DISTRIBUTION OF SPECIES OF THE *ANOPHELES GAMBIAE* COMPLEX AND THEIR PYRETHROID INSECTICIDE KNOCK DOWN RESISTANCE STATUS IN THE KUMASI METROPOLIS.

NICHOLAS AGYEPONG

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DECLARATION

I hereby declare that this dissertation is the outcome of an original research work carried out at Noguchi Memorial Institute of Medical Research, Legon, by me Nicholas Agyepong of the Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science Technology, Kumasi, except for the references to other works which have been duly cited. I also declare that this dissertation has neither in whole nor in part been presented for a degree anywhere.

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Nicholas Agyepong

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Mr. E. E. Mak-Mensah

……

Dr. F. K. N. Arthur

……

Dr. Charles A. Brown (NMIMR, UGMS)

Certified by:

……

Dr. (Mrs.). Ibok Oduro (Head, Biochemistry and Biotechnology Dept.)

DEDICATION

This work is dedicated to my mum, Madam Alice A. Frimpong, Ivy Antwi- Adu, Nana Brefo Boateng (Executive Secretary, Otumfuo Education Fund) and the rest of my family at Jamasi, Ashanti whose support and contributions have made my education this far possible.

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ABSTRACT

The *Anopheles* species play a central role in malaria transmission. Several methods are available to map out the distribution and distinction of various species of the *Anopheles* in the tropics and sub-tropical regions of the world. However the advent of molecular technology, polymerase chain reaction assay, which is faster and based on genotypic diversity of the species, simplified this research. This study was set out to identify and determine the distribution of *Anopheles gambiae* s.s, (the most prevalent malaria vector) within the selected communities in Kumasi. DNA was extracted from *Anopheles* mosquitoes larvae and used as template in polymerase chain reaction (PCR), for each specimen. Knock down resistance (Kdr) was further carried out on the samples. Seventy six out of 100 larvae collected were identified as *gambiae* s.s. Twenty six out of 50 *An. gambiae* samples were found to posses Kdr gene with remaining 24 samples being susceptible.

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ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycystidine triphosphate
dGTP	deoxyguanosine triphophate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucloside triphosphate
dTTP	deoxythymidine triphosphate
EDTA	Ethylene diamine tetraacetate disodium**.** $2H_2O$
EtBr	ethidium bromide
EtOH	ethanol
GPS	global positioning system.
H_2O	water
KOH	potassium hydroxide
M	molar (moles per litre)
ul	microlitre
uM	micromolar
ml	milliliter
Mw	molecular weight
NaOH	sodium hydroxide
PCR	polymerase chain reaction
pH	hydrogen-ion exponent
RNase	ribonuclease
Kdr	knockdown resistance
rpm	revolutions per minute
rRNA	ribosomal RNA
s.s	sensu stricto
sddH_2O	sterile double distilled water
DDT	dichlorodiphenyltrichloroethane

CHAPTER ONE

1.1 INTRODUCTION

Malaria remains one of the most devastating diseases occurring in the world today. It is estimated that about 350-500 million clinical cases occur every year with approximately 1-3 million deaths in tropical Africa alone (Bremen, 2001; WHO/UNICEF, 2003). This represents at least one death in every thirty seconds (WHO, 1996). Majority of the cases occur in children under five years (WHO/UNICEF, 2003; Greenwood *et al.*, 2005) and pregnant women are also especially vulnerable. Approximately 40% of the world's population live in regions where malaria transmission is endemic (Aultman *et al.*, 2002).

Presently, malaria is endemic in a broad band around the equator, South America, South and South-East Asia, part of Middle East and Sub-Saharan Africa where 85-90% of malaria fatalities occur (Scott, 1991). The geographic distribution of malaria within large regions is complex, and malarial and malaria-free areas are often found close to each other (Green, 1997).

In Ghana, malaria is one of the major public health problems. The disease is hyper-endemic and accounts for nearly 22% of all death and between 42 - 44% of all out-patients cases. The disease ranks fifth as the commonest cause of death in the 0-4 year of age group (Centre for Health Statistics, 1992).

Anopheles mosquitoes are the principal vector of malaria transmission in the tropical and sub-tropical areas of the world (WHO, 1999). *Anopheles* belongs to the order *Diplera,* sub-order *Nematocera,* family *culidae* and sub-family Anophelinae. The species have a worldwide distribution, occurring in both tropical and temperate

regions (Service, 1980). There are over five hundred known species of *Anopheles*, but only sixty are known to transmit malaria (Service, 1980).

In Africa, most of the important malaria vectors belong to a species complex, whose members are difficult and sometimes impossible to distinguish morphologically (Gilles and De Meillon, 1968; Culluzzi, 1984; Gilles and Coetzee, 1987; Fontenille and Lochoven, 1999). These difficulties have stimulated the development of molecular tools for precise and reliable identification of sibling species.

The *Anopheles gambiae* complex consists of six named species (Gilles and Coetzee, 1987) one unnamed species (Hunt *et al*., 1998) and several incipient species (Coluzzi *et al*, 1985; Favia *et al.*, 1997). The six named species are *An. gambiae sensu strictu, An. arabiensi, An. merus, An. melas, An. quadrianmnulatus* and *An. bwambae*. The complex varies in their ability to transmit malaria (White, 1974; Hunt *et al*., 1998) and is collectively known as *Anopheles gambiae* s.l (sensu lato).

Despite the increasing effort and international commitment to control the disease, there has been little change in the regions which are at risks since 1992 (Hay *et al*., 2004). It is also estimated that, if the prevalence of malaria stays on its present upwards course, the death rate could double in the next twenty years (Bremen, 2001). Precise statistics are unknown because many occur in the rural areas where people do not have access to hospitals or means to afford health care (Bremen, 2001).

Prevention of malaria encompasses a variety of measures that may protect individuals against infections or against the development of disease. One of the ways to protect people from the parasite is by the use of chemical prophylaxis. Anti-malarial drugs include chloroquine, doxycycline, hydroxyl chloroquine sulphate, mefloquine,

primaquine, quinine, sulfadoxine-pyrimethamine and tetracycline. Chloroquine is inexpensive and often effective, but chloroquine-resistant strains of *Plasmodium* have appeared in Africa, India and South America and are especially prevalent in Southeast Asia (WHO, 1997; NIAID, 2000). Where chloroquine-resistant strains of malaria parasites are present, more expensive drugs such as mefloquine need to be used. However, strains of *Plasmodium* resistant to multiple anti-malaria drugs are becoming problematic in endemic regions (Kashirsagar *et al.*, 2000).

Another approach for preventing malaria is to reduce the incidence of bites from *Anopheles* mosquitoes. This can be done by reducing *Anopheles* populations with chemicals such as dichlorodiphenyltrichloroethane (DDT) or introducing biological control agents such as the bacterium, *Bacillus thuringiensi* or the fungus *Lagenidium giganteum.* It can also be achieved by employing physical barriers, such as long sleeves, house screens, insect repellent creams and insecticide-impregnated bed nets.

At present, pyrethroid insecticides are the only option for impregnating bed nets for malaria control. However, resistance to this group of insecticide has been reported in some African countries including Burkina Faso, Cote d' Ivoire (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999) Ghana (Adasi *et al.*, 2000) and Kenya (Vulule *et al.*, 1994; Ranson *et al.*, 2000). The development of pyrethroid resistance may hamper the effective use of these treated bed nets (Curtis *et al.*, 1990; Curtis *et al.*, 1996) in areas where enhanced tolerance or resistance to pyrethroid has been reported. The solution to the sustained use of insecticide-impregnated bed nets could therefore be the consideration of alternative insecticide to be used in mixtures or in rotation with the existing pyrethroid in areas where resistance to the existing pyrethroid has been established (Curtis, 1985; Curtis *et al.*, 1993; Curtis *et al.*, 1998)

Currently, there is no effective vaccine for the control of malaria and this is due to the antigenic modulation exhibited by the parasites. However, recent investigation including the partial protection of rhesus monkeys against lethal *P. knowlesi* infection (Rogers *et al.*, 2001) indicate that human malaria vaccines may be developed sooner than later.

There are therefore no simple solutions to the world's malaria problem and it is unlikely that a single strategy for control will be applicable to all countries and all epidemiological situations. To ensure effective interventions for the future, a rational approach to the problem of malaria is required.

1.2 Justifications

Anopheles gambiae species are the most prevalent malaria vector in the tropics and sub-tropics. Small pools of stagnant water, small water collections in hoof prints or rubbish and stagnant/choked gutters are the characteristics of their breeding grounds. These habitats are very common in Kumasi due to its high population growth, high rate of rural-urban migration and the opening up of previously sparsely inhabited communities. These factors promote the development and distribution of the different the *Anopheles* species within the metropolis. However not much studies have been done to identify and determine the distribution of these vectors in order to control malaria transmission. The study of the distribution of specific malaria vectors is therefore pre-requisite if meaningful planning and monitoring of successful malaria control and eradication programmes are to be done. Knock down resistance (kdr) confers resistance of *An. gambiae* to pyrethroid and dichlorodiphenyltrichloroethane (DDT). Detection of kdr in Anopheles population is important, as it would help policy makers to decide on the use of alternative approach in malaria vector control programme.

