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INSECTICIDE RESISTANCE OF MALARIA MOSQUITOES FROM GUINEA CONAKRY

By

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg. May 2008.

DECLARATION

I…… declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

……..

30 May 2008

ABSTRACT

Introduction: There is only limited information on malaria vector species composition, vector status and susceptibility status in Guinea Conakry. This work provides more information from three localities (Boffa, Siguiri and Mount Nimba) with very different ecological settings.

Methods: *Anopheles* mosquitoes were collected resting inside houses. Field bioassays were performed on *An. gambiae* s.l using the standard WHO protocol. Further species identification by PCR, laboratory bioassay by WHO standards, biochemical enzyme analysis, sporozoite ELISA, *kdr* detection by PCR and pyrosequencing were performed.

Results: Of 600 specimens, 494 (82.3%) were *An. gambiae* and 2 (0.33%) *An. arabiensis*. *Plasmodium* infection rates varied from 1.25 to 5%. Resistance to 4% dieldrin and 0.1% bendiocarb need confirmation in Boffa and to 0.05% deltamethrin and 5% malathion in Siguiri. Resistance to DDT, dieldrin and bendiocarb occurred in Siguiri and Mount Nimba. F_1 progeny of wild *An. gambiae* s.s exposed to pirimiphos methyl indicated elevated esterase and monooxygenase enzymes involved in metabolic detoxification of insecticides. The *Kdr-w* mutation is present in both the M and S forms of *An. gambiae*. *Kdr* genotypes by pyrosequencing and sequencing were in agreement but the standard PCR method gave 28% false positives.

Conclusion: Both molecular forms of *An. gambiae* are implicated in malaria transmission, occur in sympatry and exhibit some degree of resistance to insecticides at all localities. The mechanism of resistance is unclear. Pyrosequencing is more accurate for detecting *kdr* than the standard PCR method. Vector control

interventions need to be tailored to each site based on the data collected by on-going monitoring and surveillance.

DEDICATION

This work is dedicated to my parents Mr/Mrs Toh Clement Bumuh and my grand mother Mama Vuwansi Tinyu who died last year 2007. It has always been their wish for their children to acquire knowledge in all disciplines in life. I promise to live up to that expectation.

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Prof. Richard Hunt carried out the field and preliminary laboratory work for AngloGold/Ashanti and BHP-Billiton as a baseline survey prior to implementation of malaria vector control. His work provided me with the specimens on which my research is based. My grateful thanks go to him for this opportunity.

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LIST OF ABBREVIATIONS

ASCHI	Acetylthiocholine iodide
α	alpha
β	beta
%	Percent
µg	microgram
µl	microliter
µM	micro molar
BLAST	Basic Local Alignment Search Tool
Bp	base pair
CDNB	1-chloro-2, 4-dinitrobenzine
DDT	1, 1, 1,-trichloro-2, 2,-bis (p-chlorophenyl) ethane/Diethyl diphenyl tricholoro ethane
DNA	deoxyribonucleic acid
DTNB	5, 5'-Dithio-bis (2-nitrobenzoic acid)
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylene diamine tetra acid (disodium salt)
et al.	and others
g	relative centrifugal force
g/l	grams per liter
h	hour(s)
HCl	hydrochloric acid

ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
kb	kilo base
KCl	potassium chloride
KAc	potassium acetate
KOH	potassium hydroxide
K_2HPO_4	potassium phosphate
M	molar
mg	milligram
Mg_2SO_4	Magnesium sulphate
$MgCl_2$	magnesium chloride
min	minute (s)
ml	milliliter
mM	millimole
NaAc	sodium acetate
NaOH	sodium hydroxide
ng	nanogram
°C	degree celsius
OD	optical density
P	probability level
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
rDNA	ribosomal DNA

rpm	revolutions per minute
s	second(s)
SDS	sodium diodeciol sulphate
UV	ultra violet
vol	volume (s)
χ^2	chi-square

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Malaria is a life-threatening disease in many tropical and subtropical areas and is endemic in over 100 countries which are visited by more than 125 million international travellers a year (WHO, 2005). Globally, malaria accounts for 300 to 500 million clinical cases and 1.5 to 3 million deaths per year (WHO, 1999), 90% of these occurring in Africa.

Vector control interventions rely mainly on the use of pesticides for treating bed nets (ITNs) or the indoor residual spraying of human habitations. Pyrethroid compounds are currently the only approved insecticides for use on ITNs. Unfortunately resistance to pyrethroids has been reported in various countries in Africa such as Benin, Kenya and Cote d'Ivoire (Tripet *et al.,* 2006), Ghana (Coetzee *et al*., 2006) and South Africa (Hargreaves *et al*., 2000). This resistance is fast becoming a serious threat to malaria control programs.

1.2 The malaria parasite

Malaria is caused by a protozoan belonging to the genus *Plasmodium* and four species (*P. falciparum*, *P. vivax, P. malariae and P. ovale*) account for human infections. *Plasmodium falciparum* causes the most severe infections and highest

mortality in Africa. *Plasmodium ovale* and *P. vivax* form resting stages in the liver and once reactivated can cause clinical relapse (Greenword *et al.,* 2005). After inoculation of the *Plasmodium* parasite into the human host by the blood sucking *Anopheline*, sporozoites invade hepatocytes and propagate rapidly by asexual reproduction producing merozoites. The liver cells then release thousands of merozoites which invade red blood cells to produce more merozoites or sexual micro and macrogametocytes which are ingested by the mosquito after a blood meal. Fertilization occurs in the mosquito, producing a zygote which develops into ookinetes. The ookinetes penetrate the midgut and form oocysts. The oocyst undergoes sporogony and releases sporozoites which invade the salivary glands and are then transferred to humans when the mosquito next feeds on human blood (Greenword *et al.,* 2005).

1.3 Malaria Vectors

The malaria parasite is transmitted by various species of *Anopheles* mosquitoes which mainly feed between sunset and sunrise (Gillies and De Meillon, 1968; WHO, 2005). In sub-Saharan Africa, *Anopheles gambiae* Giles, *Anopheles arabiensis* Patton and *Anopheles funestus* Giles are the three main vectors (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

1.3.1 The *Anopheles gambiae* complex

The *Anopheles gambiae* complex includes seven recognised species: *An*. *gambiae* Gillies, *An. arabiensis, An*. *quadriannulatus* species A Theobald (Gillies and Coetzee, 1987), species B (Hunt *et al.,* 1998), *An. merus* Dönitz, *An. melas* Theobald (Evans 1938; Muirhead-Thomson, 1947) and *An. bwambae* White (Davidson and Hunt, 1973; Davidson and White, 1972). These species are discussed below in more detail.

A) *Anopheles gambiae* Giles

Anopheles gambiae s.s is highly anthropophilic and the most efficient malaria vector amongst the members of the complex which is exophagic and endophilic.They go outdoors mainly for egg laying (Gillies and De Meillon, 1968). This fresh water species is widely distributed across Africa and the larvae are found in a variety of situations from permanent wells, and irrigation channels to sunlit temporary pools (Gillies and De Meillon, 1968).

B) *Anopheles arabiensis* Patton

In Africa, *Anopheles arabiensis* is one of the main malaria vectors (Coluzzi, 1970), occurring in sympatry and sharing the same larval habitat with *An. gambiae* s.s. in many afrotropical areas (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Anopheles arabiensis, like *An. gambiae* s.s., is anthropophilic but at a lower frequency (Gillies and Coetzee, 1987). This fresh water species also prefers other

animals apart from humans for blood meals (zoophilic) (Charlwood and Edoh, 1996). *Anopheles arabiensis* exhibits both exophilic (out door resting) and endophilic (indoor resting) behaviours (Gillies and De Meillon, 1968). This makes their control by indoor residual spraying less effective than *An. gambiae* s.s. (Gillies and Coetzee, 1987). *Anopheles arabiensis* shows seasonal variation in population density in relation to rainfall. Their numbers tend to increase by the end of the rainy season but numbers are suppressed during the dry season (White *et al*., 1972).

C) *Anopheles quadriannulatus* Theobald species A

This is a fresh water species and does not transmit malaria as it is strictly zoophilic and exophilic (Gillies and Coetzee, 1987). It was discovered in the former Transvaal region of South Africa (Gillies and De Meillon, 1968) and the larvae are found in small temporally exposed pools such as those formed from footprints and road-ruts (Gillies and Coetzee, 1987).

D) *Anopheles quadriannulatus* species B Hunt, Fettene and Coetzee

This is the East African *An. quadriannulatus* from Ethiopia. They dwell in animal and in mixed human and animal environments (Hunt *et al*., 1998). This species is mainly zoophilic and therefore of little importance in the transmission of malaria (Fettene and Temu, 2003). Crosses between *An. quadriannulatus* species A and B showed genetic incompatibility (Hunt *et al*., 1998).

E) *Anopheles melas* Theobald and *An. merus* Dönitz

Anopheles melas and *An. merus* are the salt water breeding members of the *An. gambiae* complex. *Anopheles melas* is restricted to the coast of West Africa and *An. merus* to East Africa from southern Somalia to the inland areas of South Africa, Mozambique, Swaziland and Zimbabwe (Gillies and De Meillon, 1968; Masendu *et al*., 2005). Both species are allopatric but can be found in sympatry with the other fresh water members of the complex (Muirhead-Thomson, 1951).

Anopheles melas and *An. merus* show a great degree of variation in host preference. *Anopheles melas* is strongly anthropophilic (Gillies and De Meillon, 1968) even when domestic animals are present whereas *An. merus* is mainly zoophilic. *Anopheles merus* bites humans both indoors and outdoors when domestic animals are absent (Gillies and De Meillon, 1968). Compared to *An. gambiae*, they are of lesser medical importance. *Anopheles melas* in West Africa (Gillies and De Meillon, 1968) and *An. merus* in Tanzania (Temu *et al*., 1998) have been shown to be local vectors of malaria.

F) *Anopheles bwambae* White

Anopheles bwambae is the least studied member of the *An. gambiae* complex and is found only in the Semliki forest in Uganda (Davidson and Hunt, 1973; Charalambous *et al*., 1999). Even though *An. bwambae* is anthropophilic and exophilic, it is not thought to be a major malaria vector. This is as a result of its very restricted

distribution (White, 1985). The larvae of this species are found in sunlit pools on the edges of the mineralized hot springs (White, 1974).

1.3.2 Chromosomal forms of *An. gambiae* s.s.

Polytene chromosome studies on *An. gambiae* populations have revealed five chromosomal forms which are based on differences in frequencies of paracentric inversion polymorphisms on chromosome arm 2 (2R), geographical distribution and ecological data such as aridity and breeding sites patterns (Coluzzi *et al*., 1979; Touré *et al*., 1998). The chromosomal forms, which are associated with specific ecological zones, have been designated with non- Linnean nomenclature: Mopti, Bamako, Bissau, Forest and Savanna (Coluzzi *et al*., 1979; Touré *et al*., 1983, 1998; della Torre, 2001). Based on these chromosomal rearrangements, most populations of *An. gambiae* have been found not to be in Hardy-Weinberg equilibrium in West Africa (Touré *et al*., 1998). This is as a result of notable excess of homozygotes for inversions in the right arm of chromosome two (Coluzzi *et al*., 1985).

A) *The Mopti form*

Mopti form with inversions bc and u, is the main taxon in the delta of the river Niger as well as in all irrigated areas in southern Mali eastwards to Burkina Faso.

B) *The Bamako form*

Bamako form carries jcu, and jbcu inversions. It occurs along the upper Niger basin from South Mali to Northern Guinea.

C) *The Savanna form*

Savanna form with j, b, cu and bcu inversions is widely distributed across Africa particularly in savanna areas with a riverine ecology.

D) *The Forest form*

Forest form coexists with the savanna form. It occurs in rain forest and humid savanna. It is characterised by the standard arrangement of chromosome 2 and sometimes contains inversion b or c.

E) *The Bissau form*

Bissau form carries inversion d. It occurs in coastal rice cultivated areas in Guinea Bissau, Guinea Conakry, South Senegal and in The Gambia.

1.3.3 Molecular forms of *An. gambiae* s.s.

Anopheles gambiae s.s. is also classified into M and S molecular forms which exhibit strong assortative mating (Favia *et al.,* 1997). This classification is based on the sequence analysis of both the intergenic spacers (IGS) (Favia *et al.,* 2001) and the internal transcribed spacers (ITS) (Gentile *et al.,* 2001) of their rDNA. The sequence analysis revealed a genetic difference that separated the Mopti form from the Savanna and Bamako forms. The relationship between chromosomal and molecular forms varies with their distribution. In Mali and Burkina Faso, the M molecular form corresponds to the Mopti chromosomal form and the S form to the Savanna and Bamako chromosomal forms Favia *et al*. (1997). This association breaks down in

other parts of Africa, where the M form shows chromosomal arrangements corresponding to Bissau, Forest or Savanna forms while the S form may correspond to the Savanna, Forest or Bamako chromosomal form (Della Torre *et al*., 2002). Both M and S forms have been shown to occur in sympatry with accumulating evidence in support of reproductive isolation between them. Evidence of premating barriers to gene flow exists between both forms in Mali (Tripet *et al*., 2001). The *kdr* allele occurs at high frequency in the S form but not in the M form evenwhen it occurs in both forms in Benin, most likely as a result of introgressive hybridization (Weill *et al*., 2000). M/S hybrids are absent or rare in areas where the two forms occur together as in the case of Cameroon (Wondji *et al*., 2005). Ecological differences exist between the two molecular forms. In Burkina Faso and Mali, the M form has been shown to adapt to permanent breeding sites e.g. in rice field, excavations and artificial lakes while the S form is more adapted to temporary habitats (Diabeté *et al*., 2005). The M form is predominant in the north-south transect in Cameroon with deep vegetation cover and the S form more northward where the forest is degraded (Wondji *et al*., 2005).

1.4 Methods of identification of the *An. gambiae* complex

1.4.1 Morphological analysis

Extensive studies have been carried out on the morphological identification of the members of the *An. gambiae* complex (Ribbands, 1944a, b; Muirhead-Thomson, 1945; Green, 1971; Coetzee, 1989; Lounibos *et al*., 1999). Morphological keys of

Gillies and Coetzee, (1987) can be used for identification of the group but not to species level.

1.4.2 Cross-mating experiments

Cross-mating experiments involve crossing specimens of an unknown sample with reference laboratory strains in order to identify the unknown species (Davison and Hunt, 1973; Paterson, 1962; Muirhead-Thompson, 1947). The same technique was applied by Hunt *et al*. (1998) to demonstrate that *An. quadriannulatus* species B from Ethiopia was a different species to *An. quadriannulatus* species A.

One disadvantage of this technique is that it is laborious and time consuming. As a result, it would not be suitable for large scale use. In addition, it requires laboratory stocks of all species.

1.4.3 Salinity tolerance test

Ribbands (1944) and Muirhead-Thomson (1951) made use of the salinity test to distinguish between fresh and salt water members of the *An. gambiae* complex. First instar larvae are exposed to 23.8g NaCl/L solution for two hours. The fresh water larvae die within an hour while the salt-water species survive.

1.4.4 Isoenzyme electrophoresis

Isoenzyme electrophoresis involves the analysis of enzyme migration by electrophoresis through a gel matrix such as starch or polyacrylamide under the

influence of an electric field (Miles, 1978; Mahon *et al.,* 1976). This technique requires live or liquid nitrogen preserved specimens for analysis and is labour intensive. The overlap in some diagnostic characters (Hunt and Coetzee, 1986) and the advent of new rapid less labour intensive methods such as species-specific polymerase chain reaction (PCR) assays (Scott *et al.*, 1993), has led to a decrease in the use of this method. However, it is cost effective and simple to perform.

1.4.5 Polytene chromosome banding patterns

Distinct banding patterns on the polytene chromosomes of the salivary glands of fourth instar larvae and the ovarian nurse cells of semi gravid females, are used for identification (Coluzzi and Sabatini, 1967; Hunt, 1973). Banding patterns on the X chromosome are species-specific as a result of fixed paracentric inversions. The banding patterns on the X chromosome in *An. gambiae* and *An. merus* are homosequential, but fixed inversions on arm 2R of the autosomes can be used to differentiate between these two species.

The strengths of this technique lies in the stability of intraspecific characteristics for identification, and in the fact that samples can be stored in Carnoy's fixation for later identification.

This technique is not without disadvantages. Only semi-gravid females and fourth instar larvae can be used for identification. A high level of expertise is needed for chromosomal banding pattern interpretation. It is labour intensive and the technique

is unable to differentiate between *An. quadriannulatus* species A and B (Hunt *et al*., 1998).

1.4.6 Cuticular hydrocarbon analysis

Carlson and Service (1980) investigated an alternative method of identifying either sex of adult *An. arabiensis* and *An. gambiae*, the two main vectors of malaria using chemically stable cuticular hydrocarbons. They found that the ratio of the relative abundance of n-heptacosane and hexacosane were significantly different in females of both species.

This technique is not used for identification as it is sophisticated, time consuming and expensive. This said, the technique is useful for field samples as cuticular hydrocarbons are chemically stable and can be used on dead or old adults.

1.4.7 DNA-based methods of identification

A) *DNA probes*

A DNA probe is a single-stranded DNA sequence that anneals to a sequence complementary to itself. Collins *et al.* (1987) produced a probe from ribosomal DNA (rDNA) that was used to distinguish between *An. arabiensis*, *An. melas*, and *An. gambiae*.

B) ***Species-specific polymerase chain reaction (PCR) method***

The PCR based identification technique employs ribosomal RNA gene sequences (rDNA). Firstly, rDNA is extremely useful as it is present in multicellular organisms as well as in bacteria in hundreds of tandem copies per cell nucleus. Secondly, rDNA contains intergenic spacer sequence (IGS) regions where sequences differ between closely related species and are evolutionarily reliable (Scott *et al*., 1993).

Paskewitz and Collins (1990) developed a PCR method by designing three primers that could distinguish between *An. gambiae* and *An. arabiensis*. The universal or forward primer is derived from a conserved region located at the 3' end of the 28S rDNA-coding region while the two species specific primers are derived within the intergenic spacer sequence (IGS). The PCR reaction amplified a 1.3 kb or a 0.5 kb DNA fragment, diagnostic for *An. gambiae* or *An. arabiensis* respectively. Performing this PCR using DNA from the other members of the complex, resulted in no amplification.

The above method was extended by Scott *et al*. (1993) with the design of four species-specific primers for the identification of *An. quadriannulatus* (153 bp), *An. arabiensis* (315 bp), *An. gambiae* (390 bp), *An. melas* (466 bp) and *An. merus* (467 bp). All the primers used by Scott *et al*. (1993) are different from those of Paskewitz and Collins (1990). DNA templates from *An. melas* or *An. merus* produced similar size amplicons and can not be distinguished by agarose gel electrophoresis. Their allopatric distribution ensures that this is not a major problem. The last member of the

complex, *An. bwambae*, is characterised by 390 bp and 690 bp fragments (Townson and Onapa, 1994). Currently a PCR assay developed by Favia *et al.* (1994) for the identification of *An. gambiae* M and S molecular forms is available. Fettene and Temu (2003) were able to distinguish between the *An. quadriannulatus* sibling species A and B using the PCR based assay.

The advantages of PCR based identification techniques are that mosquitoes of both sexes and any life stage can be used. Very little DNA is required, preserved samples can be used, and lastly the fact that it is rapid to perform and easy to interpret results.

The disadvantages associated with this method are that it involves staining agarose gels with ethidium bromide which is carcinogenic. In addition, the laboratory reagents and equipments are relatively expensive but have become more cost effective over the last 10 years.

1.5 Vector control

Malaria vector control forms an important part of the World Health Organization (WHO) global strategy and remains the most generally effective control method for the prevention of malaria transmission (WHO, 2006). In 1955, the WHO proposed the eradication of malaria by the use of residual house spraying (IRS) with DDT. Later in 1975, the WHO reverted from malaria eradication to malaria control mainly due to the appearance of DDT resistance in a range of mosquito vectors such as *Aedes*

tritaeniorhynchus, *Ae. solicitans* and *An. gambiae* from West Africa (Hemingway and Ranson, 2000).

1.5.1 Indoor residual spray

Spraying walls and ceilings of houses with insecticides, aims at killing indoor resting *Anopheles* mosquitoes. The effectiveness of IRS depends on the resting behaviour of the mosquitoes, susceptibility of target mosquitoes to insecticide intoxication, correct insecticide application rate, building material and design of the dwelling. DDT, deltamethrin and bendiocarb are some of the insecticides used for IRS (WHO, 2006). IRS contributes to reducing malaria transmission as the vector is killed at an early age and does not live long enough to transmit the malaria parasites (Pates and Curtis, 2005). It has successfully reduced malaria cases for example, in Bioko Island, Equatorial Guinea (Kleinschmidt *et al*., 2006) and in South Africa (Hargreaves *et al*., 2000).

1.5.2 Source reduction

Another control measure is source reduction. It is aimed at eliminating larval habitats or rendering the habitat unsuitable for the development of larvae. Knowledge of oviposition behaviour of vectors is important as it can help in the identification of breeding sites (Pates and Curtis, 2005). Source reduction can be achieved by educating the community on what to do to prevent mosquito proliferation. This involves draining or filling of pits and ponds, intermittent draining of irrigated areas as well as the maintenance of irrigation channels. This method has not been attempted

in Africa on a large scale to assess its effectiveness. This is because, it is difficult to predict when and where breeding sites will be formed as larval habitats may be small and dispersed. In many situations before the breeding sites are identified the adults have already emerged. *An. gambiae* the primary malaria vector in Africa breeds in small water pools formed after rainfall.

1.5.3 Biological control

Biological control of vectors involves the introduction of predators such as dragonfly nymphs that eat larvae and pupae of mosquitoes or entomopathogenic organisms into breeding sites. The entomopathogenic bacterium, *Bacillus thurigiensis israelensis* serotype H-14 (*Bti*) is the most widely employed biological control agent for mosquito larvae (Service, 1983). Following ingestion, *Bti* act by forming pores, disrupting membrane transport and lysing the brush border membrane of columnar epithelial cells of the gut lumen, leading to death of host (Lacey *et al*., 2001). *Bacillus sphaericus* though not widely used has some advantages over *Bti* in that it is more persistent in polluted habitats and can be recycled. Its disadvantage is that it has a narrow host range (Lacey *et al*., 2001). Biological control agents are better than chemical larvicides in that they are very species specific, environmentally safe and appear not to induce resistance when used together (Mulla *et al*., 2003). Biological control using *Bti* has been successful in many African countries such as Burkina Faso (Skovmand and Sanogo, 1999) and The Gambia (Majambere *et al*., 2006).

Mosquito fish *Gambusia affinis* and *G. holbrooki* are indiscriminate feeders that feed on tadpoles, zooplankton and aquatic insects. These fish are easily reared and are available locally (Rose, 2001). Larvivorous marsh fish are however often ineffective during periods of heavy rainfall or tidal intrusion due to immediate hatching of large numbers of mosquito eggs, dilution of the fish density and delayed increase in the fish numbers (Pates and Curtis, 2005). Success of this method in Africa is limited as the larvae of *An*. *gambiae* and *An. arabiensis* for the most part prefer temporary rain pools which can not accommodate fish (Gillies and Coetzee, 1987).

1.5.4 Genetic control

Genetic control of vectors is one of the numerous control methods currently considered for the eradication of vectors. It could be done by sterile insect techniques (SIT) in which males are sterilized (Pates and Curtis, 2005) either by subjecting the insect to gamma-irradiation or to chemosterilants, e.g. cobalt-60 (Coleman and Alphey, 2004; Davidson, 1969). The sterilized males are then released so that they mate with wild females causing them to lay sterile eggs. In theory, with the release of enough sterile males, the target population will collapse and eventually be suppressed. Apart from the sterilization approach, techniques are being tested involving the use of genetically engineered mosquitoes that interfere with ookinete invasion or oocyst differentiation at the midgut and blocking invasion of the salivary gland by the parasite (Shigeto, 2006).

Chemicals in the form of insecticides will continue to play a major role in vector control for the foreseeable future and the next chapter deals with the important issue of insecticide resistance.

CHAPTER TWO

RATIONALE AND OBJECTIVES

2.1 Insecticides used for vector control

Four classes of insecticides are currently used in the control of malaria vectors. The insecticides and their discriminating doses used in susceptibility testing (WHO 1998) when field populations are screened for resistance to these insecticides are given in Table 2.1.

