia, brain involvement, acute renal failure, and hypoglycemia. About three million cases of malaria are reported annually in nearly 45 countries, including Ethiopia, and the morbidity and death rates are rising sharply (WHO, 2023).

2.2. Malaria in Ethiopia

In Ethiopia, more than 75% areas of the country are malarious and about 60% of the population are living in the malaria risky areas (FMOH, 2020) mainly at the altitude below 2000 m a. s. l. Malaria is one of the top-ranking causes for morbidity and mortality which accounts for most outpatient visits, and it has been one of the main causes of hospitalization and deaths in the country (Graves *et al.,* 2009; FMOH, 2020). *Plasmodium falciparum* and *P. vivax* are the most dominant malaria parasites responsible for most malaria cases (FMOH, 2020), while *P. ovale* and *P. malariae* contribute to less than 1% of the cases (Alemu *et al.,* 2014). Ethiopia has about 810 districts with different levels of malaria risk with an estimated at-risk population of 50.6 million. For the expansion of malaria-free areas starting with the low and very low transmission strata, malaria elimination will be driven by epidemiological stratification aimed at regular identification of districts that are eligible to embark on interruption of residual transmission (Figure 1). Given that the burden reduction, the number of districts leaving high and moderate stratum to low and very low will keep increasing. Thus, based on epidemiological stratification, districts were regularly included and transitioned into different implementation approaches (FMOH, 2020).

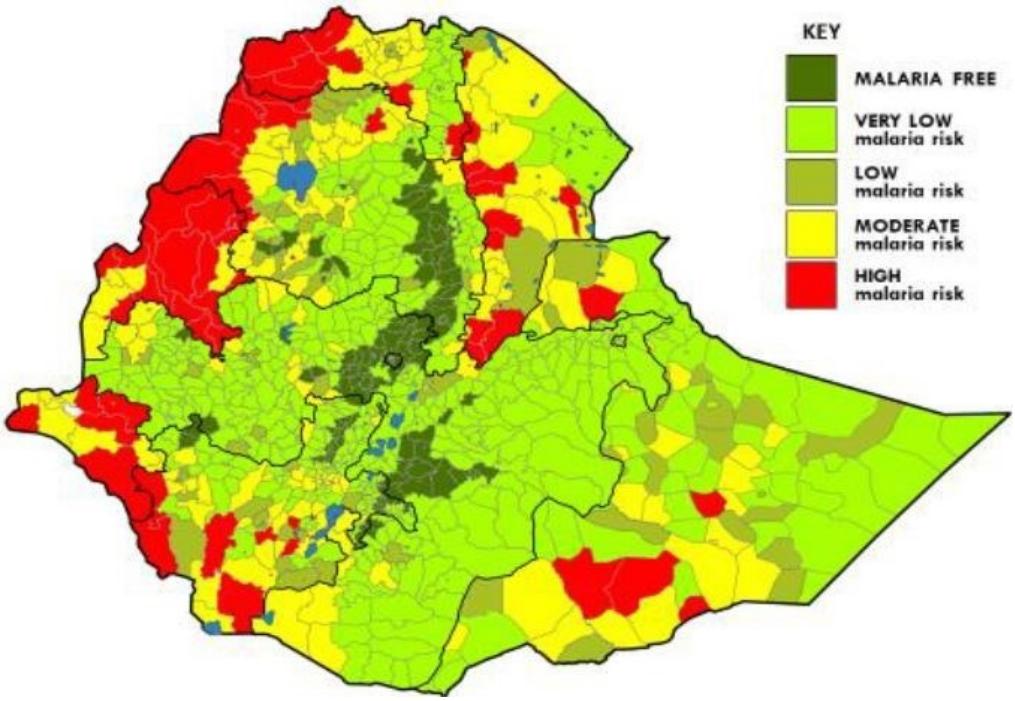


Figure 1. Map of malaria strata in Ethiopia (adapted from FMOH, 2020)

In Ethiopia, there are more than four species of *Anopheles* mosquitoes which transmit malaria (FMOH, 2017). Among them, *Anopheles arabiensis* is the principal malaria vector (Abose *et al.,* 1998). *Anopheles pharoensis, An. funestus* and *An. nili* are considered secondary human malaria transmitting vectors (Abose *et al.,* 1998; Taye *et al.,* 2006; Massebo *et al.,* 2013; FMOH, 2017). Recently, invasive *An. stephensi* was documented to be highly permissive to *P. falciparum* and *P. vivax* in Ethiopia (Tadesse *et al.,* 2021).

2.3. Biology of malaria parasite

The history of malaria involves the correlation between the cyclical infections of malaria parasite in human and female *Anopheles* mosquitoes, where they undergo different developmental stages. All types of malaria have a similar life cycle (Good *et al.,* 2005; Prudêncio *et al.,* 2006). Sporozoites, the infective stage of the malaria parasite, are injected into a human host through the saliva of an infected female *Anopheles* mosquito. These sporozoites enter the blood vessel at the site of the bite and travel through the blood stream to the liver cells within minutes, take on a new form, and multiply. When the liver cells rupture, blood stage parasites known as merozoites are released. Each merozoite invades a red blood cell, and for two days multiplies into more

merozoites. The red blood cell full of merozoites ruptures to release more merozoites. It is this stage of the life cycle that causes disease and, too often, death (Prudêncio *et al.,* 2006; Sargeant *et al.,* 2006).

The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which do not cause disease but remain in the blood until they are cleared by drugs or the immune system or taken up by the bite of mosquitoes during subsequent blood meals (Prudêncio *et al.,* 2006). The gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form, which migrates through the mosquito midgut into the hemocele, developing into an oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites, which migrate into the mosquito's salivary glands, to be injected into another human at the next bite or ready for the next transmission step (Figure 2) (Prudêncio *et al.,* 2006; Sargeant *et al.,* 2006).

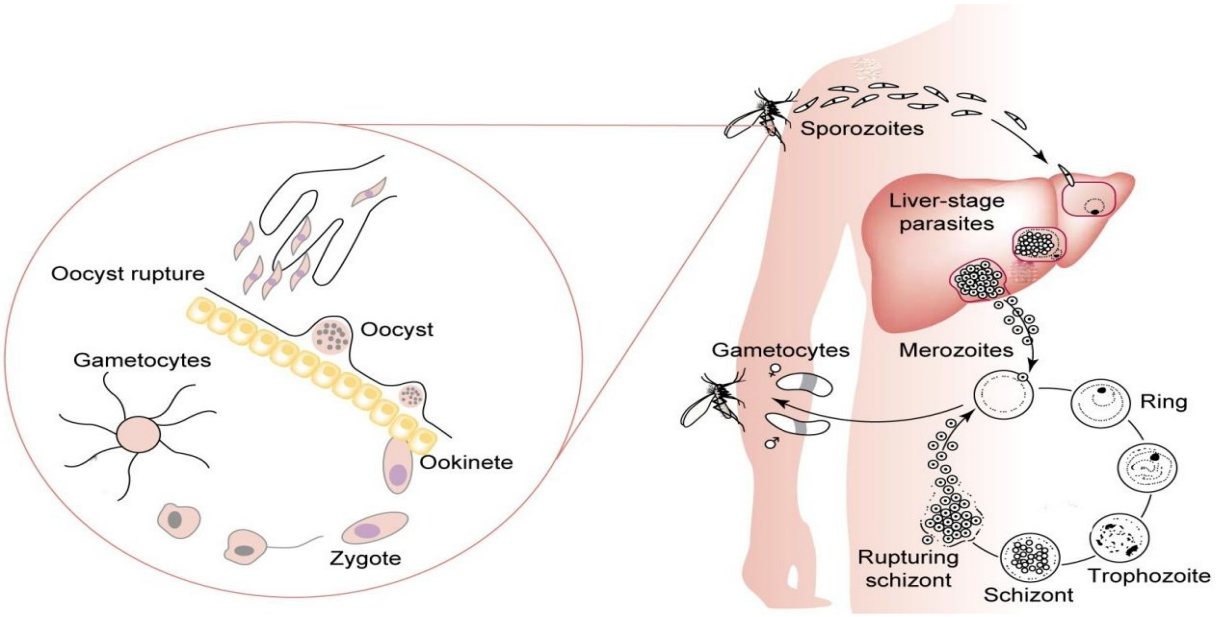


Figure 2. Life cycle of malaria parasites in humans and *Anopheles* mosquito (adapted from Targett, 2005).

2.4. Malaria and population movement in Ethiopia

The transmission of malaria infection is influenced by several factors, including susceptible population density, host immunity, human behavior, travel, and land use modification

(agriculture). Additionally, economic level, access to healthcare, climatic factors, vector capacity, and adaptation of vectors to polluted breeding sites also play a role (De Silva and Marshall, 2012). Studying travel patterns in low-malaria regions of Ethiopia revealed a concerning link between human movement and parasite transmission. This link occurs when residents from low-risk areas travel to endemic zones, get infected, and return home, or when infected individuals migrate or visit these areas (Schicker *et al.,* 2015; Haile *et al.,* 2017). Travel history increased malaria risk likely stems from movement into areas with higher transmission during mosquito biting seasons. Examples of such movements include seasonal work like crop harvesting or animal grazing, short trips to markets, visits to friends and family, or even school attendance (Schicker *et al.,* 2015; Dugassa *et al.,* 2021).

In Ethiopia, population movement has increased exposure to malaria and other vector-borne diseases that are more highly prevalent areas. With the current increase of population density in malarious lowlands of the country, areas rich with untapped natural resources, malaria will continue to be an important public health problem in the future (Haile *et al.,* 2017; Dugassa *et al.,* 2021). High population growth, scarcity of farmland in the high and midland areas and the increased investment opportunities in agriculture activities in fertile lowlands of Ethiopia have led to seasonal population mobility into lowland areas in search of work (Schicker *et al.,* 2015). In addition, the country's commitment to embark on ambitious mega-projects (especially in lowland areas including sectors, such as agriculture, transport, and power) requires movement of enormous numbers of workers to project sites. The current national malaria strategic plan aims to maintain near zero malaria deaths, reduce malaria cases by 40% and eliminate malaria from Ethiopia by 2030. Malaria among mobile and migrant labor will undoubtedly challenge the realization of this aim (Graves *et al.,* 2009; FMOH, 2020).

Travel to malarious areas has also long been recognized as a risk factor for malaria infection. Population movements can expose non-immune individuals to malaria, creating a breeding ground for transmission. Poor living conditions, high-risk work activities, and inadequate control policies in new settlements further exacerbate this risk for migrant workers (Haile *et al.,* 2017). Mobile and migrant workers may serve as carriers who transport parasites to the highlands and other locations in Ethiopia with lower malaria prevalence. The behavior and living conditions of migrant workers are major determinants of their vulnerability to mosquito bites. For example,

humans in the forested region exhibit diverse behaviors, increasing their malaria risk. These risky behaviors include commuting to high-mosquito areas, inconsistent sleep schedules due to work, and irregular use of LLINs (Schicker *et al.,* 2015; Dugassa *et al.,* 2021).

2.5. Malaria diagnostic tools

Rapid and effective malaria diagnosis not only alleviates suffering, but also decreases community transmission. The nonspecific nature of the clinical signs and symptoms of malaria may result in over-treatment of malaria or non-treatment of other diseases in malaria-endemic areas, and misdiagnosis in non-endemic areas. Moreover, delays in diagnosis and treatment are the leading causes of death (WHO, 2023). The diagnostic tools currently available for the identification of *Plasmodium* species includes light microscopy, antigen-based immunochromatographic lateral flow assays (known as Rapid Diagnostic tests (RDT)), serology, and nucleic acid amplification-based test (NAT) techniques, such as polymerase chain reaction (PCR) and Loop mediated isothermal amplification (LAMP) (Lucchi *et al.,* 2018).

2.5.1. Conventional diagnostic tools

2.5.1.1. Microscopy

Malaria diagnosis conventionally relies on examining Giemsa, Wright's, or Field’s-stained blood films under a microscope. Light microscopy is recommended by WHO as the "gold standard" involves preparing thick smears for parasite presence screening and thin smears for species identification, guiding treatment decisions (Ngasala *et al.,* 2008). The process involves staining blood smears with 10% Giemsa solution and examining them under a 100x oil immersion objective. The limit of detection for microscope is approximately 50-100 parasites per µL of blood (Mathison and Pritt, 2017). While effective for symptomatic cases, light microscopy struggles with silent infections and low parasite densities, often underestimating disease prevalence compared to molecular methods like PCR. This limitation can hinder control efforts and effective treatment (Zhao *et al.,* 2017).

2.5.1.2. Rapid diagnostic tests

Rapid diagnostic tests (RDT) are immune chromatography–based assays that are designed to detect malaria antigens where the blood is dropped into one end and the results are depicted by lines on the strip surface. Three types of antigens have been employed in this method, histidine-rich protein-2 (HRP-2), *Plasmodium* lactate dehydrogenase (LDH) and *Plasmodium* aldolase. Histidine-rich protein-2 is specific to *P. falciparum*, while LDH and *Plasmodium* aldolase are found in all species. More than 90% of commercially available RDT target HRP-2 (Mbanefo *et al.,* 2020). A 5µl blood specimen collected from the patient is applied to the sample pad on the test card along with assay buffer. The appearances of bands in a test card window after 15-20 minutes (based on the test device), indicates the presence of malaria antigens that shows whether the patient is infected with malaria parasites. Rapid diagnostic tests provide an opportunity to extend the benefits of parasite-based diagnosis of malaria beyond the confines of light microscopy, with potentially significant advantages in the management of febrile illnesses in remote malaria-endemic areas. RDTs perform poorly for active case detection of asymptomatic infections. They are ineffective in detecting low-density parasitemia (≤ 200 parasites/µL) (Mcmorrow *et al.,* 2011).

