STATUS AND MECHANISMS OF INSECTICIDE RESISTANCE IN VECTORS OF MALARIA WITHIN KILIFI COUNTY ALONG COASTAL KENYA

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DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other University.

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DEDICATION

To my loving wife, Francisca and children, Nicholas, Hope, Raphael and Sharon, for your constant encouragement. God bless all your endeavors.

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LIST OF ABBREVIATIONS AND ACRONYMS

An.	*Anopheles*
AR	*Anopheles arabiensis* primer
Bp	Base pairs
CI	Confidence interval
DDT	Dichlorodiphenyl trichloroethane
DEET	*N,N*- Diethyl-3-methylbenzamide
DNA	Deoxyribonucleic acid
dNTPS	Deoxyribonucleoside triphosphate
F_0	Wild-caught
GPS	Global positioning system
ITNs	Insecticide treated nets
IVM	Integrated vector management
Kdr	Knockdown resistance
KSM	Kisumu
LLINs	Long lasting insecticidal nets
MOH	Ministry of Health
PCR	Polymerase chain reaction
s.l	*Sensu lato*
s.s	*Sensu stricto*
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

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ABSTRACT

Vector control is key to reduction of malaria transmission. In sub-Saharan Africa, vector control programmes are mainly dependent on insecticide treated nets (ITNs) or indoor residual spraying (IRS). However, the progress, as a result of these methods, is currently under threat due to the fast and continuing evolution of insecticide resistance by malaria vectors. Knowledge on the resistance status of mosquito populations and the mechanisms involved form the basis for the development of sound plan for resistance control and management. This study investigated the resistance status of selected insecticides recommended by World Health Organization Pesticides Evaluation Scheme for control of mosquitoes. *Anopheles gambiae s.l.* larvae collected from eight study sites (Burangi, Jaribuni, Kidutani, Mangororo, Mapawa, Mbogolo, Ng'ombeni and Shibe) in Kilifi county were reared up to adulthood in the insectary. From this colony, 3-5 day old non-blood fed females were obtained and tested following the World Health Organization (WHO) procedures for susceptibility to fenitrothion (1%), bendiocarb (0.1%), permethrin (0.75%), deltamethrin (0.05%) and DDT (4%). The knockdown time (KDT) for each insecticide was recorded up to 60 minutes while final mortality was noted after 24 hours post-exposure. Polymerase chain reaction (PCR) amplification of rDNA intergenic spacers was used for identification of the sibling species of the *Anopheles gambiae* complex. In addition, 192 *Anopheles gambiae s.l* mosquitoes were used to determine the genotype constitution at amino acid 1014 of the voltage-gated sodium channel using real time polymerase chain reaction. The data for resistance was classified following World Health Organization criteria with, mortality rates of 98-100% indicating susceptibility, 90-97% possible resistance that requires further confirmation and ≤90% indicating resistance. The knockdown times at 50% and 95% of the mosquitoes were estimated using probit analysis. Final data were entered into Microsoft excel 2010 and analyzed using the R statistical software version 3.2.2. The results indicated that, *Anopheles arabiensis* was the dominant species representing 95.2% of the total collections followed by *Anopheles gambiae* s.s. at 4.8%. The percentage mortality rates indicated resistance to pyrethroids (deltamethrin and permethrin) in Burangi and organophosphate (fenitrothion) in Jaribuni villages. The L1014F knockdown (kdr) mutation was not found in both *An. gambiae s.s* and *An. arabiensis*. However, L1014S kdr mutation was discovered in *An. gambiae s.s* at an allele frequency of 3.33%. The findings from this study provide essential information to the Ministry of Health and public health stakeholders necessary for monitoring the establishment of insecticide resistance and development of management strategies to delay or mitigate its impact on vector control.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a life-endangering mosquito borne disease caused by five closely related protozoa, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale, Plasmodium vivax* and *Plasmodium knowlesi*. *Plasmodium falciparum* is the most abundant malaria parasite in Africa as well as the most virulent. It accounts for majority of the deaths caused by malaria globally (Snow, 2015; Zekar and Sharman, 2021). *Plasmodium vivax* predominates in many countries outside of sub-Saharan Africa (WHO, 2015). *Plasmodium ovale* is primarily found in sub-Saharan Africa. *Plasmodium malariae* predominantly occurs in Africa, South East of Asia and in the tropical and sub-tropical areas of Central and South America (Collins and Jeffery, 2007). *Plasmodium knowlesi* is the fifth cause of both humans and other primates' malaria (White, 2008). It is widely spread in Southeast Asia and the most common cause of human malaria in Malaysia (Amira *et al.*, 2018).

There were 229 million malaria cases in 2019 and approximately 409,000 deaths as a result of malaria globally (WHO, 2020). Ninety four percent of these malaria infections and deaths were documented in the World Health Organization African region and were mostly caused by *P. falciparum*. Most of these deaths occur among women who are pregnant and children aged five years and below due to reduced immunity (WHO, 2019). Malaria also leads to financial burden as a result of medical costs associated with its control, foregone income due to lost workdays and premature death of workers (Sachs and Malaney, 2002).

The key malaria vectors in Africa constitute three species; two *Anopheles gambiae* siblings, *An. gambiae sensu stricto(s.s)* and *An. arabiensis* and one of the *Anopheles funestus* complex, *An. funestus sensu stricto (s.s)*. These species rank globally as utmost efficient malaria vectors (Killeen *et al*., 2013). In addition to the presence of the extreme virulent parasite and efficient malaria vectors, two other factors including ideal environmental conditions for both malaria vectors and parasites and widespread larval habitats create ideal conditions for malaria to prosper in sub-Saharan Africa (Machani *et al*., 2020).

Malaria vector control has been scaled up especially with the current increase in long-lasting insecticide treated nets. The upscale of vector interventions especially the ongoing global efforts against malaria have benefitted immensely from IRS and mass distribution and widespread use of treated bednets (WHO, 2017; 2019). The use of these interventions along with improved malaria diagnosis and treatment have led to a decrease in malaria cases and malaria-related morbidity in the last two decades in the high burden African regions including Kenya (Snow *et al*., 2015; Kamau *et al*., 2020: WHO, 2021). Unfortunately, long-term use of insecticides eventually selects for insecticide resistance that may adversely affect the current gains in malaria control.

When disease vectors become resistant to a particular insecticide, the most immediate logical option is to shift to another insecticide class with a different target site. Unfortunately for malaria, this is a major challenge because pyrethroids: deltamethrin and permethrin and a limited number of alternatives, mainly pyrolles, are secured for the treatment of ITNs (WHO, 2016). Moreover, indoor residual spraying (IRS) relies heavily on pyrethroids, a similar active ingredient, which acts on a common target site enhancing the potential for the evolution of resistance (Yawson *et al*., 2004). Previous reports recorded the existence of resistance to various pyrethroids in malaria vectors

in Kenya (Ochomo *et al*., 2013; Ondeto *et al*., 2017; Kiuru *et al*., 2018; Githinji *et al*., 2020). Malaria is endemic in coastal Kenya and with the increased use and distribution of pyrethroid-treated LLINs then, up to date information on insecticide resistance in malaria vectors is vital. Therefore, this research set out to investigate the resistance status of malaria vectors and elucidate the resistance mechanisms involved in Kilifi County along coastal Kenya.

1.2 Statement of the problem

Despite the extensive utilization of ITNs and IRS in malaria control and increasing evidence that resistance to insecticide is a hefty shortcoming to the effectiveness of these strategies, monitoring of insecticide resistance has remained a low precedence for National Malaria Control Programs in Africa including Kenya (Yewhalaw and Kweka, 2016). Consequently, for many regions, the level of resistance to insecticides in vectors of malaria remains largely unknown and this has implications for malaria control. There is scanty data to show the resistance status of insecticides suggested by WHOPES for application in vector control in *Anopheles* especially after the large scale use of LLINs along coastal Kenya. This investigation was undertaken to ascertain the susceptibility level of *Anopheles gambiae s.l.* to fenitrothion (organophosphate), bendiocarb (carbamate), DDT (organochlorine), deltamethrin and permethrin (pyrethroids) in Kilifi county. It also investigated the resistance mechanisms involved in a malaria endemic area in coastal Kenya. Early detection of insecticide resistance development can facilitate implementation of strategies to minimize its negative impact on malaria control.

1.3 Justification of the study

Recent research has confirmed that, the wide-scale use of a single class, mainly pyrethroids, and related classes of mosquito insecticides has led to evolution of resistance in malaria vectors in endemic regions (Ochomo *et al*., 2014; Ondeto *et al*., 2017; Kiuru *et al*., 2018; Githinji *et al*., 2020). Insecticide resistance has been reported in most of the predominant malaria vectors including *Anopheles gambiae s.s., An. funestus s.s.* and *An. arabiensis* (Yewhalaw and Kweka, 2016). Pyrethroid resistance is the most common of all the reported cases in sub-Saharan Africa (WHO, 2020). This might be due to selection pressure following the scale up of pyrethroid-impregnated LLINs and the use of pyrethroids in most IRS programmes as well as in agricultural settings worldwide (WHOPES, 2006; Wiebe *et al*., 2017). The primary insecticides used in the treatment of ITNs are deltamethrin and permethrin. The use of these insecticides in ITNs and IRS led to reduction in malaria cases and mortality in the last two decades (WHO, 2021).

However, with the rapid evolution and spread of pyrethroid resistance and both East African (L1014S) and West African kdr mutations (L1014F) in mosquitoes (Orondo, 2016), regular monitoring of the status of resistance is required within different malaria vectors in a region. This will help understand the mechanism of resistance and thus lead to implementation of site-specific vector control strategies. The resistance mechanism should be well understood to allow for the formulation of new active ingredients with a different mode of action against the malaria vectors. The use of DDT, with the same mode of action as pyrethroids, is currently banned in Kenya. However, it was also tested in the current study with the sole purpose to explore cross-resistance in malaria vectors. It is paramount to understand that, over-reliance

on a single class of insecticides for vector control poses a big threat of increasing resistance (WHO, 2019). This study also tested the susceptibility or resistance status of bendiocarb (carbamate) and fenitrothion (organochlorine) both of which are possible substitutes for pyrethroids in vector interventions in case of control failure. The current study provided information on the resistance status of insecticides to vectors of malaria in Kilifi county along coastal Kenya after the scale up of vector control interventions.

1.4 Research questions

i. What are the main species of malaria vectors present within Kilifi County?

ii. What is the resistance status of *Anopheles gambiae s.l.* to deltamethrin, permethrin, DDT, bendiocarb and fenitrothion within Kilifi County?

iii. What is the mechanism of knockdown resistance in pyrethroid resistant *An. gambiae s.l.* mosquitoes from Kilifi County?

1.5 Hypotheses

i. The widespread use of LLINs has led to a shift in malaria vector species composition in Kilifi county along coastal Kenya.

ii. *Anopheles gambiae s.l* mosquitoes within Kilifi county are resistant to deltamethrin, permethrin, DDT, bendiocarb and fenitrothion insecticides.

iii. Pyrethroid resistant *Anopheles gambiae s.l.* mosquitoes from Kilifi county have developed knockdown resistance mutation.

1.6 General objective

To determine the status and mechanisms of insecticide resistance in vectors of malaria along coastal Kenya.

1.6.1 Specific objectives

i. To establish the main species of malaria vectors present within Kilifi county using polymerase chain reaction.

ii. To determine the resistance status of *Anopheles gambiae s.l.* to deltamethrin, permethrin, DDT, bendiocarb and fenitrothion within Kilifi county using WHO susceptibility tests.

iii. To evaluate the mechanism of knockdown resistance in pyrethroid resistant *Anopheles gambiae s.l.* mosquitoes from Kilifi county using real-time PCR.

1.7 Significance of the Study

Resistance to pyrethroid insecticides is a great hindrance to the management and control of malaria infections. The current study provided relevant information on the status and mechanisms of resistance in vectors of malaria in Kilifi county. The information generated may be used in informing both the National Malaria Control Program managers and other health partners to develop strategies to manage the spread of insecticide resistance. Furthermore, the selection and choice of insecticides for use in control of malaria vectors in any given area should be guided by the level of resistance and mechanism of insecticide resistance on public health chemicals in use.