Table 2.1: Insecticides and their discriminating doses

Insecticide Class	**Insecticide**	**Discriminating doses** (Exposure time =60 min)
Organochlorines (OC)	DDT Dieldrin	4% 4%
Organophosphates (OP)	Malathion Fenitrothion Pirimiphos-methyl	5% 1% 0.9%
Carbamates (C)	Propoxur Bendiocarb	0.1% 0.1%
Pyrethroids	Permethrin Deltamethrin Lambdacyhalothrin Cyfluthrin Etofenprox	0.75% 0.05% 0.05% 0.15% 0.5%

Insecticides were first used to control adult malaria vectors in the early 1930s in South Africa with the use of pyrethrum extracted from chrysanthemum flowers (Park Rose, 1936; Coetzee and Hunt, 1998). Pyrethrum was then mixed with kerosene and

sprayed inside houses targeting indoor resting mosquitoes with the intention of reducing the incidence of malaria (Ingram and De Meillon, 1927).

The discovery of the insecticidal properties of DDT during the 1940s, led to it being used as the main insecticide for spraying houses in an attempt to control malaria. In the 1940s and 1950s, pyrethrum was replaced by the organochlorines dichlorodiphenyl trichloroethane (DDT) and dieldrin (Pampana, 1969). The use of DDT has been opposed by house holders who dislike the marks left on walls after DDT-spraying as well as development of resistance by non-target pest such as bedbugs. The use of DDT has also been hindered by environmentalists due to its toxicity to the environment (Maharaj *et al.,* 2005). Target mosquitoes such as *An. arabiensis, An. gambiae, An. stephensi* have also been reported to have developed resistance to DDT (Brown, 1986; Coetzee *et al*. 2006; Hargreaves *et al*. 2003). The development of resistance to dieldrin and DDT by mosquitoes and the environmental impacts of these insecticides led to the total banning of dieldrin and the discontinued use of DDT in agriculture (Coetzee *et al.,* 1999). Currently DDT and pyrethriods are the insecticides of choice for malaria mosquito control in South Africa (Maharaj *et al.,* 2005; Hargreaves pers.com).

2.2 Mode of action of insecticides

All the recommended insecticides used for vector control target the central nervous system. However each insecticide class targets a unique site as demonstrated in figure 2.1.

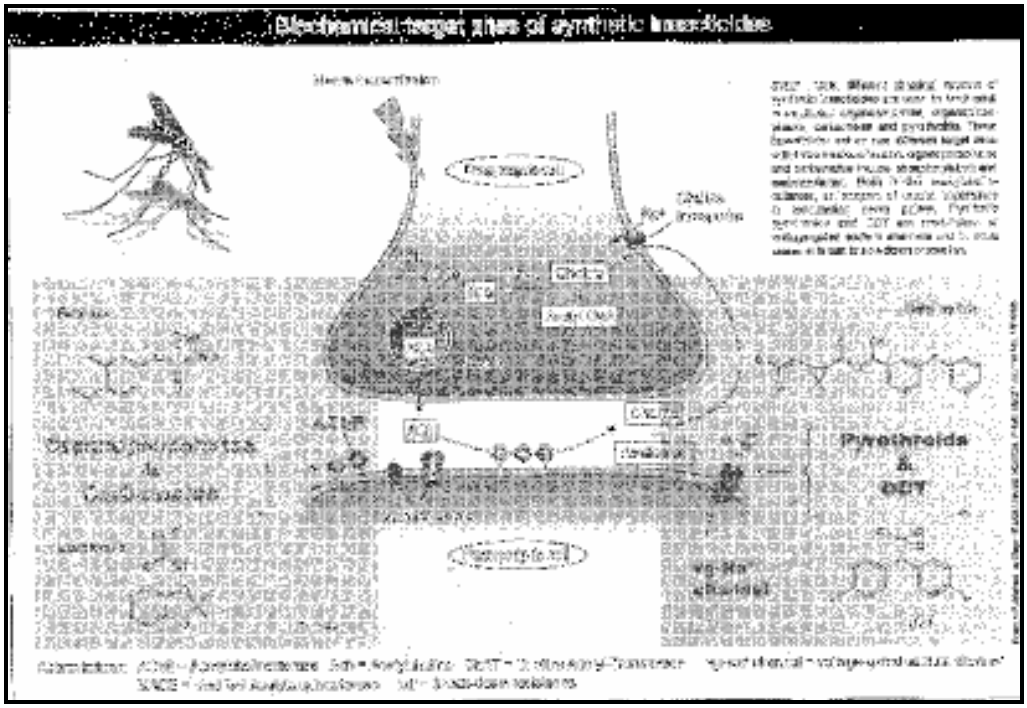


Figure 2.1: Action sites of insecticides used for vector control. (Nauen, 2006)

2.2.1 Action of Organophosphates (OPs) and Carbamates

Organophosphate and carbamate insecticides target acetylcholinesterase (AChE) in the nervous systems of insects. Acetylcholine is released after stimulation of a motor neuron by an impulse into the synaptic cleft where it binds to acetylcholine receptors on the post synaptic membrane. The combination of the neurotransmitter and the receptor results in the transmission of a nerve impulse. AChE catalyses the hydrolysis of acetylcholine to choline and acetate thereby terminating nerve impulse transmission. OP and carbamates act on AChE, inhibiting its action. Organophosphates phosphorylate and carbamates carbamylate the serine residue in AChE active site, resulting in its inability to bind to acetylcholine (Hemingway *et al*., 2004). Acetylcholine therefore binds permanently to its receptors on the post synaptic

membrane resulting to a continuous influx of sodium ions into the post synaptic knob. This depolarizes the postsynaptic cell ensuring continuous actions potential which eventually kills the insect (Hemingway *et al*., 2004).

2.2.2 Action of Pyrethroids

Pyrethroids act by targeting the voltage gated sodium channel of the nervous system (Brengues *et al*., 2003). When pyrethroid binds to the voltage gated sodium channel, they prevent the open channel from closing again. This results in the influx of sodium ions and out flux of potassium ions, preventing repolarization of the action potential leading to continuous nervous stimulation. This explains the observed tremors exhibited by poisoned insects as they lose control of their nervous system (Liu *et al.,* 2006).

2.2.3 Action of Organochlorines

DDT acts on the same target site as pyrethroids (Brengues *et al*., 2003). The mode of action of DDT has not been clearly established but it destroys the balance of sodium and potassium ions within the nerve axon, such that normal nerve impulses are prevented (Whiteacre and Ware, 2004).

2.3 Insecticide resistance

Insecticide resistance is defined by WHO as "The development of an ability in some individuals of a given organism to tolerate doses of toxicant which would prove lethal

to a majority of individuals in a normal population of the same organism" (WHO, 1998).

Resistance to insecticides has been proven in most cases, to be genetically controlled (Brown, 1986). The resistance allele may be recessive as in certain DDT-resistance cases or dominant as in organophosphate resistance or co-dominant, with the resistant-susceptible heterozygotes being intermediate as in the case pyrethroid resistance in *An. funestus* (Okoye *et al*., 2008). Resistance is not general to all insecticides but specific to the insecticide which selects the resistance. Cross-resistance to other insecticides in the same molecular group exists when the insecticides share the same target sites (Brown, 1986). It can also be mediated by metabolic detoxification even when the insecticides don't share the same target sites. Esterase based resistance to fenitrothion and deltamethrin are an example of this (Brogdon and Barber, 1990) The development of resistance by mosquitoes to larvicides and adulticides was first observed in 1949 when salt-marsh mosquitoes *Aedes taeniorhynchus* and *Ae. sollicitans* showed resistance to DDT in Florida (Brown, 1986). The development of dieldrin resistance in a population of *An. gambiae* was reported from northern Nigeria as early as 1956 (Davidson, 1956).

2.3.1 Mechanisms of insecticide resistance

Resistance to insecticides is conferred by a limited number of mechanisms in insects. This predominantly involves the metabolic detoxification of the insecticide before it reaches its target site or a change in the sensitivity of the target site such that the

insect is no longer susceptible to the insecticide (Ranson *et al.,* 2000). Of minor concern are vigour tolerance, a non specific defence mechanism to insecticides resulting from improve condition, body weight and behavioural resistance, a situation where the vector has the ability to avoid contact with an insecticides (WHO, 1970).

A) *Metabolic resistance*

Metabolic resistance mechanisms involve the detoxification of insecticides through enzymes such as esterases, glutathione S-transferases (GSTs) or monooxygenases (MOs) (Hemingway and Ranson, 2000). The activity of one or more of these three families is often elevated in resistant insects but their molecular mechanisms for resistance are not well understood. This is due largely to the fact that the metabolizing enzymes belong to large families with members having overlapping physical properties, substrate specificities and that the preparation of pure individual enzymes is difficult (Ranson *et al.,* 2000).

i) *Esterase-Based resistance*

Esterases or carboxylesterases are a group of heterogeneous enzymes present in most organisms but with diverse functions in insects that include proteolysis, nervous system function, hormone metabolism and insecticide metabolism or sequestration (Liu *et al.,* 2006). Esterases act by catalysing the hydrolysis of ester bonds in insecticides. Esterase based resistance is common to organophosphate and carbamate insecticides and produces broad spectrum resistance through rapid binding and slow turnover of insecticides, i.e. sequestration or narrow spectrum resistance through

metabolism. Elevated levels of esterase have been strongly correlated with resistance in some *Culex* mosquitoes (Bisset *et al*. 1995).

ii) ***Glutathione S-transferase based resistance***

Glutathione S-transferases (GSTs) consist of a family of multifunctional, intracellular enzymes that catalyze the conjugation of glutathione to exogenous and endogenous compounds which include chemical carcinogens, therapeutic drugs and products of oxidative stress (Morel *et al*., 2004). Eukaryotes have GSTs with different substrate specificities to accommodate their wide catalytic functions. These substrates include unsaturated carbonyls, reactive DNA bases, epoxides and organic hydroperoxides produced during oxidative stress (Vontas *et al.,* 2001). Generally, GSTs can be classified into two distantly related groups, based on their location in a cell: microsomal and cytosolic. However, a third group, the Kappa (κ) class in mammals which is structurally different from the microsomal and cytosolic GSTs is located in the mitochondria and peroxisomes (Enayati *et al.,* 2005).

GSTs are implicated in resistance to the following classes of insecticides: GST activity in the diamondback moth detoxifies OP via O-dealkylation or O-dearylation reaction (Huang *et al*., 1998). Resistance to DDT is accomplished by GSTs catalysing the dehydrochlorination of DDT forming the non-toxic DDE. This occurs through the abstraction of hydrogen by thiolate anion at the active site of GST resulting in the elimination of chlorine from DDT. This resistance mechanism has been reported in *An. gambiae* (Ranson *et al*., 2001). GSTs confer resistance to pyrethroids by

detoxifying lipid peroxidation products induced by pyrethroids or by sequestering pyrethroids (Vontas *et al*., 2002).

iii) *Monooxygenases*

Cytochrome P450s, also termed monooxygenases, is a superfamily of heme-thiolated enzymes which are common in prokaryotic and eukaryotic organisms (Schwaneberg *et al*., 1999). These enzymes are remarkable in that they are numerous and perform different functions. The P450 enzymes are also found in insects where they play a very crucial role in the biosynthetic pathways of juvenile hormones, ecdysteroids and are involved in metabolising insecticides (Feyereisen, 1999). Generally, the reaction for P450 mediated mono-oxygenation of a hydrocarbon substrate involves the addition of an oxygen atom to the substrate producing oxygenated metabolites and water (Lewis *et al.,* 1998).

Elevated levels of CYP6Z1, a type of P450, have been associated with pyrethroid resistance in *An. gambiae* from Western Kenya (Nikou *et al*., 2003) and CYP6D1 with organophosphate resistance in the housefly, *Musca domestica* (Hemingway *et al*., 2004)

Detection of metabolic resistance

Assays have been designed to quantify detoxifying enzymes activity or their levels in individual samples. Detection is based on using substrates specific to enzymes in a colorimetric reaction. The enzyme level or activity is quantified by taking optical

density readings and comparing them to a standard curve of known concentration (Penilla *et al*., 1998).

B) ***Changes in target site sensitivity***

Target site resistance mechanisms involve mutations in the acetylcholinesterase, GABA receptor genes and sodium channel (Hemingway *et al.,* 2004).

i) ***Insensitive acetylcholinesterase***.

Two acetylcholinesterase (AChE) genes (*ace*-1 and 2) have been identified in *An. gambiae* (Hemingway *et al.,* 2004). The *ace*-1 gene has been studied in different species of mosquitoes including *An. gambiae*, *Ae. Aegypti* and *Culex* and is linked to resistance (Hemingway *et al.,* 2004; Weill *et al.*, 2004). Mutations in the *ace*-2 gene that codes for the acetylcholinesterase active site, have been found to be responsible for AChE insensitivity in *Drosophila melanogaster, Musca domestica* and the olive fruit fly, *Bactrocera oleae* (Weill *et al.,* 2004).

ii) ***GABA receptor mutations***

The insect neurotransmitter γ-aminobutyric acid (GABA) receptor is the target site of cyclodiene insecticides such as dieldrin and newly introduced phenylpyrazoles such as fipronil (ffrench-constant *et al.,* 2000). Resistance to these insecticides is accomplished by the replacement of an alanine with serine or glycine residue at position 302 in the GABA receptor gene. This mutation destabilizes the insecticide's preferred conformation of the receptor thereby reducing insecticide binding (ffrench-

constant *et al*., 2004). Resistance to cyclodiene has been extensively studied in *D. melanogaster* (Hosie *et al.,* [1997] cited in Hemingway *et al.*, [2004]) and *Ae*. *aegypti* (Thompson *et al*., [1993] cited in Hemingway *et al.*, [2004]). Resistance to dieldrin in *An. gambiae* has recently been reported from Ghana (Brooke *et al.,* 2006).

iii) ***Voltage gated sodium channel***

The term knockdown resistance (*kdr*) is used in cases of resistance to DDT and pyrethroid in insects resulting from insensitivity of the voltage gated sodium channel (Liu *et al*., 2006). This type of resistance was first characterized in the house fly, *Musca domestica* (Miyazaki *et al.,* 1996).

The voltage gated sodium channel is a transmembrane protein complex forming a water filled pore through the lipid bilayer (Zlotkin, 1999). The channel consists of four homologous repeated domains (I-IV) with each domain consisting of six putative transmembrane helical segments (S1-S6) (Zlotkin, 1999). The fourth segment, S4, is conserved in all four domains and has a positively charged amino acid residue followed by two non polar residues (Zlotkin, 1999). In insects resisting knock down to pyrethroids and DDT, amino acid substitution of leucine by phenylalanine was observed in domain II segment six (IIS6) in the sodium channel gene and this resistance type was termed knock down resistance (*kdr*) (Figure1.2) (Zlotkin, 1999).

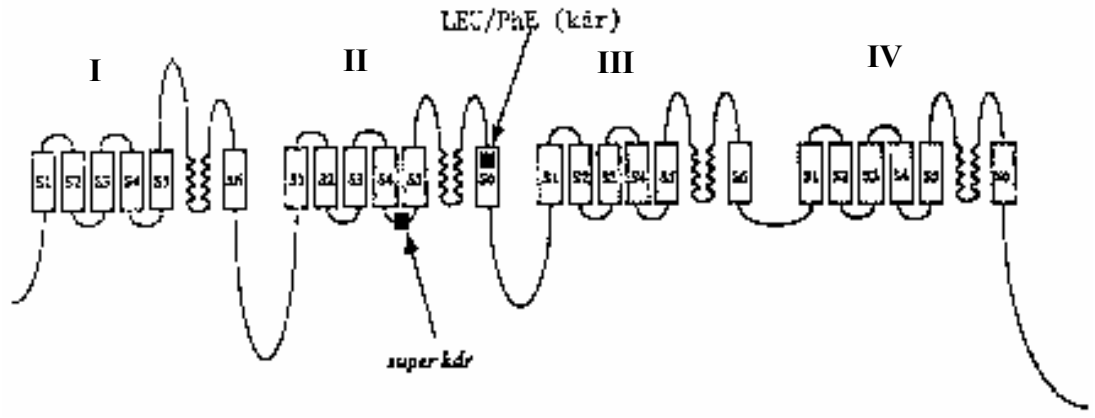


Figure 2.2: General *kdr* mutation in the para-sodium channel gene of resistant strains in medical vectors (Hemingway and Ranson, 2000). In *Anopheles gambiae*, both the West and East African *kdr* mutations occur at the same point in domain II segment 6. Both mutations have been associated to pyrethroid and DDT resistance in the malaria vector mosquito. The super *kdr* mutation between segments S4 and S5 causes a higher resistance.

In *M. domestica*, *D. melanogaster* and other insect species, *kdr* is due to a mutation in the CTT codon (position 1014) encoding for leucine resulting in TTT encoding for phenylalanine (Martinez-Torres *et al.,* 1998). In *An. gambiae* from West Africa, the single nucleotide polymorphism (SNP) in the voltage sodium channel gene is a change from leucine (TTA) at position 1014 to phenylalanine (TTT) which was first detected in the savanna chromosomal forms in Cote d'Ivoire. The East African substitution detected in Kenya involves a change from the same leucine (TTA) at position 1014 to serine (TCA) at the same codon as in West Africa (Tripet *et al*., 2006; Ranson *et al*., 2000). The West African mutation has been reported in the savanna chromosomal forms in Ghana, Mali, Benin and Burkina Faso (Tripet *et al.,* 2006). Weill *et al.,* (2000) suggested that the *kdr* mutation probably reached the M form population from the S form through introgression.

A second type of *kdr* known as a super *kdr* is found within the intracellular S4-S5 loop of domain II of the same sodium channel gene. It involves a substitution of methionine by threonine (Zlotkin 1999). The occurrence of super *kd*r alone provides low resistance but in the presence of the *kdr* in domain II segment six, causes a higher resistance and hence the name super *kdr* (Liu *et al*., 2006).

Methods for detecting kdr

Polymerase chain reaction assay

This method described by Martinez-Torres *et al.* (1998), is used for the detection of pyrethroid knockdown resistance (*kdr*) in the malaria vector *An. gambiae s.s*. Domain II of the sodium channel gene has two introns with intron 2 located 4 base pair (bp) downstream of the *kdr* mutation. To detect the West African *kdr* mutation, a PCR assay has been developed using four primers in a multiplex assay. Two primers, AgD 3 (specific for the *kdr* resistant sequence, TTT) and AgD 4 (specific to for the *kdr* susceptible sequence, TTA) are genotype specific. Primers AgD1 and AgD2 are respectively located down and upstream of the *kdr* mutation (Figure 2.3).

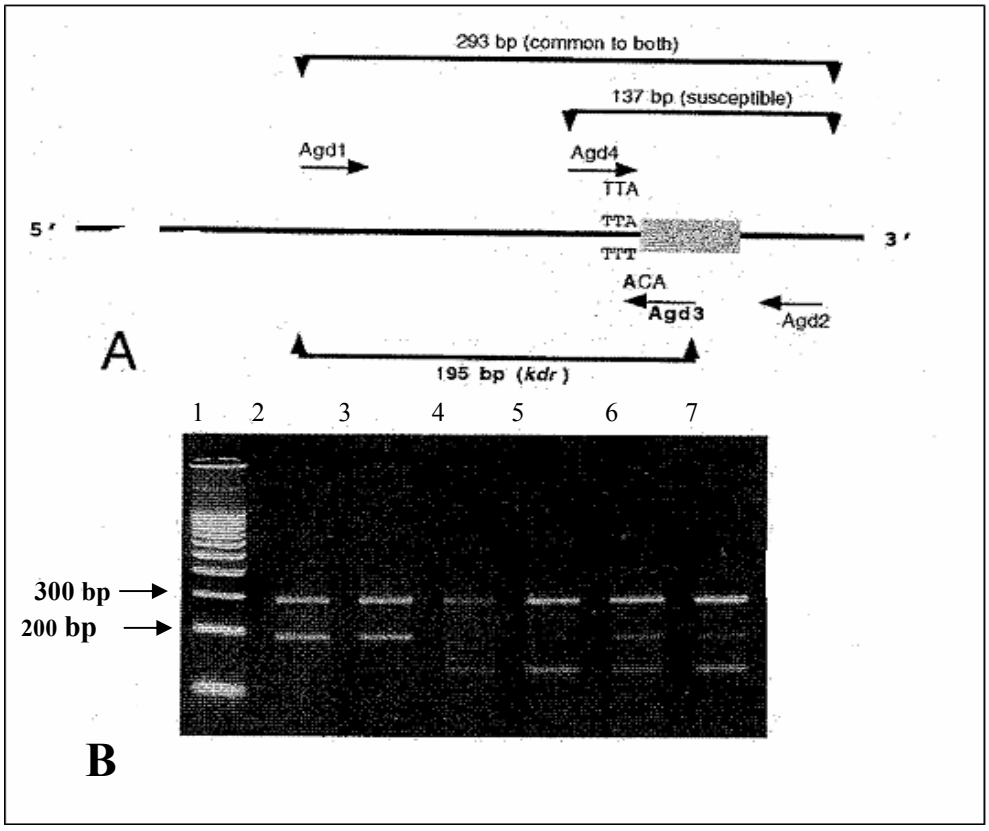


Figure 2.3 (A): Schematic representation of PCR-based diagnostic test for *kdr* detection. (B): PCR products separated on a 1.5% agarose gel (Martinez-Torres *et al*. 1998). Lane 1: molecular marker, Lane 2-3: homozygous RR, Lane 4-5: homozygous SS, Lane 6-7: heterozygous RS.

Three fragments are obtained from the PCR product. A 293 bp common amplicon together with a 195 bp fragment represents a resistant (RR) genotype. The common amplicon with a 137 bp fragment represents a susceptible (SS) genotype. The common fragment could occur together with both the 137 bp and 195 bp fragments indicating a heterozygous (RS) individual. The East African *kdr* mutation is detected in the same way as the West African *kdr* mutation, but the AgD3 primer is replaced with the AgD5 primer. Table 2.2 shows the primer sequences for this PCR.

The problems associated with the use of this method include the fact that it is not reliable as it is dependent on a single SNP and so sequencing needs to be done on the PCR products to confirm the mutation where results are inconclusive. False positive results are sometimes obtained (Matambo *et al*., 2007). For this reason alternative assays have recently been developed to simplify *kdr* detection and are discussed below.

Table 2.2: Primer sequences for detecting *kdr* genotypes (Martinez-Torres *et al*., 1998)

Primer Name	Primer sequence
AgD1	5' ATAGATTCCCCGACCATG 3'
AgD2	5' AGACAAGGATGATGAACC 3'
AgD3	5' AATTTGCATTACTTACGACA 3'
AgD4	5' CTGTAGTGATAGGAAATTTA 3'
AgD5	5' TTTGCATTACTTACGACTG 3'

Hot Oligonucleotide Ligation Assay (HOLA)

The HOLA method described by Lynd *et al.,* (2005) was developed for the detection of both East and West African *kdr* alleles in homozygous and heterozygous states. Basically, a PCR reaction is done using the primers AgD1 and AgD2 to amplify a 293 bp fragment flanking the *kdr* mutation region. PCR product is used in a hot ligation reaction with detector (5' end modified with biotin) and reporter (5'end phosphorylated and 3' end labelled with fluorescein) oligonucleotides. Four reactions are set up for each sample. A resistant and susceptible test is set up for both the West

African and the East African *kdr* alleles. The resistant and susceptible oligonucleotides are specific. Each reaction consists of a reporter and either a resistant or susceptible oligonucleotide. Where there is a ligation between the reporter and detector, an added antifluorescein antibody will bind to the 3' end of the reporter. In the presence of a substrate, the HSP-conjugated antifluorescein Ab will act on the substrate producing a bright blue colour while a negative reaction remains colourless. The reaction can be quantitatively analysed using a plate reader. Table 2.3 shows analysis of West and East African *kdr* genotypes.

Table 2.3: Analysis of both West and East African *kdr* mutation by colour change

Susceptible west	Resistant West	Susceptible East	Resistant East	Genotype
√√		√		SS-West
√		√√		SS-East
	√			RR-West
			√	RR-East
	√		√	RR-West/East
		√	√	RS-East
√	√			RS-West

This method is more reliable than the traditional multiplex PCR method, inexpensive and sensitive for heterozygote detection. However, four separate hot ligation reactions to genotype specimens for the East and West African *kdr* mutations are needed and it is more labour intensive than the PCR method.

Fluorescence Resonance Energy Transfer/Melt Curve Analysis (FRET/MCA) method

This is a real-Time PCR assay developed by Verhaeghen *et al.* (2006). This assay requires the use of a ROX labelled forward primer (AgdF-ROX), a probe KDR-FAM which is FAM labelled at its 3' end and a reverse primer AgdR. ROX and FAM are fluorescent dyes which produces emission upond excitation. Primers AgD1 and 2 (Martinez-Torres *et al*., 1998) are used to amplify a region flanking the *kdr* mutation by PCR. During a secondary PCR, primers AgdF-ROX and AgdR are used to amplify a 121 bp fragment labelled with ROX. The fluorescent probe hybridizes to the PCR products and during melting point analysis, the probe will dissociate from the amplicon and a decrease in fluorescence will occur. Changes in fluorescence appear as a peak on a plot of the first negative derivative of fluorescence against temperature.

