College of Natural Sciences

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Department of Biology

Insecticide resistance status of *Anopheles gambiae* s.l. and *Anopheles stephensi* populations to existing and some candidate insecticide formulations

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LIST OF ACRONYMS AND ABBREVIATIONS

AChE	Acethylcholinesterase
CDC	Center for Disease Control
DDT	Dichloro-diphenyl-trichloroethane
GST	Glutathione-S-transferases
HBI	Human blood index
HCH	Hexachlorocyclohexane
IRS	Indoor residual spraying
ITN	Insecticide-treated nets
KDR	Knockdown resistance
LLIN	Long-lasting insecticidal net
MCP	Malaria Control Program
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
RPM	Revolution per minute,
SIT	Sterile insect technique.
VGSC	Voltage gated sodium channel
WHO	World Health Organization

ABSTRACT

Malaria control in Ethiopia mainly relied on case management and vector control using long-lasting insecticidal nets (LLINs) and indoor residual sprayings (IRS). Anopheles arabiensis, *a member species of* An. gambiae *s.l is the major malaria vector in Ethiopia, and* An. stephensi *is an invasive species detected in recent years mainly in the eastern part of Ethiopia. Insecticide resistance in malaria vectors is a huge challenge to the national malaria elimination programe. This study was conducted to investigate the insecticide resistance status and mechanisms of resistance in* An. gambiae *s.l. and* An. stephensi *in Ethiopia*. Anopheles gambiae *s.l. (from Jimma, Oromia) and* An. stephensi *(from Awash Sebat Kilo, Afar region) were reared from larvae and pupae. Three to five days-old female adults were tested with WHO-impregnated papers (deltamethrin (0.05%), alpha-cypermethrin (0.05%), pirimiphos-methyl (0.25%), bendiocarb (0.1%), propoxur (0.1%), and clothianidin (2%)) as per WHO standard procedure. Clothianidin (10µg/ml), chlorfenapyr (100µg/ml) and a potential candidate insecticide broflanilide were tested using CDC bottle bioassays.* Anopheles gambiae *s.l. member species mosquitoes were identified by species-specific polymerase chain reaction (PCR) and screened for the presence of target site mutations* L1014F *and* L1014S *in the voltage-gated sodium channel (VGSC) gene using allele-specific PCR. The data were analysed using SPSS version 20.The results of the study showed that* An. gambiae s.l. *populations were susceptible to propoxur and pirimiphos methyl but resistant against the other existing insecticides (bendiocarb, alpha cypermethrin and deltamethrin, clothianidin.* Anopheles stephensi *population from Awash 7 kilo was resistant to almost all tested insecticides, with probable resistance to clothianidin (97%).* Anopheles gambiae *s.l. and* An. stephensi *were fully susceptible to chlorfenapyr (100µg/ml) and clothianidin (10µg/ml). Moreover, both* An. gambiae *s.l. and* An. stephensi *were susceptible to broflanilide at doses of 50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml 1.562 µg/ml and 0.78 µg/ml. Of 120* An. gambiae*.s.l. specimens tested for species identification (ID PCR), 117(97.5%) were* An. arabiensis*. The* L1014F kdr *mutation was detected in* An. arabiensis *with frequencies of 22.8%. No L1014S mutation was detected in* An. arabiensis *at the study sites. This study provides information on the insecticide resistance status of* An. arabiensis *and* An. stephensi. Anopheles stephensi *was resistant to multiple insecticides.* Anopheles arabiensis *were susceptible to pirimiphos methyl and propoxur but resistant to the other insecticides. The West African kdr mutation (*L1014F*) was detected at low frequency, and the* L1014S *kdr was not detected in An. arabiensis. Therefore, the observed resistance in* An. arabiensis and An. stephensi *to multiple insecticides is a big concern and could deeply affect the sustainability of insecticide-based interventions strategies in Ethiopia*.

Keywords: Malaria, molecular identification, insecticide resistance, insecticide resistance mechanism, Ethiopia

1. INTRODUCTION

Malaria remains a leading global health concern with over 250 million cases reported yearly (WHO, 2018). Malaria is a complex disease caused by protozoan parasites (genus Plasmodium) and transmitted by blood-feeding infectious mosquitoes (Dutta and Dutt, 1978). Over 3,500 mosquitoes have been recorded worldwide (Fang, 2010; Harbach and Kitching, 2016); however, about 537 species are *Anopheles* (Harbach, 2013) and only 70–80 is known to transmit human malaria worldwide (Robert et al., 2011). Of these, 41 are considered to be dominant vector species (Hay *et al*., 2010; Sinka *et al*., 2012) and capable of transmitting malaria by large. In Africa, there are 140 *Anopheles* species, but only twenty are known to transmit malaria to human (Hay *et al*., 2000; Sinka *et al*., 2012).

Mosquitoes as a large group of arthropods that play an important role in the transmission of many diseases to humans such as malaria, filariasis, yellow fever, dengue fever (Horsfall 1955; Tabachnick 1991; Service 2003 and Azari, 2011).

In Ethiopia, malaria remains a major public health concern with millions of cases and thousands of deaths reported annually (WHO, 2019). Unlike most of the African continent, malaria is caused by infection with *Plasmodium vivax* or *P. falciparum*. Efforts to control the transmission of malaria currently target *An. arabiensis*, the primary malaria vector in Ethiopia, as well the secondary vectors *An. funestus*, *An. pharoensis*, and *An. nili* (Ghebreyesus, 2006). *Anopheles stephensi* is an important vector that plays a major role in transmitting malaria in urban areas and is an established vector in many parts of India. The first report of *An. stephensi* in the Horn of Africa was from Djibouti in 2013 (Faulde, 2014) and this mosquito was first detected in the Somali Regional State of Ethiopia in 2016 (Carter, 2018) and subsequently been confirmed to have a broad distribution in Northeast and eastern Ethiopia (Balkew, 2020).

Transmission of malaria can be reduced by adopting vector control measures such as indoor residual spraying (IRS) with insecticides, larval control measures, and personal protection measures. The combination of tools and methods used to combat malaria now includes insect nets treated with long-lasting insecticides (LLIT) and artemisinin-based combination therapy, supported by IRS of insecticide and intermittent preventive treatment during pregnancy (WHO.,2015).

Chemical control had been the main method for combating the adult stage of malaria vector since the eradication era. Insecticide application for adult mosquito control started with organochlorines (DDT, dieldrin, and BHC) during the 1960s, followed by organophosphates (malathion and pirimiphos-methyl) for 2 decades from 1966 and continued with the carbamate, propoxur during 1977-1990, and then with pyrethroids (lambda-cyhalothrin/deltamethrin). Temephos, chlorpyriphos-methyl, and pirimiphos-methyl were used for larviciding from 1970 to 1992. In Guinea *An. gambiae s.l.* was resistant to Pyrethroid, Alpha cypermethrin, lambdacyhalothrin and DDT but susceptible to deltamethrin (Keita, 2017). In Togo *An. gambiae s.l.* found resistant against bendiocarb, deltamethrin and propoxur (Amoudji, 2019). In Ethiopia *An. gambiae s.l.* was resistant to two groups of Pyrethroid insecticides (deltamethrin and alpha-cypermethrin), but susceptible to pirimiphos-methyl, propoxur and bendiocarb (Chanyalew, 2022). Resistance of *An. stephensi* to DDT, dieldrin, and Malathion was reported for the first time in 1957, 1960, and 1976, respectively (Hemingway, 1981). *Anopheles stephensi* in Ethiopia was highly resistant to DDT, malathion, pirimiphos-methyl, bendiocarb, propoxur, deltamethrin, and permethrin (Yared, 2020). In Awash Sebat Kilo Afar region (2019, 2020) the resistance intensity of *An. stephensi* to alpha-cypermethrin, deltamethrin and permethrin was assessed through exposure to 1×, 5× and 10× the diagnostic dose (Balkew, 2021).

The various mechanisms, including metabolic resistance and site insensitivity can cause insecticide resistance (Enayati, 2006; Soltani, 2014 and Gorouhi, 2016). Metabolic resistance mechanism is based on the enzyme systems which all mosquitoes possess to help them to detoxify naturally occurring insecticides. Three categories of enzymes, namely esterases, P450s and glutathione-S-transferases are known to confer resistance to insecticides in insect pest such as malaria vectors. In Ethiopia *An. arabiensis* very high frequency of the West African *kdr* allele (L1014F), was observed with higher *kdr* allele indicating that target site resistance mechanism might contribute for the observed high level of pyrethroids (deltamethrin and alpha-cypermethrin) resistance in the population(Chanyalew, 2022). In Afghanistan *An. stephensi*, general esterases (GES), glutathione *S*-transferases (GSTs), cytochrome P450s and insensitive acetylcholinesterase (AChEs) were implicated in insecticide resistance (Safi, 2017). General esterases are involved in OPs resistance in *An. stephensi* from Pakistan (Hemingway, 1982). WHO standard insecticide susceptibility bioassays have been performed on *An. stephensi* from Afghanistan showing resistance to

organochlorines, carbamates and pyrethroid insecticides (Moph, 2014; Barwa, 2011 and Ahmad, 2016). *Anopheles stephensi* from India had increased activities of esterases and GSTs associated with deltamethrin and permethrin resistance (Ganesh, 2003 and Ganesh, 2002). Insecticides generally act at a specific site within the insect, typically within the nervous system (e.g. OP, carbamate, DDT and pyrethroids insecticides). Knockdown resistance (*kdr*) mutation is widespread in *Anopheles* species in Africa especially *An. gambiae* (Aïzoun, 2014; Ibrahim, 2014; Camara, 2018 and Thiaw, 2018). Originally, the L1014F mutation (*kdr* west) was detected in 2000; a second *kdr* mutation (*kdr* east) was detected in Kenyan *An. gambiae* (Ranson, 2000). The first report of a *kdr* L1014F resistance mechanism in *An. stephens*i was in the DUB-S strain in 2003 (Enayati, 2003). The presence of *kdr east* in *An. stephensi* from India was reported for the first time by Singh *et al.* (Singh, 2011).

Currently, most resistance monitoring is dependent on bioassays, using fixed insecticide concentrations and exposure times, and the data is reported as percentage mortality and/or Knock down (KD) effect.

WHO recommends that insecticide susceptibility status of malaria vectors should be monitored annually (WHO, 2016 and WHO, 2018). When insecticide resistance is detected, its intensity and the biochemical and molecular mechanisms should also be investigated (WHO, 2018 and WHO, 2016). Accurate information on the underlying resistance mechanisms in *An. stephensi* is needed for proper management of insecticide resistance and a better management of malaria through vector control interventions. Therefore, the aim of this study was to determine the insecticide resistance status of *An. gambiae s.l.* and *An. stephens*i to different classes of insecticides and characterize molecular resistance mechanisms in *An. gambiae s.l.*

1.1. Statement of the Problem

Malaria is a vector-borne infectious disease considered as a priority of the World Health Organization due to its enormous impacts on global health. Mosquito-borne diseases are now resurgent, largely because of the insecticide resistance that has developed in mosquito vectors. The major mechanisms responsible for the development of insecticide resistance are increased metabolic detoxification of insecticides through gene overexpression/amplification, and structural mutations of P450, esterase, and GST genes, and decreased sensitivity of

target-site proteins (AChEs, sodium channels, and GABA receptors) to insecticides through structural modification and/or mutation (point mutation). Both LLINs and IRS are designed for indoor feeding and resting vector populations. So far, remarkable decline in malaria cases reported from Ethiopia. Ethiopia has planned to eradicate malaria by 2030. However, on top of the major malaria vector, *An. arabiensis*, the occurrence of the invasive *An. stephensi* in different parts of the country and its resistance to various classes of insecticides might cause a serious problem in the eradication of malaria from Ethiopia. Therefore, it is important to study the resistance status of this invasive species for the implementation of evidence based vector control management.

1.2. General Objective

- To assess insecticide resistance status and, characterize target site mutations, in wild populations of *An. gambiae s.l.* and *An. stephensi* from different parts of Ethiopia.