CHAPTER TWO

LITERATURE REVIEW

2.1. World malaria burden

Malaria remains one of the World's most important health and development problems (WHO, 2016). It is the worst parasitic disease of human beings with close to 50% of the global human population in 107 countries being under threat of the disease (Murray *et al.*, 2012). About three million people are exposed to malaria with more than two hundred million clinical cases and over four hundred thousand deaths reported annually in the world (Buonsenso and Cataldi., 2010; WHO, 2019). In excess of 90 percent of deaths resulting from malaria arise in sub-Saharan Africa (WHO, 2020). In addition to pronounced disease occurrence and deaths, malaria also causes significant anaemia in HIV co-infected individuals, children aged five years and below and pregnant mothers. It is also linked to untimely birth defects such as preterm delivery, low birth weight, still birth and overall child mortality (WHO, 2019). In addition to morbidity and mortality effect, malaria has a significant influence on the economy especially when it occurs in conjunction with poverty. The impact of malaria is highest among the worlds' poorest countries and remains a major impediment to economic advancement. The disease presents about 0.25-1.3 percent annual reduction in economic growth rate in these countries (Gallup and Sachs, 2001). Previous studies have reported that, wherever malaria flourishes, human societies prosper least (Sachs and Malaney, 2002).

Malaria disease transmission patterns vary considerably between regions and even within individual countries (WHO, 2017). The wide diversity in malaria transmission and severe manifestation can be related to variations in mosquito populations, parasite

ecology and socio economic factors, such as poverty, access to prevention services and reliable health care (WHO, 2005; 2019).

2.2 Malaria disease status in Africa

Africa region bears incomparable brunt of malaria infections and deaths in the world (WHO, 2016; 2019). It is established that, 66% of the population in Africa are vulnerable to malaria (MOH, 2014). Additionally, over 90 percent of the total global malaria associated with mortality occurs in the World Health Organization African region (WHO, 2020). In malaria endemic regions of Africa, the worst and the most lethal *Plasmodium* species, *(Plasmodium falciparum)* is accountable for approximately 74% of all malaria cases (Korenromp *et al*., 2003; Snow, 2015; WHO, 2021). Even though the absolute figures vary, there is a general consensus that, the global malaria situation has been deteriorating over the last decade and that this negative trend will continue unless prompt and effective control measures are urgently implemented (WHO, 2017). According to World Health Organization (2005; 2020), in Africa the deteriorating malaria situation has been caused by: increased drug and insecticide resistance, high HIV infection rates, rapid population growths, civil disturbances and population movements, global warming, changes in land use patterns, breakdown of primary health care system and collapse of vector control programmes.

2.3 Malaria situation in Kenya

Malaria is the primary cause of morbidity and mortality in Kenya. It accounted for 16% of outpatient consultations with more than 75% of the Kenyan population at danger of the disease (NMCP, 2019). Malaria in Kilifi county is endemic with an acute annual transmission and entomological inoculation ranging between 30 and 100 (PMI, 2020). The distribution of malaria in Kenya fluctuates depending on altitude, rainfall patterns and humidity. *Plasmodium falciparum* prevalence is between 5-20% in coastal counties where Kilifi is located. Therefore, *P. falciparum* is the severest of the four *Plasmodium* species in Kenya. It accounts for more than 99% of all malaria infections in the country (PMI, 2021). In high malaria transmission zones, children aged five years and below and pregnant women are significantly susceptible to infections, disease and death due to lack of adequate immunity (PMI, 2019).

2.3.1 Malaria epidemiologic pattern in Kenya

Malaria endemicity levels in Kenya vary from one region to another. There is a great variation in the possibility of malaria infection mostly attributed to climatic conditions such as effects of temperature and altitude (KMIS, 2015; PMI, 2019). Based on malaria risk, Kenya is subdivided into distinct epidemiological zones as follows: endemic areas found around Lake Victoria and Coastal plain; seasonal malaria transmission areas in the hinterland adjacent to coast plain and lake Victoria; epidemic prone areas in the highlands; lowlands and the low risk and or no transmission areas in the mountains (Snow *et al.*, 1999; PMI, 2018). In the coast of Kenya, malaria is endemic and this can be attributed to the suitable climatic

conditions, the most influential components being ideal temperature and the level of precipitation (Snow *et al.*, 2015; PMI, 2020).

2.4 Global distribution of malaria parasites

Malaria disease in human beings is caused by five species of the genus *Plasmodium* namely; *falciparum*, *vivax*, *malariae, knowlesi* and *ovale* (Straat *et al.*, 2022). The species that leads to eminent sickness and mortality in Africa is *Plasmodium falciparum* due to its ability to subvert the physiology of its host during the blood stages of its development (WHO, 2000; 2017). *Plasmodium falciparum* is accountable for 85–90% of the total malaria cases in tropical Africa and Asia due to presence there of efficient mosquito vectors (*Anopheles gambiae* complex) and population movement and rapid urbanization respectively (Snow *et al.*, 2017). *Plasmodium vivax* primarily causes malaria in South America and is uncommon in sub-saharan Africa. *Plasmodium ovale* is predominantly found in tropical Africa. *Plasmodium malariae* is the least common and infrequent species all over the world (Hombhange, 1998). *Plasmodium knowlesi* is naturally maintained in macaque monkeys and causes zoonotic malaria widely in South East Asia (Sato, 2021). The disease at times can be caused either by a single or multiple infections with the five *Plasmodium* species.

2.5 Vectors of malaria in Africa

There are about 3,000 mosquito species and approximately 100 species are vectors of human diseases. About 380 species of *Anopheles* mosquitoes exist around the world

and approximately 60 species are vectors of malaria (Wiebe *et al*., 2017). Female *Anopheles* mosquitoes are the vectors of malaria and can remain alive for two to three weeks under natural conditions (Service, 1976). This longevity is important because all efficient vectors of malaria must live adequately for the parasite to undergo complete development to enhance disease transmission.

Factors responsible for sustainability of mosquito life cycles as well as parasite development and transmission to human hosts are basically ecological. Climate is paramount in reducing the transmission and spread of malaria over an extensive region (Craig *et al*., 1999). Lindsay *et al*. (1989) established a positive direct relationship between malaria and warm weather in endemic countries. It has also been observed that malaria prevalence in Kenya is determined by climatic and ecological conditions which favor survival and relative abundance of vectors such as: ideal temperatures, favourable level of precipitation as well as abundance of mosquito breeding habitats (Keating *et al*., 2005; KMIS, 2015; PMI, 2020).

2.5.1 Vectors of malaria in Kenya

Malaria is caused by parasites which are mainly vectored by *An. funestus s.s, An. arabiensis* and *An. gambiae* s.s. in western and coast provinces of Kenya (Mwangangi *et al*., 2013; PMI, 2020). These species exist all year round with their peak populations mainly during the rain seasons. In Mwea-Tebere irrigation scheme for instance, *Anopheles arabiensis* and *An. funestus s.s.* constitute the key vectors (Muturi *et al*., 2008; Orondo, 2016) while in western Kenya, *Anopheles arabiensis* and *An. gambiae* s.s represent the *An. gambiae* sibling species (Ochomo *et al*., 2013; Githinji

et al., 2020). In coast Kenya, the main vectors of malaria are *Anopheles gambiae s.s*., *Anopheles arabiensis* and *An. funestus* (Mbogo *et al*., 2003; Kiuru *et al*., 2018).

Malaria parasite infection rates in *An. funestus* are mostly less than those in *An. gambiae* (Ogola *et al*., 2018). Therefore, *An. funestus* is a secondary vector of malaria after *An. gambiae*. *Anopheles funestus* is highly susceptible to insecticides hence is easily eliminated and is slow to re-colonize the same place. In western Kenya, this species has been reduced greatly in ITN intervention areas (Gimnig *et al*., 2003; Githinji *et al*, 2020). Generally, *Anopheles gambiae s.s*. occurs in large numbers during rains while *An. funestus s.s* is common towards the close of rains and at the start of dry period (Coetzee and Fontenille, 2004). *Anopheles funestus* prefers to breed in more permanent water bodies with thick vegetation whereas *Anopheles gambiae* breeds in temporary turbid waters (Debrah *et al*., 2021).

2.6 Feeding preferences and resting behavior of mosquitoes

The development of eggs in mosquitoes requires animal or human blood meal. This meal contains important proteins required for the egg development process (Kogan, 1990). Most mosquito species show preference to feed on the blood of specific vertebrate host. Malaria spreads through a bite from a diseased female *Anopheles.* The feeding mostly occurs at night although daytime and/or early evening biting may also occur. Some mosquitoes feed indoors or outdoors in forests (WHO, 2017). Those mosquitoes that bite at night are easy to avoid than those that feed in the early evening.

Blood meal digestion and egg development takes about 2-3 days at temperature above 23 ºC (White, 1982). Therefore, blood-fed female searches for a shaded, safe place where it rests until the eggs are fully developed. Thereafter, the gravid female leaves its resting place to look for a suitable oviposition site. Some mosquito species rest in cattle sheds or houses, while others rest on vegetation or at other natural sites like crevices (Machani *et al*., 2020). According to Bhatt *et al*., (2015), various factors affect the choice of mosquitoes resting place and these include temperature, humidity and protection against sunlight, wind and predators. During rains and the wet season, vector species like *An. gambiae* and *An. funestus* may rest outside due to greater availability of shaded areas (Gillies and De Meillon, 1968). Resting behavior is also important as it governs selection of control measures; for instance, mosquitoes that rest indoors are easier target for the control by IRS and use of ITNs (Curtis *et al*, 2003) while those that rest outdoors would be difficult to control by such methods.

Anopheles gambiae and *Anopheles arabiensis* share fairly similar larval ecology, breeding in short-term, shallow small water bodies, for example, puddles in hoof prints, small ground pool sites and wheel ruts (Ondeto *et al*., 2017; Machani *et al*., 2020). *Anopheles gambiae* s.s is both endophilic and anthropophilic, making it efficient malaria vector. *Anopheles arabiensis* is exophilic and partially zoophilic making it a less likely target for LLINs (Mbogo *et al*., 2003). *Anopheles funestus* is both anthropophilic and endophilic and breeds on permanent aquatic habitats with thick vegetation especially in the grass found at the edge of such water bodies (Debrah *et al*., 2021). *Anopheles melas* and *Anopheles merus*, less important vectors, are salty water breeders on the East and West Africa coasts (Mbogo *et al*., 2003; Service, 2008).

2.7 Control of malaria

Malaria is a major global source of death and disease. However, after an infection the clinical course of the disease can be predicted. Therefore, the disease can be prevented or treated (WHO, 2003; 2017; 2019; 2020). Drugs are available for the treatment while preventive methods can be used for the control of the vectors. Over the years, WHO has adapted different control techniques to combat the disease. This is dependent on the availability of the control tools and the malaria status in endemic areas (WHO, 2006; 2015). The current World Health Organization global control strategy focuses on four main elements namely; disease management through prompt examination and treatment, use of selective and sustainable preventive measures through the use of ITNs and other appropriate local methods, early testing and prevention of epidemic, and the enhanced safeguarding and treatment of malaria in pregnant women and children in endemic areas (Nabarro and Tayler, 1998; WHO, 1998; NMCP, 2019). Vector control strategies mainly focus on adult and larval mosquito control.

2.7.1 Control of adult mosquitoes

Control of malaria entails an integrated and an all-dimensional strategy which is more reliable than a single line approach (Shea and Chesson, 2002; shea *et al*., 2002). Hence vector control should include a strategy with a combination of different vector interventions (Coleman *et al*., 2006). Control strategies for example, indoor residual spraying (IRS), insecticide treated nets (ITNs) and curtains and intradomicile application of insecticide treated materials (ITM) have so far yielded positive output as combined interventions (Naim *et al*, 2006). The major control methods that have

been broadly utilized are both untreated and treated mosquito nets such as LLINs and curtains and indoor residual spraying (IRS) (WHO, 2020). Other methods that may prove to be of great value but have been overlooked in recent years are improved housing and proper sanitation (Killeen *et al*., 2002).

2.7.1.1 Use of mosquito nets

An attractive option in control of malaria vectors is use of untreated nets. This method was used for many centuries as early as the 6th century BC in the Middle East. The use of untreated nets has shown reliable protection as long as the nets are properly tucked in and kept in good working condition. The nets should be large in size to prevent the users from making contact with them (Lindsay *et al*., 1989). However, the untreated nets can be improved by treating them with insecticides to improve their efficiency in killing or repelling potential malaria vectors.