Control melting curves are obtained for leucine to serine, leucine to phenylalanine and the wild type *kdr* mutations and their melting temperatures are noted. Samples are tested and their associated graphs compared with those of the controls. The disadvantage of this technique is that it requires real-time PCR equipment which is costly and not affordable by many resource poor laboratories.

Sequence-Specific Oligonucleotide Probe (SSOP) method

The SSOP assay starts with a PCR amplification of the region of interest containing the point of mutation. Amplification is achieved with the aid of a forward primer and a 5' end biotin labelled reverse primer as described by Kulkarni *et al,* (2006). The

PCR product is denatured and added to the wells of a streptavidin coated ELISA plate containing heated 3'end digoxigenin-conjugated SSOPs targeting the three *kdr* genotypes. The SSOP is hybridised to the PCR amplicon and visualised after substrate addition. Optical density readings for quantitative analysis using an ELISA plate reader can be carried out. Interpretation of the *Kdr* genotype alleles is similar to the HOLA method.

2.4 New technology for detecting Single Nucleotide Polymorphisms (SNPs)

Pyrosequencing is real time sequencing by synthesis, catalyzed by a cascade of four enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) (Ramon *et al*., 2003). A single-stranded DNA molecule with a sequencing primer hybridized next to the region of interest is used as template for DNA polymerase. Four different deoxynucleotides (dNTPs) (dATP, dTTP, dCTP, dGTP,) are sequentially added and then incorporated into the growing DNA strand by the polymerase. Incorporation only takes place when a dNTP is complementary to the base in the template and is followed by the release of an inorganic pyrophosphate (PP_i). The released PP_i is in a quantity proportional to the incorporated nucleotide. ATP sulfurylase converts PP_i to ATP in the presence of adenosine 5' phosphosulfate (APS) (Ramon *et al*., 2003).

The ATP produced is needed to drive a luciferase catalyzed reaction involving the conversion of luciferin to oxyluciferin with the release of light (Figure 2.4). The released light, which is directly proportional to the amount of ATP, is detected by a charged coupled device camera and displayed as a peak in the pyrogram. The peak

height is directly proportional to the number of nucleotides incorporated (Ronaghi, 2001). The apyrase functions in the continuous degradation of unincorporated dNTP and excess ATP after which, the next dNTP is added for a new pyrosequencing cycle. Pyrosequencing provides an accurate means of analysing polymorphic DNA for a large number of samples.

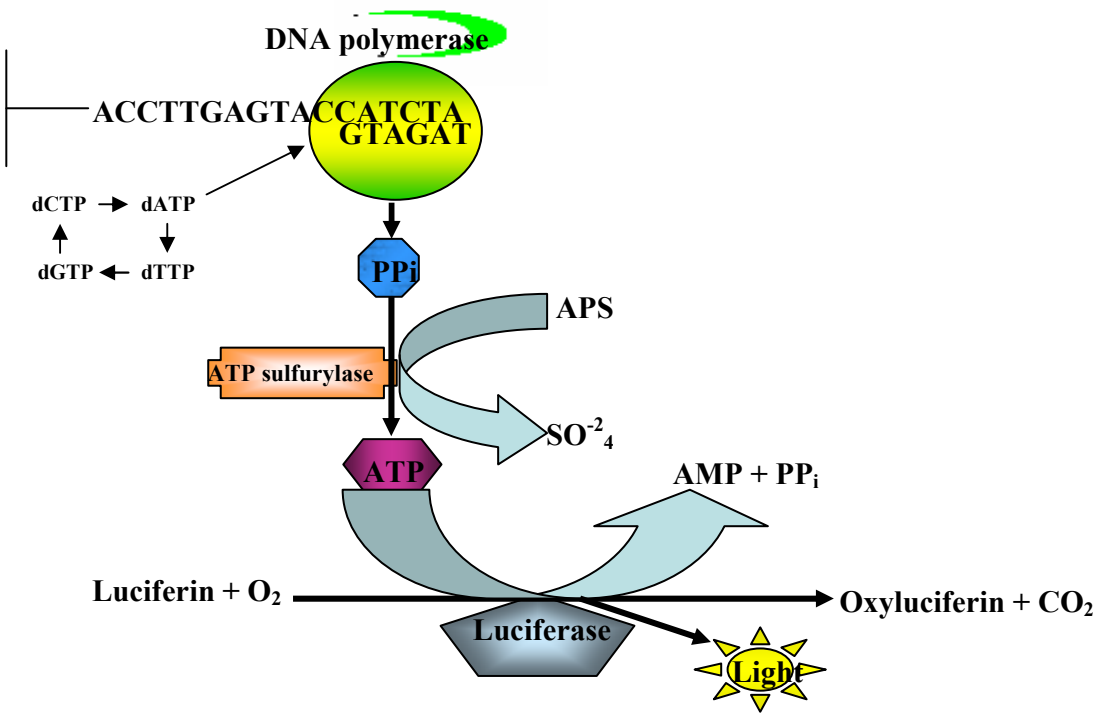


Figure 2.4: Schematic representation of pyrosequencing reaction. Pyrophosphate (PP_i) produced during DNA synthesis is converted by a series of enzymes to light. The light is seen as a peak on a program with peak height proportional to the number of nucleotides incorporated into the growing DNA strand (Adapted from Ronaghi, 2001).

Pyrosequencing has been successfully used to detect SNPs in the *rpoB* gene of *Bacillus anthraci* (Wahab *et al*., 2005); in a human leukocyte antigene (Ramon *et al*., 2003); and sequence determination of difficult secondary DNA structures (Ronaghi *et*

al., 1999). So far it has not been used for genome sequencing as a result of its limitation in read length. Currently there is no available documentation indicating its use in detecting SNPs in the sodium channel gene of *An. gambiae* s.s.

2.5 Research rationale

Success of economic development in African countries such as Guinea Conakry is inhibited by the burden of malaria. Vector control interventions are needed to reduce this burden. The use of insecticides for the control of vectors of diseases has been successful in many parts of the world but the development of insecticide resistance in malaria vectors has a major impact on the disease transmission. Baseline information on vector susceptibility to insecticides and a clear understanding of the resistance mechanisms is important in making informed decisions by vector control managers. Baseline studies to determine the vector composition, sporozoite infectivity rate and insecticide susceptibility need to be carried out in order to inform the decision makers on control strategies. It was with these in mind that two mining houses operating in Guinea Conakry contracted the services of Prof Richard Hunt (consultant entomologist in the VCRU, NICD) to carry out the baseline surveys.

Countries bordering Guinea Conakry are known to have knockdown resistance frequencies in *An. gambiae* s.s. of more than 70% in Cote d'Ivoire (Touré *et al*., 2007) and 62% in Mali (Fanello *et al*., 2003). In Cote d'Ivoire, the *kdr* frequency was found to range from 0% to 95% in six localities (Chandre *et al*., 1999). These frequencies are very high and therefore the *kdr* mutation was expected to occur in

Guinea Conakry populations for which no documentation on malaria vector susceptibility was available. Three widely separated sites were visited across Guinea Conakry. The *kdr* mutation has traditionally been detected by PCR but because of the difficulties in detecting this mutation using PCR, pyrosequencing was investigated as an alternative method.

2.6 Objectives

2.6.1 General objective

Determination of the malaria vector composition, malaria transmission status, and insecticide susceptibility in three sites (Siguiri, Mount Nimba and Boffa) in Guinea Conakry.

2.6.2 Specific objectives

1) Identify major malaria vector species and molecular forms of *An. gambiae s.s.* in three localities in Guinea Conakry.

2) Determine malaria sporozoite infection rate in the mosquito from the three localities using ELISA.

3) Determine insecticide susceptibility status of different vectors species

4) Investigate the underline resistance mechanism(s) if resistance is found

5) Investigate pyrosequencing as an alternative *kdr*–detection technology to the PCR method.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Guinea Conakry is located in West Africa bordering the Atlantic Ocean between Guinea Bissau and Sierra Leone. It is bordered by Guinea-Bissau and Senegal to the north, Mali to the north and north-east, Cote d'Ivoire to the south-east, Liberia and Sierra Leone to the west of the south (Figure 3.1). The population is 6,549,336 and has a total surface area of 246,048 km^2. The climate is tropical with wet and dry seasons. Average temperature ranges from 22-32°C and average rainfall is 430cm/annum. The major agricultural products include rice, coffee, pineapples, cattle, sheep, goats, bananas and timber. There is no formal malaria vector mosquito control program. Inhabitants' uses mosquito coils, spray cans and bed nets as control measures.

Sample collections and field susceptibility tests were done at three sites in Guinea Conakry: Siguiri, 1380ft, 11° 32.836' N, 009° 18.413' W; Mount Nimba, 1632ft, 07° 43.485' N, 008° 24.380' W and Boffa, 46ft, 010° 10.85' N, 014° 01.648' W (Figure 3.1). This was carried out by Prof Richard Hunt as part of baseline surveys for Anglogold/Ashanti and BHP Billiton prior to implementation of malaria vector control programmes at these sites.



Figure 3.1: Map of Guinea Conakry. Study sites are circled (hhtp://science.howstuffsworkers.com/map-of-guinea.htm)

3.2 Sample collection

Mosquitoes were collected resting indoors during July-August 2006. They were identified morphologically as belonging to the *An. gambiae* complex using the keys of Gillies and Coetzee (1987).

3.3 WHO Susceptibility tests

The wild *An. gambiae* s.l. were exposed to 4 % DDT, 0.05 % deltamethrin, 5 % malathion, 0.1 % bendiocarb and 4 % dieldrin in accordance with the WHO (1986)

susceptibility test protocol. A record of mortality after 24 hours of exposure was kept. Dead and alive individuals were stored separately on silica gel for laboratory analysis.

3.4 Rearing of the specimens

Live wild-caught females from Boffa were transported to the NICD in Johannesburg. Isofemale lines were reared in the Botha De Meillon insectary, which is maintained at a temperature of 25 ± 2 °C and relative humidity of 75 ± 5 %. Illumination was effected with the aid of grow lights controlled by a time switch with a 12 hour light and 12 hour dark cycle coupled with dusk/dawn transition lighting. Eggs of wild-caught females were transferred to plastic bowls 1/3 filled with distilled water. The emerging F_1 larvae were fed with a mixture of powdered yeast and dog biscuit until pupation and adult emergence. Each isofemale line was split in two, one portion being used for susceptibility bioassays and the other frozen at -70 °C for biochemical analysis.

3.5 Laboratory bioassay

The adult F_1 progeny were exposed to 0.9% pirimiphos-methyl for 24 hrs. *Anopheles arabiensis* (KGB) a laboratory colony susceptible to all insecticides was included as a control to ensure that the impregnated paper was reliable. Negative control consisted of mosquitoes exposed to untreated paper. Dead and alive mosquitoes were collected separately and preserved for laboratory analysis. Due to limited sample size only the organophosphate, pirimiphos-methyl was tested in the laboratory to provide additional information for operational decision-making.

3.6 Species identification

The *An. gambiae* complex samples from the field as well as the F_1 progeny were identified to species level by the PCR method described by Scott *et al*. (1993). A mosquito leg was used as template in the PCR reaction but in cases where PCR failed, DNA was extracted, according to the method described by Collins *et al.* (1987). Four positive controls were used: *An. gambiae s.s*, *An. arabiensis*, *An. merus* and *An. quadriannulatus* sp A from laboratory colonies maintained in the NICD insectaries. A negative control consisting of only master mix without DNA was included in each amplification experiment. The primers used are shown in the Table 3.1.

Table 3.1: Diagnostic primer sequences, melting temperature and amplicon size for the identification of the members of the *An. gambiae* complex (Scott *et al*., 1993)

Primer name	Primer sequence (5'- 3')	Tm (°C)	PCR Product size
UN	GTG TGC CCC TTC CTC GAT GT	58.3	-
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390 bp
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464 bp
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315 bp
QD	CAG ACC AAG ATG GTT AGT AT	42.7	153 bp

Species-specific primers are: GA which anneals to *An. gambiae*, ME anneals to *An. merus* and *An. melas*, AR anneals to *An. arabiensis* and QD which anneals to *An. quadriannulatus*. The universal primer (UN) anneals at the same position on the rDNA in all of the five species.

The PCR reaction consisted of the following: 1 X reaction buffer (100m M Tris-HCl pH 8.3, 500 mM KCl), 1 mM $MgCl_2$, 0.25mM dNTPs, 0.132 µM QD primer, 0.264 µM ME, GA, AR, UN primers (synthesized by Inqaba Biotechnical Industries,

Pretoria, South Africa), and 0.02U of *Taq* thermostable DNA polymerase enzyme (5U/µl) (The buffer, dNTP mix, $MgCl_2$ and Taq supplied by Takara biomedicals group Shiga Japan Cat. No. R001AM). The PCR reaction mix had a total volume of 12.5µl and contained a single mosquito leg in a PCR microcentrifuge tube. The microcentrifuge tubes and contents were centrifuged for 20 seconds at 16060 x g prior to amplification.

The cycling conditions were as follows: 95 °C for 2 minutes initial denaturation, 30 cycles of 94 °C for 30 seconds denaturation for melting of double-stranded DNA, 50°C for 30 seconds annealing of specific primers, 72 °C for 30 seconds extension (polymerization) and a final auto extension at 72 °C for 5 minutes. Amplicons were electrophoresed on an Ethidium Bromide (10mg/100 ml) (Cat. No. 15585-011, Gibco BRL, UK) stained, 2.5 % agarose gel submerged in a 1X TAE buffer. Electrophoresis was carried out until proper separation of the smaller fragments of the molecular weight marker had been achieved.

The amplicons were visualized under ultra violet light and the mosquitoes were identified by comparing the sizes of their amplicons, to 4µl of a standard molecular weight marker (GeneRuler^{TM} DNA ladder mix, Cat. No. SM0331, Fermentas Canada) loaded on the first lane of the gel. Positive controls were loaded after the marker, followed by the negative control and then the samples. The buffer and agarose gel preparations are provided in Appendix I.

3.7 Identification of *An. gambiae* molecular forms

This assay was initially described by Favia *et al* (2001) using DNA extracted from individual mosquitoes. DNA extraction is, however very time consuming and labour intensive. The Favia protocol was adapted to allow the use of a whole single mosquito leg in order to minimize time and labour cost. However, in samples where legs were absent or where the initial PCR reaction failed, DNA was extracted (see section 3.8 for details) and used for the PCR. Two positive controls were used: JS3 and G3, *An. gambiae* colonies representing the M and S forms respectively. A negative control consisting of only the master mix without a mosquito leg was included in each PCR reaction. The PCR assay was done in a total volume of 25 µl in microcentrifuge tubes.

The PCR master mix comprised of the following: 1 X S.T Gold buffer (20 mM Tris HCl p H 8, 100 mM KCl, 0.1 mM EDTA, 1 mM DDT, 0.5% nonidet and 50% v/v glycerol, 15 mM $MgCl_2$, 0.4 mM of each dNTPs, 1.45 µM primers R5 and B/S, 1.13 µM primers R3 and Mopint (Table 3.2) (primers were synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa), and 0.02U units of a 250 U/µl supertherm Gold *Taq* DNA polymerase. The S.T Gold buffer and Supertherm Gold *Taq* are supplied by Southern Cross biotechnology (Cat. No. JMR-851 UK) while the dNTPs supplied by Takara biomedical group Shiga Japan (Cat. No. R001AM).
The reaction mix was added to a microcentrifuge tube containing a whole mosquito leg. The reaction was set up while working on ice.

The cycling conditions were performed using a thermal cycler and consisted of an initial 10 minute denaturation step at 94 °C, followed by 25 cycles each consisting of 30 seconds at 94 °C, 30 seconds at 63 °C, and 30 seconds at 72 °C. The final PCR products were auto-extended for 7 minutes at 72 °C.

Twelve µl of PCR product was mixed with 2µl of bromophenol blue loading dye and loaded into the wells of the gel. The amplicons were electrophoresed on a 2.5 % agarose gel stained with Ethidium Bromide (10mg/100ml) submerged in 1 X TAE buffer for one hour at 110 volts. The gel was observed under UV light and the amplicons for the S form (500 bp) and the M form (700 bp) differentiated by comparing the sizes to a standard molecular weight marker (GeneRuler^{TM} DNA ladder mix, Cat. No. SM0331, Fermentas Canada). Bromophenol blue preparation is described in Appendix I.

Table 3.2: Primer sequences used in the identification of *An. gambiae* molecular forms (Favia *et al*., 2001)

Primer name	Primer sequence 5' to 3'
R5	GCC AAT CCG AGC TGA TAG CGC
R3	CGA ATT CTA GGG AGC TCC AG
Mopint	GCC CCT TCC TCG ATG GCA T
B/S int	ACC AAG ATG GTT CGT TGC

R5 and R3= Reverse primers, S= Savanna and B= Bamako are primers specific to respective molecular forms.

3.8 DNA extraction

DNA was extracted from adult mosquitoes according to the method described by Collins *et al*. (1987). Since only abdomens were used, all volumes of reagents used were halved. All solutions used for DNA extraction are described in Appendix I. The abdomen of a single mosquito was placed in a separate 1.5 ml microcentrifuge tube and homogenized in 100 µl of grinding buffer (0.08 M NaCl, 0.16 M sucrose, 0.06 M EDTA, 0.5 % SDS, 0.1 M Tris-Cl, pH 8.6). The homogenate was incubated at 70 °C for 30 minutes. Potassium acetate (0.98 M) was added to the heated homogenate, mixed and incubated on ice for 30 minutes. After incubation, room temperature centrifugation was carried out for 15 minutes at 16060 x g. The supernatant was removed and pipetted in to a clean microcentrifuge tube and the pellets were discarded.

Two volumes of 99.9 % Ethanol compared to the volume of homogenate was added and the solution was mixed gently by inversion. In order to facilitate DNA precipitation, the tube and contents were incubated overnight at -20 °C prior centrifugation at 16060 x g for 35 minutes. The supernatant was removed and the DNA pellet was washed with one volume (100 µl) 70 % ethanol prior to centrifugation for 10 minutes at 16060 x g. The pellets were then air dried after removing the ethanol. The DNA pellets were resuspended in 100 µl of 1 X TE buffer (0.1 M Tris and 0.01 M EDTA) and stored at -70 °C/-20 °C. A no DNA control was included in the extraction process.

3.9 *Kdr* mutation identification

The West African *kdr* mutation was detected using the method described by Martinez-Torres *et al.* (1998).

Two separate PCR reactions were set up to detect the *kdr* resistant (heterozygous and homozygous states) and susceptible alleles.The resistant PCR need primers AgD1 and AgD3 (Table 2.2) and the susceptible PCR need primers AgD2 and AgD4 (Table 2.2). For the *kdr* resistant PCR, 1 µl genomic DNA was added to a 12 µl PCR reaction containing: 1 X reaction buffer, 0.26 mM of each dNTP, 2.5 mM of $MgCl_2$, 0.07 mM of AgD1 and 0.41 mM AgD3, and 0.25 U of a 5 U/µl r*Taq* DNA polymerase (buffer, dNTP mix, $MgCl_2$ and *Taq* were supplied by Takara biomedicals group Shiga Japan (Cat. No. R001AM) and the primers were synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa. For the susceptible *kdr* PCR, primers AgD1 and AgD3 were replaced with 0.07 mM AgD2 and 0.41 mM AgD4 primers respectively and the concentration of the other reagents maintained. The cycling conditions for this PCR were: pre-denaturation at 94 °C for 2 minutes, 40 cycles each containing denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 30 seconds with final extension of the PCR products at 72 °C for 5 minutes. A positive RR control was set up using DNA template from SENN-DDT (resistant), an *An. arabiensis* laboratory colony and KGB, a susceptible *An. arabiensis* colony for SS positive control. A negative control consisted of all PCR reagents but no DNA.

Twelve µl PCR products was mixed with 2 µl bromothymol blue loading dye and loaded into the wells of an ethidium bromide stained 2.5 % agarose gel submerged in a 1 X TAE buffer in an electrophoretic tank. The first lane of the gel was loaded with a standard molecular weight marker, negative controls, positive controls followed by unknown samples. After electrophoresis at 110 volts for an hour, the gel was observed under UV light. The resistant amplicon (195 bp) and the susceptible amplicons (137 bp) were interpreted by comparing their sizes to those of the DNA ladder (GeneRuler^{TM} DNA ladder mix).

3.10 PCR for sequencing

Sequencing was done in order to confirm the *kdr* detection results obtained by PCR. To sequence the region flanking the *kdr* mutation at domain II segment 6 of the para type sodium channel gene, the PCR method described by Martinez-Torres *et al.* (1998) was used to generate a 293 bp fragment. A PCR reaction in which, 1 µl genomic DNA was added to a mix consisting of: 1 X reaction buffer, 0.238 mM of each dNTP, 1.91 mM $MgCl_2$, 0.188 mM each of primers AgD1 and AgD2, and 0.02 U of a 5 U/ µl r*Taq* DNA polymerase was set up. Double distilled water was added to give a final volume of 20.9 µl.

The cycling conditions were the same as described in section 3.9 above. PCR products were sent to Inqaba Biotechnical Industries, Pretoria, South Africa for sequencing.

3.11 Sequence analysis

Sequences were first submitted to the Basic Local Alignment Search Tool (BLAST) and only those which produced a significant alignment with the *An. gambiae* sodium channel gene (with accession number gb/AY615653.1) were aligned together using the DNASTAR, lasergene 7 (Wisconsin, USA). Each sequence was compared to the resistant SENN DDT (RR) and the susceptible KGB (SS) control.

3.12 Pyrosequencing PCR

The region of genomic DNA to be analysed was amplified by PCR to yield biotinylated products (Figure 3.2).

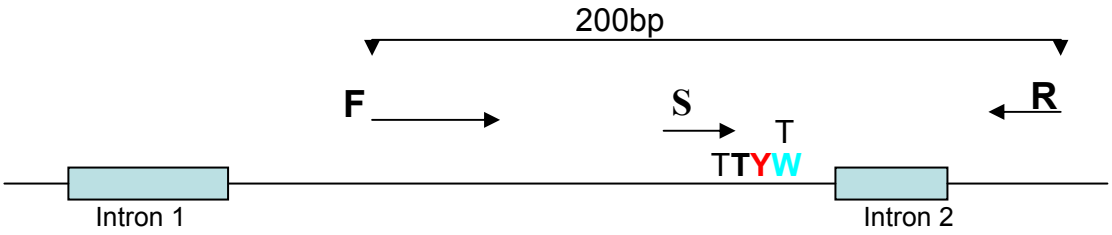


Figure 3.2: Schematic representation of a partial para sodium channel gene showing the *kdr* mutation and annealing sites of primers. F = forward primer, S = sequencing primer and R = Biotin labelled reverse primer. The Y is where the East African *kdr* mutation occurs and the W is where the West African *kdr* mutation occurs. Y can either be a T/C and W a T/A. This implies that for West Africa, the mutation is TTA to TTT and for East Africa from TCA or TTA. Y and W are International Union of Biochemistry (IUB) codes. The 200bp PCR product to be used for pyrosequencing reaction is biotin labelled.

The master mix for this PCR consisted of: 1 X Amplitaq buffer II, 2mM $MgCl_2$, 0.2 mM each of forward and universal biotinylated primer (Table 3.3) , 0.02 mM tagged, reverse primer, 0.125 mM of each dNTP, and 50U AmpliGold *taq* DNA polymerase. Twenty-three µl of this mix was transferred into a 0.2 ml PCR microcentifuge tube

and 2µl of genomic DNA was added. A no DNA negative control and positive controls (SENN-DDT for RR genotype and KGB for SS genotype) were included.

Table 3.3: Primer sequences 5' to 3' used in pyrosequencing reactions (Human genetics, NHLS Braamfontein, South Africa)

Primer	Sequence
F	CGGTGATGTATCCTGCATACCA
R.T	GATGGGACACCGCTGATCGTTTATGGTGCAGACAAGGATGATGA
Seq	GCCACTGTAGTGATAGGAAA

F= Forward, R.T = Reversed Tagged, Seq = Sequencing
Primers were synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa.

The cycling conditions consisted of a 95 ºC pre-denaturation for 5 minutes, 45 cycles of 15 seconds denaturation at 95 ºC, 30 seconds annealing at 57.8 °C and 15 seconds extension at 72 ºC. This was followed by one cycle of final extention at 72 ºC for 5 minutes. PCR products were evaluated by running 5 µl on a 2.5% agarose gel stained with ethidium bromide. Only amplicons showing a single band with high yield and no significant primer dimers were used. The buffer, $MgCl_2$ and *Taq* were supplied by Applied Biosystems, (Cat.No. 4311806, USA).

