

Epidemiological and Entomological Characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia



Tadele Emiru Abosse

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

December, 2022

Adama, Ethiopia

**Epidemiological and Entomological Characteristics of
Plasmodium falciparum Malaria in Dire Dawa City, Ethiopia**

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DECLARATION

I hereby declare that this Master Thesis entitled “**Epidemiological and Entomological Characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia**” 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 citation.

Name:

Signature

Date:

RECOMMENDATION

We, the advisors of this thesis, hereby certify that, we have read the revised version of the thesis entitled “**Epidemiological and Entomological Characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia**” prepared under our guidance by **Tadele Emiru** submitted in partial fulfillment of the requirements for the degree of Master’s of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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APPROVAL SHEET

I/we, the advisors of the thesis entitled “Epidemiological and Entomological Characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia” and developed by Tadele Emiru, 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: Hunduma Dinka (Prof) Signature _____ Date _____

Co-advisor: FitsumGirma (PhD) Signature _____ Date _____

We, the undersigned, members of the Board of Examiners of the thesis by Tadele Emiru have read and evaluated the thesis entitled Epidemiological and Entomological Characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia” 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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LIST OF ABBREVIATIONS AND ACRONYMS

AHRI	Armauer Hansen Research Institute
<i>An.</i>	<i>Anopheles</i>
API	Annual Parasite Incidence
CDC	Centers for Disease control and prevention
CSA	Central Statistics Agency
DBS	Dried Blood Spot
DDU	Dire Dawa University
EIR	Entomological Inoculation Rate
EDTA	Ethylene Diamine tetra acetic acid
FMOH	Federal Ministry of Health
HC	Health center
HEWs	Health extension workers
HH	House hold
IRS	Indoor residual spray
LLIN	Long lasting insecticidal nets
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
qPCR	Quantitative real time PCR
RDT	Rapid diagnostic test
WHO	World Health Organization

ABSTRACT

Malaria is caused by parasites of the genus Plasmodium, which is transmitted to humans by a bite of an infected female Anopheles mosquito. The South Asian malaria vector, Anopheles stephensi, was first confirmed in Djibouti in 2012, and distributed and established widely in the eastern part of urban settings of Ethiopia located on the main transportation corridor from Djibouti to Addis Ababa. The objective of this study was to assess epidemiological and entomological characteristics of imported and locally-acquired Plasmodium falciparum malaria in patients visiting Goro health center and Dire Dawa University student clinic, Dire Dawa city, Ethiopia. For this purpose a facility-based case-control study was conducted from March 01 to May 30, 2022 where a total of 55 index cases and 150 controls were recruited. An index case and controls identified at the health center were followed to their homes and their family members were tested to find other malaria parasites in family members. Malaria infection using 18S based quantitative polymerase chain reaction (qPCR) among family members of index cases (n=163) were compared with that of febrile controls (n=366). Adult and immature stages of mosquitoes were collected by prokopack aspirator and standard dipper, respectively, from the participants' homes and surrounding to identify the vector responsible for malaria transmission. A pretested structured questionnaire was used to assess socio-demographic, entomological and clinical data collection. Collected data were exported to Stata Software version 17.0 for analysis. Odds ratio with 95% CI was used as a measure of association, and variables with a p-value of ≤ 0.05 were considered as statistically significant. The prevalence of malaria among study participants by RDT, microscopy and qPCR is 13.4%, 12.4% and 19.3%, respectively. Members of the index cases were more likely to be qPCR positive (22.7%, 37/163) than members of the control household (HH) (14.8%, 54/366; odds ratio [OR]; 6.2, 95%CI, 4.2-9.2; $P < 0.001$). Being a family member of index case, spending evenings away from home, using anti-mosquito spray in home, malaria history in the past two weeks, being male and proximity to mosquito breeding sites were significantly associated with positive test result for malaria (P value < 0.00). The finding of this study showed that the principal malaria vector found was Anopheles stephensi and is contributed to current malaria transmission in Dire Dawa city. Interventions and strategies which focus on breeding sites are needed to reduce malaria infections in the area.

Keywords: *Anopheles stephensi, Case-control study, Malaria, Mosquitoes*

1: INTRODUCTION

1.1. Background

Malaria is a leading cause of mortality worldwide causing an estimated 229 million malaria cases in 87 malaria endemic countries with an estimated 215 million cases and 409,000 deaths where about 94% of malaria deaths occur in Sub-Saharan Africa. Most malaria encountered in western countries is among travelers returning from malaria-endemic areas. Sub-Saharan Africa, the greatest malaria burden worldwide, is currently a growing proportion of its population moving to urban areas (WHO, 2020).

Plasmodium falciparum (*P. falciparum*) is the most common and clinically serious of the four plasmodium parasite species that infect humans in the horn of Africa. It is a serious health risk for travelers to malaria-endemic areas and is often diagnosed on return to the town of residence. Population movement (migration and circular mobility) has also been implicated in the changing epidemiological factors of *P. falciparum* within Africa (Prothero, 1961). *Plasmodium vivax* (*P. vivax*) infection is characterized by relapses of malaria arising from persistent liver stages of the parasite (hypnozoites) and is more difficult to control and eliminate than *P. falciparum* because of its tendency to relapse after resolution of the primary infection (White, 2011).

Higher transmission of malaria in Africa is largely due to the fact that African cities tend to grow outwards with perimeters consisting of relatively underdeveloped, poorly serviced settlements (Byrne, 2007). Urban malaria transmission is influenced by population movements from rural to urban and peri-urban areas. Migrants from rural areas tend to bring their rural practices with them, creating a multitude of vector breeding sites (Fournet *et al.*, 2010), and poor quality housing provides less protection against mosquito bites (Adiamah *et al.*, 2016).

For successful malaria elimination to be achieved, identifying the origin and means of transmission of malaria parasite in urban setting is very important. Well designed and improved entomological surveillance is needed for more efficient and well-monitored vector control investments. Therefore, this study was conducted with the aim of assessing the epidemiological and entomological characteristics of imported and locally acquired *P. falciparum* malaria infection in Dire Dawa city, Eastern Ethiopia.

1.2. Statement of the problem

The South Asian malaria vector , *Anopheles stephensi* (*An. stephensi*), was first confirmed in Djibouti in 2012, marking the first confirmed report of this malaria vector from the African continent (Faulde *et al.*, 2014) and then found in the Somali region of Ethiopia in 2016 (Carter *et al.*, 2018). Since then the vector was found widely distributed in different parts of eastern Ethiopia like Afar region (Awash Sebat Kilo, Gewane, Semera), Amhara region (Bati), and Dire Dawa city. This confirms that *An. stephensi* is widely distributed and established in eastern parts of Ethiopia(Balkewet *et al.*, 2020).

An. stephensi is known to transmit both *P. falciparum* and *P. vivax* parasite species and is regarded as an efficient vector of urban malaria. The spread of this vector is a major concern for control and elimination of malaria in the Horn of Africa, as data from Djibouti indicate the presence of *An. stephensi* has been linked with dramatic increases in malaria cases. The vector has ability to breed in man-made water storage containers in urban areas and has quick adaptation to its local environment such as deep wells. Additionally it tolerates extremely high temperatures during the dry season and has shown insecticide resistance(Santi *et al.*, 2021).

The wide scale presence of immature stages of *An. stephensi* mosquitoes in man-made water features shows that these mosquitoes are well established in an urban setting in Ethiopia, situated on the main transportation corridor from Djibouti to Addis Ababa(Tadesseet *et al.*, 2021). Malaria remains a problem for many countries due to the fact that imported cases to non-endemic regions continue to pose challenges for diagnosis and management of malaria which in turn contributed to the outbreak of malaria and threatens long-term eradication goals (Tatem *et al.*, 2017).

In Djibouti city, emergence of the vector has been epidemiologically linked to an unusual resurgence in local malaria cases. Recently, there has been a substantial case build-up; increased from only 27 cases on World Health Organization (WHO)week 2 to 260 cases in week 5 and it remained unclear if the increase in cases in Dire Dawa city is strictly caused by *An. stephensi*(Seyfarthet *et al.*, 2019).

1.3. Significance of the study

The information obtained from this study would help the Federal Ministry of Health (FMoH), National Malaria Control Program to better understand malaria epidemiology and its vector in urban settings and design targeted interventions. This study also used to collect and analyze risk factors for urban malaria transmission throughout the town and to discuss their implications for control. The finding of this study would be used as baseline information for researchers that have envisioned understanding the detail characteristics of the *An. stephensi* and bionomics of the vector and thereby designing holistic interventions approach.

1.4. Research questions

- What is the source of malaria infection (imported or locally acquired) in Dire Dawa city?
- Which vector is responsible for malaria transmission in Dire Dawa city?
- What are the possible risk factors associated with malaria infection in Dire Dawa city?

1.5. Objectives

General Objective

- To assess the epidemiological and entomological characteristics of imported and locally acquired *Plasmodium falciparum* malaria infection in urban settings from patients visiting Goro health center and Dire Dawa University student clinic, Dire Dawa city, Ethiopia.

Specific objectives

- To determine source of *P. falciparum* malaria infection (imported or locally acquired) in the city.
- To identify the vector responsible for *P. falciparum* malaria infection in Dire Dawa city.
- To identify other possible risk factors of *P. falciparum* malaria infection in Dire Dawa city.

1.6. Delimitations and limitations of the study

Due to shortage of materials, molecular identification and genotyping of mosquito species to determine genetic relatedness of the infection was not done. Some constraints such as time and lack of sufficient fund hindered the need to include a large sample size that could nearly represent the total population of the city.

2: LITERATURE REVIEW

2.1. Epidemiology of malaria

Malaria is an acute febrile illness caused by Plasmodium parasites, which are spread to people through the bites of infected female Anopheles mosquitoes. Malaria occurs mostly in poor, tropical and subtropical areas of the world. Two of the five parasite species that cause malaria in humans, *P. falciparum* and *P. vivax*, pose the biggest risks. The most common and lethal malaria parasite on the African continent is *P. falciparum*. In the majority of nations outside of sub-Saharan Africa, *P. vivax* is the predominant malaria parasite. Nearly half of the world's population were at risk for contracting malaria and Sub-Saharan Africa continuing to bear the greatest burden of the disease (96% of all malaria fatalities and approximately 95% of all malaria cases in 2020)(WHO, 2021).

The history of malaria involves the correlation between the cyclical infections of malaria parasite in human and female Anopheles mosquitoes. This parasite grows and multiplies in the liver cells by destroying red blood cells and this occurs after the female Anopheline mosquito feed infected human blood. Then, female and male gametocytes are ingested during a blood meal and mate in the mosquito's gut, in which they begins cycles of growth and multiply. After 10-18 days, sporozoites migrates to the mosquito's salivary glands and the female *Anopheles mosquito* injects it in to blood stream of malaria's next victim(Cowman et al., 2016) (Fig. 1).

