EFFICACY OF CHLORFENAPYR AND CLOTHIANIDIN INSECTICIDES ON ANOPHELES MALARIA VECTOR POPULATIONS OF NYANDO, BUMULA AND NDHIWA SUB-COUNTIES, WESTERN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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DEDICATION

This work is dedicated to my family especially my wife Elizabeth Wanga and my mother Isdorah Agumba for their moral support and encouragement.

ABSTRACT

Malaria is a global health problem resulting in 435,000 deaths annually with 90% of the deaths occurring in sub-Saharan Africa. Over 70% of the Kenyan population is at risk of malaria and western Kenya is an endemic region with prevalence of 38%. The *Anopheles gambiae* complex and *Anopheles funestus* are the main vectors of human malaria in Africa with *Anopheles gambiae sensu stricto* and *Anopheles gambiae arabiensis* being the main vectors in western Kenya. *Anopheles gambiae sensu stricto* and *Anpheles arabiensis* are the predominant species in Bumula and Nyando respectively, while *Anopheles funestus* is the main vector in Ndhiwa, western Kenya. However, there is widespread resistance to the insecticides used in malaria vector control, with reports of vectors population upsurge in western Kenya. Hence there is need to evaluate alternative insecticides for mosquito control. Chlorfenapyr and Clothianidin insecticides are non-repellent, slow acting toxins that have been shown to be effective against other insects in experimental studies. This study evaluated the efficacy of chlorfenapyr and clothianidin insecticides against *Anopheles* malaria vectors of Nyando, Bumula and Ndhiwa areas. Specific objectives were to determine the diagnostic doses of chlorfenapyr and clothianidin on laboratory reared *Anopheles gambiae sensu stricto,* Kisumu strain, and to determine the susceptibility status of *Anopheles* malaria vectors of western Kenya. CDC Bottle bioassay was used to determine diagnostic doses using laboratory reared *Anopheles gambiae,* Kisumu strain, as well as susceptibility of wild mosquitoes, following WHO guidelines. A total of 6000 adult Kisumu strain female mosquitoes were exposed to a series of concentrations of each insecticide (ranging between 0-100µg/ml for chlofenapyr and 0-250µg/ml for clothianidin) for 1 hour, in four replicates. Mosquito deaths were recorded after 24_h, 48_h and 72-h recovery period respectively and survival curves were made for each insecticide to determine the diagnostic dose. For susceptibility test, both indoor collected mosquitoes and larvae samples were transported to KEMRI-CGHR insectary. Larvae reared to 3-5 days old while adult mosquitoes were allowed to rest for 48 hour before being aspirated into control bottles and test bottles coated with the determined diagnostic doses of chlorfenapyr and clothianidin for a 1 hour exposure period. The mortality rates were calculated as a percentage of individual mosquitoes that died within 72_h recovery period. Convectional PCR was used for species identification. The diagnostic doses of chlorfenapyr and clothianidin were 50 µg/ml and 150µg/ml respectively. All mosquitoes species were highly susceptible with 100% mortality at diagnostic doses of 50 µg/ml and 150 µg/ml for chlorfenapyr and clothianidin, respectively, within 72-h recovery period. The mean mortality of chlorfenapyr was 95.27% at 24 _h, 98.42% at 48_h and 72-h was 100% while clothianidin had 93.03% at 24_h, 97.82% at 48_h and 100% at 72_hwere used. These results show that chlorfenapyr and clothianidin are effective in killing *Anopheles* malaria vectors. Therefore, they should be incorporated to be used in malaria vector control, to complement existing pyrethroid in areas of high pyrethroid resistance.

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LIST OF ABBREVIATIONS AND ACRONYMS

An- Anopheles

AChE - Acetyl cholinesterase

CDC – Centers for Disease Control and Prevention

DDT - dichlorodiphenyltrichloroethane

DNA – Deoxyribonucleic Acid

IRAC – Insecticides Resistance Action Committee

IRS – Indoor Residual Spraying

ITNs- Insecticide Treated Nets

IVM - Integrated vector management

Kdr- Knock-down resistance

KEMRI- Kenya Medical Research Institute

KMIS – Kenya Malaria Indicator Survey

LLINs- Long Lasting Insecticide treated Nets

MIS - Malaria Indicator Survey

nAChR - Nicotine Acetylcholine Receptor

PCR - Polymerase chain reaction

WHO – World Health Organization

DEFINITIONS OF TERMS

Insecticide resistance (WHO): Development of ability of an insect to withstand the dosage of an insecticide that would kill majority of insect in a natural population.

Diagnostic dose: Is the concentration of insecticide that kills 100% of susceptible mosquitoes within a given time.

Susceptibility: The state of being predisposed to, sensitive to, or of lacking the ability to resist something (as a pathogen, familial disease, or a drug).

Efficacy: refers to the maximum response achievable from a pharmaceutical drug or chemical in research settings, and to the capacity for sufficient therapeutic effect or beneficial change in clinical settings.

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria remains a global health problem with a WHO estimation of 219 million cases in the year 2017, up from 216 million cases in 2016, which resulted in 435,000 deaths (WHO, 2018). Sub-Saharan Africa is the most affected with 88% of malaria cases and 90% of malaria deaths with children under age 5 years and pregnant women are the most vulnerable population (WHO, 2016). Malaria remains a major cause of morbidity and mortality in Kenya with more than 70% of the population at risk of the disease (MOH, 2014). The malaria burden in Kenya is not homogenous as areas around Lake Victoria (western Kenya) and coast region present the highest risk with western Kenya region having the highest prevalence overall at 38% as other regions remain at 5% (KMIS, 2015). Increased resistance of malaria vectors to insecticides may compromise chemical based malaria control interventions and hence threaten malaria control and elimination efforts (WHO, 2015).

Controlling the mosquito vector is the most effective way to prevent malaria transmission. The use of Long-Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) are the primary malaria vector control methods (WHO, 2018). There are 12 insecticides which belong to the four classes of insecticides (pyrethroids, organochlorines, carbamates and organophosphates) recommended for IRS against malaria vectors (WHO, 2013). However, pyrethroids is the only class approved by WHO for use on insecticide treated nettings (ITNs) because of their low human toxicity, high efficacy and does not stay long in the environment (M. A. Zaim, A. & Nakashima, N. , 2000).The effectiveness of these insecticides based vector control methods are threatened by development of resistance by malaria vectors to the insecticides used in LLINs and IRS (WHO, 2015). There are reports of pyrethriods

resistance globally including many parts of western Kenya (E. Ochomo et al., 2014; Stump AD, 2004) might have resulted in resurgence in malaria vectors, parasite prevalence and malarial disease burden despite high ownership of LLIN (Stump AD, 2004; Zhou et al., 2011).

Due to threat pose by malaria vectors developing resistance to the insecticides used in LLINs and IRS (WHO, 2015) there has been call for urgent need for safe alternative insecticides to supplement for the malaria vector control(Ranson H, 2011; M. Zaim & Guillet, 2002). Chlorfenapyr and Clothianidian insecticides have different mode of actions to the currently used insecticides, but evaluation of their efficacy on *Anopheles* malaria vectors population has not been done.

Chlorfenapyr is a pesticide, and specifically a pro-insecticide derived from a class pyrroles (Raghavendra et al., 2011). Unlike currently approved insecticides for adult mosquito control, chlorfenapyr is slow acting toxin and act by disrupting respiratory pathways and proton gradients through the uncoupling of oxidative phosphorylation in mitochondria (Black, Hollingworth, Ahammadsahib, Kukel, & Donovan, 1994). Due to its unique mode of action, chlorfenapyr have shown no cross resistance to mechanisms that confer resistance to neurotoxin insecticides against the mosquitoes *Anopheles gambiae*, *Anopheles funestus* and *Culex quinquefasciatus* (Oliver et al., 2010), bed bugs *Cimex* spp. (Tawatsin et al., 2011), or beet armyworm *Spodoptera exigua* (Che, Shi, Wu, & Yang, 2013).

Clothianidine insecticide is a neonicotinoid which is a class of insecticides that are chemically similar to nicotine. It acts on the central nervous system of insects as an agonist of acetylcholine and thus stimulates nicotine acetylcholine receptor(nAChR)(Krupke & Long, 2015),targeting the same receptor site (AChR) and activating post-synaptic acetylcholine receptors. When lowly or moderately activated it results in nervous stimulation, however, high levels over stimulate and block the receptors,(Yamamoto, 1999) causing paralysis and death (Krupke &

Long, 2015). Clothianidin can acts as an alternative to organophosphates, carbamates, and pyrethroids pesticides as it poses lower risks to mammals, including humans, when compared to organophosphates and carbamates (Simon-Delso et al., 2015). This study aimed to determine diagnostic concentrations and efficacy of chlorfenapyr and clothianidine insecticides against *Anopheles* malaria vectors populations of western Kenya.

1.2 Problem statement

Increased resistance of malaria vectors to commonly used classes of insecticides have led to resurgence of malaria cases. In addition, these insecticides are fast acting with excito-repellency effect which repels mosquitoes thus reducing the contact time with the insecticides hindering uptake of optimum dosage needed to kill them; hence there is need for safe alternative insecticide with new mode of action to help in resistance management and rotational programs.

Chlorfenapyr and clothianidin insecticides are non-repellent formulation which allows longer contact time of mosquitoes with insecticides thereby increasing the probability of picking lethal dose. However, the diagnostic concentrations and efficacy of chlorfenapyr and clothianidine insecticides against *Anopheles* malaria vectors populations has not been evaluated.

1.3 Research objectives

1.3.1: General Objective

To evaluate the efficacy of chlorfenapyr and clothianidin insecticides on *Anopheles* malaria vectors population of Nyando, Bumula and Ndhiwa sub-counties, western Kenya.

1.3.2: Specific Objectives

1. To determine the diagnostic dose of chlorfenapyr and clothianidin insecticides using susceptible *Anopheles gambiae sensu stricto,* Kisumu strain.
2. To determine the susceptibility of *Anopheles gambiae* complex and *Anopheles funestus* malaria vectors population of Nyando, Bumula and Ndhiwa to chlorfenapyr and clothianidin insecticides.

1.4 Research questions.

1. What is the diagnostic dose of chlorfenapyr and clothianidin insecticides to susceptible *Anopheles gambiae sensu stricto* Kisumu strain?
2. What is the susceptibility of *Anopheles* malaria vectors population of Nyando, Bumula and Ndhiwa, western Kenya to chlorfenapyr and chlothianidin insecticides?

1.5 Significance of the study

The emergence of malaria vector resistance to all 4 classes of insecticides used in their control necessitates the need for evaluation of new chemical compounds with modes of actions that are different from insecticides classes that are currently recommended by WHO for use in LLINs and IRS. The results of this study has shown that Anopheles malaria vectors population of Nyando, Bumula and Ndhiwa are highly susceptible to both chlorfenapyr and clothianidin insecticides. These compounds can be used to provide sustainable control and avoid insecticide resistance problem leading to achievement of malaria elimination goal. The obtained diagnostic doses are important in providing baseline data for insecticides resistance monitoring when these insecticides are employed in the field.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is the most common and devastating vector-borne diseases caused by a single-cell parasite from the genius *plasmodium* where more than 100 different species of *plasmodium* exist and widespread in the tropical and subtropical regions (WHO:, 2007). In human, malaria is caused by five species of *plasmodium (Plasmodium falciparum, Plasmodium vivax, Plasmodium malaria, Plasmodium ovale and Plasmodium knowlesi)* where it is transmitted from one person to another by infected female anopheles mosquitoes carrying malaria-causing parasites feeds on human where the parasite is injected in the form of sporozoites into the bloodstream. WHO estimated that in 2017 malaria was responsible for 219 million new case and 435,000 deaths worldwide with more than 90% of the deaths occurring in sub-Sahara Africa where *plasmodium falciparum* predominates(WHO, 2018).

