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Insecticide resistance in malaria vectors in central Sudan

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CHAPTER TWO

MATERIAL AND METHODS

2.1 THE STUDY AREA

This study was carried out in Gezira and Sennar states of central Sudan (Figure 2.1). The two states are situated in the rich savanna environment. Sentinel sites within the two states were chosen according to their history and current situation of insecticide usage in agricultural and public health.

2.1.1 Gezira state

Gezira state is the most agriculturally productive state in the Sudan. It lies between the Blue Nile and the White Nile rivers. It is bounded by Khartoum state in the North, Gadarif state in the East, White Nile state in the West and Sennar state in the South. It lies in the rich Savanna region between latitude 13-15.2^0 N and longitude 32.5 – 34^0 E. The area has a hot dry summer from April to June with daily temperature between 32-42^{0}C and relative humidity of 20%. The rainy season starts in late June and ends in October. Winter is cold and dry and is from December to February with daily temperature between 15-21^{0}C, and relative humidity of 30% (Sudan Metrological Services, 2005, unpublished data).

The Gezira Agricultural Scheme (GAS) was founded in 1913 covering an area of 153,415 hectors. It is one of the largest irrigation schemes in the world and comprises

50% of the total cultivated area in the country (about 32% of the state). It is divided into 18 sections. The main agricultural products in all the sections are cotton, sorghum (dura), wheat, groundnuts and vegetables. The canalization network consists of 5,649 km with a depth ranging from 0.50 to 0.75 meter (GAS, unpublished data). In this scheme, more than 85 % of the pesticides imported into Sudan are used for the control of cotton pests (GAS, unpublished data). The scheme receives intensive aerial spraying of pesticides from different classes every year directed against cotton, wheat and vegetables pests. Cotton spraying starts in August and ends in November (see Figure 2.2), wheat spraying from February to March and vegetables all year round according to the pests and the cultivated vegetables (GAS, unpublished report).

Nine sentinel sites within the Gezira state were chosen in this study (Table 2.1). Seven of them are located within the GAS. The other two sites which are located on the East bank of the Blue Nile are Rofaa city about 22 kilometers from El Guneid sugar factory and Wad Rawa city about 38 kilometers from El Guneid Sugar Estate.