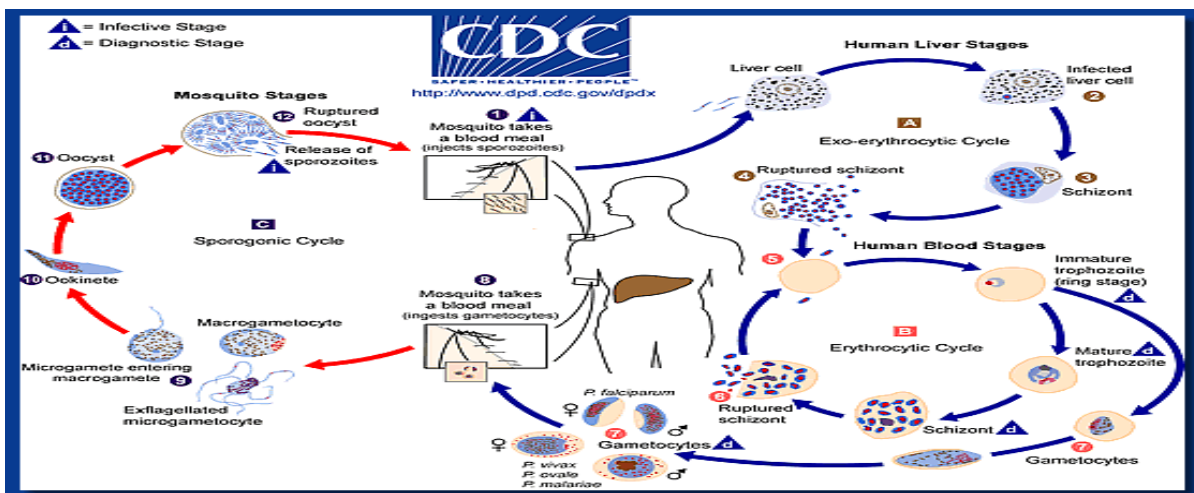


Figure 1: Life cycle of malaria parasite.

Sources: <http://www.samaritanid.com/MalariaCycle.html>

Malaria used to be endemic in Asia. Study conducted to assess epidemiological characteristics of imported and locally-acquired malaria in Singapore showed that based on the travel history, majority of the reported cases were imported (91.4% to 98.3%). Most of the imported cases originated from Southeast Asia, mostly from Indonesia, and the Indian subcontinent (Yu *et al.*, 2020). On the other hand, a study from Colombia has shown that no local transmission within the urban setup despite increased reports of malaria in urban health facilities. The report confirmed that cases are from the peri-urban areas that are investigated to be all travel related to malaria endemic area (Gómez *et al.*, 2017).

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. Malaria found all over the world, notably in Africa, south and Central America, south and Southeast Asia, and particularly in Sub-Saharan Africa with very high transmission intensity. Nearly three million cases of malaria are reported annually in nearly 45 countries, including Ethiopia, and the morbidity and death rates are rising sharply (Roll Back Malaria Partnership Report, 2015).

Study conducted in Cameroon investigated the prevalence of malaria infection and potential risk factors associated which shows the overall prevalence of malaria parasite infection in Nkongho-mbeng, Cameroon is 12%. The prevalence of malaria infection in the area was significantly associated with residing around bushy areas and areas of stagnant water, and male participants, age, location of villages and occupation (Babila *et al.*, 2021).

Despite the progress in reducing malaria infections and related deaths, the disease remains a major global public health problem in the World. The Horn of Africa (HoA), comprising Djibouti, Eritrea, Ethiopia, and Somalia, is classified as a malaria endemic area. Efforts to prevent the transmission of malaria and developing effective malaria control strategies in Ethiopia require controlling the mosquito vectors (*Anopheles*) that transmit the malaria parasite. Understanding the distribution of *An. stephensi* in Ethiopia is critical to evaluating the threat it poses to malaria control in Ethiopia and the rest of the Horn of Africa (Carter *et al.*, 2021).

In Ethiopia *P. falciparum* and *P. vivax* are the most dominant malaria parasites and are principally transmitted by the primary mosquito vector known as *Anopheles arabiensis*.

Ethiopia has about 835 districts with different levels of malaria risk with an estimated at-risk population of 50.6 million. Ethiopia implemented the revised strategies to control malaria. Among these, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the most important in malaria prevention and control strategy. A long term investments in control and prevention activities have brought substantial decline of malaria incidence in the last two decades in Ethiopia (Abossie *et al.*, 2020).

Towns in most developing nations, including Ethiopia, are becoming more urbanized without adequate planning. It is characterized with poor housing, lack of proper sanitation and poor drainage of surface water with informal settlements. Due to the vectors' adaption to the urban environment, this provides the best conditions for reproducing and aids the transmission of urban malaria (Ameyu, 2009; Mattah *et al.*, 2017).

The study conducted to assess the baseline malaria prevalence in selected districts targeted for malaria elimination in Ethiopia from October to December 2018 showed the overall prevalence of malaria as detected by rapid diagnostic tests (RDTs) in this survey was 1.17% among the total study participants. Malaria infection was reported high proportion from Harari 46(4.7%) followed by 87(3.7%) in Kersa of Jimma and 81(2.7%) and low in Misrak Badawacho, 27(1.7%) in Kolla Tembien of Tigray, 32(1.4%) in Habru and 22(1.07%) in Raya Kobo Districts of Tigray region (Negatu *et al.*, 2020).

Study conducted to assess the prevalence of urban malaria and associated factors in Gondar town, Northwest Ethiopia in 2008 shows that the prevalence of malaria in the town is high. The prevalence was strongly associated with proximity of residence to potential mosquito breeding sites (Tilaye & Deressa, 2007).

According to preliminary study conducted at Adama City, Ethiopia to determine the status of urban malaria during the minor transmission season, 3.7% (97/2590) of febrile patients who were screened for malaria during the study period were confirmed to have the disease, with 66.2% of cases being *P. vivax*, 26.5% being *P. falciparum* and 7.2% being mixed infection cases. The study also revealed that majority of the diagnosed malaria cases (65%) were from Adama city residents whereas the remaining from the neighboring rural area who visited the health facilities for malaria treatment (File & Dinka, 2020).

2.2. Risk factors associated with malaria

According to a study conducted to assess possible entomological risk factors for severe malaria in a peri-urban area of Gambia show that housing characteristics, sleeping arrangements are risk factors for urban malaria transmission (Adiamah *et al.*, 2016).

A systematic review that evaluated more than 100 articles between 1946 and 2012 on factors contributing to malaria infection, documented increasing incidence of malaria in urban areas of Sub-Saharan Africa. Among the factors that impact transmission of urban malaria are, density of susceptible population, host immunity, human behavior, land use modification/urban agriculture, economic level, coverage and utilization of vector control measures, access to health care, climatic factors, travel, vector capacity and adaptation of vector to polluted breeding sites (De Silva & Marshall, 2012).

Studies that compared malaria in urban and rural settings among African cities show clear trend of increasing malaria transmission from urban to rural settings. However, in some studies malaria transmission is higher in urban centers due to geographic factors including close proximity to water bodies, urban agriculture and/or slum conditions (Byrne, 2007; De Silva & Marshall, 2012).

The findings of baseline malaria indicator survey conducted in Amhara, Oromia and Southern Nation Nationalities and People (SNNP) regions of Ethiopia from December 2006 to January 2007 show that socio-economic factors like construction material of walls, roof and floor of house; main source of drinking water; toilet facilities are related to malaria risk. Demographic and geographic factors like gender, age, family size and region where the respondents lived also had an effect on the risk of malaria. Similarly, houses that were treated with anti-malarial spray were less likely to be affected by malaria. From the results, it was observed that households with no toilet facilities were more likely to be positive for malaria diagnosis test (Dejzmach *et al.*, 2021).

An institution-based matched case-control study conducted in Dembia district, Amhara Region from October to November 2016 shows that sleeping outdoors at night, proximity to stagnant water and bed net use were found to be statistically significantly associated with malaria infection. The finding also shows that individuals sharing a bed net with more than 3 persons

and outdoor activity were found to be at more risk for malaria infection than the counterparts. Outdoor activity is also associated with malarial illness in this study; it is possible that bed nets were not used during outdoors, decreasing their effectiveness in preventing malaria illness (Agegnehu *et al.*, 2018).

Study conducted to evaluate spatial and genetic clustering of *Plasmodium falciparum* and *Plasmodium vivax* infections in a low-transmission area of Ethiopia indicated that asymptomatic *P. falciparum* infections were clustered within index case households whilst there was no such evidence for clustering of *P. vivax* infections. The study also shows that *P. vivax* malaria infections were more genetically complex and diverse than *P. falciparum* infections, with no detectable spatial or temporal clustering (Tessema *et al.*, 2020).

The results of the study conducted to assess the prevalence of urban malaria and associated risk factors in Jimma town, south-west Ethiopia revealed that prevalence of malaria parasite was 5.2% within the town, from which 71.4% were *P. vivax*, 26.2% were *P. falciparum* and mixed infection only accounts for 2.4%. The prevalence was strongly associated with proximity of participants to potential mosquito breeding sites which indicates human activity plays a major role in urban malaria ranging from creating breeding sites, ‘importing’ cases, or through treatment-seeking choices (Alemu *et al.*, 2011).

An. stephensi, the South Asian malaria vector, was first detected in Dire Dawa in 2018. The recent detection of this vector in Ethiopia and other regions in the Horn of Africa has raised concerns about its potential impact on malaria transmission. *Anopheles stephensi* surveys conducted from August to November 2018 in ten selected urban sites including Somali region, Afar, Amhara region, and Dire Dawa city confirmed the presence of *An. stephensi* using both morphological and molecular methods (Balkew *et al.*, 2020).

A case control study conducted to assess travel history and malaria infection risk in a low-transmission setting in Ethiopia showed that human movement can transmit parasites when residents of malaria-free or low-prevalence areas travel to endemic areas, become infected, and then return to their community of origin, or when infected individuals migrate to or visit these areas from endemic region. This study also shows the relationship of travel history to malaria risk is likely due to a combination of factors including movement into areas of higher transmission during risk periods for mosquito biting, such as movement for harvesting of

crops, search of pasturage for animals, and further other short-term travel to visit nearby market areas, friends and relatives or for school attendance (Yukich *et al.*, 2013).

Study conducted in Gurage zone to assess factors affecting prevention and control of malaria in the area revealed that people in the study area use different practices to protect them from getting malaria. Among the practices, they use ITN (97%), drainage of stagnant water, and cleaning bushes (30.1%) and other significant proportion of respondents protect themselves through covering during night time, closing openings, using repellents and use of smokes(Girum *et al.*, 2017).

