Addis Ababa University

Graduate Programme

Studies on Species Composition and Behaviour of *Anopheles* Mosquitoes (Diptera: Culicidae) and Insecticide Resistance Management Option for the Control of Malaria Vectors in Selected Sites in Butajira Area, Southern Ethiopia

By

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This is to certify that the thesis prepared by Esayas Kinfe Woldesilasse, entitled: 'Study on Species Composition and Behaviour of *Anopheles* mosquitoes (Diptera: Culicidae) and Insecticide Resistance Management Option for the Control of Malaria Vectors in Selected Sites in Butajira Areas, Southern Ethiopia' submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in Biology (Insect Sciences) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Dedication

This thesis is dedicated to my beloved wife Rahel Getachew and my sons Natenael Esayas and Yabetse Esayas and to those who are dedicated to their country.

List of Abbreviation and Acronyms

AIRS	Africa IRS
AS-PCR	Allele-specific PCRs
ASTMH	American Society of Tropical Medicine and Hygiene
BBI	Bovine blood index
BCC	Behavioural Change Communication
CDC	Centre for Disease Control
CDC-LTC	CDC- light trap collection
CIPAC	Collaborative International Pesticides Analytical Council
CSPs	Circum-sporozoite protein
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetracetic acid
EHNRI	Ethiopian Health and Nutrition Research Institute
EIR	Entomological inoculation rates
ELISA	Enzyme-linked immunosorbent assay

EPHI	Ethiopian public health institute
GABA	Gamma aminobutyric acid
GST	Glutathione S-transferase
HBI	Human blood index
HCl	Hydrogen chloride
HH	Household
HPLC	High-pressure liquid chromatography
IEC	Information Education and Communication
IRS	Indoor residual spray
KD	Knockdown
kdr	Knockdown resistance
LLINs	Long lasting insecticidal nets
MOH	Ministry of health
$MgCl_2$	Magnesium dichloride
MSAC	Mouth suction aspirator collection
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PMI	Presidents malaria initiative
Pv	*Plasmodium vivax*

RNA	Ribose nucleic acid
SNNPR	Southern Nations, Nationalities, and People's Region
SSC	Space spray collection
TA	tris-acetate
TAE	tris-acetate/EDTA
UV	Ultra violet
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

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Abstract

Detection and monitoring of insecticide resistance in malaria vectors is crucial and has to be conducted together with other entomological indices for effective vector control. The aim of this study was to identify species composition of *Anopheles* mosquitoes, behaviour and feasibility of resistance management using rotation of indoor insecticides spray combined with different insecticide impregnated long lasting insecticidal nets.

Monthly larvae and adult *Anopheles* collections were undertaken from May 2013 to June 2015 in Intervention (Jolie) and Control (Gogete) study sites. Identification of *Anopheles* species were done using morphological keys under a compound microscope as well as 10% of the adult specimens identified as *Anopheles gambiae* s.l. also subjected for species-specific polymerase chain reaction (PCR) test for sibling species detection.

Abdominal status, host and resting preferences, indoor resting density, parity rate, sporozoite rate, man biting rate and entomological inoculation rate were compared among the Intervention (Jolie) and Control (Gogete) study sites to evaluate the impact of the resistance management option used in Intervention study site (Jolie). Besides, *An. gambiae* s.l. susceptibility tests result carried out every year throughout the study period (in the month of August 2013, 2014, 2015 and 2016) to determine the susceptibility status of *An. gambiae* s.l. among the Intervention (Jolie) and Control (Gogete) study sites were compared to observe the impact of the resistance management option. To detect the presence of the L1014S and the L1014F *kdr* mutation a sub-sample of *An. arabiensis* and *An. gambiae* were selected from both study sites and tested using adapted versions of the allele-specific polymerase chain reaction (from October 2016 to April 2017).

A total of 4118 third and fourth instars larvae of *Anopheles* mosquitoes used for identification, ten in Intervention study site (Jolie) and eleven in Control (Gogete) *Anopheles* species were identified. Most of *Anopheles* larvae (27.5%) were collected from marshy breeding site which served as major breeding sites during the study period, followed by irrigation canals (24.5%), rain pools (20.4%), river pockets (17.6%) and ponds (9.9%). In both Intervention (Jolie) and Control (Gogete) study sites the predominant *Anopheles* species was *An. gambiae* s.l. which is the main vector in the country. Statistically significant ($p<0.05$) seasonal variations were observed in the larval collection of *Anopheles* larvae in Intervention (Jolie) and Control (Gogete) study sites.

In line with this a total of 4461 indoor adult female *Anopheles* were caught of which 1386 (31.1%) from Intervention (Jolie) and 3075 (68.9%) from Control (Gogete) study site. Out of 1386 of female *Anopheles* mosquitoes caught from Intervention study site (Jolie) *An. gambiae* s.l. was the predominant species making up 63.9% followed by *An. pharoensis* 19%. Similarly, out of 3075 of female *Anopheles* mosquitoes caught in Control study site (Gogete) also *An. gambiae* s.l. was the predominant species (64%) followed by *An. pharoensis* (19.1%). Significant ($p<0.05$) higher number of *An. gambiae* s.l. and *An. pharoensis* were collected in the Control (Gogete) than in the Intervention (Jolie) study site. Out of 239 *An. gambiae* s.l. subjected for PCR sibling species identification 94.1% were found *An. arabiensis* and 5.9% *An. gambiae*.

At species level in Intervention study site (Jolie) *An. gambiae* s.l. showed high tendency to feed on bovine 53.8% followed by human 47.9%, unlike in Control (Gogete) *An. gambiae* s.l. showed high tendency to feed on human 57.1% followed by bovine 40.6%.

Statistically significant differences ($p<0.05$) with low HBI in Intervention (Jolie) than Control (Gogete) study site were observed in *An. gambiae* s.l.

In Intervention (Jolie) the parity rate for *An. gambiae* s.l. was 45.8 and in Control (Gogete) 52.6 determined. Statistically significant differences ($p<0.05$) of parity in *An. gambiae* s.l. and *An. pharoensis* were observed among the study sites, that is low parity rate found in Intervention (Jolie) than Control (Gogete) study site. The result of sporozoite rate of *An. gambiae* s.l. in Intervention study site (Jolie) for *Pv*-210 was (1.1) and in the Control (Gogete) study site (2.8), however for *Pf* the same result 1.1 detected in both study sites. Statistical significant differences ($p<0.05$) were observed between Intervention (Jolie) and Control (Gogete) study sites on indoor resting density in both species of *An. gambiae* s.l. and *An. pharoensis*, as well as in man-biting rate of *An. gambiae* s.l.

An. gambiae s.l. resistance to DDT, permethrin, malathion, deltamethrin and bendiocarb were detected in both study sites. However, in Intervention (Jolie) *An. gambiae* s.l. susceptible populations increased in 2016 than 2013, that is significant ($p<0.05$) improvement on mortality rates due to the tested seven insecticides observed in the Intervention study site (Jolie), while in Control (Gogete) were not.

The L1014F *kdr* mutation in *An. arabiensis* detected at a relative high frequency 41.7% in Control study site (Gogete) and 35.9% in Intervention (Jolie). Besides, two in Intervention study site (Jolie) and seven in Control (Gogete) heterozygous alleles for L1014F were found in the tested *An. arabiensis* specimens. However in *An. gambiae* both

kdr mutations were not detected in both study sites. The L1014S *kdr* alleles were not found in the two study sites.

The breeding habitats of *Anopheles* mosquitoes in the study sites particularly in the dry season were due to human activities rather than environmental causes that require larval control. In the present study *An. gambiae* was reported for the first time in addition to *An. arabiensis* in Ethiopia indicating the presence of the highly efficient vector in Ethiopia, thus establishing appropriate control strategy for this species might be necessary.

Anopheles gambiae s.l. resistance to DDT, permethrin, malathion, deltamethrin and bendiocarb insecticides in the study sites is worrying. Moreover, West African *kdr* mutation was found in a higher rate this *kdr* mutation was related with high levels of both pyrethroid and DDT resistance than East African mutation and furthermore the presence of the incomplete heterozygous state indicates the continuity of *kdr* incidence shows the needs to urgently implement resistance management strategies.

The observed significant reduction of indoor resting density, human host preference, man biting, parity rate as well as increment of susceptibility in *An. gambiae* s.l. in 2016 compared to that of 2013 clearly indicates the effectiveness of the selected resistance management option applied in Intervention study site (Jolie).

Key words: malaria vectors, insecticides resistance, *kdr* mutation, larval habitat, Ethiopia

Chapter 1 General Introduction

1.1. Global epidemiology of human malaria

Malaria is an acute disease, which typically presents with chills, fever, and profuse sweating (MOH, 2004). The clinical features of malaria vary from mild to severe, according to the species of parasite present, the patient's state of immunity (Bruce-Chwatt, 1993). It is more sever in infants, children and pregnant women. Fever may continue for several days, accompanied by headache, aching joints, and general discomfort. The sign and symptom of malaria, with chills, shivering, high fever, and sweating may not occur. The onset of a malaria attack can bear a resemblance to influenza-like illness. In infants the symptoms of malaria can be understated and quite variable and may be limited to poor appetite, restlessness, and loss of normal interest in the surrounding (WHO, 1993; Goma, 1966).

The four major species of parasites: *P. falciparum, P. vivax, P. ovalae* and *P. malariae* are responsible for human malaria; these are protozoan parasites which attack the red blood corpuscles (Yamuchi *et al,* 2007). Out of the four parasites, *Plasmodium falciparum* is the most severe, and causes fatal malaria which accounts for 80% of all malaria incidences in the world and is the most common in tropical Africa (WHO, 2000; Boyd, 1994; Goma, 1966). Human host and some of female *Anopheles* mosquitoes are essential to the propagation of the human malaria parasites. In human the parasites survive and multiply in the red-blood corpuscles, liver and possibly other organs. Females *Anopheles* mosquitoes need a blood-meal before the eggs can develop (Ishino *et al*, 2004). The parasites enter into the gut of the mosquito along with the blood- meal

from an infected person, undergo series of developmental stages and finally the sporozoites invade salivary glands. At this stage the mosquito is able to infect its next human host (Foote and Cook, 1959; King *et al*., 1960).

Malaria is not only one of the major public health problems known to cause morbidity and mortality but also have an effect on economic development activities (MOP, 2016). The ultimate damage of malaria problem is human death; the less one is severity resulting from a period of sickness which may range from a couple of weeks to stay in hospital, or home bed with treatment. Malaria epidemics usually resulting from climatic changes aggravate the risk and burden of malaria. Epidemics occur when the factors limiting transmission are distorted as a result of either temporary climatic changes such as abnormal rains, long period of increase humidity and high temperature or more permanent changes of the microclimate such as the expansion of agricultural irrigation system, mining and road constructions (Bouma *et al*., 1994; Bouma and Vander, 1996; Sherman and Irwin, 1998).

Global warming may also influence the pattern of malaria, estimating the potential impact of climatic change on malaria transmission has generated great deal of global interest due in part to the concern that this devastating disease may emerge or re- emerge in many parts of the world (Bayoh *et al*., 2010). Population movements in the globe contribute to new outbreaks and make epidemic-prone situation more spread out in the world (Cohen *et al*, 2008). A similar epidemiological pattern accompanies such development scheme as railway, road construction and large irrigation project that undertaken in highly malarious areas threaten the life of human being (Shanks *et al,* 2002). Displacement either a consequence of war or the result of natural disasters leads to

an increase in creating potential vector breeding places and human-vector contact (Packard, 1986; Loevinsohn, 1994).

In 2004, 107 countries and territories had areas at risk of malaria transmission; 3.2 billion people lived in prone areas at risk of malaria transmission. An approximated 350–500 million clinical malaria episodes occurred annually; most of these are caused by infection with *P. falciparum* and *P. vivax* (WHO, 2004). Malaria causes about 466, 000 deaths each year, out of which 90% of the malaria cases and deaths occur in Africa countries. It also contributes indirectly too many additional deaths, mostly in young children, through synergy with other infections and illnesses (WHO, 2017).

The burden of malaria increased in Africa, the reasons for this were resistance to frequently used antimalarial drugs in the past, the deterioration of primary health care services in many areas and the emergence of resistant mosquitoes to insecticides used for vector control (WHO, 2016, 2017).

1.2. Malaria disease burden in Ethiopia

The distribution of malaria in Ethiopia is interconnected to dissimilarity in altitude, topography and climate (Ayele *et al,* 2012). Malaria is common in areas where lying below 2000 meters, but highly prevalent below 1500 meters. Areas lying at altitudes of 1500-2000 meters are prone to occasional epidemics of malaria (MOH, 2006). It is believed that malaria is absent in areas above 2500 meters where the climatologically factors inhibit the survivorship of vector species and the development of the parasite in the mosquito (WHO, 2013). The country experiences three locally known climatic zones

cold "Dega", temperate "Woyna Dega and warm "Kola" (Gebre-Mariam, 1984; Tulu, 1993a).

The Cold Zone “Dega” above 2,500 meters altitude, with mean annual rainfall from 1,600 mm and mean annual temperature of 15^0c is usually free of malaria (Tulu, 1993a). In the Temperate Zone "Weyna Dega", between 1,500 – 2,500m altitudes, where mean annual rainfall is 400-2,400 mm and mean temperature 20^0c malaria does occur, but most regularly in areas below 2,000m altitude (Gebre-Mariam *et al,* 1988). Transmission may however occur in higher altitudes, sometimes up to 2,400m. In this area malaria is usually of low endemic and therefore highland populations in general usually have a low level of immunity, thus malaria in this area is very unstable (Gebre-Mariam, 1984; Tulu, 1993a).

In the Warm Zone "Kolla" below 1,500 m altitude characterized by rainfall totals of 100-900mm and mean temperatures of 20-30^0c, malaria is moderately to highly endemic (Tulu, 1993a)). In this zone the highly malarious areas of, Setit Humera, Metema, Gambella, Gode and Awash Valley (Gebre-Mariam, 1984; Tulu, 1993a).

Malaria occurs immediately after the small rainy seasons of March and April as well as following the long rain of June through September. Peak transmission occurs during the months of September to November (MOH, 2011). Malaria transmission continues less intensely in the wet seasons, several epidemics in the high land fringe areas caused numerous deaths (MOH, 2000; Tulu, 1993a).

Although, the upper limit for malaria transmission was considered as 2000 meters, periodic epidemics were recorded above this level (Covell, 1957; Chand, 1965). Consequently moderate to severe malaria epidemics was known to occur in the country

(Munga *et al,* 2013). In 1958 there was an epidemic in several regions affecting about 3 million people out of which 150,000 died (Fountain *et al*., 1961; Gebre-Mariam *et al.*, 1988). Moreover the epidemics in Ethiopia can be substantiated by its repeated events with similar feature but of lesser intensity in 1965, 1973, 1981 and 1982 (Gebre-Mariam *et al*., 1988). Recently frequent malaria epidemics of cyclical trends of variable magnitude were recorded for example in the year 1988, 1991, 1995 and 1998 in different parts of the country (MOH, 1999).

The highland populations are exposed to frequent episodes of epidemic waves; it has been consistently reported as a leading cause of morbidity and mortality in the past years (Abose *et al.,* 1998a; MOH, 1999). The magnitude of the problem of malaria burden in 2002/2003 has been worsened. The disease has been reported as the first cause of morbidity and mortality accounting for 20.4% admission and 27% in patient deaths (MOH, 2004). In non-epidemic year; 5-6 million clinical malaria cases reported from health facilities, however the number of malaria cases were reported from health facilities is only a portion of the actual magnitude (MOH, 2004). Children who seem to lack primary immunity and pregnant women are the main victims of the disease (Abose *et al.*, 1998b). The introduction of more than half a million non- immune high landers into the malaria endemic low lands has elevated malaria prevalence of the country from year 1984 to 1989. In general the problem of malaria in this country is aggravated by population movement to the lowland in agricultural and agro developmental projects, urban development as well as settlement operation (Nega and Haile-Meskel, 1991).

Ethiopia has now prioritized, national and regional elimination with a long-term ultimate goal of malaria eradication, the need to understand the ecological and epidemiological

characteristics of malaria transmission were essential for selective targeting of intervention measures. The need to analyze the distribution of specific malaria vectors is one of the prerequisites for meaningful entomological studies and for planning and monitoring of successful malaria control or eradication program. In addition, understanding the biological implications of wide-spread and long-term Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spray (IRS) use based on bionomics of mosquito is critical. Besides, a regular assessment and analysis of the country malaria vector situation in particular with relationships between transmission intensity pertain to, abundance of malaria vectors is necessary. Thus, effective resistance management strategy according to our local situation should be selected and practiced as soon as possible before the occurrence of high level.

1.3. Rationale of the study

The current malaria vector control in Ethiopia largely relied on LLINs and IRS, in fact these interventions are reliable and effective in a wide range of situations, even when the circumstances are sub-optimal and coverage is imperfect, the effect of resistance to insecticide may compromise this robustness. The observed multiple-resistance coupled with the occurrence of high *kdr* frequency in populations of *An. arabiensis* could profoundly affect the malaria vector control programme in Ethiopia. This needs an urgent call for implementing rational resistance management strategies (Balkew *et al*., 2010, 2012; Massebo *et al*., 2013b).

Furthermore, WHO recommended that all vector control interventions should include a resistance management strategy and the implementation of resistance management

measures that should not be prompted by proof of control failure or the appearance of resistance (WHO, 2009). The Insecticide Resistance Action Committee has recommended options to delay occurrence of insecticide resistance such as rotations, mosaics, combinations and mixtures. The Committee also suggested that, to make evidence based decision whether which option is appropriate, it depend on the finding of the research conducted in the local situation of each country (WHO, 2009). Thus, understanding the underlying resistance management option is important in making evidence based decision on alternative control strategies.

To summarize, this research attempt to investigate the seasonal abundance and distribution of malaria vectors. Besides, the effect and significance of combining LLINs with rotation of IRS to delay resistance were tested according to our local condition. Thus, the result of the study will provide an insight for the science world and great input for the study area in particular and Ethiopia in general.

1.4. Research objectives

1.4.1. General objective

To study species composition, feeding and resting behaviour and breeding habitats preferences of *Anopheles* mosquitoes, and investigate the impact of resistance management options towards the control of malaria vectors in selected sites in Butajira, Southern Ethiopia.

1.4.2. Specific Objectives

- To determine species composition and the principal *Anopheles* species accountable for malaria transmission in the study areas.
- To identify *Anopheles* mosquitoes breeding sites and their seasonal contribution for the transmission of malaria in the study areas.
- To evaluate density, host preference, parity and sporozoite rates of *Anopheles* mosquitoes between Intervention (Jolie) and Control (Gogete) study areas.
- To determining insecticides resistance level and kdr mutation in malaria vectors in Intervention (Jolie) and Control (Gogete) study areas.

Chapter 2 Literature review

2.1. World malaria distribution and burden

Malaria is endemic throughout 91 countries and territories in the year of 2015 causing 212 million clinical cases and 429,000 deaths the majority of which were in children aged less than five years in Africa (WHO, 2016). The majority of malaria cases in 2015 (90%) were from Africa south of Sahara mainly due to *P. falciparum* (WHO, 2016). Countries in South-East Asia and Mediterranean regions contributed only for 7% and 2% of malaria cases respectively in 2015. *Plasmodium vivax* accounted for 4% of malaria cases and distributes more in South East Asia, Eastern Mediterranean and horn of Africa. About 76% estimated malaria cases and 75% estimated malaria deaths due to *P. falciparum* occurs in 13 countries found in west, central and east Africa. India, Ethiopia, Pakistan and Indonesia accounted for 78% of *P. vivax* cases. *Plasmodium vivax* estimated to have been responsible for 3100 deaths of which 78% of deaths occurred in Ethiopia, India, Pakistan and Indonesia (WHO, 2016).

Among the five species of malaria parasites that infect human, *P. falciparum* and *P. vivax* are responsible for the causes of the greater prevalence in the world (WHO, 2015). Of these two species, *P. falciparum* is regarded as the greater threat because of high level of mortalities and its dominance on the world most malarious continent, Africa (WHO, 2015). But, regarding geographical coverage *P. vivax* has the widest geographical range distributing in temperate, tropics and subtropics zones (Gething *et al.,* 2012). Unlike its wide geographical distribution *P. vivax* is exceedingly rare in Sub-Saharan West Africa due to the absence of Duffy receptor in red cells where the merozoites of *P. vivax* use this

receptor for the invasion red blood cells (Mendez *et al.,* 2001). However, 33% and 25% of malaria cases both in Ethiopia and Eritrea are due to *P. vivax* (WHO, 2016). But only five percent of malaria cases in North Sudan, Somalia and Madagascar are also caused by *P. vivax* (WHO, 2016). *Plasmodium falciparum* is a tropical and sub-tropics species but sometimes found in temperate climate. *Plasmodium malariae* is patchily present over the same range as *P. falciparum* but much less common. *Plasmodium ovalae* is found in tropical Africa, but also occasionally found in West Pacific (Bruce-Chwatt, 1993). Recently confirmed human pathogen *P. knowlesi* is important in small geographical range in Oceania (White, 2008).

There is a substantial variation in the level of malaria transmission from country to country and even within country; many malaria endemic countries have areas with high transmission and low transmission or no transmission within a short distance of one another (WHO, 2015). Parasite rate defined as the proportion of individuals infected at given point in time is considered as a common measurement of malaria transmission intensity (Gething et *al.,* 2012). Based on parasite rate, malaria endemic area can be classified as holo-endemic, if the transmission is intense and year round. Hypo-endemic when the transmission is intense but there is a period of lower prevalence during the dry season. In meso-endemic areas, transmission is seasonal and regular whereas if the transmission is low and intermittent, the area is called hypo-endemic.

Transmissions in meso-and hypo-endemic areas are unstable where all age groups are at risk of clinical malaria. Malaria epidemics are a risk in low transmission setting due the lack of protective immunity all age groups are susceptible to malaria (Cox *et al*., 1999). However, most of malaria morbidity and mortality occurs in children less than five years

old in high transmissions countries during none epidemic years (WHO, 2016). The populations most at risk of epidemics are those living in highlands, arid and desert fringe zones (Cox *et al*., 1999).

In recent years, there is a remarkable improvement in the control of malaria worldwide. The reduction of malaria was associated with the wide scale deployment of malaria control interventions. Due to the scale up of international donors, wider coverage of LLINs, IRS and artimisinin-based treatment reduced malaria in Africa (WHO, 2016). Malaria was once a public health problem of developed countries. It is less than forty years since the final eradication of malaria in Europe and the United States was announced. The advent of DDT in malaria control, the adoption of new farming methods, mechanization, urbanization, greater access to medical care and wider use of quinine with lower prices are some of the factors that contributed to malaria eradication in Europe and America. In addition, improvements in construction methods made houses more mosquito-proof, which prevent host vector contact (Kweka, 2016; Reiter, 2008). This shows reduction of malaria in America and Europe in the past and in Asia and Latin America recently, is not only due to effective malaria control intervention, but also the real development activates that changes the living standard of the people.

2.1.1. Vectors of malaria

Among formally recognized species of *Anopheles* mosquitoes, seventy species are vectors of malaria under natural conditions and 41 of them are the dominant vector species/species complex (DVS) transmitting malaria at a level of major public health problem (Hay *et al.,* 2010). Out of around 140 species of *Anopheles* mosquitoes in Africa

(Harbach, 2013), at least 7 species are considered as dominant vector species in the continent. From these 7 species *Anopheles gambiae s.s* and *An. arabiensis* (from *An. gambiae complex*) and *An. funestus* are considered as primary vector species due to their wide distribution and efficiency in transmitting malaria in the continent. *An. melas* and *An. merus* (from *An. gambiae complex*) and *An. moucheti* and *An. nili s.l.* are considered as secondary dominate vector species with respect to distribution and vector of malaria (Sinka *et al.,* 2012).

Anopheles gambiae complex has 8 sibling species currently; *An. gambiae s.s.* Gilles, *An. arabiensis* Patton, *An. Coluzzii* Coetzee and Wilkerson, *An. bwambae* White, *An. melas* Theobald, *An. merus*, Dönitz, *An. quadriannulatus* Theobald, *An. amharicus* Hunt,Wilkerson, Coetzee and Fettene (Coetzee *et al.,* 2013). Except *An. melas* and *An. merus* that prefer salt water for breeding, others are fresh water species (Coetzee *et al.,* 2000). *An. gambiae s.s.,* is responsible for intense transmission due to its highly anthropophilic, endophilic and endophagic behaviour (Coetzee *et al.,* 2000) but recently due to mass distribution of LLINs and IRS spraying make shifting in species composition from anthropophilic and endophagic species (*An. gambiae s.s*) into opportunistic species (*An. arabiensis*) was observed in some parts of Kenya and Tanzania (Russell *et al.,* 2010; Mutuku *et al*., 2011).

However, in some areas populations of *An. gambiae s.s.,* continues to be the dominant vector because of insecticide resistant (Corbel *et al.,* 2007; N'Guessan *et al.,* 2007) and adjusting its behaviour (Sinka *et al.* 2010). The existence of *An. arabiensis* in large geographical areas compared to *An. gambiae s.s* and *An. funestus* is associated with the wider range in behaviours of the species (Sinka *et al.* 2010). For instance, its distribution

towards the arid areas Sahel and desert of Namibia and Botswana beyond those of either *An. gambiae s.s.,* or *An. funestus* showed the species tolerate drier environment. However *An. arabiensis* is absent from the humid, forested areas of western Africa (Sinka *et al.,* 2012). The adaptability and flexibility of *Anopheles arabiensis* to feed and rest outdoor on cattle or human make it difficult to control by traditional control methods such as LLINS and IRS that favour the species to become the dominate vector in areas previously considers as secondary vectors (Onyabe and Conn, 2001; Bayoh *et al.,* 2010: Mwangangi *et al.,* 2013; Lwetoijera *et al.,* 2014). On the other hand the behavioural and ecological plasticity of *An. gambiae s.s* indicated further potential speciation in the species. Cytogenetic analyses identify five chromosomal forms (Savannah, Mopti, Forest, Bamako and Bissau) whereas molecular studies found two molecular forms (M and S) (Coluzzi *et al.,* 1998; Della *et al.*, 2007). The M and S forms have different behaviours with respect to preference of larval habitat where the S form preferred temporary pools or puddles that only occur after rain whereas the M form are found in more permanent sites such as rice fields or flooded areas (Caputo *et al.,* 2008; Costantini *et al*., 2009).

The *Anopheles funestus* Giles group consists of nine species that are difficult to distinguish morphologically in adult stage. Four species, *An. funestus*, *An. vaneedeni*, *An. parensis* and *An. aruni* have identical morphology at all life stages and are known as the *Funestus* sub-group. The other species in the group are identified by difference in egg and larval stage, larval characteristics and chromosomal banding (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). All the species in *An. funestus group* are mainly zoophilic, while *An. funestus* is extremely anthropophilic (Coetzee and Fontenille, 2004)

and probably the first species to adapt itself to take blood meal from human (Charlwood *et al.,* 1995).

In addition, *Anopheles funestus* is efficient vector of human *Plasmodium* throughout its distribution and in some cases exceeding the role of other vectors in malaria transmission because of its highly anthropophilic and endophilic behaviour, longer longevity and late night biting behaviour (Gillies and De Meillon, 1968; Coetzee and Fontenille, 2004; Awolola *et al.,* 2005). For instance, earlier and recent studies showed sporozoite rate of 11% in Tanzania (Shiff *et al.,* 1995), between 2.8% and 14.6% in Burkina Faso (Costantini *et al.,* 2009) 5% in Cameroon (Antonio-Nkondjio *et al.,* 2002), 4.5% in western Kenya (McCann *et al.,* 2014) and 2.2% in Tanzania (Lwetoijera *et al.,* 2014). However, widespread use of IRS was successful in controlling *An. funestus* in area where the species was primary vector, by taking the advantage of its highly anthropohilic and endophilic behaviour (Smith 1966; Gillies and Smith, 1960).

Similarly, the introduction of impregnated mosquito nets highly reduced *An. funestus* s.s in intensive malaria transmission area in western Kenya (Gimnig *et al.,* 2003). The control measures are responsible for expansion of other members of *An. funestus* group that are more exophilic such as *An. vaneedeni*, *An. parensis*, *An. rivolurum* or *An. leesoni* which are occasionally or rarely transmit human malaria (Hargreaves *et al.,*2000). However, re-emergence of anthropophilic, endophagic and *P. falciparum* infected *An. funestus* was observed after a long period of losing its major role in transmitting malaria due to the introduction of insecticide treated nets in Asembo, western Kenya and Kilombero valley in Tanzania (McCann *et al.,* 2014: Lwetoijera *et al.,* 2014). Although, studies about the status of *An. funestus* group as a vector is highly reduced for the last 50

years (Coetzee *et al,* 2000; Sinka *et al.,* (2010) considered the species as the dominant primary vector species in Africa.

Anopheles melas in western Africa and *An. merus* in East Africa are most found in coastal brine waters where they are important specially when associated with *An. gambiae s.s.* (Lindsay and Martens, 1998). *Anopheles pharoensis* has a very wide distribution and occupies a broad variety of ecological zones. It is a vector that can maintain active transmission of malaria in areas where main vectors are not present, *An. quadriannulatus* and *An. amharicus* are markedly exophilic and zoophagic whereas endophilic tendency were reported in mixed cattle stables and human dwellings in Ethiopia (White *et al*., 1980). Though, both species has negligible malaria vectoral capacity under natural condition, although experimental infection with *P. falciparum* demonstrated its susceptibility (Taken *et al.,* 1999). The other member of *An. gambiae s.l., An. bwambae* is a much confined to a small area malaria vector in Uganda where it breeds in geothermal waters (Coetzee *et al.,* 2000). Early research in south west Uganda *An. christyi* considered as a vector of malaria. However, successive study in the area showed no additional sporozoite detection in *An. christyi* (Lindblade *et al.,* 1999).

2.1.2. Species of malaria parasites

The pathogen causing human malaria are protozoan parasites belong to the genus *Plasmodium*. *P. falciparum, P. vivax, P. malariae, P. ovalae* and *P. knowlesi* are known to cause human malaria (White, 2008). The life cycles of all species of human malaria are essentially the same. Infection begin when an infected female *Anopheles* mosquitoes inoculates sporozoites in a human host during blood meal. Majority of infective

sporozoites remain in the skin for six hours (Yamauch *et al.,* 2007) and that approximately one-third of these leaving the injection site may enter lymphatic and drain to the regional lymph nodes, other sporozoites trickle into the blood stream and traffic to the liver, resulting in multiple potential sites for sporozoite-host interaction (Ishino *et al.,* 2004). Sporozoites traverse the plasma membrane of the lymphatic and circulatory system before invading a hepotocyte, in which they developed into the next infective stage (Mota *et al.,* 2001). The sporozoite uses cell-traversal protein for passage from the circulatory system to hepatocytes through the liver sinusoidal cell layer (Kariru *et al.,* 2006).

The uninucleate extracellular stage sporozoite divides by mitosis to form the multinucleate liver stage schizont inside a parasitophorous vacuole and releases thousands free merozoite into the blood stream within to 16 days. The sporozoites of *P. vivax* and *P. ovalae* differentiate into either hypnozoites or into developing tissue schizonts in varying proportions, depending on the strain. The invasion of erythrocytes by merozoites involves multiple steps, including initial attachment, apical reorientation and junction formation followed by the entry of the merozoites into the erythrocytes, which is mediated through a connection of the ligand to the parasite's actin-myosin motor (Cowman and Crab, 2006). Some merozoites differentiate into sexual erythrocytic stages to produce male and female gametocytes.

The female *Anopheles* mosquito ingests the gametocytes which further develop into male and female gametes to form a diploid zygote (WHO, 2013). The zygote differentiates to an invasive ookinetes that penetrates the gut wall and attaches to the outer portion of the mosquito gut. Like sporozoite, the ookinete invade the mid gut epithelial cell by

microneme protein and develops into oocysts (Ishino *et al.,* 2004). When sprogony complete, the oocyst raptures, the sporozoites are released and migrate to salivary gland for injection into another host during blood meal visits. The malarial paroxysm that coincides with the release of merozoites from ruptured red blood cells (RMCS) starts with malaise, abrupt chills and fever rising to 39^0Cto 41^0C and increasing headache and nausea. The fever phase is followed by a profuse sweating that occurs over a period of 2 to 3 hours. Clinical manifestation common to all forms of malaria include jaundice, splenomegaly and malaria paroxysm (Bruce-Chwatt, 1993).

