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Abundance, Distribution and Insecticide Resistance of *Anopheles* Mosquitoes (Diptera: Culicidae) and Malaria Transmission Intensity in Relation to Agro-ecology in Sekoru District, Southwestern Ethiopia

By

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DECLARATION

I, the undersigned, declare that this thesis is my own work and has not been presented in any other University, College or Institution, seeking for a similar degree or other purposes. All source of materials used for the thesis have been duly acknowledged.

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This thesis is submitted for examination with my approval asadvisor.

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2. Dr. Delenasaw Yewhalaw Signature ________________ Date______________
3. Dr. Seth R. Irish Signature ________________ Date _____________

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Abbreviations and Acronyms

ASTMH	American Society of Tropical Medicine and Hygiene
CDC	Center for Diseases Control and Prevention
CSA	Central Statistics Authority
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immuno-Sorbent Assay
FMoH	Federal Minister of Health
HBR	Human Biting Rate
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
KDR	Knock Down Resistance
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
PSC	Pyrethroid Spray Catches
RDT	Rapid Diagnostic Test
SNP	Single Nucleotide Polymorphism
SSA	Sub Saharan Africa
USAID	United States Agency International Developments
VGSC	Voltage Gate Sodium Channel
WHO	World Health Organizations

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Abstract

Malaria is a leading cause of morbidity and mortality in several sub-Saharan African countries. Environmental/ecological changes due to anthropogenic activities are among the determinant factors for malaria transmission. Agricultural practices are among anthropogenic activities that contribute to malaria incidence and transmission. Understanding association of ecological changes due to anthropogenic activities on mosquito species composition, abundance, distribution, dynamics, insecticide resistance and malaria transmission intensity is important to plan and implement effective vector control intervention strategies. Thus, the aim of this study was to investigate species composition, abundance, distribution and infectious rate of *Anopheles* mosquitoes and their knockdown resistance (*kdr*) status in relation to agricultural practices. A longitudinal entomological study was conducted from January to December 2015 in Sekoru District, southwestern Ethiopia. *Anopheles* mosquito larvae and adults were collected using different methods from villages with different agro-ecology. The mosquitoes were identified to species level using standard keys. Molecular identification of *Anopheles gambiae* complex and detection of knockdown insecticide resistance (*kdr*) was conducted using species-specific PCR and allele specific PCR techniques. Moreover, *Plasmodium* circumsporozoite protein was detected for both *Plasmodium falciparum* and *P. vivax* using Enzyme-linked Immunosorbent Assay (ELISA). Eight *Anopheles* mosquito species (*Anophelesarabiensis,An. demeilloni*, *An. squamosus, An. garnhami, An. christyi, An. pretoriensis, An. longipalpis* and *An. marshallii*) were identified, of which *An. arabiensis* was the predominant species (46.2%; n=715). The highest number of *Anopheles* mosquitoes (66%; n=1019) was collected from the irrigated village. The infection rate of *An. arabiensis* was higher in the irrigated village (10.8 infective bites/person/month) as compared to rain fed agriculture practicing village (5.99 infective bites/person/month) and human settlement village (zero infective bite). *Anopheles gambiae*s.l. larvae were the predominant (57.4%) larval species identified. The highest larval density (2.12 larvae/dip) was recorded from the irrigated village. Only West African *kdr* mutation (L1014F) was detected with an allelic frequency of 83.88%. The distribution and frequency of *kdr* allele were significantly associated with study villages (X^2=133.85, df=2, P <0.001). The *kdr* allele frequency was 95%in the irrigated village, 78.87%in village with rain fed agriculture, and 3.89% in the human settlement village. In conclusion, *Anopheles* mosquito abundance, distribution, infection rate and insecticide resistance were significantly associated with agro-ecology. Agro-ecological practices need to be considered in the management of *Anopheles* vectors of malaria.

Keywords: *Anopheles* mosquitoes, Agro-ecology, Insecticide resistance, Irrigation, Larval habitats, Malaria, Sekoru District

Chapter 1. General Introduction

1.1. Background

Malaria is an important parasitic disease caused by protozoa of the genus *Plasmodium*. Malaria is caused by *Plasmodium* species such as *Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae, Plasmodium vivax* and *Plasmodiumknowlesi*. Malaria is transmitted by bites of infective female *Anopheles* mosquitoes (Cox, 2010). Of hundreds*Anopheles* species, only few are able to carry the parasites and be responsible for malaria transmission (Harbach, 2004).

In Ethiopia, there are more than forty species of *Anopheles* mosquitoes (O'Connor, 1967), of which *Anopheles arabiensis, Anopheles funestus, Anopheles pharoensis* and *Anopheles nili* are the malaria vectors (Krafsur, 1970; Yewhalaw *et al*., 2009; Dejenie *et al*., 2012; Jaleta *et al*., 2013). *Anopheles arabiensis* is primary malaria vector in Ethiopia. Likewise, *An. funestus* and *An. pharoensis* are secondary vectors occurring with varying population densities, limited distribution and vector competence (Kibret *et al*., 2010).

Based on the principle of National Strategic Plan of Ethiopia, malaria control programs are ongoing with various intervention strategies to reduce malaria burden to a level where it is no longer public health problem (FMoH, 2011; 2013; 2016; USAID, 2013). In spite of considerable progress in malaria control, the infection remains a severe public health problem. *Plasmodium falciparum* and *P. vivax* are the dominant malaria parasites responsible for the majority of cases in the country (FMoH, 2013; 2014).

The patterns of malaria transmission varies within and between communities/villages and season. Mosquito vector population dynamics, insecticide resistance and malaria transmission intensity are associated with land use patterns such as agriculture, deforestation and water resource developments (Ernst *et al.,* 2009; Kibret *et al*., 2010; Yewhalaw *et al.,* 2009; Stryker and Bomblies, 2012; Jaleta *et al*., 2013). Development of insecticide resistance by malaria vectors are attributable to extensive and misuse of insecticides in agriculture and public health sectors(Yewhalaw *et al*., 2014; Abuelmaali *et al*., 2013; Nkya *et al*., 2014). Several studies in Africa reported various insecticide resistance mechanisms in various geographical areas (Yewhalaw *et al.,* 2010; Kawada *et al*., 2011; Balkaw *et al*., 2012).

1.2. Statements of the problemand Rationale of the Study

Agricultural expansions such as irrigation practices affect vector population dynamics and malaria transmission in sub-Saharan Africa. In Ethiopia, malaria prevalence and the risk of transmission by *An. arabiensis* were significantly higher in irrigated sugarcane agro-ecosystem compared to non-irrigated agro-ecosystems (Jaleta *et al*., 2013; Kibret *et al*., 2010). There exists a need to develop a long-term plan for malaria control through effective vector management. To achieve a reduction in malaria transmission, before designing control options, having adequate information on vector bionomics, vector distribution and insecticide susceptibility level are important. Findings related to impacts of agricultural practices on malaria vector population dynamics are geographically and timely limited and/or varied. Therefore, periodic understanding of the association of agriculture and malaria vector abundance, distribution and insecticide susceptibility are important to implement effective interventions and to design new and effective control strategies.

Furthermore, studies on ecological distribution, species composition and vector competence of *Anopheles* mosquitoes have been conducted in different parts of Ethiopia in the past (Hunt *et al.,* 1998; Kibret *et al.,* 2010; Dejene *et al*., 2012; Jaleta *et al.,* 2013). However, there is little information on association of agricultural practice and *Anopheles* population dynamics, insecticide resistance and malaria transmission intensity.

Point mutations at Voltage Gate Sodium Channel are imperative as indicators of pyrethroids resistance in the population of *An. arabiensis* (Kawanda *et al*., 2011). Compared to other resistance mechanisms, *kdr* allele status in the populations of *An. arabiensis* was frequently reported in Ethiopia (Yewhalaw *et al.,* 2010; 2011). This is an indicator of increased resistance of *An. arabiensis* to pyrethroids. To manage pyrethroids resistanc eand evaluate efficacy of intervention options, investigations related to status of *kdr* allele and associated factors are important. However, no assessment was done as to association of agricultural practices and insecticide resistance development in malaria vectors in Ethiopia. Hence, this study was conducted to investigate status of knockdown resistance (*kdr*) and its frequency in population of *An. arabiensis* in Sekoru District, southwestern Ethiopia.

1.3. Objectives

1.3.1. General objective

- ➢ To investigate the association of agro-ecological settings and *Anopheles* mosquito population dynamics, infectious rate and the status of insecticide resistance for integrated malaria vector management in the study area.

1.3.2. Specific objectives

- ✓ To determine species composition, spatio-temporal distribution and abundance of *Anopheles* mosquitoes in the study area
- ✓ To estimate entomological inoculation rate and malaria transmission intensity in association with agro-ecological settings in the study area
- ✓ To investigate abundance and spatial distributions of *Anopheles* larvae in association with small scale irrigation in the study area
- ✓ To investigate insecticide resistance level by malaria vectors in association with agricultural practice in the study area

Chapter 2. Literature Review

2.1.Trends in malaria transmission and disease burden

Malaria is the most widespread infection caused by protozoan of the genus *Plasmodium.* Five *Plasmodium* species such as *P. falciparum, P. vivax, P. malariae, P. ovale* and P. *knowlesi* are known to carry malaria parasites (Cox, 2010). According to the report of World Health Organization (2015), globally, malaria is one of the most severe diseases with 214 million cases and about 438,000 deaths per year. The highest malaria cases and deaths were reported from African countries (88%) followed by South-East Asia Region (10%) and Eastern Mediterranean Region (2%).

The impact of malaria on human health, productivity and general well-being is profound. In endemic areas, malaria hinders children in their schooling and social development both through absence from school and permanent neurological or other damages. Malaria became a significant obstacle to socioeconomic development of the society in endemic countries (World Health Organization, 2004). Due to its direct and indirect cost, malaria has multiple impacts on economic growth and development in endemic regions of Africa. Any expenses due to malaria affect abilities of farm households to adopt new agricultural technology and improve practices. It is equally important to note indirect costs of malaria on farm productivity due to seeking health care and taking care of children and others who are troubled by malaria.

2.2.Malaria Vectors in Africa: Ecology and Distribution

Malaria is a vector borne disease transmitted from infected to healthy individuals by infective bites of female mosquitoes of the genus *Anopheles* (Harbach, 2004; Cox, 2010).The genus *Anopheles* mosquito is probably the most studied genera among medically important insects. Of the total 500 *Anopheles* species globally listed, sixty to seventy species are known to transmit human malaria. Thirty to forty *Anopheles* mosquito species are responsible for malaria transmission, of which about fifteen species are the major vectors transmitting malaria at a level of major concern to public health (Hay *et al*., 2010; Sinka, 2012). The malaria vectors in Africa include *Anopheles gambiae* s.l., *An. funestus*, *An. nili, An. pharoensis* and*An. moucheti* (Sinka *et al.,* 2010).

Due to their great contribution for malaria transmission in Africa, *An. gambiae* and *An. funestus* complexes are probably the most well studied *Anopheles* mosquito species (Sinka *et al*., 2010, 2012; Williams and Pinto, 2012). Members of the *An. gambiae* complex and *An. funestus* group are among the dominant malaria vectors species widely distributed in Africa (Figure 2.1).

Anopheles gambiae complex has been described as the most medically important insect, accounting for the majority of malaria cases and deaths (World Health Organization, 2012). It comprises about eight morphologically indistinguishable sibling species widely distributed in Africa (Sinka *et al*., 2010, 2012). The dominant malaria vectors(*An. gambiae s.s*. and *An. arabiensis*) and the minor malaria vectors (*An. melas, An. merus* and *An. bwambae*) are sibling species of *An. gambiae* complex responsible for malaria transmission in Africa. Though the dominant malaria vectors are widely distributed, the distribution of minor vectors is confined to specific geographical locations (Figure 2.1). *Anopheles melas* distributed in western while *An. merus* in eastern coastal mangroves of Africa. *Anopheles merus* are localized to South Africa, and the third *An. bwambae*, in Semliki Forest of Uganda (White, 1985). *Anopheles quadriannulatus* and *An. amharicus*, documented from the south and east Africa respectively, however, they cause no sever threat to public health, having zoophilic behavior (Hunt *et al*., 1998; Fanello*et al*., 2002). *Anopheles comorensis* is another subspecies of *An. gambiae* complex, yet their medical importance is not well documented. This species is localized to the island of Grande Comore in the Indian Ocean (Brunhes*et al*., 1997).

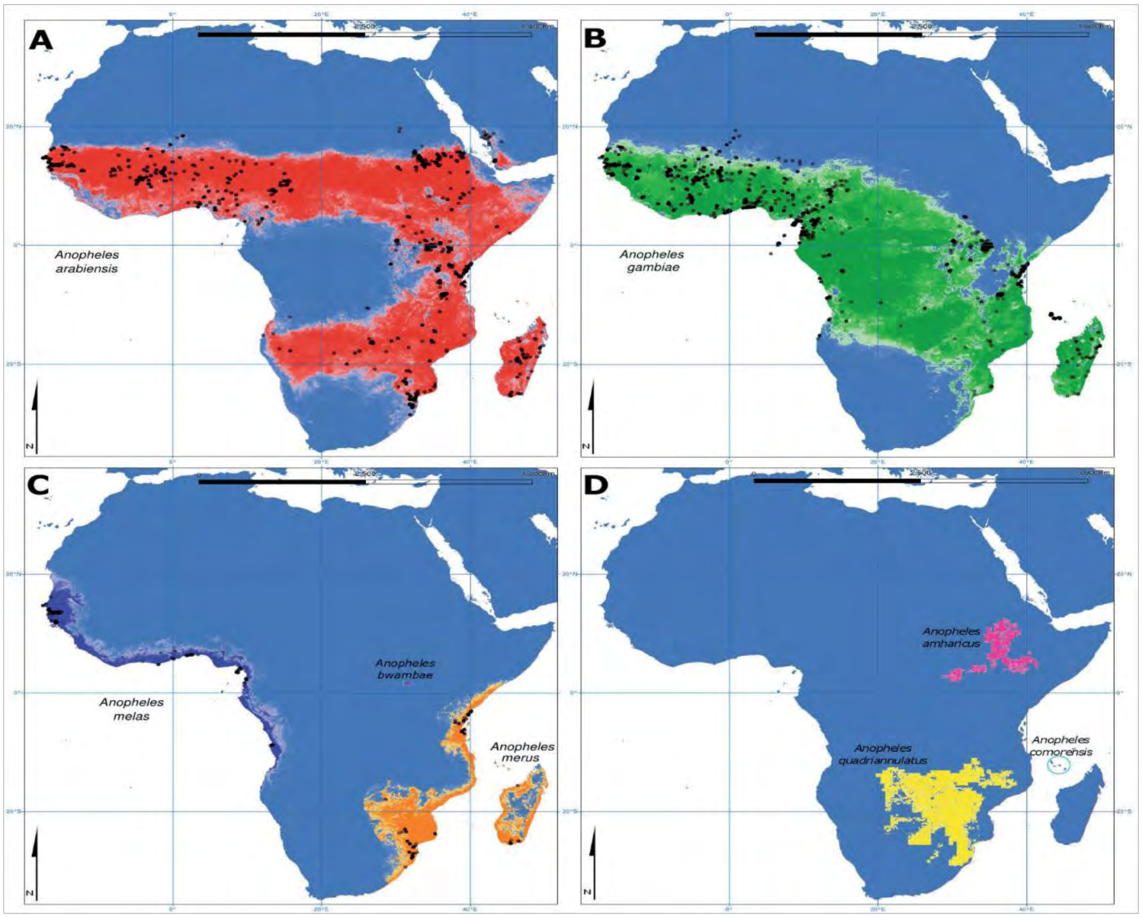

Figure 2.1:Geographical distribution of members of the *Anopheles gambiae* complex:A: *An. arabiensis* (red); B: *An. gambiae s.s.* (green); C: *An. melas* (Blue), *An. merus* (orange), and *An. bwambae* (cyan); D: *An. quadriannulatus* (former species A) (yellow), *An. amharicus* (former *An. quadriannulatus* B) (magenta) and *An. comorensis* (cyan circle): (Source: Sinka *et al*., 2010).

The second dominant malaria vector species in Africa, *An. funestus* group, comprises several sibling or closely related species of which *An. funestus* s.s. is a major malaria vector in Africa (Sinka *et al*., 2010; 2012). The ecological distributions of *An. funestus complex in* Africa are shown in figure 2.2.

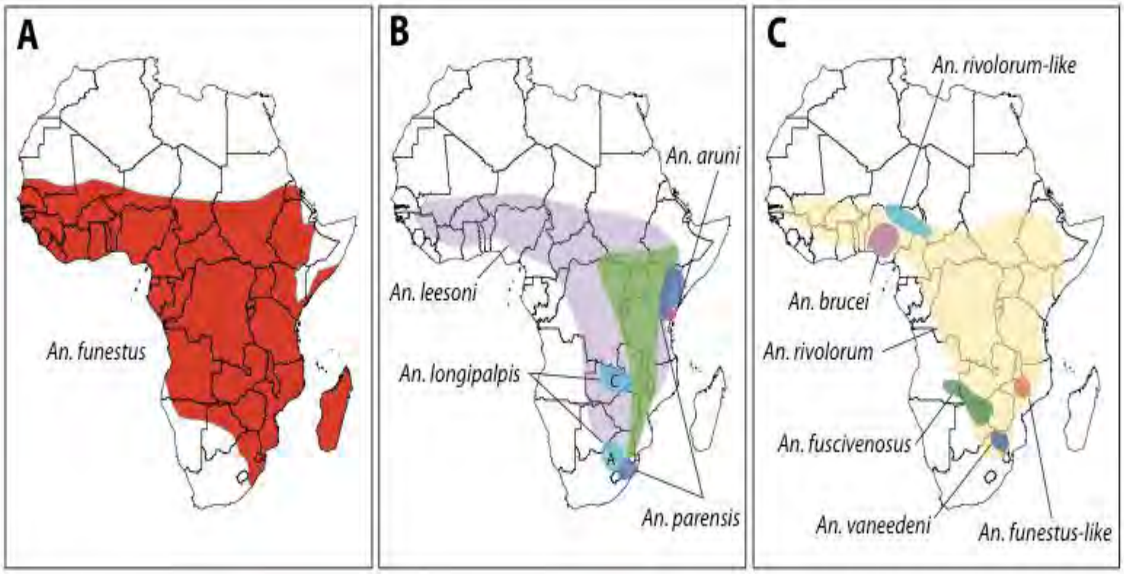

Figure 2.2:Distribution members of *An. funestus* complex in Africa, A: *An. funestus*; B: *An. leesoni*, *An. longipalpis* (type A and C), *An. aruni* and *An. parensis,* C: *An. rivolorum, An. rivolorum-like, An. funestus-like, An. vaneedeni, An. fuscivenosus* and *An. brucei:*(Source, Sinka *et al*., 2012).

The secondary malaria vectors in Africa include *An. moucheti*, *An. nili* and members of *An. gambiae* complex (*An. merus,An. melas* and *An. bwambae*). The geographic distribution of the secondary malaria vectors of Africa is indicated in figure 2.3.

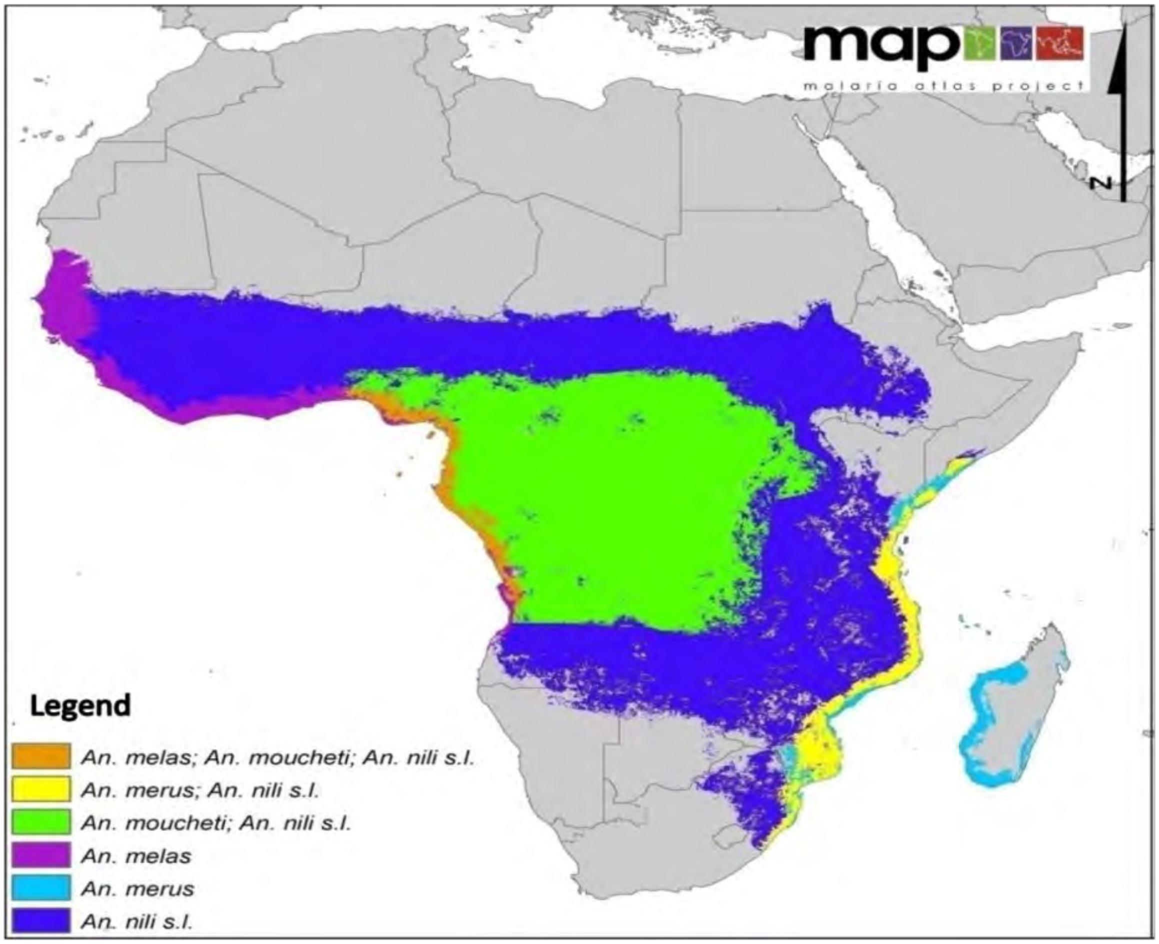

Figure 2.3:Geographicaldistribution of secondary malaria vector species in Africa: (Source, Sinka *et al*., 2010).

