CHAPTER 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS:

4.1. Mosquito sampling area:

Field populations of *Cx. quinquefasciatus* were collected from six different districts of northern region of West Bengal, India namely: Alipurduar, Coochbehar, Darjeeling, Jalpaiguri, Malda and Uttar Dinajpur owing to the prevalence of vector-borne diseases in these districts (Figure 11).

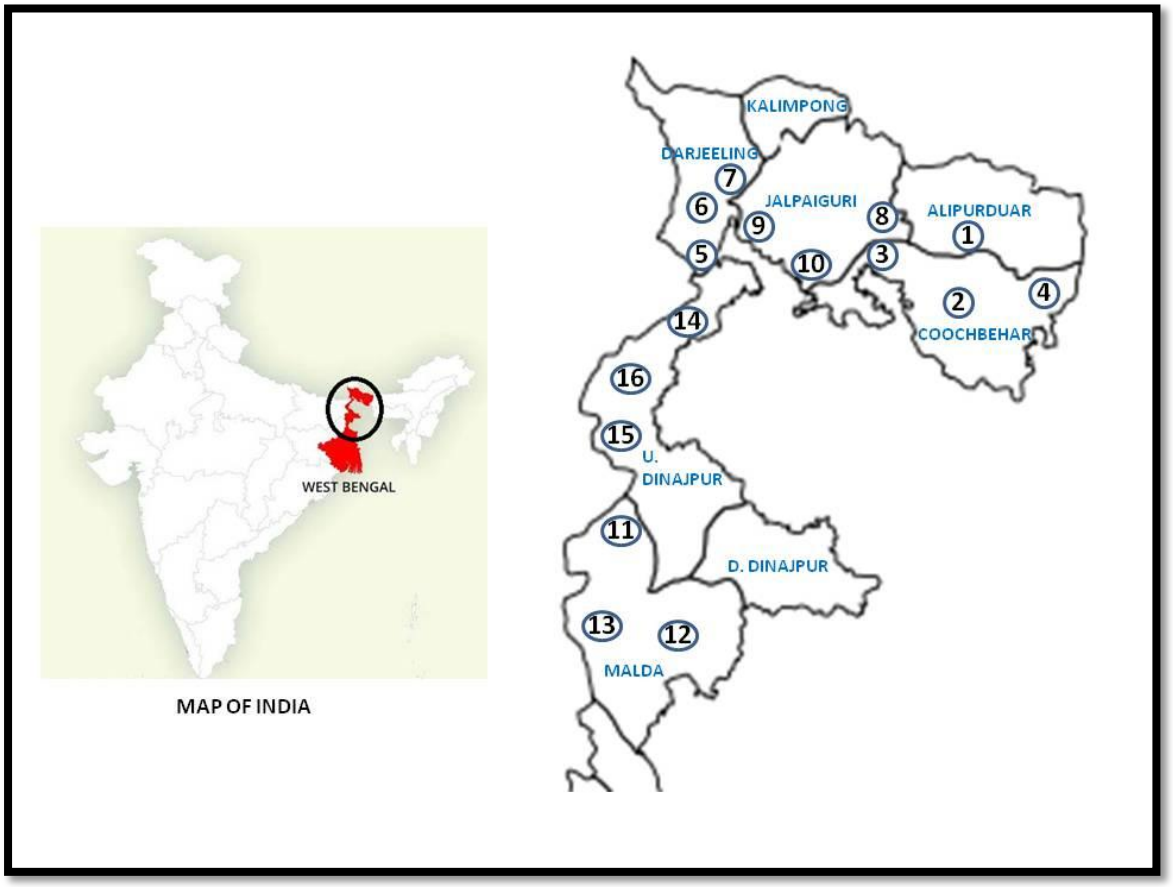

Figure 11: Different sampling sites of *Cx. quinquefasciatus* from six districts of northern West Bengal, India. 1-Alipurduar (APD); 2-Coochbehar Town (COB); 3-Mekhliganj (MEK); 4-Tufanganj (TFG); 5-Bidhannagar (BDN); 6-Shivmandir (SHM); 7-Siliguri (SLG); 8-Dhupguri (DPG); 9-Fulbari (FLB); 10-Jalpaiguri Town (JPT); 11-Harishchandrapur (HCP); 12-Malda Town (MLT); 13-Samsi (SAM); 14-Chopra (CPR); 15-Dalkhola (DLK); 16-Islampur (ISL)

Out of the six districts surveyed for *Culex* mosquitoes, Coochbehar and Malda are endemic to lymphatic filariasis apart from other vector-borne diseases. Details of the sampling districts along with other factors relevant to the present study are given in Table 7.

Table 7: Details of the sampling districts of *Cx. quinquefasciatus* from West Bengal.