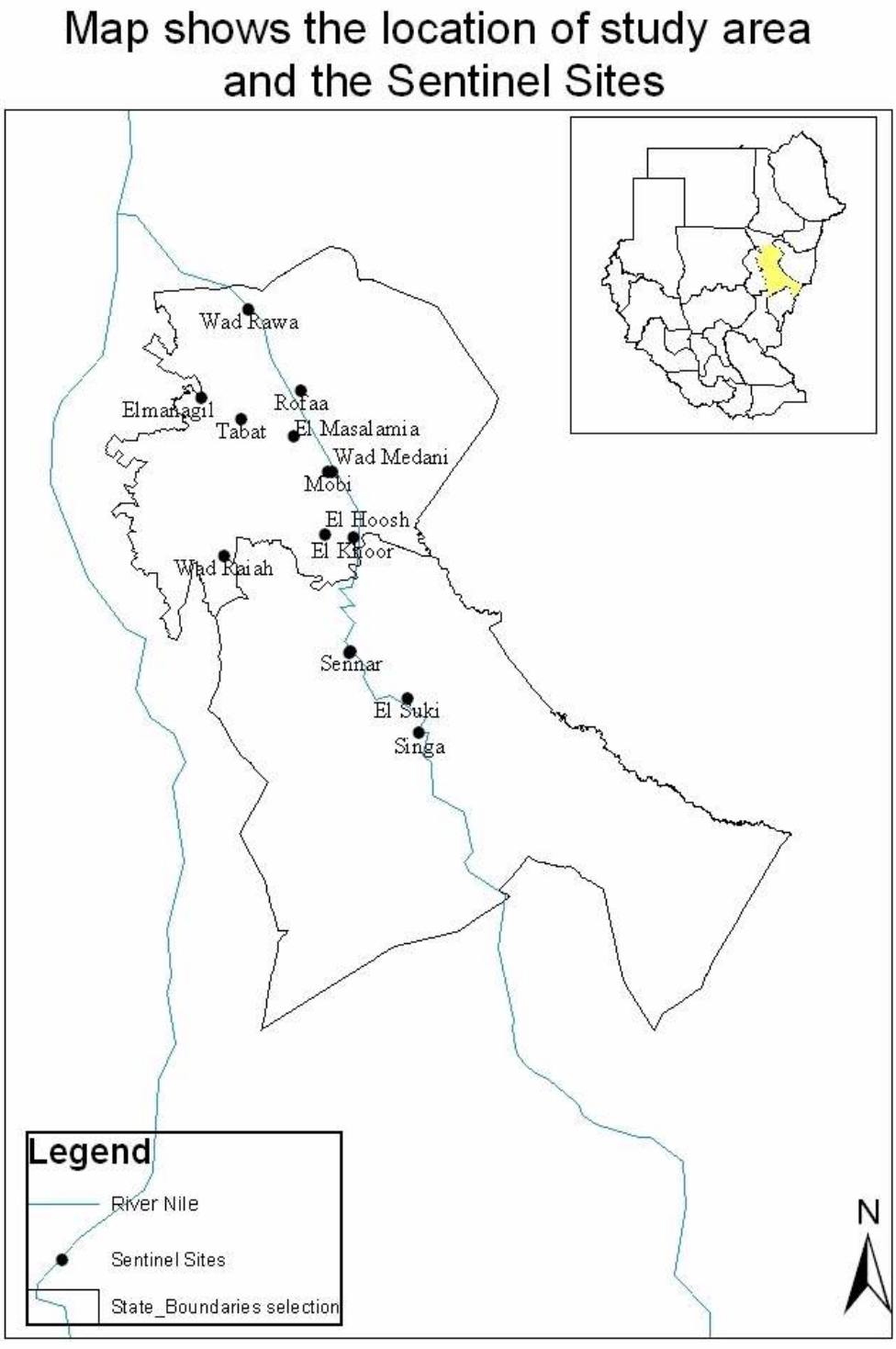


Figure 2.1 Map showing the location of the study area and the sentinel sites.

2.1.2 Sennar State

Sennar state is located in the central-east of Sudan. It Shares its borders with Gezira state in the North, White Nile and Upper Nile states in the west, Gadarif state in the east, Blue Nile state and Ethiopia country in the South. It lies also in the rich savanna region between latitude 12.5 – 14.7 ^{0} N and longitude 32.9 – 35.4 ^{0} S. Summer starts in March and ends in May, with an average daily temperature 32-40^{0}C and relative humidity 25%. The rainy season starts earlier in June and ends in September. The temperature ranges between 20-25^{0} C during winter, which starts in October (Sudan Metrological Services, 2005, unpublished).

Four sites were chosen because they are the sentinel sites for monitoring insecticide resistance in the state (Table 2.1). Sennar city in the north of the state and about 114 kilometers from Wad Medani city, El Suki city in the East, West Sennar Sugar Factory (El Booster) and Singa city the capital city in the South of the State.

The community members from all the villages within the study area are mainly farmers working for the GAS scheme. They also keep cows on a small scale. The houses in all the villages are constructed from mud with grass roofs (Figure 2.3). In the cities, the houses are constructed from bricks with either traditional roofs or cement. Community members from the cities are either employees working in different organizations or have other occupations. Each house within the study area contained 1-2 bed-nets impregnated or non-impregnated to prevent the children from being bitten by mosquito bites. The majority of the population prefers sleeping outdoors which exposes them to mosquitoes. At each sentinel site there is a malaria control unit under the umbrella of the state

malaria control programme. The houses were sprayed with deltamethrin 2.5% WP and permethrin was used for the impregnation of the mosquito nets. The geographical coordinates for all the sentinel sites were taken used a Garmin GPS machine.