2.2. Malaria parasites and vectors in Ethiopia

Malaria is believed to be the disease having long history in Ethiopia. For instance, severe epidemic occurred in 1618 in regime of emperor Suseneyos around Gorgora, which was the main reason for the shifting of emperor sitting from Gorgora to Guzaraa (Alemu, 2000). However, systematic malaria studies were started by Italian and British scientists during brief occupation of Ethiopia by fascist Italia around World War II (Cox *et al.,* 1999). Malaria is a major public health problem in Ethiopia despite comparatively low malaria prevalence compared to highly malarias countries found in west, central and east Africa (WHO, 2016). The epidemiology of malaria in Ethiopia is more variable and unstable than any other country in Africa due to extraordinary diverse topography and climatic condition (MOH, 2012). About 75% of the country's land mass comprising 68% of the country's total population is vulnerable to malaria transmission, from this 27% (>1 cases per 10000 population) and 41% (0-1 cases per 1000) of population were exposed to higher and lower malaria transmission respectively in 2015 (WHO, 20016).

Although, historically Ethiopia has been prone to periodic focal and widespread malaria epidemics that occurred in five to eight years cycle, malaria epidemics have been largely absent since 2005, after the scale up of malaria treatment and control interventions (MOP, 2008). All members of age group of the population are at risk of severe disease due to lack of immunity, unlike other countries of Africa in which adults develop partial clinical immunity to the parasite. Since, peak malaria transmission often coincides with the planting and harvesting season, and the majority of malaria burden is among older children and working adults in rural agricultural areas, malaria caused school absence and heavy economic burden in Ethiopia (MOP, 2008).

2.2.1. Distribution of *Anopheles* species and their vector status

Studies on the distribution of Anopheles species and their status as vector were done by Italian and British expatriates at the beginning of twenty century. The works of Covell (1957), Verrone (1962a and 1962b) and O'Connor (1967) have contributed a lot in the identification and distribution of malaria vector in Ethiopia. A total 45 *Anopheles* mosquitoes have been documented in Ethiopia (Gillies and Coetzee, 1987; O'Connor, 1967), among which *An. gambiae* s.l. is the most prevalent. *Anopheles arabiensis* Patton and *An. amaharicus*, previously known as *An. quadriannulatus B* are the only two species in *An. gambiae* s.l., complex that are still reported in Ethiopia (Coetzee *et al.,* 2013). *Anopheles amharicus* is zoophagic and has no role in malaria transmission (Coetzee *et al.,* 2013; Fettene, 2004). The distribution of *An. amharicus* is in the highland of South-Western region and Northern regions co-existing with *An. arabiensis* (White *et al.,* 1980). It exhibits endophilic behaviour resting in animal shades and mixed dwellings. This

species was also reported to feed on man either indoors or outdoors in the presence of cattle (White *et al.,* 1974).

Anopheles arabiensis Patton is the most important and responsible vector of malaria in Ethiopia, despite highly diversified vectors of country (O'Connor, 1967; White *et al*., 1980; Taye *et al.,* 2006; Kibret et *al.,* 2010; Animut *et al.,* 2013a). *An. arabiensis* found in most parts of the country and breeds in different types of water bodies from small sunlit temporary breeding habitat produced after rain to permanent habitat found in river margin and on the shores of lakes (Kibret *et al.,* 2010; Kenea *et al.,* 2011; Animut *et al.,* 2012; Gone *et al.,* 2014).

Anopheles pharoensis Theobald is one of the secondary malaria vectors of Ethiopia (Kibret et *al.,* 2010; Animut *et al.,* 2013a). *Anopheles pharoensis* might be responsible for the transmission of malaria in absence or low number of *An. arabiensis*, particularly in dry season (Nigatu *et al.,* 1992; Abose *et al.,* 1998b; Kibret *et al.,* 2010). The co-existence of infected *Anopheles pharoensis* with *An. arabiensis* was reported in low and mid altitude localities of Butajira area and in central rift valley in Ziway area (Kibret *et al.,* 2010; Animut *et al*., 2013b). Previous studies showed that *An. funestus* and *An. nili* are important malaria vector in Gambella (Krafsur, 1970, 1977) but currently, the role of *An. funestus* (Massebo *et al.,* 2013a; Jaleta *et al.,* 2013; Gone *et al.,* 2014) and *An. nili* (Jaleta *et al.,* 2013) are uncertain because the species are reported rarely and none of them were positive for *Plasmodium* species. Both *An. funestus* and *An. pharoensis* prefer large, permanent water bodies with emergent vegetation (Gebere-Mariam *et al.,* 1988). For instance, the swamps in Baro River in western Ethiopia provide an environment that permits *An. funestus* to outsmart *An. gambiae s.l.* in numbers throughout the year.

Similarly, higher number *An. funestus* were collected in Bahar Dar in permanent papyrus swamps in southern edge of Lake Tana compared to *An. gambiae s.l.* and other five species present in sympatric (O'Connor, 1967).

The co-existence of infected *An. gambiae s.l.* with *An. funestus* and *An. nili* were reported in earlier study in Gambella (Krafsur, 1970). The global map of dominate malaria vector by Sinka *et al.,* 2012 indicated *An. funestus* still an important vector of malaria though recent entomological studies showed negative sporozoite and the zoophagic and exophagic tendency of species (Massebo *et al.,* 2013a; Gone *et al.,* 2014; Kenea *et al.,* 2016) unlike the antropophagic, endophagic and sporozoite infected *An. funestus* studied earlier in Gambella (Krafsur, 1970; Krafsur, 1977). PCR identifications of the *Funestus* Group in Ethiopia showed, the presence of none vector species *An. parensis* (Weeto *et al.,* 2004) instead of the highly effective vector *An. funestus s.s.* indicating the species is replaced by none vector species due to the IRS spraying and LLINs utilization as seen in South Africa (Hargreaves *et al.,* 2000).

Anopheles cinereus, *An. coustani*, *An. rhodesiensis*, *An. d'thali*, *An. maculipalpis* and *An. paludis* are indicated to be susceptible to malaria parasites in other parts of Africa (O'Connor, 1967) but the vector status and their importance in the transmission of malaria is not yet known except one study reported P. *vivax infected An. coustani* in south west Ethiopia (Yewhalaw *et al.,* 2014). *Anopheles christyi* was regarded as non vector with no epidemiological importance in malaria transmission in Ethiopia (Tesfaye *et al.,* 2011; Animut *et al.,* 2013b). This species was recorded in the highland fringe of Butajira area up to 2300m a.s.l (Tesfaye *et al.,* 2011; Animut *et al.,* 2013b). Although the species is generally regarded as zoophilic (Gillies and Demellion, 1968) a blood meal analysis

showed human blood was detected in small number of the species (Adugna and Petros, 1996; Animut *et al.,* 2013b). Recent studies on the status of *P. falciparum* and *P. vivax* CSP in *An. garnahami* and *An. christyi* in southern Ethiopia showed negative result (Animut *et al*., 2013b; Massebo *et al.,* 2013b). The sporozoite rate is the proportion of *Plasmodium* infected mosquitoes in population of local vector species (WHO, 2013). Sporozoite rate is determined by three methods; dissecting the salivary glands, ELISA and PCR. The dissection method is the gold standard method where the sporozoite can be determined by dissecting the salivary glands and detecting sporozoite under microscope (WHO, 1975). However, the dissection method is labour intensive, requires trained expertise and difficult to distinguish among the *Plasmodium* species (Burkot *et al.,* 1984; Wirtz et *al.,* 1987).

The second method is an enzyme-linked immunosorbent assay (ELISA) that detects *Plasmodium* circumsporozoite protein (CSP) from sporozoites in the thoracic or salivary glands or mature oocysts on midgut (Burkot *et al.,* 1984, Wirtz *et al.,* 1987). ELISA is species-specific and can detect precisely the four or mixed species easily. It is possible to perform a single specimen separately or pooled mosquito specimens of the same species (Wirtz *et al.,* 1987). The limitations of ELISA is, it overestimate the true salivary gland infection because the method detect CSP in other mosquito tissue or detecting the CSP from the oocysts bursting, which occurs two to three days before the sporozoites actually reach the salivary glands (Fontenille *et al.,* 2001).

The limitation of microscopy is the lack of sensitivity to very low-level infections (Arez *et al.,* 2000). Due to the limitation of microscopy and ELISA molecular tools are adopted to determine sporozoite rate with better sensitivity than microscopy and ELISA though

the method has its own limitation. Multiplex PCR, real-time PCR and duplex real-time PCR uses primers designed against species specific regions in the sequences encoding the small subunit ribosomal RNA (ssrRNA) to detect all *Plasmodium* species (Bass *et al.,* 2008). Sporozoite infection rate in Ethiopia is very low and a large number of specimens need to be tested in order to determine the actual infection rate (Abose *et al.,* 1998c). For instance, Krafsur, (1970) dissected 8348 *An. gambiae s.l.* collected from Gambella and he found 156 infected mosquitoes with sporozoite rate of 1.87%. Seasonal variations with the highest sporozoite rate were observed in October (4.97%) and November (5.43%) in the study conducted by Krafsur, (1970). Nigatu *et al.,* (1992) reported sprozoite rate 0.8% (2/262) by using ELISA both for *P. falciparum* and *P. vivax* in Gambella. A sporozoite rate of 0.24% and absence CSP in *An. amaharicus* was reported in south west Ethiopia (Fettene *et al.,* 2004).

Recent study conducted in different altitudinal transect of Butajira area showed *P. falciparum* and *P. vivax* sporozoite rate in *An. arabiensis* was 0.2% and 1.7% respectively (Tesfaye *et al,* 2011). In this study, *P. falciparum* infected *An. arabiensis* is not detected in mid and high altitude localities whereas *P. falciparum* sporozoite rate was 0.3% in low altitude locality (Tesfaye *et al,* 2011). *P. vivax* sporozoite rate of 3% and 2.5% in *An. arabiensis* in lower and mid level altitude localities respectively, indicating the dominance of *P. vivax* in the area (Animut *et al.,* 2013b). Similar to the highland fringe of Butajira area, in south central Ethiopia, *P. falciparum* CSP infected *An. arabiensis* was 0.3% in relatively higher transmission area in South west Ethiopia (Massebo *et al.,* 2013b). All of *An. arabiensis* tested for *Plasmodium CSP* found to be negative in south central and south west Ethiopia (Gone *et al.,* 2014; Gari *et al*., 2016;

Kenae *et al.,* 2016). However, a higher CSP infected *An. arabiensis* (16.7%) in April but lower (1-3%) between August and November was reported in traditionally irrigated area in Western Ethiopia (Jaleta *et al.,* 2013).

Anopheles pharoensis has been naturally infected in Egypt, Nigeria and Kenya (O'Connor, 1967) but enough infection was not found throughout Ethiopia. Nigatu *et al.,* (1992) detected 0.46 % (2/436) *P. falciparum* and *P. vivax* CSP infected *An. pharoensis* in Gambella. A study conducted in south central Ethiopia, Zeway area detected *P. falciparum* sporozoite rate of 0.59 % (3/509) (Kibret *et al*., 2010). In recent study conducted in highland fringe of Butajira area, the sprozoite rate of *P. vivax* was 2.5 % (2/79) in *An. pharoensis* (Animut *et al.,* 2013b). However, all of *An. pharoensis* tested for *Plasmodium* circumsporzoite was negative in the studies conducted in central Ethiopia, Zeway area and in southern western part of Ethiopia (Massebo *et al*., 2013b; Gone *et al.,* 2014; Kenae *et al.,* 2016).

The reason for such low rates might be due the relatively low malaria endemicity prevailing in Ethiopia compared to highly endemic regions of tropical Africa (WHO, 2016). Compared to the previous studies (Krafsur, 1970; 1978; O'Connor,1967) lower sporozoites rate in *An. arabiensis* observed in recent studies (Massebo *et al.,* 2013b; Animut *et al.,* 2013b; Gari *et al.,* 2016; Kenae *et al*., 2016) might be the decrement of malaria cases due to scaling up of control intervention (WHO, 2016). The other important reason for the low sporozoite rate in Ethiopia is the presence of *An. arabiensis* as a major malaria vector in different parts of the country.

Due to its opportunistic host preference (feeding both on human and cattle) (Gari *et al.,* 2016; Animut *et al.,* 2013b; Massebo *et al.,* 2013b) feeding behaviour (endophagy and exophagy) (Kenae *et al.,* 2016) and resting behaviour (endophily and exophily) (Gari *et al.,* 2016; Massebo *et al.,* 2013b) contact between human and the vector highly is reduced unlike highly anthropophilic and endophagic *An. gambiae* or *An. funestus* (Sinka *et al.,* 2012); so dropping in the sporozoite rate for *An. arabiensis* reported. Compared to *An. gambiae s.s.* and *An. funestus,* the sporozoite rate was lower for *An. arabiensis* in a study conducted in western Kenya (McCann *et al.,* 2014). When the vector species, *An. gambiae s.s.,* and *An. funestus* was shifted feeding from human to animal feeding significant reductions in sporozoite rates were observed in Coastal area of Kenya (Mwangangi *et al.,* 2013).

Blood meals source identification was performed by precipitin test, enzyme-linked immunosorbent assay (ELISA) and PCR (WHO, 1975; Beier *et al.,* 1998; Kent *et al.,* 2005). Earlier studies used precipitin test in principle that when the serum of the blood antigen in the mosquito is put in contact with a specific antiserum, a precipitin occurs at place of contact (WHO, 1975). ELISA is rapid, more sensitive, specific, automated, quantified and easy to operate compared to precipitin test (Beier *et al.,* 1998). PCR identifies mammalian meal directly with increase speed and cost-effectiveness (Kent *et al.,* 2005). Human-specific genetic markers was also developed to identifies mixed blood meals of malaria vectors that had fed on different individuals of the same species which is important to understand human–vector contact and pathogen transmission intensity (Norris *et al.,* 2010).

These are three different types of mosquitoes based on their feeding preference. Those mosquitoes that are preferred to feed on man are called anthropophilic whereas those that feed on animals are zoophilic mosquitoes. Indiscriminate biters are those that feed on human or animal without preference (WHO, 2013). There is genetic difference on host preference on different species or sibling species in species complex. However, host availability plays a dominant role in host preference (WHO, 1975; Tirados *et al.,* 2006). Carbon dioxide, odour, heat, moisture and visual factors are some of the factors that orient the mosquito to the host. Observation showed that host specific odour carried by stream of air would enable a mosquito species to orient to a certain host at fairly long distance (WHO, 1975).

Most mosquito species rest in days in place where optimal resting conditions are fulfilled. Endophilic mosquitoes rest indoors most of the time on the proximity of the host on which they feed. Some exophilic mosquitoes rest indoors, when the indoor conditions are favourable for the species but most preferred to rest outdoors. Mosquito species which are exclusively exophilic might be found indoor before and after blood meal during the night (WHO, 2013). The need for humidity and shade make the exophilic mosquitoes to be rest in ground or a root of tree near favourable breeding sites and host. However, if the resting sites are numerous mosquitoes disperse to a large area hence a difficulty to find enough mosquitoes and underestimate outdoor resting mosquitoes (WHO, 1975). Understanding the resting behaviour of mosquitoes is important for effective malaria control interventions. The widely used control intervention (IRS and LLINS) are only effective when the mosquito are feeding and resting indoors (WHO, 2013).

The entomological inoculation rate (EIR) is the number of infectious bites per person per unit time, which is expressed per year most of the time. EIR is the product of the human biting rate (HBR) and the sporozoite rate (SR). Like parasite and spleen rate, EIR measure the endemicity of malaria and risk of epidemic development and most favoured method to assess the level of malaria endemicity (Shaukat *et al.,* 2010).

Several alternatives methods to measure the human biting rate (HBR) include Human landing catch (HLC), light traps (CDC miniature light trap) with or without bed net and indoor resting catches by pyrethrum spray catch (PSC). Human landing catch (HLC) is the most frequently used method and considered as the gold standard to determine HBR (WHO, 1975). It is the most reliable measure of human-vector contact for evaluating malaria transmission. However, it is difficult to replicate the technique and raises the ethical question for the possible risk of malaria transmission and other vector borne diseases to mosquitoes collected (Shaukat *et al.,* 2010). The method overestimates the result and bias might arise due to the variation of attractiveness between individuals for mosquito bite (Mukabana *et al.,* 2002). Like HLC each method is subjected to bias and shortcomings and, therefore, influences the result of EIR.

A CDC light trap hanged near a sleeping people under untreated bed nets can be used to estimate HBR, as it catches mosquitoes that attempt to feed on humans. However, light traps mostly collect the anthropophilic and endophagous species that enter in to houses although the presence of light attracts other species that are not anthropophilic. So collections with LT/ N should allow a good standardization (Fornadel *et al*., 2010). Specimens sampled by pyrethrum spray catches (PSC) are mostly fed females resting

indoor so the morning EIR obtained from PSC may underestimate the result because the mosquitoes leave the house before or during spraying (WHO, 2013).

Studies on host preference of *An. arabiensis* in different parts of Ethiopia showed the opportunistic behaviour of the species feeding both on human and cattle. However, there is variation in host preference of *An. arabiensis* between studies. For instance Kibret *et al.,* (2010) found the anthtropophilic behaviour of *An. arabiensis* (HBI=0.78) in Zeway area in central rift valley area. Recent study in Zeway area showed the anthrophily of the *An. arabiensis* (0.70) (Gari *et al.,* 2016). However, the zoophilic tendency of *An. arabiensis* was observed in Arba-Minch district, Gilgel-Gebe area and Derashe District in south and south west Ethiopia (Massebo *et al.,* 2013b; Yewhalaw *et al.,* 2014; Gone *et al.,* 2014).

Similar tendency for human and cattle blood was observed in Butajira and Konso in south Ethiopia (Habtewold *et al.,* 2001; Animut *et al.,* 2013b). The variations in the HBI of the vector could result from differences in the relative accessibility of hosts. However, despite in the presence of cattle 46% of resting mosquitoes collected outdoors had human blood meal in the cattle camps of Konso, where humans slept close to their cattle outdoors (Tirados *et al.,* 2006) indicating the presence of cattle did not shift the species to feed more on cattle. In the same study area placing an ox adjacent to an HLC did not reduce the catch of *An. arabiensis* whereas the catch of the highly zoophagic species *An. pharoensis* was reduced (Habtewold *et al.,* 2004). The HBI of *An. arabiensis* was lower in low altitude locality compared to mid level and high altitude localities in study conducted in different altitudinal transect in Butajira area, south central Ethiopia (Animut *et al.,* 2013b).

Although availability of host determine the feeding preference of *An. arabiensis,* high HBI in high altitude *kebele* out of the coverage of IRS and LLINS distribution in highland fringe of Butajira area might indicate the shifting of *An. arabiensis* towards cattle in the lowland due to IRS spray and LLINs distribution. In areas of intensive IRS spray in Kenya a shift from human to animal was observed in *An. gambiae s.l.* (Mwangangi *et al.,* 2013). Historically extensive use of IRS was successful in controlling *An. funestus* in area where the species was primary vector, by taking advantage of its highly anthropohilic and endophilic behaviour (Smith 1966). Similarly the introduction of LLINs highly reduced *An. funestus* s.s in intensive malaria transmission area in western Kenya (Gimnig *et al.,* 2003). *An. funestus s.s.* might be replaced by the zoophilic *An. rivulorum* or *An. parensis* due to extensive IRS spray and LLINs utilization. Molecular identification *An. funestus* s.s. collected from Ethiopia showed the *An. parensis* was the only member of the complex (Weeto *et al.,* 2004).

Except studies conducted in Zeway area (Kibret *et al.,* 2010; Gari *et al.,* 2016) zoophilic tendency in *An. pharoensis* was reported in different parts of Ethiopia (Habtewold *et al.,* 2004; Massebo *et al.,* 2013b; Animut *et al.,* 2013a; Gone *et al.,* 2014). *An. arabiensis* and *An. pharoensis* bite in the early period of the night indoor and outdoor in studies conducted in different parts of Ethiopia (Abose *et al.* 1998c; Taye *et al.,* 2006; Kibert *et al.,* 2010; Kenea *et al.,* 2016). The early time biting behaviour of *An. arabiensis* and *An. pharoensis* in these studies coinciding with the evening activities of the people in the study areas (Kibret *et al.,* 2010; Kenea *et al.,* 2016) and might be associated with long term application of IRS and LLINs utilization (Takken *et al.,* 2002; Yohannes and Boelee, 2012; Taye *et al.,* 2016). *Anopheles pharoensis* exhibited more exophagic than

endophagic behaviour in different studies conducted in Ethiopia (Krafsur, 1977; Kibret *et al.,* 2010; Kenea *et al.,* 2016). Flexibility of *An. arabiensis* in host preference, biting and resting behaviour challenge malaria control and elimination because the vector may be less vulnerable to IRS and LLINs, the two most important methods used for malaria control currently in the country (MOH, 2012).

2.2.2. Prevalence of *Plasmodium* species

All of the four human malaria parasites species occur in Ethiopia. However, *P. falciparum* is the dominant species accounting for 64% of the cases followed by *P. vivax* responsible 36% of the cases in 2015 (WHO, 2016). *P. malariae* and *P. ovalae* are rare, accounting far less than 1% of cases. Misdiagnosis of *P. malariae* and *P. ovale* with *P. falciparum* and *P. vivax* might under estimate these two species in Ethiopia. For instance, a study conducted in Dembia District in North West Ethiopia showed high discrepancy between microscopy and real-time PCR test, *P. ovale* was not reported in microscopy but higher number of *P. ovale* detected compared to *P. vivax* (Tajebe *et al.,* 2014). *P. malariae* is mainly reported from Arbaminich area, while *P. ovale* is identified from a few patients in Humera, Metemma, Gambella and Gamugofa. In Ethiopia, *P. vivax* is more dominate during the dry season, and weather this is due to active transmission or relapses has not been clearly determined (Gebre-Mariam, 1988).

2.3. Climatic and environmental factors sustaining malaria transmission

A number of inter related causes are associated with variations of malaria transmission spatially and temporally in malaria endemic areas. Factors such as climate change, Socio-economic and Scio-demographic factors, population movement, lack of effective malaria

control intervention, insecticide-resistant vectors, drug resistant parasites and human behaviour have been cited as the contributors of increasing malaria transmission at individual, household and community levels (Cohen *et al.,* 2008; Coleman *et al.,* 2007).

The impact of rainfall varies based on its amount and the topography of the area. Continuous and heavy rains causes severe flooding associated with temporary flushing of breeding places. Consequently, the breeding of vector population is greatly reduced but it was soon be re-established when normal conditions restored. Moderately frequent rainfall with fairly long periods of sunshine will increase the opportunity for prolific breeding (WHO, 1975).

Besides, drought might increase or decrease malaria transmission depending on local conditions. For instance, in areas where permanent water bodies are (River or Stream) absent malaria transmission is highly reduced due to the lack of vector breeding habitat. However, in areas with river and stream the drought create small intermittent pools in river beds, which are favourable for Anopheles breeding sites. For instance, the drought in Ethiopia which occurred in 1958, 1965, 1973-1974 and 1983-1985 overlapped with subsequent malaria epidemics in respective years. Apart from creating mosquito-breeding sites, rainfall also affects transmission though increasing humidity, which in turn will help to increase the longevity of adult vectors (Hay *et al*., 2002).

Inter annual climate variability associated with El Niño southern oscillation (ENSO) have been strongly correlated with increases malaria incidence in some parts of southern Asia, South America and East Africa (Kovats *et al.,* 2003). The eastern Africa highlands are more sensitive to climate variability due to the unstable nature of malaria (Mouchet *et*

al., 1998; Bodker *et al.,* 2000). Temperature and precipitation in the highlands are expected to rise above minimum temperature and precipitation at cyclic pattern. Rain fall peeks in east Africa correspond to El Niño southern oscillation (ENSO) years. ENSO is a periodic warming and cooling of the Pacific Ocean coupled with changes in air pressure and consequently leads to changes in precipitation, temperature and extreme rainfall events. For instance, the 1958 catastrophic malaria epidemic in Ethiopia was associated with unusually heavy and prolonged rains combined with abnormally high temperature and humidity (Cox *et al.,* 1999).

The impact of climate as the cause for malaria epidemics in the highlands of East Africa is debated between two groups of scholars (Malakooti *et al.,* 1998;: Hay *et al.,* 2002; Zhou *et al.,* 2004). According to Hay *et al*. (2002) mean temperature and rainfall did not change significantly at four locations in their study sites where malaria incidence had been increasing. As a result they concluded that the recent increase in malaria in the East African highlands could not attributed to global warming. Instead, the increase in drug resistance was considered a more likely explanation for the observed increase in malaria. Similar studies in Kericho Tea plantations by Shanks *et al*., (2002) and Omumbo *et al*., (2011) in western Kenya supported the views of Hay *et al*., (2002).

On the other hand, Zhou *et al*., (2004) found a significant temperature increase in their study sites and concluded that the increased malaria epidemic in 1990s compared to those in 1980s in East African highland was related with climate variability. They stressed the significance of concurrent analysis on the long-term time series of meteorological and parasitological data to determine the effect. Moreover, short-term variations around the mean climate state on a fine time scale could be epidemiologically more significant than

mean temperature increase. According to Pascual *et al*., (2006) a half-degree Celsius rise in temperature would increase mosquito numbers between 30% and 100% due to the effect of temperature on their developmental time. Although this increase had a marginal effect in lowland areas where mosquitoes are abundant, it is more likely to be affecting transmission rates in the highland areas, where the insects are much scarcer (Pascual *et al*., 2006).

There is large variations among-site in the abundance and temporal dynamics of malaria vector populations indicating that the risk of parasite transmission differs among sites (Katrijn *et al.,* 2010). Even in one topographic area, mosquito vectors and malaria infections may not be distributed homogeneously, and some households within the same area have a higher malaria incidence than others (Brooker *et al*., 2004). Similarly, sporozoite infection rate and annual inoculation rate varied in three villages in rural communities of western Nigeria (Oduola *et al*., 2012) and in rural and urban areas in Cameron (Bigoga *et al*., 2012). High entomological inoculation rate in dry season than rainy season was observed in perennial transmission areas of Cameron (Bigoga *et al*., 2012) whereas entomological inoculation rate and malaria cases was higher in rainy season in a low endemicity area in northern Tanzania (Oesterholt *et al*., 2006). The presence of highest indoor densities of *An. gambiae s.l.* was observed in studies conducted in Ethiopia and Kenya during the wet season (Amek *et al.,* 2012; Massebo *et al.,* 2013a; Gari *et al*., 2016).

High mosquito density is associated with high concurrent monthly rainfall in a study conducted in Tanzania where the principal malaria vector is *An. arabiensis* (Oesterholt *et al.,* 2006). However, the peak of *Anopheles* abundance was not coinciding with the peak

of precipitation months in a study conducted in Adami Tullu-Judo-Kombolcha and Arba Minch Zuriya District, south Ethiopia (Gari et *al.,* 2016; Massebo *et al.,* 2013a). Proximity to water bodies was also associated with the increase incidence of malaria (Zhou *et al*., 2012; Pullen *et al*., 2010). Living further away from the river and use of anti-insect window screens were independent protective factors for the risk of malaria infection in Tanzania (Oesterholt *et al*., 2006). A case control study in western Kenyan highland showed higher malaria risk with living <250 meter of a forest, 250 meter of a swamp, <200 meter of maize fields, in the absence of trees <200 meter, on flat land (Ernst *et al.,* 2009). A study conducted in north-central Sri Lanka, found that houses closer than 750 meters to a breeding stream had a 4.7-fold higher risk of harbouring *An. culicifacies* and a 1.5-fold higher risk of harbouring *An. subpictus* than houses at least 750 meters away (Konradsen *et al*. 2003). In western Kenya distance to water bodies was associated with increased density *An. gambiae s.l.* (Amek *et al.,* 2012).

A study in Ethiopia had shown that malaria prevalence to be higher in irrigated village in dry season while in rainy season more cases observed in non-irrigated village. However, larval and adult abundance of the malaria vectors, *Anopheles arabiensis* and *Anopheles pharoensis*, was higher in the irrigated than in the non-irrigated village throughout the study period (Kibret *et al*., 2010).

High entomological innoculation rate in dry season than rainy season was observed in perennial transmission areas of Cameron (Bigoga *et al*., 2012) whereas entomological innoculation rate and malaria cases was higher in rainy season in a low endemicity area in northern Tanzania (Oesterholt *et al*., 2006). Reduction of malaria incidence in children with increasing distance from forest was observed in high endemic rural area of Ghana

(Kreuels *et al*., 2008). Similarly low vegetation level in compound is associated with low malaria risk in urban area of Ethiopia (Peterson *et al.,* 2009).

Higher malaria transmission due to a relatively high temperature in low-altitude localities compared to highland localities was associated with higher malaria transmission in Kenya (Brooker *et al.,* 2004). High density of *An. arabiensis* was reported in low altitude in Butajira area, central Ethiopia during the dry season (Animut *et al.,* 2013a). Similarly densities of *Anopheles* mosquitoes generally decreases when the altitude increase in highland of Northern Tanzania (Kulkarni *et al.,* 2006).

Growing maize near households increases malaria transmission in Ethiopia (Kebede *et al*. 2005; Kweka *et al,* 2012) because maize pollen increase vectorial capacity by allowing more larvae to survive to adulthood, and to develop more quickly into larger and long lived vectors larvae develop to the pupal stage more rapidly and producing larger adults (Ye-Ebiyo *et al*., 2003).

Information on insecticide resistance is very important for effective vector control so detection and monitoring of insecticide resistance in malaria vectors is crucial and has to be conducted together with other entomological indices (Coleman and Hemingway, 2007). Insecticide resistance can be detected at phenotype and genotype level. Measuring phenotypic resistance using bioassays is the recommended initial step in establishing resistance levels before genotyping for target-site and metabolic resistance and biochemical assays (WHO, 2013).

A bioassay is used to determine the relationship between a physiologically of active agent and the effect that produce on tested mosquitoes. Bioassays with the dosage or the

exposure time as the variable are carried out to test the resistance status. Two types of bio-assays: the WHO diagnostic assay and the CDC bottle assays (WHO, 2013). Insecticide impregnated filter paper is used as a contact surface for exposed mosquitoes in WHO diagnostic assay of insect populations while in CDC bottle assay mosquitoes are exposed to glass bottle surfaces coated with an acetone- or alcohol-based formulation of insecticides. The assays are not used to monitor resistance gene frequencies accurately, and are not give indication of the underlying mechanisms of resistance and cannot be able to detect cross resistance between insecticides. Therefore, the resistance status detected using bioassays, can then be further studied by looking at the mechanisms responsible for resistance using biochemical and molecular assays (WHO, 2013).

Two biochemical mechanisms, target-site and metabolic resistance are known to cause resistance. Target-site resistance occurs when an insecticide fails to bind to its target (WHO, 2013). The four classes of insecticides most commonly used for contemporary malaria vector control include organochlorines, organophosphorus, carbamates, and pyrethroids have cover only two target sites (WHO, 2009).

For instance, DDT and pyrethroid insecticides are insect neurotoxins that interfere with ion flow regulation across the sodium ion channels. Ion channel modification via an amino-acid substitution leads to reduced target site sensitivity known as knockdown resistance (*kdr*). The substitution of leucine at position 1014 for either a phenylalanine or a serine has been reported in *An. gambiae s.s* and *An. arabiensis* from East Africa (Ranson *et al.,* 2000; Verhaeghen *et al.*, 2006). Metabolic resistance occurs when the insecticide is degraded by enzymes including esterases, monooxygenases, and glutathione S-transferases while carbamates block the degradation of the neuromediator

acetylcholine by inhibiting the acetylcholinesterase (Brogdon *et al.,* 1998; Brengues *et al.,* 1997). Recent advances in genomics have allowed a much more rapid identification of genes that are up or down regulated in insecticide resistant insects using microarray technology (David *et al.,* 2005).

