SPECIES COMPOSITION OF *ANOPHELES GAMBIAE* S.L., GENETIC CHARACTERIZATION OF INSECTICIDE RESISTANCE AND ASSOCIATION OF *KDR* AND *ACE-1*^{R} MARKERS WITH THE 2LA INVERSION MARKER FOR MOSQUITO RESTING BEHAVIOR IN SELECTED SITES IN KENYA

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OCTOBER, 2025

DECLARATION

Declaration by the Candidate

This work is original and authentic and has not been presented for a degree in any other university or any other award.

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DEDICATION

To my parents James Maina and Alice Maina for all their unwavering support through this work and for putting me through the best education possible.

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ABBREVIATIONS AND ACRONYMS

AChE-1	Acetyl cholinesterase-1 enzyme
Ace-1^{R}	Resistance in Acetyl cholinesterase-1 gene
AIDS	Acquired Immunodeficiency Syndrome
AIRS	Africa Indoor Residual Spray
***Alu*I**	Arthrobacter luteus-I
bp	Base pairs
BSA	Bovine serum albumin
CDC	Centre for Disease Control
DDT	Dichloro diphenyl trichloroethane
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotide triphosphates
F	Fisher's exact value
FAM	Fluorescein amidites
g	Gravitational force
GABA	γ-amino butyric acid
GPIRM	Global Plan for Insecticide Resistance Management
G119S	Substitution of glycine with serine amino acid
HIV	Human Immunodeficiency Virus
IRAC	Insecticide Resistance Action Committee
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
Kdr	Knockdown resistance
Kdr-e	East African knockdown resistance
Kdr-w	West African knockdown resistance
KEMRI	Kenya Medical Research Institute
LLINs	Long Lasting Insecticide-treated Nets
L1014F	Leucine to phenylalanine substitution at locus 1014
L1014S	Leucine to serine substitution at locus 1014
MGB	Minor Groove Binder
M	Molar
mL	Millilitres
mM	Millimolar
MOH	Ministry of Health
Ms	Microsoft
NEMA	National Environmental Management Authority
NMCP	National Malaria Control Program
n	Number of samples tested
nM	Nanomolar
NSE	Non-specific esterases
P	P-value
PASA	PCR Amplification of Specific Alleles
PBO	Piperonyl-butoxide
PCPB	Pest Control Products Board
PCR	Polymerase Chain Reaction

PMI	President's Malaria Initiative
pmol	Picomoles
qPCR	Quantitative Real-time Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
S.L.	Sensu lato
S.S.	Sensu stricto
SSC	Scientific Steering Committee
STATA	Statistics and Data
Taq	Thermus aquaticus
TBE	Tris Borate Ethylenediaminetetraacetic acid
UNEP	United Nations Environmental Program.
uL	Microlitres
U/ul	Units per microlitre
USAID	United States Agency for International Development
VGSC	Voltage gated sodium channel gene
VIC	2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
2La/a	Inversion arrangement on left arm of chromosome 2
2L+^{a}/+^{a}	Standard arrangement on left arm of chromosome 2
2La/+^{a}	Heterokaryotype arrangement on left arm of chromosome
2R	Right arm of chromosome 2

ABSTRACT

Molecular diagnostic tools have been incorporated in insecticide resistance monitoring programs to identify underlying genetic basis of resistance and develop early warning systems of vector control failure. Identifying genetic markers of insecticide resistance is crucial in enhancing the ability to mitigate potential effects of resistance. The knockdown resistance (*kdr*) mutation associated with resistance to DDT and pyrethroids, the acetylcholinesterase-1 (*ace-1*^{R}) mutation associated with resistance to organophosphates and carbamates and 2La chromosomal inversion associated with indoor resting behavior, were investigated in the present study. *Anopheles* mosquitoes were collected from four study sites representative of four out of five malaria epidemiological zones in Kenya. The collection was within the context of malaria vector surveillance in the country. Mosquitoes were collected indoors using light traps, pyrethrum spray and hand catches between August 2016 and November 2017. Mosquitoes were initially identified using morphological keys and *Anopheles gambiae* s.l. mosquitoes further identified into sibling species by the Polymerase Chain Reaction method following DNA extraction by alcohol precipitation. *Anopheles gambiae* s.s. and *Anopheles arabiensis* were analyzed for the presence of the *kdr* and *ace-1*^{R} mutations while 2La inversion was only screened for in *An. gambiae* s.s. where it is polymorphic. Chi-square statistics were used to determine correlation between the 2La inversion karyotype and *kdr* mutation. The kdr-east mutation occurred at frequencies ranging between 0.5%-65.6%. The kdr-west mutation was only found in Migori at a total frequency of 5.3% (n=124). No *kdr* mutants were detected in Tana River. The *ace-1*^{R} mutation was absent in all populations which implies that there hasn't been enough buildup of insecticidal pressure from the agricultural and domestic sector to select for this mutation. The observed phenotypic resistance to organophosphates and carbamates in the country could therefore be as a result of other mechanisms of resistance. The 2La chromosomal inversion screened in *An. gambiae* s.s. occurred at frequencies of 87% (n=30), 80% (n=10) and 52% (n=50) in Baringo, Tana River and Migori, respectively. A significant association between the 2La chromosomal inversion and the *kdr* mutation was found (Fisher's exact test statistic value, F=36.967, *P* =0.000; Likelihood Ratio=33.068, *P*=0.000). The significant association between the 2La inversion karyotype and *kdr* mutation suggests that pyrethroid resistant *An. gambiae* s.s. continue to rest indoors regardless of the presence of treated bed nets and residual sprays, a persistence further substantiated by studies documenting continued mosquito abundance indoors. Behavioral resistance by which *Anopheles* vectors prefer to exit the indoor environment may therefore not be a factor of concern in our study populations. This therefore means that we can continue deploying interventions indoors. Absence of *ace-1*^{R} implies that organophosphates and carbamates can be used as alternative insecticides to manage pyrethroid resistance.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Malaria is one amongst the most prevalent vector-borne diseases in sub-Saharan African countries (WHO, 2018). According to World Health Organization, this disease kills more than 400,000 people per year most of whom are infants in sub-Saharan Africa (WHO, 2020). In the year 2020, 241 million people in the world were infected with the disease while 627,000 succumbed to it. Africa reported about 97 million cases and 205,405 deaths (WHO, 2021).

In Kenya, the prevalence of malaria in children aged 6months – 14years was 6% (MOH, 2020) while the overall number of reported cases and deaths was 6,875,369 and 742,000 respectively (WHO, 2021). Kenya being among countries most affected by the disease (WHO, 2013) was listed for a President's Malaria focus Initiative in 2007 (PMI, 2017). As of the year 2020, 37,744,763 out of 53,771,298 Kenyans were at risk of malaria (WHO, 2021). A few years back, western and coastal Kenya regions recorded high transmission intensities in the country (Bhatt *et al*., 2015). Scale up campaigns to control malaria incidence in Kenya through Indoor Residual Spraying (IRS) and use of Long-Lasting Insecticidal Nets (LLINs) were initiated in 2000. In 2006, Long-Lasting Insecticidal Nets were distributed by the National Malaria Control Program to mothers who were expectant and to infants (Hightower *et al*., 2010; WHO, 2017; Zhou *et al*., 2011). In 2011 the ministry changed its policy and as recommended by WHO issued entire population at risk of

malaria infection with durable insecticide treated nets irrespective of gender and age (Zhou *et al*., 2014). New Long-Lasting Insecticidal Nets (LLINs) were redistributed in 2014 to replace the old nets and boost coverage.

Several studies indicate that the numbers of malaria cases and deaths have significantly reduced as a result of these mass campaigns (Fegan *et al*., 2007; Kawada *et al*., 2011; Zhou *et al*., 2016). However, these fragile gains could be watered down by the growing tolerance of *Plasmodium* parasites to antimalarial medicines, refractoriness to insecticides plus change in behavior by *Anopheles* mosquitoes and change in global climate.

There are likely chances of malaria cases and deaths increasing if the vectors' tolerance to insecticides is not managed or prevented. Snow *et al*., (2015) reports an upsurge in malaria prevalence in the Kenyan Coast despite there being vector control tools in place. According to WHO, (2022) a reduction in efficacy of interventions is an indicator for an unexpected increase in disease transmission. It is therefore suspected that resistance to insecticides is one factor that could be playing a huge role in increasing malaria prevalence in areas where gradual decrease was being reported (Trape *et al*., 2011). With genetic markers being valuable predictors of malaria incidence trends, analysing genetic variations in malaria vectors is crucial for researchers as it enables them foresee disease transmission dynamics, identify hotspots, predict drug or insecticide resistance by *Plasmodium* parasites and vectors respectively and tell in advance whether malaria control and elimination strategies are likely to fail (Ruybal-Pesántez *et al*., 2024).

The primary interventions target nocturnal blood feeding vectors. However, with the adverse insecticide pressure from these interventions a good proportion of mosquitoes may feed when humans are not protected. A good example of such is the early morning biting activity of *An. gambiae* and *An. funestus* observed by Odero *et al*., (2024) in western Kenya. The vectors bit between 0400-0700hours when people were awake and not under a net, a behavior that is contrary to the observations made in the pre-net era (Chandler *et al*., 1975). In a different study, daytime feeding was observed in *An. gambiae* and *An. funestus* of the Central African Republic. There also was a consistent higher biting event outdoors than indoors in both the nocturnal and crepuscular species (Sangbakembi-Ngounou *et al*., 2022). Several other studies report behavioral adaptations in time and place of feeding (Wamae *et al*., 2015; Sokhna *et al*., 2013; Reddy *et al*., 2011). Feeding early in the morning, early in the evening, during daytime and in the outdoors increase the opportunity for vector human contact which then drives residual malaria transmission (Sherrard-Smith *et al*., 2019).

The flactuation in densities of mosquito species can either influence the increase or decrease of malaria. For example, a decrease in the dominance of a competent malaria vector over a non-vector would lead to a decline in malaria cases (Takken, 2002). Perhaps a real case scenario would be the progressive diminishment of *An. funestus* in Benin's Dielmo Village which resulted to suppression of malaria transmission by this vector (Corbel *et al*., 2012; Fontenille *et al*., 1997; Trape *et al*., 1994).

The change in climate being experienced globally has led to a rise in concerns on whether the changes will impact efficacy of vector control measures and distribution of vector borne diseases (Ferguson and Govella, 2024). Several studies have proven that malaria transmission is highly sensitive to climate (Caminade *et al*., 2014; Kelly-Hope and Thomson, 2005; Mabaso *et al*., 2007; Reiner *et al*., 2015). A change in climate would therefore have an impact on the prevalence of disease. This is because survival, reproduction, parasite development and transmission in the African malaria vector is dependent on favourable temperature, favourable humidity and rainfall (Murdock and Thomas, 2016; Parham *et al*., 2012; Paaijmans *et al*., 2013).

It is predicted that an increase in temperature and a decrease in humidity will increase the risk of highland malaria in East Africa (Alonso *et al*., 2010; Caminade *et al*., 2014; Diouf *et al*., 2021; Ermert *et al*., 2013; Leedale *et al*., 2016; Yamana *et al*., 2016). Boyce *et al*., (2016) for instance, found out that persons living in the highlands of western Uganda had a 30% higher risk of malaria infection during extreme flooding. In addition, climate change is also likely to directly affect vector control by modifying the efficacy and quality of interventions. Climate changes that favour vector species, genotypes or vector behaviour with reduced susceptibility to interventions will have a significant impact on their efficacy. Some entomological studies indicate that residual efficacy of some insecticide-based interventions vary with temperature, humidity and other environmental variables and an effect in the efficacy of these vector control products is consequently expected with climate change (Arthur, 2013; Gerken *et al*., 2021; Ranabhat & Wang, 2020).

With all these developments challenging to render the few available vector control tools ineffective, the World Health Organization calls upon the global malaria community to develop strategies that will maintain efficacy of the vector control interventions currently in use. Amongst these strategies are vector surveillance, insecticide resistance surveillance and emerging resistance management (WHO, 2012).

Genetic markers have been used for characterization of the molecular basis of insecticide resistance and for inferring indoor resting behavior in mosquitoes. Knockdown resistance (*kdr)* and acetylcholinesterase-1 (*ace-1*^{R}) are single nucleotide polymorphic-type and restriction fragment length polymorphic-type biochemical markers, respectively, which detect changes in the amino acid sequence that brings about refractoriness to specific insecticides. *Kdr* for instance, confers resistance to pyrethroid and DDT insecticides (Ochomo *et al*., 2015) while *ace-1*^{R} mutation causes resistance to carbamates and organophosphates (Weill *et al*., 2004). The 2La inversion denoted as 2La/2La on the other hand is a molecular marker associated with adaptation to different microclimates, desiccation resistance and mosquito behaviours (Ayala *et al*., 2014).

This study analysed the composition of sibling species in the *Anopheles gambiae* complex and screened the sib species i.e *An. gambiae* s.s. and *An. arabiensis* for *kdr* and *ace-1*^{R} markers. It further screened the *An. gambiae* s.s. species for inversion 2La and determined the association of this marker with the identified genetic markers of insecticide resistance.

1.2 Problem statement

Malaria vector control measures in Kenya heavily depend on synthetic insecticides which target indoor feeding and resting mosquitoes. Due to continuous use of insecticide impregnated nets and repeated spraying of the indoor spaces in some areas, *Anopheles* mosquitoes have been subjected to intense insecticide pressure within human dwelling. This has resulted to widespread tolerance of pyrethroid insecticides by malaria vectors as revealed by phenotypic bioassays (Ondeto *et al*., 2017) and in some areas an increase in the frequency of knockdown resistance mutations (*kdr*) in the vector's insecticide target site (Mathias *et al*., 2011; Stump *et al*., 2004) thus threatening the efficacy of pyrethroid and organochlorine based interventions. Moreover there has been an observed moderate to high phenotypic resistance against carbamate and organophosphate insecticides which target the Acetylcholinesterase-1 gene (Msami, 2013; Wanjala *et al*., 2015; Wanjala & Kweka, 2018; Munywoki *et al*., 2021).

In addition to withstanding chemical toxicity, the interventions are forcing malaria vectors to adapt by exiting the targeted indoor environment so as to avoid contact with the insecticides (Githeko *et al*., 1996; Mathenge *et al*., 2001; Mutuku *et al*., 2011; Degefa *et al*., 2017). The likely consequence of this shift from indoor resting to outdoor resting is increased behavioral resistance among *Anopheles* species and persistent malaria transmission outdoors thus requiring new approaches for vector surveillance and control (Riehle *et al*., 2017).

With organophosphate and carbamate insecticides being recommended as front line alternatives to pyrethroids (USAID and PMI, 2018; Aïzoun *et al*., 2013) it is important to ascertain the status of the *ace-1*^{R} mutation among field populations of *An. gambiae* s.l. in Kenya as it is currently not known (Ondeto *et al*., 2017). Monitoring the frequency of *kdr* mutation alleles in field populations will guide the implementation of insecticide resistance management strategies.

1.3 Justification of the study

Malaria continues to be a major public health concern in Kenya. With vector control being the mainstay of malaria prevention, the alarming rate at which malaria vectors are developing resistance to insecticides is worrying. Efforts to solve this problem have led to development of new innovations like biological alternatives and transgenic insects which are under evaluation. Although these new strategies look promising, chemical compounds will still play a very significant role in managing malaria vectors. Therefore, we urgently need to develop and employ a rationale-based approach of using insecticides to ensure their continued efficacy. This begins with an understanding of insecticide resistance mechanisms, which is one of the priority aspects to monitor once phenotypic resistance is detected, followed by the deployment of insecticides with alternative modes of action or the use of alternative approaches of deployment of the insecticide where resistance is documented. In addition, alteration of insecticides target sites and behavioral change in *Anopheles* mosquitoes are two key mechanisms of resistance that may have negative impacts on the efforts made so far in decreasing incidences of malaria transmission (N'Guessan *et al*., 2007; Ochomo *et al*., 2013; Wondji *et al*., 2012). In this sense therefore, it is also paramount to study the relation

between insecticide resistance and mosquito resting behavior so as to ensure that the insecticides deployed and the methods of deployment of the insecticides take into consideration the status of resistance as well as possible changes in mosquito resting behavior resulting from previous insecticide use.

1.4 Research hypotheses

i. The knockdown resistance (*kdr*) marker of insecticide refractoriness is not present in *Anopheles gambiae* s.l. mosquitoes from Migori, Baringo, Kirinyaga and Tana River counties.

ii. The Acetylcholinesterase-1 resistance (*ace-1*^{R}) marker of insecticide refractoriness is not present in *Anopheles gambiae* s.l. mosquitoes from Migori, Baringo, Kirinyaga and Tana River counties.

iii. *Anopheles gambiae* s.l. mosquitoes from the four selected study sites in Kenya do not exhibit behavioral changes as a result of vector control interventions use.

iv. There is no association between genetic markers of insecticide resistance and resting behavior in *Anopheles gambiae* s.l. mosquitoes from Migori, Baringo, Kirinyaga and Tana River counties.

1.5 Objectives

1.5.1 Main objective

To determine the prevalence of genetic markers of insecticide resistance and resting behavior and the association of the earlier with the latter in *Anopheles gambiae* s.l. mosquitoes from Migori, Baringo, Kirinyaga and Tana River counties of Kenya.

1.5.2 Specific objectives

i. To determine the prevalence of the knockdown resistance (*kdr*) mutation associated with resistance to pyrethroids and DDT in *Anopheles gambiae* s.l. mosquitoes in Migori, Baringo, Kirinyaga and Tana River counties.

ii. To determine the prevalence of the *ace-1*^{R} mutation associated with resistance to organophosphates and carbamates in *Anopheles gambiae* s.l. mosquitoes in Migori, Baringo, Kirinyaga and Tana River counties.

iii. To determine the prevalence of 2La inversion associated with mosquito resting behavior in *Anopheles gambiae* s.l. mosquitoes in Migori, Baringo, Kirinyaga and Tana River counties.

iv. To determine whether genetic markers of insecticide resistance are associated with resting behavior in *Anopheles gambiae* s.l. mosquitoes in Migori, Baringo, Kirinyaga and Tana River counties of Kenya.

1.6 Significance of the study

By reporting species composition and frequencies of *kdr* and *ace-1*^{R} in the selected sites, this study allowed for an informed recommendation in the choice of replacement insecticide where resistance was detected at high levels while at the same time taking into consideration the vector species that was dominant. It is revealed through this study that mosquitoes targeted by insecticide intervention indoors are not behaviorally adapting to resting outdoors which then means that we can continue challenging them indoors. The study predicts efficacy of organophosphates and carbamates and thus recommends their use where pyrethroid resistance is reported and in addition guides the introduction of novel vector control interventions like Interceptor G2, Royal Guard and

PBO nets. Furthermore, the study contributes to investigations that seek to explain unexpected change in malaria disease transmission and disease burden and is expected to inform the development of other Insecticide Resistance Management (IRM) strategies in the future to help delay emergence or reduce rate of spread of insecticide resistance.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria overview

Malaria is caused by a protozoan parasite, *Plasmodium*. Infectious *Plasmodium* parasites in humans are; *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. Of the five parasites, two (*P. falciparum* and *P. vivax*) pause the greatest threat (WHO, 2017). The dominant *Plasmodium* parasite in sub-Saharan African countries is *P. falciparum*, accounting for 99.7% of malaria cases. *P. vivax* is prevalent in the Americas WHO region and is responsible for 74.1% of malaria cases (WHO, 2018).

