Journal of Communicable Diseases
Volume 51, Issue 2 - 2019, Pg. No. 22-27
Peer Reviewed & Open Access Journal

Research Article

Biochemical and Molecular Analysis of Acetylcholine Esterase to Rule out Organophosphorus Detoxification Level in Field Populations of *Culex Quiquefasciatus* Say

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DOI: https://doi.org/10.24321/0019.5138.201913

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How to cite this article:
Anju Viswan K, Pushpalatha E. Biochemical and Molecular Analysis of Acetylcholine Esterase to Rule out Organophosphorus Detoxification Level in Field Populations of *Culex Quiquefasciatus* Say. *J Commun Dis* 2019; 51(2): 22-27.

Date of Submission: 2019-04-30
Date of Acceptance: 2019-06-27

A B S T R A C T

Background & Objectives: Acetylcholinesterase, responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both organophosphate and carbamate insecticides. The current study envisage to assess acetylcholine esterase levels and mutation in the acetylcholine esterase gene1 of Cx. quinquefasciatus of Kozhikode, Cochin, Malappuram, Thrissur, and Palakkad town areas of Kerala, India.

Methods: The samples were collected from regularly insecticide spraying area and scarcely spraying area of the five locations in the year 2014, 15 and 16. The assays were done according to the protocol provided in techniques to detect insecticide resistance mechanism; Field and laboratory Manual. Site specific mutation in acetylcholine esterase gene 1 was identified by RFLP using Alu1 enzyme.

Results: The % remaining activity in propoxur inhibited fraction in the regularly and scarcely insecticide treating field populations of Cx.quinquefasciatus were higher than 30% and lesser than 30% respectively. The value greater than 30% indicates the chance to develop resistance in field populations. The RFLP using Alu1 on the ace1 also showed the presence of heterozygous genotype in all the five field populations except in Ernakulam where the Cx.quinquefasciatus population possessed homozygous resistant genotype, which is an indication to the excessive and extensive usage of Temephos as a larvicide.

Interpretation & Conclusion: The evidence of development of resistance to synthetic insecticides in mosquitoes observed in the present study points to the need of employing new or alternate insecticides which would be easily degradable and have less harmful effect on other organisms.

Keywords: Acetylcholinesterase, Ace1 Gene, Culex Quinquefasciatus, Organophosphorus Resistance

Introduction

The filariasis and Japanese encephalitis vector *Culex*, is quite widely distributed and cause common chronic symptoms in human beings. *Culex* breed is found mostly in water sources like drains, ponds, rice fields affected by pollution or any other polluted water logged areas. They are active in the night and are identified as night biters. The adults of the species can be controlled by effective indoor spraying of insecticide. The effective combating of the spread of this vector species is by decimating its larval population by using a variety of larvicides in the open drains, latrines, ditches etc. and by fumigating the closed drains.

Over the years, scientists have been experimenting with different methods to control or eradicate mosquito-borne diseases extensively. Among the numerous prevention strategies tried, chemical control using insecticides appears to be the norm for both public health and household pest control.[2] The most feasible, effective, and practical method in controlling mosquito vector species is the use of insecticides.[3] Organochlorines, organophosphates, pyrethroids, carbamates, pyrroles and phenyl pyrazole are the commonly used and widely recommended insecticides against adult mosquitoes.[4] Indoor residual spraying and long lasting nets coated with pyrethroids and DEET is being used widely these days to combat the occurrence of mosquito borne diseases.

Owing to the repeated use of the common insecticides, mosquitoes developed resistance to it and have caused a number of outbreaks of mosquito borne diseases in the recent years.[5,6] Mosquitoes have developed resistance over the years, to all kinds of insecticides including biocides.[7] In many countries, the breeding sites of *Cx. quinquefasciatus* have been sprayed with organophosphorus insecticides[8], which eventually resulted in development of resistance to them. Temephos, an Organophosphate (OP) insecticide, is widely recommended as a larvicide to control mosquitoes, midges, blackfly larvae, and other insects.[9] In India, Temephos has been recommended for controlling mosquito larvae and its use is patronized by the Government of India under their National Vector Borne Diseases Control Programme (NVBDCP).