2.2 Malaria situation in Kenya

Geographically, the country is classified into two main regions: lowland areas which include coastal region and areas around the Lake Victoria basin, and highland areas on both sides of the Great Rift Valley. Therefore, malaria prevalence varies considerably by season and across geographic regions. Malaria remains a major public health problem in Kenya and accounts for an estimated 18% of outpatient consultations and 6% of hospital admissions based on data from the routine health information system (M. o. H. MOH, 2014). Out of the five, four species of Plasmodium that infect humans occur in Kenya with *Plasmodium falciparum*, as the most common accounting for over 99% of all malaria infections in the country (MOH, 2014) .

For the purposes of malaria control, Kenya has been stratified into four epidemiological zones to address the varied risks: endemic zone of stable malaria around Lake Victoria basin in Western Kenya and the Coast; seasonal malaria transmission zone in the arid and semi-arid areas of northern and south-eastern regions: malaria epidemic prone zone of western highlands; and low-risk malaria areas in the central highlands and Nairobi (DOMC, 2010). The variation of malaria transmission and infection risk in these zones is determined largely by altitude, rainfall patterns, and temperature with 80% of the Kenyan population at risk (Noor AM, 2012). Among the population at risk, 27% (approximately 12 million people) live in areas of epidemic and seasonal malaria transmission where *P. falciparum* parasite prevalence is usually less than 5% (MOH, 2014). However, an estimated 28 million people live in endemic areas, and over a quarter (approximately 11 million people) live in areas where parasite prevalence is estimated to be equal to or greater than 20% (MOH, 2014). The 2010 Malaria Indicator Survey (MIS) indicated that malaria prevalence in the western lake endemic zone remained very high at 38% (DOMC, 2010). Table shown below indicates the projections of Kenya's vulnerable populations at risk of malaria by epidemiological zone (KMIS, 2015).

Table 2.2.1: Kenyan population at risk of getting malaria

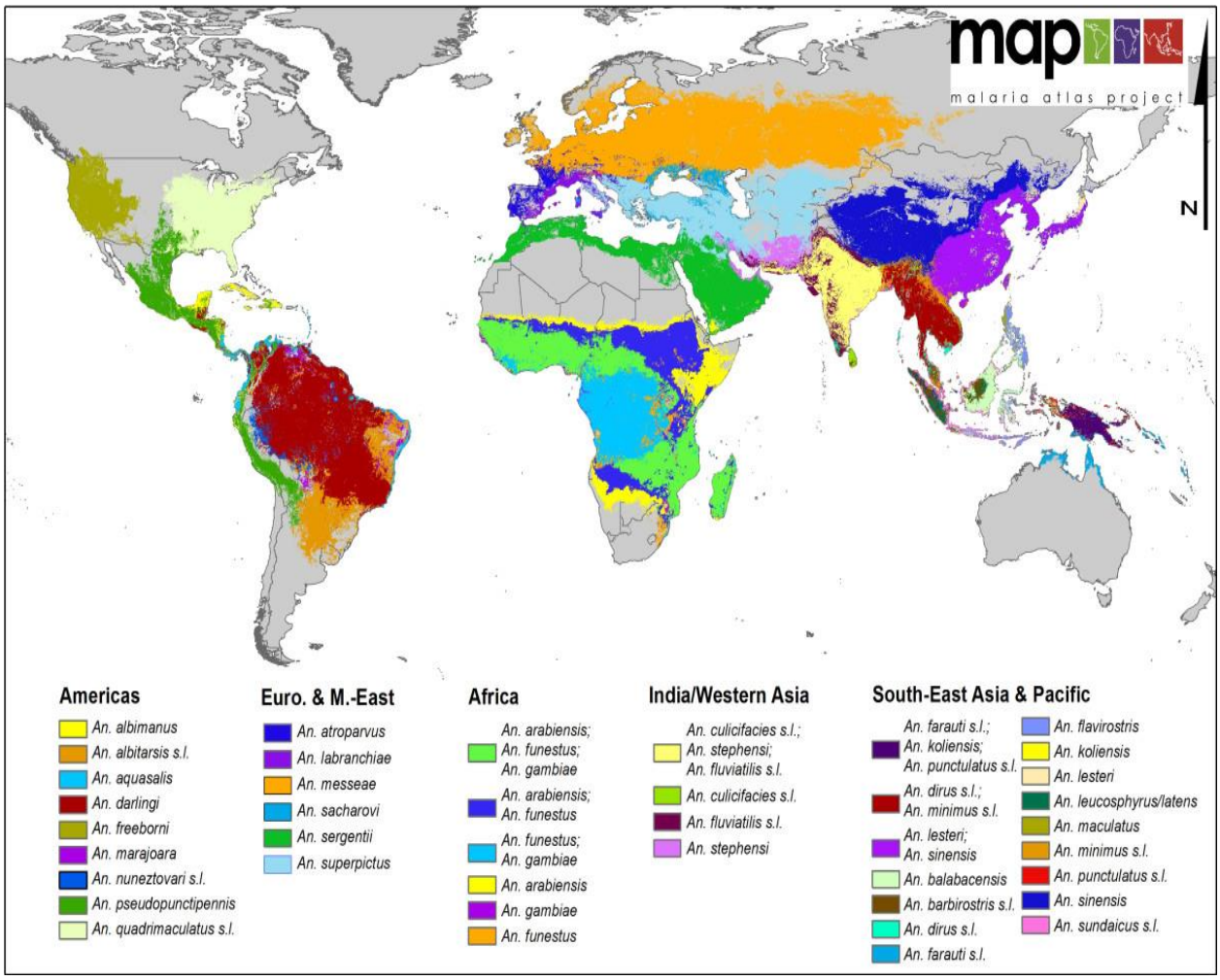
Epidemiological Strata	**Total projected population (2010)**	**Pregnant women**	**Children > 1 year**
Endemic zone	11,212,645	504,569	448,506
Highland epidemic prone zone	8,375,922	376,916	335,037
Arid/seasonal	8,007,718	360,347	320,309
Low risk zone	826,978	533,214	473,079
Total	**39,423,263**	**1,774,047**	**1,576,931**

2.3 The Malaria Vectors

Human malaria parasites are exclusively transmitted by female mosquitoes of the genus *Anopheles* Giles which feed on vertebrate blood. The identification and knowledge of the distribution of the main *Anopheles* malaria vectors is important in the implementation of vector control strategies as well as of academic interest. Anopheles vector have worldwide distribution with species like *Anopheles freeborni* and *Anopheles quadrimaculatus* found in North Amerca while *Anopheles pseudopuntipennis* and *Anopheles darling* in South Amerca (Davis, 1927; Root, 1926). *Anopheles culicifacies* and *An. minimus* are found in Asia. Africa has at least 140 *Anopheles* species, with at least eight including *An. arabiensis*, *An. gambiae s.s* and *Anopheles funestus s.l* found in the African continent and being considered to be effective vectors of malaria (M. T. Gillies & M. Coetzee, 1987; Gillies MT, 1968; G. B. White,

1974). Kenya and Afro-tropics region have two main malaria vectors: *Anopheles gambiae* complex *and Anopheles funestus* complex (Garnham, 1938; Surtees, 1970) with *Anopheles gambiae* complex considered as the most efficient vector of human malaria in the Afro-tropical region (G. B. White, 1974). These species feed predominantly on human blood (anthropophilic) and rest indoors, with relatively long and broad distribution due to ecological adaptation which is capable of sustaining development of *Plasmodium parasites* (Fahmy et al., 2015).

Figure 2.3 1: A global map of dominant malaria vector species Sinka et al; licensee BioMed Central Ltd. 2012.



2.3.1 The *Anopheles gambiae* Complex (*Anopheles gambiae* sensu lato)

The *Anopheles gambiae* complex consists of eight morphologically indistinguishable species of mosquitoes: *Anopheles arabiensis, Anopheles bwambae, Anopheles melas, Anopheles merus, Anopheles quadriannulatus A, Anopheles quadriannulatus B* (*Anopheles amharicus*), *Anopheles coluzzii and Anopheles gambiae sensu stricto* (*Anopheles gambiae s.s*) (M. T. Gillies & De Meillon, 1968). These eight species vary in their vectorial ability and ecological niche (Coetzee & Koekemoer, 2013). *Anopheles quadriannulatus A,* is widespread in southern Africa (G. B. White, 1974)and *Anopheles quadriannulatus B* (*Anopheles amharicus*), are zoophilic non-malaria vectors Ethiopia (Hunt, Coetzee, & Fettene, 1998). *Anopheles merus*

found in East Africa and *Anopheles melas* in West Africa and are both salt water breeding and therefore only important vectors in coastal regions (M. T. Gillies & De Meillon, 1968). The *Anopheles bwambae* has only been found breeding in mineral springs in the Semliki forest in Uganda (G. White, 1989).The most efficient vectors of human malaria are *Anopheles gambiae sensu stricto* and *Anopheles Arabiensis* (G. B. White, 1974). *Anopheles gambiae sensu stricto* exists in two molecular forms, denoted M and S, and was recently reclassified as two species, An*opheles coluzzii* and An*opheles gambiae s.s* based on molecular and bionomical evidence (Coetzee *et al.*, 2013). *An. quadriannulatus* is retained for the southern African populations of this species, while the Ethiopian species is named *Anopheles amharicus* (Coetzee et al., 2013). Because the seven morphologically indistinguishable species within the complex exhibit differences in vectorial ability, their identification using molecular techniques such as PCR is important for focused effort in malaria control programs(Coetzee et al., 2013)

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2.3.2 The *Anopheles funestus* Complex

Anopheles funestus group consist of nine sibling species that are distributed throughout Africa: *Anopheles parensis*, *Anopheles aruni*, *Anopheles confusus*, *Anopheles funestus s.s*, *Anopheles vaneedeni*, *Anopheles rivulorum*, *Anopheles fuscivenosus*, *Anopheles leesoni*, and *Anopheles brucei* (M. T. Gillies & M. Coetzee, 1987). In addition to morphological similarities among these sibling species their biology and vectorial competency is different(Coetzee et al., 2013). These sibling species are zoophilic except *Anopheles funestus sensu stricto*. *Anopheles funestus sensu stricto* is also the predominant species both in numbers and geographical distribution (Coetzee et al., 2013) . *Anopheles funestus* is closely associated with human dwellings, and is mainly anthropophilic and endophilic, hence it plays a critical role in malaria transmission in Africa(M. Gillies & M. Coetzee, 1987; Harbach, 2004).