Districts	Geographical coordinates	Disease endemicity	Sampling sites	Abbreviation used	Generation used
Alipurduar	26.40˚N - 26.83˚N; 89.0˚E - 89.9˚E	Dengue, Malaria, JE	Alipurduar	APD	F1
Coochbehar	25.57˚N - 26.22˚N; 88.44˚E - 89.29˚E	Dengue, Malaria, JE, Filariasis	Coochbehar Town	COB	F1
			Mekhliganj	MEK	F1
			Tufanganj	TFG	F1
Darjeeling	26.31˚N - 27.13˚N; 87.59˚E - 88.56˚E	Dengue, Malaria, JE, AES	Bidhannagar	BDN	F1
			Shivmandir	SHM	F1
			Siliguri	SLG	F1
Jalpaiguri	26.16˚N - 27˚N; 88.4˚E - 89.53˚E	Dengue, Malaria, JE, AES	Dhupguri	DPG	F1
			Fulbari	FLB	F1
			Jalpaiguri Town	JPT	F1
Malda	24.4˚N - 25.32˚N; 87.45˚E - 88.28˚E	Dengue, JE, Malaria, Filariasis	Harishchandrapur	HCP	F1
			Malda Town	MLT	F1
			Samsi	SAM	F1
Uttar Dinajpur	25.11˚N - 26.49˚N; 87.49˚E - 90˚E	Dengue, Malaria, JE, AES	Chopra	CPR	F1
			Dalkhola	DLK	F1
			Islampur	ISL	F1

*JE: Japanese Encephalitis, AES: Acute Encephalitis syndrome, F1: First filial generation

Except from Alipurduar district, more than one field population of *Cx. quinquefasciatus* were collected from three sites including different blocks and areas

from each district. A total of sixteen different populations of *Cx. quinquefasciatus* were collected from six districts of Northern West Bengal. The mosquito populations collected from a particular site was named after the name of collection area in abbreviated form. The sampling sites include: APD from Alipurduar, COB, MEK, TFG from Coochbehar, BDN, SHM, SLG from Darjeeling, FLB, DPG, JPT from Jalpaiguri, HCP, MLT, SAM from Malda and CPR, DLK, ISL from Uttar Dinajpur.

4.2. Mosquito collection:

Larvae and pupae of *Cx. quinquefasciatus* were collected from sixteen densely populated areas from six districts of Northern West Bengal, India. The sampling sites were surveyed for all possible mosquito breeding habitats with favourable environment and shady areas without direct sunlight. Sampling was conducted from February 2017 - March 2020 from various breeding habitats of *Cx. quinquefasciatus* such as drains, stagnant water, pools, plastic containers, discarded buckets, earthen pots, sewers and cemented channels (Figure 12). A 500 ml plastic beaker was used for the purpose and 8-10 dips were made at a particular sampling site. After a first stroke, initial identification of larvae and pupae collected were done and then transferred to plastic containers and brought to the laboratory. Average larval and pupal density of *Cx. quinquefasciatus* from each sampling sites were calculated. Prior permission from the land owner was taken whenever sampling was performed in private land and also from the Officer-in-charge when sampling in government areas.

Figure 12: Collection of *Cx. quinquefasciatus* larvae from its habitat.

4.3. Rearing of field caught mosquito populations:

The field collected mosquito larvae and pupae were identified to species level following standard mosquito identification keys (Tyagi *et al.*, 2015) in the laboratory and those identified as *Cx. quinquefasciatus* were reared to F1 generation under controlled laboratory conditions. Different bioassays and biochemical tests were performed with the F1 generation in order to maintain homogeneity of the population.

4.4. Rearing of susceptible reference population:

For the laboratory rearing of susceptible mosquito population to be used as a control reference in experiments, *Culex* mosquito populations were surveyed and collected from different areas in and around the University campus with no or least possibility of insecticide exposure. The field collected *Culex* larvae after identification as *Cx. quinquefasciatus* (Tyagi *et al.*, 2015) were kept in rearing cage in the laboratory under controlled temperature (25 ± 2^0C) and relative humidity (70-80%) and was referred to as F0 generation (Figure 13A). The mosquito culture was reared to F1 generation and was then subjected to insecticide susceptibility bioassays. The mosquito population that showed highest susceptibility status as compared to other populations were further reared in the laboratory up to the 10^{th} generation (F10) without the interference and exposure of insecticides.

The F0 larvae were kept in 1000 ml glass beakers and ground fish feed powder was provided for the mosquito larvae to feed upon. After pupation of the fourth instar larvae, the pupae were separately kept in another glass beaker in order to avoid overcrowding. Five percent sucrose solution soaked in cotton balls was provided as sugar source for the newly emerged adult mosquitoes. The emerged adults were then cross checked with adult identification keys for authenticity of the work. After two days of sucrose feeding, the adults were starved on the third day to make them ready for blood meal to be provided on the fourth day of emergence. Clean, trimmed and anesthetised albino rat was kept in the mosquito rearing cage for blood feeding by the female mosquitoes. Albino rat was collected from the Animal Rearing Centre, Department of Zoology, University of North Bengal. Along with the blood meal source, an egg laying apparatus for adult females was also kept inside the rearing set up.