2.5.1.3. Serological tests

Malaria diagnosis employs enzyme-linked immunosorbent assays (ELISA), which harness the specificity of antibody-antigen interactions to detect or quantify malaria-specific antibodies or antigens, acting essentially as protein hunters with enzyme-tagged flags (Marie *et al.,* 2020). There are two most used types of ELISA (depending on whether the ELISA is used to detect antigens or antibodies) (Tangpukdee *et al.,* 2009).

The first one is the detection of antigens (direct ELISA) which involves coating the surface of a solid support (typically the wells of an ELISA plate) with quantity of immunosorbent (capture antibody). The ELISA plate is subsequently loaded with a liquid sample having an unknown concentration of soluble antigen. The antigen is captured by the coated primary antibody (Tangpukdee *et al.,* 2009). The unbound antigen is washed off and an enzyme-conjugated secondary antibody is added which binds to the captured antigen. The remaining unbound

secondary antibody is once again washed off and a substrate is added resulting in the formation of a colored product which can be quantified using an ELISA reader (Marie *et al.,* 2020).

The second one is indirect ELISA (for the detection of antibodies) in which antigen is coated to a solid surface. A liquid sample containing an unknown concentration of antibodies (primary antibody) is added, which binds specifically to the coated antigen. An enzyme conjugated secondary antibody is added which binds the primary antibody. Once again, the remaining unbound secondary antibody is washed off and a substrate is added resulting in the formation of a colored product which can be quantified using an ELISA reader (Tangpukdee *et al.,* 2009; Marie *et al.,* 2020).

2.5.2. Advanced diagnostic tools

Traditional malaria diagnostic methods, microscopy and RDT, remain problematic that new laboratory diagnostic techniques for malaria parasite detection which display high sensitivity and high specificity, without subjective variation, are needed. The high sensitivity of many molecular tests makes them the tests of choice for active and reactive case detection approaches because RDT or microscopy are incapable of detecting low-density infections as many of them have limits of detection equal to or less than 2 parasites/µl (Lucchi *et al.,* 2018). A recent development in molecular biological technologies, NATs techniques: PCR and LAMP, have permitted extensive characterization of the malaria parasite and are generating new strategies for malaria diagnosis. Polymerase chain reaction and LAMP have proven specific and sensitive molecular diagnostic modalities capable of identifying asymptomatic, submicroscopic malaria infection (Ragavan *et al.,* 2018). Polymerase chain reaction can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells), compared with microscopy or RDT which can detect around 200 parasites/µl of blood (Mbanefo *et al.,* 2020).

2.5.2.1. Polymerase chain reaction

Polymerase chain reaction (PCR) is a laboratory technique used for rapidly producing (amplifying) millions to billions of copies of a specific region of DNA and offering a rapid and most sensitive means of detection of plasmodial DNA in clinical samples (Britton *et al.,* 2016). Compared with conventional methods, PCR is highly sensitive in detecting low-density infections and determining the parasite species (Hawkes and Kain, 2007). Polymerase chain

reaction-based techniques are one of the most specific and sensitive diagnostic methods than conventional microscopic examination, particularly for malaria cases with low parasitemia, asymptomatic cases or mixed infection (Morassin *et al.,* 2002). The PCR technique continues to be used extensively to confirm malaria infection, follow-up therapeutic response, and identify drug resistance (Tangpukdee *et al.,* 2009).

Asymptomatic infections and drug-resistant parasites can be detected by PCR, and it is automated to process large volumes of samples. When performed under optimal conditions, the PCR technique can detect parasites below the threshold levels of microscopy and RDT. Polymerase chain reaction can detect parasitemia as low as 1 parasite/µl of blood (Hänscheid *et al.,* 2002). The results directly dependent on the quality of the genetic material of the parasite obtained during extraction and amplification and on the quality of the reagents, and the test requires a long analysis time (Aslan *et al.,* 2007). Despite its increased sensitivity, PCR has not been established as a routine diagnostic tool in diagnostic laboratories or blood banks (Lima *et al.,* 2011).

2.5.2.2. Loop-Mediated Isothermal Amplification

The LAMP technique is claimed to be a simple and inexpensive nucleic acid-based technique for malaria-diagnostic test that detects the conserved 18S ribosomal RNA (rRNA) gene *Plasmodium* species. Loop mediated isothermal amplification have high sensitivity and specificity, for *P. falciparum, P. vivax, P. ovale* and *P. malariae*. LAMP appears to be easy, sensitive, quick, and lower in cost than PCR (Lucchi *et al.,* 2018). The isothermal diagnostic technique holds substantial promise as an alternative molecular test for malaria parasite detection because it can be carried out using limited laboratory infrastructure and many of the described assays have similar sensitivities to PCR (Britton *et al.,* 2016). Loop mediated isothermal amplification has become a forerunner in isothermal diagnostic technology for the identification of *Plasmodium* species. Loop mediated isothermal amplification has been used to identify all human *Plasmodium* species and its primers have been optimized to improve the sensitivity with which *P. falciparum* and *P. vivax* can be detected (Britton *et al.,* 2016; Lucchi *et al.,* 2018). The LAMP technique detects the conserved 18S ribosome RNA gene of *Plasmodium* species. A specialized polymerase amplifies sequences from double stranded DNA such that the products fold into

looped structures, causing the reaction mixture to appear turbid. This LAMP's unique features are the *Bacillus stereothernophilus* (*Bst*) DNA polymerase, also known as *Thermus scoticus* DNA polymerase. This assay bypasses DNA isolation by using heat-treated blood supernatants, and does not require DNA denaturation, thus eliminating the need for expensive thermal cyclers (Barazorda *et al.,* 2020).

Several malaria LAMP-based assays have been described to date, many of these have excellent diagnostic performances, detecting as few as 1 parasite/µl (*Illumi*gene LAMP), or 1–5 parasites/µl for the Loopamp malaria kit (EIKEN Chemical Co) and utilize a variety of readouts (Britton *et al.,* 2016). The malachite green LAMP is a colorimetric assay that does not require any special read-out equipment except a small portable heat block and mini centrifuge. It has a testing capacity of 38 samples per run and has been described for the detection of *P. falciparum* and *P. vivax* infection (Lucchi *et al.,* 2018; Barazorda *et al.,* 2020). One of the exciting aspects of LAMP has been its potential for field deployment by virtue of its isothermal nature, and as such efforts have been invested in improving its field readiness. The Real Amp platform in which LAMP was housed in a portable tube scanner with a built-in fluorescent detection unit able to monitor the generation of fluorophores by SYBR green in real time rather than reliance on visual colour change (Britton *et al.,* 2016; Lucchi *et al.,* 2018).

2.6. Malaria prevention and control strategies

The WHO recommendations for malaria prevention and control strategies focuses on vector control to reduce transmission of parasites from humans to mosquitoes and then back to humans, and parasite control and case management which includes prompt diagnosis and effective treatment of cases (WHO, 2023).

2.6.1. Vector control

Malaria control efforts are being threatened by competent and abundant vectors that are anthropophilic and anthropophagic (Kiszewski *et al.,* 2004) and demonstrate resistance to the available insecticides in use (Ranson and Lissenden 2016; Alemayehu *et al.,* 2017). Understanding the malaria vector populations' diversity, distribution and relevant behaviors may highlight how to select and implement more appropriate and efficient interventions. In Ethiopia,

the presence of diverse malaria vectors, dynamic bionomic traits that respond to intervention strategies, and recent reports of malaria outbreaks warrant further investigation of vector populations (Gari and Lindtjorn, 2018). Vector control is a key intervention for the interruption of malaria transmission and elimination efforts. Currently, the main vector control interventions are IRS, LLINs and larval source management (LSM). IRS and LLINs target adult mosquitoes, whereas LSM targets the aquatic stages (WHO, 2023).

Indoor residual spraying is the application of a long-lasting residual insecticide on the surfaces where the vectors might contact the insecticide (WHO, 2015). It is a powerful intervention which acts by repelling mosquitoes away from the sleeping spaces, and by killing mosquitoes that rest to digest their blood meal on sprayed surfaces (WHO, 2023). When a vector contacts a sprayed surface, it absorbs lethal doses of insecticide, thereby reducing its lifespan. This results in a progressive decline in vector density and longevity, especially among older female mosquitoes. It also reduces the overall vectorial capacity of the vectors and therefore contributes to a reduction in malaria transmission. IRS is most effective against indoor feeding (endophagic) and indoor resting (endophilic) vectors (WHO, 2015).

Long-lasting insecticide treated nets provide an effective physical and chemical barrier from exposure to the mosquito for the person who is sleeping under it (Murray *et al.,* 2014; WHO, 2023). This barrier reduces the opportunity for infectious mosquitoes biting and hence blocks the mosquito from ingesting gametocytes and becoming infectious. The impregnated insecticide also kills and repels susceptible vectors that rest on the net (Murray *et al.,* 2014; WHO, 2015).

Larval source management is one of malaria interventions that involves vector breeding habitats modification or manipulation, and chemical larviciding or biological control. The immature stages of mosquitoes are confined within aquatic habitats and cannot readily escape control measures and hence, can easily be targeted by the interventions. Thus, LSM is advantageous over adult mosquito control as adults can readily avoid intervention measurements. Hence, larval control strategies could be highly effective, and complementary to adult control (WHO, 2015).

The global fight against malaria is facing a critical threat: the increasing resistance of mosquito vectors to the insecticides currently in use. This resistance is not only rising in incidence, but also spreading geographically, jeopardizing the success of control programs worldwide (WHO, 2023). In recent years, malaria vectors across western, southern, central, and eastern Africa have displayed widespread resistance to insecticides, posing a major hurdle in our efforts to control the disease (Ranson and Lissenden 2016).

2.6.2. Malaria case management

Parasite control is a vital component of malaria control strategies which entail early diagnosis and prompt treatment of malaria cases with effective anti-malarial medicines (Argaw *et al.,* 2013). Early and effective case treatment kills the parasites before there has been a significant production of gametocytes and it also greatly reduces the infectivity of *Plasmodium* infections to mosquitoes. Thus, it is an important tool to reduce the risk of malaria burden and maintaining low levels of transmission (Murray *et al.,* 2014). The World Health Organization recommended the use of artemisinin-based combination therapies (ACTs) to treat uncomplicated *P. falciparum* malaria, which is the most effective and widely accessible drug. Chloroquine is effective for *P. vivax, P. ovale* and *P. malariae*. The use of ACTs and the increasing availability of RDT lead to a change in test and treat policy, whereby ACTs would be restricted to those with confirmed malaria (Murray *et al.,* 2014; WHO, 2023).

The growing specter of drug resistance presents a formidable challenge to global malaria control efforts. *Plasmodium* parasites have developed resistance to numerous antimalarial drugs, including chloroquine, the once-dominant treatment. This resistance is particularly concerning for ACTs, currently the most effective antimalarial drugs. The emergence and spread of artemisinin resistance, particularly in Southeast Asia, threatens to reverse decades of progress made in reducing malaria burden (Murray *et al.,* 2014). Combating this challenge requires multifaceted strategies, including robust surveillance programs, early detection and response, and investment in research and development of new antimalarial drugs and alternative treatment regimens (WHO, 2023).

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Description of the study area

This study was conducted in the Metema district of Northwestern Ethiopia (Figure 3) during major (September-December 2022) and minor (March-May 2023) transmission seasons. The district is found in the Western Gondar administrative zone of Amhara Regional State, located 1200 km Northwest of Addis Ababa, the capital of Ethiopia. The district shares boundaries with three districts (Quara, West Armachio and Chilga) and Sudan. Metema is lowland with an altitude that ranges between 500 m and 1000 m (average 750 m) above sea level (a.s.l). The mean annual rainfall ranges from approximately 850 to1000 mm. Metema district boasts one district hospital, five health centers, and 26 health posts across its 26 rural and three urban kebeles. Additionally, private healthcare options encompass 47 clinics, 5 medium diagnostic laboratories, 14 drug vendors, 9 rural drug shops, and 21 legal traditional medical sectors, complementing the public healthcare infrastructure (Metema District Unpublished Annual Health Report, 2022).