Malaria prevalence in Kenya varies across regions as presented in **figure 1**. The coastal and lake region have the highest prevalence and are thus considered endemic. The least number of cases are recorded in the central region which is classified as low risk. The arid and semi-arid regions of the country experience seasonal malaria transmission with peaks during the rainy season. The western highlands plus two sub-counties of Baringo (a seasonal risk county) are considered epidemic prone due to malaria outbreaks experienced in the rainy season (USAID, 2018). Case fatalities during epidemics can exceed those in endemic regions. Although malaria is regarded a burden in the country, it is preventable and curable, but only with the right treatment and vector control measures in place. **Figure1** shows the stratification of the country into five epidemiological zones.

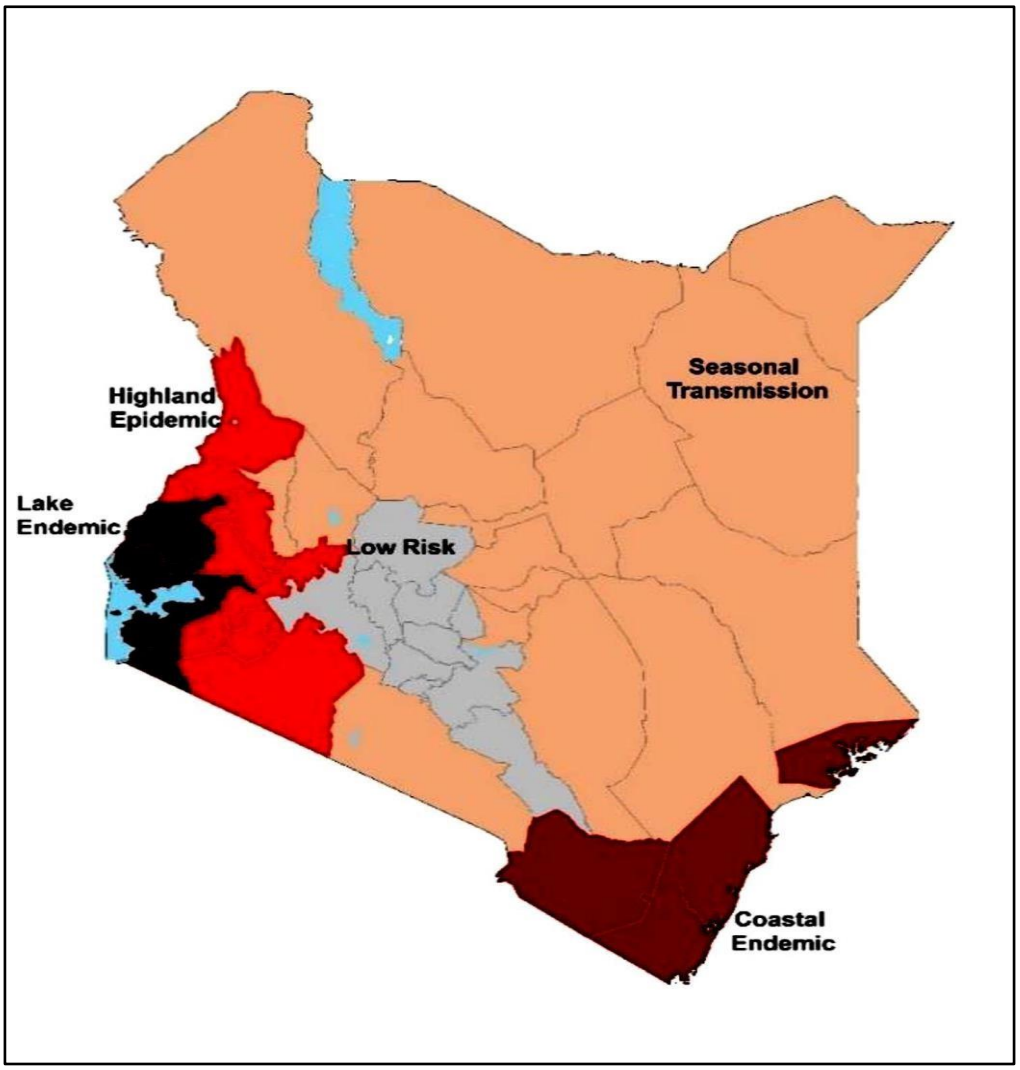

Figure 1: Map of Kenya showing malaria endemicity

Source: (The Kenya epidemiological profile, 2016)

2.2 Malaria vectors

A female *Anopheles* mosquito that injects infectious sporozoites into the blood of its human host when feeding is commonly referred to as malaria vector. There are over 300 species of *Anopheles* mosquitoes but only few are considered hosts to the causative agent of the disease. Sinka *et al*., (2010) lists *Anopheles gambiae* complex (*An. gambiae* s.s. herein **fig. 2** *An. gambiae* and *An. arabiensis*) and *Anopheles funestus* complex (*An. funestus* s.s. herein **fig. 2** *An. funestus*) as the major vector species in sub-Saharan Africa. The

distribution of these dominant sibling species of the two complexes in Africa is presented in **figure 2**.

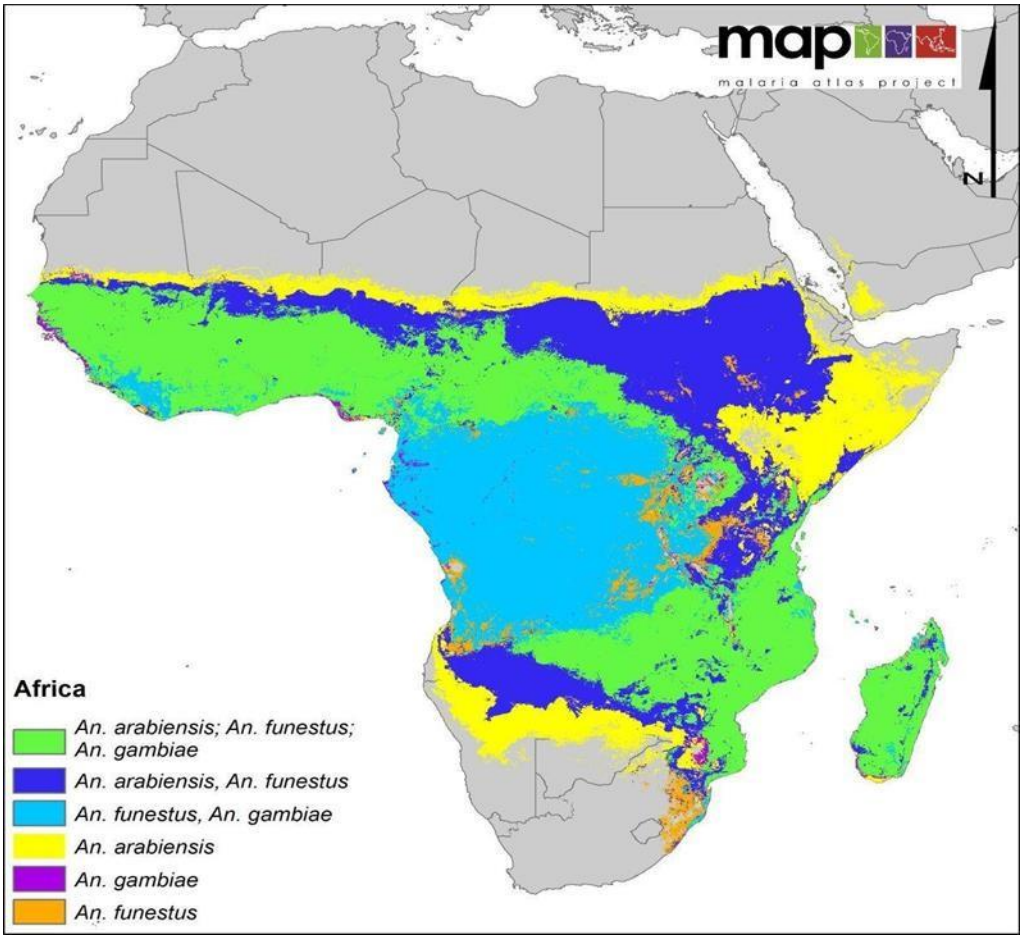

Figure 2: Distribution of dominant malaria vectors in Africa

Source: (Sinka *et al*., 2012)

In Kenya, *Anopheles arabiensis, Anopheles gambiae* s.s., *Anopheles merus* and *An. funestus* principally spread malaria (Okara *et al*., 2010). *Anopheles arabiensis* mainly feeds on cattle and only occasionally feeds on humans and preferentially rests outdoors. *Anopheles gambiae* s.s. on the other hand mainly feeds on humans late in the evening and at night and rests indoors. *An. funestus* feeds on both humans and cattle between dusk and dawn and rests indoors after a blood meal (Machani *et al*., 2020). **Figure 3** shows the distribution of these principal vectors of malaria in Kenya.

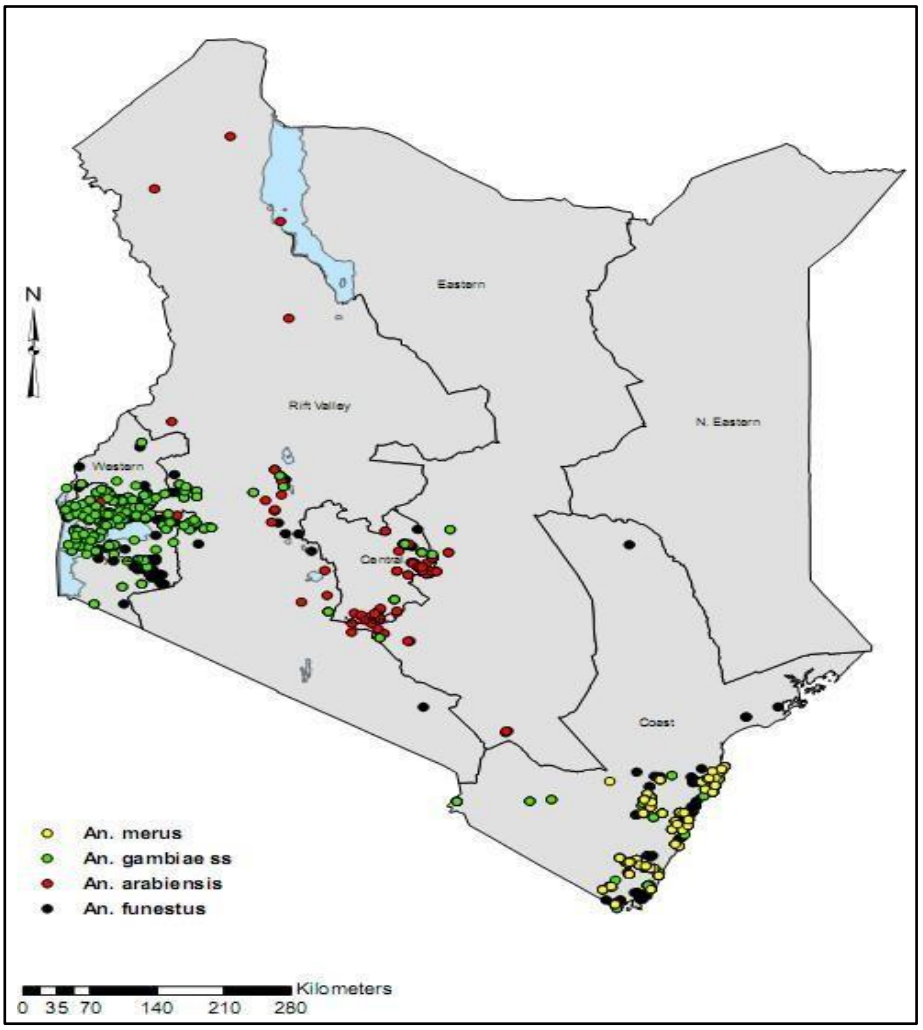

Figure 3: Distribution of major malaria vectors in Kenya

Source: (Ondeto, 2012)

2.3 Malaria vector control

2.3.1 Insecticide based control

According to Bhatt *et al*., (2015) effective control of malaria vectors is an important part of malaria incidence prevention. Synthetic insecticides produced for spraying indoors or for treating bed nets are used for such control (WHO, 2015). Indoor residual sprays of pyrethroid, organochlorine, organophosphate or carbamate classes of insecticides and pyrethroid impregnated bed nets target endophilic Anopheline species.

Dichloro-diphenyl-trichloroethane (DDT) is an organochlorine that was widely used for IRS in 1950's before debates for its elimination erupted in the late 1990's (Taverne, 1999). Although its use as an agricultural pesticide was banned worldwide under the 2001 Stockholm Convention, its use in disease vector control continues in several countries mainly due to its effectiveness in decreasing malarial transmission (Van Den Berg *et al*., 2017). Its success is attributed to its high toxicity and strong exito-repellency property which burrs mosquitoes from getting into sprayed houses thus reducing transmission due to the shorter indoor resting periods and lower mosquito blood feeding rates (Roberts & Andre, 1994). Curtis, (2002) is of the view that a combination of toxicity and exito-repellency characteristics could be what maintains DDT's efficacy even in areas where resistance has been reported against it. In Kenya, the Ministry of Health (MOH), the National Environmental Management Authority (NEMA) and the Pest Control Products Board (PCPB) opposed reintroduction of this insecticide for control of malaria vectors (Biscoe *et al*., 2004). Other organochlorines that have been used in control of malaria vectors are dieldrin and lindane. Currently, these insecticides have limited use due to high toxicity in humans (Heusinkveld & Westerink, 2012).

Pyrethroids are cost effective, have a fast knock down activity, a long residual effect and low mammalian toxicity. This explains why only they have been singled out for use on bed nets (WHO, 2006). For several years now, durable insecticide impregnated bed nets have been the cornerstone for malaria prevention and control, alongside Indoor Residual Spraying. Pyrethroid insecticides currently in use include; permethrin, deltamethrin,

lambdacyhalothrin, alphacypermethrin and bifenthrin (USAID, 2018).

In the organophosphate class, malathion and fenitrothion are the most frequently used insecticides for mosquito control (Najera, 2002). However, Pirimiphos-methyl (Actellic® 300CS), a new insecticide in the same class, was recently introduced in IRS programmes in Migori County and Homabay County in February 2017 (USAID and PMI, 2018). The downside of the potent organophosphate insecticides lies in their low residual activity.

Bendiocarb and propoxur are among the carbamate insecticides approved for disease vector control (GPIRM, 2012). These insecticides have a short residual effect which makes their use more costly compared to pyrethroids due to the many rounds of spraying needed. They also lack an excito-repellency property (WHO, 2011).

Pyrrole is a novel class of medically important insecticides. Chlorfenapyr is one of the insecticides in this class for which efficacy was observed in the control of *Anopheles gambiae* mosquitoes that exhibited resistant to pyrethroids (N'Guessan *et al*., 2007; N'Guessan *et al*., 2009). The insecticide is under review by WHOPES (PMI, 2017). If recommended, chlorfenapyr will serve as a suitable alternative to WHO-approved insecticides already faced by resistance since it shows no cross resistance with any of them (N'Guessan *et al*., 2007).

Neonicotinoid is another class of insecticides that has recently been added to the existing pool of public health insecticides (The Global Fund, 2025). This

class of insecticides targets the nicotinic acetylcholine receptor sites (nAChRs) of insects. At low concentration they cause decreased activity and incoordination while at high concentration they cause hypothermia, trembling spasms and death (Tomizawa & Casida, 2005). The neonicotinoids have high specificity for insects than vertebrates and thus have low mammalian toxicity. Clothianidin an insecticide in this class has been prequalified for control of malaria vectors through IRS using FludoraFusion™ and SumiShield™ formulations (Oxborough *et al*., 2019).

Other classes of insecticides with prequalified products for mosquito control include metadiamides e.g Tenebenal™ and butenolides e.g Flupyradifurone (Lees *et al*., 2020; Ahmed & Vogel, 2020).

Table 1 summarizes the context in which the four commonly used insecticide classes are applied while **table 2** lists insecticides approved by the PCPB for mosquito control in Kenya.

Table 1: Insecticide classes approved for LLIN and IRS products

Class of insecticide	**Approved LLIN product**	**Approved IRS product**
Pyrethroids	√	√
Organochlorines (DDT)	×	√
Organophosphates	×	√
Carbamates	×	√

√ - approved × - not approved

Table 2: Insecticides approved for public health use in Kenya

Class of insecticide	Type of insecticide
Pyrethroids	pirimiphos methyl
	alpha-cypermethrin
	deltamethrin
	cypermethrin
	tetramethrin
	pyrethrin
	permethrin
	bifenthrin
	prallethrin
	d'allethrin
	d-phenothrin
	lambda-cyhalothrin
	esbiothrin
	pynaminforte
	imiprothrin
	transfluthrin
	cyfluthrin
	etofenprox
Organochlorines	temephos
Organophosphates	diazinon
	chlorpyrifos-methyl
	malathion
	fenitrothion
Carbamates	bendiocarb
	propoxur
Neonicotinoid	clothianidin
Benzoylurea	diflubenzuron
Benzamide	diethyltoluamide(DEET)
Bio-insecticide	Coconut, Neem and soybean oils+sorbic acid
	Ethylene glucolmono-oleate+ Cetyl/Steryl alcohol polyoxyethelene

The coverage of ITNs and IRS interventions in Kenya is as presented in **Figure 4** and **figure 5** (MOH, 2016).

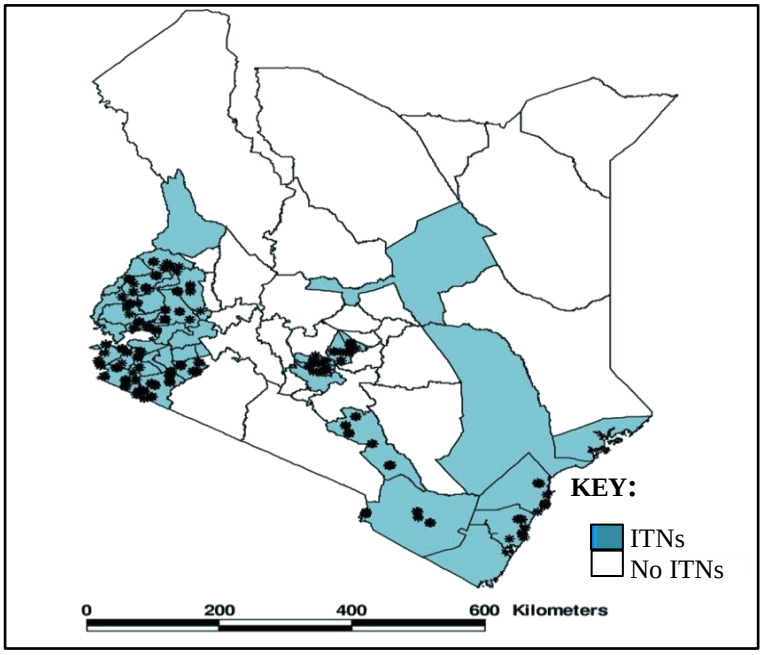

Figure 4: Counties in Kenya with a net coverage of at least two nets per person

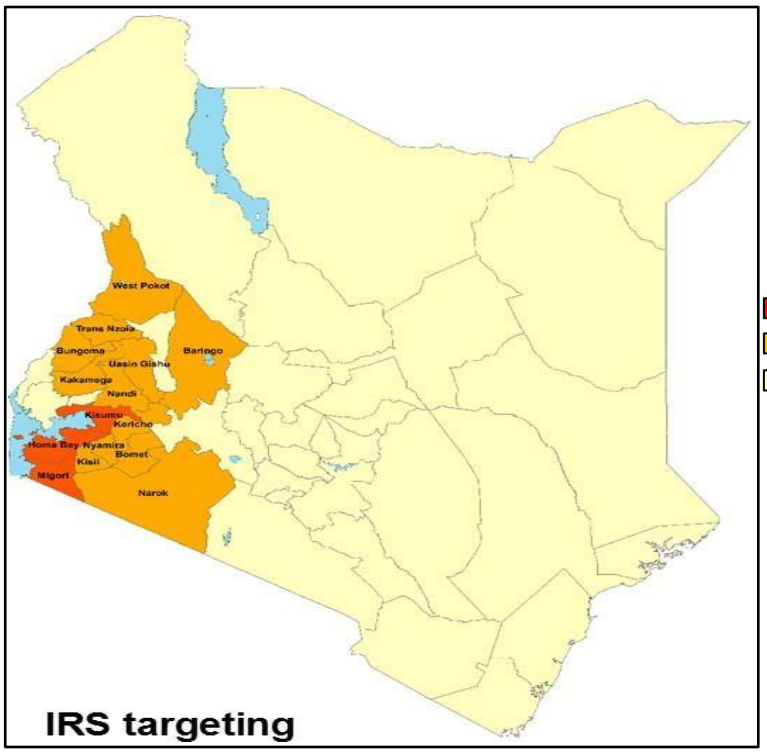

Figure 5: Coverage of IRS in Kenya since 2005

2.3.2 Novel non-insecticide based vector control

Challenges in synthetic biology for mosquito control have led to exploration of innovative strategies that do not rely on chemical-based insecticides. These innovations which include but are not limited to gene editing, sterilization and pathogenic attack are imperative in undercutting mosquito resistance to synthetic insecticides.

a) Mosquito gene editing approach

Clustered Regularly Inter-spaced Short Palindromic Repeats (CRISPR) is a genetic engineering technology currently being explored for its potential to eradicate malaria through genetic modification of the disease vector. The gene editing technology is used to either suppress the mosquito population through gene drives (where rate of gene inheritance is faster than the mendelian rate) or to create parasite resistant mosquitoes through gene knockout (Kyrou *et al*., 2018; Nourani *et al*., 2023).