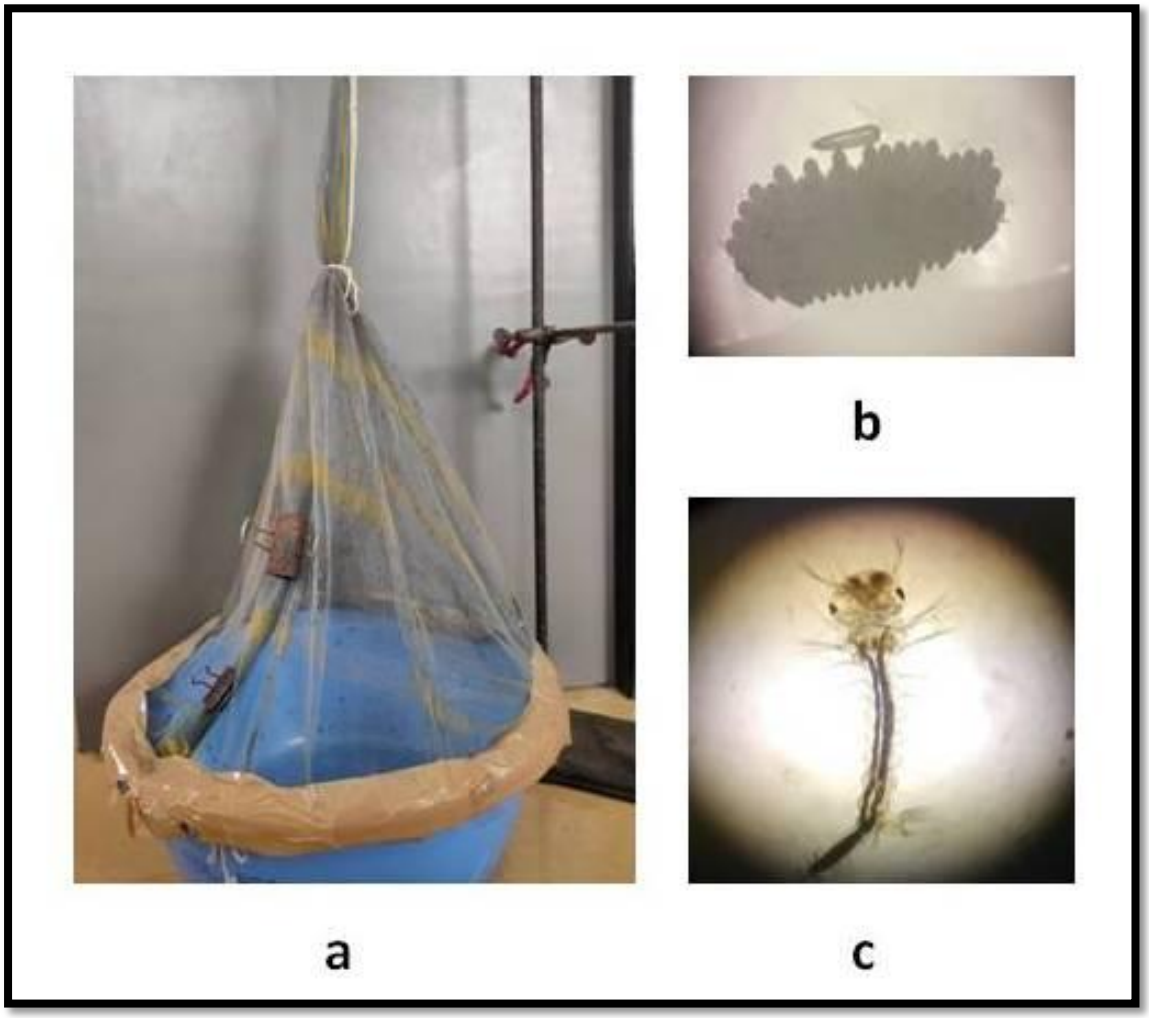

Figure 13A: Laboratory culture setup for rearing of *Cx. quinquefascaitus* (a); Egg rafts obtained from culture (b); Larva from the laboratory culture (c).

Dried hay was soaked in water and boiled till the colour of water turned brownish and turbid. The water was then allowed to cool at room temperature and then placed in glass beakers inside the rearing cage as the egg laying apparatus. The egg laying apparatus having egg rafts were taken out of the rearing set up and the egg rafts placed in enamel trays which were filled with water (Figure 13 B). Hatching of first instar larvae from the egg rafts marked the beginning of the next filial generation (Figure 14).

Figure 13B: Shifting of egg rafts from the egg laying apparatus to enamel trays for hatching and subsequent growth of larvae in the laboratory mosquito rearing.

