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Insecticide resistance in malaria vectors in central Sudan

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CHAPTER ONE

INTRODUCTION

1.1 MALARIA

Malaria is a potentially fatal disease having a significant negative impact on the health and well being of local populations and poses a risk to travelers as well as displaced people in endemic areas (WHO, 2005a). Malaria causes considerable indirect harm to economic development, productivity and quality of life. It remains today as the most important insect-transmitted human disease (Collins and Paskewitz, 1995). Malaria is endemic in more than 90 countries around the World where it kills 1 child every 30 seconds (Alnwick, 2000).

The problem of malaria has increased in the last few years due to the development and spread of resistance in *Plasmodium falciparum* to the most commonly used drugs (Greenwood and Mutabingwa, 2002). The emerging resistance of the vector (*Anopheles*) species to insecticides threatens to exacerbate the problem further (Collins *et al.*, 2000).

The highest mortality and morbidity in sub-Saharan Africa are due to malaria (Jute and Toovey, 2006). The WHO estimated that between 300 and 500 million people suffer from malaria each year resulting in 1.5 to 3 million deaths worldwide, 90% of which are in Africa, mostly in young children (Collins and Paskewitz, 1995; Snow *et al*., 2005; WHO, 1998a).

The economic development and the poor health structures play an important role in shaping the current situation (Grover-kopec *et al.*, 2006). Early diagnosis, treatment with effective antimalarial drugs and chemoprophylaxis for high risk groups, the use of insecticide-treated bednets (ITNs) and indoor residual spraying with insecticides play a significant role in malaria preventions and control (Collins and Paskewitz, 1995; Bosman *et al.*, 1999).

1.2 THE MALARIA VECTORS

Mosquitoes belong to the order Diptera, sub-order Nematocerca of the class Insecta which contain three subfamilies Toxorhynchitinae, Culicinae and Anophelinae (Coluzzi and Kitzmiller, 1975). Mosquitoes belong to the genus *Anopheles*, subfamily Anophelinae and are the exclusive vectors of human malaria because of their behaviour, physiology and the close relationship with humans (Cui *et al*., 2006; Coluzzi and Kitzmiller, 1975). Only 12% out of the almost 380 described species of anopheline mosquitoes have been implicated as malaria vectors (Alonso, 2006). In sub-Saharan Africa, where 90% of the world's malaria cases occur, *Anopheles gambiae* Giles, *An. arabiensis* Patton of the *An. gambiae* complex and *An. funestuts* Giles from the *Anopheles funestus* group are the most efficient malaria vectors (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al*., 2000).

1.3 *Anopheles gambiae* GILES COMPLEX: SIBLING SPECIES, DESCRIPTION, BIOLOGY AND DISTRIBUTION

Early entomological studies carried out by Giles based on morphological differentiations have led to the definition of the two main components of the vectorial system: *An. funestus* and *An. gambiae* (Gillies and De Meillon, 1968).

Anopheles gambiae was not a single species with ecological salt-water variants as had been initially assumed, but actually a complex of several sibling species (Gillies and De Meillon, 1968; Davidson, 1964a, b). The first evidence for the existence of *An. gambiae* as a complex was when crosses between fresh-water species and salt-water produced sterile males (Muirhead-Thomson, 1948). Paterson (1962, 1963, and 1964) concluded that *An. gambiae* is a complex with five sibling species called species A, species B, Species C, the East African and West African salt-water species *An. merus* and *An. melas* respectively.

Davidson and Hunt (1973) crossed all the five known species of the *An. gambiae* complex with *An. gambiae* populations from Bwamba country, Uganda. Studies on the F1 male reproductive system from these crosses and on the polytene chromosomes showed that this population was the sixth species in the complex (species D). Mattingly (1977) proposed the formal names of the species within the complex *An. gambiae*, *An. arabiensis* and *An. quadriannulatus* Theobald for species A, B and C respectively, *An. melas* Theobald and *An. merus* Donitz for the West and East African saltwater breeders respectively. Species D was named *An. bwambae* by White (1985). Collectively all the

members of the complex are referred to as *Anopheles gambiae s.l.* (sensu lato). Furthermore, crosses between *An. quadriannulatus* populations from Ethiopia and South Africa resulted in sterile males and females that had extensive synapsis of their ovarian polytene chromosomes (Hunt *et al.*, 1998). The population from Ethiopia was called *An. quadriannulatus* species B and yet to receive a formal taxonomic name.

While the existence of the seven species of the *An. gambiae* complex is well established, further subdivision within species of the complex may exist. Observations on the morphology on the second chromosome of *An. gambiae s. s*. populations from Mali and Gambia in West Africa revealed the existence of cryptic taxa within the species (Coluzzi *et al*., 1979, 1985; Bryan *et al.,* 1982).

1.3.1 *An. gambiae* Giles

Anopheles gambiae is the most anthropophilic and efficient malaria vector in the complex (Gillies and De Meillon, 1968). This fresh-water species is widespread in most African countries. The larvae are found in sunlit rain pools, irrigation channels and permanent wells and are known to adapt to different types of waters (Gillies and De Meillon, 1968). *Anopheles gambiae* is highly anthropophagic and rests predominantly in houses (endophilic) and within the complex it has the highest vectorial capacity.

In West Africa, polytene chromosome and molecular studies of *An. gambiae s.s.* revealed the existence of five chromosomal and two molecular forms within the species (Coluzzi *et al*., 1979; Toure *et al*., 1998; Favia *et al*., 2001; della Torre *et al*., 2001; Gentile *et al*., 2001). The five chromosomal forms are characterised by different

inversions on the second chromosome and a designated with non Linnean nomenclature (Coluzzi *et al*., 1985). According to Coluzzi *et al*. (1985) and Touré *et al*. (1998), the five forms consist of (1) the Forest form, which is characterised by the standard arrangement of chromosome 2 and sometimes found with inversions b or c, and which occurs in the rain forest areas and humid savannah. (2) Savannah, which is the most widespread form and found in the Savannah areas throughout Africa. (3) Bissau form is characterised by inversion d and is restricted to the coastal rice cultivated areas in Guinea-Bissau, Guinea Conakry, Gambia and South Senegal. (4) Bamako form has the j inversion and is found along the upper Niger River and its tributaries in southern Mali and northern Guinea Conakry. (5) Mopti is characterised by b, u and c inversions. It is found in the riverine or irrigated habitats in Mali, Ivory Coast, Burkina Faso and Guinea Conakry. The larvae of this form are known to be well adapted to all the breeding habitats especially during the dry season (Touré *et al*., 1994).

