MATERIALS AND METHODS

3. MATERIALS AND METHODS:

3.1 Selection of mosquito sampling districts:

Five different sampling districts were selected based on the dengue prevalence in northern part of West Bengal, namely, Alipurduar, Jalpaiguri, Darjeeling, Coochbehar and North Dinajpur. The mosquito samples collected from each sampling site were named as per site abbreviations. Altogether five different populations of *Aedes aegypti* were collected APD^{ae}, JPG^{ae}, DAR^{ae}, COB^{ae} and NDP^{ae} each representing five different districts namely Alipurduar, Jalpaiguri, Darjeeling, Coochbehar and North Dinajpur. The geographical coordinates and other relevant biotic and abiotic factors of the sampling districts are tabulated in Table 8.

Table 8: Details of the Sampling Districts

Districts	**Population abbreviated name**	**Geographical coordinates**	**Disease endemicity**	**Total infection in 2017 (DEN + CHIK)**	***Mosquito* Generation used in Experiments**
Alipurduar	APD^{ae}	26.69° N 89.47° E	Dengue, Malaria, JE	74	F1
Coochbehar	COB^{ae}	26.34° N 89.46° E	Dengue, Malaria, JE, Filariasis	217	F1
Jalpaiguri	JPG^{ae}	26.52° N 88.73° E	Dengue, Malaria, JE, AES	855	F1
Darjeeling	DAR^{ae}	26.71° N 88.43° E	Dengue, Malaria, JE, AES	1266	F1
North Dinajpur	NDP^{ae}	26.27° N 88.20° E	Dengue, Malaria, JE, AES	284	F1

*JE: Japanese Encephalitis, AES: Acute Encephalitis syndrome, F1: Fillial 1 generation

As abundant sample of *Aedes albopictus* could be collected so more than one population were sampled from some districts covering different blocks. Altogether eleven different populations of *Ae. albopictus* were collected namely APD[al], HAS[al], KMG[al] from Alipurduar district, JPG[al], NGK[al], NMZ[al] from Jalpaiguri district, SLG[al], NBU[al], KHR[al] from Darjeeling district, COB[al] from Coochbehar district and ISL[al] from North Dinajpur district. The demographic and disease prevalence details about the collected sites of *Ae. albopictus* have been provided in table 9.

Table 9: Epidemiological data of the study sites on vector borne diseases with special emphasis on dengue and chikungunya.

District	Site	Prevalent vector borne disease	Why considered at high risk	Population at risk	No. of Dengue cases			No. of Chikungunya cases		
					2015	2016	2017	2015	2016	2017
Darjeeling	SLG[al]	Dengue	High infection rate in with 1 death in 2017	1,44,607	65	165	1266	5	0	0
	NBU[al]	Dengue	High rate	5,405						
	KHR[al]	JE	High rate	-						
Alipurduar	APD[al]	Malaria, AES/JE	High rate	-	0	32	74	0	0	0
	HAS[al]	Dengue, Malaria, JE	High rate	2,52,776						
	KMG[al]	Malaria, JE	High rate	-						
Jalpaiguri	JPG[al]	Dengue	High rate	23,424	58	191	855	7	0	0
	NGK[al]	Dengue, JE	High rate	11,580						
	NMZ[al]	Dengue, Malaria	High rate	6,726						
Uttar Dinajpur	ISL[al]	Dengue	High rate	2,900	45	259	283	0	0	1
Coochbehar	COB[al]	Dengue	High rate	31,979	26	37	217	1	0	0