The target site of inhibition for organophosphate and carbamate insecticides is acetylcholinesterase. Acetylcholinesterase is responsible for neurotransmitter degradation at the cholinergic nerve synapse. Selection of modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism, and has been observed in numerous arthropod pest species. In the mosquito gene *ace*1, it encodes the synaptic AChE1 responsible for insensitivity to insecticides.[10]

The scope of the current study envisages to assess and analyze acetylcholinesterase levels of *Cx. quinquefasciatus* and to identify *ace*1 gene mutation of Kozhikode, Cochin, Malappuram, Thrissur, and Palakkad town areas of Kerala, India.

Materials and Methods

The larvae of *Cx. quinquefasciatus* were collected from five different areas of Kerala state, India viz, Thrissur Municipal Corporation, Kozhikode Corporation, Cochin Municipal Corporation (Ernakulam), Palakkad Municipality and Ponnani Municipality (Malappuram) in the years 2014, 15 and 16. From each of the area, depending upon the mosquito control regimes in existence, collections were made from two different locations i.e., i) regularly insectocode spraying area (T) and ii) scarcely insecticide spraying area (U). In all these areas, temephos was used as a larvicide for the control of *Culex* mosquitoes by the Municipality/corporation. Recently, they have started using *Bacillus thuringiensis* (*Bti*) as larvicide in all the five areas and alternatively, Temephos and *Bti* were predominantly used by Ponnani Municipality (as per the reports of Municipality Health Department). The district vector control unit also sprays temephos as larvicide in all these areas as a control measure for *Culex* mosquitoes. The areas of collection are denoted as MAL- Ponnani Municipality, PKD- Palakkad Municipality, TCR- Thrissur Corporation, CLT - Kozhikode Corporation and EKM - Cochin Municipal Corporation. Susceptible Laboratory Populations were collected from CRME (Centre for Research in Medical Entomology), ICMR, Madurai.

Acetylcholinesterase Assay

30 fourth instar larvae were taken from each of the samples for the assays. The assays were done according to the protocol provided in techniques to detect insecticide resistance mechanism; Field and laboratory Manual.[11] The larvae were homogenized in 200 µl distilled water using a homogenizer. The homogenate was immediately placed on ice. The assays were completed using the homogenate as rapidly as possible to ensure accuracy of results.

2X25 µl of the crude insect homogenate was taken in separate wells of microtiter plate and 145 µl of Triton phosphate buffer (1% Triton X-100 in 0.1 M phosphate buffer pH 7.8) was added to each replicate. 10 µl 0.01M DTNB (Dithiobis2-nitrobenzoic acid) was added to the mixer of each replicate. 25 µl 0.01M ASCHI (Acetylthiocholine iodide) was added to one replicate while 25 µl of 0.01M ASCHI + 0.1M Propoxur was added to the second replicate. Two or more blanks were kept per plate which contain 25 µl distilled water and 10 µl DTNB+25 µl ASCHI+145 µl triton phosphate buffer without insect homogenate. The wells were incubated at room temperature for one hour and readings were taken at 405 nm in a microplate reader as endpoint.

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.201913

Detection of Mutation in ace1 Gene by Restriction Enzyme Digestion

Two primers [Primer 1 (forward) 5´ - CGACTCGGACCCACTCGT - 3´and Primer 2 (reverse) 5´ - GACTTGCGACACGGTACTGCA - 3´] were used to amplify the partial sequence of ace1 (374bp) gene.[12] The PCR conditions were 5 min at 94°C for the first cycle, followed by 1 min at 94°C, 1 min at 60°C and 1min for 72°C for 30 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 374bp band is corresponding to ace1 partial gene. Restriction digestion was done using the Alu1 enzyme. The PCR conditions were 5 min at 94°C for the first cycle, followed by 1 min at 94°C, 1 min at 60°C and 1min for 72°C for 30 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 374bp band is corresponding to ace1 partial gene, it is the susceptible one and it is homozygous (SS) susceptible. Homozygous resistant is represented by RR with 2 bands-one at 272bp and other at 102bp. Heterozygous resistant is represented by 3 bands (SR) - bands at 374bp, 272bp, and 102bp.

Statistical Analysis

Statistical analysis was performed using Statistical package SPSS 20.0 version.