1.2.1. Specific objective

- ✓ To determine the susceptibility status of laboratory strain of *An. arabiensis, An. gambiae* s.l. *and An.stephensi* to various classes of insecticides.
- ✓ To identify member species of *An. gambiae s.l.* and *An. stephensi* using molecular assays.
- ✓ To screen target site mutations L1014F and L1014S in the *VGSC* gene of populations of *An. gambiae* s.l.

1.3. Significance of the Study

The outcome of this study provides baseline information on the susceptibility status of *An. gambia*e *s.l.* and *An. stephensi* to various classes of insecticides and resistance mechanisms in the populations of *An. gam*biae *s.l.* from Ethiopia. The data generated from this study provides evidence for various groups of stakeholders, decision-makers, and future researchers.

2. LITERATURE REVIEW

2.1. Biology of *Anopheles* Mosquitoes

The mosquito life cycle begins with an adult female laying an egg. Aquatic immature stages called larvae to emerge and develop through four molts (instars), increasing in size until the final molt when it reaches the non-feeding pupal stage inside the pupa the adult mosquito develops (either a male or female), and the terrestrial/aerial adult stage emerges from a split in the back of the pupal skin. The adult mosquitoes feed, mate and the female develop eggs to complete the cycle and begin the next generation. Some species of mosquito have only one generation per year. Others have several generations during a single season of favorable climatic conditions. Some continue to breed throughout the year but maybe more abundant in warmer seasons; this depends on the local environment, particularly temperature and rainfall (Baranitharan, 2018). Various factors like host preference, resting and feeding behavior, adult longevity and density, human biting rate, and host location strategy influence the role of mosquitoes in malaria transmission (Sindato, 2011).

2.1.1. Life cycle of *Anopheles* mosquitoes

Adult females lay 50–200 eggs per oviposition. Eggs are laid singly directly on the water and are unique in having floats on either side. The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. It can develop from an egg into an adult, which usually takes 10–14 days in tropical conditions (Gillies and de Meillon 1968). Their life span depends on temperature, humidity, and also their ability to successfully obtain blood meals while avoiding host defenses. The life cycle of a Mosquitoes includes four stages namely egg, larva, pupa, and adult. The duration of the life cycle depends on the abiotic factors (Baranitharan, 2018). The adult *Anopheles* female mates once and continues to lay eggs throughout its lifespan. The number of eggs laid by an adult female is approximately 50 to 200 eggs. Eggs are resistant to drying and within a period of 2 to 3 days, they start hatching. Although during winters or cold temperatures, the hatching process may boost up to 2 to 3 weeks.

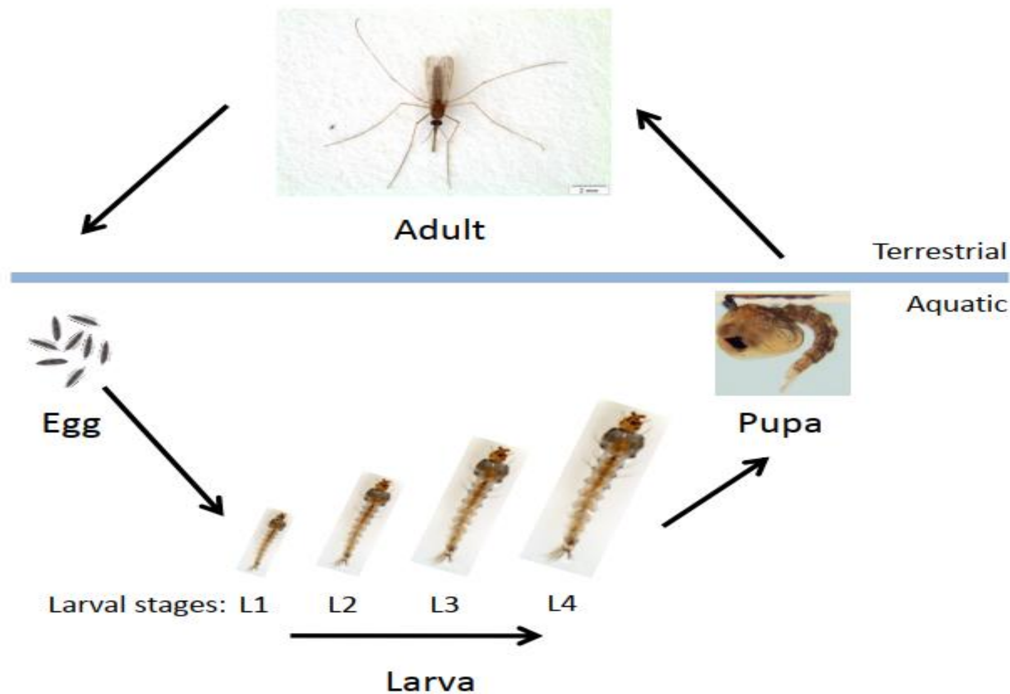

Figure 1.*Anopheles* mosquito life-cycle.

After the process of hatching, the next phase is the formation of mosquito larvae that is a well-developed head, mouth brushes, a large thorax, and an abdomen is visible without any legs up till now. The difference between other mosquitoes and *Anopheles* is that *Anopheles* does not have a respiratory siphon and hence their bodies are parallel to the surface of the water (Foster and Walker 2009). Spiracles are the only source for the breathing process for a larva. Spiracles are located on the 8th abdominal segment in their bodies. Larvae feed on algae, bacteria, and other microorganisms (Garros *et al.* 2008). *Anopheles* mosquitoes develop though four larval sizes or instars before pupating (Foster and Walker 2009). Larvae are found mainly in water marshes, mangrove swamps, rice fields, grassy ditches, streams, and rivers edges, small &temporary rain pools or pounds and artificial container. Pupae of all mosquitoes are comma-shaped when viewed from the side (CDC, 2010). In this phase, the head and thorax are much more visible and are under the abdomen covering. Similarly, like larvae, pupa also comes to the surface for breathing. After a few days, the whole covering splits and an adult mosquito emerges from it. The pupal stage is a non-feeding, mobile stage, during which the mosquito's adult body is formed.

Adult *Anopheles* has three sections like all other mosquitoes: Head, Thorax, and Abdomen. The head is specialized to get sensory information and for feeding. It contains the eyes and a pair of long many-segmented antennae. The head also has a long, straight-projecting proboscis necessary for feeding, and has two sensory palps. Both male and female adult mosquitoes feed on nectar from plants, but only the female feeds on blood of vertebrates, where she obtains nutrients for her eggs (Foster, 2009). Although adults can survive for up to one month in captivity, they usually survive around one to two weeks in the wild (CDC 2010). *Anopheles gambiae* have a variable body color, but it typically ranges from light brown to grey with pale spots of yellow, white or cream scales, and dark areas on their wings (Gillies and de Meillon 1968).

2.1.2. Breeding habitats and Biting activity of *Anopheles* Mosquitoes

Anopheles does not usually breed in swiftly moving streams or rivers, since larvae are not adapted to withstand wave action. But breeding sites can be as diverse as swamps, marshes, rice fields, temporary pools (puddles), ditches, drains, gulleys, rock-pools, tree holes, water storage containers and empty tins. *Anopheles arabiensis* breeding during the rainy season, rain pools and water bodies created by the seasonal Gash River serve as the main breeding sites (Hamza, 2016). Larvae of *An. stephensi* can be found in a wide variety of breeding habitats. In urban areas, they mainly breed in wells, cisterns, roof gutters, barrels, buckets, artificial containers and man-made ditches serve as the main breeding sites throughout the year (Kumar and Thavaselvam, 1992). *Anopheles gambiae s.l.* is considered highly anthropophilic and bite humans easily, mainly indoors (endophagic) but also outdoors (exophagic). The main biting activity occurs at night, and after blood-feeding, females leave (exophilic) or remain (endophilic) in these homes (Gillies, 1987 and Dadzie *et al*. 2013). Biting habits of *An. stephensi* actively pursues the host through the night and distinct peak activity is noticed in the early morning hours. However, some studies in Goa reported a peak activity during 2100 to 2400 h (Nandini, 2012). Multi-modal or arrhythmic biting habits are found with *An. stephensi*. The interesting finding regarding the biting behavior of *An. stephensi* is their preferences in a particular location can be influenced by the sleeping behavior of the host, microclimatic conditions, and the lunar cycle. They are crepuscular (active at dusk or dawn) or nocturnal (active at night). They feed indoors (endophagic) and or feed outdoors (exophagic) (Baranitharan, 2018).

2.1. 3. Host preference of *Anopheles* mosquitoes

Male mosquitoes feed on plant juices, nectar, and other sources of sugar while females feed only blood meal from humans (anthropophilic) and cattle (zoophilic). Mouthparts of a male mosquito are not developed for piercing and they do not suck blood but females have well-developed piercing and sucking types of mouthparts which are helpful for the blood-sucking behavior. *Anopheles gambiae* are considered to be one of the world's most important human malaria vectors because of their susceptibility to the *Plasmodium* parasite, their preference for humans as a host and their indoor-feeding behavior (CDC, 2010). Due to their short development time and their preference for developmental habitats near human dwellings, *An. gambiae s.l.* is considered effective vectors of human malaria, as well as lymphatic filariasis (elephantiasis). *Anopheles stephensi* are vector agent that carries disease-carrying viruses and parasites from person to person without catching the disease themselves. *Anopheles stephensi* can transmit the malarial parasite plasmodium (Baranitharan, 2018). The *An. stephensi* in Asia do feed on human and bovines, resting indoors and outdoors (Thomas *et al*, 2017)

2.1.4. Mating and resting behavior of *Anopheles* Mosquitoes

Adult mosquitoes usually mate within a few days after emerging from the pupal stage. In *Anopheles* the males form large swarms, usually around dusk, and the females fly into the swarms to mate. Resting places of adult mosquitoes choose to rest in houses, cattle sheds, barracks, etc., daytime resting of adults varies in the different geographical locations of the country and they typically rest inside the cattle shed and houses in large proportions. Adult mosquito rests near breeding site for a few hours after hatching. Exophilic mosquito also rests indoors for a short period of time before and after feeding. Adult mosquito disperses for feeding, finding suitable resting sites, mating and laying eggs. It can disperse through flight, air current or vehicles (Foster, 2009). *Anopheles stephensi* adults are influenced by temperature and humidity in the selection of resting place. Nightly collections of *An. stephensi* were significantly higher for indoor collections, suggesting a high degree of endophilic behavior.

2.1.5. Characteristic features of *Anopheles* mosquitoes

Anopheles can be distinguished from other mosquitoes by the palps, which are long as the proboscis and by the presence of discrete blocks of black and white scales on the wings. Adult *Anopheles* can also be identified by their typical resting position. Males and females

rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting. Adults *An. stephensi* morphologically identified to species using palps, wings, abdomen, and legs based on standard identification keys (Glick, 1992 and Gillies, 1987). *Anopheles stephensi* female wings are lateral with four dark bands on both costa and sub costa, ASP spot present. *Anopheles stephensi* female of hind femur, tibia and tarsomere 1 dark and speckled with pale scales. *An. stephensi* Female of Hindtarsomere 3 & 4 not entirely white, 5 entirely dark. Thorax Lateral View: *An. Stephensi* Female thorax lateral Character Upper proepisternal seatae absent (Faulde, 2014)

2.2. Habitat of *Anopheles* mosquitoes

Larvae of *Anopheles* mosquitoes can be found in a wide variety of breeding habitats. The *An. gambiae s.l.* breeding habitats can have varying sizes of water bodies that are natural or man-made, permanent or temporary, freshwater or saline (Rejmánková, 2013). *Anopheles gambiae s.l.* larval habitats usually occur near human habitation, and are often subject to anthropogenic activities (Munga, 2013). Larval habitat conditions of *An. stephensi* are primarily Heliophilic but known to be Heliophobic as well. Mostly freshwater but known to be salt water tolerant. Primarily clear water but found in turbid water as well. Primarily still water but known from some flowing water sources. Found in water with emergent vegetation and no vegetation. The ability of the larval stages of *An. stephensi* to develop in urban areas, Larval Habitat types are artificial containers such as domestic wells, overhead water tanks, room coolers, cisterns and roof gutters and in water bodies in construction sites and other industrial localities, brings malaria transmission into densely populated areas including the major cities of India such as Delhi (Batra, 1979 and Batra, 2006).