Biochemical assays determine resistance by measuring enzyme activity involved in resistance such as esterases glutathione-S-transferases (GST) (WHO, 2013), and cytochrome P450-dependent monoxygenases (Brogdon *et al.,* 1998). There are two ways that metabolic enzymes can produce resistance; overproduction of the enzyme, which leads to either increased metabolism or sequestration of the insecticide and an alteration in the catalytic centre activity of the enzyme, which increases the rate of insecticide metabolism by the enzyme (WHO, 2013).

Sequestration occurs when the overproduced enzyme rapidly binds and slowly metabolises the insecticide, therefore preventing it from reaching the target site within the insecticide (WHO, 2013). Molecular assay detect mutations in target site using allele-specific PCR assays. Three different primers detect the GABA receptors (Du *et al.,* 2005), the sodium channels (*kdr*) (Lynd, *et al.,* 2005), and AChE. The advantage of molecular assay is detecting resistance at lowest frequency with high-throughput and detecting heterozygous-resistant individuals that may be missed by other assays (Colema and Hemingway, 2007). The resistance of *Anopheline* mosquitoes for the available insecticides has been reported in almost all malaria endemic countries (WHO, 2012). Because the classes of insecticides used are few in number, WHO and its partners have developed a Global Plan for Insecticide Resistance Management in order to minimize the increasing trend of insecticide resistance. The Insecticide Resistance Action Committee

has recommended options to delay occurrence of insecticide resistance such as rotations, mosaics, combinations and mixtures (WHO, 2012). Rotating insecticides with different mode of action year to year; and using combinations two or more insecticide-based vector control interventions in a house (e.g. pyrethroids on nets and an insecticide of a different class on the walls are the major component of insecticide resistance management). Based on evidence, mosaics using one compound in one geographic area and a different compound in neighbouring areas and using a mixture of two or more compounds of different insecticide classes in a single product or formulation is the other alternatives for insecticide resistance management so that the mosquito is guaranteed to come into contact with the two classes at the same time (WHO, 2012).

2.4. Major malaria vector control methods

Malaria transmission is much more difficult to control in Africa than most other places in the world because the presence of efficient long-lived anthropophagic vectors, ability of vectors to avoid many domestic insecticide interventions (Trape *et al.,* 2014) inter-species differences in behaviour of the vector (Sinka *et al.,* 2012), poor health infrastructure, the potential for resistance among mosquito vectors and the parasite (WHO, 2016). Therefore, in order to achieve the target of World Health Organization and Roll Back Malaria truly integrated controls, combining all tools at our disposal, were necessary. The most commonly methods used to control and prevent malaria transmissions are those interventions that prevent host vectors contact and anti malaria drugs once the infection is occurred in the host (WHO, 2016).

2.4.1. Long lasting insecticidal treated mosquitoes nets

Long-lasting insecticidal net (LLIN) is a mosquito net that repels disables and/or kills mosquitoes coming into contact with insecticide on the netting fabric. The widely distributed net currently in Ethiopia is a long-lasting insecticidal net (MOH, 2012). The netting materials of LLINs has insecticide incorporated within or bound around the fibers and retain its effective biological activity without re-treatment for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions (WHO, 2008). LLINs act as a physical barrier preventing access by vector mosquitoes contacting the host and thus providing personal protection against malaria to the individual(s) using the nets (WHO, 2009). Insecticides, which are used to treat nets, have an excito-repellent effect that adds a chemical barrier to the physical one, further reducing human–vector contact and increasing the protective efficiency of the mosquito nets. Pyrethroid such as permethrin, deltamethrin and lamdacyhalothrin, are used universally because of their low mammalian toxicity hazard and good residual effect (WHO, 2008).

The high level of LLINs use practice caused, shifting vectors from indoor to outdoor biting (Russel *et al.,* 2010), early biting immediately after sun set and before sunrise (Fornadel *et al.,* 2010) and wide spread resistant for pyrethroid insecticide are among the major problems in using LLINs as a malaria vector control strategy (Wiebe *et al.,* 2017). LLINs are considered to be effective for controlling of *An. gambiae s.s*. and *An. funestus,* because of their anthropophagic, endophagic and endophilic characteristics, which prefer to bite at night when people are in bed (Sinka *et al.,* 2010). However, behavioural change

and insecticide resistant in these two species make LLINs ineffective (Sougoufara *et al.,* 2014).

Many of the risk factors for malaria are related to access to interventions. For instance, a study in Kenya showed mosquito density, human biting rate and EIR of indoor resting mosquitoes were reduced by more than 75% for *An. gambiae s.l.* and 92% for *An. funestus* when the bed net coverage reached 60-86%. This study also confirmed feeding choice of both vectors shifted more toward non-human vertebrates (Mutuku *et al*., 2011). A significant reduction in the abundance of *An. gambiae s.l.* over the last 20 years due to increase coverage of IRS and LLINs was also reported along Kenyan coast (Mwangangi *et al.,* 2013). However, in Senegal insecticide-treated nets quickly selected resistant mosquitoes with long lifespan and unchanged feeding behaviour (Ndiath *et al.,* 2014) so the density was not impacted by LLINs utilization. Statistically insignificant reduction of *An. arabiensis* in LLINs used households was also reported in Zambia (Chanda *et al*., 2008). Similar to entomological studies, different parasitological studies reported sleeping under LLINs reduced malaria infection (Graves *et al.,* 2009: Pullan *et al.,* 2010; Loha and Lindtjørn, 2012; Loha *et al.,* 2013; Ayele *et al*., 2012) provided that if they are used properly and regularly. However, some studies reported no differences in malaria prevalence between household that possess and utilize LLINs and those that are not (Roberts and Matthews, 2016; Gahutu *et al*., 2011).

While it is intuitively clear that LLINs provide protection to individual users, what is less obvious is the impact of widespread LLIN use at the community level. LLINs are able to reduce the density, feeding frequency and survival of mosquitoes and wide-scale use can mediate protection of all community members, even including the vulnerable portion

without a bed net (Pullan *et al*, 2010). On the other hand, it has been suggested that LLIN use could increase malaria risk for unprotected people by diverting mosquitoes away from users and concentrating their host-seeking efforts upon them (Killen and Smith, 2007). Protection of LLINs at individual level but the lack of protection of LLINs at a community level was reported in longitudinal study conducted in Chano Mille in south Ethiopia (Loha and Lindtjørn, 2012). Prolonged usage of LLINs and IRS has been linked to a shift of human exposure from occurring indoors during hours when most people are asleep, toward occurring outdoors in the evenings and mornings for the two important malaria vectors in Africa, *Anopheles gambiae s.l.* and *Anopheles funestus* (Russel *et al*., 2010).

2.4.2. Indoor residual spraying

The primary effects of indoor residual spray (IRS) are to reduce the life span of vector mosquitoes so that they can no longer transmit malaria parasites from one person to another and to reduce the density of the vector mosquitoes (WHO, 2013). The consistent application of IRS has changed the distribution of vector and the epidemiological pattern of malaria in Botswana, Namibia, South Africa, Swaziland and Zimbabwe. The major vector, *An. funestus* has been eliminated or reduced to negligible levels (Gimnig *et al.,* 2003). *Anopheles gambiae s.s* is effectively controlled in some areas where the two species rests and bites predominantly indoors. Another vector, *An. arabiensis,* which does not rest indoors as much as *An. gambiae* is less affected by IRS, even at high coverage levels and is responsible for low levels of transmission and seasonal increases and outbreaks (Beales *et al.,* 1989; Hansford, 1972; Sharp *et al.,* 1990).

Different epidemiological patterns that need different intervention might observe in a single country and situations are commonly found requiring a combination of interventions. IRS can be effective in almost all settings as long as certain conditions for implementation are met. Scientific evidence indicates that IRS is efficient to control malaria transmission if > 80% targeted communities are treated (WHO, 2006). There are specific interactions between insecticides and malaria vectors. Some insecticides tend to repel more than to kill vector mosquitoes. Changes in vector behaviour induced by insecticides may have important operational challenges, and to this effect it is important to be aware of them when selecting insecticides for IRS (WHO, 2006).

A demonstration control projects with the objectives of testing the effectiveness of residual house spraying in a range of transmission settings was carried out in Kobo Chercher (1955), the upper Awash Valley (1956), Dembia Plain (1957) and Gambela (1959). However, none of the projects achieved complete interruption of transmission but considerable reduction in malaria transmission was observed in all of pilot studies. For instance, in all of IRS demonstration sites the 1958 epidemic was not occurred whereas devastating epidemic was common in most parts of country even in localities near demonstrations sites. The success of IRS spraying in demonstration sites led to the establishment of the National Malaria Eradication Service in 1959 and spraying continued on the demonstration sites and other areas of economic significance areas but a comprehensive spraying programme was initiated in 1966 throughout the country (Cox *et al.,* 1999).

Reducing the risk of malaria infection with insecticide residual spraying was reported in different studies conducted at different parts of Ethiopia and Uganda (Ayele *et al*., 2012;

Bekele *et al.,* 2012; Steinhardt *et al.,* 2013; Roberts and Matthews 2016). In a study spraying of IRS with deltamethrin reduced malaria infection but reduction was not observed in DDT spraying in a cohort longitudinal study conducted in Chano-Mille, south Ethiopia (Loha and Lindtjørn, 2012). Statistically significant reduction of malaria morbidity for three months after IRS was sprayed but reduction of residual effect and increasing of slide positivity rate after four months was observed in retrospective study conducted in northern Uganda (Tukei *et al.,* 2017).

Historically extensive use of IRS was successful in controlling *An. funestus* in area where the species was primary vector, by taking advantage of its highly anthropohilic and endophilic behaviour (Smith 1966; Gillies and Smith1960). Similarly the introduction of LLINs highly reduced *An. funestus* s.s in intensive malaria transmission area in western Kenya (Gimnig *et al.,* 2003).

The application of insecticides in and around houses has fundamental limitations, because exhaustive coverage of all resting sites with IRS is not possible in practice (MOH, 2012). Ineffectiveness of spraying of IRS, proximity of inhabitants' house to breeding sites, high human-vector contact due to outdoor sleeping patterns and chloroquine-resistant *P. falciparum* were responsible for the increase prevalence of malaria in Pawie settlements in 1980's (Nega and Haile- Mescal, 1991). The efficacy of IRS and LLINs depends, among other things, on the proportion of the vectors to the insecticide used. It is therefore important to monitor the development and the scope of insecticide resistance in a vector population (WH0, 2011). Current malaria vector interventions such as LLINs and IRS highly reduced EIR in many malaria endemic countries of Africa (Shaukat *et al.,* 2010), however, none of these interventions achieved the minimum EIR requirement (<1ib/year)

to reduce or interrupt malaria transmission in certain area based on the finding of linear relationship between malaria prevalence and EIRs (Beier *et al*., 1999).

2.4.3. Using larvicides and environmental management

Previous to 1940 malaria control methods generally focused on control of the larvae by environmental management and regular larvicidal treatment of breeding sites. The method was effective across Europe, Asia and Americas and responsible for the suppression and even eradication of malaria from vast areas. However, the advent of dichlorodiphenyltrichloroethane (DDT) shifts the attention of malaria-control community into the idea that malaria could be controlled and even eradicated by almost exclusively targeting adult mosquitoes with synthetic insecticides (Killeen *et al.,* 2002).

Larval habitat management includes planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of environmental factors or their interaction with man with a view to preventing or minimizing vector propagation and reducing man-vector-pathogen contact. The objective of larval habitat management is to either kill the mosquito larvae or create adverse situation which is unfavourable for mosquito breeding. However, larval habitat management includes habitat modification, habitat manipulation, larviciding and biological control (WHO, 1992).

The modification of breeding site is a type of environmental management consisting of managing physical change to potential mosquito breeding areas designed to prevent, eliminate, or reduce vector habitat. The principal methods of achieving these changes include drainage, land levelling, and filling. Draining operations include the creation of ditches or drains to keep water moving and to carry the water used as breeding sites away

in a managed way. Drains may be lined or unlined and located at the surface or subsoil level (WHO 1992). In addition to complete elimination of wetlands, modification projects can involve creating channels to increase water flow in areas of standing water. Because slow-moving pools with heavy vegetation in rivers and streams can create favourable larval breeding sites for certain vector species, straightening river banks may reduce vector populations.

Modification can also involve human-made vector breeding habitats associated with water-holding structures in mini-dams and small-scale irrigation projects. The creation of a larval habitat can be avoided through careful design and collaborations with other sectors such as agriculture and construction (WHO, 1992). Habitat manipulation refers to activities that reduce larval breeding sites of the vector mosquito through temporary changes to the aquatic environment in which larvae develop. These techniques may be appropriate either when permanent removal of aquatic habitat through environmental modification is not feasible or, in the case of irrigated agriculture, when the sporadic presence of water is necessary for other activities. Techniques include changing water levels in reservoirs, flushing streams or canals, providing intermittent irrigation to agricultural fields (particularly rice), flooding and/or temporarily draining human-made or natural wetlands, and changing water salinity. Planting water-intensive tree species, such as Eucalyptus, can reduce standing water in marshy areas (WHO 1992; Sharma *et al.,* 1986).

Regular application of chemical or biological agents to kill mosquito larvae in their aquatic habitats referred as larviciding, which are feasible when the vector breeding habitats are few, fixed and accessible (WHO, 2012). Chemical and biological larvicides

were the major malaria control intervention until World War II and responsible for the eradication of *An. gambiae s.l.* from rural areas of Brazil in the 1930s (Killeen *et al.,* 2002; Walker and Lynch, 2007). In addition, effective malaria prevention programmes targeting *An. gambiae s.l.* and *An. funestus* larval habitats were implemented in several mining towns in Zambia in the 1930s and 1940s (Utzinger *et al.,* 2001).

Indoor residual spraying (IRS) and LLINs are the most powerful, reliable and practicable tools for malaria vector control in different epidemiological setting; however these two interventions are not perfect, and they cannot serve for all vector control purposes in all settings. For instance, it has often been observed in Africa that indoor transmission can be greatly reduced by careful indoor residual spraying (IRS), but outdoor transmission may persist and not prevent the complete interruption of transmission (WHO, 2012). Larviciding has the potential to overcome this problem, because it is expected to affect indoor and outdoor biting vectors equally (Kouznetsov, 1977).

However, larviciding has its own limitations; maintaining complete coverage of permanent breeding habitats scattered around the margins of larger water bodies is difficult (WHO, 2012). The other limitation of larviciding is treating all larval habitats under *Anopheles* mosquito's flight range is difficult. For example, in order to protect a small community in flight range of *Anopheles* mosquitoes (mostly 1-1.5km) breeding must be prevented within a diameter of up to 3 km, or an area of potentially more than 9 km^2. On the other hand, in larger communities, the whole area of the settlement plus a buffer region between the community and breeding sites must be covered (WHO, 2015).

In addition, during rainy season it is difficult to find every potential breeding site in large area. Due this fact and the lack of large scale trial studies on effectiveness of larviciding, WHO recommend using larviciding as a supplement to the core interventions (LLINs or IRS) measures and never be seen as a substitute for LLINs or IRS in areas with significant malaria transmission (WHO, 2012). Chemicals used as larvicides include petroleum oils, Paris green (copper acetoarsenite), monolayer surface films, DDT, organophosphate-based larvicidal formulations (temphos), synthetic pyrethroids and insect growth regulators (pyriproxyfen).

The efficacy of chemical larviciding depends on several factors including the formulation, water quality and the susceptibility of the targeted larvae. Because some chemical larvicides are toxic to non-target organisms, it is not advisable to apply those compounds to natural water bodies of environmental importance. For instance, use of Paris green (high toxicity) and DDT (high persistence, non-target effects and selection pressure for resistance) are no longer recommended. Because pyrethroids are important as bed net treatments for adult vector control, and the use of pyrethroids as larvicides could prompt vector resistance (Walker and Lynch, 2007).

The second types of larviciding, biological control refer the introduction natural enemies of larvae such as vertebrate and invertebrate predators, virus, bacteria, fungi and helimenthes into their habitat to regulate *Anopheles* populations naturally through predation, parasitism and competition. The natural enemies of mosquitoes, jointly with other regulating factors, have important role in natural balance of larval fauna by preventing a population explosion (WHO, 1975). The main biological control agents that have been successfully employed against *Anopheles* are predators, particularly fish, and

the bacterial pathogens *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* that attack the larval stages of the mosquito (Rozendaal, 1997; Fillinger *et al.,* 2003).

In addition, several fungi in the genera *Metarhizium* and *Beauveria*, nematodes of the family Mermithidae, predatory mosquitoes of the genus *Toxorhynchites,* dragonflies, small crustaceans and *Azolla*, a free floating fern that can completely cover water surfaces and prevent breeding by mosquitoes, showed a strong biological activity against *Anopheles* larvae (Rozendaal 1997;WHO, 2007). Compared to chemical larvicides biological control agents are effective at relatively low doses, safe to humans and non-target organisms, lower risk of resistance development, low toxicity and simple application procedures (Walker and Lynch, 2007).

2.4.4. Integrated vector management

The concept of integrated vector management (IVM), which is defined as a rational decision-making process for the optimal use of resources for vector control based on evidence based decision-making, integrated approaches, collaboration of health sector with other related sectors, advocacy, social mobilization and legislation, and capacity-building guarantee for rational use of available resources through a multi-disease control approach, integration of non-chemical and chemical vector control methods, and integration with other disease control measures, such as active and passive case detection and treatment (WHO, 2013). In the era of increase insecticide resistance, reliance on a single method for vector control program (IRS or LLINs) challenges the effort of malaria control intervention due to vectors resistant or the changing behaviour of the dominant *Anopheles* mosquitoes (feeding and resting outdoor and early biting behaviour of the

vectors) (Russell *et al.,* 2010; Mutuku *et al*., 2011). Because of these, a combination of two or more methods of vector control may have to be worked out for effective control depending on the local condition of the area. Adaptation of strategies and interventions to local vector ecology, epidemiology and resources, guided by operational research and subject to routine monitoring and evaluation (Beier *et al.,* 2008). Malaria control program with IVM elements brought significant reduction in vector densities and malaria transmission in different epidemiological setting of Africa (Bang *et al.*, 1975; Utzinger, 2001; Beier *et al.*. 2008: Chanda *et al.,* 2008).

2.5. Operational challenges in malaria vector control (LLINs) in Ethiopia

Typical monitoring and evaluation strategies report on indicators agreed upon and formalized by the RBM Monitoring and Evaluation Reference Group (MERG), two important core LLIN indicators for malaria control programs are the proportion of households owning (coverage) an LLIN and the proportion of vulnerable populations sleeping under an LLIN (utilization). Using these measures, many studies have shown that efforts to increase LLIN ownership have made tremendous progress; however, most sub-Saharan countries remain well below RBM targets for ownership (Noor *et al.,* 2009).

In addition, these evaluations have consistently found LLIN use by vulnerable groups sleeping under an LLIN lower than household ownership (Korenromp *et al.,* 2003; Esayas *et al.,* 2014). In order to reach the RBM goals for bed net coverage and increase LLIN use, it will be useful for programs to identify and address the determinants of LLIN use and non-use (Vanden *et al.,* 2010). An imperative for Roll Back Malaria is to monitor progress towards this target, so that shortfalls in implementation can be identify and acted

upon. Two indicators that are of potential value are (i) the proportion of households that have one or more nets and (ii) the proportion of children under 5 years of age who use (i.e. sleep under) a net (RBM, 2000). Household possession data indicate the extent to which distribution channels are enabling high coverage and may be particularly valuable at the early stages of program development and implementation.

Use of mosquito nets, however, is, of course, what affords protection and is therefore a more useful predictor of epidemiological impact. In practice, both measures will be useful for program management. If use rates are low, it is important to know whether this is due to affordability and a lack of availability, or the failure to use available nets which would suggest a need for health education (Korenromp, *et al.,* 2003). Often, there was no single reason for not using a net, and reasons overlapped or were interrelated. Some of the operational challenges which hindered the optimal net use described were, difficulty to hang and stretched correctly, inadequate space, lack of structure underneath, wrong perception, lack of feedback from beneficiaries, nets in poor condition, misinformation and lack of information, saving nets for the future, and nets being used for other purposes (Baume *et al*., 2009).

In fact, LLINs is considered as the most effective tool for malaria prevention and control with significant reduction of child morbidity and mortality. The strong effect of LLIN on malaria cases and deaths in the last decade has been well documented (Guillet *et al*, 2001; Temu *et al*, 1999; Magesa *et al*, 1991; Otten *et al*, 2009). Since 2004/2005, LLINs has been part of malaria vector control and over 41 million nets have been distributed in Ethiopia (MOH, 20011). Still there are factors that influence the intended role of LLINs

impact on malaria. For instance, net with holes provide a reduced protection or no protection for the person sleeping under the net (Irish *et al*, 2008).

Baume *et al.* (2009) in a NetMark study in Ethiopia reported 65% usage rate. Another study, in Arba Minch Zuria district of southern Ethiopia showed a slightly improved rate of 73.3% (Astatkie, 2010; Esayas, *et al.,* 2014). Besides the results of 2011 malaria indicator survey, the practice of properly utilizing nets showed 64.5% (EHNRI/MOH, 2011). A study conducted by Abebe and his colleague (Animut *et al.,* 2008), overall LLINs distribution and utilization were 97.6% and 81.6%, respectively. The majority of households (53.2%) owned a single net per family and 38% of the respondents owned two nets per household. In line with this, the result of the study conducted in southern Ethiopia (Battisso *et al.,* 2012) showed that, out of the households who owned nets, over one-third reported that had owned one mosquito net (31.8%) , half (54.1%) owned two nets, and 11% had three net. This study in addition confirmed that, the average number of nets per net-owning household was 1.86.

The current, malaria vector control in Ethiopia largely relied on LLINs and Indoor Residual Spray (IRS). These interventions are reliable and effective in a wide range of situations (Noor, *et al.,* 2009; MOH, 2011; Shargie *et al.,* 2008, 2009). Under utilization of intervention tools by the community was found to be one of the major drawbacks in the control programme whose goal is malaria elimination from low transmission areas of the country by 2015 (MOH, 2011; Dagne and Deressa, 2008; Fettene *et al.,* 2009; Gobena *et al.,* 2012, Esayas *et al.,* 2014).

In Ethiopia although different studies were conducted in the coverage and utilization of LLINs in various parts of the country, there is a limitation of data particularly on determinant factors that hinder utilization (Vanden *et al.,* 2010; Das *et al.,* 2007).

Long-lasting insecticidal nets (LLINs) were designed to have long-lasting effects in the prevention and control of malaria vectors. The nets are treated at the manufacturing level with insecticide either with incorporated into matrix of polyethylene fibers or coated with wash resistant resin on polyester fibers. The biological activity lasts as long as the useful life of the net itself, which is estimated to be 3–5 years (Guillet *et al*, 2001; Miller *et al*, 1995; WHO, 2007). Despite the use of nets as a major component of malaria vector control in almost all endemic countries, its effect on the targeted vector over time depends on textile integrity and the rate of loss of insecticide (WHO, 2013).

Chapter 3 Species composition, abundance and breeding habitat preference of *Anopheles* mosquitoes (Diptera: Culicidae) in selected sites in Butajira area, Southern Ethiopia

3.1. Introduction

Reports of different findings conducted in Ethiopia consistently incriminated that *An. arabiensis* is the primary vector responsible for the transmission of malaria where as *An. pharoensis, An. funestus* and *An. nili* are considered as secondary vectors (Ameneshewa, 1995; Abose *et al*., 1998c; Animut *et al.,* 2012; Fettene *et al.,* 2004). Recently, based on strong genetic evidence the *An. gambiae* s.s M and S molecular forms and *An. quadriannulatus* species B are assigned formal names: M form (*An. coluzzii*) S form (*An. gambiae*) and *An. quadriannulatus* species B (*An. amharicus*) (Ethiopia) (Coetzee *et al.*, 2013). As demonstrated in other studies the taxonomy of this complex species, no distinct characters are available for rapid, accurate, morphological identification of the species and thus identifications must be based on the use of molecular methods (Coetzee *et al.*, 2013).

However, the above mentioned are facts until now the vectorial roles of the rest of *Anopheles* were not yet completely known due to several limitations. Most of the currently available entomological information is emanated only from limited areas where there are easily accessible means thus the information are insufficient (Gebre-Mariam *et al*., 1988). Besides, communication problem to reach inaccessible areas has hindered the research on malaria and its vectors. For instance, *An. tenobrosus* Donitz was found positive for *P. falciparum* gametocyte in south Ethiopia with infection rates of 15.8% by

dissection and 7% by DNA hybridization (Adugna *et al*., 1998). This indicates that the need to identify other potential *Anopheles* species in the epidemiology of malaria throughout the country is unquestionable.

On the other hand, investigating the recent variations in the abundance and dynamics of malaria vector populations is also important (Animut, *et al.,* 2012). Since, the abundance and dynamics of *Anopheles* population have significant impact on the intensity of malaria transmission from place to place (Katrijn *et al.,* 2010). For example, a study conducted by Brooker *et al.,* (2004) showed that the existence of variation in the intensity of incidence of malaria within the same area. Pertinently, the density of malaria vectors has shown a great variation in the same topography even at household level within the same area.

For instance, in Ethiopia White *et al*. (1980) around Giligil Ghibe River valley reported that a variation in the density of malaria vectors with a result of less than 1 in January-March where as it go greater than 100 in July-October. But, in Gambela areas of Ethiopia, Krafsur (1970, 1978) reported that the average hut resting density was less than 30. Meanwhile, a collections made in the 1958 malaria epidemics revealed the average hut resting density were vary from 100 to 150 (Fontaine *et al*., 1961). However, Indoor Residual Spray (IRS) and LLINs are reliable and effective in a wide range of situations (Noor, *et al.,* 2009; MOH, 2011; Shargie *et al.,* 2008, 2009). Similarly, the impact of IRS in lowering indoor densities of *An. gambiae* s.l. was reported in study conducted in western foothill of Madagascar (Ratovonjato *et al.,* 2014). Therefore the aim of this study was to identify species composition, abundance and breeding habitat contribution for *Anopheles* mosquitoes in the selected study sites.

3.2. Materials and methods

3.2.1. Description of the Study sites

The study sites include Intervention (Jolie) and Control (Gogete) study sites (Kebeles = the grass root level of government administrative structure) around Butajira from Meskan and Sodo District respectively, in Gurage Zone, which is found within Southern Nation Nationalities People Region (SNNPR) of Ethiopia. Intervention (Jolie) and Control (Gogete) study sites are found below at an average altitude of 1850 meters above sea level at a distance of 120 Km south of Addis Ababa (Table 3.1). In both study sites there are few houses with corrugated iron sheet roof but dominantly houses with thatched roof (Figure 3.1). The inhabitants are engaged mainly in cultivating maize in wet season and during dry season vegetables such as onion, pepper, tomato, potato and cabbage by using irrigation (Figure 3.2). As in most parts of Ethiopia, the areas have two major seasons; the rainy season starts in June and lasts in mid-September, which then followed by the short rain in March and April. The study has been conducted from May 2013 to April 2017.

Figure 3.1. Residents near to a house with thatched roof in the Intervention study site (Jolie) during field visit

Figure 3.2. Irrigation practice in Intervention study site (Jolie) contributing for breeding habitat.

Table 3.1. Study sites, location and elevation of selected data collection sites in Southern Nation Nationality People Region (SNNPR), May 2013 to April 2017.

Zone	Districts/Wereda	Study site	Latitude/Longitude	Elevation
Gurage	Meskan	Jolie	N 08° 13.217’; E 038 28. 268’	1842 m
	Sodo	Gogete	N 08° 10.371’; E 038° 30. 449’	1846 m

3.2.2. Study design and sampling procedures

3.2.2.1. Larva collection, species identification and investigation of their habitat

Longitudinal study design was used to identify species composition, abundance and breeding habitat contribution in the study sites. Immature stage collections were carried out to supplement the data of adult collection to identify species complex and determine the principal *Anopheles* species accountable for malaria transmission in the study areas (WHO, 2013). The collections of *Anopheles* larvae were conducted in a variety of aquatic habitats of the study areas once per month by two collectors using dippers, pipettes and containers from temporary and permanent breeding sites (ponds, pools of rain water, water from broken pipes, River pocket, stagnant water in irrigation canals and marsh) in the study areas (Figure 3.3) and the number of larvae collected from each larval habitats were compared to identify the major contributor of *Anopheles* mosquitoes breeding.

The collected *Anopheles* larvae were killed in hot water (about 50^{0} C) and preserved in small vials containing 70% ethyl alcohol (WHO, 2013). All the specimens collected from a particular breeding place deposited in a labelled bottle or vial. The label were written in

pencil on a piece of paper and dropped into the specimen bottle and transported to Addis Ababa (EPHI or AAU laboratory) for identification.

Figure 3.3. Collection of *Anopheles* mosquitoes larvae from a pond using dipper, pipette and container from the Control study site (Gogete).

The preserved specimens were soaked for 12 hours in watch glasses containing distilled water. Each larva is then mounted on a glass slide separately in a drop of gum chloral mountant and covered with a piece of cover slip. Each cover slip measuring 22x22 mm is cut into four parts and one piece is used for a single specimen. The gum chloral mountant is prepared in the laboratory as described in Gordon and Lavoipierre (1969). The constituents are 25 ml distilled water, 160 gm crystal chloral hydrate, 15gm gum Arabic, 10 gm glucose syrup and 5ml glacial acetic acid which are then mixed in the above order in a water bath at a temperature of 80^0 C. At all times the mountant is kept in a dark and

well-screwed bottle. The identifications of species on immature made by using third and fourth instars larvae based on Verrone (1962b) and Gillies and Coetzee (1987) under a compound microscope.

3.2.2.2. Adult *Anopheles* collections, identification and density determination

Indoor and outdoor female *Anopheles* mosquitoes collection using mouth suction aspirators, CDC- light traps and pyrethrum spray collection methods were carried out once every months for a total of 26 months in households of the Control and Intervention group (WHO, 1992). The collected female *Anopheles* mosquitoes were sorted, counted, categorized as unfed, fed, half gravid and gravid, finally placed in a separate labelled paper cups thus all collected specimens transported to Addis Ababa (EPHI or AAU laboratory) for species identification. The procedure of collection by the three methods described below.

Pyrethrum *spray collection:* The pyrethrum spray collection method as described by WHO (2013) was employed to collect indoor resting adult *Anopheles* mosquitoes. pyrethrum spray collections of indoor resting mosquitoes were carried out once every month early in the morning beginning 6:00 am to 10:00 am. After permission is granted from the head of household or inhabitants, all human occupants, animals, domestic utensils, exposed food and water were voluntarily moved out of the dwellings prior to the spraying all household items that cannot be moved out were covered with plastic material.

Then, the entire floor were covered with white cloth sheets cut to 1.2mx2.20m, and doors and windows were closed, openings and eaves also properly covered and all entire parts

of the rooms were sprayed with aerosol (containing pyrethrum) after spraying the dwelling were left closed for 20-30 minutes to produce a knockdown effect (Figure 3.4).

Figure 3.4. Indoor resting *Anopheles* mosquitoes caught using pyrethrum spray collection method in the Control study site (Gogete).

Mouth suction aspirator collection: Mouth suction aspirator collections of *Anopheles* mosquitoes were carried out on a different day once every month, early in the morning beginning 6:00 am to 10:00 am. Mouth suction aspirator and flash light (torch) were used to collect indoor resting mosquitoes from walls, under roofs, hanging clothes, household utensils and dark corners.

CDC-Light traps collection: CDC light traps collections were used to collect *Anopheles* mosquitoes once every month; CDC light traps were positioned inside and outside of

selected houses from 06:30 pm to 6:00 am at night to collect *Anopheles* mosquito (Figure 3.5).

Figure 3.5. Installing CDC-Light trap used for outdoor collection of *Anopheles* mosquitoes in the Intervention study site (Jolie).

To identify the species of *Anopheles* mosquitoes two methods were employed. One of the methods is morphologically based on keys under a dissection microscope with a magnification power of 20x (Verrone 1962b; Gillies and Coetzee, 1987), the second is by using polymerase chain reaction (Scott *et al.,* 1993).