Insecticide-treated nets (ITNs) are manufactured in different shapes and colors. They act as human baited traps when people sleep under them by attracting and killing mosquitoes. They are important tools in the control of malaria (WHO, 2021). Presently, synthetic pyrethroids have been recommended for the treatment of nets due to their minimal lethal capability to humans and quick action on insects (WHOPES, 2006; WHO, 2017). Pyrethroids specifically have a powerful excito-repellent effect hence those lying next to individuals under the net are also protected from mosquito bites. Their ability to repel the vectors makes old and torn nets relatively viable (Mutuku *et al*., 2011). Furthermore, permethrin-treated bed nets have a community-wide suppression of mosquito population thus villages neighbouring intervention areas are also offered some protection (Gimnig *et al*., 2003).

Insecticide treated nets have also proved to reduce malarial related episodes by 50% and reduce mortality from all causes by up to 20% in Africa (O'meara *et al*., 2010). Across sub Saharan Africa, possession of at least one ITN per every two persons increased from 50% in 2010 to 80% in 2016 (WHO, 2017). This increment in ITN usage resulted in 20% reduction in the incidence rate of malaria (WHO, 2019). In Kilifi, the use of insecticide treated nets was linked to a reduction of 33% in the incidence of malaria hospitalization among children aged below 5 years and decreased malaria-related deaths (Kamau *et al*., 2020).

Recently, there has been a pronounced decrease in the global burden of malaria, morbidity and mortality (WHO, 2020). This decrease is primarily as a result of the fight against malaria using IRS and LLINs (WHO, 2021). The application of these vector control methods coupled with over dependence on one class of insecticides, the pyrethroids, largely exposes malaria vectors to selection pressure for resistance.

2.7.1.2 Indoor residual spraying

Adult mosquito control involves protection of households using insecticides and in some cases insect repellents to prevent mosquito bites. This reduces mosquito longevity, which suppresses community level transmission (Killeen *et al*., 2013). Insecticides are used to spray space and walls inside or outside human dwellings where they leave residual effect. Indoor residual spraying (IRS) entails the application of chemical insecticides with prolonged residual activity on the walls and eaves of houses. This is purposely done to eliminate the mosquitoes that perch and rest on such areas (Olanga *et al*, 2010). It is one of the methods of killing indoor resting adult mosquitoes. When applied in the right circumstances this method reduces the vector

life span, vector population size and number of humans bitten thus reducing malaria transmission (Curtis and Mnzava, 2000).

A wide variety of chemicals for indoor residual use have been assessed and proposed by the World Health Organization Pesticide Evaluation Scheme (WHO, 2016). These include organochlorines (DDT), carbamates (propoxur, bendiocarb and carbosulfan), organophosphates (pirimiphos-methyl and fenitrothion) and photostable pyrethroids such as cypermethrin, lambdacyalothrin, alphacypermethrin, permethrin, cyfluthrin, etofenprox and deltamethrin (WHO, 1993; Chavasse and Yap, 1997). The pyrethroid insecticides are fast acting and with high efficacy against mosquito vectors. In comparison to other insecticides, they are safer to humans, animals and the environment when used at the recommended dosages for they have a short residual action (WHO, 1997; Hemingway *et al*., 2014).

The use of insecticides with residual activities has successfully been utilized in the control of vectors globally. In Pare Taveta scheme along the United Republic of Tanzania-Kenya border, and the islands of Zanzibar and Pemba, house spraying abated the population of *Anopheles funestus* to negligible levels (Curtis and Mnzava, 2000). In Kisumu Kenya, 43.5% reduction in malaria incidence was recorded for all age groups after a WHO sponsored trial of spraying in 1970s. It has been documented that, resistance to insecticides by mosquitoes hampers the effectiveness of this method (Nauen, 2007; Kiuru *et al*., 2018). Resistance to pyrethroids and DDT has already been reported in members of the *An. gambiae* complex in Africa (Ochomo *et al*., 2013; Kiuru *et al*., 2018; Githinji *et al*., 2020).

2.7.1.3 Use of mosquito repellents

Repellents contain ingredients that drive away and prevent mosquitoes from biting. They are applicable on the skin, clothing or mosquito nets. Traditionally they have been widely used to keep mosquitoes and other arthropods away from homes (Mayeku *et al*., 2014). Several synthetic repellents have been made with protection duration of 2-10 hours such as N,N-Diethyl-3-methylbenzamide (DEET). They are normally in the form of creams, oils or aerosols (Wilson *et al*., 2014). They provide temporary protection against mosquitoes (Mc Cabe *et al*., 1954). Plant repellents exert their control influence through the potent constituents contained in their tissues especially the foliage. Such repellents include the neem (*Azadirachta indica*). Repellents help to minimize human-vector contact especially during odd hours when other methods like bed nets are not applicable (Das *et al*., 2003; Sangoro *et al*., 2020).

2.7.2 Control of larval mosquitoes

Larval control is a suitable practice in vector management especially in areas capable of community participation and where conditions are favorable. Locations suitable for the development of the immature stages of mosquitoes should be destroyed using both physical and chemical means as part of the integrated vector management program (Coleman *et al*., 2006). The method entails application of microbial insecticides such as BTI, Spinosad, *Lysinibacillus sphaericus* or juvenile hormone analogues and chemical insecticides to the water surface or to the breeding sites to kill mosquito larvae. They kill either as stomach poisons when ingested by larvae or through contact (Rozendaal, 1997; WHO, 2013). The earliest insecticides to be used in mosquito control were essentially inorganic chemical like Paris green and petroleum oils

(Rozendaal, 1997; Gachelin *et al.*, 2018). When applied on the water surface, oil penetrates into the trachea of the larvae and kills them either by suffocation or poisoning. Paris green is an insoluble microcrystalline green powder which floats in water surface. It is dispersed in the form of dust and it is mixed with substances like powdered charcoal, or some other locally available materials. Currently with the development of highly effective and reasonably safe organophosphorus and other larvicidal compounds, the use of Paris green has markedly reduced. A high number of larvicides are available in several formulations like wettable powder, suspension concentrate, emulsifiable concentrates, granules, pellets and briquettes (Rozendaal, 1997). Application of larvicide must be repeated at intervals corresponding to the development cycle of the targeted vector (WHO, 2013). This method is cumbersome and expensive because it entails locating all the larval habitats in a given area which is not really practical on a large region.

2.8 Insecticide resistance

Resistance is the ability of a pest population to withstand a dose of a toxicant that would prove harmful to most of the organisms in an ordinary population of a given insect species (WHO, 1957). This should be a major concern only if the recommended dose of the insecticide does not control the pest (WHO, 2002; Msami, 2013). Insecticides are either inorganic or organic (Ondeto *et al.*, 2017). Inorganic compounds contain constituents such as fluorine, phosphorus and sulfur. Organic insecticides are synthetic and of botanical origin. The synthetic insecticides are divided into: Organochlorines for example DDT and dieldrin, carbamates such as propoxur and bendiocarb, organophosphates such as malathion and pyrethroids, for

example, permethrin and deltamethrin. Insecticide resistance develops due to over use or application of insecticides within the same class or with the same mode of action.

Widespread resistance to DDT and pyrethroids in vectors of malaria has been indicated in West Africa (Awolola *et al*., 2009), in South Africa (Casimiro *et al*., 2006) and Kenya (Ochomo *et al*., 2013; 2014; Kiuru *et al*., 2018). In addition, pyrethroid resistance mechanisms that bring about cross-resistance to DDT are geographically widespread (Santolamazza *et al*., 2008). Understanding insecticide resistance at molecular level is important as the resistance gene may be detected before it spreads to sibling species that are susceptible to pyrethroids (Weill *et al*., 2000).

2.9 Mechanism of insecticide resistance

The two main mechanisms of resistance to insecticides in insects especially mosquitoes are: physiological resistance and metabolic resistance (Soderlund and Bloomquist, 1990; Panini *et al*., 2016). These are the two basic and widespread mechanisms of insecticide resistance reported in malaria vectors (Hemingway *et al*., 2016). Metabolic resistance is the most common and poses a great threat while physiological resistance is the most commonly studied mechanism in malaria vectors in Africa due to its easy experimental procedure.

2.9.1 Physiological resistance mechanism

Physiological resistance may involve reduced penetration of insecticides and/or target site insensitivity. The target sites for organophosphates and carbamates are acetyl

cholinesterase (AchE) in the nerve synapses while that of pyrethroids is the sodium channel of nerve sheaths (Brogdon and McAllister, 1998). Among the resistant strains of mosquitoes that have been studied in western Africa, knockdown resistance is the most common (Chandre *et al*., 2000). Knockdown resistance refers to resistance to both DDT and pyrethroids and is characterized by decreased sensitivity of the nervous system (Soderlund and Knipple, 2003). This mutation results in the substitution of leucine by phenylalanine and is predominantly found in West Africa (kdr-w). It may also result in leucine to serine substitution (kdr-e), initially noted in Kenya but has spread to various other countries in Africa such as: Equitorial guinea, Gabon, Cameroon, Uganda and Angola (Santolamazza *et al*., 2008; Ochomo *et al*., 2013).

The knockdown resistance occurs as a result of a simple point gene substitution in the sodium ion channel and is characterized by knockdown (Martinez-Torres *et al*., 1998). Such pyrethroid resistance is known as 'kdr' due to the knockdown resistance phenotype observed in house flies with this type of mutation (Williamson *et al*., 1996). Knockdown resistance produces cross-resistance between pyrethroids and DDT and is the only resistance common to both insecticide groups (Omer *et al*., 1980). In western Kenya, a leucine-serine transposition was found in the sodium channel of *An. gambiae* (Ranson *et al*., 2000). Alterations to target sites related to resistance are common and frequently associated with phenotypic resistance (Kwiatkowska *et al*., 2013).

2.9.2 Metabolic resistance mechanism

Metabolic resistance is caused by massive replication of genes coding for enzymes. The main enzymes involved include: glutathione-S-tranferase, monooxygenase and

esterase (Hemingway *et al*., 2016). Some enzymes detoxify or biochemically change the insecticidal compound into non-toxic form for example, cytochrome P450 monooxygenases while others such as esterases breakdown the insecticide preventing chemical reactions detrimental to normal physiology (Li *et al*., 2007). Evidence shows that P450 monooxygenases are greatly involved in pyrethroid resistance (Berge *et al*., 1998; Feyereisen, 2005). In Kenya, the function of cytochrome P450 enzymes in permethrin resistance in *Anopheles gambiae* was shown by use of P450 inhibitor, piperonyl butoxide (PBO) (Vulule *et al*., 1999). However, the potentiality of P450s to metabolise pyrethroids remains unclear.

2.10 Insecticide resistance monitoring

The resistance of malaria vectors poses a serious hindrance to reliable vector control. Pyrethroid resistance is the main setback for mosquito vector control programs (Kawada *et al*., 2011; WHO, 2013). To control pyrethroid resistance in vectors of malaria, it is paramount to regularly monitor insecticide susceptibility and establish an effective insecticide management system. World Health Organization has produced and published several guidelines and procedures for investigating the presence of resistance (WHO, 1981; 1998; 2013; 2016). In the wake of modern technology and the necessity for immediate action to prevent the spread of resistance among malaria vectors, the guidelines on interpreting WHO susceptibility results has been revised (WHO, 2013; 2016) (Table 2.1).

It is necessary to establish and apply insecticide resistance management strategies before the resistance mechanism spread and become common in a population. Otherwise, the resistant gene may not recede. These resistance management tools or

methods include: mosaic spraying, combination of different control interventions, rotations of insecticide and use of mixtures (WHO, 2012). These resistance management tools or methods may be practized in the broad settings of integrated vector management (IVM). If nothing is done and insecticide resistance is allowed to increase and spread, these would distract or reverse the gains achieved in reducing malaria transmission (Hemingway *et al.*, 2016).

Table 2.1:**Comparison between previous and current WHO insecticide resistance classification**

WHO insecticide resistance classification		
Status	**Previous**	**Current**
Susceptible	Mortality 98 – 100 %	Mortality 98 – 100 %
Possible resistance	Mortality 80 – 97 %	Mortality 90 – 97 %
Resistance	Mortality <80 %	Mortality <90 %

Source: WHO, 2013; 2016.