1.3 GENERAL OBJECTIVE

This study aims to identify species and the knock down resistance in *Anopheles gambiae* in the Kumasi metropolis using the polymerase chain reaction (PCR).

1.3.1 SPECIFIC OBJECTIVES

1. To extract the DNA from *Anopheles* mosquito larvae collected from different sites in Kumasi.
2. To identify by PCR the sibling species of *Anopheles* gambiae of each larvae.
3. To detect the presence of kdr allele in PCR positive *Anopheles gambiae* Mosquitoes.

CHAPTER TWO

LITERATURE REVIEW

2.1 MOSQUITOES

2.1.1 General description

Mosquitoes are probably the carriers of the most devastating and debilitating parasitic and viral diseases occurring in the world today. Mosquito bites can transmit diseases such as malaria, dengue, filariasis, Venezuelan equine encephalitic and yellow fever.

Mosquitoes are believed to have evolved around 170 million years ago during the Jurassic era (206-135 million years ago) with the earliest known fossils from the Cretaceous era (144-65 million years ago) (Clement, 1992). They evolved in the land mass that is now South America, spreading initially to the northern continent, Laurasia and re-entering the tropics from the north (Floore, 2000). Ancestral mosquitoes were about three times the size of the extant species and they are a sister group to the chaoboridal (biting midges) (Clement, 1992).

2.1.2 Classification, Distribution and Ecology

There are about 3300 species of mosquitoes belonging to 41 genera, all contained in the family Culicidae. This is divided into three sub-families; Toxorhynchitinae, Anophelinae (Anophelines) and Culicinae (culicines). The family Culicidae belongs to the order Diptera, sub-order Nematocerca of the class Insecta which is the most dominant group of the phylum Arthropoda (Service, 1993). Mosquitoes are readily distinguished from other similar-looking flies in the sub-order *(*Nematoerca) by their conspicuous forwardly projecting proboscis, scales on the thorax, legs, abdomen and wing veins, and fringe of scale along the posterior margin of the wings (Kettle, 1992; Service, 1993).

Mosquitoes are found throughout the tropical and temperate regions and extend their range northwards into the Artic circle. The only areas from which they are absent are Antarctica and few islands (Service, 1980; Sallum *et al*., 2000). They are found at elevation of 5500 metres and down mines at a depth of 1250 metres below sea level (Lehame, 1991). The most important pest/vector species belong to the genera Anopheles, Culex, Aedes, Ochlerotaus, Psorophora, Haemagogus and Subethes (Service 1980). Among these, Aedes have the highest proportion and are distributed throughout the world, especially in temperate countries (White, 1996). Aedes species are important vectors of yellow fever, dengue, Japanese encephalitis and many arboviruses and in few restricted areas they are also vectors of *Wuchereria bancrofti* and *Brugia malayi* (White, 1996).

Anopheles which contains by far the largest number of species has almost a worldwide distribution (Lehame, 1991; Sallum *et al.,* 2000). *Anopheles* species mainly occur in the tropics and subtropics of the world. In addition to transmitting malaria, they are vectors of filariasis (*Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*) and a few arboviruses (White, 1996). In Africa, malaria transmission is mainly due to *An. gambiae* and *An. funestus*. *Anopheles gambiae*, the most important malaria vector is a complex comprising seven species with *An. gambiae* s.s. and *An. arabiensis* as the major vectors.

2.1.3 External morphology

Mosquitoes are slender and relatively small insect usually measuring about 3-6 mm in length. In most females, the size varies but rarely greater than 15 mm (Lahame, 1991). zMost mosquitoes weigh only about 2 to 2.5 mg and the body is distinctly divided into a head, thorax and abdomen (Lahame, 1991).

The head has a conspicuous pair of kidney-shaped compound eyes. Between the eyes arises a pair of filamentous and segmented antennae. In females the antennae have whorls of short hair, but in males, with exceptions of some the antennae have many long hairs giving them a feathery or plumose appearance. Based on the antennae mosquitoes can be conveniently sexed by examination; thus the ones with feathery antennae are males, whereas those with only short and rather conspicuous antennal hairs are females (Robert and Collins, 1996).

The thorax is covered, dorsally and laterally with scales which may be dull or shining, white, brown, black in colour. It is the arrangement of black and white or coloured scales on the dorsal surface of the thorax that gives many species (especially those of the general *Aedes* and *Ochlerotatus*) distinctive patterns (Robert and Collins, 1996).

Mosquitoes possess only one pair of functional wings, the fore-wings. The hind wings are represented by a pair of small, knob-like halters. They are distinct from other flies of almost similar shape and size by the possession of:

a. conspicuous forward-projecting proboscis,
b. numerous oppressed scales on the thorax, legs, abdomen and wing vein
c. fringe of scales along the posterior margin of the wings (Lahame, 1991).

The abdomen is composed of ten segments but only the first seven or eight are visible. In mosquitoes of the subfamily Culicinae, the abdomen is usually covered dorsally and ventrally with mostly brown, blackish or whitish scales. In the anopheline, however, the abdomen is almost or entirely devoid of scales (Lahame, 1991). The last abdominal segment of the female mosquito terminates in a pair of small finger like cerci, whereas in the males a pair of prominent claspers, comprising part of the male external genitalia, is present. In unfed mosquitoes the abdomen is thin and slender, but

after females have bitten a suitable host and taken a blood-meal the abdomen becomes greatly distended and resembles an oval red balloon (Southgate, 1979).

2.1.4 Medical importance

Mosquito-borne diseases cause significant health problems, mostly in the subtropics and tropics and their incidence has increased within the last two decades (World Bank Report, 2001). They are estimated to transmit diseases to more than 69 million people annually (World Health Report, 2001).

In the United States, Australia, New Zealand, the U.K, Scandinavia and other temperate countries, mosquito bites are mostly a nuisance, they cause severe skin irritation through an allergic reaction to the mosquitos' saliva causing a red bump and itching (White, 1996).

Estimates from the World Health Organization indicate that malaria is the leading cause of mortality and morbidity in developing countries around the world especially in the tropics and subtropics (World Health Report, 2001). Malaria is exclusively transmitted by Anopheles mosquitoes and about 100-500 million clinical cases may occur every year in tropical Africa alone, where changes in the epidemiological situation in the last few years have resulted in an increased frequency of the disease (WHO Report 2001).

2.2 *ANOPHELES* MOSQUITOES

2.2.1 Life cycle and feeding habit

The mosquito undergoes complete metamorphosis, through four distinct stages (Fig. 1) in its cycle: egg, larva, pupa and adult, a process that was first described by the

Greek philosopher Aristotle (Floore, 2000). The first three stages occur in water and the length of each stage is dependent on the species and temperature.

The larvae hatch after a few days from the eggs as small "wrigglers" in puddles or in water-filled containers. These breathe air through a siphon at the tail end. The development of the larva can usually be completed in less than a week in very warm conditions with ample food (Robert and Collins, 1996). Most larvae feed on organic debris, algae and other micro-organisms within their water habitat. The larva is an active feeder and thus feed directly on the surface of the water by tilting the head 180^0 so that mouth parts directly face the surface film (Robert and Collins, 1996). Pupation typically occurs in full sunlight. The pupae are nearly as active as the larvae but breathe through thoracic "horn" attached to the thoracic spiracles. Many species of mosquito have their adult stage in roughly two weeks to two months.

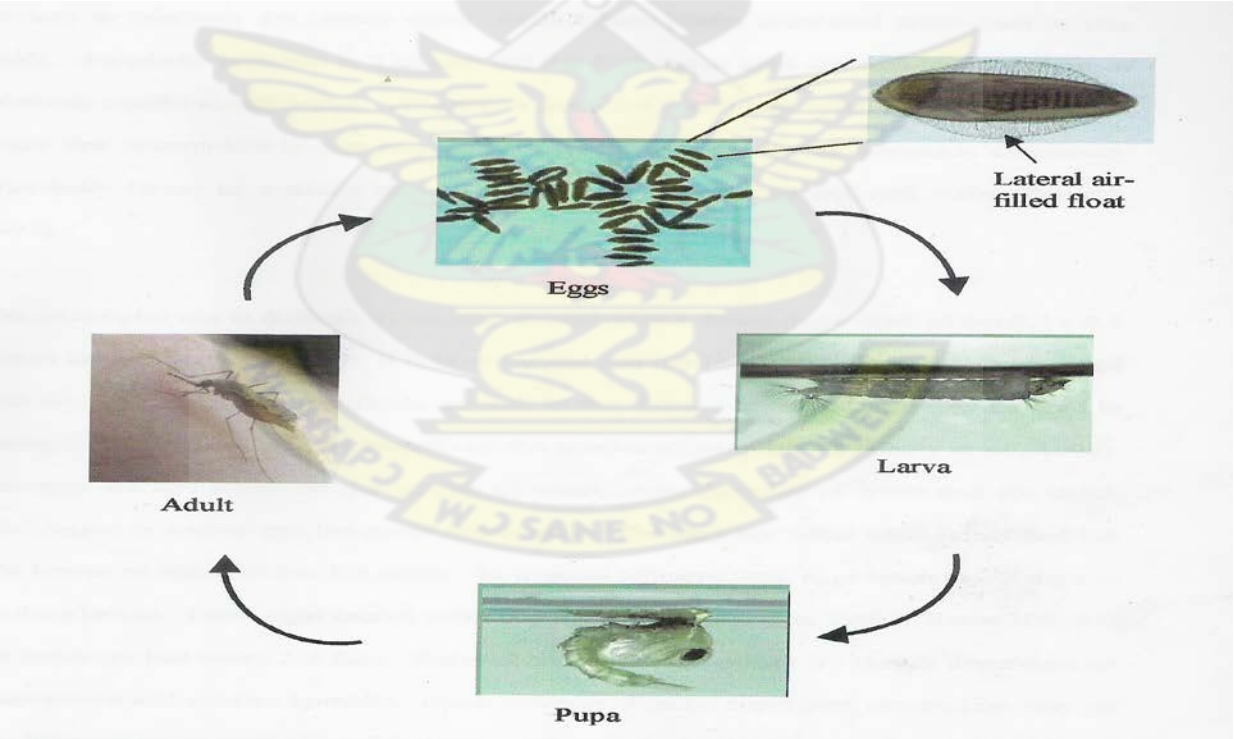

Figure: 1. The life cycle of *Anopheles* species with four developmental stages (complete metamorphosis): egg, larva, pupa and adult.