3.2.2.3. Species identification of adult *An. gambiae* complex using Polymerase Chain Reaction (PCR)

DNA extraction, amplification and gel electrophoresis procedures

Almost 10% of indoor collected and morphologically identified as *An. gambiae* s.l were selected randomly and identified to their sibling species in the complex by polymerase chain reaction (PCR), using an adapted version developed by Scott *et al.* (1993) for *Anopheles gambiae* s.l. complex discrimination based on species specific single nucleotide polymorphism (SNPs) in the intergenic spacer region (IGS) following a minor modification by Wilkins *et al*. (2006) incorporating intentional mismatch primer (IMP) to increase specifity was used for the allele amplification.

Genomic DNA was extracted from legs and/or abdomen tissues of individual mosquitoes according to the method described by Collins *et al.* (1987). The *Anopheles gambiae* s.l. legs and/or abdomen tissues of individual mosquitoes were grinded in 100µl grinding buffer solution (0.2M sucrose, 0.5% SDS, 0.1 M tris-HCL pH 7.5, 0.1 NaCl, 0.05M EDTA pH 9.1) using electric motor pestle with a sterile blue loop on centrifuge tubes until all parts remain homogeneous. The products of the grinded solution were heated at 65°C for 30 minutes. The DNAs were precipitated by adding 18µl of 5M ice-cold Potassium acetate (KAC) and incubated in a container filled with ice for 30-60 minutes, next the solutions were centrifuged at maximum speed of 13, 200 revolutions per minute at room temperature for 20 minutes.

The DNAs supernatant were gentle transferred (let alone transferring the precipitate) to a new labeled centrifuge tubes followed by adding 200µl of 100% ethyl alcohol, after that the solution mixed by shaking inverting the centrifuge tube and incubated inside a negative 20^{0}C deep-refrigerator for overnight.

In the next day the DNAs were precipitated by centrifuging the tubes at maximum speed of 13, 200 revolutions per minute for 30 minutes at 4^0C and then the supernatants were gentle discarded without disturbing the pellets. Then, 200µl of 70% ethyl alcohol added and re-centrifuged for 10 minutes at 13, 200 revolutions per minute. Finally, the 70% alcohol was disposed slowly without disturbing the pellets; the DNA sediments were allowed to dry for 30 minutes at room temperature. Lastly, the DNAs pellets were dissolved in 100µl sterilized water with gentle tapping of the tube to allow the DNAs to re-suspend for amplification process.

Four species-specific and one universl primers were used in DNA coping process, species-specific primer for *An. arabiensis* (AR-3T-R, GTG TTA AGT GTC CTT CTC CGT C); for *An. gambiae*, (GA-3T-R, GCT TAC TGG TTT GT CGG CAT GT); for *An. quadriannulatus*, (QD-3T-R, (GCA TGT CCA CCA ACG TAA ATC C); for *An merus/melas* (ME-3T-R, CAA CCC ACT CCC TTG ACG ATG) and one universal primer sequence (IMP-UN-F, GCT GCG AGT TGT AGA GAT GCG)). Mopti and Savanna rDNA were analyzed using PCR amplified rDNA by the methods of Fanello *et al.* (2002) and Favia *et al.* (1997) and furthermore by DNA sequencing.

Targeting rDNA intergenic spacer (IGS) for the *Anopheles gambiae* complex species were amplified in a multiplex reaction as illustrated by Wilkins *et al*. (2006). PCR reaction was carried out using Taq DNA polymerase (AccuStart II PCR Supermix) and the manufacturer's (Quanta Biosciences) recommended buffer at 1×concentration was used for all reactions. PCR reactions buffer consisted of a 25pmol (3.25 DI ornuclease free water, 6.25 AccuStart II PCR Supermix, 0.5 IMP-UN-F and 0.5µl of each primer) concentration and 0.5µl of DNA in a final 12µl reaction mix. The plates covers were

fasten tightly and spine down in microcentrifuge at maximum speed for two to three minutes and then amplified.

The final reaction mix, thermal cycling for all analyses was performed in a Bio-Rad iCycler®, PCR cycling consisted of melting at PCR conditions rn 1: 95^{0}C for 4' followed by 34 cycles of: 95^{0}C for 30”; 60^{0}C for 30" and 72^{0}C for 30" and a final elongation step at 72^{0}C for 5'. Agarose gel was prepared on the bases described by Wilkins *et al*. (2006) protocol. The prepared agarose gel was placed in to electrophoresis box and 1µl of molecular weight ladder was loaded into the first and the last lane of the agarose gel, then 1µl of PCR amplified DNAs (the experimental and control) were loaded into the rest wells of the agarose gel.

The electrophoresis box positive and negative ion electrodes properly pluged to the gel electrophoresis machines and subsequently allowed to run at a voltage of 90, 400amper for 90 minutes. DNAs fragments of *An. gambiae* complex species were visualized by using Benchtop UV Transilluminator machine (Figure 3.6). MultiDoc-ItTM Imaging System-Masterflex computer software was used to identify DNA fragments and capture photo of the DNA bands. The gel electrophoresis (DNA fragment) results were interpreted by using bands of the markers on the first and the last lane of the gel. *An. gambiae* s.l. sibling species were identified by comparing the DNA band with already known molecular weight ladder bands. DNA fragments having 463bp were determined to be *An. gambiae* Giles/*An. colluzzi* Coetzee and Wilkerson, 528bp *An. melas* Theobald/*An. merus* Donitz, 636bp *An. quadirianulatus* Theobald/ *An. amharicus* Hunt, Wilkerson and Coetzee and 387bp *An. arabiensis* Patton. The bp numbering is as previously designated [Genbank: AY787486].

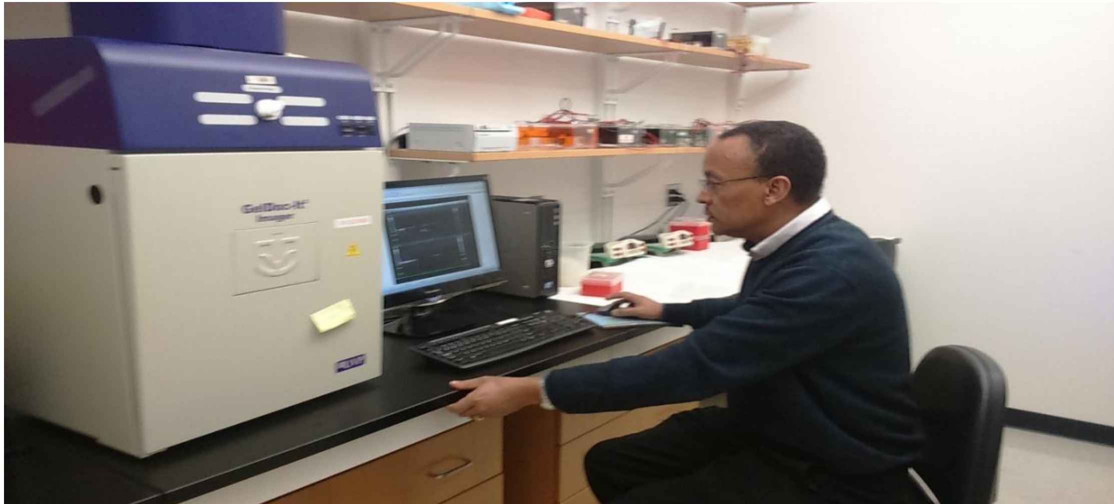

Figure 3.6. DNAs fragments of *An. gambiae* complex species visualized by using Benchtop UV Transilluminator machine in CDC Entomology Molecular Laboratory.

3.2.3. Data analysis

The collected data was computerized using Epi Info 7 and analysed using Stata/SE 11.0, percentage, mean, proportion and 95% CI was constructed to help in making inference towards the target population, for significance differences ($p<0.05$) two-sample t test with equal variance was used.

3.2.4. Ethical clearance

The proposal was presented and reviewed by the Addis Ababa University and Ethiopian Public Health Institute (EPHI) of Scientific and Ethical Review Office (SERO). In addition verbal and signed consent were obtained from the study participants for permission to carry out indoor adult female *Anopheles* mosquitoes collections using mouth suction aspirators, CDC- light traps and space spray in their houses.

3.3. Results

3.3.1. Species composition, monthly variation and breeding habitats preference of *Anopheles* larvae

A total of 4638 mosquitoes larvae were collected from different types of breeding sites, of which 4253 were *Anopheles* mosquitoes the rest 385 were culex larvae. About 135 of the larvae of *Anopheles* could not be identified because of bad preparation and mechanical damage, thus 4118 third and fourth instars larvae were used for the identification.

The majority 27.51% of *Anopheles* mosquitoes larvae were collected from marshy breeding site which served as a major breeding habitat during the dry season, followed by irrigation canals 24.50%, rain pools 20.45%, river pockets 17.63% and ponds 9.91% in the study areas (Figure 3.7).

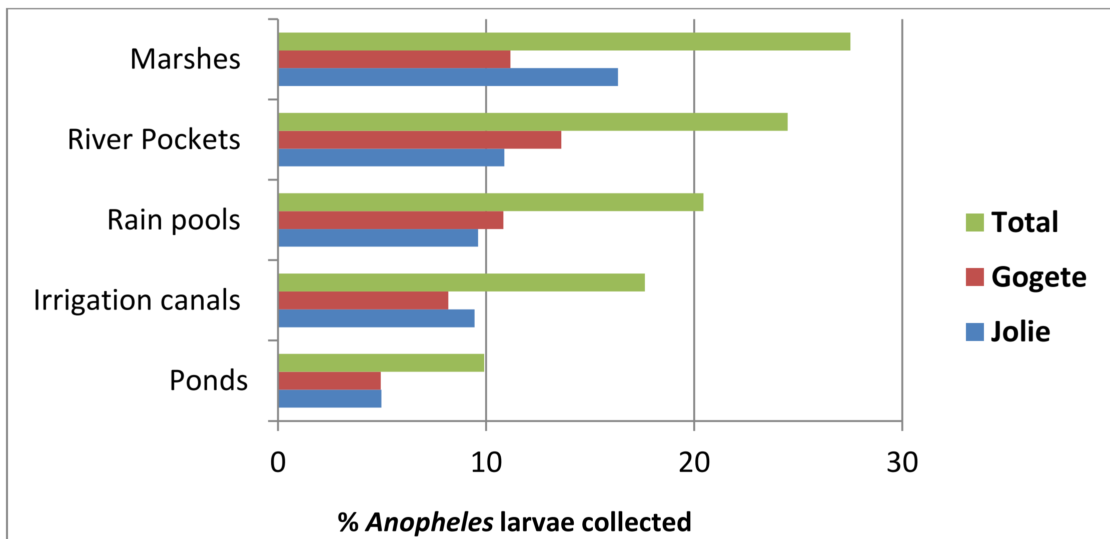

Figure 3.7. Breeding habitats and percentage of *Anopheles* larvae collected in the study areas from May 2013 to June 2015.

Immature Anopheles species composition identified

A total of 2111 and 2007 *Anopheles* mosquitoes larvae from Intervention (Jolie) and Control (Gogete) study sites collected respectively (Table 3.2). In Intervention study site (Jolie) ten *Anopheles* mosquitoes species were identified, *An. gambiae* s.l. was found the dominant species accounts for 36.1% (n=763) followed by *An. cinereus* 24.1% (n=509), *An. christyi* 23.1% (n=488) and *An. pharoensis* 7.1% (n=149) the rest are collected in small amount (Table 3.2). In Control study site (Gogete) eleven *Anopheles* mosquitoes species were identified, similarly *An. gambiae* s.l. was also found the dominant species accounts for 35.8% (n=719), followed by *An. cinereus* 23.9% (n=479), *An. christyi* 22.9% (n=459) and *An. pharoensis* 6.7% (n=135) (Table 3.2).

Table 3.2. Species composition and percentage of *Anopheles* mosquitoes identified from larval collections in the study areas collected from May 2013 to June 2015.

Species	**Number of mosquitoes larvae collected (%)**		**Total (%)**
	Jolie	**Gogete**	
An. gambiae s.l	763 (36.1)	719 (35.8)	1482 (36.0%)
An. cinereus	509 (24.1)	479 (23.9)	988 (24.0%)
An. christyi	488 (23.1)	459 (22.9)	947 (23.0%)
An. pharoensis	149 (7.1)	135 (6.7)	284 (7.0%)
An. demeilloni	113 (5.4)	101 (5.0)	214 (5.2%)
An. garnhami	37 (1.8)	29 (1.4)	66 (1.6%)
An. longipalpis	28 (1.3)	21 (1.1)	49 (1.2)
An. marshali	20 (0.9)	13 (0.6)	33 (0.8%)
An. pretoriensis	2 (0.1)	2 (0.1)	4 (0.1%)
An. sergentii	2 (0.1)	2 (0.1)	4 (0.1%)
An. squamosus	0 (0)	47 (2.4)	47 (1.1%)
Total	**2111 (100)**	**2007 (100)**	**4118 (100)**

Monthly variation in Anopheles* larvae *in study sites from May/2013 to June/2015

Statistically significant differences in Intervention (Jolie) (P=0.001) and in Control (Gogete) (P=0.001) study sites (seasonal variations) in *Anopheles* larval collection were observed (Appendix 3.1). The highest collection were done in the months of July, August, September and March accounting 141, 163, 148 and 138 respectively in Intervention study site (Jolie) and in similar months 122, 139, 129 and 129 in Control

(Gogete). The peaks of *Anopheles* mosquitoes were collected in major and minor rainy seasons (Figure 3.8).

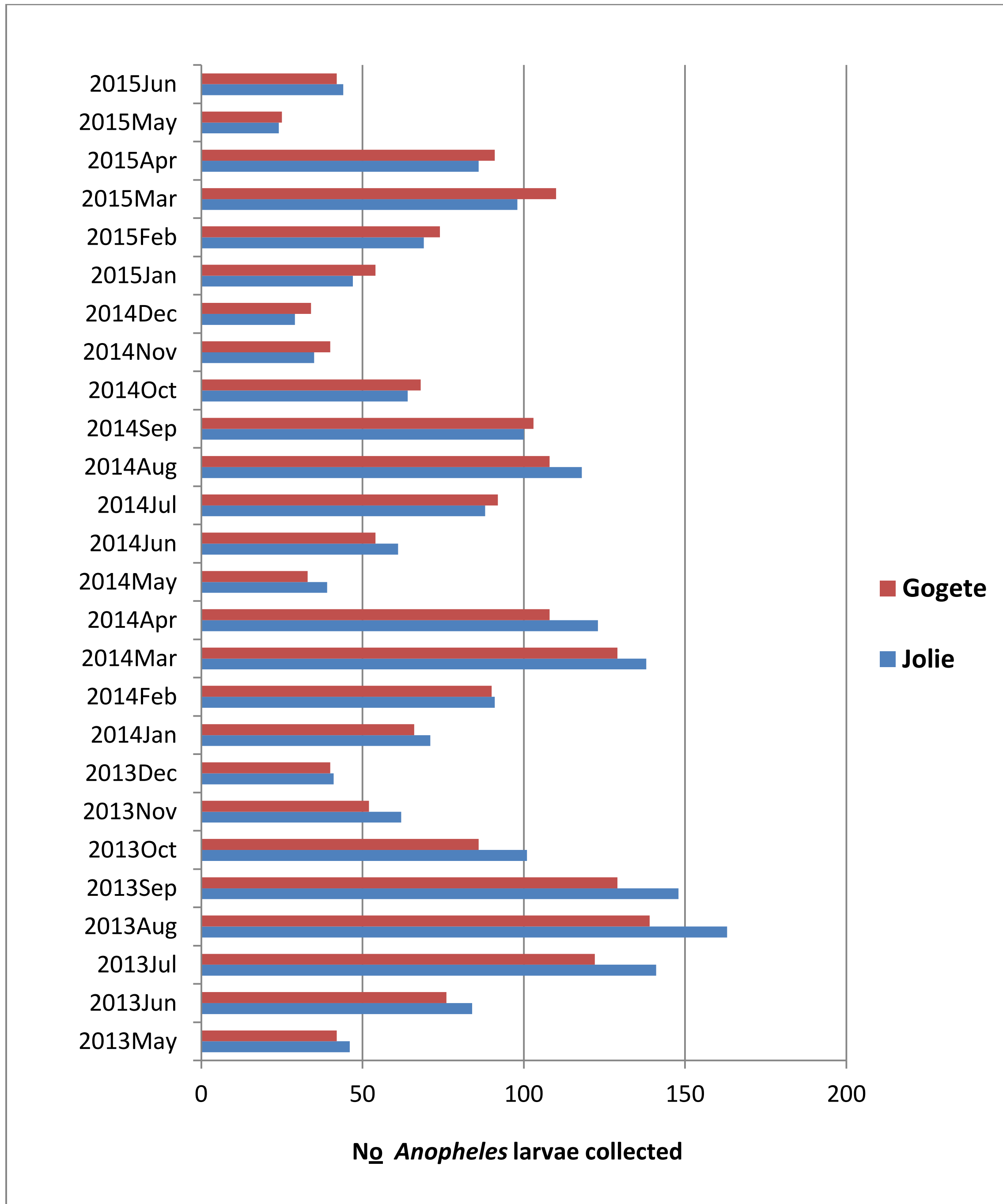

Figure 3.8. *Anopheles* larvae collected in different months in Intervention (Jolie) and Control (Gogete) study sites from May 2013 to June 2015.

3.3.2. Indoor and outdoor adult female *Anopheles* species and density

3.3.2.1. Outdoor collection using CDC light traps and mouth suction aspirators

A total of 1408 adult female *Anopheles* mosquitoes were caught using CDC light traps collection and mouth suction aspirators collection in both study sites, of which 37.7% (n=531) were from Intervention (Jolie) and 62.3% (n=877) were from Control (Gogete). The abdominal status of all *Anopheles* species caught outdoors was found Unfed.

Out of 531 adult female *Anopheles* mosquitoes caught in Intervention study site (Jolie) *An. gambiae* s.l. was found the predominant species making up 34.8%, followed by *An. pharoensis* 18.3% and *An. demeilloni* 16.6% (Figure 3.9). In Control (Gogete) out of 877 adult female *Anopheles* mosquitoes caught *An. demeilloni* was found the predominant species accounts for 31%, followed by *An. gambiae* s.l. 21.7% and *An. pharoensis* 21.1% (Figure 3.9). Statistically significant differences were observed in *An. pharoensis* collected among the study sites (P=0.015), i.e. higher number of *An. pharoensis* were collected in the Control (Gogete) than the Intervention (Jolie), but there were no significant differences in *An. gambiae* s.l. collected in both study sites (P=0.913) (Appendix 3.2)

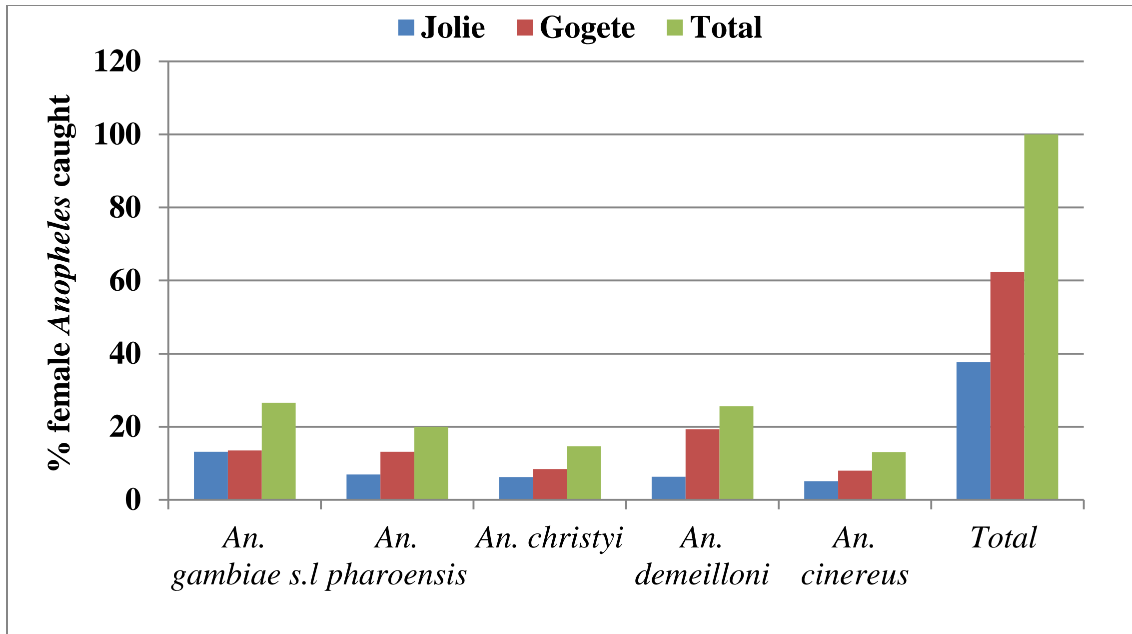

Figure 3.9. Species and percentages of *Anopheles* mosquitoes identified from outdoor collection in Intervention (Jolie) and Control (Gogete) study sites during data collection period from May 2013 to June 2015.

3.3.2.2. Indoor *Anopheles* collections using CDC light traps, space spray and mouth suction aspirators

A total of 4461 indoor adult female *Anopheles* were caught, of which 31.1% (n=1386) from Intervention (Jolie) and 68.9% (3075) from Control (Gogete). Out of 1386 of female *Anopheles* mosquitoes caught from Intervention study site (Jolie) *An. gambiae* s.l. was found the predominant species accounts for 63.9%, followed by *An. pharoensis* 19% (Figure 3.10). In Control study site (Gogete) out of 3075 of female *Anopheles* caught also *An. gambiae* s.l. was found the predominant species accounts for 64%, followed by *An. pharoensis* 19.1% (Figure 3.10). Statistically significant differences were observed in both *An. gambiae* s.l. (P=0.002) and *An. pharoensis* (P=0.001) collection among the

study sites, i.e. higher number of *An. gambiae* s.l. and *An. pharoensis* (Appendix 3.3) were collected in Control study site (Gogete) than the Intervention (Jolie).

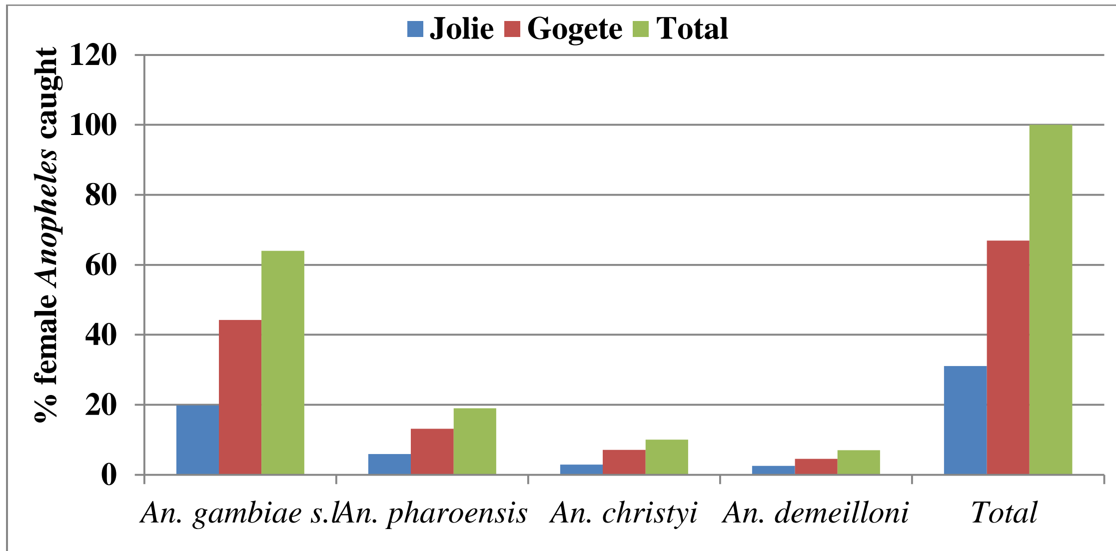

Figure 3.10. Species and percentages of adult *Anopheles* mosquitoes collected indoor by the three methods in the two study sites from May 2013 to June 2015.

Statistically significant (P=0.001) variations between wet and dry seasons were observed in the number of *An. gambiae* s.l. populations in Intervention study site (Jolie) (Appendix 3.4), i.e. higher number of *An. gambiae* s.l. collected during wet season (the number of *An. gambiae* s.l. caught in the year of 2013 and 2014 in the months of June, July and August were) than dry season (the number of *An. gambiae* s.l. caught in the year of 2013 December and 2014 January and February as well as 2014 December and 2015 January and February) presented in Figure 3.11.

Also, Statistically significant (p=0.001) variations between wet and dry seasons in the number of *An. gambiae* s.l. populations in Control study site (Gogete) were observed (Appendix 3.4), i.e. higher number of *An. gambiae* s.l. collected during wet season (the

number of *An. gambiae* s.l. caught in the year of 2013 and 2014 in the months of June, July and August) than dry season (the number of *An. gambiae* s.l. caught in the year of 2013 December and 2014 January and February as well as 2014 December and 2015 January and February) were presented in Figure 3.11.

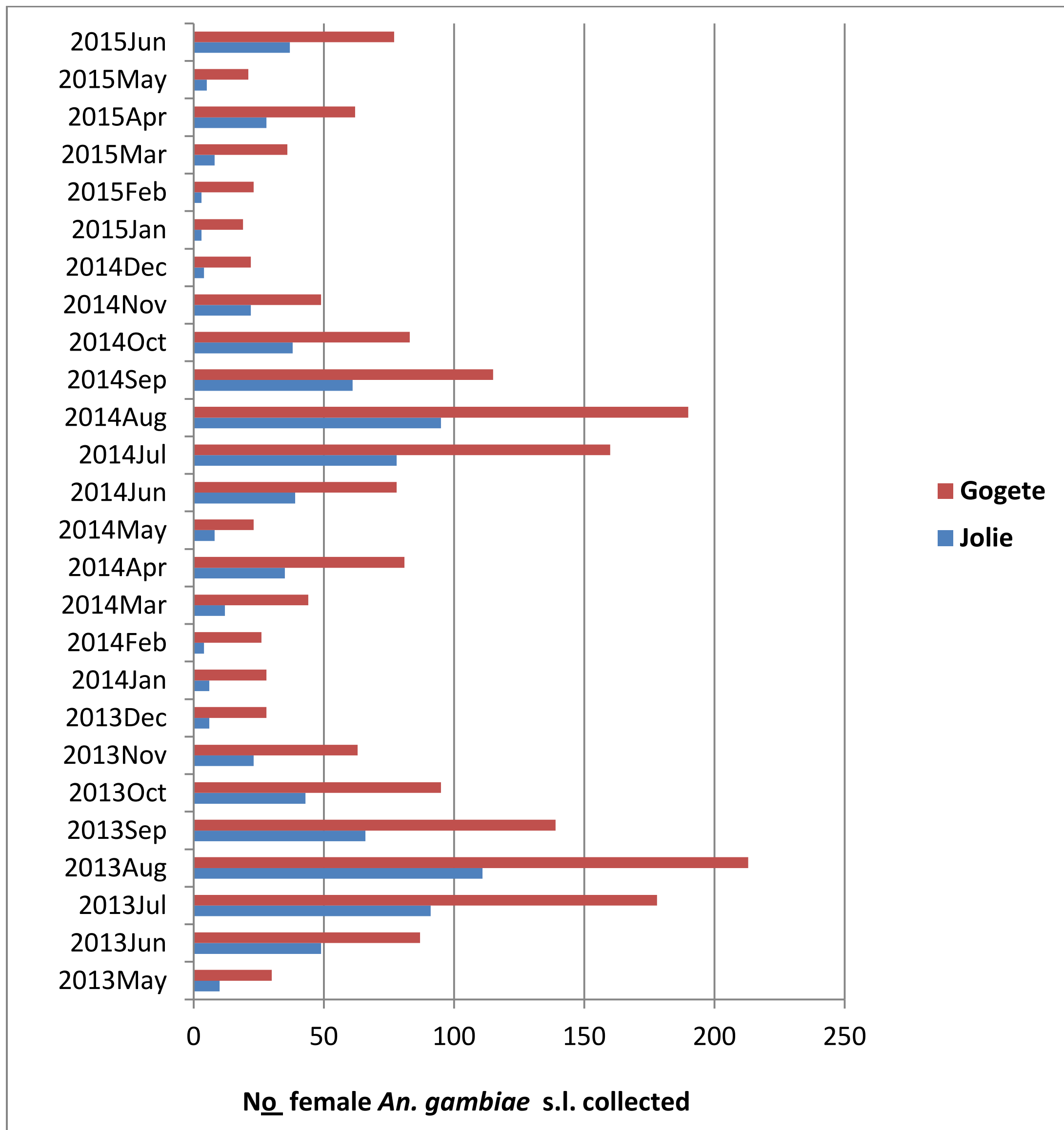

Figure 3.11. Monthly indoor collections of female *Anopheles gambiae* s.l. by three methods in Intervention (Jolie) and Control (Gogete) study sites during data collection period from May 2013 to June 2015.

3.3.3. Adult *Anopheles gambiae* complex species identified by PCR

About 272 indoor collected and morphologically identified as *An. gambiae* s.l. were subjected for PCR sibling species identification, of which due to technical problem the result of 33 specimens were rejected. Thus our result showed that, out of the total (n=239) *An. gambiae* s.l. (Figure 3.12) tested 94.1% were found *An. arabiensis* and 5.9% *An. gambiae* (Table 3.3).

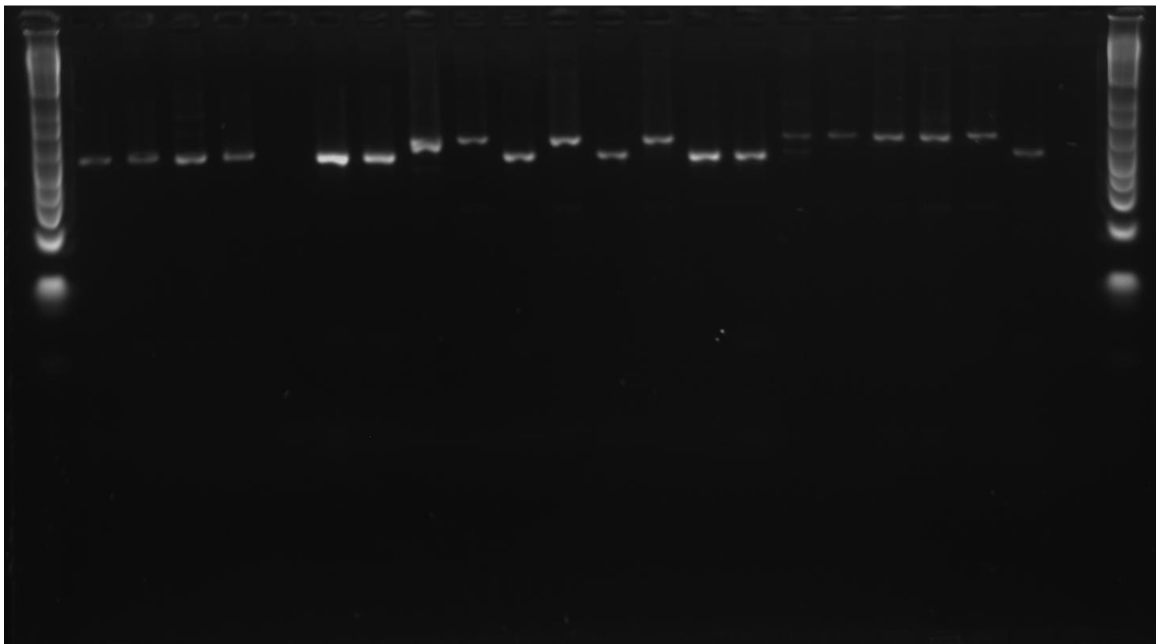

Figure 3.12. Image of PCR result of Gel electrophoresis of *Anopheles gambiae* s.l species identification comparing products run on a 2% agarose gel red. Lanes 1 and 24 1kb ladder, lanes 2-5, 6 not amplified, lanes 7, 8, 11, 13, 15 and 16 were *An. arabiensis* and lanes 9, 10, 12, 14, and 17-20 were *An. gambiae* and lanes 21-23 controls: 1µl of sample loaded and run on a 2% agarose gel red.