Two molecular forms named M and S have been identified based on the intergenic spacer (IGS) region of DNA (della Torre *et al.,* 2001) and two types called type I and type II based on studies on the internal transcribed spacer (ITS) region. The S form is associated with type I and M form with type II (Gentile *et al*., 2001).

The relationship between the M and S molecular forms and the chromosomal forms - defined according to nonrandom associations of inversions in chromosome 2 (Coluzzi *et al.*, 2002), varies according to their ecological and geographic distribution. In some areas of West Africa (for example, Mali and Burkina Faso), there is a one-to-one correspondence between the M molecular form and the Mopti chromosomal form.

Similarly, the S molecular form always corresponds to the Savanna or Bamako chromosomal form (Favia *et al*., 1997). In other areas of West Africa, this clear correspondence breaks down (della Torre *et al*., 2001).

1.3.2 *An. arabiensis* Patton

Like *An. gambiae*, *An. arabiensis* is also a significant malaria vector in Africa (Coluzzi, 1970). These species occur in sympatry and share larval habitats across most of the Afrotropical area (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Service, 1970). Although this is not always the case, *An. arabiensis* shows evidence of being better adapted to less humid environments, and it was found predominant in the arid regions in many countries such as Nigeria, Tanzania, Ethiopia and Sudan (Dukeen and Omer, 1986; White, 1972; Gillies and Coetzee, 1987). Population density of this species varies seasonally in relationship to rainfall. It increases quickly with the first rains and the maximum density is reached at the end of the rainy season (White, 1972; White and Rosen, 1973) and then decreases as the temporary breeding sites dry up.

This species is known to be anthropophilic, however, at a lower frequency than *An. gambiae* (Gillies and Coetzee, 1987). It also feeds on cattle (zoophilic), and for example, was collected feeding on humans and donkeys in Niger (Charlwood and Edoh, 1996). It is also known to have both endophilic (indoor resting) and exophilic behaviour (resting outdoor), for example eastern Sudan (Haridi, 1972) and Ethiopia (Ameneshewa and Service, 1996). These variations in the behaviour make the control of this species by indoor residual spraying difficult (Gillies and Coetzee, 1987).

1.3.3 *An. merus* Dönitz

An. merus is a salt-water breeder found along the side of the eastern continent from southern Somalia to northern Kwazulu Natal. They breed in brackish lagoons, ponds, swamps, pools and puddles, but contrary to *An. melas*, they are unable to withstand high salinity (Gillies and De Meillon, 1968; Mosha and Mutero, 1982). This species is not restricted to the coast but is also found further inland from the coast (Mahon *et al*., 1976; Mnzava and Kilama, 1986; Masendu *et al.*, 2005). The females are known to feed on humans indoors as well as outdoors in the absence of alternative hosts (Gillies and De Meillon, 1968; Coluzzi *et al.*, 1979). It is considered as a local malaria vector in Tanzania and Kenya (Temu *et al*., 1998; Mosha and Petrarca, 1983).

1.3.4 *An. melas* Theobald

Anopheles melas, is the second salt-water breeder in the complex. Its distribution is limited to the coast of West Africa from Senegal to Angola (Gillies and De Meillon, 1968; Coluzzi, 1984). *Anopheles melas* larvae can withstand very high salinity but they usually develop in waters with salinity around or lower than seawater (Akogbeto, 1995). It shows few feeding preferences: can be highly anthropophilic in the presence of humans and very zoophilic in their absence (Snow, 1983; Akogbeto, 2000). However, it has been reported as malaria vector in many localities along the West African coast (Gillies and De Meillon, 1968). It is more exophilic and zoophilic than *An. merus* (Coluzzi *et al.*, 1979).

1.3.5 *An. quadriannulatus* Theobald

Anopheles quadriannulatus, is the third fresh-water breeder and is a non-vector of malaria because it feeds mainly on cattle and rests outdoor (Gillies and Coetzee, 1987). Although recent laboratory experiments have shown that it is susceptible to *P. falciparum* (Takken *et al*., 1999). The larvae of this species are found in small, exposed, temporary pools formed in hoofprints, road-ruts and riverbeds. *Anopheles quadriannulatus* is widespread in southern Africa (Gillies and Coetzee, 1987; Ntomwa *et al*., 2006).

1.3.6 *An. quadriannulatus* species B

Crosses between *An. quadriannulatus* from Ethiopia and from South Africa resulted in sterile hybrid males and polytene chromosomes with extensive asynapsis (Hunt *et al.*, 1998). However, the new species produced the same DNA fragments as *An. quadriannulatus* from South Africa and has the same chromosomal banding patterns. *An. quadriannulatus* from South Africa are classified as "species A" and the Ethiopian population designated as "species B" (Hunt *et al.*, 1998). The status of the single record of *An. quadriannulatus* from Zanzibar is unknown.

1.3.7 *An. bwambae* White

Anopheles bwambae is found only in the Semliki forest of Bwamba Country in Uganda (White, 1985; Charamabous *et al.*, 1999). The larvae are found in sunlit pools, especially those of animal footprints around the edge of the hot mineral springs (White, 1974). The females are anthropophilic and endophilic (White, 1973) and in natural conditions some

An. bwambae adults have been found infected with both malaria sporozoites and filariae (White, 1985). However, this species 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.

1.4 IDENTIFICATION OF SIBLING SPECIES

The recognition in 1962 that *An. gambiae* was a group of sibling species lead to the development of a number of methods to distinguish between these species. These methods, from basic morphology to salinity tolerance, cross-mating experiments and the more recent molecular methods such as PCR, are described below.

