KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY KUMASI

COLLEGE OF SCIENCE

FACULTY OF BIOSCIENCES

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

KNOCKDOWN RESISTANCE (*KDR*) GENOTYPE FREQUENCIES AND DISTRIBUTION OF MOLECULAR FORMS OF *ANOPHELES GAMBIAE* IN SOME AREAS OF ACCRA METROPOLIS AND OKYEREKO

NAME: ANDREW BOAMAH-AGYEKUM

SEPTEMBER, 2009

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY OF KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE.

BY:

ANDREW BOAMAH-AGYEKUM, M. SC. BIOTECHNOLOGY

SEPTEMBER, 2009

DECLARATION

I hereby declare that this work is the result of my own original research and that no part of it has been presented for another certificate in this university or elsewhere.

....

Andrew Boamah-Agyekum Date

(Student)

....

Mr. E. E. Mak-Mensah Date

(Supervisor)

....

Dr. A. Egyir-Yawson Date

(Supervisor)

....

Prof. (Mrs.) I. Oduro Date

(Head of Department)

DEDICATION

This work is dedicated to my entire family for their care and provision up to this time.

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ABBREVIATIONS

%	percentage
µg	microgram
µl	Microlitres
µM	Micromolar
AChE	Acetylcholinesterase
BNARI	Biotechnology and Nuclear Agricultural Research Institute
Bp	Base pairs
CB	Carbamate
CYD	Cyclodienes
dd H_20	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
g	grams
GAEC	Ghana Atomic Energy Commission
GPS	Geographic Position System
HCl	Hydrochloric acid

IGS	Intergenic spacer
IMVMP	Integrated Malaria Vector Management Policy
IRS	Indoor Residual Spraying
ITN	Insecticide-Treated Nets
ITS	Internal Transcribed Spacer
KDR	Knock-down Resistance
Kdr-e	Knock-down Resistance East
Kdr-w	Knock-down Resistance West
LLIN	Long-Lasting Insecticide-treated Nets
M form	Mopti form
M	Molar
$MgCl_2$	Magnesium chloride
mL	millilitres
mM	millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaVdp	Sodium Voltage-Dependent
ng	Angstrom
NMCP	National Malaria Control Programme

NMMRI	Noguchi Memorial for Medical Research Institute
NNRI	National Nuclear Research Institute
ºC	Degree Celsius
OP	Organophosphate
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RBM	Roll Back Malaria
rDNA	Ribosomal Deoxyribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
RR	Homozygous resistant genotype
RS	Heterozygous resistant genotype
S form	Savanna form
SNP	Single Nucleotide Polymorphism
SS	Homozygous susceptible genotype
TBE	Tris-borate ethylenediamine tetraacetic acid
U	Unit
UV	Ultra violet
WHO	World Health Organization
X	Times

ABSTRACT

Malaria is one of the most contending diseases of the world with sub-Saharan Africa being the greatest sufferer. There have been several combating procedures; some focus on the causative parasite – *plasmodium* species, whilst others look into areas like vaccine technology, feasibility of large scale sterilization of the male *Anopheles gambiae* and vector control of the mosquito. A major constraint to vector control, based on indoor residual spraying and insecticide-treated materials, is the development of resistance by the mosquito vector to insecticides used for their control.

This study reports on the distribution of the molecular forms of *An. gambiae* and insecticide resistance (the *kdr* mutation) carried out by sampling mosquito larvae from five locations in the Accra metropolis, and one out-group – Okyereko. Polymerase chain reaction (PCR) was used for the several analyses, and amplified nucleotide sequences were obtained when reaction products were analyzed by gel electrophoresis.

The molecular M and S forms of *An. gambiae* were found to occur in sympatry in all locations. The M form predominated throughout locations where rice and vegetable fields existed and were undergoing irrigation, with Korle Bu having the highest frequency of 96.7 %. Labadi Wireless had the highest frequency of the S form of 66.7 %. M/S hybrids were detected in three locations, with Madina scoring 70 %. The *kdr* mutation was observed at very high frequencies within the S form (80-100 %), M form (73.9-100 %), and M/S form (85.7-100 %).

A comprehensive study of the mosquito on a large scale would ensure a properly-structured vector control programme in order to curtail the spread of insecticide resistant *An. gambiae* and also to eradicate the parasite.

Table 1: Sample Collection Data

Location	sex		collection date	molecular form			*kdr*			GPS position
	m	f		M	S	M/S	RR	RS	SS	
Kotobabi	20	20	15.05.08	33	7	0	31	8	1	05.59508^0N; 000.20871^0W
Madina	20	10	17.05.08	1	8	21	22	5	3	05.67352^0N; 000.17754^0W
Korle Bu	20	10	03.06.08	29	1	0	21	5	4	05.53604^0N; 000.23168^0W
Roman Ridge	20	10	19.05.08	17	5	8	18	7	5	05.60833^0N; 000.19593^0W
Okyereko	20	20	09.05.08	23	17	0	25	8	7	05.42118^0N; 000.60508^0W
Labadi Wireless	20	10	05.05.08	8	20	2	24	6	0	05.57304^0N; 000.16089^0W

m-male; f-female; RR-homozygous resistance; RS-heterozygous resistance; SS-homozygous susceptible

CHAPTER ONE

1.0 INTRODUCTION

Malaria is one of the most common and serious diseases of the world causing death of about 3 million people each year (Snow *et al*., 2001). It is responsible for many health problems such as fever, headache and muscular pains among others. Malaria is often described as a great imitator of other diseases, sharing various characteristics with other childhood illnesses such as influenza, tuberculosis, typhoid, brucellosis and urinary tract infection. Even more confusing, it may coexist with other diseases (Commey, 1989). Besides, it is the leading cause of morbidity and mortality in Ghana (Ahmed, 1989).

There are several malaria control programs occurring in various endemic settings, and some have been the successful trials of pyrethroid insecticide-treated nets (Binka *et al.,* 1996; D'Alessandro *et al.,* 1995; Phillips-Howard *et al.,* 2003). However, the major malaria vector of sub-Saharan Africa, *Anopheles gambiae*, has shown an increased prevalence of insecticide resistance, and therefore, hampers the complete success of the use of insecticide-treated nets (Hemingway and Ranson, 2000). Development of urban agriculture has been reported to contribute to the selection of insecticide resistance in *An. gambiae*, due to continual use of pyrethroid based agricultural pesticides in the agricultural settings (Akogbeto *et al*., 2006).

Pyrethroids act on the nervous system of *An. gambiae* by modifying the normal function of the *para*-type sodium channel, which results in a prolonged opening of the channel. This increases nerve impulse transmission, leading to paralysis and death of the mosquito (Narahashi, 1992; Soderlund and Bloomquist, 1989).

Resistance to pyrethroid insecticides was first identified in the house fly *Musca domestica* and was termed knockdown resistance or *kdr* (Busvine, 1951).

The foreseeable complete failure of all currently available insecticides has highlighted the importance of research focused on new tools for monitoring insecticide resistance in disease vectors. The monitoring of insecticide resistance in malaria vectors is of prime importance especially where control programs are planned or already running, in order to assess potential selection effects of insecticidal compounds on vector populations, and to take appropriate measures such as switching to other classes of compounds. The presence and frequency of the *kdr* mutations form valuable and useful resistance markers, mainly, to warn about resistance development as the mutation arises, even before any effect on phenotype can be detected in a population (Kelly-Hope *et al*., 2008).

1.1 OBJECTIVES

The aim of this project was to investigate the insecticide resistance status of the primary malaria vector, *Anopheles gambiae*, in some communities of the Accra metropolis by genotyping and scoring the frequencies of the *kdr* mutation.