Table 3.3. The number and percentages of *Anopheles arabiensis* and *Anopheles gambiae* identified by polymerase chain reaction (PCR) from Intervention (Jolie) and Control (Gogete) study sites.

Species	Jolie	Gogete	Total
	no (%)	no (%)	no (%)
An. arabiensis	117 (48.9)	108 (45.2)	225 (94.1)
An. gambiae	4 (1.7)	10 (4.2)	14 (5.9)
			239 (100)

3.4. Discussion

This study showed the presence of *Anopheles* larvae throughout the study period. The persistence of *Anopheles* mosquitoes throughout the year in Intervention (Jolie) and Control (Gogete) study sites were mainly associated with irrigation practices and river pocket. In addition, the existence of permanent vector breeding habitats created by poor management of water supported the survival of vector species throughout the year.

Ten *Anopheles* species in Intervention (Jolie) and eleven in Control (Gogete) study sites were identified during data collection period. In both localities the predominant *Anopheles* species was *An. gambiae* s.l. which is the main vector in the country (Animut *et al.,* 2012; Gone *et al.,* 2014). *An. cinereus* and *An. christyi* are the second dominant species in the study areas. Similar to the present study *An. cinereus* and *An. christyi* have

been reported from the neighbouring villages at about the same altitude (Animut *et al.,* 2012).

The occurrence of high number of *An. cinereus* and *An. christyi* in this study might be the presence of marsh habitat in the area which is ideal habitat for these two species (Woyessa *et al.,* 2004; Tesfaye *et al.,* 2011; Animut *et al.,* 2012). Substantial numbers of *An. pharoensis* were collected in the present study similar to the study conducted by Animut *et al.* (2012) in the neighbouring village. Irrigation activities in the study area might be associated with the existence of *An. pharoensis* throughout the year, similar to the study conducted in the central rift valley of Ethiopia (Kibret *et al.,* 2010).

However, higher numbers of Anopheles mosquitoes larvae were collected during the wet seasons both in major and minor rainy seasons. The occurrence of higher number of *Anopheles* larvae in this study during the wet season is not agree with a study conducted in neighbouring villages by Animut *et al.* (2012) where more larval collections were observed during the dry season. Frequent larval sampling during the wet season might be the reason for encountering larval positives rain polls in the present study compared to a study conducted by Animut *et al.* (2012). Local meteorological and other environmental factors might be the other explanation for the dominance of *Anopheles* larvae during the wet season.

During our study both temporary and permanent breeding habitats were dominantly occupied by *An. gambiae* s.l. throughout the wet seasons. Alike, study conducted in Butajira area near to the present study sites conducted by Animut *et al.* (2012) *An. gambiae* s.l. was found the dominant species in every types of habitat during the wet

season. The monthly variation in the quantity of *An. gambiae* s.l. in the present study might be coupled with climatic factors such as the amount of rain, temperature and relative humidity (Appendix 3.5).

The breeding habitats of *An. gambiae* s.l. in the study localities particularly in the dry season are due to human activities rather than environmental causes. That is, the water accumulated from overflow of irrigation canals, interrupted marshy areas and River pockets remain the most important breeding sites created by human activities. Similarly, studies conducted in Butajira area and western Kenya, reported that marshes, irrigation canals and river pockets were served as breeding habitat for *An. gambiae* s.l. during months of low precipitation (Animut *et al.,* 2012; Kenea *et al.,* 2011; Imbahale *et al.,* 2011; Kweka *et al.,* 2015).

The result of adult *Anopheles* collection revealed five species throughout the study period. *An. gambiae* s.l., *An. christyi, An. pharoensis, An. demellion and An. cinerus* were the species identified in adult stages whereas *An. garnhami*, *An. longipalpis*, *An. squamosus*, *An. marshali*, *An. pretoriensis* and *An. sergentii* were only collected in larval survey. The reason for this discrepancy might be the resting behaviour of *Anopheles* mosquitoes and the lack of efficient adult collection methods especially for outdoor resting mosquitoes. This is supported by the higher number of *An. christyi, An. demellion* and *An. cinerus* in larval collection compared to lower outdoor adult collection in the present study, the reason behind might be outdoors CDC-light trap may not capture enough mosquitoes in absence of host near to outdoor collection area since some species of *Anopheles* mosquitoes need CO_2 as stimulation cues in addition to light (WHO, 1975).

Of the five species identified in adult collections *An. gambiae* s.l. was also found in appreciable number, this species collected both indoors and outdoors more frequently than the other. Looking at the evidences from the present data no other malaria vector is important than *An. gambiae* s.l. in both study areas. In agreement with other studies, this species is incriminated as the principal vector of malaria in Ethiopia (Animut *et al.,* 2012; Kibret *et al.,* 2010) and elsewhere in East, West and South Africa (Kenea *et al.,* 2011; Imbahale *et al.,* 2011; Kweka *et al.,* 2015). The presence of this species in the study area during the dry months is an indication for the uninterrupted malaria transmission throughout the year. However, *An. pharoensis* was found in both study sites therefore this species can be considered as a secondary vector of malaria in the two study areas as in other parts of Ethiopia (Animut *et al.,* 2012; Kibret *et al.,* 2010).

In this study seasonal and local variations of indoor and outdoor number of collection were observed during data collection period. For instance, higher indoor and outdoor collections were observed in both study sites during the wet seasons. Similar to the present study the presence of higher indoor numbers of *An. gambiae* s.l. were observed in a studies conducted in Ethiopia and Kenya (Massebo *et al.,* 2013a; Gari *et al.,* 2016; Amek *et al.,* 2012) during the wet season. The possible explanation of higher *An. gambiae* s.l. during the wet seasons in the present study and other studies conducted in different parts of Ethiopia could be linked with the abundance of small temporary rain polls which is suitable larval habitat especially for *An. gambiae* s.l. (Munga *et al.,* 2013; Kweka *et al.,* 2012). On the contrary, higher numbers of *An. gambiae* s.l. were reported during the dry seasons in a previous study conducted near to the present study area (Animut *et al.,* 2013a). The number of *An. gambiae* s.l. and *An. pharoensis* collected

during the study period were greater in Control study site (Gogete) than Intervention (Jolie). The treatment given for the Intervention study site (Jolie) might reduce indoor and outdoor mosquitoes compared to Control (Gogete). Similar to the present study the impact of IRS in lowering indoor densities of *An. gambiae* s.l. was reported in study conducted in western foothill of Madagascar (Ratovonjato *et al.,* 2014). Besides, it was confirmed that indoor residual spray (IRS) and LLINs are reliable and effective in a wide range of situations if properly used (Noor *et al.,* 2009; MOH, 2011; Shargie *et al.,* 2008, 2009).

In the present study *An. gambiae* was identified for the first time in Ethiopia indicating the presence of more efficient vector of malaria in the *An. gambiae* complex in addition to *An. arabiensis*. Previous and recent studies conducted in different part of Ethiopia reported that *An. arabiensis* and *An. amharicus* are the only two species identified in Ethiopia (Coetzee *et al.,* 2013; Habtewold *et al.,* 2001; Animut *et al.,* 2012; Massebo *et al.,* 2013b). The absence of *An. gambiae* in the previous studies conducted in Ethiopia might be coupled with lack of accessibility to collect *Anopheles* mosquitoes, technical and technological shortcomings, however the present study was conducted in CDC, Atlanta, GA, USA thus the above mentioned problems might be solved as a result additional species identified. Besides, to verify and increase precision for the presence of *An. gambiae* in addition to PCR further confirmed by DNA sequencing.

3.5. Conclusion

Although, higher numbers of *Anopheles* mosquitoes were collected during the wet seasons both in major and minor rainy season, this study showed the persistent existence

of *Anopheles* larvae throughout the study period. The larval breeding sites in both study sites particularly in the dry season are due to human activities rather than other environmental sources. From larval collections ten *Anopheles* species in Intervention study site (Jolie) and eleven in Control (Gogete) were identified in the present study. In both study sites, the predominant *Anopheles* species was *An. gambiae* s.l. which is the main vector in the country.

From adult collections, *An. gambiae* s.l., *An. christyi, An. pharoensis, An. demellion and An. cinerus* were present throughout the study period. In the present study, 5.9% of the tested *An. gambiae* s.l. was identified as *An. gambiae* which is reported for the first time in addition to *An. arabiensis* in Ethiopia indicating the presence of the highly efficient vector in Ethiopia.

Higher numbers of *An. gambiae* s.l. were collected in the Control study site (Gogete) than Intervention (Jolie). Thus, the treatment applied as intervention with rotation of insecticides used in IRS and new LLINs in the Intervention study site (Jolie) might have resulted in reduced indoor and outdoor *An. gambiae* s.l catches compared to the Control study site (Gogete) during the study period.

Chapter 4 Blood feeding status, host preference and detection of parity and sporozoite rate in *Anopheles* mosquitoes in the study areas

4.1. Introduction

Indoor Residual Spray (IRS) and Long Lasting Insecticidal Netss are reliable and effective for malaria vector control in a wide range of situations (Noor *et al.,* 2009; MOH, 2011; Shargie *et al.,* 2008, 2009). Underutilization of these intervention tools by the community were found to be one of the major problems in malaria vector control programmes (MOH, 2011; Dagne and Deressa, 2008; Fettene *et al.,* 2009; Gobena *et al.,* 2012). Earlier studies reported that utilization of LLINs and IRS have a significant impact in reducing mosquito densities and modifying parity and sporozoite rates when properly used (Gobena *et al.,* 2012; WHO, 2007) .

Female *Anopheles* mosquitoes pick up gametocytes with the blood meal from an infected person. The extrinsic cycle of the parasite to the sporozoite stage in the mosquito vector proceeds along with subsequent blood feeding and gonotrophic development. Infective mosquitoes then bite humans and inject the sporozoites while blood feeding and transmit malaria. The blood meal digestion and egg development at different stages could be detected with change in the appearance of the abdomen of the female *Anopheles* as the result of blood digestion and ovarian development (WHO, 1975). In *Anopheles* mosquitoes, ovary maturation (egg development) occurs at the same time as blood digestion. Based on their blood digestion and egg development stages or abdominal condition, the abdominal status of *Anopheles* can be grouped as unfed, fed, half-gravid and gravid (WHO, 2013, 1975).

These blood meal digestion and ovarian development categories are indicaors of exposure of female mosquitoes to human host and thus could carry the malaria parasites. The proportions of fed and gravid females in the vector population have epidemiological significance in the level of malaria transmission and vector control in an area.

Preference of *Anopheles* mosquitoes to feed on humans can be estimated using human blood index (HBI) as the proportion of female mosquitoes fed human blood from a total of freshly fed female *Anopheles* (WHO, 1975). However, as a vector mosquito species may feed on alternative hosts depending on availability and accessibility of potential host, it remains imperative to assess their blood meal source in local settings. Tests such as enzyme-linked immunosorbent assay (ELISA) (Beier *et al.,* 1988; Beier *et al.,* 1994); precipitin test (Krafsur and Armstrong, 1978) and polymerase chain reaction (PCR) (Kent *et al.,* 2007) can be employed to identify the blood meal source of malaria vectors.

Parous females are exposed to potentially infected hosts and hence are epidemiologically dangerous since these are the most important to carry malaria parasites to human. They could have acquired malaria infections with previous blood meals from infected hosts (WHO, 2013). It is therefore epidemiologically valid to study the parous rate in malaria vector population.

Although, so far detected sporozoite rates in Ethiopia were very low the vectoral status of *Anopheles* in a given area is assessed by detection of sporozoite infection rate in mosquitoes. For instance, in *An. arabiensis* sporozoite rate was the highest one which varied from 0 to 3%, but in the three other secondary vectors namely; *An. funestus, An. nili* and *An. pharoensis* were reported with a range of 1.23-.4%, 0-1.29% and 0-0.45%,

respectively (Nigatu *et al.,* 1992). In Tanzania 17 times greater entomological inoculation rates (EIR) were observed in lowland area than highland area, though the incidence of infection was differed only 2.5 times (Maxwell *et al.,* 2003). Also, high sporozoite infection and entomological inoculation rate in dry season than rainy season in perennial transmission areas of Cameron were reported (Bigoga *et al.,* 2012). The physiological age of mosquitoes is determined by ovarian dissection method described in Detinova (1962). Ovarian dilation counts are made based on the number of ovipositions completed and age of the individual mosquito is determined by the number of dilatations as 1–parous, 2-parous, etc. the number of ovipositons so far recorded for *An. gambiae* s.l. was six (Detinova and Gillies, 1964).

Therefore the aim of this study was to determine the impact of resistance management options applied in the study sites receiving different levels of malaria vector management packages (the Intervention (Jolie) and Control (Gogete) study sites) on the man biting and resting densities, abdominal status, host preference, resting activities, parity, sporozoite rates and entomological inoculation rate of *Anopheles* mosquitoes.

4.2. Materials and methods

4.2.1. Description of study sites

As described above in section 3.2.1.

4.2.2. Study design and mosquitoes collection procedures

4.2.2.1. Application of IRS and LLINs with rotation of insecticides

Longitudinal study design was used to determine the impact of resistance management options applied in the study sites. Indoor residual spraying (IRS) using insecticide Fenitrothion WP and Pirimiphos-methyl both sprayed at a dosage of 2g a.i./m^2 in rotation, were applied alternatively during the study period (from 2013-2016) in the Intervention study site (Jolie). This was coupled and synchronized with the mixed use of Deltamethrin and Alpha-cypermethrin impregnated LLINs which were distributed in the Intervention site (Jolie) and used along with the IRS. Both the Fenitrothion WP and Pirimiphos-methyl insecticides used for rotation in IRS retain residual effect with mosquitocidal activities for 3-6 months period (WHOPES, 2003; WHO, 2006).

On the otherhand, the insecticide Bendiocarb WP was used for IRS at a dosage of 0.1–0.4g a.i. /m^2 applied for five consecutive years in the Control study site (Gogete) along with Deltamethrin impregnated LLINs which were used for three years. The insecticide Bendiocarb WP used for IRS has residual mosquitocidal efficacy 2-6 months (WHOPES, 2003; WHO, 2006).

The impact of resistance management options applied in the study sites receiving different levels of malaria vector management packages (the Intervention (Jolie) and Control (Gogete) study sites) on the man biting and resting densities of *Anopheles* mosquitoes, abdominal status, host preference, resting activities, parity, sporozoite rates and entomological inoculation rate were evaluated and used for comparison.

4.2.2.2. Adult *Anopheles* mosquito collection, processing and species identification

As described above in section 3.2.2.2

4.2.2.3. Determination of *Anopheles* abdominal status and parous rate

Female *Anopheles* mosquitoes collected by using mouth suction aspirators, CDC light traps and pyrethrum spray methods were sorted, counted, categorized as unfed, fed, half gravid and gravid, finally placed in a separate labelled paper cups until laboratory test carried out (WHO, 2013).

Unfed female *Anopheles* caught by the methods of pyrethrum spray, CDC light traps and aspirator collection were used to determine parity rate. The female *Anopheles* mosquitoes were first identified morphologically to respective species before dissection commences according to WHO (2013). The individual mosquito in a test tube was immobilised with either ethyl acetate or chloroform depending on availability of the chemicals. Each mosquito was placed in a drop of fresh physiological saline (0.85% Sodium chloride solution) on a slide and dissected under a dissection microscope.

Using two entomological needles mounted on slim wooden handle the legs and wings were first removed. With one needle pressed gently against the thorax, the last 2-3 abdominal segments were pulled by the second needle and drawn away from the body to expose the gut and ovaries. Of the two ovaries, one was left aside on the same slide to dry (to observe the skeins of the tracheoles) while the other was further dissected to see the presence or absence of ovarian dilatations and relics (Detinova, 1962; Detinova and Gillies (1964). The ovaries for parity were examined under the 10x and 40x objectives of a compound microscope.

4.2.2.4. Blood meal analysis and host preference studies

Host preferences were determined by analyzing the source of *Anopheles* mosquito-meals (Figure 4.1). The proportion of mosquitoes with human blood in vector species was used

as an indication for the degree of anthropophily of a particular species. Blood meals ELISA were used to determine the sources of blood meals (human vs. bovine) as per protocol by Beier *et al.* (1988).

Figure 4.1. Blood meal analysis to determine blood meal sources using ELISA test in Holeta Molecular Biotechnology Laboratory.

The abdomens of freshly feed mosquitoes collected by the three methods were crushed in 50µl phosphate buffer saline (PBS) and the volume was brought to 200µl with PBS buffer. Fifty micro-litters of mosquito triturate was added to two separate 96-well micro-titer plate (for human and bovine blood) simultaneously incubated overnight at room temperature. Each well was washed twice with PBS-Tween 20 and 50µl peroxidase-conjugated anti-humans IgG and peroxidase-conjugated anti-bovine IgG was added in

each well of the first and the second plate then incubated for one hour. After one hour, each well was washed three times with a PBS–Tween 20. Finally, 100µl of peroxidase substrate was added to each well and incubated at room temperature for 30 minutes and the absorbance at 405 nm was recorded with an ELISA plate reader (Beier *et al.,* 1994; WHO, 2013).

Each blood meal sample was considered positive if the absorbance value exceeded the mean plus three times the standard deviation of the four negative controls. Negative controls from a laboratory colony of *An. arabiensis* adults not fed with blood and positive controls contained human and bovine blood were included in the plates (Beier *et al.,* 1994).

4.2.2.5. Determination of *Plasmodium* sporozoite infection using Enzyme-linked immiunosorbent assay (ELISA)

Female *Anopheles* mosquitoes were first identified to respective species before the act of An Enzyme-Linked Immunosorbent Assay (ELISA) according to WHO guideline (WHO, 2013). Unfed female *Anopheles* mosquitoes caught by the three methods were subjected for ELISA test. ELISA bioassay was used to test heads and thoraces of dried unfed female *Anopheles* mosquitoes for the presence of *Plasmodium falciparum* and *Plasmodium vivax* circumsporozoite proteins (CSP) as described by (Wirtz *et al.*, 1987).

For detection of sporozoites the salivary glands were separately dissected out either attached to the head or by pulling them from the anterior thorax sometimes with the front coax. Three separate 96-well micro titer plates were coated with 50µl capture mAb of *P. falciparum*, *P.vivax*-210 and *P.vivax*-247 respectively and incubated for 30 minutes at

room temperature. The well contents were dumped and emptied, washed three times with PBS-tween 20, filled with 200µl blocking buffer (BB) and incubated at room temperature for one hour. At the same time mosquitoes were grinded individually in 50µl grinding buffer (25ml BB: 125µl Igepal CA-630) and the grinding pestle was rinsed 2 times with 100µl grinding buffer to brought the final volume 250µl (Figure 4.2).

To avoid contamination between mosquitoes the pestle were rinsed with PBS-Tween twice and the pestle was cleaned with tissue paper. After one hour of incubation, the BB was aspirated from the well and 50µl mosquitoes triturates were loaded to each of the three test wells. Similarly 50µl CSP positive sample (Well A1) as positive control and laboratory breed *An. arabiensis* (Well B1-H1) as negative controls were loaded in to three test wells. Plates were incubated for two hours and washed with PBS-Tween 20 twice. 50µl peroxidase mAB (0.05µg/10µlBB) were added to each triplicate well in plates and incubated for 1 hour.

The plates were aspirated and washed three times with 200µl PBS-Tween 20. After the enzyme activity was checked, 100µl ABTS substrates were added per well and incubated for 30 minutes. Plates were visually observed for green colour and/or by 405 nm micro plate reader. Samples were considered positives if the absorbance value (OD) greater than 2 times mean OD of negative samples. Positives samples were retested for confirmation by quantitative testing (Writz *et al.,* 1987).

Figure 4.2. Specimens prepared for ELISA test in CDC Entomology Laboratory, Atlanta, GA, USA.

4.2.3 Data analysis

Abdominal status of female *Anopheles* were expressed as percentages of unfed, fed, half gravid and gravid to the total number of mosquitoes examined. The Human Blood Index (HBI) and Bovine Blood Index (BBI) as well as parity and sporozoite rate were computed by using the WHO formula described below (WHO, 2013).

$$\textbf{Human blood index} = \frac{\textit{Number of mosquitoes which have fed on man}}{\textit{Total number of mosquitoes whose blood meals have been identified}}$$

$$\textbf{Bovine blood index} = \frac{\textit{Number of mosquitoes which have fed on bovine}}{\textit{Total number of mosquitoes whose blood meals have been identified}}$$

Note: Mixed blood meals were added to the number of human and bovine blood meals when calculating the HBI (human + mixed) and BBI (bovine + mixed) (Pappa *et al.,* 2011).

$$\textbf{Parous rate} = \frac{\text{Number of parous females}}{\text{Number of females examined}} \times 100$$

$$\textbf{Sporozoite rate} = \frac{\text{Number of mosquitoes with sporozoites}}{\text{Number of mosquito dissected}} \times 100$$

Besides, the collected data were entered into computer using Epi Info 7 and analysed using Stata/SE 11.0. Percentage, mean, proportion and 95% CI were constructed around selected proportions to help in making inference towards the target population; for significance differences ($p<0.05$) two-sample t test with equal variance and two-samples test of proportion were used.

4.3. Results

4.3.1. Abdominal status and parous rates in *Anopheles* mosquitoes

The abdominal status of female *Anopheles* mosquitoes in the Intervention (Jolie) and Control (Gogete) study sites are presented in Figure 4.3. In the Intervention study site (Jolie) out of the total 885 tested *An. gambiae* s.l. and 263 *An. pharoensis* the dominant abdominal status were fed accounts for 42.0% (n=372) and 48.3% (n=127), followed by half gravid 29.6% (n=262) and 28.5% (n=75), gravid 17.5% (n=155) and 16.0% (n=42) and unfed 10.8% (n=96) and 7.2% (n=19) respectively (Figure 4.3). In the Control study site (Gogete) out of the total 1970 tested *An. gambiae* s.l. and 585 *An. pharoensis* the

dominant abdominal status were fed accounts for 39.9% (n=786) and 42.2% (n=247), followed by half gravid 29.7% (n=585) and 28.9% (n=169), gravid 18.7% (n=368) and 17.4% (n=102) and unfed 11.7% (n=231) and 11.4% (n=67) respectively (Figure 4.3). Similar trend was observed in abdominal status of both species between Intervention (Jolie) and Control (Gogete) study sites.

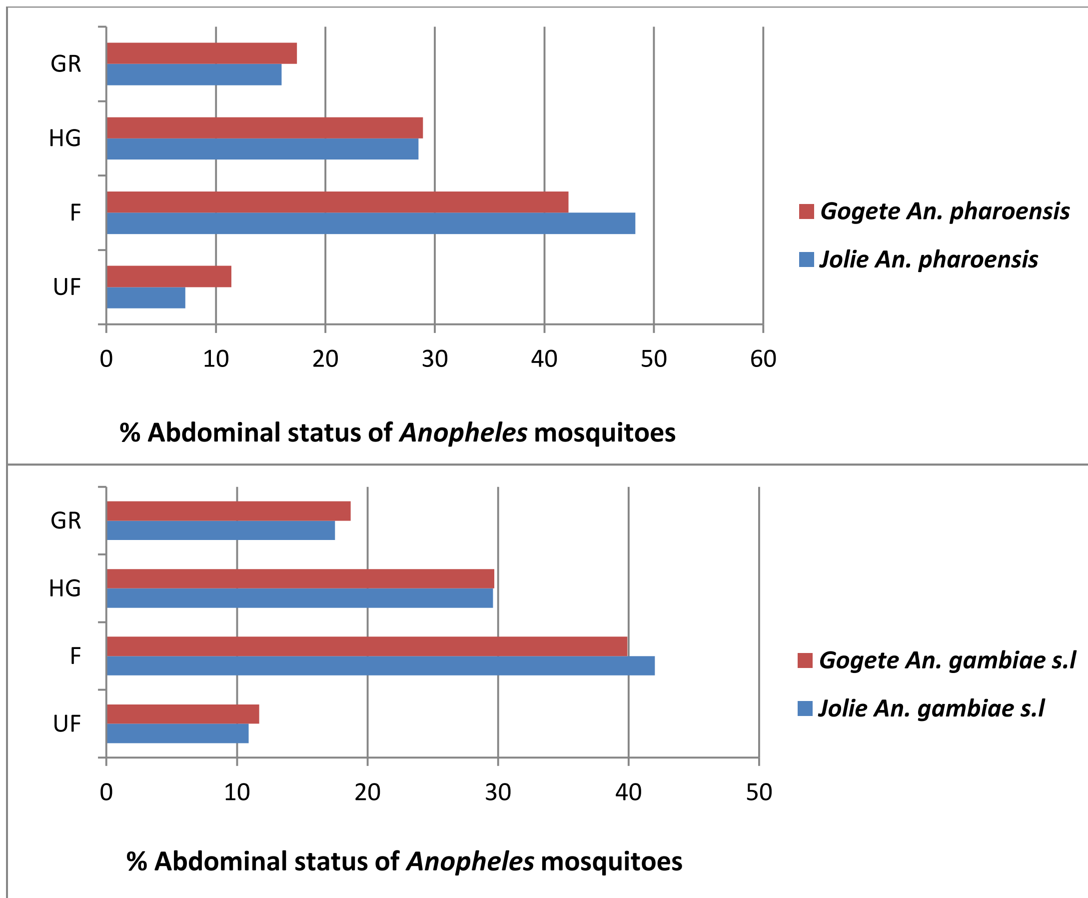

Figure 4.3. The percentage of abdominal status of indoor resting *Anopheles gambiae* s.l. and *Anopheles pharoensis* collected by the three methods in Intervention (Jolie) and Control (Gogete) study sites from May 2013 to June 2015.

A total of 413 indoor unfed *Anopheles* mosquitoes using the three methods were collected from the two study sites. Of which 96 from Intervention study site (Jolie) and

231 from Control study site (Gogete) unfed *An. gambiae* s.l. as well as 19 from Intervention (Jolie) and 67 from Control (Gogete) unfed *An. pharoensis* were dissected to determine the parity rate (PR). In Intervention (Jolie) the parity rate were 45.8 and 52.6 and in Control (Gogete) 61.9 and 62.7 respectively to the above mentioned species (Table 4.1). Statistically significant differences ($p<0.001$) in *An. gambiae* s.l. and *An. pharoensis* ($p<0.001$) were observed among the study sites, that is low parity rate found in Intervention (Jolie) than Control (Gogete) (Appendix 4.1).

Table 4.1. Percentage of parous *Anopheles gambiae* s.l. and *Anopheles pharoensis* collected in Intervention (Jolie) and Control (Gogete) study sites.

Study sites	Species of *Anopheles* mosquitoes	Parity rate
Jolie	*An. gambiae* s.l.	45.8
	An. pharoensis	52.6
Gogete	*An. gambiae* s.l.	61.9
	An. pharoensis	62.7

4.3.2. Host preference and biting activities of *Anopheles* mosquitoes collected from sites with different levels of malaria vector management packages

A total of 504 fed *Anopheles* mosquitoes using pyrethrum spray collection method were collected from the two study sites. Of which from Intervention study site (Jolie) 119 and 261 from Control study site (Gogete) freshly feed *An. gambiae* s.l. as well as 44 from Intervention (Jolie) and 80 from Control (Gogete) *An. pharoensis* were subjected for their blood meal origin test. In Intervention (Jolie) the percentages of human blood index of

An. gambiae s.l. and *An. pharoensis* accounts for 47.9 and 25, for bovine 53.8 and 65.9, for mixed 14.3 and 11.4 and unspecified 12.6 and 20.5 respectively (Figure 4.4). In Control (Gogete) HBI of *An. gambiae* s.l and *An. pharoensis* accounts for 57.1 and 36.2 for bovine 40.6 and 64.9, mixed 9.6 and 6.2, and unspecified 11.9 and 5.1 respectively (Figure 4.5).

At species level in the Intervention study site (Jolie) *An. gambiae* s.l. showed high tendency to feed on bovine 53.8%, followed by human 47.9% (Figure 4.4), unlikely in the Control study site (Gogete) *An. gambiae* s.l. showed high tendency to feed on human 57.1% (Figure 4.5), followed by bovine 40.6%. Thus, statistically significant differences ($p<0.0000$) were found among the study sites, that is low HBI in Intervention (Jolie) than Control (Gogete) was observed in *An. gambiae* s.l. (Appendix 4.2).

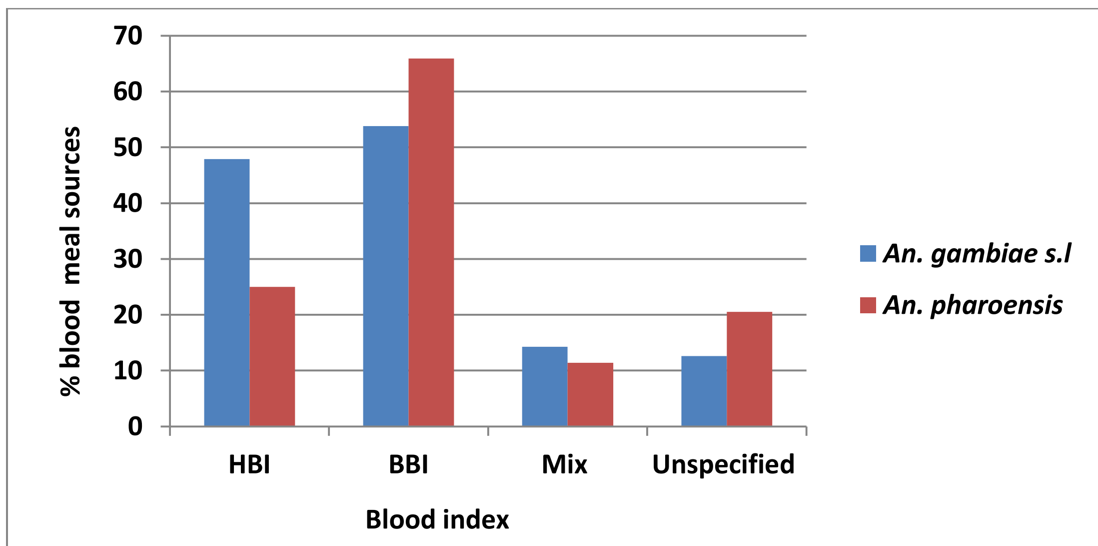

Figure 4.4. Percentages of indoor blood meal sources of *Anopheles gambiae* s.l. and *Anopheles pharoensis* in Intervention study site (Jolie) collected from May 2013 to June 2015.

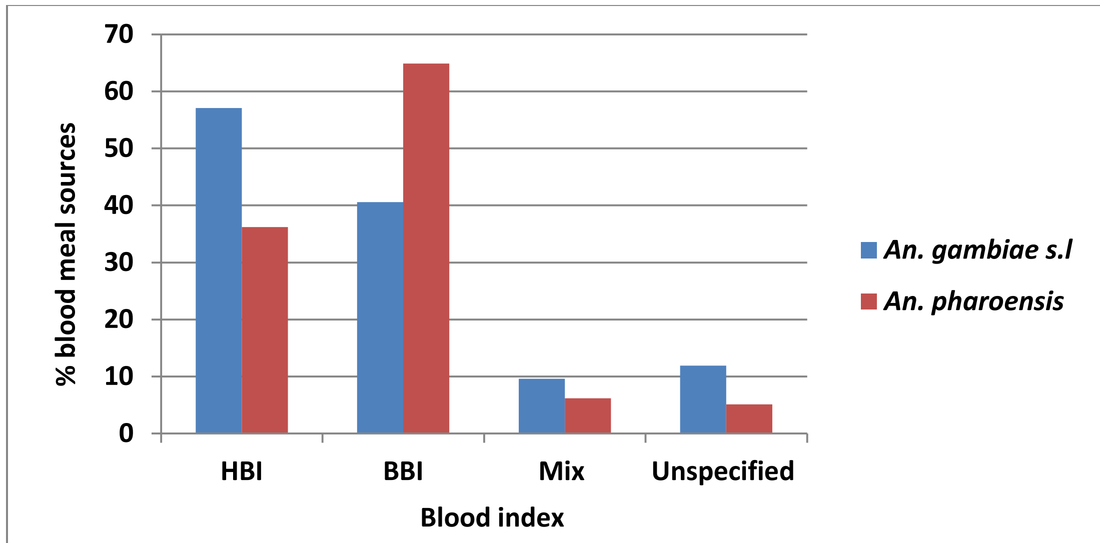

Figure 4.5. Percentages of indoor blood meal sources of *Anopheles gambiae* s.l. and *Anopheles pharoensis* in Control study site (Gogete) collected from May 2013 to June 2015.