2.3 Expansion of new malaria vector to Horn of Africa

An. stephensi, an efficient vector of both *P. falciparum* and *P. vivax* malaria, was once native to regions of Asia and Arabian Peninsula and is now a significant vector of malaria in both rural and urban settings. The first detection of the vector in African continent is in Djibouti city in September, 2012 when it was found connected to two separate unusual malaria epidemics. Since 2008, malaria incidence in Djibouti has significantly dropped(Faulde *et al.*, 2014; Santi *et al.*, 2021). Malaria reemerged in 2014 and reached an incidence of 5.9 cases/1,000 persons in 2018 and 8.1 cases/1,000 persons in 2019 (Fig 2). The epidemiologic profile of malaria in Djibouti has changed as a result of *An. stephensi*, which is a recognized vector of urban malaria in India and the Arabian Peninsula (Sinka *et al.*, 2011). The number of confirmed cases of malaria climbed to 25,319 in 2018, with more than 100,000 suspected cases (of which 64% were caused by *P. falciparum* and 36% by *P. vivax*respectively) (Santi *et al.*, 2021)

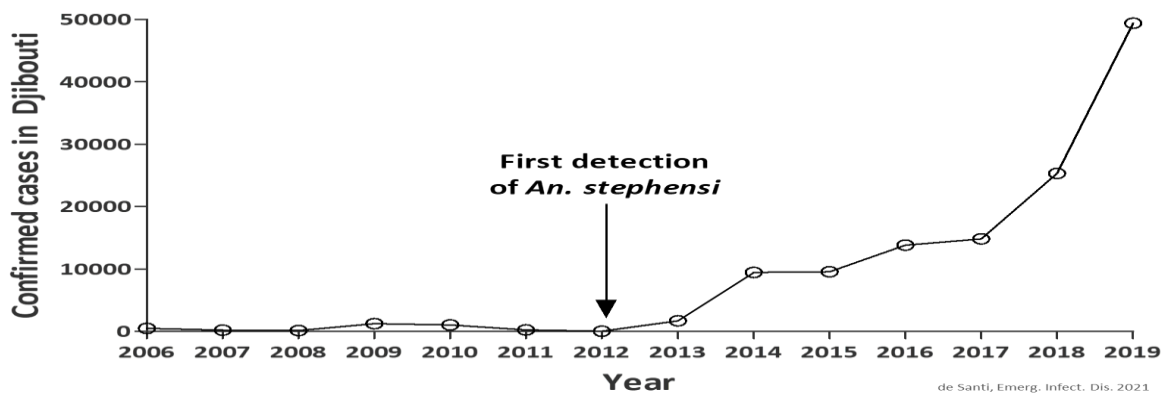


Figure 2: Distribution of confirmed malaria cases among residents, Djibouti, 2006–2019(Santi *et al.*, 2021)

The south Asian malaria vector, *An. stephensi*, was first detected in Djibouti in 2012 and since then malaria cases have increased in Djibouti (Figure 2). The vector is currently widely distributed and established in Ethiopia. In Dire Dawa city, the vector was detected in 2018 and found widely distributed in seventeen surveyed towns of Eastern Ethiopia (Balkew *et al.*, 2020).

Historically, malaria has been considered to be a disease of rural locations. But due to rapid expansion of urbanization with poor management of wastes and relatively underdeveloped settlement, malaria has been increasing in urban settings due to adaptation of the vector to the urban environment. In Ethiopia, there are more than forty species of *Anopheles* mosquitoes of which *Anopheles arabiensis*, *Anopheles funestus*, *Anopheles pharoensis*, and *Anopheles nili* are the malaria vectors. *A. arabiensis* is the primary malaria vector in Ethiopia (Kendie *et al.*, 2021).

Anopheles stephensi is an efficient malaria vector which thrives in urban settings and capable of transmitting both major malaria parasites (*P. falciparum* and *P. vivax*). The major breeding sources of the vector are containers such as overhead tanks, plastic and metal barrels, cisterns, discarded tyres and plastic containers, freshwater pools such as the margins of water streams and in irrigation ditches. In addition the vector is resistant to many classes of insecticides and feeds both human and animal blood sources (Mnzava *et al.*, 2022).

Ability of the vector to breed in man-made water storage containers in urban areas, its quick adaptation to its local environment such as deep wells, its tolerance of extremely high temperatures during the dry season, when malaria transmission typically reaches a seasonal low and its insecticide resistance makes it difficult to control. *An. stephensi* has demonstrated resistance to pyrethroids, organophosphates, and carbamates in the Horn of Africa, according to insecticide resistance data submitted to WHO (Ahmed *et al.*, 2022; WHO, 2021).

Human malaria infection may result from bite of *Anopheles* mosquitoes carrying Plasmodium sporozoites in their salivary glands. Oocyst rates are thought to be epidemiologically less helpful as a measure of the ability for a particular mosquito species to transmit malaria since earlier stage oocysts that originate in the mosquito midgut may or may not develop into sporozoites. It is crucial to distinguish between infected (just carrying oocysts) and infectious (carrying sporozoites) mosquitoes. The conventional technique for finding oocysts in the

midgut and sporozoites in the salivary glands is dissection. This procedure, however, necessitates the processing of several mosquitoes, which makes it unsuitable for low endemicity locations and necessitates the use of fresh specimens and skilled dissectors (Sutcliffe *et al.*, 2021).

Anopheles mosquitoes that harbor *Plasmodium* sporozoites in their salivary glands are potentially infectious to humans. Earlier stage oocysts that occur in the mosquito midgut may or may not develop into sporozoites, and oocyst rates are regarded as epidemiologically less informative as a measure of the potential of particular mosquito species to transmit malaria. Therefore, distinguishing between infected (oocysts only) and infective (with sporozoites) mosquitoes is important. Dissection is the traditional method for detecting oocysts in the midgut and sporozoites in the salivary glands. However, this method requires fresh specimens, experienced dissectors, and is generally unsuited to low endemicity areas where the processing of large numbers of mosquitoes is required. Methods for determining the presence of *Plasmodium* species sporozoites in the salivary glands of potential mosquito vectors are key to measuring the intensity of malaria transmission, characterizing vector species, and evaluating intervention methods (Foley *et al.*, 2012; Sutcliffe *et al.*, 2021).

Malaria vectors have been managed for the past two decades through the distribution of LLINs and IRS with significant progress in preventing malaria and related adverse outcomes. Different of African countries including Djibouti, Ethiopia, Sudan and Somalia reported of *An. stephensi* invasion and this vector has been for long a malaria vector in south-eastern Asia. These reports have been confirmed after the DNA molecular analysis (Balkew *et al.*, 2020; Kweka, 2022).

2.4. Malaria Diagnostic Tools

The WHO malaria control strategy has two key components. The first component is vector (mosquito) control through IRS and the widespread use of LLINs. The second focuses on improving diagnosis and treatment, with a particular emphasis on the increasing the use of diagnostic tools. The diagnostic tools currently available for the identification of *Plasmodium* spp. include light microscopy, rapid diagnostic tests (RDTs), Serology and Polymerase Chain Reaction (PCR) (WHO, 2021).

2.4.1. Light Microscopy

Microscopically, malaria is diagnosed by staining thick and thin blood films on a glass slide, using Giemsa stains to visualize malaria parasites. The “gold standard” for identifying malaria parasitemia is light microscopy, which allows detection, identification and quantification of various malaria causing parasites in a thick or thin smear of the patient’s blood. The technique involves preparation of thick or thin blood smear, staining it with 10% Giemsa stain and by examining malaria parasite under 100X oil immersion objective(Mathison & Pritt, 2017). LM is recommended by WHO as the "gold standard" for diagnosing symptomatic malaria. However its effectiveness in detecting silent infections (asymptomatic malaria) is often poor. LM is time-consuming and has poorer sensitivity than molecular approaches because, molecular techniques detects low parasite densities in asymptomatic infections. It has poor sensitivity in low transmission setting and in asymptomatic patients, resulting in underestimation of disease prevalence compared with the gold standard molecular diagnostic tool (PCR)(Zhao *et al.*, 2017).

2.4.2. Rapid diagnostic tests

RDTs are immune chromatography–based assays that detect malaria antigens, such as *Plasmodium falciparum*– specific histidine-rich protein 2 (HRP-2), pLDH and Plasmodium aldolase. 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 particular 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(Harvey & Bell, 2010).

RDTs have transformed malaria diagnosis by providing convenience and a quick turn-around time of about 15–20 minutes. RDTs 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/ μ L) (Mcmorrow *et al.*, 2011).

2.4.3. Serological Tests

In malaria diagnosis Enzyme-linked Immuno-sorbent assays (ELISAs) are used to detect or quantify antibodies against or antigens specific for malaria parasites. In essence ELISAs use an

enzyme linked to an antibody (or sometimes antigen) as a marker for the detection of a specific protein, typically an antigen or antibody. There are two most commonly used types of ELISA (depending on whether the ELISA is used to detect antigens or antibodies).

The first one is the detection of antigens (direct ELISA) in 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. 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 (Noedl, 2014).

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 bind 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 (Noedl, 2014; Tangpukdee *et al.*, 2009).

2.4.4 Polymerase Chain Reaction (PCR)

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. It involves using short synthetic DNA fragments called primers to select a segment of the genome to be amplified, and then multiple rounds of DNA synthesis to amplify that segment (Britton *et al.*, 2016).

To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. The original DNA is duplicated as a result of this process, with each of the new molecules comprising one old and one new strand of DNA. Each of these strands can then be used to

make two new copies, and so on, and so on. The cycle of denaturation and synthesis of new DNA can be repeated as many as 30 or 40 times, resulting to more than one billion exact copies of the original DNA segment. The entire cycling process of PCR is automated and can be completed in just a few hours. It is controlled by a machine called a thermocycler, which is programmed to change the temperature of the reaction process every few minutes in order to allow for DNA denaturation and synthesis(Rahman *et al.*, 2013).

Compared with conventional methods, PCR is highly sensitive in detecting low-density infections and determining the parasite species (Hawkes & Kain, 2007). PCR-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. PCR can detect as few as 1-5 parasites/ μ l of blood ($\leq 0.0001\%$ of infected red blood cells), whereas microscopy or RDTs can detect around 50-100 parasites/ μ l of blood (Tangpukdee *et al.*, 2009).

Asymptomatic infections and drug-resistant parasites can be detected by PCR and it is automated to process large volume of samples(Sloan & Rosenblatt, 2005). When performed under optimal conditions, the PCR technique can detect parasites below the threshold levels of microscopy and RDT. PCR can detect parasitaemia as low as 1 parasite/ μ l 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).

3: MATERIALS AND METHODS

3.1. Description of Study area

The study was conducted at Goro Health Center and Dire Dawa University (DDU) students clinic, Dire Dawa city, Ethiopia, from March 01 to May 30, 2022. Dire Dawa city is found 515 kilometers Southeast of Addis Ababa, 55 km to the north of historic city of Harar and 311 km to the West part of Djibouti. Dire Dawa is located at 9° 28' 1" N and 9 °49' 1" longitude and between 41° 38' 1" and between 43 °19' 1". In the north, east, and west the administration is bordered by the Somalia national regional state and in the south and south east by the Oromia national regional state (Fig 3)(Degife *et al.*, 2019). Dire Dawa is a commercial and industrial center located on the Addis Ababa–Djibouti railroad in the eastern part of Ethiopia which makes it a transit hub. Dire Dawa administration is character city administration that consists of 9 urban and 38 rural Kebeles. In all, the administration has a total land size of 1288 square, of which 97.73% accounts for the size of the rural area, while the remaining 2.27% covers the land size of urban areas found in the administration (UN-Habitat, 2008). The administration has a warm and dry climate with a low level of precipitation with the annual maximum and minimum temperature of 31.40 °C and 18.2°C, respectively. The administration has an average annual rain fall of 60 mm and humidity of 63%. The range of altitude of the land in the administration is between 960-2500 meter above sea level. Dire Dawa has a population of 341,834 of whom 171,461 are men and 170,461 women: 233,224 or 68.23% of the population are urban inhabitants (Mulualem & Fentie, 2015).

Malaria incidence has historically been low in Dire Dawa. Annual parasite incidence (API) has been below 5 between 2014 and 2019 (Nega *et al.*, 2021). Mosquito survey conducted from August to November 2018 in Dire Dawa city revealed that *An. stephensi* is widely distributed in the city (Balkew *et al.*, 2020). In terms of the distribution of health facilities, there are two governmental and five private hospitals, 15 health centers (eight in the city and seven in rural area), 5 higher clinics, and 12 medium clinics in the city. Goro health center and DDU student clinic were selected based on their malaria case report.