1.2 SPECIFIC OBJECTIVES

Anopheles gambiae larvae were to be collected from selected sites in Accra and fed until their growth into the mosquito stage. Mosquito DNA was to be isolated, and PCR and electrophoresis conducted to ascertain isolated *Anopheles gambiae* DNA. Frequencies of *kdr*

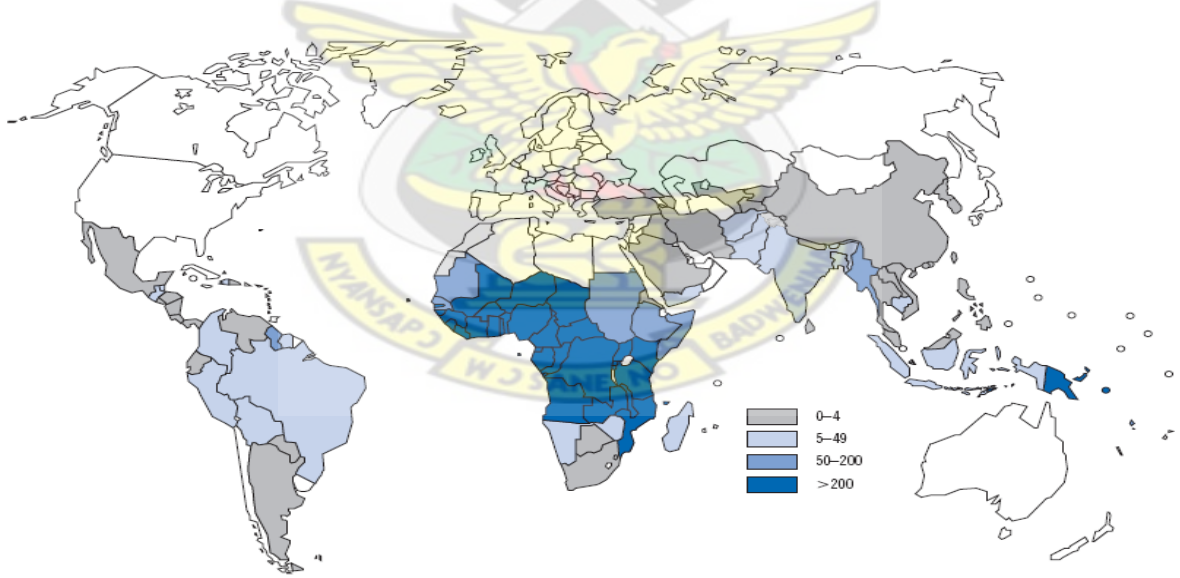
status and the spread of *Anopheles gambiae* molecular forms in some metropolis of Accra were to be determined.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 EPIDEMIOLOGY

Malaria is one of the most wide-spread diseases in the world today. It is estimated that about 3 billion of the world's population live in about 109 malarious countries, mainly in tropical Africa (Fig. 1). Each year, there are about 350-500 million reported cases, killing between 1-3 million people, the majority of whom are young children in sub-Saharan Africa (Snow *et al*., 2001), usually under 5 years (Greenwood *et al*., 2005). About 90% of the estimated reported cases live in tropical Africa. With the current population growth, even without an increase in malaria mortality rate, the number of deaths due to malaria could double to 2 million a year worldwide by the year 2010 (TDR, 1993).



Global Malaria Programme, 2007.

Figure 1: Estimated incidence of malaria per 1000 population, 2006

Malaria accounts for 20-25% of all hospital admissions in Africa although only 8-25% of persons visit health institutions (Brinkmann and Brinkmann, 1991). Ghana had an estimated 7.2 million malaria cases and 25, 000 deaths in 2006, forming 3% of the total for the African region (Global Malaria Programme, 2006). There had not been a reduction in malaria cases between 2001 and 2007, reported deaths rather increased in 2007. In 2006, reported malaria cases and deaths under 5 years were 3.2 million and 21, 000 respectively (Global Malaria Programme, 2007).

2.2 CYTOGENETIC ANALYSIS OF *ANOPHELES GAMBIAE* COMPLEX

The *Anopheles gambiae* complex consists of at least seven morphologically indistinct species (Coluzzi *et al*., 1979). Six species were later described, based on reproductive incompatibility, to give genetic distinctness, and a seventh fledgling species (Coluzzi *et al*., 1985; Favia *et al*., 1997; Hunt *et al*., 1998). The six species are: *An. gambiae* sensu stricto s.s., *An. arabiensis, An. merus, An. melas, An. quadriannulatus* and *An. bwambae*. The complex varies in their ability to transmit malaria, and is collectively referred to as *Anopheles gambiae* s.l (sensu lato) (Hunt *et al*., 1998).

Polytene chromosome analyses in *An. gambiae* s.s. from West Africa have shown a complex of naturally occurring paracentric inversions, with majority of them located on the right arm of the second chromosome, 2R (Coluzzi and Sabatini, 1967). There are five inversions designated as *j, b, c, u*, which are contiguous and not overlapping, and a fifth, designated as *d* overlapping with *u*. These inversions are shown as twelve karyotypes. The spatial distribution of these inversions depicts a strong association with ecological/climate zones (Bryan *et al*.,

1982; Coluzzi *et al*., 1979), and distribution is not random, even at the micro-geographic stage (Coluzzi *et al*., 1977). In sympatry, their relative frequencies are directly proportional to fluctuations in climate (Touré *et al*., 1998). This has been shown from field analysis of *An. gambiae* s.s. in sympatric areas, revealing that 2R inversion genotypes are not in complete conformity to Hardy-Weinberg equilibrium due to deficiency of inversion heterozygotes (i.e. there is an excess of homozygotes). Thus there is a barrier to gene flow among vector subpopulations having different 2R inversion genotypes. On this basis, *An. gambiae* s.s. has been subdivided into five chromosomal inversion forms: Bamako, Bissau, Forest, Mopti and Savannah (Bryan *et al*., 1982; Coluzzi *et al*., 1979, 1985; Coluzzi, 1984). Bamako is mostly found in Mali and north of Guinea, and has inversions *2Rjcu* and *2Rjbcu*. Bissau is predominant in The Gambia, with the inversion *2Rd*. Forest is forest breeding with occasional *b*, *c*, and *u* or *d* inversions. Mopti is also found in Mali, Guinea, Ivory Coast and Burkina Faso. Mopti is associated with irrigated fields and flooded plains, and therefore, breeds continuously throughout the year. It has 2R inversions such as *2Rbc* and *2Ru*. Savannah has been considered a typical 2R form of *An. gambiae* s.s., having the broadest geographic distribution, and therefore, spanning throughout sub-Saharan Africa. Savannah has 2R karyotypes such as *2Rbc, 2Rcu, 2Rbcd, 2Rbcu, 2Rbu* and *2Rbd*.

Population genetic studies have shown a low frequency of hybrids between Mopti and Savannah; Bamako and Savannah, but complete reproductive isolation between Bamako and Mopti forms (Touré *et al*., 1983). Cuticular hydrocarbon analyses of chromosomal forms depicted the possibility of pre-zygotic barriers to mating (Milligan *et al*., 1993), with sympatric samples from Mali and Burkina Faso showing more differentiation than samples collected allopatrically. Laboratory generation of Bamako and Mopti F_1 hybrids showed no post-mating reproductive isolation, and hybrids showed more vigour than parental strains (Di

Deco *et al*., 1980). This ambiguous pattern of population structure probably contributes to the great ecological flexibility of *An. gambiae* (Bryan *et al.*, 1982; Coluzzi *et al*., 1979, 1985; Touré *et al*., 1994), and as such their great importance as vectors of malaria, recording hundreds of millions of malaria cases per annum (Petrarca and Beier 1992). Cytogenetic analyses alone do not solve the ambiguity observed in the pattern of population structure of chromosomal forms.

2.3 MOLECULAR ANALYSIS OF *ANOPHELES GAMBIAE* SENSU STRICTO

Favia *et al*., (1994) discovered distinguishing RAPD (randomly amplified polymorphic DNA) markers between Mopti and among Bamako/Savannah chromosomal forms in Mali and Burkina Faso but were inconsistent in differentiating the various forms (Mukabayire *et al*., 2001). Further work was accomplished to sequence variations in the intergenic spacer (IGS) regions of the ribosomal DNA (rDNA) to distinguish Mopti from Bamako/Savannah. A diagnostic protocol was developed using polymerase chain reaction (PCR) amplification followed by restriction enzyme digest (restriction fragment length polymorphism, RFLP) to reliably separate Mopti from Bamako and Savannah samples collected from Mali and Burkina Faso (Favia *et al*., 1997). However, there were discontinuities between subpopulations on the definition of chromosomal forms and differences in IGS sequence. Firstly, IGS PCR-RFLP could not separate Bamako and Savannah. Moreover, field collected 'hybrid karyotypes' failed to produce a hybrid PCR-RFLP pattern; but Mopti and Bamako progenies created at the laboratory produced a hybrid pattern. Furthermore, some individuals showing Mopti karyotypes produced a Savannah/Bamako IGS PCR-RFLP pattern. These incongruities led to re-definition among forms such that karyotypes earlier fixed in one form are now shared among different forms (Favia *et al*., 1997; Touré *et al*., 1998), and 'hybrid'

identity are supposedly consequential low frequency polymorphisms in one form or another. Reproductive isolation among the re-defined chromosomal forms may be complete.

Studies conducted on *An. gambiae* populations in Mali determined karyotypes for each individual, as well as genotypes at twenty-one microsatellite loci physically mapped to polytene chromosomes. Genetic differentiation amongst chromosomal forms was smallest in markers on chromosomes 1 and 3 but large on chromosome 2. Observed divergence is consistent with a pattern of partial reproductive isolation. Three alternative hypotheses have been postulated concerning reproductive isolation (Lanzaro *et al*., 1998).

Firstly, the five chromosomal forms may be completely reproductively isolated. This is true when pattern of inversion heterozygote deficiency is marked on genomic markers irrespective of their positions to inversions. However, there could be frequent sharing of polymorphisms among the twelve genes if isolation is recent, but should obey Hardy-Weinberg equilibrium and have a strong deficiency of heterozygotes from combined chromosomal forms. Hybrids should not be observed when forms are in sympatry.