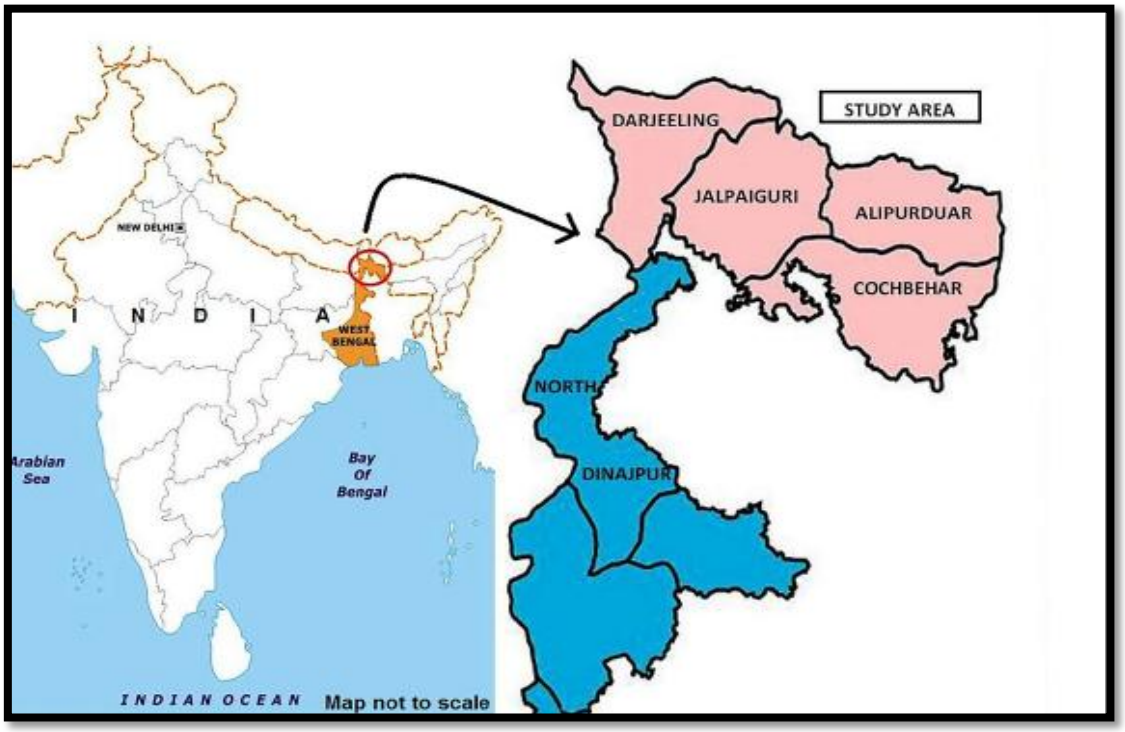
*JE: Japanese encephalitis and AES: Acute encephalitis syndrome

3.2 Collection of larva and adult mosquitoes:

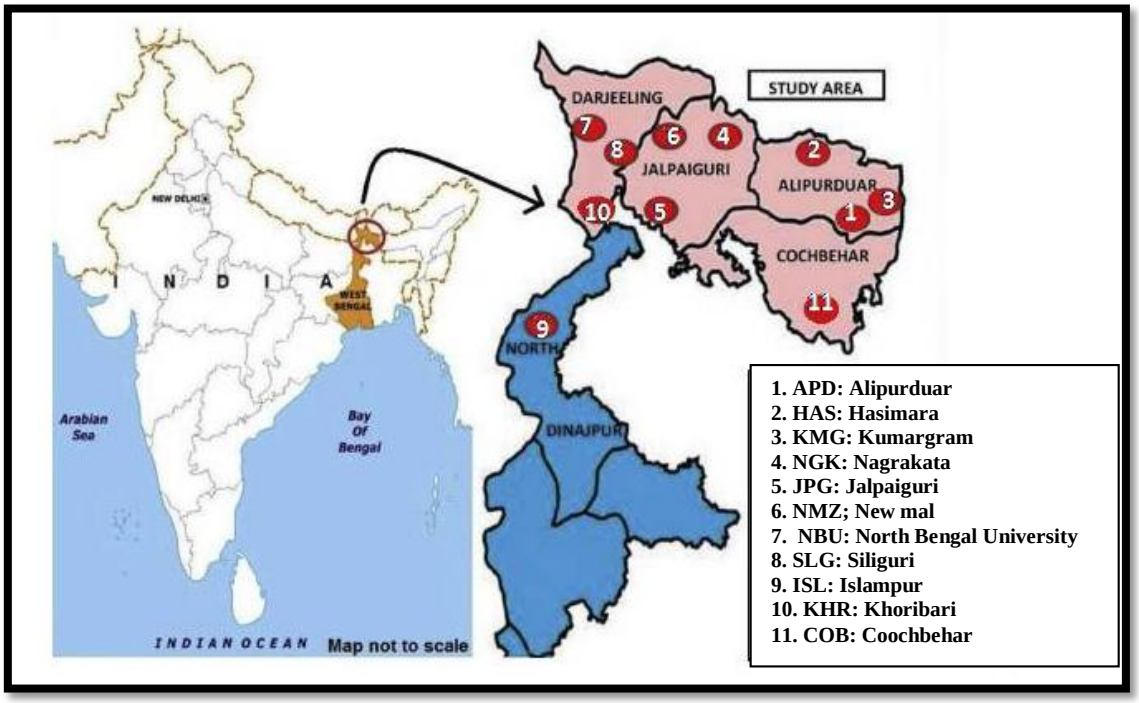
The selected sampling sites (Figure 12) were screened for the possible mosquito breeding environments, *i.e.* shady and cool environments without heavy sunlight. Such environments were critically screened for water containing larva and pupa of *Aedes* mosquitoes. Mosquito larvae or pupae were collected from different wild habitats such as artificial containers, discarded automobile tyres, earthen pots, water holding tanks, discarded buckets, aloevera plantations, tree holes, pots, discarded coconut shells, dry banana leaf containing water *etc*. After initial identification the larvae and pupae were collected and transferred to plastic containers. The samplings were done during March 2015 to August 2019. All the samplings were performed from private and governmental lands and prior permission was taken from the land owner in case of private land and head of the governmental institution in case of governmental land for mosquito collection.