In their proof of concept experiments, Simoni *et al*., (2020) used CRISPR technology to modify the sex determining gene in mosquitoes such that the male gene became dominant over the female gene. They then spread this genetic modification to subsequent generations using gene drive constructs which led to a total laboratory population collapse within 7-11 generations. In a different set of experiments, Dong *et al*., (2018) used the technology to delete the fibrinogen-related protein 1 (FREP 1) gene thus causing mosquitoes to be resistant to *Plasmodium* parasite.

Although these experiments have not been replicated in the field, laboratory and indoor caged trials show a promising potential for CRISPR technology to

replace populations of mosquitoes with vectorial capacity with those with the inability to sustain malaria transmission.

In addition, other studies have established that CRISPR can be used in functional validation of gene mutations in insecticide resistance (Grigoraki *et al*., 2021; Williams *et al*., 2022) and in reversing insecticide resistance in insect populations (order: diptera) by replacing the insecticide resistance mutant alleles with wild type susceptible alleles. Simoni *et al*., (2020) for instance, applied CRISPR-based allelic drives in replacing *kdr* mutant alleles (L1014F) with wild type alleles (L1014) in *Drosophila melanogaster* thus restoring the population's susceptibility to pyrethroids.

b) Sterile insect technique

This technique relies on mass rearing and release of male mosquitoes treated with chemosterilants or γ-rays. Their mating with wild female populations in the wild doesn't result to offsprings and this eventually leads to population suppression. This novel tool has successfully been used against field populations of *Aedes aegypti* and *Aedes albopictus* in Cuba (Gato *et al*., 2021; Tur *et al*., 2021).

c) Pathogen-based control

Some of the pathogenic microorganisms used for malaria vector control include *Bacillus thuringiensis* and *Wolbachia* bacteria.

Bacillus thuringiensis subsp. *israelensis* (Bti) is a naturally occurring bacterium that was isolated from the soil (Goldberg and Margalit, 1977) and is widely used as a biological agent against mosquito larvae in Africa, South East Asia,

Germany, Canada, USA, Sweden and France (Brühl *et al*., 2020; Schäfer and Lundström, 2014). The bacterium has been proven to be effective against larvae of the *Anopheles* species (Fillinger *et al*., 2003; Nartey *et al*., 2013; Dambach *et al*., 2014; Dambach *et al*., 2019). The mode of action of *Bacillus thuringiensis* is gut disruption. Once ingested the biocide solubilizes in the larvae's gut which leads to production of protoxins that are converted to toxins by gut bacteria and gut proteases. The toxins then bind onto the membrane of the gut and form pores thus leading to larval death (Rukmini *et al*., 2000; Vachon *et al*., 2012). Given that this biocide is selective, is less toxic and faces no resistance from field populations of mosquitoes it would be the most suitable agent to tackle malaria vectors from their source (Brühl *et al*., 2020). Moreover, the fitness cost associated with resistance to *Bti* as demonstrated in laboratory strains make it unlikely for the intervention to loose efficacy in the field (Saleh *et al*., 2003; Paris *et al*., 2011).

2.3.3 Integrated vector management

Integrated vector management (IVM) is the use of multiple interventions from across different sectors e.g public health and agriculture to control one disease vector or use of one intervention to control several disease vectors all with an aim of ensuring optimum use of available resources as schematically represented in **figure 6** (WHO, 2016). The interventions can either be chemical or non-chemical based. The five elements of IVM include integrated approach, evidence based decision making, intra and inter-sectoral collaboration, capacity building as well as advocacy, social mobilization and legislation. To adopt a country's vector control system to IVM, current and past field observations, surveillance analyses and situation analyses are needed (WHO, 2013).

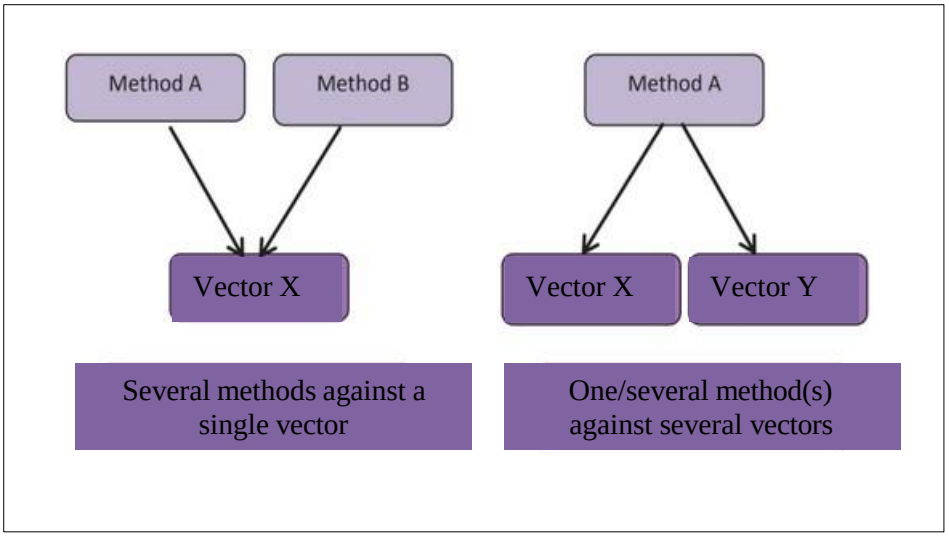

Figure 6: Schematic representation on how IVM can be implemented to achieve different outcomes

2.4 Challenges facing malaria vector control interventions

Despite their success in suppressing malaria transmission, currently used malaria interventions have been met by limitations (Karema *et al.*, 2012; Ngufor *et al.*, 2014). There is mounting evidence showing that the growing resistance by malaria vectors cuts across all the four major classes of insecticides currently in use. In addition to insecticide resistance, behavioral changes in Anopheline species and shifts in vector species composition are posing a challenge in malaria vector control (Reddy *et al*., 2011; Rusell *et al*., 2010; Sokhna *et al*., 2013; Sougoufara *et al*., 2017).

2.4.1 Insecticide resistance

The World Health Organization defines insecticide resistance as the adaptation of an insect to the effects of an insecticide by either becoming tolerant to its toxicity or by avoiding contact with the insecticide (WHO, 2012). Resistance begins with few refractory genes but with exposure to the same insecticide over time, these genes increase through genetic inheritance and become frequent in a population. **Figure 7** is an illustration of how resistance spreads

over time through genetic heritability.

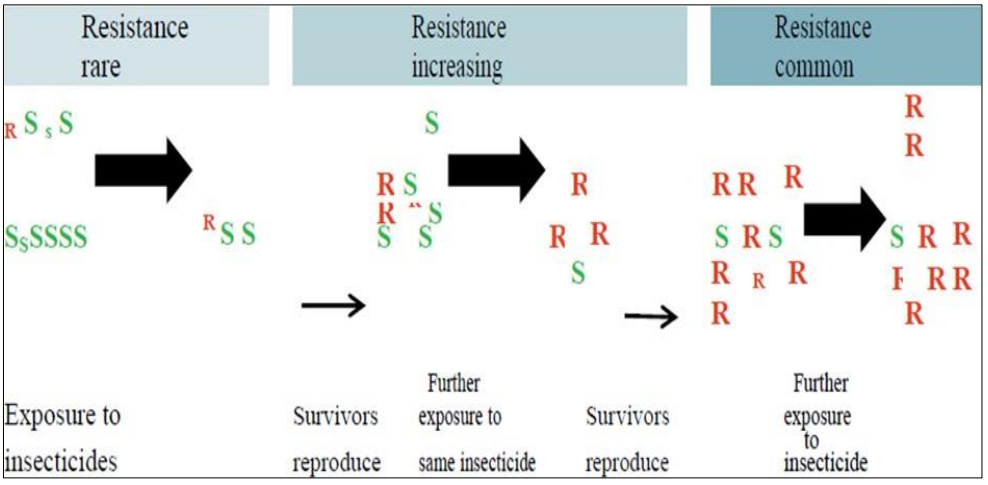

Figure 7: Increase in insecticide resistance genes with increase in insecticide pressure

The global extent of resistance to insecticides is illustrated in **figure 8.**

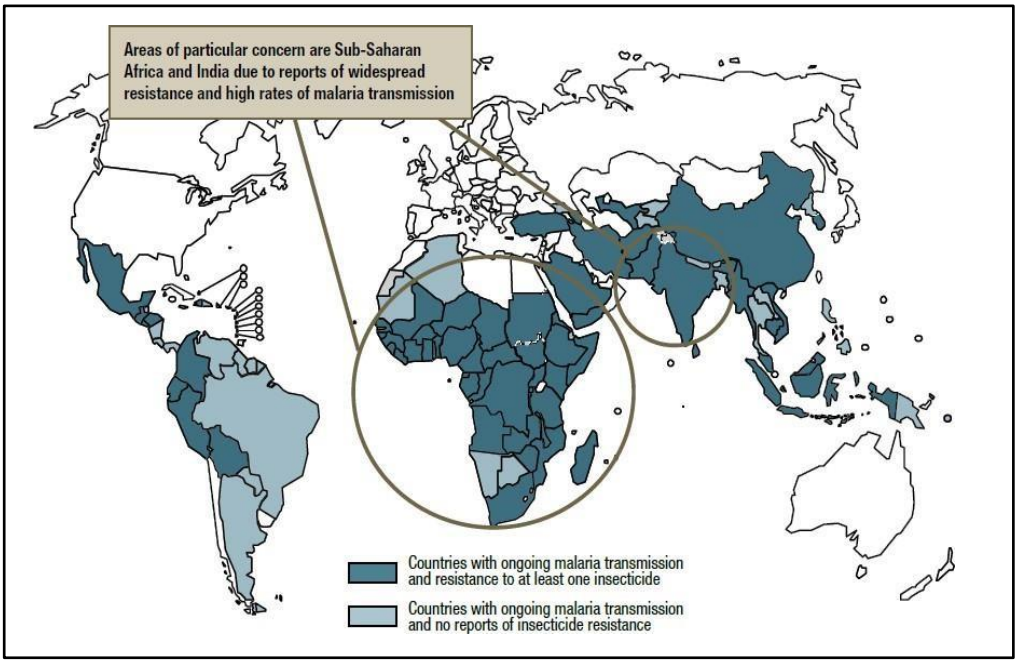

Figure 8: Countries with identified insecticide resistance and ongoing malaria transmission

Source: (GPIRM, 2012)

Although it faces a heightened concern now, insecticide resistance is not a new phenomenon. Going back in history, development of Anopheline resistance to

DDT and the negative environmental impact of this insecticide was the primary reason why political and financial support was cut off from the Global Malaria Eradication Programme initiated by WHO in 1955 (Nájera *et al*., 2011). In the late 1960s it was reported that DDT was not effective against *Anopheles gambiae* in the West, Central and East African regions (Corbel and Guessan 2013; Ranson *et al*., 2011). Even in the face of resistance, the insecticide was not banned for disease vector control until much later when concerns about its bio- magnification property in the food chain were raised by United Nations Environmental Program (UNEP). As an alternative, dieldrin was adopted for a period of time after which resistance to this insecticide was also reported (Patel *et al*., 1958; Raghavendra, 2002). By the 1980's DDT usage had been faced out by pyrethroids, organophosphates and carbamates.

Ranson *et al*., (2009) and Mnzava *et al.,* (2015) argue that refractoriness to pyrethroids by Anopheline species could be attributed to continuous deployment of LLINs and pyrethroid based IRS programs over a long period of time especially in sub-Saharan Africa.

In response to pyrethroid resistance, organophosphates and carbamates are being used for IRS as front line alternatives (AIRS, 2018; Aizoun *et al*., 2013; CDC, 2012). However, this does not seem to be the ultimate solution because already resistance to a number of insecticides in these insecticide groups has emerged in various settings. A study conducted in Muleba Tanzania, for example, reported a decline in mortality of *An. gambiae* exposed to bendiocarb (a carbamate) from 84% in 2011 to 31% in 2012. In another study conducted

in Kenya *An. gambiae* s.s. and *An. arabiensis* were found to be resistant to bendiocarb at two sites in the western region (Wanjala and Kweka, 2018). These reports suggest that bendiocarb is losing its ability to kill malaria vectors.

2.4.2 Change in vector behaviour

Use of insecticides on LLINs and for IRS is becoming less effective because vectors targeted by these tools are avoiding insecticide contact by preferring to feed and rest outdoors as opposed to the vice versa which has been the norm (Kenea *et al*., 2016; Kitau *et al*., 2012; Mutuku *et al*., 2011; Pates & Curtis, 2005; Russell *et al*., 2011; Zhou *et al*., 2011).

2.4.3 Shifts in vector species composition

Recently, there have been reports on shifts in malaria vector species composition in some parts of sub-Saharan Africa. These shifts are believed to have been caused by change in environmental conditions, change in climate, competitive displacement or introduction of new invasive species. Changes in the relative abundance of species in a vector population may alter disease transmission patterns, increase complexity of vector control strategies and reduce effectiveness of control measures. In the Kenyan coast for instance, Mwangangi *et al*., (2013) observed that *An. arabiensis* had replaced *An. gambiae* s.s. that was once a dominant vector in the region (Mbogo *et al*., 2003; Keating *et al*., 2005). The Kenyan western region is no different given that several studies have reported a decline in the abundance of *An. gambiae* s.s. and an increase in *An. arabiensis* following intensive IRS and bed net usage (Bayoh *et al*., 2010; Degefa *et al*., 2017). Similar trends have been reported in other countries like Tanzania where a remarked decrease in the

abundance of *An. gambiae* was reported as compared to *An. funestus* (Meyrowitsch *et al*., 2011) whereas in other places of the country *An. gambiae* s.s. had changed from being the most common to the most rare being replaced by *An. arabiensis* (Derua *et al*., 2012; Russell *et al*., 2010). In some parts of Uganda, scale up of vector control interventions was associated with an almost complete elimination of *An. funestus* s.l. and *An. gambiae* s.s. with a non-pronounced decline of *An. arabiensis* (Mawejje *et al*., 2021). The trend in southern Africa is that of the *An. funestus* s.s. increasingly replacing *An. gambiae* s.s. and *An. arabiensis* as the dominant and most competent vector of malaria (Msugupakulya *et al*., 2023).

2.5 Mechanisms of insecticide resistance

Development of resistance to insecticides by *Anopheles* mosquitoes is either by physiological means or behavioral means (IRAC, 2011). Physiological resistance is the ability to withstand toxicity from insecticides while behavioral resistance is the ability to avoid contact with insecticides (WHO, 2012). The physiological means of resistance has further been classified into; target site resistance, metabolic resistance and reduced cuticle penetration (Balabanidou *et al*., 2016; Hemingway, 1998; Hemingway, 2000; Plapp, 1976; WHO, 1957).

Once the specific site of action by an insecticide is tampered with, resistance to that particular insecticide ensues. Pyrethroids and organochlorines for instance target the voltage-gated sodium channel (*vgsc*) gene in *Anopheles* mosquitoes' nervous system. A mutation in this gene can either be as a result of substitution of the leucine amino acid with serine or phenylalanine amino

acid at position 1014, that is, L1014S or L1014F. This mutation, termed the Knock down resistance (*kdr*) mutation, results in cross-resistance to the pyrethroids and organochlorines insecticide classes (Silva *et al.,* 2014; Santolamazza *et al.,* 2004). L1014F is considered the West African allele (*kdr*-w) because Cote d'Ivoire, a country in the west African region, reported it first in 1998 (Martinez-Torres *et al.,* 1998) while L1014S is considered the East African allele (*kdr*-e) because Kenya, a country in the East African region, documented it first in 2000 (Ranson *et al.,* 2000). Several studies have shown that the two alleles are found in the primary malaria vector populations (Pinto *et al.,* 2006; Pinto *et al.,* 2007; Etang *et al.,* 2007, Verhaeghen *et al.,* 2006).

Another type of resistance due to alteration of the target site involves mutation in the *ace*-1 gene where glycine amino acid is substituted with serine amino acid at position 119 of the Acetylcholinesterase 1 (AChE-1) catalytic site i.e. G119S. This type of mutation causes the acetyl cholinesterase enzyme to become insensitive to organophosphates and carbamates.

An alteration in the heteromultimeric gated chloride ion channel which is the target site for dieldrin is yet another example of target site resistance. The alteration involves substitution of alanine with serine amino acid in the γ-amino butyric acid (GABA) receptor gene. This mutation results to dieldrin resistance (Wondji *et al.,* 2011).

In other cases, when a chemical compound comes into contact with a malaria vector, the vector may secrete more metabolic enzymes in response to the

chemical. The overly expressed enzyme metabolises the insecticide, sequestrates the molecules and detoxifies the insecticide such that it cannot function thus bringing about metabolic resistance. While non-specific esterases (NSE) in malaria vectors are overexpressed in response to the toxic effects of organophosphates and carbamates, cytochrome P450-dependent monooxygenases are overexpressed due to DDT and pyrethroids toxicity (González *et al.*, 2009).

Modification of the cuticle structure is a mechanism that has been observed in *Anopheles* species. Studies by Balabanidou *et al*., (2016); Bonizzoni *et al*., (2012); Jones *et al*., (2013); Wood *et al*., (2010) and Yahouédo *et al*., (2017) have suggested association between insecticide resistance and cuticle thickening. Thickening of the cuticle reduces penetration of insecticides thus contributing to resistance.

Table 3 shows mechanisms of resistance that have been identified in Kenya.

Table 3: Mechanisms of insecticide resistance in major malaria vectors in Kenya

Vector Species	**Pyrethroids**		**DDT**		**Organo-phosphates**		**Carbamates**	
	Target-site	**Metabolic**	**Target-site**	**Metabolic**	**Target-site**	**Metabolic**	**Target-site**	**Metabolic**
***An. gambiae* s.s.**	√	√	√	√	√	×	√	√
An. arabiensis	√	×	√	√	√	×	√	×
***An. funestus* s.s.**	×	×	×	√	×	×	×	√

2.6 Detection and monitoring of insecticide resistance

Currently, data on insecticide resistance is needed prior to deploying an efficacious vector control tool. Screening malaria vectors for phenotypic and genotypic insecticide resistance as well as monitoring the levels of this resistance is an evidence-based support for insecticide resistance management strategies which guide decisions on which insecticide is to be deployed where (Mavridis *et al*., 2018).