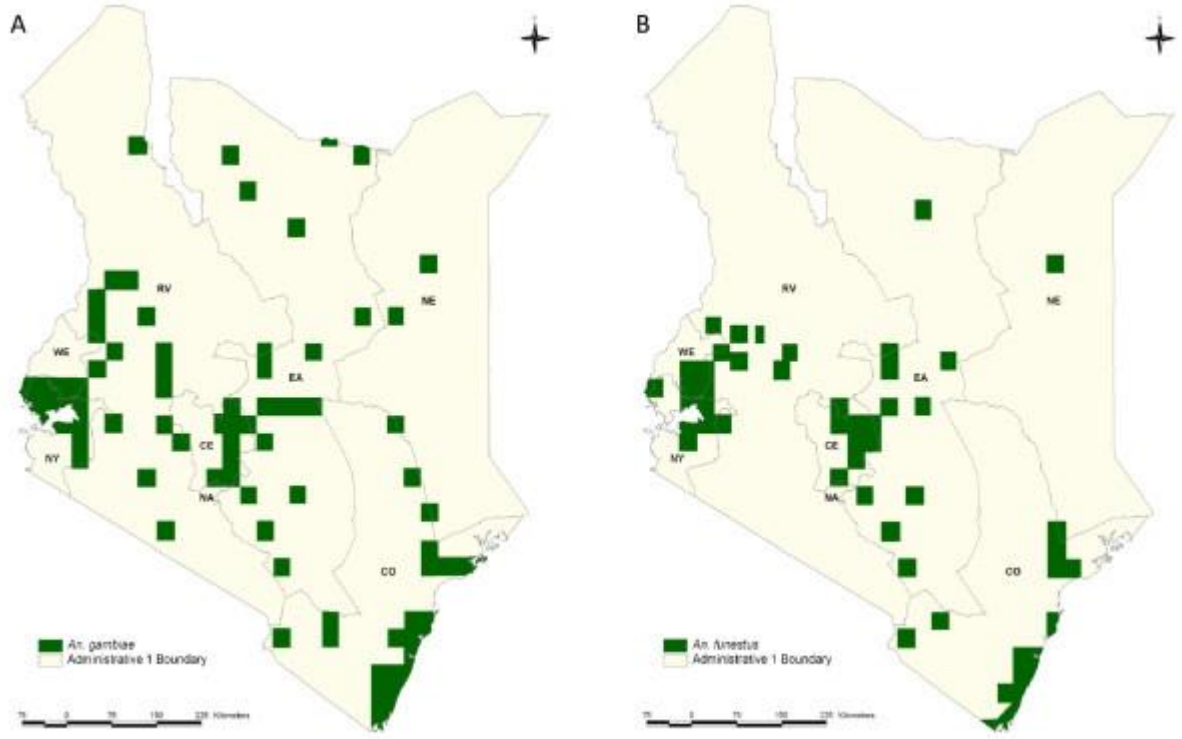
2.3.2 Ecology of members of the Anopheles Gambiae Complex in western Kenya.

The importance of studying behavior and biology of malaria vectors lies mainly in the indicators of epidemiology and control of the disease (Goriup & Van Der Kaay, 1984). *Anopheles gambiae sensu stricto* and *Anopheles arabiensis* can be regarded as the most similar species ecologically due to their common adaptation to human environments. The habitat of anopheles larvae are varying, shallow larval habitat containing algae is a definite example of *Anopheles gambiae* breeding habitat (Gimnig et al., 2002). *Anopheles gambiae* Giles prefer breeding in temporary and turbid water bodies produced by rain such as car tracks, barrow pits, hoof-prints and ditches (M. Gillies, 1988).Open habitat experience warmer temperature during daytime thus shortens larval to pupae development time while also reducing mortality associated with desiccation (M. T. Gillies & De Meillon, 1968). *Anopheles funestus* prefers breeding in more permanent water bodies (M. Gillies, 1988).

*Anopheles arabiensis*is is more common in arid areas than the rest of the *Anopheles gambiae* complex members(Hargreaves et al., 2000). The salt water species, *Anopheles merus* and *Anopheles melas* are found along the South Eastern coastal and Western coastal of Africa with *Anopheles merus* found along the coastal region of Kenya. *Anopheles arabiensis* is mainly distributed along the coast, across Western Kenya and central Kenya including the arid areas of the northern Kenya (Okara et al., 2010).*Anopheles merus* was reported to be located on the Kenyan coast reflecting the salt water larval conditions associated with this species (Okara et al., 2010). Besides the *Anopheles gambiae* complex, *Anopheles funestus* complex has distribution at the Coast, in central regions and, more frequently than other *Anopheles*, in the highland areas distal to Lake Victoria in Western Kenya. The spatial occurrence of the three most dominant vectors vary between different classifications of transmission intensity, for example, *Anopheles gambiae* was documented more often in areas with the highest transmission intensity, with less frequent reports at sites with very low transmission areas

(Okara et al., 2010). Only an *Anopheles gambiae sensu stricto* and *Anopheles arabiensis* make the *Anopheles gambiae* complex that are predominant in Western Kenya (Service, Joshi, & Pradhan, 1978)and are the most efficient vectors of malaria.

Figure 2.3 2: a and b Map of Kenya showing the distribution of Anopheles gambiae sensu lato (a) and (b) Anophels funestus(b) (Roberts, 1974).



2.4 Mosquito vector Control

Mosquito vector control has been proven to successfully reduce or interrupt malaria transmission when coverage is sufficiently high (WHO, 2016). The use of insecticide based vector interventions is the most effective measure in controlling malaria vectors (WHO, 2015). Chemical control which involves the use of insecticides in LLINs and/or IRS are the two main methods for malaria vector control (Hemingway et al., 2016). The

use of LLINs has reduced under-5 child mortality by more than 20% in both large-scale trials and under routine conditions (Lengeler, 2004; Lim SS, 2011).

However, most indoor residual spraying program was stopped in sub-Saharan Africa due to the perception that transmission was intense and that without total coverage it would not be possible to have a great impact in terms of interrupting overall malaria transmission but continued in Asia ((GMAP), 2015). The reintroduction of IRS by the US President's Malaria Initiative in 2005 has seen 55 million people per year (about 7% of those at risk from malaria) protected by IRS program in Africa (WHO, 2014). Increased coverage with LLINs and IRS preventive measures has resulted in massive reduction of malaria burden across Africa. However, the plan to sustain the efficacy of these insecticides based methods has not been put in place (Hemingway et al., 2016).

2.5 Insecticides used in malaria vector control

According to the Insecticide Resistance Action Committee ((IRAC), 2007), insecticides that are widely used in malaria vector control fall in two main groups: Group1 are those which act by inhibition of neurotransmitter, Acetyl cholinesterase and these include carbamates (bendiocarb) and organophosphates (Malathion fenitothrin and temophos; Group2 are those that are modulators of sodium channels and these include organochlorine (DDT) and pyrethroids (pyrethrins, permethrin, deltamethrin, and lambda-cyahalothrin) (Sparks & Nauen, 2015).These insecticides are both used indoors for public health in control of vectors and outdoors in agricultural purposes (Sparks & Nauen, 2015) . Most of the above named insecticides are used in IRS (M. A. Zaim, A. & Nakashima, N. , 2000).

There are 12 insecticides which belong to the four classes of insecticides (pyrethroids, organochlorines, carbamates and organophosphates) recommended for malaria vector control for use in IRS (WHO, 2013). Historically, dichlorodiphenyltrichlorethane (DDT) has been

considered the most cost effective, because it lasts longer than alternatives and therefore dwellings are sprayed less frequently, however, many residents resist spraying of DDT due to a variety of factors including its smell and staining that it leaves on walls (Komalamisra, Srisawat, Apiwathanasorn, Samung, & Kaisri, 2009). Since 80% of homes in an area must be sprayed for IRS to be effective, the resistance to DDT spraying can jeopardize IRS programs (N'guessan et al., 2007). Pyrethroids insecticides are more acceptable since they do not leave visible residues on the wall (Perry, Yamamoto, Ishaaya, & Perry, 2013). Thus, all IRS programs were using pyrethroids when the PMI was launched in 2005, and by the year 2013 nearly two-third of all IRS programs worldwide continued to rely on pyrethroids (Hemingway et al., 2016).

Pyrethroids are of two types: Type I are naturally occurring insecticidal esters of chryysanthemic acid and is comprised of permethrin and bifentrin (David M Soderlund, 2012) ; Type II pyrethroids consist of majority of insecticides such as deltamethrin, alpha-cypermethrin, permethrin, lambda-cyahalothrin and cyfluthrin (Thatheyus & Selvam, 2013) . Deltamethrin, alpha-cypermethrin, lambda-cyahalothrin and cyfluthrin are used in IRS with the exception of permethrin. Pyrethroids is the only class recommended by WHO for the treatment of LLINs because of their low human toxicity, high efficacy and does not stay long in the environment (M. A. Zaim, A. & Nakashima, N. , 2000).

Malaria vector control in Kenya has been greatly emphasized through vector control strategy. Since 2002, distribution of LLINs to pregnant women and children less than five years of age through maternal and child health clinics has been largely implemented to achieve a set target of 80% of the population at risk using appropriate malaria prevention interventions, including LLINs and IRS (MOH, 2014). The Government of Kenya plans to achieve universal ITN coverage (i.e., one net for every two people) for all groups in malaria-endemic and epidemic-

prone counties through LLINs and IRS alongside prompt diagnosis and treatment have been employed as part of government policy on malaria control (DOMC, 2010).However, Since 2010, insecticides resistance have been reported to at least one class of insecticide in 60 countries and 50 of these countries reporting resistance to 2 or more classes (WHO, 2014).

2.6 Insecticide resistance to insecticides used in controlling malaria vector

Malaria prevention heavily relies on the use of insecticides for the treatments of mosquito bed nets as vector control method (WHO, 2011). However, there are reports worldwide of strong resistance that has resulted from increased usage of these insecticides since 1950s. As per WHO, insecticides resistance can be defined as the ability of an insect to withstand the dosage of an insecticide that would kill majority of insect in a natural population (Davidson, 1957). Resistance to insecticides has been observed in nearly over 500 insect species worldwide of which more than 50 Anopheles species (Diptera: Culicidae) transmit humans malaria parasites (Hemingway & Ranson, 2000). In the framework of malaria vector control programs (Feachem & Sabot, 2008), the use of long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) are being scaled up in many endemic regions of Africa and World Malaria Report of 2013 reported that the number of ITNs distributed to malaria-endemic countries in sub-Saharan Africa increased from 6 million in 2004 to 145 million in 2010 which remained flat for two years 2011 to 2012 (Strode, Donegan, Garner, Enayati, & Hemingway, 2014) .

Pyrethroids resistance was first detected in the African malaria vectors in Sudan in the 1970s and later in west Africa in the early 1990s (Brown, 1986; Elissa N, 1993) and was probably selected for by exposure of mosquitoes to pyrethroids used to protect agricultural crops against insect damage. This resistance is caused by a target site mutation kdr (knockdown resistance), which spread rapidly across Africa. However, the level of resistance conferred by this mutation alone is low, and this led to complacency with the resistance having little or no operational effect on the efficacy of long lasting insecticide-treated bed nets (Asidi AN, 2005; Darriet F,

2000). Now, more potent resistance mechanisms have evolved, which have resulted in long lasting insecticide-treated bed nets and indoor residual spraying pyrethroids formulations that no longer kill mosquitoes in different settings including western Kenya with some blood-fed mosquitoes are increasingly being found inside long-lasting insecticide-treated bed nets or resting on newly sprayed walls (E. O. Ochomo et al., 2013).

Pyrethroids act on the insect nervous system by altering the normal function of the Para-type sodium channel, resulting in prolonged channel opening that causes increased nerve impulse transmission, leading to paralysis and death (David M. Soderlund & Bloomquist, 1989; Toshio, 1992). Pyrethroids resistance is often associated with point mutations in the Para-type sodium channel gene, which result in reduced neuronal sensitivity. This resistance mechanism was first identified in the house fly Musca domestica and was referred to as knockdown resistance or kdr (Busvine, 1951). Many analysis have demonstrated that kdr was caused by a leucine to phenylalanine replacement in transmembrane segment 6 of domain II of the sodium channel (Williamson, Martinez-Torres, Hick, & Devonshire, 1996). Two amino acid substitutions at the same position 1014 have been reported in pyrethroids resistant and this result in two forms of kdr resistance: leucine to phenylalanine substitution at position 1014 of the voltage-gated sodium channel, termed kdr west (kdr-w), has been reported in several West African countries where it is the predominant kdr mutation in *Anopheles gambiae* populations. The other is the leucine to serine substitution at the same point termed, kdr east commonly found in East African populations of *An. gambiae (E Ochomo et al., 2015)* . The latest reports show the presence of both kdr forms of resistance presence in east African countries and it was the first report of kdr-west (Vgsc-1014F) in Kenya, which appears to have emerged in both *Anopheles gambiae* and *Anopheles arabiensis.*(Kabula et al., 2014; E Ochomo et al., 2015; Verhaeghen, Van Bortel, Roelants, Backeljau, & Coosemans, 2006).