3.3 Rearing of field caught population of mosquitoes:

In the laboratory, the larvae were confirmed as *Aedes* and then again upto subspecies level following standard larval identification keys and then cross checked with adult identification keys (Farajollahi *et al.*, 2013; Tyagi *et al.*, 2014). All the collected mosquitoes were identified either to be *Aedes aegypti* or *Aedes albopictus*. The rearing protocol for these mosquitoes are provided in the next section.



a. Aedes aegypti



b. Aedes albopictus

Figure 12: Sampling sites of *Aedes* mosquitoes distributed in five districts of northern part of West Bengal

Figure 13: Sampling performed for collection of *Aedes* mosquitoes from prospective breeding habitats.

3.3.1 Rearing of susceptible reference population:

The field collected *Aedes* larvae (F_0) were then reared at controlled temperature 25±2^{0}C and 70-80% relative humidity for successive generations. The larvae were reared to F_1 generation upto adults to ensure the homogeneity of the field collected populations. The emerged adults were cross checked with adult identification keys (Farajollhi and Price, 2013; Tyagi *et al.*, 2014). To setup a susceptible laboratory culture, mosquito samples were collected randomly from areas with no insecticide exposure possibilities. The mosquito colonies after collection were reared to F1 generation and were subsequently tested for insecticide susceptibility bioassays. The mosquito population reporting the lowest level of resistance (collected from the Medicinal garden of North Bengal University campus, Siliguri, India) was chosen to be reared for successive generations (Figure 14) without any exposure to insecticides. The collected larvae (F_0) were then subjected to rearing in the laboratory maintaining the same physical factors as mentioned above. The larvae were provided with ground fish feed powder as nutrition source for all the four larval instars. Once the larvae grew into pupae, they were shifted to beakers. Emerging adults were provided with 5% glucose soaked in cotton balls for two days. Then the adults were starved for one day and then provided with anesthetised albino rat (collected from animal rearing centre, North Bengal University campus, Siliguri, India) as a source of blood for the female. After two days of blood feeding, the set up was provided with blotting papers soaked in water containing beakers to serve as the egg laying apparatus for the female mosquitoes. The laid eggs (Figure 15a) were shifted to enamel trays filled with water. The whole setup was covered with mosquito net (Figure 15b). The presence of hatched larvae in the trays marked the onset of next generation. The whole cycle (Figure 14) was repeated for successive generations to be used as the laboratory reared control/ susceptible

population (SP) after at least ten generations. The F1 larvae and adults were used for bioassays and detoxifying enzyme activity studies.

