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GENOTOXICITY OF ACEPHATE AND PROFENOFOS ASSESSED BY PCR ASSAY

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Abstract: The present study was undertaken to evaluate the genotoxicity of organophosphate pesticides acephate and profenofos by polymerase chain reaction (PCR) using the genetic material of a mosquito *Culex quinquefasciatus* taken as an experimental model. For this purpose, second instar larvae were treated with LC_{20} dose of the pesticides for 24 h and the nucleotide sequence variations in the partial *16S* gene sequences of freshly hatched unfed control and treated individuals were studied. From the sequence alignment data of control and treated individuals, induced mutations in the form of deletion, insertion and substitution of bases were observed in acephate and profenofos treated individuals. It was found that after acephate treatment the *16S* gene sequence suffered 4 deletions, 7 insertions and 19 substitutions while profenofos induced 3 deletions, 5 insertions and 15 substitutions. This study indicates that both the pesticides had significant potential to cause DNA damage for which PCR is a highly sensitive and reliable technique to detect sequence specific errors.

Keywords: Genotoxicity, Acephate, Profenofos, PCR, *Culex quinquefasciatus*

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INTRODUCTION

Pesticides are widely used throughout the world in agriculture to protect crops and in public health to control diseases. As a consequence of their residual accumulation in the environment, a variety of harmful effects have been found in the non-target living systems including man. Organophosphate pesticides are heavily used throughout the world for the control of various pests. The toxicity of organophosphate pesticides is attributed specifically to the inhibition of the enzyme acetylcholinesterase, involved in the regulation of neurotransmission by hydrolysis of the neurotransmitter, acetylcholine. Subsequent accumulation of acetylcholine and consequential over stimulation of acetylcholine receptors has been the chief mechanism of their acute toxicity. [1] Acephate and profenofos are broad spectrum organophosphate pesticides which have been shown to induce chromosomal aberrations, micronuclei, and necrosis in a number of prokaryotes, eukaryotes and mammalian cells. [2-4]

For the evaluation of genotoxic potential of pesticides a variety of test models are being used because the structure and functions of DNA is same in all the experimental organisms, therefore a genotoxic agents would affect them by reacting with certain sites of DNA and modifying it in number of ways such as cleavage of phosphodiester bonds, insertions, deletions and substitutions. In the recent years there had been an increase concern towards reducing the number of higher laboratory animals for research due to ethical issues. This has lead to more emphasis on the use of alternative animal models and in reference to this the present study involves the use of mosquito *Cx. quinquefasciatus* as a test system. Although it differs from the rest in terms of metabolism, DNA repair and physiological processes affecting chemical mutagenesis, yet the universality of DNA and the genetic code provides reasonable rationale to predict the action of mutagens on the genomic integrity of the effected individuals. In this context, flies have been found to be equally as sensitive to toxicants as mammals because some studies have shown that flies and mammals have a similar dose-response relationship. [5-7] An appreciable number of tests and protocols have been developed to measure chromosome and gene mutations. Recent developments in molecular biology have offered new possibilities for detecting DNA damage at the nucleotide level by the application of PCR technique. [8,9] In relevance to this, the present PCR based investigations were undertaken to evaluate the genotoxicity of acephate and profenofos by using the genetic material of a mosquito *Culex quinquefasciatus* taken as an experimental model in which the effect was studied on the partial sequence of *16S* gene. For this, the larvae were treated with LC_{20} of the pesticides and the nucleotide sequence changes were studied from the control and treated sequence alignment

data in which the mutations in the form of insertions, deletions and substitutions were recorded.

MATERIALS AND METHODS

Test chemicals

For the present study, technical-grade acephate (75% SP) and profenofos (50% EC) manufactured by Scientific Fertilizers Co. Pvt. Ltd., Coimbatore, India, were used. In order to assess the toxicity of a chemical, it is always crucial to determine a suitable dose for its effective action in the test system. Accordingly, LC_{20} was found to be an ideal dose which was standardized by probit analysis [10] that gave these values as 5 µg/ml and 5.19µl/ml for acephate and profenofos respectively.