Different mosquito species have varying feeding habits; some bite only animals (zoophilic) and others humans (anthropophilic/anthropophagic) (Robert and Collins, 1996). They may also live and bite outside (exophilic and exophagic respectively) or enter houses and bite (endophilic) and endophagic respectively (Robert and Collins, 1996). In most female mosquitoes, the mouth parts form a long proboscis for piercing the skin of mammals to suck their blood. The females require proteins for egg development and laying, and since the normal mosquito diet consist of nectar and fruit juice, which has no protein, most must suck blood.

2.2.2 Anopheles mosquitoes vector of humans malaria

Anopheles gambiae,s arabiensis ands *funestus* transmit most of human malaria and are found in Africa (Besanky *et al*., 2004). *Anopheles gambiae,* the most famous and significant of these three, is one of the sixty anopheline mosquitoes able to transmit malaria to humans (Budiansky, 2002). *Anopheles gambiae sensu stricto* and *An. arabiensis*, the two most anthropohilic species are responsible for more than 75% of the world *falciparum* inoculations (Favia and Louis, 1999) and are thus the most efficient vectors of malaria in sub-Saharan Africa. However *gambiae s.s.* is the primary malaria vector, and this may be due to its relatively long life, strong anthropophily and endophily, compared to *An arabiensis* (Besansky *et al.*, 2004). Adult mosquitoes of the *Anopheles* normally rest during the day inside human habitats and emerge to feed at night (Hunt *et al*., 1998). Their larvae tend to develop in temporary water bodies, such as those typically found near agricultural sites or even in flooded hoof print (Vogel, 2002). These characteristics combine to make *An. gambiae* a successful malaria vector in sub-Saharan Africa (Mekuria, 1983).

A comparison of the entomological inoculation rate (EIR) of infectious mosquitoes in Asia or South America to sub-Saharan Africa indicates that in Asia or South America a person's EIR rarely exceeds five bites per year. However in sub-Saharan Africa it may exceed thousand bites, per year (Greenwood and Mutabingwa, 2004). Greenwood and Mutabingwa (2004) also reported that during a single night in sub-Saharan Africa hundreds of mosquitoes typically collected in a room occupied by human 1-5% of these were infectious (infected with *Plasmodium* parasites).

2.2.3 *Anopheles gambiae* complex

Morphologically, identical mosquito species are found to be different since genetic exchange between them is not possible or leads to sterile hybrids (Davidson, 1962). Such mosquitoes may be found in the same region (sympatric mosquitoes) (Coluzzi, 1964). One can often differentiate sibling species by electrophoresis. Proof of speciation is based on the lack of heterozygosity of the diagnostic iso-enzymes in species which breed in the same environment, and also by culture experiments (Davidson and White, 1972). In this way six closely related species have been identified in the most important African vector, *Anopheles* gambiae (Gilles and Coetzee, 1987).

2.2.3.1 *Anopheles* gambiae sensu strictu

Anopheles gambiae sensu strictu, is an anthropophagic and endophilic fresh water mosquito which flourishes preferentially in moist regions (Coluzzi *et al.*, 1979) and is also the main malaria vector in sub-Sahara Africa (Service, 1980). It is extremely versatile regarding tolerance to wide variety of micro- and macro-environmental conditions as evidenced by its broad geographic distribution. It is widespread in

Africa, though better adapted to water regions than to savannah areas (Lindsay *et al*., 1979).

The vectorial potency of An. gambiae s.s. stems from its strong association with humans, i.e. its preference for biting humans which is exacerbated by its capacity to adopt to changes to its natural habitat induced by humans (Della-Torre *et al*., 2001).

2.2.3.2 *Anopheles arabiensis*

This is the member species of the complex with the widest geographical distribution. It is found in the Afro-tropical region, Sahel, on the plateaux of Southern Africa, Madagascar, in the countries bordering the Red Sea and Aden Gulf, and most of the islands in the Indian Ocean (Abdullah and Merdan, 1995). *Anopheles* arabiensis is one of the main malaria and bancroftian filariasis vectors in Afro-tropical region together with *An. gambiae* s.s. and *An. funestus* (White, 1996). It is anthropophilic, but in the presence of livestock this mosquito prefers cattle.

2.2.3.3 *Anopheles quadriannulatus*

Anopheles quadriannulatus is restricted to Eastern and Southern Africa. It is, unlike the other species, relatively tolerant to the highland cold conditions and has been found in the highlands of Ethiopia and extensively in Southern Africa (White, 1974). It is zoophagic and exophilic and no control measures are necessary for this fresh water mosquito since it is not a malaria vector (White, 1974).

2.2.3.4 *Anopheles bwambae*

This exists only in a very smaller area, in the Semliki forest in Rift valley (White, 1989). It is an anthropophagic mosquito which breeds in geothermal fresh water streams (White, 1989). *Anopheles bwanbae* is not suspected to play an important epidemiological role in the transmission of pathogenic agents of humans or domestic animals because of its very restricted distribution (White, 1974).

2.2.3.5 *Anopheles melas*

Anopheles melas, an anthropophagic and endophilic, occurs on the coast of West Africa (Coluzzi, 1984). Its capacity as a vector is considerably lower than that of *An. gambiae* s.s. and *An. arabiensis* (Bryan, 1983). It plays a negligible role in malaria transmission, except perhaps when it is very abundant (Diop *et al*., 2002**).**

2.2.3.6 *Anopheles merus*

This breeds on the coast of East and South Africa both in fresh and brackish water**,** and is more exophilic than endophilic (Coluzzi, 1984). It transmits malaria and bancroftian filariasis perfectly in the laboratory (Hunt and Gunders, 1990). However, it has been indicated that it is a bad vector for bancroftian filariasis in natural conditions because of its short life span and feeding habits (Southgate and Bryan, 1992). The sporozoitic indices of An. merus are always smaller than that of *An. gambiae* s.s., although *An. merus* may still play a role as secondary vector in localities where it is abundant (Bryan, 1983).

2.3 IMPACT OF MALARIA

2.3.1 Epidemiology

For thousand of years malaria has been a deadly scourge, and it remains so to date. Malaria causes about 350-500 million infection in human and approximately 1-3 million deaths annually (WHO, 1996), and this represents at least one death every thirty seconds. The majority of cases occur in children under the age of five years (WHO, 1996).

Despite an increasing effort to reduce the transmission and enhance treatment, there has been little change in malaria endemic areas since 1992 (Hay *et al.*, 2004). It is estimated that if the prevalence of malaria stays on its present upward course, the death rate could double in the next two decades (Sachs and Maloney, 2002) Precise statistics are unknown because many cases go unreported since most people do not have access to hospitals or means to afford health care (WHO, 1996). Many cases are also diagnosed in clinics where functional microscopes are not available (Green and Mutabingwa, 2002). Approximately 40% of the world population lives in regions where malaria transmission is endemic, mainly tropical and sub-tropical regions (Aultmen, 2002).

In the temperate regions of the world, malaria has been successfully controlled and effectively eliminated (Sachs and Malaney, 2002). Measures such as change in agricultural and constructional practices, reduced availability of standing water and targeted vector control using insecticides such as DDT were control strategies adopted (Greenwood and Mutabingwa, 2002). Also industrialization and improved housing conditions were instrumental in the elimination of the disease in temperate countries

(Budiansky, 2002). Additionally, the more severe season of the temperate regions present another factor for the successful eradication programs. The role of the mosquito in the life cycle of P. *falciparum* requires that the parasite be able to maintain an extended infection in order to ensure transmission during the following season (Kyes *et al*., 2001). Therefore severe seasonality results in lower basal levels of reproduction (Sachs and Malaney, 2002).

In the developing world, urbanization is one of the factors responsible for migration of people. Sub-Saharan Africa is the most rapidly urbanizing region in the world (World Bank Report, 1996). Urbanization and congestion of the urban areas leads to poor housing and sanitation, lack of proper drainage of surface water and use of unprotected water reservoirs. This increases human-vector contact and vector breeding thereby leading to an increase in the transmission of the disease.