Table 2.1: shows the geographical coordinates for the sentinel sites in Gezira and Sennar states

The state	Location	Sentinel site	Geographical coordinate
Gezira	North	El Masalamia	33^0 20' E, 14^0 34' N
		Tabat	33^0 05' E, 14^0 39' N
	Central	Wad Medani	33^0 31' E, 14^0 24' N
		Mobi	33^0 30' E, 14^0 24' N
	South	El Hoosh	33^0 29' E, 14^0 06' N
		El Khoor	33^0 37' E, 14^0 05' N
	East	Rofaa	33^0 22' E, 14^0 41' N
		Wad Rawa	33^0 08' E, 14^0 47' N
	West	El Managil	32^0 59' E, 14^0 40' N
Sennar	North	Sennar	33^0 37' E, 13^0 35' N
	East	El Suki	33^0 53' E, 13^0 20' N
	West	El Booster	33^0 36' E, 13^0 32' N
	South	Singa	33^0 55' E, 13^0 10' N



Figure 2.2 Cotton spraying at the Gezira Irrigation Scheme.



Figure 2.3 Typical houses at one of the villages in Gezira state.

2. 2 MOSQUITO COLLECTIONS

Mosquitoes from the sentinel sites were collected 2-3 times per week from August 2005 to January 2006 by indoor resting and larval collections. The methods were chosen according to the WHO criteria (WHO, 1998b), and are listed below.

2.2.1 Indoor collections

Blood-fed adult female *Anopheline* mosquitoes were collected from inside houses using an aspirator in the morning. They were kept alive in clearly labeled paper cups and offered 10% sugar solution. Mosquitoes were transferred to the insectary in the Blue Nile Research and Training Institute (BNRTI) where they were identified to species group using the morphological keys of Gillies and De Meillion, (1968) and Gillies and Coetzee, (1987). Only members of the *An. gambiae* complex were used in this study. All blood fed females were preserved in labeled microcentrifuge tubes containing blue indicated silica gel as desiccant. Preserved mosquitoes were used for molecular identification, sporozoite and blood meal identification using enzyme linked immuno-sorbent assay (ELISA).

2.2.2 Larval Collections

It is recommended that susceptibility tests are conducted using 1-3 days-old unfed female mosquitoes. In the field, this can only be obtained through rearing of collected mosquito larvae (WHO, 1998b). Collected larvae were separated from other species larvae and predators and kept in buckets for transferred to the BNRTI insectary where

they reared through to adults. When sufficient number were obtained (100 female mosquitoes per test, as recommended by the WHO), they were identified morphologically to *An. gambiae s.l.* using the keys of Gillies and De Meillion, (1968) and Gillies and Coetzee, (1987) and used in the susceptibility tests.

2.3 INSECTICIDE SUSCEPTIBILITY TESTS

The choice of the insecticides, which were used in the study, was according to their chemical classification, their usage, the status of insecticide resistance in the area and to cover at least one insecticide in each class of insecticides. The WHO susceptibility tests were carried out in this study to determine the current status of insecticide susceptibility or resistance to permethrin, DDT, malathion and bendiocarb. The test kits were obtained from the WHO Eastern Mediterranean Regional Office (EMRO) in Cairo, Egypt. Table 3.2 shows the discriminating concentrations of insecticides which were used in the tests (WHO, 1998b).