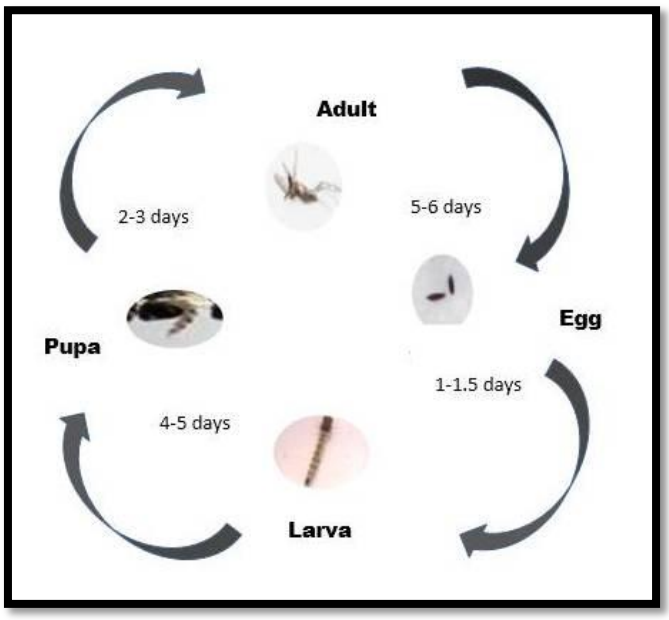


Figure 14: Life cycle of *Aedes* mosquito studied under laboratory condition

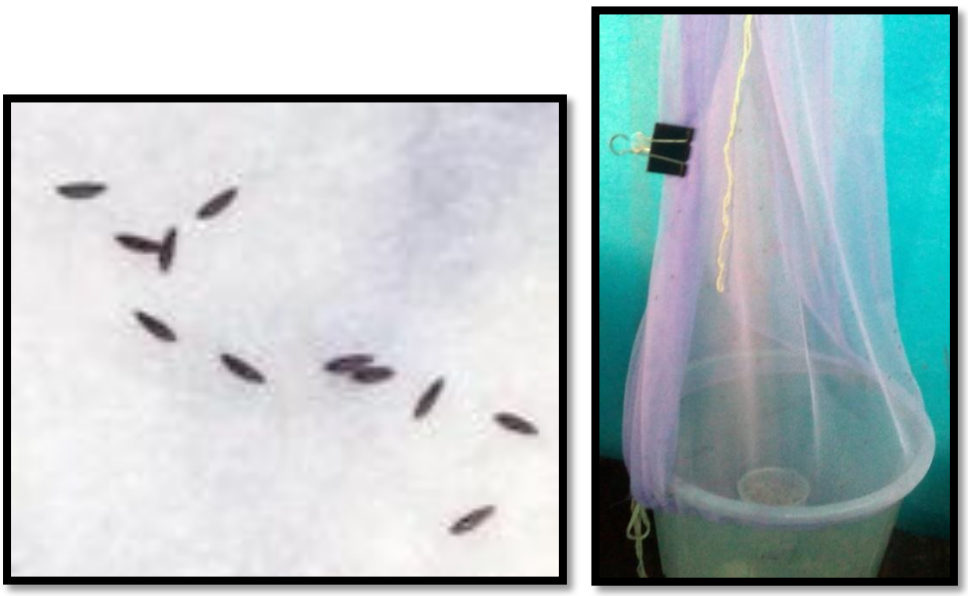


Figure 15: a. *Aedes* eggs laid in laboratory **b.** Laboratory adult mosquito chamber

3.4 Surveying of larvae:

All the sites were screened once in a two month interval for larval stages of mosquitoes belonging to genus *Aedes.* Both private as well as public sector lands/premises were investigated for the study. At least 50 house/premises were selected randomly and screened for presence of possible *Aedes* mosquitoes breeding habitats (both indoor and outdoor). Mosquito breeding habitats such as, Tyres, cemented tanks, artificial containers, thermocol boxes, plant axils, tree holes, refrigerator trays, discarded buckets *etc* were scanned thoroughly for the presence of larval stages. Larvae were collected through random strokes in the breeding habitat and were collected in plastic containers and brought to the laboratory. Entomological indices were determined in this study that helps to assess the risk posed by these mosquitoes in dengue infection throughout the area. Indices such as, Percentage positivity, *i.e.* percentage of total number of *Aedes* infested breeding habitat type, Larva density index, *i.e.* total number of larvae / total number of premises examined, mean no. of larvae per habitat per positive *Aedes* breeding habitat and the preferred habitat for each species were determined.

3.5 Insecticide source:

Single larvicide namely temephos was used in this study. Amongst the adulticide, an organophosphate: 5% Malathion; Synthetic pyrethroid: 0.05% Deltamethrin, 0.05% Lambda cyhalothrin and 0.75% Permethrin; an organochlorine: 4% DDT; a carbamate: 0.1% Propoxur were used. All the insecticides were purchased from Vector control unit, Universiti sains Malaysia (Penang, Malaysia, a WHO Collaborating Centre) as 156.25g/ml solution of temephos and insecticide impregnated papers of adulticides.