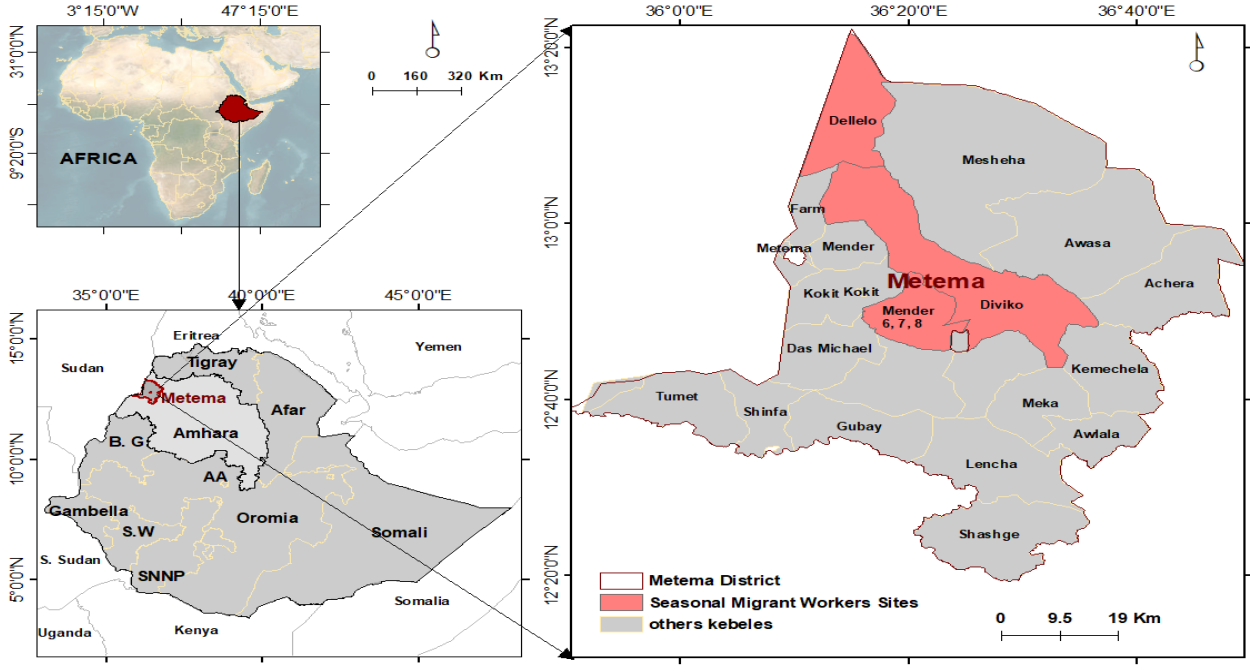


Figure 3. Location of Metema district in the map of Ethiopia and kebele included in the study.

The selection of the district is based on known presence of seasonal migrant workers and historical high malaria incidence. Metema is one of the nine agricultural investment (development corridors) districts in the lowlands of Northwestern Ethiopia with a total permanent resident population of 154,618 (CSA, 2014). The district receives a huge number of seasonal migrant workers, a temporary residential destination, each year with an estimated 400,000–500,000 migrant workers mainly from the Amhara region with various climatic zones: highland, midland, and lowland (FMOH, 2021). These seasonal migrants are mostly engaged in farmland preparation, site clearing, farming, weeding, and harvesting of sesame, sorghum, and cotton products at their destination. Site clearing, farming, planting, and weeding take place from May to the second week of September. Harvesting of sesame occurs from the end of September to December. Then, few migrants will remain at their destination from 1 month to 6 months to collect Sorghum and cotton. In Metema district, the Guwang and other seasonal rivers are creating numerous river pockets and pools that support vector populations. These rivers run through the district and persist until the end of December, serving as a water source, and drain to Sudan.

3.2. Study design

To understand malaria transmission dynamics among seasonal migrant workers in the Metema district of Northwestern Ethiopia, this study employed two complementary approaches. Firstly, cross-sectional targeted parasite surveys were conducted from September to December 2022 to assess malaria prevalence within the worker population. Secondly, entomological assessments were carried out during the same period (September-December 2022) and repeated later (March-May 2023) to investigate vector and human behavioral drivers of malaria transmission.

3.3. Study population

The study population for the targeted parasite survey includes participants in the selected seasonal migrant workers present at the selected farms at the time of the surveys. The participants were managed according to the best clinical practice in Ethiopia, by attending health professionals. Treatment for malaria infection depends on the parasite species: artemether-lumefantrine for *P. falciparum* and chloroquine for *P. vivax*, as per 2020 national guidelines

(FMOH, 2020). Entomological surveys were done in structures where seasonal migrants live in the farms area where the targeted parasite survey was taking place.

3.4. Sample size determination

3.4.1. Targeted parasite surveys

The required sample size for targeted parasite surveys was calculated using the single population proportion formula ($n = z^2p\ (1\text{-}p)\ x\ deff/d^2$), assuming a 95% confidence interval, 3% margin of error, and a design effect of 2. Based on 18.4% *plasmodium* prevalence from previous research by Aschale et al. (2018), and a 10% anticipated non-response rate, the calculation yielded a final sample size of 1597 individuals surveyed. Out of 158 farms, a proportional sample of 32 was chosen, and 50 workers were recruited from each selected farm. Farm and workers lists were obtained from owners, and when unavailable, a census was conducted prior to the survey. This sampling strategy ensured adequate representation of migrant workers across the study area.

3.4.2. Entomological assessment

Entomological surveys were conducted by using hourly CDC Light Trap (CDC-LT) collections and human behavior observations (HBOs) in the Metema district around seasonal migrant workers the farms areas where targeted parasite survey was taking place. To assess entomological and human beavior drivers of malaria transmission among seasonal migrant workers, 16 households/farm structures (8 for CDC-LT and 8 for HBOs) within and near their dwellings at Dellelo farm sites were randomly selected for entomological collections and observations.

3.5. Study variables

Dependent variables

Malaria infection (the presence or absence of malaria parasites), *Anopheles* mosquitoes and human behavior.

Independent variables

Socio-demographic characteristics (age, gender, family size), malaria prevention methods (LLINs, IRS), sleeping sites at night, human presence (inside/outside), time to sleep, *Anopheles* biting activity, human-vector exposure, major and minor malaria transmission seasons.

3.6. Inclusion and exclusion criterias

3.6.1. Inclusion criteria for parasite surveys

Study subjects (individuals) must fulfill all the following inclusion criteria to be eligible for the study:

- Working as a seasonal worker in a selected farms at the time of the survey,
- Willing and available to provide consent to participate in the study.

3.6.2. Exclusion criteria for parasite surveys

Subjects at the farms who did not meet any of the following criteria were excluded from the parasite survey:

- Unable to communicate for various reasons,
- Seriously ill person,
- Unwilling or unavailable to provide consent to participate in the study,
- Subjects that using antimalarial drugs.

3.7. Data collection and Laboratory procedures

3.7.1. Questionnaire

Standard targeted parasite survey questionnaires (Annex II) were used at farm sites to collect participant's socio-demographic characteristics, ownership, and utilization of LLIN and other risk factors for malaria infection. Entomological data for HBOs participant's farm structure and camp characteristics were collected using entomological questionnaire observations, smartphone or tablet-based forms, paper-based forms.

3.7.2. Field and Laboratory data

3.7.2.1. Rapid diagnostic tests

Rapid diagnostic tests were used during the targeted parasite surveys to detect malaria infection among seasonal migrant workers. RDT was performed on the site/field by the attending healthcare provider to determine malaria infection status and was performed on all consenting individuals. Rapid diagnostic tests were performed according to the manufacturer's protocol (Boyce and O'Meara, 2017). Study nurses/community health workers (CHW) performed the RDT tests, and results were available within 20 minutes according to manufactures instruction. Rapid diagnostic tests results were provided to the participant or their parent/guardian verbally and were recorded on the data collection tablets. Participants who test positive for malaria were treated on site by a study nurse/CHW according to national treatment guidelines (FMOH, 2012). The RDT type that used were the standard of care in the country, i.e., the RDT used by the NMEP at the time of the study. Currently, the standard RDT in use at the study area are CareStart™ Malaria HRP2/pLDH (*Pf/Pv*) from Access Bio, Inc.

3.7.2.2. Dried blood sample (DBS) collection

Blood spots were collected onto filter paper for molecular analysis. Whatman 3MM filter paper was pre-cut into individual squares, stamped with ink to demarcate four circles, and stapled to a thick card that was serve as its cover. A volume of approximately 25 µl per blood spot (4 blood spots per card) was collected. Filter paper samples were labelled with the individual's unique identification number on the card stock cover and were allowed to dry at ambient temperature and relative humidity before closing the card over the filter paper. DBS samples were placed into a stock card box with desiccant and humidity indicator cards and stored in a freezer at -20ºC at the health facility and then transported to the laboratory at Armauer Hansen Research Institute (AHRI) in Addis Ababa for further molecular analysis.

3.7.2.3. Molecular testing (DNA extraction and quantitative PCR)

Genomic DNA was extracted using the Chelex method with Tween 20 extraction method. DBS were punched using a 6 mm hole-puncher into 1.5 ml microcentrifuge tubes. One mL of 0.5% Tween 20 in 1 PBS was added into the tube containing DBS punches and incubated overnight at

4 °C. The samples were briefly centrifuged, Tween-PBS was removed, and the punches were washed with 1 mL of 1 × PBS and incubated for 30 min at 4 °C. The samples were briefly centrifuged at 14,000 rpm for 10 minutes, PBS removed and 150 µL of 10% Chelex 100 resin in water were added to each sample, ensuring the DBS punches were covered with the Chelex solution and incubated for 10 min at 95 °C. The tubes were centrifuged at 14,000 rpm for 10 minutes and the supernatant was transferred to 0.6 mL microcentrifuge tubes and centrifuged at 14,000 rpm for 5 minutes. The extracted DNA was then transferred to a 96-well plate and stored at − 20 °C until undergoing PCR testing using primer to confirm malaria status with 18S based qPCR at the AHRI laboratory.

Quantitative PCR (qPCR) for parasite detection and quantification was performed by targeting the 18S small subunit rRNA gene for *P. falciparum* and *P. vivax* using primer and probe sequences described by Wampfler et al. (2013). The following sequences of primers and probes were used. Pf18S forward primer sequence 5'- GTA ATT GGA ATG ATA GGA ATT TAC AAG GT-3', reverse sequence 5'TCA ACT ACG AAC GTT TTA ACT GCA AC-3' and probe sequence 6FAM-AACAATTGGAGGGCAAG–MGBNFQ and Pv18S forward primer sequence 5'-GCT TTG TAA TTG GAA TGA TGG GAA T-3', reverse sequence 5'-ATG CGC ACA AAG TCG ATA CGA AG-3' and probe sequence HEX-AGC AAC GCT TCT AGC TTA - MGB-BHQ. All reactions were performed on BioRad CFX96^{TM} real-time system (BioRad) using 20µl total reaction volumes, 10 µl of 2X TaqMan Fast Advanced Master Mix (Applied Biosystems), 5µl of DNA elute, forward and reverse primers at final concentrations of 833nM each, and probe concentration of 110nM. The following cycling parameters were used: holding at 50°C for 2 minutes, initial denaturation at 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. Asexual *P. falciparum* parasites were quantified using standard curves generated from a serial dilution of NF54 ring stage parasites (10^6 – 10^3 par/ml). For *P. vivax* parasite quantification was done using plasmid constructs to infer copy numbers by running serial dilutions (10^7 – 10^3 copies/µl) of plasmids containing the amplicon in duplicate on each plate. For mixed infection parasite quantification was done by simultaneous detection of both *P. falciparum* (using a serial dilution of NF54 ring stage parasites) and *P. vivax* (plasmid constructs) DNA in the same sample.

3.7.3. Entomological data

During the study period, adult mosquitoes were collected by hourly indoor and outdoor CDC-LT and HBOs were conducted during the major (September to December 2022) and minor (March to May 2023) malaria transmission seasons. CDC LT, powered by 6 V batteries, hung indoors in the study participant's room close to their sleeping area at approximately window height. Traps were set in the early evening at 18:00 hours and left to run throughout the night until about 07:00 hours the following morning. A total of 52 collection nights per village during major, and 40 collection nights per village during minor transmission seasons were conducted. Each collection night extended from 18h00 to 06h00. In each selected structure, CDC-LTs were positioned indoors (near the sleeping area of the inhabitants) and outdoors (~10 meters away from the house entrance). A two-person entomology team per house manually changed CDC-LT collection cups hourly. The entomology teams were closely supervised to verify the timing and consistency of mosquito collection. Adult mosquitoes were stored in individual clearly labelled collection cups, then killed by freezing or alcohol to sort by sex and genus. Female *Anopheline* mosquitoes were individually preserved in Eppendorf tubes with silica gel, labelled with date, household ID, location, and hour of collection and stored for further analysis.

3.7.3.1. Species identification

Morphological species identification of *Anopheles* mosquitoes was done using the key developed by Gillies and Coetzee (1987). Next, a subset of randomly chosen morphologically identified anopheline mosquito specimens were confirmed to species-level by sequencing the ribosomal DNA ITS2 and mitochondrial DNA cox1 loci as previously described by Laurent et al. (2016).

Genomic DNA was extracted from the head and thorax of *Anopheles* specimens using a cetyltrimethylammonium bromide (CTAB) technique. The ribosomal DNA ITS2 was amplified from genomic DNA using the ITS2A (5.8 rDNA) (5′-TGTGAACTGCAGGACACAT-3′) and ITS2B (28 rDNA) (5′-TATGCTTAAATTCAGGGGGT-3′) primers (Beebe and Saul, 1995). The 25-µL PCR mixture contained 2.5 µL of 10× buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.2 mM MgCl2, 0.5 units of Taq DNA polymerase, 0.75 µL of 10 pmol/µL each of forward and reverse primers, and 1 µL of DNA template prepared as above. The thermocycling conditions were as follows: initial denaturation at 94°C for 5 minutes, 30 cycles

of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension at 72°C for 5 minutes.