The worsening malaria problem has increased the risk of infection of visitors to endemic areas. Imported malaria from tourists, business travellers and immigrants is an increasing problem in Europe and the United States (Martens and Hall, 2000). For example, there has been an increase from 803 in 1987 to 1,165 in 1995 in cases of imported malaria to the United Kingdom among residents around airport. With the enormous and continuing increase in air traffic, cases of airport malaria may increase (Martens and Hall, 2000).

The incidence of malaria has also increased due to insecticide resistance of the vector and the resistance of parasites to anti-malaria drugs. The failure of the WHO's malaria eradication program was, to a significant degree, due to increased resistance to DDT and the refusal of people to spray their houses (Greenwood and Mutabingwa, 2002). The current, most widely employed technique for vector control is bed nets treated

with the insecticide, pyrethroid. However, it is only a matter of time before pyrethroid, like DDT loses its efficacy (Curtis *et al.*, 1990; Curtis, 1996). The viability of introducing genetically modified mosquitoes, which are either unable to transmit malaria to humans or are sterile, is being investigated (Alonso and Dgedge, 1999).

Two of the four human malaria parasites (P. *falciparum* and P. *vivax*) have been reported to have developed resistant to anti-malaria drugs (Bloland, 2001). *Plasmodium falciparum h*as developed resistance to nearly all anti-malaria drugs currently in use and *P. vivax* has been shown to be resistant to chloroquine and/or primaquine (Bloland, 2001). The resistance to anti-malaria drugs has occurred through spontaneous mutation that confer reduced sensitivity to a given drug or class of drugs. For some drugs only a single point mutation is required to confer resistance, while for others, multiple mutations appear to be required (Bloland, 2001). The resistance of *P. falciparum* to chloroquine however is believed to be related to an increased capacity for the parasite to expel chloroquine at a rate that reduces its levels required for inhibition of haem polymerization (Foley and Tilley, 1997).

2.3.2 Socio economic impact of malaria

Malaria is a disease devastating millions of people each year. Despite the strong effort to eradicate malaria, the disease burden is still on the rise. Estimates indicate that the number of cases could double in the next twenty years if development of new methods for control is not taken (Sachs and Malaney, 2002).

Apart from the human tragedy, it produces an economic disaster for the stricken countries. Gallup and Sachs (2001) reported that, during the period 1945-1990, the

annual growth rates in malaria endemic countries were 1.3% lower compared to non-endemic countries. This corresponds to a 50% decrease in per capita gross domestic product (GDP) (Gardener, 2002). Malaney and Sachs (2002) also hypothesized that there is an apparent correlation between poverty and malaria. Increase in population in malaria endemic regions, compounded by poor public health system in developing countries, climate changes (Hay *et al*., 2004), new agriculture practices such as irrigation and dam construction (Sachs and Malaney, 2002), make control of the vector difficult and consequently promoting vector-insecticides resistance (Bozdech *et al.,* 2003).

2.3.3 Aetiology and Symptomatology of Malaria

Human malaria results from infection with *Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale or Plasmodium malariae. Plasmodium falciparum* causes a large majority of the clinical cases and it is responsible for about 80% percent of infection and 90% of deaths (Bozdech *et al.*, 2003). These malaria parasites are transmitted to humans by bites on the mosquitoes of the genus Anopheles. The mosquito picks up the parasite during feeding on a human with parasitaemia.

The clinical symptoms of malaria is cyclical occurrence of sudden coldness followed by rigor, then fever and sweating lasting for four to six hours, occurring every two days in *P. falciparum*, P. *vivax and P. ovale* infection, and in every three days for *P. malariae* (WHO, 1998). Malaria has been found to cause cognitive impairments especially in children and those who have frequent malaria exhibit abnormal posturing, a sign indicating severe damage to the brain (Lindsay *et al.,* 2000). It causes widespread anaemia and direct brain damage from cerebral malaria to vulnerable children (Boivin, 2002).

A plasmodium falciparum infection usually occurs 6-14 days after mosquito bites (Trampuz *et al.,* 2003). Cerebral malaria is characterized by coma and may result in death if untreated (WHO 1998), young children and pregnant women are especially vulnerable (Lindsay *et al.,* 2000). Splenomegaly, severe headache, cerebral ischemia, hepatomegaly and hemoglobinuria with renal failure may occur, which can progress extremely rapidly and cause death within hours or days (Trampuz *et al.,* 2003). In the most severe cases of the disease fatality rate can exceed 20%, even with intensive care and treatment (Kain *et al.,* 1998). In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malara can be as high as one in ten (Mockenhaup*t et al.,* 2004).

Chronic malaria is seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. In this case the disease can relapse months or years after exposure, due to the presence of latent parasites in the liver. Describing a case of malaria as cured by observing the disappearance of parasites from the bloodstream can therefore be deceptive. The longest incubation period reported for a *P. vivax* infection is thirty years (Trampuz *et al.,* 2003), and approximately one in five of *P. vivax* malaria cases in temperate areas involves over wintering by hypnozoites (Adak *et al.,* 1998).

2.3.4 Life Cycle of Malaria Parasite

Malaria parasites spread by infecting successively two types of hosts: humans and the female Anopheles mosquito. The transmission of plasmodium involves an exogenous sexual phase (sporogony) with multiplication in Anopheles mosquitoes and endogenous asexual phase (schizogony) with multiplication in the human or the vertebrate host.

The parasite first enters the bloodstream through the bite of an infected female Anopheles mosquito. As she feeds, the mosquito injects a small amount of saliva containing an anticoagulant along with small haploid sporozoites. The sporozoites in the bloodstream immediately enter hepatic cells of the liver. In the liver, they under go multiple asexual fission [schizogony] and produce merozoites. After being released from the liver cells, apart from the *P. falciparum* the merozoites either infect other liver cells, thus continuing the pre-erythrocytic state, or attach to erythrocytes and penetrate these cells.

Once inside the erythrocyte, the plasmodium begins to enlarge as uninucleate, cell called trophozoite. The trophozoite's nucleus then divides asexually to produce a schizont that has 6-24 nuclei. The schizont divides and produces mononucleated merozoites (Aikawa and Seed, 1980). This erythrocytic stage is cyclic and repeats itself approximately every 48 to 72 hours or longer, depending on the species of plasmodium involved. Occasionally merozoites differentiate into macro and micro gametocytes, which do not rapture the erythrocyte. When these are ingested by a mosquito, they develop into female and male gametes respectively.

In the mosquito's gut, the infected erythrocytes lyse and gametes fuse to form a diploid zygote known as ookinete (Aikawa and Seed 1980). The ookinete migrates to the mosquito's gut wall, penetrates and forms an oocyst on its outer surface. In the process called sporogony, the oocyst undergoes meiosis and forms sporozoites that migrate to the salivary glands of the mosquito. The mosquito inoculates another human when it bites, thus the cycle is repeated

VECTOR	PARASITE	HOST
Anopheles mosquito	**Plasmodium**	**Human**

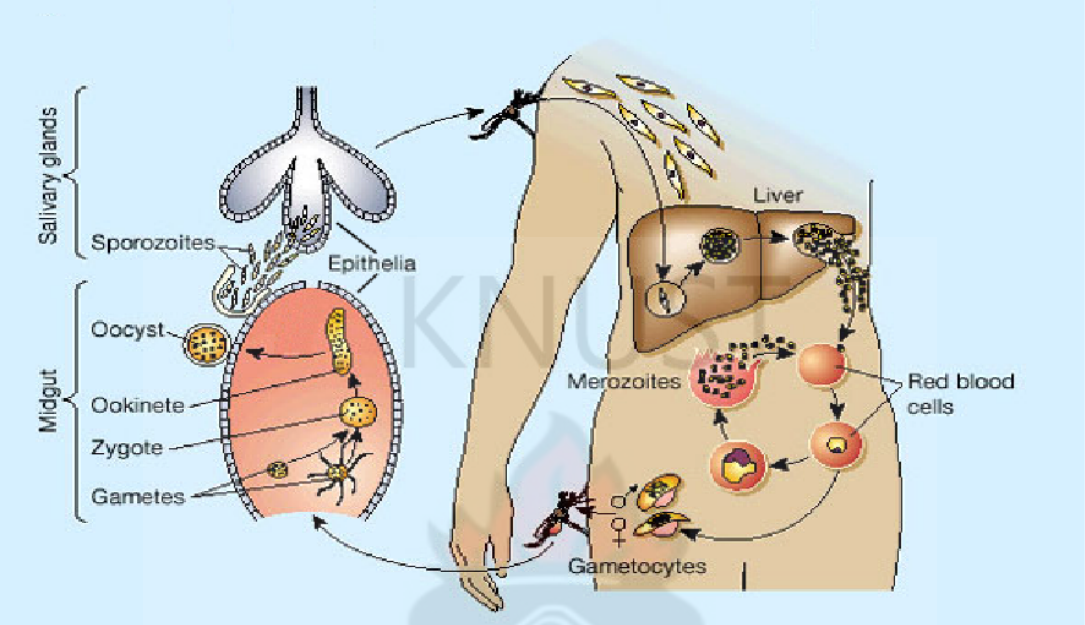

Fig. 2, The diagrammatic representation of the life cycle of malaria parasite in the human host.