1.4.1 Morphological identification

Morphological identifications are the baseline of any identification technique. Basic descriptions of anopheline mosquitoes and keys for identification have been established by Gillies and De Meillon (1968) and later improved by Gillies and Coetzee (1987).

Members of the *An. gambiae* complex are difficult to morphologically identify (Gillies and Coetzee, 1987; Fettene *et al.*, 2004; Coetzee, 2004) and the only reliable morphological characters are found between the two salt-water and the fresh-water species by using the number of antennal sensillae and the palpal index (Coluzzi, 1964).

1.4.2 Salinity tolerance

Ribbands (1944) and Muirhead-Thomson (1951) distinguished between the fresh water breeder *An. gambiae* and the two salt water species *An. melas* and *An. merus* using a salinity tolerance assay. The first instar larvae were placed in a solution equivalent to 75% sea water (23.5 g/litre sodium chloride) and only *An. melas* and *An. merus* survived for more than 1 hour.

1.4.3 Cross-matting experiments

The recognition of all the sibling species of the *An. gambiae* complex are based on results from crossing experiments (Davidson *et al*, 1967; Hunt *et al*, 1998). The first recorded cross between populations of *An. gambiae* was by Muirhead-Thomson (1948) in Lagos, Nigeria; sterile males were produced when fresh-water species were crossed with salt-water species, but his results were ignored. In 1962, Paterson indicated that *An. merus* was a distinct biological species. This technique is time consuming and not suitable to be used for a large-scale.

1.4.4 Cytogenetic analysis

This method classifies the mosquitoes based on the detection of differential banding patterns of the polytene chromosomes. These chromosomes can be found in either the fourth instar larvae or in the ovaries of semi/ half gravid females. Cytogenetic observations carried out by Coluzzi and Sabatani (1967, 1968 ,1969) on the five species of the *An. gambiae* complex (Species A, B, C, *An. melas* and *An. merus*) showed that the fresh water species differ in paracentric inversions on the X chromosome while the salt-

water species are characterized by inversions on the autosomes. The disadvantages of this method include the requirements of expertise to carry out the assay, it is limited to early fourth instar larvae or semi-gravid females and it can not be used to identify large numbers of samples (Gale and Crampton, 1987).

1.4.5 Isoenzyme analysis

Isoenzyme analysis is a biochemical method where the protein molecules in a gel matrix are exposed to an electrical field (Brewer, 1970). The method was described by Mahon *et al*. (1976) to distinguish between four members of the *An. gambiae* complex and in 1978 Miles published a Biochemical Key to identify six members of the complex. It is recommended that before applying the method on a population with unknown genotype, it should be correlated with another method, e.g. cytogenetics (Coetzee *et al.*, 1993). The advantage of the isoenzyme electrophoresis technique over cytogenetic analysis is that it does not require a specific sex or larval stage (Miles, 1978). However, the technique is time consuming and requires specimens to be stored in liquid nitrogen, which makes the collection in the field very difficult (Miles, 1979).

1.4.6 Polymerase Chain Reaction (PCR)

The PCR is an *in vitro* method developed for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Saiki *et al.,* 1985). The first PCR method to distinguish members of the *An. gambiae* complex was originally developed by Paskewitz and Collins (1990). This method was based on species-specific nucleotide

sequences in the ribosomal DNA (rDNA) intergenic spacer regions which is useful in identifying species within the complex regardless of life stage and sex using either extracted DNA or fragments of a specimen. Scott *et al*. (1993) improved the method by developing four species-specific primers to include the five most widespread members of the *An. gambiae* complex: *An. quadriannulatus*, *An. gambiae*, *An. arabiensis*, *An. melas* and *An. merus*. Later, the method was extended to include other members within the complex such as *An. bwambae* from Uganda (Townson and Onapa, 1994), *An. quadriannulatus* species B (Fettene *et al.,* 2002).

Validity of the method has been tested by a number of laboratories and currently this is the most frequently used method for species identification (Cornel *et al.*, 1997). Results obtained from the assay are easy to interpret, the storage of samples is much simpler and does not require liquid nitrogen, it is relatively inexpensive and, importantly large samples can be easily processed (Paskewitz and Collins, 1990; Scott *et al.*, 1993).

1.5 MALARIA CONTROL: HISTORY, INSECTICIDES AND INSECTICIDE RESISTANCE

1.5.1 Malaria control (history and current)

The 1930's represent the beginning of vector control in Africa by introducing the use of organic chemicals extracted from plants such as nicotine, rotenone and pyrethrum. Good results were achieved in controlling malaria vectors with pyrethrum in southern Africa (De Meillon, 1936). This was replaced with the organochlorine dichloro-diphenyl-trichloroethane (DDT) in the 1940's (Kumar, 1984). In 1955, the World Health

Organization assembly launched the malaria eradication initiative with the use of (DDT) as the primary tool (Hemingway and Ranson, 2000). Following the use of DDT, cyclodiene insecticides such as benzine hexachloride (BHC) and dieldrin were introduced for malaria control. The success of these insecticides in controlling malaria has been achieved in many parts of the world, for example in 1950s-60s the near eradication of malaria in the islands of Zanzibar (Curtis and Mnzava, 2000) and the highlands of Madagascar (Curtis, 2002; Joncour, 1956) was achieved by DDT and BHC indoor spraying. This evolution of insecticides soon ended as a result of insecticide resistance; in 1976 the WHO changed the policy from malaria eradication to malaria control (Hemingway and Ranson, 2000).

Currently, application of insecticides through indoor residual-house spraying and impregnation of mosquito nets are the main means of controlling malaria vectors (McCarroll and Hemingway, 2002; WHO, 2005b). Roll Back Malaria partnership aims to reduce the overall malaria mortality by 50% by the year 2010 (WHO, 2005b). Pyrethroids are the widest used insecticides for the above applications due to low mammalian and environmental toxicity. However, the development of insecticide resistance, particularly pyrethroid resistance in *An. gambiae*, *An. arabiensis* and *An. funestus* in many African countries such as Ivory Coast, Burkina Faso, Kenya, Tanzania, Uganda, Mozambique and South Africa threaten the effectiveness of these control measures (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Diabaté *et al.*, 2004; Kulkarni *et al*., 2006; Verhaeghen *et al*., 2006; Casimiro *et al*., 2006; Hargreaves *et al*., 2000, 2003).

