1st December, 2024

Original Article

CROSS-RESISTANCE TO DIFFERENT CLASSES OF INSECTICIDES IN PYRETHROIDS-SELECTED *CULEX QUINQUEFASCIATUS*

Prerana Bhujel[1] and Dhiraj Saha[1*]

[1]Insect Biochemistry and Molecular Biology Laboratory, Department of Zoology, North Bengal University, Raja Rammohanpur, Dist. Darjeeling, West Bengal, India 734013.

Received : 27th October, 2024

Accepted : 17th November, 2024

ABSTRACT

Background: *Culex quinquefasciatus* is the vector of several life-threatening diseases. In addition to spreading disease, it is a source of irritation due to its relative abundance. Vector control strategies particularly chemical control are employed to combat the spread of vector-borne diseases. Synthetic pyrethroids such as permethrin (Type I) and alphacypermethrin (Type II) are recommended for indoor residual spray, long-lasting insecticidal treated nets, outdoor fogging and liquid vaporizer mosquito repellent.

Methods: In this study, three strains of mosquitoes namely alphacypermethrin-selected (AS), permethrin-selected (PS), and susceptible (S) strains were reared for

**Corresponding Author:*

Prof. Dhiraj Saha; dhirajsaha@nbu.ac.in

Cite this article as:

Bhujel Prerana, Saha Dhiraj. Cross-resistance to different classes of insecticides in pyrethroids-selected *Culex quinquefasciatus*. *J Med Arthropodol & Public Health.* 2024; 4(2): 45-62.

20 generations under selection pressure of alphacypermethrin, permethrin and in the absence of insecticide respectively. The susceptibility status of these three strains against different classes of insecticides with and without the use of synergists was analysed. Synergists were used to examine the involvement of metabolic resistance mechanisms. Biochemical assays and allele-specific polymerase chain reactions were conducted to study the resistance mechanism involved.

Results: It was found that both the selected strains showed high resistance and cross-resistance to pyrethroids and other classes of insecticides as the mortality percentage was less than 90% except in the case of malathion. Even the use of synergists could only partially restore the susceptibility. Biochemical assays showed higher activities of monooxygenases and fixation of L1014F *kdr* mutation in the selected strains.

Conclusion: Both metabolic detoxification and *kdr* mutation were involved in the resistance in pyrethroid-selected strains, resulting in cross-resistance. Hence, monitoring the insecticide susceptibility status before the application of a particular insecticide is extremely essential to know its efficiency in controlling the vector. Moreover, rotation of insecticides with different modes of action is highly encouraged to increase the effectiveness of vector control.

Keywords: Alphacypermethrin, permethrin, WHO Tube test, Synergist test, Biochemical assays, kdr genotyping

INTRODUCTION

Culex quinquefasciatus is the vector of several life-threatening diseases such as lymphatic filariasis (LF), Japanese encephalitis (JE), West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), Usutu virus, avian malaria, dog heartworm, Rift valley fever, etc. In addition to spreading diseases, *Culex* mosquitoes are a source of nuisance. *Culex quinquefasciatus* is the most abundant mosquito, found in tropical and subtropical regions[1]. In India, it is the predominant vector of the nematode worm *Wuchereria bancrofti*, that causes bancroftian filariasis[2]. LF is endemic to 345 districts from 21 States and Union Territories (NCVBDC, accessed 20 October 2024). Mass drug administration (MDA) of a combination of three drugs namely ivermectin, diethylcarbamazine citrate (DEC) and albendazole in regular intervals has been the primary control method of LF[3]. However, poor

community participation and low compliance with MDA have created a barrier to its complete eradication in India. In this situation, vector control aids in combating the spread of diseases.

Vector control mainly depends upon the chemical control method, especially during epidemic conditions for the quick reduction of vector population and subsequent control over the disease. Organochlorines, organophosphates, carbamates, synthetic pyrethroids, neonicotinoids, phenylpyrazoles, butenolides, diamides and insect growth regulators are different classes of chemical insecticides commonly used for vector control. Synthetic pyrethroids, used as indoor residual spray, long-lasting insecticidal treated nets, outdoor fogging, and liquid vaporizer mosquito repellent have dominated the field of chemical control due to their fast action and low mammalian toxicity[4]. Pyrethroids disrupt the functioning of the voltage-gated sodium channel in both insects and mammals[5]. They are divided into type I and type II depending upon the structure (absence and presence of α cyano group respectively), symptoms of poisoning (hyperexcitation, prostration, body tremors, and stimulus-dependent nerve depolarization) and electrophysiological responses (repetitive discharge of action potential and disruption of action potential)[6]. Some pyrethroids show intermediate functions and are difficult to distinguish into types[7]. Alphacypermethrin is a type II synthetic pyrethroid used as an agricultural pesticide[8] as well as for vector control[9]. Permethrin is a type I synthetic pyrethroid used against various insect pests and vectors[10].