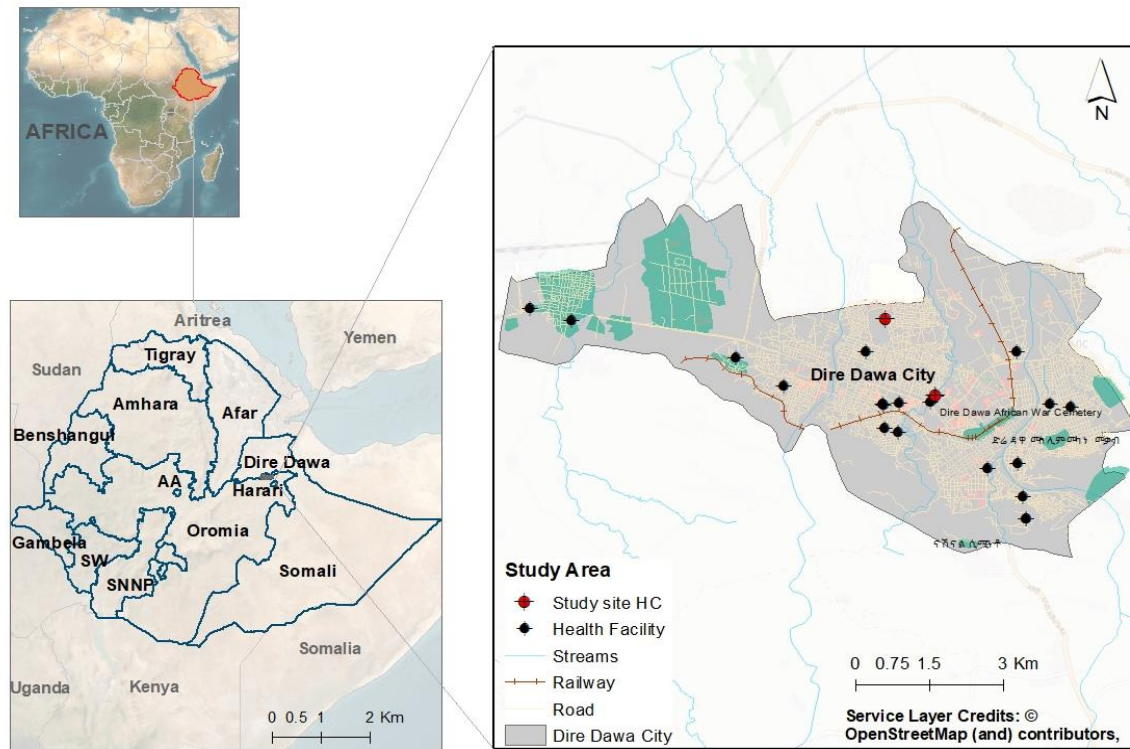


Figure 3: Map of Study Site, Dire Dawa City, Ethiopia (ArcGIS version 10.3.1)

3.2. Study design

The study was a case-control study in which identified *P. falciparum* malaria cases (index cases) and their family members were tested to investigate other malaria cases in the family members. The index cases were followed to their homes to test all family members for malaria. Individuals who attended the health center with negative microscopy result and with matched age group and gender as of index case were taken as a control and the same procedure was followed as of the index case (Fig 4). Blood sample was collected from finger prick of family members and tested by Rapid Diagnostic Test (RDT) (Abbott Bioline malaria Ag/Ab test kit, India), slide smear and Dried Blood Spot (DBS) was prepared and the remaining whole blood was kept in ethylene diamine tetra acetic acid (EDTA) tube. During the study period, adult mosquitoes were collected from homes of cases and their controls using CDC light traps catches and prokopack aspirator. Collected mosquitoes were morphologically identified by expert entomologists at Dire Dawa University (DDU) entomology laboratory, preserved in Eppendorf tube with silica gel (desiccant) and transported to Armauer Hansen Research

Institute (AHRI) for *Circumsporozoite* protein and blood meal analysis. Immature mosquitoes were also collected in 100meter radius from participants' homes and reared to adults.

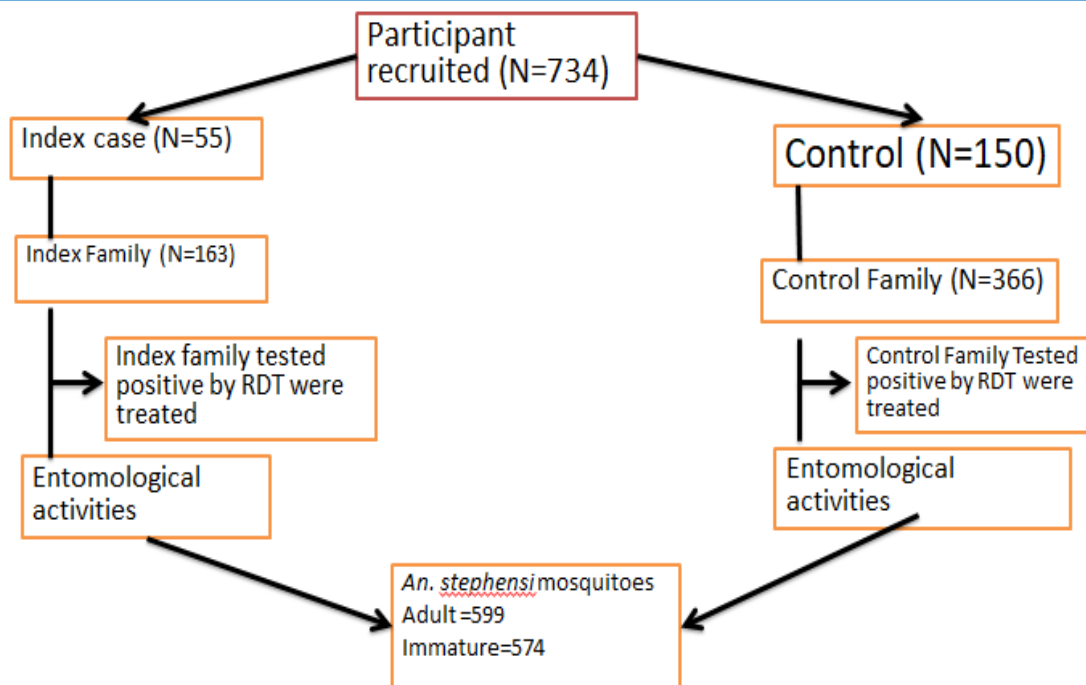


Figure 4: Flow chart of the participants included in the study.

3.3. Sample size determination and sampling Techniques

The sample size was determined using unmatched case control study formula by Epi-info 7.2 version considering confidence interval of 95%, Power 80%, case to control ratio 1:3, previously reported prevalence of malaria by qPCR (21.6%) and to detect an odds ratio of 2.7(Tessema et al., 2020). Goro Health Center and Dire Dawa University student clinic in Dire Dawa city were selected purposefully considering health facility with higher malaria report. A total of 734 participants were enrolled during the study period (55 index cases, 150 controls, 529 family members). The calculated sample size was divided to both health facilities based on their catchment population proportion.

3.4. Study variables

Dependent variables

Malaria infection

Independent variables

Socio-demographic characteristics (age, gender, educational status, occupation, family size), LLINs utilization, IRS, LLINs sharing, housing condition, water source for domestic use, sleeping outdoors at night, travel history to other malaria endemic areas, sleeping sites.

3.5. Data Collection

3.5.1 Questionnaire: Household questionnaire (Annex I) was used to collect participant's socio-demographic characteristics, quality of housing, travel history; ownership and utilization of LLIN, coverage of IRS and other risk factors for malaria infection. Entomological data including type and characteristics of mosquito breeding habitats, distance from participant's household and household characteristics were collected using entomological questionnaire (Annex II).

3.5.2 Laboratory data

Blood Sample collection:

Blood sample (~300µL) was collected from, a febrile patients visited Goro health center and DDU student clinic, a single finger prick of index case, controls and their respective family members in 1ml EDTA-coated microtainer tubes. Thick and thin smears were prepared for microscopic investigation of malaria parasites. Dried Blood Spots (DBS) samples containing three drops of 20µl each on filter paper was prepared, air dried at room temperature, packed in plastic ziplock bag with Silica gel and ≈200µl of the remaining whole blood was kept in EDTA tubes and stored in -20 °C freezer for further investigation.

Smear microscopy

Thick and thin blood smears were taken from all participants (from index case, controls and their respective family members) and prepared on the same slide for the detection and quantification of malaria parasites during the study period. The smears was stained

immediately with 10% Giemsa for 10–15 min for initial screening and examined by light microscopy immediately. Parasite density/ul was calculated by complying to standard formula shown below (WHO, 2016):

$\text{Parasite density (per } \mu\text{l)} = \frac{\text{Number of parasites counted} \times 8000}{\text{Number of leukocytes counted (approximately 200)}}$

Rapid diagnostic test (RDT):

RDTs were used to detect malaria infection in the family members according to manufacturer’s procedure(Boyce & O’Meara, 2017). Participants with positive test result were treated as per national treatment guideline(FMOH, 2012).

DNA extraction and quantitative PCR

Blood samples in EDTA tubes were used to extract DNA using MagMax DNA multi-sample kit. It was based on MagMAX™ magnetic bead technology on the KingFisher Flex robotic extractor machine (Thermo Fisher™). 50µl of EDTA preserved blood sample digested by proteinase K at optimum temperature before adding lysis buffer. After lysis, samples were mixed with isopropanol, then combined with paramagnetic beads with a DNA binding surface. The magnetic beads have a large available binding surface, allowing thorough nucleic acid binding, washing, and elution. The beads, with bound DNA, were immobilized on magnets and washed to remove proteins and other contaminants and then the DNA was eluted using a 150ul low-salt elution buffer.

Quantitative PCR (qPCR) for parasite detection and quantification was performed by targeting the 18S rRNA small subunit gene for *P. falciparum* and *P. vivax* using primer and probe sequences described by Hermsen and Wampfler, respectively(Hermsenet al., 2001; 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™ 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: 50°C for 2 minutes, 95°C for 10 min and 45 cycles of 95°C for 15 s, and 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.

3.5.3. Entomological data:

During the study period, adult mosquitoes were collected from homes of index cases and their control groups using Prokopack aspirator to collect and identify species of mosquito responsible for malaria transmission in Dire Dawa City. CDC light traps, powered by 6 V batteries, hung in the study participant's room and student's dormitory close to their beds at approximately window height. Traps were set in the early evening between 18:30 and 21:00 hours, depending on the numbers of homes to be visited each day and left to run throughout the night until about 07:00 hours the following morning. The Prokopack aspirator operated on a 6Vdry-cell battery placed in a custom-made pouch and attached to a belt around the collector's waist. Aspiration was carried out in the morning between 06:00 and 08:00 and conducted indoors (in all rooms of the house) and outdoors, moving the aspirator across walls, ceiling, near furnitures, near lockers and cupboards. Collected adult mosquito was taken to the DDU entomology laboratory for further species identification, counting and transported to AHRI for further blood meal analysis and sporozoite infection. When mosquito breeding site found in 100 meter radius from the control or index case home, immature mosquitoes were collected, reared to adults. Adults emerged from pupa were collected from the rearing cages, and morphologically identified at DDU entomology laboratory, preserved in Eppendorf tube with silica gel desiccant to determine the larval density and identification of vectors.

3.5.4 Malaria Secondary data from health facilities

A secondary malaria data of 4 years was collected from 34 government and private health facilities across Dire Dawa city administration (Figure 5) to evaluate the trend of malaria cases

from 2019 to May 2022. The data was retrieved directly from laboratory registration logbook using prepared data capturing sheet. The purpose of collecting the data was to see whether the malaria case increased or decreased after the detection of *An. stephensi* vector in Dire Dawa city. The summarized data was used to examine the contribution of *An. stephensi* to the recent malaria case increases in Dire Dawa city administration.