Adult *An. stephensi* are predominantly endophilic and endophagic, however are known to feed on animals and humans outside under warmer conditions. There is evidence implicating this species as primarily anthropophillic with opportunistic zoophilic behavior (Thomas *et al*, 2017). Changes that occur in the environment especially in climate have a great bearing on breeding habitats of different mosquito species that influences the population density of adult mosquitoes (Bashar, 2013).

In rural areas, the larvae of *An. stephensi* may exist in many aquatic habitats, such as ponds, streams, swamps, marshes, and other sources of standing water (Foley, 2008) They may also

occupy smaller environments, such as tree holes, leaf axils, and man-made containers. (Harbach, 2007). The larvae of *A. s. mysorensis* exclusively prefer to occupy stone pots and earthenware containers (Sinka, 2011). This species is also able to endure high levels of salinity, and have been found to breed readily in water where the salinity is equal to or even surpassing that of seawater (Sinka, 2011). Furthermore, *An. stephens* breeds in a number of different water-bodies in urban areas, but predominantly in artificial containers, walls, overhead tanks, and ground-level water tanks (Jeyabalan, 2003).

Most larvae feed on microorganisms and particle matter suspended in water (Harbach, 2007). However, later in development, adult males feed on the nectar of flowers, whereas females take blood meals, which help produce viable eggs. *Anopheles stephensi* larvae have been found indoors, larvae are typically found outdoors. In urban areas, *An. stephensi* can be found all year long, however, peak activity occurs during the summer months (June to August). Adult *An. stephensi* are predominantly endophilic and endophagic, however are known to feed on animals and humans outside under warmer conditions. There is evidence implicating this species as primarily anthropophilic with opportunistic zoophilic behavior (Wolff, 2018).

2.3. Seasonal Abundance of *Anopheles* mosquitoes

Seasonal abundance of *An. gambiae s.l.* varies depending on location, but generally the population decreases during the dry season and peaks during the wet season (Gillies and de Meillon 1968, Yaro *et al*. 2012). Populations begin to increase as the rainy season commences, peak in mid-season, and decline as water levels stabilize and aquatic predators establish themselves (Gillies and de Meillon, 1968). The type form is predominantly an urban mosquito while *mysorensis* is largely rural in distribution. In southern India, more number of *An. stephensi* was recorded in April and May than the other months though breeding facilities existed throughout the year. *Anopheles stephensi* populations were highest during the rainy and summer seasons. Especially in Pondicherry well breeding is found in most months of the year except monsoon seasons. In all mainland zones of the country, the presence of both types of form and variety is noticed. The *type* form is domestic in all seasons of the year whereas the *mysorensis* variant occupies the outdoor niche during monsoon and post-monsoon seasons, with spillover into domestic sites during the summer-ecological stress period. During the monsoon period (July–September) *mysorensis* variant occupies outdoor

niches and is zoophagic. However, in urban areas throughout the country, both the variants occupy domestic habitats and are involved in the transmission of malaria.

2.4. Vector Control

Awareness of the epidemiology of vectors and vector-borne diseases is necessary to develop integrated control measures needed to reduce the impact of vector-borne diseases (WHO. 2015) generally; the main goal of any control operation is downsizing the vector population, which in turn reduces the transmission rate. Malaria control is based on two pillars of rapid diagnostic tests and artemisinin-based combination therapies, and indoor vector control by using ITNs and IRS. Consequently, residual malaria transmission, defined as the forms of transmission that can persist after achieving full universal coverage with effective ITNs and IRS (Killeen, 2014), can be maintained due to mosquito behavioral variation, for example, malaria vectors biting mainly outdoors or when people are active. Thus, even an improved coverage and efficacy of IRS and ITNs will not achieve malaria elimination across most of the tropics due to a non-negligible proportion of vectors avoiding fatal contact with these interventions (Killeen, 2014). The residual transmission also maintains the possibility to cause a resurgence of malaria when the vector control program is weakened or withdrawn.

Five classes of insecticides are currently recommended for vector control measures: carbamate, organochlorines, organophosphates, neonicotinoids, and pyrethroids, with only the latter being approved for use in ITNs (WHO, 2019). Following reports of insecticide resistance in malaria vectors, routine resistance monitoring has been set up in several countries

In urban areas, control of *An. stephensi* induced malaria is primarily dependent on anti-larval methods and space spraying of insecticides. However, it has developed resistance to DDT, Malathion, and dieldrin in many areas of the country. This resistance may be due to the use of these insecticides in indoor residual spraying activity by the National vector-borne disease control Programme in rural areas. Even though DDT and HCH are not directly used against this vector in urban areas, their use in peri-urban and rural areas has induced resistance in this species. In Mangalore city of Karnataka, it has been found resistant to malathion, but susceptible to DDT and permethrin. Resistance to DDT was reported in Kerala, Rajasthan, Gujarat, and Madhya Pradesh (Singh, 2014, Dykes, 2016 and Hariprasad, 2016). In Rajasthan however, this species is susceptible to malathion. *Anopheles stephensi* resistance to different

classes of insecticide poses threat shortly that it would increase in the fold and expand throughout the country and in addition causing serious failure in vector control programs.

The species is resistant to multiple insecticides as a result indoor residual spraying is not used for control. Instead recommended control measures are (i) source reduction, (ii anti-larval methods including chemical and biological larvicides, (iii) application of larvivorous fish, i.e., guppy and gambusia, (iv) aerosol space spraying for control of adult vector populations and (v) legislative bylaws for preventing mosquito breeding (National Vector Borne Disease Control Programme, 2012). In the face of rapid urbanization, unplanned growth and mushrooming of urban slums, rationed water supply, and unsafe water storage practices; urban malaria is a growing problem presently accounting for >10% of reported malaria cases in the country (Akhtar, 2010). The first report of *An. stephensi* in the Horn of Africa was from Djibouti in 2013 (Faulde, 2014) and was recently confirmed to be persisting in the country (Seyfarth, 2019). *An. stephensi* was detected in Ethiopia for the first time in 2016 in Kebri Dehar (Somali Region) but it remains unclear how broadly distributed the species is in the rest of the country (Carter, 2018). The high susceptibility of this species to *Plasmodium falciparum* and its tolerance to urban habitats may challenge the global malaria control and elimination programs in the future (WHO. 2018

2.5. Insecticide Resistance in *Anopheles* mosquitoes

According to the WHO, resistance is defined as the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations (Davidson, 1957). Resistance has been observed in more than 500 insect species worldwide among which more than 50 *Anopheles* species (Diptera: *Culicidae*) are responsible for the transmission of malaria parasites to humans (Hemingway, 2000). Resistance is a heritable character that relies on a genetic basis. Resistance results from the selection of a genetic modification in one or several genes occurring by migration and/or mutation. Based on WHO criteria that suggested (98–100% mortality indicates susceptibility, 90–97% mortality indicates resistance candidate (more investigation is needed), and less than 90% mortality suggests resistance (WHO, 2013). Insecticide resistance to a range of insecticides in *An. arabiensis* has been reported in several different countries in Africa (Yewhalaw, 2011 and Balkew, 2021). In Sudan Malathion, DDT, dieldrin, bendiocarb, deltamethrin, and permethrin were reported (Abuelmaali, 2013 and Yagoop, 2013). In Jaffna

(Sri Lanka), they invaded *An. stephensi* population is highly resistant to DDT (4%), malathion (5%) and deltamethrin (0.05%) (Surendran, 2019). In Afghanistan, there was evidence of resistance in *An. stephensi* to DDT, malathion, bendiocarb, deltamethrin, and permethrin in 2011 (Ahmad, 2015). Resistance to DDT, dieldrin, and malathion is reported in *An. stephensi* in Iran, Iraq, Saudi Arabia, and the Indian subcontinent (Busvine, 1969 and Abai, 2008).

In Ethiopia, *An. stephensi* from Kebri Dehar town site were resistant to insecticides of all major classes. Mortality after exposure to the discriminating concentrations of malathion, pirimiphos-methyl, bendiocarb, propoxur, permethrin, and deltamethrin were 32%, 14%, 23%, 21%, 53%, and 67%, respectively, revealing relatively high resistance to all those insecticides (Yared, 2020).

2.6. Insecticide resistance mechanisms

The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories including metabolic resistance, target-site resistance, reduce penetration and behavioral avoidance. Resistance can involve several physiological and/or behavioral changes.

2.6.1. Target-Site resistance

During target-site resistance, the binding site of an insecticide is modified (mutated) or lost and catalyzing the target-site is incompatible for activation (Bass, 2011). Changes in the insecticide target site that reduce its binding to insecticides (known as target site resistance) are the best-understood type of resistance mechanism and molecular diagnostics to detect this resistance mechanism are integrated into insecticide resistance monitoring strategies in malaria control programs of some malaria endemic countries (Sharp *et al*, 2007 and Ridl, 2008). Target-site insensitivity results from the structural modification or mutation (point mutation) of genes encoding target proteins that interact with insecticides (Casida, 2005). Insecticides such as dichlorodiphenyltrichloroethane (DDT) and pyrethroids target sodium channels. After binding to the sodium channels, they cause the insect's nervous system to repetitively discharge and its nerve membranes to depolarize (Narahashi, 1988), resulting in death. Acetylcholinesterase (AChE) is a key enzyme in the nervous system, hydrolyzing acetylcholine neurotransmitters and terminating nerve impulses, and is the target for both OP

and carbamate insecticides. Cyclodiene and fipronil insecticides target the γ-aminobutyric acid (GABA) receptors (Cole, 1993 and 1995, and Cuamba , 2010).

2.6.2. Metabolic resistance

Metabolic resistance is the detoxification of chemicals/insecticides that occurs in insects. The over-production of some enzymes breaks down insecticides before reaching and binding the target sites. The over-produced enzymes in insect have the capacity to develop protection against insecticides (WAR *et al,* 2012 and RANE, 2016). Enhanced insecticide metabolism that lowers the amount of insecticide reaching the target site (known as metabolic resistance) is more complex but recent advances have identified key enzymes responsible for insecticide detoxification, paving the way for the development of molecular markers for this type of resistance mechanism (Stevenson, 2011 and Mitchell *et al* ,2012). The detoxification of insecticides in mosquitoes involves three major metabolic detoxification gene families: cytochrome P450s (P450s), esterases, and glutathione S-transferases (GSTs). P450s are one of the largest gene families in all living organisms and fulfill a highly diverse group of physiological and biochemical functions. P450s are critical for the detoxification and/or activation of xenobiotics and endogenous compounds (Feyereisen, 2005). GSTs are soluble dimeric proteins that are involved in the metabolism, detoxification, and excretion of a large number of endogenous and exogenous compounds (Ranson, 2005 and Ketterman, 2011). A significant feature of insect P450s and GSTs is their transcriptional up regulation, which results in increased levels of protein production and enzymatic activities. This in turn enhances the metabolic detoxification of insecticides and plant toxins in insects and leads to the development of insecticide resistance (Scott, 1999 and Feyereisen, 2005) and tolerance to plant toxins (Schuler, 2007 and Li 2007). The amplification/duplication of genes encoding P450s has also been reported to be involved in the development of insecticide resistance (Riveron, 2012). Esterase, a group of heterogeneous enzymes, are present in most organisms. Their overproduction has been studied extensively, as the amplification and/or occasional overexpression of esterase genes increases the production of detoxification proteins (Raymond, 1998).