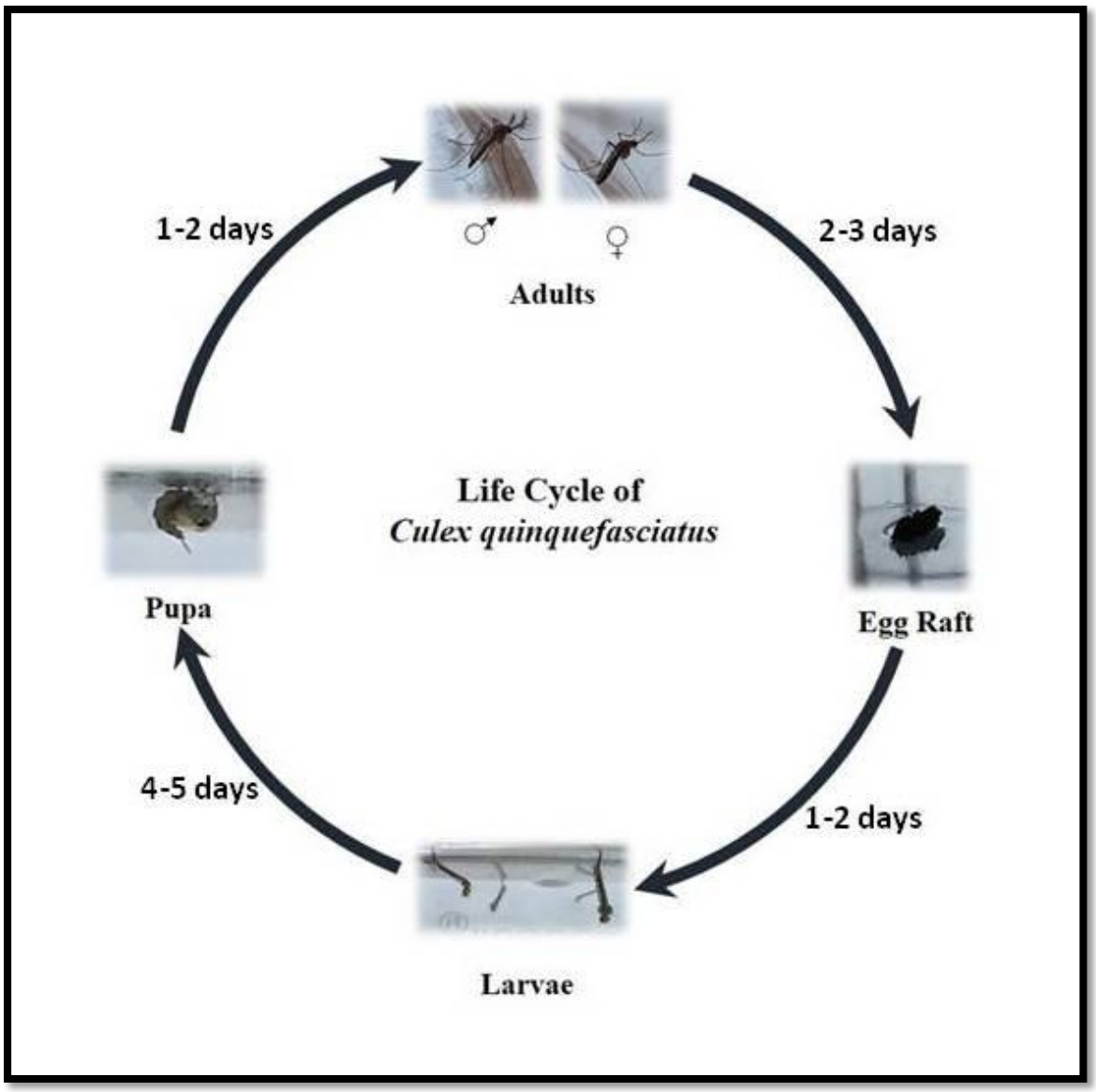

Figure 14: Life cycle of laboratory reared *Cx. quinquefasciatus* under controlled room temperature and relative humidity.

The entire process was repeated for several generations till F10 generation of susceptible population of *Cx. quinquefasciatus* was obtained. The laboratory reared F10 generation were considered as laboratory reared susceptible population (SP) and used as control for insecticide susceptibility bioassays and biochemical studies involving the metabolic enzymes.

4.5. Survey of mosquito larvae:

Mosquito larvae belonging to genus *Culex* were collected from mosquito breeding habitats like drains, cemented tanks, channels, artificial containers, muddy pools, stagnant water bodies, puddles *etc.* The sampling sites were screened once in every two months for the presence of *Culex* larvae. Residential areas with both proper and poor drainage system were visited for the purpose of mosquito sampling. A minimum of 50 houses or premises were included for random collection of mosquito larvae in a particular sampling site. Drains along highways and roads were also screened for the presence of *Culex* larvae. Larvae collection was done using a 500 ml plastic beaker and 8-10 random dips were made at a particular sampling site. Total larvae were kept in plastic containers and brought to the laboratory for identification. Average larval density index (total number of larvae collected / total number of random dips made at a sampling site) was calculated for each sampling site. Preferred breeding habitat of *Culex* mosquitoes and co-existence of other insect vectors and insect species were also studied in the laboratory from the sample collected.

4.6. Insecticide susceptibility assays (Bioassays):

4.6.1. Insecticide source:

Larval bioassay for insecticide susceptibility test was performed against one commonly used larvicide *i.e.*, temephos which is an organophosphate insecticide. Six different adulticides belonging to four different classes of insecticides for adult bioassay were used:

i) Malathion (5%) an organophosphate,

ii) Deltamethrin (0.05%), Lambdacyhalothrin (0.05%) and Permethrin (0.75%) – synthetic pyrethroids,

iii) Propoxur (0.1%) a carbamate and

iv) DDT (4%) an organochlorine.

