# Insecticide Susceptibility/ Resistance status and distribution pattern of *kdr* genotype in *Culex quinquefasciatus* of Palakkad District, Kerala

Anju Viswan,K, Evangeline Surya Hermon, Pushpalatha, E.

*Biopesticides & Toxicology Laboratory, Department of Zoology, University of Calicut, Malappuram Dist, Kerala, 673635, India*

***Abstract*— *Culex quinquefasciatus* Say is the principal vector of filariasis and a major biting nuisance. Palakkad district in Kerala has had a long history of Lymphatic filariasis and there has been a sudden increase in the number of filarial cases reported in the district from 2014. The present study aims at assessing the resistance status of *Cx. quinquefasciatus* in the district towards Organophosphates, and Pyrethroids and to investigate the occurrence of *kdr* mutation in the field populations of *Cx. quinquefasciatus* collected from 4 different sites. Susceptibility to organophosphates and pyrethroids were tested by WHO susceptibility kit using insecticide impregnated papers (Malathion - 5%, Cyfluthrin - 0.15%, and Deltamethrin - 0.05%). Biochemical analyses were done to identify the levels of detoxifying enzymes. All the 4 populations showed resistance to the three insecticides tested and higher resistance shown towards pyrethroids. Biochemical assays showed the presence of elevated enzyme levels and molecular assays using AS-PCR confirmed the presence of *kdr* mutations in the 4 populations of mosquitoes tested.**

***Keywords*— *Culex quinquefasciatus*, insecticide resistance,detoxification enzymes, *kdr* mutation**

## I. INTRODUCTION

*Culex quinquefasciatus* is one of the common and abundant mosquito species found in India and South-eastern Asia. *Cx. quinquefasciatus* attacks human hosts both indoors and outdoors. In Southern India, it is highly anthropophilic with 50-76% of them feeding on human beings. The females of the species also feed on amphibians, pigs, horses, cattle, sheep, and birds. It is the principal vector of *Wuchereria bancrofti*, the causative organism of Lymphatic filariasis.

Palakkad is the largest district in Kerala and is situated in the middle-east region of the state near the Tamil Nadu border. Many earlier studies have shown high prevalence of LF in the district. Recent parasitological surveys shows the Palakkad town and 16 panchayats in the district to be positive for LF with an overall microfilaria (mf) rate of 4.08%. All the microfilariae reported from Palakkad till date belongs to *W. bancrofti* (Nair, 1966; Regu *et al*, 2016) and *Cx. quinquefasciatus*, which is ubiquitous in the district is the principal vector of bancroftian filariasis.

Vector control plays an important role in the eradication or control of the disease. The National Filaria Control Programme (NFCP), launched in 1955 gave due importance to vector control by anti-larval measures in urban areas and indoor residual spraying in rural areas. Development of resistance in vector species, and emergence of new resistant genotypes among the vector population has been a major setback in global efforts to control vector-borne diseases (Hemingway & Ranson, 2000).Due to the emergence of resistance in vector species to all major and commonly used insecticides, studies must be done from time to time to evaluate the resistant status of vector species and necessary changes must be brought in the strategies for vector control for the effective management of vector-borne diseases.

## II. MATERIALS & METHODS

*Study sites and sample collection*

The study was conducted from May to August of 2017. Four different study sites were selected in the Palakkad municipality for sample collection namely Thottingal, Othungode, Big Bazaar and Melamuri. *Cx. quinquefasciatus* larvae were collected from drains in the four selected localities by dipping method and reared in separate cages until adults emerged. The larvae and emerged adults were identified as *Cx. quinquefasciatus* species according to standard diagnostic keys on characteristic morphological features. Susceptible laboratory population were collected from CRME (ICMR), Madurai and reared in our laboratory without exposure to any chemical insecticides.

*Biochemical assays*

Biochemical assays were used to quantify levels of monooxygenases, and non-specific esterases, as well as to detect the presence of elevated acetylcholine esterase in 4$^{th}$ instar larvae of the F1 generation. 30 fourth instar larvae were taken from each of the four samples for the assays. The assays were done according to the protocol provided in Techniques to detect insecticide resistance mechanism Field and laboratory Manual, WHO (1998) [Techniques to detect insecticide resistance mechanisms, WHO/CDS/CPC/MAI:: /98;6 .]