2.3.Malaria status and Vector mosquitoes in Ethiopia

Malaria is the leading causes of outpatient attendance, the most frequently reported and the principal causes of morbidity and mortality in Ethiopia (FMoH, 2015). Malaria epidemiologic profile revealed wide distribution of the disease in different parts of the country except some highland areas (USAID, 2013). More than 75% of the land in Ethiopia is malariaous and about 60% (52 million) of the population are at risk of malaria transmission. In Ethiopia, the incidence and prevalence of malaria are determined by climatological and topographical factors of which the most important is altitude (FMoH, 2011).

Most cases of malaria are due to *P. falciparum* and *P. vivax*, the former causing the most severe symptoms though their relative prevalence rate varies according to localities and seasons. The other *Plasmodium* species, *P. malariae* accounts for less than 1% (FMoH, 2016).

Malaria cases were decreased from 2.6 million cases in 2011 (FMoH, 2011; Richards *et al.,* 2012) to 2,174,707 cases in 2015 (FMoH, 2016). Previously, *P. falciparum* and *P. vivax* cased 60% and 40% malaria infection, respectively. However, current report indicated that among all malaria confirmations 1,188,627 (63.7%) were *P. falciparum* while 678,432 (36.3%) were *P. vivax* (FMoH, 2016).Regarding regional epidemiologic distribution, the highest number of malaria cases were reported from Amhara (610,486), followed by Oromia Region (430,969 cases) while highest number of malaria deaths was from Oromia (214 deaths) followed by Southern Nation Nationality and people of Ethiopia (166 deaths). The recent reports revealed that the monthly malaria pattern indicated an increased malaria cases in the first five months (September to January) of the fiscal year with the peak in November. Out of all malaria cases reported, 1,867,059 (85.9%) were confirmed by either rapid diagnostic tests (RDT) or microscopy (FMoH, 2016).

2.3.1. Malaria Vectors in Ethiopia

In Ethiopia, about 42 to 44 species of *Anopheles* mosquitoes have been documented so far (O’Connor 1967; Hunt *et al*., 1998; FMoH, 2014). Of all *Anopheles* mosquito species in Ethiopia, *An. arabiensis, An. funestus*, *An. pharoensis* and *An. nili* are the medically important malaria vectors in Ethiopia (Dejene *et al*., 2012; Kibret *et al*., 2010; Yewhalaw *et al*., 2013; Jaleta *et al*., 2013).The distribution of the vectors in Ethiopia is summarized in figure 2.4.

Anopheles arabiensis is a major malaria vector widely distributed in Ethiopia (Mekonnon *et al*., 2005; Woime, 2008; Dejene *et al*., 2012; Jaleta *et al*., 2013). The other common vectors of malaria dominating in malaria endemic areas are *An. funestus* and *An. pharoensis* (O'Connor, 1967; Krafsur, 1970; Taye *et al.,* 2006; Woime, 2008; Woime, 2008). *Anopheles nili* is the least common and more localized species and not adequately studied. It is found in the southwestern, western and northwestern parts of Ethiopia (Krafsor, 1970; Jaleta *et al*., 2013).

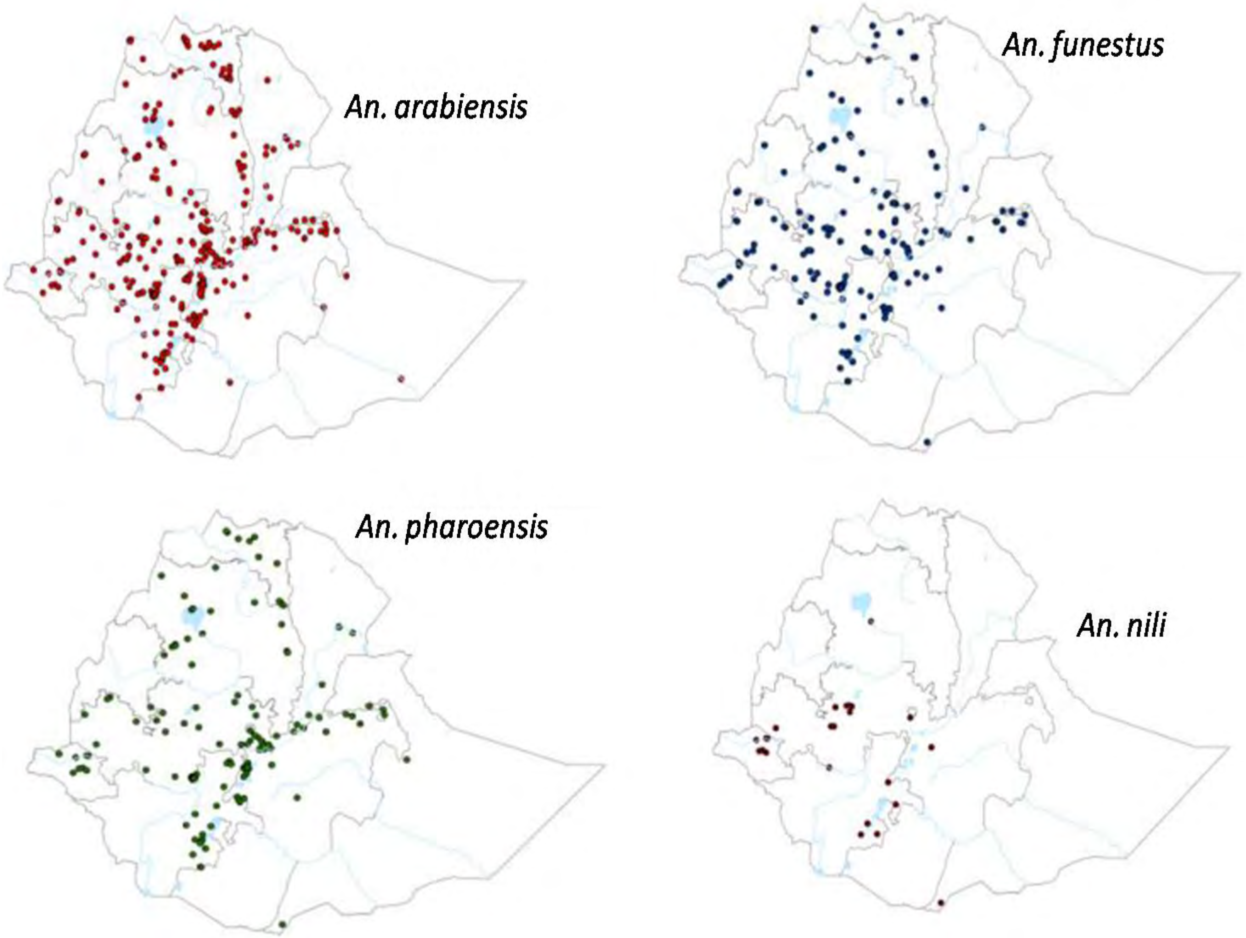

Figure 2.4: Distribution of the malaria vector species in Ethiopia: Data and map adopted from FMoH, (2014).

2.3.2. Malaria Control Strategies and Challenges in Ethiopia

In Ethiopia, malaria control strategies focus on intensifying vector control, environmental management and increasing malaria case detection and prompt treatment (USAID, 2013). Malaria vector control strategies relied on scaled up use of Long Lasting Insecticidal Nets and Indoor Residual Spraying (IRS). In 2015, 17.2 million LLITNs were distributed in the country that was in excess of the amount distributed in 2014 (11.7 million). In 2015, Ethiopian Minister of Health has had a plan of covering 5.9 million unit structures by IRS. However, 89.8% (5.3 million unit structures) in endemic areas were sprayed which was by 3.9 million households higher than 2014 (FMoH, 2016). Furthermore, admittance to care for malaria patients and appropriate diagnosis and effective and prompt treatment using Arthemesinin Combination Therapy (ACT) is another malaria control strategy in the country (FMoH, 2013).

Budget constraints to conduct IRS activities, delay in replacement of old insecticide treated nets were limitations hindering implementation of successful malaria controls. Furthermore, inadequate knowledge, attitude and practice (KAP) of the community to ward malaria control interventions and utilization of LLINs were among the challenges of malaria control (FMoH, 2016).

2.4.Factors determining vector distribution and malaria transmission

Biological factors (insecticide resistance of the vector, drug resistance of the parasite and immunity of the host), environmental factors (altitude, meteorological variables) and anthropogenic activities (land use patterns, interventions, population movement and settlement, socioeconomic activities, livestock and health access) are major determinant factors of malaria epidemiology (Protopopoff *et al.,* 2009). Studies showed that *Anopheles* mosquito distribution and abundance and malaria incidence and prevalence were associated with land use patterns such as agricultural practices (Muturi *et al*., 2008; Dejene *et al*., 2012; Abuelmaali*et al*., 2013; Jaleta *et al*., 2013), water resource developments (Yewhalaw *et al*., 2009, Kibret *et al*., 2010) and population settlements (Degefa *et al*., 2015).

2.4.1. Land use patterns and Malaria

Irrigation

Land use patterns such as agricultural activities, water resource development, urbanization, deforestation, plantations, logging, road construction and mining are created because of wide variety of human activities (Dixon *et al*., 2001).Irrigated agro-ecosystem contributed for augmented malaria incidence and prevalence. Following irrigation, the number of mosquitoes usually increases often leading to a rise in malaria incidence and transmission (Muturi *et al*., 2008; Kibret *et al*., 2010; Jaleta *et al*., 2013). Spatial malaria transmission is proportional to the mosquito density in a given area.

Several studies blamed irrigation schemes for malaria risk and vector population dynamics in sub-Saharan Africa (Table 2.1). For example, in Ethiopia, where malaria transmission intensities vary seasonally, incidence of the infection and density of the vector is influenced by irrigation schemes. Malaria prevalence and the risk of transmission by *An. arabiensis* were significantly higher in irrigated sugarcane agro-ecosystem (49% in modern irrigation and 28% in traditional irrigation) compared to non-irrigated agro-ecosystems (23%) in western Ethiopia (Jaleta *et al*., 2013). Kibret *et al.*(2010) reported that higher *Anopheles* mosquito density in irrigated villages (94%) compared to the non-irrigated (6%). Similarly, 19% and 16% malaria prevalence was recorded from irrigated and non-irrigated villages, respectively. Boelee *et al.* (2002) reported that operation of irrigation scheme was blamed for increased malaria prevalence in Zimbabwe.

Similarly, Gezira-Managil irrigation scheme of Sudan extended the length of the region's longest malaria transmission season creating new mosquito breeding grounds (Keiser *et al*, 2005). According to the report, before irrigation development, the land of Gezira was mostly left unsown during winter months as the same time malaria was not a problem during any part of the year. However, the Gezira irrigation scheme led to a 20% increase in the local malaria infection rate, and the disease began to plague the population year-round. Similar observation was reported in Rusizi valley of Burundi. Irrigated areas in the valley had higher malaria prevalence rates and a 150-fold higher vectorial capacity of *An. arabiensis* when compared to neighboring non-irrigated villages (Boelee *et al.*, 2002).

Though the negative effects of irrigation schemes on malaria transmission are pronounced, several controversial conclusions regarding irrigation and malaria prevalence were investigated. As exemplified in Table 1, in sub Saharan Africa, studies reported that irrigation schemes either reduced or has no impact on malaria incidence and transmission and *Anopheles* mosquito population dynamics. Mutero *et al.* (2004) reported that irrigated areas were found to have lower prevalence of malaria though they had a 30–300 times higher abundance of the local malaria vector compared with areas without irrigation in Kenya. Authors investigated that the density of *An. arabiensis* was highest in the irrigated rice village and lowest in the non-irrigated village while *An. funestus* density was higher in the non-irrigated village than in irrigated ones in the region. However, the human blood index (HBI) for both *An. arabiensis* and *An. funestus* was significantly higher in the non-irrigated village compared with irrigated villages (Muturi*et al.,* 2008).

In Tanzania, 2.6 times lower EIR and low malaria transmission was reported in irrigated villages compared to control villages (Ijumba *et al.,* 2001). According to Ijumba *et al.*

(2002), though vector density was four times higher in irrigated vicinity, 61 to 68% lower infective bites were observed. In the Kou valley, Burkina Faso, malaria prevalence rates ranged from 16-58% in an irrigated village, compared to 35-83% in a non-irrigated villages (Boudin *et al*., 1992; Sanchez-Ribas *et al*., 2012). Report from Mali revealed a two-fold reduction of annual malaria incidence after the implementation of irrigation scheme (Sanchez-Ribas *et al*., 2012; Sissoko *et al*., 2004; Sogoba *et al*., 2008; Diuk-Wasser*et al*., 2007).

Malaria and irrigated agro-ecosystem relationship were partially explained by enhanced incomes that facilitate better protective measures to be taken (Sanchez-Ribas *et al*., 2012). An increased income from agricultural development for the community is likely to improve access to malaria treatment and may support an increased use of malaria preventive devices.

Table 2.1: Impact of irrigation on entomologic parameters and malaria transmission in sub-Saharan Africa

Country	Impact on mosquito density	Impact on malaria prevalence/incidence	Reference
Burkina Faso	-	decreased malaria prevalence	(Boudin *et al*., 1992; Erhart *et al*., 2004; Keisare *et al*., 2004)
Burundi	increased vectorial capacity of *An. arabiensis*	Increased malaria incidence	(Coosemans, 1985; Protopopoff *et al*., 2009)
Cameroon	decreased human biting	decreased malaria transmission	(Keisar *et al*., 2004; Sanchez-Ribas *et al*., 2012; Tanga *et*

	rate		*al*., 2011)
Ethiopia	increased vector density	increased malaria transmission	(Kibret *et al*., 2010; Jaleta *et al.,* 2013; Dejenie*et al*., 2012; Kibret *et al*., 2014)
Ivory Coast	decreased human biting rate	decreased malaria transmission	(Keisar *et al*., 2004; Sanchez-Ribas *et al*., 2012; Henry *et al*., 2003; Briet *et al*., 2003)
Kenya	increased vector density	malaria prevalence	(Mutoro *et al*., 2004; Mwangangi *et al*., 2006; 2010; Jacob*et al*., 2007; Muturi *et al*., 2008)
Mali	-	decreased malaria incidence	(Sissoko *et al*., 2004; Diuk-Wasser*et al*., 2007; Sogoba *et al*., 2008)
Sudan	-	increased malaria prevalence	(Keisar *et al*., 2004; 2005; Sanchez-Ribas *et al*., 2012; Ageep *et al*., 2009; Eltayeb *et al*., 2015)
Tanzania	-	decreased malaria transmission in rice field but increased in sugarcane field	(Sanchez-Ribas *et al.,* 2012)

Maize cultivation

Cultivation of crops ensures food security in sub Saharan Africa. However, cultivation of some crops may increase malaria incidence and disease transmission. For instance, maize cultivation affects *Anopheles* mosquito distribution, abundance and malaria transmission. Maturing maize produces a copious amount of wind-borne pollen that is nutritious enough and produced over a sufficient period to support the development of at least one generation of anopheline mosquitoes (Ernst *et al.*, 2009; Kebede *et al*., 2005).

Ye-Ebiyo *et al*. (2000) reported that larvae of *An. arabiensis* readily ingest the pollen grains themselves. An aqueous extract of maize pollen markedly accelerates the rate at which larvae ingest inert particles. The feeding ability of *Anopheles* mosquito larvae on maize pollen in turbid water is enhanced by water-soluble phago-stimulatory components released from pollen grains. Larvae develop to the pupal stage more rapidly and produce larger adults where maize pollen is abundant than do those that have little access to this food (Ye-Ebiyo *et al*., 2003). Larger adult body size contributes to greater longevity and reproductive success. Additionally, maize pollination season coincides with *Anopheles* mosquito breeding time due to warmer temperatures and higher humidity. Therefore, cultivation of high yield maize contributes to both higher life expectancy and density of mosquito and consequently to malaria transmission.

Rice cultivation

Rice cultivation is associated with malaria risk providing suitable breeding habitat for vectors (Service, 1991; Hunter *et al*., 1993). Many species of *Anopheles* mosquitoes favor open sunlit habitat that rice fields provide ideal breeding site for members of *An. gambiae* complex in sub-Saharan Africa (Carnevale *et al*., 1999). *An. gambiae* complex mosquitoes rapidly colonize recently flooded rice field (Lindsay *et al*., 1991). Irrigated rice cultivation increases the number of cropping cycles (Assi *et al*., 2013)that may also extend the breeding seasons of these vectors consequently increasing transmission season. Although *An. gambiae* complex is generally linked with rice field, *An. funestus* also thrives in rice field. Unlike *An. gambiae* complex, *An. funestus* occurs in rice field during later season extending transmission time (Diuk-Wasser *et al*., 2007). Reports from sub Saharan Africa indicated that rice cultivation affected population density of malaria vector, malaria incidence and transmission (Table 2.2).

Rice cultivation creates conducive breeding habitats for *Anopheles* mosquitoes and increase malaria transmission in local communities. However, several reports revealed that rice cultivation has no effect or reduce malaria risk in local communities (Ijumba *et al*. 2001; 2002; Assi *et al.*, 2013).A comparative study conducted in non-irrigated, rice agro-ecosystems to investigate impact of rice cultivation on vector population dynamics and malaria incidence and transmission in Kenya indicated highest density of *An. arabiensis* in the planned rice cultivation and lowest in the non-irrigated areas(Muturi *et al*., 2008). Nevertheless, lower anthropophllic behavior of *An. arabiensis* and *An. funestus* was observed in rice cultivating villages compared to the controlled villages. The annual infective bites for the vectors were not significantly different among study villages.

Table 2.2: Impact of crop cultivation on *Anopheles* mosquito population dynamics and malaria incidence and transmission in different regions of sub-Saharan Africa

Crop type	Location	Impacts on malaria/vectors	Vector species	Citations/ reference
Maize	Ethiopia	increased malaria incidence	*An. arabiensis*	(Kebede *et al.*, 2005; Ye-Ebiyo *et al.*, 2000; 2003)
Rice	The Gambia	increased vector density	*An. gambiae s.l.*	(Lindsay *et al.*, 1991; Sanchez-Ribas *et al.*, 2012)
	Tanzania	increased vector density but decreased malaria transmission	*An. gambiae s.l.*	(Ijumba *et al.*, 2001; 2002)
	Mali	increased vector density and decreased malaria rates	*An. funestus*	(Sogoba *et al.*, 2008; Diuk-Wasser *et al.*, 2007; Klinkenberg *et al.*, 2003; Diuk-Wasser *et al.*, 2007)
	Kenya	decreased HBI and malaria transmission but increased *An. arabiensis* density	*An. funestus* and *An. arabiensis*	(Muturi *et al.*, 2008; Mwangangi *et al.*, 2006; 2010)
	Uganda	increased malaria infection	*An. gambiae s.l.*	(Staedke *et al.*, 2003; Assi *et al.*, 2013)
	Ivory cost	increased human biting rate and malaria transmission	*An. gambiae s.l.* and *An. funestus*	(Keiser *et al.*, 2004; Sanchez-Ribas *et al.*, 2012; Lindblade *et al.*, 2000; Ernst *et al.*, 2009; Dossou-Yovo *et al.*, 1994)
	Ivory cost	no impact on malaria incidence	_	(Krefis *et al.*, 2011; Assi *et al.*, 2013)

2.4.2. Water resource development and malaria transmission

Several studies in sub-Saharan African countries reported impacts of dams on malaria vector distribution and abundance, malaria incidence and transmission (Table 2.3).Water resource projects (dams) which are constructed for hydro-electric power and irrigation purpose are blamed for year-round malaria transmission and increased incidence in Ethiopia (Lautze *et al*., 2007; Kibret *et al*., 2010; Dejene *et al*., 2012; Yewhalaw *et al.*, 2009; 2013).