However, in response to the insecticidal selective pressure, mosquitoes are parallelly developing resistance against these insecticides making them less effective. Insecticide resistance has been reported profoundly in the *Culex quinquefasciatus* population across the globe as they are continuously exposed to the insecticides directly and indirectly[6,11–13]. Resistance to insecticides is achieved through different mechanisms individually or in combination. Target-site mutation and metabolic resistance are the major resistance pathways for pyrethroid resistance[14]. Resistance against pyrethroids is mainly conferred by the knockdown resistance (kdr) mutation in voltage-gated sodium channel gene and overexpression of Cytochrome P450 monooxygenases[6]. Insecticide resistance is a major barrier in the process of vector control. The present study was conducted utilizing the 20th generation of Culex quinquefasciatus, which were reared under the selective pressure of two synthetic pyrethroids: alphacypermethrin and permethrin. The aim

was to investigate the susceptibility status of these pyrethroid-selected strains toward various classes of insecticides, both with and without the application of synergists, as well as to explore the resistance mechanisms involved. This study intends to simulate field conditions characterized by continuous insecticide selection pressure and to evaluate the effectiveness of insecticide rotation. Alphacypermethrin and permethrin were specifically chosen due to their prevalent use in vector control measures, available in the market in forms such as chalks, coils, bed nets, and aerosols, catering to household, public health, and agricultural applications. The incorporation of synergists aimed to assess their preliminary role in metabolic resistance and to determine whether their use enhances the efficacy of insecticides against an already resistant mosquito population.

MATERIALS AND METHODS

Sample collection

Larvae and pupae of the wild population of *Culex quinquefasciatus* were collected from the high drains of the Shivmandir area in November 2021. The samples were then brought to the laboratory and morphologically identified following pictorial identification keys by Das[15] and Tyagi *et al*.,[16].

Insecticides

Alphacypermethrin (technical grade, 97%) and permethrin (technical grade, 98%) were purchased from Heranba Chemicals (Mumbai, India). Insecticide-impregnated papers (5% malathion, 4% DDT, 0.4% dieldrin, 0.05% alphacypermethrin, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.75% permethrin, 0.1% bendiocarb, and 0.1% propoxur) were purchased from Vector Control Research Unit, Universiti Sains Malaysia.

Larval Bioassay

A larval bioassay test against alphacypermethrin and permethrin was conducted using the standard procedure by the World Health Organisation[17] to obtain the sub-lethal concentrations. 100ppm stock solution of both insecticides was prepared from the purchased chemicals. From this, five concentrations yielding 10%-90% mortality were prepared to determine the 50% lethal concentration (LC_{50}). Four replicates of 25 late third and early fourth instar larvae were exposed to 100ml

serially diluted five insecticide concentrations in a 250ml glass beaker for 24 hours along with a control setup with no insecticide. Mortality percentages were recorded after 24 hours. Larvae showing no or faint movement when touched were considered dead or moribund. If 5-20% mortality was observed in the control set-up, it was corrected by using Abbott's formula [18] given below:

$$\text{Observed mortality} = \frac{\text{Test mortality} - \text{Control mortality}}{100 - \text{Control mortality}} \times 100$$

Insecticide selection and rearing setup

After obtaining the LC_{50} values, collected larvae were divided into three groups. One group was selected against a 50% sub-lethal concentration of alphacypermethrin and named as 'AS' strain, the second group was selected against a 50% sub-lethal concentration of permethrin and named as 'PS' strain and the third group was susceptible devoid of any insecticide treatment and named as 'S' strain. The selection was carried out by exposing approximately 2000 late third and early fourth instar larvae of each generation to 50% sub-lethal concentration for 24 hours. The larvae that survived after 24 hours of exposure were then shifted to enamel trays (25cm ×30cm ×5cm) filled with tap water. They were provided with powdered fish food till they started pupating. The pupae were separated manually in a beaker and kept inside the mosquito cages (30cm ×30cm ×30cm) for the emergence of adults which were fed with 10% sucrose solution soaked in cotton. After 3-5 days of emergence and successful mating, females were separated and starved for 24 hours before providing an EDTA-treated broiler's blood meal for two hours. The blood meal was again replaced by a 10% sucrose solution. The egg-laying apparatus was set after three days of blood meal. Egg rafts laid were kept individually in separate enamel trays filled with water. The larvae hatched were provided with ground fish food till pupation. The rearing was continued for 20 generations following this procedure.