2.6.3. Reduced Penetration

Cuticular resistance is mediated by a thickening of or a change of chemical composition of insect cuticle to reduce penetration of insecticides into the insect body. Reduced penetration

in turn can facilitate the action of metabolic enzymes by providing more time for detoxification as well. Cuticular resistance is considered to confer cross-resistance to multiple insecticides since most of the insecticides are lipophilic in nature (Scott, 1989 and Nkya *et al*., 2013).

2.6.4. Behavioral resistance

Insecticide resistance in mosquitoes is not always based on biochemical mechanisms such as metabolic detoxification or target site mutations, but may also be conferred by behavioral changes in response to prolonged exposure to an insecticide. Behavioral resistance does not have the same "importance" as physiological resistance but may be considered to be a contributing factor, leading to the avoidance of lethal doses of an insecticide (Roberts, 1997 and Chandre, 2000)

3. METHODOLOGY

3.1. Study area

The study was conducted at Awash Sebat kilo and Jimma towns. Awash Sabat kilo is located in Afar region, 220 km south-east of Addis Ababa. The town's geographical coordinates are Latitude: 8.989149" N Longitude: 40.164715 " E. The town is found at an average altitude of about 916 m above sea level. The town has an estimated total population of 24,700; the semi-arid climate is dominated by a major rainy season (July-August) and short intermittent rains (April/May), average temperature of 25.8^{o}C (17.3^{o}C-33.6^{o}C). The Awash River Valley is the most irrigated area in the country with extensive river-fed agricultural production. Malaria transmission is perennial in the area surrounding the town with annual parasite incidence of 536 per 1000 population in 2019 (five-year trend summarized in supplemental notes) (Tadesse, 2021). Entomological surveys conducted in 2018 detected the occurrence of *An. stephensi* in Awash Sebat Kilo town (Balkew, 2020).

On the other hand, Jimma town is located 353km south-west of Addis Ababa. The town's geographical coordinates are approximately 7°41' N latitude and 36° 50'E longitude. The town is found in an area of average altitude of about 1780 m above sea level. It lies in the climatic zone locally known as Woyna Daga which is considered ideal for agriculture as well as human settlement. The town is generally characterized by warm climate with a mean annual maximum temperature of 30°C and a mean annual minimum temperature of 14°C. The annual rainfall ranges from 1138 mm to 1690 mm. Maximum precipitation occurs during the three months period, June to August, with minimum rainfall in December and January. From a climatic point of view, abundant rainfall makes this region one of the best watered of Ethiopian highland areas, conducive for agricultural production. Bacho bore Kebele is relatively malarious and has abundant mosquito breeding habitats. The area is malaria endemic with moderate transmission and the principal malaria parasites are *P. falciparum* and *P. vivax. An. arabiensis* is the major malaria vector in the area.

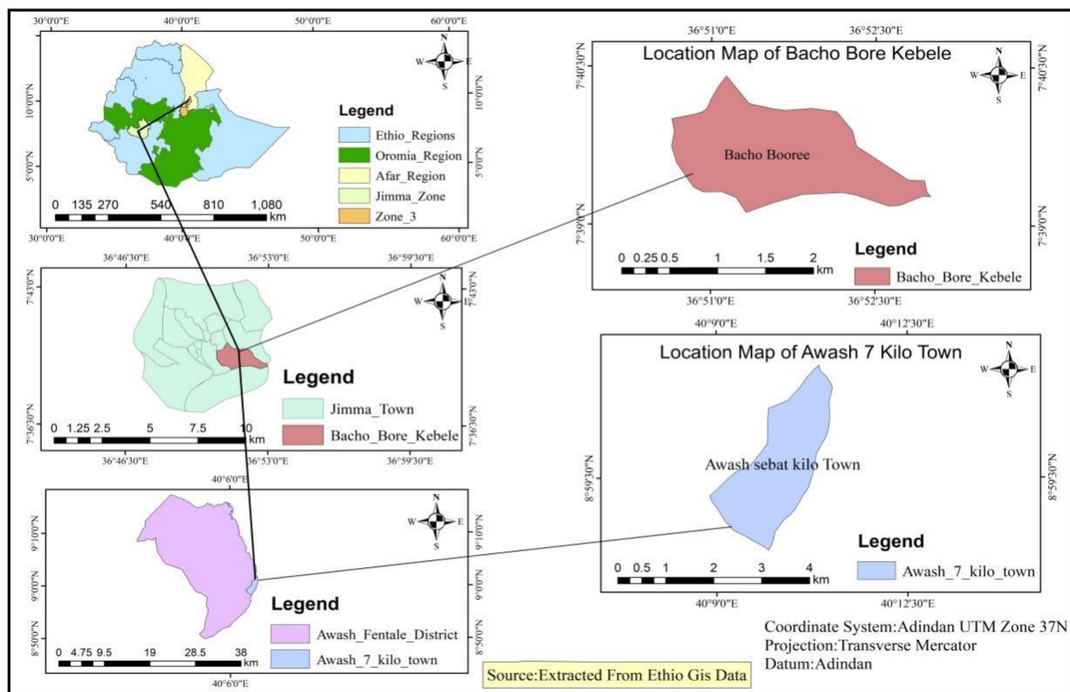

Figure 2. The map of study sites

3.2**. Mosquito collection and identification**

From May 2022 to February 2023, wild *Anopheline* larvae and pupae were collected by dipping in Jimma town, Bocho Bore Kebele, and Awash Sebat Kilo town, from various natural habitats and man-made places, respectively. Trained entomology technicians collected and reared the immature mosquitoes. *Anopheles arabiensis a* susceptible strain was obtained from the Tropical and Infectious Disease Research Centre at Jimma University. *Anopheles gambiae* s.l. larvae were collected from natural habitats including swamps, marshes, rice fields, temporary pools (puddles), ditches, drains, gulleys, rock pools, tree holes, water storage containers, and empty tins in Bacho bore Kebele Jimma town, Oromia region, Ethiopia. *Anopheles stephensi* larvae were collected from artificial habitats, including domestic wells, overhead water tanks, room coolers, cisterns, roof gutters, plastic containers, irrigation ditches, and water bodies in construction sites and other industrial localities in Awash sabat Kilo town, Afar region, Ethiopia. The characteristic of larvae breeding site was annotated, namely: Geographic location (GPS coordinates, name of the locality), type of breeding site (permanent, semi-permanent, temporary), origin of the water (*e.g.* rain, river, lagoon, man-made), nature of the water collection (*e.g.* puddle, rice field, ditch), exposure to sunlight (shaded, sunlit). Presence of vegetation (emergent, submerged, floating), characteristics of the water (*e.g.* clear, turbid, polluted, dark).

Plastic container containing larvae from a breeding site were labeled with the number of the breeding site that was annotated in the notebook. Mosquito larvae was transported to the insectary room and reared to adults feeding them yeast powder and sailfarm food supplements. The immature mosquitoes were kept at 25 ± 2 °C, 12:12 light: dark photo cycle, and relative humidity at 75 ± 10 %. Pupae were transferred into adult emergence cages and after two to three days, the pupae emerge to adults and the cages were put in a safe place protected from contamination and ants. The Collected mosquitoes were identified morphologically (Coetzee, 2020).

3.3. Insecticide Susceptibility Tests

3.3.1. WHO Tube test

Susceptibility test was done using WHO tube test following standard protocol (WHO, 2013). Briefly, the mosquitoes were exposed to papers impregnated with a lethal concentration (discriminating dose) of a given insecticide. The WHO tube test kit is made up of two plastic tubes that are 44mm in diameter and 125mm long. One end of each tube has a 16-mesh screen. One of the tubes is marked with a red dot and is used as exposure tube as it is lined with insecticide impregnated filter paper held in place with two copper clips. The other tube, with a green dot, serves as a holding tube, which has its inner walls lined with plain paper held in place by two steel clips. The holding tube is attached to a slide unit which has a 20mm hole through which mosquitoes are introduced using as aspirator. Connect the holding tube with slide unit.

Blood unfed 3-5 days old female *An. gambiae s.l.*, *An. stephensi* and laboratory strain *An. arabiensis* were used for the bioassays. A total of 100 wild mosquitoes of each species were tested in four replicates for each insecticide. For each insecticide, two replicate of control was also run in parallel. The insecticide impregnated papers used in the study were pirimiphos-methyl 0.25% (organophosphate), 0.1% propoxur and bendiocarb (carbamate), 0.05% deltamethrin and alpha-cypermethrin (pyrethroids) and 2% clothianidin (neonicotinoids). The assay was conducted following WHO standard protocol (WHO, 2013). Mosquitoes surviving insecticide exposure were provided with 10% sucrose solution in WHO holding tubes, kept in holding room at 27±2°C and 80±10% relative humidity for 24 h. Mortality was determined by counting the dead and alive mosquitoes at the end of the 24 h post exposure. Then, dead mosquitoes were scored and percent mortality was calculated as follows.

$$\% \text{ Mortality} = \frac{\text{Total No. of dead mosquitoes}}{\text{Total mosquitoes exposed}} \times 100$$

All survived and dead specimens by the WHO tube test were kept individually in Eppendorf tubes over silica gel for further molecular assays.

Resistance was defined according to WHO guideline which suggests that 98–100 % mortality indicates susceptibility, 90–97% indicates the possibility of resistance that needs to be confirmed, and < 90 % indicates resistance (WHO 2013).

3.3.2. CDC bottle bioassay

3.3.2.1. CDC bottle bioassays for clothianidin and chlorfenapyr

The CDC bottle bioassay relies on time mortality data, which are measures of the time it takes an insecticide to penetrate a vector, traverse its intervening tissues, get to the target site, and act on that site. CDC bottle bioassays for three insecticides (different concentration of broflanilide, 100 µg/bottle of chlorfenapyr and 10 µg/bottle of clothianidin) were conducted according to established guidelines (Brogdon, 2013). Chlorfenapyr Stock solutions were prepared by diluting technical grade insecticide in 50 mL of acetone. Clothianidin Stock solutions were prepared by diluting technical grade insecticide in 50 mL of Acetone + MERO solution (800ppm), making sure that bottles and caps were completely dry. Caps were removed from the bottles. Disposable pipettes were used; label one pipette as 'solvent only' for the control bottle, and another pipette as 'insecticide solution' for the test bottles. One ml of acetone or meron + acetone was added to the control bottle. In the first test bottle 1ml insecticide stock solution was added and similar trend was repeated for the other three test bottles. Swirled the contents inside the bottle so that the bottom is coated and inverted the bottle and twisted the bottles to coat the inside of the cap. Gently rotated while rocking the bottle so that the sides all the way around were coated. The caps were removed and continue rolling bottles on their side until all visible signs of the liquid are gone from inside and the bottles are completely dry. Left bottles on their sides and covered with papers that kept them protected from light.

3.3.2.2. CDC bottle bioassay Serial dilution for Broflanilide

Broflanilide insecticide Stock solution was prepared taking 2mL of Acetone + MERO solution (800ppm) and added to a glass beaker. One mg technical grade broflanilide was

added red into the bottle containing the acetone + MERO solution (800ppm). Tightly closed the lid and mixed carefully until no solid particles can be seen in solution. These create a solution with a broflanilide concentration of 500µg/ml. Nine mL of Acetone + MERO solution (800ppm) was added to a new glass beaker**.** Using a pipette removed 1mL of dilution series stock solution (500µg/mL) and added it to the new glass beaker with 9mL of Acetone + MERO solution**.** Tightly closed the lid and mixed to create a 10mL solution with a broflanilide concentration of 50µg/ml.

The Wheaton bottles were laid in a row in order of dilution (the first bottle is the most concentrated and the last bottle is the least concentrated) and loosen all the lids to facilitate adding the delivery volume. Wheaton bottles were kept in a row on a table in order and coded A, B, C, D, E, F and G (fig. 3).