Chlorfenapyr and Clothianidin insecticides mode of actions differ from those of organophosphates, carbamates, pyrethroids and organochlorines furthermore, clothianidin can display a high level of activity against pest insects that have developed resistance to these existing compounds (Uneme, Konobe, Akayama, Yokota, & Mizuta, 2006). The present study was designed to evaluate the efficacy of chlorfenapyr and chlothianidin insecticides against pyrethroid resistant *Anopheles gambiae sensu lato* in western Kenya following WHO guidelines

2.7 Chlorfenapyr Insecticide.

Chlorfenapyr is a pro-insecticide which belongs to pyrrole class of insecticides which works by targeting the oxidative pathways in the insect's mitochondria thus disrupting ATP production (Black et al., 1994). Due to its unique mode of action, chlorfenapyr have shown no cross resistance to mechanisms that confer resistance to neurotoxin insecticides against the mosquitoes *Anopheles gambiae, Anopheles funestus* and *Culex quinquefasciatus* (Oliver et al., 2010), bed bugs *Cimex* spp. (Tawatsin et al., 2011), or beet armyworm *Spodoptera exigua* (Che et al., 2013) and can also been introduced as an alternative to synthetic pyrethroids.

2.8 Clothianidin Insecticide

Clothianidin insecticide is developed by Takede Chemical Industry and Bayer AG. It is a neonicotinoid which is a class of insecticides that are chemically similar to nicotine and acts on the central nervous system of insects as an agonist of acetylcholine hence stimulates nicotine acetylcholine receptor(nAChR) (Krupke & Long, 2015), targeting the same receptor site (AChR) and activating post-synaptic acetylcholine receptors but do not inhibit AChE (Krupke & Long, 2015). Its activation will result in nervous stimulation, however, high levels over stimulate and block the receptors,(Yamamoto, 1999) causing paralysis and death (Krupke & Long, 2015). Clothianidin can acts as an alternative to

organophosphates, carbamates, and pyrethroids pesticides as it poses lower risks to mammals, including humans, when compared to organophosphates and carbamates (Simon-Delso et al., 2015)

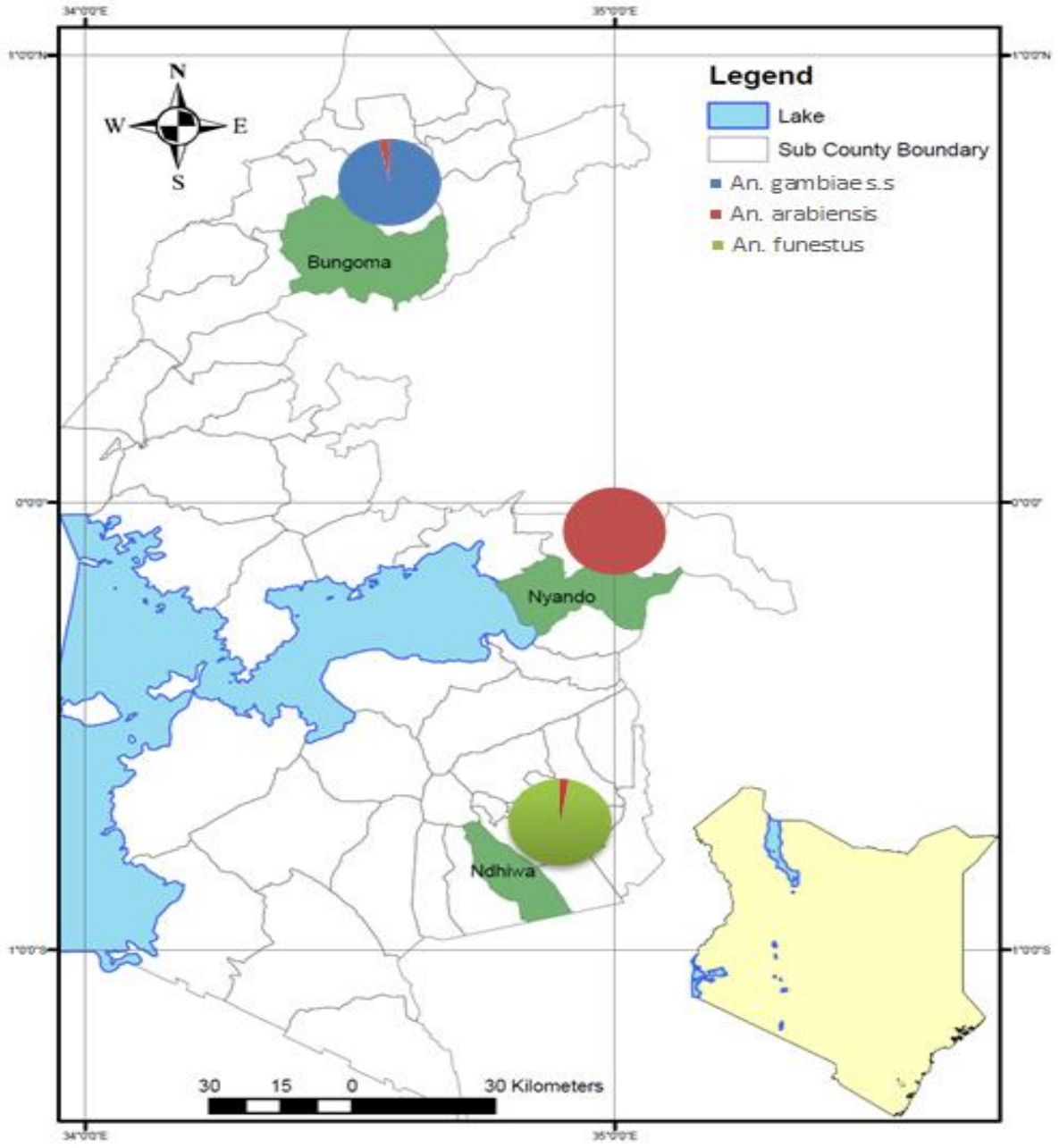
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

The study was conducted in three malaria endemic sub counties: Nyando sub County in Kisumu County, Bumula sub County in Bungoma County andNdhiwa sub County in Homa Bay County, western Kenya. These are malaria endemic areas with high and perennial seasonal peaks between April to July and November to December coincident with the long and short rainy seasons respectively (Hamel et al., 2011). These sites have high coverage of LLINs and practice agriculture as the major economic activity. Ndhiwa Sub County grows mainly sugarcane and maize with *Anopheles funestus* as the main malaria vector (unpublished data). Bumula Sub County is centered on the cash crops such as sugar cane and tobacco as well as horticulture farming while Nyando Sub County is rice growing area which provide breeding habitat throughout the year as *Anopheles arabienis* considered to be the predominant vector. Bumula and Nyando are sites with confirmed pyrethroids resistance in Western Kenya (E. O. Ochomo et al., 2013). Malaria prevalence is averagely at 38% which predominantly affecting the ages of 6 months to 14 years of age (KMIS, 2015). Figure below shows map of the study area.

Figure 3.1 1: Map of the study areas.



3.2 Sampling of indoor resting mosquitoes populations

Sampling was done in two sub-locations per Sub County: Unga and lower Kabonyo in Ndhiwa Sub county Homabay County, Siloba and syombe in Bumula Sub county Bungoma County. Indoor-resting *Anopheles* was sampled from 5th October 2017 to 20th February 2018 using mouth aspiration as described by (Gimnig *et al.*, 2003). Sampling was done in 26 houses across

an area of 25 km^2 in Ndhiwa, 30 houses across an area of 37km^2 in Bumula sub County and 16 houses across an area of 10 km^2. All the mosquito collected were morphologically identified as *Anopheles gambiae sensu lato* and *Anopheles funestus sensu lato.* Collected mosquitoes were sorted and allowed to rest for 48 hours after which they were exposed to the insecticides and only those which were not injured during collections were retained for bioassays.

3.2.1 Larval sampling and rearing

The larvae were collected from their breeding habitat using dippers sorted in trays and transferred in plastic tins, labeled and transported to KEMRI/CGHR insectary for rearing under standard conditions (Temperature of 29-32°C, photo-period of 12:12 hours (light: dark). Larvae from each container were transferred to a larval tray where they were sorted into different larva instars (1^{st}, 2^{nd}, 3^{rd} and 4^{th}) and then labeled with same sample label. Larval trays contained water depth of 1cm. Larvae were fed on grounded TetraMin™ or KOI'S CHOICE® premium pond fish food. On pupation, all pupas from the same larval tray were transferred to the same pupa cup/bowl. Pupa cups were then placed inside individual cages (30cm by 30cm by 30cm) for emergence into adult. Both pupa cups and cages were labeled with the same sample label. Freshly emerged adults were fed on 5-10% sugar solution and maintained at a temperature of 27 ± 2^0C and a relative humidity of 80± 10% after which they were ready for assays (3-5 days old). The larval rearing technique used as one described in (Das, Garver, & Dimopoulos, 2007)

3.3 Insecticides

Two technical-grade insecticides were used in this study: Pyrrole (Chlorfenapyr, purity 99.1%)) and Neonicotinoid (Clothianidin, purity 99.5%) all from Chem Service, Inc., West Chester, PA, USA. Lethal concentrations for Chlorfenapyr and Clothianidin were prepared for the *Anopheles gambiae,* Kisumu strain using technical grade of these insecticides. All

insecticide dilutions were prepared in absolute ethanol, stored in glass bottles, wrapped in aluminum foil, and kept at 4 °C while not being used.

3.3.1 Preparation of insecticides stock solutions

Developing a stock solution of a known concentration from a source of insecticide active ingredient (AI) was done. All desired concentrations included as treatments in the bioassay were made from the original stock solution. In brief, 100mls of a 1000 µg/ml solution was prepared as a stock solution and several dilutions were prepared from this initial concentration. Adjustment to the amount of technical grade AI to be weighed based on the percent purity.

Amount to be weighed = (volume to make) X (desired concentration (µg / ml) solution) X (100/% purity)

For example, it would require 101522.84 µg of technical AI to make 100 ml of a 1000 µg/ml solution of a technical grade insecticide of 98.5% purity:

(100 ml) X ([1000µg/ml] / 0.985) = 101522.84 µg

Working solution of different concentrations of chlophenapyr and chlothianidin insecticides were prepared from stock solution of 1000µg/ml. The insecticide was labeled and stored at 4°C in dark until use. Dosage(s) recommended on the manufacturer's label or that found to provide at least 100% mortality in small-scale testing was the basis for determining diagnostic dose.

3.3.2 Preparation of insecticide concentrations for tests

The insecticide concentrations (at least 5) should give a range of 5-100% insect mortality based on a preliminary replicated test. The serial dilution starts from the highest to the lowest concentration. From the stock solution, preparation of serial dilutions using the equation $C_1V_1 = C_2V_2$, where:

C_1= initial concentration, V_1= initial volume, C_2= Final concentration, V_2 = final volume

To prepare 2 ml of 100µg/ml from 1000 µg/ml stock solution, the volume needed of the stock solution using the formula above will be:

(1000µg/ml) (x) = (100 µg/ml) (2 ml) =

1000x =200 = 0.2 ml stock solution + 1 8ml absolute ethanol.

Continue the serial dilutions using the above equation or do a 1:1 dilution of 10 concentrations. Secure the cap of the vials with parafilm to minimize evaporation. Store the prepared insecticide dilutions in a refrigerator (4^0C) or freezer (preferably -20^0C). Replace and dispose properly the pipette tips after preparation of an insecticide.