Customarily, resistance phenotypes are detected through bioassays which use synergists or impregnated papers with discriminating or diagnostic insecticide concentrations. With bioassays it is difficult to tell the basis of resistance and again they cannot detect incipient resistance (Donelly *et al*., 2016).

Molecular assays complement phenotypic bioassays in that they can detect resistance before it even becomes phenotypically observable (Brogdon and McAllister, 1998). This comes in very handy because one of the recommendation strategies towards Global Insecticide Resistance Management is early detection of insecticide resistance. Moreover, molecular assays can tell the cause of resistance. Target site resistance due to mutations for example, is detected through this technique which determines whether or not individual vectors possess mutant alleles. Examples of molecular assay techniques include Polymerase Chain Reaction based methods like amplification of specific alleles- PASA-PCR (Yan *et al*., 2014) and Restriction Fragment Length Polymorphism- PCR-RFLP (Singh *et al*., 2009).

Resistance due to elevated enzymes like the glutathione S-transferases, esterases and mixed function oxidases is detected through immunologic and biochemical assays.

One of the reliable indicators of insecticide resistance level in pests of medical importance is the frequency of mutant alleles in field populations (Feng *et al.*, 2015). It is therefore necessary to monitor these alleles because they influence the choice of vector control intervention to be deployed.

2.7 Status of insecticide resistance in Kenya

Kenya reported its first case of insecticide resistance by malaria vectors in its western region in 1994 (Vulule *et al*., 1994). Today, insecticide resistance is common and has been reported in four classes of insecticides. A review conducted by Ondeto *et al*., (2017) reported that *Anopheles arabiensis* and *Anopheles gambiae* s.s. tolerated pyrethroids and carbamates. In the same report, resistance to organophosphates and organochlorines was revealed in *An. gambiae* s.l. and *An. funestus* s.s. respectively. Unlike refractoriness to organophosphates, organochlorines and carbamates, pyrethroid resistance is widespread in Kenya and this could be attributed to overreliance on pyrethroid impregnated nets and pyrethroid-based IRS programmes in malaria endemic regions in addition to selection pressure from agricultural pesticides and industrial wastes with pyrethroid insecticidal properties (Blakey *et al*., 2013; Kamau and Vulule 2006; Stump *et al*., 2004).

One of the main causes of insecticide resistance among *Anopheles* species in Kenya of this kind of resistance reported in was in an *Anopheles gambiae*

population and was due to an L1014S *kdr* mutation (Ranson *et al*., 2000). In his review, Ondeto *et al*., (2017) indicates that target site resistance due to substitution of amino acids in the *ace-1*^{R} gene has not been documented in Kenya. They recommend that presence or absence of this gene be determined and that the status be reported, especially because insecticides that would trigger this kind of resistance have been recommended as alternatives in a strategy to manage pyrethroid resistance (Aïzoun *et al*., 2013; AIRS, 2018).

Most of the research on insecticide resistance mechanisms in Kenya has focused on the *kdr* mutation with only limited research on *ace-1*^{R} gene mutation and on metabolic resistance (Stump *et al*., 2004; Kamau and Vulule 2006; Kawada *et al*., 2011; Mathias *et al*., 2011; Ochomo *et al*., 2015). There is therefore need for further research on the *ace-1*^{R} gene mutation and metabolic resistance mechanism in the country. **Figure 9** shows the distribution of L1014S and L1014F alleles in Kenya.

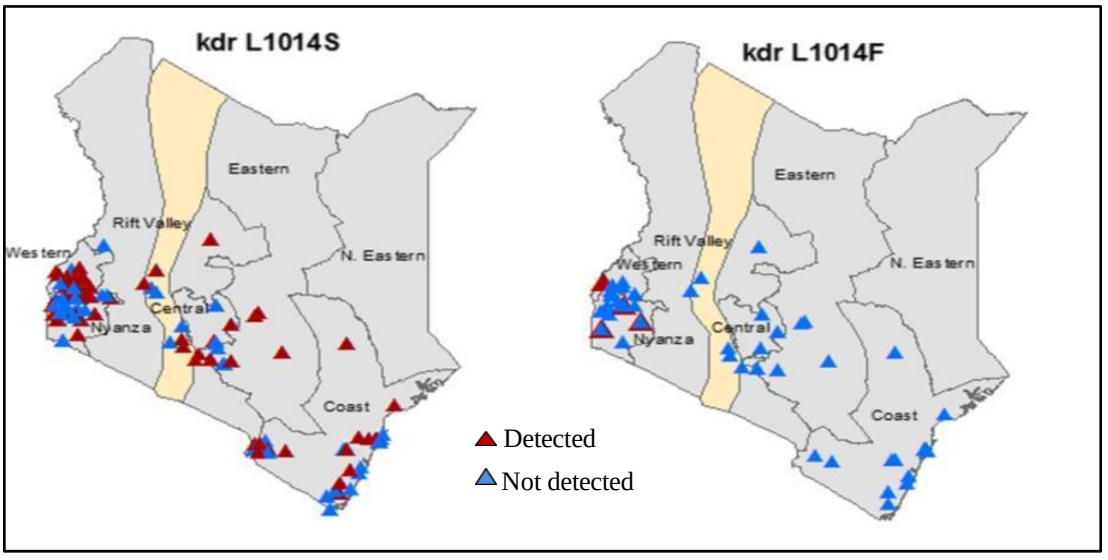

Figure 9: Distribution of the *Kdr* mutation in *Anopheles* species in Kenya between 1994 and 2015

Source: (Ondeto *et al*., 2017)

Apart from physiological changes (target site resistance & metabolic resistance), behavioral changes in *Anopheles* mosquitoes in Kenya have also contributed significantly to insecticide resistance. In his study, Githeko *et al.,* (1996) found that *An. gambiae* s.s. had shifted from being endophilic to being exophilic after implementation of permethrin treated curtains, but still retained their anthropophagy. Fergusson *et al.,* (2010); Sokhna *et al.,* (2013) and Takken, (2002) explain that few studies on behavioral resistance have been conducted since the mechanism was identified in 1956, and further reason that this could be due to the difficulty that comes with designing experiments to investigate this kind of resistance.

Inversions in chromosomes of *Anopheles* species are believed to be an adaptive mechanism that enables these malaria vectors to respond to insecticide pressure by changing their behavior. Change in behavior can range from shifts in feeding and resting preference, host preference and change in feeding time.

2.8 Chromosomal inversions

Chromosomal inversions occur when a chromosome breaks and in the event of repair the fragment is re-inserted in the reverse orientation. White *et al.,* (2007a) describes the three kinds of karyotypes that arise due to these chromosomal inversions, that is, one where the inversion is none existent (standard karyotype), another where the inversion exists (inverted karyotype) and the third which is a hybrid of the two (heterokaryotype). The inversions are an adaptive mechanism that occurs in fungi, plants, mammals and insects

and are important because they contribute to ecological success of a species (Dobzhansky, 1937; Hoffmann *et al.,* 2004; Hoffmann and Rieseberg, 2008; Krimbas and Powell, 1992). *An. gambiae* s.s. originally inhabited the rainforest but over time, this species has invaded the savannas and the polymorphism of its chromosomal inversions is thought to be responsible for this ecological flexibility (Ayala *et al.,* 2014; Fouet *et al.,* 2012). Apart from adaptation, chromosomal inversions are also responsible for formation of new distinct species and sex chromosome evolution (Feder *et al.,* 2003; van Doorn and Kirkpatrick 2007; Hoffmann and Rieseberg, 2008).

Common inversions in *An. gambiae* occur on chromosome 2. On one arm of the chromosome we have 2La inversion while on the other we have 2Rj, 2Rb, 2Rc, 2Rd and 2Ru inversions (Ayala *et al.,* 2014). The current study is particularly interested in the 2La inversion.

2.8.1 2La chromosomal inversion

An. gambiae s.s. has a paracentric inversion on chromosome's 2 left arm denoted as 2La. The inversion in this sub-species is polymorphic (White *et al.,* 2007b) with a standard homokaryotype arrangement ($2L+^{a}/+^{a}$), an inverted heterokaryotype arrangement ($2La/+^{a}$) and an inverted homokaryotype arrangement-2La/a (Coluzzi *et al*., 2002; Perlman and Wahlgren, 2003). While *Anopheles gambiae* s.s. is polymorphic for the 2La inversion, *An. arabiensis* and *An. merus* are fixed for the inverted homokaryotype arrangement and *An. melas*, *An. bwambae* and *An. quadriannulatus* are fixed for the standard arrangement (Sharakhov *et al*., 2006).

2La chromosomal inversion has been linked to indoor mosquito feeding and resting behavior (Ayala *et al*., 2014; Sharakhov *et al*., 2006) among other traits like susceptibility to *Plasmodium* infection (Blandin *et al*., 2004; Osta *et al*., 2004; Riehle *et al*., 2006; Riehle *et al*., 2017; Slotman *et al*., 2007; Petrarca and Beier 1992), resistance to desiccation in adult female mosquitoes (Coluzzi *et al*., 1977; Gray *et al*., 2009), thermal tolerance in larvae (Rocca *et al*., 2009) and adaptation to arid conditions (Fouet *et al.,* 2012). The frequency of inversion 2La is nearly fixed in arid zones and almost absent in humid rainforests (White *et al*., 2007; Wondji *et al*., 2005).

One of the challenges that species living in xeric habitats have to deal with is dehydration stress. They therefore have to develop physiological or behavioral adaptations to survive. Studies conducted by Gray *et al.,* (2009), Rocca *et al.,* (2009) and Fouet *et al.,* (2012) revealed that carriers of the inversion survive longer under heat stress compared to non-carriers due to less loss of water. Since the 2La inversion has been correlated with aridity, it is postulated that genes close to or inside the breakpoints of the inversion cause homeostatic responses that act against the negative effects of temperate stress thus preventing water loss.

Studies have also linked the 2La inversion to dieldrin and fipronil resistance in *An. gambiae* laboratory colonies (Brooke *et al*., 2000; 2002; 2006). In this case individuals who are heterokaryotypic for 2La have an advantage over those that have a homokaryotypic inversion arrangement (Ayala *et al*., 2014).

This association is further explained by Holt *et al*., (2002), who found out that the rdl locus linked to dieldrin and fipronil refractoriness is located within the inversion. Unlike the adaptive traits mentioned earlier and which are triggered by inversion 2La, the latter -insecticide resistance, is not due to the inversion; instead, the inversion provides a stable mechanism for the continual inheritance of insecticide resistance genes (Brooke *et al.,* 2002).

Resting behavior of malaria vectors remains an important aspect for consideration when choosing a vector control measure as this will determine its effectiveness. Coluzzi *et al.,* (1979), Petrarca and Beier, (1992) and Sharakhov *et al*., (2006) established that carriers of the 2La inversion are most likely to rest indoors while those without the inversion are more likely to be found outdoors. In this study the inversion-2La was used to determine whether behavioral adaptation of malaria vectors is likely to have a negative impact on the efficacy of vector control interventions currently in place.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

This study was conducted in four counties; Kirinyaga, Baringo, Tana River and Migori.

Kirinyaga in the central region of Kenya is a low risk malaria zone. Baringo being a semi-arid region experiences seasonal malaria transmission. However outbreaks are known to occur in its highland regions during the rainy seasons. While Tana River experiences seasonal malaria transmission, Migori experiences constant and intense malaria transmission throughout the year.

These sites which were selected for study are shown in **figure 10**.

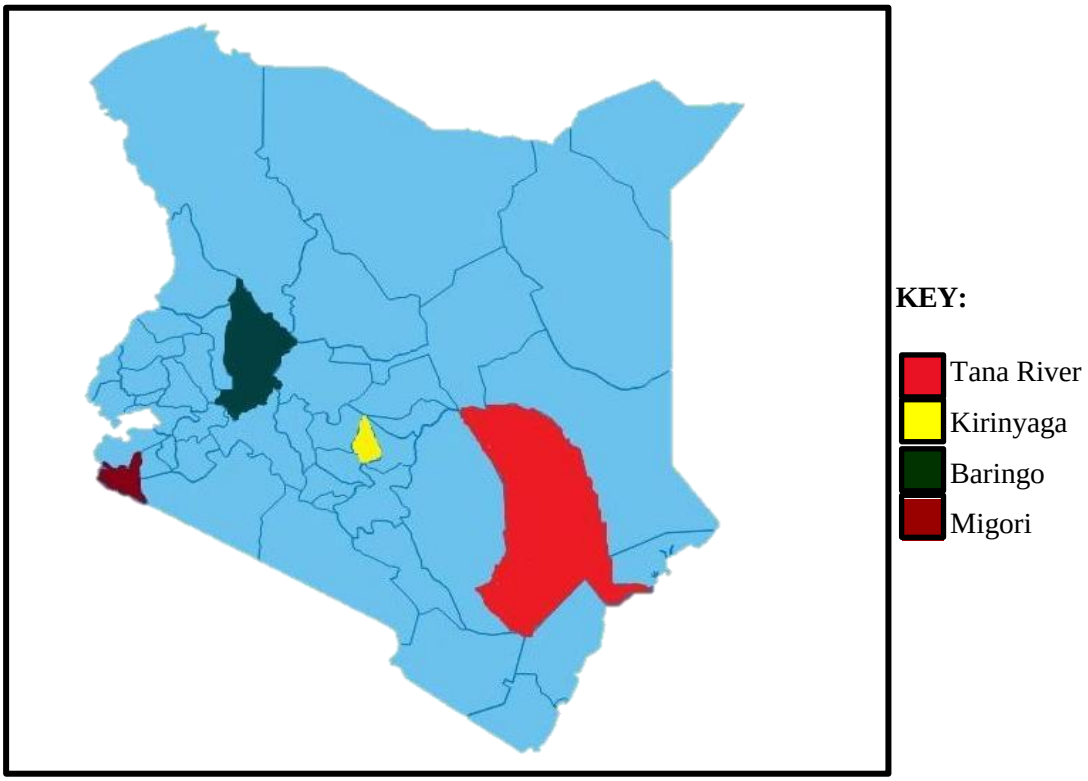

Figure 10: A Kenyan map showing selected study sites

Kirinyaga is located North-East of Nairobi, Kenya and lies between longitude 37.3827°E and latitude 0.6591°S. The area receives long rains between March and May while short rains occur between October and November. Temperatures are 16°C – 26.5°C while relative humidity is between 52% – 67% (Asimeng and Mutinga 1993; Mutero *et al*., 2004). Climatic conditions in this area have largely contributed to rapid development and survival of malaria vectors. Furthermore, the area has vast rice irrigation fields which provide suitable breeding grounds for the mosquitoes. Abundance of malaria vectors in this region is influenced by the rice growing cycle and rainfall pattern (Rapuoda, 2012).

Baringo County is located within the Rift Valley region. It lies between longitude 35.602°–36.277°E, and latitude 0.541°–0.723°N and is 870 m-2499 m above sea level. On the basis of height above sea level the county is divided into four zones, that is; riverine, lowland, midland and highland zone (Omondi *et al.*, 2017). The lowland and riverine zones are characterized by lakes, rivers and poorly drained soils that are liable to flooding. Malaria transmission in these zones is at its peak during the rainy season which is in March, June and November (Omondi *et al*., 2017). While there are no cases of malaria transmission in the highland zone, few cases are reported in the midland zone. County fact sheets indicate that malaria incidence was 12% as of December 2011 (Open data Kenya demographic, 2012).

Tana River County is located in the upper coast of Kenya (1.5868° S, 39.4424° E). Bordering it are Kilifi, Lamu, Taita Taveta, Kitui, Isiolo and

Garissa counties. Rainfall ranges between 400mm-750mm annually while temperature ranges between 30^0C and 33^0C. Like Kirinyaga, Tana River County is characterized by vast irrigation schemes. The presence of Bura and Hola irrigation schemes has contributed to the amplification of malaria prevalence. Tana River was one of the counties that received LLINs in the year 2015 through a net roll out program supported by the government (MOH, 2016). According to Omar Berhe, the County Malaria Control Programme Coordinator then, malaria infections had increased in the county because the nets in use were worn out and hence ineffective. In 2017, Tana River was listed as one of the beneficiaries of the government's LLINs roll out program that saw new nets get distributed to replace the old ones (MOH, 2017). Bednets are the most practical tool for preventing vector-human contact in this county.

Migori County is located in the Western region of Kenya and lies between longitude 34.4198° E and latitude 0.9366° S. It borders river Kuja, Migori River and Lake Victoria. Annual rainfall here averages 1369 mm while temperature is 21.2 °C on average with a relative humidity of 54%. Malaria, diarrhea, HIV/AIDs and urinary tract infections are the most prevalent diseases in the county. Results from a health policy project conducted in the region indicate that malaria incidences had increased from 33.1% in 2012 to 49.0% in 2015 (Migori County-Health policy project, n.d). The county is one of the priorities for increased malaria control investment by the Kenyan government (Okoyo *et al*., 2015). In a campaign to increase ITN ownership

coverage the government in partnership with the Ministry of Health distributed LLINs in the county in 2014 (MOH, 2016). In 2017 and 2018, the county benefited from an IRS program that saw more than 200,000 structures sprayed (MOH, 2017; AIRS, 2018).

3.2 Mosquito specimens

Anopheles gambiae s.l. mosquitoes that had been preserved in anhydrous calcium sulphate and stored at room temperature were used in this study. The archived specimens were collected between August 2016 and November 2017 within the context of a malaria survey conducted by Kenya Medical Research Institute (KEMRI) and the Kenya National Malaria Control Program (NMCP). Collection of the mosquitoes was conducted inside selected houses using mouth aspirators, pyrethrum spray hand catches and CDC light traps. CDC light traps were set up at the foot side of the bed, 1 m off the ground between 1800h - 1900h and were collected the following morning between 0600h and 0700h. Indoor resting mosquitoes were collected using mouth aspirators and pyrethrum spray hand catches between 0700h and 0900h.

The mosquitoes were identified by use of morphological keys (Gillies and Cootzee 1987). A summary of the morphological keys used to distinguish between *Anopheles gambiae* s.l. and *Anopheles funestus* s.l. are provided in **table 4**.

Table 4: Distinguishing characteristics between *An. gambiae* and *An. funestus* complexes

Feature	***Anopheles gambiae* s.l.**	***Anopheles funestus* s.l.**
Protarsi	Speckles present on protarsi	Speckles absent on protarsi
Palp	2 broad and 1 narrow pale bands on the palp	3 narrow pale bands on the palp

Feature	***Anopheles gambiae* s.l.**	***Anopheles funestus* s.l.**
Wing	Tip of vein 6 has a fringe spot	Tip of vein 6 lacks a fringe spot
Abdomen	Scaling is scanty and is confined to tergum VII and VIII	Scaling is heavy especially on tergum VI and VII

3.3 Sample size

The method of Collins and Schwartz, (2002) was used to determine sample size. By this method, screening a minimum of 65 chromosomes is required to detect a 5% polymorphism at the 95% confidence level. Thus, a minimum of 33 samples (representing 66 chromosomes) were target for the analysis of each of the genetic markers.

3.4 Identification of *Anopheles gambiae* sibling species

Sibling species of the *An. gambiae* complex that are morphologically indistinguishable were identified using a standard Polymerase Chain Reaction (PCR) based assay (Scott *et al.*, 1993).