Amplification of mitochondrial DNA cytochrome oxidase subunit 1 (Cox1) was conducted by adapting the procedure described by Folmer et al. (1994) using light cycle oil (LCO) and heavy cycle oil (HCO) primers. Briefly the primers used were LCO 1490 (5′-GGTCAACAAATCA TAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCA GGGTGACCAAAAAATCA-3′). The 25-µL PCR mixture contained 2.5 µL of 10× buffer, 0.2 mM of each dNTP, 1.2 mM $MgCl_2$, 0.5 units of Taq DNA polymerase, 0.75 µL of 10 pmol/µL each of forward and reverse primers, and 1 µL of DNA template prepared as above. The thermocycling conditions were as follows initial denaturation at: 94°C for 5 minutes, then 30 cycles of denaturation at 94°C for 40 seconds, annealing at 51°C for 1 minute, and extension at 72°C for 1 and half minutes, with a final extension at 72°C for 5 minutes.

The amplified fragments were visualized by electrophoresis on a 1% agarose gel. The PCR product was purified using an enzyme cleanup; 2 units of exonuclease 1 (USB Corporation, Cleveland, OH), 1 unit of Shrimp Alkaline Phosphatase (USB Corporation), and 1.8 µL of double distilled H_2O were added to 8 µL of PCR product. This mixture was incubated at 37°C for 15 minutes, followed by 15 minutes at 80°C to inactivate the enzymes. The PCR products were sequenced directly (with one of the PCR primers) using Sanger sequencing on ABI 3730xl DNA Analyzer platform (PE Applied Biosystems, Warrington, England). A subset of specimens was independently confirmed using species-diagnostic PCRs for *An. funestus* and *An. gambiae* (Scott *et al.,* 1993).

3.8. Data management and analysis

3.8.1. Data quality assurance and quality control

All data collectors were trained in the objectives, methods of effective communication with study participants, collection of high-quality data, and principles of ethics in human subject research. Data collectors received additional training specific to the tasks they performed including completing questionnaires, use of tablet devices and collecting blood samples. Electronic questionnaires were programmed with internal consistency and completeness checks to avoid

data entry errors. Standard operating procedures (SOPs) were written for all activities and manuals of all relevant documents were provided to each member of the study team.

3.8.2. Data analysis

The data was collected using tablets with data forms developed in REDCap software version 11.0.3 (Harris *et al.,* 2019). The data were subsequently sent to the AHRI data server on daily basis. The collected data were cleaned for completeness and consistencies, coded, and transported into the Stata software version 17.0 (Stata Corp. 2021; Stata Statistical Software: Release 17; College Station, TX: StataCorp LLC) for further analysis. The results were organized, summarized, and presented using texts, tables, and graphs. Simple descriptive statistics and chi-squire tests were used to assess different variables. P-value <0.05 was considered as statistically significant result.

3.8.2.1. Targeted parasite surveys

Descriptive statistics were used to determine the number and percent of participants infected with malaria using RDT onsite and qPCR in the laboratory. The distribution of parasite densities was also described through density plots and histograms.

3.8.2.2. Analysis of entomological and human behavior data

Hourly indoor and outdoor CDC LT catches enable the assessment of present vector species, and *Anopheles* and species-specific occurrence per hour (indoors/outdoors). The hourly human-biting rate (HBR, bites per person per hour): HBR indoors and HBR outdoors were calculated for each hour from 18h00 to 06h00 and for overall *Anopheles*to discern overall *Anopheles* biting trends (biting times, peak biting time, and biting location (inside/outside)) throughout the night. The HBR data were integrated with the HBO data by calculating the hourly adjusted HBR, as outlined in Monroe et al. (2020). The hourly adjusted HBR is the product of the proportions of people observed doing specific activities (awake outside and not under net, awake inside and not under net, asleep/resting inside under net) per hour with the hourly human-biting rate (HBR). The adjusted HBR allowed the quantification of the spatial and temporal protection by LLINs, as well as the identification of specific human-exposure points (gaps in protection). Further, the hourly adjusted HBR shed light on more granular patterns in vector exposure throughout the

night. Spatiotemporal vector behaviors and the hourly mosquito capture rate both indoors and outdoors were calculated for each hour from 18h00 to 06h00.

3.8.2.3. Sequence analysis

Two genetic regions, ITS2 and cox1, were used to identify different species in the samples. Variations in the DNA sequences, like single nucleotide changes and missing or added pieces, and grouped similar sequences together. The ITS2 region, requiring 98% similarity for grouping, compared to 95% for cox1. Finally, the groups were double-checked and compared to a large database of National Center of Biotechnology Information- nucleotide sequence to confirm which species they belonged to (Lemma *et al.,* 2019).

3.9. Ethical considerations

Ethical approval for the study was obtained from the National Research Ethical Review Committee (NRERC), Addis Ababa, Ethiopia, reference number: 02/256/630/14 and the institutional review boards of AHRI/ALERT Ethics Review Committee (protocol number: P0-08-22) Addis Ababa, Ethiopia (Annex III). The study procedures were explained and written informed consent/assent was sought from each study participant or his/her parents or legal guardian in case of children under 18 years of age for study participation prior to studying procedure (Annex I**)**. Assent for adolescents 12-17 years of age was obtained in addition to consent from a parent or guardian. All methods used in this study were performed in accordance with the relevant guidelines and regulations. It was noted that the DBS samples were collected only for the purpose of malaria diagnosis and treatment and all cases of malaria were treated immediately according to the national malaria treatment guideline. Household owners, village administrators and district authorities were informed by formal letters, written permission was also sought prior to the study and before mosquito collection. Informed consent was also obtained from the head of each household/farm structures for CDC light trap mosquito collection.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Socio-demographic characteristics of seasonal migrant agricultural workers

A total of 1,597 seasonal migrant agricultural workers were enrolled and tested by RDT from the 32 farm sites. Their mean age was 27.5 ± 5.4 years, and the median was 25 years (IQR = 8), 712 (44.58%) were in the age range of 15–24. The majority (96.81%) of the study participants were males. Only 8.02% of seasonal migrant workers possessed LLINs (Table 1).

Table 1. Description of study participants enrolled in targeted parasite survey in Metema district, Northwestern Ethiopia, September to December, 2022

Variables	Category	Number (n)	Percentage (%)
Sex	Male	1,546	96.81
	Female	51	3.19
Median age in years (IQR)	25 (20-32)		
Age (Years)	15-24	712	44.58
	25-34	556	34.82
	+35	329	20.60
Own LLIN	Yes	128	8.02
	No	1,469	91.98
Slept under net last night	Yes	105	6.57
	No	1,492	93.43
Total		**1,597**	

IQR = Interquartile range, LLIN= Long-lasting insecticide net

In the current study, the ownership of LLINs was very low among the study subjects. This finding is comparable to the study from Amhara region reporting nearly 13% (Aschale *et al.,* 2018). However, this LLIN ownership was lower than studies conducted in different parts of Ethiopia: 32.4% in Northwestern Ethiopia (Alemu *et al.,* 2014), 64% at the national level (EPHI, 2016), 31% in South-central Ethiopia (Deressa *et al.,* 2017), and 29% among Ethiopian Army members (Gebru *et al.,* 2019). This might be associated with low access to LLINs, and

most of the seasonal migrant workers did not bring their LLINs from home to the farm areas (Dugassa *et al.,* 2021).

4.2. Prevalence of malaria cases

The prevalence of malaria infection among seasonal migrant agricultural workers was 23.22% (371/1597) by RDT and 38.32% (612/1597) by PCR. *Plasmodium falciparum* dominated among malaria infections in seasonal migrant workers followed by *P. vivax* and mixed infections (Table 2).

Table 2. Prevalence of malaria and infecting *Plasmodium* species using RDT and qPCR in Metema district, Northwestern Ethiopia, September to December, 2022

Parasite Species	**Parasite detection diagnostic tool**			
	RDT n (%)		qPCR n (%)	
	No.	%	No.	%
Plasmodium falciparum	340	21.29	549	34.38
Plasmodium vivax	20	1.25	36	2.25
Mixed infection	11	0.69	27	1.69
Total Positive	371	23.23	612	38.32
Total Negative	1,226	76.77	985	61.68
Grand Total	**1,597**		**1,597**	

n = Number, RDT = Rapid Diagnostic Tests, qPCR = Quantitative polymerase chain reaction

This study investigated malaria infection in seasonal migrant workers, finding a high prevalence 23.2% using RDT and 38.4% by qPCR. This high malaria prevalence at the farm sites agreed with a study conducted in West Armachiho District in Northwestern Ethiopia (Aschale *et al.,* 2018) and Dilla town in southern Ethiopia (Molla *et al.,* 2015). The prevalance of malaria incidence from this study was lower than the study conducted in East Shewa zone, Oromia, Ethiopia (Tadesse *et al.,* 2018), Nigeria (Fana *et al.,* 2018) and Tanzania (Sumari *et al.,* 2017). However, malaria prevalence in this study was much higher than the malaria prevalence survey conducted among seasonal migrant workers during the plantation and weeding season in the Amhara Region of Ethiopia (Schicker *et al.,* 2015) and in Gondar Zuria district of Northcentral

Ethiopia (Minwuyelet *et al.,* 2020). The possible reasons for the difference could be differences in study design, geographical location, nature of study population, sample size, tool used, study period, and the implemented malaria control program in the study area. Moreover, this study found a high prevalence of malaria among seasonal migrant farmworkers. This could be due to repeated exposure in endemic areas or development of partial immunity without symptoms (Alemu *et al.,* 2014; Gryseels *et al.,* 2015). These malaria cases might be responsible for spreading malaria in areas where they are returning home and their communities. A study conducted in villages around Lake Tana, Northwestern Ethiopia, indicated that travel to farms in the lowlands was significantly associated with the risk of malaria infection and imported malaria to the villages (Malede *et al.,* 2018).

The present study revealed a significantly ($p < 0.0001$) high prevalence of *P. falciparum* and a relatively very low proportion of *P. vivax* in the study sites. This finding is comparable with a prior study conducted in Northwestern Ethiopia (Minwuyelet *et al.,* 2020), but higher than the study findings in Southwest Ethiopia (EPHI, 2016). Traveling away from home for seasonal farm activities might have exposed seasonal migrant workers to high risk of *P. falciparum* infection. Harvest activities in the evening, no or little use of malaria prevention methods (Solomon *et al.,* 2019), and low malaria immunity could have exposed more seasonal migrant workers to malaria infection at farm sites (Malede *et al.,* 2018). Moreover, the country's strategy for malaria prevention and control, which does not include a focus on seasonal migrant workers (Schicker *et al.,* 2015), and exclusion of agricultural farm workers in a malaria elimination program might have increased their exposure to malaria infection. Seasonal migrant workers who traveled from lowland malarious areas to study areas could also contribute to increase malaria prevalence at destination sites (Schicker *et al.,* 2015; Malede *et al.,* 2018).

4.3. Comparison of the malaria parasite detection by RDT and qPCR

Among 612 malaria cases confirmed by PCR, *P. falciparum* reigned supreme, causing 89.7% (549/612) of infections compared to just 5.9% (36/612) and 4.4% (37/612) detection for P. *vivax* and mixed infections, respectively. Out of a total of 371 RDT detected malaria cases, 91.6% (340/371) were due to *P. falciparum*, 5.4% (20/371) were due to *P. vivax*, and 3.0% (11/371)

were mixed infections. While RDT and qPCR agreed on parasite detection for 80.32% (298/371) of cases, qPCR significantly ($p < 0.0001$) identified 19.68% (73/371) of missed infections (RDT-negative cases), underscoring its critical role in accurate diagnosis and effective control (Table 3).