3.4 Insecticide bioassays.

Bioassay data was scored according to the guidelines by WHO, where populations with mortality >98% were regarded as susceptible, populations with 90 - 98% mortality were suspected to be resistant pending further tests while populations with <90% mortality were considered resistant. Insecticide susceptibility assays were carried out following the World Health Organization (WHO) protocol (Brogdon & Chan, 2010) using *Anopheles gambiae sensu lato* and *Anopheles funestus* adults collected from houses and larvae collected from their habitat and reared to adult of 3-5 days old. Both unfed and blood fed females from indoor collection were allowed to rest for 48 hours at the KEMRI-CGHR insectary before exposure to the insecticides and only those which were not injured during collections were retained for bioassays. Following exposure the mosquitoes were transferred to a clean holding paper cups with 10% sugar solution, and mortality was monitored for 72 hours with recording after every 24 hours.

3.4.1 CDC Bottle Bioassay technique

CDC Bottle Bioassay technique uses transparent colorless 250ml Wheaton bottles with screw lids. A set of five bottles were used; one for control and the remaining four for the test

replicates. CDC-bottle bioassay is a tool for evaluating insecticides efficacy as well as detecting resistance to insecticides (Brogdon & Chan, 2010). 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 (Brogdon & Chan, 2010). The assay was done in Bioassay laboratory at KEMRI-CGHR. The temperature and humidity were maintained at 27± 2^0 C, and 70±10% respectively as sub-optimal temperatures or relative humidity can cause large variability in bioassay response.

Figure 3.4 1: Showing arrangement and numbering of CDC bottle prior to coating

3.4.2 Cleaning and drying of the CDC bottle.

The 250ml Wheaton bottles were first soaked in warm soapy water for 12 hours then washed thoroughly and rinsed in tap water where they were left to stand for 2 hour. This was to ensure that no traces of insecticides remained in the bottle. They were then left to air dry completely

for 48 hours after which some susceptible mosquitoes (Kisumu strain) were introduced to ensure that no traces of soap or other chemicals was present.

3.4.3 Coating of CDC Bottle with insecticides

The 250ml Wheaton bottles were marked using masking tape and numbered (1-4) for test and one marked as control(C). Absolute ethanol (1ml) was added to the control bottles while the four remaining bottles was coated with 1ml of the prepared insecticide solution. The bottles were inverted swirled to coat the inside of the cap, then bottles were placed on their side for a moment to let the contents pool (Brogdon & Chan, 2010). Gently, the bottles were rotated for ten minutes to make sure that the entire inner surface and the lid were evenly covered with the insecticide. This was done until no trace of liquid was visible. The bottles were left on their sides and caps removed, covered to protect them from light for overnight air dry before the mosquitoes were introduced for exposure (Brogdon & Chan, 2010) .

3.5 Determination of diagnostic doses

Insecticides diagnostic dose determination was done using CDC-Bottle assay in which 250ml Wheaton bottle coated with 1ml of the insecticide and then mosquitoes are exposed to the insecticides coated surface for sixty minutes and mortality recorded after 24_h (Brogdon & Chan, 2010). Diagnostic dose is the concentration of insecticide that kills 100% of susceptible mosquitoes within a given time. Diagnostic dose was determined for each of the insecticide using the KEMRI-CGHR laboratory reared susceptible *Anopheles gambiae sensu stricto,* Kisumu strain.

Only female mosquitoes between the ages of 3-5 days old were subjected to bioassays. One ml of each insecticide was used to coat the bottle and allowed to dry properly for at least 12 hours. The experiment was carried out between 0800hrs and 1100hrs. Total of 25 susceptible female *Anopheles gambiae,* Kisumu strain were introduced into the previously coated bottle,

knockdown monitored every 10 minutes for 1 hour. After which the exposed mosquito were gently removed and transferred into clean paper cups, fed on 5-10% sugar solution and mortality observed for 72 hours with mortality count recorded after every 24hours. This was done for different concentrations (chlorfenapyr 10-100 µg/ml while clothianidin 50-250 µg/ml) until 100% mortality is achieved. Total of 400 mosquitoes were tested for each concentration for every insecticide.

3.6 Evaluation of efficacy of clothianidin and chlorfenapyr insecticides

Evaluation of clothianidin and chlorfenapyr insecticides efficacy was done according to WHO guidelines for testing mosquito adulticides (WHO, 2006) using CDC- Bottle Assay. The previously determined diagnostic doses of Clothianidin (150µg/ml) and Chlorfenapyr (50µg/ml) using laboratory colony of *Anopheles gambiae sensu stricto*, Kisumu strain were used.

Approximately 14 hours after coating bottles with insecticide, 10–25 female adult mosquitoes of were aspirated and gently blown into each bottle. This was applied to both *Anopheles* malaria vectors collected as indoor resting collection and larvae collected from breeding sites. Mosquitoes were aspirated into the control bottle first, followed by the four insecticide-coated bottles. Once mosquitoes had been aspirated into the bottle, the timer was started and recorded as time zero. The numbers of live and knockdown mosquitoes were recorded at 0, 10, 20, 30, 40, 50 and 60 minutes. All bottles were held vertically for the duration of the experiment. After exposure period was over, the mosquitoes were gently aspirated from the bottle into clean paper cups where they were provided with 10% sugar solution soaked in cotton wool during recovery period. The lethal concentrations causing 100 % mortality for both chlorfenapyr and clothianidin insecticides after one hour exposure were observed until 72 hours was reached. The dead mosquitoes were packed in 1.5µl eppendorf tubes for species identification using PCR.

3.7 Identification of Anopheles malaria vectors population into species.

Polymerase chain reaction (PCR)(Scott, Brogdon, & Collins, 1993) technique was used to identify the sibling species of *anopheles gambiae* and *An. funestus* complexes after exposure to the insecticides. Morphological identification was done during sample collection to differentiate *Anopheles gambiae* from *An. funestus* species. Preparation of the PCR master mix and the reaction mixture calculations is described in table 2.

3.7.1.1 Preparation of reagents for DNA extraction.

Homogenized buffer consist of 0.1M NaCl, 0.2M sucrose, 0.01M EDTA, and 0.03M Tris Base. The resulting solution will be adjusted to PH8.0.

Lysis buffer consist of 0.25M EDTA, 2.5% ^{W}/v SDS and 0.5M Tris Base all mixed to a PHof 9.2.

Grinding buffer was prepared by mixing homogenization and lysis buffers in the proportions of 4:1 (Homogenization: Lysis)

TE Buffer consists of 0.001M EDTA, 0.01M Tris –HCl at PH 8.0.

3.7.1.2 DNA Extraction.

DNA was extracted from the whole mosquitoes using the alcohol precipitation method (Collins *et al.*, 1987) with few modifications. In brief, before starting the actual DNA extraction, a 65^0C water bath was prepared. The frozen sample were placed individually in sterile centrifuge tubes and crashed in 100µl of grinding buffer. Once ground, the sample was incubated at 65^oC water bath for 30 minutes. A volume of 14µl potassium acetate was then added and the sample vortexed to mix. The mixed samples were incubated on ice for 30 minutes during which tubes were labeled and supernatant transferred. After 30 minutes, the samples were spinned for 10 minutes at 13,200 rpm. Supernatant was then transferred to the newly labeled sterile vials. Ice cold absolute ethanol [volume of 200µl] then added and samples placed at -20^0C for 20

minutes. A final spin is done for 20 minutes at 13,200 rpm to pellet the DNA and then vials washed first in 200µl of 70% ethanol, then in the same volume of absolute ethanol. The tubes were then inverted to dry overnight. The samples were then reconstituted in 100µl of TE buffer and the DNA stored at -20^{0}C until DNA amplification.

Table 3.7 1: PCR mixture set up.

100 Samples	**1 sample**	**Reagent(G/A)**	**Reagent(F/L)**
498	4.98	Sterile water	Sterile water
300	3.0	5x Buffer	5x Buffer
180	1.8	$MgCl_2$	$MgCl_2$
100	1.0	UN-F (10 pmol/µl)	UN-F (10 pmol/µl)
100	1.0	AR-R (10 pmol/µl)	F-R (10 pmol/µl)
100	1.0	GA-R(10 pmol/µl)	LEE-R (10 pmol/µl)
114	1.14	DNTPs (2.5 Mm mix)	DNTPs (2.5 Mm mix)
8	0.08	Taq polymerase	Taq polymerase

Thermocycler program for amplification

Anopheles gambiae/ arabiensis	***Anopheles funestus***
95ºC/ 5 x 1 cycle94^{0}C 4 min x cycle	
95^{0}C/30 sec: 56ºC/30 sec 72ºC/ 30 sec] x 35 cycle	95^{0}C/30 sec: 56ºC/30 sec 72ºC/ 30 sec] x 35 cycle
72ºC/ 5 min x 1 cycle	72ºC/ 5 min x 1 cycle
4ºC hold	4ºC hold

3.7.1.3 DNA Amplification

Amplification of the DNA molecule was done to obtain several copies of the extracted DNA samples of the mosquitoes (Scott et al., 1993). The primers used were: Forward universal primer (5'-GCT GCGAGT TGT AGA GAT GCG -3'), Reverse *Anopheles gambiae* primer

(AG)- (3'GCT TAC TGG TTT GGT CGG CAT GT-5') and the reverse *Anopheles arabiensis* primer- (3'GCT TAC TGG TTT GGT CGG CAT GT-5') (DNA chemistry section, Biotechnology core facility branch, Division of scientific resources, centers for Disease Control and Prevention, Atlanta, USA). dNTPs 5X PCR, and Taq enzyme polymerase (Promega, Madison, WI, USA) and $MgCl_2$ (KEMRI Nairobi, Kenya) was used at recommended Standard concentrations (Scott et al., 1993). The PCR program used comprised of the following the steps: The PCR mixture undergone an initial heating for 10 minutes at 95^o C to completely denature complex DNA so that the primers could anneal to the template as the reaction cools. Subsequent cyclic denaturation was done at 95^{o}C for 30 seconds. Primer annealing was set at 64^{o}C for 30 seconds and extension done at 72^{o}C for 45 seconds. The reaction ran for 35 cycles then a final extension at 72^{o}C for 5 minutes to promote completion of partial extension product and annealing of single stranded complementary products after which samples were held infinitely at 4^o C.

3.7.1.4 Agarose gel electrophoresis for separation of the amplified DNA molecules.

The gel was prepared by dissolving the agarose powder (3g) in150ml of TBE (Tris Boric Acid EDTA) buffer was used in electrophoresis. The agarose was dispersed in the buffer before heating it to near-boiling point. The melted agarose was allowed to cool sufficiently before pouring the solution into a cast. A comb was placed in the cast to create wells for loading sample.

3.7.1.5 Loading the Sample.

After PCR time was over the plate containing the samples were removed from the thermo-cycler machine and oil at the bottom blotted out. The gel was set and comb removed creating wells where DNA samples were loaded. The plate sealer removed and a total of 16µl of amplicon loaded into the gel wells and allowed to run for 10minutes after the currents is

switched on to flow. The formed bands were read and the result of the species recorded in the data sheet.

3.7.1.6 Band visualization and reporting of result

The gel was removed and placed on a UV illuminator slab. The main light switched off as the UV source is switched on. Film cartridges for the camera are kept at 4^{o}C and have were loaded 1 hour before use to attain room temperature. The camera was placed on the gel and photo taken. The lower paper was pulled followed by the upper larger one. The film was allowed to rest for 1 minute then photograph was peeled out and visualized and results scored using the loading map according to the gel image.