Result

Acetylcholinesterase Activity

Acetylcholinesterase in the nerve synapses is the target of organophosphate and carbamate insecticides. Altered or elevated levels of acetylcholinesterase in the nerve synapse are a major mechanism which contributes to organophosphate resistance. Activity of acetylcholinesterase was found out in uninhibited and propoxur - inhibited fractions of mosquito homogenate from the four field strains and was compared with that of laboratory strain. 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 same insect and multiplying it by 100. A percentage value greater than 30% indicated chance of development of resistance.

Table 1, shows the percentage of remaining activity in the propoxur - inhibited fraction of the *Cx. quinquefasciatus* of field and laboratory sample for a period of three years. The % of remaining activity in propoxur inhibited fraction in the LAB population was less than ten in the years 2014, 15 and 16. In all the five populations, the sample collected from scarcely treated area was less than 30. MAL T population had a value of 32.24±1.8, 31.54±1.2, 33.88±1.4, PKD T had 36.64±1.6, 38.86±2.1, 38.88±2.2, TCR T had 42.36±1.4, 44.54±1.2, 45.88±1.4, CLT T had 38.88±1.5, 44.56±1.4, 45.56±3.2 and EKM T 56.62±2.4, 64.43±1.8, 72.58±2.6% respectively.

Table 1.% Remaining activity in Propoxur inhibited fraction in the field and laboratory populations of Cx.quinquefasciatus

Sample	Year		
	2014	2015	2016
LAB	8.86±1.2	6.98±0.08	7.96±1.1
MAL U	12.54±2.2	12.48±1.4	13.56±1.2
MAL T	32.24±1.8	31.54±1.2	33.88±1.4
PKD U	16.54±1.4	15.68±1.5	17.66±1.2
PKD T	36.64±1.6	38.86±2.1	38.88±2.2
TCR U	15.58±2.1	16.88±1.3	17.66±1.2
TCR T	42.36±1.4	44.54±1.2	45.88±1.4
CLT U	16.66±1.2	18.42±1.2	19.54±1.5
CLT T	38.88±1.5	44.56±1.4	45.56±3.2
EKM U	27.36±1.6	28.42±2.2	28.65±2.1
EKM T	56.62±2.4	64.43±1.8	72.58±2.6

Table 2.Genotypes of Cx. quinquefasciatus of laboratory and field populations obtained by RFLP using Alu I enzyme on the partially amplified ace I gene

Area	Total no. of samples	No. of Bands formed regions after digestion with Alu1		
		SS (374bp)	SR (374,272,102)	RR (272,102)
LAB	15	15	-	-
CLT	15	8	7	-
EKM	15	6	6	3
MPM	15	13	2	-
PKD	15	10	4	-
TCR	15	9	6	-

Isolation of Partial ace1 Gene and RFLP using Alu I Enzyme

DNA was isolated from the samples collected from five different locations, and subjected to amplification of partial portion of ace1 gene. The amplified portion is then digested using Alu1 enzyme to detect the site-specific mutation. 15 samples were taken from each area and three different patterns of bands were formed. A single band in 374bp position indicated there is no mutation and it is homozygous susceptible. A band formed in the 374 position and one band 272 and another band at 102 indicates that one is heterozygous resistant and the band formed in 272bp and 102bp position indicates that sample was homozygous

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.201913

resistant. After digestion with Alu1, the number of bands formed are detected and represented in the Table 2. The homozygous susceptible population i.e., the number of bands formed in the 374bp in CLT, EKM, MPM, PKD and TCR were 8, 6, 13, 10 and 9 and the heterozygous resistant population i.e. the bands formed in 374bp, 272bp and 102bp position were 7, 9, 2, 4 and 6 respectively.