Test organism

Culex quinquefasciatus Say, used as an experimental insect for the present investigations was collected in the early morning from the cattle sheds and human dwellings. The gravid females of the species were held in the test tubes where they were allowed to oviposit on a strip of wet filter paper. A larval colony was raised from these eggs in a BOD incubator by feeding the stocks with a diet consisting of finely powdered dog biscuits and yeast tablets. [11,12] The treatment was given to the second instar larvae for which they were kept in standardized dose of pesticide for 24 h after which they were transferred to pesticide free water for further growth up to adult stages. For each set of experiments separate stocks of parallel controls were also maintained under similar conditions and freshly hatched unfed adults were stored in separate eppendorf tubes at –20°C for DNA extraction.

Extraction of DNA and Polymerase Chain Reaction

The DNA was extracted from individual adult mosquitoes by using Phenol-Chloroform extraction method [13] and the purity of extracted samples was determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The isolated DNA from the control and treated samples was resolved on 0.8% agarose gel containing ethidium bromide in 1X TAE (Tris–acetate–EDTA) buffer at 85 V and the DNA bands were visualized and photographed on UV transilluminator. Partial sequence of *16S* gene was amplified using forward and reverse primers *viz*: 5'-CGCCTGTTTATCAAAAACAT-3' and 5'-CTCCGGTTTGAACTCAGATC-3' respectively. [14] PCR amplification was performed by using 25µl reaction mixture containing 0.2mM dNTP mix, 1X buffer, 1mM $MgCl_2$, 1U Taq polymerase, 0.2µM primers and 2µl of DNA template. The amplification process was carried out as per the protocol of Williams *et al*. [15] according to which, 25 µl of reaction mixture was loaded in a thermocycler which was programmed for the initial one cycle for denaturation of DNA at 94°C for 10 mins followed by 35 cycles each of denaturation, annealing of primer and extension of DNA at 94°C for 1min,

56°C for 1 min and 72°C for 1 min respectively terminating with a final extension at 72°C of 5 mins. In all such amplifications, a negative control consisting of all the components of reaction mixture except the DNA was also carried out so as to rule out the experimental errors. The PCR products and DNA ladder were electrophorased on 2% agarose gel containing ethidium bromide and visualized on ultraviolet transilluminator. These amplified products were sequenced and the DNA sequences were aligned using ClustalW multiple sequence alignment program.

RESULTS AND DISCUSSION

As the result of the treatment with pesticides, three different types of mutations in the form of deletions, insertions and substitutions of bases were encountered in the partial sequences of *16S* gene. In the aligned sequences from control and treated individuals asterisk (*) show the identical bases. Dashes (–) in the control sequence indicate insertion of bases while dashes in treated sequence mark the deletions. The rest of the places show differences in the complementary bases in the two types of sequence. These were the regions where substitutions had taken place due to transitions and transversions wherein transitions are the loci where purines were replaced by purines or pyrimidines by pyrimidines while transversions were the sites where purines were replaced by pyrimidines and vice versa (Figures 1 and 2). According to these parameters of sequence comparison *16S* amplicon from control individuals yielded sequence of 543 bp whereas acephates and profenofos treated individuals consisted of sequence of 546 and 545 bp respectively. The sequence of acephate treated individual suffered as many as 30 different types of mutations which included 4 deletions, 7 insertions and 19 substitutions in which there were 6 transitions and 13 transversions. There was a stretch of 4 bases GAAA which was deleted from position 8 to 11 while a stretch of 7 bases AAGAAAC was inserted at the end of the sequence whereby the total length of the treated sequence increased by three bases. It was observed that acephate caused maximum deletions and insertions of adenine base (Table 1, Figure 1). Profenofos treatment resulted in 23 mutations which included 3 deletions, 5 insertions and 15 substitutions consisting of 5 transitions and 10 transversions. Out of the 3 deletions, 2 with a sequence CG were located at the position of first two bases while T was deleted from a position 15 in the sequence. A stretch of 5 bases ATCGC got inserted at the end of the sequence which increased the length of the treated sequence by two bases (Table 2, Figure 2). From this data it was evident that acephate treatment caused more mutations as compared to pofenofos. With this it was also noticed that these pesticides had greater tendency of substitution of thymine with guanine and adenine i.e. T→G and T→A.