Secondly, the five chromosomal forms may be partially reproductively isolated. This is true when gene flow occurs only in season or certain geographic locations. Hybrids could exist but rare, as well as shared polymorphisms among forms. Heterozygote deficiency during sympatry should be lesser than allopatry.

Thirdly, the five chromosomal forms may not be reproductively isolated. This is true when genomic markers are homogeneous among sympatric groups, and in consistency with Hard-Weinberg equilibrium (Lanzaro *et al*., 1998).

Designations M and S were proposed to indicate the two IGS types. Studies in Mali and Burkina Faso always showed correspondence of M with Mopti, and S with Savanna. However, this linkage got broken outside Mali and Burkina Faso (Della Torre *et al*., 2001; Gentile *et al*., 2001). A considerable research to sequence DNA in the development of chromosomal form-specific diagnostics has been on-going, especially sequencing nuclear genes such as *white* gene on X-chromosomes (Besansky *et al*., 1997), the *tryptophan oxygenase* gene (Mukabayire *et al*., 1996) on 2R, *pKM*2 on 2L (Gentile *et al*., 2001), the *gua introns VIII, V, VI, F72* and *Gambif1* on chromosome 3 (Gentile *et al*., 2001). None of these produced a difference among the chromosomal forms (Gentile *et al*., 2001; Mukabayire *et al*., 2001). Typing of the mitochondrial gene *CO II* also failed to produce a difference among the forms (Gentile *et al*., 2001). However, Wang *et al*. (2001), in a microsatellite study, confirmed a slight distinction between M and S, except for those closely linked to the rDNA region of the X chromosome. Mukabayire *et al*. (2001) found credible partitioning between Mopti and Bamako/Savannah in the internal transcribed spacer (ITS) of the rDNA (Fig. 2), and showed that two ITS genotypes (ITS I and ITS II) were in complete linkage disequilibrium with the S and M IGS types, respectively. There were conformity of ITS results of populations outside of Mali and Burkina Faso (Gentile *et al*., 2001). These S and M molecular forms, thus far cover all populations in Africa (Della Torre *et al*., 2001, 2002). The results of these studies are consistent with the hypothesis of partial reproductive isolation among the five chromosomal forms.

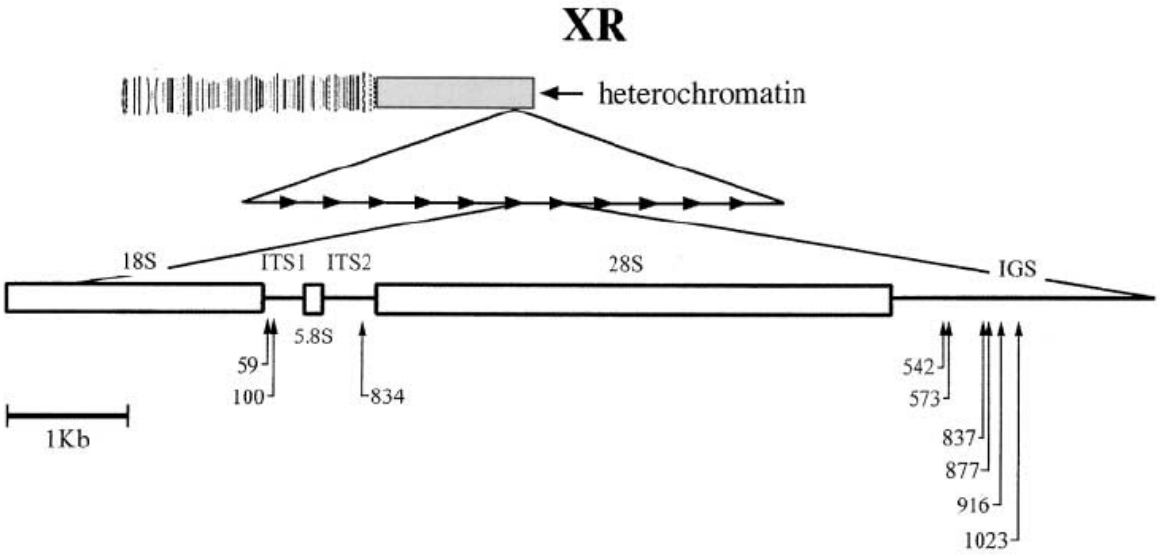


Figure 2: Location and organization of the rDNA in *An. gambiae.*

The rDNA locus consists of one very long array of repeat units (500–700 repeats per genome) located in the heterochromatic region of the X chromosome (Collins *et al*. 1989). Each repeat is 9 kb long and consists of the genes for the 18S, 5.8S, and 28S rDNA (open rectangles). These genes are separated by spacers (solid lines), the internal transcribed spacers (ITS1 and ITS2), and the intergenic spacer (IGS). Arrows point to the three diagnostic sites in the ITS (type I and type II) and the six diagnostic sites in the IGS (M and S). The ITS and IGS sites are 5 kb apart (Gentile *et al*., 2001).

2.4 CONTROL OF MALARIA

These programmes took the form of chemoprophylaxis and vector control in order to ameliorate the precarious malaria situation. There are currently no vaccines that prevent malaria but this area of research is actively ongoing.

2.4.1 CHEMOPROPHYLAXIS

During the 17th century quinine was used as prophylaxis against malaria (Adjei, 1989). But more effective alternatives such as chloroquine and primaquine have been used in the 20th

century to reduce the reliance on quinine and have resulted in the decline of malaria incidence rates (WHO, 1997). In 1950 proguanil (paludrine) was used for prophylaxis but resistance developed later (Neequaye, 1989). Pyrimethamine (Daraprim) was introduced in 1952 as the therapeutic triumph but its extensive use made *P. falciparum* develop resistance to it. This was observed in some semi-immunes in Kenya developing resistance after 6 months of use (Neequaye, 1989). Resistant strains to sulfadoxine/pyrimethamine combination (fansidar) appeared in South East Asia, South America (Peterson *et al*., 1991) and Ghana (Akanmori *et al*., 1989; Mak-Mensah *et al*., 2007).

2.4.2 CURRENT VECTOR CONTROL MEASURES

Malaria is killing more people than any other tropical disease (WHO, 1997). The best protection against malaria is prevention, which includes individual protection such as the use of insecticide-treated bed nets, and community measures such as insecticide spraying, control of mosquito breeding sites and drainage. In 1998 the World Health Organization, WHO launched a global partnership initiative known as the Roll Back Malaria, RBM. Its main objective is to reduce malaria morbidity and mortality by half by the year 2010, and a further reduction of 50% in morbidity and 75% in mortality by 2015 until malaria ceases to become a public health issue. Several initiatives have been stemmed up in Ghana following the launching of the RBM partnership. In 2007 Integrated Malaria Vector Management Policy, under the National Malaria Control Programme, NMCP have laid emphasis on vector control and the sole use of pyrethroids, and have outlined interventions such as adulticiding using Indoor Residual Spraying (IRS) and larviciding, biological control, environmental management and insecticide-treated materials (Global Malaria Programme, 2007).

There has been a marked increase in insecticide-treated net (ITN) use in Ghana over the past five years. ITN use in children under five years increased from 3.5 % in 2003 to 22 % in 2006. ITN use in pregnant women increased from 3.3 % in 2003 to 46.5 % in 2006. In 2006 Long-lasting insecticide-treated bed nets, LLINs numbering 1.9 million were distributed through an integrated child vaccination/ITN campaign. Plans were made to increase the supply to 2.1 million LLINs in 2007 through a similar child health/ITN campaign (Global Malaria Programme, 2006).

Since 2007 National Policy for Vector Control has included IRS into the integrated vector control programme, tailored IRS implementation model of the AngloGold Ashanti Mining Company in Obuasi, which had been in existence since 2004. An important part of these initiatives has been the regular monitoring of insecticide resistance because historically, heavy use of insecticides, including DDT, on cocoa, cotton, and other crops resulted in high levels of insecticide resistance (Global Malaria Programme, 2007). N'Guessan *et al.* (2007) recorded that pyrethroid resistance (*kdr*) had implications on the failure of LLINs and IRS in experimental huts in south Benin. However, insecticide bioassays performed on *An. gambiae* from south-western Ghana had revealed that there was no evidence of *kdr*-causing resistance in the midst of widespread use of pyrethroids and DDT for pest control on crops in the vicinity (Kristan *et al*., 2003).