***3.6 Larval bioassay**:*

The susceptibility of *Ae. aegypti* and *Ae. albopictus* larvae against an organophosphate larvicide temephos was tested following the WHO guidelines. For larval bioassays, two different doses were selected: 1) 0. 020 mg/L, *i.e.* WHO diagnostic dose and 2) 0.0125 mg/L, *i.e.* National Vector Borne Disease Control Programme (prime governmental vector control organisation in India) recommended dose. Twenty-thirty early fourth instar or late third instar larvae of each *Aedes* population (field caught and laboratory reared susceptible population) were exposed to test vials containing both dosages of temephos in water (Figure 16a). The bioassays were set in triplicate along with a set of control (using equal volume of solvent only) under standard laboratory parameters. Mortality percentages were calculated after 24 hours exposure to temephos. When the larvae failed to evoke any response when stimulated/touched they were considered dead/ moribund (WHO, 2005).

Each population were also tested for the mortality percentages against six dosages lower than the above two for the determination of lethal concentrations LC_{50} and LC_{90} (Concentration at which 50% or 90% larvae are found dead) for each population. Around 20-25 larvae from each population (including susceptible) were exposed to different concentrations of temephos in a vial containing the doses (Marcombe *et al.*, 2014).

Against each concentration three to four replicates and 4-6 serial concentrations selected through random trial (0.0001 - 0.01 mg/L) in the range of the insecticide causing 10% - 90% mortality were set to determine LC_{50} and LC_{90} values. Two sets of control containing the pure solvent (*i.e.* ethanol) only in water were also run. Larval mortality were calculated after 24 hours of temephos exposure following the same criteria for

discrimination between dead and live larvae. If greater than 10% mortality was recorded in controls, the whole set was discarded and set again. Whenever mortality in controls were 0-10%, Abotts correction was applied. If after 24 hours, any larvae moulted into pupae, it was not considered for the calculation of mortality percentages.

***3.7 Adult bioassay**:*

Adult bioassays were also performed following standard WHO protocol (WHO, 2006). The method is similar to larval bioassay except for the fact in adult bioassays insecticide is exposed for 1 hour only. Around 2-3 days old non blood-fed adults, twenty-thirty in number, were introduced in the experimental tubes and exposed to insecticide impregnated papers with WHO recommended diagnostic dose of insecticide (5% malathion, 0.05% deltamethrin and 0.05% lambda cyhalothrin, 4% DDT, 0.1% propoxur and 0.75% permethrin) for 1 hour. After the stipulated time, the mosquitoes were transferred to another tube carrying cotton balls soaked in 10% glucose solution (Figure 16b). The whole set was maintained at laboratory conditions for 24 hours. Mortality percentages were recorded 24 hours post-exposure. For control, mosquitoes were placed in tubes containing papers impregnated with acetone (in case of OC and SP insecticides) and ethanol (for OP and CB insecticides). The whole set was performed along with three replicates. Mortality percentages were calculated as the mean of all set of insecticidal assays.

a.

b.

Figure 16: Set up for testing **a.** larval and **b**. adult insecticide susceptibility

3.8 Synergism test:

For the evaluation of the role of insecticide detoxifying enzymes conferring insecticide resistance, synergism tests were conducted for the field populations using enzyme inhibitors. Two types of inhibitors were used namely, 1. Piperonyl butoxide (PBO, 90%, Sigma from Sigma-Aldrich, Switzerland), a CYP450s inhibitor and 2. Triphenyl phosphate (TPP, 99%, from Sigma-Aldrich, Germany), a CCE inhibitor. In this test, the sub-lethal doses (doses which effectively inhibit the corresponding enzymes

without hampering the suvivality of the mosquitoes) of the synergists *i.e.*4% and 10% for PBO and TPP respectively were used. In case of larval synergism test, the protocol was quite similar to the larval insecticidal bioassays, except that the insecticide was mixed with synergist prior to the test and the larvae were exposed to this mixture for 24 hours. In case of adult synergism test, each population was exposed for one hour to the enzyme blocker prior to insecticide exposure. After PBO/TPP exposure the mosquito populations were exposed to insecticides for 1 hour. Insecticide susceptibility bioassays in WHO bioassays section (exposure to insecticide only) served as positive control for the synergistic assay while bioassays without insecticide were used as negative control.