The results obtained in the current investigation are in the agreement with the findings of many workers, who have shown that these pesticides are able to induce a variety of changes in the genomic integrity of the affected individuals. For example, it was seen that acephate caused chromosomal aberrations and micronuclei in the bone marrow cells and peripheral blood erythrocytes of chicks [16] and intercalary heterchromatic linkages in the larval polytene chromosomes of *Anopheles subpictus*. [3] A significant increase in sister chromatid exchange along with the decreased mitotic index in human peripheral lymphocytes were also observed. [17] As for the genotoxic impact of profenofos it has also been reported to induce different types of chromosomal aberrations in the germ cells of mice [18] and apoptosis, necrosis and chromatid breaks in cultured human peripheral blood lymphocytes. [4] In one of the recent studies in this laboratory to assess the effects of neonicotinoid pesticides imidacloprid and thiamethoxam on the sequence of internal transcribed spacer (ITS2) of *Anopheles stephensi*, a significant increase in the incidence of induced nucleotide mutations were observed at several loci along the amplified sequences. [19] Results obtained in the present and in the related other studies have shown that acephate and profenofos are DNA damaging chemicals as organophosphate pesticides are known to act as alkylating agents and alkylation of DNA bases either directly or indirectly via protein alkylation is involved in the DNA disintegration. The phosphorus moiety in such organophosphates acts as a good substrate for nucleophilic attack which cause phosphorylation of DNA. As for the possible action of pesticides it is claimed that most of these chemical formulations significantly increase the cellular reactive oxygen species (ROS) leading to modifications in the DNA in the form of base pair errors and strand breaks. [20-22]

In conclusion, the present data demonstrate that acephate and profenofos could induce mutations in living organisms. This study finds the use of PCR as a reliable and highly sensitive technique for detecting pesticide related sequence specific DNA damage. PCR assay can be used in combination with other tests for screening mutagenic effect of chemicals and for investigating the implications of DNA damage that can provide information at molecular level which may be used to determine the potential of a chemical to induce carcinogenicity.

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Table 1: Mutations in *16S* gene sequence of acephate treated *Cx. quinquefasciatus*.

Type of mutation	Total number of mutations	Type of bases mutated	Position of mutated bases in the sequence
Deletion	4	GAAA	8-11
Insertion	7	AAGAAAC	after 543
Transition	6	A→G	22
		G→A	155, 483
		C→T	68, 414
		T→C	541
Transversion	13	A→T	259
		T→A	401, 402
		T→G	14, 15, 209
		G→T	527
		C→G	3, 31
		G→C	370
		C→A	104, 198
		A→C	437

Table 2: Mutations in *16S* gene sequence of profenofos treated *Cx. quinquefasciatus*.

Type of mutation	Total number of mutations	Type of bases mutated	Position of mutated bases in the sequence
Deletion	3	CG	1, 2
		T	15
Insertion	5	ATCGG	after 543
Transition	5	A→G	9, 499
		G→A	76, 213
		C→T	-
		T→C	391
Transversion	130	A→T	170
		T→A	280, 315, 519
		T→G	349, 376
		G→T	-
		C→G	409
		G→C	-
		C→A	198, 435
		A→C	205

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CLUSTAL 2.1 multiple sequence alignment

CONTROL         CGCTGTTGAAAATTTAAGTCTACCTGCCCACTGATATAAATTAAAGGGCCGCAGTATTTT 60
TREATED         CGGTGTT----ATGGAAGTCTGCCTGCCCAGTGATATAAATTAAAGGGCCGCAGTATTTT 56
                ** ****    **  ****** ******** ******************************

CONTROL         GACTGTGCGAAGGTAGCATAATCACTAGTCTTTTAATTGGAGGCTTGTATGAATGGTTGA 120
TREATED         GACTGTGTGAAGGTAGCATAATCACTAGTCTTTTAATTGGAGGATTGTATGAATGGTTGA 116
                ******* ********************************** ****************

CONTROL         ATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTAATTAAAAAGTT 180
TREATED         ATGAGATATATACTGTCTTTTTTAAAATTATATAAAATTTTATTTTTTAATTAAAAAGTT 176
                ********************************** ************************

CONTROL         AAAATAAAATTAAAGGACGAGAAGACCCTATAGATCTTTATTTTTGTTATTTATAAATTA 240
TREATED         AAAATAAAATTAAAGGAAGAGAAGACCCGATAGATCTTTATTTTTGTTATTTATAAATTA 236
                ********************* ********** ********************************