WHO insecticide susceptibility tube tests

The females from 20th generation aged 3-5days old were exposed to discriminating concentrations (DCs) of one organophosphate (5% malathion), two organochlorines (4% DDT and 0.4% dieldrin), four pyrethroids (0.05%

alphacypermethrin, 0.05% deltamethrin, 0.05% lambdacyhalothrin, and 0.75% permethrin,) and two carbamates (0.1% bendiocarb and 0.1% propoxur) according to the procedure by WHO[19]. WHO[20] has released lesser DCs (0.025% deltamethrin, 0.025% lambdacyhalothrin, and 0.25% permethrin) for *Culex quinquefasciatus*. But since these laboratory strains were under continuous selection pressure the DCs recommended for *Anopheles* were tested. Twenty five non-blood fed females were kept in the 6 holding tubes with green dots containing clean white paper rolled inside the tube fastened with steel spring wire clip for 1 hour (Figure 1).

Fig. 1. Insecticide susceptibility tube test

Any dead or moribund mosquitoes were removed after 1 hour. Silicon oil-treated papers were rolled in yellow dotted tubes and insecticide-impregnated papers were in red dotted exposure tubes fastened with copper spring wire clip. The mosquitoes were then gently transferred to these tubes for 1 hour with regular monitoring at intervals of 10 minutes for recording the knocked-down mosquitoes. After 1 hour of exposure, the mosquitoes were again shifted to green-dotted holding

tubes with no paper this time and provided with a 10% sucrose solution soaked in cotton. Mortality was recorded after 24 hours based upon which susceptibility status was determined.

WHO Synergist Bioassay

To determine the involvement of metabolic enzymes in developing resistance, a synergist bioassay was performed according to the protocol by WHO [19]. Female *Culex quinquefasciatus* were exposed to 4% piperonyl butoxide (PBO) and 10% triphenyl phosphate (TPP) impregnated papers for 1 hour before exposing to insecticide-impregnated papers and rest protocol was like that of susceptibility bioassay test.

Biochemical assays

Thirty non-blood-fed, 3-5days old mosquitoes were homogenized in 200 µl of ice-cold Sodium phosphate buffer (pH 7.2) by Teflon micro-pestle in a 0.5ml microcentrifuge tube followed by centrifugation at 13000 rpm for 2 minutes. The supernatant was used for biochemical assays.

Carboxylesterase assay: It was measured with α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) as a substrate with a minor modification in the method of Van Asperen [21]. The staining agent was Fast Blue BB salt (FBBS). The absorbance (540 nm) was taken by a microplate reader (SPECTROstarnano, BMG Labtech). Blanks contain the same reaction mixture except for the homogenate. Standard curves of α- and β- naphthol were created to estimate the esterase activity.

Cytochrome P450 monooxygenase assay: It was measured by using a substrate namely tetramethyl benzidine (TMBZ) and stained with hydrogen peroxide (H2O2) solution in a microtitre plate well WHO[22]. After 2 hours of incubation, the plate was read at 630 nm in a microplate reader (SPECTROstarnano, BMG Labtech). By using a standard curve of cytochrome C, the activity of CYP450 monooxygenases was calculated.

Glutathione-S-transferase (GST) assay: It was measured with minor modification in the method of Habig et al.,[23]. The homogenate was mixed with 65mM 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in methanol and 10mM of

reduced glutathione (GSH) dissolved in 0.1M phosphate buffer (pH 6.5) and subjected to kinetic assay in SPECTROstarnano, BMG Labtech. The absorbance values were taken for 5 minutes followed by the calculation of GST activity (mM mg protein-1 min-1).

Protein Bioassay: The total protein concentration of the individual mosquito was determined by taking absorbance at 630 nm using SPECTROstarnano, BMG Labtech according to the method of Lowry et al., [24] and compared with Bovine Serum Albumin (BSA) standard curve to nullify size differences and obtain specific enzyme activity.