Table 3. Comparison of the malaria parasite detection by RDT and qPCR, among seasonal worker camps Metema District, Northwestern Ethiopia, September to December, 2022

RDT Result	**PCR Results**				
	Mixed %, (n/R)	***P. vivax* %, (n/R)**	***P. falciparum* %, (n/R)**	**Negative %, (n/R)**	**RDT Total**
Mixed Infection	27.3 (3/11)	18.2 (2/11)	54.6 (6/11)	0.0	0.7 (11/1,597)
P. vivax	25.0 (5/20)	55.0 (11/20)	20 (4/20)	0.0	1.3 (20/1,597)
P. falciparum	3.5 (12/340)	0.6 (2/340)	83.6(284/340)	12.4 (42/340)	21.3 (340/1,597)
Total positive	5.4 (20/371)	3.0 (11/371)	91.6 (340/371)	0.0	23.2 (371/1,597)
Total Negative	0.6 (7/1,226)	1.7 (21/1,226)	20.8 (255/1,226)	76.9 (943/1,226)	76.8 (1226/1,597)
qPCR Total	**1.7 (27/1,597)**	**2.3 (36/1,597)**	**34.4 (549/1,597)**	**61.7 (985/1,597)**	**1,597**

R = RDT positive; n = qPCR positive; RDT = Rapid Diagnostic Tests; qPCR = Quantitative polymerase chain reaction

This study, compared to previous studies in Ethiopian, reported higher overall malaria positivity rates. Santana-Morales et al. (2012) in southwest Ethiopia found 7.4% RDT and 10.6% qPCR positivity, while Yohannis et al. (2022) reported 15.1% RDT and 24.4% qPCR in Northwest Ethiopia. These discrepancies could stem from varying factors like study populations (potential higher risk groups in this study), transmission seasons, and diagnostic techniques (specific RDT and qPCR kits with differing sensitivities and specificities) (Neupane *et al.,* 2021). Crucially, qPCR detected 15% more infections than RDT in this study, highlighting its potential to identify cases missed by RDT and provide a more comprehensive picture of malaria prevalence. This advantage stems from qPCR's ability to detect both parasitemic (active infection) and non-

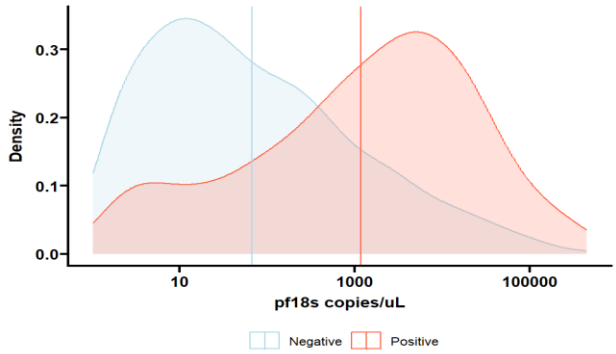
parasitemic (parasite DNA) cases, compared to RDT which primarily target parasitemic infections. Additionally, RDT can be susceptible to false negatives due to factors like temperature, storage conditions, and parasite strain variations (Neupane *et al.,* 2021).

Underestimating malaria prevalence through sole reliance on RDT can have significant public health consequences, potentially leading to inadequate control measures and delayed treatment for low-density infections, as documented in studies across resource-limited settings (Santana-Morales *et al.,* 2012; Neupane *et al.,* 2021; Yohannis *et al.,* 2022). Fortunately, incorporating qPCR alongside RDT offers a more accurate picture of malaria burden, informing targeted interventions and potentially accelerating elimination efforts, as demonstrated by research in Ethiopia and other malaria-endemic regions (Santana-Morales *et al.,* 2012; Yohannis *et al.,* 2022). This complementary approach holds promise for a more precise understanding and effective control of malaria, ultimately contributing to improved public health outcomes.

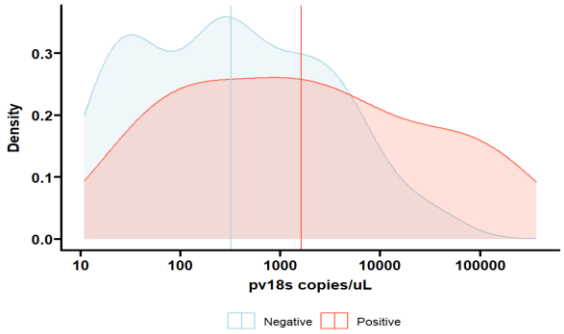
4.4. Parasite density in *Plasmodium*-infections

The median parasite density/µl for *P. falciparum* infections 3913 (IQR: 1–447,214) is significantly higher than for *P. vivax* infections 979 (IQR: 10–365,125) among seasonal migrant workers district (Figure 4). This indicates there was evidence of a difference in parasite density between *P. falciparum* and *P. vivax* qPCR detected malaria infections according to the Mann–Whitney test ($p < 0.0001$).

A. Parasite density plot for *P. falciparum*



B. Parasite density for plot *P. vivax*



C. Parasite copies by species

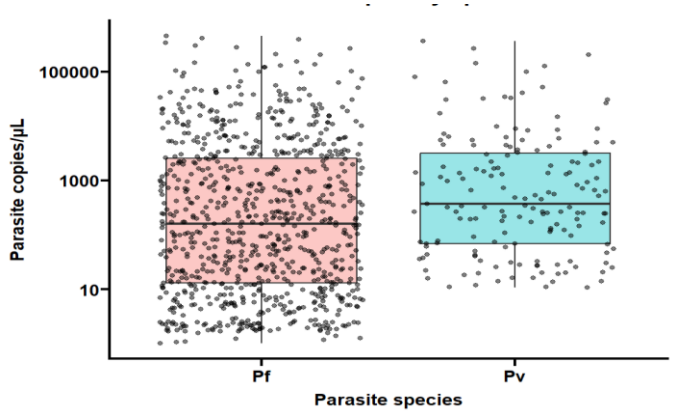


Figure 4. Parasite density plots for *Plasmodium*-infections by qPCR, among seasonal workers Metema District, Northwestern Ethiopia, September to December, 2022. Pf: *P. falciparum; Pv: P. vivax;* µL: microliter.

The finding from this study aligns with previous Ethiopian studies that highlight the higher *P. falciparum* parasite density than *P. vivax* density by qPCR, the observed parasite load levels are notably higher (Kifle *et al.,* 2020). This likely stems from this study focus on seasonal migrant workers, potentially exposed to higher transmission intensity, and could influence public health strategies by highlighting the need for targeted interventions to control *P. falciparum's* faster multiplication and transmission potential (Kifle *et al.,* 2020). Further research into factors contributing to parasite density variability is crucial for tailoring control measures to specific settings.

4.5. Vector species composition and relative abundance

A total of 532 adult *Anopheles* mosquitoes morphologically belonged to nine *Anopheles* species were captured by CDC-LT over 368 collection nights of the survey period. *Anopheles arabiensis* was the most abundant species and accounted for 76.69% (408 of 532), followed by *An. pretoriensis* 10.34% (55 of 532) and *An. demeilloni* 4.32% (23 of 532). Seasonal variation of *Anopheles* species in the study area indicated that species compositions were heterogeneous between major and minor transmission seasons. The highest species diversity and abundance (97.93%; 521 of 532) in study camps were recorded during the major transmission season (September to December), whereas only 2.07% (11 of 532) of mosquito were trapped during minor malaria transmission season (Table 4).

Table 4. *Anopheles* mosquito species composition and seasonal abundance in the seasonal migrant agricultural workers camps Metema District, Northwestern Ethiopia, Major (September to December, 2022) and Minor seasons (March to May, 2023)

***Anopheline* species**	**Malaria transmission Season**		
	Major transmission season n (%)	**Minor transmission season n (%)**	**Total n (%)**
An. arabiensis	403 (77.35)	5 (45.46)	408 (76.69)
An. pretoriensis	52 (9.98)	3 (27.27)	55 (10.34)
An. demeilloni	20 (3.84)	3 (27.27)	23 (4.32)
An. fuscivenosus	18 (3.46)	0.0	18 (3.38)
An. dancalicus	8 (1.54)	0.0	8 (1.50)
An. rufipes	7 (1.34)	0.0	7 (1.32)
An. pharoensis	6 (1.15)	0.0	6 (1.13)
An. salbaii	4 (0.77)	0.0	4 (0.75)
An. tenebrosus	3 (0.58)	0.0	3 (0.56)
All *Anophelines*	**521 (97.93)**	**11 (2.07)**	**532**

The current entomological survey revealed variations in the *Anopheles* species composition and abundance in between the two seasons. The high *Anopheles* mosquito species diversity may result from the presence of varied ecological and climatic factors favoring the larval development of different species (Coetzee *et al.,* 2000; Graves *et al.,* 2009; Tesfaye *et al.,* 2011).

Moreover, the diversity and variation in *Anopheles* mosquito species composition are mostly related to human activities (e.g., farming, vegetable crops) like observations elsewhere (Ouldabdallahi *et al.,* 2016; Musiime *et al.,* 2019; Esayas *et al.,* 2020). *Anopheles arabiensis* was the most abundant *Anopheles* species in the study site. This concurs with other studies from different parts of Ethiopia that this species plays a principal role in malaria transmission (Abose *et al.,* 1998; Massebo *et al.,* 2013; Esayas *et al.,* 2020). This finding allows for better tailoring of interventions for primary malaria vectors in the study sites. The presence of secondary vectors *An. pretoriensis* and *An. demeilloni* in the study site was also demonstrated. Although *An. pretorienis* has not been incriminated as a vector of malaria in Ethiopia, it has been widely reported from Eastern, Southwest, and Northern parts of the country (Krafsur, 1971; Kindu *et al.,* 2018; Carter *et al.,* 2019). A study from Zambia showed *An. pretoriensis* was positive for *P. falciparum* (Lobo *et al.,* 2015), suggesting it is important to understand the vectorial capacity of this species in Ethiopia. In general, the diverse species composition and abundance in the study sites highlights the importance of conducting routine entomological surveillance across the different parts of the country to monitor changes across time and location for better tailoring of interventions.

In the present study, despite variability in species composition and abundance throughout the collection period. Seasonal variations were observed in study site with an increase in mosquito populations following *kiremt*, and a decrease towards *belg,* which may relate to the presence of breeding habitats in the study areas. This is in line with previous observations from different parts of Ethiopia (Massebo *et al.,* 2013; Esayas *et al.,* 2020; Dugassa *et al.,* 2021). It is important to note that the peak of the agricultural activities in lowlands coincided with the peak *Anopheles* mosquito densities, making the economic significance of malaria important to consider.

4.6. Morphological and molecular identifications of *Anopheles* species

By analyzing two key genetic regions (ITS2 and cox1) and comparing them to a database, researchers found a new species that would have been missed by simply looking at its physical appearance (Lemma *et al.,* 2019). The importance of using molecular techniques to complement morphological identifications was validated with the documentation of possible new *Anopheles* species. Internal transcribed spacer region 2 and cox1 sequencing of 266 randomly chosen

samples (across sites and trapping periods) demonstrated the presence of ten species. The primary *Anopheles* identified was *An. arabiensis* (n = 148). An unknown species, misidentified as *An. fuscivenosus, An. salbaii* and *An. tenebrosus* was called *Anopheles* sp. KHH1 (n = 9). This species consisted of 3.4% of the total mosquito population collected from the study sites (Figure 5).

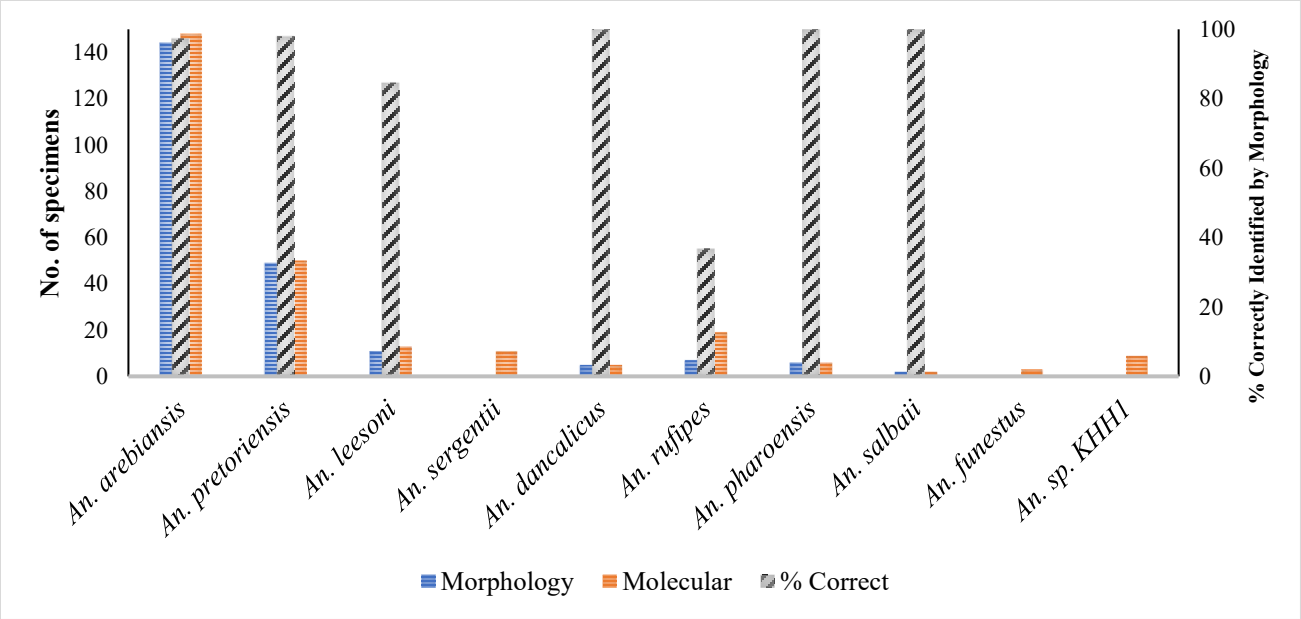


Figure 5. Comparison of morphological and molecular identifications of *Anopheles* species, Metema District, Northwestern Ethiopia, Major (September to December, 2022) and Minor seasons (March to May, 2023). The number of specimens identified as specific *Anopheles* species by morphological and molecular techniques (n = 266), and percentage accuracy of morphology compared to molecular identity are presented. An: *Anopheles.*

While past Ethiopian studies, have shed light on hidden *Anopheles* mosquito diversity through molecular approaches, this study in Metema pushes the envelope further with the potential discovery of new species (Adugna *et al.,* 2020). Notably, *An. arabiensis* remained the dominant player, mirroring national trends. This highlights the crucial role of combining morphology and molecular tools for a complete picture of *Anopheles* diversity, as it informs targeted control strategies in specific regions like this study with potentially unique mosquito fauna and potentially novel malaria vectors demanding further investigation.