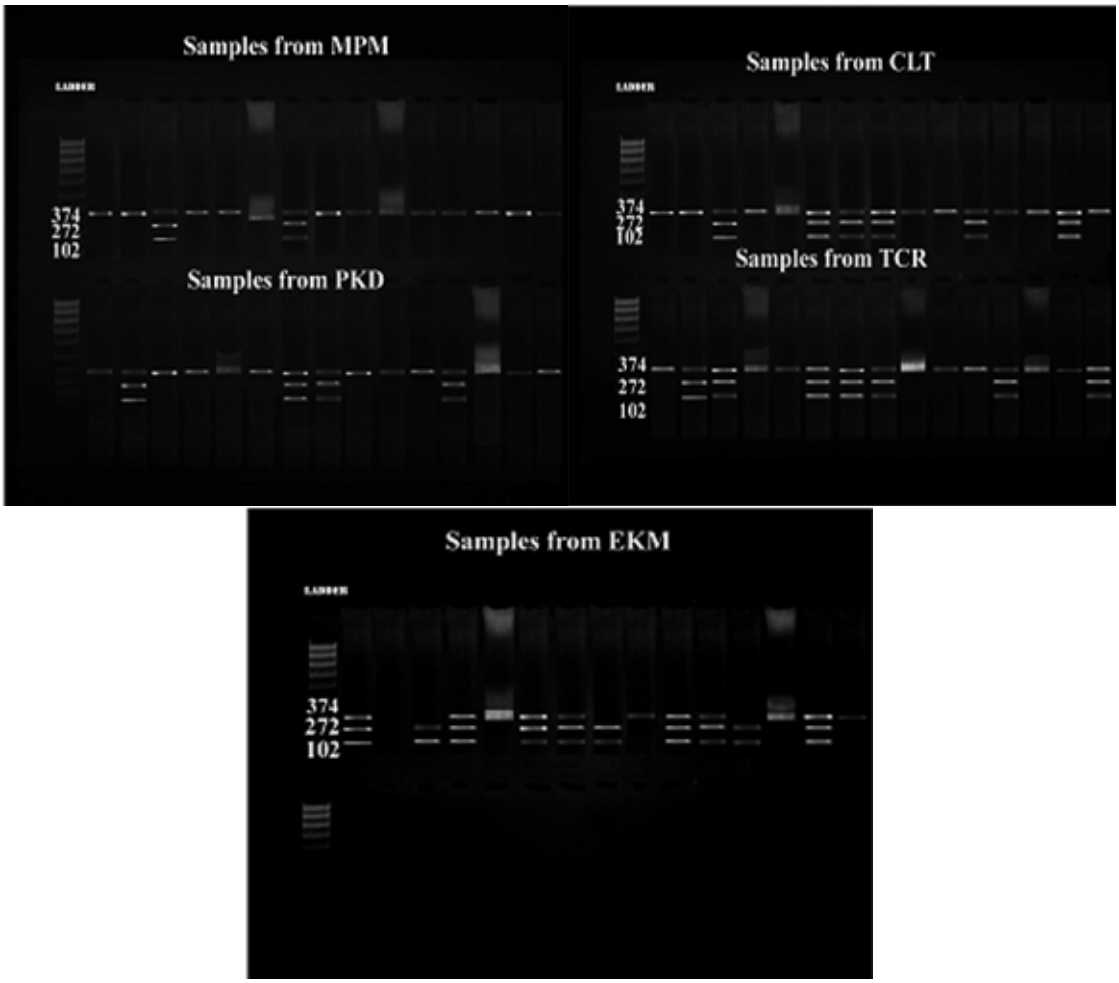

Figure 1.Agarose gel showing ace1 genotypes in the field collected Cx. quinquefasciatus obtained after RFLP using Alu1 enzyme

Discussion

Temephos belongs to the group of organophosphorus insecticides and is a widely used synthetic insecticide in the mosquito control programmes almost throughout Kerala. Hence it is highly imperative to study the progression of the development of resistance in native species of mosquitoes to this insecticide. Owing to the extensive use of this chemical pesticide in the field applications throughout Kerala by the local authorities, it became easy to study the development of resistance as the supply of wild population exposed to Temephos became abundant with steady supply from the areas selected for sampling.

Acetylcholinesterase responsible for neurotransmitter degradation at the cholinergic nerve synapses, is the target of both organophosphate and carbamate insecticides. Selection of a modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism. In natural populations of mosquitoes, high level of resistance to carbamate and organophosphates is provided by insensitive acetylcholinesterase.