DNA Extraction

Genomic DNA was extracted from 24 mosquitoes from each strain by High Salt protocol Barik et al., [25] with minor modification. Individual mosquito was homogenized in a 1.5ml microcentrifuge tube using 98µl digestion buffer to which 2µl of 0.2mg/ml proteinase K was added and the mixture was kept in a water bath for incubation at 55°C for at least 2hours. 40 µl of 6M sodium chloride and 157 µl of chloroform were added to the sample and mixed for 20 minutes by gentle shaking followed by centrifugation at 10000rpm for 5 minutes. Only the upper phase, leaving the whitish lower phase, of the supernatant was transferred to the new microcentrifuge tube to which 140 µl of isopropanol was added and put on a shaker for 5 minutes followed by centrifugation at 8000rpm for 15 minutes. A black-colored pellet of DNA could be seen at the bottom of the centrifuge tube. The supernatant was discarded and 150 µl of chilled 70% ethanol was added and subjected to centrifugation at 8000rpm for 5 minutes. The supernatant was discarded and the tube was left to dry. After which, the pellet was dissolved in 20 µl of autoclaved distilled water and stored at -20°C for further use. The ratio of Optical Density at 260nm and 280nm was measured by SPECTROstarnano (BMG Labtech, Germany) to check the purity of the extracted DNA. Genomic DNA with values of a ratio between 1.8 and 2 were considered for kdr genotyping.

Genotyping kdr mutation

The extracted DNA was used to genotype the kdr mutation L1014F in voltage-gated sodium channel gene through allele-specific PCR (AS-PCR) with minor

modifications in the procedure of Martinez-Torres et al., [26] and Sarkar et al., [27]. The primers used were Cgd1 (5'-GTGGAACTTCACCGACTTC-3'), Cgd2 (5'-GCAAGGCTAAGAAAAGGTTAAG-3'), Cgd3 (5'-CCACCGTAGTGATAGGAAATTTA-3') and Cgd4(5'-CCACCGTAGTGATAGGAAATTTT-3'). Three PCR reactions were simultaneously run: Cgd1 and Cgd2 primers were combined in the first reaction to amplify voltage-gated sodium channel gene, Cgd2, and Cgd3 primers in the second reaction to amplify 1014L gene while Cgd2 and Cgd4 primers in the third reaction to amplify 1014F gene. Each reaction was performed in a 25 µl volume mixture of 25ng/µl extracted DNA, 1 µl each of forward and reverse primers, 12.5µl of GoTaq Mastermix (Promega), and 6.5 µl of nuclease-free water. PCR conditions were an initial denaturation at 95°C for 15 minutes, followed by 35 cycles at 94°C for 45seconds, 49°C for 45seconds, 72°C for 45seconds, and a final extension at 72°C for 10 minutes. The PCR products were then analyzed in 2% agarose gel stained with ethidium bromide in a UV Transilluminator (Himedia). The first reaction would yield a product size of 540 base pairs while the second and third reactions a band of 380 base pairs.

Data Analysis

Based upon the mortality percentage against each insecticide calculated, the mosquito population was termed susceptible with ≥ 98% mortality, possible resistant with ≥ 90% but < 98% mortality, and confirmed resistant with < 90%mortality WHO[20]. Abbott's formula was used for the correction of mortality if the mortality in the control group was ≥ 5% but < 20%. The data obtained from larval bioassays and knockdown time (KDT) were analyzed using log-probit analysis Finney *et al.*,[28] in IBM SPSS version 21. The differences in the enzymatic activity between the three strains were checked through analysis of variances (ANOVA).

Results

The larval bioassay of wild *Culex quinquefasciatus* mosquitoes showed LC_{50} of 0.014ppm (0.01-0.018) against alphacypermethrin and 0.035ppm (0.027-0.043) against Permethrin which increased to 1.016ppm (0.784-1.283) and 1.456ppm (1.138-1.846) in 20th generation respectively (Table 1).

Table 1. Sub-lethal concentration (LC_{50}) of different strains of *Culex quinquefasciatus*.

	AS	PS
F0	0.014 (0.01-0.018)	0.035 (0.027-0.043)
F20	1.016 (0.784-1.283)	1.456 (1.138-1.846)
SF20	0.007 (0.004-0.013)	0.011 (0.006-0.018)
RR50	145.14	132.36

Data shows 50% lethal concentrations with a confidence interval in the parenthesis. F0- field population, F20- laboratory reared 20th generation, SF20- susceptible strain at 20th generation RR_{50}- Resistance ratio calculated by dividing LC_{50} of selected strains divided by LC_{50} of susceptible strain.

Both AS strain and PS strain at 20th generation of selection showed 0% mortality against 4 synthetic pyrethroids (0.05% alphacypermethrin, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.75% permethrin), one organochlorine (4% DDT) and one carbamate (0.1% propoxur), 27% and 40% mortality against 0.1% bendiocarb while 66% and 68% mortality against 0.4% dieldrin indicating resistant status against these insecticides. Possible resistance was seen against 5% malathion in both the AS and PS strains with 90% and 94% mortality respectively (Table 2).