*Insecticide Susceptibility Test*

2 – 3 days old adult female mosquitoes from the field populations and the known susceptible strain were exposed to

insecticide impregnated papers containing diagnostic doses of insecticides to check the susceptibility status of the field population, according to standardWHO protocol. Bioassays were done for Malathion – 5%, Cyfluthrin - 0.15%, and Deltamethrin - 0.05%. 3 batches of 15 – 25 adult female mosquitoes were exposed for one hour to insecticide-impregnated papers. Knockdown was recorded every 10 minutes and final mortality was recorded after a 24 hour holding period after exposure where the mosquitoes were transferred to clean tubes and allowed to recover. A 10% sucrose solution was made available to the mosquitoes during this recovery period. Control assays were run along with the tests and included exposure of 25 mosquitoes per study site to untreated papers.

Survivors and non-survivors from each study site from the test with pyrethroids (Cyfluthrin and Deltamethrin) were stored separately at -20°C for further molecular characterization. DNA of 2 non-survivors and 20 survivors (10 deltamethrin survivors and 10 cyfluthrin survivors) from each study site were isolated by Phenol - Chloroform - Isoamyl alcohol extraction and was used for AS-PCR assay for determining the presence of kdr genotype.

*Allele - specific PCR (AS-PCR) for detecting knock down resistance (kdr) mutation*

*Primer selection*

Four primers (Primer 1, 2, 3 and 4) were selected from the region II of para-type voltage gated sodium channel (vgsc) gene of *Cx. pipiens*. Primer 1 (forward) 5´-GTGGAACTTCACCGACTTC- 3´and Primer 2 (reverse) 5´-GCAAGGCTAAGAAAAGGTTAAG- 3´ amplified the fragment of sodium channel gene containing the *kdr* mutation site. Primer 3 and Primer 4 were allele-specific primers used in genotyping of knockdown susceptible and knockdown resistant alleles by Allele-specific PCR assay. Primer 3 (forward) 5´-CCACCGTAGTGATAGGAAATTTA- 3´ corresponds to the susceptible (S) allele and Primer 4 (forward) 5´-CCACC GTAGTGATAGGAAATTTT- 3´ corresponds to the resistant (R) allele.The allele-specific primers were identical except at the 3´-OH end where 'A' in Primer 3 was replaced by 'T' in Primer 4. Both primers 3 and 4 could amplify a 380bp corresponding region.

*AS-PCR Assay*

The PCR was performed according to Martin-Torres *et al*, with modifications to detect kdr mutation in the mosquito population. Two PCR reactions were run in parallel. One reaction contained the primers 1, 2 and 3 (10 pmol each). In the other reaction primer 3 was replaced by primer 4. 10 ng of mosquito DNA was added as template in each reaction. The reaction mixture contained 10 µl 1x PCR smart mix, and the final reaction volume was 20 µl. The PCR conditions were 5 min at 94 °C for the first cycle, followed by 1 min at 94 °C, 2 min at 49 °C and 2 min for 72 °C for 29 cycles, and 10 min at 72 °C for the final extension. The DNA fragments were separated by electrophoresis on 1.5% agarose gel and were visualized by ethidium bromide staining under UV light. The presence of 380 bp band corresponding to resistant and susceptible specific primers revealed the genotype of the mosquitoes.

## III. RESULTS

*Adult Susceptibility Bioassay*

Percentage mortality was calculated and WHO guidelines were used for evaluating resistance or susceptibility. According to WHO criteria for resistance (WHO 1998), mortalities below 80% represented definite, strong resistance, whereas mortalities ranging from 80 to 98% represented varying degrees of resistance; generally described as tolerance, and those above 98% represented definite susceptibility. Table 1 shows percentage mortalities of the four different field populations against the susceptible laboratory mosquitoes. All the four populations studied were highly resistant to pyrethroids, both cyfluthrin and deltamethrin, with a mortality ranging from 35% to 65%. The lowest mortality rate was shown in response to deltamethrin (35%).