The insecticides mentioned above were purchased from Vector control unit, Universiti Sains Malaysia (a WHO Collaborating Centre). Temephos was purchased as 156.25 g/ml solution while the adulticides were purchased as insecticide impregnated papers.

4.6.2. Larval bioassay:

Larval susceptibility to temephos in *Cx. quinquefasciatus* from different districts of northern West Bengal were tested in the laboratory following WHO guidelines (WHO 2005). WHO recommended dose of 0.02 ppm and National Vector Borne Disease Control Programme recommended dose of 0.0125 ppm were prepared in test vials having 200 ml water for assessing the susceptibility status of *Cx. quinquefasciatus* larvae collected from different districts. Thirty late third – early fourth instar larvae of *Cx. quinquefasciatus* from each sampling site were exposed to two different concentrations of temephos in the test vials (Figure 15a). The experiment was performed in triplicate to avoid any handling error and a control was set up using the laboratory reared susceptible population with the exposure to ethanol in water only. After 24 hours of temephos exposure, mortality percentage of larvae was calculated. The larvae were considered dead or moribund if they failed to respond when stimulated or touched by a fine brush (WHO, 2005).

The larval population of *Cx. quinquefasciatus* were also tested against other different dosages of temephos in water for determination of lethal concentration LC_{50} (concentration at which 50% larvae are dead) and LC_{90} (concentration at which 90% larvae are dead) values of a particular population. For determination of LC_{50} and LC_{90} values of each population, six dosages lower than the above mentioned dosages of

temephos were prepared. To test the mortality percentage of mosquito larvae, 25-30 late third instar – early fourth instar larvae from each field collected population and laboratory reared susceptible population were kept in test vials containing six different concentrations of temephos. The experiment was repeated thrice and a set of control was run where mosquito larvae were exposed to ethanol (pure solvent) in water only.

Figure 15: Setup for insecticide susceptibility assays. a. Larval bioassay; b. Adult bioassay.

After 24 hours of temephos exposure, larval mortality was calculated. During the exposure period if any larva pupated, the pupa was not included in the mortality calculation. In the control set up, if larval mortality was less than 5% then Abbott's correction was not applied.

4.6.3. Adult bioassay:

The mosquito adult bioassay tests were performed following standard WHO protocol (WHO, 2006; WHO, 2016b). Twenty five to thirty, 2-3 days old adult non blood-fed mosquitoes from each field population and also from SP were exposed for an hour to particular insecticide-treated paper placed inside bioassay tube (Figure 15b). Insecticide dose for adult exposure was used following the WHO recommended dose *i.e.*, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.75% permethrin, 5% malathion,

0.1% propoxur and 4% DDT. After 1 hour of insecticide exposure the adults were shifted to a retention tube and cotton balls soaked with 10% sucrose solution was provided as food source. Mortality percentage was calculated after 24 hours of post insecticide exposure period in the retention tube. A control was set up where mosquitoes were exposed to acetone-impregnated filter papers (control for synthetic pyrethroid and organochlorines) and ethanol–impregnated filter papers (control for organophosphate and carbamate insecticides). Three replicates were run for each experiments and the experimental set up was left undisturbed at laboratory condition. Mean value of three experiments performed for each insecticide was taken as mortality percentage of a mosquito population against that insecticide.

For the calculation of knockdown time (KDT) of the synthetic pyrethroids and DDT in adult mosquitoes, knocked down mosquitoes (paralysed and unable to retain flight) were calculated after every 10 minutes during the 1 hour exposure to insecticides.

4.7. Synergist assays:

The synergist assays were conducted using two most commonly used synergists Piperonyl butoxide (PBO, 90% from Sigma-Aldrich, Switzerland) – cytochrome P_{450}s (CYP_{450}s) inhibitor and Triphenyl phosphate (TPP, 99% from Sigma-Aldrich, Germany) – carboxylesterases (CCEs) inhibitor. The test was conducted in order to study the effectiveness of synergists in increasing the mortality rate of field collected adult mosquitoes against insecticides by affecting the detoxifying enzymes. Synergists were used in their sub-lethal dose *i.e.,* 4% PBO and 10% TPP which does not harm the mosquito's survival rate but inhibits detoxifying enzymes in conferring resistance to insecticides. Thirty non-blood fed adults were exposed to synergist-impregnated paper for an hour in bioassay tubes and then were exposed to insecticide-impregnated paper

for an hour. After an hour of insecticide exposure the mosquitoes were then shifted to retention tube like in the adult bioassay test and mortality counted after 24 hours post exposure. The insecticide bioassay tests were taken as positive control and the control used in adult bioassay test as negative control.

4.8. Major insecticide detoxifying enzymes' activity:

Thirty non blood fed adult *Cx. quinquefasciatus* mosquitoes from each sampling site were taken and homogenized individually in a 1.5 ml centrifuge tube. Each mosquito was homogenized in 100 µl 0.02 M sodium phosphate buffer (pH 7.2) with a teflon micro pestle and then the pestle was washed with additional 100 µl 0.02 M sodium phosphate buffer (pH 7.2) to make a total solution of 200 µl homogenate. After homogenisation of individual mosquitoes, the homogenate was centrifuged at 5,000 rpm (rotation per minute) for 10 minutes in a high speed refrigerated centrifuge (SIGMA 3K.30). The supernatent was collected in a fresh and autoclaved 1.5 ml centrifuge tube and stored at -20˚C for further use in detoxifying enzyme quantification assays.