CONTROL         AAAAGAATTTTAAAATTTATAATTTAATAAAAAATTTTATTGGGGTGATATTAAAATTTA 300
TREATED         AAAAGAATTTTAAAATTTTTAATTTAATAAAAAATTTTATTGGGGTGATATTAAAATTTA 296
                ****************** ******************************************

CONTROL         AAAAACTTTTAAAATTTATTAACATAAATATATGAATAAATGATCCAGTTTTATTGATTA 360
TREATED         AAAAACTTTTAAAATTTATTAACATAAATATATGAATAAATGATCCAGTTTTATTGATTA 356
                ************************************************************

CONTROL         AAAATTTAAGTTACCTTAGGGATAACAGCGTAATTTTTTTTTAGAGTTCATATCGACAAA 420
TREATED         AAAATTTAACTTACCTTAGGGATAACAGCGTAATTTTTTTAAAGAGTTCATATTGACAAA 416
                ********* ******************************  ************ ******

CONTROL         AAAGATTGCGACCTCGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAGTTT 480
TREATED         AAAGATTGCGACCTCGCTGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAGTTT 476
                *************** ******************************************

CONTROL         AGGTCTGTTCGACCTTTGAATTCTTACATGATCTGAGTTCAAACCGGAGATGATCTGAGT 540
TREATED         AGATCTGTTCGACCTTTGAATTCTTACATGATCTGAGTTCAAACCGTAGATGATCTGAGT 536
                ** ****************************************** *************

CONTROL         TCA------- 543
TREATED         CCAAAGAAAC 546
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Figure 1: Multiple sequence alignment of *16S* gene sequences of control and acephate treated *Cx. quinquefasciatus*.

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CLUSTAL 2.1 multiple sequence alignment

CONTROL         CGCTGTTGAAAATTTAAGTCTACCTGCCCACTGATATAAATTAAAGGGCCGCAGTATTTT 60
TREATED         --CTGTTGGAAATT-AAGTCTACCTGCCCACTGATATAAATTAAAGGGCCGCAGTATTTT 57
                  ****** ***** ********************************************

CONTROL         GACTGTGCGAAGGTAGCATAATCACTAGTCTTTTAATTGGAGGCTTGTATGAATGGTTGA 120
TREATED         GACTGTGCGAAGGTAACATAATCACTAGTCTTTTAATTGGAGGCTTGTATGAATGGTTGA 117
                ********************************************************

CONTROL         ATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTAATTAAAAAGTT 180
TREATED         ATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTATTTAAAAAGTT 177
                ************************************************** **********

CONTROL         AAAATAAAATTAAAGGACGAGAAGACCCTATAGATCTTTATTTTTGTTATTTATAAATTA 240
TREATED         AAAATAAAATTAAAGGAAGAGAAGCCCCTATAAATCTTTATTTTTGTTATTTATAAATTA 237
                ***************** ****** ******* **************************

CONTROL         AAAAGAATTTTAAAATTTATAATTTAATAAAAAATTTTATTGGGGTGATATTAAAATTTA 300
TREATED         AAAAGAATTTTAAAATTTATAATTTAATAAAAAATTTTAATGGGGTGATATTAAAATTTA 297
                ************************************** ********************

CONTROL         AAAAACTTTTAAAATTTATTAACATAAATATATGAATAAATGATCCAGTTTTATTGATTA 360
TREATED         AAAAACTTTTAAAAATTATTAACATAAATATATGAATAAATGATCCAGGTTTATTGATTA 357
                ************** ********************************** ***********

CONTROL         AAAATTTAAGTTACCTTAGGGATAACAGCGTAATTTTTTTTTAGAGTTCATATCGACAAA 420
TREATED         AAAATTTAAGTTACCGTAGGGATAACAGCGCAATTTTTTTTTAGAGTTGATATCGACAAA 417
                ************** ************** ***************** ***********

CONTROL         AAAGATTGCGACCTCGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAGTTT 480
TREATED         AAAGATTGCGACCTAGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAGTTT 477
                ************** ************************************************

CONTROL         AGGTCTGTTCGACCTTTGAATTCTTACATGATCTGAGTTCAAACCGGAGATGATCTGAGT 540
TREATED         AGGTCTGTTCGACCTTTGGATTCTTACATGATCTGAGTACAAACCGGAGATGATCTGAGT 537
                ****************** ****************** ********************

CONTROL         TCA----- 543
TREATED         TCAATCGG 545
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Figure 2: Multiple sequence alignment of *16S* gene sequences of control and profenofos treated *Cx. quinquefasciatus*.