4.7. Human behavior observations and *Anopheles* mosquitoes biting behavior

4.7.1. Human behavior observations

Human behaviors around LLIN use and sleeping patterns inside and outside homes were examined alongside CDC-LT. Across the two collection seasons in the study area, most people were observed awake inside and outside up to 21h/22h when they went to sleep, after which over 60% of people observed were slept outside their homes. Most inhabitants went to sleep between 23h – 24h, and several household members rose in the early morning hours (4h – 5h) for farm activities (Figure 6).

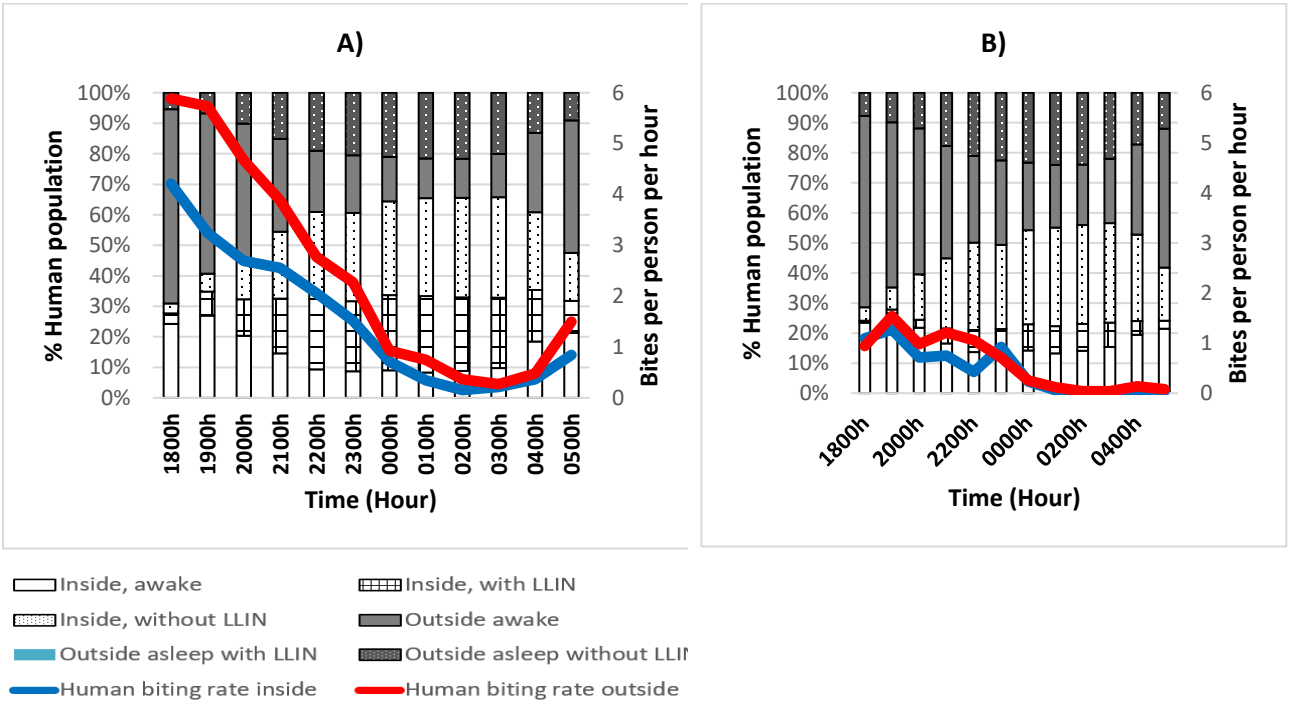


Figure 6. Proportion of human population observed sleeping or awake, inside, or outside, under or not under an LLIN, superimposed with *Anopheles* mosquitoes hourly HBR in A) major (September to December, 2022) and B) Minor seasons (March to May 2023) in the Metema District, Northwestern Ethiopia.

Malaria transmission occurs when people are exposed to infectious vector bites because they are not adequately protected by the interventions in place, if any. Thus, the efficacy of malaria vector control interventions depends on how the intervention interacts with local vectors populations. In

this study, entomological collections were integrated with HBOs to assess the appropriateness of LLINs for reducing exposure to local *Anopheles*, and to identify and quantify remaining gaps in protection. Consequently, knowledge of vector bionomic traits, species composition, and abundance and human behaviors (Mwema *et al.,* 2022) are vital to implement an effective control strategy. This study fills an important knowledge gap by investigating entomological parameters in the seasonal migrant worker sites. Moreover, the findings from this study will inform the malaria control program through targeted prevention messaging and infection prevention interventions.

4.7.2. *Anopheles* mosquitoes biting behavior

Across the two malaria transmission seasons in the camps of the seasonal migrant workers, *Anopheles* mosquito biting activity was recorded throughout the night, both inside and outside, with an overall preference for outdoor biting. Indoor landing rates fluctuated between 18.92 bites per person, per night (bpn) (September to December, major season) and 7.22 bpn (March to May, dry season), while outdoor landing rates extended from 20.14 bpn (major transmission season), and 9.22 bpn (minor transmission season). In general, peak indoor and outdoor biting activity in the study area occurred during the early evening hours (18h00-22h00) (Figure 6).

Understanding the local vector behavior is important to evaluate how a vector contributes to risk of exposure, how interventions may function, as well as to guide tailoring and targeting of interventions. In addition, the biting behavior of mosquitoes is an important risk factor for infection with malaria parasites (Braack *et al.,* 2015). Regarding species-specific vector bionomic traits, this study documented outdoor and early in the evening biting behaviors in the lowlands. The outdoor early evening peak biting times of *Anopheles* mosquito present a challenge for the protection of seasonal migrant workers from infectious bites. This indicates there might be high risk to people working at night and an increased level of malaria transmission outdoors (Dugassa *et al.,* 2021). Thus, the primary interventions used for protection in the country, LLINs and IRS, might fall short due to outdoor biting behavior in the study site. Hence, malaria prevention and control measures should ideally consider the spatial and temporal heterogeneity of exposure profiles (Mwema *et al.,* 2022).

4.8. Human behavior adjusted biting rates

Directly measured HBRs were adjusted to account for human presence (inside, outside), time inhabitants went to sleep, and LLIN use, i.e., HBO-adjusted HBR. In the study site among seasonal migrants, for both transmission seasons, human-vector exposure to *Anopheles* mosquito was much higher outdoor (major: 70.7%; minor: 67%) (awake or asleep without bed nets) than indoor exposure (major: 29.3%; minor: 33%). The total potential human-vector exposure, and outdoor exposure occurred primarily from 18h/19h to 23h. Indoor vector exposure while asleep without LLINs accounted for about 13.4% for major and 14.9% for minor seasons from the total potential exposure to biting (Figure 7).

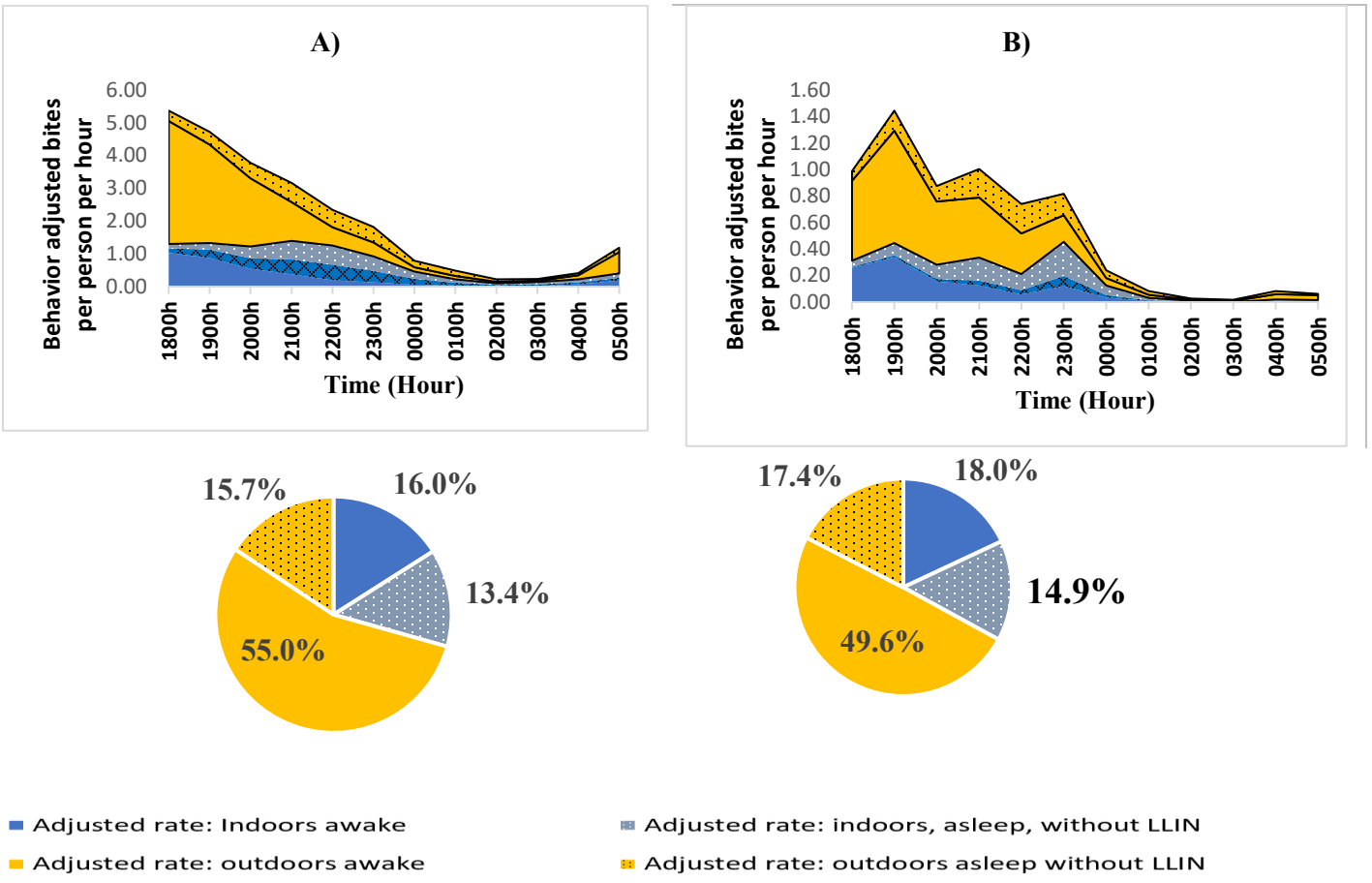


Figure 7. Hourly HBO-adjusted HBR from 18h00 to 06h00 to account for human presence (inside/outside), time to sleep, and LLIN use (top), and percent vector exposure by activity (bottom pie chart), in A) major (September to Dececember, 2022) and b) Minor seasons (March to May 2023) in the Metema District, Northwestern Ethiopia.

In the current study, upon integration of *Anopheles* landing rates (i.e., HBR) with the HBOs, nuanced and contrasting human-vector exposure profiles were observed in both major and minor collection seasons. The HBO-adjusted HBR which quantifying human-vector exposure points indicates the overall *Anopheles* HBRs remained substantially higher in study site. *Anopheles* host-seeking activity was recorded both indoors and outdoors throughout the entire night. *Anopheles* landing rates were notably higher outdoors than indoors, and generally higher towards the earlier evening hours, than later in the night (Lobo *et al.,* 2015; Ávila *et al.,* 2021). Both outdoor and indoor vector exposures were higher in major season than in minor season. Elevated outdoor vector exposure (70% of total potential vector exposure) in the current study area left seasonal migrant workers as the community most vulnerable to exposure to infectious *Anopheles* bites. These human-vector exposure profiles likely resulted from human behavior differences rooted in distinctive lifestyle habits that inhabitants of Metema district spent more time socializing outdoors throughout the evening. HBO-adjusted HBRs measured in this investigation demonstrate that it is critical to factor in human behavioral data when estimating the impact of interventions on human-vector exposure profiles, because it allows national malaria elimination program to quantify the actual protection conferred by interventions in place and the remaining gaps in protection (Monroe *et al.,* 2020).

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In conclusion, seasonal migrant agricultural workers in this study encountered a confluence of factors ripe for malaria transmission. They exhibited low ownership and use of LLINs, with only 8.02% possessing and utilizing them effectively. Furthermore, a concerningly high prevalence of the *Plasmodium* parasite was detected, at 23.2% by RDT and 38.3% by more sensitive qPCR. Additionally, their outdoor work exposed them to mosquito bites at a high rate, with 70.7% experiencing frequent bites in environments where mosquitoes thrive. Quantitative polymerase chain reaction's ability to detect more infections than RDT underscored the potential for undetected cases fueling future outbreaks. The mosquito picture was equally concerning, with *Anopheles arabiensis* being the most abundant mosquito species and molecular techniques revealing potential new species. Human behavior adjusted biting rates suggested that most of the exposure occurred outdoors (awake or asleep without bed nets) and the highest risk of being bitten was early in the evening. Clearly, malaria control in this setting demands immediate action, targeting LLIN use, outdoor protection, and mosquito vectors. This trifecta of risk factors necessitates a multi-pronged approach to protect migrant workers and prevent the resurgence of malaria in their home communities and beyond.