CHAPTER THREE

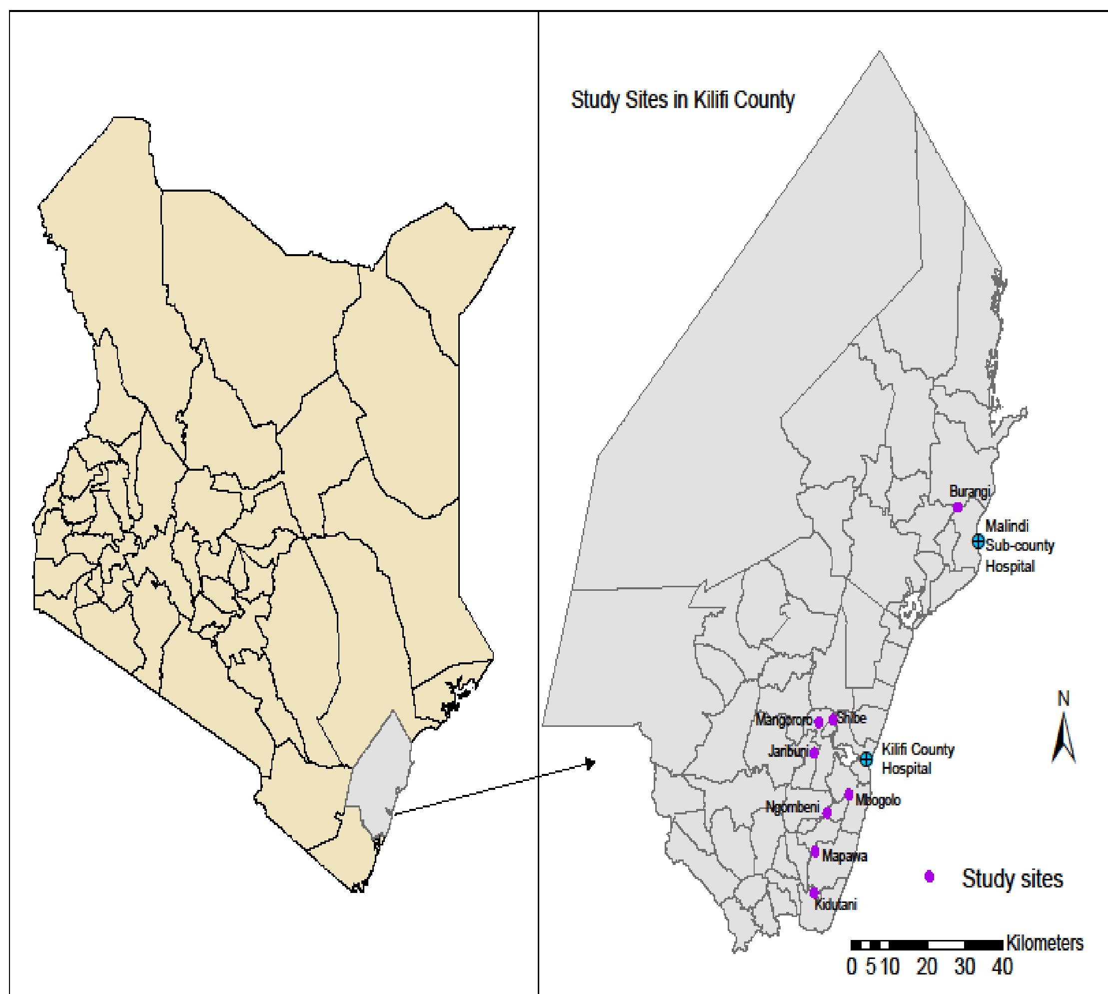
MATERIALS AND METHODS

3.1 Study sites

This study was carried out in Kilifi county in the coastal region of Kenya. The county is found North and North East of Mombasa county on the coastline. It borders Taita Taveta, Tana River, Kwale and Mombasa counties. It consists of several sub-counties namely, Kilifi, Ganze, Malindi, Magarini, Rabai and Kaloleni. The county has an area of 12,609 Km^2 and the human population exposed to infection risk is 1,446,856 (KNBS, 2019). The study was conducted in eight study sites purposively selected based on malaria vector abundance and ITNs distribution profile. The latitude and longitude of each of the study sites was recorded by use of the global positioning system (GPS) device type [Garmin International Inc., Olathe, Ks]. The eight villages were: Ng'ombeni (Latitude -3.73208 Longitude 39.76491), Mbogolo (Latitude -3.69806, Longitude 39.81706), Jaribuni (Latitude -3.62054 Longitude 39.73354), Kidutani (Latitude -3.88209 Longitude 39.71819), Shibe (Latitude -3.55840 Longitude 39.77921), Mangororo (Latitude 03.56366 Longitude 39.74507), Burangi (Latitude -3.09828 Longitude 40.04817) and Mapawa (Latitude -3.80452 Longitude 39.73641). A map depicting the eight sampling sites was then developed to show the exact location of the study sites as shown in figure 3.1 (a) and (b).

The coast region of Kenya often experiences two rain seasons annually. The long rains usually fall from April to July while short rains from October to November. The average annual precipitation ranges between 750 and 1,200 mm. The average daily temperature varies from 22-30 °C (Mwangangi *et al.*, 2012). Majority of the houses

are mud-walled and thatched with palm leaves. Between the walls and the roof there is an open space that facilitates entry of mosquitoes. Residents mainly practice small scale farming where little or no chemical fertilizers and pesticides are applied. Small-scale irrigated agriculture is practised close to river Sabaki and Jaribuni. This irrigation system of agriculture uses pesticides, mainly of the class organophosphates (Mwangangi *et al*., 2013).



3.1 (a) **3.1(b)**

Figure 3.1:(a) A map of Kenya showing the location of Kilifi county

(b) A map of Kilifi County showing the study sites

3.2 Study design

The study utilized a longitudinal design approach where data was obtained at various distinct times on the same set of cases and variables. The dependent variable was measured at several time points for each subject as described by Skinner *et al*. 2007. Briefly this involved: mosquito collection in various breeding sites, carrying out susceptibility bioassay tests and determination of knockdown resistance patterns as outlined in figure 3.2. Field collected larvae were reared in the insectary to produce F_0 generation for the test procedures which were outlined as follows: a) rearing mosquitoes in the insectary (b) performing the susceptibility tests (c) storage of individual mosquito specimens and (d) molecular analysis or testing.

3.3 Sample size

The sample size was determined following the World Health Organization guidelines. According to WHO (2013; 2016), 150 female mosquitoes of age 3-5 days are used to conduct a single set of WHO susceptibility test per insecticide. The mosquitoes should be non-blood fed. This is because physiological statuses such as blood-fed, gravid and semi-gravid affect the susceptibility of an insecticide (WHO, 1998; 2013). For this study, one hundred mosquitoes were used in four replicates of twenty five each per single test (WHO, 2016). For the positive control, 25 susceptible *Anopheles gambiae* Kisumu (KSM) strain was subjected to WHO tubes with treated papers while in the negative control, 25 field collected females were subjected to the WHO tubes with untreated papers.

Field collected larvae were reared in the insectary to produce F_0 generation. These were categorized into:

a) Test population: Field mosquitoes subjected to the insecticide treated bioassay papers.

b) Negative control: Field mosquitoes subjected to untreated bioassay papers.

c) Positive control: Susceptible KSM strain exposed to treated bioassay papers.

Bioassay testing was done following the WHO standard procedure (WHO, 2016)

a) Non-blood fed female mosquitoes of the age 3-5 days were used per test.

b) Mosquitoes were subjected to bendiocarb, fenitrothion, DDT, deltamethrin and permethrin.

c) Knockdown time was registered at 10 minute interval for one hour.

d) Bioassay tests were done at temperature of 26-29°C and 74-82% relative humidity.

After bioassays, both surviving and dead mosquitoes were;

a) Each stored in a mosquito vial, labeled and kept in desiccated silica gel.

b) Specimens for molecular analysis were kept at -70°C.

Mosquito identity and kdr detection

a) DNA was extracted from the legs and wings and used for species identification and Knockdown resistance analysis.

Figure 3.2: WHO guidelines for mosquito susceptibility and molecular testing (WHO, 2016)

3.4 Larval mosquito collection

Larval mosquitoes were collected in June (long rain season), August (during dry season) and November (short rain) in the year 2012 and again in July (immediately after the long rains) 2013. The collections were carried out over the various seasons to ensure sampling of a wide variety of mosquitoes in the larval habitats. Larvae were collected from stagnant waters sampled in each study site by use of the standard dipping technique (Service, 2004). In each village, five collection sites were sampled once per week. The five collection sites were purposively chosen in order to collect many larvae. The habitats consisted of ponds, sand pits, roadside ditches and river banks. The standard 350ml dipper was used to make 10 dips per suitable larval habitat (Figure 3.3). However, in larval habitats with few larvae, the number of dips was increased to 15 in order to collect as many larvae as possible. The Anopheline larvae were separated from Culicine larvae and other aquatic arthropods using dropper pipettes. The larvae were separated based on the abdomen posture position in water and siphon condition. Anopheline larvae do not have a siphon and stays parallel to the water surface while Culicine larvae possess a siphon and stay inclined to the water surface. They were then put in whirl-pak® bags and transported to an insectary in KEMRI, Kilifi for rearing.

Figure 3.3: Larval collection from water pool in a habitat in Kilifi county

3.4.1 Rearing the F0 adult mosquitoes

The field collected larvae and pupae were placed in larval pans or plates in the insectary (Figure 3.4). Tetramin® baby fish was used as the larvae rearing diet. The temperatures were kept between 25°C-27°C while the relative humidity was maintained between 74 and 82%. Checking of the larval pans was done daily and pupae collected twice and put into plastic cups in the mosquito rearing cages (Figure 3.5). The adults (F_0) emerging were categorized using morphological keys (Gillies and Coetzee, 1987). Briefly, the morphological characteristics considered included: position of the *Anopheles* mosquito at rest and the body colour pattern. The F_0 adults were kept as cohorts of same age and later used for insecticide susceptibility testing.

Figure 3.4: Mosquito larval trays

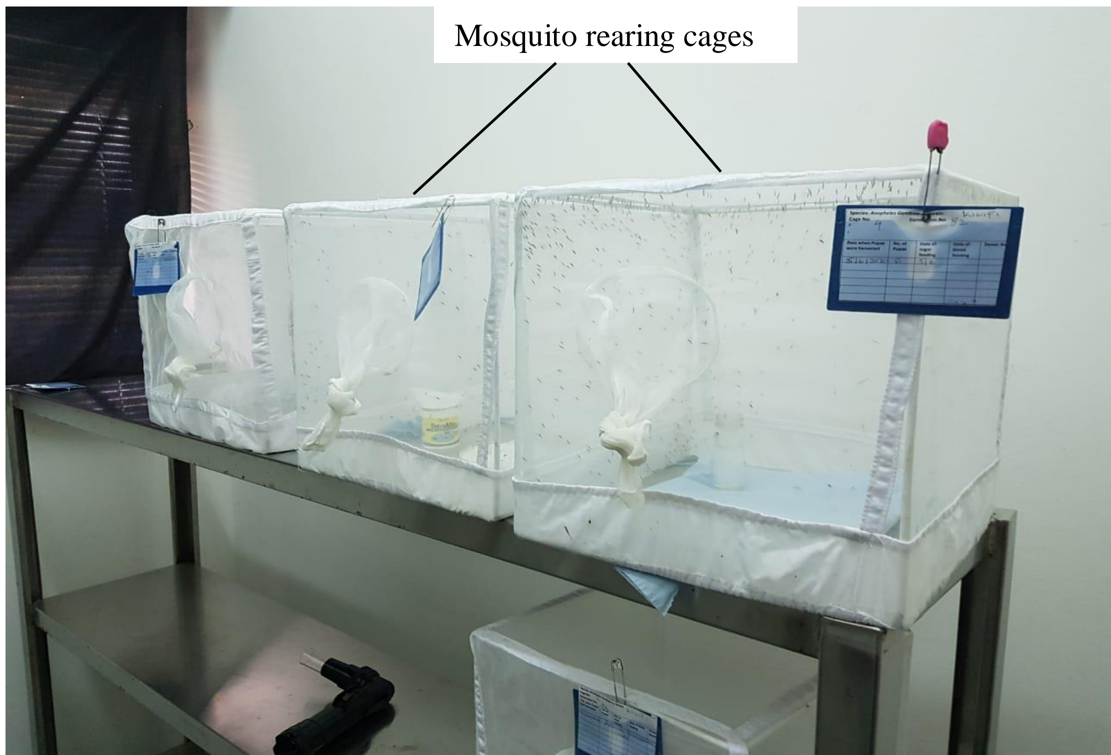


Figure 3.5: Adult mosquito rearing cages.

