Genotoxicity Evaluation of Acephate and Profenofos by the PCR-RFLP Assay

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ABSTRACT

Objectives: In this study we have evaluated the genotoxic potential of pesticides acephate and profenofos by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay with the mosquito *Culex quinquefasciatus* taken as experimental model. **Material and Methods:** Second instar larvae were treated with LC_{20} of each pesticide for 24 h and induced mutations in the sequence of mitochondrial *16S* rRNA gene were studied from restriction patterns generated with *Pac*I and *Psi*I restriction endonucleases. **Results:** Variations in the number and size of digested fragments were recorded from treated individuals compared with controls showing that the restriction enzymes created a cut at different locations. In addition, sequences of the *16S* gene from control and treated individuals were also used to confirm the RFLP patterns. From the sequence alignment data, it was found that mutations caused the destruction and generation of restriction sites in the gene sequence of treated individuals. **Conclusion:** This study indicates that both the pesticides had significant potential to induce mutations in the *16S* gene of *Culex quinquefasciatus*.

Key words: Acephate, culex quinquefasciatus, genotoxicity, pcr-rflp, profenofos

INTRODUCTION

Organophosphorus insecticides are among the most widely used synthetic chemicals for the control of agricultural and domestic insect pests. The rampant use of these pesticides has created a chemical environment which is proving harmful to the living system. Organophosphate exposures have been associated with genotoxicity, neurotoxicity and reproductive toxicity.[1-3] As a consequence of this, constant monitoring of their genotoxicity has become the priority areas of research. For the evaluation of genotoxic action of pesticides a number of tests or protocols have been developed by using bacteria, yeast, insects and mammals as experimental models. 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 *Culex 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.[4-7]

In relevance to this, the present PCR-RFLP based investigations were undertaken for genotoxicity assessment of two organophosphate pesticides acephate and profenofos by using the genetic material of a mosquito *Cx.*

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quinquefasciatus taken as an experimental model. The procedure helped in measuring the extent of mutations which tend to alter a restriction endonuclease recognition sequence. It involves the PCR amplification of a specific region of DNA followed by restriction enzyme digestion of the PCR products. Mutations are detected by the loss or generation of a restriction site which are seen in the form of variation in the number and size of restriction fragments. In the present study, a region of the mitochondrial *16S* rRNA gene was amplified from control and pesticide treated individuals which was then digested with *Pac*I and *Psi*I restriction endonucleases and the RFLP patterns generated from control and treated individuals were compared.

MATERIALS AND METHODS

Test chemicals

For the present study, acephate (75% SP) and profenofos (50% EC) manufactured by Scientific Fertilizers Co. Pvt. Ltd., Coimbatore, India, were purchased from market. 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, LC20 was found to be an ideal concentration and the LC_{20} values for acephate and profenofos as calculated by probit analysis were 5 and 5.19 μl/ml, respectively.

Test organism

Cx. 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 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.[8] The chemical treatment was given to the second instar larvae for which they were kept in standardized dose of the pesticide for 24 h after which they were transferred to pesticide free water for further growth up to adult stages. Freshly hatched unfed adults were stored in separate Eppendorf tubes at –20°C for DNA extraction.

Amplification

The DNA was extracted from individual adult mosquitoes by following the protocol of Ausubel *et al.*[9] according to which each specimens of freshly hatched unfed adult were homogenized. A portion of the *16S* gene was amplified using forward primer 5'-CGCCTGTTTATCAAAAACAT-3' and reverse primer 5'-CTCCGGTTTGAACTCAGATC-3'.[10] PCR amplification was performed in a 25 μl reaction volume containing 0.2 mM dNTP mix, 1X buffer, 1 mM $MgCl_2$, 1U Taq polymerase, 0.2 μM primers and 2 μl of DNA template. The amplification reactions was performed as described by Williams *et al.*[11] according to which, each of the 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 min. This was followed by 35 cycles each of denaturation, annealing of primer and extension of DNA at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, respectively. This was followed by final extension at 72°C of 5 minutes. 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 the ClustalW multiple sequence alignment program.