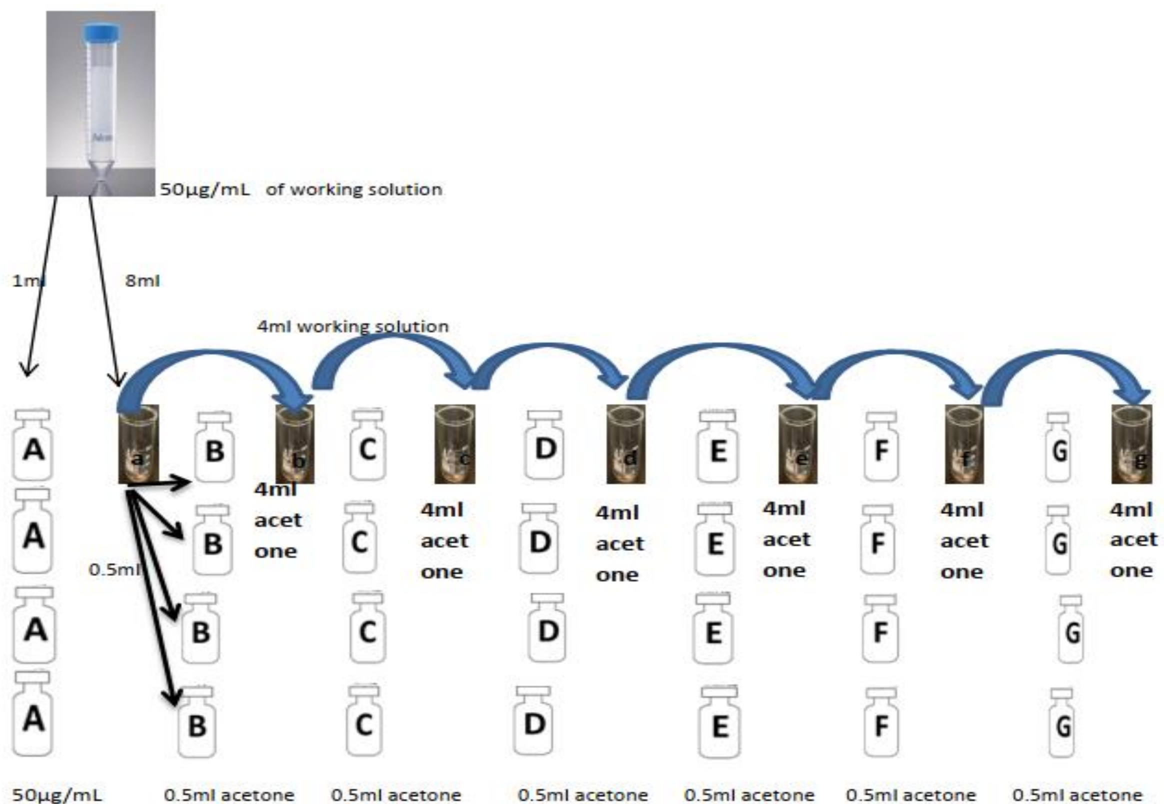

Figure 3. Serial dilution of Broflanilide insecticide

From total mosquitoes, 2400 female were selected for broflanilide insecticide serial dilution test. Using an aspirator, 25 non-bloods fed 3-5 days old adult mosquitoes were introduced, into the control bottle (two replicate). Twenty-five non-blood fed mosquitoes were introduced

into each insecticide dose bottle (four replicated) using a mouth aspirator and mortality was recorded at 15 min intervals in 60 min exposure time.

3.4. Molecular identification and detection of target site mutations

3.4.1. DNA extraction

Whole genome mosquitoes were extracted using DNAzol reagent (MRCgene, USA) following established protocol (Asghar, 2015). Extraction was made on individuals (50 surviving and 10 dead mosquitoes) exposed to deltamethrin and 10 from the control. Mosquitoes were individually grounded in 100µl of DNAzol reagent. The resulting thicker mixture was incubated for 2-3 minutes and then centrifuged at 13,000 rpm for 20 min (4°C). Two hundred µl 100% ethanol were then added to the supernatant (later transferred in new Eppendorf tube 1.5 ml) and the mixture was centrifuged again at 13,000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was rinsed with 100 µl 70% ethanol. Dried pellets were re-suspended in 100 µl TAE buffer. The DNA was used for species ID and genotyping target site resistance mutations.

3.4.2. Molecular identification of *Anopheles gambiae s.l.* and *An. stephensi* using PCR

Molecular identification of the *An. gambiae s.l.* was carried out by species-specific polymerase chain reaction (PCR) following established protocol (Safi, 2019). PCR using species-specific primer pairs was used to confirm identification. Based on the PCR identification methods, from the total tests of *An. gambiae s.l.* and *An. stephensi* female, 120 females *An. gambiae s.l.* and 39 *An. stephensi* were randomly selected for molecular identification. *An. gambiae s.l.* 50 alive and 10 dead in the deltamethrin test and 10 females in each insecticide tested used as control were selected for molecular identification was considered as representative of the mosquito population being tested. *An. stephensi.* 30 alive and 5 dead in the deltamethrin test and 4 females in insecticide tested used as control were selected for molecular identification was considered as representative of the mosquito population being tested. Molecular species identification and kdr allele detection was conducted at the Tropical and Infectious Diseases Research Centre (TIDRC) of Jimma University. DNA was re-suspended in 25 ml sterile TE-buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). Molecular identification of *An. gambiae s.l.* was carried out by species-specific PCR using primers for *An. gambiae s.l.,* and *An. arabiensis* laboratory strain, according to Scott *et*

al. (Scott , 1993), with modifications (Yewhalaw 2011, Fettene,2003). Briefly, genomic DNA was mixed with primers AR (5′-AAGTGTCCTTCTCCATCCRA-3′; specific for *An. arabiensis*), AG (5′-CTGGTTTGGTCGGCACGTTT-3; specific for *An. gambiae*.), QD (R, 25 pmol/µl) [CAGACCAAGATGGTTAGTAT] specific for *An. quadriannulatus* and UN (5′-GTGTGCCCCTTCCTCGATGT-3′; common for all species) in a 25 µl reaction volume. Amplification reactions contained 1 µl of DNA, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100, 200 µM of dNTPs (Amersham, Buckinghamshire, United Kingdom), 25 pmol of primers AR, AG, QD-b and UN and 0.25 U of SilverStar DNA polymerase (Eurogentec, Seraing, Belgium) (Verhaeghen , 2006). PCR reaction conditions are described (Scott *et al.* 1993 and Wilkins *et al.* 2006). Amplified PCR products were visualized on 1.5% agarose gels, stained with ethidium bromide. After PCR identification of the species the specimens were used for *Kdr* test.

For the polymerase chain reaction (PCR) species ID of *An. stephensi* for the I*TS2* assay, a 400–500 bp region including the gene was PCR amplified using primers from Singh (2022). For 1 Reaction volume, genomic DNA was mixed with primers (0.5 µl of forward primer (of 10 mol *ITS2A*), 0.5 µl of reverse primer (of 10 mol *ITS2B*), 9.5 µl of PCR Nuclease free water, 12.5 µl of Master mix (Hot start 2X master mix), and 1-2 µl Template DNA. The PCR reaction condition was denaturation at 95 °C for 30 s, 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; followed by 72 °C for 5 min. Amplificon was subjected to electrophoresis on 2% agarose gels.

3.4.3. Detecting resistant alleles (L1014F and L1014S) in *An.arabiensis*

Detection of the *kdr* allele was carried out using allele-specific PCR following a standard protocol (Singh, 2011). To find out if target-site mutation was responsible for resistance in the mosquitoes, after the WHO tube assay were performed; PCR genotyping of *kdr* and *ace-1* was carried out. A total of 58 female *An. arabiensis* from the dead and alive specimens were genotyped for *kdr*. The allele specific PCR procedure for *kdr* genotyping was intended to detect the West African *kdr allele,* L1014F, using the protocol and primer sequence described earlier (Martinez-Torres *et al.* (1998). The L1014F gene mutation was the only one analysed because it is the most common in West Africa, whereas the *L1014S* mutation is confined to eastern Africa (Santolamazza, 2008). Allele specific (AS) PCR (a conventional PCR) was chosen for *kdr* mutation detection in *Anopheles* mosquitoes, although real time (RT) PCR is the most sensitive and specific assay to use. This PCR was, however, chosen on the basis of

its relatively lower cost and reports of a small number of failed reactions and incorrect scores (Bass, 2007). The primers AgD1 (5′-ATA GAT TCC CCG ACC ATG-3′) and AgD3 (5′-AAT TTG CAT TAC TTA CGA CA-3′) amplified the resistant allele, yielding 195bp fragments. The susceptible allele was assayed using primers AgD2 (5′-AGA CAA GGA TGA TGA ACC-3′) and AgD4 (5′-CTG TAG TGA TAG GAA ATT TA-3′), which amplified a 137bp fragment. The primer set AgD1 and AgD2 amplified a common fragment of 293 bp for control. The reaction condition of the *kdr* PCR was as follows: denaturation at 94 °C for 3 min followed by annealing; 35 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 10 s). The extension was set at 72 °C for 5 min. Amplified PCR products were visualized on 2% agarose gels, stained with ethidium bromide.

3.5. Data analysis

The WHO bioassay knockdown was recorded every 10 min for 1 h and final mortality was recorded at 24 h for all test runs with corresponding negative and positive controls. In the case of mortality, when the control mortality was between 5% to 20% it was corrected by Abbott's formula (Abbott, 1965). Error bars for each mortality were calculated based on the statistical method at α=5%. The lethal Time for 50% and 90% mortality (LT50 and LT90) values and their 95% confidence intervals, as well as probability regression line parameters, were determined with the Finney method, and then the regression line of all Insecticides was plotted using Microsoft Excel. The molecular analysis data was calculated based on the frequency of *kdr* mutations in wild *An. arabiensis* populations from dead or alive specimens following bioassays. Hardy Weinberg equilibrium software was used for analysis between different *kdr* genotypes (Livak, 1984).

3.6. Ethical Considerations

An ethical letter was obtained from Jimma University College of Natural Sciences Department of Biology for the concerned bodies (Bacho-bore Kebele and Awash 7 Kilo Town) for data collection from the community.

4. **RESULTS**

4.1. Immature mosquito collection and species identification

A total of 6150 female *Anopheles* mosquitoes were reared from larvae and pupa collected from Bacho bore Kebele, Jimma town Oromia region, and Awash 7 kilo Town, Afar region, Ethiopia. The breeding sites of *An. gambiae s.l.* were predominantly natural habitats and depend on seasonal conditions (Palate-1a). The breeding sites of *An.stephensi* were artificial habitats (Plate- 1b). According to morphological identifications, each *Anopheline* larvae species is identified morphologically during collection. Anopheline larvae do not possess a siphon and they rest parallel to the water surface).

Table 1. Characterization of *Anopheles* mosquitoes breeding habitats in Jimma and Awash 7 kilo towns, Ethiopia

S/N	*Breeding site*	*An. gambiae s.l.*	*An. stephensi*
1	Geographic location	Jimma Town (Bacho Bore)	Awash 7 kilo town, Afar
2	Type of breeding site	temporary	Permanent or semi-permanent
3	Origin of the water	rain	man-made
4	Nature of the water collection	Puddle, ditch	Ditch, container, over tank
5	Exposure to sunlight	shaded, sunlit or partial light	shaded, sunlit or partial light
6	Presence of vegetation	emergent, submerged, floating	floating
7	Characteristics of the water	Clear, turbid	clear

a.

Plate 1. Breeding habitats of *Anopheles* mosquitoes (a. habitat of *An. gambiae s.l.* at Bacho bore Kebele, Jimma Town, Oromia region, Ethiopia b. Habitat of *An. stephensi* at Awash 7 Sebat Afar region, Ethiopia)

All adult *An. gambiae s.l.* and *An. stephensi* reared from larvae and pupa in the laboratory morphologically identified using standard key (Glick, 1992)(Fig.4).