3.8 Data analysis

Time-response survival curves were made for each insecticide by plotting time on the X-axis against percentage mortality on the Y-axis (Brogdon & Chan, 2010). A diagnostic dose was determined to be the lowest dose tested that caused 100% mortality within 72 hours after 1 hour exposure period. Mortality was calculated by summing the number of dead mosquitoes across all the exposure replicates and expressed as a percentage of the total number of exposed mosquitoes and a cutoff point of 98% was used to score resistance for susceptibility test.

$$\textbf{Observed mortality} = \frac{\textbf{Total number of dead mosquitoes}}{\textbf{Total sample size}} \times 100$$

Assessment of efficacy outcome of field collected anopheles mosquitoes to both chlorfenapyr and clothianidin insecticides were done following WHO guidelines, where populations with mortality >99% were regarded as susceptible, populations with 90 - 98% mortality were suspected to be resistant pending further tests while populations with <90% mortality were considered resistant.

3.9 Ethical Consideration

Permission to carry out this study was granted by Maseno University School of Post Graduate Studies while ethical approval was given by Maseno University ethical review committee. Mosquitoes sampling was done after household heads were briefed on the study and permission granted. During collection of mosquito samples fieldworkers were wearing protective clothing and gum boots that protected them from any harm. Upon collection, the mosquitoes were placed in cooler box with a lid tightened and then transported to the laboratory for assay. During exposures of samples to bioassays, all laboratory safety rules were adhered to. Data from this study was stored in a secure database and accessed only by authorized personnel.

3.9.1 Limitations and Biases

The research was conducted in three counties which are far from each other and this needed good financial support. Seasonal variation and availability of vector abundance also affected our sample size where we could not get enough mosquitoes to expose especially in Bumula. Second, the time constrain is also another challenge as it needed much time to get the needed sample size. Bias may be in the choosing of study site which would have included many areas but the experts said research is done on specific locality to represent a large population.

CHAPTER FOUR

RESULTS

4.1 Determination of chlorfenapyr diagnostic concentration.

A dose-response survival curve for each of chlorfenapyr and clothianidin insecticides was created following (Brogdon & Chan, 2010). A total of 4000 *An. gambiea s.s,* Kisumu strain were exposed to varying concentrations of chlorfenapyr insecticides with each concentration having 400 mosquitoes. The insecticide test range was between concentrations of 10µg/ml - 100µg/ml with 72_h observation period. There was an increase of 10µg/ml interval until 100% mortality was achieved at 50µg/ml within 48_h and recorded as the LC100. There was no knockdown rate was observed during the 1_h exposure period. The proportion of mortality for the LC100 at 24 hours was 97.75% and 100% mortality achieved at 48 hrs. Concentration of 50µg/ml was the lowest concentration that gave 100% mortality of the susceptible population within 48 hrs hence it deemed suitable as the diagnostic dose to discriminate susceptible from resistant ones in a population. Figure below shows the concentrations response curve of susceptible *Anopheles gambiae,* Kisumu strain.

Figure 4.1 1: Dose-response curve of Anopheles gambiae, Kisumu strain to chlorfenapyr insecticide.

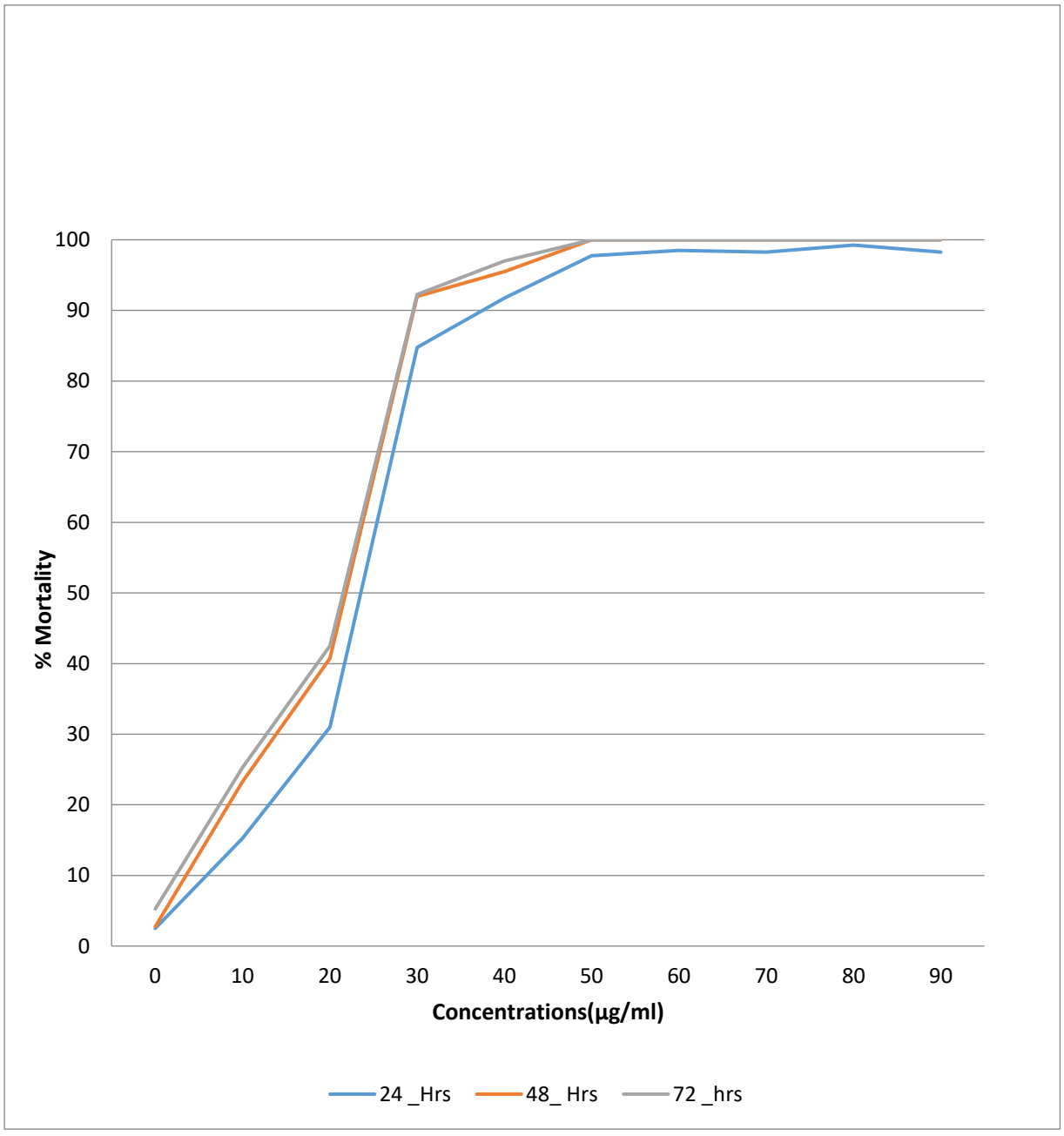


Table 4.1 1: Anopheles gambiae, Kisumu strain response to varying concentrations of chlorfenapyr insecticides.

Concentrations (µg/ml)	**Sample size (N)**	**(n) % Mortality**		
		@ 24 hours Dead (%)	**@ 48 hours Dead (%)**	**@ 72 hours Dead (%)**
0	400	10 (2.5)	11 (2.75)	21 (5.25)
10	400	74 (18.50)	92 (23)	100 (25)
20	400	128 (32.00)	163 (40.75)	170 (42.5)
30	400	329 (82.25)	358 (89.50)	364 (91)
40	400	367 (91.75)	381 (95.25)	387 (96.75)
50	400	391 (97.75)	400 (100)	400 (100)
60	400	394 (98.50)	400 (100)	400 (100)
70	400	393 (98.25)	400 (100)	400 (100)
80	400	397 (99.25)	400 (100)	400 (100)
90	400	393 (98.25)	400 (100)	400 (100)
100	400	387 (96.75)	398 (99.5)	400(100)

4.2 Determination of diagnostic concentration of clothianidin insecticide

A total of 2000 *Anopheles gambiea s.s*, Kisumu strain were exposed to varying concentrations of clothianidin insecticide. Each concentration of clothianidin had 400 mosquitoes exposed to it. The insecticide test was at interval of 50µg/ml until 100% mortality achieved. Concentration ranges were from 50-250µg/ml with 72_h observation period. 100% mortality was achieved with 150µg/ml which had responses of 96.5% mortality at 24_h, 99.5% at 48_h and 100% at 72-h. During the exposure period of one hour there was knockdown observed at concentrations of 50, 100 and 150 µg/ml with mean KD_30 minutes of 0.6875. The concentration of 250µg/ml achieved a mean KD of 0.6875 at 20 minutes. This increased with the increase in concentration and time. Concentration of 150µg/ml was the lowest concentration that gave 100% mortality of the susceptible population within 72_h hence it deemed suitable as the diagnostic dose to discriminate susceptible from resistant ones in a population. The graph and table below show the mosquito responses to varying concentrations of clothianidin insecticide.

Figure 4.2 1: Dose- response curve of Anopheles gambiae, Kisumu strain to clothianidin insecticide.

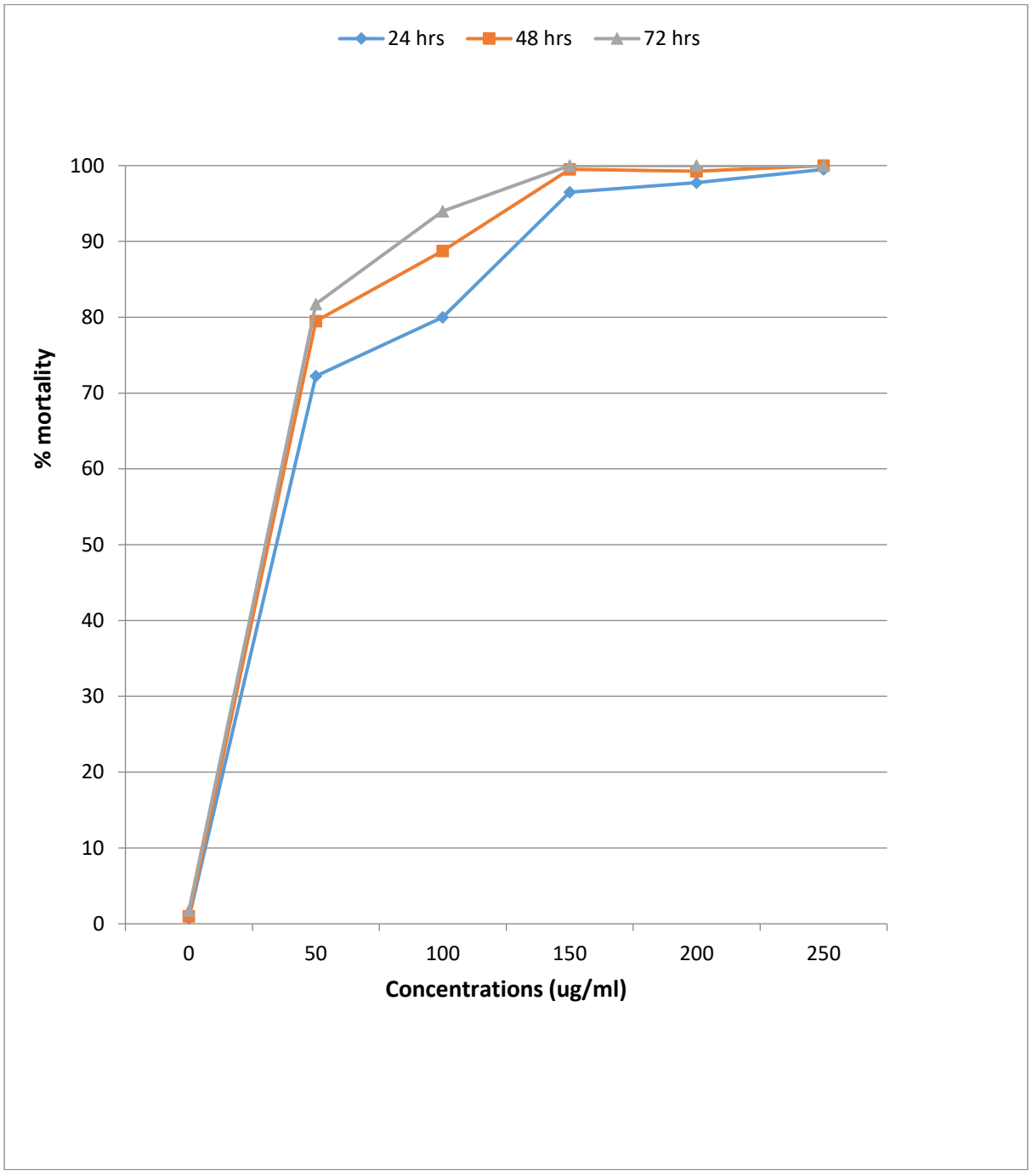


Table 4.2 1: Response of Anopheles gambiae, Kisumu strain to varying doses of clothianidin insecticide.