The remaining 20µl biotinylated PCR product was immobilized onto streptavidin-coated beads and double stranded DNA separated by denaturation in NaOH for one minute. The denaturation buffer was removed by vacuum and DNA washed twice in 150 µl of washing buffer followed by the resuspension of the DNA in 50 µl of

annealing buffer. Forty-µl per well of mixed DNA was transferred into a 96-well microtitre plate and 5 µl of 3 µM sequencing primer (**GCCACTGTAGTGATAGGAAA**) was added, resulting in 45 µl reaction volume. The sequencing primer was allowed to anneal to the template while the plate was set at 80 ºC for 2 minutes and then was left to cool to room temperature. During this cooling step, the following: dNTP (dATP, dCTP, dGTP and dTTP), substrate and pyrosequencing enzymes were added into separate wells of the pyrosequencer cartridge as per amount specified by PSQ 96 MA 2.1 (Biotage, Sweden) software. A nucleotide dispensation order (**GTCATACGTCGT)** was generated by the software based on necessary information for the run. The PSQ plate and the cartridge were placed into the pyrosequencer and run started.

3.13 Enzyme-Linked Immunosorbent Assay (ELISA)

To detect the vector status of wild caught mosquitoes, a sporozoite ELISA was performed according to Wirtz *et al.* (1992). Four buffers (Grinding buffer, washing buffer, blocking buffer and phosphate buffer saline) were prepared for this assay, and full protocols are provided in Appendix I.

The head and thorax of individual *Anopheles* female mosquito was separated from the abdomen with the aid of a blade and forceps and placed in a sterile 1.5 ml microcentifuge tube. The blade and forceps were rinsed twice in methylated spirit solution and wiped dry. The sample was homogenised in 50 µl grinding buffer with a pestle and the pestle washed with 150 µl blocking buffer to give a total volume of 200 µl. A positive control, Pf 2+ (Cat. No. Pf-PC Washington DC, USA) consisted of a

synthetic peptide standardised against *P. falciparum* and was prepared fresh on the day of the ELISA. Negative controls, consisted of seven unfed female *An. gambiae s.s.* prepared as described above and samples were stored in -70 ºC until use (less than two months).

Each well of a 96 well microplate was coated with a solution of 0.200µg/50 µl monoclonal antibody, Pf 2A10-CDC O1 (Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA) and the plate was wrapped with aluminium foil before incubating overnight at 4 ºC. The microtitreplate was aspirated and filled with blocking buffer followed by one hour incubation at room temperature. During this incubation, a positive control was prepared by mixing 100 pg of *P. falciparum* with 50 µl Blocking buffer.

The wells were aspirated and 50 µl of each mosquito homogenate loaded per well. Well A1 of the plate, was designated for the positive control and the last seven wells (H6 to H12) for negative controls. This was followed by 2 hour incubation and washing of wells twice with PBS-Tween 20. Fifty µl of a 9 mg / µl Peroxidase labelled monoclonal antibody (Cat. No. 37.00-24-4, Kirkegaard and Perry Laboratories, Maryland, USA) was added into each well and the plate incubated for one hour at room temperature. The wells were washed 3-4 times with PBS-Tween 20 and 100 µl of freshly prepared ABTS peroxidase substrate (2,2'-azino-di-3 ethyl-benzthiazoline) (Cat. No.50-60-18, Gaithersburg, Maryland USA) was added to each well. The plate was incubated in the dark at room temperature for 30-60 minutes to

allow the peroxidase reaction to occur. Optical density was measured using a plate reader at a wavelength of 405 nm. Samples with an optical density value greater than twice the average of the optical density values of the negative controls, were considered positive. All positive samples were repeated for confirmation.

High infectivity rate in Boffa was investigated by PCR technique described by Tassanakajon *et al*. (1993). DNA was extracted from ELISA homogenates according to Collins *et al*. (1987). The PCR master mix comprised of the following: 1 X reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.7µM of forward and reverse primers, 2.5 units *Taq* DNA polymerase and 1µl DNA. The reaction was carried out in a total volume of 12 µl. The Buffer, *Taq* and $dNTP_S$ were supplied by Takara biomedicals group Shiga Japan (Cat. No. R001AM). A positive control consisting of DNA extracted from *Plasmodiun falciparum* and a no DNA negative control was included. The cycling conditions consisted of : Pre denaturation at 95°C for 5 minutes, 40 cycles each consisting of denaturation at 92°C for 30 seconds, annealing at 45°C for 30 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 5 minutes.

3.14 Biochemical Enzyme Assays

Biochemical assays designed to determine the levels of expression of glutathione-S-transferase, monooxygenase, non-specific esterase and to determine the relative proportion of acetylcholinesterase inhibition by propoxur in individual F_1 progeny *An. gambiae* s.s. were performed according to Penilla *et al*., (1998). Each microtitre

plate was loaded with F_1 progeny samples as well as a KGB base line control sample for comparative purposes.

3.14.1 Preparation of homogenate

One to six days old mosquitoes from the F_1 generation of isofemale lines were transferred by means of forceps into a microtitre plate on ice. Each well contained one mosquito. Samples were homogenised in 50 µl of distilled water per well using a grinding pestle. The pestle was then rinsed with 170 µl of distilled water so that a total volume of 220 µl homogenate was obtained per well. A laboratory strain of *An. arabiensis* from Kanyemba (KGB) in Zimbabwe, susceptible to all insecticides, was used as a reference sample on all plates and was prepared as above. Negative controls consisted of wells containing only reagents without mosquito homogenate. All assays were performed according to Brogdon *et al*. (1988), Lee (1990) and Penilla *et al*. (1998).

3.14.2 Glutathione S-transferase (GST) assay

GST activity, which plays an important role in the detoxification of DDT, was determined according to the method described by Penilla *et al* (1998) and Lee (1990). In this assay chlorodinitrobenzine (CDNB) and reduced glutathione (GSH) were both used as substrates for GST. Ten µl of homogenate was added to each well of a 96 well micro titre plate in duplicate followed by the addition of 200 µl of: 0.0093 M glutathione reduced into 0.1 M sodium phosphate buffer pH 6.5 plus 0.077 M 1-chloro-2, 4-dinitrobenzine dissolved in 1 ml ethanol. The mean GST activity of each

individual mosquito was determined spectrophotometrically on a UV microtitre plate reader at 340 nm kinetically for 5 minutes.

3.14.3 Monooxygenases assay

The activity of monooxygenases was assayed according to the protocols described by Brogdon *et al*. (1988) and Lee (1990). This assay measures the amount of cytochrome P^{450} which is a measure of the amount of haem present in a non blood fed mosquito. Twenty µl of mosquito homogenate was placed in a microtitre plate in duplicate and the following added: 80 µl of 0.0625 M potassium phosphate buffer pH 7.2, 200 µl of: 0.0016 M 3,3-5,5-tetramethyl benzidine dissolved in 5 ml methanol, plus 15 ml of 0.25 M sodium acetate buffer pH 5, and 25 µl of 3% hydrogen peroxide to each well. The plate was incubated for 2 hours at room temperature and read at 650 nm for endpoint analysis.

3.14.4 General Esterase assay

The method described by Brogdon *et al.* (1988) was employed. Each well of a microtitre plate contained: twenty µl of mosquito homogenate in duplicate, 200 µl of: 30 mM alpha naphthyl acetate dissolved 13 ml 0.02 M sodium phosphate buffer pH 7.2 to one duplicate and to the other duplicate, 200 µl of: 30 mM beta naphthyl acetate dissolved into 13 ml 0.02 M sodium phosphate buffer pH 7.2. The plate was incubated on ice for 30 minutes and 50 µl of a mixture of: 0.023 g of fast blue, 2.25 ml distilled water, 5.25 ml 5 % SDS sodium phosphate buffer pH 7.2 was added to

each well to stop the reaction. The plate was left on ice for 5 min and optical density (OD) read at 570 nm.

3.14.5 Protein assay

This assay enables the measurement of the protein concentration of each mosquito which was then used to calculate comparative values for GST, monooxygenases and esterase data. Ten µl of each mosquito homogenate was added to each micro titre plate well, in duplicate. This was then followed by the addition of 300 µl of diluted Bio-Rad protein solution (Cat. No. 500-0006, Bio-Rad laboratories, South Africa) to each well. The plate was left on ice for 5 min and OD was read at 570 nm.

3.14.6 Acetylcholinesterase assay

Twenty five µl of mosquito homogenate was placed in duplicate into a micro titre plate followed by the addition of the following: 145 µl of 1% triton phosphate buffer pH 7.8 , 10 µl of 0.0113 M 5,5-dithio-bis (2-nitrobenzoic acid) dissolved in 2 ml 0.1 M phosphate buffer pH 7.0 , 25 µl of 0.02g acetylthiocholine iodide dissolved in 5 ml double distilled water containing 5 µl propoxur to one replicate and 25 µl of 0.02g acetylthiocholine iodide dissolved in 5 ml double distilled water without propoxur, to the other replicate. The absorbance values read at 405 nm for 5 minutes. Percentage enzyme inhibition by propoxur was calculated using the OD readings of the well containing propoxur and the well without propoxur for each mosquito.

3.14.7 Data analysis

A comparison of the mean enzyme activity between F_1 progeny and the susceptible colony (KGB) assayed on the same plate was statistically analysed using a student *t*-test assuming equal variance and following adjustment for total protein content. This adjustment was required because the mosquitoes used were of different sizes and therefore varying protein content. Also, laboratory temperature fluctuations possibly affect the rate of the enzyme activity. Significance levels were determined at a probability level of 95 % confidence. Altered acetylcholinesterase was determined by calculating the percentage inhibition of this enzyme by propoxur. If the percentage inhibition was found to be lower than 70% then, altered acetylcholinesterase was operating in the population.

CHAPTER FOUR

SPECIES COMPOSITION, ROLE IN TRANSMISSION AND INSECTICIDE RESISTANCE

4.1. *Anopheles* species in the study sites

A total of 1295 mosquitoes were collected from the three sites in Guinea Conakry and were identified as members of the *Anopheles gambiae* complex according to the morphological keys (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The majority of the samples were collected from Mount Nimba (n = 521, 40 %), followed by Siguiri (n = 425, 33 %) and Boffa (n = 349, 27 %).

Species-specific identification by PCR (Figure 4.1) was carried out on 600/1295 (46%) *An. gambiae* complex mosquitoes. The randomly selected 600 samples were necessitated by time and budget constraints of the project. A large portion of the samples 494/600 (82.3 %) were identified as *An. gambiae* s.s. followed by 2/600 (0.33 %) *An. arabiensis* and 104/600 (17.33 %) were unidentified probably due to degradation of the DNA during storage. Species-identification based on locality are summarized in Table 4.1.

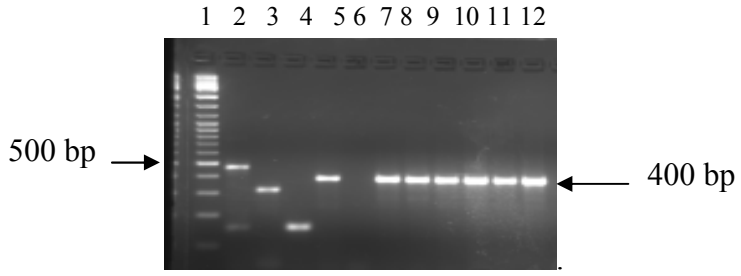


Figure 4.1: PCR identification of members of *An. gambiae complex* s.l. The amplicons were electrophoresed with positive controls on a 2.5% agarose gel stained with ethidium bromide. Lane 1: DNA molecular weight marker. Lane 2: *An. merus* positive control. Lane 3: *An. arabiensis* positive control. Lane 4: *An. quadriannulatus* positive control and Lane 5: *An. gambiae* s.s. positive control. Lanes 6: Negative control and Lanes 7 to 12 represent individual samples identified from Siguiri as *An. gambiae* s.s.

Table 4.1: Summary of species-identification of *Anopheles* mosquitoes based on locality

	Locality			
	Siguiri	**Mount Nimba**	**Boffa**	**Total**
Sample size	177	241	182	600
An. gambiae	157 (89%)	206 (85%)	131 (72%)	494
An. arabiensis	2 (1%)	-	-	2
No ID	18 (10%)	35 (15%)	51 (28%)	104

(-) = No sample, No. ID = No identification

4.2 Identification of *An. gambiae* molecular forms

A total of 494 *Anopheles gambiae* s.s samples were assayed and results obtained for 465 samples. Figure 4.2, shows amplicons derived using single legs and Figure 4.3 using DNA extracted from individual mosquitoes as template in PCR. In Siguiri and Boffa, majority of the samples identified were of the S molecular form whereas in Mount Nimba, they were of the M form (Table 4.2).

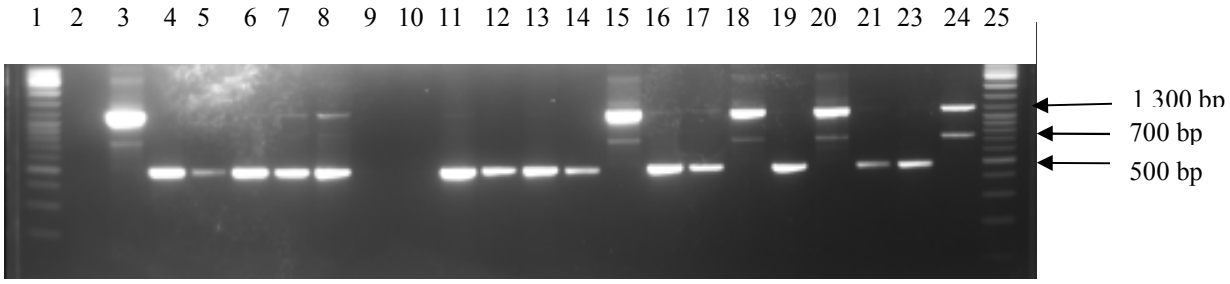


Figure 4.2: **PCR identification of molecular form in *An. gambiae* using legs as template. Lanes 1 and 25: DNA molecular weight maker. Lane 2: negative control. Lanes 3 and 4: positive controls for M and S forms respectively. Lanes 5-8, 11-14, 16-17, 19 and 21-23: S-molecular form. Lanes 15, 18, 20 and 24: M-molecular form. Note: No amplification occurred for samples in Lanes 9 and 10.**

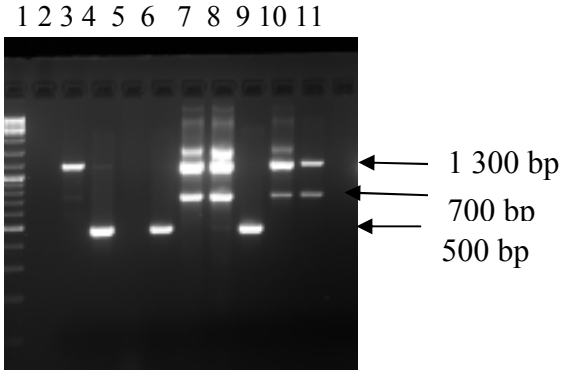


Figure 4.3: **PCR identification of molecular forms in *An. gambiae* using extracted DNA as template. Lane 1: DNA molecular weight maker. Lane 2: negative control. Lane 3: *An. gambiae* amplicon. Lanes 4, 6 and 9: S- molecular form. Lanes 7, 8, 10 and 11: M-molecular form, Lane 5: No amplification.**

Table 4.2: *Anopheles gambiae* s.s molecular forms based on locality

	Locality			
	Siguiri	**Mount Nimba**	**Boffa**	**Total**
Sample size	154	197	114	465
S Form	147 (95%)	75 (38.1 %)	111(97.4%)	333
M form	7 (5%)	121 (61.4%)	3 (2.6%)	131
Hybrid	-	1 (0.5%)	-	1

(-) = no sample

4.3 Insecticide bioassays

Susceptibility status of wild mosquito populations

Results of susceptibility tests on *An. gambiae* s.s from all three sites are summarized in Tables 4.3, 4.4 and 4.5. In Siguiri and Mount Nimba, samples were resistant to DDT, bendiocarb and dieldrin. Suspected resistance to deltamethrin and malathion was observed in Siguiri. Here, two *An. arabiensis* were completely susceptible to DDT. Insecticide susceptibility tests carried out on samples collected from Boffa, showed possibility of resistance to dieldrin and bendiocarb. Complete susceptibility to deltamethrin, DDT and malathion was observed in this site.

Exposed samples (dead and survivors) were preserved on desiccated silica gel 24hr post-exposure and these were later identified to species level using the polymerase chain reaction (PCR) assay of Scott *et al*. (1993). The susceptibility status of these samples is given in Table 4.6 and further broken down into M and S forms in Table 4.7.

Table 4.3: Summary of susceptibility status of unidentified *An. gambiae* complex from Siguiri

Insecticides	Sample size (n)	% Mortality 24hrs post exposure	WHO interpretation
0.05% Deltamethrin	100	91	Resistance suspected
4% DDT	104	40.4	Resistant
0.1% Bendiocarb	66	30.3	Resistant
4% Dieldrin	54	63	Resistant
5% Malathion	101	91	Resistance suspected

Table 4.4: Summary of susceptibility status of unidentified *An. gambiae* complex from Mount Nimba

Insecticides	Sample size (n)	% Mortality 24hrs post exposure	WHO interpretation
0.05% Deltamethrin	89	98.9	Susceptible
4% DDT	98	79.6	Resistant
0.1% Bendiocarb	109	77.1	Resistant
4% Dieldrin	116	69	Resistant
5% Malathion	109	100	Susceptible

Table 4.5: Summary of susceptibility status of unidentified *An. gambiae* complex from Boffa

Insecticides	Sample size (n)	% Mortality 24hrs post exposure	WHO interpretation
0.05% Deltamethrin	90	100	Susceptible
4% DDT	58	98.3	Susceptible
4% Dieldrin	42	83.3	Resistance suspected
0.1% Bendiocarb	54	94.4	Resistance suspected
5% Malathion	105	100	Susceptible

Table 4. 6: Insecticide susceptibility status of PCR identified *An. gambiae* s.s and *An. arabiensis* from the three study sites.

Site	Sample size	Insecticides	Phenotype	*An. gambiae*	% Mortality	*An. arabiensis*	No ID
Siguiri	177	Deltamethrin	Resistant	8	86.7	-	-
			Susceptible	52		-	-
		DDT	Resistant	61	34.6	-	10
			Susceptible	36		2	8
Mount Nimba	241	Deltamethrin	Resistant	3	91.7	-	-
			Susceptible	33		-	-
		DDT	Resistant	20	71.8	-	-
			Susceptible	51		-	27
		Bendiocarb	Resistant	22	77.8	-	3
			Susceptible	77		-	5
Boffa	182	Deltamethrin	Resistant	-	100	-	-
			Susceptible	82		-	18
		DDT	Resistant	-	100	-	-
			Susceptible	31		-	20
		Bendiocarb	Resistant	-	100	-	-
			Susceptible	3		-	2
		Dieldrin	Resistant	6	60	-	5
			Susceptible	9		-	6

Table: 4.7: Insecticide susceptibility of PCR identified S and M molecular forms of *An. gambiae* s.s from the study sites

Sites		Insecticides											
		DDT			Deltamethrin			Bendiocarb			Dieldrin		
		Susceptible	Resistant	% Mortality	Susceptible	Resistant	% Mortality	Susceptible	Resistant	% Mortality	Susceptible	Resistant	% Mortality
Siguiri	S form	31	59	34.4	49	8	86	-	-	-	-	-	-
	M form	1	3	*	3	-	*	-	-	-	-	-	-
Mount Nimba	S form	11	10	52.4	19	-	100	18	17	51.4	-	-	-
	M form	37	9	80.4	14	1	93.3	55	5	91.7	-	-	-
Boffa	S form	16	-	100	76	-	100	1	3	25	9	7	56.3
	M form	-	-	-	3	-	*	-	-	-	-	-	

* = M form samples are too small for meaningful analysis.

Susceptibility status of laboratory reared *Anopheles gambiae*

Table 4.8 shows that, 6/23 (26%) families (45, 53, 56, 64, 69 and 73) were resistant. Complete susceptibility was observed in 12/23 (52%) families (19, 32, 34, 51, 52, 55, 59, 60, 61, 63, 66 and 78). Resistance was suspected in the rest of the families 5/23 (22%).

Table 4.8: Summary of susceptibility status of F_1 progeny of *An. gambiae* from Boffa to 0.9% pirimiphos methyl

Family number	Sample size	% Mortality 24 hrs post exposure	WHO interpretation
19	22	100	Susceptible
32	10	100	Susceptible
34	17	100	Susceptible
41	25	96	Resistance suspected
45	17	24	Resistant
46	15	93	Resistance suspected
49	20	90	Resistance suspected
51	24	100	Susceptible
52	22	100	Susceptible
53	14	64	Resistant
54	19	94	Resistance suspected
55	21	100	Susceptible
56	9	44	Resistant
59	20	100	Susceptible
60	25	100	Susceptible
61	28	100	Susceptible
63	6	100	Susceptible
64	19	68	Resistant
66	29	100	Susceptible
69	21	76	Resistant
71	15	93	Resistance suspected
73	13	61	Resistant
78	13	100	Susceptible

An average percentage mortality of 87% was obtained from all exposed mosquitoes.

4.4 Malaria sporozoite rate in mosquito collected

Figure (4. 4) clearly shows the colour change in A1 after addition of substrate. Visual colour change in well A8, B4, E12 and F9 of wild-caught samples was confirmed by optical density readings and were repeated twice.

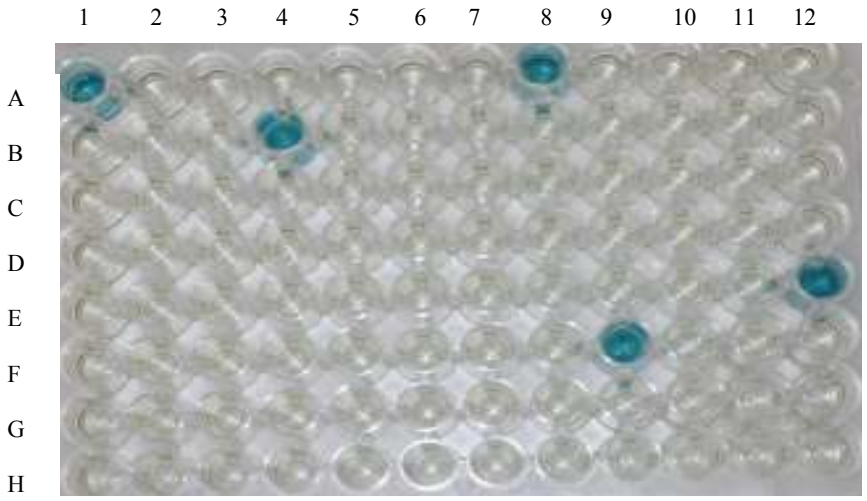


Figure 4.4: ELISA microtitre plate indicating positive control in well A1, negative controls in wells H6 to H12 and samples from Siguiri in the remainder of the wells. The green colour in wells A8, B4, E12 and F9 indicated positive samples. Samples were considered positive when their OD values were more than 2 times the mean OD values of the negative control. All seven negative controls did not change colour

Table 4.9 summarizes *An. gambiae* s.s ELISA results in relation to molecular forms. The two *An. arabiensis* from Siguiri were ELISA negative and are not included in Table 4.9. The highest infectivity rate was observed in Boffa where a total of 28/131 samples were found to be infected with the *P. falciparum* circumsporozoite antigen giving an overall sporozoite infectivity rate of 21%. Of the 111 S form individuals in this site, 20.7% were positive (the M form sample size was too small for meaningful

analysis). The high infectivity rate in this site was further investigated by PCR according to Tassanakajon *et al*. (1993). PCR revealed that only 3/12 (25%) samples, all of them S form, were positive..

Table 4.9: **Summary of ELISA results of all identified *An. gambiae* s.s.**

Site	Sample size	POSITIVE				NEGATIVE			
		M	S	H	No form	M	S	H	No form
Siguiri	157	2 (28.6 %)	6 (4.1%)	-	-	5	141	-	3
Mt Nimba	206	6 (5.0 %)	5 (6.8 %)	-	2	115	68	1	9
Boffa	131	1 (33.3 %)	23 (20.7%)	-	4	2	88	-	13
Total	494	9 (6.9 %)	34 (10.5%)	-	6	122	289	1	25

S and M = molecular forms, H = one hybrid, No form = molecular form not identified.

Percentages represent number of positive per molecular form

4.5 Biochemical analysis

Biochemical assays designed to determine the levels of expression of glutathione-S-transferase, monooxygenase, non-specific esterase and to determine the relative proportion of acetylcholinesterase inhibition by propoxur in individual F_1 progeny *An. gambiae* s.s. were performed according to Penilla *et al*., (1998). Each microtitre plate was loaded with F_1 progeny samples as well as a KGB base line control sample for comparative purposes. The mean protein OD readings were standardized by equalizing each family with the corresponding KGB OD readings by an appropriate factor and adjusting the OD values of the families in all other assays accordingly. This adjustment was required because the mosquitoes used were of different sizes and therefore varying protein content. Also, laboratory temperature fluctuations possibly affect the rate of the enzyme activity. With the aid of a two sample *t-test*, the adjusted mean enzyme activity of each family was compared to the corresponding control KGB mean value for each assay.