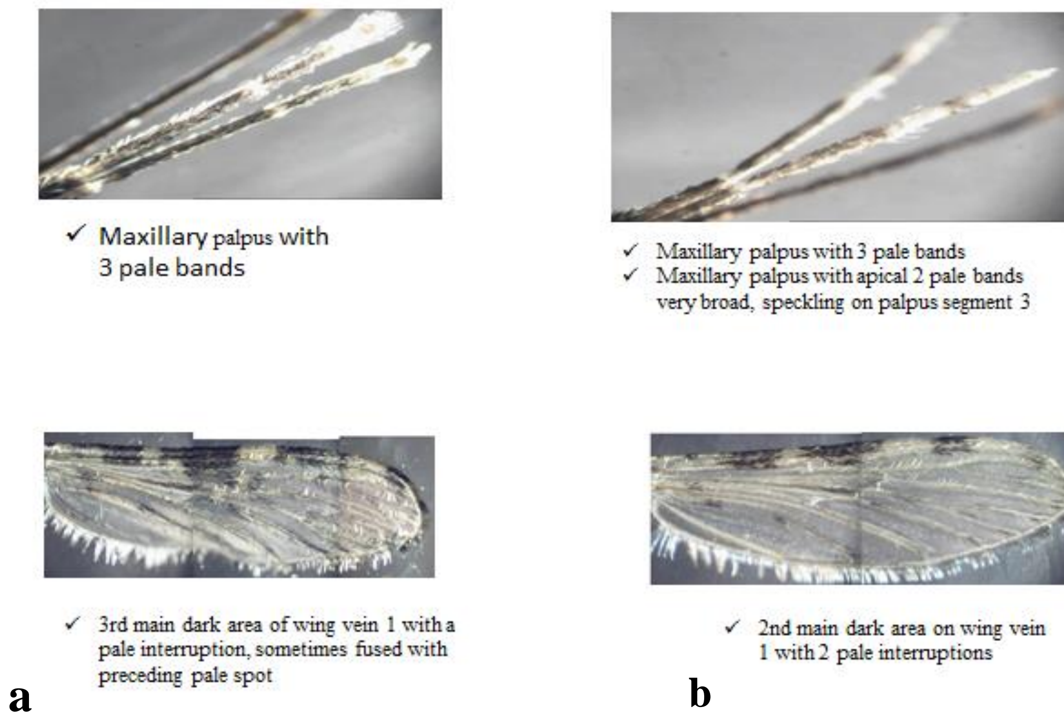

Figure 4. Morphological identification of adult *An. gambiae s.l.* and *An. stephensi*

4.2. Bioassays

4.2.1. WHO tube test

A total of 900 *An. gambiae s.l.* (n = 600 exposed and n = 300 control) were tested for susceptibility against pirimiphos methyl 0.25% , bendiocarb 0.1%, deltamethrin 0.05% , alpha-cypermethrin 0.05%, clothianidin 2% and propoxur 0.1%.

Plate 2. Insecticide susceptibility test using WHO Tube test

Based on the WHO criteria, *An. gambiae s.l.* from Jimma area displayed resistance to four of the six tested insecticides: pyrethroids (deltamethrin and α-cypermethrin), carbamate (bendiocarb) and neonicotinoid (clothianidin). Mortality rates ranged between 18% and 73%, which are far below the susceptibility threshold of 90%. For pyrethroids insecticides, the mortality rates were 18% for deltamethrin and 43% for α-cypermethrin. The mortality rate was 63% for bendiocarb and 73% for clothianidin. *An*opheles *gambiae s.l.* species were susceptible to propoxur and pirimiphos methyl (fig.5). From the result of *An. gambiae s.l.* susceptibility between insecticides, pyrethroids (deltamethrin and α-cypermethrin) have high resistance.

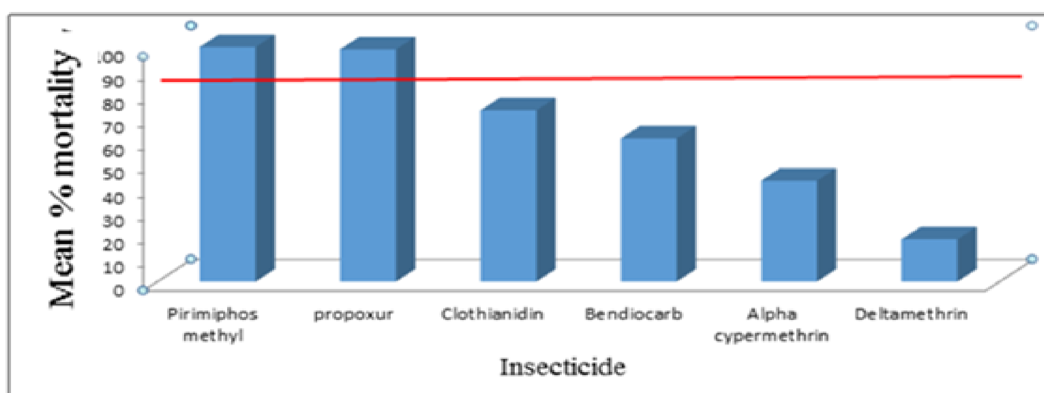

Figure 5. Mean mortality rates of *An.gambiae* s.l using WHO tube test (Pirimiphos methyl(0.25%) propoxur(0.1%), Clothianidin(2%), Bendiocarb(0.1%), Alpha cypermethrin (0.05%) and Deltamethrin (0.25%) *(solid line shows cutoff point as per WHO, 2016).*

The population of *An. stephensi from* Awash 7 Kilo Town displayed resistance to all the insecticides. The mortality rates of *An. stephensi* after 24 hours post-exposure were 97%, 31%, 23%, 9%, 8%, and 7% for clothianidin, propoxur, pirimiphos methyl, alpha cypermethrin deltamethrin, and bendiocarb, respectively (Fig. 6). Considering the possible resistant threshold of a mortality rate above 90%, population *An. stephensi* from Awash 7 kilo showed possible resistance to candidate clothianidin (97% mortality). *Anopheles stephensi* has high resistance to all insecticide groups except clothianidin in our study compared to *An. gambiae s.l.*

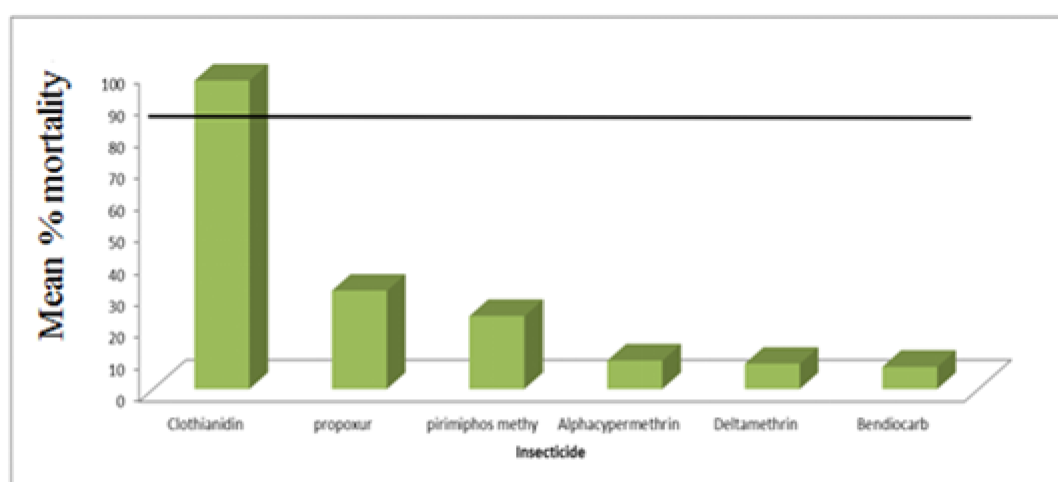

Figure 6. Mean mortality rate of *An. stephensi* using WHO tube test *(solid line shows cutoff point as per WHO, 2016)*

4.2.2. CDC bottle bioassay for chlorfenapyr and clothianidin

A total of 100 adult female *An. gambiae* s.l. and *An. stephensi* were exposed to chlorfenapyr (100µg/ml) and observed that all mosquitoes were fully susceptible to the insecticide Similarly, female *An. stephensi* exposed to clothianidin (10µg/ml) and female *An.gambiae s.l.* exposed to clothianidin (4µg/ml) were fully susceptible (Fig.7). The results compared from the WHO test (diagnostic dose) for clothianidin, *An. gambiae s.l.*, and *An. stephensi* population resistance, but the CDC bottle bioassays for clothianidin, *An. gambiae s.l.,* and *An. stephensi* population were fully susceptible.

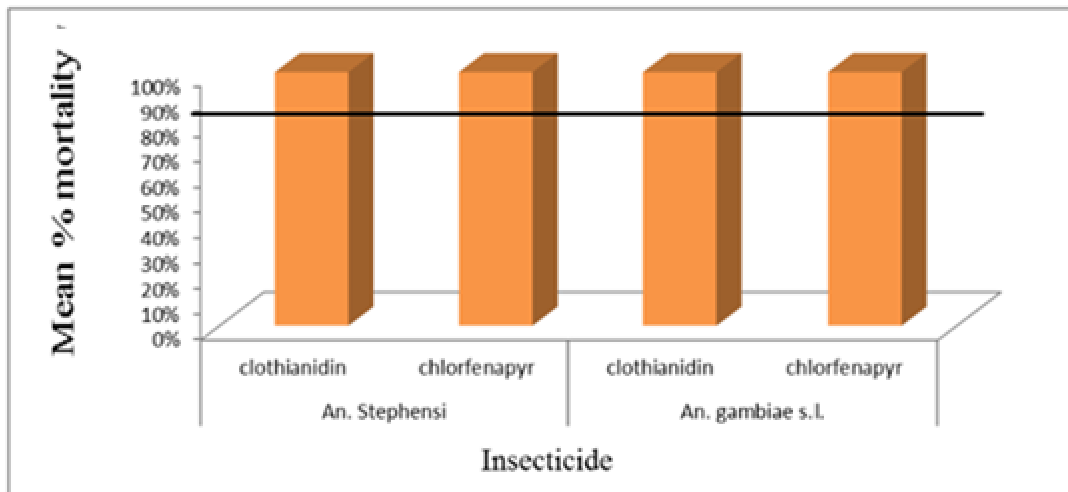

Figure 7. Mean mortality rates of *An.gambiae* s.l., and *An. stephensi* 24 exposed to Chlorfenapyr and Clothianidin using CDC bottle bioassay *(solid line shows cutoff point as per WHO, 2016)*

4.2.3. CDC bottle bioassay test for serial dilution of broflanilide

A total of 700 adult female *An. gambiae* s.l. was exposed to new candidate insecticide, broflanilide of different dose (50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml 1.562 µg/ml and 0.78 µg/ml) and observed that all the exposed mosquitoes died 24 hr post exposure (Fig.8)

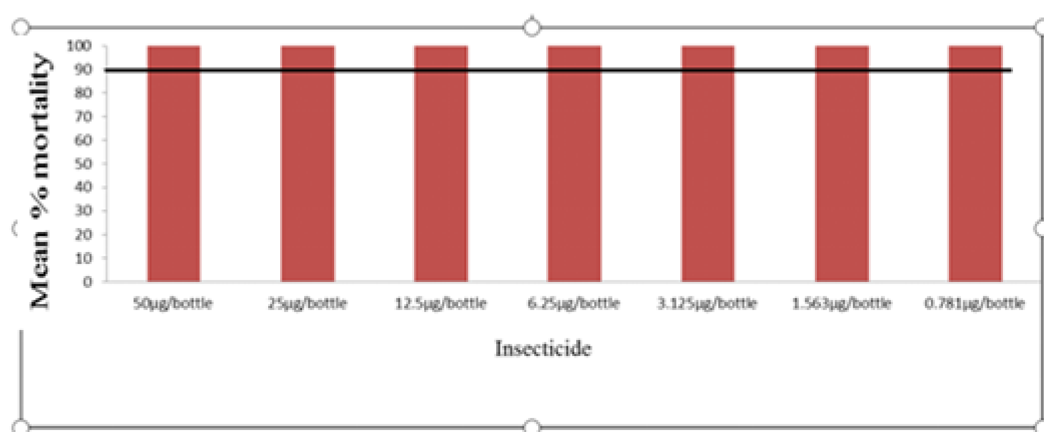

Figure 8. Mean mortality rates of *An.gambiae* s.l. 24 hr post-exposure to broflanilide using serial dilution of CDC bottle bioassay test *(solid line shows cutoff point as per WHO, 2016)*

A total of 700 adult female *An. stephensi* were exposed to a new candidate broflanilide (50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.563 µg/ml and 0.78 µg/ml) and observed that all mosquitoes were fully susceptible to the insecticide, but the last two concentrations were 99% susceptible (Fig. 9). In contrast, in both the CDC bottle bioassay discriminate dose (chlorfenapyr and clothianidin) test and the CDC bottle bioassay serial dilution for broflanilide test, *An. gambiae s.l.* and *An. step*hensi were fully susceptible except for the last two concentrations (1.563 µg/ml and 0.781 µg/ml) in *An. stephensi* (99%), *Strains of An. arabiensis* were fully susceptible to all insecticides tested. From the result, a high concentration (50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml) is more susceptible than the last two concentrations.