The enzyme activity assays were conducted using a single substrate each for cytochrome P_{450} ($CYP_{450}s$) monooxygenases and glutathione S-transferases (GSTs) and two substrates for carboxylesterases (α-CCEs and β-CCEs). Two technical replicates were run for all of the enzyme quantification assays.

4.8.1. Total soluble protein estimation:

Total protein content of individual mosquitoes was calculated following the protocol of Lowry *et al.*, 1951 from the homogenate / enzyme source. For protein estimation alkaline solution was prepared freshly by mixing solution A (2% sodium carbonate and 0.1 N sodium hydroxide) and solution B (0.5% copper sulphate and 1%

sodium potassium tartarate) in a 50:1 ratio. In a microplate with 96 wells, 2 µl of the homogenate was added for individual mosquito followed by 250 µl of the alkaline solution. The mixture was left for incubation at room temperature for 10 mins and then 50 µl of Folin Ciocalteau reagent was added as the staining solution (Figure 16). After 30 mins of incubation, absorbance was taken at 630 nm (Spectrostar Nano, BMG Labtech). Two blanks were prepared with distilled water in place of the mosquito homogenate. Bovine serum albumin in different concentrations was taken as a standard in the assay for preparation of a standard curve.

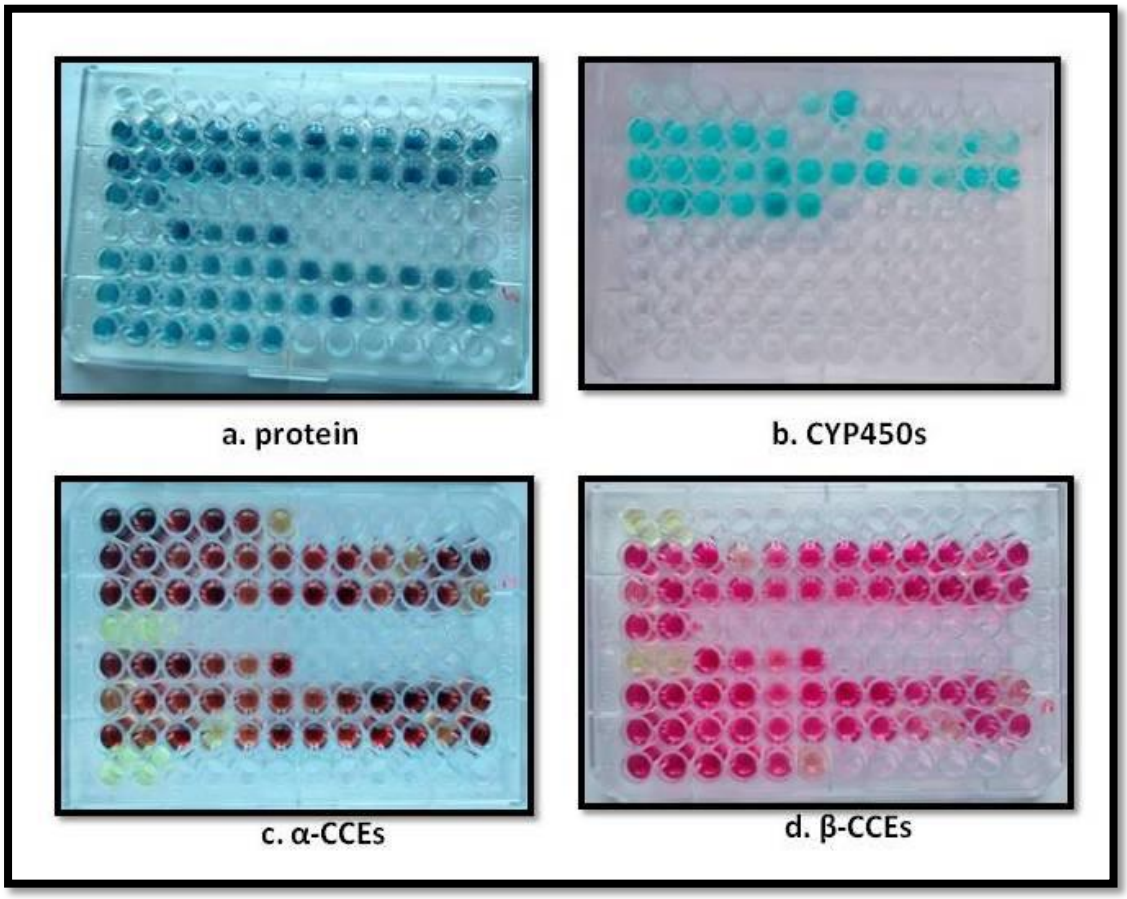

Figure 16: Enzyme assays performed in 96 wells microplate.

Total protein content of individual mosquitoes from each sampling site was calculated in order to cancel out the size differences of each mosquito and to get a precise estimation of enzyme activity or quantification. Therefore, the enzyme activities were expressed as per mg protein.