3.4.1 DNA extraction

Using the method of Collins *et al.*, (1987), DNA was extracted from the preserved Anopheline mosquitoes. First, each mosquito was split into three sections; i) head and thorax ii) legs and wings and iii) abdomen. Legs and wings were placed in a 2ml centrifuge tube and using pestles they were ground in a 100µl maceration buffer consisting of homogenizing and lysis buffer in the ratio of 4:1. The homogenate was placed on a hot water bath at 65^{o}C for 30minutes then allowed to cool on ice for 30 minutes before adding 14µl of 8M Potassium acetate. To ensure proper mixing the contents were vortexed. This was followed by centrifuging the mixture at 11700rpm (14600g) for 10minutes. The resulting

supernatant was transferred to a sterile 1.5ml centrifuge tube and 200µl of 100% ethanol added after which the contents were vortexed and stored in the freezer overnight. The contents were then centrifuged a second time at 11700rpm (14600g) for 20minutes and absolute ethanol poured off. To wash the DNA, 200µl of 70% ethanol was added and poured off before adding another 200ul of 100% ethanol which was also poured off. The centrifuge tube containing the DNA pellet at the bottom was inverted on a clean paper towel and DNA was left to air dry overnight. 100µl of DNase/RNase-free water was added to the dry DNA to re-suspend it. Lastly the tube was sealed and DNA samples stored at -20^{o}C as they waited further processing.

3.4.2 Polymerase Chain Reaction (PCR) assay

The procedure in the PCR assay described by Scott *et al.,* (1993) was followed during the identification of sibling species of the *Anopheles gambiae* complex that were of interest to this study. A PCR mixture was prepared by adding 5.86µl sterile water, 1.8µl of 25mM $MgCl_2$, 3µl of 5X PCR buffer, 0.3µl of 2.5mM dNTPs mix i.e. (dGTP, dATP, dTTP and dCTP), 0.26µl of 25pmol/µl forward universal primer, 0.26µl of 25pmol/µl AR reverse primer, 0.26µl of 25pmol/µl GA reverse primer, 1.2µl BSA and 0.06µl Taq DNA polymerase (5 U/µl) in a 1.5ml eppendorf tube. These one sample volumes were adjusted upwards to fit the number of samples analysed per run. The PCR mix was then aliquoted equally into 0.2ml PCR tubes. In to each PCR tube containing PCR mix, 2 µl of sample DNA template prepared as described above was added.

The PCR tubes were placed on a thermo cycler for amplification. The reaction began with 1cycle of activation at 95^{0}C for 5minutes and was followed by 30

cycles of; denaturation at 95°C for 30seconds, annealing at 50°C for 30seconds and extension at 72°C for 30seconds. The reaction ended with a final elongation of 5minutes at 72^0C followed by a hold at 4°C. After cooling, the amplified products were electrophoresed through an ethidium bromide stained agarose gel (3%) and later scored by eye under ultra violet light. DNA bands on the gel were compared to positive controls initially identified as *An. arabiensis* and *An. gambiae* s.s. and with sizes 315bp and 390bp respectively.

Table 5 shows the names of primers, their sequences and expected sizes of their target fragments.

Table 5: Sequences of *Anopheles gambiae* oligonucleotide primers

Primer	**Sequence (5'-3')**	**Amplicon size (bp)**
Universal	GTG TGC CCC TTC CTC GAT GT	415
Anopheles arabiensis	AAG TGT CCT TCT CCA TCC TA	315
Anopheles gambiae s.s.	CTG GTT TGG TCG GCA CGT TT	390

3.5 Molecular detection of *Kdr* mutation

Kdr genotypes at amino acid position 1014 were determined for *Anopheles gambiae* s.s. and *Anopheles arabiensis* using a modified Real-time Polymerase Chain Reaction (qPCR) protocol by Bass *et al*., (2007). Samples were analysed using wild type, heterozygote and mutant allele probes of sequences 5'-CTTACGACTAAATTTC-3', 5'-ACGACTGAATTTC-3', 5'-ACGACAAAATTTC-3' labeled with VIC, 6-FAM and 6-FAM fluorescent dyes respectively.

Each Real time-PCR reaction included 2.75µl sterile water, 5.0µl 2X TaqMan mix, 0.2µl kdr-forward primer (800nM) CATTTTTCTTGGCCACTGTAGTGAT, 0.2µl kdr-reverse primer (800nM) CGATCTTGGTCCATGTTAATTTGCA, 0.2µl VIC-labeled TaqMan MGB wild type probe (200 nM) CTTACGACTAAATTTC, 0.15µl FAM labeled TaqMan MGB KdrE probe (200 nM) ACGACTGAATTTC and 0.15µl TaqMan MGB KdrW probe (200nM) ACGACAAAATTTC labeled with FAM. ROX was used as the reference dye. Positive controls initially identified for all three genotypes and a negative control were included in triplicate for each plate. PCR conditions began with a 10 minutes melting step at 95°C followed by 45 cycles of 95°C for 25 seconds and 64°C for 1 minute. Stratagene MxPro qPCR software was used to visualize the reaction curves. Guided by Bass *et al*., (2007), genotypes were scored by eye. A sample showing substantial increase in VIC fluorescence was scored as wild type while that showing a substantial increase in FAM fluorescence was scored as a homozygous mutant. Samples that showed an intermediate increase in both signals were scored as heterozygotes. The order of sequences and target regions of primers and probes used in this protocol are shown in **table 6**.

Table 6: Sequence and allele targets of probes and primers

Probe & Primer name	**Sequence**	**Target**
VIC labeled Taqman probe	CTTACGACTAAATTTC	Wild type allele (L1014)
6-FAM labeled Taqman probe	ACGACTGAATTTC	Kdr-e allele (L1014S)

Table 6: Sequence and allele targets of probes and primers

Probe & Primer name	Sequence	Target
6-FAM labeled Taqman probe	ACGACAAAATTTC	Kdr-w allele (L1014F)
Kdr forward primer	CATTTTTCTTGGCCACTG TAGTGAT	-
Kdr-reverse primer	CGATCTTGGTCCATGTTA ATTTGCA	-

3.6 Molecular detection of G119S (*ace-1^R*) mutation

The G119S mutation was screened for using a PCR-Restriction Fragment Length Polymorphism (RFLP) assay by Weill *et al.,* (2004). A 24µl volume of PCR master mix was prepared by mixing the following reagents in order: 16.65µl PCR water, 2.5µl 10X PCR Buffer, 1µl dNTPs (2.5 mM mix), 1.25µl Moustdir1 (25pmol/µl), 1.25µl Moustrev1 (25pmol/µl) and 0.15µl Taq DNA polymerase (5U/µl). To this mixture 2µl template DNA was added to make up a final volume of 26µl. The thermo cycler was set to run at temperature cycling conditions of 93^{o}C for 5minutes in a single cycle, 35cycles of 93^{o}C for 1minute, 53^{0}C for 1minute and 72^{0}C for 1.5minutes.1 cycle for the final elongation step was ran for 10minutes at 72^{0}C and was followed by cooling at 4^{0}C. Primers created amplicons of size 194bp. The PCR step was followed by a restriction digestion process which began with testing the activity of A*lu*I enzyme on substrate DNA (lambda). The enzyme was confirmed fit for use when substrate DNA (lambda) was digested into multiple restriction fragments. To digest the amplicons previously obtained, fresh PCR master mix was prepared on ice by adding 2µl of distilled water, 2µl of 10 X Tango buffer

and 1µl A*lu*I enzyme in one vial. Into this mix, 15µl of PCR product was added and the contents were allowed to incubate on the thermocycler at 37°C for 16 hours. This was followed by a heat inactivation step of the enzyme at 65^{o}C for 20minutes.

Samples were run through an electrophoresis box containing TBE buffer and an Ethidium Bromide-stained agarose gel prepared at 3%. Bands in the gel were compared to a 100bp DNA ladder. Individuals with two fragments of size 74bp and 120bp were considered homozygous resistant and were scored as G119S while those with a base pair size of 194 were considered wild types and scored as G119.

Table 7 shows the sequence and names of primers used in this protocol.

Table 7: Primers for *ace-1^{R}* mutation and their sequences

Primer name	**Sequence**
MOUSDIR1	CCGGG**N**GC**S**AC**Y**ATGTGGAA
MOUSTREV1	ACGAT**M**ACGTTCTC**Y**TCCGA

3.7 Molecular detection of 2La inversion

A conventional PCR assay described by White *et al*., (2007b) was used to screen for the 2La inversion. Primers 23A2, 27A2 and DPCross5 designed by White *et al*., (2007) and of sequences CTC GAA GGG ACA GCG AAT TA, ACA CAT GCT CCT TGT GAA CG, GGT ATT TCT GGT CAC TCT GTT GG respectively were used. A 24µl volume of PCR master mix was prepared for each sample by mixing 9.65µl distilled H_2O, 5µl 5X PCR buffer, 2µl dNTPs (2.5 mM concentration), 1µl 23A2 reverse primer, 1µl 27A2 forward

primer, 1µl DPCross 5 forward primer, 2µl $MgCl_2$ (25 mM) and 0.15µl Go-Taq DNA polymerase (5U/µl). 1µl of template DNA was added to 24ul of the prepared PCR mix to total up to a 25ul reaction volume. PCR cycle began to run with an activation step at 94^0C for 2 minutes then proceeded with 35 cycles of the following; 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, 45 seconds of extension at 72°C and ended with 1 cycle of final elongation at 72^oC for 10 minutes. The amplicons were held at 4^0C before being electrophoresed through an ethidium bromide stained agarose gel. Bands were visualized through a trans-illuminator and scored against 100bp DNA ladder. DNA bands of size 492bp were recorded as inversion arrangements (2La/a) while those of size 207bp were recorded as standard karyotypes ($2L+^a/+^a$).Where DNA bands of the two sizes were present, the sample was recorded as a heterokaryotype ($2La/+^a$). The sequence of primers targeting specific 2La karyotypes are shown in **table 8**.

Table 8: Primers for inversion and standard 2La allele, their sequences and target regions

Primer name	**Sequence**	**Target**
23A2	CTCGAAGGGACAGCGAATTA	Universal
27A2	ACACATGCTCCTTGTGAACG	2La
DPCross5	GGTATTTCTGGTCACTCTGTTGG	$2L+^a$

The target regions of primers used to generate the different 2La karyotype fragments are schematically represented in **figure 11**.

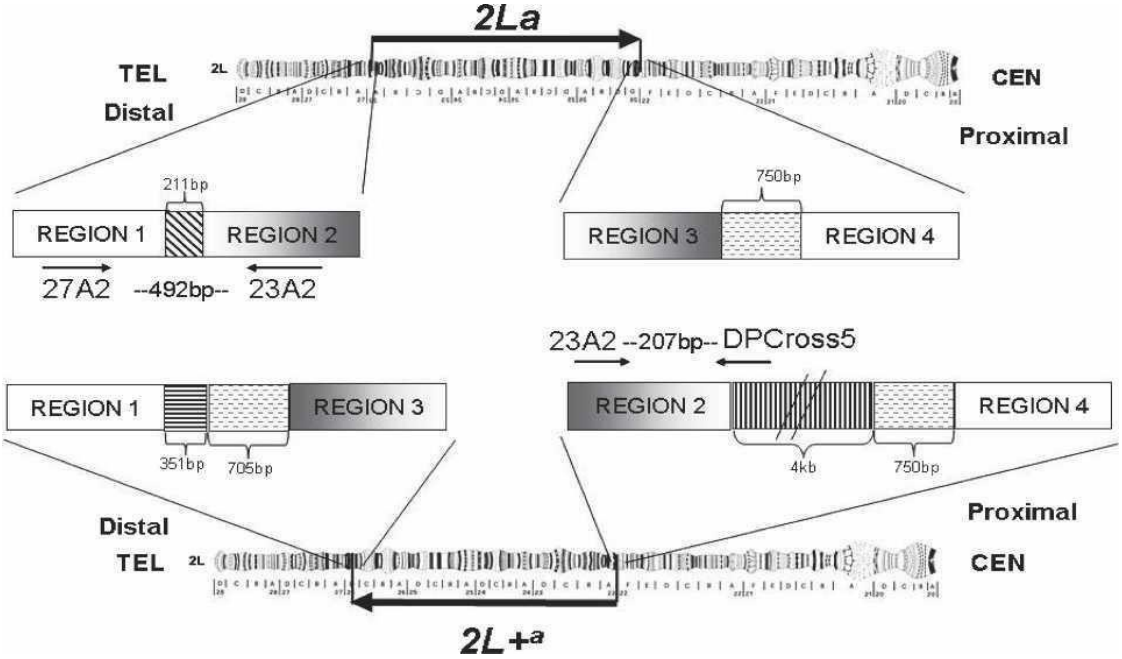

Figure 11: A model representation of the generation of three 2La inversion karyotype fragments

Source: (Sharakhov *et al*., 2006)

KEY:
Region 1-Region 4: Sequences flanking breakpoints
Arrows labeled 23A2, 27A2, DPCross: Target region and orientation of primers
Hatched boxes: Repetitive DNA
Stippled boxes: Sequences present at one breakpoint of an inversion fragment and duplicated at both breakpoints of the standard
TEL:Telomere
CEN: Centromere

3.8 Data management and analysis

Data was recorded in laboratory data forms, entered in Microsoft Excel version 10, cleaned and then transferred to STATA software version 14.2 for analysis.

The distribution of *Anopheles gambiae* species was determined by generating percentage frequencies of each species. Difference in species composition between the study sites was also examined.

Frequency of the different *kdr* mutant and 2La inversion alleles was calculated (Kawada *et al*., 2011) by;

$$\frac{\text{Occurrence of an allele}}{\text{Total number of alleles}} \times 100$$

Allele frequencies for each species and for each site were entered into a table on Microsoft Excel that was then used to generate graphs.

Association between the insecticide resistance markers and the 2La chromosomal inversion was determined based on chi-square statistics Fisher tests using the STATA software version 14.2 (Kabula *et al*., 2014; Hemming-Schroeder *et al*., 2018; Kiuru *et al*., 2018).

3.9 Ethical consideration

This work was part of a larger project whose ethical approval was sought from the KEMRI Scientific Steering Committee, protocol approval number: KEMRI/SSC/1677

CHAPTER 4

RESULTS

4.1 Distribution of *Anopheles gambiae* sibling species

731 *An. gambiae* s.l. were subjected to sibling species identification where 91.1% (n=666) successfully amplified while 8.9% (n=65) failed to amplify. 90.5% (n=603) of the successful amplifications were identified as *An. arabiensis* and 9.5% (n=63) as *An. gambiae* s.s.. Both *An. arabiensis* and *An. gambiae* s.s. sub species were found to exist in sympatry in Baringo (86.5% versus 13.5%), Migori (72% versus 28%) and Tana River (95.2% versus 4.8%) respectively. In Kirinyaga, only *An. arabiensis* (100%) was found present.

Figure 12 shows an agarose gel with diagnostic fragments of 390bp and 315bp.

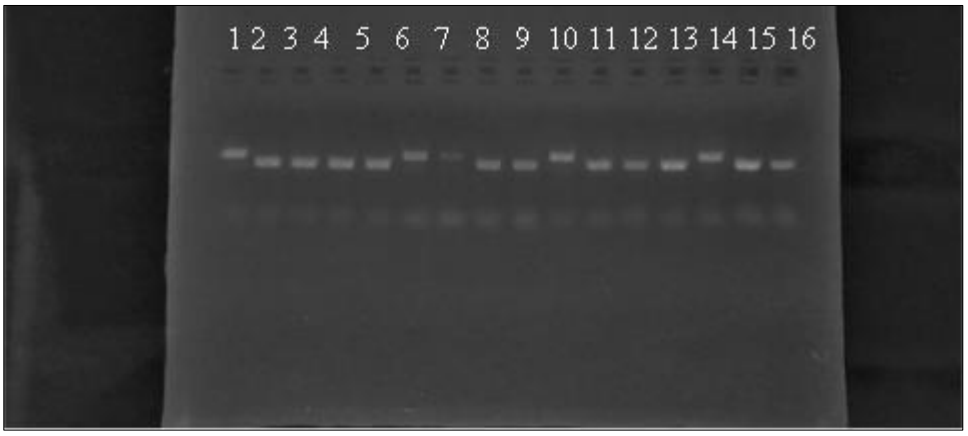

Figure 12: Gel image showing diagnostic fragments of *An. gambiae s.s.* and *An. arabiensis*

Lane 1: *An. gambiae* s.s. +ve control, Lanes 2-15: Test samples,

Lane 16: *An. arabiensis* +ve control

4.2 Distribution of the insecticide resistance mutations

4.2.1 East and West African *kdr* alleles

Frequency of the East African *kdr* (L1014S) alleles varied across the different study sites with Migori County recording a frequency of 15.2% while Kirinyaga and Baringo recorded a frequency of 0.51% and 1.6% respectively. The East African allele was not recorded in Tana River County. The L1014S allele frequencies also varied between species; *An. gambiae* s.s. (75%) and *An. arabiensis* (2.4%). The West African *kdr* (L1014F) allele was only recorded in Migori County at a frequency of 2.4%. **Table 9** is a summary of the frequencies of east and west *kdr* alleles for each sibling species across different study sites.

4.2.2 G119S (*ace-1*^{R})

Substrate DNA lambda was digested into multiple fragments. This confirmed that *Alu*I enzyme was active. G119S mutant alleles were not identified in any of the study sites since the 194bp amplification products were not digested by the *Alu*I enzyme. **Figure 13** is the resulting gel image of electrophoresis after PCR-RFLP assay.

4.3 Frequency of the 2La chromosomal inversion

An. gambiae s.s. in Baringo, Tana River and Migori counties recorded 2La inversion allele frequencies of 87% (n=30), 80% (n=10) and 52% (n=50) respectively. All *Anopheles arabiensis* that were randomly selected for screening were 100% (n=50) inverted for 2La/a inversion. **Figure 14** shows the diagnostic fragments of the 2La inversion as they appeared on the gel while **table 10** shows the frequencies at which the inversion occurred.