2.4 PREVENTION AND CONTROL OF MALARIA

Prevention of malaria encompasses a variety of measures that may protect against infection or against the development of disease in individuals. Measures that protect against infections are directed against the mosquito vector. These can be personal (individual or household) protection measures, which include the use of protective clothing, repellents, bed nets or community/population protection measures through the use of insecticides or environmental management to control transmission. Measures which protect against disease but not against infection include chemoprophylaxis. There are currently no vaccines that prevent malaria, but this area of research is actively being pursued.

2.4.1 Environmental Management

Environmental management for vector control includes those procedures that specifically modify the habitats of the target or humans to make those habitats unfavourable for the vector and therefore reduce human-vector pathogen contact (WHO, 1980). Efforts to eradicate malaria by eliminating mosquitoes have been successful in some areas such as the temperate regions. Malaria was once common in United State and Southern Europe, but the draining of wetland breeding grounds and better sanitation, in conjunction with the monitoring and treatment of infected human eradicated it from effluent regions (Najera and Zaim, 2002). Malaria was eliminated from the northern parts of the USA in the early twentieth century, and the use of the pesticide DDT wiped it out from the south by 1957 (WHO 1980).

In the 1950s and 1960s, there was a major public health effort to eradicate malaria worldwide by selectively targeting mosquitoes in areas where malaria was rampant (Gladwell and Malcolm, 2001). Despite its success in the temperate zones, it is still difficult adopting environmental management in the tropical zones especially in Africa (Mitchell, 1996). This is because of conditions such as deforestation, development projects, the lack of proper understanding of the behaviour of vector populations and the agricultural production systems promote vector breeding and engender malaria transmission.

Brazil, Eritrea, India and Vietnam have, unlike many other developing nations, successfully reduced the malaria burden. Factors responsible for the success are conducive country conditions, a targeted technical approach using a package of effective tool, data-driven decision-making, active leadership at all levels of government, involvement of communication, decentralized implementation and

control of finances, skilled technical and managerial capacity at national and sub-national levels, hands-on technical and programmatic support from partner agencies and sufficient and flexible financing (Barat, 2006).

2.4.2 Indoors residual spraying

The use of DDT in combating mosquitoes has been a subject of considerable controversy. While some argue that DDT deeply damages biodiversity, others contend that DDT is the most effective weapon in combating mosquitoes and hence malaria.

The WHO recommends a series of alternative insecticides such as pyrethroids, carbonates and organophosphates, which are used in areas where mosquitoes are DDT-resistant and to slow the evolution of resistance (WHO, 1996). The public health use of small amount of DDT is permitted under the Stockholm convention of persistent organic pollutants (POPs), which prohibits the agricultural use of DDT for large-scale field spraying (http://www.Who.int/malaria/docs/10thingsonDDT.pdf). However, because of its legacy, many developed countries discourage DDT use even in small quantities (http:/www.pops.int).

2.4.3 Mosquito nets and bedclothes

The most effective solutions for malaria control efforts in the developing countries are mosquito nets and mosquito nets treated with insecticides. Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets in preventing mosquito bites, and offers greater than 70% protection compared with no net (Hull and Kevin, 2006). The distribution of mosquito nets impregnated with insecticide (often permethrin or deltamethrin) has been shown to be an extremely effective method of malaria control, and it is also one of the most cost-effective methods of prevention. The cost of a mosquito net is often unaffordable to people in developing countries

especially for those most at risk. Only one out of 20 people in Africa own bed net (Hull and Kevin, 2006). A study among Afghan refugees in Pakistan found that treating top-sheets and cheddars (head coverings) with permethrin has similar effectiveness to using a treat net, but is much cheaper (Rowland *et al*., 2000).

2.4.4 Chemoprophylaxis and Treatment

Several drugs, most of which are also used for treatment of malaria, can be taken as prophylaxis. Generally, these drugs are taken daily or weekly, at a lower dose than would be used for treatment. The use of prophylactic drugs is seldom practical in full-time residents' malaria endemic areas, and their use is usually restricted to short term visitors and travellers to these regions. This is due to cost and negative side effects from long-term use (Bloland, 2001).

In the seventeenth century doctors used quinine as a prophylactic against malaria. The development of more effective alternatives such as chloroquine and primaquine in twentieth century has reduced the reliance on quinine in endemic countries and has resulted in a dramatic decline in malaria incidence rates (WHO, 1997). However after years of use and abuse, chloroquine-resistant malaria parasites have evolved (NIAID, 2000). From the 1950's to present, chloroquine resistant *Plasmodium falciparum* has gradually spread to nearly all endemic regions (WHO, 1997; NIAID, 2000).

Morden drugs for preventive purpose include mefloquine [lariam ®], doxycline and the combination of atovaquone and proguanil hydrochloride [malarone ®]. The choice of these drugs in an area depends on the parasites resistance and side effect.

2.4.5 Vaccination

Potential vaccines for malaria are being developed. The first promising studies that demonstrated the potential for malaria vaccine were performed in 1967 by immunising mice with live, radiation-attenuated sporozoites. It provided protection to about 60% of the mice upon subsequent injection with normal, viable sporozoite (Nussenzweig *et al*., 1967).

Vaccine development is focusing on pre-erythrocyte stage, blood stage and transmission-block targets. For pre-erythrocytic-stage vaccines, immunization strategies under consideration include inducing antibodies that will prevent sporozoites from entering the liver cells and inducing T-cell responses that will attack parasite-infected liver cells (Nussenzweig *et al.*, 1967). Strategies for the creation of blood-stage vaccines include the induction of antibodies that will keep merozoites from entering the blood. Transmission-blocking vaccines would create antibodies that would immobilise plasmodium in the mosquito during the gamete or ookinete stages, prevent the parasite from being transmitted to another host and consequently limit the spread of the disease [McGregor, 1964].

2.5 RESISTANCE OF ANOPHELES TO INSECTICIDES

Resistance is said to develop whenever a species survives in an area despite the use of the compound that would normally be lethal to individuals of the same species (WHO, 1992). Malaria vector control is primarily based on the use of insecticides. Appropriate monitoring of vector resistance to insecticides is an integral component of planning and evaluation of insecticide use in malaria control programmes. Pyrethroids and DDT are two important insecticides used for vector control. They block the nerve –impulse conduction by preventing a para-type sodium channel from returning to the closed-gated configuration after an action potential (Vais *et al.,* 1997)

An important mechanism that confers resistance to pyrethroids and DDT, known as knock down resistance (kdr), was first described in the housefly *Musca domestica (*Brown, 1958). It has been reported that a single mutation in the S6 transmembrane segment of domain II in the para –type sodium channel sequence is the molecular basis of the kdr in *Musca domestica* (Brown, 1958). At present, the para –type sodium channel gene has been characterised in *An. gambiae*. Mutations in this gene have been linked to knockdown resistance and a diagnostic PCR test has been developed for the detection of the kdr-mutation in *Anopheles gambiae* (Saiki, 1998). The resistance to both DDT and pyrethroid is due to a mutation in the gene that encodes the voltage gate sodium channel. In most resistant *Anopheles*, there is leucine (TTA) to phenylalanine (TTT) substitution in the S6 hydrophobic segment of domain II (Williamson *et al*., 1996; Martinez-Torres *et al*., 1998). The kdr resistance has also been reported in the chromosomal forms of *An. gambiae* s.s. and found to occur in high frequencies in the S form but not in the M form (Chandre *et al*., 1999), although both are subjected to heavy pyrethroid pressure**.**

2.6 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is an *in vitro* enzymatic synthesis and amplification of specific DNA sequences of interest using oligonucleotide primers. The technique was invented by Kary Mullis (Mullis *et al*., 1986; Mullis and Faloona, 1987). It was originally used to amplify human **(ß)** globin DNA and for prenatal diagnosis of sickle –cell anemia (Saiki *et al.*, 1985; Saiki *et al* 1986; Embury *et al* 1987). The PCR mimics the *in vivo* process of DNA replication and therefore begins theoretically with one molecule of the double-stranded DNA called the target or template DNA to be amplified. A typical amplification reaction medium includes a thermo stable DNA polymerase, oligonucleotide primers, deoxyribonucleoside

triphosphates (dNTPs), reaction buffer, magnesium/optional additives and a template DNA. The reaction takes place in an automated thermal cycler which takes the reaction through a series of different temperatures per varying time durations. Each cycle consists of three phases, i.e. DNA denaturation of the target sequence, primer annealing and strand extension with thermo stable polymerase enzyme. The primers are designed to anneal to opposite strands of the target sequence so that they are extended by the addition of nucleotide to their 3'ends. After successful completion of the cycles, the minute quantities of double-stranded DNA would have been synthesized. The PCR amplification products are separated by electrophoresis and are directly visualized after staining with ethidium bromide.

