JEB
Journal of Environmental Biology

Journal Website : www.jeb.co.in
E-mail : editor@jeb.co.in
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Chlorpyrifos induced gene mutations in *Culex quinquefasciatus*

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Publication Info

Paper received:
16 April 2013

Revised received:
22 September 2013

Re-revised received:
16 December 2013

Accepted:
05 May 2014

Abstract

Mutagenicity of chlorpyrifos was evaluated by using the genetic material of mosquito *Culex quinquefasciatus*. Second instar larvae were treated with LC_{20} of chlorpyrifos for 24 hrs and induced mutations in the sequence of mitochondrial *16S* rRNA gene (543bp) were studied from restriction patterns generated with *Alu*I, *Pac*I and *Psi*I restriction endonucleases. Variation in the number and size of digested fragments were observed from treated individual when compared with control, showing that the restriction enzymes created a cut at unrelated locations. In addition, sequences of *16S* gene from control and treated individuals were also used to confirm the RFLP patterns. When *16S* gene sequences of control and treated individuals were compared, induced mutations in the form of deletion, insertion and substitution of bases were observed in chlorpyrifos treated individual. It was found that after chlorpyrifos treatment, *16S* gene sequence suffered 4 deletions, 8 insertions and 23 substitutions.

Key words

Chlorpyrifos, *Culex quinquefasciatus*, Mutagenicity

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Introduction

Chlorpyrifos is one of the most widely used organophosphate pesticides for the control of various pests (Donaldson *et al.*, 2002). The primary mechanism of action of chlorpyrifos involves inhibition of acetylcholinesterase resulting in a wide range of neurotoxic effects (Costa, 2006). Chlorpyrifos has been shown to induce mitotic abnormalities and cytotoxicity (Vera-Candioti *et al.*, 2014), immunologic abnormalities (Elelaimy *et al.*, 2012) and reproductive toxicity (Joshi *et al.*, 2007). In addition to that, chlorpyrifos also caused changes in some haematological and biochemical parameters (Kazmi *et al.*, 2003; Jacobson *et al.*, 2004).

The genotoxic effects of pesticides are determined by a number of tests using bacteria, yeast, insects and mammals as experimental models (Giri *et al.*, 2002; Siddique *et al.*, 2005; Esteve *et al.*, 2009). In recent years, there has been an increased concern towards reducing the number of higher laboratory animals for research due to ethical issues (Siddique *et al.*, 2005). This has lead to more emphasis on using alternative animal models, and in reference to this the present study involved the use of *C. quinquefasciatus* mosquito as a test system (Gupta *et al.*, 2010; Mishra and Tiwari, 2011). Although it differs from the rest in terms of metabolism, DNA repair and physiological processes affecting chemical mutagenesis, yet the universality of DNA and genetic code provides reasonable rationale to predict the action of mutagens on the genomic integrity of the affected organisms.

An appreciable number of protocols have been developed to measure chromosome and gene mutations. The developments in molecular biology have offered new possibilities for detecting DNA damage at the nucleotide level by applying PCR technique (Suenaga *et al.*, 2013; De Wolf *et al.*, 2004). Taking these considerations into account, the aim of this research was to evaluate the mutagenicity of chlorpyrifos by PCR-RFLP assay, using the genetic material of *C. quinquefasciatus* in which the effect was studied on partial sequence of *16S* gene.

Materials and Methods

In this study, chlorpyrifos (40% EC) manufactured by Scientific Fertilizers Co. Pvt. Ltd., Coimbatore, (India), was used as test chemical. 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. The dose should neither be too strong to considerably reduce the population of test organisms nor too weak to give misleading data about its safe use. Accordingly, LC_{20}

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was found to be an ideal dose which was standardized by probit analysis (Finney, 1971). In the present study LC_{50} value was 3.46 l ml^{-1}. *Culex quinquefasciatus*, used as an experimental insect for the present investigations, was collected in early morning from cattle sheds and human dwellings. The gravid females of the species were held in 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 (Singh *et al*., 1975; Clements, 1996). The treatment was given to the second instar larvae which were kept in standardized dose of pesticide for 24 hrs. They were then 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 eppendorf tubes at –20°C for DNA extraction.