3.6. Circumsporozoite protein (CSP) analysis

All collected adult mosquitoes were tested for presence of sporozoites, using Circumsporozoite protein (CSP) magnetic-bead immunoassay (MAGPIX[®]-Luminex) technology. The heads and thoraces from all morphologically identified fed adult mosquitoes were separated from legs, wings, and abdomens between the second and third legs using a scalpel (Foley et al., 2012). These heads and thoraces were assayed to detect antibodies against the CSP of *P. falciparum*, *P. vivax*-210 (Pv-210) and *P. vivax*-247 (Pv-247) according to the protocol described in the manual (Luminex, 2018; Sutcliffe *et al.*, 2021). The beads used in this process were received from CDC, Atlanta, USA.

3.7. Ethical approval

Ethical approval was obtained from the institutional review boards of AHRI-ALERT Ethics Review Committee (protocol number PO/07/19) Addis Ababa, Ethiopia. The information provided was confidential and used only for the purpose of the research. Participant confidentiality of information was secured and potential identifiers like name, mobile number and specific address was not be displayed. Written informed consent/assent was sought from each participant or his/her guardian in case of children for study participation. Parents or caretakers were asked to provide informed consent for child below 18 years of age (Annex III).

3.8. Data analysis

Collected data was cleaned for completeness and consistencies, coded and entered in to REDcap software version 11.0.3 and transported into the Stata software version 17.0 for further analysis. The results were organized, summarized and presented using texts, tables, and graphs. Simple descriptive statistics and chi-square tests were used to assess different variables. P-value <0.05 was considered as statistically significant result.

4: RESULTS AND DISCUSSION

4.1. Socio-demographic study results

A total of fifty five index cases and one hundred fifty controls, with seven hundred thirty four family members were enrolled in the study from March to May, 2022. Among the total participants, 35.3% (two hundred fifty nine participants) were from Goro Health Center and 64.7% (four hundred seventy five participants) were from Dire Dawa University (Table 1). Among the index cases 16 (29%) were from DDU and 39 (71%) were from Goro health center (Table 1).

Table 1: Description of participants enrolled in case control study in selected health facilities in Dire Dawa City (N=734)

Study Sites	Index case	Control	Index Family	Control family	Total
Goro Health center	39	103	112	221	475
Dire Dawa University student clinic	16	47	51	145	259
Total	55	150	163	366	734

Among 734 participants enrolled in the study, 470 (64%) were males while 264 (36%) were females. Among the index cases, 29% (16/55) were from DDU and 71% (39/55) were from Goro Health center. Regarding their age composition, 45 (6.1%) were 01-4 age group, 320 (43.6%) were 5-14 age group and 369 (50.3%) were greater than 15 age group respectively. Majority of participants were greater than 15 years old. Educational level of majority of participants was higher education. 367 (50%) of them had family size of greater than 5.

Table 2: Socio-demographic data from patients included in case control study in selected health facilities in Dire Dawa City, from March to May 2022 (N=734)

Variables	Category	Index family N=218(%)	Control family N=516(%)	Total (N=734)
Sex	Male	136 (62)	334(65)	470 (64%)
	Female	82 (38)	182(35)	264 (36%)
Age	0-4	13 (6)	32 (6)	45(6.1%)
	5-14	81(37)	239(46)	320 (43.6%)
	≥15	124 (57)	245(47)	369(50.3%)
Occupation	Government	13 (6)	39 (8)	52 (7%)
	Private	50 (23)	65(13)	115(16)
	House wife	19 (9)	46(9)	65(9)
	Daily laborer	4(2)	36(7)	40(5)
	NGO worker	0(0)	1(0.00)	1(0.00)
	Student	113 (52)	311(60)	424 (58)
	Other	17 (8)	20(4)	37 (5)
	Illiterate	26(12)	55(11)	81 (11%)
Educational level	KG	10 (5)	33(6)	43 (6%)
	Primary school	61(28)	115(22)	176 (24%)
	Highschool	35(16)	67(13)	102 (14%)
	Higher Education	86(39)	246(48)	332 (45%)
Family size	≥5	103	264	367 (50%)
	<5	115	252	367 (50%)

4.1. Malaria infection by age group

In this study, *P. falciparum* malaria infection was detected across all age groups and higher malaria prevalence rate was observed among people aged above 15 years 127 (21.5%) followed by 5-14 years old and 0-4 years old with prevalence rates of 15 (17.7%) and 4 (9.8%), respectively (Table 2). This may be related to the fact that they participate in outdoor activities since they are in charge of taking care of the family, and that young children are less likely to be exposed to mosquitoes because of parental or guardian awareness of malaria prevention and control measures, which reduces the likelihood that they will be. This finding is supported by study conducted in Dembia district, Northwestern Ethiopia (Agegnehu *et al.*, 2018). But in contrast to this result, study conducted in Shebe Sambo and Omo Nada districts of Jimma

Zone, Ethiopia, where the prevalence of malaria in 5-14 age group was found to be higher (Zemene *et al.*, 2018).

4.2. Parasite detection by RDT, Microscope and qPCR

Among the total participants, the prevalence of malaria among study participants by RDT, microscopy and qPCR was 13.4%, 12.4% and 19.3%, respectively. There were 91 individual participants confirmed of malaria positive by microscopy of which 90 were *P. falciparum* mono infection, and only 1 participant was positive for *P. vivax* and no mixed infection found by microscopy, whereas 141/734 (19.3%) found to be positive by the qPCR, which was different from the microscope result (Table 3). Detection and identification of malaria parasite at the species level between microscopy and qPCR was initially congruent for 80.5% of the cases.

Table 3: Comparison of the parasite detection by RDT, Microscope and qPCR, Dire Dawa city, from March to May 2022

Parasite detection tool	Negative		Positive		<i>P. falciparum</i>		<i>P. vivax</i>	
	No	%	No	%	No	%	No	%
RDT	636	86.6	98	13.4	97	13.2	1	0.1
Microscopy	634	86.4	91	12.4	91	12.4	0	0
qPCR	592	80.7	142	19.3	141	19.2	1	0.1

4.3. Risk factors associated with malaria positivity

Among the potential determinants explored regarding the positivity for malaria being a family member of index case, spending evenings away from home during night, using anti-mosquito spray in home, being male and proximity to mosquito breeding sites are significantly associated with positive test result for malaria (P value <0.00) (Table 4).

Table 4: Risk factors associated with malaria positivity by qPCR in Dire Dawa city, from March to May 2022

Variables	Category	Index family N(%)	Control family N(%)	Odds ratio	P value
Being a family member of index case		89 (41)	52(10%)	6.15	0.001
Spending evenings away from home	Yes	13 (6)	18(3%)	1.72	0.020
	No	76 (35)	34(6%)		
Sex	Male	59 (27)	47(9)	0.53	0.002
	Female	30 (14)	5(1%)		
Age	<5	3(1.4)	0(0)	2.01	0.060
	5-14	13(6)	1(0.2)		
	≥15	73(34)	51(10)		
Proximity to mosquito breeding sites (<100m)	Yes	15 (7)	0 (0)	6.04	0.004
	No	74(34)	52(10%)		
Using anti mosquito spray	Yes	8(3.7)	2(0.4%)	0.50	0.040
	No	79(36)	49(9.5%)		
Travel history	Yes	6(2.8)	5(1)	0.82	0.55
	No	83(38)	47(9.1)		
Malaria history in past Two weeks	Yes	4(2)	10(2)	3.20	0.025
	No	87(40)	50(10)		
Sleeping place	Indoor	2 (1)	2(0.4)	2.10	0.22
	Outdoor	4(2)	9(2)		
Bed net ownership	Yes	33(15)	6(1.2)	0.82	0.35
	No	56(26)	46(9)		
Bed net use	Always	11 (5)	1(0.2)	1.2	0.29
	Occasionally	11(5)	3(0.6)		
	Never	67(31)	48(9.3)		
Bed net sharing	Yes	17(7.8)	2(0.4)	0.98	0.95
	No	16(7.3)	4(0.8)		

Being a family member of the index case was significantly associated with malaria infection. The odds of being qPCR positive was six times higher among index family compared to the control family members (odds ratio [OR]; 6.03, 95%CI, 3.99-9.14; P<0.001). This might be

due to the fact that family members who have previous history of malaria infection can spread the disease to other family members by acting as a reservoir for plasmodium parasites. Our finding shows higher odds of malaria infection than study results conducted in Northwest Ethiopia (Negatu et al., 2020).

Participants who were living in 100m near to natural and man-made mosquito breeding habitat were more likely to have positive test result for malaria than the counterparts (2.04% 15/734; OR 2.55, 95%CI, 1.3-4.9; P value <0.004). This might be due to mosquito breeding sites being an ideal environment for Anopheles mosquitoes to reproduce. Mosquito breeding sites identified were natural habitats like Butuji stream and man-made habitats like cemented cisterns, discarded tires, ditches at the side of roads and plastic drums. This finding is in line with the case-control study conducted from October to November 2016, in Dembia district of Northwest Ethiopia (Agegnehu et al., 2018; Alemu et al., 2011).

Among the total, only 230 (31%) of the participants had bed nets and about half (50%) of the participants share their bed nets with other family members. Most participants (56.1%) did not use bed net in the previous night. The most widely identified reason for not using the bed net was; discomfort primarily due to excessive heat and not being able to hang the mosquito net due to high roof of the sleeping rooms (Table 2).

Spending evenings away from home significantly associated with malaria infection (4.2%, 31/734; odds ratio [OR]; 1.7, 95%CI, 1.1-2.7; P value <0.020). The finding is comparable with study conducted in Zanzibar. Understanding when and where individuals are exposed to malaria vectors is necessary for targeting interventions for malaria prevention (Monroe et al., 2019). The reported reason for staying and spending evenings away from living home are occupation related like security guards, students searching for internet access outside of dormitory and staying at restaurants.

Use of anti-mosquito spray in homes is significantly associated with malaria infection (1.40%, 10/734; OR 0.50, 95%CI, 0.25-0.97; P value < 0.040). Eighty eight (12%) participants use anti-mosquito spray in their houses. This can be justified as using anti-mosquito spray may decrease the risk of getting malaria by directly killing the mosquitoes. The finding presented here is consistent with study conducted in Hawassa, Ethiopia (Fikrie et al., 2021).

A total of 470 males and 264 females were enrolled in the study. Male sex is strongly associated with malaria infection (P value <0.002), which indicate that high malaria infection rate among males. The prevalence of malaria infection in males and females is 106/734 (14.4%) and 35/734 (4.8%), respectively. However, the difference in malaria prevalence among gender groups was not significant. The high malaria prevalence rate among males may be due to the fact that males usually stay in outdoor activities more frequently than females and hence are more exposed to mosquito bites. The finding agree with study conducted by the Carter Center in three regions (Oromiya, Amhara and SNNP) of Ethiopia during 2006-2007(Ayele et al., 2013).

Variables such as travel history, sharing bed net with other family members, sleeping places and house structure were not significantly associated (P value >0.05) with malaria infection. On the contrary to other studies, travel history is not associated with malaria infection in our findings. This suggests that the malaria infection in Dire Dawa city is not travel acquired and it was locally acquired infection which is in agreement with the previous report by(Hoogen et al., 2021) in Haiti.