2.5 EVOLUTION OF INSECTICIDE RESISTANCE

Several studies throughout Africa reported the existence of anopheline populations which were resistant to various insecticides (Awolola *et al*., 2007; Chouiabou *et al*., 2008; Corbel *et al*., 2007; Kristan *et al*., 2003; N'Guessan *et al*., 2007; Tia *et al*., 2006). Insecticides impair the function of molecular targets which are crucial for life. Insecticide resistance is induced

when mutation modifies the physiology of mosquitoes in a way that targets remain at least partially functional. This may be attributed to decreased insecticide penetration, increased metabolism of insecticidal compounds, and insecticide target-site insensitivity due to single nucleotide polymorphisms (SNP) (Hemingway *et al*., 2004). Resistance due to behavioural avoidance of insecticide (Lockwood *et al*., 1984; Priester and Georghiou, 1980) had been reported in mosquitoes, even though genetic studies have not been done.

Metabolic resistance to pyrethroids is usually accompanied with increased oxidase and esterase activities; resulting in an insecticide detoxification (Vulule *et al*., 1999). There have been reported cases of metabolic-based resistance on pyrethroid efficacy in mosquito apart from West Africa, and even conferring cross-resistance to carbamate insecticides in the vector *An. funestus* (Brooke *et al*., 2001; Hargreaves *et al*., 2000). There have also been some reported cases in *An. gambiae* populations from Cameroon (Etang *et al*., 2003, 2005), and Kenya (Vulule *et al*., 1999) as well. The genes encoding for elevation of the activity of detoxifying enzymes such as the cytochrome P450s, the glutathione S-transferases or carboxylesterases have been grouped into supergene families; but the individual genes that are up-regulated or amplified in insecticide resistance are yet to be identified (David *et al*., 2005).

The targets of insecticides are important molecules of the nervous system of mosquitoes. For instance, organophosphorus (OP) and carbamate (CB) insecticides target synapse acetylcholinesterase (AChE), the GABA receptor of the GABA-gated chloride channels are targeted by cyclodienes (CYD), which blocks the channels whilst avermectins activate them, and sodium voltage-dependent (NaVdp) channel is targeted by DDT and pyrethroids (Clark

et al., 1995). Target-site insensitivity emanate from point mutations in the voltage-gated sodium channel gene (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000), also known as *kdr* (knock-down resistance), which influences a cross resistance to DDT as well.

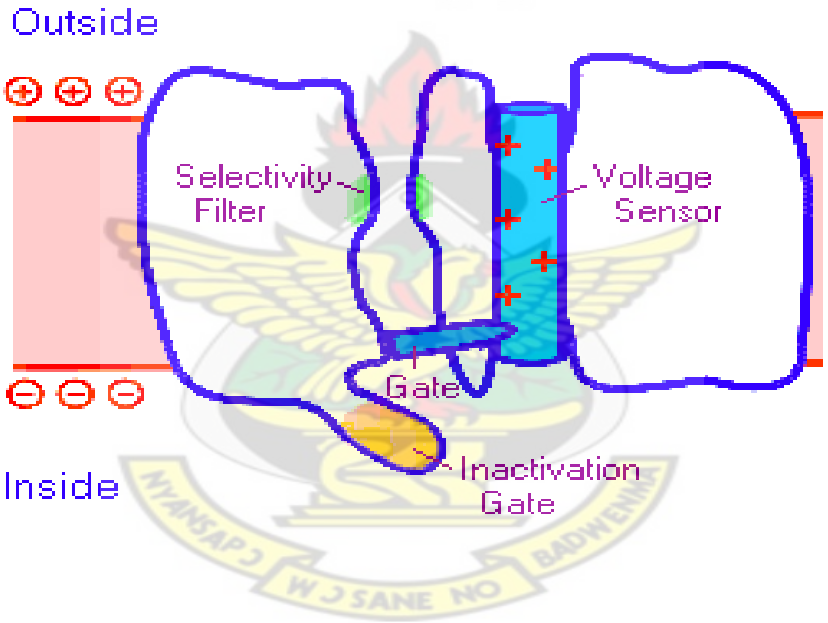
Sequence analysis has shown two different mutations in *An. gambiae*. In many West African countries the predominant *kdr* mutation in *An. gambiae* populations is the change from leucine to phenyalanine amino acid (L104F), also known as *kdr* west (*kdr*-w). However, in the East African countries the leucine is substituted with serine (L104S), also termed *kdr* east (*kdr*-e), (Awolola *et al*., 2005; Della Torre *et al*., 2001; Diabaté *et al*., 2004; Fanello *et al*., 2003; Stump *et al*., 2004; Verhaeghen *et al*., 2006). Heterozygote individuals for both the *kdr*-w and *kdr*-e alleles have also been reported (Etang *et al*., 2003; Verhaeghen *et al*., 2006).

2.6 ACTIVITY OF PYRETHROID INSECTICIDES

Insecticide resistance can be defined as the reduction in the sensitivity of insect population to an insecticide. This is observed by the continual failure of the insecticide to achieve the desired level of control when used according to recommendations for that pest species (World Health Organization, 1997).

Pyrethroids act on the nervous system of insects such as the mosquito vector by modifying the normal function of the *para*-type sodium channel (Fig. 3), which will result in a prolonged opening of the channel that increases transmission of nerve impulse, leading to paralysis and death (Narahashi, 1992; Soderlund and Bloomquist, 1989). Nerve cell membranes have a specific electrical charge. Altering the amount of ions (charged atoms)

passing through ion channels causes the membrane to depolarize, which, in turn, causes a neurotransmitter to be released. Neurotransmitters assist communication in nerve cells, and electrical messages sent between nerve cells allow them to generate response, like a movement in the mosquito. Pyrethrins affect the nervous system of mosquitoes generating multiple action potentials in the nerve cells, which delay the closing of the ion channels (Costa, 1997). Pyrethrins and pyrethroids act as contact poisons, which affects the nervous system of insects (Klaassen *et al*., 1996; Tomlin, 1994). Figure 4 shows the amplification regions of primers on sodium channel.



Costa, 1997

Figure 3: Sodium ion channel

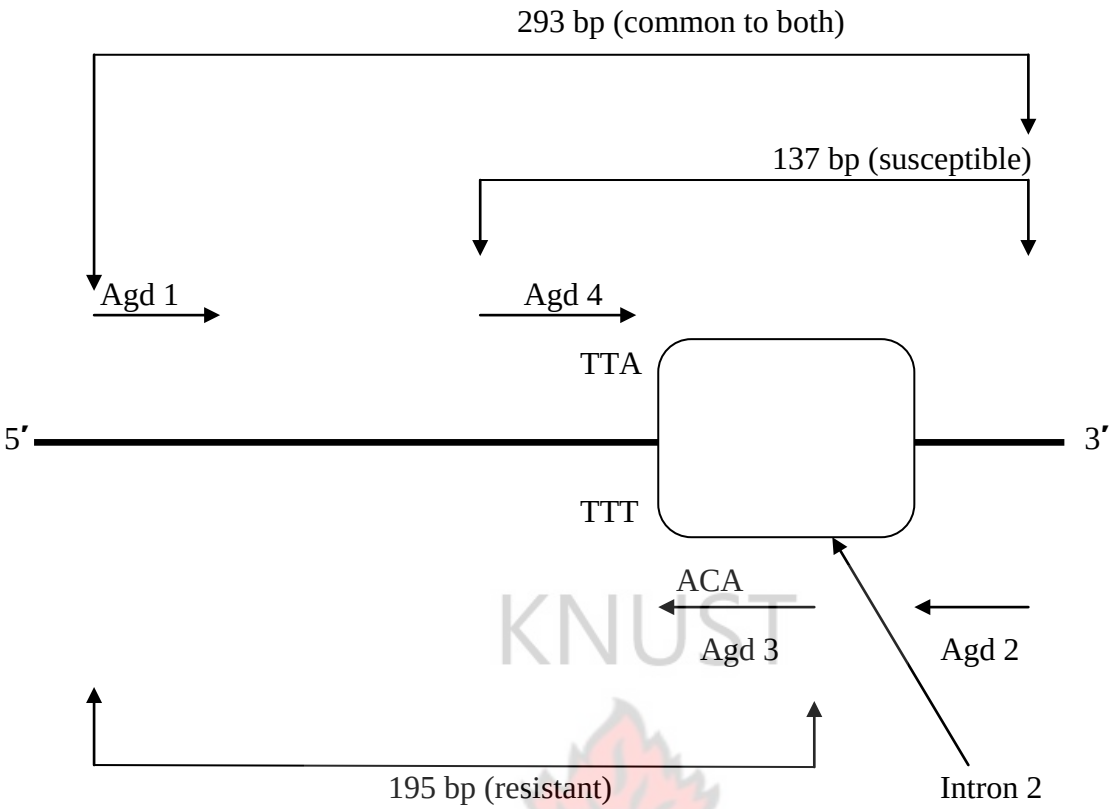


Figure 4: Schematic representation of PCR amplification of sodium channel region involved in pyrethroid resistance

Thick black line represents IIS4-IIS6 region of sodium channel. Leucine (TTA) – observed in susceptible mosquitoes. Phenyalanine (TTT) – observed in *kdr* mosquitoes. Agd 1 + 2 – both genotypes (293 bp). Agd 1 + 3 – amplifies resistant genotype (195 bp). Agd 4 + 2 – amplifies susceptible genotype (137 bp). Square on thick black line represents position of intron 2 (Martinez-torres *et al*., 1998).