4.3.3. Sporozoite rates in *Anopheles* mosquitoes collected from sites with different levels of malaria vector management packages

The result of sporozoite rate of *An. arabiensis* collected from both study sites to detect *Plasmodium* infections were subjected for ELISA test. In Control study site (Gogete) the highest (Figure 4.6) Pv-210 sporozoite rates 2.8 (n=176) were observed compared to Intervention study site (Jolie) 1.1 (n=176). Whereas, the same result of Pf sporozoite rates (1.1) were obtained in both study sites (Figure 4.7).

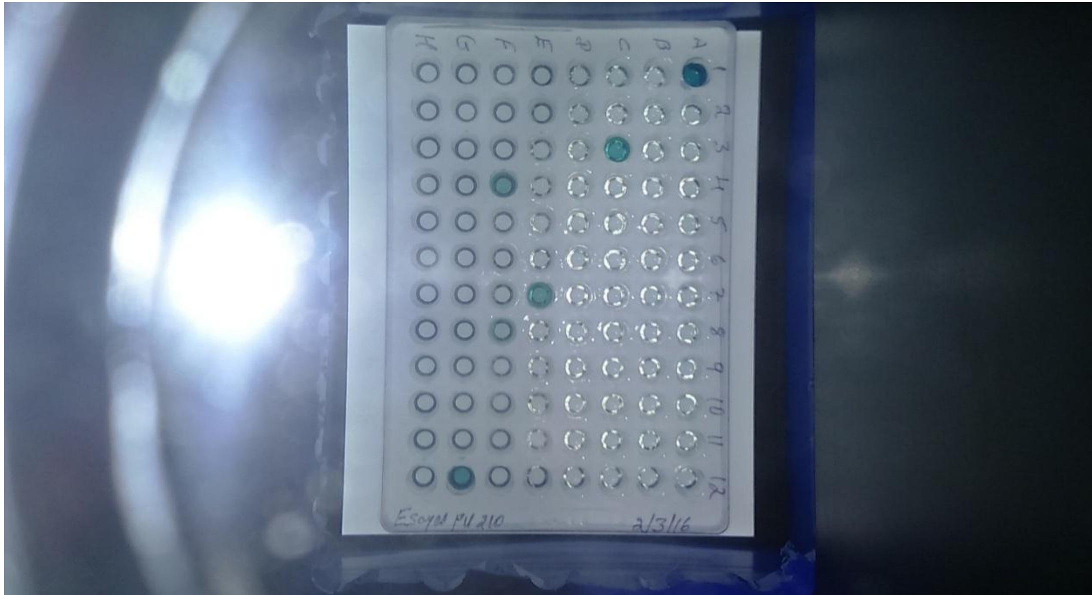

Figure 4.6. Image taken by 405 nm micro plate readers that show the presence of *Plasmodium vivax* from the Control study site (Gogete) tested specimens of *An. arabiensis* in CDC Entomology Laboratory, Atlanta, GA, USA.

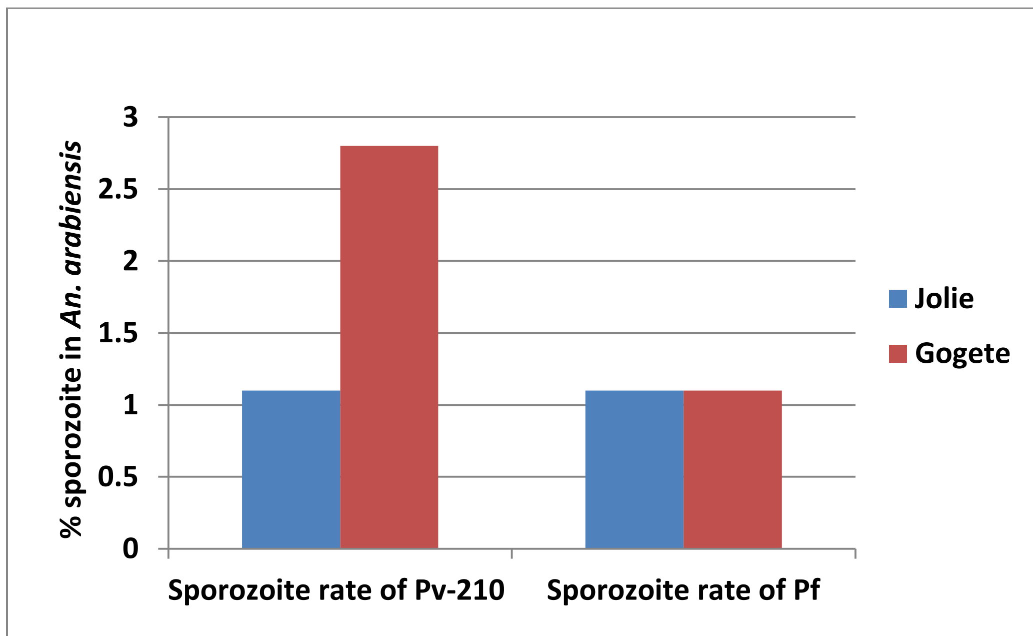

Figure 4.7. Percentage of sporozoite in *Anopheles arabiensis* collected from Intervention (Jolie) and Control (Gogete) study sites collected from May 2013 to June 2015.

4.4. Discussion

In the present study, abdominal conditions, host preference, parity and sporozoite rate were determined in Intervention (Jolie) and Control (Gogete) study sites in order to evaluate the impact of insecticide resistant management on malaria vectors in selected study sites of southern Ethiopia. However, unlike abdominal conditions and sporozoite rate statistically significant differences were observed on parity rate and host preference among Intervention (Jolie)) and Control (Gogete) study sites.

The overall HBI of *An. gambiae* s.l. in Intervention study site (Jolie) was (47.9%) lower compared to the HBI (57.1%) in control study site (Gogete) in the present study. Similarly, higher HBI was reported in *An. arabiensis* in studies conducted in south central Ethiopia in the rift valley region (Kibret *et al.,* 2010; Gari *et al.,* 2016). However, *An. arabiensis* had similar host preference for bovine and human in the nearby village conducted by Animut *et al.* (2013b). In addition, findings from different parts of Ethiopia showed *An. arabiensis* preferred bovine blood than human blood (Habtewold *et al.,* 2001; Massebo *et al.,* 2013b; Yewhalaw *et al.,* 2014). Such host preference significant differences in study sites situated on the same ecological and meteorological conditions and human behaviour clearly indicated the treatment given in Intervention (Jolie) reduced feeding on human.

Similarly, a shift from human to animal feeding in *An. gambiae s.l* was reported in area of intensive IRS spraying in Kenya (Mwangangi *et al.,* 2013) like the present study where the Intervention study site (Jolie) was sprayed by effective insecticides four times during study period. The other explanation for the overall lower HBI of *An. gambiae* s.l. in

Intervention (Jolie) might be associated with the freely new distributed LLINs impregnated with different kinds of insecticides in Intervention (Jolie) than the existing LLINs of in the Control (Gogete) study site.

Similarly, change in species composition from anthropophilic and endophagic species (*An. gambiae s.s*) into opportunistic species (*An. arabiensis*) after mass distribution of LLINs was observed in Kenya and Tanzania (Mutuku *et al.,* 2011; Russell *et al.,* 2010). The HBI was lower in sites that had fewer numbers of cattle than sites that have higher number of cattle (Habtewold *et al.,* 2001) in study conducted in Konso in southern Ethiopia. However, in the present study human-cattle ratio was the same in Intervention (Jolie) and Control study site (data not shown).

Statistically significant differences ($p<0.001$) were found among the study sites, in the Intervention (Jolie) *An. gambiae* s.l. showed high tendency to feed on bovine 53.8% followed by human 47.9%, unlikely in the Control (Gogete) *An. gambiae* s.l. showed high tendency to feed on human 57.1%, followed by bovine 40.6%. Likewise, different studies reported that LLINs and IRS have a powerful impact on mosquito density, parity and sporozoite rate if properly used (Temu *et al.,* 1999; Quinones *et al.,* 1998; Magesa *et al.,* 1991).

Since, both study sites have similar climatic, household and environmental factors in this study the lower parous rate might be associated with the treatment given in Intervention study site (Jolie). Besides, under utilization of LLINs and indoor residual spray by the community were found to be one of the major drawbacks in the Control study site (Gogete). However, indoor residual spray and LLINs are reliable and effective in a wide

range of situations (Noor, *et al.,* 2009; MOH, 2011; Shargie *et al.,* 2008, 2009) under utilization of these intervention tools by the community were found to be one of the major problem in malaria vector control programme (MOH, 2011; Dagne and Deressa, 2008; Fettene *et al.,* 2009; Gobena *et al.,* 2012).

The lack of significant differences in sporozoite rate in Intervention (Jolie) and Control (Gogete) might be the overall small sporozoite rate in Ethiopia (Ameneshewa, 1995; Abose *et al.,* 1998b). Similarly, significant differences were not found in parous rates and sporozoite rates between treated and untreated villages in *An. pharoensis* in a study conducted in Kenya (Snow *et al.,* 1987).

The presence of *P. falciparum* and *P. vivax* infected *An. arabiensis* indicated the species is the sole vector of the area. Similar to the present study both *P. falciparum* and *P. vivax* sporozoite infected *An. arabiensis* was reported in nearby village and other areas of Ethiopia (Animut *et al.,* 2013b; Nigatu *et al.,* 1992). Besides, higher *P. falciparum* infected *An. arabiensis* in line with the present study was reported in relatively higher transmission area in South west Ethiopia (Massebo *et al.,* 2013a). However, unlike the present study all of *An. arabiensis* tested for *Plasmodium CSP* found to be negative in south central and south west Ethiopia (Gone *et al.,* 2014; Gari *et al.,* 2016; Kenea *et al.,* 2016).

4.5. Conclusion

In this study, the overall HBI and parous *An. gambiae* s.l. in Intervention study site (Jolie) were lower compared to the Control study site (Gogete). Such host preference and parity rate significant differences situated in the same ecological, meteorological conditions and human behaviour clearly indicates the treatment given in Intervention study site (Jolie) reduced feeding on human and the existence of old *Anopheles* mosquitoes.

Chapter 5 Evaluation of biting and resting activities and vector status of *Anopheles* mosquitoes (Diptera: Culicidae) in relation to malaria transmission

5.1. Introduction

The abundance and dynamics of malaria vector populations have significant impact on the variations of intensity of malaria transmission from place to place. For example, a study conducted by Brooker *et al.* (2004) showed that the existence of disparity in the intensity of incidence of malaria within the same area. Pertinently, the density of malaria vectors has shown a great discrepancy in the same topography, even at household level within the same area. For instance, in Ethiopia, White *et al*. (1980) around Giligil Ghibe River Valley reported that a variation in the density of malaria vectors with a result of less than 1 in January to March where as it goes greater than 100 in July to October. But, in Gambela areas of Ethiopia,(Krafsur, 1970, 1977) reported that the average hut resting density was less than 30.

Meanwhile, a collections made in the 1958 malaria epidemics revealed the average hut mosquitoes resting density were vary from 100 to 150 (Fontaine *et al.,* 1961). This figure remains the highest in published reports in Ethiopia. In Tanzania 17 times greater entomological inoculation rates (EIR) were observed in lowland area than highland area, though the incidence of infection were differed by 2.5 times (Maxwell *et al.,* 2003). Also, high sporozoite infection and entomological inoculation rate in dry season than rainy season in perennial transmission areas of Cameron were reported (Bigoga *et al.,* 2012).

An. arabiensis and *An. pharoensis* bite in the early period of the night indoor and outdoor in study conducted in different parts of Ethiopia (Abose *et al.,* 1998b; Taye *et al.,* 2006; Kibret *et al.,* 2010; Kenea *et al.,* 2016). The early time biting behaviour of *An. arabiensis* and *An. pharoensis* in these studies coinciding with the evening activities of the people in the study areas (Kibret *et al.,* 2010; Kenea *et al.,* 2016) and might be associated with long term application of IRS and LLINs utilization (Takken *et al.,* 2002; Yohannes and Boelee, 2012; Taye *et al.,* 2016). *Anopheles pharoensis* exhibited more exophagic than endophagic behaviour in different studies conducted in Ethiopia (Krafsur, 1977; Kibret *et al.,* 2010; Kenea *et al.,* 2016). Flexibility of *An. arabiensis* in host preference, biting and resting behaviour challenge malaria control and elimination because the vector may be less vulnerable to IRS and LLINs, which are the two most important methods used for malaria currently in the country (WHO, 1998).

The other factors, from the number of facts to be cited for the variation, a study conducted in Kenya showed malaria vector abundance was influenced by the type of house construction and the presence of domestic animals in the household (Mutuku *et al.*, 2011). Proximity to the water bodies was also another factor associated with high incidence of malaria (Zhou *et al.,* 2012; Pullan *et al.,* 2010; Peterson *et al.,* 2009). Besides, in Ethiopia Nega and Haile-Meskal (1991) reported that ineffectiveness of indoor residual spraying, proximity of inhabitants' house to breeding sites and outdoor sleeping patterns have created high human-vector contact, thus boost in the prevalence of malaria in Pawie settlements in 1980's were occurred. Besides, Esayas *et al.* (2016) reported that the pooled average content of LLINs in the four regions was 4.55 mg/m^2 which were less than the WHO recommended dosages of deltamethrin for the reason that

the vector may be less vulnerable to LLINs. Reduction of malaria incidence in children with increasing distance from forest was observed in high endemicity rural area of Ghana (Kreuels *et al.,* 2008). Similarly, presence of vegetation in a compound is associated with high malaria risk in urban area of Ethiopia (Peterson *et al.,* 2009). Besides, the findings of Ye-Ebiyo *et al*. (2003) showed that growing maize near households increases malaria transmission, this is because maize pollen allows larvae to develop into larger pupal stage more rapidly and producing bigger long lived adults that enable the vector to have high vectorial capacity (Ye-Ebiyo *et al.,* 2003; Kebede *et al.,* 2005). Therefore the aim of this study was to study the density, biting, resting activities and annual entomological inoculation rate (EIR) and the data used for comparison of the presence of significant differences between the Intervention (Jolie) and Control (Gogete) study sites (WHO, 2013).

5.2. Materials and methods

5.2.1. Study sites

As described in section 3.2.1.

5.2.2. Study design and procedures

Longitudinal study design was used to determine the impact of resistance management options applied in the study sites.

5.2.2.1. Adult female *Anopheles* mosquitoes collection and processing

As described in section 3.2.2.2

5.2.2.2. Species identification, density, biting and resting activities

Indoor female *Anopheles* mosquitoes collection using pyrethrum spray collection (PSC) method was carried out once every month during data collection period, thus an overall indoor resting density of all *Anopheles* mosquitoes species were determined (WHO, 1992). Besides, the total numbers of *An. gambiae* s.l. collected by PSC method from households in the Intervention (Jolie) versus Control (Gogete) study sites were compared to evaluate the presence of significance differences on indoor resting density (WHO, 2013).

Due to ethical point of view currently WHO do not recommend to collect *Anopheles* mosquitoes using human land catch. For this reason, we used space spray collections to calculate indirect man-biting rate of all collected species of *Anopheles* mosquitoes (WHO, 2013). However, the obtained result of *An. gambiae* s.l. man-biting rate was used for comparison among the Intervention (Jolie) and Control (Gogete) study sites.

Resting habit is an important index to assess the success of indoor residual spraying (IRS) in interrupting transmission (WHO, 2013). The aim of residual spraying is to reduce the probability of infected vectors reaching an infective stage. Thus, in this study we have used *An. gambiae* s.l collected by pyrethrum spray collection (PSC) method for comparison of the presence of significant differences on the proportion of blood-meals taken on humans and followed by indoors resting between the Intervention (Jolie) and Control (Gogete) study sites (WHO, 2013).

5.2.2.3. Determination of annual entomological inoculation rate (EIR)

The number of infective bites per person per night known as the Entomological Inoculation Rate (EIR) assuming no protection against mosquito bites had been used

(among all *An. gambiae* s.l. female that could have taken their blood-meals on the person during a day/month/or year, how many would have been effective). To find the value of EIR sporozoite rate and the human-biting rate are required.

5.2.3. Data analysis

The indoor resting density, man-biting rate, resting habit and EIR calculated by using the formula described below (WHO, 2013).

Indoor resting density (D) = *Total number of females of a particular species*

Total number of houses inspected (used for the SSC)

Man-biting rate (M) = *Total number of freshly fed mosquitoes of the particular species (F)*

Total number of human occupants in houses used for collection

(w)

* Nevertheless, in some vector species such as *An. arabiensis* a significant proportion (up to 30%) can feed on animals and may be found resting in human dwellings. The results may need to be adjusted accordingly by multiplying "*M*" by the proportion of female found that had fed on human blood.

Resting habit (f) = kHD/NPM

Where k = a correction value of 1.16

Human blood index (H) = *Number of mosquitoes which have fed on man*

Total number of mosquitoes whose blood meals have been identified (from SSC)

Indoor resting density (D) = $\frac{\textit{Total number of females collected}}{\textit{Number of houses used for the spray sheet collection}}$

M = human-biting rate

Duration of resting indoors after feeding, in days (*P*) = 1 + *G / F*,

Where G is the total number of half-gravid and gravid female (spray sheet collections) and F is the number of freshly fed females (spray sheet collections)

N = average number of persons per house (household size)

Entomological inoculation rate (EIR) = Human-biting rate x Sporozoite rate (%) / 100

* The monthly EIRs in each study sites were summed up to obtain the annual EIR (Kent *et al.,* 2007).

Besides, the collected data was computerized using Epi Info 7 and analysed using Stata/SE 11.0, percentage, mean, proportion and 95% CI was constructed around selected proportions to help in making inference towards the target population, for significance differences ($p<0.05$) two-sample test of proportion was used to compare indoor resting density.

5.3. Results

5.3.1. Indoor resting densities of adult *Anopheles* mosquitoes

A total of 619 *Anopheles* mosquitoes using PSC method were collected, of which 213 from Intervention study sites (Jolie) and the rest 406 from Control (Gogete). The average indoor resting density of *Anopheles* mosquitoes collected by PSC method in Intervention

study sites (Jolie) the leading species was *An. gambiae* s.l. accounted an indoor resting density of 2.5 (n=119), followed by *An. pharoensis* 0.9 (n=44), *An. christyi* 0.6 (n=28) and *An. demeilloni* 0.5 (n=22). Similarly, in Control study site (Gogete) *An. gambiae* s.l. accounted an average indoor resting density of 5.4 (n=261), followed by *An. pharoensis* 1.7 (n=80), *An. christyi* 0.8 (n=41) and *An. demeilloni* 0.5 (n=24) (Figure 5.1). Statistical significant differences ($p<0.001$) were observed in indoor resting density of *An. gambiae* s.l between the study sites, i.e. less indoor resting density in *An. gambiae* s.l. were observed in Intervention (Jolie) than in Control (Gogete) study site (Appendix 5.1).

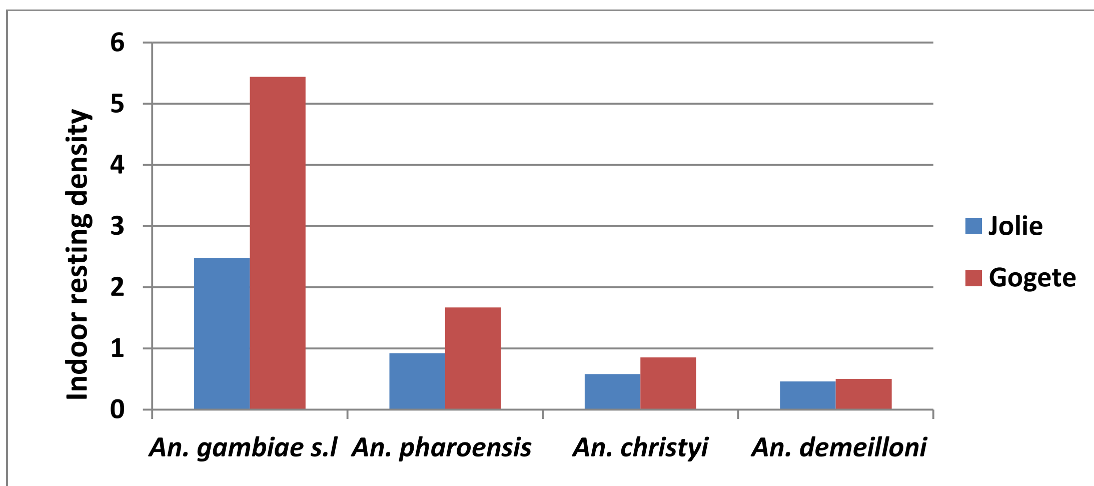

Figure 5.1. Average number of indoor resting densities of *Anopheles* collected by SSC methods in Intervention (Jolie) and Control (Gogete) study sites from May 2013 to June 2015.

Note: *In each month 48 houses were visited for one collection in one study area, 48 houses/visiting were determined.*

5.3.2. Man biting activities by *Anopheles gambiae* s.l. mosquitoes

A total of 380 freshly fed *An. gambiae* s.l. using PSC method was collected from the two study sites, of which 119 and 261 from Intervention (Jolie) and Control (Gogete) study sites respectively. The monthly man biting rate of *An. gambiae* s.l. in both study areas presented in Figure 5.2. The average annual man biting rate of *An. gambiae* s.l. in Intervention study site (Jolie) accounts for 0.64 and in Control (Gogete) 1.54. Statistical significant differences ($p<0.014$) were observed in man biting rate of *An. gambiae* s.l. between the Intervention (Jolie) and Control (Gogete) study sites (Appendix 5.2).

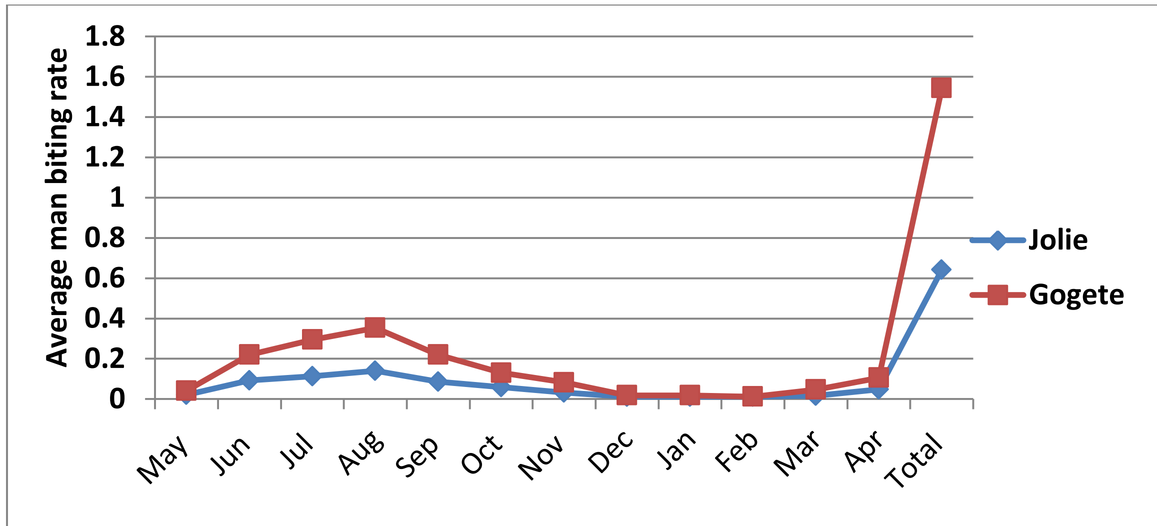

Figure 5.2. Average monthly man-biting rate of *Anopheles gambiae* s.l. in Intervention (Jolie) and Control (Gogete) study sites collected by SSC method from May 2013 to June 2015.

Note: *In each month 48 houses were visited. The mean number of people per household were found 5.8 and 5.3 in Intervention (Jolie) and Control (Gogete) study sites respectively thus, hence in Intervention (Jolie) 278 (48x5.8) and Control (Gogete) 254 (48x5.3) occupants were determined.*Annual Man-biting rate = the sum of all months man-biting rate.*

5.3.3. Resting site preferences in *Anopheles gambiae* s.l mosquitoes

The resting habit of *An. gambiae* s.l. was calculated using the formula mentioned in section 5.2.3. Hence, from the following table, it can be seen that in Intervention (Jolie) and Control (Gogete) study sites the percentage of *An. gambiae* s.l feeding on human and rested indoor were 12 and 20 respectively (Table 5.1). Thus, the tendency of exophilic resting behaviour of *An. gambiae* s.l observed in Intervention (Jolie) than Control (Gogete) study site.

Table 5.1. Proportion of blood-meal sources from human blood and followed by indoor resting *Anopheles gambiae* s.l. in Intervention (Jolie) and Control (Gogete) study sites.

Characteristic/	***An. gambiae* s.l (Jolie)**	***An. gambiae* s.l (Gogete)**
H (human blood index)	0.34	0.57
Total No of *An. gambiae* s.l (collected by SSC)	274	646
D (indoor resting density)	2.48	5.44
Fed	119	261
Half gravid	79	188
Gravid	47	121
P (indoor resting post feeding)	1 + (79+47)/119 = 2.10	1 + (188+121)/261 = 2.18
M (human biting rate)	0.64	1.54
N (average number of persons per house/household size)	5.8	5.3
f = 1.16 (D) H / (N) (M) (P)	**0.12**	**0.20**

5.3.4. Entomological inoculation rate (EIR) and vectorial status

The entomological inoculation rate was calculated as the product of the man biting rate and circumsporozoite antigen rate as confirmed by ELISA. However, a small number of mosquitoes were found sporozoite positive on both study sites this resulted in low EIR estimates for *An. gambiae* s.l. in Intervention (Jolie) and Control (Gogete) study site accounts for 0.015 and 0.53 respectively.

5.4. Discussion

In Intervention study area (Jolie), *An. gambiae* s.l. still remained dominant in abundance. The second abundant species was *An. pharoensis*, but it has never outnumbered *An. gambiae* s.l. even in the dry months as it has been documented in the Zeway area (Abose *et al*., 1998b).

Studies conducted in Ethiopia reported that *An. arabiensis* prefer to rest in cow sheds and ditches (Abose *et al*., 1998b, Ameneshewa and Service, 1996). In this study *An. gambiae* s.l. demonstrated behavioural variation in its indoor and outdoor resting behaviour within the study sites, the number of the exophlic population was higher than the endophilic in Intervention study area (Jolie). As the result, the densities of the *An. gambiae* s.l. in households of Control (Gogete) exceed that of the Intervention (Jolie), this could be attributed to the efficient coverage and utilization of LLINs and IRS at this site so the species might be shifted in to outdoor biting and resting activities.

Similar to the present study spraying of insecticide indoors shifts the mosquito resting outside due to excito-repellence effect on the indoor resting mosquitoes. Besides, it was

confirmed that mosquitoes tend to develop behavioural avoidance of human dwellings with LLINs (WHO, 2013).

Even though, data is not available on the density of this species before and after in these two study sites to compare with the results produced in this study it can be speculated that the four-year interruption using effective LLINs and rotation of insecticides appropriately have effect in the density of indoor resting population. It is obvious that in households without LLINs *An. gambiae* s.l. population outnumber those of households with LLINs (Chanda *et al*., 2008).

In addition, a significant reduction in the abundance of *An. gambiae* s.l. over the last 20 years due to increased coverage of IRS and LLINs were also reported along Kenyan coast (Mwangangi *et al.,* 2013). However, in Senegal insecticide treated nets quickly selected resistant mosquitoes with long lifespan and unchanged feeding behaviour (Ndiath *et al.,* 2014) so the density was not impacted by LLIN utilization. In addition, Esayas *et al.* (2016) less dosage of insecticide used for impregnation of nets induce insecticide resistance and reduce the vulnerability of the malaria vectors.

A major reduction in the vectorial capacity of the vector population is the most desirable effect of using an insecticide for malaria control (WHO, 2013). Insecticide treated bed nets may be able to provide this as well as giving personal protection. Some studies of insecticide treated bed nets have shown reductions in survivorship, sporozoite rate and density of the mosquito vector population at a village level (Snow *et al*., 1987; MacCormack and Snow, 1986; Robert and Carnevale, 1991; Aikins *et al.,* 1994).

Besides, statistically insignificant reduction of *An. arabiensis* in LLINs used households was also reported in Zambia (Chanda *et al*., 2008).

To summarised, *An. gambiae* s.l. (most are *An.arabiensis*) is the most prevalent species in both study sites. Statistical significant differences were observed in indoor resting density and man biting rate of *An. gambiae* s.l among the study sites, besides a tendency of exophilic resting behaviour of *An. gambiae* s.l observed in Intervention study site (Jolie) than Control (Gogete) these might be attributed to the efficient coverage and utilization of LLINs coupled with rotation of insecticides had impact on *An. gambiae* s.l. reduction of indoor density, biting and a tendency of exophilic resting behaviour at this Intervention study site (Jolie).

5.5. Conclusion

The result of the present study revealed that the indoor resting and biting rate of *An. gambiae* s.l. in the Control study site (Gogete) exceed that of the Intervention (Jolie). Besides, *An. gambiae* s.l. a tendency of behavioural avoidance of human dwellings (exophilic) in Intervention study site (Jolie) observed. This could be attributed to the impact of the treatment given in Intervention study site (Jolie).

Chapter 6 Evaluation of malaria vector management practices and insecticides resistance in *Anopheles* mosquitoes (Diptera: Culicidae) in selected sites in Butajira area, Southern Ethiopia

6.1. Introduction

In accordance with Roll Back Malaria (RBM) guiding principles to decrease the disease burden, Ethiopia has made a significant pace in expanding coverage of key malaria interventions tools throughout the country (WHO, 2004; Jima *et al.,* 2005). The 2008 global fund impact assessment survey carried out by the Ethiopian Health and Nutrition Research Institute (EHNRI) and FMOH showed 100% LLIN coverage (EHNRI/MOH, 2008). Among pastoralists regions of Ethiopia in Afar it was reported that only 86.1% of respondents owned at least one LLIN (Negash *et al*., 2012). Besides, studies conducted in Ethiopia reported 91% coverage (Baume *et al.,* 2009) and another study elsewhere also found, 90.1% of households in the poor villages owned at least one LLIN (Neeta *et al.,* 2009).

Baume *et al.* (2009) in a NetMark study reported 65% usage rate. Another study, in Arba Minch Zuria district of southern Ethiopia showed a slightly improved rate of 73.3% (Astatkie, 2010). In addition, it was reported that 86.7% owned at least one net and 73.2% of nets had someone slept under them every night (Esayas *et al.,* 2014). Besides, the results of 2011 malaria indicator survey the practice of properly utilizing nets showed 64.5% (EHNRI/MOH, 2011). A study conducted by Animut *et al.* (2008), reported the overall LLINs distribution and utilization were 97.6% and 81.6% respectively.

Under utilization of intervention LLINs and IRS by the community was found to be one of the major drawbacks in the control programme whose goal is malaria elimination from low transmission areas of the country by 2015 (MOH, 2011; Noor *et al.,* 2009; Dagne and Deressa, 2008; Fettene *et al.,* 2009; Gobena *et al.,* 2012; Shargie *et al.,* 2008, 2009).

In line with this, the result of the study conducted in southern Ethiopia Battisso *et al.* (2012) showed that, out of the households who owned nets over one-third reported that had owned one mosquito net (31.8%) , half (54.1%) owned two nets and 11% had three net. This study in addition confirmed that, the average number of nets per net-owning household was 1.86. In Ethiopia although different studies were conducted in the coverage and utilization of LLINs in various parts of the country there is a limitation of data particularly on determinant factors that hinder utilization (Vanden *et al.,* 2010; Das *et al.,* 2007).

The nets are treated at the manufacturing level with insecticide either with incorporated into matrix of polyethylene fibers or coated with wash resistant resin on polyester fibers (WHO, 2013). The biological activity lasts as long as the useful life of the net itself which is estimated to be 3–5 years (Guillet *et al.,* 2001; Miller *et al.,* 1995; WHO, 2007). Despite the use of nets as a major component of malaria vector control in almost all endemic countries its effect on the targeted vector over time depends on textile integrity and the rate of loss of insecticide.