A major set back encountered in the original PCR procedure was that the DNA polymerase (*Escherichia coli* DNA polymerase I, Klenow fragment) was replenished after every cycle because it was not stable at the high temperature needed for denaturation (Siaki *et al.*, 1985; Mullis and Facoona, 1987). This problem was solved in 1987 with the discovery of a heat-stable DNA polymerase called Taq (Saiki *et al.*, 1988), an enzyme isolated from the thermophilic bacterium, *Thermus aquaticus*, which inhabits hot springs (Chien *et al.*, 1976).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY AREA

Mosquito larvae were collected from five different communities, Tafo Anyano, Kwadaso, Ahodwo, Ayeduase and Abuakwa all within the Kumasi metropolis. All the communities lie approximately between (5°55´ and 7º10´N) and (1º25´ and 2º12´ W) and cover an area of about 8000 km^2 (Fig 3). The area falls within the equatorial climate zone (Walker, 1962) with a rain fall regime which is typical of the moist semi-deciduous forest zone of the country. There are two well defined rainfall seasons. The major season occurs from mid- March to the end of July with a peak fall in June. The minor rains commence in September and end in mid November. The mean annual rainfall ranges between 1457 and 1488 mm. The mean monthly temperature is between 26 and 30 ^{0}C and the relative humidity ranges between 62 and 78 % (Walker, 1962).

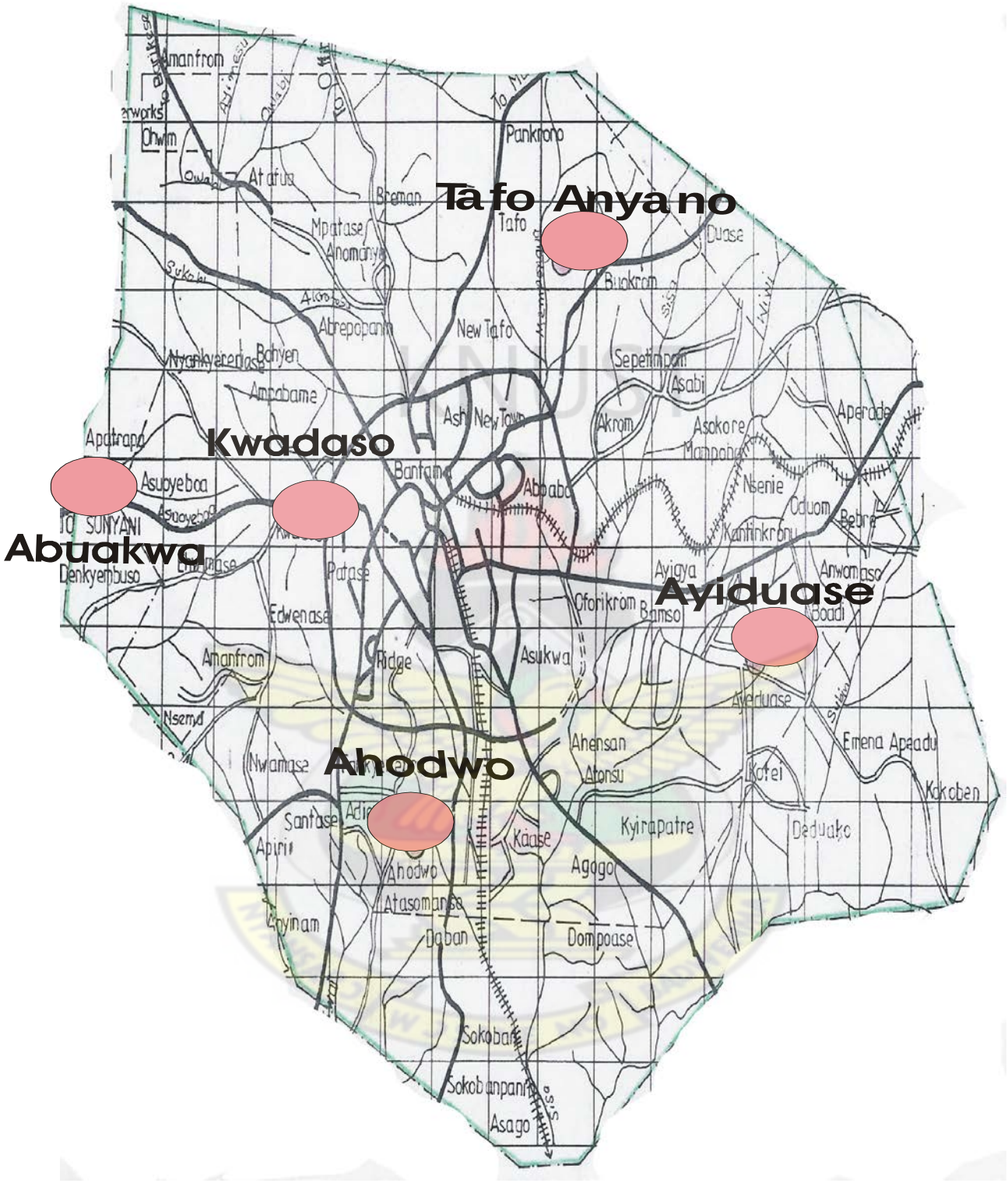

FIG. 3 Map showing the five communities within the Kumasi metropolis from which mosquito larvae were collected.

3:2 **Sample collection and identification of the *Anopheles* larvae**

Mosquito larvae were collected, using a ladle, from diverse habitats (fig. 4-8) including fresh shallow pools of water, construction and sand winning sites, gutters, vegetable farming sites and slow running streams. Most of the habitat had floating vegetation.

Fig: 4 Sampling site at Ayiduase (sand winning site with temporary collected pool of rainwater)

Figure: 5. Mosquito collection site at Abuakwa (small shallow pool of stagnant water in an irrigation farm).

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Figure: 6 Mosquito collection site at Kwadaso (narrow stretch of water flowing from a broken water pipe).

Figure: 7. Mosquito collection site at Tafo Anyano (slow flowing polluted effluent from surrounding houses)

Figure 8. Sample collection site at Ahodwo (open drain with stagnant water exposed to sunlight)

The larvae were transported to the laboratory in wide mouthed labeled containers which were loosely covered to prevent suffocation, and under suitable condition. The larvae were transferred into white labeled plastic trays. *Anopheles* larvae were identified by their characteristic horizontal position below the surface of the water. The larvae were gently picked using a 3 ml rubber Pasteur pipette into a labeled 2.0 ml eppendorf tubes containing absolute ethanol for storage prior to the DNA extraction.

3.3 MOLECULAR STUDIES

3.3.1 DNA extraction

The DNA extraction method of Collins *et al. (*1987), slightly modified, was used. Each mosquito larva was placed in a 1.5 ml Eppendorf tube in 100 ul Bender buffer (Appendix II) (pre- heated to 65 °C) and homogenized using a sterile polypropylene rod. This was followed by incubation at 65ºC for 30 minutes followed by the addition of 15 µl of pre-chilled 8M potassium acetate to each tube and mixed well by tapping

the tube. Each was then incubated on ice for 30 minutes and then centrifuged at 14000 rpm for 10 minutes for 10 minutes and supernatants transferred separately into fresh tubes. A volume of 250 µl of pre-chilled absolute ethanol was added to each supernatant and mixed well by inverting the tubes several times to precipitate the DNA. Each tube was then incubated at -40°C for two hours followed by centrifugation at 14000 rpm for 5 minutes to pellet the DNA and the supernatants discarded. The DNA pellets were washed with 70% ethanol (200µl) by centrifugation at 10000 rpm for 5 minutes. The supernatants were discarded and the tubes inverted over a paper towel and left to dry by evaporation. The dried DNA pellets were re-dissolved in 25 µl TE + RNAse and then stored in the -20ºC freezer until ready for use.

3.3.2 Polymerase chain reactions

3.3.2.1 Molecular identification of *Anopheles gambiae*

The PCR method of Scott *et al*., (1993) with species-specific oligonucleotide primers was used for the identification of the *An.gambiae*. The amplification process for the purpose of this work utilized one universal primer and three species-specific primers.

The sequence details of the primers that were abbreviated UN, GA, ME and AR with corresponding band sizes of the PCR products after electrophoresis on agarose gel is shown in Table 1. The universal primer UN anneals to the same position on the rDNA of each of the species of the *An. gambiae* complex apart from *An. quandriannulatus* of whose primer was not included. The GA anneals specifically to *An. gambiae s.l* ME to both *An. merus* and *melas*, and AR to *arabiensis*.

Table 1: An. gambiae s.l primer sequences, melting temperature and expected band sizes of the PCR amplified DNA products (Scott *et al.*, 1993)

Primer	sequences (5'-3')	Tm (ºC)	Band size (bp)
UN	GTGTGCCCCTTCCTCGATGT	56	468
GA	CTGGTTTGGTCGGCACGTTT	62	390
ME	TGACCAACCCACTCCCTTGA	90	464s
AR	AAGTGTCCTTCTCCATCCTA	78	315

3.3.2.2 PCR Amplification

The contents of the PCR reaction mix was thoroughly mixed, centrifuged briefly at 10000 rpm for 5 minutes and over laid with oil to avoid evaporation and refluxing during thermo cycling. The amplification was carried out using a 100 PTC thermal cycler (MJ Research Inc., USA). The temperature profile for the reaction was 94 ºC for 3 minutes (initial denaturation) followed by 35 cycles of 94 ºC for 30 seconds (denaturation), 50 ºC for 30 seconds (annealing), 72 ºC for 1 minute (extension) and final cycle of 72 ^{0} C for 10 minutes followed by 4^{0} C for cooling. For each set of reactions a control which contained no DNA template was included.