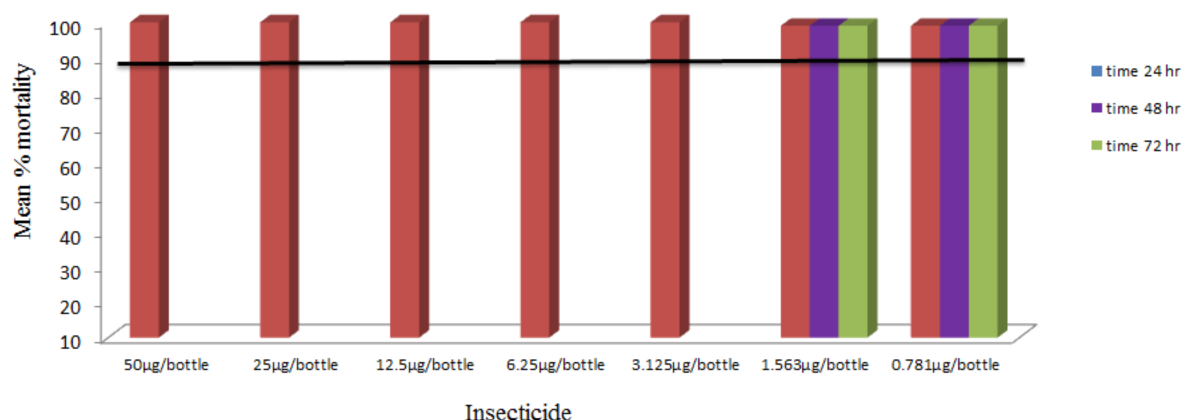

Figure 9. Mean mortality rates of *An.stephensi* 24 hr. post-exposure to broflanilide *by* serial dilution of CDC bottle bioassay test *(solid line shows cutoff point as per WHO, 2016)*

A total of 700 adult female *strain of An.arabiensis* was exposed to broflanilide (50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml 1.562 µg/ml and 0.78 µg/ml) and observed that all mosquitoes were fully susceptible to the insecticide (Fig.10)

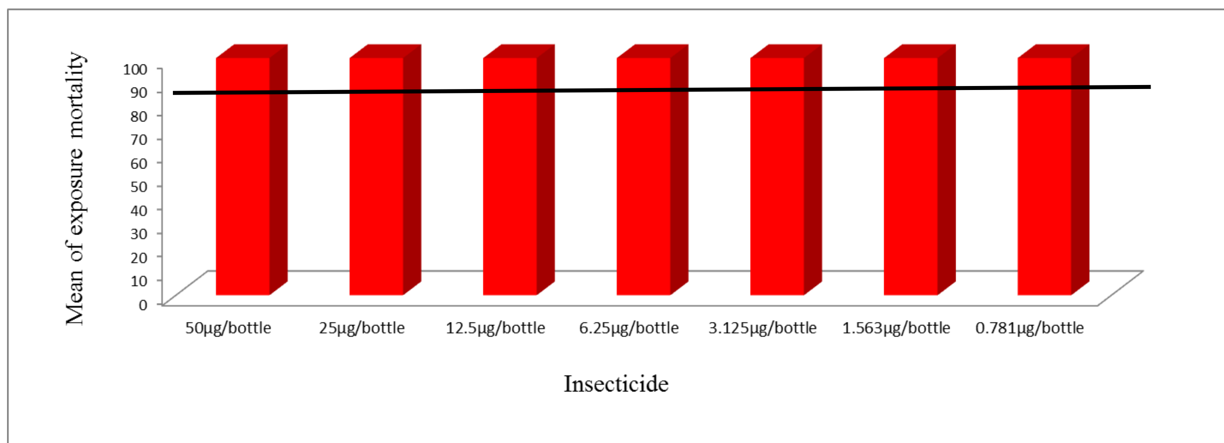

Figure 10. Mean Mortality rates of *An.arabiensis* 24hr post-exposure to broflanilide using serial dilution of CDC bottle bioassay test (*solid line shows cutoff point as per WHO, 2016)*

4.3. Molecular identification of *An. gambiae s.l.* and *An. stephensi* using PCR

A total of 117 (97.5%) of *An. gambiae s.l.* were successfully amplified and all were *An. arabiensis* (Fig.11).

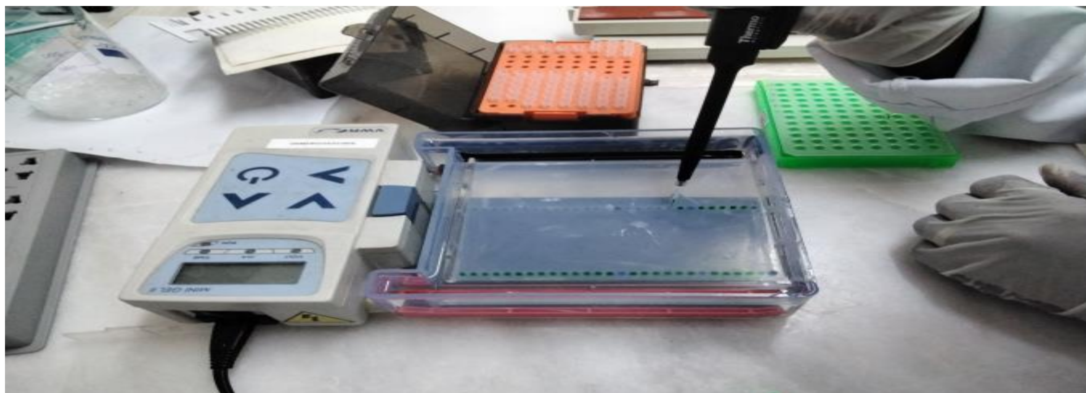

Plate 3. Molecular identification of *Anopheles* mosquitoes using PCR

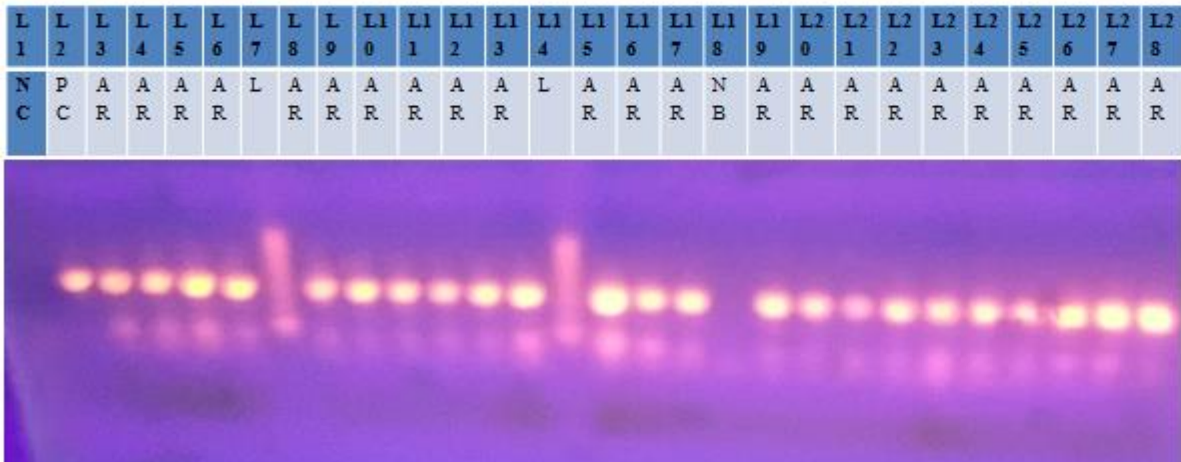

Figure 11. Agarose gel showing the amplification products generated from the As- PCR using DNA extracted from individual mosquitoes of known species. L1-L28: lanes, NC: negative control, PC: positive control, AR: *Anopheles arabiensis* and L: 100 base pair (BP) DNA ladder

Of the 900 female *An. stephensi* used for the phenotypic detection of insecticide resistance, 39 were used for molecular identification. Of these, 16(41.02%) were determined to be *An. stephensi* and the remaining 23 (58.97 %) were not amplified (Fig.12).

Figure 12. Molecular identification of *An.stephensi* using PCR; NC: negative control, PC: positive control, An.st: *Anopheles stephensi*, NO: No band and L: represent 100 base pair (BP) DNA ladder.

4.4. Detecting resistant alleles (L1014F and L1014S) in *An.arabiensis*

The L1014F *kdr* (West Africa *kdr*) mutation was detected in *An. arabiensis* in Bacho bore kebeles study sites with frequencies 22.8 %(13 heterozygotes and 0 homozygotes)(Fig.13). Out of the 57 specimens analyzed 22.8 %(13/57) were heterozygous (RS). No homozygous resistant (RR) detected. The mutation frequency of *kdr* (L1014F) is shown in (table 2). No *Kdr* east (L1014S) mutation was detected *An. arabiensis* in the study sites.

Table 2. Summary of L1014F *kdr* genotype frequency in survived and dead *An. arabiensis* exposed to pyrethroids, RR: homozygous resistances: heterozygous resistance and SS: homozygous susceptible from Bacho bore kebeles, Jimma town Ethiopia.

An.arabiensis	Number tested	RS	RR	SS	*Kdr* allele frequency
Dead	8	0	0	8	0
Alive	49	13	0	36	0.26
total	57	13	0	44	0.26

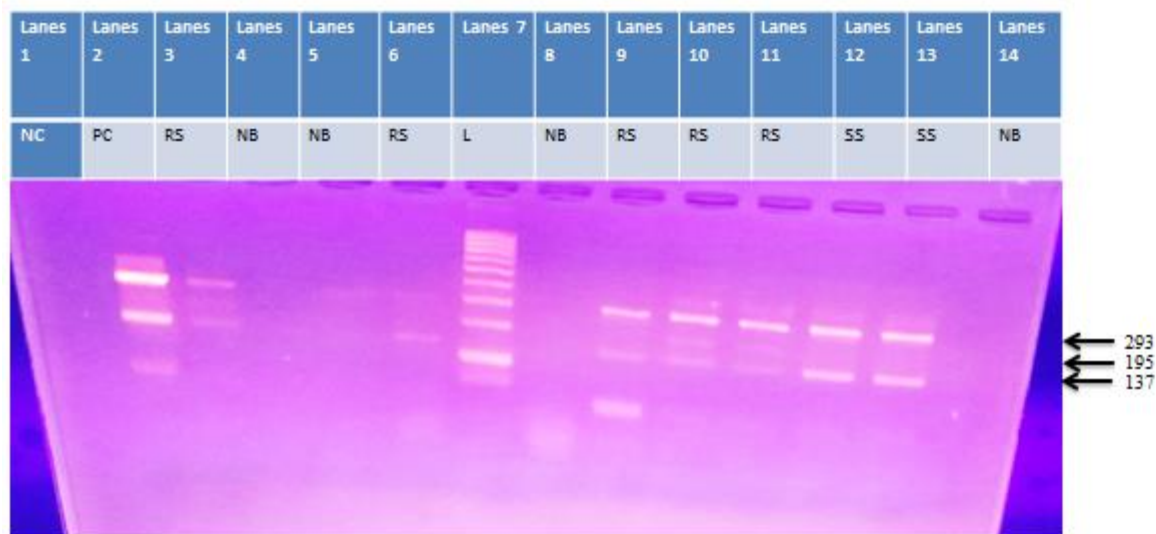

Figure 13. Agarose gel of the West *Kdr* of amplified PCR of *An. arabiensis*; NC: negative control, PC: positive control, RS: heterozygote resistance, SS: homozygote susceptible, NB: No band and L: represent 100 base pair (BP) DNA ladder.