Table 9: Kdr allele frequencies of *An. gambiae* sibling species at different sites

Study site	**Species**	**n**	**Kdr-East**				**Kdr-West**			
			L.L	L.S	S.S	Frequency % (*Kdr*–e)	L.L	L.F	F.F	Frequency %(*Kdr*-w)
Migori	*An. arabiensis*	46	46	-	-	-	44	2	-	2.2%
	An. gambiae s.s.	16	5	1	10	65.6%	15	1	-	3.1%
	Total	**62**	**51**	**1**	**10**	**65.6%**	**59**	**3**	**-**	**5.3%**
Baringo	*An. arabiensis*	80	78	1	1	1.9%	80	-	-	-
	An. gambiae s.s.	16	14	1	1	9.4%	16	-	-	-
	Total	**96**	**92**	**2**	**2**	**11.3%**	**96**	**-**	**-**	**-**
Kirinyaga	*An. arabiensis*	98	97	1	-	0.5%	98	-	-	-
	An. gambiae s.s.	-	-	-	-	-	-	-	-	-
	Total	**98**	**97**	**1**	**-**	**0.5%**	**-**	**-**	**-**	**-**
Tana River	*An. arabiensis*	47	47	-	-	-	47	-	-	-
	An. gambiae s.s.	5	5	-	-	-	5	-	-	-
	Total	**52**	**52**	**-**	**-**	**-**	**52**	**-**	**-**	**-**
ALL SITES	*An. arabiensis*	271	268	2	1	2.4%	269	2	-	2.2%
	An. gambiae s.s.	37	24	2	11	75%	36	1	-	3.1%
	Total	**308**	**292**	**4**	**12**	**77.4%**	**305**	**3**		**5.3%**

KEY:
L.L- Leucine wild type
L.S- Leucine to serine heterozygous *kdr*-east mutant
S.S- Homozygous *kdr*-east mutant due to substitution of leucine with serine
L.F- Heterozygous *kdr*-west mutant due to substitution of leucine with phenylalanine
F.F-Leucine to phenylalanine homozygous *kdr*-west mutant
n -number of samples tested

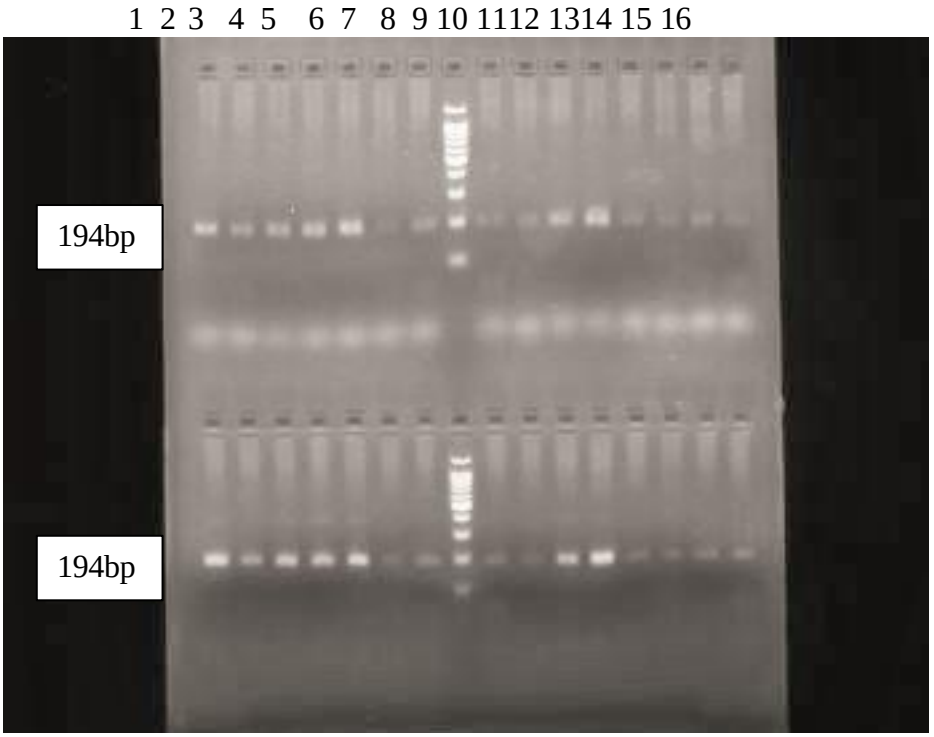

Figure 13: A gel image showing 194 bp DNA fragments after restriction digestion of PCR amplicons
Lanes 1-7 and 9-16 are test samples and lane 8 is a 100bp DNA ladder

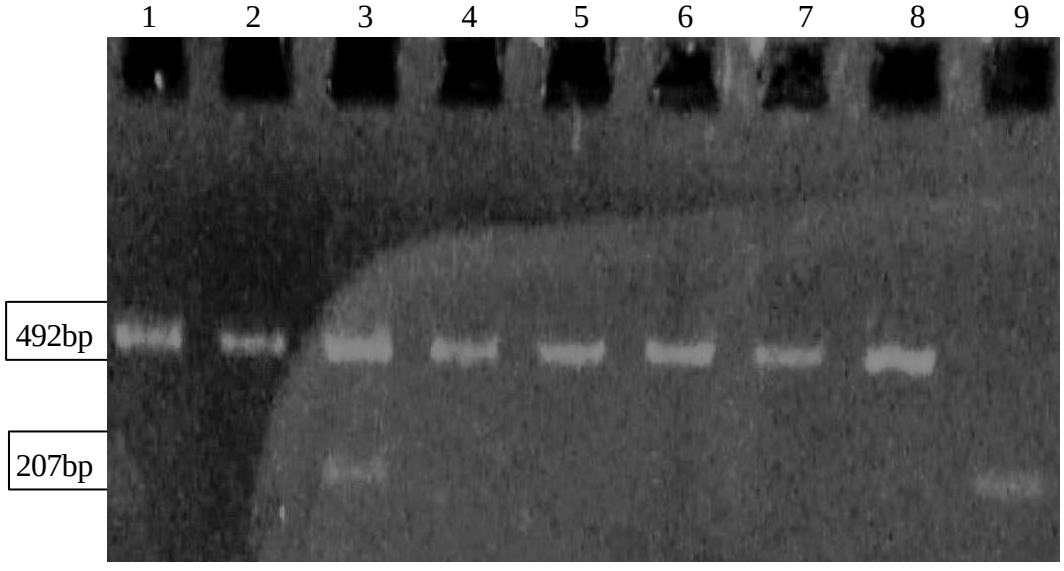

Figure 14: A gel image showing diagnostic fragments of inversion 2La in lanes 2-8 of test samples and positive controls of the inverted karyotype (492bp) and standard karyotype (207bp) in lanes 1 and 9 respectively.

Table 10: Frequencies of 2La inversion karyotypes

Study Site	No. specimens (n)	Total no. of alleles	No. Standard karyotype $2L+^a/2L+^a$	No. Hetero-karyotype $2La/2L+^a$	No. Inversion karyotype 2La/2La	%2La inversion allele
Baringo	15	30	2	0	13	87%
Tana River	5	10	1	0	4	80%
Migori	25	50	11	2	12	52%

4.4 Association between inversion polymorphism (2La) and knockdown resistance (*kdr*)

Chi-square tests used to seek association between the 2La inversion karyotype and knock down resistance revealed a significant association between the *kdr*-East mutation and the 2La inversion (Fisher's exact test statistic value, F=36.967, *P* =0.000; Likelihood Ratio=33.068, *P*=0.000).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

In this study *An. gambiae* s.l. sibling species from four malaria epidemiological zones in Kenya were screened for presence of the 2La chromosomal inversion and mutant alleles in the voltage gated sodium channel (*kdr*) and the Ache-1 catalytic site (*ace-1*^{R}) at codon 1014 and 119 respectively. While the 2La inversion is associated with mosquito resting behavior, *kdr* and *ace-1*^{R} mutations are associated with insecticide resistance. The study also aimed at establishing whether an association existed between the mutant alleles and the 2La inversion and thus infer whether mosquito resting behaviour is associated with insecticide resistance. In summary, results from this study showed absence of the *ace-1*^{R} mutation in the study populations and a variation in allele frequencies of *kdr* and inversion 2La across the different sites.

Identifying malaria vectors to species level is of critical importance in malaria vector control because species individually differ in terms of abundance, vector competence and host preference, factors which determine malaria transmission. Furthermore, data on vector species composition in sites of study is usually important as it helps vector control programmes implement control interventions that target the major vector. In Kirinyaga county only *An. arabiensis* was present which agrees with other studies that have reported it as the only species in existence (Kamau & Vulule, 2006). This sole existence is an evident case of competitive exclusion of other species. It is likely that this

species is less affected by pyrethroid treated nets commonly used in the county as it preferentially feeds on cattle and is less endophilic (Takken & Verhulst, 2013; Mayagaya *et al*., 2015). This gives it a competitive advantage over *An. gambiae* s.s. which is endophagic, anthropophagic and endophilic in nature and is thus thought to be potentially killed by LLINs (Day, 2005; Kitau *et al*., 2012).

An. gambiae s.s. and *An. arabiensis* were found to co-exist in Migori, Baringo and Tana River counties but at varying frequencies. Sympatric habitation of different species of a complex has previously been reported (Coetzee *et al*., 2000; Mathenge *et al*., 2001). The presence of the two *An. gambiae* s.l. sibling-species in Migori, Baringo and Tana River confirms their ecological adaptability and ability to reproduce in these sites. Coetzee *et al*., (2000) describes the two sibling species as the most efficient vectors of malaria. This study assumed that all *An. gambiae* s.s. were of the S molecular form since M form has not been reported in East Africa yet (Lehman *et al*., 2003). The sub-species of *An. gambiae* complex that is most commonly found in the western region of Kenya as opposed to other regions is *An. gambiae* s.s. (Mutuku *et al*., 2009; Okara *et al*., 2010; Wamae *et al*., 2010). This continues to be the case as is reflected in Migori County where it appeared more frequently. The frequent occurrence of *An. arabiensis* in Migori, Baringo, Tana River and Kirinyaga has been observed in previous studies by Ondiba *et al*., (2018); Muturi *et al*., (2008); Okara *et al*., (2010) and Mala *et al*., (2011) who describe this sibling species as a vector dominant to the arid, semi-arid and irrigated areas of Kenya. Poor DNA quality or extremely low DNA concentrations are

likely to have caused failure in amplification leading to lack of species identification in a small number of samples. It is also possible that the none-identified samples belonged to sibling species that are not targeted by the primers used in speciation. It is important to have this position confirmed through testing with relevant primers.

Kdr-e and *kdr*-w mutations occurred in both *An. gambiae* s.s. and *An. arabiensis* as is the case in other studies (Dabiré *et al*., 2014; Mathias *et al*., 2011; Verhaeghen *et al*., 2006) and is an indication that both of these species have received exposure to vector control interventions that induce insecticidal pressure which selects for knock-down resistance. The variation in *kdr* allele frequency in the two *An. gambiae* s.l. species and across the three study sites are likely due to differences in their behavioral attributes and differences in environmental characteristics such as pre-existing pesticide exposure, presence or absence of natural xenobiotics in breeding sites and differences in temperature (Stump *et al*., 2004). Vector populations that have been pre-exposed to pesticides may have resistance genes existing at low frequencies. Exposure of such populations to insecticides therefore increases prevalence of the resistance genes (Chouaïbou *et al*., 2016; Nkya *et al*., 2014). When present in breeding sites, natural xenobiotics are fed on by larvae. Once ingested, these compounds begin to affect mosquito metabolism by modulating their ability to tolerate insecticides and might even cross-select resistance mechanisms to pyrethroids (Nkya *et al*., 2013) thus causing a relative impact on the response of mosquitoes to pyrethroids (Kim and Muturi, 2012). A study by Ibrahim *et al*., (2021) demonstrated that extremely high

temperatures (44°C) are likely to harden larval and adult forms of mosquitoes which in turn facilitates insecticide resistance. An increased tendency to rest and feed outdoors is a behavioral attribute which enables disease vectors avoid contact with insecticides thus reducing the chances of selection for resistance alleles (Kawada *et al*., 2011; Russell *et al*., 2011).

While some studies have reported the occurrence of *kdr*-e in East Africa only (Matowo *et al*., 2015; Santolamazza *et al*., 2008) and *kdr*-w in West Africa only (Santolamazza *et al*., 2008), others have reported co-occurrence of both alleles in these regions (Dabiré *et al*., 2014; Ondeto *et al*., 2017). The latter is in agreement with this study which reports presence of *kdr*-w and *Kdr*-e in Migori County-East Africa. Kenya first documented the *kdr*-w mutation in 2012 (Ochomo *et al*., 2015), just a few years after it was reported in West Africa. Uganda (Verhaeghen et al., 2006), Tanzania (Kabula *et al*., 2014), Ethiopia (Yewhalaw *et al*., 2010) and Sudan (Yewhalaw *et al*., 2010) are other East African countries that have documented presence of the West African allele (*kdr*-w). The lack of confinement of the two mutations within the geographical regions where they were first reported suggests shifts in *kdr* allele frequencies in malaria endemic countries (Himeidan *et al*., 2007; Santolamaza *et al*., 2008). Although the *kdr*-w mutation detected in Migori County occurred at low frequencies, in concordance with previous studies (Kawada *et al*., 2011; Ochomo *et al*., 2015; Stump *et al*., 2004), its presence in East Africa is indicative of gene flow. However, it is important to note that its occurrence still remains predominant in West Africa (Diabate *et al*., 2004; Stump *et al*., 2004).

As opposed to previous studies by Chen *et al*., (2008), this study reports presence of *kdr-e* allele in Kirinyaga which is in agreement with a recent study by Mutunga, (2018). This finding gives the impression that *kdr-e* allele is spreading to areas where it was previously reported as missing.

The lack of detection of *kdr* alleles in Tana River is consistent with other studies that have reported absence of resistance in areas with existing pyrethroid-based interventions (Kamau & Vulule, 2006; Kamau *et al*., 2008; Stump *et al*., 2004) and could mean that insecticidal pressure from public health in the county has not buildup to an extent where it drives resistance genes. The nomadic lifestyle characterised by temporary house structures by some of the communities living in this region (Mutero, 2002) does not support constant deployment of insecticide treated nets. In addition, other ancient methods used to reduce mosquito bites such as burning cow dung and herbs are rarely used and may lack insecticidal properties (Muriuki, 2014). Again, the levels of use of pyrethroid and organochlorine insecticides for agricultural and domestic purposes are likely to be below what would select for naturally occurring resistance in the *Anopheles* species.

The low frequency of *kdr* in Baringo and Kirinyaga may be beneficial to malaria control as it suggests that a larger proportion of the vector population in these sites is still susceptible to pyrethroid and organochlorine insecticides. However the pyrethroids should be used judiciously guided by monitoring procedures to prevent increase in frequency of resistance alleles due to selective insecticide pressure. The occurrence of *kdr* mutation alleles in low

frequencies have similarly been reported in other non-irrigated and irrigated areas of Kenya (Kiuru *et al*., 2018; Orondo *et al*., 2021; Stump *et al*., 2004).

Kdr-e mutation observed at high frequency in Migori however, could be attributed to selection by historic intensive pyrethroid spray programs. Since the county falls under the stable malaria endemic classification, it remained a beneficiary of IRS programs with pyrethroids only from the year 2010 to 2012 under support by United States' Presidential Malaria Initiative (AIRS, 2017; MOH, 2016). The previous plus current heavy use of nets impregnated with pyrethroids could also be a contributing factor. In their study, Stump *et al*., (2004) observed that *kdr-e* allele frequencies in *An. gambiae* increased from 4% prior to ITN introduction to 8% following a large scale implementation of the permethrin-impregnated ITNs in Asembo western Kenya. In a different study in West Africa, Padonou *et al*., (2012) reported a significant increase in the frequency of *kdr* gene in the intervention area during the three years of IRS and ITNs scale up in Benin and a stable *kdr* gene frequency in the non-intervention control area. Elsewhere in Central Africa's Equatorial Guinea the *kdr* gene frequency increased from 0% before 2001 to 55.8% after 2001 following increased antimalarial efforts that involved intense application of pyrethroid aerosols from trucks and by hand (Berzosa *et al*., 2002; Reimer *et al*., 2005).

Frequencies of the *kdr* mutation will be expected to gradually increase and spread in field populations of mosquitoes for as long as pyrethroid pressure from IRS, bed nets, agricultural pesticides, industrial waste and detergents of

domestic use is present. This is likely to have a negative impact on the efficacy of malaria vector control tools that rely on insecticides from the pyrethroid class and other insecticides from a different class but with the same mode of action and on the reduction of malaria incidence. Loss of pyrethroid efficacy for example was observed when field populations of *Anopheles gambiae* s.l. in Guinea survived ten times the insecticidal concentration required to kill susceptible individuals (Collins *et al*., 2019). A malaria transmission dynamics model by Churcher *et al*., (2016) predicts that pyrethroid resistance would increase malaria incidence due to reduced mosquito mortality and lower overall community protection with bednets. Further, a study whose aim was to determine whether insecticide resistance was associated with loss of LLINs efficacy and an upsurge in malaria cases in Cameroon found out that pyrethroid treated bednets had lost their efficacy against field populations of *An. gambiae* where high *kdr* resistance was reported and that high malaria transmission was associated with high pyrethroid resistance rate (Ndjeunia-Mbiakop *et al*., 2025). Other studies have demonstrated that resistance mutations increase susceptibility of the resistant vector to *Plasmodium* infection which is likely to potentiate malaria transmission (Alout *et al*., 2014). Breaking the cycle of resistance due to *kdr* mutation and retaining the insecticidal activity of pyrethroid-based malaria vector control interventions is thus paramount and can be achieved through rotation in time and in space of insecticides with different modes of action or through their simultaneous use as mixtures (Chouaïbou *et al*., 2017).

This study reports the absence of *ace-1*^{R} mutation. The observed moderate phenotypic resistance to organophosphates and carbamates in the country

(Ngala *et al*., 2015; Wanjala & Kweka, 2018) could therefore be due to a metabolic resistance mechanism, such as overexpression of nonspecific esterases (NSE), Glutathione-S-transferase or elevation of detoxification enzymes (Adolfi *et al*., 2019; Ngangue-Siewe *et al*., 2022). Moreover, it is likely that there hasn't been a high enough buildup of insecticidal pressure from organophosphates and carbamates to drive *ace-1*^{R} mutation in the selected study sites. Thus, the absence of *ace-1*^{R} mutation in the study sites suggests that organophosphates and carbamates are viable alternatives to pyrethroids in IRS use.

The *ace-1*^{R} mutation has been reported in other parts of Africa such as in West Africa where it is widely spread (Dabiré *et al*., 2008; Dabiré *et al*., 2009; Djogbénou *et al*., 2008;2009;2011; N'Guessan *et al*.,2003), in Central Africa where it exists in low to high frequencies (Elanga-Ndille *et al*., 2019; Bandibabone *et al*., 2021) and in East Africa where its frequencies are low (Nsengimaana, 2022). Until 2019, the only case of *ace-1*^{R} mutation in a Kenyan population was in an *An. gambiae* strain from Kisumu coded AcerKis which was obtained in the laboratory through introgression of the mutation by over 19 generations of backcrosses between the insecticide susceptible and insecticide-resistant *An. gambiae* from Kisumu and Bobo-Dioulasso region of Burkina Faso respectively, followed by selection with propoxur (Djogbénou *et al*., 2007). More recent studies by Kitungulu *et al*., (2022), Owuor *et al*., (2021) and Orondo *et al*., (2021) have reported presence of *ace-1*^{R} mutation at low frequencies in Kakamega, Bungoma and Homabay counties respectively.

One of the components of natural malaria transmission system is the 2La inversion which should be appreciated because it influences vector behavior and *Plasmodium* susceptibility. This study found *Anopheles gambiae* s.s. to be polymorphic for the 2La inversion while *An. arabiensis* was fixed for the inversion, consistent with other studies (Matoke-Muhia *et al*., 2016; Sharakhov *et al*., 2006). Baringo and Tana River are characterised by arid conditions which seem to favor the 2La inversion as is the case in other studies (White *et al*., 2007; Wondji *et al*., 2005). In addition, the high prevalence of the 2La inversion arrangement in the two study sites compared to that of the standard and heterokaryotype arrangements implies that most of the *An. gambiae* s.s. mosquitoes are not exiting the targeted human dwelling space after blood feeding since this inversion is associated with indoor resting where there is a moisture deficit at night. The implication of this behavior in malaria vector control cannot be ignored. Vector control interventions in these two counties should be carefully chosen to target indoor spaces since the probability of contact with insecticides on them would be high. In the absence of resistance thereof, LLINs and IRS in Baringo and only LLINs in Tana River should be effective.