Table 2: PCR reaction mix for *An. gambiae* s. l. species identification

Reagent	Volume(µl)	Final concentration
Sterile water	20.28	
10xReaction buffer	2.5	1x
20mM dNTPs	0.4	0.2mM
Primers: 10µMGA	0.3	0.3µM
10µMME	0.3	0.3µM
10µMAR	0.3	0.3µM
10µMUN	0.3	0.3µM
Taq polymerase (5U/µl)	0.13	0.5U
DNA template	0.5	
Final volume	25.0	

3.3.2.3 Analyses of PCR products

Gel electrophoresis was done with 2% agarose gel containing 0.5 mg/ml of ethidium bromide. After hardening of the gel, the comb was removed and the gel placed in an electrophoretic tank. One times TAE buffer was poured into the tank till the gel was totally submerged. An amount 8 µl of each PCR product was mixed with 1µl of 10x Bromophenol blue loading dye on a strip of Para film. Using either a mini or maxi-gel system the gels were run at 100V for 20 minutes. The bands were then visualized on an ultra violet trans-illuminator (UVP Dual-intensity Trans-illuminator, Upland, CA, U.S.A). A Polaroid direct screen instant camera fitted with an orange filter, a hood and a Polaroid type 667 film was used to take photograph of the bands. The film was processed as recommended by the manufacturers (Polaroid Inc., USA). The sizes of the PCR products were estimated by comparing with the mobility of a standard 100 bp ladder (Sigma, USA).

3.4 Detection of knock down resistance (kdr) alleles in *Anopheles gambiae.*

The Martinez –Torres *et al*., (1998) method was used to detect kdr genes in the mosquitoes. A total of 50 samples, (10 for each site) positive for *Anopheles gambiae* s.s. by PCR were selected for the kdr detection. The kdr primers used were $Agd_{1,}$ $Agd_{2,}$ Agd_3 and Agd_4. The detail of the primer sequence is indicated in table 3. The contents of the PCR reaction mix was thoroughly mixed, centrifuged briefly at 1000 rpm and over laid with oil to avoid evaporation and refluxing during thermo cycling. Polymerase chain reaction products were analyzed as described under section 3.3.2.3.

Table 3: Details of kdr primer sequences and their respective temperatures (Martinez-Torres et *al*., 1998).

Primer	sequence 5' – 3'	Tm (^{0}C)
Agd1	ATAGAT TCC CCG ACC ATG	54
Agd2	AGA CAA GGA TGA TGA ACC	64
Agd3	AAT TTG CAT TAC TTA CGACA	40
Agd4	CTG TAG TGA TAG GAA ATTTA	52

Table 4: Kdr-PCR reaction mix for detection of the Kdr gene

Reagent	Volume(µl)	Final concentration
Sterile water	15.48	
10xReaction buffer	2.50	1X
20mM dNTPs	0.40	0.2mM
Primers 10µM Agd_1	0.25	0.25µM
10µM Agd_2	0.25	0.25µM
10µMAgd_3	0.25	0.25µM
10µM Agd_4	0.25	0.25µM
Taq polymerase (5U/µl)	0.13	0.5U
DNA template	0.50	
Final volume	20.0	

CHAPTER FOUR

RESULTS

4:1 MOLECULAR STUDIES

4.1.1 PCR identification of siblings' species of *An. gambiae* s.l. of the larvae.

A total of 100 mosquitoes larvae were used for the PCR after their genomic DNA have been extracted. These comprise 20 samples from each of the five different sites used in this study. Out of the 100 larvae used, PCR amplification for species identification was successful for 76% while the other 24% were unsuccessful. The 76 specimen were identified as *An.gambiae* s.s. as revealed by the size of 390 base pair fragment (Fig. 10).

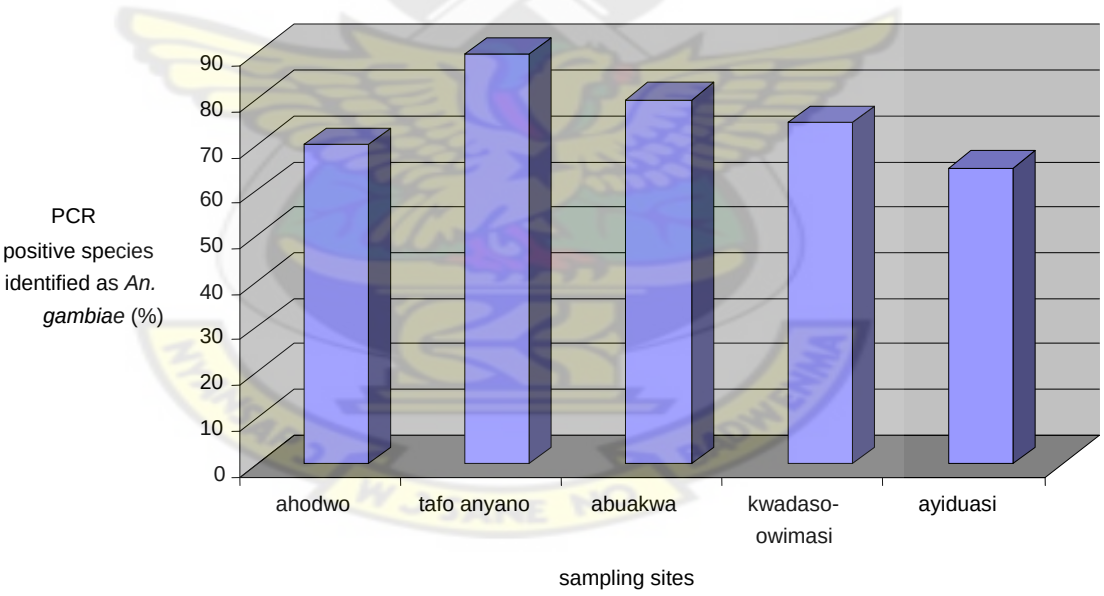

Fig. 9. The percentage of PCR positive species identified as An. gambiae for all the sampling sites.

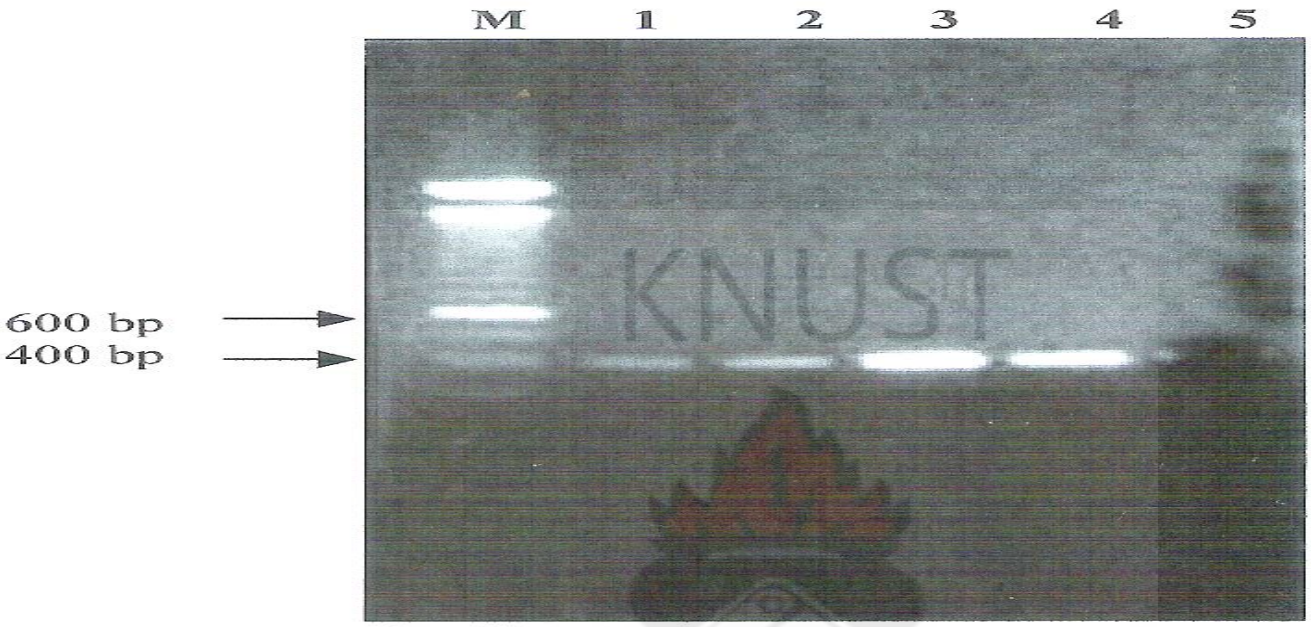

Figure 10: An example of 2.0% agarose gel electrophoresis of PCR amplified rDNA sequences of An gambiae ss larvae. Lane M = DNA molecular weight marker, lane 1-4 An. gambiae larvae, lane 5 = negative control.

Table.5. The Kdr status of *An. gambiae* s.s.