Earlier studies reported that LLINs have a powerful impact on mosquito density, parity and sporozoite rate (Temu *et al.,* 1999; Quinones *et al.,* 1998; Magesa *et al.,* 1991). In contrary to this finding another study showed that there was no significant difference

between the proportion of mosquitoes entering a room and taking a blood meal after and before net use (Doannio *et al.,* 1999). The same author indicated that sporozoite rate, entomological inoculation rate (EIR) and the transmission rate of malaria parasites were not much different compared with villages without net (Doannio *et al.,* 1999). Similarly, a study in Burkina Faso also found that field performance of the nets was not quite as good as anticipated (Müller *et al.,* 2002).

In fact, LLIN is considered as the most effective tool for malaria prevention and control with significant reduction of child morbidity and mortality (WHO, 2007). The strong effect of LLIN on malaria cases and deaths in the last decade has been well documented (Guillet *et al.,* 2001; Temu *et al.,* 1999; Magesa *et al.,* 1991; Otten *et al.,* 2009). Since 2004/2005, LLINs has been a part of malaria vector control and over 41 million nets have been distributed in Ethiopia (MOH, 2011).

Still there are factors that influence the intended role of LLINs impact on malaria vector control programme. For instance, Esayas *et al.* (2016) reported that out of the examined 4138 nets 44.07% were with holes. Nets with holes provide a reduced protection or no protection for the person sleeping under the net (Irish *et al.,* 2008).

Related to the physical longevity of bed nets, many investigations overestimate the spans of time during which bed nets remain intact (Kilian *et al.,* 2008). A study demonstrated that the nets were effective until 3–4 years after they have been installed in rural Tanzania (Maxwell *et al.,* 2002) and another study showed a life span of LLIN is 3–5 years (Feilden, 1996). Thus, local evidence is required related to some of those

characteristics prior to programme implementation and revising post implementation strategies (Onwujekwe *et al.,* 2005).

The functional life of nets is affected by the number and the size of holes as well as damages. However, it is difficult to compare the ranges of damages from different studies as various definitions were applied by various researchers. For instance, it ranged from 45% severely damaged (> 7 holes larger than 2 cm) in Tanzania (Erlanger *et al.,* 2004) to 33.2% (> 5 holes) after two years in Sudan (Ritmeijer *et al.,* 2007) and to 28% with at least one hole of 40 cm^2 in Uganda (Spencer *et al.,* 2004). Another important factor is washing which is believed to affect insecticide dosage on the nets and rate of use. A study showed clean nets are very important to some net users (Kweka *et al.,* 2016; Feilden, 1996).

Another important issue is the number of wash and persistence of insecticide after repeated wash. A study in five sub-Saharan African countries reported that 50–77% of nets were washed at least once *per* month, with little difference between urban and rural areas except in Senegal (66 vs. 43%) and Nigeria 71 vs. 83% (Ordonez-Gonzalez *et al.,* 2002). Other studies in southern Ethiopia found that most (75%) of the household heads confirmed that they had washed their nets on average 3.2 times per quarter (Batisso *et al.,* 2012) and about half of surveyed nets (43.7%) had been washed during their lifetime (Dagne and Deressa, 2008). However, regular washing eventually reduce the insecticide amount below an effective level (Adams *et al.,* 2002; Miller *et al.,* 1999).

Evidences showed that a significant loss of insecticides following repeated washes. For example, a multi-country survey confirmed that 72.1% of the baseline insecticide has

been lost after 20 washes for PermaNet 2.0® (Graham *et al.,* 2005; Yates *et al.,* 2005). In addition, in the Columbia study six PermaNet® nets were washed by local women during five months and then used by these families for another 2.5 years after 20 washes (Kroeger *et al.,* 2004). Furthermore, Asidi *et al.* (2004) tested PermaNet 1.0® in late 2000, after five washes using an experimental hut design and found no difference to conventionally treated nets. Still, Graham *et al.* (2005) tested first generation PermaNet® in three countries using experimental hut trials and found mortality on the LLIN after 15 to 21 washes above 90% but median time to knockdown and blood feeding inhibition only significantly better than conventionally treated nets in one trial. Besides, in Ethiopia reported that the result of LLINs bio-cone tests mortality rates in susceptible *Anopheles arabiensis* and field collected wild *Anopheles gambiae* s.l were 71.5 and 41.5 respectively (Esayas *et al.,* 2016).

Following the large-scale malaria epidemic during 2002/3 Ethiopia introduced globally proven interventions like LLINs and indoor residual spray for vector control and artemesinin-based-combination therapies (ACT) (Otten *et al.,* 2009). This approach is based on the 'Scale-up for Impact' (SUFI) approach recommended by the World Health Organization, which overlapped with reduction of malaria burden in the country (EHNRI/MOH, 2011). The Ministry of Health of Ethiopia documented a promising result in reducing malaria burden and absence of major outbreaks until recently. Although the coverage of LLINs between 2007 (53%) (Jima *et al.,* 2005) and 2011 (65%) (EHNRI/MOH, 2011), was not that much improved its importance as personal protection and community level intervention remain clears (Animut *et al.,* 2008).

The major goal of MOH of Ethiopia is to attain a universal coverage of LLIN to households located in malaria endemic areas (MOH, 2006). Although more than 41 million nets are distributed in the country so far 100% coverage is not yet achieved (MOH, 2011). Insecticide resistance is an important concern for national malaria control programs the main vector control tools long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) rely on the efficacy of the insecticide used for effective control (WHO, 2006, 2011a). Ethiopia has a long history of IRS beginning with DDT in 1959, which continued until 2009, and malathion was used in selected areas during the 1980s and 1990s (Balkew *et al.*, 2003).

When DDT resistance was found to be widespread, deltamethrin was used as an IRS treatment. Quite quickly, deltamethrin resistance led to use of bendiocarb and propoxur in recent years (Gebreyesus *et al.*, 2006; Abose *et al.*, 1998c; Balkew *et al.*, 2003 and 2012; PMI/Africa IRS (AIRS), 2016). In southern Ethiopia, different studies (Fettene *et al.*, 2013; Yemane *et al.*, 2016; Massebo *et al.*, 2013b) reported resistance to DDT and deltamethrin in *Anopheles gambiae s.l.* but susceptibility to propoxur and bendiocarb. Besides, Esayas *et al.* (2016) reported that *An. gambiae* s.l. was resistant to DDT, permethrin, deltamethrin, malathion and bendiocarb but susceptible to propoxur.

Changes in the sites of insecticidal target and increasing the rates of insecticide metabolism are the two major kinds of resistance mechanisms. Knockdown resistance is induced by a single mutation resulting in a leucine-to-phenylalanine (L1014F, West African mutation) or leucine-to-serine (L1014S, East African mutation) change. Although both mutations are thought to provide DDT and pyrethroid resistance, the L1014F *kdr* mutation might give a higher level of pyrethroid resistance than the L1014S

kdr mutation (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). Besides, the incomplete heterozygous state indicates the continuity of kdr occurrence (Martinez-Torres *et al.,* 1999).

In many sites where study conducted in Uganda the *kdr* allele frequency rose above 30% which is much higher than the 8% observed in Kenya (Katrijn *et al.,* 2010). In addition, in the same study the lowest L1014S *kdr* frequencies were confirmed in the permethrin and DDT susceptible *An. gambiae* s.s. populations of western Uganda areas where coffee, tea and tobacco plantations are present. The highest L1014S *kdr* frequencies occurred in cotton-growing areas of Uganda. Furthermore, in *An. gambiae* s.s. populations of Burkina Faso, the L1014F *kdr* frequency was high in cotton-growing and urban areas and low in areas with limited insecticide selection pressure from agriculture (e.g., rice fields) (Diabaté *et al.,* 2002).

Since, *kdr* frequencies can increase rapidly (Protopopoff *et al.,* 2008) this longevity advantage might have significant impact on malaria transmission and might put at risk the current resistance management strategies. There are facts that at least some of these resistant vectors genes have the potential to threaten the effectiveness of current malaria vector control interventions in Ethiopia (Balkew *et al.,* 2010; Yewhalaw *et al.,* 2012). Studies have shown that, insecticide resistance in *Anopheles arabiensis* from villages in central, northern and south west Ethiopia were detected and this study also confirmed the presence of the knockdown resistance *(kdr)* mutation of leucine to phenylalanine in the sodium ion channel gene in populations from Gorgora and the Ghibe River Valley (Balkew *et al.,* 2010).

Besides, Yewhalaw *et al*. (2012) reported that *An. arabiensis* was resistant to DDT, permethrin, deltamethrin and malathion, but susceptible to propoxur, this study also confirmed that the presence of the West African *kdr* allele in Ethiopia. Additionally, in South Ethiopia Massebo *et al*. (2013b) reported that a high proportion of *An. arabiensis* were resistant to the pyrethroid insecticides with a mortality rate of 56% for lambdacyhalothrin, 50% for cyfluthrin and alphacypermethrin, 47% for deltamethrin. In addition, Balkew *et al.* (2010) reported bendiocarb resistant populations were also detected from a few localities.

The observed multiple-resistance together with the occurrence of high *kdr* frequency in populations of *An. arabiensis* could profoundly affect the malaria vector control programme in Ethiopia. This needs an urgent call for implementing rational resistance management strategies (WHO, 2010; Balkew *et al.*, 2010, 2012; Yewhalaw *et al.*, 2010, 2012).

Furthermore, WHO recommended that all vector control interventions should include a resistance management strategy and the implementation of resistance management measures that should not be prompted by proof of control failure or the appearance of resistance (WHO, 2010). The Insecticide Resistance Action Committee has recommended options to delay occurrence of insecticide resistance such as rotations, mosaics, combinations and mixtures. The Committee also suggested that, to make evidence based decision whether which option is appropriate it depend on the finding of the research conducted in the local situation of each country (WHO, 2010). The aim of this study was to measure susceptibility of malaria vectors in the study sites, to detect the

presence of *kdr* mutations and understanding the impact of resistance management option for alternative control strategies.

6.2. Materials and methods

6.2.1. Description of study sites

As described above in section 3.2.1.

6.2.2. Study design and procedures

A community based cross-sectional study design was used, using purposive and multi-stage sampling technique in Southern Nations Nationalities and Peoples region (SNNPR) of Ethiopia purposively selected for the study.

Selection of Study sites: This study used two study sites and it was done by random selection from the list of malarious *Kebeles* (localities) in the district health office. Thus, study was carried out in Intervention study site (Jolie) and Control study site (Gogete) from Meskan and Sodo districts respectively. The study sites were the enumeration areas where the size of their sample population was determined.

Sample size determination: The sample size was determined using Epi-Info software package, on the bases of less than ten thousands population with the assumption of 44% long lasting insecticidal nets (LLINs) utilization (Jima *et al.,* 2007) and 95% confidence level (confidence limit of 0.05) with 5.0% adjustment for non response. Thus, in each study site 383 households and in both a total of 766 (383 x 2) were included in the sample size. However, almost 10% (71) of the data was rejected due to poor quality hence data collected from 695 households was used for the analysis.

Selection of households: Household (HH) was defined as a single person lives alone or a group of persons voluntarily living together having a common housekeeping arrangements for supply of basic living needs. The mean number of people per household was considered to be five with an assumption that the family size might range from a single HH to an extended family of greater than seven (MOH, 2009, 2010).

The sampling frame was the list of households of each study sites. Households in the enumerated study sites were the study units. The participating households were selected using systematic random sampling of one in every 6th HH from the list of eligible households in each study site selection and enrolment of households were based on the criteria: they need to be residents of the study site, having at least one LLINs and the head of the HH is willing to participate in the study.

In the first study site all households with new LLINs + rotation of IRS (as described in section 4.2.2.1) service provision were involved as an Intervention group (Jolie) in this study and in the second study site (at least 5-10 kms far from the first to provide buffer zone) all households with routine LLINs + IRS services were involved as a Control group (Gogete).

The core indicator data collection tool was prepared to evaluate the household LLIN coverage level, the practice of regular LLIN use and net shape as well as colour preferences of the community in the study areas were collected from all households included in the sample size.

To detect bio-activity and chemical residue a mini-sample size was determined (WHO, 2011), 48 LLINs (WHO, 2011) having 24-36 months old were randomly selected from a

roster of nets and collected by researchers for bioassay test and 10 LLINs (WHO, 2009) for chemical analysis in the laboratory. In line with this, to detect resistance level of malaria vectors among the study areas using a standard WHO susceptibility test kit and procedure (WHO, 1998) laboratory reared wild *An. gambiae* s.l. collected from the two study areas were tested every year (2013-2016) in month of August. Preliminary susceptibility test were conducted in the two study areas to use as a baseline data that will be used for comparison. Sub-samples of *An. gambiae* s.l. killed and surviving during susceptibility test were randomly selected from Intervention (Jolie) and Control (Gogete) study sites and then subjected for knockdown resistance (*kdr*) detection.

6.2.3. Assessment of utilization, coverage and preferences of LLINs

6.2.3.1. Coverage and use of LLINs

The core indicator data collection tool was prepared to evaluate the household LLIN coverage level, the practice of regular LLIN use and net shape as well as colour preferences of the community. The questionnaire covered background characteristics of the respondent and household level information, the number of LLINs owned and preference of the household in terms of LLIN shape and colour, and utilization practices.

The questionnaire was prepared in English and translated into each local language of study participants (Amharic and Guragegna) then, it was pre-tested by trained data collectors outside the study *Kebeles* (localities) but with similar settings. The necessary revision was made based on the feedback obtained during the pre-test. The experience gathered in the pre-testing was used in organizing the study properly. Net utilization was

documented by trained local assistants visiting in person the HHs every other morning to observe the status of net use.

6.2.3.2. Assessment of community net colour and shape preference

As described in section 6.2.3.1.

6.2.4. Evaluation of bio-activity and chemical residue

6.2.4.1. WHO bio-cone test processing of LLINs

Using templates of 30×30 cm samples were cut out of the nets marked with the date and the net ID number, packed in aluminium foil and stored at a temperature of 4^0 C and transported to the respective laboratories for analysis. Samples from each net were always taken from lower quarter, lower middle quarter, upper middle quarter, top quarter and roof. Aquatic forms of *An. gambiae* s.l. from both study areas were collected from different breeding sites reared to adults in big cages and identified to the species level using identification keys (Gillies and Coetzee 1987; Verrone, 1962a, 1962b). Five non bloods fed 2–5-day old female *An. gambiae* s.l. were exposed to the net sample (30×30 cm) using standard WHO cone for 3 minutes (Figure 6.1). The numbers of *An. gambiae* s.l. knockdown after 60 minutes exposure and mortality after 24 hours of recovery period were counted and recorded.

After which they were hold for 24 hours with a piece of moist cotton wool on the gauze end with access to sugar solution and in a wooden box with large holes for ventilation and covered with a damp towel. Besides, the same number of *An. gambiae* s.l. exposed to

untreated nets was also used as controls for each net. Four replicates for the experiment and two replicate for control were carried out.

Figure 6.1. Bio-cone test carry out in the laboratory of Ethiopian Public Health Institute using WHO procedures.

An average knock down and mortality value for each treatment group were then calculated based on the number of nets for each group using Abbot's formula. If mortality for a given day was between 5% and 20% on the control net it was corrected by Abbott's formula (Abbott, 1925): Adjusted mortality (%) = 100 x (X – Y) / (100 – Y), Where X is the percentage mortality in the treated sample and Y is the percentage mortality in the untreated control sample. If mortality for a given day was > 20% all bio-cone test carried out on that day were rejected and then repeated. The tests were carried out at a temperature of 27 ± 2°c and 80 ± 10% RH (WHO, 1998).

6.2.4.2. Laboratory evaluation of chemical residue in LLINs materials

A sample of 10cmx10cm cut from positions lower quarter, lower middle quarter, upper middle quarter, top quarter and roof using sharp scissors. Each sample were rolled up and placed in a labelled new clean aluminium foil for storage in a refrigerator (+4°c) before dispatch to a quality control laboratory for chemical assay. Insecticide residues were measured with HPLC using the standard procedure of Collaborative International Pesticides Analytical Council (CIPAC) the principle of this method was proposed in 2006 for adoption (WHO, 1998; CIPAC, 2009). The values of the samples collected from each net were combined to provide the average target concentration of the insecticide on the nets.

The chemical analysis was carried out in collaboration with the Factory of Adami Tulu Pesticide Processing Share Company (Figure 6.2, 6.3). Surface area and weight of each 10cm×10cm sample was measured then cut into small pieces of <2cm, subsequently the sample introduced into a 100 ml Erlenmeyer flask. Deltamethrin was extracted into solution using a mixture of solvents iso-octan plus 1, 4 dioxan with 0.15% HPLC grade water (80/20, v/v). Dibutyl phthalate were added as the internal standard. The extraction bottle was sonicated in a water bath set at 80°C and then shaken vigorously for at least 15 minutes. A proper volume of solution was filtered through 0.45 micrometer membrane syringe filter into a vial. A volume of 5μl of filtered solution was injected into a normal phase isocratic HPLC equipped with PDA/UV detector and deltamethrin was quantified using an internal standard calibration curve.

Figure 6.2. Samples of LLINs prepared for HPLC test in the laboratory of Adami Tulu Pesticide Processing Share Company.

Figure 6.3. The principal investigator conducting chemical analysis to measure the insecticide residue in the net by using HPLC in the laboratory of Adami Tulu Pesticide Processing Share Company.

6.2.5. Procedure of susceptibility test

Collection and rearing of mosquitoes: *Anopheles* mosquitoes were collected as larvae using dippers and pipettes in a variety of aquatic habitats including ponds, rainwater pools, water from broken pipes, rivers pockets, stagnant water in irrigation canals and marshes. Mosquitoes were reared in field insectaries until they were pupae at which point they were put into cups placed in cages to emerge. Adults of *Anopheles gambiae* s.l were maintained on sugar solution until they were 2 to 3 days old at which point they were used for testing (O'Connor, 1967; Hargreaves *et al.*, 2000).

Susceptibility tests: Mosquitoes were tested following WHO procedures (WHO, 1998). One hundred mosquitoes were used per test and 50 for control. Mosquitoes were exposed to treated papers for 60 minutes at which point they were blown gently into the untreated tube for monitoring where they were provided with access to sugar solution in cotton wool. Sixty minutes after the test the number of mosquitoes that were unable to fly and lay on the floor of the tube was counted as "knockdown" and 24 hours after the test the numbers of dead mosquitoes were counted to determine mortality.

Control tubes were run simultaneously to ensure that all mortality was due to the insecticide. If any of the control tubes had mortality of 5% or more, Abbott's correction was used (Abbott, 1925) but if the mortality was greater than 20%, the entire series of tests was discarded. We have used the pooled number of the tested *Anopheles* mosquitoes. The tested insecticides concentrations were: DDT (4%), permethrin (0.75%), malathion (5%), fenitrothion (1%), deltamethrin (0.05%), propoxur (0.1%) and

bendiocarb (1%). The insecticidal papers were procured from the University Sains Malaysia (Penang, Malaysia) as recommended by the WHO.

6.2.6. Molecular analysis of knockdown resistance (*kdr*) in *Anopheles* mosquitoes

Sub-samples of *Anopheles gambiae* s.l species killed and surviving the bioassays selected from Intervention (Jolie) and Control (Gogete) study sites subjected for mmolecular analysis of knockdown resistance (*kdr*). Following the protocol developed by Scott *et al.,* (1993) for *Anopheles gambiae* s.l. complex discrimination based on species specific single nucleotide polymorphism (SNPs) in the intergenic spacer region (IGS) with a minor modification by Wilkins *et al*. (2006) incorporating intentional mismatch primer (IMP) to increase specifity was used for the allele amplification. To detect the presence of *kdr* mutations of the L1014S (East African *kdr*) described by Ranson *et al*. (2000) and the L1014F (West African *kdr*) illustrated by Martinez-Torres *et al*. (1998) methods were used for *kdr* mutations assay.

The reactions were carried out in a 12µl final volume using GoTaq (Promega) and the primers in the exact concentrations as described in the manual MR4/BEI available at (https://www.beiresources.org/Publications/MethodsinAnophelesResearch.aspx).

The master mix was prepared by the manufacturer's (Promega®) recommended buffer at 1x concentration. For East African kdr, 7.8µl distilled water, 5µl GoTaq PCR Buffer, 2.5µl dNTP (2.5mM concentration), 0.5µl MgCl2 (25mM concentration), 2µl IPCF, 2µl altRev, 2µl East F and 2µl WTR [5pm] and 0.2µl Go-Taq, for West African *kdr*, 7.8µl distilled water, 5µl GoTaq PCR Buffer, 2.5µl dNTP (2.5mM concentration), 0.5µl MgCl2 (25mM concentration), 2µl IPCF, 2µl altRev, 3µl West F, 2µl WTR [25pm] and 0.2µl

Go-Taq. In centrifuge tubes for both of *kdr* mutation types (L1014S and L1014F) were used. 0.5µl of DNA and 1µl of the master mix were added into separately labeled PCR plate wells for both *kdr* mutation types.

Two independent amplification reactions for each specimen of East African and West African *kdr* mutations were used, the amplification conditions for East African mutation were: 95°C/5min; 35 cycles of: 95°C for 30sec, 57°C for 30sec along with a final elongation period at 72°C for 5min and for West African mutation 95°C/5min; 35 cycles of: 95°C for 30sec, 59°C for 30sec along with a final elongation period at 72°C for 5min . Finally, the PCR products were visualized with UV light in 2% agarose gels stained with gel red as described by Wilkins *et al*. (2006).

All reactions included specific controls of mosquito reference strains for the susceptible, resistant and heterozygote allele variants and negative control. The reactions containing a band of 314bp were considered as successful amplification and visualized, but reactions without DNA band not matching with 314bp were considered as negative reactions or not amplified; DNA bands of 156bp were considered as homozygous susceptible genes or wild type genes; bands with 214bp were considered as homozygous resistant genes while DNAs having both 214pb and 156bp bands were considered as heterozygous genes.

6.2.7. Data analysis

All the data were double-entered using Epi info 3.5.1 (Centre for Disease Control, Atlanta, USA) and transferred to STATA 11 (Stata Corporation, College Station, Texas, USA) for analysis. After appropriate data preparation percentage for cumulative coverage, utilization, net colour and shape preference were determined. Besides, for the

chemical residue analysis the all samples pooled result were calculated and compared with the recommended dosage by WHO Pesticide Evaluation Scheme (WHOPES, 2003).

In addition, to help in making inference towards the target population for significance differences ($p<0.05$) two-sample test of proportion was used to compare among study sites.

Pertain to bio-cone test on the LLINs and susceptibility test the KD and mortality rate calculated using Abott's formula as described by Abbott (1925) below:

$$\textbf{Control mortality (C)} = \frac{\text{Number of dead mosquitoes}}{\text{Total Number of mosquitoes in control tube}}$$

$$\textbf{Exposure mortality (E)} = \frac{\text{Number of dead mosquitoes}}{\text{Total Number of mosquitoes in experimental tube}}$$

If control mortality is greater than or equal to 5% and less than or equal to 20% the value for exposure mortality (E) corrected by using the following formula (Abbott's formula):

$$\textbf{Corrected exposure mortality (\%)} = \frac{E - C}{100 - C} \times 100$$

6.3. Results

6.3.1. Possession, utilization and net preference of LLINs

6.3.1.1. Possession and utilization rate of LLINs

Overall LLINs Ownership and Use: Out of the surveyed HHs in Intervention study site (Jolie) 85% owned at least one net and 79% used every night. In Control study site (Gogete) 84% owned at least one net and 77% used every night (Table 6.1). The total numbers of nets owned by households were 881 with an average of 1.3 nets per net-owning household.

Table 6.1. The percentage of LLINs possession and utilization in Intervention (Jolie) and Control (Gogete) study sites.

Possession and Utilization	Jolie n (%)	Gogete n (%)	Total n (%)
Possession	n = 348	n = 347	n = 695
No	52 (14.9)	56 (16.1)	108 (15.5)
Yes	296 (85.1)	291 (83.9)	587 (84.5)
Utilization	n = 296	n = 291	n =587
Used every night	235 (79.4)	225 (77.3)	460 (78.4)
Used occasionally	53 (17.9)	55 (18.9)	108 (18.4)
Not used completely	8 (2.7)	11 (3.8)	19 (3.2)

6.3.1.2. Colour and shape preference

Colour and Shape Preferences: The existing owned most (89%) LLINs in the study sites distributed by the government were white and most 93.4 of LLINs were rectangular, followed by 5% circular and a few 1% a combination of both circular and rectangular

(data was not presented). However our finding showed, the percentage of colour preference, out of the surveyed HHs in Intervention study site (Jolie) 43.6 preferred blue, followed by 43.5 green, 4 white colour and 8.8 preferred other colours. In Control (Gogete) 44.3% preferred blue, followed by 44 green, 3.8% white colour and 7.9 preferred other colours (Table 6.2). The percentage of shape preference, out of the surveyed HHs in Intervention (Jolie) 65.2 preferred rectangular, followed by 24.3 circular and 10.5 have not specific choice. In Control (Gogete) 68 preferred rectangular, followed by 23 circular and 8.9 have not specific choice (Table 6.2).

Table 6.2. The percentage of LLINs colour and shape preference at households' level in Intervention (Jolie) and Control (Gogete) study sites.

Preferences	**Jolie n (%)**	**Gogete n (%)**	**Total n (%)**
Colour	**n = 296**	**n = 291**	**n = 587**
Blue	129 (43.6)	129 (44.3)	258 (43.9)
Green	129 (43.5)	128 (44)	257 (43.8)
White	12 (4)	11 (3.8)	23 (3.91)
Other	26 (8.8)	23 (7.9)	49 (8. 3)
Shape	**n = 296**	**n = 291**	**n = 587**
Rectangular	193 (65.2)	198 (68)	391 (66.6)
Circular	72 (24.3)	67 (23)	139 (23.6)
No specific choice	31 (10.5)	26 (8.9)	57 (9.71)

Note: *n = number of HHs owned at least one net*

6.3.2. Bioactivity and insecticide residue on field collected LLINs

6.3.2.1. Bio-cone test

The percentage of the results of samples of LLINs after the use of 24-36 months bio-cone test done in 2013 on *An. gambiae* s.l. in Intervention study site (Jolie) showed that a knockdown and mortality of 36 and 43 respectively, in Control study site (Gogete) were 34 and 39 respectively (Table 6.3). Whereas, the results of bio-cone test done in 2016 the result of knockdown and mortality were 90 and 97 respectively in Intervention (Jolie) as well as in Control (Gogete) 36 and 49 respectively.

Table 6.3. The result of bio-cone tests on *Anopheles gambiae* s.l. by study site after 60 minutes exposure for knockdown and 24 hours for mortality in August 2013 and 2016 in Intervention (Jolie) and Control (Gogete) study sites.

	August 2013	
Study sites	**Knock down and mortality rate**	**%**
Jolie	Knockdown rate after 60 min	36
	Mortality rate after 24 hours	43
Gogete	Knockdown rate after 60 min	34
	Mortality rate after 24 hours	39
	August 2016	
Jolie	Knockdown rate after 60 min	90
	Mortality rate after 24 hours	97
Gogete	Knockdown rate after 60 min	36
	Mortality rate after 24 hours	49

** No of An. gambiae* s.l. *(n) for each study area 5, 280 (4, 800 for experimental and 480 for the control) were used for the test. The mortality rate in the control group was less than 5%.*

6.3.2.2. Chemical residue available in LLINs fabrics

As shown in Table 6.4, the result of insecticide residues available in the nets were measured using High Performance Liquid Chromatography (HPLC) the range of the deltamethrin in the study sites vary from 0.4 to 3.6 mg/m^2. The pooled average deltamethrin content found in Intervention study site (Jolie) was 2.3 mg/m^2 and in Control study site (Gogete) 2.0 mg/m^2, the average content in both study sites was 2.17 mg/m^2, thus the result showed less than the recommended dosage of WHO.

Table 6.4. Average study site deltamethrin residue in mg/m^2 detected by using High Performance Liquid Chromatography (HPLC) in Intervention (Jolie) and Control (Gogete) study sites.

Study sites	Code of sample net	Sample weight in mg/m^2	Delta content in mg/m^2
Jolie	002	0.5500	3.2
	007	0.6258	2.4
	018	0.5922	0.5
	019	0.5428	2.1
	024	0.5416	3.3
	Total average	**0.5705**	**2.3**
Gogete	026	0.5500	0.9
	027	0.6139	0.4
	030	0.5678	3.2
	035	0.5456	3.6
	09	0 .5543	2.1
	Total average	**0.5663**	**2.04**
Grand total average in both study sites		**0.5684**	**2.17**

6.3.3. Susceptibility of *Anopheles gambiae* s.l. to insecticides in use for LLINs and IRS

A total of 2, 100 reared adults of *An. gambiae* s.l. collected from Intervention (Jolie) and Control (Gogete) study sites were used for susceptibility test in a laboratory. As shown in Table 6.5 in Intervention study site (Jolie) our result of the mean KD in August 2013, 2014, 2015 and 2016, were for DDT 33, 49, 58 and 69; for perimethrin 42, 61, 77 and 82; and for deltamethrin 45, 81 respectively to the above mentioned years. In Control study site (Gogete) the mean KD in August 2013, 2014, 2015 and 2016, were for DDT 52, 51, 53 and 54; for perimethrin 62, 63 and 66; and for deltamethrin 62, 65, 67 and 69 respectively to the above mentioned years (Table 6.5).

Table 6.5. Mean KD of *Anopheles gambiae* s.l. after 60 minutes exposure to the three types of insecticides in Intervention (Jolie) and Control (Gogete) study sites.

Study site	**Year tested**	**Exposed and KD**	**DDT**	**Permethrin**	**Deltamethrin**
Jolie	Aug,	Exposed	100	100	100
	2013	KD	33	42	45
	Aug,	Exposed	100	100	100
	2014	KD	49	61	64
	Aug,	Exposed	100	100	100
	2015	KD	58	77	81
	Aug,	Exposed	100	100	100
	2016	KD	69	82	84
Gogete	Aug,	Exposed	100	100	100
	2013	KD	52	61	62
	Aug,	Exposed	100	100	100
	2014	KD	51	62	65
	Aug,	Exposed	100	100	100
	2015	KD	53	63	67
	Aug,	Exposed	100	100	100
	2016	KD	54	66	69

The result of mean mortality for DDT, permethrin, malathion, fenitrothion, deltamethrin, propoxur and bendiocarb in both study sites conducted within the consecutive four years presented below (Table 6.6). As shown in Table 10, our result of the mean mortality rate in Intervention study site (Jolie) in August 2013, 2014, 2015 and 2016, were for DDT 9, 12, 19 and 28; for permethrin 30, 37, 46 and 63; for malathion 58, 67, 70 and 73; fenitrothion 100, 100, 100 and 100; for deltamethrin 47, 54, 59 and 77; propoxur 97, 98, 99 and 100; and for bendiocarb 90, 92, 94 and 97 respectively for the above mentioned years.

While, in Control study site (Gogete) in August 2013, 2014, 2015 and 2016, were for DDT 13, 15, 14 and 17; permethrin 35, 39, 37 and 40; malathion 62, 64, 64 and 59; fenitrothion 100); 100, 100 and 100; deltamethrin 53, 56, 61 and 51); propoxur 99, 97, 97 and 96 and bendiocarb 98, 93, 92 and 89 respectively for the above mentioned years (Table 6.6).

Thus, in Intervention (Jolie)) *An. gambiae* s.l susceptible population increased in 2016 than 2013 that is the mean mortality rates were improved due to DDT, permethrin, malathion, deltamethrin as a result statistical significant differences (for DDT P<0.001, for permethrin P<0.001, for malathion P<0.006 and deltamethrin P<0.001) in the mortality of *An. gambiae* s.l were observed in Intervention study site (Jolie) (Appendix 6.1), conversely in Control (Gogete) improvement in the mean mortality were not obtained.

Table 6.6. Mean mortality and resistance status of *Anopheles gambiae* s.l. for the seven insecticides in Intervention (Jolie) and Control (Gogete) study sites tested in August 2013 to 2016.

