IMPACT OF USING LONG LASTING INSECTICIDAL TREATED NETs (LLIN) AND INDOOR RESIDUAL SPRAYING (IRS), SINGLE AND IN COMBINATION ON INSECTICIDE RESISTANT *Anopheles arabiensis* PATTON (DIPTERA: CULICIDAE) IN REPUBLIC OF THE SUDAN

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by

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LIST OF ACRONYMS AND ABBREVATIONS

µL	microliter
AChE	Acetylcholinesterase
BHC	benzene hexachloride
bp	base pair
DDT	diethyldiphenyl trichloroethane
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleic triphosphate
EDTA	Etheylenediamine tetracetic acid
GABA	gamma –aminobutyric acid
GEF	Global Environment Facility
GMP	Global Malaria Programme
IRS	Indoor Residual Spraying
kb	kilo base
kdr	knockdown resistance
kdr-east	East African *kdr* mutation
kdr-west	West African *kdr* mutation
LLIN	Long-lasting insecticidal treated nets
M	Molar
MgCl2	magnesium chloride
ml	millilitre
mM	millimole
MO	monooxygenase
NaCL2	sodium chloride
NMCP	National Malaria Control Programme
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
RBM	Roll Back Malaria
rpm	revolutions per minute
SMS	Sudan Metrological Services
Taq	*Thermus aquaticas*

KESAN MENGGUNAKAN KOMBINASI ALAT KAWALAN VEKTOR LLIN-IRS DALAM RINTANGAN RACUN SERANGGA *Anopheles arabiensis* PATTON (DIPTERA: CULICIDAE) DI SUDAN

ABSTRAK

Pengawalan vektor malaria di Sudan bergantung kepada penggunaan kelambu yang diimpregnasikan dengan insektisid yang tahan lama (LLINs) dan penyemburan residu dalam rumah (IRS) di kawasan perumahan. Kajian ini dijalankan di 140 kluster (kampung) di Sudan untuk memantau daya rintangan *Anopheles arabiensis* (Patton) terhadap racun serangga. Kesemua 140 kluster telah menggunakan LLIN dan sebahagian daripadanya (70 kluster) mendapat penambahan rawatan IRS dengan campuran bendiocarb dua kali setahun selama empat tahun . Kajian ini adalah sebahagian daripada percubaan bebrapa negara secara dua hala berkelompok besar dan rawak oleh WHO di Sudan untuk mengukur kebersanan gabungan LLIN dan IRS dalam mengenalpasti hubungan kerintangan racun serangga dan mekanisma yang terlibat.Garis panduan bioesei daripada WHO telah digunakan untuk menentukan status daya rintangan *An. arabiensis*terhadap deltamethrin, DDT dan bendiocarb. Komplek spesies *Anopheles* telah dikenalpasti melalui proses rDNA-base, *kdr* dan ACHE telah disaring oleh SNP genotyping dengan menggunakan TaqMan assay (RT-PCR). *Anopheles arabiensis* (93.4%, n = 2411/2580) merupakan ahli tunggal daripada komplek *Anopheles gambiae* (Giles) yang terdapat dikesemua kawasan dan populasi daripada semua kawasan menunjukkan species ini rentan kepada bendiocarb, tetapi rintang kepada deltamethrin dan DDT. Kerintangan terhadap deltamethrin adalah tinggi di New Halfa (kematian= 55.6, 95% CI : 50.2-61.1, $p<0.05$). Terdapat peningkatan yang

ketara pada tahap kerintangan dalam tempoh empat tahun (p<0.001), dengan penurunan purata peratusan kematian terhadap deltamethrin daripada 81.0% (95% CI, 77.6-84.3) pada tahun 2011 kepada 47.7% (95% CI : 43.5- 51.8) pada tahun 2014. Kerintangan DDT tertinggi direkodkan di New Halfa (61.7%, 95% CI: 56.8-66.7), namun, tiada trend yang konsisten *kdr*-west telah dikesan di sepanjang tempoh kajian dijalankan di semua kawasan dan kekerapannya adalah tinggi di Galabat (0.487, 95% CI: 0.469-0.504) dan di New Halfa (0.382, 95% CI: 0.367-0.396). Terdapat penurunan ketara dalam trend kekerapan *kdr* daripada 0.318, (95% CI; 0.296- 0.341) pada tahun 2010 kepada 0.250, (95% CI: 0.227 - 0.272) pada tahun 2014. Tiada bukti *ace-1R* dalam semua sample yang telah diuji (n=2590). Hubungan diantara kematian menggunakan deltamethrin dan kekerapan *kdr*-west pada tahap populasi adalah negatif (r = 0.54, p<0.0001). Walaupun terdapat peningkatan dalam rintangan fenotip deltamethrin di dalam kedua-dua penglibatan, tetapi masa yang diambil untuk menghasilkan rintangan adalah lebih lama (p= 0.023) dengan 2.1% % (95% CI: 0.25 - 3.9) menggunakan gabungan LLIN dan IRS (7.3% setiap tahun) berbanding menggunakan hanya LLIN (9.3% setiap tahun), tetapi gabungan rawatan tidak membantut kerintangan *kdr* (p = 0.345). . Oleh itu, kajian ini mencadangkan frekuensi *kdr* boleh digunakan untuk meramalkan kematian kerentanan nyamuk. Penemuan yang paling penting daripada kajian ini adalah menggabungkan intervensi untuk memperlahankan pembentukan kerintangan. Kesimpulannyarintangan terhadap racun serangga dan mekanisma terhadap populasi *An. arabiensis*di Sudan telah didokumentasikan. Gabungan rawatan IRS dengan campuran bendiocarb dan LLIN telah membuktikan bahawa ia mampu melambatkan kerintangan pyrethroid dengan ketara apabila dibandingkan dengan menggunakan LLIN sahaja. Oleh itu, kombinasi kawalan vektor haruslah digunapakai jika sumber boleh diperolehi. Kajian