REFERENCES

1. Costa LG: Current issues in organophosphate toxicology. Clin Chim Acta. 2006; 366: 1-13.

2. Rahman MF, Mahboob M, Danadevi K, Saleha Banu B and Grover P: Assessment of genotoxic effects of chlorpyriphos and acephate by the comet assay in mice leucocytes. Mutat Res. 2002; 516: 139-1347.

3. Chaudhry A, Anand PK, Geeta, Singh S and Lovleen: Ectopic pairing of the intercalary heterochromatin in the organophosphate pesticide treated mosquito chromosomes (Culcidae: Diptera). Cytologia, 2006; 71: 431-437.

4. Prabhavathy Das G, Shaik AP and Jamil K: Cytotoxicity and genotoxicity induced by the pesticide profenofos on cultured human peripheral blood lymphocytes. Drug Chem Toxicol. 2006; 29: 313-322.

5. Siddique HR, Chowdhuri DK, Saxena DK and Dhawan A: Validation of *Drosophila melanogaster* as an *in vivo* model for genotoxicity assessment using modified alkaline Comet assay. Mutagenesis, 2005; 20: 285–290.

6. Gupta SC, Mishra M, Sharma A, Deepak Balaji TG, Kumar R, Mishra RK and Chowdhuri DK: Chlorpyrifos induces apoptosis and DNA damage in *Drosophila* through generation of reactive oxygen species. Ecotoxicol Environ Saf. 2010; 73: 1415-1423.

7. Mishra N and Tewari RR: Cytotoxic and genotoxic effects of mercury in house fly *Musca domestica* (Diptera: Muscidae). Cell Mol Biol. 2011; 57: 122-8.

8. Savva D: Use of DNA fingerprinting to detect genotoxic effects. Ecotoxicol Environ Saf 1998; 41: 103-6.

9. De Wolf H, Blust R and Backeljau T: The use of RAPD in ecotoxicology. Mutat Res. 2004; 566: 249-62.

10. Finney DJ: The computing of probit analysis. University Press, Cambridge, Second edition, 1971.

11. Singh KRP, Patterson RS, La-Brecque GC and Razdan RK: Mass rearing of *Culex pipiens fatigans* Weid. J Com Dis. 1975; 7: 31-53.

12. Clements AN: The biology of mosquitoes. Chapman and Hall, London, 1996.

13. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K: Short protocols in molecular biology. John-Wiley and Sons, Inc, New York, Fourth edition, 1999.

14. Shouche YS and Patole MS: Sequence analysis of mitochondrial *16S* ribosomal RNA gene fragment from seven mosquito species. J Biosci. 2000; 25: 361-366.

15. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990; 18: 6531-6535.

16. Jena GB and Bhunya SP: Mutagenicity of an organophosphate insecticide acephate an *in vivo* study in chicks. Mutagenesis, 1994; 9: 319-324.

17. Ozkan D, Yuzbasioglu D, Unal F, Yilmaz S and Aksoy H: Evaluation of the cytogenetic damage induced by the organophosphorus insecticide acephate. Cytotechnology, 2009; 59: 73-80.

18. Fahmy MA and Abdalla EF: Genotoxicity evaluation of buprofezin, petroleum oil and profenofos in somatic and germ cells of male mice. J Appl Toxicol. 1998; 18: 301-305.

19. Bhinder P, Chaudhry A, Barna B and Kaur S: Imidacloprid and thiamethoxam induced mutations in internal transcribed spacer 2 (ITS2) of *Anopheles stephensi*. Toxicol Int. 2012; 19: 201-206.

20. Poovala VS, Kanji VK, Tachikawa H and Salahudeen AK: Role of oxidant stress and antioxidant protection in acephate-induced renal tubular cytotoxicity. Toxicol Sci. 1998; 46: 403-409.

21. Saleha Banu B, Danadevi K, Rahman MF, Ahuja YR and Kaiser J: Genotoxic effect of monocrotophos to sentinel species using comet assay. Food Chem Toxicol. 2001; 39: 361-366.

22. Hreljac I, and Filipic M: Organophosphorous pesticides enhance the genotoxicity of benzo(a)pyrene by modulating its metabolism. Mutat Res. 2009; 671: 84-92.