The susceptibility tests were carried out following the WHO procedures (1998b) using standard kits under optimum conditions (temperature $\pm 26^0$C and 70 - 80% relative humidity). Each test had five replicates and a control tube with oil-treated paper, i.e. without insecticide. The holding tubes marked with green dots contained clean papers and had steel clips to hold the papers in position against the wall of the tube. The exposure tubes containing the impregnated papers were marked with red dots and had copper clips. The test tubes were placed vertically on the bench. Twenty to twenty five mosquitoes were transferred into each holding chamber where they were allowed to rest

for one hour. Thereafter they were transferred to the exposure chambers for a one-hour exposure period. The number of mosquitoes lying on their backs or sides at the bottom of the tube were recorded on the susceptibility test form (Appendix I), after 10, 15, 20, 30, 40, 50 and 60 minutes of exposure, just before transfer to the holding tube, to record rate of knockdown (KD) of mosquitoes. After one hour, all knock-down and surviving mosquitoes were transferred back into the holding chambers and a 10% sucrose solution was offered. Final mortality was recorded after a 24 h recovery period. Those mosquitoes that survived the susceptibility test were stored separately from the dead mosquitoes in eppendorf tubes with silica gel and clearly labeled.

Table 2.2: Insecticides used in the study and their discriminating concentrations

Insecticide (name and class)	**Discriminating doses**	**impregnation Date**	**Expiry date**	**Impregnated by**
DDT (Organochlorine)	4%	March 2003	March 2008	WHO
Malathion (Organophosphates)	5%	June 2003	June 2007	WHO
Bendiocarb (Carbamates)	0.1%	August 2005	August 2008	WHO
Permethrin (Pyrethroids)	0.75%	August 2005	August 2006	WHO

2.4. *ANOPHELES GAMBIAE* SPECIES-SPECIFIC POLYMERASE CHAIN REACTION (PCR)

The PCR was carried out according to the method described by Scott *et al*., (1993) who designed five species-specific primers for the identification of the five common members of the *An. gambiae* complex. The sequence details of these primers and the expected sizes of the PCR products are given in Table 2.3. Species standards were used with each PCR reaction as positive controls. These standards were obtained from colonies maintained at the National Institute for Communicable Diseases at the vector Control Reference Unit, in the Botha De Meillon insectaries as follow: *Anopheles quadriannulatus* (SKUQUA from Skukuza in Kruger National Park, South Africa), *An.*

arabiensis (SENN, from Sennar city, Sudan), *An. gambiae* (JS3 from Iworo, Nigeria) and *An. merus* (MAF, from Kruger National Park, South Africa). One leg of a mosquito was placed into 12.5µL PCR reaction mixture. This mixture contained the following: 1.25µL of 10X reaction buffer (100mM Tris-HCl pH8.3, 500mMKCl), 1.25µl of 0.25mM dNTPs, 0.5µL of 1mM $MgCl_2$. 0.15µM of UN, ME, AR, GA and QD primers (synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa) and 5 units of Taq thermostable DNA polymerase. Finally, double distilled water was added to make the total volume 12.5µl and the reaction mixture was centrifuged for 20 seconds at 16 K. rpm. ($MgCl_2$, Buffer, Taq and dNTPs supplied by Takara Shuzo CO., LTD Japan, cat. No: R001AM).

Negative controls containing PCR reaction mixtures without DNA were added to each PCR experiment, PCR reaction conditions were as follow: 30 cycles of denaturation at 94^0 C for 30 seconds, annealing at 50^0 C for 30 seconds, extension at 72^0 C for 30 seconds and final auto extension at 72^0 C for 5 min. A volume of 10 µL of the PCR product was mixed with 3µL of ficoll dye (50% sucrose, 0.05M EDTA pH7, 0.1 % bromophenol blue, 10% ficol powder) and loaded on 2.5% agarose gel stained with 12 µL Ethidium Bromide (10mg/100ml) (Cat. No. 15585-011, Gibco BRL, UK), submerged in 1XTAE buffer and electrophoresed at 100 volts for one hour. 5µL of molecular marker (GeneRuler^{TM} DNA ladder Mix, Cat. No. SM0331) was loaded on the first and last wells of the agarose gel, negative control on the second well, positive controls in the next four wells followed by the wild samples. The DNA fragments were visualized under Ultra Violet (UV) light and the size of the products was confirmed

using the molecular ladder. Preparation of buffers and agarose gel are shown in Appendix IIa.