5. DISCUSSION

This study was carried out to determine the levels of susceptibility of *An. gambiae s.l. and An.stephensi* population from Ethiopia to organophosphate, carbamate, pyrethroids, neonicotinoids, isoxazolines (broflanilide) and pyrroles (chlorfenapyr) insecticides. A number of genetic, physiological, behavioral, and ecological factors, as well as the quantity and frequency of insecticide applications, play a direct and indirect role in the development of insecticide resistance (Zhu *et al.,* 2016). In this study, as per the WHO-impregnated paper test, the procedural test showed that *An. gambiae* s.l. populations have full susceptibility to organophosphate (pirimiphos methyl) insecticides, which were collected from Bacho bore Kebele, Jimma town. This study is more in line with the previous reports from Gambella, Southwestern Ethiopia (Chanyalew, 2022), Sudan (Korti, 2021), Burkina Faso (Soma, 2021), and Ghana (Baffour, 2016), in which *An. gambiae* s.l. was fully susceptible to pirimiphos-methyl, but it contradicts the study conducted in Western Kenya (Kitungulu, 2022), because, the species showed great resistance to these insecticides. The results of this study suggest that the susceptibility of *An. gambiae* s.l. to insecticides may vary depending on location and environmental factors.

Additionally, the *An. gambiae s.l.* population showed full susceptibility to propoxur but resistance status to bendiocarb insecticides. According to research reports from Lake Tana, northwest Ethiopia, and Faranah, Guinea, the majority of *An. gambiae* s.l. population showed comparable resistance to insecticide bendiocarb (Kendie, 2023; Stica, 2019). However, research done in south-eastern Benin showed that wild populations of *An. gambiae* s.l. were completely susceptible to bendiocarb, with a 100% mortality rate recorded. This study did not find this to be the case (Sovi, 2020).According to the present findings, in the pyrethroid insecticide class, both deltamethrin and alpha-cypermethrin resistance were observed i*n An. gambiae* s.l. similarly, a research report from Gambella, Southwestern Ethiopia, showed that *An. gambiae* s.l. has demonstrated potential candidate resistance to both deltamethrin and alpha-cypermethrin (Chanyalew, 2022).

These varying results suggest that the resistance of *An. gambiae* s.l. to bendiocarb insecticides may be location-specific and influenced by local factors such as insecticide usage and mosquito control practices. Further research is needed to fully understand the extent and causes of this resistance in different regions. Besides these, the inhibition of acetylcholinesterase by the

insecticides results in continuous stimulation of the post-synaptic nerve membrane, leading to the death of the mosquitoes. Alteration in AChEs in resistant mosquitoes results in a decreased sensitivity to inhibition of the enzymes by these insecticides. This can lead to reduced efficacy of insecticide-based interventions and the need for alternative control strategies. Additionally, understanding the genetic basis of resistance can inform the development of new insecticides or genetic modification techniques to combat resistant populations.

On the other hand, *An. gambiae* s.l. resistance to the neonicotinoids class, including clothianidin insecticide. In disagreement with this study, *An. gambiae* s.l. showed full susceptibility to Clothianidin insecticide, as per the previous report from Faranah, Guinea (Stica *et al*, 2019). These findings suggest that the resistance of *An. gambiae* s.l. to insecticides is not uniform across different classes and locations. Therefore, it is important to regularly monitor and evaluate the effectiveness of insecticides used for malaria vector control in different regions.

In a WHO-impregnated paper test, *An. stephensi* populations showed tolerance resistance to clothianidin, but in another insecticide, resistance to alpha-cypermethrin, deltamethrin, propoxur, pirimiphos-methyl, and bendiocarb was found in Awash, who died in the 7 kilo Afar region, Ethiopia. In Kebri Dehar town, Somali region, Ethiopia reports that *An. stephensi* resisted bendiocarb, deltamethrin, propoxur, and pirimiphos-methyl insecticide (Yared, 2020). The current results are higher than the resistance reported in the previous study conducted at Awash Sebat Kilo, Meki, Metehara, and Godey in Ethiopia in 2020. The study found that *An. stephensi* exhibited resistance to the three pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin), bendiocarb, propoxur, and pirimiphos-methyl (Balkew, 2021). In contrast, *Anophelese stephensi* is much more resistant to insecticides than *An. gambiae* s.l.. This defense against numerous. This resistance to multiple insecticides is concerning as it may limit the effectiveness of current malaria control strategies.

All *An. gambiae* s.l. and *An. stephensi* were fully susceptible to the insecticides candidate clothianidin (10%), chlorfenapyr (100%), and new candidate broflanilide (different doses) in the CDC bottle bioassay test. The WHO standard operating procedure for testing the insecticide susceptibility of adult mosquitoes in the CDC bottle bioassay protocol changed the insecticide concentration used in this test in January 2022. Based on this standard operating procedure, insecticide bottle bioassays with clothianidin (10 g/ml) and chlorfenapyr (100 g/ml) were carried out at both sites and on species at full susceptibility. Report on

widespread full susceptibility to chlorfenapyr (100 g/ml) in Africa (Tchouakui, 2023). This change in concentration was made to ensure that the results obtained are more accurate and reliable. The report on widespread full susceptibility to chlorfenapyr in Africa highlights the need for continued monitoring and evaluation of insecticide resistance in mosquitoes.

The study's findings raised questions about the comparability of the CDC bottle and WHO-impregnated bioassay tests for detecting insecticide resistance. The results of the clothianidin-impregnated paper test were superior in both areas of resistance compared to the WHO-impregnated paper test. For the prevention of the *Anopheles* mosquito species, the CDC bottle bioassay test was more crucial. The discriminating dosages are set at twice the insecticide dose in the CDC bottle bioassay test, which results in 100% mortality of the least susceptible *Anopheles* mosquitoes, which was recorded. All *An. gambiae* s.l. and *An. stephensi* mosquitoes in study areas are fully susceptible to the insecticides clothianidin and chlorfenapyr, and the insecticide doses are adjusted to control resistant mosquitoes. This is one of the strategies for controlling mosquitoes that depend on resistance. In both instances, the insecticides used to control mosquitoes are still active; chlorfenapyr insecticide in WHO-impregnated paper was not tested. In Ethiopia, there are more insecticide-resistant mosquito species. Increasing the insecticide's concentration will reduce this problem.

However, in WHO-impregnated paper tests with all species in all sites, resistance to clothianidin (2%) was recorded within 24 hours. In contrast, complete mortality was observed in the CDC bottle bioassay test with all species in all sites to clothianidin (10%) and chlorfenapyr (100%) within 24 hours. The broflanilide insecticide test was very effective at controlling *An. gambiae* s.l. and *An. stephensi* in the CDC bottle bioassay test. Ethiopian mosquito populations of *Anopheles* are not tested with this insecticide. All concentrations of the broflanilide insecticide (50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml, 3.125 g/ml, 1.562 g/ml, and 0.78 g/ml) cause fully susceptibility in all species, but the last two concentrations only in *An. stephensi* susceptibility. This suggests that broflanilide may be a promising insecticide for controlling *An. stephensi* populations, particularly in areas where this species is the primary vector of malaria transmission. Further research is needed to determine the efficacy of broflanilide against other mosquito species and in field settings.

Anopheles arabiensis, the main malaria vector, was tested in this study for both East and West African *kdr* in Bacho bore kebeles Jimma town, Ethiopia. No East African *kdr* mutation

was found in the current study. Only L1014F *Kdr* west causes the substitution in *An. arabiensis.* There were none of the *Kdr* east (L1014S) mutations found in *An. arabiensis*. The *Kdr* east (L1014S) mutation in *An. arabiensis* does not result in a Leu to ser substitution. Contrary to the earlier report from eastern Ethiopia, a very low frequency of the West African *kdr* mutation was seen (Carter, 2022). Although I concur with the report from Santiago, Cabo Verde, regarding the prevalence of the West African *Kdr* mutation, it disagrees with the report regarding the East African *Kdr* mutation. For the observed high or low frequency of the kdr mutations that have programmatic implications, analysis of the *kdr* trait variation provides crucial evolutionary historical context. Deltamethrin resistance levels remained low, indicating that resistance is only provided by the comparatively feeble *kdr* mechanism. The phenotypes of *An. arabiensis* in this study demonstrated high deltamethrin insecticide resistance but genotypically low *Kdr* mechanisms. There are probably other mechanisms at play in the development of resistance, especially given the lack of association between mosquito bioassay mortality and the presence or absence of a *Kdr* mutation.

Due to a lack of primer, *An. stephensi Kdr* mutation was not completed. To fully comprehend the profile of insecticide resistance in Ethiopia, more research on resistance is required. A thorough resistance management plan should now be created to address potential issues moving forward, before resistance issues start to reduce the effectiveness of malaria control, in light of the findings of the development of insecticide resistance in *An. arabiensis* at these sites. Future research should examine the biochemical makeup and susceptibility of mosquitoes from more locations, examine samples for evidence of additional resistance mechanisms, and use CDC bottle bioassays to assess the "intensity" of *Anopheles* species resistance to various insecticide doses.

6. **CONCLUSION and RECOMMENDATION**

6.1. Conclusion

This study provides up-to-date details on the prevalence of insecticide resistance in the study region of Ethiopia among the malaria vector species *An. gambiae* s.l. and *An. stephensi*. According to this study, *An. stephensi* in the Afar Region's Awash Sebat Kilo Town was almost invulnerable to every test currently in use. However, pirimiphos-methyl and propoxur were found to be effective against *An. gambiae* s.l. Chlorfenapyr and broflanilide were effective against *Anopheles stephensi*, but the mosquito displayed potential clothianidin resistance. *Anopheles gambiae* s.l. was however sensitive to clothianidin, broflanilide, and chlorfenapyr. The findings of this study suggest that insecticide resistance in *An. stephensi* is a major concern in the study area and requires immediate attention. Further research is needed to determine the mechanisms of resistance and develop effective control strategies. The observed insecticide resistance in *An. gambiae* s.l. and *An. stephensi* is therefore a major concern and may have a significant impact on the long-term viability of insecticide-based intervention strategies in Ethiopia. In order to choose appropriate insecticides and other complementary techniques for vector control interventions toward the eradication of malaria, it is necessary to regularly monitor insecticide resistance. This will help to identify any changes in resistance patterns and inform the development of new strategies that can effectively control the spread of malaria. It is also important to consider alternative approaches, such as genetic modification of mosquitoes or the use of biological control agents, to ensure long-term success in the fight against malaria.

6.2. Recommendation

An insecticide resistance management strategy should be used in the study areas due to the high levels of *An. stephensi* population resistance as well as the presence of resistant *An. gambiae* s.l.. For the design and execution of malaria vector control strategies in Ethiopia, these findings are crucial. Future research should be done in larger areas to properly assess the effectiveness of the candidate insecticides (clothianidin and chlorfenapyr) and the new candidate insecticide (broflanilide), which showed higher efficacy in populations of *An. stephensi* and *An. gambiae* s.l. in the study sites. Moreover, it is important to investigate the underlying mechanisms of resistance in these mosquito populations to inform the

development of new control methods. Additionally, community engagement and education programs should be implemented to ensure the sustained success of vector control efforts.

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