Understanding how inversions facilitate the spread of insecticide resistance alleles could have direct implications on the success of malaria control programs (Ayala *et al*., 2014). Associations between inversion polymorphisms and insecticide resistance have previously been documented in field populations of *An. coluzzii* from Nigeria and in a laboratory colony, resulting from *An. gambiae* collected from Nigeria, whereby loci within the

inversion region on the left arm of chromosome 2 were found to be associated with phenotypic dieldrin resistance (Adeogun *et al*., 2019; Brooke *et al*., 2000; Brooke *et al*., 2002). In a different study, association between the 2La inversion and phenotypic pyrethroid resistance was documented in *An. gambiae* s.l. (Ibrahim *et al*., 2021). Association of the inversion with a genetic mutation (A296S) conferring resistance to dieldrin is also implied by the mapping of this mutation onto a chromosomal position within the 2La inversion (Brooke *et al*., 2006; Du *et al*., 2005; Holt *et al*., 2002). Although the *kdr* mutation is not located within the 2La inversion region, we found a significant association between the *kdr-east* mutation and the 2La inversion associated with indoor resting. This suggests that *An. gambiae* s.s. mosquitoes harboring the *kdr* mutation still have a higher propensity to rest indoors suggesting that behavioural resistance may not be an important factor of concern in our study population.

5.2 Conclusions

i. *Anopheles arabiensis* has an ecological advantage over *Anopheles gambiae* s.s. in all the four study sites.

ii. While no *kdr* mutation was detected in Tana River suggesting that LLINs will remain effective, the mutation was found at high frequencies in Migori and Baringo counties and at a low frequency in Kirinyaga which highlights the need for Insecticide Resistance Management (IRM) strategies in the three study sites.

iii. With absence of the G119S allele, organophosphates and carbamates can be used for malaria vector control in the four study sites but alongside regular monitoring of the allele.

iv. Despite having insecticide treated nets and residual spray interventions in place, the study population continues to display persistent indoor resting behavior which implies that behavioral resistance by which vectors prefer to rest outdoors is not an important factor of concern in our study sites.

5.3 Recommendations

a) Recommendations from this study

i. Outdoor control interventions should be deployed in the four study sites to target the predominant *An. arabiensis* which has higher tendencies to rest outdoors as compared to the *An. gambiae* s.s.

ii. While Tana River should retain standard pyrethroid only LLINs, Migori and Baringo should implement use of the next generation LLIN-Interceptor G2 (contains a mixture of pyrethroid alpha-cypermethrin and a pyrrole chlorfenapyr) and further adopt IRS with SumiShield® (has a neonicotinoid clothianidin) as a combination tool with the Interceptor G2 LLIN and IRS with Fludora® Fusion (contains mixture of pyrethroid deltamethrin and a neonicotinoid clothianidin) as a preventive measure for disease outbreaks respectively. Kirinyaga on the other hand should introduce the next generation LLIN-Royal Guard (contains mixture of pyrethroid alpha-cypermethrin and an Insect Growth Regulator pyriproxyfen) together with PBO LLINs to prevent rapid increase in the frequency of *kdr*-east mutations.

iii. Organophosphate insecticides like Actellic® (contains pirimiphos-methyl) and carbamate insecticides like propoxur can be used as alternatives to pyrethroids and DDT in the four study sites.

iv. The indoor environment should continue to be targeted for malaria vector control in the four study sites. Where *kdr* mutation has been detected, non-pyrethroid based indoor control interventions should be deployed or the pyrethroid should be deployed as a mixture with an insecticide from a different class and with a different mode of action.

b) Recommendations for further study

i. There is need to carry out further screening for *kdr* mutants in Migori, Baringo and Kirinyaga counties to determine how levels of this marker of insecticide resistance are changing and its potential impact on vector control interventions.

ii. There is also need for continued insecticide resistance surveillance for *kdr* in Tana River and *ace-1*^{R} in all the four study sites to ensure that once resistance due to these mutations is detected, it is managed in a rational approach.

iii. There is need to determine whether other mechanisms of resistance other than G119S are responsible for the observed phenotypic resistance to organophosphates and carbamates in the country.

iv. The effectiveness of outdoor control interventions such as attractive toxic sugar baits, veterinary insecticides, eave tubes, insecticidal clothing and vapor emanators should be explored against malaria vectors that display more outdoor biting and resting activity.

REFERENCES

Adeogun, A. O., Brooke, B. D., Olayanju, D. R., Adegbehingbe, K., Oyeniyi, T. A., Olakiigbe, A. K., & Awolola, T. S. (2019). Test for association between dieldrin resistance and 2La inversion polymorphism in *Anopheles coluzzii* from Lagos, Nigeria. *Tropical Biomedicine*.

Adolfi, A., Poulton, B., Anthousi, A., Macilwee, S., Ranson, H., & Lycett, G. J. (2019). Functional genetic validation of key genes conferring insecticide resistance in the major African malaria vector, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1914633116

Ahmed, M. A. I., & Vogel, C. F. A. (2020). Toxicological evaluation of novel butenolide pesticide flupyradifurone against *culex quinquefasciatus* (Diptera: Culicidae) mosquitoes. *Journal of Medical Entomology*. https://doi.org/10.1093/jme/tjaa118

Aïzoun, N., Aïkpon, R., Gnanguenon, V., Oussou, O., Agossa, F., Padonou, G. G., & Akogbéto, M. (2013). Status of organophosphate and carbamate resistance in *Anopheles gambiae* sensu lato from the south and north Benin, West Africa. *Parasites and Vectors*. https://doi.org/10.1186/1756-3305-6-274

Alonso, David Bouma, Menno J Pascual, M. (2010). Epidemic malaria and warmer temperatures in recent decades in an East African highland. Proceedings of the Royal Society B-Biological Sciences. *The Royal Society*, *278*(1712), 1661–1669. https://doi.org/10.1098/rspb.2010.2020

Alout, H., Krajacich, B. J., Meyers, J. I., Grubaugh, N. D., Brackney, D. E., Kobylinski, K. C., Foy, B. D. (2014). Evaluation of ivermectin mass drug administration for malaria transmission control across different West African environments. *Malaria Journal*. https://doi.org/10.1186/1475-2875-13-417

Arthur, F. H. (2013). Dosage rate, temperature, and food source provisioning affect susceptibility of *Tribolium castaneum* and *Tribolium confusum* to chlorfenapyr. *Journal of Pest Science*. https://doi.org/10.1007/s10340-013-0495-9

Ayala, D., Ullastres, A., & González, J. (2014). Adaptation through chromosomal inversions in *Anopheles*. *Frontiers in Genetics*. https://doi.org/10.3389/fgene.2014.00129

Bandibabone, J., McLoughlin, C., N'Do, S., Bantuzeko, C., Byabushi, V., Jeanberckmans, M., Messenger, L. A. (2021). Investigating molecular mechanisms of insecticide resistance in the Eastern Democratic Republic of the Congo. *Malaria Journal*. https://doi.org/10.1186/s12936-021-04002-8

Bayoh, M. N., Mathias, D. K., Odiere, M. R., Mutuku, F. M., Kamau, L., Gimnig, J. E., Walker, E. D. (2010). *Anopheles gambiae*: Historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. *Malaria Journal*, *9*(1).

Boyce, R., Reyes, R., Matte, M., Ntaro, M., Mulogo, E., Metlay, J. P., Siedner, M. J. (2016). Severe Flooding and Malaria Transmission in the Western Ugandan Highlands: Implications for Disease Control in an Era of Global Climate Change. *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jiw363

Brooke, B. D., Hunt, R. H., Chandre, F., Carnevale, P., & Coetzee, M. (2002). Stable chromosomal inversion polymorphisms and insecticide resistance in the malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae). *Journal of Medical Entomology*, *39*(4), 568–573. https://doi.org/10.1603/0022-2585-39.4.568

Brooke, B. D., Hunt, R. H., & Coetzee, M. (2000). Resistance to dieldrin + fipronil assorts with chromosome inversion 2La in the malaria vector *Anopheles gambiae*. *Medical and Veterinary Entomology*. https://doi.org/10.1046/j.1365-2915.2000.00222.x

Brooke, B. D., Hunt, R. H., Matambo, T. S., Koekemoer, L. L., Van Wyk, P., & Coetzee, M. (2006). Dieldrin resistance in the malaria vector *Anopheles gambiae* in Ghana. *Medical and Veterinary Entomology*, *20*(3), 294–299. https://doi.org/10.1111/j.1365-2915.2006.00639.x

Brühl, C. A., Després, L., Frör, O., Patil, C. D., Poulin, B., Tetreau, G., & Allgeier, S. (2020). Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis subsp. israelensis* (Bti). *Science of the Total Environment*. https://doi.org/10.1016/j.scitotenv.2020.137800

Caminade, C., Kovats, S., Rocklov, J., Tompkins, A. M., Morse, A. P., Colón-González, F. J., … Lloyd, S. J. (2014). Impact of climate change on global malaria distribution. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1302089111

Chandler, J. A., Highton, R. B., & Hill, M. N. (1975). Mosquitoes of the Kano Plain, Kenya. I. Results of indoor collections in irrigated and nonirrigated areas using human bait and light traps. *Journal of Medical Entomology*. https://doi.org/10.1093/jmedent/12.5.504

Chouaïbou, M. S., Fodjo, B. K., Fokou, G., Allassane, O. F., Koudou, B. G., David, J. P., … Bonfoh, B. (2016). Influence of the agrochemicals used for rice and vegetable cultivation on insecticide resistance in malaria vectors in southern Côte d'Ivoire. *Malaria Journal*. https://doi.org/10.1186/s12936-016- 1481-5

Churcher, T. S., Lissenden, N., Griffin, J. T., Worrall, E., & Ranson, H. (2016). The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *ELife*. https://doi.org/10.7554/eLife.16090

Collins, Julianne S. Schwartz, C. E. (2002). Detecting Polymorphisms and Mutations in Candidate Genes. *The American Journal of Human Genetics*, *71*, 1251–1252. Retrieved from https://www.cell.com/ajhg/pdf/S0002-9297(07)60428-5.pdf

Collins, E., Vaselli, N. M., Sylla, M., Beavogui, A. H., Orsborne, J., Lawrence, G., … Messenger, L. A. (2019). The relationship between insecticide resistance, mosquito age and malaria prevalence in *Anopheles gambiae* s.l. from Guinea. *Scientific Reports*.

Corbel, V., Akogbeto, M., Damien, G. B., Djenontin, A., Chandre, F., Rogier, C., … Henry, M. C. (2012). Combination of malaria vector control interventions in pyrethroid resistance area in Benin: A cluster randomized controlled trial. *The Lancet Infectious Diseases*. https://doi.org/10.1016/S1473- 3099(12)70081-6

Dambach, P., Baernighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Louis, V. R. (2019). Reduction of malaria vector mosquitoes in a large- scale intervention trial in rural Burkina Faso using Bti based larval source management. *Malaria Journal*. https://doi.org/10.1186/s12936-019-2951-3

Dambach, P., Louis, V. R., Kaiser, A., Ouedraogo, S., Sié, A., Sauerborn, R., & Becker, N. (2014). Efficacy of *Bacillus thuringiensis* var. *israelensis* against malaria mosquitoes in northwestern Burkina Faso. *Parasites and Vectors*. https://doi.org/10.1186/1756-3305-7-371

Day, J. F. (2005). Host-seeking strategies of mosquito disease vectors. In *Journal of the American Mosquito Control Association*. https://doi.org/10.2987/8756-971x(2005)21[17:hsomdv]2.0.co;2

Degefa, T., Yewhalaw, D., Zhou, G., Lee, M., Atieli, H., Githeko, A. K., & Yan, G. (2017). Indoor and outdoor malaria vector surveillance in western Kenya : implications for better understanding of residual transmission. *Malaria Journal*, 1–13. https://doi.org/10.1186/s12936-017-2098-z

Derua, Y. A., Alifrangis, M., Hosea, K. M., Meyrowitsch, D. W., Magesa, S. M., Pedersen, E. M., & Simonsen, P. E. (2012). Change in composition of the *Anopheles gambiae* complex and its possible implications for the transmission of malaria and lymphatic filariasis in north-eastern Tanzania. *Malaria Journal*. https://doi.org/10.1186/1475-2875-11-188

Diouf, Ibrahima Adeola, Abiodun M. Abiodun, Gbenga J. Lennard, Christopher Shirinde, Joyce M. Gbobaniy, Pascal Yaka Jacques-André Ndione, Gbobaniy, E. O. (2021). Impact of future climate change on malaria in West Africa. Theoretical and Applied Climatology. *Theoretical and Applied Climatology*, 853–865.

Elanga-Ndille, E., Nouage, L., Ndo, C., Binyang, A., Assatse, T., Nguiffo-Nguete, D., … Wondji, C. S. (2019). The G119s acetylcholinesterase (Ace-1) target site mutation confers carbamate resistance in the major malaria vector *Anopheles gambiae* from cameroon: A challenge for the coming irs implementation. *Genes*, *10*(10). https://doi.org/10.3390/genes10100790

Ermert, V., Fink, A. H., & Paeth, H. (2013). The potential effects of climate change on malaria transmission in Africa using bias-corrected regionalised climate projections and a simple malaria seasonality model. *Climatic Change*. https://doi.org/10.1007/s10584-013-0851-z

Ferguson, Heather M. and Govella, N. J. (2024). Impacts of climate change on malaria vector control in Africa (pp. 387–421). https://doi.org/10.3920/9789004688650_015

Fillinger, U., Knols, B. G. J., & Becker, N. (2003). Efficacy and efficiency of new *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* formulations against Afrotropical Anophelines in Western Kenya. *Tropical Medicine and International Health*. https://doi.org/10.1046/j.1365- 3156.2003.00979.x

Fontenille, D., Lochouarn, L., Diagne, N., Sokhna, C., Lemasson, J. J., Diatta, M., … Trape, J. F. (1997). High annual and seasonal variations in malaria transmission by Anophelines and vector species composition in Dielmo, a holoendemic area in Senegal. *American Journal of Tropical Medicine and Hygiene*. https://doi.org/10.4269/ajtmh.1997.56.247

Gerken, A. R., Scully, E. D., Campbell, J. F., & Morrison, W. R. (2021). Effectiveness of long-lasting insecticide netting on *Tribolium castaneum* is modulated by multiple exposures, biotic, and abiotic factors. *Pest Management Science*. https://doi.org/10.1002/ps.6134

Goldberg, L.J. Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles-sergentii*, *Uranotaenia-unguiculata*, *Culex-univitattus*, *Aedes-aegypti* and *Culex-pipiens*. *Mosquito News*, *37*(3), 356–358. Retrieved from https://www.biodiversitylibrary.org/content/part/JAMCA/MN_V37_N3_P355- 358.pdf

Hemming-Schroeder, E., Strahl, S., Yang, E., Nguyen, A., Lo, E., Zhong, D., Yan, G. (2018). Emerging pyrethroid resistance among *Anopheles arabiensis* in Kenya. *American Journal of Tropical Medicine and Hygiene*, *98*(3), 704–709. https://doi.org/10.4269/ajtmh.17-0445

Holt, R. A., Mani Subramanian, G., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Hoffman, S. L. (2002). The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science*. https://doi.org/10.1126/science.1076181

Ibrahim, S. S., Mukhtar, M. M., Muhammad, A., & Wondji, C. S. (2021). 2La paracentric chromosomal inversion and overexpressed metabolic genes enhance thermotolerance and pyrethroid resistance in the major malaria vector *Anopheles gambiae*. *Biology*. https://doi.org/10.3390/biology10060518

Kabula, B., Kisinza, W., Tungu, P., Ndege, C., Batengana, B., Kollo, D., … Magesa, S. (2014). Co-occurrence and distribution of East (L1014S) and West (L1014F) African knock-down resistance in *Anopheles gambiae* sensu lato population of Tanzania. *Tropical Medicine and International Health*, *19*(3), 331–341. https://doi.org/10.1111/tmi.12248

Kamau, L., Agai, D., Matoke, D., Wachira, L., Gikandi, G., & Vulule, J. M. (2008). Status of insecticide susceptibility in *Anopheles gambiae* sensu lato and *Anopheles funestus* mosquitoes from Western Kenya. *Journal of Insect Science*, *8*. https://doi.org/10.1673/031.008.1101

Kamau, L., & Vulule, J. M. (2006). Status of insecticide susceptibility in *Anopheles arabiensis* from Mwea rice irrigation scheme, Central Kenya. *Malaria Journal*, *5*. https://doi.org/10.1186/1475-2875-5-46

Kawada, H., Futami, K., Komagata, O., Kasai, S., Tomita, T., Sonye, G., Takagi, M. (2011). Distribution of a knockdown resistance mutation (L1014S) in *Anopheles gambiae* s.s. and *Anopheles arabiensis* in Western and Southern Kenya. *PloS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0024323

Keating, J., Mbogo, C. M., Mwangangi, J., Nzovu, J. G., Gu, W., Regens, J. L., Beier, J. C. (2005). *Anopheles gambiae* s.l. and *Anopheles funestus* mosquito distributions at 30 villages along the Kenyan coast. *Journal of Medical Entomology*. https://doi.org/10.1093/jmedent/42.3.241

Kelly-Hope, L and Thomson, M. (n.d.). *Climate and infectious diseases. In: Climate, Climatic Change and Human Health Workshop* (Advances in Global Change Research.). Wengen, Switzerland.

Kenea, O., Balkew, M., Tekie, H., Gebre-Michael, T., Deressa, W., Loha, E., Overgaard, H. J. (2016). Human-biting activities of *Anopheles* species in south-central Ethiopia. *Parasites and Vectors*. https://doi.org/10.1186/s13071- 016-1813-x

Kitau, J., Oxborough, R. M., Tungu, P. K., Matowo, J., Malima, R. C., Magesa, S. M., Rowland, M. W. (2012). Species shifts in the *Anopheles gambiae* complex: Do LLINs successfully control *Anopheles arabiensis*? *PLoS ONE*. https://doi.org/10.1371/journal.pone.0031481

Kitungulu, N., Guyah, B., Webale, M., Shaviya, N., Machani, M., Mulama, D., & Ndenga, B. (2022). Resistance of *Anopheles gambiae* sensu lato to Pirimiphos-methyl Insecticide in Kakamega County, Highlands of Western Kenya. *African Health Sciences*. https://doi.org/10.4314/ahs.v22i1.68

Kiuru, C. W., Oyieke, F. A., Mukabana, W. R., Mwangangi, J., Kamau, L., & Muhia-Matoke, D. (2018). Status of insecticide resistance in malaria vectors in Kwale County, Coastal Kenya. *Malaria Journal*, *17*(1). https://doi.org/10.1186/s12936-017-2156-6

Leedale, J., Tompkins, A. M., Caminade, C., Jones, A. E., Nikulin, G., & Morse, A. P. (2016). Projecting malaria hazard from climate change in eastern Africa using large ensembles to estimate uncertainty. *Geospatial Health*. https://doi.org/10.4081/gh.2016.393

Lees, R. S., Ambrose, P., Williams, J., Morgan, J., Praulins, G., Ingham, V. A., Malone, D. (2020). Tenebenal: a meta-diamide with potential for use as a novel mode of action insecticide for public health. *Malaria Journal*. https://doi.org/10.1186/s12936-020-03466-4

Mabaso, M. L. H., Craig, M., Ross, A., & Smith, T. (2007). Environmental predictors of the seasonality of malaria transmission in Africa: The challenge. *American Journal of Tropical Medicine and Hygiene*. https://doi.org/10.4269/ajtmh.2007.76.33

Machani, M. G., Ochomo, E., Amimo, F., Kosgei, J., Munga, S., Zhou, G., Afrane, Y. A. (2020). Resting behaviour of malaria vectors in highland and lowland sites of western Kenya: Implication on malaria vector control measures. *PLoS ONE*, *15*(2). https://doi.org/10.1371/journal.pone.0224718

Mala, A. O., Irungu, L. W., Shililu, J. I., Muturi, E. J., Mbogo, C. M., Njagi, J. K., Githure, J. I. (2011). *Plasmodium falciparum* transmission and aridity: A Kenyan experience from the dry lands of Baringo and its implications for Anopheles arabiensis control. *Malaria Journal*. https://doi.org/10.1186/1475- 2875-10-121

Mathenge, E. M., Gimnig, J. E., Kolczak, M., Ombok, M., Irungu, L. W., & Hawley, W. A. (2001). Effect of permethrin-impregnated nets on exiting behavior, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in western Kenya. *Journal of Medical Entomology*, *38*(4), 531–536. https://doi.org/10.1603/0022-2585-38.4.531

Mathias, D. K., Ochomo, E., Atieli, F., Ombok, M., Nabie Bayoh, M., Olang, G., Gimnig, J. E. (2011). Spatial and temporal variation in the kdr allele L1014S in *Anopheles gambiae* s.s. and phenotypic variability in susceptibility to insecticides in Western Kenya. *Malaria Journal*, *10*. https://doi.org/10.1186/1475-2875-10-10

Mawejje, H. D., Kilama, M., Kigozi, S. P., Musiime, A. K., Kamya, M., Lines, J., … Staedke, S. G. (2021). Impact of seasonality and malaria control interventions on *Anopheles* density and species composition from three areas of Uganda with differing malaria endemicity. *Malaria Journal*. https://doi.org/10.1186/s12936-021-03675-5

Mayagaya, V. S., Nkwengulila, G., Lyimo, I. N., Kihonda, J., Mtambala, H., Ngonyani, H., … Ferguson, H. M. (2015). The impact of livestock on the abundance, resting behaviour and sporozoite rate of malaria vectors in southern Tanzania. *Malaria Journal*.