Table 2.3: *Anopheles gambiae* complex species diagnostic primers sequences and their melting temperature (T_m)* **(**Scott *et al.,* 1993).

Primer	Primer sequence (5' to 3')	(T_m)(^{0} C)	Expected amplified DNA size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	58.3	468
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315
QU	CAG ACC AAG ATG GTT AGT AT	42.7	153

*UN primer anneals to the same position of the rDNA of all the five species, GA anneals specifically to *An. gambiae*, ME anneals to both *An. merus* and *An. melas*, AR anneals to *An. arabiensis* and QD anneals to *An. quadriannulatus*.

2.5 DETECTION OF THE *KDR* MUTATIONS

A diagnostic PCR developed by Martienz-Torres *et al*., (1998) was used for the detection of the leucine-phenylalanine *kdr* mutation in *An. gambiae* from West Africa. Ranson *et al*. (2000) adapted this PCR for the detection of the leucine-serine *kdr* mutation in the Kenyan *An. gambiae* population. The two methods were used in this study for the detection of the West and East African mutations in *An. arabiensis* permethrin /DDT susceptible and survivor specimens.

2.5.1 DNA extractions

DNA extraction was done according to the method of Collins *et al.*, 1987 using single mosquitoes. All solutions for DNA extractions are described in Appendix IIb. For each set of DNA extraction, a negative control containing all reagents without mosquito was included. Each mosquito was homogenised in 200µl grinding buffer and then incubated at 70 ^{0}C for 30 minutes. Twenty eight microliters of 8M Potassium Acetate was added to a final concentration 1M and the mixture was incubated on ice for 30 minutes. This step was followed by centrifugation at 13,000 rpm for 15 minutes and the supernatant was transferred to a new tube and the old tube with the pellet was discarded. 400µl of ice cold 100% ethanol was added to the supernatant and mixed gently by inversion. To facilitate precipitation of the DNA, the tube was incubated overnight at -20^{0}C and the mixture centrifuged for 15 minutes at 13,000 rpm. The ethanol was removed without disturbing the pellet and 200µl of 70% ice cold ethanol was added to wash the pellet to remove excess salts and centrifugation was done at 13,000 rpm for 15 minutes. The pellet was air dried by opening the tube and resuspended in 200µl 1X TE buffer and stored at -20^{0}C until needed.

2.5.2 Polymerase Chain Reaction for detection of *kdr* mutations

Two separate master mixes were prepared for the detection of the resistant alleles (heterozygous and homozygous states) and the susceptible alleles. Each master mix contained 1.25µl of 10 X buffer (100 mM Tris-HCl, ph 8.3, 500 mM KCl), 0.75µl of 25 mM $MgCl_2$, 1.25µl of 2.5 mM dNTPs and 0.1µl of Taq DNA polymerase. For the detection of West African *kdr*, 0.25µl of Agd1 and 1.5µl of Agd3 primers were added to

each 12µl PCR reaction mix. Agd3 was substituted with 1.5µl Agd5 primer for the East African *kdr* detection. For the detection of the susceptible alleles, 0.25µl Agd2 and 1.5µl Agd4 primers were used for each 12µl PCR mix. The volume was made up to 12µl by adding distilled water. (The primers sequences are shown in Table 2.4).

The condition was 35 cycles consisting of 95^0C for 1 minute, 48^0C for 1 minute and 72^0C for 1.5 minutes and a final extension step at 72^0C for 10 minutes. The amplified fragments were analyzed using 1.5% agarose gel stained with ethidium bromide and visualized under UV light (see Appendix IIa).

Primers Agd3, Agd4 and Agd5 are allele specific. Agd3 and Agd5 bind only to the West and East African *kdr* allele respectively. A 195 bp fragment was obtained for Agd1 and Agd3 (West African) or Agd5 (East African) primer pairs. Agd4 binds only to the susceptible allele and amplified a 137 bp fragment when paired with Agd2. Heterozygote individuals produced two bands of 137 bp (susceptible) and 195 bp (resistant) fragments.