Table 2. Mean Mortality percentages against nine insecticides among three strains of *Culex quinquefasciatus*

INSECTICIDES	ASF20 (N-100)		PSF20 (N-100)		SF20 (N-100)	
	M% ± SE	Status	M% ± SE	Status	M% ± SE	Status
BENDIOCARB	27.00±2.51	R	40.00±1.63	R	100.00±0.00	S
PROPOXUR	0.00±0.00	R	0.00±0.00	R	99.00±1.00	S
ALPHACYPERM-ETHRIN	0.00±0.00	R	0.00±0.00	R	98.00±1.15	S
DELTAMETHRIN	0.00±0.00	R	0.00±0.00	R	99.00±1.00	S
LAMBDACYHAL-OTHRIN	0.00±0.00	R	0.00±0.00	R	98.00±1.15	S
PERMETHRIN	0.00±0.00	R	0.00±0.00	R	98.00±1.15	S

INSECTICIDES	ASF20 (N-100)		PSF20 (N-100)		SF20 (N-100)	
	M% ± SE	Status	M% ± SE	Status	M% ± SE	Status
MALATHION	90.00±2.58	PR	94.00±2.58	PR	100.00±0.00	S
DDT	0.00±0.00	R	0.00±0.00	R	98.00±1.15	S
DIELDRIN	66±2.58	R	68.00±1.63	R	100.00±0.00	S

N- Number of mosquitoes tested, M%- Mortality percentage, S.E- Standard Error, R-Resistant (M%<90), PR- Possible resistance (98%<M%>90%), S- Susceptible (M%≥98%).

Upon treating the strains with synergist PBO and TPP which are cytochrome P450 inhibitor and carboxylesterase inhibitor respectively before exposing them to the insecticides, mortality percentages increased but could not bring full susceptibility. Since, the mortality percentages with synergists followed by insecticides were less than 98% but greater than mortality percentage without synergist, it implies that enzyme-based resistance mechanism accounts only partially for the expression of resistant phenotype (Figure 2).

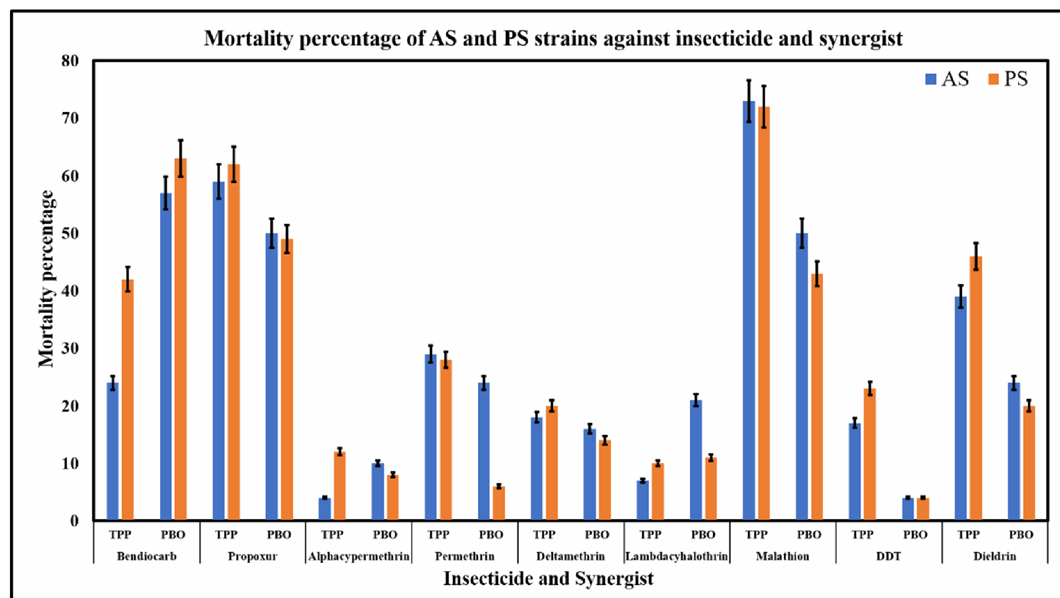

Fig. 2. Graph depicting mean mortality percentages of three strains of *Culex quinquefasciatus* against insecticides and synergists

The duration at which 50% and 90% of mosquitoes were knocked down are designated as KT_{50} and KT_{90} respectively. AS and PS strains showed the lowest KT_{50} against malathion and the highest against dieldrin (Table 3). KDT values for other insecticides could not be calculated as there were no knocked-down mosquitoes within exposure time.