ini juga menekankan tentang pentingnya sistem pemantauan kerintangan racun serangga sebagai sebahagian daripada program kawalan malaria supaya strategi pengurusan kerintangan racun serangga boleh dilaksanakan.

IMPACT OF USING LONG LASTING INSECTICIDAL TREATED NETs (LLIN) AND INDOOR RESIDUAL SPRAYING (IRS) SINGLE AND IN COMBINATION ON INSECTICIDE RESISTANT *Anopheles arabiensis* PATTON (DIPTERA: CULICIDAE) IN REPUBLIC OF THE SUDAN

ABSTRACT

Malaria vector control in Sudan depends on the deployment of long-lasting insecticidal treated nets (LLINs) and indoor residual spraying (IRS) in the domestic environment. This study was designed to monitor insecticide resistance in *Anopheles arabiensis* (Patton) from 140 clusters (villages) in Sudan. All 140 clusters received LLIN, while half (70 clusters) had the addition of IRS (with bendiocarb) in campaigns of two rounds/year for 4 years. This work is part of a WHO-coordinated multi-country cluster randomized trial, which in Sudan measured the effectiveness of a combination of LLIN+IRS in relation to resistance profiles of local vectors, and how these interventions may have affected the evolution of insecticide resistance. Standard WHO bioassay test kits were used to determine the susceptibility status of *An. arabiensis* to deltamethrin, DDT and bendiocarb. *Anopheles* species complex was identified by rDNA-base, while *kdr* and ACHE were screened by SNP genotyping using Real-time TaqMan assay (RT-PCR). *Anopheles arabiensis* (93.4%, n= 2411/2580) was the sole member of the *Anopheles gambiae* (Giles) complex present in all study areas. The populations from all areas were resistant to deltamethrin and DDT, but susceptible to bendiocarb insecticide. Deltamethrin resistance was significantly higher in New Halfa (mortality =55.6, 95% CI: 50.2-61.1, $p<0.05$). There was a significant increases in resistance level over the four years ($p < 0.001$), with overall mean % mortality to deltamethrin declining from

81.0% (95% CI, 77.6 - 84.3) in 2011 to 47.7% (95% CI: 43.5 - 51.8) in 2014. The highest DDT resistance was evident in New Halfa (61.7%, 95% CI: 56.8 - 66.7), however, no consistent trend in DDT resistance. The *kdr*-west was detected throughout the study period in all areas. The frequency was significantly higher in Galabat (0.487, 95% CI; 0.469 - 0.504) and New Halfa (0.382, 95% CI: 0.367 - 0.396). There was a marked decrease in *kdr* frequency trends over years, from 0.318, (95% CI: 0.296 - 0.341) in 2010 to 0.250, (95% CI: 0.227 - 0.272) in 2014. No evidence of *ace-1R* in all samples assayed (n = 2590). There was significant negative correlation ($r = -0.54$, $p<0.0001$) between deltamethrin mortality and *kdr*-west frequency at the population level. Although there was an increase in deltamethrin resistance in both interventions but, the speed of resistance development was significantly ($p= 0.023$) lower by 2.1% (95% CI: 0.25 - 3.9) in the LLIN+IRS intervention (7.3% per annum) than in the LLIN only (9.3% per annum) and did not retard the *kdr* frequency ($p = 0.345$). In conclusion, the spatial and temporal resistance of insecticide and the underlying mechanism in the Sudanese *An. arabiensis* populations were documented. Combination of IRS with bendiocarb plus LLIN significantly slowed down the speed of pyrethroid resistance compared to LLIN alone. Therefore, combination vector control strategy should be deployed if resources are available. The findings also highlight the urgent need for the establishment insecticide resistance monitoring system as a part of malaria control programme so that insecticide resistance management strategies can be implemented