2.7 KNOCKDOWN RESISTANCE (*KDR*) GENE AND INTROGRESSION BETWEEN M AND S MOLECULAR FORMS

Research into genetic differentiation of M and S rDNA types resulted in the study of the insecticide resistance gene, *kdr*, which is not associated with genes on X chromosome (Chandre *et al*., 1999; Della Torre *et al*., 2001; Weill *et al*., 2000), and is found on the left arm of the second chromosome, 2L, location 20C (Ranson *et al*., 2000). The extent of reproductive isolation between *An. gambiae* molecular forms can be described inferring from the on-going spread of insecticide resistance in them. In Ivory Coast *kdr* was only present in

the S molecular form (Chandre *et al*., 1999), thus aligning with complete reproductive isolation hypothesis. However, within one year, *kdr* had been found in M individuals as well in neighbouring Benin.

Sequence in an upstream intron from the S and M types suggested that *kdr* in M type arose through introgression rather than as an independent, new mutation (Weill *et al*., 2000). Sequences from 90 *kdr* positive individuals from Benin, Ivory Coast and Burkina Faso have shown a loss in genetic differentiation in the intron upstream of the *kdr* locus; suggesting that *kdr* locus together with areas of genome of proximal decent have been hitchhiked, also known as genetic sweep (Weill *et al*., 2000). It can be argued that introgression has recently occurred between M and S forms because of the pattern of gene flow with *kdr* locus in focus, together with its proximal genomic region, therefore, supporting the hypothesis of residual gene flow in areas of sympatry. However, molecular studies from Ivory Coast showed that *kdr* broke off with molecular IGS type but in Benin it occurred in both M and S types of the Forest and Savannah forms (Della Torre *et al*., 2001; Fanello *et al*., 2000). Diabaté *et al*. (2003) found *kdr* mutation in the M form from Burkina Faso. *Kdr* resistance gene has only been reported from M form populations which are in sympatry with the S form Savannah or Forest populations where between-form gene flow could be on-going. Identification of *kdr* in M form could be caused by selective introgression between forms rather than independent mutations, even though it is not empirically supported.

The occurrence of introgression without visible post-mating barriers threatens the maintenance of genetic identity between chromosomal forms. Genetic analyses of *An. gambiae* females and the sperm extracted from their spermatheca showed positive assortative

mating within forms, even though a small percentage of matings were between forms (females mated with wrong males) (Tripet *et al*., 2001, 2003). In a study to ascertain the survival of hybrids at different developmental stages, Tripet *et al*. (2001) investigated Mopti and Bamako forms in Mali, and found cross-mating frequency equal to 0.00839, but the frequency of adult hybrids was lower, 0.00303, suggesting that selection along development acts against hybrids. But in some complex populations of Africa, current gene flow between forms known to occur has been identified. Those are the groups where M/S form hybrids have been found (Tripet *et al*., 2001).

In Ghana, polytene chromosome analysis of *An. gambiae* has revealed the existence of three chromosomal forms: the Forest, typical of moist semi-deciduous forest, with inversion arrangements 2*Rb*, 2*Rd* and 2*La*; the Savannah, typical of more arid areas of the coastal and interior savannas, characterized by inversions 2*Rbc* and 2*Rbcd*; the Mopti characterized by inversion 2*Rbc*/2*Ru*, and sympatric with the Savannah form in the drier zones of both coastal and interior savannas (Appawu *et al*., 1994). M forms exist in the northern part of Ghana (Donnelly *et al*., 2004; Lehmann *et al*., 2003; Yawson *et al*., 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY AREAS

An. gambiae s.s. samples were collected from six different locations: Korle-Bu, Kotobabi, Labadi Wireless, Madina, Roman Ridge, all within Accra metropolis of the Greater Accra Region except Okyereko, which is an out-group in the Central Region. Samples from Korle-Bu (05.53604^{o}N, 000.23168^{o}W), Kotobabi (05.59508^{o}N, 000.20871^{o}W), and Roman Ridge (05.60833^{o}N, 000.19593^{o}W) were collected close to vegetable farms, and Okyereko (05.42118^{o}N, 000.60508^{o}W) from an irrigated rice field. Samples from Labadi Wireless (05.57304^{o}N, 000.16089^{o}W) and Madina (05.67352^{o}N, 000.17754^{o}W) were collected from areas without any agricultural activity. Sample sites were located in southern Ghana and are characterized by strand and mangrove, and coastal savannah zones. In these zones relative humidity is high (65-75%) throughout the year, with highest monthly mean temperature of 30^{o}C occurring between March and April, and lowest of 26^{o}C in August. The major rainfall season is in June and July. Vegetation is mainly grass with sparse trees and isolated patches of shrub.

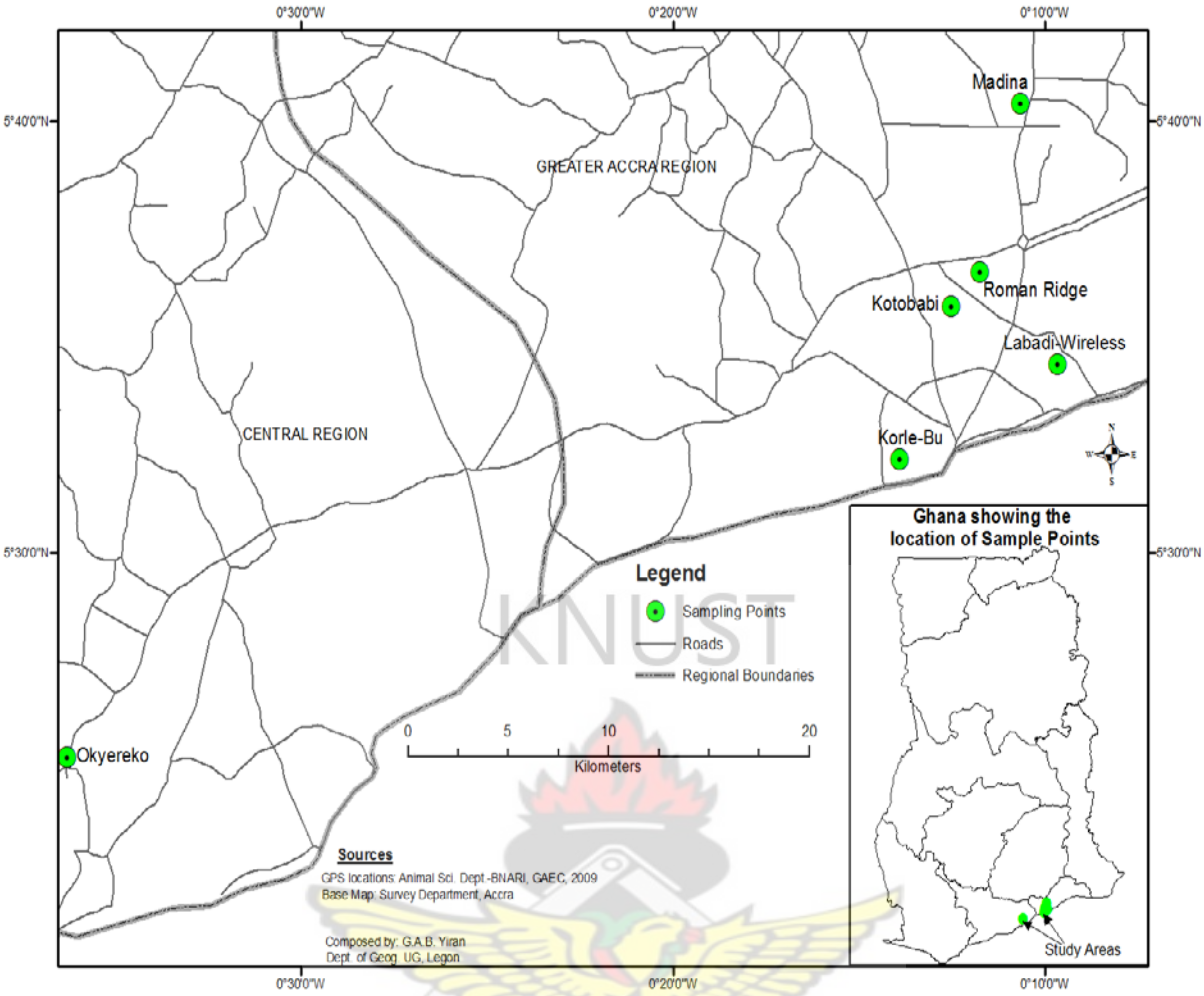


Figure 5: Map showing sampling sites of *Anopheles gambiae* larvae

3.2 METHODS

3.2.1 SAMPLE COLLECTION

Mosquito larvae were collected using standard procedure from the various localities under study. The larvae were sent to the laboratory where they were reared to adults in trays. The larvae were supplied with water and fed on fish food flakes until they metamorphosed into the pupae stage. The mosquitoes that emerged from the pupae were kept in 1.5 ml eppendorf tubes and frozen at -20ºC to kill them.