Majority of the participants (93%) resided in houses constructed of brick or cement block walls with metal roofs and 4% lived in houses of mud or grass walls and grass roofs. Housing structure has no association with malaria infection. This finding is in contrary to the study conducted in Southern Zambia, which indicated association of house structure with *P. falciparum* infection. The study shows compared to low-quality houses constructed of mud or grass walls with grass roofs, residing in a medium- or high-quality house (houses with brick/cement block walls) was associated with significantly reduced odds of malaria (OR: 0.26, 95% CI: 0.09–0.73, P = 0.01) (Ippolito et al., 2017).

4.4. Identification of the vector responsible for malaria transmission

This case-control study conducted between March to May, 2022 in the Dire Dawa city of Eastern Ethiopia examined the association between *P. falciparum* malaria infection and presence of *An. stephensi* mosquito. This may be the first scientific evidence that shows strong linkage of the vector with malaria infection in Ethiopia. The female Anopheline mosquito carrying the malaria parasite requires a blood-meal after mating in order for eggs to develop.

As the female leaves the breeding site in search of a suitable human host, it is logical that individuals who are closer to where the search starts have a higher likelihood of being targeted.

Adult mosquitoes were collected from participant's homes, manholes, animal shelter, empty tankers in DDU compound and student's dormitory by prokopack aspirator. All collected mosquitoes were morphologically identified at species level at DDU entomology laboratory and transported to AHRI for further blood meal analysis and sporozoite infection. Of 619 adult mosquitoes collected, 599 (97%) were morphologically confirmed as *An. stephensi* (Table 5). This finding is comparable with study conducted in different towns of Eastern Ethiopia including Dire Dawa city (Carter et al., 2021).

Table 5: Adult mosquitoes collected from different resting sites at Dire Dawa city, from March to May 2022

Resting sites	<i>An. stephensi</i>	<i>An. gambiaes.l</i>	Other species	Total
Home/ dormitory	41	2	2	45
Animal Shelter	465	2	1	468
Manhole	51	12	1	64
Empty water tankers	42	0	0	42
Total	599	16	4	619

Boiled (at 100°C) head and thoraces of collected adult *Anopheles* mosquitoes (n=619) were tested for *Plasmodium* infections using a multiplex bead-based assay that targets the circumsporozoite protein. Only 4 mosquitoes were infected with *P. falciparum*, and 2 with *P. vivax*. Mixed species infection was not detected in all mosquitoes. *Anopheles stephensi* were positive for CSPs and hence are important vectors of malaria in the study area. This finding is compared to study conducted in Southwest Ethiopia (Abraham et al., 2017).

Immature stages of mosquitoes (larvae and pupae of *Anopheles*) were dipped from potential larval breeding habitats. These include man-made water containers, freshwater pools, stream margins, discarded tires, plastic containers and drainage ditches at the side of roads. Because of shortage of water in the city, water storage for household use and construction is common in

the city. These include metal and plastic tanks and barrels. To make brick for construction purpose, they store water in cemented cistern (constructed from cement and brick-stone).

In total, 694 larval and pupal samples were collected and reared to adulthood in DDU entomology laboratory. Each adult mosquito emerged from the pupa were identified to species level using morphological characters under a dissecting microscope. Accordingly, morphological analysis confirmed that 83% (574/694) were *Anopheles stephensi*(Table 6). This finding was supported by study conducted in ten towns of Eastern Ethiopia, including Dire Dawa city, in 2020 of which 2149 immature stages and 82 adult mosquitoes collected were all identified as *Anopheles stephensi*(Balkew et al., 2020). Our current study also confirms the wide scale distribution of the vector with its contribution in malaria transmission in the city.

Table 6: Number of mosquito species emerged from pupal stages at Dire Dawa city, March to May 2022

Habitat type	Mosquito species identified				Total
	<i>An. stephensi</i>	<i>An. gambiaes.l.</i>	<i>An. pretoriensis</i>	<i>An. turkhudi</i>	
Plastic and metal barrel	222	0	0	0	222
Stream edge	160	67	42	11	280
Water treatment pond	101	0	0	0	101
Plastic drum (roto)	52	0	0	0	52
Cistern	23	0	0	0	23
Ditches	16	0	0	0	16
Total	574	67	42	11	694

4.5. Malaria Secondary data from health facilities

A secondary malaria data of 4 years was collected from 34 government and private health facilities across Dire Dawa city administration to evaluate the trend of malaria cases from 2019, to May 2022 (Fig 5). The summarized data was used to examine the contribution of *An. stephensi* to the recent malaria case increases in Dire Dawa city administration. *An. stephensi*

was first detected in Dire Dawa in December 2018 and since then there is an increase of malaria cases in the city. The dominant malaria parasite was *P. falciparum*.

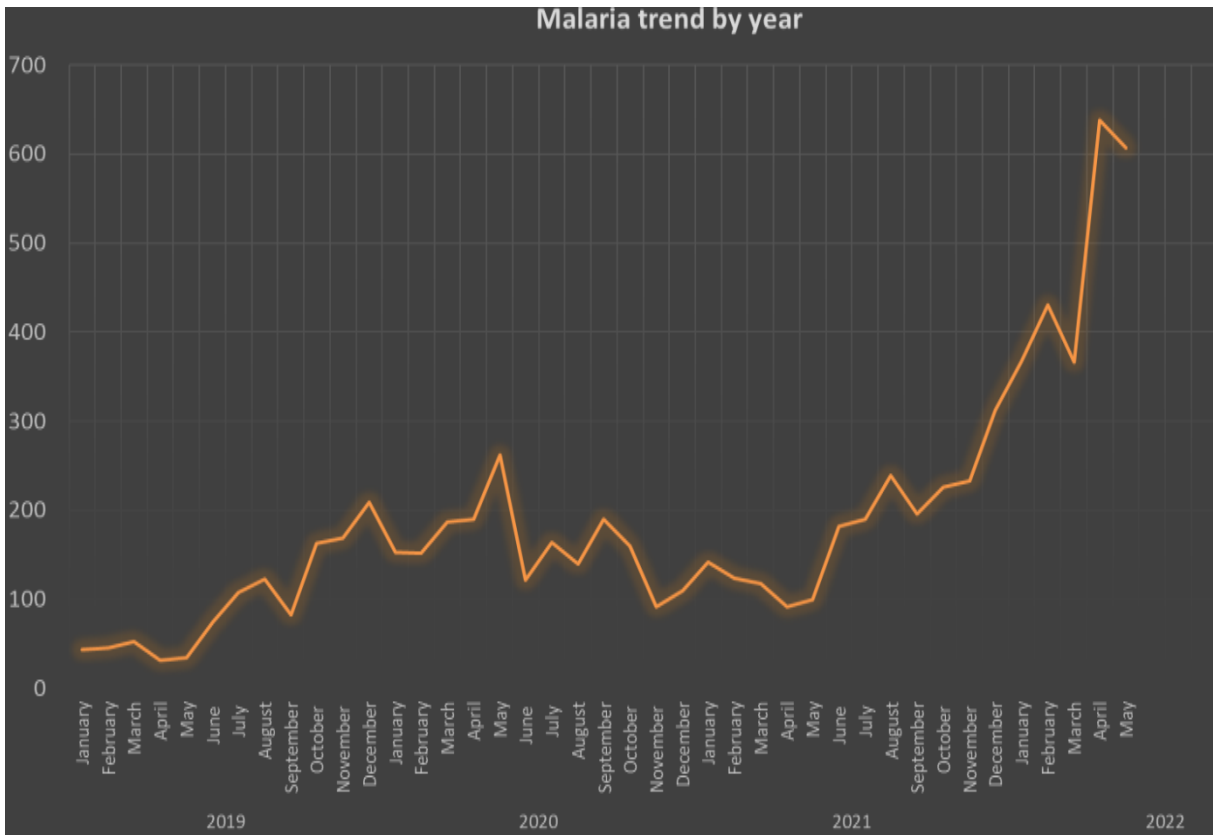


Figure 5: Trend of *P. falciparum* malaria cases since the detection of *An. stephensi* in Dire Dawa city from 2019 to 2022.

5: CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study describes that the majority of malaria infection in Dire Dawa city is locally acquired and driven by the vector *An. stephensi*. The identification of *An. stephensi* in Ethiopia has important implications for understanding malaria transmission in Ethiopia. Malaria infection may become a much bigger problem and vector poses a threat to malaria control and elimination in Ethiopia cities if the mosquito continues to spread and uncontrolled. The results of this study contribute for the broader understanding of malaria vector composition in Dire Dawa city, which indicate the need for vector management and control.

In addition, *P. falciparum* was identified as the most virulent species and the main cause of infections in the region. Individuals with male sex, family member of the index cases, individuals having family history of malaria in the past two weeks, individuals who live closer to the mosquito breeding sites and who spend evenings away from home were at higher risk of contracting malaria.

Water treatment pond in DDU compound, Butuji water stream around Goro health center, uncovered water containers like plastic drums, barrels, discarded tires, ditches at the side of roads are among the identified potentials mosquito breeding habitats in Dire Dawa city. Animal shelters, manholes, empty water tankers and plastic drums are among the identified mosquito resting sites. Based on the above conclusion we would like to forward the following recommendations:

- The wide scale distribution of *An. stephensi* in Dire Dawa city suggests that Dire Dawa Health Bureau should establish larval source management and enhance surveillance to control the vector.
- Covering any water containers tightly like barrels, plastic drums, cemented cisterns, cleaning animal shelters regularly, keep using bed nets and indoor sprays can also help to prevent the mosquitoes from laying eggs in the containers.
- To expand and sustain the combined application of ITNs, IRS and larval source management in the city

- Since infected family members serve as sources of infection for the rest of the family members, it is important to encourage infected family members to seek timely treatment in order to reduce the possibility of spreading malaria infection to other family members.
- Water treatment pond around DDU buildings, as it is the potential breeding habitat for *Anopheles stephensi*, needs to be managed and sprayed with chemicals.
- There is a need for collaborative and team work to organize capacity building for laboratory and field entomologists in identification of *An. stephensi* mosquitoes.

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7. APPENDICES

Annex-I:Form-1-Questionnaire:Epidemiological and Entomological characteristics of Plasmodium falciparum Malaria in Dire Dawa city of Ethiopia.