4.8.2. Non-specific esterase assay:

Non-specific esterase or carboxylesterases (CCEs) activity was assayed following the protocol of Van Asperen (Van Asperen, 1962) with a slight modification of using 96 wells microplate. Two substrates – α-napthyl acetate and β-napthyl acetate were used for measuring the activity of α-CCEs and β-CCEs respectively. Substrate solution was prepared in acetone and 0.1 M sodium phosphate buffer. Twenty (20) µl of the homogenate was mixed with 200 µl of substrate solution. The mixture was incubated at room temperature for 15 mins and 50 µl staining solution (Fast Blue B salt in 5% SDS solution) was added. SDS in the staining solution acts as a reaction stopping agent. After 30 mins of incubation (Figure 16), absorbance was taken at 540 nm in microplate reader (Spectrostar Nano, BMG Labtech). For blank preparation, distilled water was used instead of the homogenate.

Standard solution was prepared using α- and β- napthol in varying concentration (0.1 µM – 1 µM) for obtaining a standard curve with an absorbance at 540 nm. The unknown concentrations of esterase enzyme in *Cx. quinquefasciatus* mosquitoes were determined by plotting the optical density data on the standard curves.

4.8.3. CYP_{450}s monooxygenases estimation

Standard protocol of Brogdon *et al*., 1997 that estimated heme peroxidase activities was followed for the estimation of CYP_{450} monooxygenases. 3,3',5,5'-Tetramethyl benzidine (TMBZ) in sodium acetate buffer was used as the substrate working solution and 200 µl of the working solution was mixed with 20 µl of the homogenate. Thereafter, 25 µl of 3% H_2O_2 was added as the staining solution and the mixture was incubated for 2 hours (Figure 16). Absorbance was taken at 630 nm in microplate reader. Different concentrations (0.0025 nM – 0.0200 nM) of cytochrome C horse heart type VI (Sigma Aldrich) was used for the preparation of standard curve to estimate the total CYP_{450} concentration expressed as equivalent units (EUs) per mg protein of individual mosquitoes. Blanks were prepared with distilled water instead of using the mosquito homogenate.

4.8.4. Glutathione S-transferases (GSTs) estimation:

Estimation of GSTs activity was performed following the standard protocols (Habig *et al*., 1974; WHO, 1998). CDNB-GSH conjugate was used as the substrate working solution. Ten (10) µl of the homogenate was mixed with 200 µl of the working solution in a quartz cuvette. Then 2.7 ml of distilled water was added in order to make the total volume of 2.91 ml. Absorbance of cuvette was recorded at 340 nm for 5 mins at an interval of every 1 min. Change in absorbance per minute and rate of GSTs activity (µM mg $protein^{-1}$ min^{-1}) was calculated using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. For preparation of blanks, 10 µl of distilled water was used in place of the mosquito homogenate.

4.9. Analysis of α-CCEs and β-CCEs through Electrophoresis:

Native PAGE (Polyacrylamide Gel Electrophoresis) of both field collected and laboratory reared populations of *Cx. quinquefasciatus* was carried on using equal amount of protein in tris-glycine (pH 8.3) buffer at 120V for 4-5 hours at 4˚C. 8% polyacrylamide gel was prepared to visualize the pattern of elevated esterase isozymes present in different field collected and laboratory reared populations of *Cx. quinquefasciatus*. Individual mosquitoes were homogenized in 100 µl 0.02 M sodium phosphate buffer and then centrifuged at 5,000 rpm for 5 minutes. The homogenate was taken as the protein source and equal amount was loaded in each lane of the polyacrylamide gels.

The gels were thereafter stained following the staining protocol of Carvalho *et al*., 2003. For staining, the gels were first incubated in a staining box having 0.02 M sodium phosphate buffer (pH 7.2) for 15 mins. For analysis of α-CCEs isozymes, the gels were transferred to another staining box containing 20 mg α-napthyl acetate in 5 ml acetone and 50 ml of 0.02 M sodium phosphate buffer (pH 7.2). For analysing the isozymes of β-CCEs, β-napthyl acetate was used instead of α-napthyl acetate. The gels were incubated for 15 mins after which the working solution was drained off and 5% Fast Blue B salt (FBBS) solution was added for staining. The relative mobility (Rm) of various bands designated as isozymes of α-CCEs and β-CCEs were calculated based on mobility of esterases from anode to cathode using the formula: individual band position divided by dye font. The bands on polyacrylamide gels were designated as α-EST I, II, β-EST I, II and so on based on the calculated Rm values.

4.10. DNA extraction:

Genomic DNA of 10 adult mosquitoes from each field collected mosquito population that were alive even after 24 hours post exposure to synthetic pyrethroids and DDT was extracted following the High Salt protocol (Barik *et al.,* 2013) with minor modifications. Individual mosquito was homogenized in a 1.5 ml micro-centrifuge tube using digestion buffer. Twenty (20) µl proteinase K was added and the samples incubated at 55 - 60˚C in a water bath for at least 2 hours. The sample was centrifuged at 14000 rpm for 15 minutes after addition of chloroform and sodium chloride solution. The supernatant was transferred to a new micro-centrifuge tube, chilled 70% ethanol added and centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and pellet was suspended in autoclaved distilled water and stored at –20˚C as stock solution (25-30 ng/µl) for further use. DNA concentration was measured using a spectrophotometer at 260 nm.