The construction and operation of dams in Senegal River increased anopheline densities, malaria transmission intensity and prevalence (Dia *et al*., 2008). Study in Burkina Faso exposed significant association between small reservoirs and malaria risks (Boelee *et al*., 2009).

Table 2.3: The impact of water resource development on malaria vectors and transmission in sub-Saharan African countries

Country	Purpose of the projects	Impact on malaria and/ or vector density	Source/ citation
Ethiopia	Irrigation and HEP	increased *An. arabiensis* and *An. pharoensis* density and malaria transmission	(Lautz *et al.,* 2007; Kibret *et al.,* 2010; Dejenie *et al*; 2012; Jaleta *et al*., 2013; Yewhalaw *et al*., 2009; 2012)
Sudan	irrigation	increased malaria prevalence	(Keisare *et al*., 2004; 2005; Sanchez-Ribas *et al*., 2012; Ageep *et al*., 2009; Eltayeb *et al*., 2015)
Burundi	irrigation	increased vectorial capacity of *An. arabiensis*	(Coosemans, 1985; Sanchez-Ribas *et al*., 2012)
Kenya	irrigation	decreased vector density and malaria prevalence	(Mutero *et al*., 2004; Muturi *et al*., 2008)
		Increased vector density	(Mwangangi e*t al*., 2006; 2010; Jacob *et al*., 2007; Tatem *et al.,* 2013; Mutero *et al*., 2000)
Tanzania	irrigation	increased malaria in rice field but decreased in sugarcane field	(Ijumba *et al*., 2001; 2002; Keisare *et al*., 2004; Sanchez-Ribas *et al*., 2012)
Burkina Faso	irrigation	decreased malaria prevalence	(Boudin *et al*., 1992; Sanchez-Ribas *et al*., 2012)
	irrigation and HEP	increased malaria infection and mosquito bites	(Keisar *et al*., 2004; Sanchez-Ribas *et al*., 2012)
Mali	irrigation	decreased malaria incidence	(Sogoba *et al*., 2008; Klinkenberg *et al*., 2003; Assi *et al.,* 2013; Sanchez-Ribas *et al*., 2012)
Senegal	HEP	increased vector density and malaria incidence	(Sow*et al*., 2002; Dia *et al*., 2008; Tatem *et al*., 2013)

2.4.3. Insecticide resistance and underlying mechanisms in malaria vectors

Synthetic insecticides play an essential role in malaria vector control (Raghavendra *et al*., 2011). However, the development of resistance by malaria vectors to insecticides such as DDT has become a serious challenge to malaria control effort in endemic areas (Yewhalaw *et al*., 2010; 2011b; Asale *et al*., 2014). Therefore, malaria control programmers make efforts to find other effective insecticide classes to which malaria vectors are susceptible. Following the removal of DDT as insecticide of choice in many countries, pyrethroids were considered to play a central role in malaria control strategies such as ITNs (Yewhalaw *et al*., 2010; Kawada *et al.,* 2011; Okia *et al.,* 2013).

However, recently, studies in Africa are clearly revealing that malaria vectors developed resistance against pyrethroids too. For instance, studies in East Africa revealed that *An. gambiae s.s.* and *An. arabiensis* have developed pyrethroids resistance (Yewhalaw *et al*., 2010; 2011; Balkaw *et al*., 2012; Asale *et al*., 2014; Ohashi *et al*., 2014 and Matowo *et al*., 2015). Simlarly, Jenkins *et al*. (2014), Niang*et al*. (2016) and Toé *et al*. (2014) reported pyretroid resistance development in the population of *An. gambiae s.s.* and *An. arabiensis* in West Africa.

Anopheles mosquitoes developed resistance to pyrethroid insecticides by producing/modifying detoxifying enzymes that metabolize insecticides before reaching their target sites in the nervous system (Perry *et al*., 2011). Metabolic resistance involves modification of enzymes such as Glutathion-S-transferase, Oxidases P450 and Esterases (Montella *et al*., 2007, Kawada *et al.,* 2011).

The other insecticide resistance mechanism of malaria vector is inhibiting effects of the insecticide by modifying their molecules to which it binds. Such resistance mechanism is target site mutation (Huestis *et al*., 2011). Neurotoxic insecticides have ultimate target molecules in central nervous systems of the insects. Insecticides such as carbamates and organophosphates binds to acetylcholine while DDT and pyrethroids bind to voltage gated sodium channel (VGSC).

Two point mutations at the same foci (L1014) of the VGSC linked to DDT and pyrethroids knockdown resistance in *An. gambiae s.l*. The first mutation is a substitution of leucine (TTA) by phenylalanine (TTT) at 1014 point of the gene, which is referred to West African *kdr* mutation (L1014F). The second mutation involves a replacement of leucine (TTA) by serine (TCA) in the same gene, which is East African *kdr* mutation (L1014S) (Kawada *et al*., 2011). In Ethiopia, only L1014F has been detected in populations of *An. arabiensis* examined from different parts of the country (Yewhalaw *et al*., 2010; 2012; Balkaw *et al.,* 2012).

Chapter 3.General Materials and Methods

3.1.Descriptions of study area

The study was conducted in three villages in Sekoru District, southwestern Ethiopia from January-December 2015. The total number of inhabitants of Sekoru Districtis 136, 320 with 68,469 males, 67,851 females(CSA, 2015). The area has typically one dry season (November to March) and two rainy seasons: a long rainy season from June to September, with the peak rainfall in July and August and a short rainy season from April to May. The areas receives1940.5 mm mean annual rainfall, having the heavy rains during June to August and short rains September to December. The mean annual temperature is 13°C to 20°C. Sekoru District experiences coldest and hottest temperatures in August and February respectively (Ethiopian National Meteorological Agency, unpublished report).

The local populations mainly practicemixed farming that involves both cultivation of crops and rearing of livestock. Maize *(Zea mays)*, teff (*Eragrostis tef)* and sorghum (*Sorghum bicolor*) are some of the crops cultivated during the main rainy season (June to September) and the short rainy season (April to May).

The study villages were Ayetu, Chafe and Toli (Figure 3.1).These villages have similar eco-topographical settings and inhabitants have socio-economic conditions but are with different agro-ecological settings and are 5-17km far from each other. Ayetu and Toli are located in agricultural fields and the households are engaged in subsistence agriculture of mixed crop and livestock farming systems. However, Chafe village is mainly a human settlement.

Ayetu village is located at07°49.115′N and 037°20.646′Egeographical coordinates and elevation of 1675-1680ma.s.l.The inhabitants are farmers, mainly cultivating crops, including maize, sorghum, sugarcane, vegetables and fruits on a subsistence scale and practice small-scale irrigation based agriculture with some rearing cattle. The village is located in the centre of the irrigation scheme with houses surrounding the agricultural fields in close proximity to irrigation fields.

The irrigation field was watered by water canal system. The irrigation canal system was constructed by support of world vision in collaboration with Ethiopian government (personal comm. Sekoru District Health Office). The irrigation system is supplied by diverting Chilalo River into open lined canals (ditches). This river is also used for water pump and hand watering system. It is common for these canals to be filled with standing water or for leakage and water pockets from the canals to occur. The depressions formed during watering system, ditches and canals form potential water pool during the long rains when they are not in use.

Toli has geographical coordinates of 07°45.680′ N, 037°19.297′E and an elevation of 1740m a.s.l. Toli is a village practicing cattle rearing and with rangeland and rain fed agriculture but without any irrigation practice. Maize, sorghum, millet and mixed vegetables and fruits are crops cultivated in the village.

The third study village Chafe is a village with human settlement with altitude of1700 to 1740m a.s.l., latitude 07°50.615-07°50.556′N and longitude 037°22.042-037°21.833′E.Chafe is a village clustered as urban setting and population in the area are mainly civil servants and merchants.Chafe is found 5-8km far from Ayantu and 17km far from Toli.

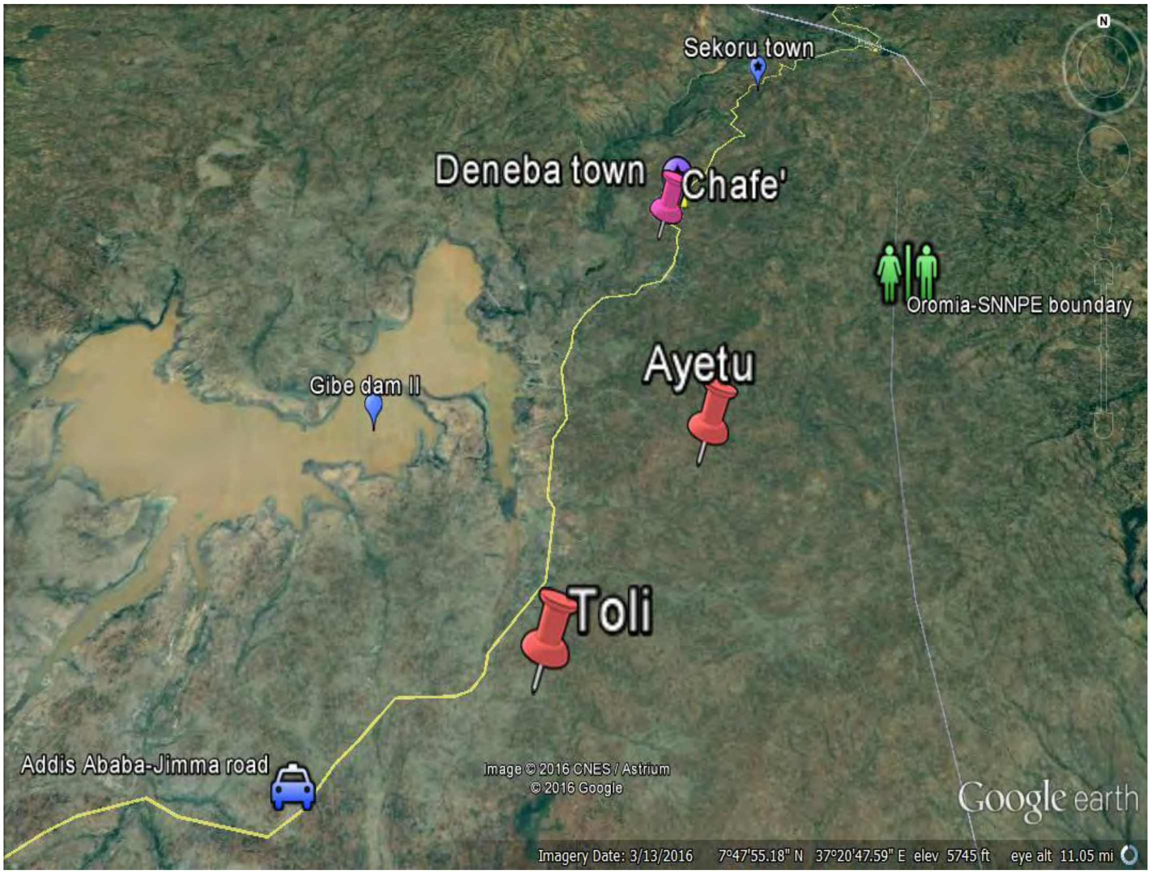

Figure 3.1: Map of the study area

3.2.Entomological data collection

3.2.1. *Anopheles* mosquito larvae collection

Larvae breeding sites such as river fringes, paddle/farming field ditches, irrigation channel, sewerage/drainage ditches, swamp, footprints and stagnant water pools were visited for larvae collection in the villages (Plate3.1). *Anopheles* larvae were sampled twice a month from June-October 2015 using standard dippers (350 ml).The larval densities were estimated by calculating the number of mosquito larvae per dip (350ml volume). The presences of larvae at high or low densities were determined by dipping. From every potential breeding site up to 10 dips were taken with a standard dipper (Satller *et al.,* 2005).

Larvae collected from each type of breeding habitats were transferred to separately labeled vials. Then collected larvae were immediately preserved in 75% ethanol. Then, the 3rd and 4th instars larvae were identified to species level using standard keys (Verrone, 1962b and Zvantsov *et al*., 2003).

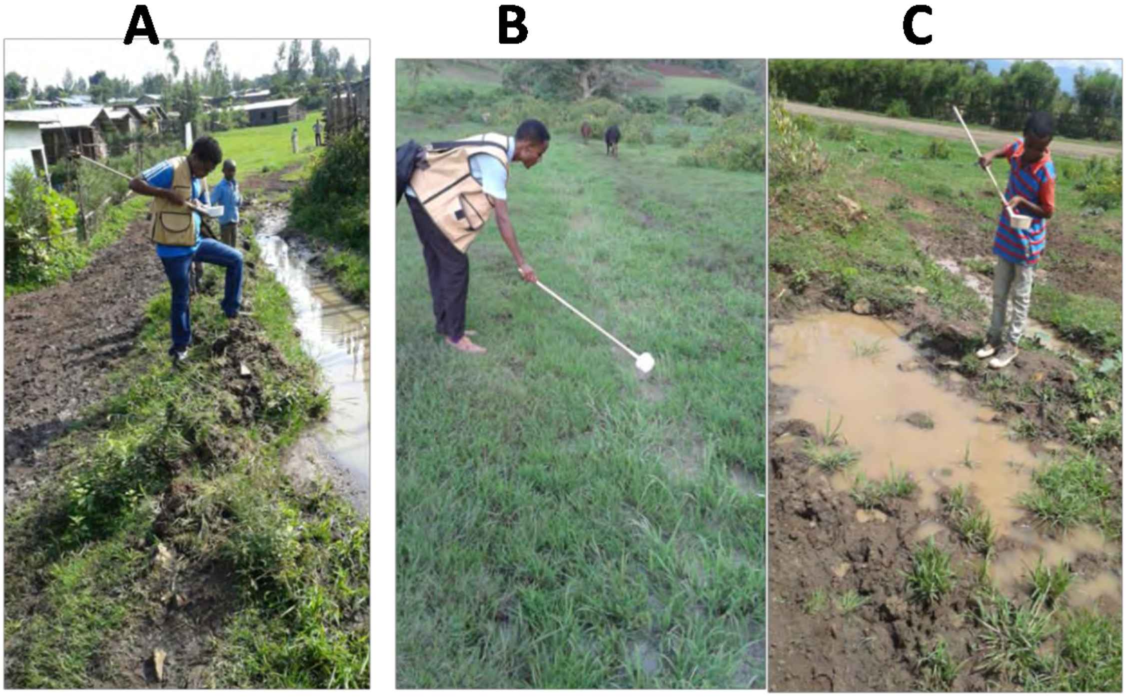

Plate 3.1: Larvae collection from different breeding sites in the study area: (A) drainage ditch, (B), Swamp and (C), stagnant water

3.2.2. Adult *Anopheles* mosquito collection

Adult *Anopheles* mosquitoes were collected using CDC light traps and Pyrethrum Spray Catch (PSC) from selected houses in each village. CDC light traps were placed indoors from 6:00PM in the evening to 06:00AM in the morning to collect endophagic female *Anopheles* mosquitoes (Plate 3.2). Furthermore, adult *Anopheles* mosquitoes were collected from outdoor by using the same technique (CDC light trap).

Additionally, indoor-resting mosquitoes were collected in the mornings (6:00 to 9:00 AM) from ten other houses by using the PSC technique. All food items, feeding utensils and animals were removed from houses, and all openings and eaves of windows and doors were closed with pieces of cloths. Finally, the floors were covered with white sheets before spraying the houses with Baygon aerosol (SC. Johnson & Son. Inc, USA). Fifteen minutes after spraying, *Anopheles* mosquitoes that had been knocked down were collected by using forceps, paper cups and torch light. The number of occupants and animals who had slept in each house the previous night was recorded.

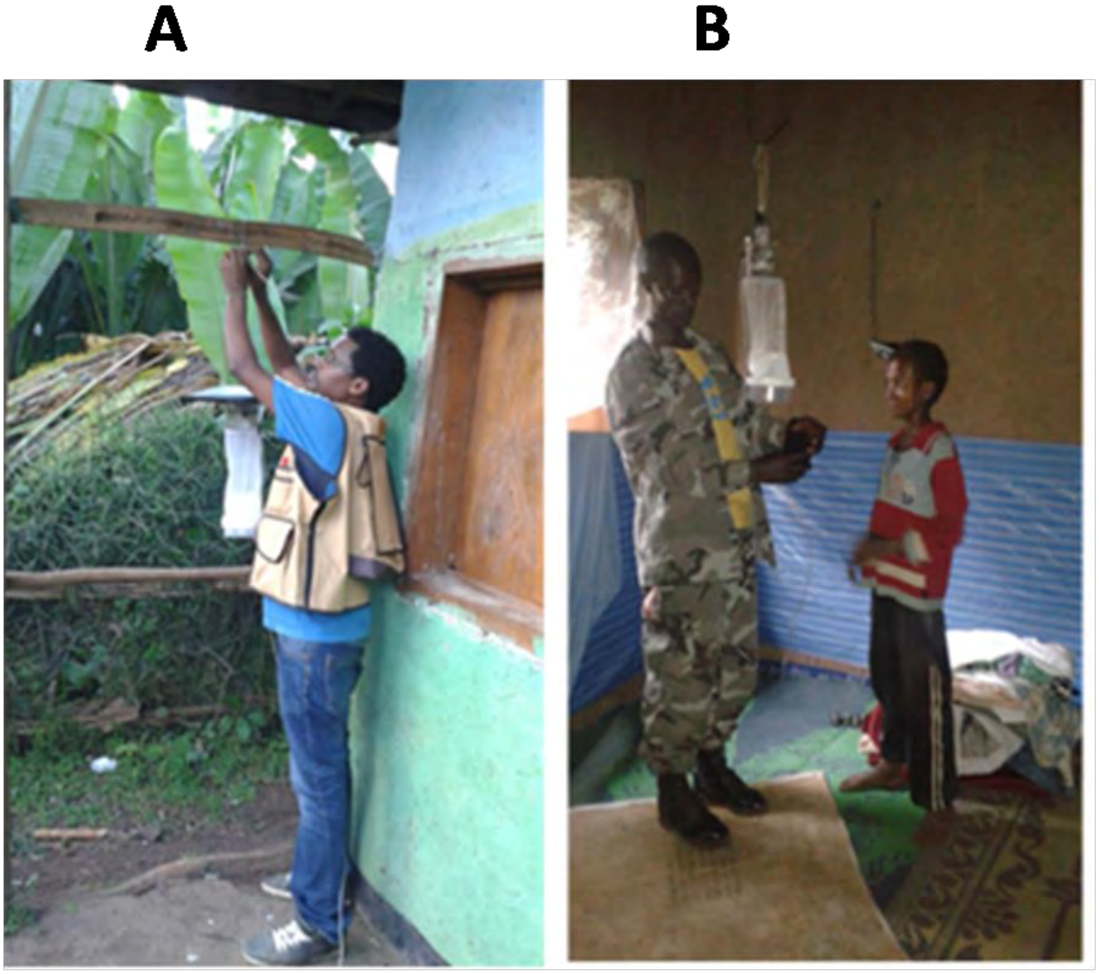

Plate3.2: Adult mosquito collection using CDC light trap

3.3. Adult *Anopheles* Mosquito Processing and Species Identification

Adult *Anopheles* mosquitoes were anesthesed by chloroform, sorted by sex, abdominal status (Plate3.3) and identified morphologically to genus and species level using taxonomic keys (Verrone 1962a; Gillies and Coetzee 1987). *Anopheles gambiae* complex were preserved with silica gel in eppendorf tubes. Then the specimens were transported to Center for Disease Control and prevention (CDC), Atlanta, Georgia, USA for species identification using PCR techniques (Wilkins *et al*., 2006). Detail procedures of molecular techniques for species identification are described in Appendix 1.

Plate3.3: Sorting, preserving and labeling *Anopheles* mosquitoes for further processes

3.4.Data Analysis

Entomological parameters such as *Anopheles* mosquito abundance, distribution, sporozoite infectivity and *kdr* levels in all agro-ecological settings were entered in to excel computer program and analyzed using IBM SPSS version 20 soft ware packages.

Mean difference in human biting rate (HBR), sporozoite rate (SR) and entomological inoculation rate (EIR) among the study villages were assessed using ANOVA. Mean mosquito density difference among the study villages were tested using Chi square (X^2). All statistic tests were performed at 0.05 significance level.