Table 3. Knockdown times (95% confidence interval) of ASF20 and PSF20 strains

INSECTICIDES	STRAINS	KDT50 (CI) (MINUTES)	KDT90 (CI) (MINUTES)	R^2	CHI SQUARE
MALATHION	ASF20 (N-100)	45.62 (39.12-56.37)	106.24 (78.03-201.99)	0.96	1.32
	PSF20 (N-100)	43.70 (40.05-47.85)	64.69 (57.12-80.70)	0.88	4.80
DIELDRIN	ASF20 (N-100)	82.68 (64.90-793.76)	138.03 (87.24-14856.84)	1	1.27
	PSF20 (N-100)	99.83 ($69.36-3.06*10^{10}$)	185.94 ($96.52-3.08*10^{18}$)	0.99	0.39
BENDIOCARB	ASF20 (N-100)	57.99 (48.56-81.53)	134.10 (91.27-344.30)	0.95	1.04
	PSF20 (N-100)	50.46 (42.37-66.87)	128.80 (88.32-301.20)	0.88	2.37

KT_{50}- Duration at which 50% mosquitoes were knocked down, KT_{90}- Duration at which 90% mosquitoes were knocked down, CI- Confidence interval, N- number of mosquitoes tested.

Monooxygenase activity was increased to 2.99-fold and 3.06-fold respectively in AS and PS strains in contrast to the S strain. Carboxylesterase activity and GSTs activity were found not to be significantly different among the selected strains in comparison to that of the susceptible one (Table 4).

Table 4. Enzymatic activity among three strains of *Culex quinquefasciatus*

Strains	***N***	***Monooxygenases (µM EU/mg protein ± SE)***	***Alphaesterases (mM/min/mg protein ± SE)***	***Betaesterases (mM/min/mg protein ± SE)***	***GSTs (µM/min/mg protein ± SE)***
AS	30	38.97±3.37[a]	0.15±0.01[a]	0.011±0.002[a]	1.49±0.54[a]
PS	30	39.89±3.51[a]	0.16±0.02[a]	0.012±0.003[a]	1.67±0.33[a]
S	30	13.03±1.74[b]	0.14±.011[a]	0.010±0.001[a]	1.38±0.11[b]

Values with different alphabets are significant (ANOVA, p≤0.05). SE- Standard Error, N- number of mosquitoes tested. AS- Alphacypermethrin-selected, PS- Permethrin selected, S- susceptible strain.

ASPCR revealed the fixation of L1014F mutation in the selected strains with the total absence wild type genotype (LL) and heterozygous resistant genotype (FS) but only the presence of homozygous resistant (FF). In contrast, this susceptible strain showed 83.34% LL, and 8.34% each of LF and FF respectively (Table 5). The absence of insecticide selection pressure for 20 generations (approximately 1 year 6 months) in susceptible strains still contained the homozygous and heterozygous resistant genotypes.

Table 5. Genotype frequency and allele of three strains of *Culex quinquefasciatus*

STRAINS	**Genotype frequency (%) (N=24)**			**Allele frequency**	
	LL	**LF**	**FF**	**L**	**F**
AS	0	0	100	0	1
PS	0	0	100	0	1
S	83.34	8.34	8.34	0.88	0.13

DISCUSSION

Culex quinquefasciatus not only transmits various mosquito-borne diseases but is a constant source of irritation due to its high abundance owing to the ability to breed in highly polluted water[11] There have been several studies worldwide to monitor the insecticide resistance status in field population of *Culex quinquefasciatus* [1,6,12,13,29,30]. However, fewer to negligible studies have been carried

out in laboratory-selected strains of *Culex quinquefasciatus* [4,31–33]. Hence this study was conducted to evaluate the susceptibility status of two pyrethroids-selected laboratory strains (AS and PS) of *Culex quinquefasciatus* at 20th generation against four classes of insecticides with and without synergists. The study simulates the field condition of *Culex quinquefasciatus* which is under continuous selection pressure and investigates the efficiency of vector control through the use of chemicals. It was observed that AS showed 145-fold resistance and PS showed 132-fold resistance than that of the S strain after 20 generations of selection against alphacypermethrin and permethrin respectively. Both the selected strains showed resistance to synthetic pyrethroids as well as cross-resistance to organophosphates, organochlorines, and carbamates. Moreover, the use of synergists, before exposure to the insecticide, also could not restore the susceptibility in resistant strains though the mean adult mortality rate was slightly higher than that of insecticide alone. It indicates that the detoxification by the metabolic enzyme is not the major insecticide resistance mechanism involved. This finding contrasts with other studies where a significant increase in the mortality rate and restoration of susceptibility was observed[11,34]. On the other hand, a study by Lucas *et al*.,[35] also reported the partial involvement of metabolic enzymes in the resistance status of *Culex quinquefasciatus*. Biochemical assays showed higher activities of monooxygenases in selected strains. Moreover, fixation of kdr mutation L1014F was found in selected strains. Overexpression of cytochrome P450s and presence of L1014F mutation has been reported in various previous studies[4,13,31,33,35]. Detoxifying enzymes can act individually or synergistically, sequentially metabolizing the same or different insecticides and often work together with target-site mutations which leads to soaring resistance levels along with cross-resistance. This might be the reason for the cross-resistance observed in our study. Additionally, pyrethroid resistance may have pleiotropic effects, influencing multiple biochemical pathways, including those involved in organophosphates, organochlorines and carbamate resistance. Moreover, repeated exposure to pyrethroids may have selected mosquitoes with pre-existing resistance to other insecticides. This study highlights the need for the development of novel insecticides with different modes of action.