4.11. Detection of kdr mutation:

Allele-specific PCR (AS-PCR) reactions were performed using the extracted genomic DNA individually to detect the presence of two kdr mutations at the sodium channel gene *i.e.,* L1014F and L1014S, with minor modifications in the protocol of Martinez-Torres *et al.,* (1999) and Sarkar *et al.,* (2009). Five different primers were used for the experiment namely: Cgd1, Cgd2, Cgd3, Cgd4 and Cgd5 (Table 8). Four PCR reactions were run in parallel. Cgd1 and Cgd2 primers were combined in the first reaction for the amplification of kdr region in the sodium channel, Cgd2 and Cgd3 in the second for detection of L1014F mutation, Cgd2 and Cgd4 in third reaction analysing the wild L1014L genotype and Cgd2 and Cgd5 primers combined in the fourth reaction for detection of L1014S mutation. Each reaction was performed in 25 µl volume having 25-

30 ng genomic DNA, 0.25 μM primer, 1.5 mM $MgCl_2$, 0.2 units Taq polymerase (Promega, USA. Cat no. M7401), 200 μM dNTP mixture (Promega, USA. Cat no. U1511) and 1x PCR buffer (Promega, USA. Cat no. D2301). PCR conditions were an initial denaturation at 95˚C for 15 min, followed by 30 cycles at 94˚C for 45 seconds, 49˚C for 45 secs, 72˚C for 45 secs, and a final extension of 10 mins at 72˚C. The amplified fragments were loaded on 3% agarose gel, run in TAE buffer at 90-100 V for an hour and analysed under UV light with ethidium bromide as stain. The amplified products were differentiated based on differences in size because of primers with varying lengths of GC-rich tails. Cgd1 and Cgd2 amplified bands at 540 bp while Cgd3-Cgd5 amplified at 380 bp (Sarkar *et al.*, 2009). To confirm the presence of mutations, two Cgd1 and Cgd2 amplified PCR products from each population were sequenced (Bioserve Biotechnologies (I) Pvt. Ltd.).

Table 8: Details of primers used in the knockdown resistance (kdr) mutation genotyping of L1014F and L1014S mutations in *Cx. quinquefasciatus* from northern districts of West Bengal

Sl no.	Primer name	kdr mutation	Primer sequence	Annealing temperature	Product size (bp)
1	Cgd1 (Forward)	kdr mutation site	5- GTG GAA CTT CAC CGA CTT C -3	49˚C	540
2	Cgd2 (Reverse)		5- GCA AGG CTA AGA AAA GGT TAA G -3		540
3	Cgd3 (Forward)	L1014F	5- CCA CCG TAG TGA TAG GAA ATT TA -3		380
4	Cgd4 (Forward)	L1014L	5- CCA CCG TAG TGA TAG GAA ATT TT -3		380
5	Cgd5 (Forward)	L1014S	5- CCA CCG TAG TGA TAG GAA ATT C-3		380

4.12. Calculation of data and statistical analysis:

The mortality values of larval bioassay were calculated and the respective LC_{50} and LC_{99} values were estimated by putting log dose and mortality percentage against probit at 95% confidence interval in SPSS 21.0 software. The linear regression coefficient (r^2) obtained from the calculation was then used to assess the linearity of the experimental data for all of the field collected populations and laboratory reared susceptible population (SP). Twice the value of LC_{99} (LC_{99} x 2) for each population was taken as the recommended diagnostic dose / discrimination dose for that particular mosquito population. Resistance ratio 50 (RR_{50}), an indirect measurement of insecticide resistance was calculated by dividing the LC_{50} value of each field population with the LC_{50} value of susceptible population.

Mortality percentages of larval and adult bioassay tests were calculated and populations with <90% mortality were marked as resistant population, 98 – 100% were taken as susceptible population and populations showing mortality percentages between 90 – 98 were considered as incipient / unconfirmed resistance with the probability of either becoming resistant or susceptible (WHO, 2016). KDT_{50} and KDT_{90} values of synthetic pyrethroids and DDT were calculated by subjecting the time dependent knockdown percentage to probit analysis in SPSS 21.0 software version at 95% confidence level. The linear regression coefficient (r^2) obtained from the analysis was used to check the linearity of data.

In the insecticide assays, in case of mortality exceeding 10%, the data of mortality percentage was corrected using Abbott's correction. The average values and standard errors were calculated using Microsoft Excel, MS Office 2016.

Comparision of means obtained from all sampling sites were subjected to One Way ANOVA and Tukey's test in SPSS 21.0 and Addinsoft XLSTAT 2021.1.1. Correlation of the observed resistance status in larvae and adults along with the studied enzyme activity and kdr mutations in different field collected populations was analysed by Principal Component Analysis in Addinsoft XLSTAT 2021.1.1.