3.2.2 DNA EXTRACTION

The DNA extraction method of Collins *et al*. (1988) was slightly modified and used. Whole mosquitoes were homogenized individually in 100 µl lysis buffer. This was followed by incubation in water bath at 65 ºC for about 30 minutes. Fourteen microlitres of 3 M potassium acetate was added to the incubated samples and vortexed. This was followed by incubation on ice for 30 minutes. Samples were centrifuged at 12000 x g for 10 minutes, and the upper aqueous phase transferred into a new tube. About 200 µl of 95% ethanol was added to the pellet and vortexed, followed by incubation at -20 ºC for at least 1 hour. The contents were centrifuged at 12000 x g for 15 minutes, and the upper phase (ethanol) discarded. Two hundred microlitres of 70% ethanol was added and vortexed and centrifuged at 12000 x g for 10 minutes. The ethanol was discarded and samples were dried at room temperature. DNA was resuspended in 50 µl sterilized standard double distilled water (dd H_20) and stored at -20 ºC in the freezer.

3.2.3 IDENTIFICATION OF *ANOPHELES GAMBIAE* BY POLYMERASE CHAIN REACTION (PCR)

The PCR method of Scott *et al*. (1993), which is based on species-specific nucleotide sequences in the intergenic spacer (IGS) regions of ribosomal DNA (rDNA) was used for the molecular identification of *An. gambiae*. Amplification was carried out in a total volume of 25 µl PCR mix containing 1 µl of the genomic DNA of an individual mosquito; 5 µl of 5X PCR Buffer (Promega, USA); 1 µl of 25 mM $MgCl_2$ (Sigma-Genosys, UK); 2.5 µl of 2.5 mM of mixed dNTPs (Bioline, UK); 1 µl each of 6.25 ng of primer GA, 12.5 ng of primer UN, 18.75 ng of primer AR, 12.5 ng of primer ME (Sigma-Genosys, UK), 0.2 µl of 5 U/µl *Taq* DNA polymerase (Promega, USA).

The cycling conditions were as follows: a 5 minute 94 ºC denaturation step followed by 35 cycles of 20 seconds at 94 ºC, 30 seconds at 55 ºC, and 1 minute at 72 ºC; there was a final extension step of 10 minutes at 72 ºC. PCR reactions were carried out in 0.2 mL eppendorf tubes using Techne, TC 412 thermal cycler. Following the PCR cycles, 8 µl of PCR product from each reaction were electrophoresed in a 2.0 % agarose (Eurogentec, the Netherlands) in 10 X TBE buffer containing 1.0 µg/mL ethidium bromide (Sigma, Germany). Electrophoresis was carried out at 130 volts for 30 minutes. The amplified PCR fragments were visualized and photographed on Hoefer, Macro Vue UV-25 ultra violet transilluminator and band size of 390 bp was obtained.

Sequences of primers used for the PCR and their expected band sizes (base pairs, bp) are as follows:

UN: 5'- GTG TGC CCC TTC CTC GAT GT -3'	468 bp
GA: 5'- CTG GTT TGG TCG GCA CGT TT -3'	390 bp
AR: 5'- AAG TGT CCT TCT CCA TCC TA -3'	315 bp
ME: 5'- TGA CCA ACC CAC TCC CTT GA -3'	464 bp

The PCR products identified for *An. gambiae* s.s from the protocol of Scott *et al*. (1993) were digested to identify their M- and S-forms using the restriction enzyme *Cfol* ((10 U/µL), Bioline, UK) (Fanello *et al*., 2002). About 0.1 µl (*Cfol*) in 2.7 µl 10 X Buffer B (Promega, U.S.A) was added to the individual mosquito PCR products and then digested at 37 ºC for about 3 hours. After the digestion, 8 µl of PCR product from each reaction were electrophoresed in a 2.0 % agarose gel in 10 X TBE buffer containing 1.0 µg/mL ethidium bromide. Electrophoresis was carried out at 130 volts for 30 minutes. The amplified PCR fragments were visualized using ultra violet transilluminator and pictures taken.

3.2.4 DETECTION OF *KDR* ALLELE IN *AN. GAMBIAE*

Following the method of Martinez-Torres *et al*. (1998), a total volume of 25 µl for each reaction contained 1 µl of genomic DNA of an individual mosquito, 5.0 µl of 5 X PCR Buffer (Promega, USA), 1.5 µl of 25 mM $MgCl_2$ (Bioline, UK), 0.5 µl of 2.5 mM mixed dNTPs (Bioline, UK), 0.625 µl of 5 µM each of Agd1 and Agd2 primers, 0.313 µl of 2.5 µM each of Agd3 and Agd4 primers obtained from the Parasitology Unit, Noguchi Memorial Institute for Medical Research, Legon-Ghana, and 0.125 µl of 5 U/µL of *Taq* DNA polymerase (Promega, USA). All positive controls were provided by the Vector Genetics Laboratory of the Animal Science Department, Biotechnology and Nuclear Agricultural Research Institute (BNARI), Ghana Atomic Energy Commission, GAEC.

The cycling conditions were as follows: a 15 minutes 95 ºC denaturation step followed by 35 cycles of 30 seconds at 94 ºC, 30 seconds at 54 ºC and 1 minute at 72 ºC; there was a final extension step of 10 minutes at 72 ºC. Amplified fragments were analyzed by electrophoresis on a 2.0% agarose gel (Eurogentec, the Netherlands) and visualized by ethidium bromide (Sigma, Germany) staining under ultraviolet light. Sequences of primers used for PCR are as follows:

Agd 1: 5'- ATA GAT TCC CCG ACC ATG -3'
Agd 2: 5'- AGA CAA GGA TGA ACC -3'
Agd 3: 5'- AAT TTG CAT TAC TTA CGA CA -3'
Agd 4: 5'- CTG TAG TGA TAG GAA ATT TA -3'

CHAPTER FOUR

4.0 RESULTS

4.1 DISTRIBUTION BY SEX IN *AN. GAMBIAE* AT SAMPLE AREAS

The numbers, Geographic Position System (GPS), and date of collection of *An. gambiae* have been shown in Table 1. A total of 200 samples were collected from the various sampling sites and analyzed. Of these samples 120 were males (60%) and 80 females (40%) as shown in Table 1 and 2. A total of 40 samples each consisting of 20 males and 20 females were collected from Kotobabi and Okyereko. Thirty (30) samples each were collected from Madina, Korle-Bu, Roman Ridge and Labadi Wireless. The female to male composition of these samples is shown in Table 2. In Table 2, Korle-Bu and Roman Ridge samples showed higher numbers of M form in males than in females. Labadi Wireless and Okyereko samples had higher numbers of S form in males than in females. Madina and Roman Ridge showed M/S form both in males and females. Meanwhile, Labadi Wireless did not have any male M/S form. Again, Korle-Bu, Labadi Wireless, Madina and Roman Ridge showed higher numbers of *kdr* allele in males than in females. But Okyereko showed an equal number of *kdr* alleles in both sexes (Table 2). *Anopheles gambiae* were also identified at species level (Fig. 6) by PCR.

Table 2: Frequency of mosquito sexes in both forms and *kdr* gene mutation for all locations

		Male (m)			Female (f)		
Location		M form	S form	M/S form	M form	S form	M/S form
Korle-Bu	RR	12	-	-	8	1	-
(m=20; f=10)	RS	5	-	-	0	0	-
	SS	3	-	-	1	0	-
Kotobabi	RR	11	4	-	14	2	-
(m=20; f=20)	RS	4	0	-	3	1	-
	SS	1	0	-	0	0	-
Labadi W.	RR	1	15	-	3	4	1
(m=20; f=10)	RS	4	0	-	0	1	1
	SS	0	0	-	0	0	0
Madina	RR	1	5	9	-	2	5
(m=20; f=10)	RS	0	0	3	-	1	1
	SS	0	0	2	-	0	1
Roman R.	RR	8	2	4	3	0	1
(m=20; f=10)	RS	2	0	1	1	2	1
	SS	1	1	1	2	0	0
Okyereko	RR	4	10	-	5	6	-
(m=20; f=20)	RS	2	1	-	6	0	-
	SS	3	0	-	3	0	-