3.9 Major insecticide detoxifying enzymes' activity:

Single adult non blood fed *Aedes* mosquitoes were homogenized in 100 µL of 0.1M sodium phosphate buffer (pH 7.2) with a teflon micro-pestle in a 1.5 mL centrifuge tube. The pestle was washed with another100 µL of 0.1M sodium phosphate buffer (pH 7.2) to make the whole solution 200 µL. The homogenate was then subjected to centrifugation at 12,000 rpm (revolutions per minute) for 15 minutes in a centrifuge (Sigma 3K30, Sigma United Kingdom). The supernatant to be used as enzyme source for detoxifying enzyme activity assays was collected and stored at -20^{0}C and was used freshly (within 3-4 days).

For every enzyme assay, a minimum of thirty mosquito individuals were used. The whole enzyme assays were run in two technical replicates. In this study, for each enzyme class, a single substrate (two for CCEs) was used for evaluation of the enzyme activity levels. Since, an enzyme group may have many substrates, the substrates used were chosen as per the substrates used in standard protocols (WHO, 1998).

3.9.1 Non-specific esterase (Carboxylesterase) assay:

The activity of Carboxylesterases (CCE) were assayed using α- and β- napthyl acetate as the substrate (α- napthyl acetate α- CCEs and β- napthyl acetate for β-CCEs) for hydrolysation by these enzyme group following the method of van Asperen, 1972 (van Asperen, 1972) with few modifications for use in 96 well microplates (WHO, 1998). Twenty (20) µl of the enzyme source was mixed with 200 µl of substrate working solution and incubated for 15 minutes. Fifty (50) µl of Fast blue B salt (staining agent) mixed with 5% SDS solution (reaction stopping agent) was then poured onto the wells. After 15 minutes, the absorbance was recorded at 540 nm using microplate reader (Biotek, model: ELx800, United States of America) (Figure 17a, b). Blanks were also set following the same method only the enzyme source was replaced with 0.1M sodium phosphate buffer.

Standard curves of α- and β- napthol were prepared for the estimation of CCE activity. Standard solutions of α- and β- napthol were prepared (0.1µM – 1 µM) and their absorbance were recorded at 540nm. A standard curve was prepared from the absorbance values plotted against the known concentrations. Unknown concentrations were then determined plotting the values of absorbance on the standard curves.

3.9.2 CYP450s assay:

The activity of CYP450 monoxygenases were also measured according to standard protocol estimating the approximate heme peroxidise activities (Brogdon *et al.*, 1998) using 3,3',5,5'-Tetramethyl benzidine (TMBZ) as a substrate and H_2O_2 as the peroxidising agent. 20µl of the enzyme was mixed with working solution of TMBZ in sodium acetate buffer. Then 25µl of 3% H_2O_2 solution was added to each well and the whole set was incubated for 2 hours (Figure 17c). Absorbance was then recorded at

630nm using the microplate reader. Blanks were set replacing the enzyme source with 0.1M sodium phosphate buffer. A standard curve for the heme peroxidase activity was prepared using different concentrations of cytochrome c (0.0025 nM to 0.0200 nM) for horse heart type VI (Sigma Aldrich). The total CYP450 was expressed as CYP450 equivalent units (EUs) in mg protein.