1.5.2 Classification of insecticides

Insecticides used for malaria control can be classified into four groups: organochlorines, organophosphates, carbamates and pyrethroids. Table (1.1) shows insecticides used for malaria control.

Table 1.1: Insecticides used for IRS in malaria control, their classes, dosage and effective action as recommended by the WHO Pesticide Evaluation Scheme (WHO, 2001).

Product	**Class group***	**Dosage (g/m^2)**	**Duration of effective action (months)**
DDT	OC	1-2	>6
Malathion	OP	2	2-3
Fenitrothion	OP	2	3-6
Pirimiphosmethyl	OP	1-2	2-3
Bendiocarb	C	0.1-0.4	2-6
Propoxur	C	1-2	3-6
Alphacypermethrin	P	0.02-0.03	4-6
Cyfluthrin	P	0.01-0.05	3-6
Deltamethrin	P	0.01-0.025	2-3
Etofenprox	P	0.1-0.3	3-6
Lambdacyhalothrin	P	0.02-0.03	3-6

*OC= Organochlorines; OP= Organophosphates; C= Carbamates; P= Pyrethroids.

1.5.3 Insecticide Resistance

The first report of insecticide resistance in mosquitoes was in the salt marsh *Aedes tritaeniorhynchus* and *Ae. solicitans* when they became resistant to DDT in Florida in 1947 (Brown, 1986). Since then more than 100 mosquito species globally are reported to be resistant to several insecticides and more than 50 of these species are anophelines.

The World Health Organization defined insecticide resistance as: "The ability in a population to tolerate doses of insecticide which would prove lethal to the majority of individuals in a normal population of the same species, developed as a result of selection pressure to the insecticide" (WHO, 2001).

1.5.4 Mode of action of insecticides and resistance mechanisms

The characteristics of insecticide resistance are inherited and are due to genetic factors (Brown, 1986). The two major mechanisms by which insects acquire resistance are metabolic resistance and target site insensitivity. Each one of these mechanisms consists of several biological mechanisms and these are discussed below (Brogdon and McAllister, 1998). Another type of resistance is called behavioural resistance, where the "exito-repellent" effect of the insecticide prevents the insect from resting on insecticide-sprayed surfaces. Compounds such as pyrethroids and DDT are good examples as they have a repellency effect. This causes behavioural changes, for example in reducing the number of mosquito coming indoors to feed, increasing the rate of early exit, changing the biting times by causing mosquito to feed early in the evening and influencing mosquitoes to seek alternative hosts for feeding (Lines *et al.*, 1987; Mbogo *et al.*, 1996; Mathenge *et al.*, 2001).

1.5.4.1 Target site resistance

Each insecticide targets a specific molecule in the nervous system of the mosquito. However, mutations within these sites may reduce the sensitivity of the site resulting in resistance (Scott, 1995).

Insensitive acetylcholinesterase (AChE)

The neurotransmitter acetylcholine plays an important role in the transmission of the external stimuli which enables the nervous system to translate it into effective action. When the appropriate message is successfully passed, the acetylcolinesterase (AChE) terminates the nerve impulses by catalyzing the hydrolysis of the acetylcholine (Mutero *et al.*, 1994; Walsh *et al.*, 2001). Organophosphate and carbamate insecticides target AChE by phosphorylating or carbamoylating the active-site serine and preventing it from hydrolysing the acetylcholine. Therefore, this causes the continuous action of the acetylcholine and finally the death of the insect (Vontas *et al.*, 2002).

Insensitive acetylcolinesterase was reported for the first time in spider mites (Smitssaert, 1964). Since then it has been reported from several insect species such as *Myzus persicae*, *Myzus nicotianae*, *Aphis gossypii* and *Rhyzopertha dominica* (Moores *et al*., 1994; Silver *et al*., 1995; Guedes *et al*., 1997; Walsh *et al.*, 2001). In *Drosophila melanogaster* a single gene has been described (ace-2) with five point mutations found in three resistant strains (Mutero *et al.*, 1994). The insensitivity of AChE in *An. gambiae*, *An. albimanus* and *Culex pipiens* is due to a high level of another ace gene (*ace*-1) (Weill *et al.*, 2004).

GABA Receptor mutation

The GABA neurotransmitter receptors contain five subunits found around the central ion channel (Hemingway and Ranson, 2000). Each subunit has an extracellular cysteine loop and four transmembrane domains (M1 – M4). The transmembrane domain two (M2) is the most important one because it forms the ion channel and contains the conserved alanine residue 302. Binding to its ligand GABA, the receptor increases the flow of chloride through the membrane.

Cyclodiene insecticides (e.g. dieldrin and BHC) block the GABA receptor thus preventing the inhibition of neuroal activity, leading to the death of the insect. Amino acid substitutions from alanine to serine or glycine within the second transmembrane region of the RDL subunit at position 302 are found associated with resistance to dieldrin in many insects (Hemingway *et al.*, 2004; Du *et al.*, 2005). An Ala → Ser substitution was found in *Drosophila melanogaster, D. simulans*, *Aedes aegypti, An. stephensi* and *An. gambiae* (ffrench- Constant *et al.*, 1993, 2000; Du *et al.*, 2005). In *An. arabiensis*, the resistance was found to be due to an alanine-glysine substitution at the same position (Du *et al*., 2005).

Mutations in the voltage-gated sodium channel

The voltage-gated sodium channels play an integral role in the transmission of nerve impulses. Pyrethroid and DDT analogs are known to be directly toxic to the nervous system, and while the two insecticides are from different groups, both target the voltage-gated sodium channel by interfering with the normal functioning of these channels (Beeman, 1982; Martinez-Torres *et al*., 1997; Hemingway and Ranson, 2000). The sodium

channel comprises of four homologous domains (I–IV). Each domain consists of six transmembranes (S1–S6) (Zlotkin, 1999). When the membrane is at resting position (inactivation state) the channel is closed and when it becomes depolarized (activation state) this causes the channel to produce a sodium current.