Chapter 4. Species Composition, Abundance and *Plasmodium* Infection Rate of *Anopheles* Mosquitoes in Sekoru District, Southwestern Ethiopia

4.1.Introduction

A change in either mosquito density or sporozoite rate or both affects the malaria transmission intensity. Similarly, vector population abundance and distribution is widely varied across geographical areas and seasons. In Ethiopia, in spite of considerable progress in malaria control, the disease remains an important public health problem. About 75% of the total land is malarious and 60% of the total population is at risk of infection (FMoH, 2013). The two predominant malaria parasites in Ethiopia are *P. falciparum* and *P. vivax*, accounting for about 63.7% and 36.3% of malaria cases, respectively (FMoH, 2016). Of the African malaria vector suchas *Anopheles gambiae* complex, *An. funestus* group, *An. nilli, An. pharoensis and An. Moucheti* (Krafsur, 1970; Sinka *et al.,* 2010), the first four vectors species are responsible for transmission of malaria in Ethiopia (FMoH, 2014).*Anopheles arabiensis* (member of *An. gambiae* complex) is principal malaria vector in Ethiopia and southwestern part of the country (Jaleta *et al*., 2013; Yewhalaw *et al*., 2013; 2014).

However, studies revealed that the distribution and abundance of the vectors vary according to location and season (Jaleta *et al*., 2013; Yewhalaw *et al*., 2013; Degefa *et al*., 2015). Thus, understanding of vector bionomics such as distribution and correct identification of the target species, abundance and their behavior helps to implement successful vector control measures. Hence, this study was conducted to investigate the species composition, abundance, spatiotemporal distributions of *Anopheles* species and infective bites in Sekoru District, southwestern Ethiopian.

4.2.Materials and methods

4.2.1. Descriptions of study area

The study was conducted in Sekoru district, which is located at the distance of 255 km from Addis Ababa, the capital of Ethiopia. The study was conducted in three villages having different agro-ecology. The villages include Ayetu (irrigated agriculture practicing village), Toli (rain fed agriculture-practicing village) and Chafe (human settlement). Study areas and designs are described in Chapter 3, Section 3.1.

4.2.2. Entomological data collection

Adult *Anopheles* mosquitoes were collected using CDC light traps and Pyrethrum Spray Catch (PSC) from selected houses in each village. The data collection was conducted monthly in three study sites. CDC light trap collections were conducted both indoor and outdoor in five houses in each villages (Chapter 3, Section 3.2.).

4.2.3. *Anopheles* mosquito species identification

Mosquitoes belonging to the genus *Anopheles* were identified from non-*Anopheles* using their wings and palps (Verrone, 1962a). *Anopheles* mosquito species were identified morphologically using taxonomic keys (Gillies and Coetzee, 1987).

Molecular identification of *Anopheles gambiae* complex

Identification of *Anopheles gambiae* complex sibling species were conducted using PCR techniques (Collins *et al*., 1987; Wilkins *et al*., 2006) in Molecular Laboratory of Entomology at CDC, Atlanta, Georgia, USA.

DNA extraction and purification: Genomic deoxyribonucleic acids (DNAs) were extracted individually as described by Collins *et al*. (1987) for identification of *An. gambiae* complex species by molecular techniques. Either full or parts of the mosquitoes were ground 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)with a sterile blue Konte's pestle in centrifuge tubes until all parts remain unidentifiable. The grinding products were heated for 30 minutes at 65°C. After an overnight precipitation in 100%ethanol and washed in 70% ethanol, DNA pellets were dissolved in 100µl sterilized water.

DNA amplification: the ribosomal region targeting specific SNPs for the *Anopheles gambiae* complex was amplified in a multiplex reaction as described by Wilkins *et al*., (2006). PCR reaction was carried out using AccuStartII PCR Supermix (Quanta Biosciences)(Pleasanton, California, USA) in a final 12µl reaction mix, containing 0.3µl of each primer in a 25pmol concentration and 0.5µl of DNA. PCR conditions were 95°C for 4' followed by 34 cycles of 95°Cfor 30"; 60°C /30" and 72°C for 30" and a final elongation step at 72°C for 5'. PCR products were visualized with UV light in 2% agarose gels stained with gelred. All reactions included specific controls from the *Anopheles gambiae* complex and a negative control. Details of PCR technique procedures are indicated in Appendix 1.

4.2.4. Circumsporozoite Protein Detection

The head-thorax regions of 532female *Anopheles* mosquitoes were checked for circum-sporozoite protein (CSP) by Enzyme Linked Immuno-Sorbent Assay (ELISA)techniques as illustrated by Wirtz*et al.,*(1992) in molecular laboratory of Entomology at CDC, Atlanta, Georgia, USA. The head-thorax region of each female *Anopheles* mosquito was ground by electric-motor operated pestle using 100µl gridding buffer (BB-NP40) (Plate 4.1).

All reactions included specific positive controls of *Plasmodium* with CSP antigen for *P. falciparum*, *P. vivax*210 and *P.viva*247and negative controls. Spectra MAX 340 plate reader with the help of SoftMax 5.4.5 computer program was used to detect the presence of *Plasmodium* CSP. Details of ELISA procedures are indicated in Appendix 2.

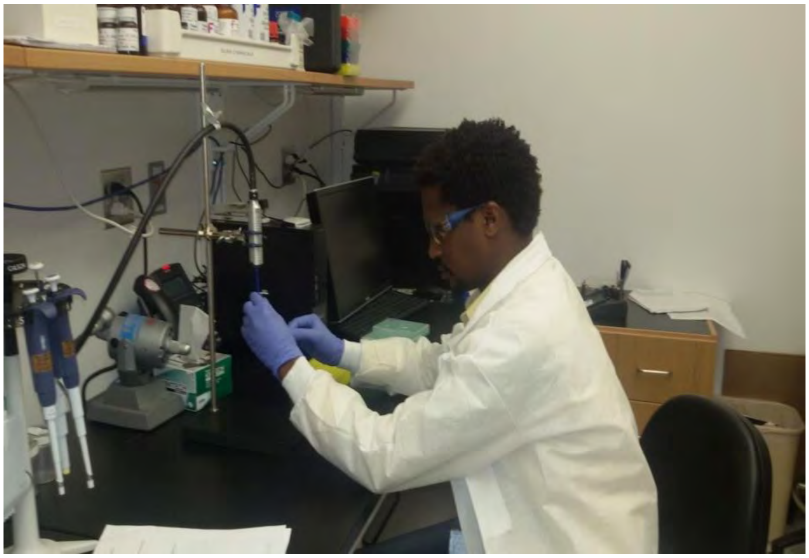

Plate 4.1: Grinding head-thorax region of female *Anopheles* mosquitoes using eclectic motor pestle in Molecular Entomology Laboratory at CDC, Atlanta, Georgia, USA

4.2.5. Statistical Analysis

Anopheles mosquito Data such as species composition, density, biting rate, sporozoite rate and entomological inoculation rates were entered in to excel computer program and analyzed using SPSS version 20 (SPSS, Inc., Chicago, IL).*Anopheles* mosquito density, species composition and spatio-temporal distribution in relation to study sites were analyzed using chi-square (X^2 using IBM SPSS version 20 (SPSS, Inc., Chicago, IL) statistical soft ware package). Human biting rate was calculated as the number of CDC light trap catches mosquitoes/person/night as described by Lines *et al.,* (1991). The sporozoite rates were estimated as the ratio of sporozoite ELISA positive mosquitoes to all mosquitoes tested for CSP. The Entomological Inoculation Rate (EIR) was calculated by multiplying the mean number of human biting rate (mosquito bites/person/night) by the proportion of sporozoite positive mosquitoes (Drakeley*et al*., 2003; World Health Organizations, 2013).All statistic tests were performed at 0.05significance level.

4.3.Results

4.3.1. Species composition and abundance of *Anopheles* mosquito

A total of 1,546 *Anopheles* adult female mosquitoes were collected from three villages by light traps and PSC collection techniques (Table 4.1). Eight species of *Anopheles* mosquitoes (*Anopheles arabiensis,An. demeilloni*, *An. squamosus, An. garnhami, An. christyi, An. pretoriensis, An. longipalpis* and *An. marshallii)* were identified from the three villages, of which *An.* was the predominant (46.2%; n=715). As shown in Table 4.1, higher numbers of *Anopheles arabiensis* mosquitoes were collected by CDC light traps (92%; n=1421) compared to Pyrethrum Spray Catches (PSC) (8%; n=125).

Table 4.1: Species composition and number of *Anopheles* mosquitoes collected and identified in the study area (January-December 2015)

	Collection methods							
	CDC Light Traps							
	Indoor collections		Outdoor collections		Pyrethrum Spray Catches		Total	
Anopheles species	No.	(%)	No.	%	No.	%	No.	%
An. arabiensis	242	48.11	419	46	54	43.2	715	46.2
An. squamosus	42	8.34	74	8	6	4.8	122	8
An. marshallii	24	5	32	3.5	11	8.8	67	4.3
An. longipalpis	21	4.2	30	3.3	3	2.4	54	3.5
An. pretoriensis	13	2.6	25	2.7	6	4.8	44	3
An. christyi	18	3.6	35	4	9	7.2	62	4
An. demeilloni	99	20	227	24.7	25	20	351	22.7
An. garnhami	44	9	76	8.3	11	8.8	131	8.5
Total	503	100	918	100	125	100	1546	100

4.3.2. Spatio-temporal distribution of *Anopheles* mosquitoes in different agro-ecological settings

The total numbers of *Anopheles* mosquitoes collected and identified in the three study villages were presented in Figure 4.1. 1019 female *Anopheles* mosquitoes (65.9%) were collected from a village practicing small-scale irrigation (Ayetu), while, 432 *Anopheles* mosquitoes (27.9%)were collected from rain fed agriculture village (Toli) and 95 female *Anopheles* mosquitoes (6.1%) were collected and identified in the human settlement villages (Chafe), respectively. The total numbers of *Anopheles* mosquitoes collected in irrigated village were significantly higher than mosquito collected from rain fed agriculture practicing village and human settlement ($X^2 = 8.543$, df = 2, $P < 0.001$).

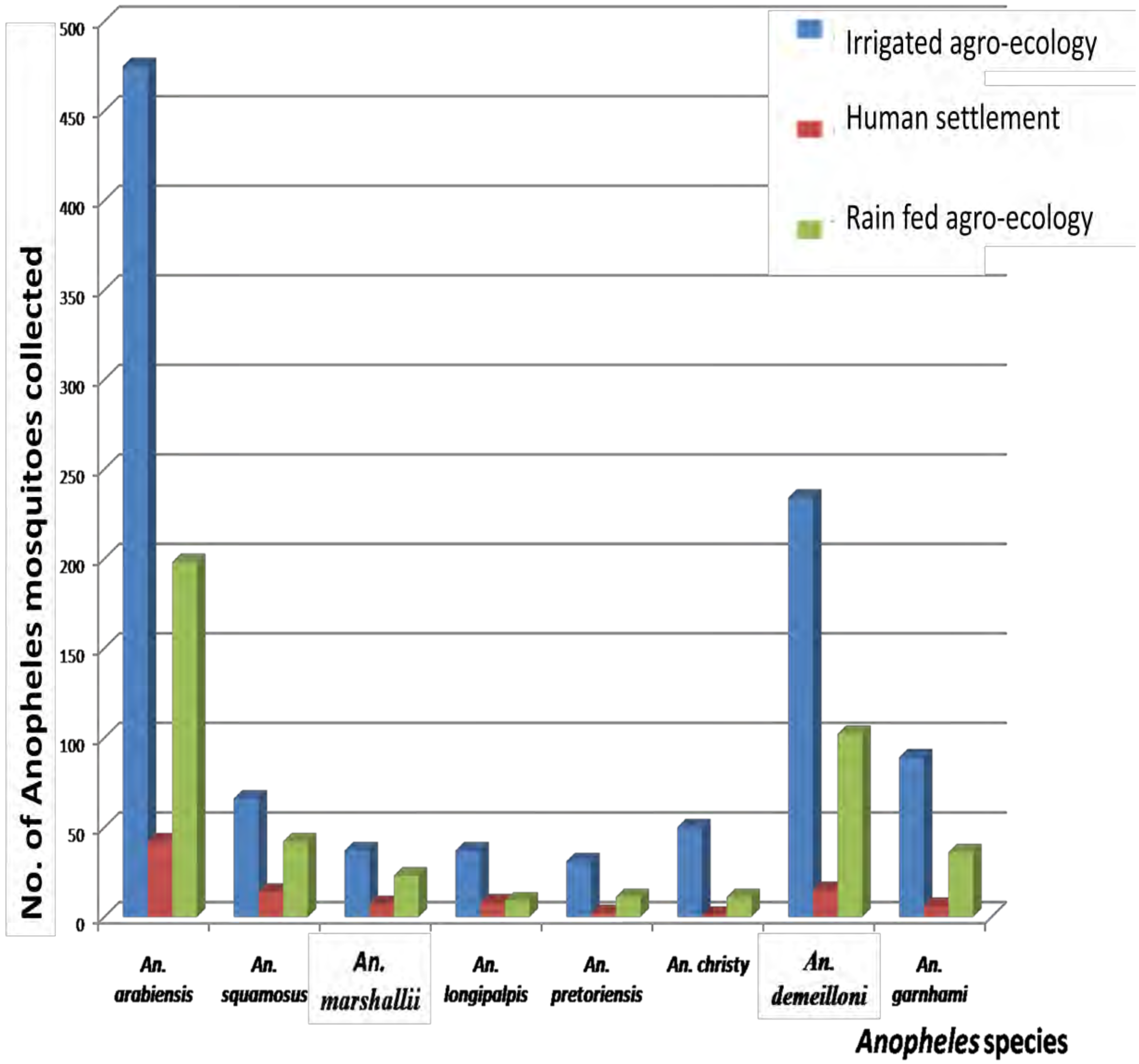

Figure 4.1: Species and Number of *Anopheles* mosquitoes collected from three villages with different agro-ecological settings

The total numbers of *Anopheles* mosquitoes collected and identified in the three study villages in different months are presented in Figure 4.2.

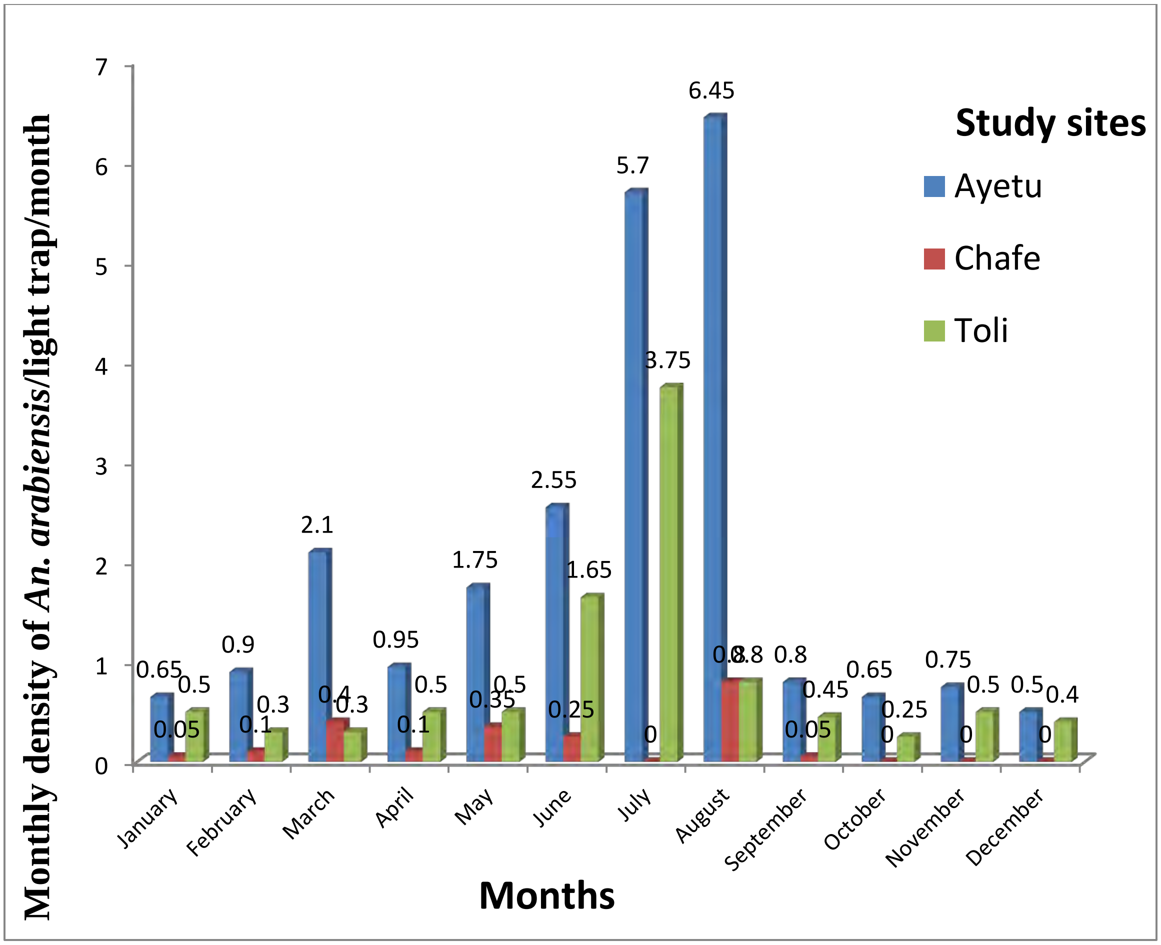

Figure 4.2: Number of *Anopheles* mosquitoes collected from villages with different agro-ecological settings in different months in the study area

Highest *Anopheles* mosquito numbers (25.5%; n=394) was recorded in August with mean density of 6.57 mosquitoes/light trap while least mosquito abundance (3.5%;n=54)was recorded in February with mean density of 0.9 mosquitoes/light trap. After August (peak mosquito catches), monthly mosquito catches reduced, thereafter until short rainy season to the wet season during which an increase in the *Anopheles* population was observed (Figure 4.2).

Three hundred fifty two specimens (49.2%) of the total *An. gambiae* complex were further identified to sibling species by PCR techniques, of which 316 (90%) were successfully amplified and identified. However, 36 (10%) specimens were not amplified. According to the PCR analysis, the entire group of successfully amplified *An. gambiae* s.l. in this study was *An. arabiensis*. Hence, all of the *An. gambiae* s.l. species collected in this study were determined as *An. arabiensis*.

Out of 715 female *An. arabiensis* collected 661 (92.4%) were collected by CDC light traps, while 54 (7.6%) were collected by PSC collections. As indicated in Appendix 3, highest number, (n=475; 66.43%) of *An. arabiensis* was collected from Ayetu village (irrigated agro-ecosystem), followed by Toli (rain fed agro-ecosystem) (n=198; 27.7%). However, lowest number of *An. arabiensis* (n=42; 5.87%) was observed in Chafe (human settlement). The abundance and distribution of *Anopheles* mosquitoes were significantly associated to agro-ecological settings (X^2=11.15, df=2, P=0.003).

Anopheles arabiensis distributions were associated with months in each village. Generally, peak *An. arabiensis* (n=189; 26.4%) were recorded in July, while lowest (n=18; 2.5%) *An. arabiensis* were recorded in October. The distribution of *An. arabiensis* were significantly associated with months (X^2 =33.15, df=11, P<0.001).

In addition to *An. arabiensis*, the predominant *Anopheles* mosquito species collected and identified in the study area are indicated in Figure 4.4.*Anopheles demeilloni, An. garnhami* and *An. squamosus* were the predominant species accounting for 351 (22.7%), 131(8.5%)and 122(7.9%), respectively.