4.5.1 Glutathione S-transferase

Figure 4.5 summarizes the results of GST assays carried out on unexposed subsamples of the F_1 progeny of wild caught females from Boffa. GST activity was significantly elevated in 3/28 (11 %) families (38, 55, and 61) ($p < 0.05$). None of the resistant families (45, 56, 64, 69 and 73) had significantly elevated GST activity. There was no bioassay information for families 38, 42, 44, 47 and 57.

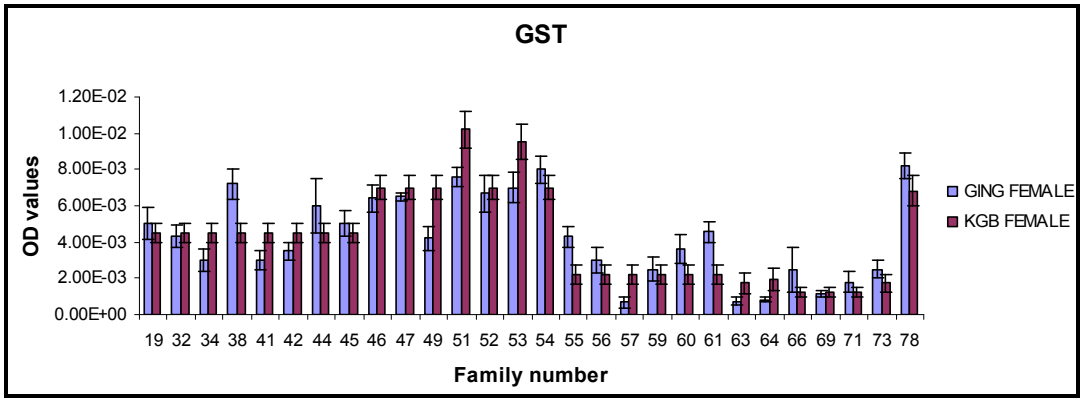


Figure 4.5: Average levels of GST activity in F_1 progeny *An. gambiae* s.s. females by family compared to KGB baseline colony assayed on the same plate.

GST optical density values for 2-3 days old F_1 female progeny of wild caught *An. gambiae* s.s. families were plotted against percentage mortality as measured by bioassay exposures to pirimiphos-methyl Figure 4.6. The susceptible mosquitoes (% mortality > 80%), showed a wide range of enzyme activity as measured by optical densities which ranged from 0.0075 to 0.0082. The resistant samples (% mortality < 80) had enzyme activities which ranged from 0.000833-0.007. When a regression line was fitted to these data it was found out that there is no significant correlation between absorbance values for the enzyme data and mortality rate ($R^2 = 0.0135$, $p < 0.05$).

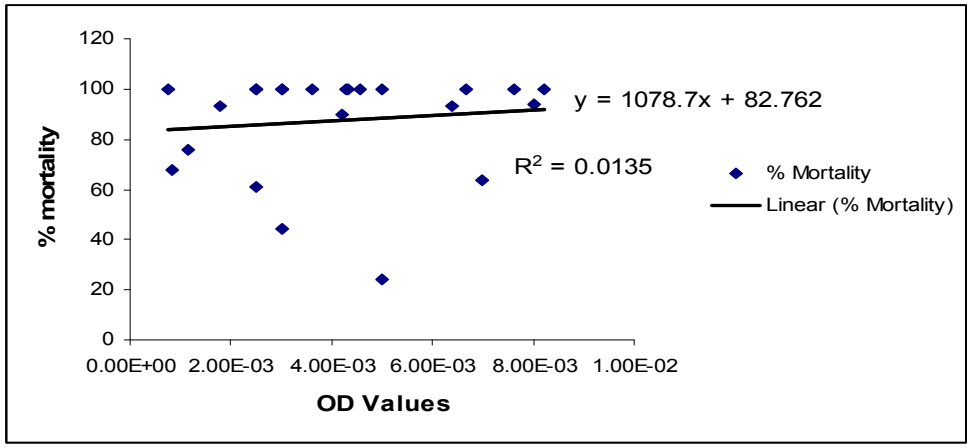


Figure4.6: **Scatter plot analysis of percentage mortality versus GST activity of F_1 female progeny of wild caught *An. gambiae* s.s. exposed to pirimiphos methyl. Each point represents a family and the solid line represents regression with 95% confidence interval.**

Based on data from males, GST activity was significantly elevated in 5/28 (18%) families (55, 56, 59, 63 and 69) when their mean OD values were compared to those of the KGB ($p < 0.05$) (Figure 4.7). However, only family 56 amongst these families with significant levels of GST is resistant to 0.9 % pirimiphos-methyl. Families 45, 53, 64 and 73 with no significant levels of GST were resistant to the same insecticide.

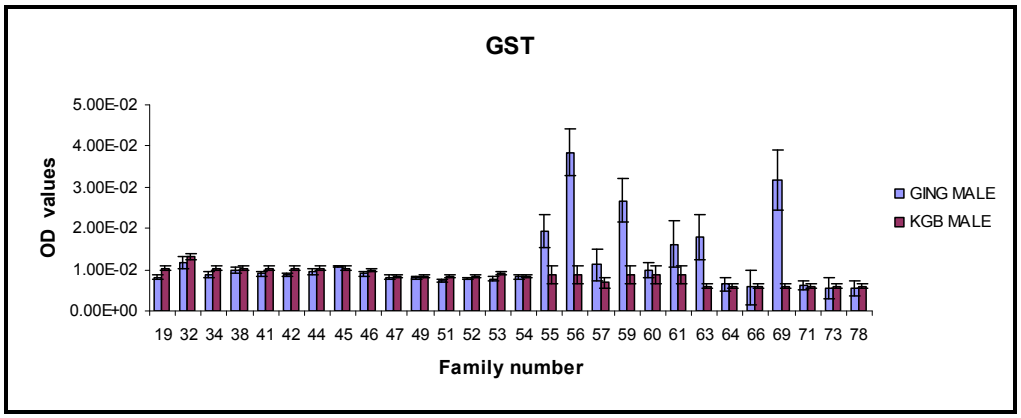


Figure 4.7: Average levels of GST activity in F_1 progeny *An. gambiae* s.s. males by colony family line compared to KGB baseline assayed on the same plate.

Scatter plot analysis of bioassay and absorbance values for 3-5 days old F_1 male progeny of *An. gambiae* s.s. was performed as shown in Figure 4.8. The majority of

the susceptible 16/17 (94%) and 4/6 (67%) resistant families showed very low enzyme levels i.e. OD < 0.02. A linear model was fitted to the data and a regression line was obtained indicating that, there is a significant but weak correlation between the GST activities compared with the bioassay data of these families ($R^2 = 0.0613$, p = 0.0000).

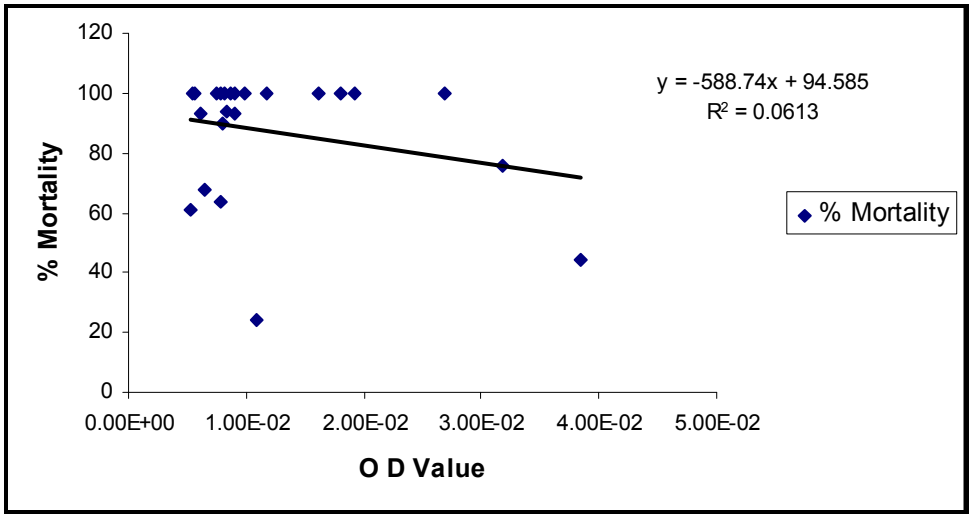


Figure 4.8: **Scatter plot analysis of percentage mortality versus GST activity of F_1 male progeny of wild caught *An. gambiae* s.s. Each point represents a family.**

4.5.2 Monooxygenase assay

Figure 4.9 shows biochemical assay results performed on F_1 female progeny of families which were cultured from wild caught adults from Boffa. Monooxygenase activity was significantly elevated in 23/28 (82%) families (19, 32, 41, 42, 44, 45, 46, 47, 49, 51, 52, 53, 54, 55, 56, 57, 59, 61, 63, 64, 66, 69 and 73) when compared with the insecticide susceptible KGB baseline strain assayed on the same plate ($p < 0.05$).

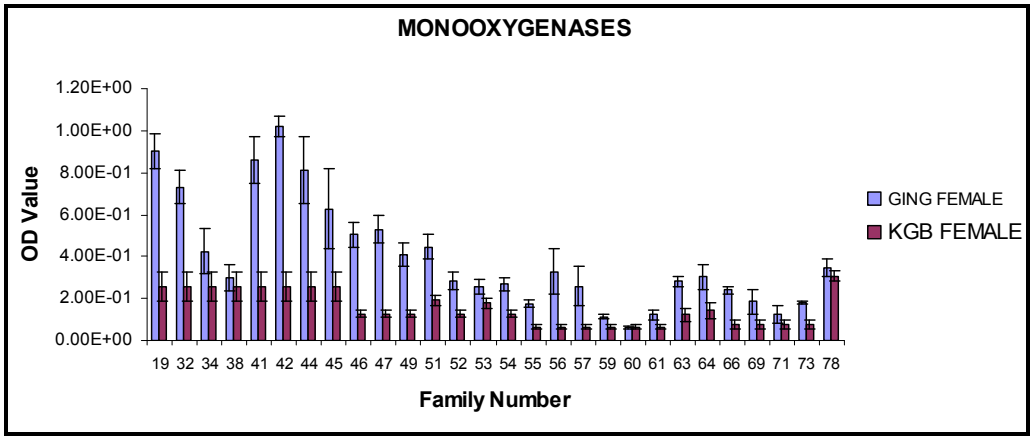


Figure 4.9: Average levels of monooxygenase activity in F_1 *An. gambiae* s.s. progeny females by family compared to KGB baseline colony assayed on the same plate

An analysis of bioassay results and monooxygenase activity of F_1 female progeny of wild caught *An. gambiae* s.s. families by scatter plot was performed to investigate correlation. A regression line was fitted to the data and families showed a weak correlation between bioassay mortality and elevated monooxygenase activity (R^2 = 0.0005, $p < 0.05$).The susceptible families showed OD values between 0.00638-0.9 and the resistant families gave values between 0.181 – 0.626 Figure 4.10. Families 38, 42, 44, 47, and 57 had no bioassay data, and therefore could not be tested for correlation.

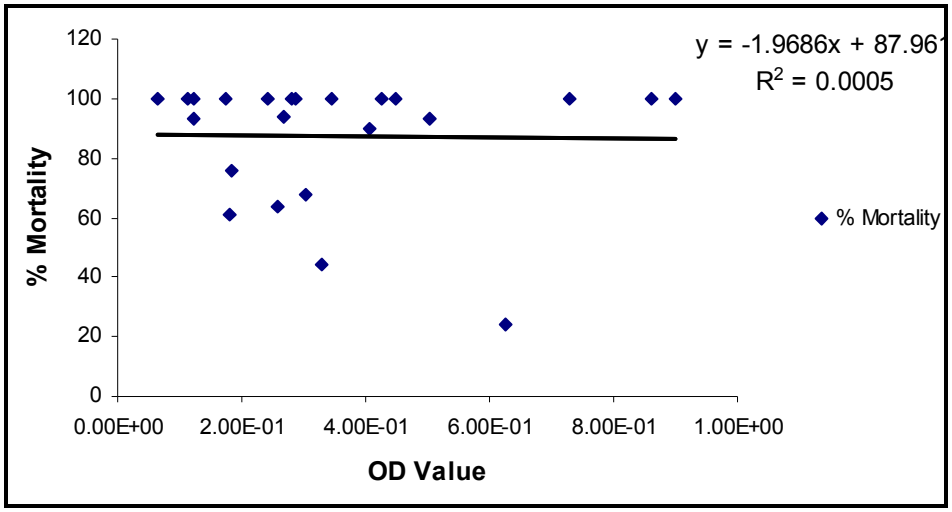


Figure 4.10: **Scatter plot analysis of percentage mortality versus monooxygenase activity of F_1 female progeny of wild caught *An. gambiae* s.s. Each point represents a family.**

Figure 4.11 shows the monooxygenase activity of male F_1 progeny reared from wild caught adults from Boffa compared to the KGB base line insecticide susceptible strain, assayed on the same plate. The result reveals that 24/28 (86%) families (19, 32, 34, 38, 41, 42, 44, 45, 46, 47, 49, 51, 52, 53, 54, 56, 57, 59, 63, 64, 66, 69, 71, and 73) showed significant elevation of monooxygenase activity compared with the KGB reference baseline ($p < 0.05$). Families 38, 42, 44, 47 and 57 had no bioassay data.

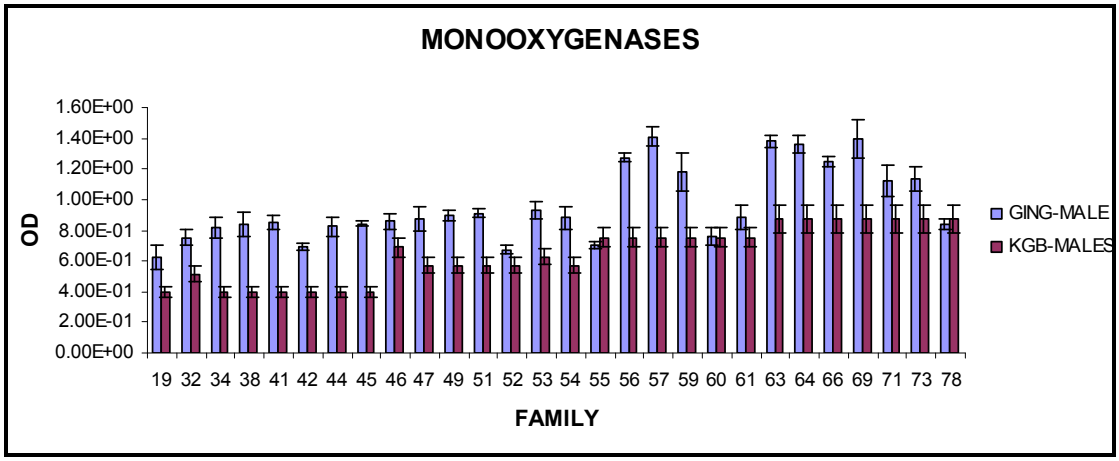


Figure 4.11: Average levels of monooxygenase activity in males F_1 progeny *An. gambiae* s.s. by family compared to KGB baseline colony assayed on the same plate.

Analysis of bioassay and monooxygenases data of F_1 male progeny of wild *An. gambiae* s.s. by scatter plot with inserted regression line revealed that, there is a weak negative correlation between bioassay and mortality data ($R^2 = 0.093$, $p < 0.05$) Figure 4.12.

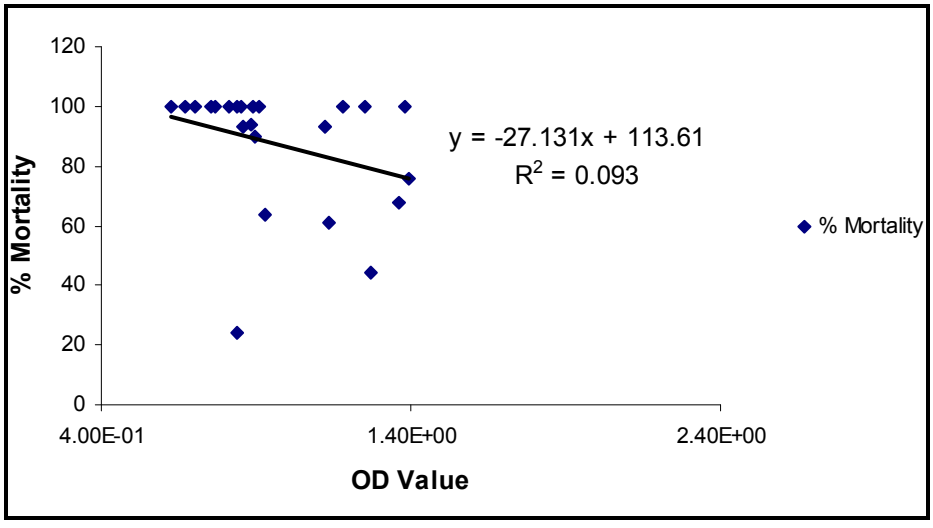


Figure 4.12: Scatter plot analysis of percentage mortality versus monooxygenase activity of F_1 male progeny of wild caught *An. gambiae* s.s. Each point represents a family.

4.5.3 Non-specific esterase activity

The non-specific esterase activities monitored using alpha and beta naphthyl acetate respectively as substrates, are shown in Figures 4.13 and 4.15 respectively.

Esterase activity monitored using the substrate α naphthyl acetate was significantly elevated in 16/28 (57%) families (19, 34, 38, 42, 45, 49, 53, 55, 56, 57, 59, 60, 66, 69, 71, and 73) when compared with the KGB base-line reference strain assayed on the same plate ($p < 0.05$). The elevated enzyme activity in the resistant families (45, 53, 56, 69 and 73) corresponds to their phenotypic resistant characteristic. Family 64 with enzyme activity similar to that of the KGB susceptible strain was resistant to pirimiphos-methyl exposure.

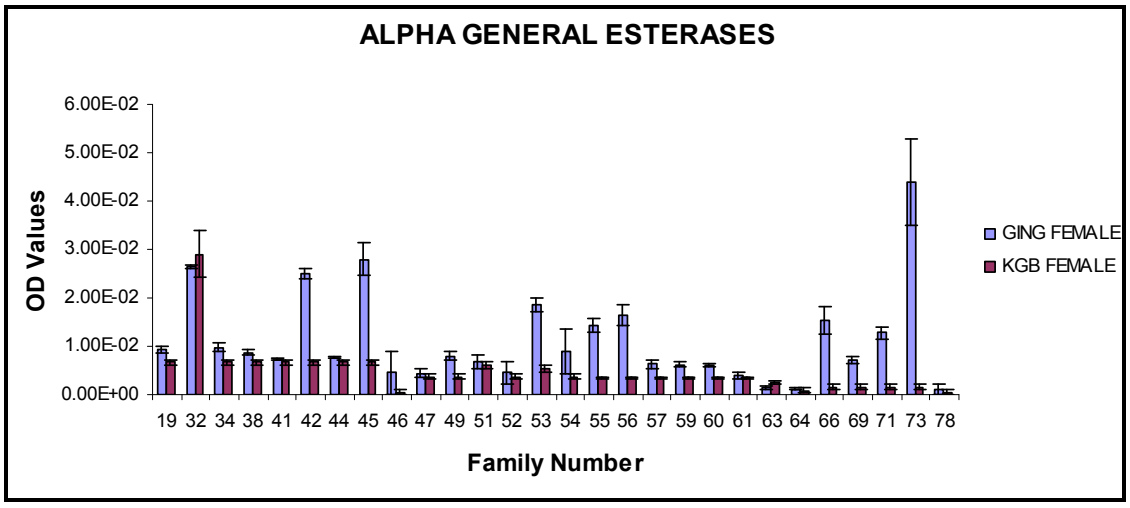


Figure 4.13: General esterase activity of female *An. gambiae* s.s. mosquitoes compared to KGB base line colony using alpha-naphthyl acetate as substrate

Analysis of bioassay and alpha esterase activity by scatter plot revealed that there is a negative correlation between bioassay and mortality data (R^2=0.295, p= 0.008) Figure 4.14

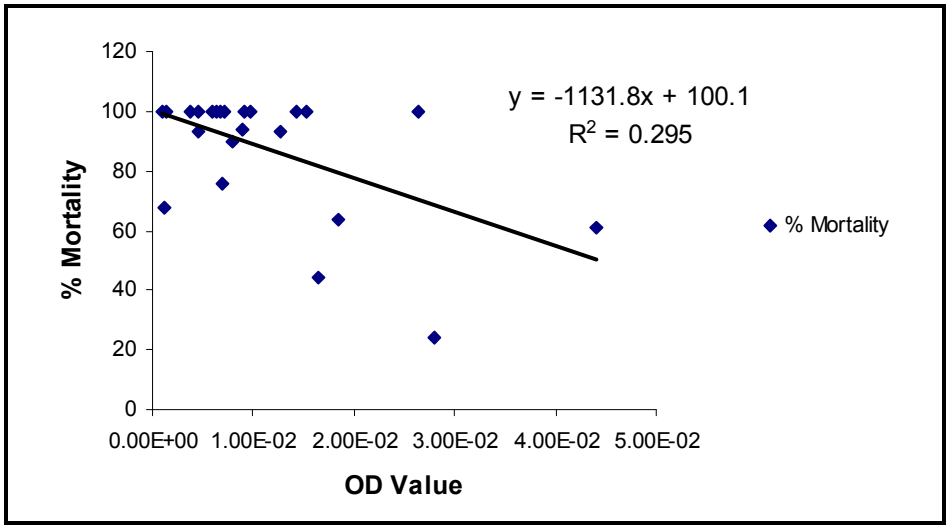


Figure 4.14: Scatter plot analysis of percentage mortality versus alpha esterase activity of F_1 female progeny of wild caught *An. gambiae* s.s. Each point represents a family.

General esterase activity monitored using β naphthyl acetate as a substrate was significantly elevated in 10/28 (36 %) female families (32, 34, 42, 45, 46, 49, 55, 56, 66 and 73) when their OD values were compared to the OD values of the KGB reference baseline strain assayed on the same plate ($p < 0.05$) (Figure 4.15). Amongst the families with significant esterase activity, only families 45, 56 and 73 were resistant to 0.9 % pirimiphos-methyl.

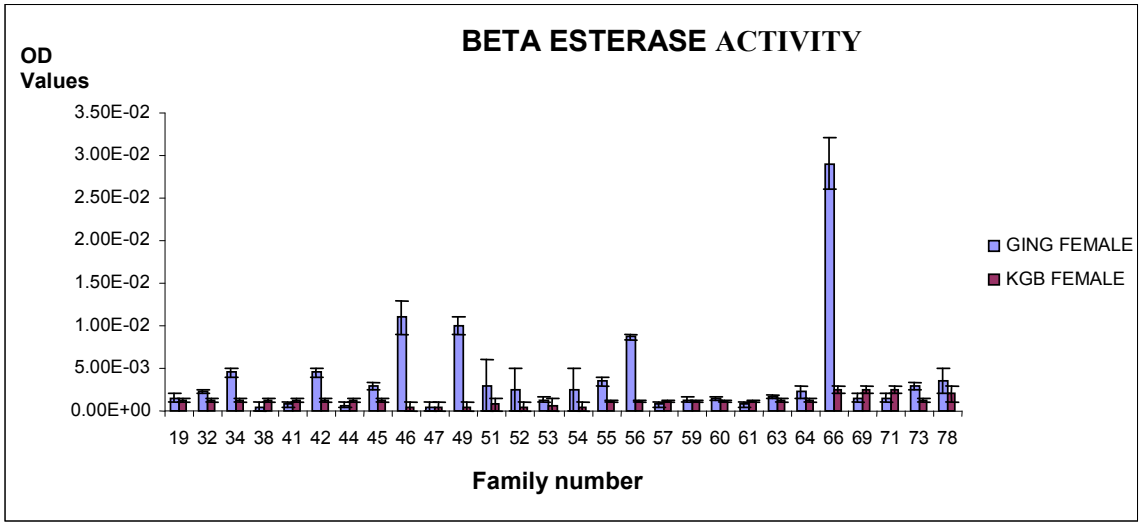


Figure 4.15: General esterase activity of female *An. gambiae* s.s. mosquitoes compared to KGB base line colony using beta-naphthyl acetate as a substrate.

Figure 4.16 shows analysis of bioassay and beta esterase data by scatter plot analysis of F_1 female progeny of wild *An. gambiae* s.s. A fitted regression line shows that there is a very weak correlation between bioassay and biochemical data ($R^2 = 0.001$; p = 0.0036).