DNA was extracted from individual adult mosquitoes by using Phenol-Chloroform extraction method (Ausubel *et al*., 1999) and the ratio of 260 and 280 nm was used to determine the quality of extracted DNA. The isolated DNA from 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 on UV transilluminator. Partial sequence of *16S* gene was amplified using forward and reverse primers *viz*: 5'-CGCCTGTTTATCAAA AACAT-3' and 5'-CTCCGGTTTGAACTCAGATC-3' respectively (Shouche and Patole, 2000). PCR amplification was performed using 25 l reaction mixture containing 0.2mM dNTP mix, 1X buffer, 1mM $MgCl_2$, 1U Taq polymerase, 0.2M primers and 2l of DNA template. The amplification process was carried out following the protocol of Williams *et al.* (1990) according to which, 25 µl of reaction mixture was loaded in a thermocycler which was programmed for 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 DNA was also carried out so as to rule out the experimental errors. After amplification, 4 µl PCR product was digested with sufficient units of selected restriction enzyme (*Alu*I, *Pac*I or *Psi*I) in 2µl of buffer for 5 hrs at 37°C. Reactions were terminated by incubation at 70°C for 15 min, after which the digested fragments were resolved on 2% agarose gel with ethidium bromide staining and photographed on ultraviolet transilluminator. The amplified products were also sequenced and DNA sequences were aligned using ClustalW multiple sequence alignment programme.

Results and Discussion

In the present PCR-RFLP analysis, the DNA band patterns generated from control and treated individuals were compared. The *in silico* restriction enzyme analysis of *16S* gene sequences with NEBcutter software helped in obtaining the actual fragment number and fragment size. The fragment size of each fragment obtained from NEBcutter 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 from control and treated individuals were used to confirm the RFLP pattern. The RFLP pattern generated from non-treated *C. quinquefasciatus 16S* amplicon indicates that there was no restriction site for *Alu*I, one nicking site for *Pac*I which resulted in the production of two bands of 171 and 372 bp, and 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. The *16S* amplicon of chlorpyrifos treated specimen, when digested with *Alu*I, produced two bands of 212 and 335 bp, as against the control sequence in which *Alu*I had no restriction site. This happened due to a mutation which resulted in the generation of a new site in the treated sequence when substitution from adenine to cytosine (A→C) took place at base 214. *Pac*I yielded two bands of 318 and 229 bp, which differed from two bands generated from the *16S* amplicon of untreated individual as a transversion from A→ T at base number 170 destroyed the restriction site previously present in the normal sequence while a transversion of T→ A at base position 317 generated a new restriction site for *Pac*I in the treated sequence. *16S* amplicon of the treated individual, after digestion with *Psi*I, remained unaffected as both the restriction sites for this enzyme were destroyed by mutations at bases 236 and 259, where A was replaced with C and T, respectively (Table 1, Fig. 1).

As a result of chlorpyrifos treatment, three different types of mutations in the form of deletions, insertions and substitutions of bases were encountered in the partial

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

Type of sample	PCR product size (bp)	PCR-RFLP product size (bp)		
		AluI	PacI	PsiI
Control	543	543*	372, 171	284, 233, 26
Chlorpyrifos treated	547	335, 212	318, 229	547*

* PCR product not digested (no restriction site)

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CLUSTAL 2.1 multiple sequence alignment

CONTROL          CGCTGTTGAAAATTTAAGTCTACCTGC---CCACTGATATAAATTAAAGGGCCGCAGTAT 57
TREATED          CG----TAAAAGTGGAACGCGAGCCATGAACCACTGATATAAATTAAAGGGCCGCAGTAT 56
                 **     * *** *  **   * * *       ******************************

CONTROL          TTTGACTGTGCGAAGGTAGCATAATCACTAGTCTTTTAATTGGAGGCTTGTATGAATGGT 117
TREATED          TTTGACTGTGCGAAGGTAGCATAATCACTAGTCTTTTAATT GGAGGCTTGTATGAGTGGT 116
                 ******************************************************** ****

CONTROL          TGAATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTT TTAATTAAAAA 177
TREATED          TGAATGAGATATATACTGTCTTTTTTAAAATTATATAGAATTTTATTTTTTATTTAA AAA 176
                 ***************************************************** *******

CONTROL          GTTAAAATAAAATTAAAGGACGAGAAGACCCTATAGATCTTTATTTTTGTTAT TTATAAA 237
TREATED          GTTAAAATAAAATTAAAGGACGAGAAGACCCTAT AGCTCTTTATTTTTGTTATTTATACA 236
                 ************************************ ********************* *

CONTROL          TTAAAAAGAATTTTAAAAT TTATAATTTAATAAAAAATTTTATTGGGGTGATATTAAAAT 297
TREATED          TTAAAAAGAATTTTAAAATTTTTAATTTAATAAAAAATTTTATTGGGGTGATATTAAAAT 296
                 ********************* ***************************************

CONTROL          TTAAAAAACTTTTAAAATTTATTAACATAAATATATGAATAAATGATCCAGTTTTATTGA 357
TREATED          TTAAAAAACTTTTAAAA TTAATTAACATAAATATATGAATAAATGATCCAGTTTTATTGA 356
                 ******************* *****************************************

