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

Preety Bhinder, Asha Chaudhry

Department of Zoology, Punjab University, Chandigarh, India

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 LC20 of each pesticide for 24 h and induced mutations in the sequence of mitochondrial 16S rRNA gene were studied from restriction patterns generated with PacI and PsiI 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. 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.

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.
*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 *Pst*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 LC20 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 kept 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. 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.* according to which each specimens of freshly hatched unfed adult were homogenized. A portion of the 16S gene was amplified using forward primer 5′-CGCCTGTTTATCAAAAAACAT-3′ and reverse primer 5′-CTCCGTTTGAACCTAGATC-3′. PCR amplification was performed in a 25 µl reaction volume containing 0.2 mM dNTP mix, 1X buffer, 1 mM MgCl₂, 1U Taq polymerase, 0.2 µM primers and 2 µl of DNA template. The amplification reactions was performed as described by Williams *et al.* 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 for 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 electrophoresed 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 *Pst*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 *Pst*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, 238 and 26 bp. Due to its small size, the 26 bp band was not visible on 2% agarose gel. In the acephate-treated individual *Pst*I produced two bands of 167 and 379 bp. The change in the expected length of fragments from *Pst*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.
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) | PacI       | PsiI       |
|---------------------|-----------------------|------------|------------|
| 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

Figure 1: RFLP pattern obtained after PacI (a) and PsiI (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

Figure 2: Restriction sites of PacI (TAAATTAA) and PsiI (TAATTA) in 16S gene sequences 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
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 heterochromatic 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.\[^{16}\]

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. The phosphorous moiety in organophosphorus pesticides acts as a good substrate for nucleophilic attack leading to DNA damage and alkylation. The phosphorous moiety in organophosphorus pesticides acts as a good substrate for nucleophilic attack leading to DNA damage and alkylation.

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**Figure 3:** RFLP pattern obtained after *PacI* (a) and *PsiI* (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

| Restriction Enzyme | Sequence of CONTROL | Sequence of TREATED | Lane |
|--------------------|--------------------|--------------------|------|
| *PacI*             | CGGTGTTGAAAAATTTTGAAGTCCTACCTGGCCACTGATAATAATTTAAGGCGCAGTATTTT 60 |
|                    | CTGTGTTGAATTTAAGGTCCTACCTGGCCACTGATAATAATTTAAGGCGCAGTATTTT 57 |
| *PsiI*             | GACTGTCCGAAGTGCTATAATACACTCTCTTTTTATTGGGCTTGTATGATGTTGTA 120 |
|                    | GACTGTCCGAAGTGCTATAATACACTCTCTTTTTATTGGGCTTGTATGATGTTGTA 117 |
|                    | ATGAGATATATACCTGTTTTTTAAAATTATAGTTTTATTTTTATAATTTTTATTTTT 180 |
|                    | ATGAGATATATACCTGTTTTTTAAAATTATAGTTTTATTTTTATTTTTATTTTT 177 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 240 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 237 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 300 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 297 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 360 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 357 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 420 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 417 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 480 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 477 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 540 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 537 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 540 |
|                    | AAAAAATTTAAAAAGGACGGAGAGACCCTTAGACCTTTATTTTTTTGTTATTTTTATTTTT 537 |

**Figure 4:** Restriction sites of *PacI* (**TAATTA**) and *PsiI* (**TATA**) in 16S gene sequences of control and profenofos treated *Cx. quinquefasciatus*. 

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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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