The value for the well with propoxur divided by that without propoxur multiplied by 100 gives the % remaining activity in propoxur inhibited replicate rate. Populations with more than 70% remaining activity after inhibition can be characterized as homozygous resistance (RR) with respect to altered AChE mechanism. Populations with 30-70% and less than 30% remaining activity can be categorized as heterozygous (RS) and homozygous susceptible (SS) respectively.[13] Results of assay conducted to identify the insensitivity of AChE to insecticide inhibition by propoxur are presented in the table. In this experiment, all the samples collected from areas treated regularly by insecticides have a value greater than 30% and it indicates the resistance status of those mosquitoes. All the values were highly

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.201913

significant with a P-value less than 0.001. The present results have provided strong evidence on the role of insensitive acetylcholinesterase in the development of organophosphate and carbamate resistance in EKM *Cx. quinquefasciatus.*

The % increase in activity was higher in CLT in the year 2015 as compared with that of 2014. In the year 2016, the activity was higher at EKM field where the *Cx. quinquefasciatus* was collected as compared to the year 2015. From 2014 to 2016, there has been a hike in the activity % of enzymes at EKM. The activity of both CLT collected populations showed similar pattern of increase from the year 2014 to 2016 and the values were 17.287 for scarce treating area and 17.181 for regular treating area.

Acetylcholinesterase, a key enzyme of the central nervous system, is the target of both organophosphate and carbamate insecticides. The common resistance mechanism in numerous arthropod pests is the selection of modified AChE less sensitive to carbamate and organophosphate compounds. Most other insects, other than fruit flies, studied so far possess two ace genes, ace1 and ace2. When insecticide insensitivity has been confirmed functionally, it has been attributed to a point mutation in the *ace*1 gene. To date, only few positions, all lining the active site of *ace*1, have been shown to be involved in insensitivity, suggesting a high structural constraint of this enzyme.[14]

In mosquitoes, only three ace1 amino acid substitutions involved in resistance have been identified so far: G119S, F290V and F331W [numbering according to the *Torpedo californica* AChE nomenclature. The G119S substitution was selected in several species including *An. gambiae, An. albimanus*, *Cx. vishnui* and *Cx. pipiens* and was shown to be widespread in *Cx. pipiens* natural populations.[10, 15]

Number of bands formed while using Alu1 restriction enzyme confirms the level of resistance in each population (Figure 1). Only the EKM population exhibited homozygous resistant mosquitoes. Of the 15 mosquitoes from EKM population, 6 are homozygous susceptible and heterozygous resistant. *Culex* mosquitoes collected from CLT have 8 SS and 7SR and from TCR have 9SS and 6SR genotypes. PKD and MPM *Culex* showed 10SS, 5SR and 13 SS, 2SR genotypes of the 15 mosquitoes collected respectively. The genotypes indicate the urgent need of opting alternate control measures against the field populations of *Culex* mosquitoes.

The PCR diagnostic assay that was performed in the present study detected *ace*1mutations (G119S) in some of the mosquito populations. Nevertheless, very low frequencies of homozygote resistance were found. Expectedly, high frequencies of ace1 mutations were found in mosquitoes in areas where the bioassay test showed the mosquitoes to be resistant to organophosphate and carbamate insecticides, suggesting the involvement of the mutation in the resistance of the mosquito population to the insecticides. Acetyl cholinesterase, the target site for organophosphates and carbamates, is a synaptic enzyme that hydrolyzes the neurotransmitter acetylcholine to terminate nerve impulses, thereby blocking nervous transmission and leading to the death of the insect. Selection of a modified acetylcholinesterase less sensitive to these insecticides has been shown to be a common resistance mechanism in mosquitoes.[15] The low frequency of homozygote resistance can be explained by the high fitness cost that is associated with *ace*1 mutation, such as long development time and decreased male reproductive success.[17] Despite *ace*1 mutations being reported to provide cross resistance to organophosphates and carbamates[16], the resistance level greatly varied between the two classes of insecticides. However, some studies have suggested that *ace*1 mutations have a greater impact on carbamate than organophosphate resistance.

In the present study only at EKM we got homozygous resistant mosquitoes. In all the other populations heterozygous resistant mosquitoes are present. The least number of resistant mosquitoes are seen in MPM and it supports the results of bioassay and biochemical assays.

Conclusion

As a conclusion of the present study, the results from the biochemical assay and PCR assay showed an association between enzyme levels and the degree of insecticide resistance among the *Culex* mosquitoes. The evidence of development of resistance to synthetic insecticides in mosquitoes observed in the present study points to the need of employing new phytochemicals, bio-control measures and IVM strategies in the field as an alternative to synthetic chemical pesticides which would be easily degradable and have less harmful effect on other organisms.

Acknowledgement

We are thankful to UGC- BSR, Delhi, India for the financial support and UGC-SAP for the instrumentation facilities.

Conflict of Interest: None

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ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.201913