CONTROL          TTAAAAATTTAAGTTACCTTAGGGATAACAGCGTAATTTTTTTTTAGAGTTCATATCGAC 417
TREATED          TTAAAAATTTAAGTTACCTTAGGGAAAACAGCGTAATTTTTTTTTAGAGTTCATATCGGT 416
                 ************************* ********************************

CONTROL          AAAAAAGATTGCGACCTCGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAG 477
TREATED          ACAAAAGATTGCGACCTCGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTTAAAG 476
                 * ****************************** ****************************

CONTROL          TTTAGGTCTGTTCGACCTTTGAATTCTTACATGATCTGAGTTCAAACCGGAGATGATCTG 537
TREATED          TTTAGGTCTGTTCGACCTTTGAATTCTTACATGATCTGAGTTCAAAACGGAGATGATGTG 536
                 ********************************************** * ********* **

CONTROL          AGTTCA----- 543
TREATED          AGTTCAATCCG 547
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Fig. 1 : Multiple sequence alignment of *16S* gene sequences of control and chlorpyrifos treated *C. quinquefasciatus*. restriction sites of *Alu*I (AGCT), *Pac*I (TTAATTAA) and *Psi*I (TTATAA)

sequences of *16S* gene. In aligned sequences from control and the treated individuals, asterisk (*) showed identical bases. Dashes (–) in the control sequence indicated insertion of bases while dashes in the treated sequence marked deletions. The rest of the places showed difference in the complementary bases in two types of sequence. These were the regions where substitutions had taken place due to transitions and transversions. Transitions are the loci where purines were replaced by purines or pyrimidines by pyrimidines, while transversions are the sites where purines were replaced by pyrimidines and vice versa (Fig. 1). According to these parameters of sequence comparison *16S* amplicon from control individuals yielded sequence of 543 bp, whereas chlorpyrifos treated individual consisted of sequence of 547 bp. The sequence of chlorpyrifos treated individual suffered as many as 35 different types of mutations which included 4 deletions, 8 insertions and 23 substitutions, in which there were 8 transitions and 15 transversions (Table 2, Fig 1).

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Table 2 : Mutations in *16S* gene sequence of chlorpyrifos treated *C. quinquefasciatus*

Type of mutation	Total number of mutations	Type of bases mutated	Position of mutated bases in the sequence
Deletion	4	CTGT	3-6
Insertion	8	GAA	27-28
		ATCCG	after 543
Transition	8	A→G	12, 113, 416
		G→A	8, 26
		C→T	27, 417
		T→C	25
Transversion	15	A→T	170, 259
		T→A	317, 383
		T→G	14, 15, 19, 21
		G→T	-
		C→G	23, 535
		G→C	18
		C→A	524
		A→C	214, 236, 419

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

The studies carried out so far on the genotoxic activity of chlorpyrifos showed that chlorpyrifos caused increased frequency of mosaic wing spots, sex-linked recessive lethals and apoptosis in *Drosophila melanogaster* **(**Gupta *et al*., 2010). In addition, an increased rate of chromosomal aberrations, sister chromatid exchange and mean comet tail length in leucocytes and spleen cells of mice and toad were also observed (Rahman *et al*., 2002; Ismail *et al*., 2014). It has also been reported that repeated administration of chlorpyrifos in rats lead to inhibition of DNA synthesis, suppressed expression of genes and transcription factors which were required for differentiation (Crumpton *et al*., 2000). A dose dependent increase in DNA damage in liver and brain cells of rats, gill cells of *Channa punctatus* and erythrocytes of *Bufo bufo gargarizans* tadpoles have been also reported earlier (Mehta *et al*., 2008; Ali *et al*., 2009; Yin *et al*., 2009). Bhinder and Chaudhry (2013) assessed the effect of organophosphate pesticides acephate and profenofos on *16S* gene sequence of *C. quinquefasciatus*, a significant increase in the incidence of induced mutations was observed. As for the possible action of pesticides, it is claimed that most of these chemical formulations significantly increased the cellular reactive oxygen species (ROS), leading to modifications in DNA in form of base pair errors and strand breaks (Saleha Banu *et al*., 2001; Hreljac and Filipic, 2009).

The present study indicated that chlorpyrifos is a DNA damaging chemical and the use of PCR-RFLP is a highly sensitive technique for detecting pesticide related sequence specific DNA damage.

Acknowledgments

The author is grateful to Dr. Asha Chaudhry, retired professor of Zoology, Punjab University, Chandigarh for her guidance through all the phases of this research work. Thanks also to the Chairperson, Department of Zoology, Punjab University, Chandigarh for providing necessary facilities to carry out the present research work under the Centre of Advance Studies (CAS) Programme of the University Grants Commission (UGC), New Delhi, India.

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