Pyrethroid and DDT insecticides modify the gating kinetics of the sodium channel by slowing both activation and inactivation of the channel, resulting in prolongation of the inward sodium (Na^+) current and suppression of the outward potassium (K^+) current (Lund and Narahashi, 1983; Beeman, 1982). The wide use of pyrethroid and DDT in agriculture and public health has led to the rapid development of insecticide resistance in several insect species (Martinez-Torres *et al.*, 1997). Mutations in the gene that encodes the voltage-gated sodium channel known as knockdown resistance (*kdr*), have been found associated with resistance to pyrethroid and DDT, (Martinez-Torres *et al.*, 1998). In insects, the voltage-gated sodium channel is encoded by the *para* gene (Loughney *et al*., 1989). Studies on the *para* gene in houseflies and cockroaches identified a single amino acid substitution, leucine to phenylalanine, within the IIS6 transmembrane segment that associated with pyrethroid resistance (Dong, 1997; Miyazaki *et al.,* 1996; Williamson *et al.,* 1996). Another two substitutions at the same position also confer resistance to DDT and pyrethroids: a leucine-histidine substitution is associated with pyrethroid resistance in *Heliothis virescens* (Park and Taylor, 1997) and a leucine-serine substitution confers DDT resistance and low levels of permethrin resistance in a strain of *Culex pipiens* from China (Martinez-Torres *et al*., 1999; Jamroz *et al*., 1998). Secondary mutations (*super-kdr*) have also been identified within the domain II region of the channel, including M918T in housefly and horn fly (Guerrero *et al.,* 1997; Williamson *et al*., 1996), T929I

in diamondback moth and head louse (Lee *et al*., 2000; Schuler *et al*., 1998) and L925I in tobacco whitefly (Morin *et al*., 2002). These mutations are found in combination with the L1014F *kdr* mutation and give high resistance levels of up to 1000-fold for certain pyrethroids (Soderlund and Knipple, 2003; Vais *et al*., 2001); the exceptions being L925I which appears as a single mutation in tobacco whitefly and T929I in head lice where it is found in combination with a fifth mutation in this region, L932F (Lee *et al.,* 2000). These five mutations are all located in the domain II (S4-S6) region of the sodium channel.

Two different types of the *kdr* mutations have been found in *An. gambiae* and *An. arabiensis* (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Diabate *et al.*, 2004). The first mutation, leucine-phenylalanine substitution at position 1014 of the sodium channel gene (L1014F), in the S6 hydrophobic segments of domain II, has been found in the Ivory Coast and Burkina Faso in West Africa (Martinez-Torres *et al.*, 1997, 1998; Chandre *et al.*, 1999a,b, 2000). The second mutation at the same position causes a leucine-serine substitution (L1014S). This mutation has been reported from Western Kenya in East Africa (Ranson *et al.*, 2000).

1.5.4.2 Metabolic resistance

Metabolic resistance to insecticides occurs when the metabolic pathways of the insect become modified in ways that detoxify the insecticide or prevent the metabolism of the insecticide into its toxic form. There are three enzyme classes involved in this type of resistance glutathione S-transferases (GSTs), Carboxylesterase and Cytochrome P450 monooxygenase (P450s) (Devonshire *et al*., 1992).

Glutathione S-transferase

The Glutathione S-transferases (GSTs) are a large family of detoxification enzymes found in almost all living organisms (Ranson *et al.*, 2001). They are cytosolic dimeric proteins with two subunits, consists of two domains, each containing two binding sites, the G site and the H site. The G site binds the tripeptide glutathione and the H site or the substrate binding site (Hemingway *et al.*, 2004; Ding *et al.*, 2003).

The GSTs are classified according to their location in the cell i.e. microsomal or cytosolic (Enayati *et al.*, 2005). Six classes of the insect GSTs have been identified Delta, Epsilon, Omega, Sigma, Theta and Zeta (Hemingway *et al.*, 2004). The two classes Delta and Epsilon are the most important because of their role in insecticide resistance to the major classes of insecticides (Ding *et al.*, 2003). For example, organophosphate resistance in the diamondback moth, *Plutella xylostella* is caused by elevated levels of Epsilon-GSTs (Ku *et al*., 1994; Huang *et al*., 1998), whereas permethrin resistance in the brown plant-hopper *Nilaparvata lugens* is associated with over expression of *nlGST1-1*, a class of Delta GSTs (Vontas *et al*., 2001). GSTs, catalyse the reaction of the dehydrochlorination of DDT to the non-toxic form DDE, where the thiolate anion in the active site of the GST acts as a base and abstracts a hydrogen from DDT resulting in the elimination of chlorine to generate DDE. This type is found in *An. gambiae* and *Aedes aegypti* (Ortelli *et al.*, 2003; Ranson *et al.*, 2001).

Resistance to organophosphates is due to increases in GST detoxification rates by O-dealkylation or O-dearylation reaction (Hemingway *et al.*, 2004). GSTs also protect the insect against the toxicity of pyrethroids either by detoxification of the lipid peroxidation

products induced by the insecticide or by sequestering the insecticide (Kostaropoulos *et al.*, 2001; Vontas *et al.*, 2002).

Carboxylestarase

Carboxylesterases are a large group of enzymes with different substrate specificity. According to Aldridge (1953), they are classified as A or B esterases according to their preference for the substrates α or β-naphthyl acetate.

Esterases produce resistance either by rapid-binding and slow turnover of the insecticide (elevated esterase) i.e. sequestration, or metabolism of the insecticide by catalyzing the hydrolysis of carboxylic and phosphotrieste bonds in a wide range of insecticides such as organophosphate, carbamates and pyrethroids (Scott, 1995; Brogdon and McAllister, 1998; Hemingway and Karunaratne, 1998). Gene amplification has been found associated with elevated esterases that function by sequestration, for example in the aphid *Myzus persicae* and *Culex* mosquitoes, *C. quinquefasciatus* and *C. tritaeniorhynchus* (Vaughan and Hemingway, 1995; Claudianos *et al.*, 1999; Hemingway, 2000). However, studies of the elevated estα1 in C. *pipiens* from France showed that there was no involvement of gene amplification (Raymond *et al.*, 1998). The elevated estrases of *C. quinquefasciatus* from Sri Lanka has both the sequestration and metabolic functions (Peiris and Hemingway, 1990).