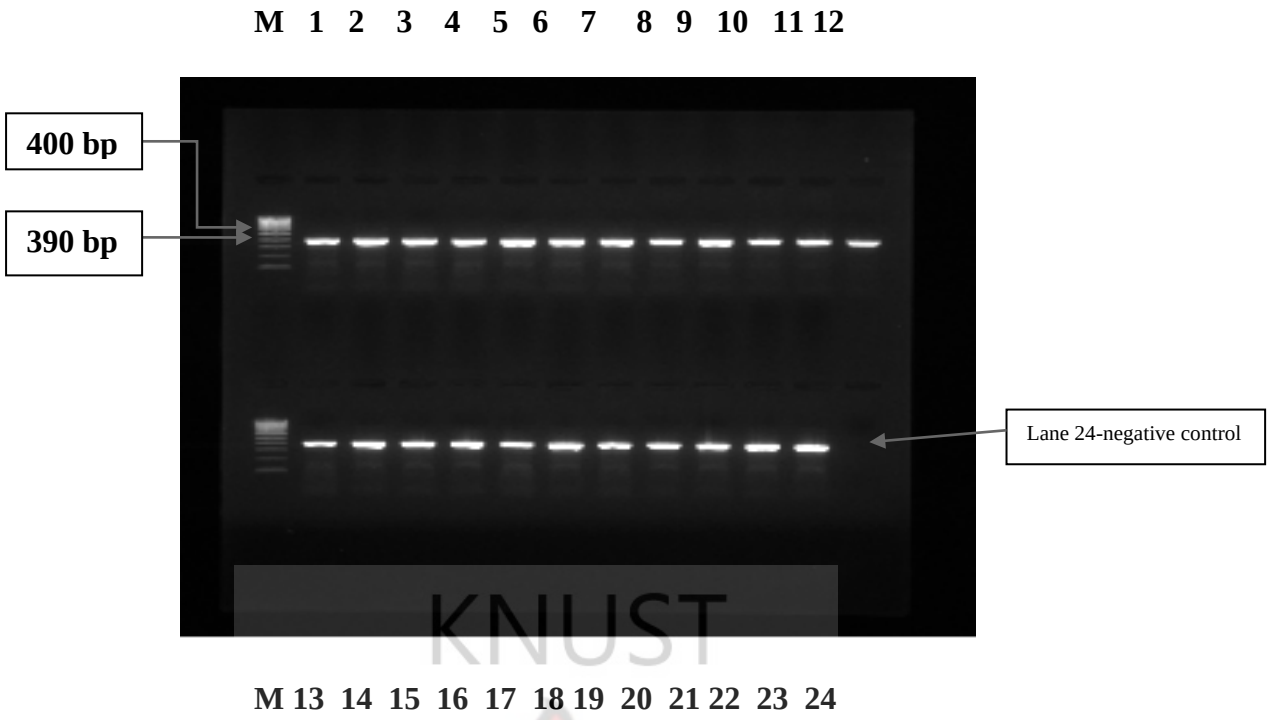


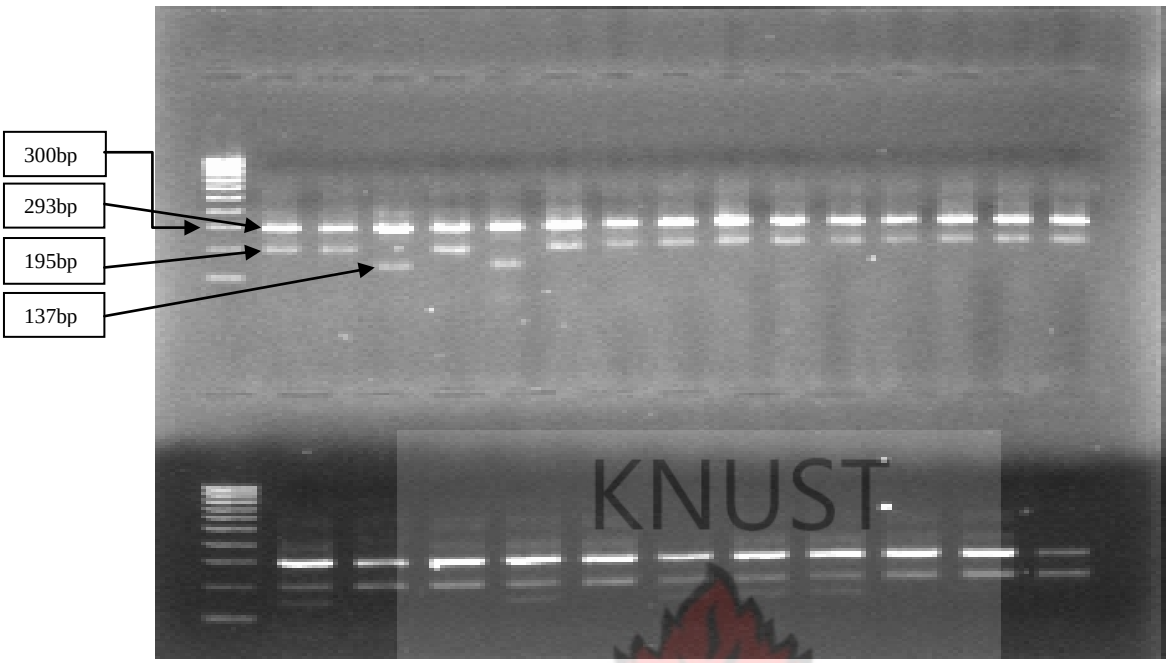
Figure 6: Species identification of *An. gambiae* DNA on 2.0% agarose gel

Lane M – 100 base pairs (bp) DNA molecular weight marker. Lane 1- positive control (Vector Genetics Lab, BNARI, GAEC, Ghana) for *An. gambiae* s.s. Lane 2-11 – *An. gambiae* isolates, Lane 24 – negative control.

4.2 DISTRIBUTION OF *KDR* MUTATION IN *AN. GAMBIAE*

Out of the 200 samples, 180 (90%) had the *kdr* allele, with 141 (Table 4) showing a homozygous resistant gene (*RR*) (70.5%), and 39 as heterozygous resistant gene (*RS*), 19.5%. High frequencies of the *kdr* gene were recorded at all individual locations (73.9 % - 100 %; Table 6). Labadi Wireless had a total of 100 % for *kdr* allele in all samples collected, including hybrids; whilst Madina scored 100 % for only M and S forms (Table 6). There was no significant difference in *kdr* gene among all locations at $p \leq 0.01$ but different at $p \leq 0.05$, except Roman Ridge and Okyereko, which were not different (Table 7). *Kdr* genotypes were determined on a molecular level as shown in Figure 7.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



M 16 17 18 19 20 21 22 23 24 25 26

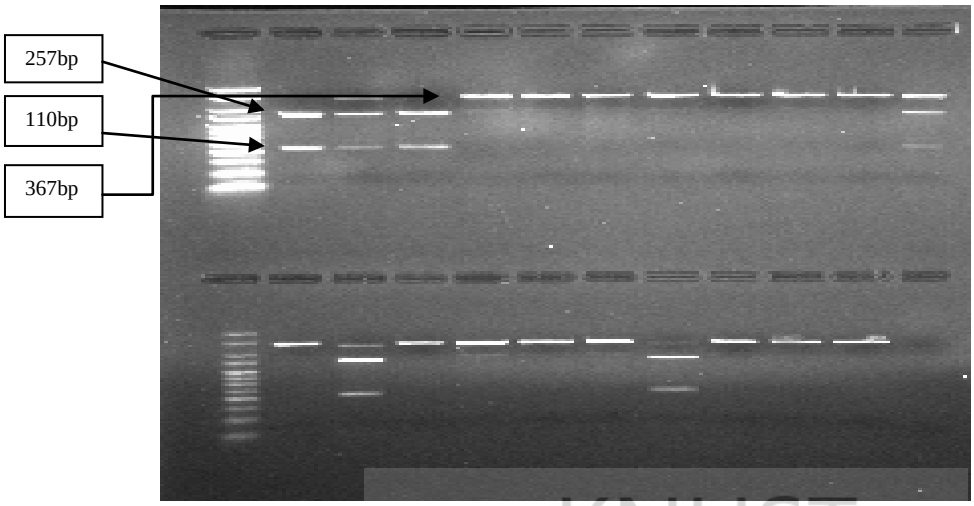
Figure 7: Gel electrophoregram of PCR detection of the *kdr* gene

Lane M – 100 bp DNA marker. Lane 1 – positive control for *RR* genotype. Lane 3 – positive control for *SS* genotype. Lane 16 – positive control for *RS* genotype.

4.3 DISTRIBUTION OF MOLECULAR FORMS IN *AN. GAMBIAE*

Out of the 200 samples M forms represented a frequency of 111 (55.5 %), S forms 58 (29 %), and M/S forms 31 (15.5 %) as shown in Table 3. Among the individual locations, Madina had the lowest M forms (3.3 %; Table 5) and highest M/S forms (70 %; Table 5). Korle-Bu had the highest M forms (96.7 %; Table 6) and lowest S forms (3.3 %; Table 6). Korle-Bu, Kotobabi and Okyereko among all locations did not record M/S forms. Labadi Wireless had the least number of M/S forms and highest S forms. Identification of molecular forms has been shown in Figure 8.