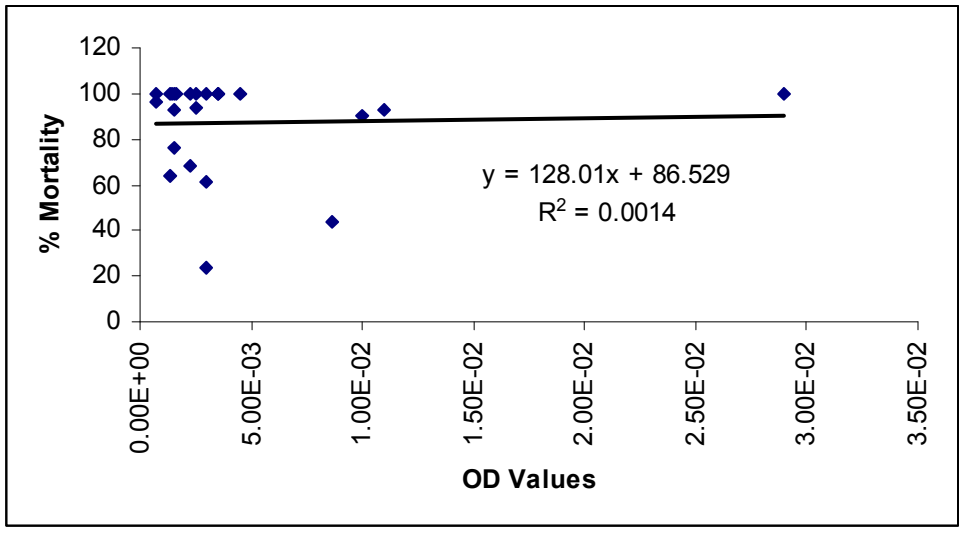


Figure 4.16: Scatter plot analysis of percentage mortality versus beta esterase activity of F_1 female progeny of wild caught *An. gambiae* s.s. Each point represents a family.

General esterase activity monitored using alpha naphthyl acetate in male F_1 progeny by family was significantly elevated in 11/28 (39 %) families (19, 52, 55, 56, 57, 59, 60, 64, 66, 69 and 73) when compared with the KGB base line strain ($p < 0.05$) (Figure 4.17). Families 56, 64, 69 and 73 were resistant to 0.9 % pirimiphos-methyl. No bioassay data was available for family 57.

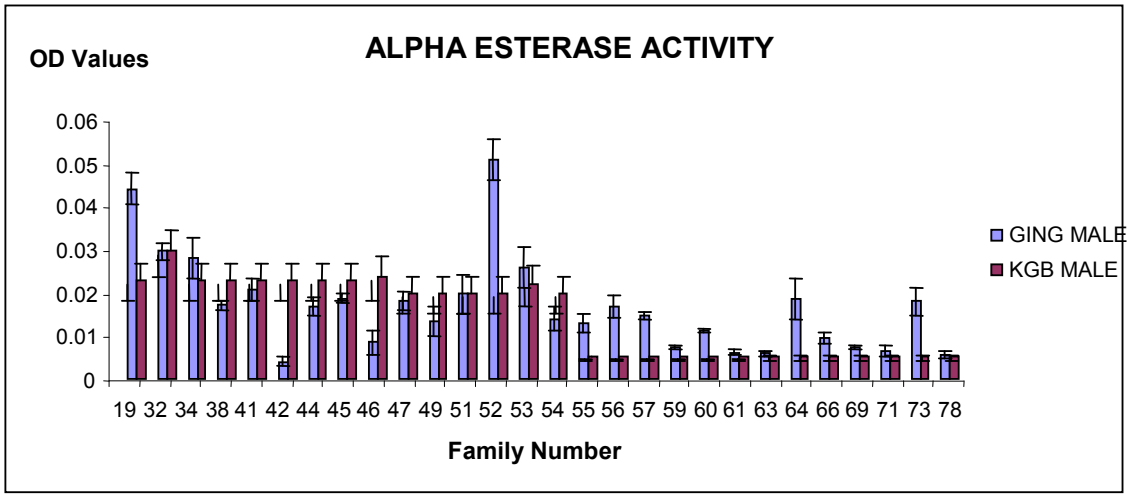


Figure 4.17: General esterase activity of F_1 male *An. gambiae* s.s. mosquitoes by family compared to KGB baseline colony using alpha-naphthyl acetate as substrate.

All the resistant families showed had enzyme activity lower than 0.025 as shown in Figure 4.18. Regression analysis showed that there is only a weak correlation between mortality bioassay and biochemical enzyme data ($R^2 < 0.001$; $p < 0.001$).

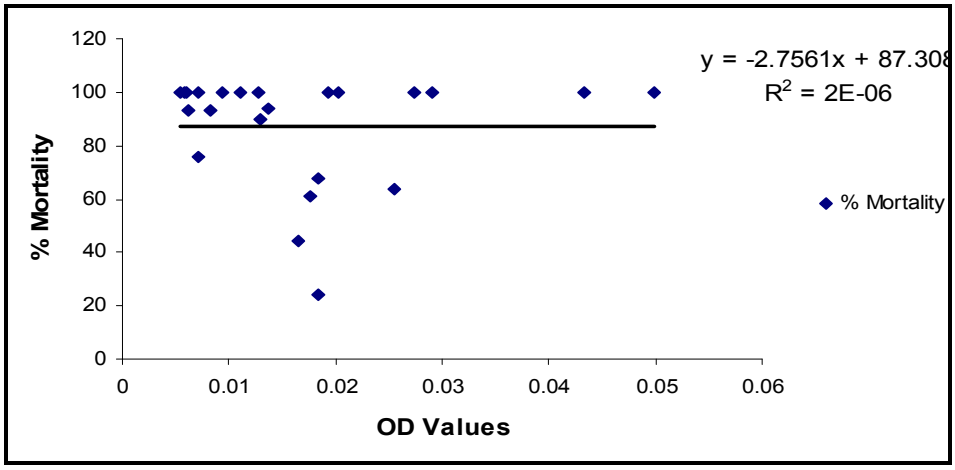


Figure 4.18: Scatter plot analysis of percentage mortality versus alpha esterase activity of F_1 male progeny of wild caught *An. gambiae* s.s. Each point represents a family.

Esterase activity in male *An. gambiae* s.s. monitored by family using beta naphthyl acetate as substrate was significantly elevated in 7/28 (25 %) families (19, 49, 51, 54, 56, 57 and 60) compared to the KGB base line male strain ($p < 0.05$) (Figure 4.19). Of these, only family 56 was resistant to 0.9 % pirimiphos-methyl exposure. Families 45, 64, 69 and 73 were all resistant to the insecticide but showed no significant elevation in their enzyme activity.

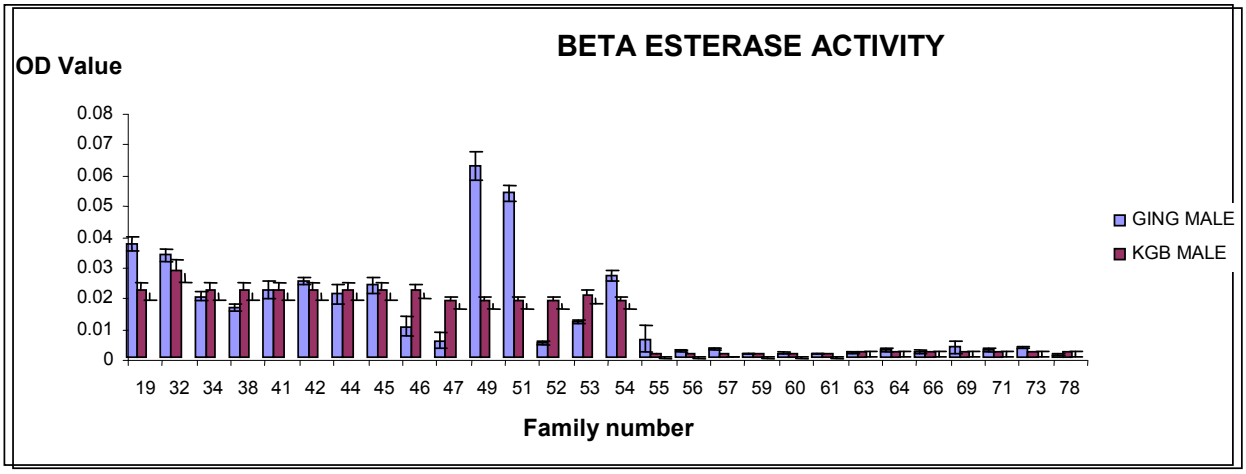


Figure 4.19: Average general esterase activity in male F_1 progeny of *An. gambiae* s.s. families compared to the KGB base line colony using beta naphthyl acetate as a substrate.

Figure 4.20 shows analysis of bioassay and beta esterase activity of F_1 male progeny of wild caught *An. gambiae* s.s. families by scatter plot. The fitted regression line shows a weak positive correlation between mortality bioassay and biochemical assay data ($R^2 = 0.0084$; $p = 0.0011$).

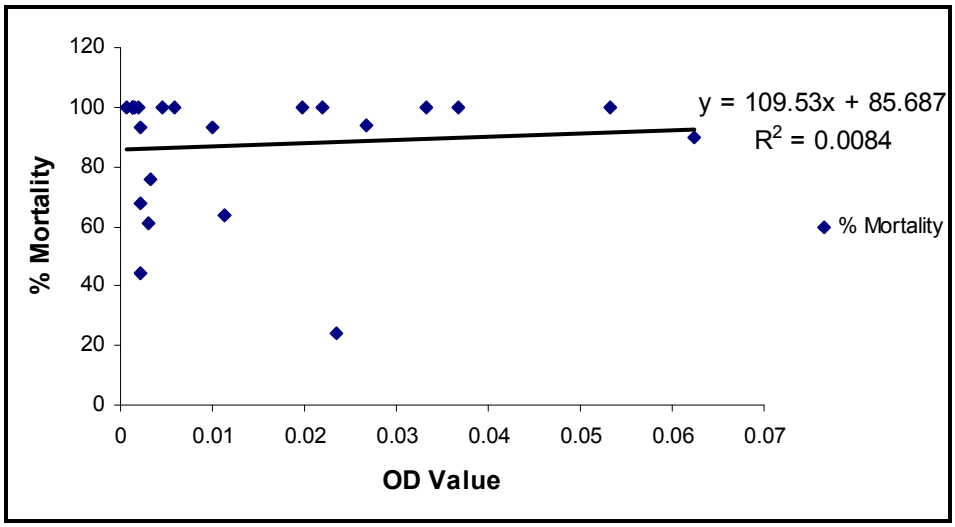


Figure 4.20: Scatter plot analysis of percentage mortality versus beta esterase activity of F_1 male progeny of wild caught *An. gambiae* s.s. families. Each point represents a family.

4.5.4 Effect of propoxur on acetylcholinesterase

The mean percentage inhibitions of AChE for the familial F_1 progeny of wild caught *An. gambiae* are shown in Figure 4.21. AChE inhibition rates by propoxur ranged from 42.3% to 99% compared to an average of 83% for KGB. Out of 27 female families, nine (19, 42, 44, 45, 55, 56, 60, 63, and 64 (33.3%) showed percentage inhibition below the recommended 70% inhibition criterion (Penilla *et al*., 1998) which indicates the presence of an altered AChE.

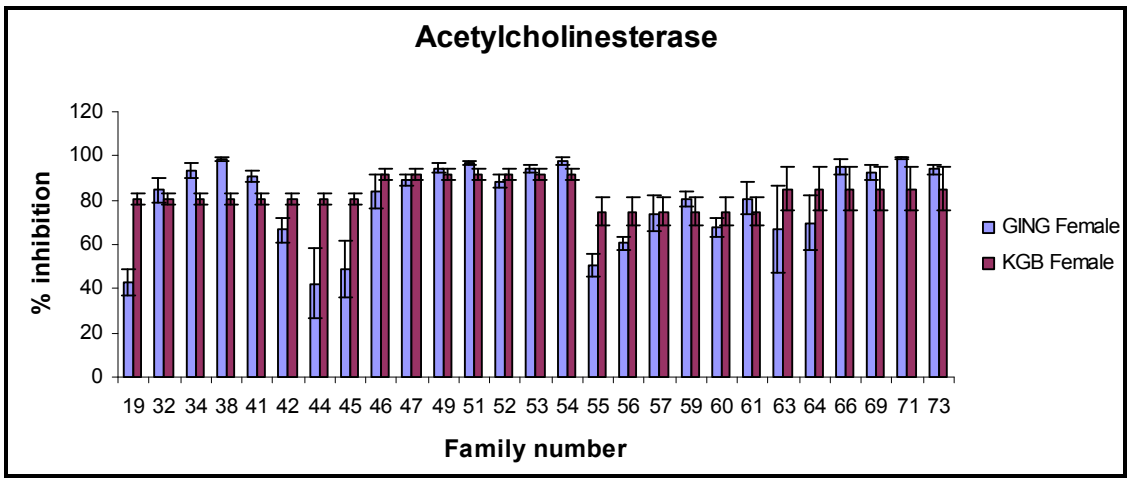


Figure 4.21: Mean percentage acetylcholinesterase inhibition by propoxur in F_1 progeny of female *An. gambiae* s.s. reared from wild caught females compared to the KGB baseline colony.

Figure 4.22 shows the mean percentage inhibition of AChE for male familial F_1 progeny of wild caught *An. gambiae* .The percentage inhibition of AChE by propoxur ranged from 82.9% to 94.1% compared to an average of 91.8% for the KGB baseline reference colony. The biochemical assay reveals that none of the male families assayed showed percentage inhibition below the recommended 70 % inhibition criterion.

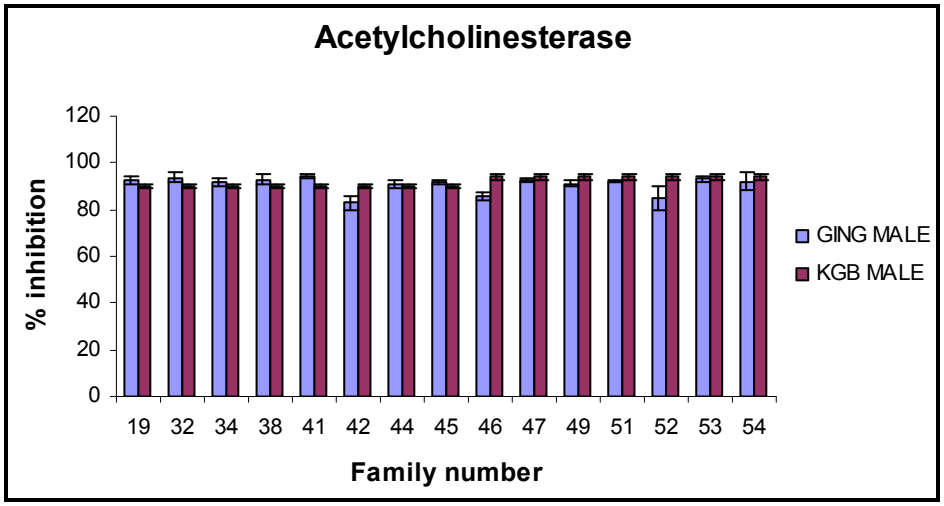


Figure 4.22: Mean percentage acetylcholinesterase inhibition by propoxur in F_1 progeny of male *An. gambiae* s.s. reared from wild caught females compared to the KGB baseline colony.

Acetylcholinesterase activity data obtained for female family 78 and male families 55, 56, 57, 59, 60, 61, 63, 64, 66, 69, 71, 73 and 78 were inconclusive. They were therefore not included in the analysis.

CHAPTER FIVE

KNOCKDOWN RESISTANCE AND PYROSEQUENCING

5.1 *Kdr* PCR detection (West African type)

The diagnostic PCR described by Martinez-Torres *et al.* (1998) was used to distinguish between resistant and susceptible *kdr* alleles. The results of the *kdr* PCR revealed three genotypes: a 195 bp *kdr* band (RR), a 137 bp susceptible band (SS) and an RS genotype with both bands for a single sample (Figure 5.1).

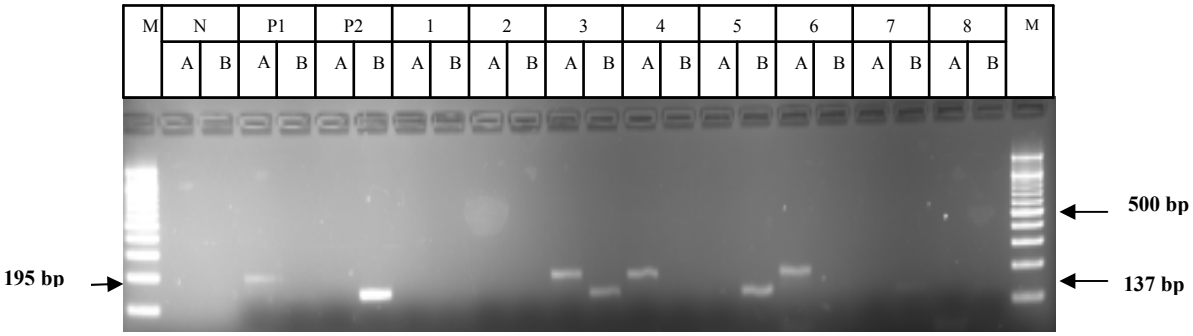


Figure 5.1: A) 2.5 % agarose gel showing amplification of the 195 bp *kdr* resistant and 137 bp *kdr* susceptible bands. M: Molecular marker. N: No DNA negative controls using resistant and susceptible PCR master mix respectively. P1 and P2: Positive controls. Numbers represents individual mosquitoes. A: *kdr* resistant allele PCR. B: *kdr* susceptible allele PCR. 3: Heterozygous resistant (RS). Mosquitoes 4 and 6: Homozygous resistant (RR). Mosquito 5: Homozygous susceptible (SS). Mosquitoes 1, 7 and 8: No amplification. DNA from a *kdr* RR mosquito (SENN DDT) and *kdr* SS mosquito (KGB) was used in both P1 and P2.

Table 5.1-5.3 shows summary of *kdr* mutation PCR results of *An. gambiae* s.s. samples from all three sites. Association test done using statistical 7 package showed that there is an association between *kdr* and bioassays phenotype in DDT resistant and susceptible populations in Mount Nimba ($\chi 2 = 67.02$, $\mathbf{p} < 0.05$) and Siguiri ($\chi 2$

=115.55, **p** < 0.05) as well as Deltamethrin resistant and susceptible populations in Siguiri ($\chi 2 = 31.96$, **p** < 0.05).

Table 5.1: Summary of *kdr* genotypes of *An. gambiae* s.s. from Boffa

Insecticide	**Bioassay phenotype**	***Kdr* genotype**			**Allele frequency**	
		RR	RS	SS	R	S
DDT (n=23)	Susceptible (n=23)	-	2	21	**0.4**	**0.96**
Deltamethrin (n=47)	Susceptible (n=47)	3	3	41	**0.1**	**0.9**

Table 5.2: Summary of *kdr* genotypes of *An. gambiae* s.s. from Mount Nimba

Insecticide	**Bioassay phenotype**	***Kdr* Genotype**			**Allele frequency**	
		RR	RS	SS	R	S
DDT (n = 46)	Susceptible (n = 40)	-	1	39	**0.01**	**0.99**
	Resistant (n = 6)	3	-	3	**0.5**	**0.5**
Deltamethrin (n = 31)	Susceptible (n = 31)	-	5	26	**0.08**	**0.92**

Table 5.3: Summary of *kdr* genotypes of *An. gambiae* s.s. from Siguiri

Insecticide	**Bioassay phenotype**	***Kdr* Genotype**			**Allele frequency**	
		RR	RS	SS	R	S
DDT (n = 62)	Susceptible (n = 33)	-	4	29	**0.06**	**0.94**
	Resistant (n = 29)	13	12	4	**0.66**	**0.34**
Deltamethrin (n = 38)	Susceptible (n = 31)	6	11	14	**0.37**	**0.63**
	Resistant (n = 7)	4	1	2	**0.64**	**0.36**

The relationship between *kdr* genotypes and molecular forms of *An. gambiae* s.s per study site irrespective of the insecticide, to which samples were exposed, is shown in Table 5.4.

Table 5.4: *kdr* genotypes of the molecular forms in all three study sites

Locality	Sample size	Molecular form	*kdr* genotype			Allele frequency	
			RR	RS	SS	R	S
Siguiri	53	M	-	-	2 (4%)	**-**	**1**
		S	23 (43%)	25 (47%)	3 (6%)	**0.7**	**0.3**
Mount Nimba	73	M	-	3 (4%)	39 (53%)	**0.04**	**0.96**
		S	3 (4%)	3 (4%)	25 (34%)	**0.15**	**0.85**
Boffa	59	M	-	-	3 (5.1%)	**-**	**1**
		S	2 (3.4%)	2 (3.4%)	52 (88.1)	**0.05**	**0.95**

5.2 Sequence confirmation of PCR-genotypes

Sequencing was necessary to confirm the *kdr* allele results obtained by PCR.

The region flanking the *kdr* mutation was amplified by PCR according to Martinez-Torres *et al*. (1998) using primers AgD1 and AgD2 (Figure 5.2). The 293 bp PCR products (Figure 5.2) were sequenced by Inqaba biotec Pretoria South Africa and chromatograms are shown in Figure 5.3.

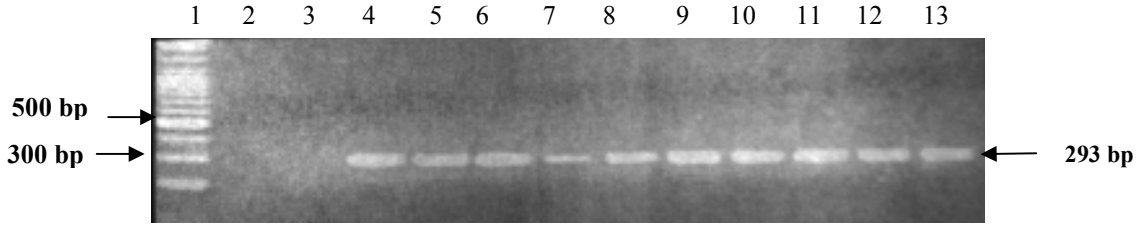
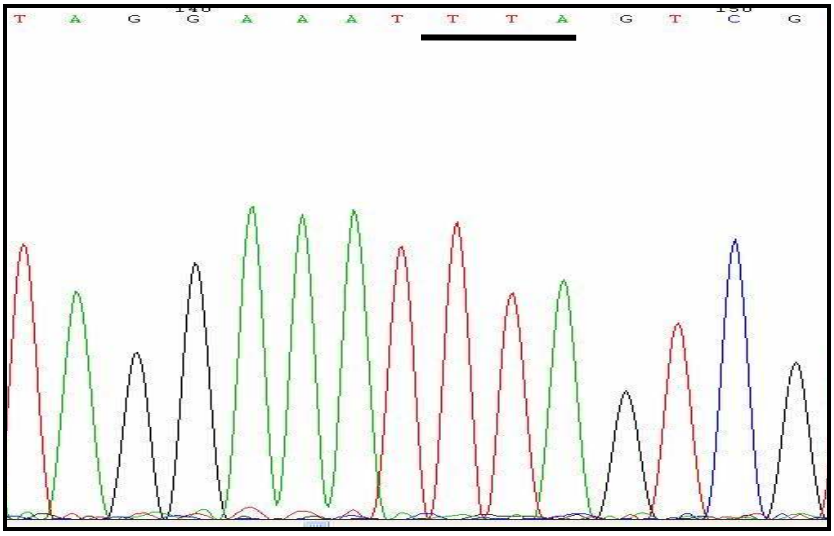


Figure 5.2: PCR amplicon (293 bp) amplified for sequencing. Lane 1: Molecular marker. Lane 2: negative control. Lane 3: No sample. Lane 4: SENN DDT positive control. Lane 5: KGB positive control. Lanes 6-9: RR samples and Lanes 9-13: SS samples.