2.5.3 Sequencing

PCR assay with Agd1 and Agd2 primers was used to flank the region containing the *kdr* mutation and amplified A 293 bp fragment of the IIS6 segment containing the *kdr* mutation from genomic DNA. The PCR products were sent to Inqaba Biotechnical Industries, South Africa, for sequencing in order to cross-check the PCR results.

Table 2.4: Sequences of the primers used in the *kdr* detection (*Martinez-Torres *et al*, 1998; **Ranson *et al*, 2000).

Primer	Sequence
*Agd1	5′ ATA GAT TCC CCG ACC ATG 3′
*Agd2	5′ AGA CAA GGA TGA TGA ACC 3′
*Agd3	5′ AAT TTG CAT TAC TTA CGA CA 3′
*Agd4	5′ CTG TAG TGA TAG GAA ATT TA 3′
**Agd5	5′ TTT GCA TTA CTT ACG ACT G 3′

2.6 BLOOD MEAL ANALYSIS USING ENZYME LINKED IMMUNO-SORBENT ASSAY (ELISA)

Blood meal identification assays from wild caught female mosquitoes were carried out according to Beier *et al*., (1988). All the chemicals used are described in Appendix IIc. Head and thoraxes of blood fed female mosquitoes, preserved on silica gel were, separated from the abdomens using a blade and forceps. After dissecting each mosquito, the blade and forceps were washed three times using PBS-Tween 20 and once in 1M NaOH solution and wiped dry before dissecting the next mosquito. The abdomens were kept in a numbered sterile 1.5ml microfuge tubes (corresponding head and thorax were kept for *P. falciparum* detection). Each individual abdomen was ground in 50µl phosphate buffer saline (PBS) (0.01M Phosphate Buffered Saline and Polyoxyethylenesorbitan Monolaurate, Cat. No. 9005-64-5, Sigma Chemicals Co., St Louise, USA) using a plastic pestle. The homogenate that remained on the pestle was

rinsed with more grinding buffer until a final volume of 450µl. Fifty µl of the triturated abdomens were added to each of the 88 well of the micro titre plate (F96 MaxiSorb, Cat. No. 442402, Nalge-Nunc International, Denmark). Positive control (similarly human and bovine fed *An. arabiensis* mosquitoes) was added to well A1. Negative controls (male mosquitoes from the insectary) were added to the last four wells in the plate. The plate was incubated at room temperature for 3 hours and washed three times with washing buffer (PBS Tween-20). The host conjugate, goat anti-Human IgG (H + L) peroxidase labeled (Cat. No. 074-1006, Kirkegaard and Perry Laboratories, Maryland, USA) was diluted 2,000 times in 0.5% boiled casein (C-0376, Sigma Chemicals Co., St Louise, USA), and goat anti-Bovine IgG (H+L) phosphate labeled (Cat. No. 15-12-06, Kirkegaard and Perry Laboratories, Maryland, USA) was diluted 250 times using boiled casein. After 1 hour incubation, wells were washed three times with PBS-Tween 20 and 100µl of ABTS (2,2'-azino-di-3[ethyl-benzthiazoline sulfonate]) peroxidase substrate (1 component), (Cat. No. 50-66-01, Kirkegaard and Perry Laboratories, Maryland, USA), was added to each well. Absorbance values (optical densities) were read after 45 minutes on a microtitre plate reader. Positive samples were determined by the multiplying the mean plus three times the standard deviation of the negative controls.

2.7 DETECTION OF *PLASMODIUM FALCIPARUM S*POROZOITES IN *ANOPHELES* MOSQUITOES BY ELISA

The ELISA method used was that described by Wirtz *et al.* (1987). All the solutions are described in Appendix IIc. The head and thorax of an individual *Anopheles* female mosquito was separated from the abdomen using blade and forceps. The blade and

forceps were rinsed three times in PBS- Tween 20 (washing buffer), once in 1M NaOH solutions and wiped dry. The mosquito parts were then placed in a numbered sterile microfuge tube. Numbering was correlated with species identified and blood meal source. Abdomens were placed back into original microfuge tubes containing silica gel.