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is one of the most serious public health problems in the world. So far it is a tropical and sub-tropical disease and it is endemic in 97 countries. These countries are inhabited by 3.2 billion people, constituting more than 50% of the world's population at risk of malaria infection (WHO 2015a). Despite the remarkable reduction in the global burden of malaria over the past 15 years, malaria is still high in many parts of the globe, particularly in sub-Saharan African. The latest report by WHO (WHO 2015a) showed that the global malaria cases were reduced from 262 million cases in 2001 to 214 million in 2015. Deaths due to malaria have also been reduced by half, from 839,000 deaths in 2001 to 438,000 in 2015. The largest proportion of malaria incidence (88%) and deaths (90%) occur in sub-Saharan African, and most of these cases and deaths occur in children under 5 years old. Approximately 80% of malaria deaths occurred in Africa and reported in just 15 countries (WHO 2015a). The high burden of malaria in Africa, however, is related to many factors, including the presence of *Plasmodium falciparum* (Welch) the most deadly malaria parasite species (Snow et al. 2005), co-existing with *Anopheles gambiae* s.s. (Giles), *An. arabiensis* and *Anopheles funestus* (Giles), which are the most extremely efficient vector species in the world (Coetzee 2004, Sinka et al. 2012).

Diagnosis and treatment of clinical malaria cases, intermittent preventive treatment and preventing transmission via effective vector control are the main strategies available to fight against malaria (WHO 2015b). The recent progress in

reducing malaria morbidity and mortality, particularly in African region was achieved through massive scale-up of long-lasting insecticide treated nets (LLIN), indoor residual spraying (IRS) and Artemisinin-based combination therapy (ACT). Recent study by Bhatt et al. (2015) revealed that, 79% of the reduction in malaria cases were attributed to LLIN and IRS, and the remaining 21% were related ACT (Bhatt et al. 2015). Historically, malaria has been successfully eradicated from several geographical settings except in SSA countries by DDT IRS, (Karunamoorthi 2011, van den Berg et al. 2012). Even today, vector control is considered as a milestone in the malaria control and elimination campaigns (Hemingway 2014), and so far it is one of the three pillars of the global technical strategy for malaria 2016-2030 (WHO 2015b).

Whilst, significant progress have been achieved in malaria control since 2001, the emerge and spread of insecticide resistance in the major African malaria vectors undermine the effectiveness of insecticide base malaria vector control programmes (Ranson and Lissenden 2016). According to the WHO (2012) insecticide resistance in malaria vectors have been documented in 64 countries, of these 27 are in the African continent. Insecticide resistance commonly occur via alterations in the target site of the active ingredient, termed "knockdown resistance" (*kdr*) and increased metabolic detoxification of insecticides (Hemingway et al. 2004). In *An. gambiae* s.s., two site mutations at amino acid position 1014 of the voltage-gated sodium channel (VGSC) gene have been characterized, resulting in change from leucine-to-phenylalanine, named West African *kdr* mutation (here after referred as *kd*r-west) (Martinez-Torres et al. 1998), or from leucine-to-serine, known as East African *kdr* mutation (here after referred as *kdr*-east) (Ranson et al. 2000), both mutations were associated with pyrethroid and DDT resistance in the field populations. The two *kdr*

mutations described in *An. gambiae* s.s. have also been reported in *An. arabiensis* by independent investigators in widely disperse locations. It has been reported in *An. arabiensis* populations from Ethiopia (Yewhalaw et al. 2010), Cameroon (Nwane et al. 2011), Chad (Witzig et al. 2013), Senegal (Ndiath et al. 2015) and Uganda (Mawejje et al. 2013). Whereas, the target site insensitivity to carbamates and organophosphates is acetyl-cholinesterase (AChE) at codon 119 (G119S) (Ranson et al. 2000). This type of mutation has been reported in *An. gambiae s.s.* population from various locations (Djogbénou et al. 2007, Edi et al. 2012). Increased metabolic detoxification is the most common mechanism of insecticide resistance (Hemingway et al. 2004). Three enzymes are responsible for detoxification of insecticides: Cytochrome P450, carboxylesterases (CCEs), and glutathione S-transferases (GST's) (Hemingway et al. 2004). Enzymes from all three families have been implicated in conferring resistance to all classes of insecticide in many insect species (Li et al. 2007, Liu 2015). The elevated levels of monooxygenases (P450s) are the most frequently observed in pyrethroid resistant *An. gambiae s.s and An. arabiensis* (Awolola et al. 2009, Mitchell et al. 2012, Mitchell et al. 2014, Ibrahim et al. 2016). Esterase mediated detoxification has been described in pyrethroid resistant populations of *An. gambiae* and *An. arabiensis* from Mozambique (Casimiro et al. 2006). Whilst, metabolic resistance due to GSTs expression have been implicated in most reports of DDT resistance in both *An. arabiensis* and *An. gambiae* s.s (Riveron et al. 2014, Wilding et al. 2015).

1.2 The rationale of the study

Insecticide-based vector control interventions such as LLIN and IRS are well proven interventions for malaria vector control (Lengeler 2004, Pluess et al. 2010, Bhatt et al. 2015). Moreover, insecticide applications are also currently the prime method for

control of leishmaniasis, dengue and yellow fever. Presently, only four classes of insecticide are approved for malaria vector control, with all having only two mode of action; pyrethroids and organochlorines act on sodium channel, while carbamates and organophosphates act on acetyl-cholinesterase (Hemingway et al. 2004, WHO 2006). Unfortunately, the continuous and intensive use of insecticides resulted in the development and spread of resistance, which made insecticide use ineffective and limits its availability for vector control options (WHO 2011, van den Berg et al. 2012). The emergence of resistance not only shortens the lifespan of currently available insecticides but tends to undermine the efficacy of newly discovered or developed insecticides owing to cross and multiple resistance mechanisms (Ranson and Lissenden 2016).