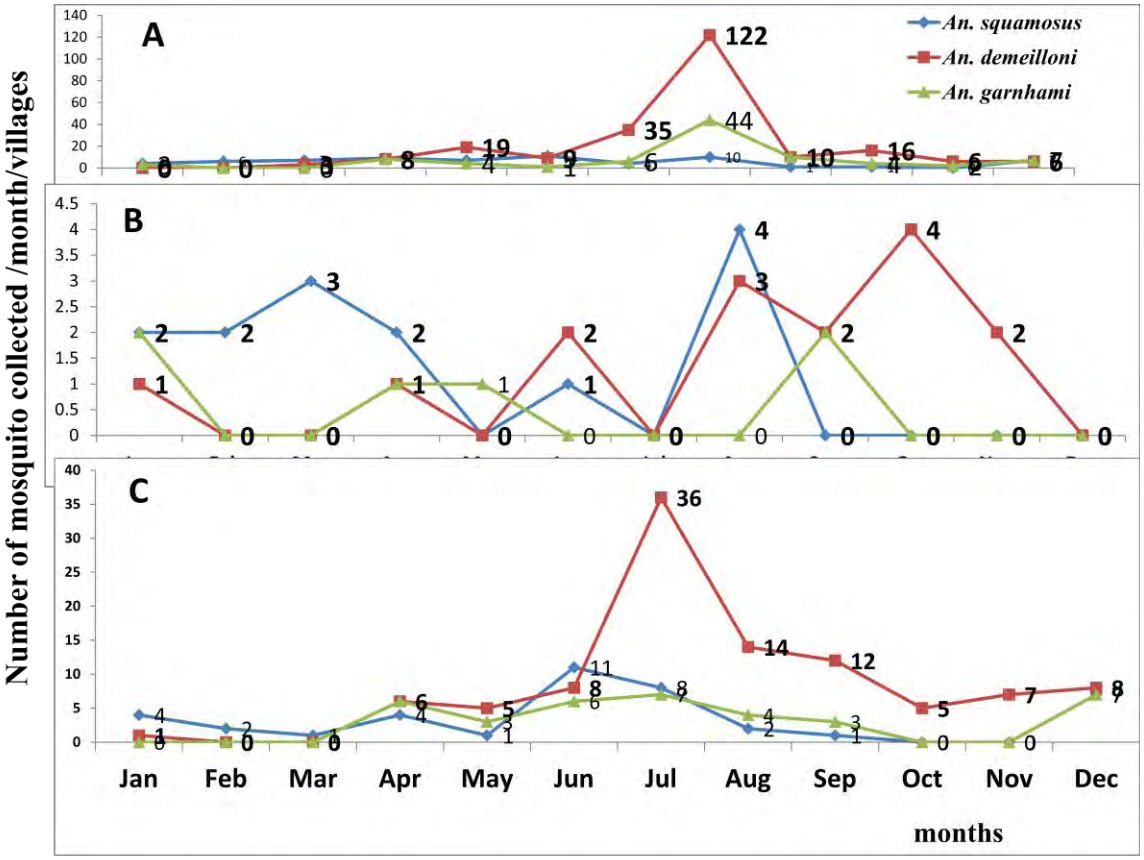

Figure 4.4: *Anopheles demeilloni*, *An. squamosus* and *An. garnhami* collected in villages with different agro-ecological settings: (A) irrigated agro-ecology, (B) human settlement and (C) rain fed agro-ecology

4.3.3. Density of Host seeking *Anopheles* mosquitoes

Monthly indoor and outdoor hosts seeking *Anopheles* mosquitoes in all study villages are presented in Figure 4.5. Outdoor host seeking mosquito density was higher (2.49 mosquitoes/CDC light trap) than indoor host seeking mosquito density (1.46 mosquitoes/CDC light trap). Statistically, there was no significant difference in human biting density of *Anopheles* mosquitoes among villages (P>0.05).

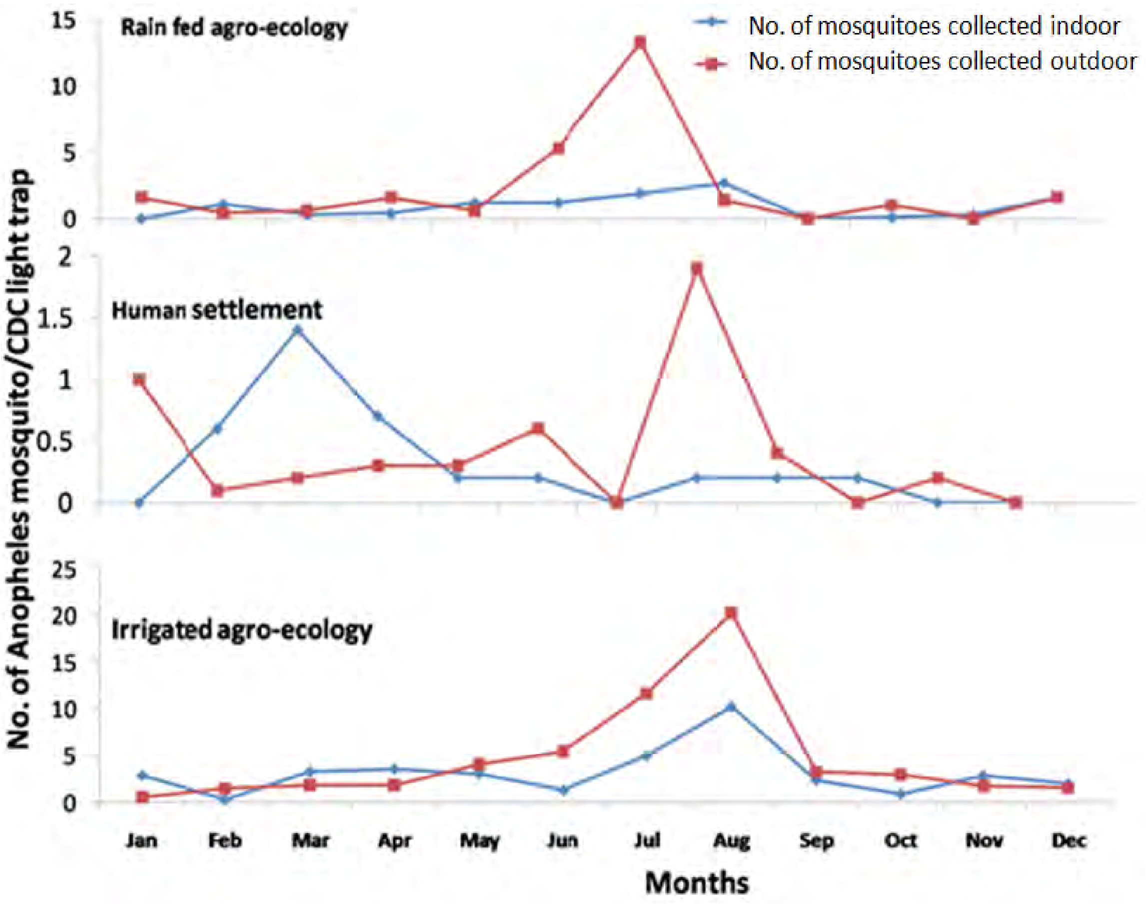

Figure 4.5: Monthly indoor and outdoor *Anopheles* mosquito densities in three agro-ecological settings in the study area during the study period

4.3.4. Biting Rate, Sporozoite Rates, Entomological Inoculation Rate

Monthly human biting rates in the three villages are indicated in Figure 4.6. An overall estimated biting rate was 11.87 *An. arabiensis* bites/person/month. Human-biting rate of *An. arabiensis* was associated with agro-ecological settings. Monthly human biting rates in a village with small-scale irrigation ranging from 0.05 to 0.65 was higher than biting rate in a village with rain fed agriculture ranging from 0 to 0.5 and human settlement village ranging from 0 to 0.08.In all villages, biting rate was seasonally varied increasing from April to August followed by gradual decline reaching dip values during the dry season.

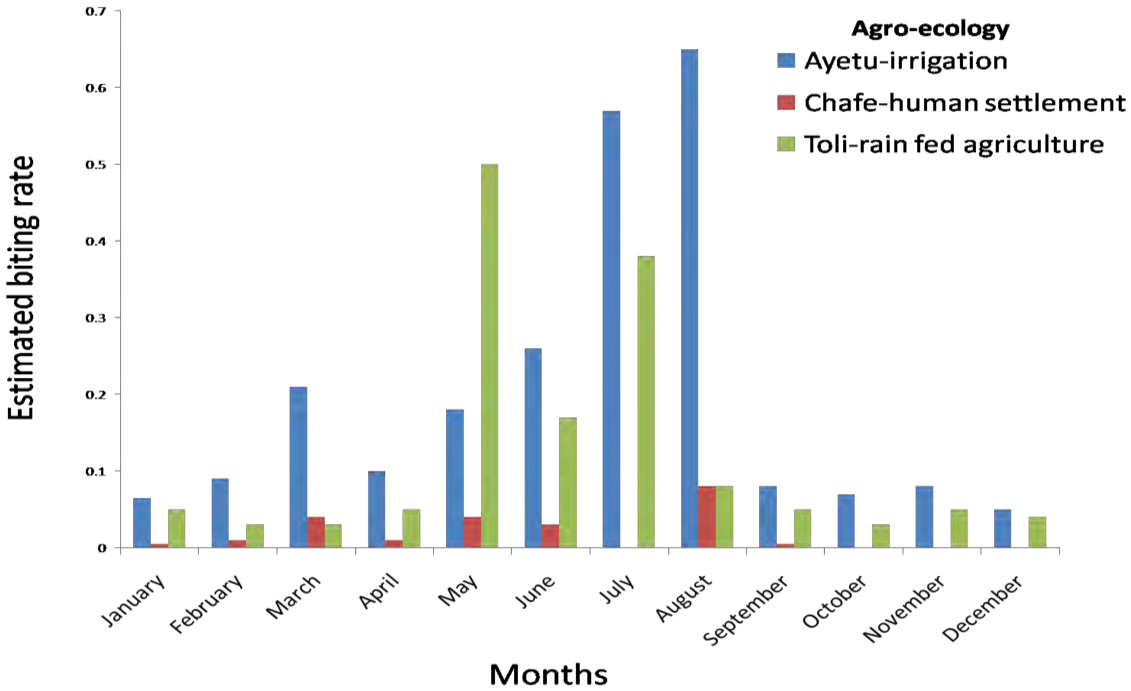

Figure 4.6: Overall monthly density and estimated human biting rate by *Anopheles* mosquitoes in three different agro-ecosystems in the study area

The sporozoite rate for *An. arabiensis* collected and tested from the study villages is shown in Table 4.2. Out of 532 *An. arabiensis* examined for *Plasmodium* Circum Sporozoite-Proteins (CSP), five specimens were positive for the antigen. Therefore, overall sporozoite rate for *An. arabiensis* was 0.94%. Of those, 60% (n=3) were positive for *P. falciparum* and 40% (n=2) were positive for *P. vivax*210. None of the mosquitoes tested was positive for *P. vivax247* antigen. *Plasmodium* sporozoite rates for mosquitoes from indoor LTCs and outdoor LTCs were 20% (n=1) and 80% (n=4), respectively.

As shown in Table 4.2, the sporozoite rates for *An. arabiensis* were varied among the three villages. In a village practicing irrigation-based agriculture (Ayetu), sporozoite positive specimens were recorded during January and August. In Toli, a village conducting conventional agricultural (rain fed agriculture), sporozoite positive malaria vectors were detected during August. In contrast, no infective *An. arabiensis* was recorded from human settlement village (Chafe). Generally, sporozoite rates for *An. arabiensis* in both agriculture-practicing villages were summed up for 0.94 infective bites.

Table 4.2: Number and distribution of sporozoite positive *Anopheles arabiensis* in three villages practicing different agro-ecological settings in the study area: Ayetu-Irrigated village, Toli-rain fed village and Chafe- human settlement

Study village	No. mosquitoes tested	*Plasmodium* species					
		P. *falciparum*		*P.vivax210*		*P. vivax247*	
		No. +ve	Sporozoite rate (%)	No. +ve	Sporozoite rate (%)	No. +ve	Sporozoite rate (%)
Ayetu	328	3	0.91	0	0	0	0
Toli	165	0	0	2	1.21	0	0
Chafe	39	0	0	0	0	0	0
Total	532	3	0.6	2	0.4	0	0

The Entomological Inoculation Rates in the study area is presented in Table 4.3. Annual Entomological Inoculation Rate (EIR) for *An. arabiensis* in the study areas were 11.2 infective bites per year.EIR for *P. falciparum* and *P. vivax* were 3.34 and 2 infective bites/person/month, respectively. EIR in village practicing irrigation, rain fed agriculture and human settlement were10.8, 5.99 and zero infective bites/person/year, respectively.

Table 4.3: Biting rate, sporozoite rate and EIR in the three villages with different agro-ecological settings in the study area (January-December, 2015)

Agro-ecology	Entomological parameters		
	Biting rate	Sporozoite rate (%)	EIR (infective bite/year)
Irrigation	11.87	0.91	10.8
Rain fed agriculture	4.95	1.21	5.99
Human settlement	1.05	0	0
Total	11.9	0.94	11.2

Note: Over all human biting rate was calculated as "total mosquitoes (715)/no. of catches (720/2days=360)/average number occupants during collection (5) multiplied by number of days in the month (30)" (Drakeley*et al*., 2003).

4.4.Discussion and conclusions

In the present study, eight species of *Anopheles* mosquito were identified in three different agro-ecological settings in Sekoru District, southwestern Ethiopia. Of all *Anopheles* species identified, *An. arabiensis* were found to be the predominant *Anopheles* species (46.2%; n=715). This result was similar with previous studies in different parts of Ethiopia: in central (Kibret *et al*., 2010), northern (Dejene *et al*., 2012), western (Jaleta *et al*., 2013),southern (Massebo., *et al*., 2013),southwestern (Yewhalaw *et al*., 2014; Degefa *et al*., 2015) and south-central Ethiopia (Kenea *et al*., 2016). Furthermore, this investigation was similar with results in Kenya (Amek *et al*., 2012) and Tanzania(Kigadye*et al*., 2010).

Anopheles mosquitoes were collected from outdoor and indoor with higher outdoor host seeking densities (n=918; 64.6%). This investigation agrees with reports of Degefa *et al*., (2015) and Kenea *et al*., (2016) in Ethiopia. This result is an indicator for behavioral changes of *Anopheles* mosquitoes against vector control interventions. This study determined that malaria vectors were active outside house. On the other hands, malaria vector intervention strategies such as IRS and ITNs mainly targeted indoor active *Anopheles* mosquitoes (USAID, 2013; FMoH, 2016). Thus, it is possible to deduce that *Anopheles* mosquitoes are responding to indoor-targeted vector control and prevention strategies in Ethiopia.

In this study, *Anopheles* mosquito densities were determined invillages practicing different agro-ecosystems in Sekoru District.1019 *Anopheles* mosquitoes (65.9%) were recorded from village conducting small-scale irrigation scheme, while 432 were collected and identified in a village practicing rain fed agriculture (27.9%), and 95*Anopheles* mosquitoes were collected and identified from human settlement(6.1%).This finding was in line with previous reports in Ethiopia (Kibret *et al*., 2010; 2014;Dejenie; *et al*., 2012; Jaleta *et al*., 2013),in Kenya (Mutoro *et a*., 2004; Mwangangi *et al*., 2006; 2010; Jacob*et al*., 2007; Muturi *et al*., 2008; 2013)and in Sudan(Keisar *et al*., 2005; Ageep *et al*., 2009; Eltayeb *et al*., 2015). It could be due to configuration of suitable ecological and climatic conditions for breeding and survival of malaria vector mosquitoes because of environmental changes and habitat manipulation for surface irrigation.

Anopheles mosquito distributions were found to be associated with collection seasons. The highest numbers of *Anopheles* mosquitoes were recorded during wet months (May to August). The peak *Anopheles* mosquitoes (n=394; 25.5%) were collected in August declining starting from September to throughout the dry months. This result agrees with reports of Jaleta *et al*. (2013), Yewhalaw *et al*., (2013) and Taye*et al*., (2016) in Ethiopia.

The reduction of *Anopheles* mosquito abundance starting from September could be associated with national strategic plan and implementation of malaria control interventions. In Sekoru District, the annual malaria vector control interventions, particularly, IRS was implemented from August 20-30/2015 (personal observation). Furthermore, meteorological factors and temporary breeding habitats may have affected seasonal distribution of *Anopheles* mosquitoes (Koenraadt and Takken, 2003; Ye *et al*., 2007 and William and Pinto, 2012). Hence, shortage/absence of aquatic habitats during dry months may have affected *Anopheles* mosquito seasonal distribution and abundance.

In the current study, an estimated EIR from all study villages was 11.2 infective bites/person/year. This result was lower than reports by Degefa *et al*., (2015) and Messobo *et al*., (2013) which was 15.23 and 17.1 infective bites, respectively. This variation could be due to land use systems and ecological factors. In the current study, adult mosquito collection was done only by PSCs and CDC light traps that collection methods could also be a factor for the different EIR result observed. In this study, EIR was calculated only from CDC Light trap catches. Overall PSCs catches were small in number 6% and no *An. arabiensis* from PSCs catches were detected to carry any *Plasmodium* sporozoite.

The highest EIR (10.8) was *An. arabiensis* tested from irrigated village compared to (5.99) EIR from *An. arabiensis* tested in rain fed agriculture practicing village and (zero) EIR from *An. arabiensis* tested in a human settlement. Three out of five (60%) *P. falciparum* sporozoite positive mosquitoes were recorded and all of them were from mosquitoes collected from irrigated village. Two (40%) out of all sporozoite positive mosquitoes were positive for *P.vivax*210 and both of them were from rain-fed agriculture practicing village.

Generally, this finding indicates higher transmission intensity of malaria in irrigated village as compared to non-irrigated villages. Such difference in infectivity rates at local level could be due to vector density and host exposures to infective bites.

In conclusion, the distribution and abundance of *Anopheles* species was associated with season. *Anopheles arabiensis* was the dominant malaria vector species with wide distribution in Sekoru District, southwestern Ethiopia in the current study. Infective malaria vectors distributions and abundances were associated with study sites. Malaria transmission intensity in the study areas was fluctuated over space and time, in which high transmission intensity was detected in a few hot spots such as agriculture practicing areas.

Chapter 5. Impact of agro-ecological settings on Abundance and Distribution of *Anopheles* Mosquito Larvae in Sekoru District, Southwestern Ethiopia

5.1.Introduction

In several parts of sub-Saharan African countries where more than 70% of the residents live in rural and rely on agriculture, the main concern is increasing agricultural yield. Raising irrigation increases agricultural productivities and enhance the self-sufficiency of rapidly increasing human population in the region (Keiser *et al*., 2004). Accordingly, in Africa, irrigation probably increases agricultural outputs and many governmental and nongovernmental organizations considered irrigation schemes as critical to future economic development.

Yet, according to the report of World Health Organizations, (2015), 88% of estimated annual global deaths (660,000) caused by malaria are in Africa. Agricultural practices can play a central role in malaria transmission (Kanzaria, 2003; Keiser *et al*, 2005). The main association between agriculture and malaria is irrigation practices. Availability and accessibility of suitable breeding habitats are among main factors for successful reproduction of *Anopheles* mosquito. Therefore, malaria vector breeding habitat affects vector density and consequently, malaria transmission and burden in a given geographical location.

Irrigation could create conducive environments to *Anopheles* mosquitoes. According to studies in sub-Saharan African countries, irrigation increases malaria risk and vector density. For example, in Ethiopia, incidence of the infection and density of the vector is influenced by irrigation. Malaria prevalence and the risk of transmission by *An. arabiensis* were higher in irrigated sugarcane agro-ecosystem as compared to non-irrigated agro-ecosystems (Jaleta *et al*. 2013). Similarly, Kibret *et al*.,(2010) reported higher *Anopheles* mosquito density and malaria prevalence in irrigated villages as compared to the non-irrigated villages. Furthermore, in Zimbabwe, operation of irrigation scheme was reported to be cause for increased malaria prevalence (Boelee *et al.*, 2002).

In general, irrigated agro-ecosystems favor *Anopheles* reproduction and increase vector abundance due to increased density of aquatic stages consequently enhancing human-vector contacts (Ijumba and Lindsay,2001). This is attributable to establishment of new and suitable breeding sites and micro-climatic conditions for reproduction due to habitat manipulations for irrigation. For instance, surface irrigation creates temporary shallow water bodies, which form ideal breeding sites for malaria vectors. Among *Anopheles* species described globally as potential vectors, several breed predominantly in temporary habitats such as irrigation cannels (Petrarca *et al.,* 2000).

Hence, larvae target malaria vector control strategies should be designed and established based on anthropogenic activities of local community, ecological settings, and habitat productivity and oviposition behaviors of particular species. Factors that determine larval density and distribution in part affect adult *Anopheles* vector population dynamics and hence malaria transmission. The objective of this study was to investigate the spatial distribution and abundance of *Anopheles* mosquito larvae in association with habitat types and agro-ecological settings in Sekoru District, southwestern Ethiopia.

5.2.Materials and methods

5.2.1. Study area descriptions

The study was conducted in three villages such as Chafe, Ayetu and Toli in Sekoru district, southwestern Ethiopia. *Anopheles* larvae were collected from different breeding habitats twice a month in each village from June to October 2015. The larvae were collected by using standard dipper (350ml). Ten dips were taken from each breeding habitat. The details of larvae collection are described in Chapter 3, section 3.1).

5.2.2. Collections, processing and identification of *Anopheles* larvae

Anopheles mosquito larvae were collected from various breeding habitats using standard dippers (350 ml). Collections of larvae were carried out once a month from June-October 2015. Different breeding habitat types were visited and examined for *Anopheles* larvae species productivity. *Anopheles* larvae were collected from swamps, irrigation cannels, animal footprints, paddles or farm ditches, sewerage ditches, river fringes and taps temporary pools by using standard sampling techniques.

Larvae were counted/estimated and transferred to separately labeled vials and preserved in 75% ethanol for identification. Mosquito larvae were identified morphologically by dissecting microscope using standard identification keys (Verrone 1962b; Zvantsov *et al*., 2003). Details of larvae collection, processing and identifications are described in Chapter 3, section 3.2.