CONCLUSIONS

This study provides valuable insight into the importance of monitoring insecticide susceptibility status before the application of a particular insecticide to

know its efficiency in controlling the vector. With the rise in resistance against insecticides, integrated vector management methods for the control of mosquito vectors must be encouraged such as the use of biological control agents, breeding habitat destruction and most importantly mass awareness among the public about sanitation and vector-borne diseases. Insecticide use should be minimized and kept for emergency outbreaks so that the epidemic outbreak can be controlled at the earliest. For successful vector control, coordinated works of various organizations and individuals are required which includes researchers, chemical industries, control agencies, national and international governments, and local populations, and successful implementation at the ground level.

ACKNOWLEDGEMENTS

The authors express sincere gratitude to the Head, Department of Zoology for allowing access to the laboratory facility. The first author is thankful to University Grants Commission, New Delhi, India Dated 10 DEC 2019) for financial support through JRF Award Letter [F. No. 16-9(June 2019)/2019(NET/CSIR), UGC Ref. No.: 687/(CSIR-UGC NET JUNE 2019)]. The funder had no role in the preparation of this manuscript.

REFERENCES

1. Yadouléton A, Badirou K, Agbanrin R, et al. Insecticide resistance status in *Culex quinquefasciatus* in Benin. Parasit Vectors 2015;8(1).
2. Agrawal VK, Sashindran VK. Lymphatic filariasis in India: Problems, challenges and new initiatives. Med J Armed Forces India. 2006;62(4):359–362.
3. Ramaiah KD, Das PK. Mass drug administration to eliminate lymphatic filariasis in India. Trends Parasitol. 2004;20(11):499-502.
4. Ramkumar G, Shivakumar MS. Laboratory development of permethrin resistance and cross-resistance pattern of *Culex quinquefasciatus* to other insecticides. Parasitol Res 2015;114(7):2553–2560.
5. Narahashi T. Nerve Membrane as a Target of Pyrethroids. Pestic Sci. 1976;7(3):267-72.
6. Scott JG, Yoshimizu MH, Kasai S. Pyrethroid resistance in Culex pipiens mosquitoes. Pestic Biochem Physiol. 2015;120:68–76. https://doi.org/10.1016/j.pestbp.2014.12.018
7. Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016;133:1–12.

8. Baruah P, Chaurasia N. Ecotoxicological effects of alpha-cypermethrin on freshwater alga Chlorella sp.: Growth inhibition and oxidative stress studies. Environ Toxicol Pharmacol 2020;76:103347.
9. Ngufor C, Agbevo A, Fagbohoun J, Fongnikin A, Rowland M. Efficacy of Royal Guard, a new alpha-cypermethrin and pyriproxyfen treated mosquito net, against pyrethroid-resistant malaria vectors. Sci Rep 2020;10(1):12227. https://doi.org/10.1038/s41598-020-69109-5
10. Low VL, Chen CD, Lee HL, Lim PE, Leong CS, Sofian-Azirun M. Current susceptibility status of Malaysian *Culex quinquefasciatus* (Diptera: Culicidae) against DDT, propoxur, malathion, and permethrin. J Med Entomol 2013;50(1):103–111.
11. Omotayo AI, Dogara MM, Sufi D, et al. High pyrethroid-resistance intensity in *Culex quinquefasciatus* (Say) (Diptera: Culicidae) populations from Jigawa, North-West, Nigeria. PLoS Negl Trop Dis 2022;16(6).
12. Rai P, Bharati M, Saha D. Assessment of insecticide resistance in *Culex quinquefasciatus* Say with first report on the presence of L1014F mutation from northern districts of West Bengal, India. Int J Trop Insect Sci 2019;39(4):301–309.
13. Talipouo A, Mavridis K, Nchoutpouen E, et al. High insecticide resistance mediated by different mechanisms in *Culex quinquefasciatus* populations from the city of Yaoundé, Cameroon. Sci Rep 2021;11(1).
14. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. In: Insect Biochemistry and Molecular Biology. 2004; p. 653–665.
15. Das BP. Pictorial Key to Common Species of Culex (Culex) Mosquitoes Associated with Japanese Encephalitis Virus in India. In: Mosquito Vectors of Japanese Encephalitis Virus from Northern India. Springer India; 2013;25-42.
16. Tyagi BK, Munirathinam A, Venkatesh A. A catalogue of Indian mosquitoes. A catalogue of Indian mosquitoes. Int J Mosq Res. 2015;2(2):50-97.
17. World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides (No. WHO/CDS/WHOPES/GCDPP/2005.13). 2005.
18. Abbott BS. A method of computing the effectiveness of an insecticide. J econ Entomol. 1925;18(2):265-267.
19. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Global Malaria Programme. 2016
20. World Health Organization. Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays. 2022.
21. Van Asperen K. A study of housefly esterases by means of a sensitive colorimetric method. J insect physiol. 1962;8(4):401-416.