The common feature across all vector-borne diseases is that whilst there is extensive experimental evidence of resistance there is little assessment on how best to mitigate its development. Most of the research efforts have been to document changes in resistance frequencies rather than proactively guide insecticide resistance management strategies to make better use of limited resources of insecticide. Therefore, it is very important for vector control programmes to develop insecticide resistance management (IRM) approaches to preserve or recover insecticide efficacy thereby sustaining the impressive public health gains (WHO 2012).

In recent years there have been suggestions that combination of interventions with different classes of insecticide for IRS and LLIN may be able to delay the development of resistance (WHO 2011, 2012). To develop the evidence-based IRM strategy, WHO initiated a large cluster randomized trial (CRT) study implemented in five countries and Sudan being one of these, to provide quantitative evidence of the impact of combination of interventions on IRM. The present study was designed to

assess the combination treatment (LLIN; deltamethrin +IRS; bendiocarb) within selected clusters to determine whether it would offer the greatest potential impact on delaying resistance development in malaria vectors. The study will also monitor and compare changes in resistance genes frequencies of vector populations per intervention/area. The research findings will provide evidence and lessons that could be shared with the other project countries and globally.

1.3 General objective

The overall objective of this study is to assess and compare the impact of different insecticide-based vector control intervention strategies, combination (LLIN-deltamethrin plus IRS-bendiocarb) on the development and/or delay of insecticide resistance in the primary malaria vector, *An. arabiensis* populations from four endemic areas of Sudan.

1.3.1 Specific objectives

1. To monitor and compare the susceptibility level of *An. arabiensis* to deltamethrin, DDT and bendiocarb, in selected sentinel sites per study interventions (LLIN-deltamethrin/LLIN plus IRS-bendiocarb) per area.
2. To estimate and compare the allele frequency of target-site resistance mutations L1014F and L1014S pyrethroid/DDT at baseline and throughout the study period, per study interventions (LLIN-deltamethrin/LLIN plus IRS-bendiocarb) per area
3. To screen for the presence of the acetylcholinesterase target-site resistance mutations G119S (ace-1R) for bendiocarb phenotype, per study interventions (LLIN-deltamethrin/LLIN plus IRS-bendiocarb) per area
4. To quantify the changes in phenotypic and *kdr* allele frequencies in relation to vector control interventions stratified by area.

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria parasite

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* and transmitted from person to person by female *Anopheles* mosquitoes. Among the four human malaria parasite species, *Plasmodium falciparum* (Welch) is the most prevalent species, accounting for 78% of global malaria infection, out of which 90% occurred in the African continent (Snow et al. 2005, Guinovart et al. 2006). It is also responsible for most malaria-related deaths worldwide. *Plasmodium vivax* (Grassi) is the second most major cause of malaria, mainly in Asia and the Americas (Price et al. 2007). The other two species, *Plasmodium ovale* (Stephens) and *Plasmodium malariae* (Grassi) are responsible for a very small percentage of the total cases worldwide in Central and South America. Recently, a fifth species, *Plasmodium knowlesi* (Knowles) has been incriminated as human malaria parasite in some jungle areas of South-East Asia (Vythilingam et al. 2006).

2.2 Malaria vectors in African

Globally, malaria is transmit by 41 *Anopheles* mosquito vectors, of these *An. gambiae* complex and *An. funestus* are the principal malaria vectors in the African continent (Coetzee 2004, Sinka et al. 2012). *Anopheles gambiae* complex contains seven species of mosquitoes that are morphologically indistinguishable but have distinct genetic and behavioural differences which are reflected in their ability to transmit malaria (Coetzee et al. 2000). The seven species include; *An. gambiae s.s.*, *An. arabiensis*, *Anopheles merus* (Dönitz), *Anopheles melas* (Theobald), *Anopheles quadriannulatus* (Theobald) *species* A and B *and Anopheles bwambae* (White).

Out of these, two species, *An. gambiae s.s*., and *An. arabiensis* are commonly known as the most efficient vector species in the world due to their high anthropophilic and endophilic behaviour. *Anopheles gambiae s.s*. is well adapted to wet humid environments, therefore, it has a wide geographical distribution in all forested areas of the African territories where the rainfall >1000 mm. This species is responsible for approximately 80 - 85% of global malaria transmissions (Levine et al. 2004). Opposite to *An. gambiae s.s*., *An. arabiensis* is dominant in arid desert fringes (Coetzee et al. 2000, Sinka et al. 2012). The two salt-water breeder; *An. merus* and *An. melas* in addition to *An. bwambae* the hots springs water breeder in Uganda are known to be localized vectors of malaria in the coastal region of East and West Africa and Uganda respectively (Coetzee et al. 2000). *Anopheles quadriannulatus* species A and B both are zoophilic and exophilic species and have no medical importance (Coetzee et al. 2000). With regards to *An. funestus*, many varieties are known, but only the typical form share the same importance as *An. gambiae s.s.,* and *An. arabiensis* in being efficient vectors and present in almost all of the African countries (Sinka et al. 2010, Sinka et al. 2012).