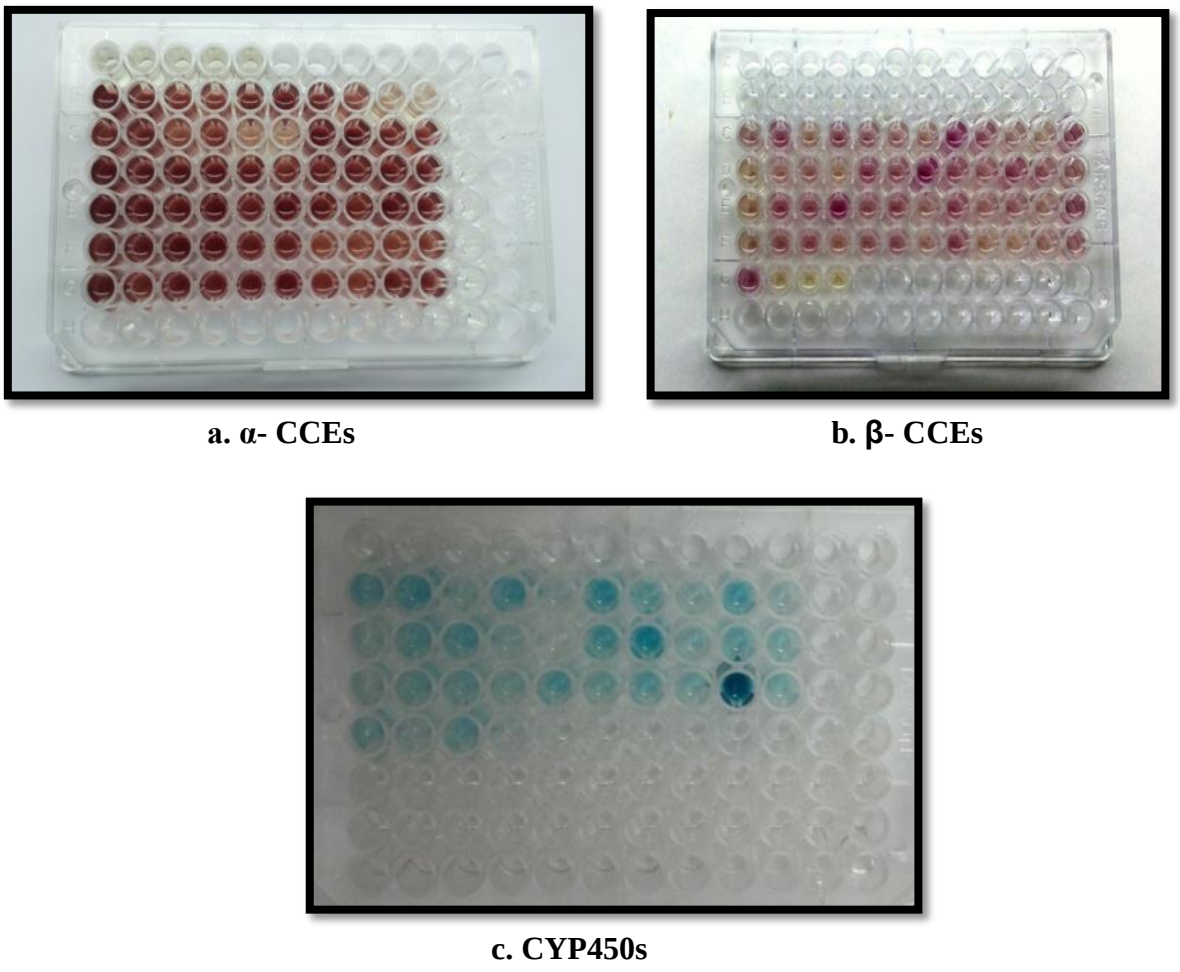
3.9.3 Glutathione S-transferase (GST) assay:

GST activity was assessed following the standard protocol using CDNB and GSH conjugate as the working solution (Habig *et al.,* 1974; WHO, 1998). To 10 µL of homogenate, 200 µL of CDNB-GSH working solution was added in a quartz cuvette. Blanks were set with 10 µL of distilled water mixed with with 200 µL of working solution. 2.7 ml of distilled water was added to the cuvette to make the final volume 2.91 ml. The cuvette absorbance was read at 340 nm continuously for 5 minutes. An extinction coefficient 9.6 $mM^{-1}cm^{-1}$ was used to calculate the change in absorbance per minute to rate of GSTs activity (µM mg protein-^{1} min^{-1}).

3.9.4 Total soluble protein content:

To negate any size differences amongst the mosquito individuals and for the precise estimation of enzyme activity, total protein of each individual was determined according to Lowry *et al.,* 1951 and the activities of the detoxifying enzymes were expressed as per mg protein. To 20 µl of the enzyme source 250 µl of freshly prepared alkaline solution {2% sodium carbonate in 0.1N sodium hydroxide (A) mixed with 0.5% copper sulphate in 1% potassium sodium tartarate (B) in 50:1 ratio, (v:v)} was added. After 10 mins of incubation, 50 µl of folin ciocalteau reagent was added. Absorbance was read after 30 minutes at 630 nm. Blanks were set using 0.1M sodium

phosphate buffer. Standard curve was prepared using different concentrations of bovine serum albumin.



a. α- CCEs **b. β- CCEs**

c. CYP450s

Figure 17: Microplates showing the end point of insecticide detoxifying enzyme's assay