Restriction digestion

After amplification 4 μl PCR product was digested with sufficient units of selected restriction enzymes in 2 μl of buffer for 5 hours at 37°C. Reactions were terminated by incubation at 70°C for 15 minutes. Digested fragments were resolved on 2% agarose gel with ethidium bromide staining and photographed on ultraviolet transilluminator.

RESULTS AND DISCUSSION

In the present PCR-RFLP analysis, *16S* amplicon from both control and treated stocks were digested with *Pac*I and *Psi*I restriction endonucleases and the resulting digested PCR products were then isolated by using 2% agarose gel. The DNA band patterns generated from control and treated individuals were compared. This was followed by *in silico* restriction enzyme analysis of *16S* gene sequences with NEBcutter software for validation of results. NEBcutter helped in obtaining the actual fragment number and fragment size. The fragment sizes obtained from NEBcutter software and those observed experimentally showed congruency in the results. The only difference encountered in some cases was the lack of one very small fragment that was difficult to discern on agarose gel. In addition, sequences of the *16S* gene from control and treated individuals were used to confirm the RFLP pattern. The RFLP pattern generated from non-treated *Cx. quinquefasciatus 16S* amplicon indicates that there was one nicking site for *Pac*I which resulted in the production of two bands of 171 and 372 bp while there were two sites for *Psi*I that yielded three fragments of 284, 233 and 26 bp. Due to its small size, the 26 bp band was not visible on 2% agarose gel. In the acephate-treated individual *Pac*I produced two bands of 167 and 379 bp. The change in the expected length of fragments from *Pac*I digestion was due to the rearrangement in the sequence which occurred due to deletion of four bases from the sequence from position 8 to 11. Digestion with *Psi*I produced two bands of 229 and 317 bp length as one of its restriction sites was destroyed by a mutation that replaced adenine with thymine (A → T) at base 259 [Table 1, Figures 1 and 2]. The PCR product of profenofos treated individual remained undigested by enzyme *Pac*I as a transversion from A → T at base 170 destroyed the restriction site previously present in normal sequence while *Psi*I yielded 289, 230 and 26 bp fragments whose length changed due to rearrangement in the sequence which occurred due to deletion of three bases at position 1, 2 and 15 [Table 1, Figures 3 and 4].

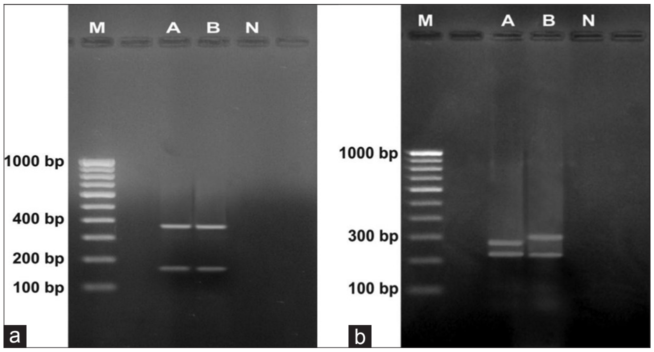

Figure 1: RFLP pattern obtained after *Pac*I (a) and *Psi*I (b) digestion of the *16S* amplicon of control and acephate-treated *Cx. quinquefasciatus*. Lane M: gene ruler, Lane A: RFLP pattern from control individual, Lane B: RFLP pattern from treated individual, Lane N: negative control

This investigation has shown that both the pesticides induced mutations which were evident from the variations in the restriction pattern of treated individuals from control individuals. These differences resulted from base substitutions, insertions, deletions or sequence rearrangements within the restriction enzyme recognition sequences. From the sequence alignment data it was found that mutations caused the destruction and generation of restriction sites in the *16S* gene sequence of treated individuals. The presence of undigested DNA fragments indicated that a mutation had destroyed a restriction site previously present in the normal sequence. When a mutation generated a new restriction site, the sequence was cleaved by the specific restriction endonuclease while the

Table 1: PCR-RFLP product sizes of the 16S gene sequence of control and treated *Culex quinquefasciatus*