Concentrations (µg/ml)	**Number exposed**	**Mortality_24 hour**		**Mortality_48 hour**		**Mortality_72 hour**	
		Count	**%**	**Count**	**%**	**Count**	**%**
0	200	3	1.5	4	2	7	3.5
50	400	289	72.25	318	79.5	327	81
100	400	320	80	355	88.75	376	94
150	400	386	96.5	398	99.5	400	100
200	400	391	97.75	297	99.25	400	100
250	400	398	99.5	400	100	400	100

Table 4.2 2: Probit analysis result of chlorfenapyr and clothianidin insecticides

	Chlorfenapyr	**Clothianidin**
Number exposed	400	400
$LD_{50\%}$(95% CI[a])	16.849 (13.576 – 19.906)	27.576 (5.76– 41.24)
$LD_{99\%}$ (95% CI[a])	55.371 (42.761 – 85.647)	143.540(100.176-503.917)
Diagnostic dose	50 µg/ml	150 µg/ml
Chi-square (X^2)	117.454	13.380
P value	0.001	0.040

4.3 Efficacy of chlorfenapyr, Clothianidin and Deltamethrin insecticides against malaria vectors populations of western Kenya

Efficacy of three insecticides was evaluated against field collected *anopheles* populations from three sub counties with a total of 2379 mosquitoes used in the bioassay; 407 from Nyando, 200 from Bumula and 343 from Ndhiwa were exposed to chlorfenapyr at a concentration of 50 µg/ml. For clothianidin insecticides a dose of 150µg/ml was used in the bioassay; 415 mosquitoes from Nyando, 200 from Bungoma and 303 from Ndhiwa. Deltamethrin concentration of 12.5µg/ml was evaluated against 531 mosquitoes. A total of 400 mosquitoes from Ndhwa (200) and Nyando (200) while Bumula had 131 mosquitoes were exposed to deltamethrin insecticide. *Anopheles* populations of western Kenya showed 100% mortality in

CDC-Bottle bioassays for 72 hours holding periods to both chlorfenapyr and clothaindin insecticides with resistance observed in deltamethrin insecticide. Both adult collected as indoor resting collection and larvae raised to adult of 3-5 days old had 100% susceptibility to both chlorfenapyr and clothianidin insecticides. The mean mortality of chlorfenapyr at 24 h was 95.27%, 48-h was 98.42% and at 72-h was 100% while the mean mortality for clothianidin at 24-h was 93.03%, 48-h was 97.82% while at 72-h was 100%. Deltamethrin (pyrthroids) had a mean mortality of 76.8% for Nyando, 45.1% for Bumula and 87% for Ndhiwa with 24-h recovery period. The 4.3.1 below shows summary of efficacy result from CDC Bottle Bioassay.

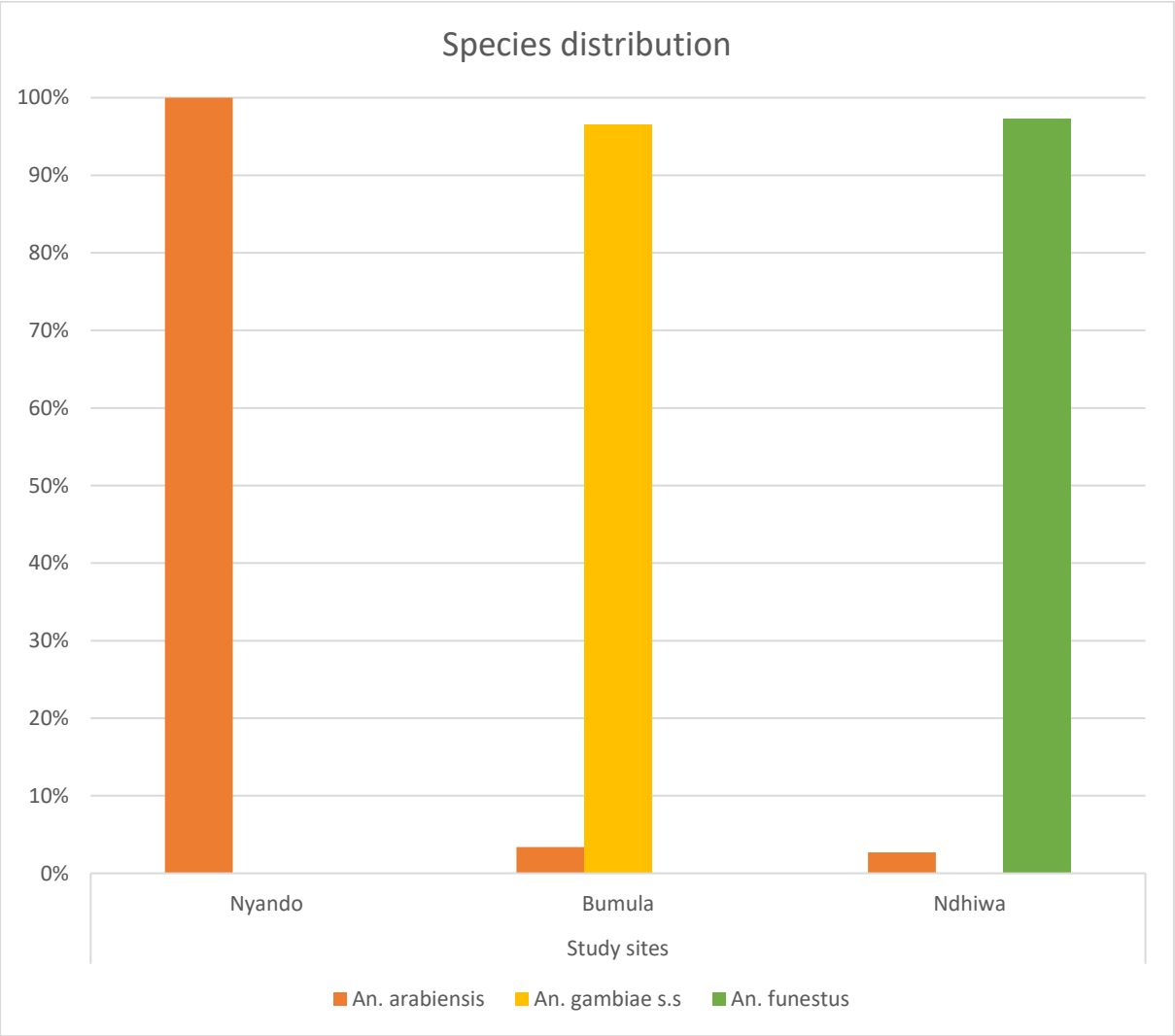
Table 4.3 1: CDC bottle bioassay result for efficacy of chlorfenapyr and clothianidin insecticides.

Site/Population	Insecticide	Sample size (N)	% Knockdown at 60 min	% Mortality		
				24 hrs	48 hrs	72 hrs
Nyando/*An. arabiensis*	Chlofernapyr	407	0	94.6	96.1	100
	Clothianidin	415	12.5	92.8	99.3	100
	Deltamethrin	200	90.5	83	88	90
Ndhiwa/ *An. funestus*	Chlofernapyr	343	0	98	99.4	100
	Clothianidin	303	4.3	89	97	100
	Deltamethrin	200	86	89.1	91.5	94.5
Bumula/ *An. gambiae s.s*	Chlofernapyr	200	0	92.7	98	100
	Clothianidin	200	0.5	98.1	99.5	100
	Deltamethrin	ND	ND	ND	ND	ND

4.4 Molecular identification of the *An. gambiae* complex and An. funestus complex

Convectional PCR was used for identification of 2510 *Anopheles gambiae* and *Anopheles funestus* complexes to species. Nyando Sub County had (1133/1133) 100% *Anopheles arabiensis* while Bumula had high population of *Anopheles gambiae sensu stricto* at (517/531) 97.4% with (14/531) 2.6% *Anopheles arabiensis*. Ndhiwa Sub County had high population of *Anopheles funestus* at (827/850) 97.3% with *Anopheles arabiensis* at (23/400) 2.7%had a distribution in all study sites. A graph of species distribution is shown in figure 4.4.1

Figure 4.4 1: Anopheles malaria vector distribution in the study sites



CHAPTER FIVE

DISCUSSION

Diagnostic dose is the concentration of insecticide that kills 100% of susceptible mosquitoes within a given time (Brogdon and Chan, 2010). During the determination of diagnostic doses of chlorfenapyr and clothianidin insecticides against *Anopheles gambiae s. s,* Kisumu susceptible reference strain both insecticides showed slow mode of action for the 1 hour exposure period. There was no knockdown observed in chlorfenapyr (50 µg/ml) while clothianidin (150 µg/ml) showed a low mean knockdown at 60 minutes of 11.2%. Knockdown effect therefore, cannot be used to determine the strength of mosquito's phenotype resistance when exposed to chlorfenapyr and clothianidin insecticides. Determination of diagnosis doses of these insecticides is very important as they provide baseline data in monitoring insecticides resistance when deploying these insecticides.

The significance of this study was further emphasized when the insecticidal activity of these insecticides were evaluated against the field collected mosquitos. The obtained diagnostic doses of both chlorfenapyr and clothianidin had extended killing effects of up to 72 hours with 100% mortality on the field collected pyrethroid resistance malaria vectors of western Kenya. The *Anopheles* malaria vector populations of western Kenya are mainly *Anopheles gambiae s.s, Anopheles arabiensis* and *Anopheles funestus*. Prolonged mortality rates with chlorfenapyr and clothianidin observed for three days indicates that when these insecticides are deployed in LLINs or IRS may returd the development of insecticide resistance to the members of Anophelines malaria vectors. Chlorfenapyr and clothianidin can therefore be deployed as IRS or in LLINs treatment to complement existing pyrethroids in areas of high pyrethroid resistance. The Global Plan for Insecticides Resistance Management (GPIRM) has four main strategy (rotation of insecticides, combination of intervention, mosaic spraying and mixtures)

for vector control therefore the results of this study put these two insecticides as better candidates to be deployed in either of the strategies.