2.3 Malaria vector control

Over years, malaria vector control remains the most powerful approach to fight against malaria (Hemingway 2014). Long-lasting insecticidal treated nets (LLINs) and indoor residual spraying (IRS) are the most widely implemented methods of malaria vector control. Both interventions are considered as key elements for interrupting malaria transmission by reducing the life-span of adult mosquito vector (Okumu et al. 2011, Hemingway 2014, Bhatt et al. 2015). However, owing to technical and operational cost associated with IRS campaigns, LLIN has been the

more widely applied intervention in sub-Saharan Africa (SSA) (WHO 2015a). Between 2013 and 2015 more than 446.5 million LLIN have been distributed free of charge in SSA resulting in an overall coverage of 50% of population at risk had access to an LLIN (WHO 2015a).

Indoor residual spraying (IRS) is the second most widely malaria vector control method in all malaria endemic countries specifically in SSA (WHO 2015a). this method has traditionally been used in countries with strong malaria control programme, where it has been highly successful in controlling malaria and reducing transmission (Maharaj et al. 2005). During the 1900s, malaria has been successfully eradicated from Eurasia, northern America, most of northern Africa, and Australia by means of IRS (Karunamoorthi 2011, van den Berg et al. 2012). In 2013, 88 countries adopted and implemented IRS worldwide, of these 42 in SSA. Out of 88 countries implementing IRS, 53 countries used pyrethroid insecticides for IRS, carbamates were used by 12 countries and in 9 countries DDT was sprayed (WHO 2014). However, it is very important to note that the real success of an IRS campaign fundamentally depends upon the efficacy of the choice insecticide and quality of the spray. In fact, most of the current malaria vector control methods heavily depends on one class of insecticides, the pyrethroids (van den Berg et al. 2012).

Recently, malaria vector control by means of larval source management (LSM) was encouraged. WHO recommends larviciding only in settings where mosquito breeding sites are few, fixed, feasible and are easy to access for treatment (WHO 2013b). Presently about 38 countries are using LSM to control malaria vector, of these 27 countries used chemical larviciding (WHO 2014). Larval source management (LSM) such as larviciding, biological control, proper water

management and intermittent irrigation have also shown to have a significant impact on malaria transmission (Keiser et al. 2005, Tusting et al. 2013). The best examples of these are the successful stories of Dar es Salam (Castro et al. 2009), Khartoum Malaria Free initiative (El Khalifa et al. 2008) and the eradication of *An. gambiae* from both the Amazon River in Brazil (Killeen et al. 2002) and Egypt (Shousha 1948).

2.4 Insecticides used for vector control

The current malaria control practice is mainly depending on chemical insecticides. At the moment, only 12 insecticides belonging to 4 classes are recommended by WHO for vector borne disease control including; organochlorines, organophosphates, carbamates and pyrethroids (Table 2.1) (WHO 2006). For IRS, all the twelve insecticides are recommended. However, pyrethroid is the only class of insecticides approved for LLINs because they are fast-acting, long-lasting and demonstrate relatively low toxicity to mammals (WHO 2006). Deltamethrin, alpha-cypermethrin, bifenthrin, cyfluthrin, lambda-cyhalothrin and etofenprox are all pyrethroids used for LLINs (WHO 2006). Recent report showed that insecticides use for vector control were dominated by pyrethroids in terms of surface area treated (81%) and by organochlorines (DDT) in terms of quantity applied (71%) (van den Berg et al. 2012).

Table 2.1: List of chemical insecticides approved by the WHO for malaria vectors control (WHO 2006).

Class	Insecticide	Mode of action
Organochlorine	DDT	Contact
Organophosphate	Malathion	Contact
	Fenitrothion	Contact & airborne
	Pirimiphos-methyl	Contact & airborne
Carbamate	Bendiocarb	Contact & airborne
	Propoxur	Contact & airborne
Pyrethroid	Deltamethrin	Contact
	Lambda-cyhalothrin	Contact
	Alpha-cypermethrin	Contact
	Bifenthrin	Contact
	Cyfluthrin	Contact
	Etofenprox	Contact

2.5 Insecticides mode of action

Most insecticides are neurotoxicants, and the majority of these neurotoxic insecticides act on three types of neuroreceptors and ion channels. This is exemplified by DDT and pyrethroids that act on voltage-gated sodium channels (VGSC), carbamates and organophosphates those act on acetylcholine ion (AChE) and cyclodiene class (e.g. dieldrin) on gama-aminobutyric acid (GABA) receptors of the chloride ion (Rinkevich et al. 2013).