Participant Information		FORM-1
1. Patient ID _____	2. Health Center _____	3. Date of visit __/__/____
4. Age (years) _____	5. Sex <input type="checkbox"/> Male <input type="checkbox"/> Female	6. Kebele _____
7. Microscopy result <input type="checkbox"/> Neg <input type="checkbox"/> PF <input type="checkbox"/> PV <input type="checkbox"/> Mixed		8. Temperature: _____ °C
9. Treatment given and Duration: <input type="checkbox"/> Coartem 4 or 3days <input type="checkbox"/> Coartem 4 or 3days + PQ stat <input type="checkbox"/> Chloroquine for 3days <input type="checkbox"/> CQ for 3days + PQ for 14 days <input type="checkbox"/> other _____		Phone No. __

2. Malaria related

Questions	Responses	
1. Have you been sick with malaria in the past 2 weeks?	(1) <input type="checkbox"/> Yes	(1) <input type="checkbox"/> No
2. If Yes, Have you treated?	(1) <input type="checkbox"/> Yes	(1) <input type="checkbox"/> No
3. If Yes, Name of Drug/s and duration _____		
4. Had fever in the last 48 hours?	(1) <input type="checkbox"/> Yes	(1) <input type="checkbox"/> No

B. Household Characteristics

1. What is the main source of water for the household?

(0) <input type="checkbox"/> Piped water	(3) <input type="checkbox"/> rainwater	(6) <input type="checkbox"/> surface water (River, Lake, Pond, Stream)
(1) <input type="checkbox"/> dug well	(4) <input type="checkbox"/> tanker truck	(7) <input type="checkbox"/> bottled water
(2) <input type="checkbox"/> water from spring	(5) <input type="checkbox"/> cart with small tank	(8) <input type="checkbox"/> other (specify) _____ (9) <input type="checkbox"/> NA

2. What kind of toilet facility do members of your household usually use?

(0) <input type="checkbox"/> Flush or Pour	(2) <input type="checkbox"/> Pit latrine	(4) <input type="checkbox"/> No facility/Bush/Field
(1) <input type="checkbox"/> Flush Toilet	(3) <input type="checkbox"/> Bucket toilet	(5) <input type="checkbox"/> Other (Specify)

3. Do you share toilet facility with others? (1) Yes (2) No

4. What type of fuel does your household mainly use for cooking? (Multiple responses)

(0) <input type="checkbox"/> Electricity	(2) <input type="checkbox"/> Wood	(4) <input type="checkbox"/> Animal Dung	(6) <input type="checkbox"/> other (specify) _____
(1) <input type="checkbox"/> Kerosene	(3) <input type="checkbox"/> Charcoal	(5) <input type="checkbox"/> LPG	(7) <input type="checkbox"/> NA

5. Does any member of your household own any agricultural land? (1) Yes (2) No (3) NA

6. What kind of waterbody exists in your neighborhood?

(0) <input type="checkbox"/> None	(2) <input type="checkbox"/> Stagnant Water	(3) <input type="checkbox"/> Swamp	(4) <input type="checkbox"/> Lake
(5) <input type="checkbox"/> River	(6) <input type="checkbox"/> Pond	(7) <input type="checkbox"/> Stream (8) <input type="checkbox"/> others (Specify) _____	

7. How far (in Minute) is the nearest water body located from your house on walking?

(0) <input type="checkbox"/> 5-10	(2) <input type="checkbox"/> 15-30	(4) <input type="checkbox"/> 45-60
(1) <input type="checkbox"/> 10-15	(3) <input type="checkbox"/> 30-45	(5) <input type="checkbox"/> > 60

8. How far (in minutes) is the nearest health facility from your house on walking?

(0) <input type="checkbox"/> 5-10	(2) <input type="checkbox"/> 15-30	(4) <input type="checkbox"/> 45-60
(1) <input type="checkbox"/> 10-15	(3) <input type="checkbox"/> 30-45	(5) <input type="checkbox"/> > 60

Form 2--Household information,

Index Case ID _____ Control ID (For Controls only) _____ Address: Woreda/ city _____ Kebele _____

GPS coordinate: Latitude _____ Longitude _____ Altitude _____

<i>Now I would like to ask some information about the people who usually live in your household or who are staying with you now.</i>									
Participant Code									
S / N	Usual residents and visitors	Name 1 (Index Case/ Control)	Name 2	Name 3	Name 4	Name 5	Name 6	Name 7	Name 8
1	Please give me the names of the persons who usually live in your household and guests of the hh who stayed here last night, starting with the head of the household.								
2	What is the relationship of (NAME) to the head of the household?*	1=Head of HH	1=Head of HH	1=Head of HH	1=Head of HH	1=Head of HH	1=Head of HH	1=Head of HH	1=Head of HH
		2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband	2=Wife/ Husband
		3=Child	3=Child	3=Child	3=Child	3=Child	3=Child	3=Child	3=Child
		4=Brother/ Sister	4=Brother / Sister	4=Brother / Sister	4=Brother / Sister	4=Brother/ Sister	4=Brother / Sister	4=Brother/ Sister	4=Brother/ Sister
		5=Relative	5=Relativ	5=Relativ	5=Relativ	5=Relative	5=Relativ	5=Relative	5=Relative
		6=Other (Specify) __	6=Other (Specify)	6=Other (Specify)	6=Other (Specify)	6=Other (Specify) __	6=Other (Specify)	6=Other (Specify)	6=Other (Specify) __
3	Is (NAME) available now to participate in the study?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No	2=No
4	Sex	1=Male	1=Male	1=Male	1=Male	1=Male	1=Male	1=Male	1=Male

		2=Female	2=Female	2=Female	2=Female	2=Female	2=Female	2=Female	2=Female
5	Age								
6	Temperature (in degree Celcius)								
7	Occupation								
8	Educational level	<i>0=Illiterate-</i>	<i>0=Illiterat</i>	<i>0=Illiterat</i>	<i>0=Illiterat</i>	<i>0=Illiterate-</i>	<i>0=Illiterat</i>	<i>0=Illiterate</i>	<i>0=Illiterate-</i>
		<i>1=Kindergar</i>	<i>1=Kinder</i>	<i>1=Kinder</i>	<i>1=Kinder</i>	<i>1=Kinderga</i>	<i>1=Kinder</i>	<i>1=Kinderg</i>	<i>1=Kinderga</i>
		<i>ten</i>	<i>garten</i>	<i>garten</i>	<i>garten</i>	<i>rten</i>	<i>garten</i>	<i>arten</i>	<i>rten</i>
		<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>	<i>2=Primary</i>
		<i>School (1-8)</i>	<i>School (1-</i>	<i>School (1-8)</i>	<i>School (1-</i>	<i>School (1-8)</i>	<i>School (1-</i>	<i>School (1-</i>	<i>School (1-8)</i>
9	Does (NAME) usually live here?	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES
		2=NO	2=NO	2=NO	2=NO	2=NO	2=NO	2=NO	2=NO
10	If Yes, Did (NAME) stay here last night?	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES	1=YES
		2=NO	2=NO	2=NO	2=NO	2=NO	2=NO	2=NO	2=NO

Completed by: _____ Date (DD/MM/YYYY): _____

Checked by: _____ Date (DD/MM/YYYY): _____

Form 3- Intervention and Risk factor assessment

	Participant Code							
S. N	Characteristics	Name 1 (Index/Control	Name 2	Name 3	Name 4	Name 5	Name 6	Name 7
1	Name of Family							
2	Do you have LLIns for your own?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
3	Do you share your LLIns with a family member?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
4	If yes, Does your LLIns have holes?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
5	Observe the size of the LLIN holes?							
6	How often do you sleep under a mosquito net?	1=Always	1=Always	1=Always	1=Always	1=Always	1=Always	1=Always
		2=Occasionally	2=Occasionally	2=Occasionally	2=Occasionally	2=Occasionall	2=Occasionally	2=Occasionally
		3=Never	3=Never	3=Never	3=Never	3=Never	3=Never	3=Never
7	Slept under LLINs last night?	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No
8	If no why? **	0=Too hot	0=Too hot	0=Too hot	0=Too hot	0=Too hot	0=Too hot	0=Too hot
		1=Slept outdoors	1=Slept outdoors	1=Slept outdoors	1=Slept outdoors	1=Slept outdoors	1=Slept outdoors	1=Slept outdoors
		2=Unable to hang net	2=unable to hang net	2=unable to hang net	2=unable to hang net	2=unable to hang net	2=unable to hang net	2=unable to hang net
		3=Don't like	3=Don't like	3=Don't like	3=Don't like	3=Don't like	3=Don't like	3=Don't like
		4=others__	4=others__	4=others__	4=others__	4=others__	4=others__	4=others__
9	What times do you usually go to							

	sleep?LT							
10	What times do you usually wake up? LT							
11	Where did you typically sleep:	1=Indoors	1=Indoors	1=Indoors	1=Indoors	1=Indoors	1=Indoors	1=Indoors
		2=Outdoors	2=Outdoors	2=Outdoors	2=Outdoors	2=Outdoors	2=Outdoors	2=Outdoors
12	Did you travel away from home in last one month?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
13	If yes, where did you travel? Please specify the region, zone/ woreda/place name (NAME) traveled to.	Region____	Region____	Region____	Region____	Region____	Region____	Region____
		Zone____	Zone____	Zone____	Zone____	Zone____	Zone____	Zone____
		Woreda__	Woreda__	Woreda__	Woreda__	Woreda__	Woreda__	Woreda__
		Kebele__	Kebele__	Kebele__	Kebele__	Kebele__	Kebele__	Kebele__
14	Reason for travel?*							
15	Did you spend any evenings away from home in the last one month?	1=Yes;	1=Yes;	1=Yes;	1=Yes;	1=Yes;	1=Yes;	1=Yes;
		2=No;	2=No;	2=No;	2=No;	2=No;	2=No;	2=No;
		3=Don't know	3=Don't know	3=Don't know	3=Don't know	3=Don't know	3=Don't know	3=Don't know
16	If yes, where did you go?	1=school;	1=school;	1=school;	1=school;	1=school;	1=school;	1=school;
		2=church;	2=church;	2=church;	2=church;	2=church;	2=church;	2=church;
		3=outdoor bar/restaurant;	3=outdoor bar/restaurant;	3=outdoor bar/restaurant;	3=outdoor bar/restaurant;	3=outdoor bar/restaurant;	3=outdoor bar/restaurant;	3=outdoor bar/restaurant;
		4=Shopping	4=Shopping	4=Shopping	4=Shopping	4=Shopping	4=Shopping	4=Shopping
		5=Someone's house;	5=Someone's house;	5=Someone's house;	5=Someone's house;	5=Someone's house;	5=Someone's house;	5=Someone's house;
		6=Other specify	6=Other, specify	6=Other, specify	6=Other, specify	6=Other, specify	6=Other, specify	6=Other, specify
17	Specify name or exact location of							

	the venues							
18	Have you been ill with Malaria at any time in the last 1 year?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
19	If yes, how many times							
20	Have you been ill with Malaria at any time in the last three months?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
21	If yes, when exactly?							
22	Have you been ill with Malaria at any time in the last 2wks?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
23	If Yes, at any time during the illness, have you provided blood for examination?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
24	If yes, what is your examination result?	0=Positive	0=Positive	0=Positive	0=Positive	0=Positive	0=Positive	0=Positive
		1=Negative	1=Negative	1=Negative	1=Negative	1=Negative	1=Negative	1=Negative
		2=Don't Know	2=Don't Know	2=Don't Know	2=Don't Know	2=Don't Know	2=Don't Know	2=Don't Know
25	Did you seek treatment	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No	1=Yes 2=No
26	If Yes, Where did you seek treatment	0=Public	0=Public	0=Public	0=Public	0=Public	0=Public	0=Public
		1=Private	1=Private	1=Private	1=Private	1=Private	1=Private	1=Private
		2=Others __	2=Others __	2=Others __	2=Others __	2=Others __	2=Others __	2=Others __
27	What drugs did you take? Record all							

28	Did you take all your medicine as the health care provider told you	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
		3=Don't Know	3=Don't Know	3=Don't Know	3=Don't Know	3=Don't Know	3=Don't Know	3=Don't Know
29	Have you been ill with a fever at any time in the last 2wks?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
30	For Eligible Women (15-49yrs), ask:- are you currently pregnant?	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes	1=Yes
		2=No	2=No	2=No	2=No	2=No	2=No	2=No
		3=NA	3=NA	3=NA	3=NA	3=NA	3=NA	3=NA
		4=Don't Know	4=Don't Know	4=Don't Know	4=Don't Know	4=Don't Know	4=Don't Know	4=Don't Know