5.2.3. Data analysis

Data were entered into excel computer program, checked for completeness and analyzed by using IBM®SPSS® statistics 20 (SPSS Inc., Chicago, IL, USA). Mean larval density difference among the study villages were tested using Chi square (X^2). All statistic tests were performed at 0.05significance level. Larval density in all breeding habitats and study villages were calculated as *Anopheles* mosquito larvae per dip (Sattler *et al*., 2005).

5.3.Results

5.3.1. Species composition and abundance of *Anopheles* mosquito larvae

A total of 2,665*Anopheles*larvae were collected from different breeding sites during the study period. As shown in Figure5.1, five *Anopheles* mosquito species larvae were identified from different breeding sites. The *Anopheles* species larvae collected included *An. gambiae s.l., An. deimilloni, An. garnhami, An. squamosus*and *An. funestus*. Of all *Anopheles* species collected, *An. gambiae s.l.* and *An. demeiloni* were predominant accounting for 1,531 (57.4%) and 788 (29.5%), respectively.

Anopheles gambiae s.l. was found in all study villages and breeding habitats except in paddle. The highest number of *An. gambiae s.l.* was found in Ayetu (n=936; (61.1%) with mean density of 1.34 larvae/dip. Nevertheless, in Toli and Chafe, 413 (27%) and 182 (11.9%)*An. gambiae* s.l. larvae with mean density of 0.59 and 0.26 larvae/dip were recorded, respectively.

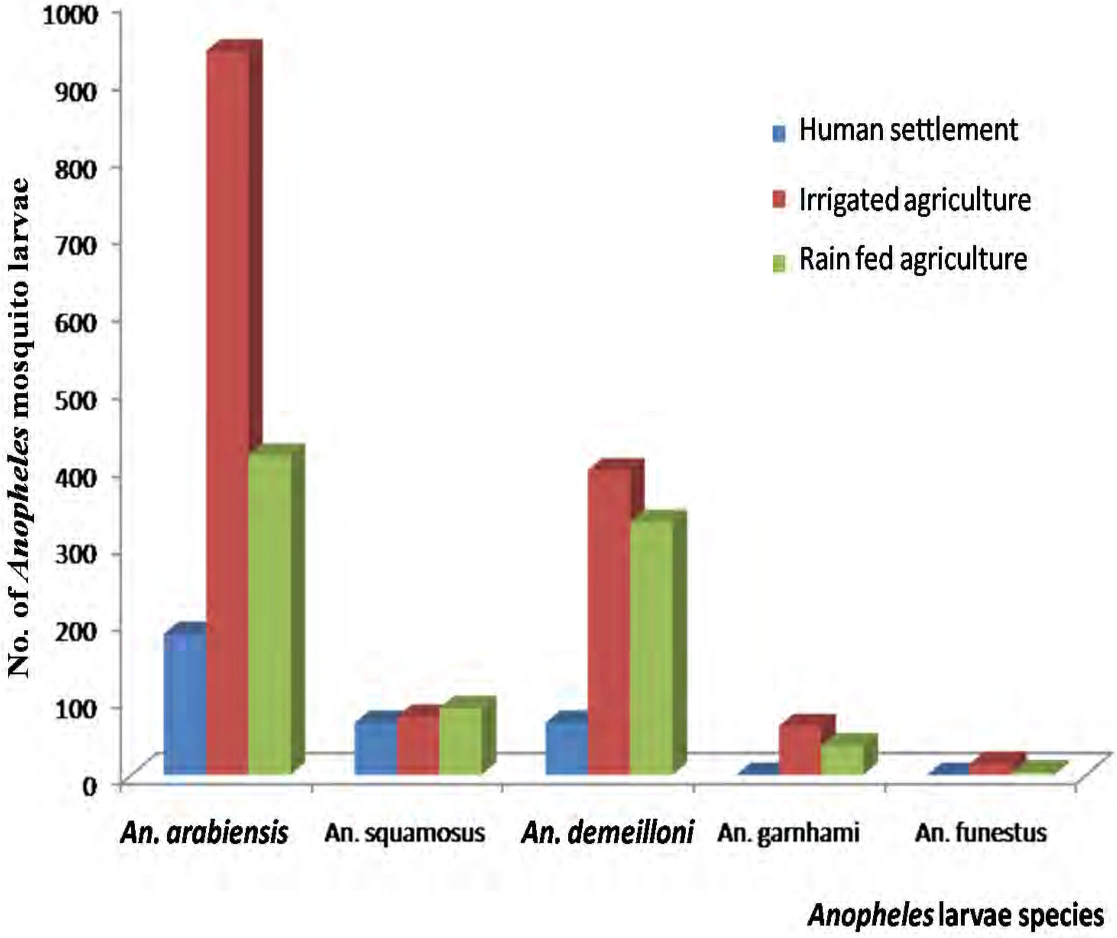

Figure 5.1: Species and number of *Anopheles* mosquito larvae identified from three different agro-ecological settings in the study area (June-October 2015)

5.3.2. Spatio-temporal distribution of *Anopheles* mosquito larvae

As shown in Figure 5.2, higher number of *Anopheles* larvae (n=1482; 55.6%) were collected from Ayetu (village with irrigated agro-ecology) with mean density of 2.12 *Anopheles* larvae/dip. In Toli (a village with rain fed agro-ecology) and Chafe (a village without agriculture or human settlement) (n= 867; 32.55%)and (n=316; 11.85%) larvae were collected with mean density of 1.24 and 0.45 larvae/dip, respectively. The associations between villages with different agro-ecological settings and larval density were statistically significant (X^2=84.76, df=2, P<0.01).

Highest number of *Anopheles* mosquito larvae were collected in August (n=890; 33.3%) followed by July (n=746; 28%) with monthly mean density of 2.12 and 1.77 larvae/dip, respectively. However, the lowest *Anopheles* larvae were recorded in October (n=150; 5.6%) with monthly mean density of 0.35 larvae/dip.

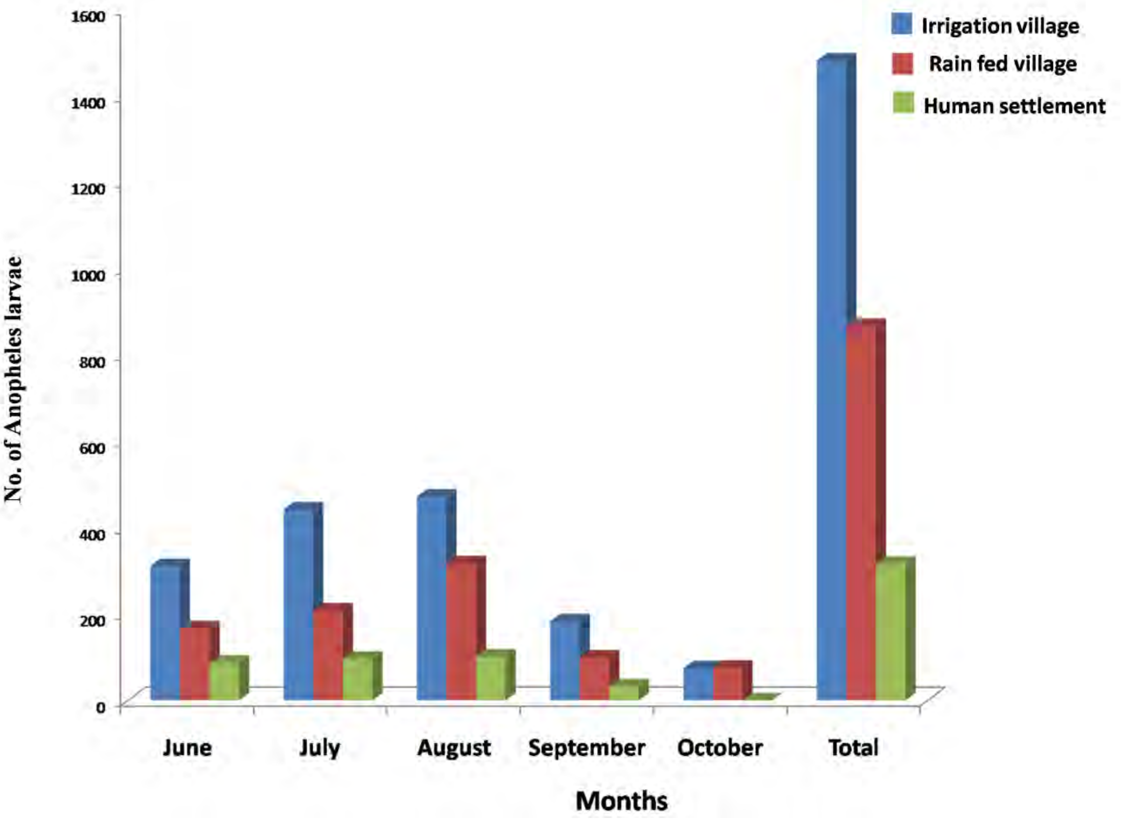

Figure 5.2: *Anopheles* mosquito larvae collected from different agro-ecology during and immediately after the long rainy season: Irrigated (Ayetu), human settlement (Chafe) and rain fed agriculture (Toli)

5.3.3. Breeding site types and the number of larvae collected

Anopheles mosquito larvae collected and identified from different breeding sites in the study are shown Figure 5.3. *Anopheles* larvae were collected from river fringes, paddle (farm field ditches), irrigation channel, sewerage (drainage ditches), swamp, animal footprints and stagnant water /pools. The most productive habitats were swamps (n=665; 24.9%) with larvae/dip followed by sewerage ditches (n=567; 21.2%), while paddles were detected to be least productive (n=20; 0.7%) for *Anopheles* mosquito larvae with mean density of 2.22, 1.89 and 0.07 larvae/dip, respectively.

In small-scale irrigation practicing village (Ayetu), irrigation cannels were the most productive breeding habitat for *Anopheles* larvae with mean density of 5.06 larvae/dip followed by water pool with mean density of 2.45 larvae/dip while no *Anopheles* larvae was recorded from paddle. However, in a village practicing rain fed agriculture, swamp was the most productive habitat for *Anopheles* larvae with mean density of 3.55 larvae/dip followed by animal footprints 2.73 larvae/dip and river fringes 2.05 larvae/dip but no larvae was recorded from paddle/ farm ditches and tap. In human settlement village, swamp was the most productive for *Anopheles* larvae with mean density of with 1.44 larvae /dip.

Anopheles mosquito species larvae collected from different breeding habitats during the study period in the study area are presented in Table 5.1. Highest number (n=375; 1.25larvae/dip) of *An. gambiae* s.l. larvae was collected and identified from animal footprint, while *An. gambiae* s.l. larvae was not found from paddle/farm ditches.

Table 5.1: *Anopheles* larvae collected and identified from different breeding habitats in different agro-ecological settings in the study area

Anopheles mosquito species	*Anopheles* mosquito breeding habitat types							Total n(density)
	Swamp n(density)	River Fringes n(density)	Paddle n (density)	Tap n (density)	Irrigation n (density)	Ditches n(density)	Footprints n (density)	
An. gambiae	292 (0.97)	203 (0.68)	11 (0.04)	283 (0.94)	126 (1.26)	241 (0.80)	375 (1.25)	1531 (0.81)
An. squamosus	54 (0.18)	47 (0.16)	0	19 (0.06)	48 (0.48)	38 (0.13)	28 (0.09)	228 (0.12)
An. demeilloni	242 (0.81)	125 (0.42)	9 (0.03)	19 (0.06)	57 (0.57)	250 (0.83)	86 (0.29)	788 (0.41)
An. garnhami	24 (0.08)	22 (0.07)	0	1	0	40 (0.13)	19 (0.06)	103 (0.05)
An. funestus	15 (0.05)	0	0	0	0	0	0	15 (0.01)
Total	665 (2.22)	395 (1.32)	20 (0.07)	327 (1.09)	221 (2.21)	569 (1.89)	470 (1.57)	2665 (1.4)

5.4.Discussion and conclusions

Five *Anopheles* species larvae (*An. gambiae s.l., An. deimilloni, An. garnhami, An. squamosus* and *An. funestus*) were collected and identified in three agro-ecological settings in Sekoru district, southwestern Ethiopia. This study revealed that larvae of *An. gambiae s.l.* were found predominantly (0.73 larvae/dip). The occurrences of *An. gambiae s.l.* larvae in all breeding habitats in all villages were in line with previous reports in Ethiopia (Kenea *et al.*, 2013; Animut *et al.*, 2012) and Kenya (Minakawa *et al.*, 1999). Furthermore, it was previously reported that *An. arabiensis* usually breeds in semi-permanent pools of rainwater or overflow pools and better adapted to severely dry environments (Petrarca *et al.,* 2000). Note that *An. gambiae* complex larvae were not identified to subspecies in the current study. However, adults collected from the same study villages and periods were identified using PCR techniques, and all successful amplifications were identified as *An. arabiensis* (Chapter 4, section 4.1). With the intention that all *An. gambiaes.l.* adults were *An. arabiensis*, the larvae of the species collected in this study were presumably *An. arabiensis*.

In this investigation, *Anopheles* larvae abundance and distribution were in association with habitat types. *Anopheles gambiae s.l.* larvae were recorded from various breeding habitats. Swamps, river fringes, irrigation channels, stagnant water, ditches, paddle and animal footprints were productive *Anopheles* larvae habitats. This finding was in line with reports of Kenea *et al*., (2013) in Ethiopia where all of these breeding sites were reported to be productive breeding habitats except irrigation cannels. Similarly, the current investigation was in line with study in Kenya (Mwangangi *et al*., 2010).

Highest *Anopheles* mosquito larval densities were recorded from a village conducting small-scale irrigation scheme (2.12 larvae per dip)as compared to non-irrigation scheme (1.24 larvae per dip) and human settlement (0.45 larvae per dip). This finding was in line with reports of Mwangangi *et al*. (2010). The presence and abundance of *Anopheles* mosquito larvae in small-scale irrigation scheme practicing village could be due to formation of suitable eco-climatic conditions for breeding and survival of malaria vector mosquitoes.

Irrigated agriculture increases the number of aquatic breeding habitats and extends breeding duration of the vectors because of environmental changes and habitat manipulation for irrigation; consequently, extending transmission seasons (Kibret *et al*., 2010). Yewhalaw *et al*., (2009), Kibret *et al.,* (2010) and Dejene *et al*., (2012) reported that water resource projects such as construction of reservoir and irrigations channels determine malaria incidence and transmission in Ethiopia. This could be due to formation of breeding habitats such as shallow surface water, water pockets and water leaking from irrigations.

In conclusion, environmental manipulations in irrigation scheme increases availability and suitability of vector breeding sites insuring continued reproduction throughout the year. Hence, irrigation practices lead to increased vector abundance and consequently malaria incidence and transmission in the study area. Thus, effective vector monitoring and control strategies are needed in the area of water resource projects such as irrigation practices.

However, other reports indicated that there is less malaria in communities living in close proximity to irrigation schemes when compared with populations living further away, which is partially explained by enhanced incomes that facilitate better protective measures to be taken (Ijumba *et al*., 2002). Accordingly, agricultural development resulting in increased income for the community is likely to improve access to malaria treatment and may support an increased use of malaria preventive devices. This is so-called paddies paradox. For instance, Mutero *et al.* (2004) reported that irrigated areas were found to have lower prevalence of malaria though they had a 30–300 times higher prevalence of the local malaria vector compared with areas without irrigation in Kenya.

Furthermore, studies in Burkina Faso, Senegal, Mali, and Tanzania reported similar result (Keiser *et al.*, 2005; Mutero *et al*., 2004). Effective vector control programs, effective water management, and prevention interventions in the irrigated communities are among the several factors accounted for malaria prevalence reduction.

Chapter 6.Frequency of Knockdown Resistance (*Kdr*) Alleles in Populations of *Anopheles arabiensis* Patton (Diptera: Culicidae) in Sekoru District, Southwestern Ethiopia

6.1.Introduction

Chemical insecticides are playing an essential role for pest control in agriculture and vector control in public health sectors (Raghavendra *et al*., 2011). However, due to extensive and misuse of insecticides, various agro-ecosystems became a reason for insecticide resistance development in medically important insects such as malaria vector mosquitoes greater than before (Soko *et al.*, 2015). Malaria control measures are still getting difficulty due to insecticide resistance developments in *Anopheles* vectors because of repeated insecticide-insect contacts in agricultural areas. Hence, an existence of insecticide resistant strains associated with agricultural practices may affect the effectiveness of malaria vector control strategies.

Malaria vectors may become resistant to insecticides by either one or multiple mechanisms. Insecticide resistance mechanisms in malaria vectors include target site modification, behavioral changes and alterations of integuments (Yewhalaw *et al.,* 2010; Kawada *et al.,* 2011; Okia *et al.,* 2013). Malaria vectors may develop cross-resistance and multiple resistance mechanisms. Therefore, resistance development to one insecticide class may cross to other insecticide due to cross-resistance mechanisms. Furthermore, malaria vectors could develop resistance against multiple insecticide classes simultaneously. For instance, *An. gambiae* s.s. and *An. arabiensis* were investigated that they have developed resistance mechanisms such as *kdr* mutation and P450 Oxidases, Glutathion-S-transferase, and Esterases enzymes (metabolic resistance). Hence *An. gambiae* s.s. and *An. arabiensis* were resistant against organochlorines, pyrethroids, organophosphates and carbamates insecticide families simultaneously (Montella *et al*., 2007; Muller *et al*., 2008; Kawada *et al.,* 2011).

Knockdown resistance (*kdr*) is modifications of the Voltage Gated Sodium Channel (VGSC) in central nervous systems of the insects (O'Reilly*et al*., 2006 and Ranson*et al.,* 2011). VGSC is pyrethroid and organochlorine target site (Huestis *et al*., 2011). Hence, VGSC modification (*kdr*) is defense mechanisms against dichlorodiphenyltrichloroethane (DDT) and pyrethroids. Knock down insecticide resistance (*kdr*)is the most common resistance mechanisms of malaria vectors in Africa (Ohashi *et al*., 2014; Niang*et al*., 2016; Nkya *et al*., 2014; Kawanda *et al*., 20114b; Matowo *et al*., 2015).

In Ethiopia, insecticides play a critical role in public health and agricultural sectors. Yet, insecticide resistance development by vectors and pests questioned their efficacy, probably due to mistreatment and extensive use of insecticide in pest and vector control (Yewhalaw *et al*., 2010). For instance, following its success in vector and pest control, credit had been given to DDT for increased agricultural production and human health improvements in the past. Later on, because of reduced susceptibility of insects and public health concerns, DDT was banned from the agricultural and health sectors (Soko *et al*., 2015). The legendary era of DDT had passed and it was erased from agenda of functional insecticide lists of World Health Organizations (WHO). Yet, as they have similar mode of actions (VGSC), DDT affected the effectiveness of insecticides such as pyrethroids due to cross-resistance mechanisms. Pyrethroids resistance is a major problem as they are one of the main insecticide classes used for malaria vector control in

Ethiopia and other African countries (Yewhalaw *et al*., 2010; Kawada *et al.,* 2011; Okia *et al.,* 2013).

In Ethiopia, malaria vector control strategies rely on indoor residual spraying (IRS) and large-scale distribution of insecticide-treated nets (ITNs). Pyrethroids are the only insecticide classes used for ITNs in Ethiopia (FMoH, 2016). However, because of resistance developments by malaria vector populations against pyrethroids, it is becoming less effective than ever. No feasible insecticide molecules that can substitute pyrethroids are approved. Therefore, resistance development against pyrethroids is foremost challenge of malaria vector control programs. To establish new effective vector control strategies, timely assessment and monitoring of susceptibility of the vector to existing insecticides is essential. Hence, adequate information regarding factors associated with pyrethroids efficacy is required to manage the problem and to design effective malaria control strategies. This study was conducted to investigate the status of knockdown resistance (*kdr*) allele mutation in the populations of *An. arabiensis* and the likely association of agricultural practices with increased pyrethroid resistance in Sekoru District, southwestern Ethiopia.

6.2. Materials and methods

6.2.1. Descriptions of study area

The study was conducted in three villages of Sekoru District, southwestern Ethiopia from January-December 2015. The three villages include Ayetu, Chafe and Toli. The study villages have similar geo-topographical setting, and the inhabitants of the villages have similar socio-economic condition. However, the study villages have different agro-ecological settings. The details of the study site description are indicated in Chapter 3, section 3.1.

6.2.2. *Anopheles* mosquito collection

Adult *Anopheles* mosquitoes were collected from the three study villages monthly. Adult *Anopheles* mosquitoes were collected using CDC light traps and Space Spray Catch (SSC) from selected houses in each village. Adult *Anopheles* mosquitoes were collected twice a month in each study village from January to December 2015. The collection included both indoor and outdoor feeding *Anopheles* mosquitoes. Details of *Anopheles* collections are indicated in Chapter 3, section 3.2.