Pyrethroids and DDT are structurally different from each other but, their mode of action has been shown to be very similar modulating the activity of sodium channels (Narahashi 2002). Both insecticides induce their insecticidal toxic effects primarily by binding to the voltage-gated sodium channels in the nerve tissue, altering its gating properties, and keeping it open for an unusually long time (Rinkevich et al. 2013). The modifications in the sodium channel structure, due to either point mutations or amplifications, lead to insensitivity to DDT and pyrethroids

in the sodium channels of the nervous system via a reduction in or an elimination of the binding affinity of the insecticides to enzymes (Narahashi 2002).

Another important feature of the action of DDT and pyrethroids is negative temperature dependence. of nerve sensitivity to both insecticides (Soderlund 2012). It has shown that these insecticides are more potent in killing insects at low temperature than at high temperature (Narahashi 2002). Although the mechanism that underlies this phenomenon is unclear a long time but, it has recently become clear that the most critical factor is the prolongation of sodium current, more accurately that of charge transfer (Soderlund 2012).

Whilst, organophosphorus and carbamate insecticides are represented by a wide variety of structure which has different chemical and physical properties (WHO 2006). The toxicity of these insecticides to insects and mammals is attributed to their ability to inhibit acetylcholinesterase (ACHE, .choline hydrolase) (Fukuto 1990). ACHE is a class of enzymes present in all animals including insects. It is responsible for the rapid hydrolysis of the neurotransmitting agent acetylcholine (ACh) into inactive products of choline and acetic acid (Fukuto 1990). Thus, the inhibition of AChE by organophosphate or carbamate, the enzyme is no longer able to hydrolyze acetylcholine; resulting in high concentration of ACh, and continuous stimulation of the muscle or nerve synapse occurs, resulting eventually in exhaustion and tetany leading to paralysis or death,

2.6 Resistance definition

Resistance is a genetically inherited characteristic which increases in the vector population as a direct result of the selective effects of the insecticide. WHO define resistance as “the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the species” (WHO 2012). Nevertheless, resistance is governed by multiple factors including; genetics, physiological and/or behavioral, operational factors (Hemingway et al. 2004).

2.7 Resistance mechanism

Four major types of insecticide resistance mechanisms have been documented namely: 1) reduced penetration, 2) behavioral resistance, 3) metabolic resistance, and 4) target site insensitivity resistance (Hemingway et al. 2004, Ranson et al. 2011).

2.7.1 Reduced penetration

Reduced penetration is also known as “cuticular resistance”, cuticle being a major route of insecticide penetration in insects. Thus, modifications in the outer layer cuticle of the insect as well as in the digestive tract linings that prevent or slow down the penetration and/or absorption of insecticides is known as cuticular resistance (Ranson et al. 2011). In malaria vector control, insecticides where often applied on walls or on LLINs and curtains, the ordinary uptake of insecticides is through penetration. Therefore, an increase in the thickness of the outer layer cuticle, or altered to lesser permeability to insecticides, could have a significant impact by reducing the entry rate of insecticide into the vector body (Ranson et al. 2011, Karunamoorthi and Sabesan 2013).

Recent studies have shown that some cuticular protein genes (e.g. CPLCG8 and CPLCG3), were implicated in pyrethroid metabolism in *An. gambiae* s.s, (Awolola et al. 2009), *Anopheles stephensi* (Liston) (Vontas et al. 2007) and *Culex pipiens* (Linnaeus) (Fang et al. 2015). However, more investigations are needed in order to understand the significant role of cuticular resistance on malaria vector control (Ranson et al. 2011).

2.7.2 Behavioral resistance

This type of resistance occurs due to modification in the insect behaviour to avoid coming in contact with insecticides (Ranson et al. 2000, Gatton et al. 2013). Due to excito-repellency of DDT, change in their resting behaviour from indoor to completely outdoor observed in *An. gambiae* complex after DDT was sprayed (Asidi et al. 2005, Chandre et al. 2010). Similar observations were reported in *An. arabiensis* in Sudan (Haridi 1972b). In Ethiopia, the intensive and continuous use of DDT for IRS for more than four decades resulted in increased *An. arabiensis* exophily as well as shift in bite time (Yohannes and Boelee 2012). Another field trial study in East Africa showed that the continuous usage of LLIN resulted in dramatic change from indoor to outdoor feeding behaviour in *An. gambiae s.s, and An. funestus* (Russell et al. 2011).

2.7.3 Metabolic resistance

This type of resistance mechanism is associated with biochemical transformation of the enzymatic systems, through which insect can detoxify toxicant substrates (Hemingway and Ranson 2000, Hemingway et al. 2004, Liu 2015). Metabolic resistance occurs when increased or modified activities of an enzyme preventing the insecticide from reaching its targeted site of action (Hemingway 2000). It involves

the overproduction of specific enzymes through sequestration, metabolism, and/or detoxification of the insecticide. Generally, three major enzyme families have been identified in insect; I) cytochrome P450s or mixed-function-oxygenase (MOF), II) carboxyl/cholinesterases or esterase (CCEs), and III) glutathione-S-transferases (GSTs) (Hemingway and Ranson 2000, Hemingway et al. 2004). In fact, metabolic resistance is the most complex and intensive form of insecticide resistance (Hemingway 2000). Although metabolic resistance is important for all classes of insecticide, but each enzyme has specific affects against specific class of insecticide (Hemingway 2000, Hemingway and Ranson 2000, Hemingway et al. 2004).