3.5 Determination of the resistance status of *Anopheles gambiae s.l* to different insecticides in Kilifi county

Bioassays were done on field-collected female mosquitoes reared in the insectary using World Health Organization susceptibility tubes and impregnated papers. The insecticides used included: dichlorodiphenyltrichloroethane (DDT), deltamethrin, permethrin, fenitrothion and bendiocarb. A set of 25 mosquitoes from the collection sites was subjected to untreated papers as the negative control while 25 susceptible *Anopheles gambiae s.s* KSM acted as the positive control. The knockdown time (KDT) for each insecticide was noted after every 10 minutes for one hour. The mosquitoes subsequently were moved to a separate tube containing 10% sucrose. The terminal mortality was noted after the expiry of 24 hours. All bioassays were done at 25-27 ºC and 74 - 82% relative humidity as adopted from the WHO protocols (WHO, 2016).

After recording mortality 24 h post exposure, the live mosquitoes were aspirated using mouth aspirator into paper cups, kept at -20°C for 30 minutes in order to die and then stored individually in labeled eppendorf tubes (Figure 3.6). The dead mosquitoes were also stored for species identification use. The labels showed the insecticide tested, study site, date of the susceptibility test and whether the mosquito died or survived after 24hr post exposure. Specimens for molecular analysis were preserved at -70°C.

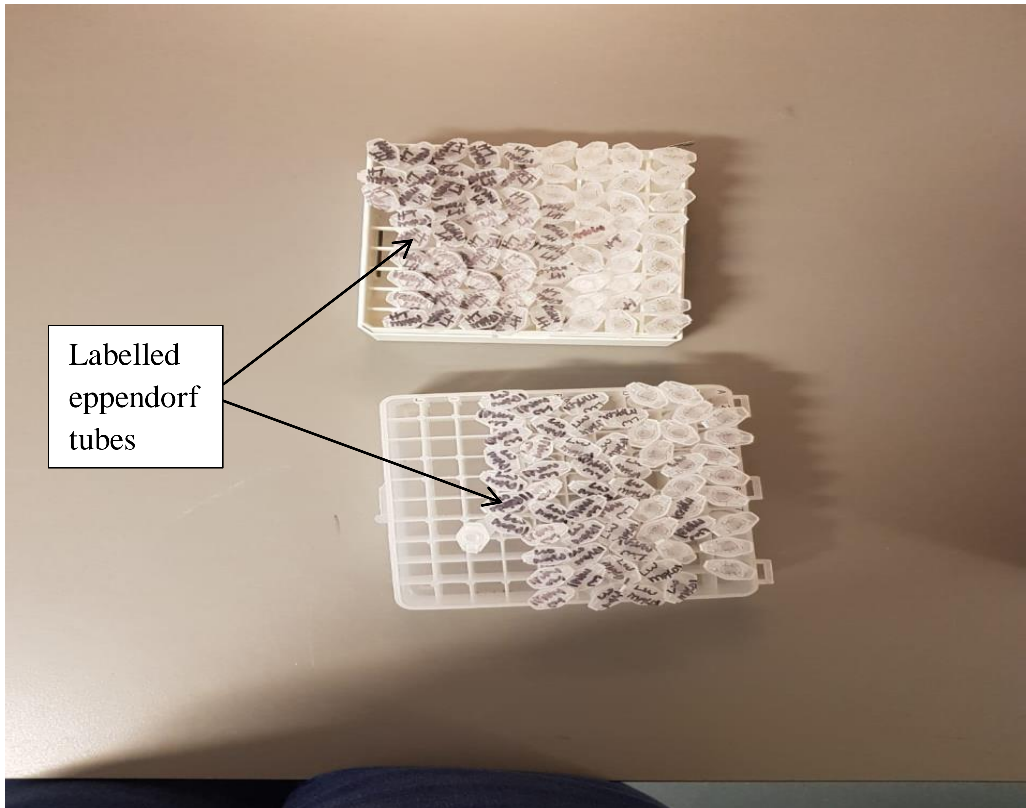


Figure 3.6: Labelled eppendorf tubes with mosquito specimens for storage

3.6 Molecular techniques

Molecular techniques involve isolation and analysis of DNA and other macromolecules. The isolation of genomic DNA entails separating DNA from protein and other cellular components by use of alcohol precipitation method (Scott *et al.*, 1993). Polymerase chain reaction (PCR) was used for detection and amplification of a specific DNA sequence. The current study focused on molecular techniques such as: DNA extraction, PCR, detection of point mutation and gel electrophoresis.

3.6.1 DNA extraction

Only 20% of the test mosquito population and ninety five survivors from the eight study sites were used for the molecular techniques which are in line with the WHO recommendation for such tests (WHO, 2013). The specimens were taken randomly from each of the study sites including any survivors 24 hr post exposure to pyrethroids. Altogether, 975 mosquitoes were subjected to the DNA extraction protocol. Using this method, deoxyribonucleic acid was obtained from the wings and legs of the adult specimens following the alcohol precipitation method (Scott *et al*., 1993).

Each mosquito was ground in 100µl grinding buffer (Appendix 1) in a 1.5ml microfuge tube which was then maintained at a temperature of 65°C for 40 minutes. At least, 14µl of 8M potassium acetate (58.89g potassium acetate and 75ml deionised water) was added, mixed thoroughly and placed in ice water to thaw for about 30 minutes. The samples were then centrifuged at top speed of 14,000 revolutions per minute (rpm) at a temperature of 4°C for 10 min. The supernatant was then put into unused sterile microfuge tube and the precipitate discarded. Cold ethanol measuring 200µl was put in the supernatant and the samples chilled at -20°C overnight. The samples were microfuged again at top speed for 20 minutes and at a temperature of 4°C. The cold ethanol was poured off and 200µl of 70% ethanol added to the wash. A third wash was carried out using 200µl of 95% ethanol and the supernatant discarded. The tubes were placed upside down on an absorbent material and kept overnight to dry. The contents were then resuspended in 100µl of PCR grade water, vortexed and kept at -20°C awaiting species identification.

3.6.2 Sibling species identification

The *Anopheles gambiae* complex was further differentiated through polymerase chain reaction (PCR) amplification of ribosomal DNA intergenic spacers (Scott *et al.*, 1993). Deoxyribonucleic acid amplification was conducted in 15µl reaction volume consisting of; 13µl master mix (appendix 2), 2µl DNA template and 625.2 pmole/µl of GA primer, 579.8 pmole/µl of UN primer and 607.0 pmole of AR primer. Therefore, in every reaction there was a requirement of 5.86µl PCR water, 3µl of 5 x green reaction buffers which contains the blue and the yellow dye acting as the loading dye. There was also 1mM MgCl2 and 100µl (25 pmole/µl) of each dinucleotide triphosphates (dNTPs) which consisted of cytosine (dCTP), thymine (dTTP), guanine (dGTP) and adenine (dATP) and 0.25 units of Taq polymerase. Bovine Serum albumin (BSA) was used to enhance the yield of PCR amplification. The BSA was prepared by dissolving of 0.01g albumin serum in 1ml of distilled water.

Anopheles gambiae s.l. amplification was done in a Perkin Elmer 9600 Cetus Thermo cycler under the following thermo cycling conditions: 94°C for a 5-minute duration, then 30 revolutions at 94°C for 30 seconds, 50°C for 30 seconds, 72 °C for 30 seconds and a final extension step at 72 °C for 10 seconds. The primer set used in this study and associated characteristics are shown in table 3.1.

Table 3.1: **Primer details for the identification of *Anopheles* species**

NAME OF PRIMER	SEQUENCES 5'-3'	MELTING TEMP. T_m(°C)	AMPLIFIED DNA(bp) SIZE
UN	GTG TGC CCC TTC CTC GAT GT	58.3	468
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390

* UN primer anneals at the same site of the rDNA for all species, AR - For *An. arabiensis*, GA – For *An. gambiae*

3.6.3 Analysis of PCR products by gel electrophoresis

In this study amplification of deoxyribonucleic acid (DNA) was scored using 3% agarose gel electrophoresis. Visualization was done under ultra violet (UV) radiation. The agarose gel was mixed with ethidium bromide (EtBr) to enhance visualization. To make the 3% gel, 1.5g agarose was heated in 50 ml of tris borate EDTA (TBE) buffer (Appendix 3) in a microwave for 45 seconds. The solution was cooled but not allowed to solidify. At least 1.3µl of ethidium bromide was further added and swirled before being poured into the gel electrophoresis chamber which had been prepared and the combs put in place. The solution was left to solidify after which the combs were removed.

The tank was flooded with electrophoresis buffer (TBE) and the sample amplicons loaded into the wells. Where the DNA size marker was used, the 100bp DNA ladder

was prepared using 4µl PCR water, 1µl × 6 blue loading dyes and 1µl DNA ladder giving a total volume of 6µl. This mixture was vortexed and approximately 5µl was added to designated wells in the gel. The electrophoresis chamber was connected to the electricity and allowed to run for 30-minutes duration. The fragments were examined under UV illumination and scoring done.

3.6.4 Knockdown resistance (kdr) gene analysis

Knockdown resistance (kdr) was performed by use of 192 mosquitoes either dead or alive after bioassays. Knockdown resistance data form was used to record information for this molecular test (Appendix 4). The genotype constitution at amino acid 1014 was determined using real-time PCR using the method of Bass *et al*. (2007) and a modification done by Mathias *et al*. (2011).

The master mix (MM) was prepared by mixing PCR water, 2x TaqMan mix (TaqMan® Gene expression MM), forward and reverse primers, wild type probe (LL) and the kdr allele under investigation; either kdr east (SS) or kdr west (FF). The following probes: 5’-CTTACGACTAAATTTC-3’, 5’-ACGACTGAATTTC-3’ and 5’-ACGACAAAATTTC-3’ were used to genotype the susceptible, 1014S kdr and 1014F kdr alleles respectively.

Samples and controls were loaded on a 96-well PCR plate. The controls were included in the final four wells of the plate which consisted of FAM positive control, heterozygote control, HEX positive control and non-template control (NTC). FAM dye (blue) was used to identify the mutant allele while the HEX dye (green) detected the susceptible (wild type) allele. The reporter dye used was ROX. The temperature profile was set at 95 °C for 10 minutes for initiation and then 40 cycles of denaturing

at 95 °C (for kdr-west) or 92 °C (kdr-east) for 15 seconds and annealing at 60 °C for 1 minute on a Strategene® MxPro 3000 real-time PCR machine.

3.7 Data analysis

During field work, data on larval mosquito collections were entered into the larval field collection forms (Appendix 5). Data on deoxyribonucleic acid (DNA) extraction and species identification were entered in the laboratory processing forms (Appendix 6). The information was transferred into Microsoft (Ms) Excel sheets after every field activity day.

The WHO criteria was followed to ascertain resistance where a mortality rate between 98 and 100% reveals susceptibility, 90 to 97% suggests possible resistance that should be investigated further while less than 90% is considered resistant. The paired t-test was used to examine the knockdown rates at 10 minute interval for the Kilifi mosquito in comparison to *An. gambiae s.s.* KSM strain. The knockdown rates between different samples were compared using Analysis of variance (ANOVA) at 0.05 significance level. Knockdown time (KDT_{50} and KDT_{95}) was estimated by employing probit analysis (Finney, 1971; Kamau and Vulule, 2006). To determine and confirm resistance using resistance ratios, the resistance ratios (RR) were scaled and interpreted as follows: Resistance (RR≥3), resistance suspected (RR=2) and susceptible (RR=1) (Hinzoumbe *et al*., 2008; WHO, 2016). The allelic frequencies within a population were calculated using the formula:

$$\frac{2\,(RR) + 1\,(RS)}{N*2}$$

Where: RR - Homozygous genotype

RS – Heterozygous genotype

N - Sum of the population

Final data were captured in Microsoft Excel 2010 and analyzed using R© statistical package, Version 3.3.2.

3.8 Ethical consideration

This study involved larval mosquito collections only. Therefore, written permission was obtained from the location Chief and village elders. In order to collect larvae from individual land and other private property, permission was sought from the household heads. The consent sought was restricted to larval mosquito collections in the various selected villages. Ethical clearances were received from Kenya National Ethical Review Committee of the Kenya Medical Research Institute (KEMRI), Ministry of Higher Education, Science and Technology and Kenyatta University (Appendix 7).