M 1 2 3 4 5 6 7 8 9 10 11



M 12 13 14 15 16 17 18 19 20 21 22

Figure 8: Identification of molecular forms of *An. gambiae* **on 2.0% agarose gel**

Lane M - 1000 bp DNA marker. Lane 1 – positive control for S form. Lane 2 – positive control for M/S form. Lane 4 – positive control for M form.

CHAPTER FIVE

5.0 DISCUSSION

Heavy patronage of pesticides for agricultural purposes and the use of insecticides have been implicated in the generation of *kdr* mutation in *An. gambiae* molecular forms. Therefore, continual monitoring of distribution of the mutation is paramount.

The two molecular (M and S) forms of *An. gambiae* were sympatric in all selected locations in the Accra metropolis, southern Ghana, which is consistent with observations of Yawson *et al*. (2004). Samples from all locations were collected during the rainy season. The M form was predominant in all the selected locations. This was because four out of the six locations namely: Korle Bu, Kotobabi, Roman Ridge and the out-group Okyereko had their samples collected in close proximity to vegetable and rice fields with permanent breeding conditions for M forms (Table 5). *An. gambiae* M forms are found to be associated with flooded and irrigated sites, where extensive rice cultivation is being undertaken, whereas the S forms are markedly associated with rain-dependent breeding sites (Diabaté *et al*., 2003; Touré *et al*., 1998). S forms predominated over their sympatric M forms both in Madina and Labadi Wireless. Hybrid M/S forms were found at Labadi Wireless, Madina and Roman Ridge (Table 5), with frequencies of 6.7 %, 70 % and 26.7 % respectively out of the total numbers analyzed. Della Torre *et al*. (2001), Taylor *et al*. (2001), and Tripet *et al*. (2001) also recorded a similar pattern. The remainder of the sample sites did not record M/S hybrid forms.

The *kdr* mutation was observed in all molecular forms namely; M, S, and M/S hybrids from all selected sites in the Accra metropolis (Table 6). Out of the total samples collected and analyzed only 10 % lacked the *kdr* allele (Table 4). Coluzzi (1982) first reported the

widespread of *kdr* mutation in all molecular forms and attributed it to gene interactions in the environment, which includes the repercussions of environmental modification due to human activity, especially related to the establishment of more permanent settlements and agriculture. Guillet *et al*. (2001) reported high prevalence of *kdr* mutation in the S form and also attributed it to selection pressure resulting from long usage of pesticides in agriculture. Results from this work also showed high levels of *kdr* mutation in the S form (Table 6). Frequencies in the M forms were relatively high (73.9 % - 100 %; Table 6). *Kdr* frequency in the M form was reported to be 78 % in Benin (Corbel *et al*., 2004). Previous reports suggested rather low frequency of the *kdr* in M forms (Yawson *et al*. 2004). It has been proposed that urban agriculture and the extensive use of pesticides could select for resistance to pesticides (Diabaté *et al*., 2002). Results as shown in Table 6 seem to support this proposal. The out-group, Okyereko, previously recorded *kdr* frequency in the M form as 3.38 %, and S form, 95 % (Yawson *et al*., 2004); however, this work has shown an increase in the M form (73.9 %); with S form as 94.1 % (Table 6). This indicates a possible selection for the *kdr* allele in the M form. Moreover, samples from Korle-Bu, Kotobabi, Roman Ridge and Okyereko were collected in vegetable and rice fields where heavy use of insecticides may have subjected *kdr* allele to positive selection, which is reflecting in their high *kdr* frequencies. However, these observations are in contrast to findings by Kristan *et al*., (2003) done in other regions where pyrethroid insecticides had been used heavily on crops as pest control. The high numbers of *kdr* frequency (%) in M/S hybrid forms in Labadi Wireless, Madina and Roman Ridge suggest that there could be numerous adaptive and epidemiologically important genetic exchanges between forms. Even though, introgression could be highly probable further work is necessary to ascertain its occurrence in samples from selected sites.

CHAPTER SIX

6.0 CONCLUSION

Results of this study at selected areas of the Accra metropolis indicated that *kdr* mutation, could be responsible for pyrethroid-based insecticide resistance and cross-resistance to DDT in *An. gambiae*, is widespread in the molecular forms.

Analyses have revealed M form preponderance of frequency 111 (55.5 %) over S form, scoring a frequency of 58 (29 %), and hybrid M/S form, 31 (15.5 %). High *kdr* frequency (%) was represented in S form (80 % - 100 %), M form (73.9 % - 100 %), and M/S form (85.7 % - 100 %) from all selected sites. Samples collected at Okyereko had an increase in *kdr* in M forms (73.9 %) in 2009 as compared to 3.36 % in 2004.

6.1 RECOMMENDATION

- Much more comprehensive studies carried out over a wider range of locations at different periods of the year would be required to assess accurately the distribution and frequencies of *kdr* mutation gene in the molecular forms, and seasonal variation in abundance of the forms.
- Further work will be necessary to confirm whether *kdr* mutation genotype in the M form has been through introgression.
- Future work should include *kdr*-e alleles, and also the application of some alternative techniques that have recently been described to eliminate inconsistency in *kdr* genotyping by polymerase chain reaction (PCR) e.g. Hot Oligonucleotide Ligation Assay (HOLA).
- Effective monitoring of malaria control strategies could adopt some conventional insecticide bioassay tests (WHO standards), and the use of molecular tests that can detect metabolic resistance as well.

CHAPTER SEVEN

7.0 REFERENCES

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CHAPTER EIGHT

8.0 APPENDIX I

8.1 DNA EXTRACTION AND PCR BUFFERS

Tris – borate ethylenediamine tetraacetic acid (EDTA, 0.5 M) (TBE) buffer (pH 8.0):

108 g Tris

55 g boric acid

7.4 g EDTA

1000 ml distilled water

Tris – EDTA, 0.5M buffer (pH 8.0)

12.1 g Tris

3.72 g EDTA

1000 ml distilled water

Lysis buffer (pH 8.0)

0.1 M Tris – HCl, pH 8.6

- 1 M Tris – HCl (pH 8.6)

  157.6 g tris – HCl

  500 ml dd H_20

  Adjust pH to 8.6 with NaOH

  Top up volume to 1 liter.

- 5 M NaCl

  292.2 g of NaCl ⟶ 292.2 g salt in 1000 ml dd H_20

  800 ml dd H_20

  Top up to 1 liter with dd H_20

- 0.5 M EDTA (pH 8.0)

  186.1 g EDTA

  500 ml double distilled water (dd H_20)

  Adjust pH to 8.0 with 10 M NaOH

  Top up volume to 1 liter with dd H_20

- 10 M NaOH (100 ml vol.)

  40 g NaOH

  50 ml dd H_20

  Top up to 100 ml with dd H_20

2.0 % Agarose Gel

1 g agarose powder

50 ml (10x TBE buffer)

4 µl ethidium bromide

8.2 APPENDIX II

Table 3: Frequency of molecular forms for all locations

	Frequency	Percent	Cumulative percent
M-form	111	55.5	55.5
S-form	58	29	84.5
M/S-form	31	15.5	100
Total	200	100	

Table 4: Frequency of *kdr* gene mutation for all locations

	Frequency	Percent	Cumulative percent
RR	141	70.5	70.5
RS	39	19.5	90
SS	20	10	100
Total	200	100	

Table 5: Frequencies of the molecular forms of *An. gambiae* s.s. from sampled sites

		Percentage of molecular forms		
Location	n	M	S	M/S
Korle Bu	30	96.7	3.3	-
Kotobabi	40	82.5	17.5	-
Labadi Wireless	30	26.7	66.7	6.7
Madina	30	3.3	26.7	70
Roman Ridge	30	56.7	16.7	26.7
Okyereko	40	57.5	42.5	-

n – total number of collected samples.

Table 6: Frequency distribution of *kdr* gene mutation from selected sites

	Kdr frequency (%)					
	M-form		S-form		M/S-form	
Korle Bu	29	86.2%	1	-		
Kotobabi	33	97%	7	100%	-	
Labadi Wireless	8	100%	20	100%	2	100%
Madina	1	100%	8	100%	21	85.7%
Roman Ridge	17	82.4%	5	80%	8	87.5%
Okyereko	23	73.9%	17	94.1%	-	

Percent values refer to percentage score of kdr mutation in total number of the form identified.

Table 7: Comparison of distribution of *kdr* mutation in the different locations

Location	*Kdr* Frequency
Korle Bu	86.67±6.67 ab
Kotobabi	97.50±2.50 bc
Labadi	100.00±0.00 c
Madina	90.00±3.33 abc
Roman Ridge	83.34±33.34 a
Okyereko	82.50±2.50 a

Values represent mean values ± SE, (CI, 5%)