6.2.3. Mosquito processing and species identification

Indoor and outdoor collected adult mosquitoes were anesthesed by chloroform, sorted by genus, species, sex and abdominal status. All mosquitoes belonging to genus *Anopheles* were morphologically identified using identification keys for Ethiopian and East African mosquitoes (Verrone, 1962a; Gillies and Coetzee, 1987).

Morphologically confirmed *Anopheles gambiae* s.l. was transported to Entomology Molecular Laboratory at Center for Diseases Control and Prevention (CDC), Atlanta, Georgia, USA for molecular processes. Genomic deoxyribonucleic acids (DNAs) were extracted individually for identification of *An. gambiae* s.l. sibling species by molecular techniques as described by Collins *et al*., (1987). Details of molecular techniques were described in Appendix 1.

6.2.4. Detection of *kdr* alleles

The knockdown resistance (*kdr*) alleles were amplified following the protocol developed for *Anopheles gambiae* s.l. The assay uses an intentional mismatch primer method (Wilkins *et al*., 2006)to detect the East (Ranson *et al*., 2000) and West African (Martinez-Torres *et al*., 1998) *kdr* mutations in two independent amplification reactions for each specimen. The reactions were carried out in a 12µl final volume using GoTap (Promega) and the primers in the exact concentrations presented in the MR4/BEI manual (https://www.beiresources.org/Publications/MethodsinAnophelesResearch.aspx).

PCR master mix was prepared by mixing 1x (7.8µl distilled water, 5µl GoTaq PCR Buffer, 2.5µl dNTP (2.5mM concentration), 0.5µl MgCl2 (25mM concentration), 2µl IPCF, 2µl altRev, 2µl WTR [5pm] (for East kdr) or 2µl WTR [25pm] (for west *kdr*), 2µl East F (for east kdr) or 3µl WEST F (for west *kdr*) and 0.2µl Go-Taq) in centrifuge tubes for both of *kdr* mutation types (L1014S and L1014F). 1µl of the master mix and 0.5µl of DNA was added into separately labeled PCR plate wells for both *kdr* mutation types. Amplification conditions were: 95°C/5min; 35 cycles of: 95°C for 30sec, 57°C(east)/59°C(west) for 30sec and a final elongation period at 72°C for 5min.

Finally, PCR products were visualized with UV light in 2% agarose gels stained with gelred (Wilkins *et al*., 2006). All reactions included specific controls of mosquito reference strains for the resistant, susceptible and heterozygote allele variants and a negative control. 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 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 heterozygous genes.

6.2.5. Data Analysis

Data of *An. arabiensis* distribution and abundance and *kdr* allele mutation frequency were entered into excel computer program and checked for completeness. Association between agro-ecology (irrigation agriculture, rain fed agriculture and human settlements), the abundance and distribution of *An. arabiensis* and *kdr* mutation frequency were analyzed using IBM SPSS version 20 statistical software package. To estimate the associations between vector parameters and agro-ecological factors, chi square test (X^2) was analyzed. Univariate analysis was performed to estimate interaction between study variables such as vector density, *kdr* mutation levels, agro-ecosystems and seasonal variations. P values ≤ 0.05 were considered to reveal significant association between the variables.

6.3.Result

6.3.1. Knock down resistance (*kdr*) mutation frequency

Out of a total of 316 *An. arabiensis* screened for *kdr* alleles, 304 specimens successfully amplified the target region. As shown in Plate 6.1, all observed *kdr* allele mutations were West African (L1014F) type mutations with 76.31% (n=232) and 15.13% (n=46) homozygous and heterozygous gene composition, respectively, while 8.55% (n=26) of the tested *An. arabiensis* were carrying wild type gene. A total of *kdr* allele mutation frequency was ranging from 83.88% (n=510). As shown in Figure 6.1, no *An. arabiensis* were carrying East African *kdr* allele mutation (L1014S).

6.3.2. Distributions and frequency of *kdr* alleles among various agro-ecological settings

The frequencies of *kdr* allele in different agro-ecosystems in Sekoru District, southwestern Ethiopia were shown in Table 6.1. The *kdr* allele frequencies in village with irrigated agro-ecosystem (n=378; 95%) were 3.4 times higher than *kdr* allele mutation frequency (n=112; 78.87%) in village with rain fed agro-ecosystem (P=0.016) and 19 times higher than *kdr* allele mutation frequency (n=20; 3.89%) in human settlement village (P<0.001). The association between agro-ecosystem and *kdr* allele mutation frequency was statistically significant (X^2=133.85, df=2, *P* <0.001).

Table 6.1: Distribution and frequency of *kdr* allele mutation in *An. arabiensis* among various agro-ecological settings in the study are

Type of agricultural practices	genes tested # (%)	RR # (%)	Rs # (%)	SS # (%)	*kdr* allele frequencies # (%)
Irrigated agro-ecosystem	199 (67)	182 (91.45)	14(7.05)	3(1.50)	378(95)
Non irrigated agriculture	71 (22)	49(69)	14 (19.71)	8(11.29)	112(78.87)
Human settlement	34 (11)	1(2.94)	18(52.94)	15(44.1)	20 (3.89)
Total	304 (100)	232 (76.31)	46 (15.13)	26 (8.55)	510(83.88)

Note: **RR**-Homozygous resistant, **RS**-Heterozygous resistant and **SS**-homozygous susceptible

Monthly *kdr* allele mutation frequencies and gene composition is indicted in Table 6.2.

Table 6.2: Monthly distribution and frequency of *kdr* alleles in the population of *An. arabiensis* in the study area

Months	Total specimens tested	Gene status Homozygous resistant	Heterozygous resistant	Homozygous susceptible	*Kdr* allele frequency
January	14	10	1	3	75%
February	16	9	5	2	72%
March	27	19	7	1	83.33%
April	10	9	0	1	90%
May	9	7	2	0	88.88%
June	23	20	3	0	93.47%
July	18	16	1	1	91.66%
August	156	118	25	13	84.93%
September	11	11	0	0	100%
October	6	6	0	0	100%
November	8	7	0	1	87.50%
December	6	0	2	4	8.33%
Total	304	232	46	26	83.88%

West African *kdr* allele monthly status and frequency in the population of *An. arabiensis* in different agro-ecological settings in the study area is indicated in Figure 6.2.The *kdr* distribution among months was statistically significant (X^2=59.91, df=2, P<0.001).

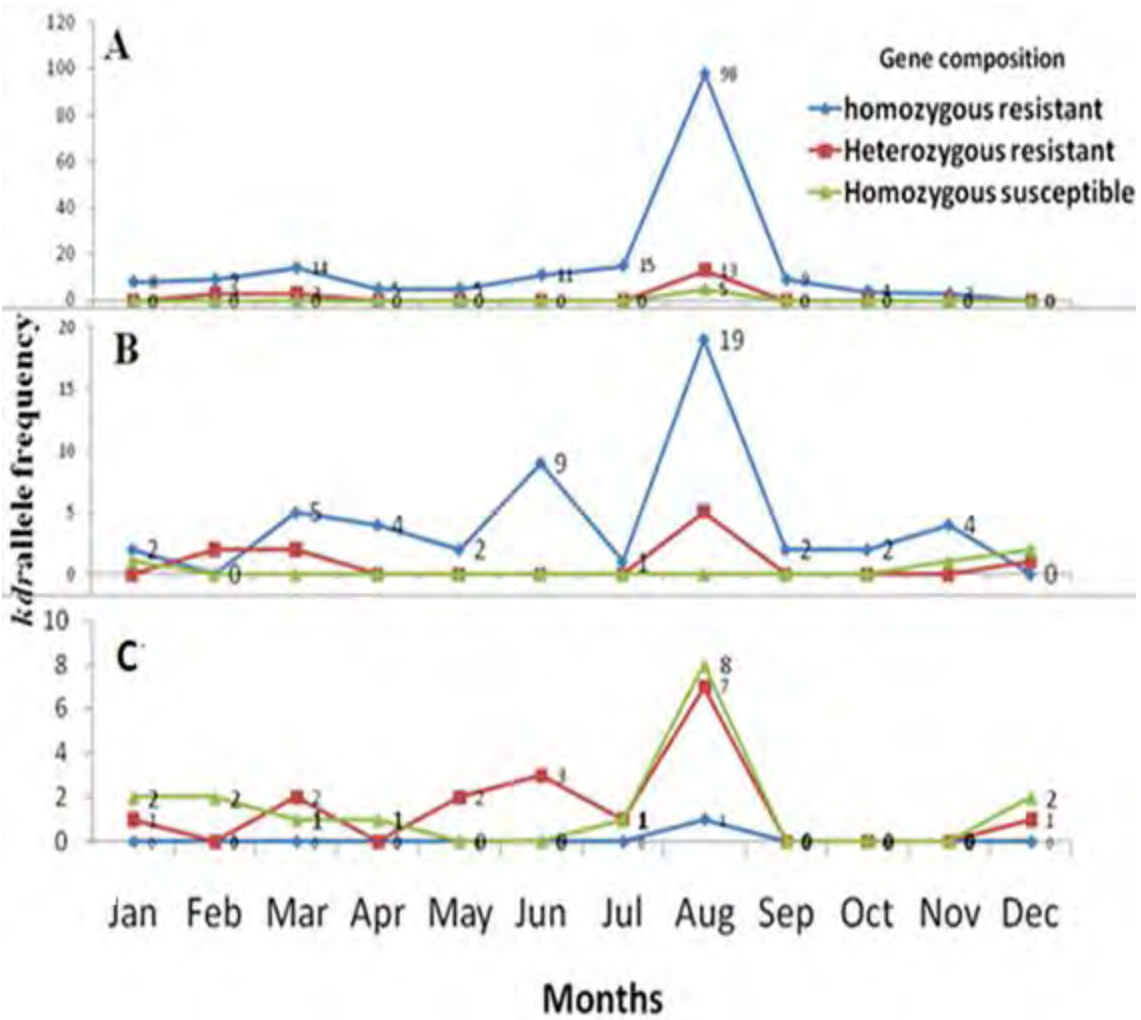

Figure 6.2: Monthly *kdr* allele frequency across different agricultural practicing villages of Sekoru District, southwestern Ethiopia: (A) irrigated agro-ecosystem, (B) Rain fed agriculture and (C) human settlement

6.4. Discussion and conclusions

In this study, 83.88% (n=510) overall *kdr* alleles frequency was detected in the populations of *An. arabiensis.* So that the vectors were investigated to be resistant against pyrethroids, the one among primary insecticide class in use for malaria control options, ITNs in Ethiopia. This investigation agrees with previous studies (Yewhalaw *et al*., 2010; 2011; Asale *et al*., 2014) in Ethiopia, (Chen *et al*., 2008; Kawada *et al*., 2014a; Ohashi *et* *al*., 2014; Ochomo *et al*., 2014)in Kenya, (Okia *et al.,* 2013) in Uganda and (Edi *et al*., 2012; 2014; Koffi *et al*., 2013) in Cote d'Ivoire.

The high *kdr* allele mutation frequency in the current study might be due to natural selection of the vectors related to long and extensive use of insecticides such as DDT in near past and pyrethroids for ITNs right now. According to personal communication with Sekoru District health office, malaria control department, though it was banned, DDT had been in use as far as recent days intentionally or unintentionally in the area. Thus, due to frequent exposure, vector mosquitoes could have developed phenotypic resistance against DDT and consequently, against pyrethroid insecticide classes that share target site (VGSC).

A total of 304 *An. arabiensis* was tested for both East African (L1014S) and West African (L1014F) *kdr* allelic mutations. No *An. arabiensis* were detected to develop East African *kdr* allele mutation. All of *kdr* mutations were West African type (L1014F) with 83.88% allelic frequencies. This result agreed with previous reports of Yewhalaw *et al*., (2010) in Ethiopia and Protopopoff *et al*., (2013) in Tanzania. However, it was not in line with other reports (Kawada *et al*., 2011)in Kenya, (Kulkarni *et al*., 2006) in Tanzania, (Matambo *et al*., 2007) in Sudan, (Chouaibou *et al*., 2008) in Cameroon, (Fanello *et al*., 2003) in Mali and (Diabate *et al*., 2004) in Burkina Faso. Those reports revealed absent or low-moderate frequencies of West African *kdr* mutation (L1014F) in *An. arabiensis*.

This point mutation cause resistance against pyrethroids and organochlorines (DDT) insecticide classes (Brooke, 2008). Hence, high frequency of L1014 point mutations (*kdr)* caused insecticide resistance (target site modification) putting efficacy of pyrethroids in question. For instance, investigation in Benin revealed that high frequency of West African *kdr* mutation associated with reduced efficacy of pyrethroids treated nets and indoor residual spraying (IRS) (N'Guessan *et al*., 2007).

Finally, this study investigated distribution, genotype status and frequency of *kdr* allele in populations of *An. arabiensis* in different agro-ecological settings in Ethiopia for the first time. *An. arabiensis* collected from irrigated agro-ecosystems were detected to have highest frequency of West African *kdr* allele mutations (L1014F) (n=378; 95%) as compared to those collected from village practicing rain fed agriculture (n=112; 78.87%) and human settlement villages (n=20; 3.89%).The association of year round active agriculture practicing areas and increased pyrethroid resistant *An. arabiensis* could be due to frequency of pesticides' application in agricultural fields. According to Sekoru district health office report (not published), in the current study area, DDT has been extensively and frequently in use in agricultural pest control for irrigation based agricultural crops for long time. DDT had also been the primary insecticide for malaria vector control in southwestern Ethiopia for long time.

The extensive and year round contact of vector larvae and insecticides accumulated in irrigation field and water pockets in the irrigation cannels either after or during the era of DDT might cause natural selection in the populations of the vectors against related insecticide classes. Furthermore, chemical insecticides (DDT) leaching from agricultural fields to nearby breeding habitats could also increase the chance of vector-insecticide repeated contacts leading to natural selection. So that the exposure of *An. arabiensis* larvae to agricultural pesticides such as DDT can select them for resistance against existing insecticides during adult stage (Nkya *et al.*, 2014).

In general, extensive and miss use of DDT for agricultural pest control in the past could have left a bench for pyrethroids resistance in the population of *An. arabiensis* because of cross-resistant mechanism. Thus, abundance and wide distribution of *An. arabiensis* strains resistant against pyrethroids in the area of year round active agro-ecosystems could be due to evolution (natural selection) of DDT resistant mosquitoes in the past.

In conclusion, agricultural activities are associated with malaria vector population abundance and distributions in Sekoru District, southwestern Ethiopia. Abundance and distribution of *An. arabiensis* strains having allelic point mutation at L1014 gene foci was associated with agricultural practices the study sites. Consequently, irrigated agro-ecosystems elevate insecticide resistance developments in the population of malaria vectors, so that malaria incidence and transmission intensity in the community.

Chapter 7. General discussion, Conclusion and Recommendations

Anopheles mosquitoes' species composition, distribution, abundance and *kdr* status and frequency level in the population of *An. arabiensis* were investigated. The study revealed association of agricultural practices and *Anopheles* mosquito's population dynamics and species composition and infectious rate in Ayetu, Chafe and Toli villages.

A total of eight *Anopheles* mosquito species were investigated in the current study. The species include *An. gambiae sl.*, *An. demeiloni, An. garnhami, An. squamosus, An.marshali,An. longipalpis, An. pretoriensis* and *An. christyi,* of which *An. gambiae* s.l. was predominant. This result is similar to previous results reported from different parts of Ethiopia (Kibret *et al*., 2010; Yewhalaw *et al*., 2009; Jaleta *et al*., 2013).

Highest *Anopheles* mosquito densities (4.46mosquitoes/CDC light traps) were recorded in village conducting small-scale irrigation as compared to 1.79 mosquito/CDC light traps and 0.40 mosquito/CDC light traps in a village practicing rain fed agriculture and human settlement, respectively. It could be due to configuration of suitable ecological and climatic conditions for breeding and survival of malaria vector mosquitoes because of environmental changes and habitat manipulation for surface irrigation.

Higher outdoor feeding density (2.5*Anopheles* mosquitoes/CDC light trap/night) of *Anopheles* mosquitoes were recorded as compared to 1.8 mosquitoes/CDC light trap/night indoor host seeking mosquitoes. This investigation agrees with reports of Degefa *et al*., (2015) and Kenea *et al*., (2017) in Ethiopia. This could be related to malaria control interventions and mosquito behavioral changes. *Anopheles* mosquitoes may have changed their endophagic behaviors to exophagic responding to actively ongoing indoor-based control strategies (IRS and LLITNs).

In the current study, 11.2 infective bites/person overall annual EIR was estimated. The highest EIR 10.8 was detected in Ayetu as compared to 5.99 and zero EIR in Toli and Chafe, respectively. Hence, higher malaria transmission intensity was found to be in agriculture practicing villages compared to human settlement. Such difference in infectivity rates at local level could be due to vector density and host exposures to infective bites.

Pyrethroids are insecticides playing a central role in malaria control strategies (ITNs) in Ethiopia in general and in southwestern Ethiopia in particular (Yewhalaw *et al*., 2010; FMoH, 2016). In current study, levels of pyrethroid target site (voltage gated sodium channel) mutations in population of *An. arabiensis* were evaluated in association with agricultural practices in Sekoru District, southwestern Ethiopia.

In this study, out of a total of 304 *An. arabiensis* amplified using allele specific PCR, 76.31% (n=232) specimens were carrying homologous resistant genes; 15.13% (n=46) *An. arabiensis* were detected to have heterozygote resistant gene while 8.55% (n=26) *An. arabiensis* were detected to be wild type or susceptible to pyrethroid. Overall, high frequencies of (*kdr*) mutation alleles (pyrethroid target site resistance) ranging from 83.88% (n=510) were detected in the population of *An. arabiensis* showing elevated insusceptibility of malaria vectors to pyrethroid in the study area. All of *kdr* mutation alleles were detected to be West African type (L1014F). This result was supported by previous reports of Yewhalaw *et al*., (2010) in southwestern Ethiopia and Protopopoff *et al*., (2013) in Tanzania.

The high *kdr* mutation allele's frequencies in the current study might be due to natural selection of the vectors related to long and extensive use of insecticides such as DDT in near past and pyrethroids up-to-date for LLITNs. Therefore, high frequencies of West African kdr allele mutation (L1014F) cause pyrethroid resistance (N'Guessan *et al*., 2007).

This investigation revealed association of agro-ecological settings with developments of insecticide resistance and *kdr* allelic frequency in *An. arabiensis* in Ethiopia for the first time. This study investigated *kdr* allele status and frequency in the population of *An. arabiensis* in association with irrigated agriculture, rain fed agriculture and human settlements in Sekoru District, southwestern Ethiopia.

Anopheles arabiensis collected and tested from village practicing small-scale irrigation scheme had over all *kdr* mutation allele frequency ranging from 95% (n=378). In those vectors 91.45% (n=182) homozygous and 7.05 %(n=14) heterozygous resistant genes were detected; but out of all specimens, only 1.5% (n=3) were wild type carrying homozygous pyrethroid susceptible gene. Thus, *An. arabiensis* collected from houses in the vicinity of irrigated agro-ecosystems were investigated to be resistant to pyrethroids.

Anopheles arabiensis collected and examined from the agro-ecological settings with rain fed agriculture were detected to have over all *kdr* mutations allele frequency ranging from 78.87% (n=112). The genomic DNAs of these specimens were investigated to carry

68% (n=49) homozygous, 19.71% (n=14) heterozygous pyrethroid resistant genes while only 11.29% (n=8) specimens were carrying wild type gene.

Anopheles arabiensis collected from human settlement village were detected to have over all *kdr* allele frequency ranging from 3.89% (20). Among *An. arabiensis* tested from human settlement, only 2.94% (n=1) mosquito was having homozygous resistant gene while 52.94% (n=18) and 44.11% (n=15) were heterozygous resistant and wild type respectively.

In general, this study showed that *kdr* allele frequency levels and pyrethroid susceptibility were associated with agro-ecological settings. This result may be due to active uses of DDT in vector and pest control in the past. DDT has been extensively in use in agricultural pest control for irrigation based crops such as green pepper (*Capsicum annuum*), kat (*Katha edulis*) for long time in the current study sites. Vector larvae in irrigated land use patterns had a chance of year round and repeated contact with pesticide chemicals such as DDT accumulated in cannels and water pockets due to irrigation. Furthermore, chemical insecticides such as DDT leaching from agricultural fields to nearby breeding habitats could also increase chance of vector-insecticide repeated contacts leading to genotypic and phenotypic adaptation. So that the exposure of *An. arabiensis* larvae to agricultural pesticides can select the resistance strain to survive insecticides during adult stage Nkya *et al.*, (2014).

7.1. Conclusions

Anopheles arabiensis is the dominant *Anopheles* species widely distributed in the current study area. Malaria vector density, feeding behavior and infectivity were in association with agricultural practices. Numbers of infective bite of *An. arabiensis* in the study sites were associated with agro-ecosystems. Hence, malaria transmission intensity in the study areas was associated with agricultural practice and high transmission was detected in a few hot spots.