CHAPTER FOUR

RESULTS

4.1 The main species of malaria vectors in Kilifi county

The entire population of adult mosquitoes collected and raised in the insectary was 4,401. Out of these, 800 were sampled from Jaribuni, 600 each from Shibe, Kidutani and Ng'ombeni, 500 each from Mapawa, Mbogolo and Burangi and 301 from Mangororo (Figure 4.1). According to World Health Organization (2013), the age and gender of mosquitoes are all factors that can influence the results of the susceptibility tests. The use of male mosquitoes is not recommended for resistance studies because they are usually smaller and more fragile than female. They tend to have higher control mortalities (WHO, 2016). Therefore, in this study only 3683 female *Anopheles* mosquitoes were used to carry out the susceptibility tests. The rest, 718 were discarded for either being males or getting damaged before the susceptibility tests. Overall, 99.9% of the mosquitoes collected belonged to the *Anopheles gambiae* complex.

The results of PCR analyses (Plate 4.1) demonstrated that, *Anopheles arabiensis* was the dominant species, accounting for 91.49% of the total collection, followed by *An. gambiae s.s* accounting for 5.02%. The rest of the mosquitoes, 3.49% either did not amplify or showed potential of introgression between the two species (Table 4.1). Mosquitoes collected in three sites namely; Mangororo, Shibe and Mbogolo were all *Anopheles arabiensis* (Mangororo: 100%, Shibe: 96.8%, and Mbogolo: 99.01%) respectively. However, in the other remaining study sites (Burangi, Ng'ombeni, Jaribuni, Mapawa and Kidutani) majority of the mosquito species were *An. arabiensis* (Table 4.1). A chi-square test performed revealed significance difference in relative

distribution of *An. gambiae* s.s. and *An. arabiensis* in the remaining five sites ($\chi 2$=65.73, df=5, $p > 0.05$).

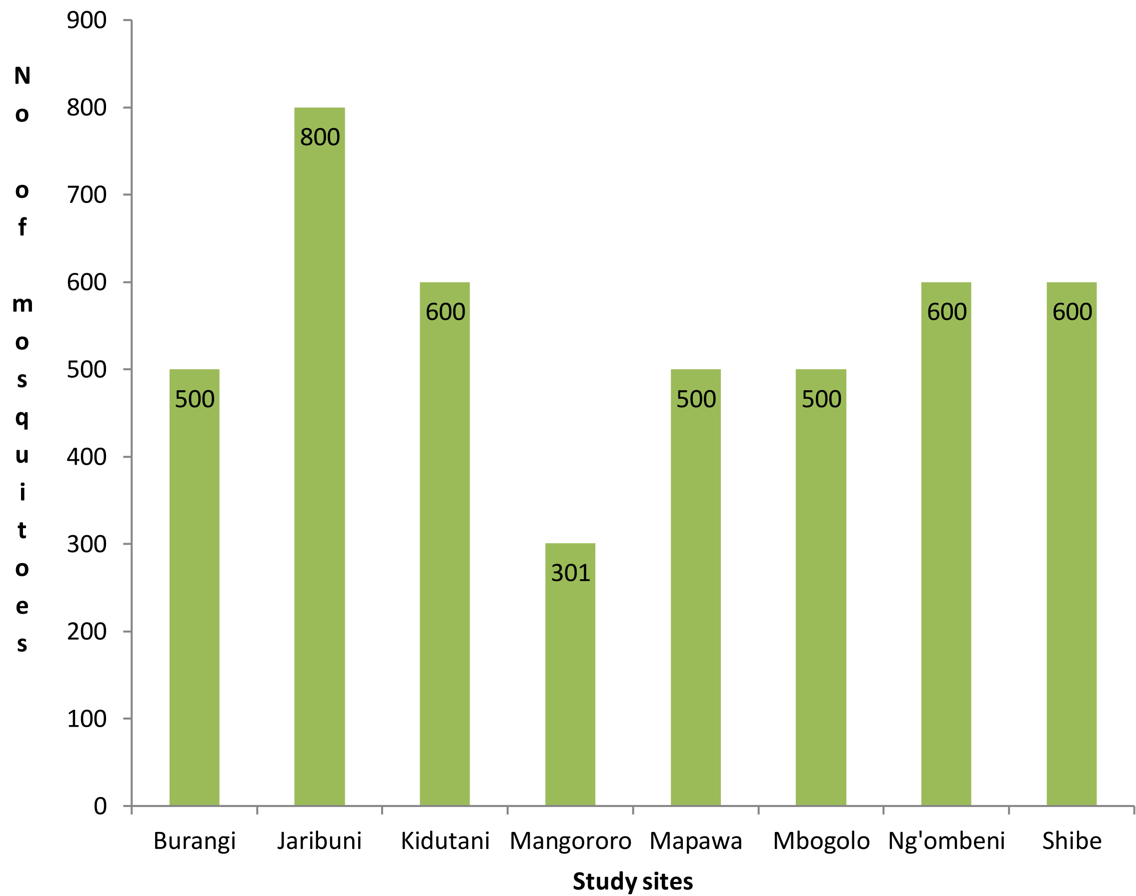


Figure 4.1: Number of field-collected and raised *Anopheles* mosquitoes per study site in Kilifi county.

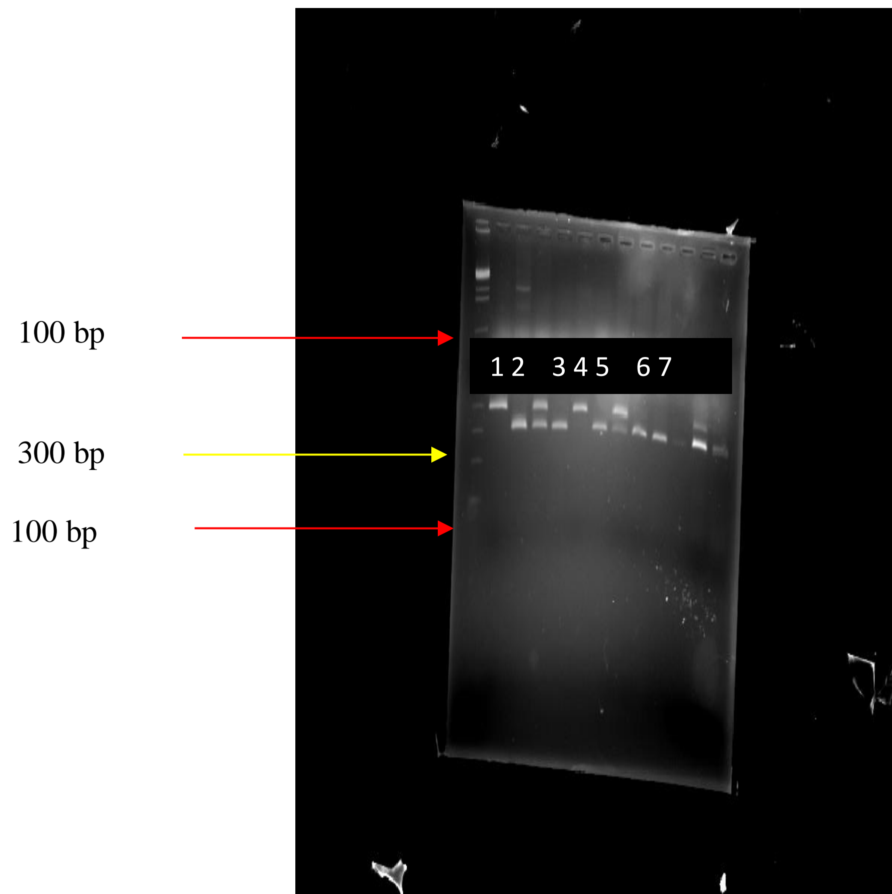


Plate 4.1: Gel images after amplification and electrophoresis with 100bp ladder.

Bands: 1,4 - *Anopheles gambiae s.s.*

Bands: 2,3,5,6,7 – *Anopheles arabiensis*

Table 4.1: Proportions of the *Anopheles gambiae* species in the eight study sites

Village	n	*An. arabiensis*	*An. gambiae s.s.*	N/a
Burangi	163	153(93.86%)	8(4.91%)	2(1.23%)
Jaribuni	172	148(86.04%)	12(6.98%)	12(6.98%)
Kidutani	124	114(91.94%)	6(4.84%)	4(4.00%)
Mangororo	60	60(100%)	0(0.00%)	0(0.00%)
Mapawa	108	95(87.96%)	5(4.63%)	8(7.41%)
Mbogolo	101	100(99.01%)	0(0.00%)	1(0.99%)
Ng’ombeni	123	102(82.93%)	18(14.63%)	3(2.44%)
Shibe	124	120(96.77%)	0(0.00%)	4(3.23%)
Total	975	892(91.49%)	49(5.05%)	34(3.49%)

n- Samples genotyped for species identification

N/a – Not amplified

4.2 Resistance status of An*opheles gambiae s.l.* to different insecticides in Kilifi county

Insecticide resistance was tested against permethrin, deltamethrin, DDT, fenitrothion and bendiocarb in all villages except in Mangororo where the tests were performed against deltamethrin, permethrin and bendiocarb only. This was mainly determined by the number of mosquitoes available for use. A single susceptibility test should have sufficient mosquito numbers, that is, 150 non-blood female mosquitoes constitute the recommended sample size (WHO, 2016). The percentage mortality for *Anopheles gambiae s.s.* KSM species turned out to be 100% for insecticides tested (Table 4.2). In addition, the negative control experiment which utilized field collected *Anopheles gambiae s.l.* mosquitoes exposed to untreated bioassay papers did not yield any mortality; therefore correction of natural causes of mortality by Abbott's formula was not necessary.

The collected female mosquitoes showed they were 100% susceptible to deltamethrin in all the study villages except in Burangi where mortality was 45.5%. Permethrin percentage mortality also showed resistance at Burangi (mortality 48%). On the other hand, mosquitoes from the other seven study villages namely: Jaribuni, Mangororo, Shibe, Mapawa, Kidutani, Mbogolo and Ngombeni were susceptible to permethrin (mortality 100%). All the mosquitoes tested against DDT (4%) were susceptible with a mortality >99% in the seven study sites (Table 4.2).

The adult mosquitoes recorded a mortality of 88% against fenitrothion in Jaribuni and possible resistance at Ngombeni (mortality 97%), Mapawa (mortality 96%) and Mbogolo (mortality 94%). However, there was susceptibility to fenitrothion at Burangi (mortality 99%), Kidutani (mortality 100%) and Shibe (mortality 100%).

Susceptibility to bendiocarb was recorded in 6 out of the 8 study sites namely; Jaribuni (mortality 100%), Shibe (mortality 100%), Kidutani (mortality 100%), Mangororo (mortality 100%), Burangi (mortality 100%) and Mapawa (mortality 100%). Possible resistance was registered in Mbogolo (mortality 93%) and Ng'ombeni (mortality 97%) against bendiocarb (Table 4.2).

Table 4.2: Resistance status (% mortality) of *An. gambiae s.l.* subjected to various insecticides in Kilifi county

Study sites	Deltamethrin	Permethrin	DDT	Fenitrothion	Bendiocarb
Burangi	100(45.5%)	100(48%)	100(100%)	100(99%)	100(100%)
Jaribuni	100(100%)	100(100%)	100(99%)	100(88%)	100(100%)
Kidutani	100(100%)	100(100%)	75(100%)	100(100%)	100(100%)
Shibe	100(100%)	100(100%)	100(100%)	100(100%)	100(100%)
Ng'ombeni	100(100%)	100(100%)	100(99%)	100(97%)	90(97%)
Mapawa	100(100%)	100(100%)	75(100%)	100(96%)	100(100%)
Mbogolo	80(100%)	100(100%)	100(100%)	100(94%)	75(93%)
Mangororo	100(100%)	100(100%)			88(100%)

4.3 Comparison of mean knockdown rates between test population and control per study site

Deltamethrin recorded a low mortality rate in Burangi at 45.5% and 100% mortality in the other seven collection sites namely Jaribuni, Shibe, Mapawa, Ng'ombeni, Mbogolo, Kidutani and Mangororo. There was no significance difference in the percentage knockdown rates at ten minute interval in comparison with the positive control in all the study sites except in Burangi (paired t = 2.650, df = 6, P=0.010) (Table 4.3).