Each head and thorax was homogenized using a grinding pestle, in 1.5ml microfuge tube with 50µl grinding buffer (50µl blocking buffer, 0.5% Nonidet P-40, Igepal CA 630, Sigma Chemicals Co, St Louis, USA). Nonidet P-40, improves the sensitivity of the assay. The pestle was rinsed with 150µl grinding buffer onto the homogenate bringing the final volume of the homogenate to 200µl. The homogenates were stored frozen at –70^{o}C until used.

A volume of 40µl (1µg/µl) of Monoclonal antibody capture (Mab), Pf 2A10 – CDC O1 (Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA) designated to detect *Plasmodium falciparum*, was diluted in 5ml of 0.01M PBS solution and 50µl of the solution was then added to each well of a-96 well plate and the plate was covered with glad wrap and incubated overnight at 4^{o}C.

The capture Mab was removed and the wells filled with 200µl of blocking buffer (BB) and incubated for one hour at room temperature. The plate was emptied and 50µl of the mosquito homogenate was added to each well. Positive control (Pf 2+, cat. No. Pf-PC Washington DC, USA) was diluted in 50µl of blocking buffer and added to A1 well. Male mosquitoes obtained from the insectary were used as negative controls and added to H6 to H12 wells. These were removed after two-hour incubation at room temperature, and the wells were washed two times with washing buffer (PBS containing Tween 20).

The plates were incubated for 1 hour in darkness in 10µl (0.25mg/0.5ml) of stock peroxidase labeled Pf2A10-11(Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA). Wells were emptied and washed three times with PBS-Tween 20 washing buffer. Finally, 100µl of freshly prepared ABTS peroxides substrate (2,2'-azino-di-3[ethyl-benzthiazoline sulfonate]) were added to each well.

The results were read visually and scored 30 – 60 minutes after the substrate was added and then measured spectrophotometrically at 405 nm. Samples were considered positive if their color changed to green and had absorbance values of twice the average of the optical density values of the negative samples. All the positive samples were repeated to confirm the initial results.

2.8 SELECTION FOR DDT RESISTANCE

A laboratory colony of *An. arabiensis* (SENN) has been established at the Malaria Training Centre, Sennar city in central Sudan and stabilized in the insectary of the National Institute of Communicable Diseases (NICD), Johannesburg and a DDT resistant strain (sub-colony) has been selected from this colony and reared through 17 generations.

The newly emerged mosquitoes were separated by gender and kept for a minimum of 2 days and offered 10% sugar water. Replicate of 25 mosquitoes per experiment were exposed to 4% DDT for 1 hour following the WHO susceptibility test protocol (1998b). Knockdown was recorded after 1 hour and final mortality was recorded 24 hour post exposure and during this period survivors were offered 10% sugar solution. Male and

female survivors were reared under standard insectary conditions (25^0C, 80% relative humidity). This procedure has been repeated through 17 generations and mosquitoes from each generation were exposed to DDT 4%.

2.8.1 Biochemical Analysis

The biochemical assays were carried out using the method described by Penilla *et al*. (1998) and Hemingway *et al*. (1997). One to three day old fresh mosquitoes were individually homogenised in 200µl of distilled water in flat bottomed microtitre plate. The plate was kept on ice during the assays to maintain enzyme activities. Samples from the unselected and selected strains were assayed on the same 96 well microtitre plate in order to allow for direct comparison of optical density values between unselected and selected samples following adjustment for total protein content. Each sample duplicated on the same microtitre plate. Negative controls included all the reagents without the mosquito homogenate. All the buffers used in the following assays are described in Appendix IId.