Esterases which produce resistance by metabolism of the insecticide are associated with a single amino acid substitution in the structural genes (Hemingway *et al.*, 2004). This type of mechanism is often involved with malathion resistance, for example in *An.*

arabiensis from Sudan, *An. culicifacies* from Sri Lanka and *An. stephensi* from Pakistan (Hemingway, 1982, 1983; Herath *et al*., 1987)

Cytochrome P450 monooxygenase (P450s)

Cytochrome P450 monooxygenases are hydrophobic, heme containing enzymes. The P450 family is one of the largest gene super-families and is found in all the living organisms. The insect P450s are involved in insect growth, development, reproduction and insecticide resistance (Feyereisen, 1999). The P450s play an integral role in the metabolism of endogenous and exogenous compounds such as steroids, fatty acids and xenobiotics (Scott and Wen, 2001; Rongnoparut, 2003; Hemingway *et al*., 2004).

Monooxygenase enzymes are named as CYP followed by a number, a letter and a number respectively for example CYP6D1 (Scott and Wen, 2001). In insects, an increase in the elevated levels of P450 results in metabolic resistance (Feyereisen, 1999). For example, the CYP6D1 is over-produced in the house fly, *Musca domestica*, which results in a high level of permethrin resistance, and a similar situation is CYP6A1 which is associated with organophosphate resistance (Kasai and Scott, 2000; Hemingway *et al.*, 2004).

In mosquitoes, elevated levels of oxidase have been reported from several species, for example, CYP6Z1 was found associated with pyrethroid resistance in *An. gambiae* from Western Kenya (Nikou *et al*., 2003), and CYP6P5 and CYP6AA2 have been cloned from *An. minimus* resistant to deltamethrin in Thailand (Rongnoparut *et al.*, 2003). Another CYP6 gene is CYP6F1 which has been reported from a permethrin-resistant strain of *Culex quinquefasciatus* (Kasai *et al*., 2000).

1.6 MALARIA IN SUDAN: HISTORY AND CURRENT SITUATION

1.6.1 Demographical profile

Sudan is the largest country in Africa and has a special geopolitical location bonding the Arab world to Africa south of the Sahara. It has an area of 2.5 million km^2 extending between latitudes 4º and 22º North and latitudes 22º to 38º East. Its north-south extent is about 2 000 km, while its maximum east-west extent is about 1 500 km. On the north-east it is bordered by the Red Sea and it shares common borders with nine countries: Eritrea and Ethiopia in the east, Kenya, Uganda and the Democratic Republic of Congo in the south, the Central African Republic, Chad and the Libyan Arab Jamahiriya in the west, and Egypt in the north.

The arable area is estimated at about 105 million ha (42 percent of the total land area). The forest resources of Sudan cover approximately 27 percent of the total country's area. The main forest types include arid and semi-arid shrubs; low rainfall savannah; high rainfall savannah; special areas of mountainous vegetation in Jebel Marra, the Red Sea Hills and the Imatong Mountains. Sudan is under federal rule with 26 States. Each State is governed by a Wali (Governor) with 7 to 10 State Ministers, 4 to 5 Commissioners for the different provinces and a number of localities. Sudan has a tropical sub-continental climate, which is characterized by a wide range of variations extending from the desert climate in the north through a belt of summer rain climate to an equatorial climate in the extreme south. The average annual rainfall is 416 mm, but ranges between 25 mm in the dry north and over 1600 mm in the tropical rain forests in

the south. The mean temperature ranges from 30 ºC to 40 ºC in summer and from 10 ºC to 25 ºC in winter. Sudan's population is 34.3 million (2004) with an annual growth rate of 2.2 percent Population density is 14 inhabitants/km^2 and 60 percent of the total population is rural. Most of the population lives along the Nile and its tributaries, and some live around water points scattered around the country.

1.6.2 Malaria in Sudan

Malaria is by far the leading cause of morbidity and mortality in Sudan. It is responsible for 20 - 40% of all outpatient attendance and around 30% of hospital admissions (WHO, 2005c). Malaria is estimated at 7.5 million cases and 35,000 deaths annually. This represents 50% and 70% of all the malaria cases and deaths in the WHO countries of the Eastern Mediterranean Region (EMRO) respectively (WHO/EMRO, 2001, unpublished report). Possible factors leading to this situation include floods, drought, famine, widely extended irrigated schemes without due consideration to the health component as well as population movement (internal displacement and influx refugees) (Malik *et al.*, 2006). The situation is further complicated by the spread of insecticide and drug resistance (Himeidan *et al.*, 2004).

The endemicity of malaria varies from hypo-endemic in the north to hyper-to-holo-endemic in the south (Figure 1.1) (MOH 2003, unpublished report).