5.2. Recommendations

Based on the above conclusions we would like to forward the following recommendations:

1. To protect both migrant workers and their communities from malaria's grasp, prioritize a comprehensive farm-based approach: widespread education, accessible on-site screening, and swift treatment.
2. To shield migrant workers from outdoor mosquito bites, implement a multifaceted approach combining targeted insecticide spraying, repellent, and community mobilization campaigns for comprehensive awareness and risk reduction strategies.
3. To ensure comprehensive case detection and targeted interventions in malaria control programs, integrate qPCR alongside RDT, bolstering surveillance across communities while optimizing qPCR for resource-limited settings.
4. To combat the threat of novel vectors, implement a comprehensive pronged approach encompassing sustained surveillance for population tracking, in-depth research into transmission potential, and targeted interventions tailored to vector ecology and behavior.
5. For precise evaluation and targeted intervention against malaria, integrate human biting observations with mosquito landing rates to pinpoint behavioral risk gaps and optimize intervention effectiveness.

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7. ANNEXES

Annex I: Consent Forms (English Version)

Cross sectional survey Informed Consent for Adults/Appropriate guardian

Introduction

My name is _____. I am a researcher working with the Federal Ministry of Health, the Armauer Hansen Research Institute (AHRI) and the University of California in San Francisco (UCSF). UCSF is a university and AHRI is a research center also working to improve health around the world.

The study's name is: "Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys."

We are inviting you to participate in a study about malaria prevention in Amhara region, Ethiopia. This is a medical research study, and you do not have to take part.

In this locality, some populations like migrant workers are at high risk of malaria. You/(the minor you consent for) are being invited to take part in this study because you are a member of one of these populations or live near where they work.

Why is this study being done?

Some populations and places in Ethiopia have more risk of malaria. For example, migrant agricultural workers and places near where they work. They may need specific interventions to prevent malaria infection. We are conducting a research study to understand how much malaria these groups have. We also want to know which interventions to prevent malaria are used or could be used.

What will happen if I/(the minor I consent for) take part in this study?

If you agree to be in this study, our team will ask a few questions about your/(the minor you consent for) experiences with malaria, prevention of malaria and travel history. We will record the answers. We will collect your/(the minor you consent for) name, address, and contact information so that we can follow up with you in the future if needed. We may also ask you/(the minor you consent for) about co-workers within your social circle. We will collect 2 to 4 drops of blood from your/(the minor you consent for) finger. We will conduct a malaria test and give you the result immediately. We would also like to use the blood for malaria research. We would like to keep it for 15 years for future malaria research. It will never be used for anything else. The blood will be kept and analyzed at AHRI.

If your/(the minor you consent for) test today is positive for malaria, you/(the minor you consent for) will be given a medicine to cure malaria. If needed, we will refer you/(the minor you consent for) to a health facility to receive another treatment.

How long will I/(the minor I consent for) be in this study?
Taking part in this study will take you only 30 minutes. We will ask you/(the minor you consent for) questions and take blood by finger prick.

Can I/(the minor I consent for) stop being in this study? What other choices do I/(the minor I consent for) have if I/(the minor I consent for) do not take part in this study?
You are free to choose whether to take part in the study. If you decide to take part, you/(the minor you consent for) may leave the study at any time. If you would like to withdraw your/(the minor you consent for) information and/or blood samples, you can contact us. You will not lose any of your regular benefits and it will not affect your medical care. If the minor you consent for does not wish to participate, he/she is allowed to refuse, even if you agree that he/she participates.

Are there risks from taking part in this study?
There are minimal risks from participating in the interview.

- The questionnaire does not ask sensitive questions. We will keep the information that identifies you/(the minor you consent for) confidential. There's a risk that someone who is not authorized may see your/(the minor you consent for) personal information by mistake. If this happens, we will tell you.
- The needle stuck on the finger to obtain some drops of blood may hurt a little bit.
- There is a small risk of bruising, swelling, fainting, and a rare risk of infection. The risk is minimal because the process will be performed by well-trained personnel.

Are there benefits to taking part in this study?
The results of this research may help the Federal Ministry of Health develop better strategies to prevent malaria. Taking part in this study will not benefit you/(the minor you consent for) personally.

Will you keep information about me/(the minor I consent for) private?
We will do our best to protect the information we collect from you/(the minor you consent for). We will also keep the information that identifies you/(the minor you consent for) secure and restricted. We will destroy this information when the study is over. However, we might need to share personal information if required by law. If information from this research is published or presented at scientific meetings, we will not use your/(the minor you consent for) name or other identifiers. The following organizations may look at information about you in your research records: Federal Ministry of Health, AHRI, UCSF and the Bill and Melinda Gates Foundation. The data we collect in this study will be shared with the funder. It may be made open to the public so that others can learn from it. If data are shared publicly, they will not be linked to you/(the minor you consent for) personally.

How will my/(the minor I consent for) specimens and information be used?
Researchers will use the blood and information you/(the minor you consent for) provide to help develop strategies to prevent malaria. We may share the blood sample and information that you/(the minor you consent for) provide with other researchers, academics and educators. They

might use it in the future for other studies, academics or education related to malaria. We will not ask you/ (the minor you consent for) additional permission to share the information. If you agree, we will store your/(the minor you consent for) blood for up to 15 years at AHRI. Samples will be tested at the AHRI laboratory. Some samples might also be sent to the UCSF laboratory, where they will be analyzed and stored. Genetic testing of the malaria parasite will be conducted, but no human genetic testing will be done. The blood and information you/(the minor you consent for) provide will not be used for commercial use or profit. You/(the minor you consent for) can still take part in the study even if you don't wish your blood to be saved and used for future studies. In that case, we will not keep blood from you/(the minor you consent for). We will not share your name or any other personal information that would let the researchers know who you/(the minor you consent for) are. We will remove all links to you/(the minor you consent for) on your /(the minor you consent for) blood sample. Thus, once the samples are stored at UCSF, we will not be able to know which sample is yours/(the minor you consent for). If you change your mind about not wanting us to store your/(the minor you consent for) sample, we will not be able to destroy it.

Are there costs or payments associated with taking part in this study?

You/(the minor you consent for) will not be charged for any of the study procedures. You/(the minor you consent for) will not be paid for taking part in this study.

Who can I/(the minor I consent for) contact in case of injury or if I/(the minor I consent for) have future questions about this study?

If you have any questions about this study or your participation, you may contact the Study Coordinator: Dr. Endalamaw Gadisa, AHRI, Addis Ababa, at + 251 (911) 868827. You can ask questions about the study or your rights as a research participant to someone other than the researchers. Please call the AHRI Ethics review committee at 0118962183.

CONSENT

You have been given a copy of this consent form to keep. PARTICIPATION IN RESEARCH IS VOLUNTARY. You have the right to decline to participate or to withdraw at any point. There will be no penalty or loss of benefits to which you or the minor you consent for are otherwise entitled. By signing this form, you are giving permission for you or the minor(s) you consent for to participate in the study. If you wish to participate in this study, you should indicate how you would like to participate and sign below.

Number of minors (<18 years old) whom I consent may participate in this study: _____

I agree to be interviewed today. □ Yes □ No

I agree for the minor I consent for to be interviewed today. □ Yes □ No

I agree to provide a blood sample for malaria testing today and be saved for up to 15 years for future malaria research. □ Yes □ No

I agree for the minor I consent to provide blood sample for malaria testing today and be saved for up to 15 years for future malaria research. □ Yes □ No

Date	Name of adult or Guardian	Signature or thumbprint if unable to sign

Name(s) of minor(s) (if indicated, list names of additional minor(s) on the back of this form):

Name of minor 1	Name of minor 2
Name of minor 3	Name of minor 4 (etc)

Date	Name of Person Obtaining Consent	Signature of person obtaining consent

WITNESS (only necessary if participant is illiterate)

I witness that this consent form has been read to the participant in the language recorded below. I confirm that the participant has agreed to voluntarily enroll in the study. Language that consent was conducted in:_____.

Date	Name of Witness	Signature of Witness

Cross sectional - Youth (12-17 years) Informed Consent Assent

Introduction

My name is _____. I am a researcher working with the Federal Ministry of Health, the Armauer Hansen Research Institute (AHRI) and the University of California in San Francisco (UCSF). UCSF is a university and AHRI is a research center also working to improve health around the world.

The study's name is: "Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys."

This is a research study about malaria. In Ethiopia, some groups of people have a lot of malaria. We want to understand how much malaria these groups have. We also want to know which interventions to prevent malaria are used or could be used.

You can say yes or no to taking part in this study. If you say no, nothing bad will happen to you. Since you are younger than 18 years old, we also need to ask a parent or guardian. Even if your parent or guardian agrees, you still can say no.

What will happen if I take part in this study?

If you agree to be in this study:

- We will ask you questions about your experiences with malaria.
- We will ask your name, address, and contact information so that we can contact you in the future.
- We will take 2 to 4 drops of your blood from your finger. We will conduct a test for malaria. If the test is positive, we will give you an antimalarial drug to cure you.
- We want to keep the blood for malaria research. It will never be used for anything else. If you do not want your blood stored for future research, you can still participate but we will not keep blood.
- The study should take about 30 minutes of your time.

Are there risks from taking part in this study?

- When we take blood drops from your finger, you may feel some pain. You may have bruising, swelling, or fainting. It is very rare, but sometimes an infection can happen from finger prick. We do not think this will happen because we use highly trained people to take the blood.
- Answering our questions will take some time, but there are no other risks.
- We will keep your personal information safe and private. If we take blood, it will not have your name. We may share your study information and blood sample with other researchers. But they will not be able to identify you.
- There is a small risk that someone by accident may see your personal information. If this happens, we will tell you and your parents/guardian.
- Some groups who oversee the study can look at our study records. They will keep your information confidential.

Are there benefits to taking part in this study?
If you have malaria, you will be treated with an antimalarial drug. This study may help others in the future.

Who can answer my questions about the study?
If you have any questions about this study or your participation, you may contact the Study Coordinator: Endalamaw Gadisa, AHRI, Addis Ababa, at + 251 (911) 868827. You can ask questions about the study or your rights as a research participant to someone other than the researchers. Please call the AHRI Ethics review committee at 0118962183.

If you want to be in this study, just tell us.

Assent will be documented on the form below. The signature of the person conducting assent discussion is required below.

I have explained the study to _____ (print name of minor here) in language he/she can understand, and the minor has agreed to be in the study.

_____	_____	_____
Date	Signature of person	Name of minor (printed) obtaining assent

WITNESS (only necessary if participant is illiterate)
I witness that this consent form has been read to the participant in the language recorded below. I confirm that the participant has agreed to voluntarily enroll in the study. Language that consent was conducted in:_____

_____	_____	_____
Date	Name of Witness	Signature of Witness

Entomological assessment – CDC Light traps Informed Consent

Introduction

My name is _____. I am a researcher working with the Federal Ministry of Health, the Armauer Hansen Research Institute and the University of California in San Francisco (UCSF). UCSF is a university and AHRI is a research center also working to improve health around the world.

The study's name is: "Malaria Transmission among Seasonal Migrant Agricultural Workers in Metema District, Northwestern Ethiopia: An Entomological and Parasitological Surveys."

We are inviting you to participate in a study about malaria prevention in Amhara region, Ethiopia. This is a research study, and you do not have to take part.

We would like to collect mosquitoes within and near houses in this area and assess the numbers of mosquitoes found. Mosquitoes will then be examined to determine the species, whether they carry malaria parasites and whether they are resistant to insecticides. We will use the results to help develop strategies for malaria control.

Why is this study being done?

Some populations in Ethiopia have more risk of malaria. For example, migrant agricultural workers. They may need specific interventions to prevent malaria infection. We are conducting a research study to understand what the gaps in protection from malaria in this area are. For example, we want to know where and when people are being bitten by mosquitoes and what type of mosquitoes there are.

What will happen if I take part in this study?

If you agree to be in this study, our team will install traps to collect mosquitoes, one trap within and one trap outside your house. We will come on four different occasions for one year. Every time we come; we will collect mosquitoes for seven nights in a row. We will ask you to sign the consent form the first time. On the following visits, we will ask again for your verbal consent.

Traps contain a small light to which mosquitoes are attracted and a fan which sucks them into a bag. This trap is called a Centers for Disease Control (CDC) light trap and operates from connection to a 6-volt battery. Mosquitoes are primarily active at night, so the traps are usually set to operate from around 5-6pm to 6am. For the traps installed indoors, we will hang them near someone's sleeping space, so that mosquitoes are attracted. We will provide a bed net for everybody in the household to be protected from mosquito bites. We will ask everyone to use the bed nets during collection nights. Every day at the designated time we will connect the battery and collection will begin. A team member will empty the trap every hour to collect the mosquitoes.