2.7.3 (a) Cytochrome (P450s) based resistance

Cytochrome P450s (CYP genes), are constitute one of the largest gene families in all living organisms, and are a highly diverse group of physiological and biochemical functions. Their essential common feature is the absorbance peak at 450 nm of their carbon-monoxide-bound form for which they are named (Werck-Reichhart and Feyereisen 2000). P450 enzymes are best known for their monooxygenase role, catalyzing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water (Li et al. 2007, David et al. 2016).

Moreover, P450s based detoxification has the potential to induce multiple resistance to insecticides independent of their target sites (David et al. 2016). In African malaria vector *An. gambiae* a total of 111 putative P450s genes have been isolated (Ranson et al. 2002). Of these only five genes have been identified (CYP4C27, CYP4H15, CYP6Z1, CYP6Z2, and CYP12F1) that involved in high DDT and pyrethroids resistance (Hemingway et al. 2004, Penilla et al. 2007, Chiu et al. 2008, Stevenson et al. 2011). In addition to oxidation mechanism, P450s, capable

to mediate insecticides resistance via gene amplification and/or duplication (Ranson et al. 2002). In some situation such as *An. arabiensis* populations in Sudan, carboxylesterase resistance to malathion has been associated with a qualitative change in amino acid genes resulted in increase the rate of hydrolysis of the enzyme (Hemingway 1985). Recent a study in Chadian *An. arabiensis* populations revealed that a CYP6P4 gene was main caused of permethrin and bifenthrin detoxification. However, no activity was observed against deltamethrin, bendiocarb, propoxur and malathion insecticide (Ibrahim et al. 2016).

2.7.3 (b) Glutathione S-transferases (GSTs) based resistance

Glutathione-S-transferases (GSTs) are a major family of detoxification enzymes mainly involved in insecticide detoxification. GSTs-based resistance is commonly associated with organophosphate, organochlorine and pyrethroid insecticides (Enayati et al. 2005, Ranson and Hemingway 2005, Che-Mendoza et al. 2009). To date, approximately 31 GSTs genes belonging to six classes (Delta, Epsilon, Omega, Sigma, Theta and Zeta) have been identified in *An. gambiae*, of these a very few (GSTe1 GSTe2 GSTe3 and GSTe4) have been implicated in insecticide detoxification (Ranson et al. 2002, Che-Mendoza et al. 2009). The GSTs detoxify organophosphorus class via glutathione conjugation, organochlorines class; through glutathione peroxidase and dehydrochlorination and pyrethroids; via sequestration mechanism (Enayati et al. 2005, Ranson and Hemingway 2005). In Uganda, a microarray study showed that over expression of GSTe4 gene has been the main caused of pyrethroids insecticide resistance in both *An. arabiensis* and *An. gambiae* s.s (Wilding et al. 2015).

2.7.3 (c) Carboxylesterases (CCEs) based resistance

Carboxylesterases (CCEs) based resistance is involve in organophosphorus and carbamate insecticides, and to a lesser extent, pyrethroid (Hemingway et al. 2004). In *An. gambiae* a total putative of 51 CCEs genes have been identified, but only two major esterase genes, Estα2 and EstB2 have been implicated in this type of mechanism (Hemingway 2000). At least there are two insecticide detoxification mechanisms have been characterized including; non-elevated and elevated esterase-based mechanism (Hemingway et al. 2004, Liu 2015). Non-elevated esterase-based mechanism is based on the mutation in carboxylesterase causing the loss of its carboxylesterase activity but gaining hydrolase activity. While, elevated esterase-based mechanism is to elevate carboxylesterase through gene amplification, protecting insects by binding and sequestering insecticides (Hemingway 2000, Hemingway et al. 2004, Liu 2015).

In organophosphates and carbamates resistant mosquitoes, Estα2 and EstB2 enzymes, encode for detoxifying the insecticide through non-elevated esterase-based mechanism has been numerously reported in *Culex* mosquito vector (Li et al. 2007, David et al. 2016). In the elevated esterase-based mechanism, the overproduced esterase enhances the sequestration of organophosphate insecticide with high hydrolysis activity. Such enzymatic activity has been observed in malathion-resistant *An. arabiensis* populations from Sudan, in which qualitative change in amino acid genes has been associated with in increase the rate of hydrolysis of the carboxylesterase enzyme (Hemingway 1985). Recent studies have shown that none-elevated was implicated for pyrethroid resistant in *An. arabiensis* and *An. gambiae* from Tanzania and Mozambique (Casimiro et al. 2006, Matowo et al. 2010).