Type of sample	PCR product size (bp)	PCR-RFLP product size (bp)	
		*Pac*I	*Psi*I
Control	543	372, 171	284, 233, 26
Acephate treated	546	379, 167	317, 229
Profenofos treated	545	545*	289, 230, 26

* PCR product not digested (no restriction site), RFLP = Restriction fragment length polymorphism

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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 2: Restriction sites of *Pac*I (TTAATTAA) and *Psi*I (TTATAA) in *16S* gene sequences of control and acephate treated *Cx. quinquefasciatus*

normal sequence remained unaltered. Studies carried out so far on the mutational activity of acephate and profenofos have shown that these pesticides were able to induce a variety of changes in the genomic integrity of the affected individuals. For example, acephate has been reported to increase the incidence of chromosomal aberrations and micronuclei in bone marrow and peripheral blood erythrocytes of chicks[12] and intercalary heterchromatic linkages in the polytene chromosomes of treated larvae of *Anopheles subpictus*.[13] A significant increase in sister chromatid exchange along with the decreased mitotic index in human peripheral lymphocytes was also observed.[14] Profenofos has been reported to induce different types of chromosomal aberrations in the germ cells of mice.[15] It also induced apoptosis, necrosis, chromatid breaks and single-strand breaks in cultured human peripheral blood lymphocytes.[16]

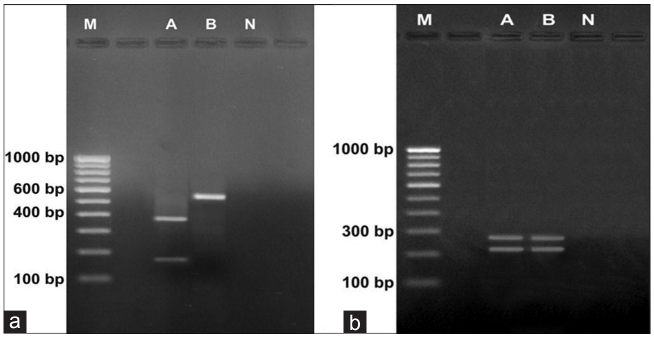

Figure 3: RFLP pattern obtained after *Pac*I (a) and *Psi*I (b) digestion of the *16S* amplicon of control and profenofos-treated *Cx. quinquefasciatus.* Lane M: gene ruler, Lane A: RFLP pattern from control individual, Lane B: RFLP pattern from treated individual, Lane N: negative control

Results obtained from the present research work and studies carried out so far showed that acephate and profenofos are DNA-damaging chemicals. It is known that major biological reactions of organophosphate pesticides are phosphorylation and alkylation. The phosphorous moiety in organophosphorus pesticides acts as a good substrate for nucleophilic attack leading to DNA damage and alkylation of DNA bases either directly or indirectly via protein alkylation is responsible for DNA disintegration. It has been reported that most of the pesticides which produce genotoxic effects have been known to form reactive oxygen species (ROS) as well as electrophilic free-radical metabolites which interacts with DNA to induce DNA strand breaks.[17-19]

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

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

CONTROL         ATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTAATTAAAAAGTT 180
TREATED         ATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTTATTTAAAAAGTT 177
                ************************************************* **********

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

CONTROL         AAAAGAATTTTAAAATTTATAATTTAATAAAAAATTTATTGGGGTGATATTAAAATTTA 300
TREATED         AAAAGAATTTTAAAATTTATAATTTAATAAAAAATTTAATGGGGTGATATTAAAATTTA 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 4: Restriction sites of *Pac*I (TTAATTAA) and *Psi*I (TTATAA) in *16S* gene sequences of control and profenofos treated *Cx. quinquefasciatus*

In conclusion, findings of this investigation indicated that acephate and profenofos could induce mutations in living organisms. The present study advocates the use of the PCR-RFLP assay as an efficient, rapid and sensitive technique for the detection of genotoxic effects of pesticides and also suggestive of the fact that sufficient caution is required in the use of these pesticides in agricultural and non-agricultural arenas.

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