Mbogo, C. M., Mwangangi, J. M., Nzovu, J. G., Gu, W., Yan, G., Gunter, J. T., Beier, J. C. (2003). Spatial and temporal heterogeneity of *Anopheles* mosquitoes and *Plasmodium falciparum* transmission along the Kenyan coast. *American Journal of Tropical Medicine and Hygiene*. https://doi.org/10.4269/ajtmh.2003.68.734

Meyrowitsch, D. W., Pedersen, E. M., Alifrangis, M., Scheike, T. H., Malecela, M. N., Magesa, S. M., … Simonsen, P. E. (2011). Is the current decline in malaria burden in sub-Saharan Africa due to a decrease in vector population? *Malaria Journal*. https://doi.org/10.1186/1475-2875-10-188

MOH. (2020). Kenya Malaria Indicator Survey 2020.

Msami, J. E. (2013). *Monitoring Insecticide Resistance among Malaria Vectors in Coastal Kenya*. Retrieved from https://erepository.uonbi.ac.ke/bitstream/handle/11295/52382/Msami_Monitoring Insecticide Resistance among Malaria Vectors in Coastal Kenya.pdf?sequence=3

Msugupakulya, B. J., Urio, N. H., Jumanne, M., Ngowo, H. S., Selvaraj, P., Okumu, F. O., & Wilson, A. L. (2023). Changes in contributions of different *Anopheles* vector species to malaria transmission in east and southern Africa from 2000 to 2022. *Parasites and Vectors*. https://doi.org/10.1186/s13071-023- 06019-1

Munywoki, D. N., Kokwaro, E. D., Mwangangi, J. M., Muturi, E. J., & Mbogo, C. M. (2021). Insecticide resistance status in *Anopheles gambiae* (s.l.) in coastal Kenya. *Parasites and Vectors*. https://doi.org/10.1186/s13071-021- 04706-5

Murdock, C. C., Sternberg, E. D., & Thomas, M. B. (2016). Malaria transmission potential could be reduced with current and future climate change. *Scientific Reports*. https://doi.org/10.1038/srep27771

Mutuku, F. M., King, C. H., Mungai, P., Mbogo, C., Mwangangi, J., Muchiri, E. M., … Kitron, U. (2011). Impact of insecticide-treated bed nets on malaria transmission indices on the south coast of Kenya. *Malaria Journal*, *10*. https://doi.org/10.1186/1475-2875-10-356

Mwangangi, J. M., Mbogo, C. M., Orindi, B. O., Muturi, E. J., Midega, J. T., Nzovu, J., … Beier, J. C. (2013). Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. *Malaria Journal*, *12*(1). https://doi.org/10.1186/1475-2875-12-13

Nartey, R., Owusu-Dabo, E., Kruppa, T., Baffour-Awuah, S., Annan, A., Oppong, S., … Obiri-Danso, K. (2013). Use of *Bacillus thuringiensis* var *israelensis* as a viable option in an Integrated Malaria Vector Control Programme in the Kumasi Metropolis, Ghana. *Parasites and Vectors*. https://doi.org/10.1186/1756-3305-6-116

Ngala, J., Kamau, L., Mireji, P., Mburu, J., & Mbogo, C. (2015). Insecticide resistance, Host preference and *Plasmodium falciparum* parasite rates in *Anopheles* mosquitoes in Mwea and Ahero rice schemes. *Journal of Mosquito Research*. https://doi.org/10.5376/jmr.2015.05.0014

Ngangue-Siewe, I. N., Ndjeunia-Mbiakop, P., Kala-Chouakeu, N. A., Bamou, R., Talipouo, A., Djamouko-Djonkam, L., … Antonio-Nkondjio, C. (2022). Bendiocarb and Malathion Resistance in Two Major Malaria Vector Populations in Cameroon Is Associated with High Frequency of the G119S Mutation (Ace-1) and Overexpression of Detoxification Genes. *Pathogens*. https://doi.org/10.3390/pathogens11080824

Nkya, T. E., Akhouayri, I., Poupardin, R., Batengana, B., Mosha, F., Magesa, S., … David, J. P. (2014). Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: A case study in Tanzania. *Malaria Journal*. https://doi.org/10.1186/1475- 2875-13-28

Nsengimaana, B. (2022). Phenotypic and genetic susceptibility patterns of *Anopheles gambiae* s.l. mosquitoes to carbamates in selected districts of Uganda. Retrieved from https://www.researchgate.net/publication/376358430

Ochomo, E., Subramaniam, K., Kemei, B., Rippon, E., Bayoh, N. M., Kamau, L., … Mbogo, C. (2015). Presence of the knockdown resistance mutation, Vgsc-1014F in *Anopheles gambiae* and *An. arabiensis* in western Kenya. *Parasites and Vectors*, *8*(1). https://doi.org/10.1186/s13071-015-1223-5

Odero, J. I., Abong'o, B., Moshi, V., Ekodir, S., Harvey, S. A., Ochomo, E., Monroe, A. (2024). Early morning Anopheline mosquito biting, a potential driver of malaria transmission in Busia County, western Kenya. *Malaria Journal*. https://doi.org/10.1186/s12936-024-04893-3

Ondeto, B. M., Nyundo, C., Kamau, L., Muriu, S. M., Mwangangi, J. M., Njagi, K., … Mbogo, C. M. (2017). Current status of insecticide resistance among malaria vectors in Kenya. *Parasites and Vectors*, *10*(1). https://doi.org/10.1186/s13071-017-2361-8

Orondo, P. W., Nyanjom, S. G., Atieli, H., Githure, J., Ondeto, B. M., Ochwedo, K. O., …Yan, G. (2021). Insecticide resistance status of *Anopheles arabiensis* in irrigated and non-irrigated areas in western Kenya. *Parasites and Vectors*.https://doi.org/10.1186/s13071-021-04833-z

Owuor, K. O., Machani, M. G., Mukabana, W. R., Munga, S. O., Yan, G., Ochomo, E., & Afrane, Y. A. (2021). Insecticide resistance status of indoor and outdoor resting malaria vectors in a highland and lowland site in Western Kenya. *PLoSONE*. https://doi.org/10.1371/journal.pone.0240771

Oxborough, R. M., Seyoum, A., Yihdego, Y., Dabire, R., Gnanguenon, V., Wat'Senga, F., … Dengela, D. (2019). Susceptibility testing of *Anopheles* malaria vectors with the neonicotinoid insecticide clothianidin; Results from 16 African countries, in preparation for indoor residual spraying with new insecticide formulations. *Malaria Journal*. https://doi.org/10.1186/s12936-019- 2888-6

P J Berzosa, J Cano, J Roche, J M Rubio, L García, E Moyano, A Guerra, J C Mateos, V Petrarca, V Do Rosario, A. B. (2002). Malaria vectors in Bioko Island (Equatorial Guinea): PCR determination of the members of *Anopheles gambiae* Giles complex (Diptera: Culicidae) and pyrethroid knockdown resistance (kdr) in *An. gambiae* sensu stricto. *Vector Ecology*. Retrieved from https://pubmed.ncbi.nlm.nih.gov/12125862/

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology*. https://doi.org/10.1111/gcb.12240

Padonou, G. G., Sezonlin, M., Ossé, R., Aizoun, N., Oké-Agbo, F., Oussou, O., Akogbéto, M. (2012). Impact of three years of large scale Indoor Residual Spraying (IRS) and Insecticide Treated Nets (ITNs) interventions on insecticide resistance in *Anopheles gambiae* s.l. in Benin. *Parasites and Vectors*. https://doi.org/10.1186/1756-3305-5-72

Parham, P. E., Pople, D., Christiansen-Jucht, C., Lindsay, S., Hinsley, W., & Michael, E. (2012). Modeling the role of environmental variables on the population dynamics of the malaria vector *Anopheles gambiae* sensu stricto. *Malaria Journal*. https://doi.org/10.1186/1475-2875-11-271

Paris, M., David, J. P., & Despres, L. (2011). Fitness costs of resistance to Bti toxins in the dengue vector *Aedes aegypti*. *Ecotoxicology*. https://doi.org/10.1007/s10646-011-0663-8

Pates, H., & Curtis, C. (2005). Mosquito behavior and vector control. *Annual Review.of.Entomology*.https://doi.org/10.1146/annurev.ento.50.071803.130439

Paulette Ndjeunia-Mbiakop, Idriss Nasser Ngangue-Siewe, Abdou Talipouo, Arnold Princewil Chi Nji, Roland Bamou, Joel Djoufounna, Jeannette Tombi, C. A.-N. (2025). Malaria transmission, insecticide resistance and efficacy of insecticide-treated nets in Belabo and Ouami, two localities of the East Region of Cameroon. *Current Research in Parasitology & Vector-Borne Diseases*,*7*. Retrieved from https://doi.org/10.1016/j.crpvbd.2025.100266

Ranabhat, S., & Wang, C. (2020). Effect of Moisture on Efficacy of Selected Insecticide Dusts against the Common Bed Bug, *Cimex lectularius* (Hemiptera: Cimicidae). *Journal of Economic Entomology*. https://doi.org/10.1093/jee/toaa122

Reddy, M. R., Overgaard, H. J., Abaga, S., Reddy, V. P., Caccone, A., Kiszewski, A. E., & Slotman, M. A. (2011). Outdoor host seeking behaviour of *Anopheles gambiae* mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea. *Malaria Journal*. https://doi.org/10.1186/1475-2875-10-184

Reimer, L. J., Tripet, F., Slotman, M., Spielman, A., Fondjo, E., & Lanzaro, G. C. (2005). An unusual distribution of the kdr gene among populations of *Anopheles gambiae* on the island of Bioko, Equatorial Guinea. *Insect Molecular Biology*. https://doi.org/10.1111/j.1365-2583.2005.00599.x

Reiner, R. C., Geary, M., Atkinson, P. M., Smith, D. L., & Gething, P. W. (2015). Seasonality of *Plasmodium falciparum* transmission: A systematic review. *Malaria Journal*. https://doi.org/10.1186/s12936-015-0849-2

Rukmini, V., Reddy, C. Y., & Venkateswerlu, G. (2000). *Bacillus thuringiensis* crystal δ-endotoxin: Role of proteases in the conversion of protoxin to toxin. *Biochimie*. https://doi.org/10.1016/S0300-9084(00)00355-2

Russell, T. L., Govella, N. J., Azizi, S., Drakeley, C. J., Kachur, S. P., & Killeen, G. F. (2011). Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria Journal*, *10*.

Russell, T. L., Lwetoijera, D. W., Maliti, D., Chipwaza, B., Kihonda, J., Charlwood, J. D., … Killeen, G. F. (2010). Impact of promoting longer-lasting insecticide treatment of bed nets upon malaria transmission in a rural Tanzanian setting with pre-existing high coverage of untreated nets. *Malaria Journal*, *9*(1). https://doi.org/10.1186/1475-2875-9-187

Ruybal-Pesántez, S., McCann, K., Vibin, J., Siegel, S., Auburn, S., & Barry, A. E. (2024). Molecular markers for malaria genetic epidemiology: progress and pitfalls. *Trends in Parasitology*. https://doi.org/10.1016/j.pt.2023.11.006

Saleh, M. S., El-Meniawi, F. A., Kelada, N. L., & Zahran, H. M. (2003). Resistance development in mosquito larvae *Culex pipiens* to the bacterial agent *Bacillus thuringiensis* var. *israelensis*. *Journal of Applied Entomology*. https://doi.org/10.1046/j.1439-0418.2003.00703.x

Sangbakembi-Ngounou, C., Costantini, C., Longo-Pendy, N. M., Ngoagouni, C., Akone-Ella, O., Rahola, N., … Ayala, D. (2022). Diurnal biting of malaria mosquitoes in the Central African Republic indicates residual transmission may be "out of control." *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.2104282119

Schäfer, M. L., & Lundström, J. O. (2014). Efficiency of Bti-based floodwater mosquito control in Sweden – four examples. *Journal of the European Mosquito Control Association*.

Sherrard-Smith, E., Skarp, J. E., Beale, A. D., Fornadel, C., Norris, L. C., Moore, S. J., … Churcher, T. S. (2019). Mosquito feeding behavior and how it influences residual malaria transmission across Africa. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1820646116

Sokhna, C., Ndiath, M. O., & Rogier, C. (2013). The changes in mosquito vector behaviour and the emerging resistance to insecticides will challenge the decline of malaria. *Clinical Microbiology and Infection*. https://doi.org/10.1111/1469-0691.12314

Stump, A. D., Atieli, F. K., Vulule, J. M., & Besansky, N. J. (2004). Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene*, *70*(6), 591–596. https://doi.org/10.4269/ajtmh.2004.70.591

Takken, W. (2002). Do insecticide-treated bednets have an effect on malaria vectors? *Tropical Medicine and International Health*. https://doi.org/10.1046/j.1365-3156.2002.00983.x

Takken, W., & Verhulst, N. O. (2013). Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*. https://doi.org/10.1146/annurev- ento-120811-153618

The Global Fund. (2025). List of Indoor Residual Sprays (IRS) that Meet GF QA Requirements for Use Against Malaria Vector. Retrieved from https://www.theglobalfund.org/media/utjkftpy/psm_indoorresidualsprayirsgf_list_en.pdf

Tomizawa, M., & Casida, J. E. (2005). Neonicotinoid insecticide toxicology: Mechanisms of selective action. *Annual Review of Pharmacology and Toxicology*. https://doi.org/10.1146/annurev.pharmtox.45.120403.095930

Trape, J. F., Rogier, C., Konate, L., Diagne, N., Bouganali, H., Canque, B., Da Silva, L. P. (1994). The Dielmo project: A longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. *American Journal of Tropical Medicine and Hygiene*.

USAID and PMI. (2018). Kenya End of Spray Report: February 12-March 24 2018. Rockville, MD. The PMI Africa IRS (AIRS) Project, Abt Associates Inc.

Vachon, V., Laprade, R., & Schwartz, J. L. (2012). Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. *Journal of Invertebrate Pathology*. https://doi.org/10.1016/j.jip.2012.05.001

W. Du, T. S. Awolola, P. Howell, L. L. Koekemoer, B. D. Brooke, M. Q. Benedict, M. Coetzee, A., & Zheng, L. (2005). Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *Anopheles arabiensis*. *Insect Molecular Biology*, *14*(2), 179–183. https://doi.org/10.1111/j.1365-2583.2005.00544.x

Wamae, P. M., Githeko, A. K., Otieno, G. O., Kabiru, E. W., & Duombia, S. O. (2015). Early biting of the *Anopheles gambiae* s.s. and its challenges to vector control using insecticide treated nets in western Kenya highlands. *Acta Tropica*, *150*, 136–142. https://doi.org/10.1016/j.actatropica.2015.07.008

Wanjala, Christine L., Mbugi, J. P., Ototo, E., Gesuge, M., Afrane, Y. A., Atieli, H. E., … Yan, G. (2015). Pyrethroid and DDT resistance and organophosphate susceptibility among anopheles spp. Mosquitoes, western Kenya. *Emerging Infectious Diseases*, *21*(12), 2178–2181. https://doi.org/10.3201/eid2112.150814

Wanjala, Christine Ludwin, & Kweka, E. J. (2018). Malaria Vectors Insecticides Resistance in Different Agroecosystems in Western Kenya. *Frontiers in Public Health*, *6*(March). https://doi.org/10.3389/fpubh.2018.00055

Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M., & Raymond, M. (2004). The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Molecular Biology*, *13*(1), 1–7. https://doi.org/10.1111/j.1365-2583.2004.00452.x

WHO. (2013). *Handbook for integrated vector management*. *Outlooks on Pest Management*.

WHO. (2016). A toolkit for integrated vector management in sub-Saharan Africa WHO Library Cataloguing-in-Publication Data A toolkit for integrated vector management in sub-Saharan Africa. *WHO*.

WHO. (2021). World Malaria Report 2021.

World Health Organization. (2020). *World Malaria Report: 20 years of global progress and challenges*. *World Health Organization*.

World Health Organization. (2022). Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. *Organização Mundial Da Saúde*.

Yamana, T. K., Bomblies, A., & Eltahir, E. A. B. (2016). Climate change unlikely to increase malaria burden in West Africa. *Nature Climate Change*. https://doi.org/10.1038/nclimate3085

Zhou, G., Afrane, Y. A., Vardo-Zalik, A. M., Atieli, H., Zhong, D., Wamae, P., … Yan, G. (2011). Changing patterns of malaria epidemiology between 2002 and 2010 in western kenya: The fall and rise of malaria. *PLoS ONE*, *6*(5). https://doi.org/10.1371/journal.pone.0020318

APPENDICES

Appendix I: Grinding buffer

a) 100ml homogenizing buffer at PH 8.0

Reagent	**Weight (g)**
0.1M Sodium chloride	0.59
0.2M Sucrose	6.84g
0.1M EDTA	0.37g
0.03M Trizma base	0.36g
Add 100ml sterile water and stir	

b) 100ml lysis buffer at PH 9.2

Reagent	**Weight (g)**
0.25M EDTA	9.28g
1.88g 2.5% w/v SDS	1.88g
6.03g 0.5M Trizma base	6.03g
Add 100ml sterile water and stir	

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

Appendix II: TBE buffer

Reagent	**Weight (g)**
Boric acid	5.5
Trizma base	10.8
EDTA	0.93
Add 1L distilled water and stir	

Appendix III: Conventional PCR assay laboratory data sheet

LABORATORY DATA SHEET

USER NAME_____
DATE_____
PURPOSE:_____

PROGRAM Name: _____

PCR Cycle: Denature- _____; Anneal-____; Extend -_____Auto extend-_____
No. of Cycles: _____

	1X	**X_____**
dH_2O		
5X /10X PCR BUFFER		
DNTPS		
$MGCL_2$		
PRIMERS		
BSA		
TAQ POLYMERASE		
DNA TEMPLATE		

Lane 1		**Lane 2**		**Lane 3**		**Lane 4**	
Specimen ID	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**
1.		1.		1.		1.	
2.		2.		2.		2.	
3.		3.		3.		3.	

Appendix IV: RT-PCR data sheet

Mosquito ID	***KDR* East**		***KDR* West**	
	RT- PCR Date	***KDR* result**	**RT- PCR Date**	***KDR* result**