A mortality of 68.3% - 90% was shown in Malathion, with the lowest mortality (68.3%) shown by the BB population and the highest (90%) shown by TH. Very high resistance was shown towards pyrethroids, with mortality ranging from 35% to 65%. In the test with Cyfluthrin, the lowest mortality (41.67%) was shown by the BB population followed by ME population (43.33%), and OT population (50%) while the highest mortality was shown by the TH strain (65%). Of all the three papers tested, the highest resistance was shown to Deltamethrin, where the lowest mortality was 35% (BB strain) and the highest mortality was 42% (TH strain). In all the three insecticides tested, *Cx. quinquefasciatus* collected from TH showed the least resistance while the highest resistance was shown by individuals collected from BB closely followed by those collected from ME.

Table 1: Percentage mortality after exposure of *Culex quinquefasciatus* F1 adults collected from four localities in the Palakkad Municipality to diagnostic doses of different insecticides.

| Insecticides | Percent Mortality | | | |
|---|---|---|---|---|
| | Area of Collection | | | |
| | TH | BB | ME | OT |
| Malathion 5% | 90% (100)* | 68.3% (108) | 73.33 % (100) | 83.33% (102) |
| Cyfluthrin 0.15% | 65% (100) | 41.67 (108) | 43.33 % (100) | 50% (100) |
| Deltamethrin 0.05% | 42% (106) | 35% (108) | 35.43 % (100) | 38.33% (100) |

* ( ) = sample size** Test with control papers showed 0 mortality

Temephos (organophosphate/ common name: Abate) is the commonly used larvicide for the control of mosquitoes in Palakkad municipality areas. Larvicides are regularly sprayed

in the ditches in BB and ME while in OT and TH which are both residential areas, only infrequent spraying is done. The BB and ME strains showed only 68 – 73% mortality in the test with Malathion which is an organophosphate. This result shows that these populations are developing resistance against organophosphates. The regular exposure to organophosphate insecticides might be the reason for this resistance. Though the OT and TH populations cannot be considered as completely susceptible as they show only 83% and 90% mortality respectively, they are l more susceptible than the other two strains.

All the four populations are showing alarmingly high resistance to Pyrethroids with mortality of 35 - 65%. BB showed the least mortality (Cyfluthrin- 41.67% and Deltamethrin – 35%) while TH showed the highest mortality (Cyfluthrin – 65% and Deltamethrin – 42%). This high resistance to pyrethroids must be considered seriously as pyrethroids are used for Indoor Residual Spraying (IRS) and Indoor Space Spraying (ISS) for the immediate control of mosquito populations in areas where diseases like Dengue, Chickungunya and Malaria were reported, and areas with high incidence of LF.

Fig1. shows the α- and β-esterase activity of laboratory strain and the four field strains of *Cx. quinquefasciatus*. The mean α-esterase and β-esterase activity in the four field strains of *Cx. quinquefasciatus* obtained from Palakkad ranged from 0.19 – 0.30 nmol α-naphthol/min/mg protein, and 0.18 – 0.27 nmol β-naphthol/min/mg protein respectively. The α and β esterase levels in all field strains were significantly different ($p < 0.05$) from the laboratory strain by one-way ANOVA ($F = 2857.08011$, $p < 0.00001$; The result is significant at 95% confidence level). There was a significant increase in the α and β esterase activity in the four field populations. Elevated esterase activity accounts for resistance to organophosphate, carbamate, and pyrethroid insecticides (Terriere, 1984; Brogdon, 1989; Hemingway and Karunaratne, 1998). The esterases either produce broad spectrum insecticide resistance through rapid binding and slow turnover of insecticide or narrow spectrum resistance through metabolism of a very restricted range of insecticides containing a common ester bond.