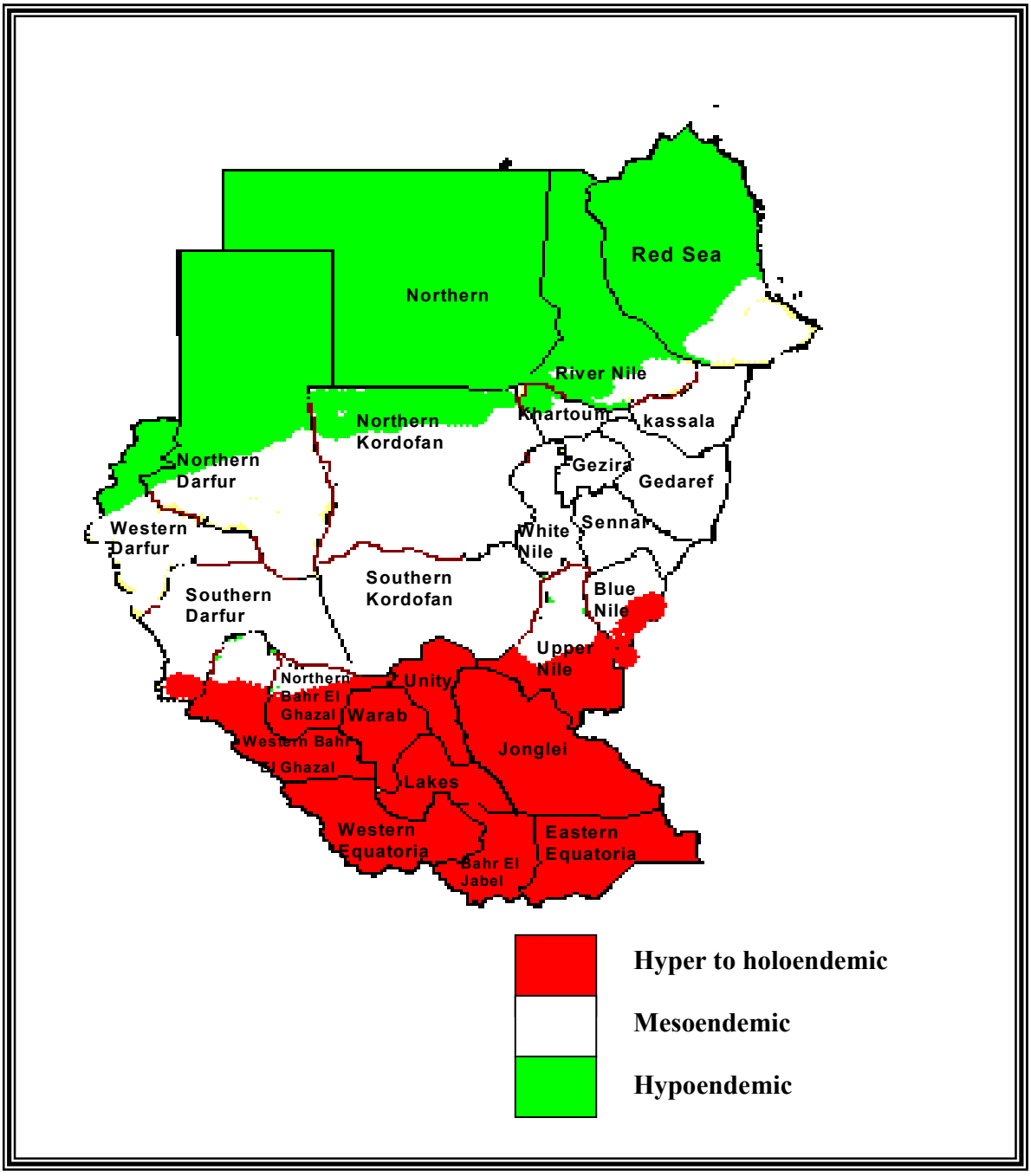


Figure1.1: Map showing the malaria endemicity levels in Sudan, (MOH 2003, unpublished report).

1.6.3 Malaria vectors and parasites

Mosquitoes of the *Anopheles gambiae* Giles complex are the main vectors of malaria in Sudan. *Anopheles funestus* Giles also contributes to malaria transmission in southern and South-Eastern Sudan. The only other malaria vector of any known importance in Sudan is *An. nili* (Theobald) (Lewis, 1958). *Anopheles pharoensis* Theobald is widespread but, in view of its largely zoophilic and exophilic habits, seems to be of little or no importance in malaria transmission (Petrarca *et al.*, 2000).

Identification of sibling species comprising the *Anopheles gambiae* complex have been based on crossing experiments (Haridi, 1972; Akiyama, 1973) and cytotaxonomy (Dukeen and Omer, 1986), showing that *An. arabiensis* is the most predominant malaria vector in northern and central parts of Sudan (for example Gezira state).

All surveys carried out during the Blue Nile Health Project (BNHP) in the Gezira area showed that *P. falciparum* remains the dominant species followed by *P. vivax* while *P. malariae* is very rare and *P. ovale* was not reported (BNHP report, 1989, Elsayed, *et al*., 2000).

1.6.4 History of malaria situation in central (Sudan)

The Gezira Irrigation Scheme (GAS) was founded in 1913 and lies between the Blue Nile and the White Nile with an area of 153,415 hectare (about 32% of the state and 50% of the total cultivated area in the country) under gravity irrigation through a canal system from Sennar Dam on the Blue Nile (GAS-unpublished data). The introduction of irrigation in the arid areas of central Sudan produced drastic changes in the ecology. As a

result of these changes, Gezira is currently the largest area in Sudan with the highest number of permanent mosquito breeding sites (El Gadal *et al*., 1985).

At the beginning of the scheme only cotton was cultivated. Moreover, the irrigation was under control and the transmission of malaria was seasonal with few mosquito breeding sites during the rainy season. In 1960, the scheme introduced wheat, sorghum, groundnuts and vegetables. This resulted in the introduction of new agricultural and irrigation practices. As a result of this the malaria transmission changed from seasonal to perennial and malaria endemicity from mesoendemic to hyperendemic. Furthermore the migrant labour from inside and outside the country complicated the problem of malaria transmission in Gezira (El Gadal *et al*., 1985).

1.6.5 Control activities in central Sudan

The pre-insecticide era (1930 – 1950)

During this time simple control measures, usually source reduction were used. These included larval control measures with the application of paris green and diesel oil to mosquito breeding sites. Water management and control of irrigation practices were also used by the farmers and by the management of the scheme. There were no malaria epidemics experienced in the area during this period (El Gadal *et al.*, 1985).

The insecticide era (1950 – 1965)

The first insecticide to be introduced for indoor residual spraying was BHC (benzene hexachloride). An entomological survey conducted by the malaria pre-eradication

programme in 19961 showed that there was a reduction in malaria cases with 1.9% malaria prevalence, which led to a relaxation in the use of mosquito larvae control methods (El Gadal *et al.*, 1985).

The beginning of insecticide resistance (1966 -1970)

DDT for indoor residual spraying replaced BHC when resistance to the latter was reported in 1965. A remarkable reduction in malaria cases was observed. However, DDT was used only for four years before mosquito vectors developed resistance to it in 1969. By 1970 resistance to DDT had become widespread in the area (Haridi, 1972).