Sample sites	Resistance (%)	Susceptible (%)
Ahodwo	(6) 60	(4) 40
Tafo Anyano	(8) 80	(2) 20
Abuakuah	(5) 50	(5) 50
Kwadaso	(7) 70	(3) 30
Ayiduasi	(1) 10	(9) 90

N = 10

Where, N is the number of specimen per site

4.1.2 PCR determination of kdr mutation in the An. gambiae

A total of 50 PCR positive *An. gambiae* s.s. specimen (10 from each site) were selected for detection of the kdr status. Out of the 50, 27 (54%) were identified having the kdr gene whilst 23 (46%) were susceptible. An amplification product of 195 bp was revealed as positive for Kdr (resistant strain of *An. gambiae* s.s.) and 137 bp product also identified as negative (susceptible strain of *An. gambiae* s.s) with an internal control of 293 bp, as shown in the electrophoregram in figure: 11.

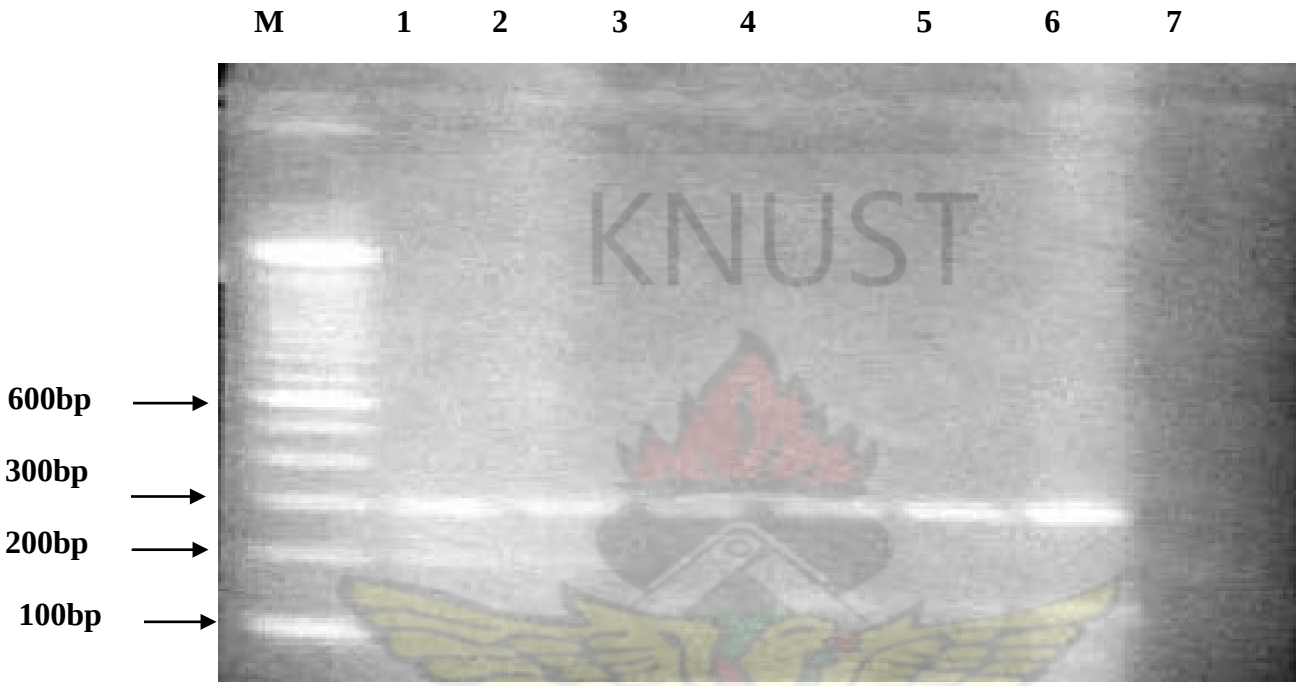

Figure 11: Gel electrophoregram of PCR detection of the kdr gene.

Lane M= molecular weight marker, lane 1 = kdr positive control, lane 2-5 = wild type An. gambiae s.s, lane 6= susceptible positive control, lane 7= negative control

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Polymerase chain reaction was used to determine the distribution of *Anopheles gambiae* species and to further determine the kdr status of the species from the selected communities within the Kumasi metropolis. The use of PCR for species identification depends on the quality of DNA that could be extracted and this in turn depends on the methods used for preservation. Only *An. gambiae s.s* was identified at all the sites. This result is consistent with previous work reported by some researchers (Adasi *et al*., 2000, Midega, 2001) and (Sobomana *et al.*, 2002), all of which identified *An.* gambiae s.s as the only species in the selected areas of Greater Accra region which has similar climatic and environmental conditions with those of the present study sites in Kumasi.

A total of 76 out of 100 samples were identified as *An. gambiae* s.s. by PCR (Fig.9). The other 24 specimens were unsuccessful. This could have been due to DNA degradation prior to storage. All the five sites have relatively high percentages of *An. gambiae* s.s. ranging between 70 and 90% (14 to 18 out of 20 samples for each site) fig. 9. The high numbers of the *Anopheles gambiae* s.s. which were identified in all the sites could be due to the species association with rain-dependent temporary sites than with permanent water bodies. These temporary fresh water collection sites were common to all the sampling sites. Again the annual relative humidity is between 62 and 78 % which is high and thus supports the growth of *An. gambiae s.s.* population (Adasi *et al*., 2000 and Midega, 2001). Also the *An. gambiae* s.s. is associated with

the human habitat (anthropophilic) (Robert and Collins, 1996) and since Kumasi is urban, this therefore could have been responsible for their high numbers.

Fifty four percent (27 out of 50 PCR positive for *An. gambiae,.s.s.*) possessed the kdr allele. Except Ayiduasi, which had the lowest value of 10% (1 out 10 samples) (table 5), the other sites had relatively high percentages (50-80%) of kdr mutation gene which conferred on them the resistance to insecticides. This may be due to the indiscriminate use of pyrethroid for agricultural purposes and for the control of household pests (Darriet, 1997; Curtis *et al*., 1998; Chandre *et al.,* 1999). The latter may be the case in these communities where there was high rate of urbanization and its consequent use of insecticides.

Twenty four percent (24 out of 100) did not give PCR product (fig. 9). The primers used in this study annealed to phenylalanine codon in the pyrethroid resistant *An. gambiae.* Some of the *An. gambiae* templates may harbour serine instead of phenylalanine and they would avoid detection by the PCR assay (Martinez-Torres *et al.,* 1998 and Ranson *et al.,* 2000).

There were relatively high *An. gambiae* populations in these sampled communities (70-85%) fig. 9. This may be due to indiscriminate use of insecticides favoring selection of resistant *An. gambiae* species (WHO, 1999). The mosquito population could be reduced by pouring oil on the surface of water bodies. This was not practised in the communities.

5.1.1 CONCLUSION

Only *An. gambiae* s.s was identified (76%) using the available diagnostic oligonucleotide primers. Fifty four percent (27 out of 50 PCR positive samples) possess the kdr allele which confers on them the resistance to insecticides.

CHAPTER SIX

6. 1 **RECOMMENDATION**

Therefore to ensure meaningful planning and successful malaria control measures in the Kumasi, metropolis, future studies should be done with larger sample sizes and determination of insecticides resistance among the *An. gambiae* s.s. population should be evaluated in other communities to provide data necessary for the planning of the city wide malaria control.

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APPENDIX 1

DNA extraction and PCR reagents

PCR core kit (Roche)

Oligonucleotide primers (Bioserve)

Absolute ethanol (Sigma)

Mineral oil (Sigma)

Chelex-100 (Sigma)

Orange G dye (Kanto)

Agarose molecular biology grade (Sigma)

Ethanol (Sigma)

Tris hydrocymethane aminomethane (Trizma base, Sigma)

Ethylene diamine tetraacetate disodium acid (EDTA, Sigma)

Ethidium bromide (Sigma)

100 bp molecular weight marker (Invitrogen)

Appendix II
Preparation of standard solutions

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121 ^{O}C in. for 15 minutes in an Eyela Autoclave (Rikikkaki, Tokyo).

1. Solutions for DNA extraction

Bender buffer 0.1 M NaCl, 0.2M sucrose, 0.1M Tris- HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS. Stored at 4 ^{O}C.

0.5M EDTA (pH 8.0) 186.1 g/l in water, pH adjusted with NaOH pellets and stored at room temperature.

EtBr (10mg/m) 1g of EtBr was completely dissolved in 100 ml sddw and Stored in the dark at room temperature.

KAc (8M) 60 ml of 5M KAc and 11.5 ml glacial acetic acid in 28.5ml distilled water

RNase 10 ml/mg in water. Sterilized by filtration and stored in -20^{O}C.

TE (pH 8.0) 10 mM Tris- HCl (pH 8.0), 1 mM EDTA (pH 8.0 stored at room temperature.

TE+ RNase (5 µg/ml) 5 µl of RNase (10 mg/ml) solution, 99 µl of TE (pH 8.0). Stored at -20^{O}C.

2. Solutions of Electrophoresis

i. For agarose gels

10 x TAE buffer, 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with sddw

0.5 M EDTA (pH 8.0) 186 g of EDTA, dissolved in 800 ml sddw, pH adjusted with NaOH pellets and stored at room temperature.

ii. Gel loading buffers

5x Orange G 20% (W/V) Ficoll, 25 mM EDTA, 2.5% (w/v) orange G. Stored at $4^{O}C$.

6x Bromophenol blue 0.25 % bromo phenol blue, 40% sucrose in water. Stored at $+4^{O}C$.