3.10 Isolation of DNA and kdr genotyping:

Genomic DNA was extracted following the SDS extraction method (Barik *et al.*, 2013). DNA concentration was measured using a spectrophotometer at 260 nm. Stock solutions were prepared at a concentration of 25 ng/µl and used for Allele specific-PCR (AS-PCR) genotyping. The primers used and their annealing temperature is provided in Table 10. Each reaction was performed in a 25 µl volume consisting of 1.5 mM MgCl2, 1x PCR buffer (Promega, USA), 0.25 µM common primer, 0.125 µM each mutation specific primer, 200 µM

dNTP mixture (Promega, USA), 0.2 units Taq polymerase (Promega, USA) and 25 ng genomic DNA. The thermal cycling condition was set with an initial DNA denaturation step for two minutes at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at mentioned temperature (Table 10) at and extension at 30 sec at 72°C. PCR amplification products were loaded onto a 3% agarose gel and run for 1 hr at 100 V in TAE buffer. Since, the primers used had GC-rich tails of varying lengths, amplified products could be differentiated by size, 93 bp, 113 bp for F1534C mutation and 60 bp, 80 bp for V1016G (Yanola *et al.,* 2011; Stenhouse *et al.,* 2013; Aguirre-Obando *et al.,* 2017) and the results could be accordingly interpreted. Allele frequency calculations were done following the standard methods (Aguirre-Obando *et al.,* 2017).

Table 10: Details of primers used for kdr genotyping of F1534C and V1016G mutations

Species	Kdr mutation	Primer sequence	Annealing temperature	Product size (bp)	Reference
Ae. aegypti	F1534 (Reverse)	5'-TCTGCTCGTTGAAGTTGTCGAT-3'	60°C	--	Yanola *et al.*, 2011
	F1534F (Forward 1)	5'-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3'		93	
	F1534C (Forward2)	5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3'		113	
Ae. aegypti	V1016 (Forward)	5′-ACCGACAAATTGTTTCCC-3′	55°C	--	Stenhouse *et al.*, 2013
	1016V (Reverse1)	5′- GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA-3′		80	
	1016G (Reverse2)	5′-GCGGGCAGGGCGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-3'		60	
Ae. albopictus	F1534 (Reverse)	5'-TCTGCTCGTTGAAGTTGTCGAT-3'	60°C	--	Aguirre-Obando *et al*., 2017
	F1534F (Forward 1)	5'-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3'		93	
	F1534C (Forward 2)	5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3'		113	

3.11 Electrophoretic analysis of α- and β-esterases:

Native Polyacrylamide Gel Electrophoresis (PAGE) of different *Ae. aegypti* populations *i.e.* field collected as well as from laboratory reared control populations were carried out in 8% gels in tris-glycine (pH 8.3) buffer system. Field collected *Aedes* population as well as laboratory reared control mosquitoes were homogenized freshly in 100 µl of 0.1 M sodium phosphate buffer. To run the gels equal amounts of protein were loaded onto the gel in tris–glycine (pH 8.3) at 100 V for 5–6 h at 4°C.

To stain the gels for α –CCEs, standard staining protocol was followed with minor modifications (Steiner and Johnson 1973; Carvalho *et al.* 2003). The gels were first incubated in sodium phosphate buffer (0.1M, pH 7.2) for 15 mins in dark staining box. Then the gels were transferred to a solution containing 20 mg of α –Napthyl acetate in 5 ml of acetone mixed with 50 mL of sodium phosphate buffer (0.1M, pH 7.2). After 15 mins of incubation, the gels were provided with 5% solution of fast blue B salt for 30 minutes. Same procedure was followed for staining of β-CCEs replacing the substrate with β-Napthyl acetate. The gels were then preserved between transparent plastic sheets for future reference. Bands designated as isozymes of α-CCEs and β-CCEs were designated as Est I, II, III, IV, V based on mobility from anode to cathode and the Rf (Retardation factor) values were calculated.

3.11 Calculation and statistical analyses of the data:

In the bioassays, $LC_{50,}$ LC_{90} and LC_{99} were estimated at 95% confidence interval by putting log dose against probit in SPSS 16.0 software and the obtained linear regression coefficient (r^2) was used to assess the linearity of the whole data set. Double the extrapolated value of LC_{99} (2 X LC_{99}) was taken as the recommended discriminating dose/ diagnostic dose of the insecticide for that specific region/area. The population

with mortality percentages when > 98 is said to be susceptible, 80-97 is assessed as resistance not confirmed (= incipient resistance) and <80 as resistant (WHO, 2006). Resistance ratio 50 *i.e.* RR_{50}, which is an indirect measurement of insecticide resistance development was also determined as the LC_{50} of sampling site divided by the LC_{50} of the susceptible population. Calculation of the average values and standard errors and the comparisons of the mean of different data sets were performed using Graphpad Instat 3.05 at p=0.05.