Form 4-Malaria parasite measurement & serological survey

	Name 1	Name 2	Name 3	Name 4	Name 5	Name 6	Name 7	Name 8
SAMP LE RDT	0=Done	0=Done	0=Done	0=Done	0=Done	0=Done	0=Done	0=Done
	1=Not present	1=Not Present	1=Not Present	1=Not Present	1=Not Present	1=Not Present	1=Not Present	1=Not Present
	2=Refused	2=Refused	2=Refused	2=Refused	2=Refused	2=Refused	2=Refused	2=Refused
	3=Other_	3=Other_	3=Other__	3=Other__	3=Other_	3=Other__	3=Other___	3=Other__
RDT RESU LT	0=Neg	0=Neg	0=Neg	0=Neg	0=Neg	0=Neg	0=Neg	0=Neg
	1=PF	1=PF	1=PF	1=PF	1=PF	1=PF	1=PF	1=PF
	2=PV	2=PV	2=PV	2=PV	2=PV	2=PV	2=PV	2=PV
	3=Mixed	3=Mixed	3=Mixed	3=Mixed	3=Mixed	3=Mixed	3=Mixed	3=Mixed
	4=Invalid	4=Invalid	4=Invalid	4=Invalid	4=Invalid	4=Invalid	4=Invalid	4=Invalid
Sympt om	0=None	0=None	0=None	0=None	0=None	0=None	0=None	0=None
	1=Headache	1=Headache	1=Headache	1=Headache	1=Headache	1=Headache	1=Headache	1=Headache
	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs	2=Fever in the last 48 hrs
	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite	3=Loss of appetite
	4=Vomiting	4=Vomiting	4=Vomiting	4=Vomiting	4=Vomiting	4=Vomiting	4=Vomiting	4=Vomiting
	5=Joint pain	5= Joint pain	5= Joint pain	5= Joint pain	5= Joint pain	5= Joint pain	5= Joint pain	5= Joint pain
	6=Other__	6=Other__	6=Other__	6=Other__	6=Other__	6=Other__	6=Other__	6=Other__
TREA TMEN T	0=None	0=None	0=None	0=None	0=None	0=None	0=None	0=None
	1=CoArtem	1=CoArtem	1=CoArtem	1=CoArtem	1=CoArtem	1=CoArtem	1=CoArtem	1=CoArtem
	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ	2=CoArtem +Stat PQ
	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ	3=CQ+ 14days PQ
	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ	4=CQ+ 14days PQ
	5=Referral	5=Referral	5=Referral	5=Referral	5=Referral	5=Referral	5=Referral	5=Referral

FORM-2-A-Household Information

1.Participant ID _____ 2.Date of visit ____/____/____	3.Address:Region _____ Woreda/Town _____ Kebele _____ Site _____
4) GPS coordinate: Latitude _____ Longitude _____ Altitude _____	

B. Household Characteristics

1. Number of people in the family _____
2. No. of people slept in the house the previous night _____
3. Do you have LLINs in your house:- (1) Yes (2) No
4. If yes, how months ago did your household most recently get the LLINs? _____
5. How many LLINs do you have currently: _____
6. How many people slept under LLINs the previous night _____
7. Observe the LLINs brand (1) MAGNet (2) PermaNet 2.0 (3) DuraNet (4) Other _____
8. Observe if the LLINs were hanged? (1) Yes (2) No
9. Does this household own any livestock, herds, other farm animals, or poultry? (1) Yes (2) No
10. If yes, how many of the following animals does your household have in your compound?

(1) <input type="checkbox"/> Sheep ____ (2) <input type="checkbox"/> goat ____ (3) <input type="checkbox"/> cattle ____ (4) <input type="checkbox"/> donkey ____ (5) <input type="checkbox"/> Horse/Mule ____ (6) <input type="checkbox"/> camel ____ (7) <input type="checkbox"/> Chickens ____ (8) <input type="checkbox"/> Dog ____ (9) <input type="checkbox"/> Cat ____ (10) <input type="checkbox"/> others (Specify) ____

11. Measure the distance (in meters) of animals shelter from the house _____
 GPS Coordinate: Latitude _____ Longitude _____

12. Observe main materials of floor.

(0) <input type="checkbox"/> Earth/Sand (3) <input type="checkbox"/> palm/bamboo (2) <input type="checkbox"/> Wood planks (6) <input type="checkbox"/> Ceramic tiles (8) <input type="checkbox"/> Carpets (1) <input type="checkbox"/> Dung (4) <input type="checkbox"/> Parquet or polished wood (5) <input type="checkbox"/> vinyl or asphalt strips (7) <input type="checkbox"/> Cement (9) <input type="checkbox"/> Other _
--

13. Observe main materials of walls.

(0) <input type="checkbox"/> No Walls	(3) <input type="checkbox"/> Bamboo with mud	(6) <input type="checkbox"/> Cardboard	(9) <input type="checkbox"/> Bricks
(1) <input type="checkbox"/> Cane/trunks	(4) <input type="checkbox"/> Stone with mud	(7) <input type="checkbox"/> Uncovered adobe	(10) <input type="checkbox"/> Reused wood
(2) <input type="checkbox"/> Dirt	(5) <input type="checkbox"/> Plywood	(8) <input type="checkbox"/> Cement	(11) <input type="checkbox"/> other _____

14. Observe main material of roof?

(0) <input type="checkbox"/> no roof	(3) <input type="checkbox"/> card board	(6) <input type="checkbox"/> wood	(9) <input type="checkbox"/> Ceramic tiles
(1) <input type="checkbox"/> thatch/palm leaf/ straw	(4) <input type="checkbox"/> Palm/bamboo	(7) <input type="checkbox"/> Cement	(7) <input type="checkbox"/> Other _____
(2) <input type="checkbox"/> rustic mat	(5) <input type="checkbox"/> metal	(8) <input type="checkbox"/> roofing shingles	

15. Observe main material of Ceiling?

(0) <input type="checkbox"/> no ceiling	(2) <input type="checkbox"/> ceiling	(3) <input type="checkbox"/> Sheet
(4) <input type="checkbox"/> Wooden	(5) <input type="checkbox"/> Fiber	(6) <input type="checkbox"/> other _____

16. Observe the eaves (open or closed)? (1) open (2) Closed (3) Partially opened

17. Has your household been sprayed with insecticide in the last 12 months? (1) Yes (2) No

18. If yes, when exactly does it sprayed? _____

19. Do you use anti-mosquito spray in your house? (1) Yes (2) No

20. If yes, how often do you use anti-mosquito spray in your house?

Always (2) Sometimes (2) Never

21. Did you burn or smoke repellents to prevent mosquito entrance to your house last night?

(1) Yes (2) No

22. If yes Q16, did you use anti-mosquito spray in your house last night? (1) Yes (2) No

23. Mosquito collection method _____ Mosquito collection time _____

24. Mosquito Collection place :- (1) Indoor (2) Outdoor _____

25. Mosquito collected (Species) 1=Female Anopheles _____ 2=Culex _____ 3=Aedes _____

26. Species of Anopheles Mosquitoes 1=*An.stephensi* _____ 2=*An.gambiaes.l.* _____

27. Abdominal status:- 1=Unfed _____ 2=Fed _____ 3=half gravid _____ 4=gravid _____

Annex-III-Consent and Assent Forms

Form 1-Consent Form for Adults and household head (≥18 Years)

Title: Epidemiological and Entomological characteristics of *Plasmodium falciparum* Malaria in Dire Dawa city of Ethiopia.

Participant ID: _____

I have been invited to participate in a study that aims to assess the current distribution and risk factors of urban malaria. I read/have been read and understood the information sheets attached. I had the opportunity to discuss the study and ask questions.

I understand that, if I do not wish to participate in the study I will still receive the standard health care that I deserve. I am also aware that I can withdraw my consent at any time on my own.

I agree to participate in the study and get diagnosed for malaria as per the protocol; get my sociodemographic data documented, my finger prick samples be transported to AHRI/ALERT for further analysis with highly sensitive tools and report immediately on any discomfort I might experience.

I also agree for the study team to collect mosquitoes by installing mosquito collection tools to collect adult and immature stages of mosquitoes from inside and around my house.

Category	Name	Signature/ fingerprint	Date (GC)
Name of participant			
Name of Person Obtaining Consent			

WITNESS (only necessary if participant is illiterate or incapable by law)

I witnessed that this consent form has been read to the participant in the language the participant and I understand, and the participant has agreed to enroll in the study.

	Name	Signature/ fingerprint	Date (GC)
Name of witness			

Form 2-Certificate of consent from the child`s family/guardian (12-17 years)

Title: Epidemiological and Entomological characteristics of *Plasmodium falciparum* Malaria in Dire Dawa city of Ethiopia.

Participant ID: _____

I have been invited for my child to participate in a study that aims to assess the current distribution and risk factors of urban malaria in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child to participate in this study.

I agree my child to participate in the study and get diagnosed for malaria as per the protocol; get his/her sociodemographic data documented, his/her finger prick samples be transported to AHRI/ALERT for further analysis with highly sensitive tools and report immediately on any discomfort my child might experience.

Name of child _____

	Name	Signature/ fingerprint	Date (GC)
Parent/Guardian			
Person Obtaining Consent			

WITNESS (only necessary if participant is illiterate or incapable by law)

I witnessed that this consent form has been read to the participant in the language the participant and I understand, and the participant has agreed to enroll in the study.

	Name	Signature/ fingerprint	Date (GC)
Name of witness			

Form 3: Consent Form for Parents or Guardians (For Children <12yrs)

Title: Epidemiological and Entomological characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, of Ethiopia.

Participant ID: _____

My child has been invited to participate in a study that aims to assess the current distribution and risk factors of urban malaria. I understood the information sheets, and had the opportunity to discuss the study and ask questions.

I understand that my child will get a finger prick for malaria tests today and to use for further analysis with highly sensitive tools. I understand that, if my child do not wish to participate and/or I do not want my child to participate in the study I/my child will still receive the standard health care I/my child deserve. I am also aware that I/my child can withdraw my/his or her consent at any time on my/her or his own or the decision of the physician/health professional.

I agree my child to participate in the study and get diagnosed for malaria as per the protocol; get his/her sociodemographic data documented, his/her finger prick samples be transported to AHRI/ALERT for further analysis with highly sensitive tools and report immediately on any discomfort my child might experience.

Name of child _____

	Name	Signature/ fingerprint	Date (GC)
Parent/Guardian			
Person Obtaining Consent			

WITNESS (only necessary if participant is illiterate or incapable by law)

I witnessed that this consent form has been read to the participant in the language the participant and I understand, and the participant has agreed to enroll in the study.

	Name	Signature/ fingerprint	Date (GC)
Name of witness			

Form 4: Assent form for participant age 12-17 years

Title: Epidemiological and Entomological characteristics of *Plasmodium falciparum* Malaria in Dire Dawa City, Ethiopia.

Participant ID: _____

I have been invited to participate in a study that aims to assess the current distribution and risk factors of urban malaria. I understood the information sheets, and had the opportunity to discuss the study and ask questions.

I understand that I will get a finger prick for malaria tests today and to use for further analysis with highly sensitive tools. I am aware that, if I do not wish to participate in the study I will still receive the standard health care I deserve. I also noted that I could withdraw my consent at any time.

I thus agreed to participate in the study and report on any discomfort I might experience immediately.

	Name	Signature	Date (GC)
Person Obtaining assent			
Child in the study (only if child assents)			