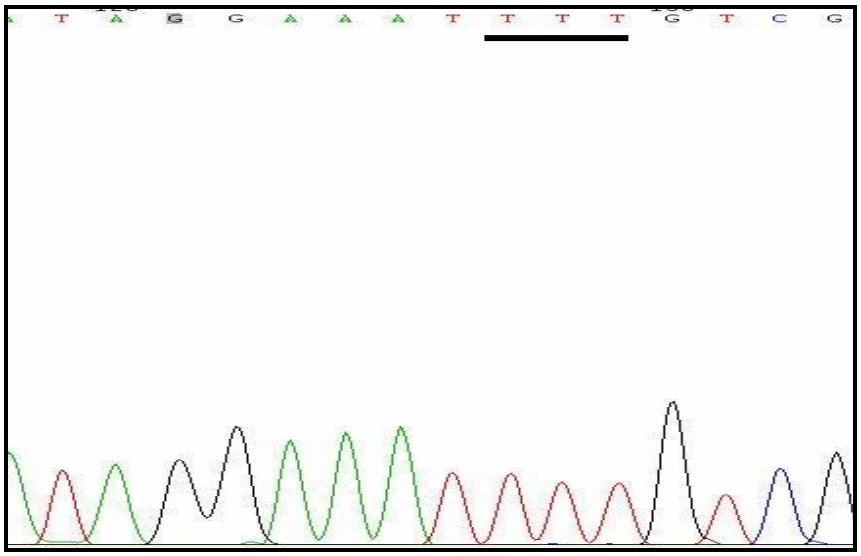
Homozygous susceptible (SS)

A



Homozygous resistant (RR)

B



Heterozygous (RS)

C

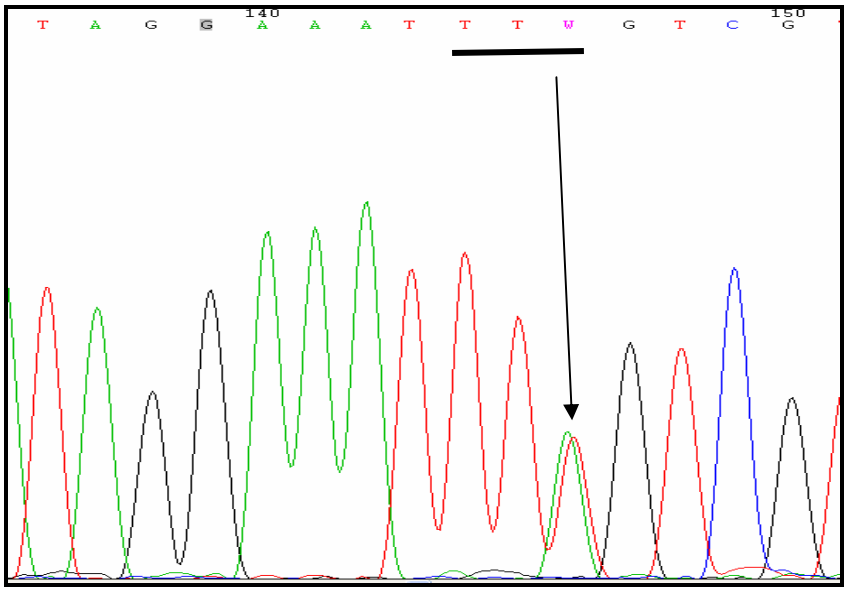


Figure 5.3: Chromatogram obtained by sequencing PCR products from A) Homozygous susceptible (SS), B) Homozygous resistant (RR) and C) Heterozygous resistant (RS) individual. The underlined nucleotide sequences indicate *kdr* region. The letter W in the underlined *kdr* nucleotide sequence of the heterozygote is the IUB code for A/T nucleotide. The arrow on the chromatogram indicates heterozygote situation (A and T peaks).

To confirm that PCR amplified the correct region flanking the *kdr* mutation of domain II of the para sodium channel gene using oligonucleotide primers AgD1 and AgD2, the sequences were submitted to the National Center for Biotechnology Information (NCBI) data base using Basic Local Alignment Search Tool (BLAST). (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences produced significant alignment (100%) (Figure 5.4) with the *An. gambiae* sodium channel gene (accession number gb\AY615653.1).

```
Query  4    ATAGATTCCCCGACCATGATCTGCCAAGATGGAATTTTACAGATTTCATGCATTCCTTCA  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  516  ATAGATTCCCCGACCATGATCTGCCAAGATGGAATTTTACAGATTTCATGCATTCCTTCA  575

Query  64   TGATTGTGTTCCGTGTGCTATGCGGAGAATGGATTGAATCAATGTGGGATTGTATGCTTG  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  576  TGATTGTGTTCCGTGTGCTATGCGGAGAATGGATTGAATCAATGTGGGATTGTATGCTTG  635

Query  124  TCGGTGATGTATCCTGCATACCATTTTTCTTGGCCACTGTAGTGATAGGAAATTTAGTCG  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  636  TCGGTGATGTATCCTGCATACCATTTTTCTTGGCCACTGTAGTGATAGGAAATTTAGTCG  695

Query  184  TAAGTAATGCAAATTAACATGGACCAAGATCGTTTTTACATGACATTGTTTTGCAGGTGC  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  696  TAAGTAATGCAAATTAACATGGACCAAGATCGTTTTTACATGACATTGTTTTGCAGGTGC  755

Query  244  TTAACCTTT  252
            |||||||||
Sbjct  756  TTAACCTTT  764
```

Figure 5.4: Sequence alignment of query (sequence from inqaba biotechnology) and sequence from NCBI data base (accession number gb\AY615653.1) using BLAST. The sequence had identities of 249/249 (100%). This confirms that the PCR amplified the correct region of the gDNA. The red nucleotide is the point where west African *kdr* mutation occurs.

The nucleotide sequences of the amplified fragment from individual mosquito were then aligned together (Figure 5.5). This enabled the rapid identification of the point of mutation in the test samples.

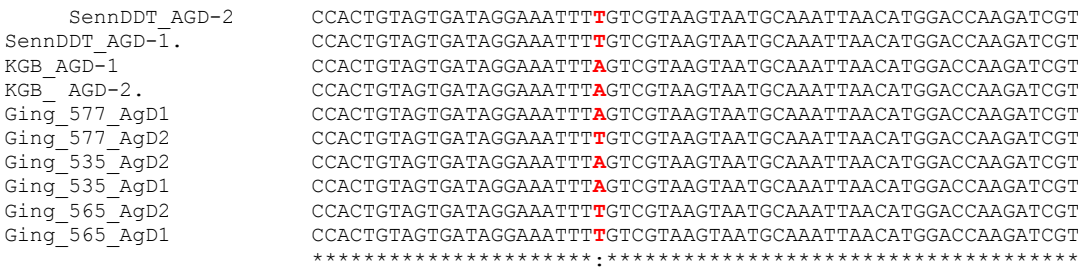


Figure 5.5: Alignment of partial sequences of the sodium channel gene associated with pyrethroid knockdown resistance in *An. gambiae* s.s. The nucleotides in red indicates the site of polymorphism. The control sequences SENN-DDT and KGB are RR and SS respectively. The genotype of sample Ging 535 is homozygous susceptible (SS), Ging 577 is heterozygous resistant (RS) and Ging 565 is homozygous resistant (RR). AgD1 and AgD2 represent the forward and reverse primers respectively, used for sequencing the PCR products.

Twenty six randomly selected DDT susceptible and DDT resistant samples from Siguiri with known *kdr* genotype by PCR were reamplified and their PCR products sent for sequencing. Only the West African *kdr* mutation was observed after analysing the sequences. Table 5.5 shows a comparison between the *kdr* genotypes obtained by PCR and those obtained by analysing the sequences.

Table 5.5: Comparison of *kdr* genotypes obtained by PCR and sequencing, using DDT susceptible and resistant *An. gambiae s.s.* n = number of sample, S = susceptible allele and R = resistant allele.

		***kdr* Genotype**	
Bioassay phenotype	**n**	***kdr* genotype by PCR**	***kdr* genotype by sequence analysis**
Susceptible	14	12 SS	12 SS
		2 RS	2 SS
Resistant	12	6 RR	3 RR and 3 RS
		6 RS	1 RS and 5 SS

5.3 Pyrosequencing PCR for amplification of West and East African *kdr* mutations

The region of genomic DNA flanking both West and East African *kdr* mutations was amplified yielding 200 bp PCR products Figure 5.6.

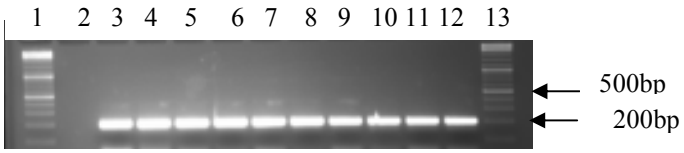


Figure 5.6: **PCR amplicons amplified for pyrosequencing (200 bp). Lanes 1 and 13: Molecular marker. Lane 2: negative control. Lanes 3-4: SENN DDT and KGB positive control and Lanes 5-12: individual samples.**

The generated PCR products were sequenced using a PSQ ^{MT} 96MA pyrosequencer system. Figure 5.7-5.10 shows pyrograms obtained with peak height proportional to number of nucleotide incorporated. The higher T peak in the yellow region of figure 5.8 to 5.10 reflects the incorporation of three consecutive Ts nucleotides. The A peak in figure 5.8 and 5.10 represents the incorporation of an A nucleotide. Three nucleotides, TTA forms the codon where the *kdr* mutation occurs. The second T represents position 1 where it is substituted by C in the case of the East African *kdr* mutation. Position 2 represented by A is substituted by T in the case of the West African *kdr* mutation. Both positions are located in the highlighted yellow region in the programs.

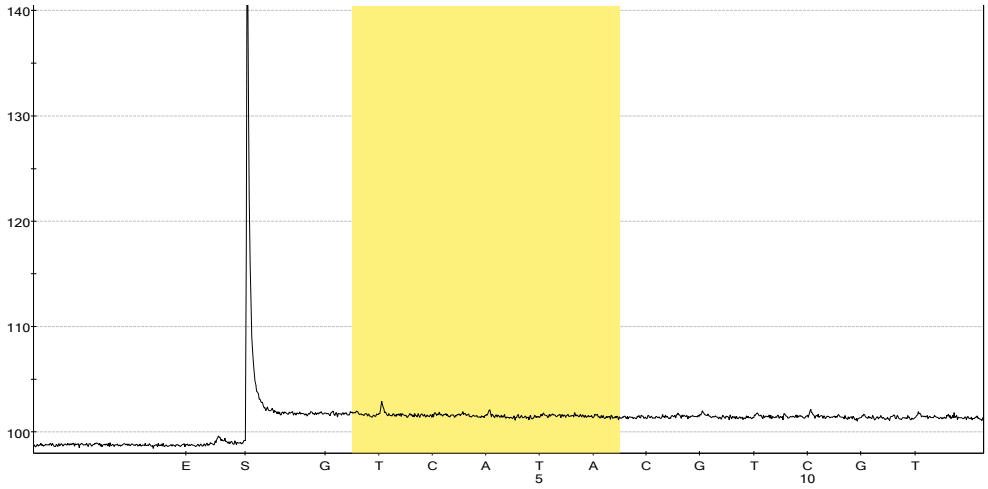


Figure 5.7: Pyrogram of PCR negative control generated from pyrosequencing reaction in one well of a 96 well plate. Initial addition of enzyme (E), substrate mixture (S) and sequential addition of nucleotides are shown on the x axis. The y axis (peak height) represents the number of nucleotide incorporated.

Figure 5.8 shows a pyrogram for PCR product generated using DNA template from KGB a susceptible mosquito. The first T peak represent the incorporation of a T nucleotide at position 1 and second peak, an A nucleotide in position 2. This gives a nucleotide sequence of TTA representing an SS genotype. The program was generated from a pyrosequencing reaction in one well of a 96 well plate.

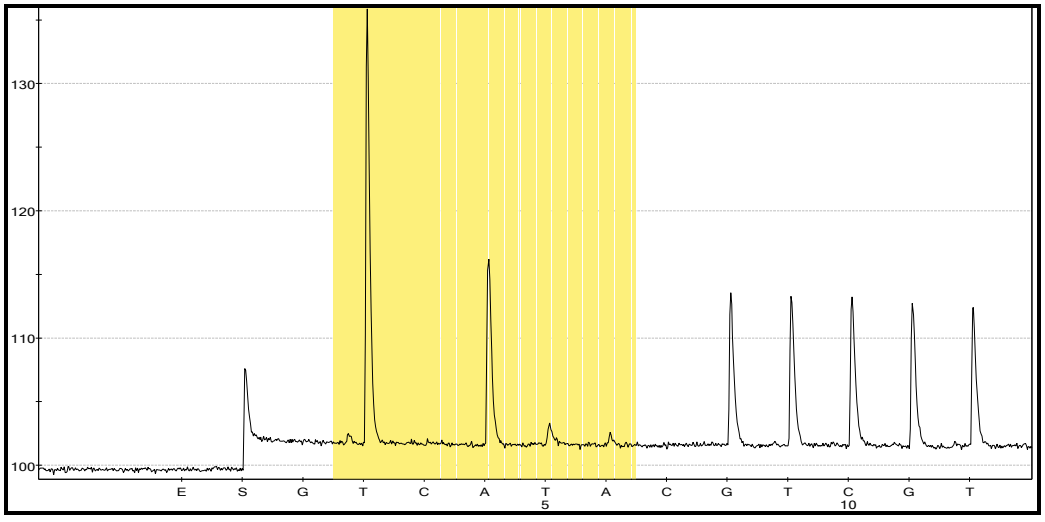


Figure 5.8: Pyrogram for homozygous susceptible (SS) positive control.

Figure 5.9 shows a pyrogram for PCR product generated using DNA template from SENN-DDT, a DDT resistant mosquito. A T is incorporated in positions 1 and 2. This gives a nucleotide sequence of TTT representing a RR genotype. Incorporation of identical consecutive nucleotide gave peaks higher than those generated when a single nucleotide was incorporated

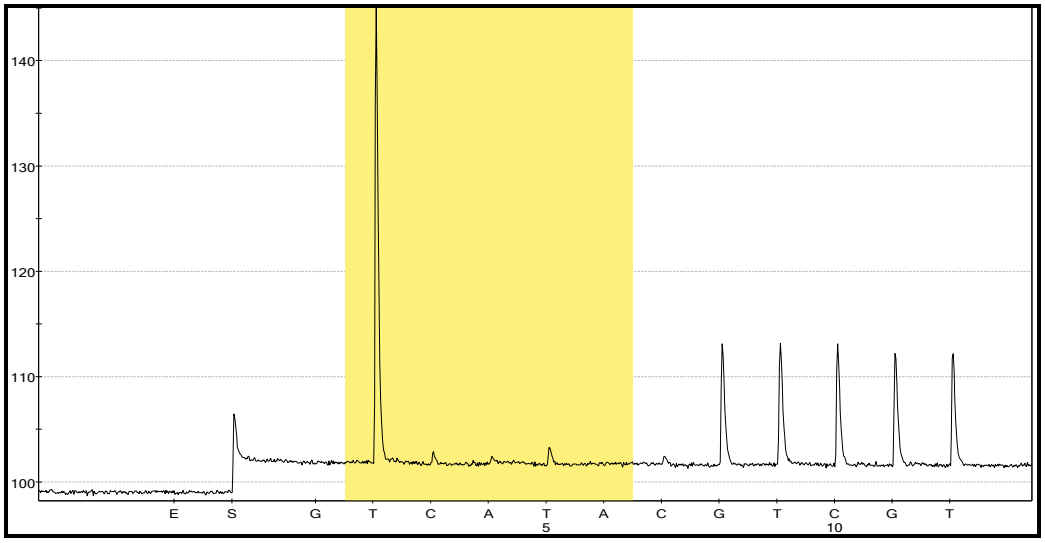


Figure 5.9: Pyrogram for homozygous resistant (RR) positive control.

Figure 5.10 represents a pyrogram generated using DNA template from a field collected mosquito. The three peaks in the highlighted region represent the incorporation of T nucleotides in position 1 together with an A and a T nucleotide in position 2. This gives a nucleotide sequence of TTA/T representing a RS genotype.

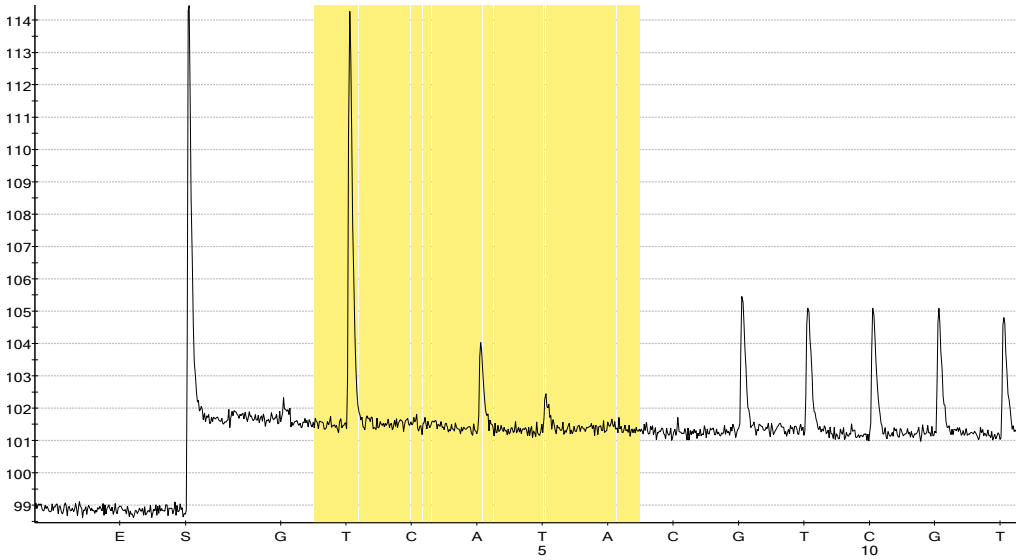


Figure 5.10: Pyrogram for heterozygous resistant (RS) positive control.

5.4 Evaluation of pyrosequencing compared to PCR assay.

Twenty-two samples with *kdr* genotypes obtained by pyrosequencing were amplified and sequenced. This was necessarily to check out the reliability of pyrosequencing. A detailed breakdown of the result is shown in Tables 5.6 and 5.7.

Table 5.6: Comparison of *kdr* genotypes obtained by PCR and by pyrosequencing.

Bioassay phenotype	Sample size	Genotype by PCR	Genotype by pyrosequencing
DDT susceptible	32	31 SS	31 SS
		1 RS	1 SS
DDT resistant	15	8 RR	6 RR, 2 RS
		5 SS	4 SS, 1 RS
		2 RS	2 RS
Deltamethrin susceptible	38	2 RR	1 SS, 1 RS
		33 SS	32 SS, 1 RS
		3 RS	1 RS, 2 SS

Table 5.7: Comparison of *kdr* genotypes obtained by pyrosequencing, PCR plus inqaba sequencing

Bioassay phenotype	Genotype by pyrosequencing	Genotype by sequencing	Genotype by PCR
Deltamethrin susceptible	5 SS	5 SS	1 RR, 3 SS, 1 RS
	2 RS	2 RS	1 RS, 1 SS
Deltamethrin resistant	3 RR	3 RR	2 RR, 1 RS
DDT susceptible	5 SS	5 SS	5 SS
DDT resistant	2 RS	2 RS	2 RR
	1 SS	1 SS	1 SS
	4 RR	4 RR	4 RR

All *kdr* genotypes generated by pyrosequencing match those obtained from conventional sequencing by 100 % (Table 5.7) whereas only 89% and 72 % Tables 5.6 and 5.7 respectively, PCR generated genotypes matched with pyrosequencing genotypes. *Kdr* genotypes obtained by pyrosequencing DDT/pyrethriod resistant and susceptible samples irrespective of site are shown in Table 5.8. The distribution of this mutation in the molecular forms of *An. gambiae* s.s. per study site, is shown in Table 5.9.

Table 5.8: *kdr* genotype obtained by pyrosequencing using DDT and deltamethrin susceptible and resistant *An. gambiae* s.s. from all three study sites. S = susceptible allele and R = resistant allele.

Bioassay phenotype	Sample size	*Kdr* genotype by pyrosequencing
DDT susceptible	38	37 SS
		1RR
DDT resistant	46	25 SS
		11 RS
		10 RR
Deltamethrin susceptible	56	51 SS
		3 RS
		2 RR
Deltamethrin resistant	4	3 RR
		1 SS

Table 5.9: *kdr* genotypes by pyrosequencing versus molecular forms in all three study sites

Locality	Sample size	Molecular form	*kdr* genotype			Allele frequency	
			RR	RS	SS	R	S
Siguiri	76	M	-	-	4	**-**	**1**
		S	12	11	49	**0.24**	**0.76**
Mount Nimba	64	M	1	-	30	**0.03**	**0.97**
		S	1	7	25	**0.14**	**0.86**
Boffa	6	M	-	-	-	**-**	**-**
		S	-	-	6	**-**	**1**

CHAPTER SIX

GENERAL DISCUSSION

6.1 Mosquito Collections

The majority of mosquitoes were collected from Mount Nimba followed by Boffa and lastly Siguiri. Mount Nimba and Boffa have high equatorial temperatures and rainfall which is different to the climatic conditions in Siguiri. These environmental factors, which are conducive for mosquito breeding, together with the absence of effective control measures results in massive vector populations. The mosquito environment in Boffa is dominated by the tidal river estuary which is frequently flushed by tidal flooding and heavy rains. This disturbs the breeding sites and may reduce the population size in comparison to Mount Nimba (Hunt pers comm). The personal use of anti-mosquito measures by the local human populations in Siguiri and Boffa may also account for the smaller number of mosquitoes collected at these two localities.

6.2 Species-specific identification

Identification of members of the *An. gambiae* complex is very important for malaria control because they are morphologically identical and two, or often more, members can occur in sympatry (Coluzzi *et al.,* 1979). Several techniques have been employed for identifying individual members of the *An. gambiae* complex. The PCR based technique used in this study had been successfully used by Scott *et al*. (1993) to

distinguish individuals of the *An. gambiae* complex for over 15 years. It is also used to identify interspecies hybrids.

Majority of specimens were identified as *An. gambiae* s.s except for two individuals from Siguiri that were identified as *An. arabiensis*. The absence of *An. arabiensis* in collections from Mount Nimba and Boffa may be due to the fact that samples were collected during the rainy season from July/August and *An. gambiae* s.s populations increase after the rains start. These results are similar to those from Libreville, Gabon, where samples collected during the rainy season were all identified as *An. gambiae* s.s. (Pinto *et al*., 2006). A similar result was also found in Madagascar (Tsy *et al*., 2003).

A total of 104/600 (17%) samples could not be identified to species level even when PCR analysis was repeated twice. It is possible that this was due to human error either in the laboratory or in the field. More likely is the fact that 86/104 (83%) of these mosquitoes were susceptible to insecticide which may have resulted in the mosquitoes DNA degrading after they died while waiting for the 24 hrs period needed to record percentage mortality before being stored on silica gel. In areas of high humidity, degradation of the DNA under these circumstances is highly possible.

6.3 Identification of *An. gambiae* molecular forms

Anopheles gambiae s.s. has been split recently into two molecular forms commonly referred to as M and S forms, which are assumed to be reproductively isolated (Favia

et al., 2001). Favia *et al*. (2001) developed a diagnostic PCR technique that allows the separation of these molecular forms.

The dominance of molecular forms has been associated with breeding sites. The M molecular form is associated with flooded/irrigated sites that provide permanent breeding conditions and the S form to rain-dependent breeding sites (Diabate *et al*., 2003). Most of the breeding sites in Siguiri and Boffa are mainly rain-dependent whereas in Mount Nimba, they are mostly flooded sites (Appendix II).

One hybrid M/S form (0.5%, 1/197) was detected from Mount Nimba where both M and S form occurred in sympatry at fairly high numbers. Our result is similar to findings reported by della Torre *et al*. (2001), who observed 0.4% (1/236) hybrids in Benin, 0.6% (1/181) in Ivory Coast and 1.4% (1/67) at Sombili in Guinea Conakry. Here the S form of *An. gambiae* predominated over the M form. This clearly indicates that low levels of hybridization between S and M forms, occur in the natural population. The difference in frequencies observed by our study and that of della Torre *et al*. (2001), in Guinea Conakry can probably be explained by the small sample size of their study. From this study, though, it is difficult to make definitive conclusions about gene flow and the presence of the hybrid requires further investigations. The possibility of contamination of samples is ruled out because each specimen was stored individually in separate vials. A negative control was included in the DNA extractions as well as in all PCR reactions and no contamination was observed.

6.4 Insecticide bioassays

Routine WHO bioassays carried out on *An. gambiae* s.l. collected from Siguiri and Mount Nimba indicated a level of tolerance to DDT, bendiocarb and dieldrin. Detection of resistance at these sites even with the use of mosquitoes of mixed ages indicates the presence of resistance genes in this population. The insecticide resistance observed in the field from this study is similar to that which was observed in Benin in West Africa. Here *An. gambiae* and *Culex quinquefaciatus* were resistant to DDT, bendiocarb, dieldrin and permethrin from four sites (Corbel *et al*., 2007). Resistance to dieldrin and bendiocarb in Boffa and also to deltamethrin and malathion in Siguiri was suspected and needs investigation. This implies that these insecticides can not be used for vector control in this study sites before a thorough investigation is done. In Mount Nimba, the mosquitoes were susceptible to deltamethrin and malathion. However, *An. gambiae* S form were more resistant to DDT and bendiocarb than the M form. More research is required to understand the underlining factors responsible for this discrimination.