Abundance and distribution of *Anopheles* mosquito larvae are associated with agricultural activities, particularly irrigation. Environmental manipulations such as irrigated agro-ecosystems increase availabilities and suitability of breeding habitats leading to increased vector abundance and consequently increasing malaria incidence and transmission.

High frequency level of West African *kdr* allele mutations were detected in the population of *An. arabiensis* in the study area. Abundance and distribution of *An. arabiensis* carrying *kdr* alleles in the study sites were in association with agro-ecological settings. The West African *kdr* mutation (L1014F) status and frequency level were associated with land use patterns particularly, agro-ecological settings.

Generally, entomological parameters such as vector density, human biting density, infectivity and insecticide resistance level were associated with structures and agro-ecosystems such as small-scale irrigation based agriculture in Ethiopia.

7.2. Recommendations

Anopheles mosquito distribution and abundance was determined to be associated with agricultural practice. Irrigation increase number of breeding habitats increasing frequency of gonotrophic cycle and host-vector contact.

Thus, malaria control strategies should be planned based on land use patterns, local vector species, population characteristics and efficacy of ongoing vector management options and strategies. Planning and monitoring of effective vector control interventions are needed before/during operation of water resource projects for irrigation. Similarly, irrigation practices are suggested to be planned in the manner that they do not cause malaria risk; and local communities should be given priority regarding community awareness, education related to malaria control.

In the current study area, the dominant malaria vector was *An. arabiensis*, a vector with dynamic breeding, feeding and resting behavior. IRS and ITNs, the malaria vector control strategies in Ethiopia are mainly targeting endophilic, anthropophillic and endophagic mosquitoes (USAID, 2013; FMoH, 2016). However, *An. arabiensis* were investigated to have developed response mechanisms to these vector control strategies. Therefore, the malaria vector control interventions in the study area and areas with similar land use patterns are recommended to be planed and implemented in line with vectors' behavior.

To reduce insecticide resistance and implement effective vector control measures, it is important to work in collaboration with sectors responsible for water resource development projects and land use patterns. Furthermore, the high *kdr* frequency in the study area also highlights a need for new and effective alternative control options instead of pyrethroid for ITNs. Currently, as no appropriate alternative insecticide for ITN is approved, resistance to pyrethroids is forecasted to be a foremost problem for the malaria vector control program. An establishment of insecticide resistance monitoring and management (IRMM) schemes, formulation of new effective insecticide and implementation of Integrated Vector Managements (IVM) are essential for effective malaria vector control.

Developments of other insecticide resistance mechanisms along *kdr* alleles leading to multiple-resistance may significantly affect efficacy of malaria control options. Hence, further investigations related to association of agricultural practices and insecticide resistance mechanisms such as metabolic resistance, cuticlar resistance and behavioral adaptation are appreciated.

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Appendix

1. *Anopheles gambiae* s.l. molecular identification techniques

DNA extraction and purification: Out of 715 *Anopheles gambiae sl.,* 352 (50%) were extracted for identification of the sibling species. DNA of 352 adult *An. gambiae sl.* were extracted following Collins *et al.* (1987) protocol in CDC, Atlanta, Georgia, USA molecular laboratory for entomology. Either full or parts of the mosquitoes were grinded with a sterile blue Konte's pestle in centrifuge tubes until all parts remain unidentifiable. The grinding products were heated for 30 minutes at 65°C 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). After an overnight precipitation in ethanol 100%, and wash in 70%, DNA pellets were dissolved in 100µl sterilized water. DNAs were precipitated by adding 18µl of 5M ice-cold Potassium acetate (KAC) and incubated in ice for at least 30 minutes. The solutions were centrifuged at maximum speed of 13,200revolutions/minute at room temperature for 20 minutes. The entire DNA supernatants were carefully transferred (avoiding transferring the precipitate) to newly labeled centrifuge tubes followed by adding 200µl of 100% ethyl alcohol. The solutions were mixed by inverting the centrifuge tubes and incubated in -20^{o}c freezer overnight. On the following day, DNAs were precipitated by centrifuging the tubes at maximum speed of 13,200rpm for 30 minutes at 4^{o}c and supernatants were removed without disturbing the pellets. Then, 200µl of 70% ethyl alcohol was added and re-centrifuged for 10 minutes at 13,200rmp. Finally, the alcohol was removed without disturbing the pellets; the DNA sediments were allowed to dry at room temperature (for at least 30 minutes). DNA pellets were dissolved in 100µl sterilized water with gentle tapping of the tube to allow the DNAs to re-suspend for amplification process.

DNA amplification

The ribosomal region targeting specific SNPs for the *Anopheles gambiae* species complex was amplified in a multiplex reaction as described Wilkins *et al*., (2006). PCR reaction was carried out using AccuStart II PCR Supermix (Quanta Biosciences) in a final 12µl reaction mix, containing 0.3µl of each primer in a 25pmol concentration, and 0.5µl of DNA. The primers for GA-3T-R (*An. gambiae s./An.culluzzi*), AR-3T-R *(An. arabiensis*), ME-3T-R *(An. melas/An. merus*)andQD-3T-R *(An. quadriannulatus/An. amharicus*) were used in DNA coping process. The plates were well covered and spine down in micro-centrifuge at maximum speed for two to three minutes and amplified.PCR conditions were: 95ºC for 4' followed by 34 cycles of: 95ºCfor 30”; 60ºC /30" and 72ºC for 30"; and a final elongation step at 72ºC for 5'.

Gel electrophoresis

Agarose gel was prepared by following Wilkins *et al*., (2006) protocol. 1.5g of agarose powder was added to 75ml of 1xTAE and mixed in a microwavable flask. The solution was melted in microwave for two minutes. After cooling down the mixture, 7.5µl gel red was added and homogenized, so that it binds to the DNAs and allow the DNA to be visualized under UV light. The gel was prepared by pouring agarose solution on to midsized gel tray with well comps in place. The gel was left for 20-30 minutes at room temperature so that it was completely solidified.

Gel tray was placed in to gel box and 1xTAE buffer having gel red was added until it overflows on the gel tray. 1µl of molecular weight ladder was loaded into the first and the last lane of the gel. 1µl of PCR amplified DNAs were loaded into additional wells of the gel. Setting the positive and negative ion electrodes properly, the gel electrophoresis was allowed to run at voltage of 90volt, current of 400amper for 90 minutes.

DNA fragments of *An. gambiae* complex were visualized by using Benchtop UV Transilluminator machine. MultiDoc-It^{TM} Imaging System-Masterflex computer software was used to detect DNA fragments and capture photo of the DNA bands. The visualization of the gel electrophoresis result was determined based on the DNA size (the shorter run faster). The DNA fragments were interpreted by using bands of the markers on the first and the last lane of the gel. *An. gambiae sl.* sibling species were identified by comparing the DNA band with already known molecular weight ladder bands. DNA fragments having 464bp, 529bp, 637bp and 388bp were determined to be *An. gambiae s.* Giles/*An. culluzzi* Coetzee and Wilkerson*, An. melas*Theobald/*An. merus* Donitz and *An. quadirianulatos* Theobald/ *An. amharicus* Hunt, Wilkerson and Coetzee and *An. arabiensis* Patton respectively.

2. Circumsporozoite Protein Detection

The head-thorax regions of female *An. gambiae* s.l. mosquitoes were checked for circum-sporozoite protein (CSP) by Enzyme Linked Immuno-sorbent Assay (ELISA) techniques following the protocol described by Wirtz*et al.,*(1992) in the Molecular Entomology Laboratory at CDC, Atlanta, USA. The head-thorax region of each female *Anopheles* mosquitoes was ground using electric-motor operated pestle. 100µl gridding buffer (BB-

NP40) prepared in the ratio of 1ml blocking buffer to 5ul NP-40 was used as gridding solution.

Coating and Blocking the Wells of ELISA Plate

A mixture of 500µl of 1x PBS and 40µl primary *Pf* capture Monoclonal Antibody with lot number 2016pfcab was prepared in 15ml Falcon tube for *P. falciparum CSP*. Similarly, solution of 500µl of 1x PBS and 20µl primary Pv210 and Pv247Monoclonal Antibody capture (mAbcap) with lot number 2016pv210 and 2016pv247cab were prepared for *P. vivax*210and *P.vivax*247, respectively. Then the solution was mixed using vortex and poured into reservoir. Using multichannel pipette, 50µl of the solution was added into each well of the ELISA plate. Covering with aluminum foil, the ELISA plate was incubated for 30 minutes at room temperature.

After the incubation, contents of ELISA well were aspirated using vacuum system and banged upside down five times onto the diaper pad. The wells were filled by blocking buffer using blocking buffer stat-Matic, and incubated for one hour at room temperature.

Positive Control Preparation and Sample Testing

At 45 minutes of the incubation, positive controls were prepared for each antigen. For *P. falciparum*, *P. vivax*210 and *P.viva*247, frozen positive controls with lot numbers *Pf* Feb/2016, Pv210 June/2016 and Pv247 June/2016 respectively were thawed and mixed using vortex. 490µl of blocking buffer was mixed with 10µl of Pf-PC, Pv210-PC and Pv247-PC added into three respective 1.7ml centrifuge tubes. The positive controls in the centrifuge tubes were mixed using vortex.

The foil cover was removed from ELISA plates; well contents were aspirated by vacuum system and banged upside down five times onto the diaper pad. Using new pipette tips for each, 50µl of each positive control were added into A1, and negative controls were added into B1-H1of their respective ELISA plates. Similarly, 50µl of test sample were added in to the rest wells of the three plates (Plate 1a). The ELISA plates were covered with aluminum foil and incubated for two hours at room temperature (Plate 1b).

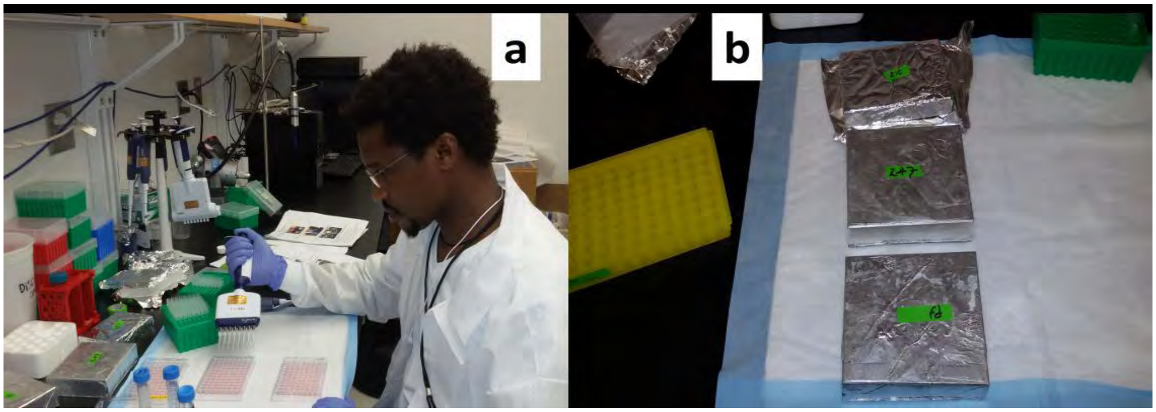


Plate 1: Sample testing for *Plasmodium* circum-sporozoite protein

Fifteen minutes to end of ELISA plate incubation period, secondary antibodies or Peroxidase (HRP-conjugates) were prepared. 500µl of blocking buffer was added into 15ml Falcon tubes labeled PfHRP2°, Pv210HRP2° and Pv247HRP2°. Similarly, 10µl of PfHRP (peroxidase-mAb lot number 2013PfHRP), Pv210HRP (peroxidase-mAb lot number 060414) and Pv247HRP (peroxidase-mAb lot number 150355-2015HRP) conjugates were added into their respective Falcon tubes (already containing blocking buffers). The Falcon tubes were covered with aluminum foil and put aside for later use.

The covers of ELISA plates were carefully removed and 50µl of sample from each ELISA plates were placed back into the original sample tubes using adjustable multichannel pipettes for other uses. Then the whole contents of each wells of the ELISA plates were aspirated using vacuum system attached to 8-well manifold and banged upside down five times onto diaper pad. Then each well was filled with 1xPBS+tween20 by using 1xPBS+tween20 stat-Matic. The whole contents of the wells were aspirated and banged five times on diaper pad. This washing step was repeated one more time.

The cover on the Falcon tubes containing HRP-conjugate solution was removed and the contents were poured in to respective reservoir followed by transferring 50µl of the solution in to ELISA plate wells using multichannel pipettes. The ELISA plates were covered by aluminum foil and incubated for one hour at room temperature.

After incubation, the whole contents of the wells were removed by aspirating and washed three times using 1x PBS+Tween20 and 100µl of ABTS (Accupenser Junior, Nichryo A and B) substrate were added into each well; the plates were covered by aluminum foils and incubated for 30 minutes at room temperature.

The presence of *Plasmodium* CSP was detected using Spectra MAX 340 plate reader instrument. SoftMax 5.4.5 computer program was used to visualize the result. The plate reader was set at spectra max 250 & 340 and connection COM1 settings. The ELISA plates were carefully placed in the right direction in the plate reader (Plate2a) and read at 405 wavelengths. The result appeared on the screen was copied and pasted onto excel computer software (Plate2b). Circum-sporozoite protein positive samples were estimated by calculating cutoff value which is average absorbance value of all negative controls, multiplied by 2. Any samples from wells with absorbance value higher than or equal to the cutoff value were considered positive and any value less than cutoff value were considered negative (Figure 2c).

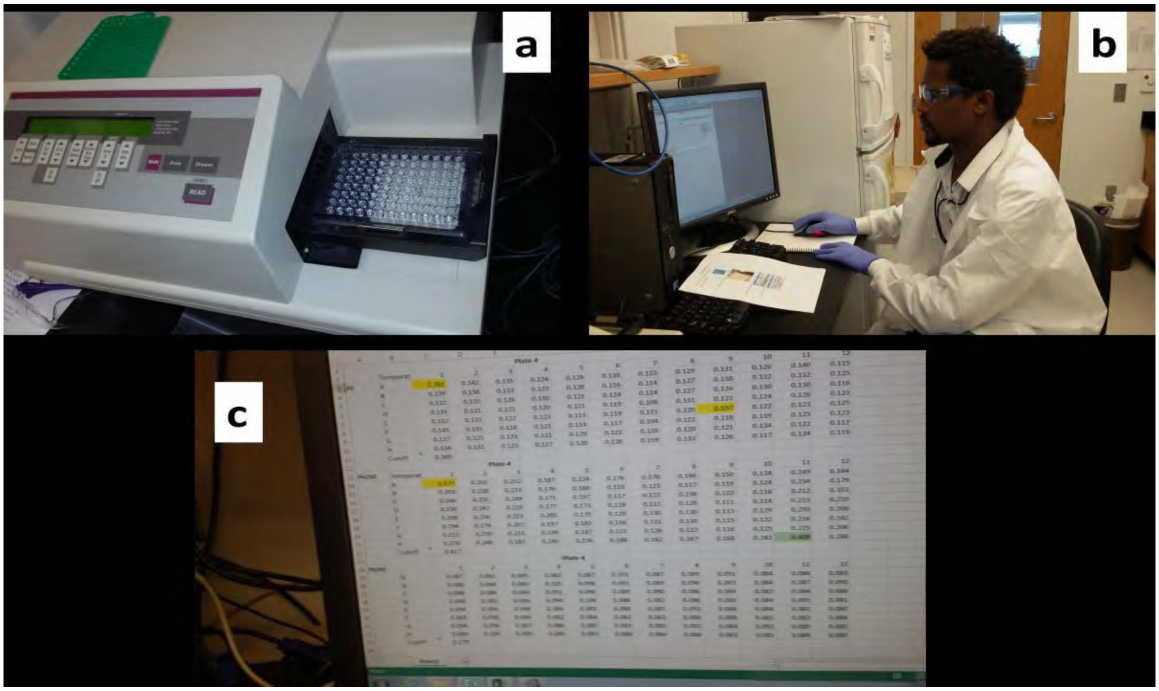
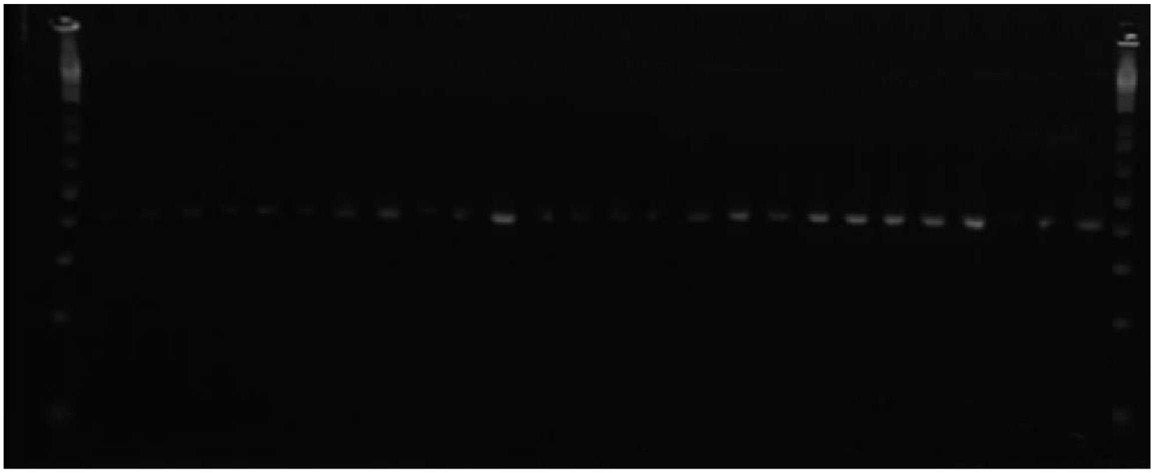
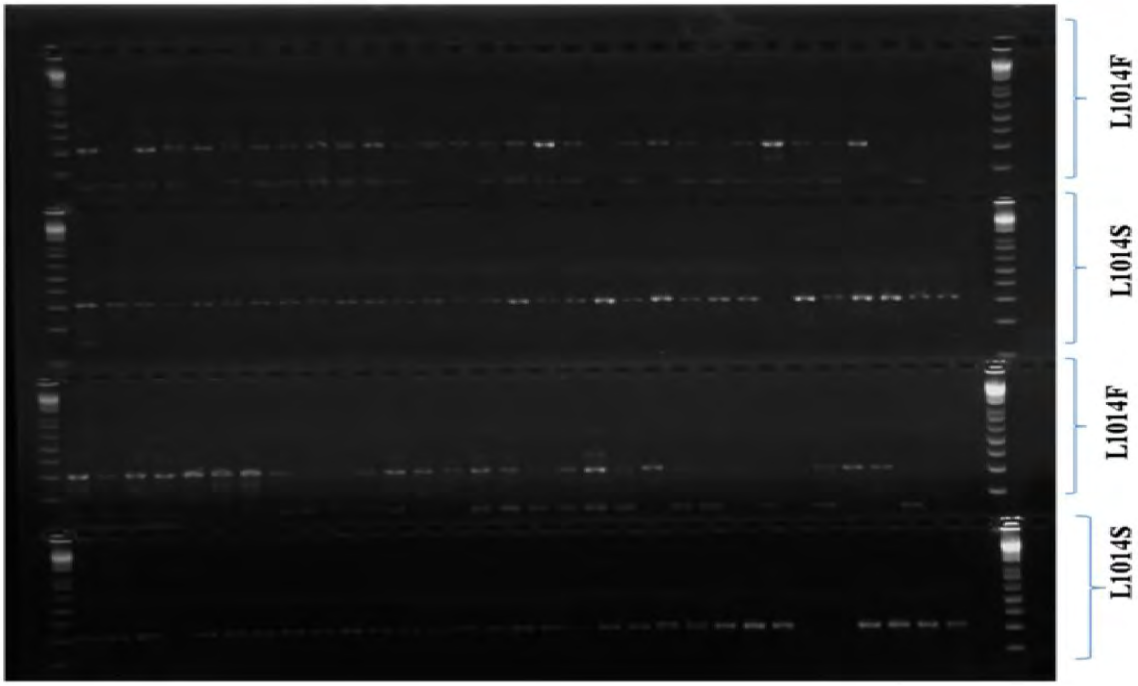


Plate 2: Visualization of sporozoite ELISA test

Finally, malaria transmission intensity was estimated by determination of entomological inoculation rate (multiplication of human biting rate and sporozoite rate).



Appendix 3: *Anopheles arabiensis***:** lanes 1, 32 1kb ladder, lanes 2 not amplified, lanes 3-27*An. arabiensis* and lanes 28-31 controls:0.7µl of sample loaded and run on a 2% agarose gelred gel.



Appendix 4: Gelred stained agarose gel electrophoresis showing amplification of kdr allele in *An. arabiensis*