Table 4.3: **Knockdown times for 50 and 95% of the *Anopheles gambiae s.l.* from Kilifi County to 0.05% deltamethrin insecticide**

Site	Sample size	KDT_{50} (min)	95% CI	KDT_{95} (min)	95% CI	KDT_{50} ratio (RR)	Status
Jaribuni	200	18	40.9-55.1	43	91.6-97.9	1.1	S**
Shibe	200	16	43.9-58.1	25	79.3-89.7	0.9	S**
Kidutani	200	18	42.9-57.3	37	88.5-96.1	1.1	S**
Ng,ombeni	200	23	41.9-56.2	40	91.0-97.6	1.4	S**
Mapawa	100	32	40.8-61.1	53	81.2-94.4	1.9	S**
Mbogolo	100	25	39.8-60.2	35	57.9-77.0	1.5	S**
Burangi	200		*		*		R***
Mangororo	100	30	39.8-60.2	50.95	82.4-95.1	1.8	S**
KSM strain	50	17	31.8-60.7	30	83.5-98.8	1.0	

*- Lower and upper limit rating not applicable as a result of large g value (Finney., 1971), **- deviation not significant, ***deviation significant, 95% CI- 95% confidence interval, S –susceptible, R –resistance.

Permethrin also recorded 100% mortality rate in all the seven study sites as deltamethrin did except in Burangi where a low mortality of 48% was obtained. Percentage knockdown rate at ten minute intervals showed significant difference for permethrin at Burangi (paired $t = 2.167$, $df = 6$, $P = 0.025$) (Table 4.4).

Table 4.4: **Knockdown times for 50 and 95% of the *Anopheles gambiae s.l.* from Kilifi county to 0.75% permethrin insecticide**

Site	Sample size	KDT_{50} (min)	95% CI	KDT_{95} (min)	95% CI	KDT_{50} ratio (RR)	Status
Jaribuni	100	18	39.9-60.2	50	87.4-97.8	1.0	S**
Shibe	100	21.5	37.9-58.2	44.5	84.8-96.5	1.2	S**
Kidutani	100	35.5	38.9-59.2	50.9	83.6-95.8	2.0	PR**
Ng'ombeni	100	30	40.8-61.1	50	88.7-98.4	1.7	S**
Mapawa	100	20	39.8-60.2	50	88.7-98.4	1.1	S**
Mbogolo	100	27	38.9-59.2	50.9	83.6-95.8	1.5	S**
Burangi	100	60	37.9-58.2	*	*	3.3	R***
Mangororo	100	30	39.8-60.2	51	82.4-95.1	1.7	S**
KSM strain	50	18	35.5-64.5	50	86.3-99.5	1.0	

*- Lower and upper limit approximation not applicable due to a large g value (Finney., 1971), **- deviation not significant, ***deviation significant, PR –possible resistance, R –resistance.

DDT recorded 100% mortality in seven study sites for the insecticides tested. When compared with the positive control, the knockdown rates at 10 min interval showed no statistical significant difference in all study sites (paired t, P>0.05) (Table 4.5).

Table 4.5: **Knockdown times for 50 and 95% of the *Anopheles gambiae s.l.* from Kilifi county to 4% DDT insecticide.**

Site	Sample size	KDT_{50} (min)	95% CI	KDT_{95} (min)	95% CI	KDT_{50} ratio (RR)	Status
Jaribuni	200	30.5	40.9-55.2	50	89.75-96.9	2.1	PR**
Shibe	100	15	37.9-58.2	34	84.8-96.5	1.0	S**
Kidutani	100	18	39.8-60.2	40	88.7-98.4	1.2	S**
Ng'ombeni	100	27.3	39.8-60.2	47.2	87.4-97.8	1.9	S**
Mapawa	100	31.5	39.8-60.2	51.3	77.6-92.1	2.2	PR**
Mbogolo	100	25	39.8-60.2	51.3	78.8-92.9	1.7	S**
Burangi	100	24.4	36.9-57.2	40	87.4-97.8	1.7	S**
Mangororo	-	-	-	-	-	-	-
KSM strain	50	14.5	35.5-64.5	33	83.5-98.8	1.0	

**- deviation not significant, 95% CI- 95% confidence interval, KDT_{50} and KDT_{95} – time taken for 50% and 95% of the test population to be knocked down, RR - KDT_{50} of tested group divided by control, S –susceptible, PR –possible resistance.

A comparison of the percentage knockdown rates at 10 minute interval for bendiocarb showed no significant difference between test population and susceptible Kisumu strain in the eight study sites (paired t, P>0.05) (Table 4.6).

Table 4.6: **Knockdown times for 50 and 95% of the *Anopheles gambiae s.l.* from Kilifi county to 0.1% bendiocarb insecticide**

Site	Sample size	KDT_{50} (min)	95% CI	KDT_{95} (min)	95% CI	KDT_{50} ratio (RR)	Status
Jaribuni	200	19.2	43.9-58.1	34	89.8-96.9	1.0	S**
Shibe	100	19.3	39.8-60.2	42	73.1-89.0	1.0	S**
Kidutani	100	22	39.8-60.2	50	87.4-97.8	1.2	S**
Ng'ombeni	100	26.9	39.8-60.2	50	88.7-98.4	1.4	PR**
Mapawa	100	30	39.8-60.2	51	82.4-95.1	1.6	S**
Mbogolo	100	23	37.9-58.2	*	*	1.2	PR**
Burangi	100	24	37.9-58.2	45	82.4-95.1	1.3	S**
Mangororo	100	30	40.8-61.1	51	83.6-95.8	1.6	S**
KSM strain	50	19	37.4-66.4	33	83.5-98.8	1.0	

*- Rating lower and upper limits impossible because of large g value (Finney., 1971), **- deviation not significant, 95% CI- 95% confidence interval, KDT_{50} and KDT_{95} – time taken for 50% and 95% of the test population to be knocked down, RR –KDT_{50} of tested group divided by control, S –susceptible, PR –possible resistance.

Table 4.7: Knockdown times for 50 and 95% of the *Anopheles gambiae s.l.* from Kilifi county to 1.0% fenitrothion insecticide

Site	Sample size	KDT_{50} (min)	95% CI	KDT_{95} (min)	95% CI	KDT_{50} ratio (RR)	Status
Jaribuni	100	46	37.9-58.3	*	*	2.1	R**
Shibe	100	23.5	39.8-60.2	44.5	83.6-95.8	1.1	S**
Kidutani	100	40	42.8-63.1	51	80.0-97.8	1.9	S**
Ng'ombeni	100	31	38.9-59.2	52.3	87.4-97.8	1.5	S**
Mapawa	100	21.5	37.9-58.2	57.5	84.8-96.5	1.0	S**
Mbogolo	100	46.3	37.9-58.2	52.6	76.5-91.4	2.2	PR**
Burangi	100	36	36.9-57.2	55.3	76.5-91.4	1.7	S**
Mangororo	-	-	-	-	-	-	-
KSM strain	50	21.3	35.5-64.5	45.6	70.9-92.8	1.0	

*- Rating lower and upper limits impossible because of large g value (Finney., 1971), **- deviation not significant, 95% CI- 95% confidence interval, KDT_{50} and KDT_{95} – time taken for 50% and 95% of the test population to be knocked down, RR –KDT_{50} of tested group divided by control, S –susceptible, PR –possible resistance.

Knockdown rates at ten minutes interval for fenitrothion were also not statistically significant in all the study sites (paired t, P>0.05) (Table 4.7).

4.3.1 Knockdown times and ratio at 95% CI

The resistant ratio at KDT_{50} for permethrin ranged from 1.0 to 3.3, deltamethrin from 0.9 to more than 3, fenitrothion from 1.0 to 2.2, bendiocarb from 1.0 to 1.6 and DDT from 1.1 to 2.2 (Tables 4.3-4.7). This indicates susceptibility because the susceptible range for KDT_{50} ratio varies from 1.0 to 1.7 in the susceptible control group.

Based on the knockdown ratio (KDT_{50} R), mosquitoes collected from Burangi were resistant to deltamethrin at $KDT_{50}R<3$. However, mosquito populations from all the other study sites namely; Shibe, Jaribuni, kidutani, Ng'ombeni, Mbogolo, Mapawa and Mangororo were susceptible to deltamethrin with a KDT_{50} resistance ratio between 0.9 and 1.9 (Table 4.3). In Burangi, the knockdown ratio ($KDT_{50}R=3.3$) indicated resistance to permethrin. The KDT_{50} ratio showed possible resistance in Kidutani, KDT_{50} R=2.0 in respect to permethrin. However, the KDT_{50} ratio was within the susceptible range in the other six sites namely: Mbogolo, Jaribuni, Mapawa, Shibe, Ng'ombeni and Mangororo (Table 4.4). There was possible resistance to DDT in both Mapawa and Jaribuni with $KDT_{50}R=2.1$ and $KDT_{50}R=2.2$ respectively. Mosquitoes sampled from Shibe, Kidutani, Mbogolo and Burangi were susceptible to DDT with KDT_{50} range (1.0-1.9) (Table 4.5).

The KDT_{50} ratio indicated susceptibility to bendiocarb in the eight sites with KDT_{50} ratio range from 1.0 to 1.6. Therefore, based on resistance ratio, mosquitoes sampled from each of the eight study sites were susceptible to bendiocarb (Table 4.6). The mosquito species were susceptible to fenitrothion (KDT_{50} resistance ratio range 1.0 to 1.9) in five study sites namely; Shibe, kidutani, Ng'ombeni, Mapawa and Burangi.

However, resistance was recorded in Jaribuni and possible resistance in Mbogolo with KDT_{50} ratio of 2.2 (Table 4.7).

4.4 Determination of mechanism of knockdown resistance in *Anopheles gambiae s.l.* from Kilifi county

Tests for knockdown genotype mutation were performed on 192 samples. Neither L1014F nor L1014S knockdown resistance alleles were obtained in *An. arabiensis* species. Fifteen *An. gambiae s.s.* had resistant phenotype while one hundred and seventy seven had the susceptible phenotype upon exposure to either deltamethrin or permethrin for 24 hours. The L1014S genotypic alleles were revealed in four *Anopheles gambiae s.s*. Out of the four mosquitoes with the L1014S mutation, two of them indicated homozygous (RR) trait while the other two were heterozygous (RS). Among the fifteen resistant mosquitoes, only one was found to possess the heterozygous resistant genotype for the L1014S characteristic. The rest of the *Anopheles gambiae s.s.* indicated a recessive genotype (SS) (Table 4.8).

Table 4.8: Kdr allele frequency among *Anopheles gambiae s.s.* in Kilifi county

Mosquito status	**n**	**L1014S**			**F**
		RR	RS	SS	
Resistant	15	0	1	14	0.0333
Susceptible	177	2	1	174	0.0141

n - Mosquitoes genotyped, R -resistant allele, S -susceptible allele and F -kdr allele frequency. Resistant -mosquitoes alive in 24hr post exposure while susceptible -dead mosquitoes 24h post-exposure to either permethrin or deltamethrin.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The key malaria vectors in the coastal region of Kenya belong to *An. gambiae* complexes. In this study, the prevalent species in the eight villages sampled in Kilifi county was *An. arabiensis* followed by *An. gambiae s.s*. Previously, *An. gambiae s.s.* had been reported as the most prevalent sub species along coastal and many other parts of Kenya while *Anopheles arabiensis* was termed a minor vector (Mbogo *et al*., 2003). However, a reverse in malaria vector trends has been realized which may be accounted for by the up scaling of control methods particularly the continuing extensive implementation and adoption of insecticide-treated nets (ITNs) along the coast region of Kenya (Ondeto *et al*., 2017; Kiuru *et al*., 2018).

The upward trend in population of *An. arabiensis* and downward trend in *An. gambiae s.s*. might be possibly attributed to their ecological adaptations. The *An. gambiae s.s* species is endophilic, endophagic and anthropophilic in behavior (Degefa *et al*., 2017) which means more contact hours with treated nets. This prolonged exposure to insecticides might have negatively affected their lifespan leading to population decrease. On the other hand, *An. arabiensis* is naturally both exophilic and zoophilic. This is consistent with earlier studies by Bayoh *et al*. 2014 that indicated that, *An. arabiensis* mosquitoes are exophilic vectors. It is a more adaptable species compared to *An. gambiae s.s*. This may have led to shortened contact time with insecticide-treated bednets and insecticides for IRS, hence promoting its upward trend (Russel *et al*., 2011). This change in *An. gambiae* complex composition has implications on malaria vector management and control methods. It calls for the application of both

indoor and outdoor vector control methods in order to minimize the upward trend in *An. arabiensis* species population.