Laboratory bioassay of F_1 progeny which were reared from wild *An. gambiae* collected from Boffa showed resistance to pirimiphos-methyl in 6/23 (26%) families and possible resistance in 4 (17%) families. Resistance found in pirimiphos methyl compared with complete susceptibility observed in malathion in the field is not surprising. Although these insecticides belong to the same class, resistance to malathion is as a result of a point mutation in the enzyme E3 esterase resulting in Trp – Leu substitution at position 251. Resistance to other organophosphates such as

pirimiphos methyl is caused by a point mutation resulting in the substitution of Gly-Asp at position 137 in the same enzyme (Hemingway and Ranson, 2000).

6.5 Biochemical analysis

Biochemical analysis on F_1 progeny was done to investigate the possible use of pirimiphos-methyl in the control of malaria vectors. The parents, from which the F_1 were generated, were collected from Boffa at the same time as the field bioassay specimens and should be of a good reflection of the enzymes systems present in the field population.

Insect GST's are of importance as they are thought to play a vital role in insecticide resistance. Their over expression has been implicated in the organophosphate fenitrothion and DDT resistance in *Musca domestica* and *An. subpictus* (Hemingway *et al.*, 1991). GSTs have not been linked to resistance to pirimiphos-methyl in *Anopheles gambiae*. In this study there was low GST activity and there was no correlation between bioassay data and GST activity.

Significantly increased levels of monooxygenase activity ($p < 0.05$) was found in the majority of both sexes (23/28 females and 24/28 males families) of F_1 progeny of the wild caught material from Boffa. The biochemical data showed a weak negative correlation with bioassay data in females ($R^2 = 0.005$, $p < 0.05$) and a negative correlation in the males ($R^2 = 0.093$, $p < 0.05$). The up regulated P450 may be as a result of environmental factors not necessarily linked to resistance as bioassays

showed complete susceptibility to deltamethrin, DDT and malathion except to the organophosphate pirimiphos methyl. However, monooxygenases are seldom implicated in organophosphate resistance (Brooke pers comm)

In this study alpha and beta esterases were significantly elevated in some families of both sexes of F_1 *An. gambiae* progeny reared from wild caught *An. gambiae* s.s. females. Comparatively, expression of alpha esterase was greater than that of beta esterase. Both alpha and beta esterase activity was over expressed more in females than males. This result is parallel to that obtained by Ferrari and Georghiou (1990) where organophosphate susceptible *Cx. quinquefasciatus* females had higher esterase activity when compared to males. A possible reason is that the females are most exposed to OP, pyrethriods and other insecticide selection pressures in their oviposition sites or indoors during blood meals. In this study, there was a weak correlation between biochemical data of both alpha and beta esterase with bioassay results, making the results inconclusive.

Elevated esterases and altered acetylcholinesterases are associated with resistance to organophosphate and carbamates insecticides in insects (Bisset *et al*., 1990). Altered AChE in *An. albimanus* from Central America has being implicated as the most common resistance mechanism to organophosphates and carbamates (Ayad and Georghiou, 1979). In this study, females from 9/28 families (33.3%) and none of the male progeny had percentage inhibition by propoxur less than the recommended 70% (Penilla *et al*. 1998). Percentage inhibition above 70% implies that there is no

mutation at the active site of AChE allowing the insecticide inhibitor to block enzyme activity. In this situation the substrate acetylthiocholine iodide will not be fully metabolized. Percentage inhibition below 70% means that there may be a mutation at the enzyme active site preventing the inhibitor (propoxur) from binding to the AChE. This allows the substrate to bind to the AChE active site and be metabolized. Our results suggest that there is an altered acetylcholinesterase operating in the wild population in Guinea Conakry. Earlier results by Djogbenou *et al*. (2008) confirmed the presence of altered acetylcholinesterase gene in *An. gambiae* in Burkina Faso, West Africa. Involvement of an altered AChE in pirimiphos methyl and carbamate resistance in our study is unclear and would need to be investigated.

6.6 Vector status

Malaria transmission and endemicity levels vary across Africa. High transmission occurs in area such as West Africa, Madagascar coast and Comoro Islands throughout the year (Macdonald. 1957). In these regions *An. gambiae* s.s. and *An. funestus* are the major vectors (Mouchet *et al*., 1993). Low transmission occurs in areas such as southern Africa and the highlands of Madacascar where it is highly seasonal. The main vectors here are *An. funestus* and *An. arabiensis* (Mouchet *et al*., 1998). This variation has been associated with factors such as vector density, survival, host contact and their feeding preference (Bogh *et al*., 2001). Knowledge of the sporozoite infectivity rate is very important as it helps in identifying main vectors to be targeted for malaria control. In this study, the sporozoite infectivity rate was investigated on samples species-specifically identified as members of the *An. gambiae* complex.

The ELISA technique has been used successfully for detecting the presence of *P. falciparum* in the salivary glands of infected female mosquitoes for many years (Wirtz *et al.,* 1992). ELISA has advantages over the standard method of dissecting and microscopically examining mosquito salivary glands for sporozoite infection. ELISA tests are cheap to run, can be repeated easily and can distinguish between the human species of *Plasmodium*. However, a problem with ELISA is that it overestimates the number of infected mosquitoes (Beier *et al*., 1990). Some studies have shown an overestimation of sporozoite infection rate by up to 1.1 to 1.5 times (Sokhna *et al.*, 1998, quoted by Lochouarn and Fontenille, 1999). Similarly, a study carried out in Senegal, a neighbouring country to Guinea Conakry, detected a 0.32% false positives in *An. gambiae* (Lochouarn and Fontenille, 1999). False ELISA positive results have also been reported in non vectors by Koekemoer *et al*. (2001) and Mouatcho *et al*. (2007).

In this study mosquitoes were found to be infected with *Plasmodium falciparum* and infection rates varied across all the sites. Of the 12 ELISA positives that were retested, a false positive rate of 75% was detected by PCR. The reminder of the positive samples could not be tested by PCR because no homogenate remained after the ELISA. If the ELISA results from Boffa (21% positive) are reduced by this factor, an infection rate of 5.25% is obtained which is much more in line with other published rates for West Africa (Gillies and De Meillon, 1968). In Siguiri and Mount Nimba, the sporozoite rates were 5% and 6% respectively and are reduced to 1.25%

and 1.5% if the 75% false positive rate is applied. Although ELISA is cheap for mass screening, this study recommends that all ELISA positives are checked by PCR.

6.7 *Kdr* mutation and pyrosequencing

Malaria mosquito control has mainly focused on the use of insecticides such as organochlorines, pyrethroids, organophosphates and carbamates through indoor residual spraying (IRS). The use of insecticide treated bed nets (ITN) has reduced childhood malaria morbidity in Gambia, Ghana and Kenya by 50% and global mortality by 20-30% (as quoted in Diabete *et al*., 2002). However resistance to insecticides used for both ITN'S and IRS limit the success of such interventions.

Knockdown resistance (*kdr*) involves amino acid changes in the sodium channel gene confering resistance to DDT and pyrethroids (Martinez-Torres *et al*., 1998). Two separate mutation events have been recorded: Leu to Phe (L1014F) in West Africa (Martinez-Torres *et al*., 1998) and Leu to Ser (L1014S) in East Africa Ranson *et al*. (2000). The frequency of the *kdr* allele is significantly increasesd in resistance selected samples in our study indicating an association between *kdr* and resistant phenotype. Extensive research has been done on the distribution of these *kdr* mutations across Africa by Santolamazza *et al*. (2008). In their study, they showed that Africa can be divided into three geographical areas based on *Kdr* distribution. The Western area where L1014F occurs exclusively and at times reaches frequencies as high as 50%. The West-Central area, where both L1014F and L1014S mutations are present at variable frequencies. Lastly, the East Africa area where the L1014S

occurs exclusively but at low frequencies. It is worth noting that *Kdr* frequencies above 90% have been recorded in West Africa (Fanello *et al*. (2003). Our study confirmed the presence of only the L1014F mutation. Results showed that *kdr* mutation was present in the S form in two sites. Frequencies of 24% and 14% were recorded in Siguiri and Mount Nimba respectively.This tallies with the study of Santolamazza *et al*. (2008) who showed that L1014F is the only mutation in West Africa (Figure 6.1). We also recorded a single L1014F heterozygote in the M form in Mount Nimba. Though this is a rare occurence in West Africa, a similar result was recorded in Benin where the first case of L1014F in the M form was reported (Akogbeto and Yakoubou, 1999). It is hypothesised that L1014F in M form is spreading eastward and westward (Diabete *et al.,* 2002) although this may be simply a matter of sampling bias.

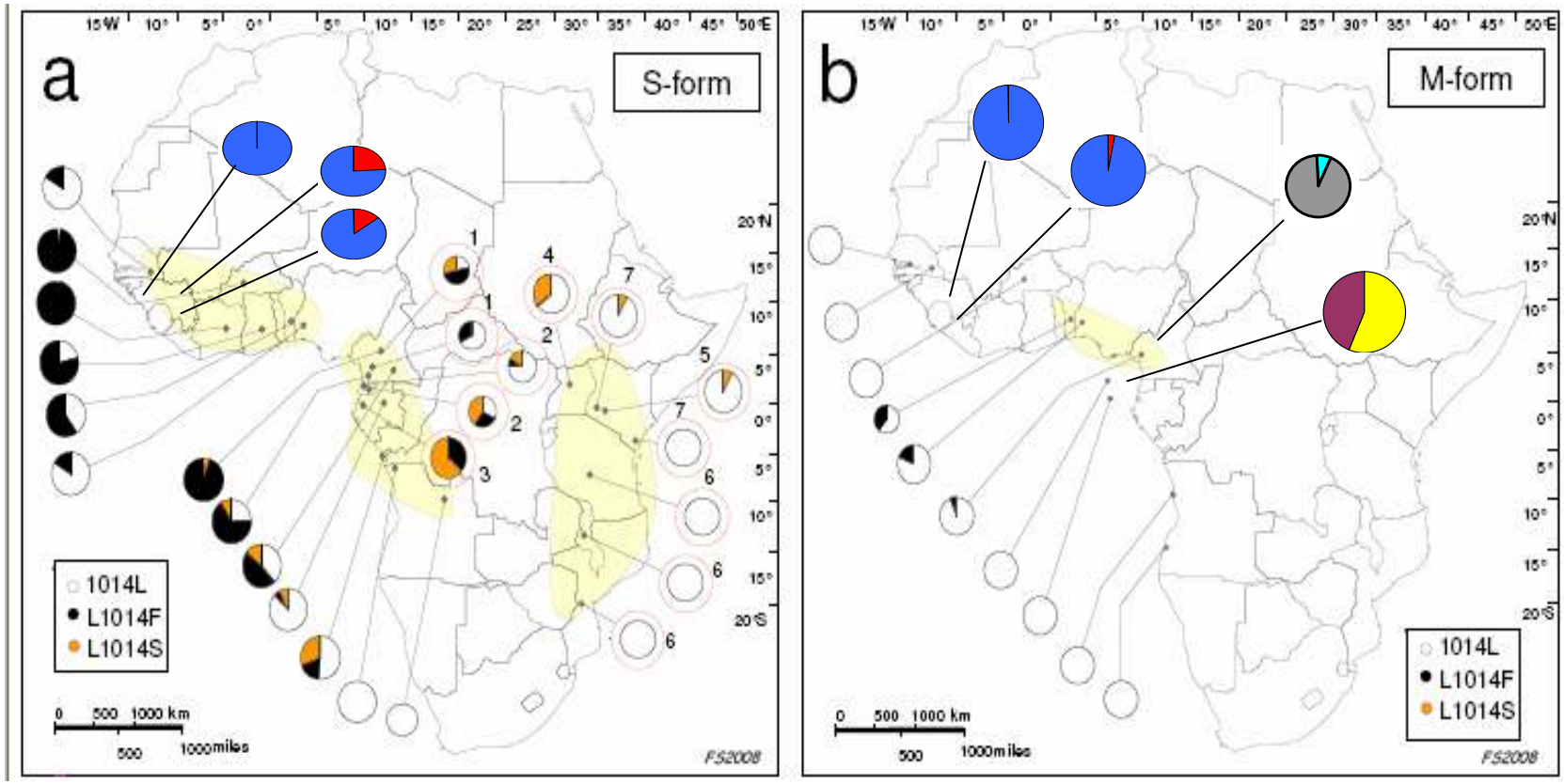


Figure 6.1: Distribution of *kdr* mutation in *An. gambiae* molecular forms across Africa (Santolamazza *et al*., 2008). Findings from our study are superimposed on the map with blue section of pie chart representing the 1014L allele and the red representing the L1014F (*kdr* mutation). *Kdr* mutation frequencies in the M form according to Reimer *et al*. (2005) and Etang *et al*. (2006) are shown in yellow and green respectively in the pie chart.

This study highlights the long held view that PCR based methods for detecting single point mutations are not reliable (Matambo *et al.,* 2007). By comparing *kdr* genotypes obtained by PCR and sequencing in this study 38.5% were inaccurate.

Results comparing the pyrosequencing and conventional sequencing methods, however, correlated 100%. Based on these results it is clear that pyrosequencing is more accurate than the conventional PCR technique of Martinez-Torres *et al.* (1998). It has other advantages in that it is quick (96 samples can be analysed in 40 minutes), nonradioactive, simple and can screen both West and East African *kdr* mutation in a single run using a single pyrosequencing primer. The disadvantages, however, are that the pyrosequencer machine is expensive and processing one sample will cost 2US $ compared with 1 US$ for conventional PCR.

Finally, an interesting aspect of the *kdr* results from the Siguiri S form is the fact that the allele frequencies calculated from the PCR results (R = 0.7, S = 0.3) are the opposite of those obtained from the pyrosequencing (R = 0.24, S = 0.76). Furthermore, if Hardy-Weinberg equilibrium is calculated on the gene frequencies of both data sets, only the pyrosequencing results show a significant deficit of heterozygotes ($\chi^2 = 10.79$, $p< 0.05$). The results for the PCR data set are in Hardy-Weinberg equilibrium ($\chi^2 = 0.47$, $p>0.05$). The implication of these results is unknown and further studies are required at Siguiri.

CHAPTER SEVEN

CONCLUSION

The mosquitoes used in this study were collected during surveys to specifically inform control programs on what vectors were present, what their role in transmission was, and what their insecticide susceptibility status was. These objectives were achieved with some interesting results.

Anopheles gambiae s.s. was the main malaria vector in all the study sites and occurred in sympatry with *An. arabiensis* only in Siguiri. In Siguiri and Boffa, the S molecular form predominated over the M form while in Mount Nimba the M form was marginally more abundant than the S form. Estimates of *Plasmodium falciparum* infectivity rates in *An. gambiae* S form at Boffa were out by 75% according to PCR verification of ELISA-positives specimens. Adjusted results gave rates of 5.25% at Boffa, 1.25% at Siguiri and 1.5% at Mount Nimba. The M form at Mount Nimba (the only locality where sufficient numbers were collected) showed similar infectivity rates to the S form.

Field insecticide susceptibility tests indicated that wild populations of *An. gambiae* s.s in Siguiri and Mount Nimba were tolerant to DDT, bendiocarb and dieldrin. Wild populations of *An. gambiae* s.s in Boffa were fully susceptible to deltamethrin and malathion. Suspected resistance to deltamethrin and malathion was observed in

Siguiri and to dieldrin and bendiocarb in Boffa. Laboratory bioassay showed that resistance to the organophosphate pirimiphos-methyl occurred at a low frequency in Boffa.

The contribution of elevated esterases and monooxygenases to resistance to pirimiphos-methyl in the F_1 families is unclear as these enzymes where also elevated in the susceptible families. More work needs to be done to confirm the resistance mechanism. However, there is presence of *kdr* mutation with correlation to resistance selection, but its involvement in resistance is not clear as only resistance to DDT and not pyrethroid was detected by field bioassay. This needs to be investigated further.

Based on bioassay results, pyrethroids and organophosphates could be the insecticides of choice, for indoor residual spraying (IRS) to control malaria vectors in these sites. Long-lived deltamethrin can be applied during January in the dry season followed nine months later by a short-lived organophosphate such as pirimiphos methyl. Repeating this rotational strategy will help prevent the development of resistance to these insecticide (Hunt pers comm). A further investigation of the role of *kdr* mutation and the suspected resistance to insecticides in this study is recommended. Pyrosequencing provides an alternative method of detecting the *kdr* mutation compared with the conventional PCR assay, and is therefore recommended for detecting the West African *kdr* mutation.

APPENDIX I

LABORATORY METHODS

A) Preparation of PCR solutions Sambrook *et al.* (1989)

- **TAE (Tris Acetic EDTA) Buffer 50X (pH 8)**

  242g Tris

  37.2 g Na_2 EDTA.$2H_2O$

  57.1M glycial acetic acid

  Make up to 1 L

- **Agarose gel**

  For a 2.5% agarose gel, dissolve 10g agarose in 400ml 1X TAE

- **Ethidium bromide**

  Dissolve 10mg EtBr crystals in 1ml distilled H_2O

- **Ficoll dye**

  50% sucrose

  1ml 0.05M EDTA (pH7.0)

  0.1% Bromophenol blue

  10% ficoll

- Super Therm Gold Buffer

  (20 mM Tris HCl p H 8,

  100 mM KCl, 0.1 mM EDTA,

  1 mM DDT, 0.5% nonidet and 50% v/v glycerol

B) **DNA extraction solution** (Collins *et al*., 1987)

- **8M KAc**
- **Grinding buffer**

  0.08M NaCl

  0.16M Sucrose

  0.06M EDTA

  0.5% SDS

  0.1M Tris-HCl

- **TA (Tris EDTA) Buffer** (Sambrook *et al.,*1989)

  100ml 1M Tris (pH)

  20ml 0.5M EDTA

  Make up volume to 1L.

C) Preparations of sporozoite ELISA solutions (Wirtz *et al.,* 1987)

- **Phosphate Buffer Saline (PBS) 10X**
- **Blocking Buffer (BB)**

  2.5% Casein

  50ml 0.1N NaOH

  450ml 0.01M PBS pH 7.4

  0.002% Phenol red

- **Phosphate Buffer Saline Tween 20 (PBS-Tween 20)**

  500µl Tween 20 plus 1 litre 1 X PBS

- **Grinding Buffer (BB-NP40)**

50ml BB plus 250 µl NP-40

D) Preparation of the buffers used in the biochemical analysis (Penilla *et al*. (1998) and Hemingway *et al*. (1997)

- **0.1 M Na_2HPO_4 pH 7.4**

  Na_2HPO_4 14.2 g/1litre dH20–dibasic

  Na_2PO_4 12 g/1L dH_20-monobasic

  Add monobasic to dibasic to adjust pH

- **0.05M Na_2HPO_4 pH 7.4**

  350ml 0.1M buffer plus 350ml dH_2O. Monobasic and dibasic solutions

  Add monobasic to dibasic to adjust pH

- **0.02 M Na_2HPO_4 pH 7.2**

  100ml 0.1M buffer plus 400ml dH_2O. Monobasic and dibasic solutions

  Add monobasic to dibasic to adjust p H

- **0.1M Na_2HPO_4 pH 6.5**

  As * but adjust pH to 6.5

- **0.1 M Na_2HPO_4 5% SDS pH 7.0**

  As * but adjust pH plus 25g SDS per 500ml buffer

  SDS (Sodium dodecyl sulphate)

- **0.1 M Na_2HPO_4 1% Triton pH 7.8**

  As * but adjust pH plus 1ml Triton per 100ml buffer

  Triton X-100

- **0.25 M $NaC_2H_3O_2$ (Sodium acetate) buffer pH 5.0**

  10.2537 g/500ml dH_2O plus HCl to adjust pH

- **0.0625 M K_2HPO_4 pH 7.2**

  2.7219g K_2HPO_4/250ml dH_2O Dibasic

  2.1265g/K_2HPO_4/250ml dH_2O Monobasic

  Adjust pH by adding monobasic to dibasic

- **30M α- Naphthyl acetate and β-Naphthyl acetate**

  0.055g α or β NA in 10ml acetone

- **0.1M propoxur in 10ml acetone**

D) Pyrosequencing solutions (www.biotage.com)

- **Annealing buffer (1XAB), pH 7.6**

  -20m M Tris-Acetate (2.42 g/L)

  -2 mM Mg-Acetate (0.43 g/L)

  Dissolve chemicals in 900 ml Milli-Q (18.2MΩ x cm) water

  Adjust pH to 7.6 with 4 M acetic acid at 22 °C ± 1°C

  Fill up to 1000 ml with Milli-Q (18.2MΩ x cm) water.

- **Denaturation solution, 0.2 M NaOH**

  Dissolve 8g NaOH in 950 ml Milli-Q (18.2MΩ x cm) water.

  Fill up to 1000ml with Milli-Q (18.2MΩ x cm) water.

- **Washing buffer, pH 7.6**

  -10 mM Tris-Acetate

Dissolve 1.21g Tris-acetate in 900 ml Milli-Q (18.2MΩ x cm) water

Adjust pH to 7.6 with 4 M acetic acid at 22 °C ± 1 °C

Fill up to 1000 ml with Milli-Q (18.2MΩ x cm) water

- **0.5 M NaOH for denaturation of DNA**

  Dissolve 20g NaOH in 950 ml Milli-Q (18.2MΩ x cm) water

  Fill up to 1000 ml with Milli-Q (18.2MΩ x cm) water

- **Binding buffer pH 7.6**

  -10 mM Tris-HCl

  -2 M NaCl

  -1 mM EDTA

  -0.1% Tween 20

  Dissolve 1.21g Tris acetate, 117g, NaCl and 0.292g EDTA in 900ml Milli-Q (18.2MΩ x cm) water

  Adjust pH to 7.6 with 1 M HCl at 22 °C ± 1 °C

  Add 1ml Tween 20 and fill up to 1000 ml with Milli-Q (18.2MΩ x cm) water

APPENDIX II

Pictures of *An. gambiae* breeding sites



A



B

Typical *Anopheles gambiae* breeding sites in Siguiri. A. Informal mining operations. B. Road ruts close to village habitations.



Typical housing in Gbakore village in Mount Nimba



Actual breeding site in Gbakore village in Mount Nimba



Field insecticide susceptibility tests

SUSCEPTIBILITY TESTS FORM

Date:

Area information
Country: Province:
Sample information
Species tested: Species control: Sex: Age (days) :
Collection method :
Human Landing Indoor ☐ Resting night Indoor ☐
Human Landing Outdoor ☐ Resting night Outdoor ☐
Cattle Collection ☐ Larval collection ☐
Resting morning indoor ☐ Colony ☐
Physiological stage Non-blood fed ☐ Blood fed ☐ Semi-gravid ☐ Gravid ☐
Insecticide information
Insecticide tested: Concentration:
Impregnated papers prepared by: Date of impregnation:
Date of expiry: Date paper removed from pack ☐☐☐☐☐☐
Number of times the paper is used:
Storage conditions: Room temperature ☐ Refrigerated ☐

Test conditions

	Temperature °C	Relative humidity
Exposure period: Start		
Holding period: after 12 hours		
Holding period: End		

Test results: **Period of exposure (minutes)**

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Control
No.exposed						

Number of knocked down mosquitoes after exposure for minutes :

	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Control	
	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.
START												
10'												
15'												
20'												
30'												
40'												
50'												
60'												

Number of killed mosquitoes at the End of holding period

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Control
After 24 hours:						

APPENDIX IV

PRESENTATIONS:

1. Scientific talk

A) VEZENEGHO, S. B., KOEKEMOER, L. L., HUNT, R. H., and COETZEE, M.

Pyrosequencing: an alternative method for identifying knockdown resistant mutations in malaria vector mosquitoes National Institute for Communicable Diaeases (NHLS) scientific presentation. James gear auditorium, Sandringham. 12 March 2008.

B) VEZENEGHO, S. B., KOEKEMOER, L. L., HUNT, R. H., and COETZEE, M.

Baseline surveys of malaria vector mosquitoes from Guinea Conakry, West Africa. XXIII International Congress of Entomology, ICE 2008- Durban, South Africa.

2. Poster

VEZENEGHO, S. B., KOEKEMOER, L. L., HUNT, R. H., and COETZEE, M.

Baseline surveys of malaria vector mosquitoes from Guinea Conakry, West Africa. Witwatersrand facuty of health science research day (Abstract submitted.)

ARTICLES:

1. VEZENEGHO, S. B., KOEKEMOER, L. L., HUNT, R. H., and COETZEE, M.

Baseline surveys of malaria vector mosquitoes from Guinea Conakry, West Africa (In prep.)

2. VEZENEGHO, **S. B., KOEKEMOER, L. L.**, **HUNT**, **R. H., and COETZEE, M.**

Pyrosequencing: an alternative method for identifying knockdown resistant mutations in malaria vector mosquitoes (In prep.)

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