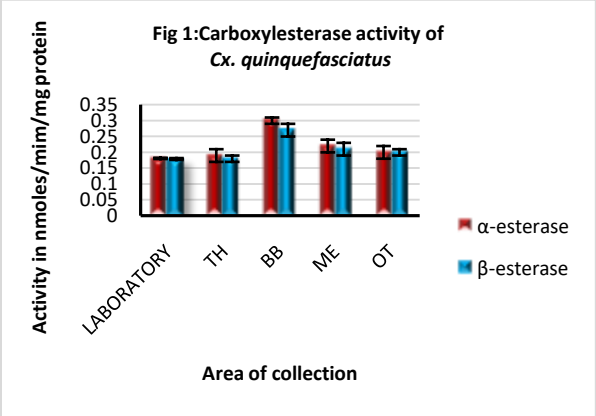
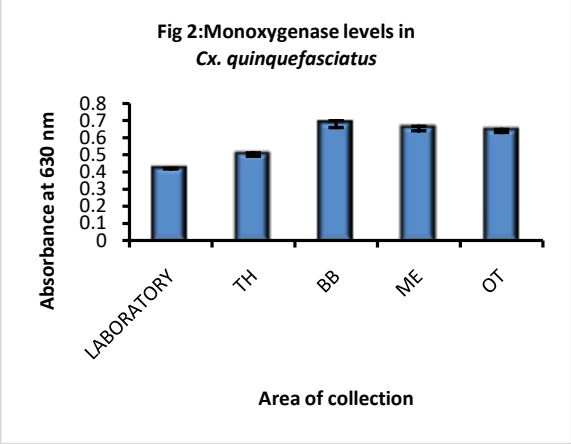
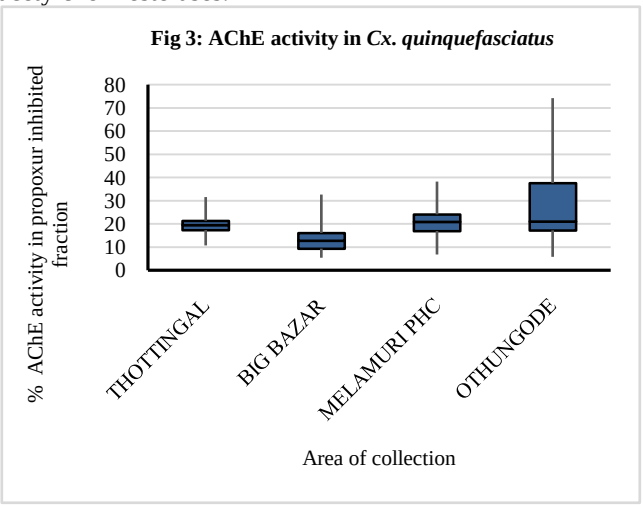



Fig 2. shows the P450 Monooxygenase activity of Laboratory strain and the four field strains of *Cx. quinquefasciatus* collected from Palakkad. The mean optical density (OD) of the elevated oxidase activity in the four field strains of *Cx. quinquefasciatus* ranged from 0.502 – 0.68. Monooxygenase levels in all field strains were significantly different ($p < 0.05$) from the laboratory strain by one-way ANOVA ($F = 2857.08011$,$p < 0.00001$;The result is significant at 95% confidence level).




The percentage remaining activity in Propoxur – inhibited fraction was found out by dividing the absorbance for the well with propoxur by that without propoxur for the sameinsect and multiplying it by 100. A percentage value greater than 30% indicated resistance. Fig 3. shows the percentage remaining activity in the Propoxur – inhibited fraction of the four field strains of *Cx. quinquefasciatus* from Palakkad. More than 30% of the BB strain has percentage activities greater than 30% with the maximum value being 74.18%, indicating that elevated levels of acetylcholinesterase has an important role in conferring organophosphate resistance in this population. Only less than 10% of the other three field strains showed percentage activities greater than 30% suggesting that organophosphate resistance in these field strains were not associated with elevated acetylcholinesterases.



The AS-PCR assay revealed the presence of leucine – phenylalanine kdr mutation in the field strains of *Cx. quinquefasciatus*. PCR assay showed three genotypes, identified by the characteristic 380 bp band corresponding to resistant and susceptible specific primers. The 380 bp PCR product with both the knock down specific [kds (primer3) and kdr (primer4)] primers in an individual mosquito indicates heterozygous condition (SR). The appearance of this band only in susceptible-specific primer (kds - primer3) indicates homozygous susceptible (SS) and in resistant specific primer (kdr - primer4) indicates homozygous resistant (RR).