The malaria crisis era (1970 – 1975)

As a result of resistance to the two insecticides, malaria took an epidemic form during the period between 1970 and 1975. Also the cost of the alternative insecticide (malathion) was three times that of DDT and it could not be provided by the local government. This resulted in farmers resorting to the application of diesel oil and environmental management to control malaria. It was from this time that malaria indeed became a public health problem (El Gadal *et al.*, 1985).

Externally assisted control efforts (1975 -1979)

In 1975 the government secured a grant through the WHO for the purchase of malathion for 5 years from Kuwait and Saudi Arabia (El Gadal *et al.*, 1985). The malaria control programme staff used abate for mosquito larval control and also applied malathion for indoor residual spraying with one spraying cycle per year at 2 g/m^2. The malaria

prevalence was dropped from 20% to 7.7% after the first round of spraying in 1975. In 1979 an increase in the malaria cases was observed (data not available) as a result of resistance of the vectors to malathion (El Gadal, 1985; Akood, 1980).

The Blue Nile Health Project (1979 – 1990)

The Blue Nile Health Project (BNHP) was established in 1979 as a joint venture between the Sudan Government and the World Health Organization to develop strategies to control the major water-associated diseases in the irrigation schemes (El Gadal, 1985). During this period fenitrothion was used indoors in one spraying cycle/year at a 1 g/m^2 (El Gadal *et al.*, 1985).

Malaria vector control (1991 to date)

By the end of the BNHP the malaria control programme had reverted to larval control due to unavailability of fenitrothion. Currently the malaria vector control programmes in Gezira and Sennar states depend largely on indoor residual spraying with deltamethrin (2.5% wettable powder WP), permethrin for space spraying (25% emulsion concentrate EC), larviciding with ABATE (50% EC) and the distribution of insecticide-treated bed nets (ITNs). The coverage rate for use of ITNs is still very low. The objectives of the malaria control programme are to make the Gezira State malaria free by 2010 (Gezira Malaria free initiative - unpublished report, 2005).

1.7 RESISTANCE MANAGEMENT

Insecticide resistance is a growing problem affecting the sustainability of any vector control programme. Resistance management is designed to delay or prevent insecticide resistance (WHO, 1992). An important part in the resistance management strategies, is identifying the resistance and mechanisms involved (Rose, 2001). The aim is to obtain the baseline information about the vector susceptibility, detection of resistance in the early stage and monitoring its frequency levels over time (WHO, 1992). The rotational use of insecticides with different modes of actions, for example organophosphates and pyrethroids, could be used as a part of a resistance management strategy (Rose, 2001). Furthermore, biological control agents that attack the larval stages of the mosquitoes, for example larvivorous fish such as *Gambusia affinis*, the bacterial pathogens *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Bs), fungal pathogens, the nematode *Romanomermis culcivorax* (Das and Amalraj, 1997; Lacey and Lacey, 1990) and environmental management can also be used within the resistance management strategy (Bruce-Chwatt, 1985).

Several methods are now available for the detection of resistance, including the bioassay tests and molecular and biochemical techniques.

1.7.1 The WHO standard protocol for insecticide susceptibility

The method is based on using diagnostic concentrations to detect the presence or absence of resistance among specific populations. A diagnostic concentration, as defined by the WHO, is the concentration of a given insecticide which results in 100% mortality after 1

hour exposure. The method can only detect the overall levels of resistance. When more than 5% of the samples survive the test after the 24 hours recovery period, resistance is said to be suspected and requires further investigation. When >20% survive, resistance is confirmed. Molecular and biochemical assay can be used to detect the mechanism/s involved (WHO, 1998b).

1.7.2 Biochemical techniques

The biochemical techniques are used to detect elevated enzymes that are associated with resistance in an individual mosquito, such as esterases, P-450s and glutathione S-transferases (Hemingway *et al*., 1997; Penilla *et al*., 1998). The advantages of this method include the detection of low levels of resistance and the ability to detect multiple resistance mechanisms per mosquito (Brown and Brogdon, 1987). The disadvantages are that fresh or frozen mosquitoes are needed and enzymes can be elevated for reasons other than resistance.

1.7.3 Molecular methods

Molecular methods were developed for the detection of resistance associated with mutations in the target-sites. These are PCR based methods for the detection of cyclodiene resistance mutations in the GABA channels (ffrench-Constant *et al*., 1995) and *kdr* mutations within the sodium channel (Martinez-Torres *et al*., 1998).

New techniques were developed recently that are focused on a more efficient detection of the resistance *kdr* mutations because of the important and wide use of pyrethroid insecticides in malaria control. These methods include the hot ligation oligonucleotide

assay (HOLA) (Lynd *et al*., 2005), the fluorescence resonance energy transfer/melt curve analysis (FRET/MCA) (Verhaeghen *et al*., 2006) and the sequence-specific oligonucleotide probes/ELISA (SSOP-ELISA) (Kulkarni *et al*., 2006). All these methods were modified from the two methods described by Martinez-Torres *et al*. (1998) and Ranson *et al*. (2000). The advantage of the molecular techniques over the bioassay and biochemical methods is that, they can detect resistance genes before the problem becomes widespread in a population.

1.8 AIM AND OBJECTIVES OF THE STUDY

1.8.1 General objective

Insecticide resistance is one of the major causes of failure of many vector control programmes in the world. In the Gezira area of Sudan, resistance of the main malaria vector *Anopheles arabiensis* to insecticides is well established. This study will therefore attempt to determine the degree to which this problem prevails in this irrigated part of central Sudan with the view to recommending effective strategies to mitigate its impact on malaria transmission.

1.8.2 Specific objectives

1. Identify *An. arabiensis* the main malaria vector species in the area using a molecular method.
2. Determine the sporozoite infection rate in the malaria vectors in the study area using ELISA.
3. Determine host blood meal sources in the vectors using ELISA.

4. Determine insecticide resistance in *An*. *arabiensis* using the WHO susceptibility tests for DDT, malathion, permethrin and bendiocarb.
5. Determine the mechanisms involved in the resistance using molecular and biochemical assays.