2.7.3(d) Other detoxification mediated enzymes

In addition to the three major insecticides detoxification enzymes mentioned previously, another two detox-related transcripts of enzymes accumulated differentially in insects including mosquitoes have been identified: symbiont-mediated resistance and ABC mediated resistance (Roth et al. 2003, Ferrari and Vavre 2011). Bacterial symbionts are present in all insects and animals, and are transmitted vertically with their hosts' genes, and hence extend the heritable genetic variation present in one species (Ferrari and Vavre 2011). However, the mechanisms by which symbionts protect their hosts from natural enemies are diverse. Some produce substances with anti-microbial properties such as isatin and tyrosol. While, others deter predators from their hosts through the production of toxic substances such as bryostatin, pederin and theopederins, which can inhibit eukaryotic protein biosynthesis (Brownlie and Johnson 2009). Recent study showed that a toxin produced by the bacteriophage APSE (*Acyrthosiphon pisum* secondary endosymbiont) in the symbiont *Hamiltonella defensa* protects its aphid host from parasitoids (Moran et al. 2005). Research is currently underway that suggests that the presence of the symbionts in the mosquito may activates the immune response against *Plasmodium* in addition to having a direct effect on the mosquito resistance evolution to the natural xenobiotics.

The ABC (ATP-binding cassette) proteins family is known as one of the largest transporter families in all living organisms (Buss and Callaghan 2008, Dermauw and Van Leeuwen 2014). The majority of ABC proteins function as primary multidrug-resistance proteins (MDRs) or P-glycoproteins (P-gps). The P-glycoproteins is utilized to produce several amino acids that are required for

metabolism toxicant and then eliminated out of cells by an ATP-dependent mechanism (Dermauw and Van Leeuwen 2014). Many insecticides, drugs and other xenobiotics are transported out of the cell by P-gps which makes them important regulators of the cellular toxicity of these chemicals. Genome sequencing analysis of *An. gambiae* has identified 41 ABC families, of these only ABCC subfamily is protecting these insects from the large variety of different toxic chemicals they encounter in their environment (Roth et al. 2003). Recently, a comparative study on deltamethrin resistant and susceptible *An. gambiae* form Kenya has demonstrated that, the ABC transporter proteins were accumulated significantly at higher levels in resistant than in susceptible mosquitoes (Bonizzoni et al. 2012). While in DDT resistant *An. arabiensis*, various numbers of ABC transporter genes (ABCB and ABCG) were shown to be expressed at very high levels (Jones et al. 2012b).

2.7.4 Insensitive target site resistance

Insensitive target site resistance termed "knock-down resistance or kdr" is related to the insecticide molecule no longer binding tightly to its target. In mosquito vectors three target sites have been identified including voltage-gated sodium channel (VGSC), Acetylcholinesterase (ACHE) and gamma-amino butyric (GABA) receptors gene (Hemingway et al. 2004, Ranson et al. 2011). These resistance mutations can occur each one alone or in combination, the later result in an extremely high level of resistance to all insecticides class (Hemingway et al. 2004).

2.7.4(a) Knock-down resistance (*kdr*)

Voltage-gated sodium channels (VGSC) as intracellular transmembrane are playing critical role in transporting signals between cells (Wang et al. 2015). Therefore, sodium channels are the primary target site of a variety of naturally occurring and

synthetic insecticides, particularly pyrethroids and DDT insecticides (Hemingway and Ranson 2000). The sodium channel contains four homologous domains (I–IV). Each domain consists of six trans-membranes (S1–S6) segments (Martinez-Torres et al. 1998, Ranson et al. 2002). When the membrane is at resting state the channel is closed and when it becomes depolarized the channel open (i.e. activation state), this led the channel to produce a sodium wave currents. Both pyrethroid and DDT insecticides change the VGSC kinetics properties, and keeping it open for an unusually long time (Martinez-Torres et al. 1998, Ranson et al. 2000). This resulted mutations in the gene that encodes the voltage-gated sodium channel known as knockdown resistance or *kdr* mutation (Ranson et al. 2002).

Up to date at least seven *kdr* mutations have been identified, and so far they have been detected in 13 dominant malaria vector species from Africa; *An. gambiae*, *An. arabiensis),* Asia; *An. sinensis* (Theobald), *Anopheles stephensi* (Liston), *Anopheles subpictus* (Grassi), *Anopheles sacharovi* (Favre), *Anopheles culicifacies* (Giles), *Anopheles sundaicus* (Rodenwaldt), *Anopheles aconitus* (Dönitz), *Anopheles vagus* (Dönitz), *Anopheles paraliae* (Sandosham), *Anopheles peditaeniatus* (Leicester) and *Anopheles albimanus* (Wiedemann) in American continent (Silva et al. 2014). Among these, two *kdr* mutations which have been described in *An. gambiae* s.s. is the most common repeated mutation. A leucine-to-phenylananine (L1014F) change in the VGSC at positions 1014 was first screened in *An. gambiae s.s.* from West Africa, named West African *kdr* mutation (*kdr*-west) *(*Martinez-Torres et al. 1998). Whilst, leucine-to-serine (L1014S) change at the same site positions 1014 was identified in *An. gambiae s.s.* from East Africa and referred as East Africa *kdr* mutation (*kdr*-east) (Ranson et al. 2000). In addition to *kdr*-west and