Resistance to malaria vectors to the major classes of insecticides currently in use is a potential threat that soon may contribute to absolute failure of the control interventions being employed. This already evident with the reversal of gains made in the fight against malaria as already presented in the latest WHO reported increase of malaria cases(WHO, 2018). Pyrethroids (Deltamethrin) susceptibility test in two study sites showed some level of resistance. In Nyando *(Anopheles arabiensis*) exposure to deltamethrin insecticide had mortality between 89%, 88% and 95% for 24, 48 and 72 hours observation period respectively while Ndhiwa (*Anopheles funestus*) mosquitos' exposure to deltamethrin showed mortality at 89%, 92% and 95% for 24, 48 and 72hrs respectively. Despite extended observation period of 72hrs for deltamethrin, there is still resistance. This results confirms the previous findings in other studies (E. O. Ochomo et al., 2013). This is a clear indication of potential threat to the efficacy of pyrethroids which is used intensively in controlling malaria vector for LLINs treatments as well as in IRS. A proactive approach should be adopted so as to delay the spread or arrest resistance in areas with pyrethroids resistance deterring the effectiveness of the already available insecticides.

CDC bottle bioassay principle is to determine the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control. Anything that prevents or delays the compound from achieving its objective of killing the arthropods contributes to resistance. Diagnostic dose is the concentration of insecticide that kills 100% of susceptible mosquitoes within a given time (Brogdon & Chan, 2010). The diagnostic doses were determined using CDC bottle bioassay. The chlorfenapyr (50µg/ml) and clothianidin (150µg/ml) doses were checked on the *Anopheles gambiae,* Kisumu susceptible reference strain before being applied to field populations according to the CDC protocol (Brogdon & Chan, 2010). The solubility of chlorfenapyr was

immediate in absolute ethanol while clothianidin had a poor solubilty in absolute ethanol of up to two days at 4^0C. During diagnostic dose determination of clothianidin insecticide the knockdown time varied with increase of concentration, 50µg/ml had knockdown at 40, 50 and 60 minutes with a mean KD of 0.1075 at 60 minute while 250 µg/ml had knockdown at 20, 30, 40, 50 and 60 minutes with a mean knockdown of 0.4075 at 60 minutes. Chlorfenapyr insecticide had no knockdown during exposure period.

Resistance to malaria vectors to the major classes of insecticides currently in use is a potential threat that soon may contribute to absolute failure of the control interventions being employed. This already evident with the reversal of gains made in the fight against malaria as already presented in the latest WHO reported increase of malaria cases (WHO, 2017). Deltamethrin susceptibility test in two study sites showed some level of resistance. In Nyando exposure of anopheles mosquito to deltamethrin insecticide had mortality between 80%and 75% for larvae collected from the habitat and indoor resting collected mosquitoes respectively while Ndhiwa sample exposure to deltamethrin showed mortality at 87% and Bumula had mortality of 45.1%. This result confirms the previous findings in other studies (E. O. Ochomo et al., 2013) .This is a clear indication of failure on the pyrethroids which is used intensively in controlling malaria vector for LLINs treatments as well as in IRS. A proactive approach should be adopted so as to delay the spread or arrest resistance in areas with pyrethroids resistance deterring the effectiveness of the already available insecticides. Based on the WHO susceptibility assay by use of CDC bottles bioassay exposures of mosquitoes collected from three study sites yielded a mortality of 100% within 72 hours for both chlorfenapyr and clothianidin insecticides. The populations used in the assays were mainly composed of *Anopheles gambiae* complex where *Anopheles arabiensis* and *Anopheles gambiae sensu stricto* were predominantly found Nyando and Bumula respectively while *Anopheles funestus* was the main malaria vector in Ndhiwa.

Chlorfenapyr is a novel insecticide with a registration in 19 countries for the control of various insect and mite pests on cotton, ornamentals and a number of vegetable crops (Rand GM 2004). Chlorfenapyr seems suggested to be a good alternative candidate insecticide for malaria vector control in areas with pyrethroid-resistant *Anopheles gambiae sensu lato* (N'guessan et al., 2007) and *Anophels funestus* (Hargreaves et al., 2000). The results of this study also indicated that chlorfenapyr can be used effectively for vector control as well as management of insecticide-resistant malaria vector species especially pyrethroid resistant vectors. Clothianidin insecticide has proved to be a very good candidate for malaria vector control. This study result shows that clothianidin insecticide is a slow acting with extended killing effect of 72 hours post exposure. Clothianidin can be used to control pyrethroid resistance anopheles malaria vector as it has different mode of action as compared to the currently used vector control insecticides (Uneme et al., 2006) . Due to its slow acting nature it can be used as IRS insecticides in areas with high prevalence of malaria with high pyrethroids insecticides resistance.

Anopheles arabiensis was the only *Anopheles gambiae* complex member found to be present in every study site and exclusive to Nyando Sub County area. Bumula Sub County had the three main malaria vectors with *Anopheles gambiae sensu stricto* as the predominant malaria vector species at 96.6% and a small proportion of *Anopheles arabiensis* at 3.4% while Ndhiwa Sub County had high proportion of *Anopheles funestus* but much lower numbers of *Anopheles arabienis*. The presence of *Anopheles arabiensis* in all the study sites could be because of its zoophilic and exophilic nature which make it be considered as less efficient vector (Bayoh et al., 2010) compared to *Anopheles gambiae sensu stricto* More focus should be therefore put on controlling *Anopheles arabiensis* as its behaviour could increase outdoor biting thus spread of malaria disease.

CHAPTER SIX

SUMMARY OF RESULTS, CONCLUSION AND RECOMMENDATION

6.1. Summary of results and conclusion

Chlorfenapyr (50µg/ml) and clothianidin (150µg/ml) were observed to be very effective in killing anopheles malaria vectors of western Kenya and therefore should be incorporated in malaria vector control. They can either be used for IRS or in the treatments of bed nets. Chlorfenapyr and clothianidin insecticides have showed potential of improving the control of malaria transmission compared to pyrethroids which are commonly used in either IRS or LLINs treatment. There is an urgent need for a crucial pyrethroids resistance management strategy to avoid failure of the control interventions in place.

6.2 Recommendation

1. Evaluation of new insecticides for malaria vector control should be incorporated as part of insecticides resistance monitoring to help in maintaining gains that have been made in the fight against malaria. The diagnostic doses of 50µg/ml and 150µg/ml of chlorfenapyr and clothianidin insecticides respectively obtained should be used as baseline when employing these insecticides.
2. Due to prolonged mortality rates with chlorfenapyr and clothianidin observed for three days these insecticides should be used for IRS as they may require less frequent applications to cover transmission seasons compared to some currently available non-pyrethroid IRS insecticides.

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APPENDICES

Appendix 1: Map of resistance status

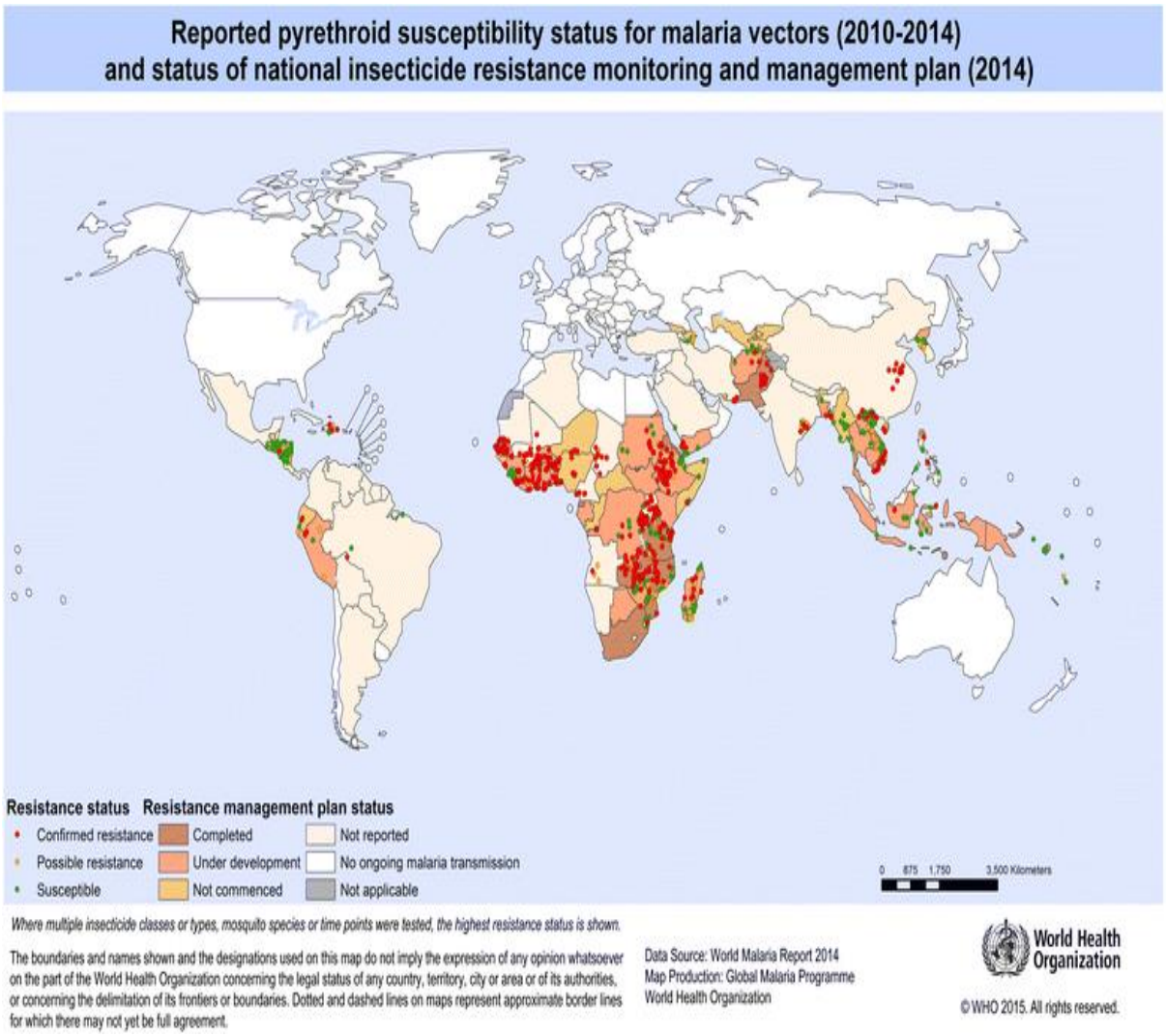

Figure 6.1 1: Global map of malaria vector resistance status to commonly used classes of insecticides.

Appendix 2: CDC-Bottle Bioassay Mortality data form

Date: _____ Mosquito species: _____

Insecticide: _____ Location of mosquito collection: _____

Diagnostic dose: _____ Diagnostic time: _____

Temperature: _____ Humidity: _____

Bottle ID	**No. exposed**	**Knockdown(KD)**														**Mortality**		
		0 min		**10 min**		**20 min**		**30 min**		**40 min**		**50 min**		**60 min**		**24 hrs**	**48 hrs**	**72 hrs**
		Alive	KD	Alive	KD	Alive	KD	Alive	KD	Alive	KD	Alive	KD	Alive	KD	Dead	Dead	Dead
1																		
2																		
3																		
4																		
Control																		

Table 5.1 1: CDC bottle bioassay mortality data recording form

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 11th September, 2017

TO: Silas Okoth Agumba
PG/MSc/PH/0050/2015
Department of Biomedical Science and Technology
School of Public Health and Community Development
P. O. Box, Private Bag, Maseno, Kenya

REF:MSU/DRPI/MUERC/00451/17

RE: Evaluation of the Efficacy of *Chlorfenapyr* and *Chlothianidin* Insecticides on Anopheles Malaria Vector Populations of Ahero and Bungoma, Western Kenya. Proposal Reference Number: MSU/DRPI/MUERC/ 00451/17

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 11th day of September, 2017 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 10th September, 2018. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th August, 2018.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th August, 2018.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to the MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES 11 SEP 2017 MASENO UNIVERSITY

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Figure 6.2 1: Approval latter by Maseno Univesity Ethical Review Committee.