2.8.1.1 Mixed function oxidase assay

This assay was used to measure the total amount of cytochrome P450 (monooxygenase) in each individual mosquito. Twenty µl of each mosquito homogenate was transferred to a new microtitre plate. Eighty µl of 0.0625M potassium phosphate buffer pH 7.2 was added to each replicate. This was followed by adding 200µl of a tetramethyl benzidene solution (0.01 g 3,3',5,' tetramethyl benzidene dissolved in 5ml of methanol prior to mixing with 15ml of 0.25 M sodium acetate buffer pH 5.0). Twenty five µl of 3%

hydrogen peroxide was added to each well and the plate was then inculpated at room temperature for 2 hours. The absorbance was read at 650 nm.

2.8.1.2 Altered Acetylcholinesterase assay (AchE)

The AChE in each mosquito homogenate was solubilized by adding 145µl of Triton phosphate buffer pH 7.8 to each well. Ten µl of DTNB solution (0.01M dithiobis 2-nitrobenzoic acid in 0.1M phosphate buffer pH 7.0) and 25µl of the substrate ASCHI (0.01M acetylthiocholine iodide) were added to one replicate of each mosquito homogenate. The latter solution was substituted for ASCHI containing 0.2% of the inhibitor propoxur (0.1M) for the second replicate. Two control wells contained all reagents and 25 µl dH_2o with and without propoxur were included. The kinetics of the enzyme reaction were monitored continuously at 405 nm for 5 minutes in a kinetic Multiskan RC microtitre plate reader. The percentage of acetylcholinesterase inhibition by propoxur was calculated for each individual by comparing the OD readings for uninhibited wells to inhibited wells.

2.8.1.3 Naphthyl acetate assays

The two substrates α and β naphthyl acetate were used in these assays to measure the esterase activity directly in each individual. In resistant mosquitoes the elevated esterases are generally active in both substrates. Twenty µl of each mosquito homogenate was transferred to a separate plate and 200µl of α-naphthyl acetate solution (100µl of 30 mM α-naphthyl acetate in acetone diluted in 10ml of 0.02M phosphate buffer pH 7.2) was added to one replicate and 200µl β-naphthyl acetate solution

(prepared as for α-naphthyl acetate) was added to the other replicate. The reaction was left for 30 minutes at room temperature. Fifty µl of Fast blue stain solution (22.2 mg Fast blue in 2.25ml distilled water, then 5.25ml sodium dodecyl sulphate in 0.1M phosphate buffer pH 7.0) was added to each well to stop the reaction. Two negative controls containing all the reagents barring homogenate were included. Enzyme activity was read at 570 nm as an end point reading.

2.8.1.4 Glutathione-S-transferase (GST) assay

Chlorodinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates were used in this assay to measure the GST activity for each mosquito. Ten µl of each mosquito homogenate was transferred to a separate plate and 200µl of GSH/CDNB working solution (10mM GSH in 0.1M phosphate buffer pH 6.5 and 63mM CDNB diluted in methanol) were added to each well. Enzyme activity rates were measured at 340 nm for 5 minutes.

2.8.1.5 Protein assay

This assay was used to measure the protein concentrations for each mosquito and they were then used in the calculation of absolute values for esterase and glutathione-S-tranferase data. Ten µl of each mosquito homogenate was transferred to a separate plate. Three hundred µl of Bio Rad protein solution, diluted in distilled water (1Bio Rad: 4 dH_2o), was added to each replicate. The plate was left for 5 minutes and OD was read at 570 nm.

2.9 Data analysis

Spectrophotometer (Labsystems, m ultiskan RC vl. 5-0) with Genesis software version 3.03 was used in measuring the optical densities of the enzymes involved in the metabolic resistance and results of blood meal and sporozoite ELISA. STATISTIX 7.0 Analytical Software was used in the biochemical results analysis. Sequences were analyzed using Lasergene software version 6, DNASTAR, Inc, Madison, WI.