We will also record a few basic structural details of your house such as wall construction material and roofing type. We will also ask how many people sleep in your house and whether you normally use anti-mosquito measures such as bed nets or repellents. We will also collect your name, address, and contact information so that we can follow up with you for the subsequent visits. The mosquitoes we collect will be analyzed in the laboratory at AHRI. Some mosquitoes might also be sent, analyzed, and kept at UCSF.

How long will I be in this study?

We will be collecting mosquitoes four times over the following year. Every time we come; we will collect mosquitoes for seven nights in a row.

Can I stop being in this study?

You are free to choose whether to take part in the study. If you decide to take part, you may leave the study at any time. If you would like to withdraw your information, you can contact us. You will not lose any of your regular benefits and it will not affect your medical care.

Are there risks from taking part in this study?

Potential risks and discomforts to participating households include loss of privacy. The study team will ensure that this is minimized. Care will be taken to protect the privacy of your household. Study personnel will be instructed to interact with your households in a courteous and respectful manner to limit this possible discomfort. The CDC traps are low voltage devices and present minimal risk. However, we request that you do not touch the battery or electrical connections when running to avoid any possibility of a mild electrical shock. The light from the traps is quite dim and the fan only moderately loud. The noise might disturb light sleepers. We will demonstrate the operation to you so that you can decide whether this might affect you or your household adversely. CDC light traps will use a person sleeping under a bed net to attract mosquitoes. This could increase the risk of mosquito bites. However, the risks associated with mosquito bites are likely to be low as the mosquito net will protect the person sleeping under it.

Are there benefits to taking part in this study?

The benefit to your household is that we will collect the mosquitoes in your house so you may have fewer flying insects for a few days. The results of this research may help the Federal Ministry of Health develop better strategies to prevent malaria. Taking part in this study will not benefit you personally.

Will you keep information about me and my household private?

We will do our best to protect the information we collect from you and your household. We will also keep the information that identifies you secure and restricted. We will destroy this information when the study is over. If information from this research is published or presented at scientific meetings, we will not use your name or other identifiers. The following organizations may look at information about your household in their research records: Federal Ministry of Health, AHRI, UCSF, and the Bill and Melinda Gates Foundation. The data we collect in this

study might be shared with the funder. It may be made open to the public so that others can learn from it. If data are shared publicly, they will not be linked to you personally.

How will my information be used?

Researchers will use the information you provide to help develop strategies to prevent malaria. We may share it with other researchers, academics, and educators. They might use it in the future for other studies, academics or education related to malaria. We will not ask you for additional permission to share the information. We will not share your name or any other personal information that would let the researchers know who you are. Collected mosquitoes will be stored at AHRI for some time. Some might also be shipped to UCSF and stored there. We will remove all links to you on the samples.

Are there costs or payments associated with taking part in this study?

You will not be paid for taking part in this study.

Who can I contact in case of injury or if I have future questions about this study?

If you have any questions about this study or your participation, you may contact the Local Study Coordinators: Endalamaw Gadisa, AHRI, Addis Ababa, at + 251 (911) 868827. You can ask questions about the study or your rights as a research participant to someone other than the researchers. Please call the AHRI Ethics review committee at 0118962183.

CONSENT

You have been given a copy of this consent form to keep. PARTICIPATION IN RESEARCH IS VOLUNTARY. You have the right to decline to participate or to withdraw at any point. There will be no penalty or loss of benefits to which you are otherwise entitled. By signing this form, you are giving permission for your household to participate in the study. If you wish to participate in this study, you should indicate how you would like to participate and sign below.

I agree to be interviewed today. ☐ Yes ☐ No

I agree that mosquito traps are installed within or outside my household during all collection nights. ☐ Yes ☐ No agree that mosquito traps are emptied every hour during collection nights. ☐ Yes ☐ No

Date	Name of adult	Signature or thumbprint if unable	Name of Person Obtaining Consent

WITNESS (only necessary if participant is illiterate)

I witness that this consent form has been read to the participant in the language recorded below. I confirm that the participant has agreed to voluntarily enroll in the study. Language that consent was conducted in:_____.

Date	Name of Witness	Signature of Witness

Annex II: Targeted Parasite Survey Questionnaire

Section 1: Study Eligibility and consent			**Skip Pattern**
ELIGIBILITY CRITERIA FOR SURVEY RESPONDENTS **All respondents must:** • **Identify current primary occupation as migrant agricultural worker.** • **Be willing and able to provide consent (ie mentally fit)**			
1.	What is your current primary occupation?	1. Migrant agricultural worker 2. Other	If migrant agricultural worker, continue, else, end interview*
CONSENT: Read the informed consent form to the participant and make sure they understand the information. The participant needs to sign ONE paper copy of the informed consent for them to keep. Participant will sign in tablet for study records.			
*This individual does not meet eligibility criteria to participate in the survey. Please thank them for their time and end the interview.			
2.	I agree (for the minor I consent for) to be interviewed today?	1. Yes 0. No	
3.	I agree (for the minor I consent for) to provide a blood sample for malaria testing today?	1. Yes 0. No	
4.	I agree (for the minor I consent for) to provide a blood sample for malaria testing and be saved for up to 15 years for future malaria research?	1. Yes 0. No	
5.	Signature		

Section 2: Interview context			
6.	Name of interviewer	Drop down menu of interviewers	
7.	Date of interview	DD/MM/YYYY	
8.	Health facility catchment area	Drop down menu of study health post catchment areas	
9.	Farm (worksite) QR code	Scan QR Code	
10.	Manual farm (worksite) QR code		
11.	Location of the farm	District_____ Kebele	
12.	Name of farm owner/employer		

13.	What type of crop is cultivated at this farm		
14.	Participant Individual QR code	Scan QR Code	
15.	Manual Participant Individual QR code		
16.	GPS coordinates	Auto from tablet	

Section 3. Demographics			
17.	Participant gender	1. Male 2. Female	
18.	What is your age? (years)	_____ (Number)	
19.	Participant date of birth	DD/MM/YYYY	
20.	Besides your current work as a **[insert eligible occupation]**, what other occupations do you do? *Select multiple or selecting none in also allowed.*	1. Agricultural worker 2. Cattle herder 3. Gold miner 4.Professional/technical/managerial 5. Teacher 6. Student/learner 7. Small business/retail 8. Government staff 9. Hunting 10. Fisherman 11. Private security personnel 12. Construction 13. Housewife 777. Other: Specify: _____	
21.	What is the highest level of education that the participant has completed?	1. No formal education 2. Primary school 3. Secondary school 4. University 5. Other (specify) 888. Don't know	
22.	For how many years have you been in Ethiopia?	1. Less than one year 2. One year or more	If Q20 not Ethiopia
23.	Is your main place of residence (i.e. where you maintain a household and live most of the year) in this district (Woreda) of (Name from Q3)?	1. Yes 0. No	
24.	Village (Kebele) of main residence	_____text	If Q22=1
25.	For how long have you been in this district (Woreda) of (Name from Q3)?	1. Less than a month 2. Between 1 and 3 months 3. Between 3 and 6 months 4. Between 6 months - 1 year 5. More than 1 year 888.Don't know/don't remember	If Q22=0
26.	How many trips do you make to this district (Name from Q3) from your main residence (name of Kebele Q25) between June and December?	_____ (Number)	If Q23=0

27.	When was the last time you visited your main residence (name of Kebele Q25)?	DD/MM/YYY	If Q28=1
28.	Village of current residence in this district (Name from Q3)		If Q24=0
Section 4: Occupational travel and worksite conditions			
Thank you for your answers. Now I would like to ask you some questions about your work.			
4a. Worksite characteristics			
29.	When did you begin working for your current employer?	DD/MM/YYYY	
30.	Do you work for this employer seasonally (only during certain seasons) or year round?	1. Seasonal 2. Year round 777. Other (explain)	
31.	How many employers/worksites in the study area do you work for between June and December	___ (Number)	
32.	Over the entire year, what months do you work in this area?	1. January, 2. February, 3. March, 4. April, 5. May, 6. Jun 7. July, 8. August, 9. September 10. October, 11. November, 12. December	
33.	How many farms have you worked at as a seasonal worker since June 2021?	_____ (Number)	
Now, I am going to ask you some questions about farms you worked in as a seasonal worker since June 2021, starting with the current one.			
34.	What is the name of the farm	Text_____	
35.	Where is the farm located	Region Woreda Kebele (or closest village)	
36.	Is this a permanent worksite? *Define: A worksite that workers return to and sleep at every year; has at least some permanent sleeping structures.*	1. Yes 0. No	
37.	Are you currently working at this **[PLACE]**?	1. Yes 0. No	If YES -> skip to Q47
38.	When did you complete your work there?	DD/MM/YYYY	
39.	The last time you were working at this place, how long did you stay there?		
40.	How many trips do you typically make to this location between June and December, working as a **migrant worker**?	_____ (Number)	
41.	How many agricultural workers were working at this **[PLACE]** while you were there the last time?	_____ (Number)	
42.	How many of these agricultural workers were migrant workers?	_____ (Number)	

43.	How many other **migrant workers** did you travel with?	_____ (Number)	
44.	How many farms are within a 15 minute walk of **[PLACE]**?	_____ (Number)	
4b. Malaria risk at worksite			
45.	Did you work outside as a **migrant worker**, between the hours of 6pm and 6am, while at this **[PLACE]**?	1. Yes 0. No	
46.	Besides **migrant agricultural work**, did you do any other types of work while you were at this **[PLACE]**?	1. Yes 0. No	
47.	Were any of these activities done between 6pm and 6am?	1. Yes 0. No	
48.	What, if anything, did you do to protect yourself from mosquitos while you WORKED at **[PLACE]**? ***Do not probe for different answers.*** ***Record whatever the participant says, even if you do not believe it helps prevent malaria.*** ***Select all that apply.***	1. Chemoprophylaxis/Medicine (specify) 2. Bed net 3. Mosquito repellent on skin 4. Mosquito repellent on clothes 5. Burned mosquito coil 6. Fire 7. Wore clothes that covered skin 8. Nothing 777. Other (specify) 888. Don't know / don't remember 999. Decline to answer	
49.	Did you sleep outside of any structure while you were working at this **[PLACE]**?	1. Yes, frequently 2. Yes, occasionally 3. Yes, rarely 0. No, never	
50.	Do you know of any migrant agricultural worker working within 15 minutes of **[PLACE]** who were diagnosed with malaria (by rapid diagnostic test or microscopy) while you were working there or shortly after?	1. Yes 0. No	

Section 5. Clinical Section			
51.	RDT result	1. Negative 2. Positive Pf only 3. Positive Pf + other 4. Invalid - *Repeat the test and record the result below* 5. RDT not conducted	

52.	Please specify why RDT was not conducted.	1. Participant refused 2. Inadequate blood flow 3. Other (specify): _____	If RDT not conducted
53.	(If first RDT result invalid) Repeat RDT result	1. Negative 2. Positive Pf only 3. Positive Pf + other	If RDT result invalid
54.	Was a DBS collected?	1. Yes 0. No	
55.	Number of FULL DBS spots collected?	_____ (Number)	
56.	Why was DBS not collected?	1. Participant refused 2. Inadequate blood flow 3. Other (specify): _____	If DBS not collected
57.	Was the RDT cassette saved and labelled?	1. Yes 0. No	

Medical History

Use the Ministry of health treatment eligibility form to assess for potential criteria restricting participant from receiving antimalarial medication:

58.	Is the participant eligible for malaria treatment?	1. Yes 0. No	
59.	Was dose #1 directly observed?	1. Yes 0. No	
60.	Why was Dose #1 not directly observed?	_____	

Closing statement

Thank you very much for answering these questions. Your answers will help us understand who gets malaria and why.

If you suspect you have symptoms of malaria in the future, such as having a fever or body aches, please go to the closest clinic when you feel sick and ask for a malaria test.

Do you have any questions for me about malaria or this research?

If you have any questions about this study in the future please contact the Study Coordinator.

[Show the participant where to find their phone numbers on the consent form.]

Interviewer: Any comments?

Annex III: Ethical Approval Letter

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ቀን/DATE: 29 AUG 2022
ቁጥር/REF NO: 02/256/163 d/14

Armanuer Hansen Research Institute (AHRI)
Addis Ababa

Subject: ***Letter of Approval***

The Ministry of Education (MoE) via its National Research Ethics Review Committee has reviewed **"Assessing the contribution of seasonal migrant workers in selected districts of Northwest Amhara Region, Ethiopia to malaria transmission in midlands and highlands"** project protocol in an expedited manner. We are writing to advise you that MoE has granted full approval to the above named project, for a period of **one year (August 25, 2022- August 24, 2023).**

All your most recently submitted documents have been approved for use in this study. The study should comply with the international and national scientific and ethical standard guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to MoE. Please ensure that you submit biannual progress report to MoE once in six months and annual renewal application 30 days prior to the expiry date.

We are confident that your esteemed organization and the PI (copied) of the project should monitor the ethical implication of the project as stipulated in the latest approved document.

Sincerely

Samuel Kifle Kidane (PhD)
State Minister

Cc

- State Minister for Higher Education Development Sector
- CEO, Research and Community Engagement Affairs
- Research Ethics Desk

  Ministry of Education
- Mr. Assefaw Getachew (PI)
- Dr Endalamaw Gadisa(PI)

  AHRI