2 non-survivors and 20 survivors were taken from each of the four study sites for the AS-PCR assay. All the non-survivors screened had the SS genotype. The result of AS-PCR for pyrethroid survivors from each area is shown in Table 6.

Table 2. Genotypes predicted by AS-PCR

| Area of Collection | Genotypes shown | | |
|---|---|---|---|
| | **RR** | **RS** | **SS** |
| BB | 10 | 8 | 2 |
| ME | 6 | 10 | 4 |
| OT | 4 | 10 | 6 |
| TH | 4 | 12 | 4 |

From the table we can see that upto 50% of the pyrethroid resistant individuals screened from the BB strain have the RR genotype, while in ME it is 30%, and in OT and TH strains it is 20%. Most of the resistant individuals are heterozygous for the allele. The presence of the kdr allele in such high magnitude has serious consequences for mosquito control programmes.

This result confirms with the bioassay and biochemical assay results of these strains, where the BB and ME strains showed high resistance to pyrethroids in terms of mortality rates and the enzyme levels while OT and TH strains showed lesser resistance when compared to the former strains.

The high incidence of kdr mutation must also be considered seriously as pyrethroids are used for IRS and ISS for the immediate control of mosquito populations in areas reporting high incidence of LF cases. The kdr mutation renders the *Cx. quinquefasciatus* populations resistant to the common household control measures used, as pyrethroids are the common constituent of mosquito mats, coils and repellents.

The best strategy for controlling disease vectors is the rotational use of insecticides of different modes of action altogether, rather than merely alternating members of one chemical class or different chemical classes that address the same target site. For example, the presence of kdr resistance renders DDT and pyrethroids less effective, whereas carbamates, such as bendiocarb, or organophosphates can still be used – in the absence of Modified Acetylcholinesterase (MACE), they can be used in rotation. Such a strategy might increase the chance of regaining pyrethroid susceptibility, but it has to be carefully monitored.

## ACKNOWLEDGMENT

Authors are thankful to UGC BSR SAP for providing financial supportto carry out this work.

## REFERENCES

[1]. Brogdon, W. G., & McAllister, J. C. (1998). Insecticide resistance and vector control. *Emerging infectious diseases*, *4*(4), 605.

[2]. Finney, D. J. (1971). *Probit Analysis*. Cambridge University press, Cambridge, England.

[3]. Hemingway, J., & Karunaratne, S. H. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and veterinary entomology*, *12*(1), 1-12.

[4]. Hemingway, J., & Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual review of entomology*, *45*(1), 371-391.

[5]. Martinez-Torres, D., Chevillon, C., Brun-Barale, A., Bergé, J. B., Pasteur, N., & Pauron, D. (1999). Voltage-dependent Na+ channels in pyrethroid-resistant Culex pipiens L mosquitoes. *Pest Management Science*, *55*(10), 1012-1020.

[6]. Nair, C. P. (1966). Filaria survey of Palghat Town, Kerala State. *Bulletin of the Indian Society for Malaria & Other Communicable Diseases*, *3*(3), 198-206.

[7]. National Vector Borne Disease Control Programme, Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India. Website: Available from: http://nvbdcp.gov.in/filariasis-new.html [Cited on 2010 Jan 13].

[8]. Regu K., Rajendran R.,Showkath M. K.,Mohanan, W., Tamizharasu, V. M., Ravindranath, T.G., Thomas, S. K., Jain, S., Venkatesh (2016) Current Status of Bancroftian Filariasis in Palakkad District, Kerala . *Journal of Communicable Diseases*.48(4): 21-25.

[9]. Terriere, L. C**.** (1984). Introduction of detoxification enzymes in Insects. *Annual review of Entomology* , *29*(1),71-78.

[10]. World Health Organisation (1998) Techniques to detect insecticide resistance mechanism (Field and laboratory Manual), Geneva.