Resistance to insecticides is a notable setback on reliability of LLINs and IRS in the fight against malaria. The findings of the current study revealed pyrethroid resistance in *Anopheles gambiae s.l.* in Burangi village along the Kenyan Coast. The study indicated a fairly high phenotypic pyrethroid resistance of 45.5% and 48% in deltamethrin and permethrin respectively. Similar resistance levels have been recorded in West Africa in countries such as Burkina Faso and Ivory Coast where mortality was lower than 40% (Diabate *et al*., 2002) while in Southern Benin mortality ranged between 30-40% (Yadouleton *et al*., 2009; N'Guessan *et al*., 2010; Koudou *et al*., 2011). Similar studies done along the Kenyan Coast had also indicated high pyrethroid resistance levels of 83-93% (Msami, 2013). This increase in the resistance levels might be attributed to a combination of factors selecting for insecticide resistance such as: increased use of LLINs along the Kenyan Coast and the use of pyrethroids in agriculture to control pests.

Resistance attributed to the continued use of insecticides in agriculture has been observed in Burkina Faso (Diabate *et al*., 2002), Cameroon (Chouaibou *et al*., 2008), Uganda (Brogdon and Barber, 1990; Ramphul *et al*., 2009; Verhaeghen *et al*., 2010) and Kenya (Chen *et al*., 2008; Kawada *et al*., 2011; Mathias *et al*., 2011; Ochomo *et al*., 2014; Kiuru *et al*., 2018; Githinji *et al*., 2020). In addition, the increased use of insecticides in public health sector has also worsened the condition of resistance to pyrethroids (Ondeto *et al*., 2017). The increased use of pyrethroids for treatment of LLINs and in IRS and agriculture has subjected the mosquito vectors to selection pressure, hence, increasing their probability of resistance (Reid and MCkenzie, 2016). The resistance of *Anopheles gambiae s.l.* to pyrethroids in Burangi is further

supported by the high median knockdown time (KDT_{50}) of more than three compared with other study sites. High KDT_{50} value of 2.0 was also recorded in Kidutani which had mosquitoes with possible resistance to pyrethroids. The knockdown times for *Anopheles gambiae s.l.* population from the other seven study sites namely: Jaribuni, Kidutani, Mangororo, Mapawa, Mbogolo, Ng'ombeni and Shibe were within a comparable range with *Anopheles* categorized as susceptible (Chandre *et al.*, 2000; Diabate *et al.*, 2002; Kristan *et al.*, 2003; Etang *et al.*, 2003; Chouaibou *et al.*, 2008; Githinji *et al.*, 2020). The results obtained in the current study concur with previous work carried out in Tanzania (Kabula *et al.*, 2012) where resistance to pyrethroids was marked by high median knock down times. High KDT_{50} values in the test populations have been put forth to show the existence of kdr mechanism of resistance (Chandre *et al.*, 2000).

Based on KDT_{50}, there was possible resistance to dichlorodiphenyltrichloroethane (DDT) in Mapawa and Jaribuni villages. The potential resistance to DDT based on KDT_{50} might be partly due to cross-resistance from pyrethroids (Ochomo *et al.*, 2014) and/or the occurence of recessive genes in the mosquitoes in those regions (Msami, 2013). In Kenya, DDT was lastly used in 1986. Currently, it is not authorized for use especially in the public health sector. However this chemical insecticide has a long residual effect which contaminates mosquito larval habitats. Studies have documented evidence on residues of DDT found in soil and water collected from the Indian Ocean, river Sabaki and Kiwaya Bay along the coastline (Lalah, 1993). Therefore, this characteristic residual effect of DDT cannot be ignored as a cause of this potential resistance in Jaribuni and Mapawa villages in Kilifi County.

The mosquito test population showed possible resistance that requires further investigation based on mortality to bendiocarb in Ngombeni and Mbogolo. In all other study sites, the *Anopheles* mosquitoes indicated susceptibility to bendiocarb. Percentage mortality at 10 min interval showed no significant difference when compared with KSM strain. Based on the KDT_{50} ratio, there was susceptibility to bendiocarb in all the villages. Carbamates such as carbofuran and bendiocarb are used in agriculture to control pests (Wandiga *et al*., 2003). Some agricultural practices such as irrigation provide favorable habitats for mosquito breeding (Ondeto *et al*., 2017). Therefore, the possible resistance noted in the current study may be as a result of evolutionary pressure caused by contamination of the larval collection sites by carbamates used in agricultural settings. This possible resistance to bendiocarb recorded in the current research is a threat because a carbamate insecticide is evaluated for IRS and/or alternative control tool against pyrethroid-resistant vectors (Aizoun *et al*., 2013).

The mosquito populations in Jaribuni were resistant to fenitrothion based on mortality rate. There was also possible resistance to fenitrothion in mosquitoes collected from Mbogolo based on KDT_{50} ratio. The occurrence of both resistance and possible resistance to fenitrothion revealed in this study may be linked to the use of organophosphate pesticides by subsistence farmers as well as exposure of the mosquitoes to this insecticide during sugar feeding or outdoor resting. The pesticides contaminate the larval mosquito habitats selecting for resistance in this particular study sites. Previous studies have documented evolution of resistance to fenitrothion following its use for agricultural purposes (Sharp *et al*., 2007; Ngala *et al*., 2015). Organophosphates are potential substitutes for IRS in vector control especially for mosquitoes which are resistant to pyrethroids. Therefore development of resistance to

this insecticide class poses a great risk to their application in IRS and as substitute to pyrethroids.

The presence of phenotype resistance to deltamethrin and permethrin act as an indication of the presence of target site insensitivity (WHO, 2016). This study showed overall absence of knockdown resistance allele in *An. arabiensis*. These findings are the same as those obtained in studies done in western Kenya by Mathias *et al*. 2011. However, L1014S knockdown resistance tested positive in *Anopheles gambiae s.s.* from Burangi village. The allele frequency recorded was 3.33% in the resistant population and 1.41% in the susceptible test population. This shows a low allele occurrence which is consistent to other studies done along Kenyan coast (Orondo, 2016; Kiuru *et al*., 2018). In contrast, earlier studies had indicated elevated frequentness and vast spread of L1014S mutation among *An. arabiensis* in western Kenya (Kawada *et al*., 2011; Ochomo *et al*., 2013).

The variations in resistance allele frequency may be due to several factors such as: movement of the variant genes from the selection pressure regions, deletion repeats in the genomic sequence of the mosquito population and type of species. Although earlier studies indicated evolution of knockdown resistance in the neighboring Kwale County, this may not practically be the source of the mutant genes. Target site resistance is jointly linked with cross resistance to pyrethroids and DDT in mosquito vectors (Diabate *et al*., 2002; Matambo *et al*., 2007). Cross resistance complicates insecticide resistance which is already a great danger to vector control. The development of cross resistance has been documented in *An. funestus* (Djouaka *et al*., 2011), *An. arabiensis* (Yewhalaw *et al*., 2011) and *An. gambiae s.s.* (Edi *et al*., 2012; Kwiatkowska *et al*., 2013) in Africa.

The application of pyrethroids in vector control through IRS and ITNs, alone, is faced by the problem of unsustainability. The inclusion of other methods such as environmental management (Imbahale *et al.*, 2011), physical barriers (Kirby *et al.*, 2009), application of chemicals with different mode of action (Blanford *et al.,* 2011) and larviciding (WHO, 2013) is an idea to bank on. Integrated Vector Management (IVM) provides a more sustainable and effective vector control management.

5.2 Conclusions

i. The study revealed that, *Anopheles arabiensis* is the most dominant vector of malaria accounting for 91.49% of the total collection within Kilifi county, followed by *An. gambiae s.s.* at 5.05%. *Anopheles funestus* was not detected in this study.

ii. *Anopheles gambiae s.l.* mosquitoes collected from Burangi were resistant to both deltamethrin and permethrin insecticides. Evidence of phenotypic resistance in *An. gambiae s.l.* to fenitrothion was also seen in Jaribuni. However, all *Anophele gambiae s.l.* mosquitoes tested were susceptible against bendiocarb and DDT.

iii. Occurrence of knockdown resistance mechanism involving kdr-east gene (L1014S) was found in *An. gambiae s.s* only at a low allele frequency of 3.33%. However, kdr-west gene (L1014F) was not detected in both *An. arabiensis* and *An. gambiae s.s.*

iv. This study has contributed to knowledge of the status of insecticide resistance in *An. gambiae s.l.* along coastal Kenya. This information is paramount in the planning and management of malaria vectors along the Kenyan coast.

5.3 Recommendations

i. Combination of mosquito collection methods targeting larvae and adults as well as indoor and outdoor resting mosquitoes should be used in studies that aim to determine the distribution and composition of mosquitoes in a given region.

ii. Continuous and regular monitoring of the resistance status of mosquitoes in malaria endemic areas is needed in order to detect its development and prevent the spread which might impart negatively on malaria control.

5.3.1 Suggestion for further research

i. There is need to determine the mechanisms of resistance in all insecticide groups by conducting molecular tests using PCR as well as biochemical assays for the detection of metabolic enzymes.

ii. Monitoring of insecticide resistance should also include the use of CDC bottle bioassay and synergist assays. These assays determine the duration taken to kill adult mosquitoes exposed to a known concentration of insecticide and assess the contribution of detoxifying enzymes towards production of resistant phenotypes respectively.

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APPENDICES

Appendix 1: Grinding buffer

Homogenization buffer

0.59g 0.1M sodium chloride

6.84g 0.2M sucrose

0.37g 0.01M EDTA

0.36g 0.03M trizma base

100ml distilled water

Lysis buffer (100ml at PH 9.2)

9.28g Ethylenediamine tetraacetic acid (EDTA)

1.88g Sodium dodecyl sulfate

6.03g trizma base

100 ml distilled water

Mix the homogenization buffer and lysis buffer at a ratio of 4:1 to make the grinding buffer.

Appendix 2: Master Mix (MM)

Reagent	**Concentration for 1 sample**	**Volume for 1 sample**	**Volume for 20 samples**
PCR Water	N/A	7.025 µl	140.5 µl
5 × PCR buffer (No $MgCl_2$)	**1**×	3 µl	60 µl
2mM dNTP mix	0.2mM of each	1.5 µl	30 µl
25mM $MgCl_2$	1 mM	0.6 µl	12 µl
Primer GA	1 µM	0.6 µl	12 µl
Primer AR	1 µM	0.6 µl	12 µl

Appendix 3: Tris Borate EDTA buffer

To make 1 litre of TBE buffer

5.5g boric acid,

10.8g trizma base,

0.93g of EDTA

1 litre of distilled water.

The solution is then stirred until all the solutes dissolve.

APPENDIX 4: KDR DATA FORM

KDR LAB DATA SHEET

STUDY SITE…………………… CODE…………………DATE…………

Mosquito Id	Test	Susceptibility Date	Status (Dead/Alive)	DNA Extraction Date	KDR East/West	
					PCR Date	KDR Result

Appendix 5: Larval field collection form

STUDY SITE…………………… DATE………………

HABITAT TYPE	GPS POSITION	WATER TURBIDITY	PREDATORS PRESENT (YES/NO)	EXPOSED TO SUNLIGHT (YES/NO)	EARLY INSTARS (L1/L2)	LATE INSTARS (YES/NO)

APPENDIX 6: LABORATORY PROCESSING FORM

MOSQUITO ID	COLLECTION DATE	SPECIES	DNA EXTRACTION DATE	SPECIES ID

Appendix 7: Kenyatta University authorization letter

OUR REF: 156/22795/11 DATE: 17th January, 2014

The Permanent Secretary,
Ministry of Higher Education, Science & Technology,
P.O. Box 30040,
NAIROBI

Dear Sir/Madam,

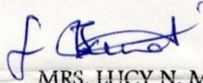
RE: RESEARCH AUTHORIZATION FOR MR. DANIEL NZIOKA MUNYWOKI REG. NO.156/22795/11

I write to introduce Mr. **Munywoki** who is a Postgraduate Student of this University. He is registered for M.Sc. Degree programme in the **Department of Zoological Sciences in the School of Pure and Applied Sciences.**

Mr. Munywoki intends to conduct research for a proposal entitled, **"Insecticide Resistance Monitoring in Malaria Vectors in Kilifi County, Along the Coastal Kenya."**

Any assistance given will be highly appreciated.

Yours faithfully,



MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

LNM/cao