22. Hemingway J. Insecticide resistance mechanisms (field and laboratory manual). World Health Organization:Geneva, Switzerland. 1998:1-39.
23. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-Transferases: the first enzymatic step in mercapturic acid formation. J biol chem 1974;249(22):7130-9
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. protein measurement with the folin phenol reagent. J biol chem. 1951;193(1):265-75.
25. Barik S, Barik SK, Kumar Hazra R, Prusty MR, Rath A, Kumar Kar S. A simple, rapid and very efficient protocol for DNA isolation from mosquito species. Protoc Exch 2013;
26. Martinez-Torres D, Chevillon C, Brun-Barale A, Bergé JB, Pasteur N, Pauron D. Voltage-dependent Na ‡ channels in pyrethroid-resistant Culex pipiens L mosquitoes. Pestic Sci. 1999;55(10):1012-1020.
27. Sarkar M, Borkotoki A, Baruah I, Bhattacharyya IK, Srivastava RB. Molecular analysis of knock down resistance (kdr) mutation and distribution of kdr genotypes in a wild population of *Culex quinquefasciatus* from India. Tropical Medicine and International Health 2009;14(9):1097–1104.
28. Finney, D.J., 1952. A statistical treatment of the sigmoid response curve. Probit analysis. Cambridge University Press, London, 633.
29. Delannay C, Goindin D, Kellaou K, Ramdini C, Gustave J, Vega-Rúa A. Multiple insecticide resistance in *Culex quinquefasciatus* populations from guadeloupe (French west indies) and associated mechanisms. PLoS One 2018;13(6).
30. Lopes RP, Lima JBP, Martins AJ. Insecticide resistance in *Culex quinquefasciatus* Say, 1823 in Brazil: A review. Parasit Vectors. 2019;12(1). https://doi.org/10.1186/s13071-019-3850-8
31. Yanola J, Chamnanya S, Lumjuan N, Somboon P. Insecticides resistance in the *Culex quinquefasciatus* populations from northern Thailand and possible resistance mechanisms. Acta Trop 2015;149:232–238.
32. Nazni, WA, Lee, HL, Sa'diyah I. Rate of resistance development in wild *Culex quinquefasciatus* (Say) selected by malathion and permethrin. Southeast Asian J Trop Med Public Health, 1998;29(4):849-55.
33. Tabbabi A, Laamari A, Cheikh R Ben, Jha I Ben, Daaboub J, Cheikh H Ben. Resistance development and insecticide susceptibility in Culex pipiens pipiens, an important vector of human diseases, against selection pressure of temephos and its relationship to cross-resistance towards organophosphates and pyrethroids insecticides. Afr Health Sci. 2018;18(4):1175–81.
34. Bisset J, Rodriguez M, Soca A, Pasteur N, Raymond M. Cross-Resistance to Pyrethroid and Organophosphorus Insecticides in the Southern House Mosquito (Diptera: Culicidae) from Cuba [Homepage on the Internet]. 1997; Available from: https://academic.oup.com/jme/article/34/2/244/922685

35. Lucas KJ, Bales RB, McCoy K, Weldon C. Oxidase, esterase, and kdr-associated pyrethroid resistance in *Culex quinquefasciatus* field collections of collier county, Florida. J Am Mosq Control Assoc 2020;36(1):22–32. https://doi.org/10.2987/19-6850.1