Study sites	Year	Insecticide tested and percentage of mortality						
		DDT*r	**Per*r**	**Mal*r**	**Fen*s**	**Del*r**	**Pro*s**	**Ben*pr**
Jolie	Aug 2013	9	30	58	100	47	97	90
	Aug 2014	12	37	67	100	54	98	92
	Aug 2015	19	46	70	100	59	99	94
	Aug 2016	28	63	73	100	77	100	97
Gogete	Aug 2013	13	35	62	100	53	99	98
	Aug 2014	15	39	64	100	56	97	93
	Aug 2015	17	40	64	100	61	97	92
	Aug 2016	14	37	59	100	51	96	89

Note: *WHO recommendation on interpretation of susceptibility test results for adult malaria vectors *s = 98-100% mortality indicates susceptibility; *pr = 80-97% mortality suggests the possibility of resistance that needs to be confirmed; *r = < 80% mortality suggests resistance.*

6.3.4. Detection and allelic frequency of knockdown resistance (*kdr*) in *An. arabiensis* and *An. gambiae* in the study areas

A total of 239 *An. gambiae s.l.* species were subjected for *kdr* laboratory test. The L1014F *kdr* mutation in our finding present at a relative high frequency in both study sites (Figure 6.4), out of the tested *An. arabiensis* (n = 117) in Intervention study site (Jolie) accounted 35.9% and in Control study site (Gogete) 41.7% (n = 108), however *An. gambiae* (n = 14) were susceptible for both L1014F (West African mutation) and L1014S (East African mutation) in the two study sites. In Intervention (Jolie)) two and in Control

(Gogete) seven heterozygous for L1014F were detected in *An. arabiensis*. The L1014S *kdr* alleles were not found in both study sites (Table 6.7).

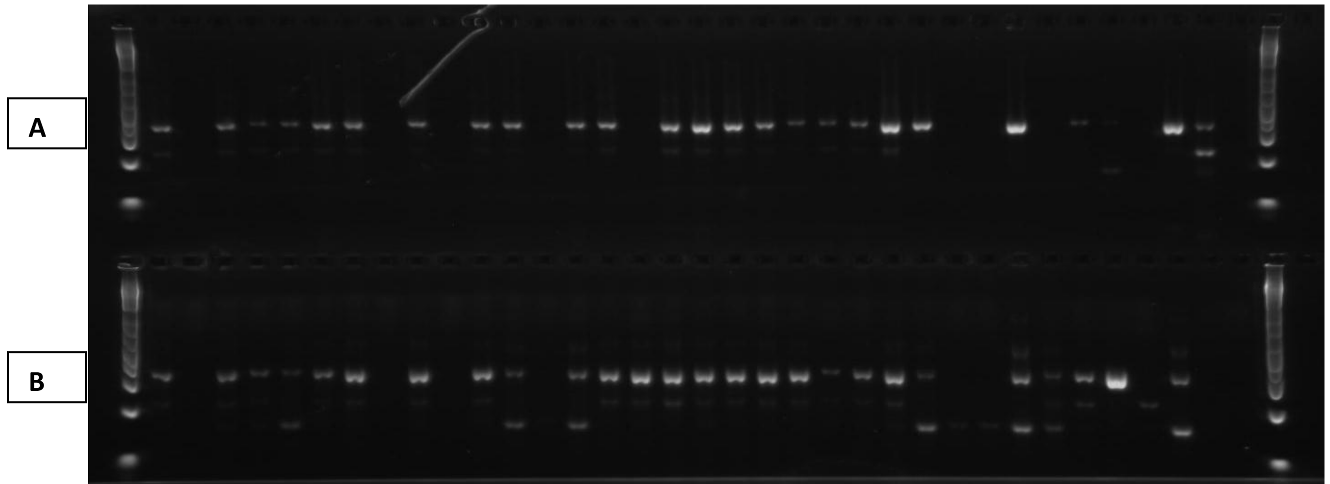

Figure 6.4. Image captured using Benchtop UV Transilluminator machine that shows (A) the absence of L1014S *kdr* and (B) presence of L1014F *kdr*. (Lanes 6, 13, 15, 26, 29 and 30 were positive for L1014F *kdr*; lane 31 Heterozygous for L1014F *kdr*, lanes 33-36 were control and lanes 1 and 37 1kb ladder).

Table 6.7. Frequency of the L1014S and L1014F *kdr* detected in *Anopheles arabiensis* and *Anopheles gambiae* in Intervention (Jolie) and Control (Gogete) study sites.

Study sites	**Species**	Frequency of L1014S kdr, Heterozygous and susceptibility			Frequency of L1014F kdr, Heterozygous and susceptibility		
		kdr	Heterozygou	Susceptible	*kdr*	Heterozygou	Susceptible
Jolie	*An. arabiensis*			117	42	2	73
	An. gambiae			4			4
Gogete	*An. arabiensis*			108	45	7	56
	An. gambiae			10			10

6.4. Discussion

This study found an average between eight to nine out of ten surveyed households located below 2000m above sea level (malaria endemic or potentially suitable for malaria transmission). Our present finding showed highest (85%) LLIN coverage is in line with a study that found 86.1% in pastoralist areas of Afar Regional State (Negash *et al*., 2012). High LLIN coverage in Ethiopia also reported in an earlier in-depth survey carried out by Net-Mark (Baume *et al*., 2009) and Shargie *et al.,* (2009).

However, other study found lower (55.2%) LLINs ownership (EHNRI/MOH, 2011) and in the same study it was reported that owning at least one net per household increased in Tigray, followed by SNNPR, but Oromia had the lower achievement in net ownership (EHNRI/MOH, 2011; Dagne and Deressa, 2008). As Ethiopia, targets *P. falciparum* malaria elimination in low transmission areas by 2015 (MOH, 2011; Shargie *et al.,* 2008) it needs to increase its coverage pace hand in hand with LLIN replacement in order to sustain the success attained in malaria control and to achieve the planned targets, even though 41 million nets has been distributed throughout the country to date (MOH, 2011). For instance, Madagascar has scaled up and have already achieved a 90% net coverage for malaria control activities with a goal of elimination by 2009 (MOH, 2011; Shargie *et al.,* 2008) surpassing the global target at an earlier date.

Multiple LLIN ownership and increasing indoor residual spray coverage for effective protection of all household members should be considered (Noor *et al.,* 2009). The malaria control strategy in Ethiopia is to distribute LLINs with the target of making ownership of two LLINs per household (MOH, 2011). However, the current finding

showed that the mean LLIN ownership was 1.3 nets per HH which was nearly similar with a study conducted by NetMark (Baume *et al*., 2009) and malaria consortium in SNNPR (Battisso *et al.,* 2012) report of a mean household ownership of 1.8 nets, per household.

The result of our study on utilization showed 78% which is consistent with the finding of previous longitudinal study in Southern Ethiopia (Astatkie, 2010). Although, the objectives of 2011- 2015 plan of the FMOH were to distribute 100% LLINs and achieve utilization rate of 80% (MOH, 2011)] there is a gap between possession and usage making the applicability of this key vector control tool less effective than desired (Fettene *et al.,* 2009). This indicates a need for more Information Education and Communication or Behavioural Change Communication (IEC/BCC) work at a community level by the malaria control programme.

The finding related to preferences for shape and colour is an important aspect of the present study. So far, similar studies indicated rectangular shape and light blue colour are more preferred (Das *et al*., 2007). Unlikely, this present finding showed about 87% of the respondents preferring blue and green, this is not in agreement with the freely distributed net colour where the vast majority of nets (89%) were white. This result is consistent with the report of NetMark where the vast majority of nets owned (84%) were the blue rectangular LLINs (Baume *et al*., 2009). In connection with this, it was reported that Light-blue rectangular polyester LLINs of different sizes was preferred by respondents in India and Nepal in large-scale intervention trial in the prevention of visceral leishmaniasis (Das *et al*., 2007).

Predominantly, the shape of net owned by the community was rectangular (93.4%). However our finding showed that circular shape was preferred by 66% of the community, while 24% preferred rectangular. Similarly, a study conducted in eastern Ethiopia also identified that LLINs colour and shape preference were barriers to bed nets use the blue and cylindrical shape LLINs were more preferable than the white and rectangular (Gobena *et al.,* 2012).

This indicating that the distributed nets shape and the preferences of the community was not harmonized. Major reason behind for preferring different shapes seems to go with the size and shape of the house the number of holes require to bore into the wall to hang a net properly that is four holes in a rectangular shape and only one in a circular shape net (Baume *et al*., 2009; Gobena *et al.,* 2012).

Besides, the cultural background of the community giving more consideration for neatness of their house wall especially during annual ceremony days was one of the problems. In addition, the long-established round houses (huts) becomes a problem to hung nets (Baume *et al*., 2009). The round structure with a pointed roof makes it difficult to find four hanging points for a rectangular net. The other problem is the net itself where rectangular nets have not enough height during stretching. Due to this, the net does not reach the sleeping mat and is not enough to tuck underneath (Gobena *et al.,* 2012). Due to these and other reasons, some families are obliged not to hang nets at all. Therefore shape and inadequate height of LLINs may inhibit utilization.

In addition, our study evaluated the bioactivity and measured the concentration of insecticide available in the textile of net after two to three years use by laboratory test.

Our result showed below the WHO recommended standards of bioactivity and doses of insecticides were found in samples of nets collected from both study sites.

The present finding is in line with previous studies that reported the lowest concentration of deltamethrin after distribution in Africa (Graham *et al.,* 2005; Yates *et al.,* 2005; Kroeger *et al.,* 2004; Asidi *et al.,* 2004). While the present finding is not in agreement with a previous study that found the highest concentration of deltamethrin in 2-3 years after distribution (Maxwell *et al.,* 2002; Feilden, 1996).

More interestingly, our finding combined variables like laboratory analysis of insecticide concentration and bioactivity of nets to give comprehensive results. This is believed to give precise information how nets are deteriorating in rural areas before they give the intended goal of protecting for long duration (Graham *et Al.,* 2005; Yates *et al.,* 2005; Kroeger *et al.,* 2004; Asidi *et al.,* 2004).

When we thought on the longevity of protection one might anticipate the duration of the insecticidal effect, but it is only one part. The WHO recommended LLINs induced 95% knockdown and 80% mortality rate were not met, that is we can understand that locally collected *An. gambiae* s.l were highly resistant to pyrethroids. Similarly, a report from Burkina Faso (Müller *et al.,* 2002) after 12 and 18 months use reported that a mortality rate of 54% and 7% respectively. Besides, Kilian *et al.* (2008) reported that, vector mortality and knockdown rate dropped by 18% and 70% respectively for permanet first generation LLIN after six months.

In addition to the above elements measuring the insecticide available in the fabrics was necessary to reach a conclusion. Thus, the result of insecticide residues available in the

nets was measured using High Performance Liquid Chromatography (WHO, 1998; CIPAC, 2009). The ranges of the deltamethrin in the study sites vary from 0.4 to 3.6 mg/m^2. The pooled average deltamethrin content was 2.17 mg/m^2 this indicate that at least the minimum recommended dosage of 5 mg/m^2 by WHOPES was not met (WHOPES, 2003). Similarly, a study conducted in Uganda (Kilian *et al.,* 2008) shown that, baseline concentration of deltamethrin was rapidly lost in the first generation PermaNet® LLINs with median of 2.5 mg/m^2 after six months. Besides, with respect to actual duration of insecticide protection in the field initial data from Burkina Faso indicated that performance was not quite as good as expected (Müller *et al.,* 2002). The relationship between laboratory bioassay results and field efficacy of nets under household way of life is not sufficiently understood.

Laboratory insecticide-induced mosquito mortality might not give us clear answers about effectiveness of nets. As a result, it is difficult to wrap up whether the practical insecticide effect is sufficient to kill mosquitoes or protect against biting in a community actual living settings. Even if it is not to say the very last field studies under community real living situation can provide us clue to understand the situation of malaria prevention and control strategy.

The resistance to DDT, malathion and pyrethroids found in this survey are consistent with other susceptibility tests done in SNNPR Fettene *et al*. (2011) found resistance to deltamethrin in Mirab Abaya and resistance to deltamethrin and DDT in Boloso Sore, both areas are near to our sites where this study conducted. Besides, Massebo *et al*. (2011) found low levels of mortality with lambdacyhalothrin, alphacypermethrin, cyfluthrin and deltamethrin. In addition, Yewhalaw *et al.* (2012) reported that *An.*

arabiensis was resistant to DDT, permethrin, deltamethrin and malathion, but susceptible to propoxur.

In the most recent report presidential malaria initiative/Africa IRS (PMI/AIRS, 2016) on insecticide susceptibility by the AIRS project (Abt Associates) *An. gambiae* s.l. was resistant to DDT, deltamethrin and permethrin in Arba Minch. While DDT and pyrethroids are not used for IRS in Ethiopia LLINs continue to be distributed and the effects of pyrethroid resistance on the effectiveness of LLINs should be further studied.

In our susceptibility test result indicated incomplete mortality after exposure to bendiocarb and propoxur (less than 98% mortality) in both study sites show the potentially an early sign of resistance signs for the efficacy of this insecticide. Similarly, Balkew *et al.* (2010) reported bendiocarb resistant populations were also detected from a few localities. While considerably less bendiocarb is being used than in previous years due to short residual life on walls (Yemane *et al.*, 2016), the emergence of resistance to bendiocarb appears to be a recent phenomenon. Fettene *et al.* (2011) found no bendiocarb resistance in the two sites tested in SNNPR in 2011. While, the PMI/AIRS report do not indicate resistance to bendiocarb even in the 2016 results from Arba Minch.

More concerning are the incomplete mortalities to propoxur, an insecticide which is still widely used for IRS in Ethiopia. There is a need for implementation of resistance management strategies as well as new insecticides and vector control tools to ensure continued gains in reducing malaria.

In Intervention (Jolie)) *An. gambiae* s.l susceptible population increased in 2016 than 2013 that is the mean mortality rates were improved due to DDT, permethrin, malathion,

deltamethrin however in Control (Gogete) improvement in the mean mortality were not obtained. Similarly, Djènontin *et al.* (2009) in the study of managing insecticide resistance in malaria vectors by combining carbamate-treated plastic wall sheeting and pyrethroid-treated bed nets reported a moderate effect against wild populations of *Cx. quinquefasciatus*, which were strongly resistant to pyrethroid. Such difference in these study sites clearly indicates that the effectiveness of treatment given in Intervention (Jolie).

Changes in the sites of insecticidal target and increasing the rates of insecticide metabolism are the two major kinds of resistance mechanisms (Martinez-Torres *et al.,* 1998, 1999). Knockdown resistance is induced by a single mutation resulting in a leucine-to-phenylalanine (L1014F, West African mutation) or leucine-to-serine (L1014S, East African mutation) change (Scott *et al.,* 1993). There are facts that at least some of these resistant vectors genes have the potential to threaten the effectiveness of current malaria vector control interventions in Ethiopia (Yewhalaw *et al.,* 2012; Balkew *et al.,* 2010).

The L1014F *kdr* mutation in our finding present at a relative high frequency in Control (Gogete) in the tested *An. arabiensis* accounts for 41.7% however in Intervention (Jolie) accounts for 35.9% and the L1014S *kdr* (East African mutation) alleles were not found in both study sites. Similarly, Yewhalaw *et al.* (2012) reported that West African *kdr* allele was found in 280 specimens out of 284 with a frequency ranged from 95% to 100%. Besides, Balkew *et al.* (2010) reported the West African *kdr* mutation in *An. arabiensis* from Ghibe and Gorgora. In addition, West African knockdown resistance (*kdr*) with allelic frequency of 98.5% was reported in population of *An. arabiensis* from south-

western Ethiopia (Yewhalaw *et al.,* (2010). In line with this, alike to our result both authors Yewhalaw *et al.* (2010) and Balkew *et al.* (2010) were not reported the presence of the L1014S *kdr* (East African mutation) alleles in Ethiopia.

In addition, our result revealed that two in Intervention (Jolie) and seven in Control (Gogete) heterozygous for L1014F *kdr* allele were detected in *An. arabiensis*. Similarly, Yewhalaw *et al.* (2012) reported that however the majorities (96.4%) of the tested individuals were homozygous and 3.6% were found heterozygous for L1014F *kdr* allele.

Moreover, in *An. gambiae* s.s. populations of Burkina Faso the L1014F *kdr* frequency was high in cotton-growing and urban areas and low in areas with limited insecticide selection pressure from agriculture (e.g., rice fields) (Diabaté *et al.,* 2002). Although, the lowest L1014S *kdr* frequencies were confirmed in the permethrin and DDT susceptible *An. gambiae* s.s. populations of the neighboring western Uganda areas where coffee, tea, and tobacco plantations are present (Katrijn, *et al.,* 2010).

Since, *kdr* frequencies can increase rapidly (Protopopoff *et al.,* 2008) this longevity advantage might have significant impact on malaria transmission and might put at risk the current resistance management strategies. Besides, the incomplete heterozygous state indicates the continuity of *kdr* occurrence (Martinez-Torres *et al.,* 1999).

Furthermore, as shown in our result West African *kdr* mutation was found in a higher rate this West African *kdr* mutation was related with high levels of both pyrethroid and DDT resistance than East African mutation (Martinez-Torres 1998; Ranson *et al.,* 2000), besides, the presence of incomplete heterozygous state indicates the continuity of *kdr*

occurrence, thus the need to be carefully followed and urgently establish resistance management strategies in the study areas might be necessary.

6.5. Conclusion

The study found the highest net possession and better usage with the varying shape and colour preferences among the households. The LLIN procurement could take the preference of varying community probably. The lower bio-activity and chemical content below the recommended dosage of World Health Organization might be due to unevenly treatment and persistence of the insecticide in the fabrics, however maintaining the nets by the community and procuring effective nets required.

Anopheles mosquitoes resistance to the insecticides is worrying most of the tested insecticides showed reduced mortality even to the insecticides currently used in Ethiopia for IRS. Susceptibility tests should continue as a part of insecticide resistance monitoring in the study areas as well as research to determine the effects of resistance on control programme.

In Intervention study site (Jolie) *An. gambiae* s.l susceptible population increased in 2016 than 2013 but not in Control study site (Gogete). Such difference in these study sites clearly indicates that the effectiveness of treatment given in Intervention study site (Jolie).

The West African *kdr* mutation was found in a higher rate and the presence of heterozygous alleles indicate that the needs to be carefully followed and urgently to establish resistance management strategies as soon as possible.

Chapter 7 General Conclusions and Recomendatios

7.1. Conclusions

The breeding habitats of *An. gambiae* s.l. in the study localities particularly in the dry season are due to human activities rather than environmental causes. Water accumulated from overflow of large canals, interrupted canals, marshy areas and ponds remain the most important breeding sites created by human negligence.

From larval collection eleven *Anopheles* species in Control (Gogete) and ten in Intervention (Jolie) study sites were identified during data collection period. In both localities the predominant *Anopheles* species was *An. gambiae* s.l. which is the main vector in the country. From adult *Anopheles* mosquitoes collection five *Anopheles* species *An. gambiae* s.l., *An. christyi, An. Pharoensis, An. demellion and An. cinerus* are the species identified in both study sites. Higher number of *An. gambiae* s.l. and *An. pharoensis* were collected in the Control study site (Gogete) than Intervention (Jolie). Thus, treatment given for the Intervention study site (Jolie) might reduce indoor mosquitoes density compared to Control (Gogete).

However, in the present study *An. gambiae* was reported for the first time in addition to *An. arabiensis* in Ethiopia indicates the presence of the highly efficient vector in the Ethiopia. The absence of *An. gambiae* in the previous study conducted in different parts of Ethiopia might be the lack of sensitivity and specificity of molecular assay coupled with technical and technological short coming, however the present study was conducted in entomology laboratory of CDC, Atlanta, Georgia and with expertise and thus resulting

in the identification of *An. gambiae* as a new record in Ethiopia and for better precision in the presence of this species is further confirmed by sequencing.

Unlike abdominal conditions and sporozoite rate statistically significant difference was observed on parity rate and host preference in Intervention (Jolie) and Control (Gogete) study sites. The overall HBI of *An. gambiae* s.l. in Intervention study site (Jolie) was lower compared to the Control (Gogete). Such host preference differences situated on the same ecological, meteorological conditions and human behaviour clearly indicated the treatment given in Intervention study site (Jolie) shifts the behaviour of *An. gambiae* s.l. feeding more on bovine than the Control (Gogete).

In line with this, the indoor density and biting of the *An. gambiae* s.l. in Control study site (Gogete) exceeds that of the Intervention (Jolie). Besides, tendency of exophilic resting behaviour of *An. gambiae* s.l observed in Intervention study site (Jolie) than Control (Gogete). This could be attributed to the efficient coverage of LLINs as well as rotation of insecticides had impact on *An. gambiae* s.l. reduction of indoor density, biting and resting behaviour at this site Intervention (Jolie).

Besides, the study found the highest net possession and better usage with the varying shape and colour preferences. Furthermore, LLIN procurement could take the preference of varying community probably. The lower bio-activity and chemical content below the recommended dosage of World Health Organization, might be due to unevenly treatment or/and less dosage treatment, however maintaining the nets by the community and procuring effective nets required.

Anopheles resistance to the insecticides is worrying, most of the tested insecticides showed reduced mortality to insecticides currently used in Ethiopia for IRS. In Intervention study site (Jolie) *An. gambiae* s.l susceptible population increased in 2016 than 2013 that is the mean mortality rates were improved due to DDT, permethrin, malathion, deltamethrin however, in Control (Gogete) improvement in the mean mortality were not obtained, this clearly indicates that the effectiveness of treatment given in Intervention study site (Jolie).

Furthermore, as shown in our result West African *kdr* mutation was found in a higher rate, indicates that the needs to be carefully followed and urgently to implement resistance management strategies as soon as possible, for the reason that this *kdr* mutation was related with high levels of both pyrethroid and DDT resistance than East African mutation, furthermore the presence of incomplete heterozygous state indicates the continuity of *kdr* occurrence in the study areas.

7. 2. Recommendations

The modification and manipulation of *Anopheles* breeding site to prevent, eliminate, or reduce vector habitat by achieving drainage, land levelling, filling and creating channels to increase water flow in areas of standing water are necessary. Since, in the study areas the vector breeding habitats are few, fixed and findable regular application of chemical or biological agents to kill mosquito larvae in their aquatic habitats is the other option to do.

However, in the present study *An. gambiae* was reported for the first time in addition to *An. arabiensis* in Ethiopia indicating the presence of the highly efficient vector in the Ethiopia, thus establishing appropriate control strategy might be necessary.

LLIN procurement could take in consideration the LLINs colour and shape preference of community and health education to maintain the nets by the community for effective nets use required. Furthermore, there is a gap between possession and usage making the applicability of this key vector control tool is less effective than the desired outcome. This indicates a need for more Information Education and Communication or Behavioural Change Communication (IEC/BCC) work at a community level by the malaria control programme.

The poor performance of bioactivity and less insecticide residue might be due to unevenly treatment or/and less dosage treatment, washing and and drying effects, procuring effective nets based on evidence (using research data) is essential.

In the study areas the result of susceptibility tes indicates bendiocarb and propoxur early signs of resistace which are currently in use for IRS. Thus, a need for continuous monitoring and implementation of resistance management strategies as well as to create or propose new insecticides and vector control tools to ensure continued gains in reducing malaria.

Furthermore, West African *kdr* mutation was found in a higher rate this *kdr* mutation was related with high levels of both pyrethroid and DDT resistance than East African mutation and the presence of incomplete heterozygous state indicates the continuity of *kdr* occurrence that the needs to be carefully followed and urgently to implement resistance management strategies as soon as possible.

Statistically significant differences were observed on indoor biting, resting activities and density, parity rate and host preference in Intervention (Jolie) and Control (Gogete) study

sites. Besides, significant increments of knockdown and mortality rate in Intervention study area (Jolie) on the tested *An. gambiae* s.l. were observed. That is, the susceptible population increased in 2016 than 2013. Even if it is not to say the very last, such difference in these study sites situated on the same ecological, meteorological conditions and human behaviour clearly indicates that the effectiveness of the treatment given in Intervention study site (Jolie). Therefore, using effective LLINs plus rotation of insecticides for IRS have impact on the control of *An. gambiae* s.l. this might be help full to use as a resistance management strategies.

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Appendixes

Appendix 3.1. *Anopheles* mosquitoes larvae collected during wet and dry seasons in Intervention (Jolie) and Cntrol (Gogete) study sites.

Jolie study site

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	4	129.25	13.90069	27.80138	85.0118	173.4882
1	4	38.75	3.567796	7.135592	27.39568	50.10432

Degrees of freedom = 6, Pr(T < t) = 0.9996 Pr(|T| > |t|) = 0.0007 Pr(T > t) = 0.0004

Gogete study site

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	4	121.5	7.511103	15.02221	97.59632	145.4037
1	4	37.25	2.212653	4.425306	30.20835	44.29165

Degrees of freedom = 6, Pr (T < t) = 1.0000 Pr (|T| > |t|) = 0.0000 Pr (T > t) = 0.0000

Appendix 3.2. Adult *An. gambiae* s.l. and *An. pharoensis* outdoor collections in the Control (Gogete) and Intervention (Jolie) study sites.

An. gambiae s.l

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	7.115385	1.279816	6.525807	4.479554	9.751215
1	26	7.307692	1.21246	6.182357	4.810584	9.8048

Degrees of freedom = 50, Pr(T < t) = 0.4568 Pr(|T| > |t|) = 0.9136 Pr(T > t) = 0.5432

An. pharoensis

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	3.730769	.6458333	3.293116	2.400651	5.060888
1	26	7.115385	1.181039	6.022139	4.68299	9.547779

Degrees of freedom = 50, Pr(T < t) = 0.0076 Pr(|T| > |t|) = 0.0152 Pr(T > t) = 0.9924

Appendix 3.3. The collection of indoor adult *An. gambiae* s.l. and *An. pharoensis* in the Intervention (Jolie) and Control (Gogete) study sites.

An. gambiae s.l

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	34.03846	6.298835	32.11788	21.06577	47.01116
1	26	75.76923	11.30178	57.62798	52.49278	99.04568

Degrees of freedom = 50, Pr(T < t) = 0.0011 Pr(|T| > |t|) = 0.0022 Pr(T > t) = 0.9989

An. pharoensis

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	10.46154	1.166495	5.94798	8.059098	12.86398
1	26	23.53846	3.422204	17.44988	16.4903	30.58662

Degrees of freedom = 50, Pr(T < t) = 0.0003 Pr(|T| > |t|) = 0.0007 Pr(T > t) = 0.9997

Appendix 3.4. Adult *Anopheles gambiae* s.l. collected indoor during wet and dry seasons in Intervention (Jolie) and Control (Gogete) study sites.

Jolie study site

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	6	77.16667	11.40882	27.94578	47.83937	106.494
1	6	4.333333	.5577734	1.36626	2.899531	5.767135

Degrees of freedom = 10, Pr(T < t) = 1.0000, Pr(|T| > |t|) = 0.0001, Pr(T > t) = 0.0000

. **Gogete study site**

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	6	151	22.80351	55.85696	92.38172	209.6183
1	6	24.33333	1.47573	3.614784	20.53985	28.12682

Degrees of freedom = 10, Pr(T < t) = 0.9999, Pr(|T| > |t|) = 0.0002, Pr(T > t) = 0.0001

Appendix 3.5. Average precipitation, maximum and minimum temperature collected around the study sites meteorology station.

Year	Month	AV-MAXT	AV-MINT	AV-PRICIP
2013	5	29.0	10.3	6.9
2013	6	26.6	12.2	109.1
2013	7	26.2	12.0	145.3
2013	8	25.3	11.9	116.1
2013	9	23.9	11.1	136.1
2013	10	24.8	9.5	67.2
2013	11	26.3	7.5	2.1
2013	12	26.3	8.5	0.2
2014	1	26.6	6.9	27
2014	2	27.6	6.2	7
2014	3	27.2	10.5	94
2014	4	27.2	11.7	220.7
2014	5	25.6	11.8	266.9
2014	6	25.9	11.4	166.1
2014	7	24.5	11.5	394.8
2014	8	25.4	10.3	169
2014	9	26.4	10.9	274.6
2014	10	27.3	11.0	133.7
2014	11	26.2	11.0	29.8
2014	12	26.8	7.5	0
2015	1	27.1	8.7	3
2015	2	26.6	11.8	53.4
2015	3	27.5	11.2	176.1
2015	4	26.4	11.2	324.8
2015	5	27.3	10.2	98.9
2015	6	26.3	11.8	229.2

Source: Butajira police station meteorology data collection site (EG_GH_ID; SHBUTA23)

Appendix 4.1. Comparison of parity rate in *Anopheles gambiae* s.l. and *An. pharoensis* species between the Intervention (Jolie) and Control (Gogete) study sites.

An. gambiae s.l

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	1.692308	.4216058	2.149776	.8239942	2.560621
1	26	5.5	.8862193	4.518849	3.674797	7.325203

Degrees of freedom = 50, Pr(T < t) = 0.0002 Pr(|T| > |t|) = 0.0003 Pr(T > t) = 0.9998

An. pharoensis

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
0	26	.3846154	.1367415	.6972473	.1029911	.6662397
1	26	1.615385	.3640664	1.856382	.8655757	2.365194

Degrees of freedom = 50, Pr(T < t) = 0.0013 Pr(|T| > |t|) = 0.0026 Pr(T > t) = 0.9987

Appendix 4.2. Comparison of HBI in *Anopheles gambiae* s.l. species between the Intervention (Jolie) and Control (Gogete) study sites.

x: Number of obs = 119

y: Number of obs = 261

Variable	Mean	Std. Err.	z	P>|z|	[95% Conf. Interval]	
x	.336	.0432992			.2511351	.4208649
y	.571	.0306356			.5109553	.6310447

Pr (Z < z) = 0.0000   Pr (|Z| < |z|) = 0.0000   Pr (Z > z) = 1.0000

Appendix 5.1. Comparison of indoor resting density of *Anopheles gambiae* s.l. species between Experimental (Jolie) and Control (Gogete) study sites.

x: Number of obs = 119

y: Number of obs = 261

Variable	Mean	Std. Err.	z	P>|z|	[95% Conf. Interval]	
x	.248	.0395878			.1704094	.3255906
y	.544	.0308292			.483576	.604424

Pr (Z < z) = 0.0000 Pr (|Z| < |z|) = 0.0000 Pr (Z > z) = 1.0000

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Appendix 5.2. Indoor man biting rate of *Anopheles gambiae* s.l. species between Intervention (Jolie) and Control (Gogete) study sites.

x: Number of obs = 119

y: Number of obs = 261

Variable	Mean	Std. Err.	z	P>|z|	[95% Conf. Interval]	
x	.064	.0224365			.0200253	.1079747
y	.154	.0223422			.1102102	.1977898

Pr (Z < z) = 0.0071 Pr (|Z| < |z|) = 0.0141 Pr (Z > z) = 0.9929

Appendix 6.1. The mortality of tested *Anopheles gambiae* s.l. for DDT, permethrin, malathion and deltamethr in Intervention study site (Jolie).

DDT					
Group	**Obs**	**Mean**	**Std. Err**	**Std. Dev**	**[95% CI]**
0	4	2.25	.25	.5	1.45 - 3.04
1	4	7	.41	.82	5.70 - 8.29
Degrees of freedom = 6 Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0001 Pr(T > t) = 1.0000					
Permethrin					
0	4	7.5	.64	1.23	5.45 - 9.55
1	4	15.75	.63	1.23	13.75 - 17.75
Degrees of freedom = 6, Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0001 Pr(T > t) = 1.0000					
Malathion					
0	4	14.5	.87	1.73	11.74 - 17.26
1	4	18.25	.25	.5	17.45 - 19.05
Degrees of freedom = 6, Pr(T < t) = 0.0030 Pr(|T| > |t|) = 0.0059 Pr(T > t) = 0.9970					
Deltamethrin					
0	4	11.75	.48	.96	10.23 - 13.27
1	4	19.25	.63	1.26	17.25 - 21.25
Degrees of freedom = 6, Pr(T < t) = 0.0000 Pr(|T| > |t|) = 0.0001 Pr(T > t) = 1.0000					