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Malathion Prompted Genotoxicity Assessment in rDNA ITS1 and ITS2 Sequence of *Anopheles stephensi*

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Abstract

Indiscriminate use of pesticides in agricultural practices has developed its toxicity in humans and animals exposed to it. Malathion is a non-systemic insecticide belonging to organophosphate class. It is used in controlling Mediterranean flies, bugs, and aphids in fields. The present study was aimed to evaluate the malathion induced genotoxicity in Internal transcribed spacer, ITS 1 and 2 sequences of rDNA of mosquito, *Anopheles stephensi*. For this, second instar larvae of mosquito were exposed to 2.54 ppm (LC20) of malathion for an acute period of 24h. Post-treatment, larvae were allowed to develop into adults, and Internal transcribed spacer (ITS) sequences of rDNA were amplified by Polymerase chain reaction (PCR). PCR amplification revealed significant point mutations in form of transition, transversion, deletion and insertion in treated ITS 1 and 2 sequences compared to control. ITS 1 sequence showed deletion of 26 bases, insertion of 141 bases, and substitutions of 236 bases compared to control. While, treated ITS 2 sequence suffered 48 deletions, 54 insertions, and 117 substitutions of nucleotide compared to non-treated mosquito sequences. ITS 1 was found to be more affected by malathion toxicity with lowered GC content. Thus, present study details the toxicity of pesticide in the mosquito, *Anopheles stephensi*, contributing to the field of toxicology.

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1. Introduction

The exploding population has developed the concept of increased productivity through the worldwide use of fertilisers and pesticides. Pesticides play its role by controlling pests, weeds, insects, microbes, fungus, and weeds in the fields and on the crops. India accounted for annual production of approximately 217, 000 metric tons in 2019 with 3rd position in chemical production in Asia [1]. Organophosphates are an important class of pesticides that act by inhibition of neuromuscular enzyme, acetylcholinesterase. Malathion is a non-systemic insecticide used in agricultural fields controlling aphids, bugs, Mediterranean flies, etc.

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Malathion has been reported to cause toxicity in animals, fish, and insects[2, 3, 4,5]. It is also used as a topical treatment for head lice, fumigation, veterinary practices, domestic and public health purpose [6,7]. Because of its low mammalian toxicity malathion becomes one of the most commonly used organophosphate compounds and hence a major source of environmental hazard [8,9]. In the environment, it raises concern due to persistence for a longer period having hydrolysis half-life of 10 days [10].

Pesticide interacts with the DNA of living organisms by conversion to more reactive intermediates or electrophiles affecting genetic material, cells, and tissues. This has created an urgent need to evaluate the harmful effects of these chemicals on the structure and function of biological systems as they possess clastogenic activities [11,12].

Amplification of sequences from whole DNA using primers aide in the assessment of genotoxicity. rDNA gene is conserved sequences used for molecular profiling and toxico-genomics. rDNA comprises of 18S, 5.8S, and 28S gene coding for rRNA [13]. ITS spacer sequences are located in the form of tandem repeated multigene families in the eukaryotic rDNA gene [14]. Mature rDNA gene is produced by processing of large precursor from which different transcribed regions are sequentially removed through an elaborate pathway of cleavage steps [15]. Although they form part of introns yet the transcribed spacer regions are known to play an important role in the coding of required rRNA and for the control of ribosome biogenesis [16]. Keeping these in view, present research work was carried out to evaluate the genotoxicity in ITS 1 and 2 sequences of *Anopheles stephensi* on exposure to malathion.

2. Material and Methods

2.1. Test organism, collection and rearing

Larvae of *Anopheles stephensi* belonging to the family Culicidae (Order Diptera) were collected from the river Ghaghar, Chandigarh. The collection was done in seasons of high availability (September to December). The second instar larvae were fed on a protein-rich diet, consisting of finely powdered dog biscuit and yeast extract in the ratio of 6:4 [17,18].

2.2. Toxicant used and treatment

Malathion (C10H1906PS2) (50%EC) belonging to organophosphate class was purchased from Scientific Fertilizers Co. Pvt. Ltd., Coimbatore, India. For the effective dose determination, LC20 was determined using Probit analysis [19]. For this, second instar larvae (n=20) each was exposed to successive serial dilution (10-2 to 10-11 µl/ml) of malathion for 24 h. Simultaneously, parallel controls were maintained in pesticide free distilled water. Triplicates of each dilution were maintained and the percentage mortality was calculated from the survival rate of treated larvae. LC20 value of malathion for *Anopheles stephensi* came to be 2.54 X 10-4 µl/ml with regression equation (Y=10.62+0.52X) using SPSS-18. Finally, second instar larvae were treated with 2.54 X 10-4 µl/ml of malathion for 24 h and larvae were then transferred to the pesticide-free distilled water for the completion of their life cycle and to develop as adults.

2.3.DNA extraction, PCR amplification, sequencing of ITS 1 and ITS 2

The DNA was extracted from the whole-body homogenate of the mosquitoes by the phenol-chloroform extraction method of Ausubel et al. [20] and stored at -20°C till further use. Each set of PCR amplification reactions contained DNA template, oligonucleotide primers for ITS 1 and 2 (Table 1), 1X PCR buffer, Taq polymerase (1U), double distilled water, MgCl2, and dNTPs mix. PCR amplification was carried out in Thermocycler (Biometra Personal) programmed for 37 cycles under detailed conditions (Table 2). PCR products were analyzed on horizontal gel electrophoresis with a gene ladder (100bp). The amplified sample of DNA was sent for sequencing to Chromos Biotech Pvt. Ltd, Bangalore. These were sequenced with the help of the Big Dye terminator cycle sequencing kit (version 3.1) of Applied Biosystems. Finally, DNA sequences were aligned using Clustal W, and results were interpreted.

Table 1. Details of primers used for amplification of ITS 1 and ITS 2

	Primer	Sequence	Reference
ITS1	Forward	5'-CCTTTGTACACACCGCCCGT-3’	Shouche and Patole [21]
	Reverse	5’-GTTCATGTGTCCTGCAGTTCAC-3’	
ITS2	Forward	5’-TGTGAACTGCAGGACACAT-3’	Porter and Collin [22]
	Reverse	5’-TATGCTTAAATTCAGGGGGT-3’	

Table 2. Details of PCR conditions

Steps	Process	Time	Temperature
1.	Initial denaturation of DNA (1 cycle)	10minute	94℃
2.	36 cycles steps		
	Denaturation of DNA	1minute	94℃
	Annealing of primers	1minute	ITS1-56℃,ITS2-59℃
	Extension	1minute	72℃
3.	Final extension (1cycle)	10minute	72℃

3.Result and Discussion

ITS 1 and ITS 2 sequence of rDNA of control and treated individuals of *Anopheles stephensi* subjected to PCR amplification elucidated alterations in the form of insertion, deletions, transition, and transversion of bases. ITS 1 and ITS 2 PCR amplification generated product of approx. 600 and 400bp respectively (Fig. 1 and 3). Clustal W aligned sequence generated congruent results, and loci marked with the asterisk (*) depicted identical bases in both the sequences. While, loci marked with dashes (-) in control sequence indicated bases differing due to insertion, and dashes in treated sequences exhibited deletion. The region of base sequences not indicated by the asterisk or dash indicated transition and transversion (Fig. 2 and 4). Comparative study of the two sequences provided considerable incidence of mutation induced by malathion. A comparison of total base-pair length of ITS 1 sequence from control individual produced a band of 585bp as compared to 700bp from the treated sequences. The average G: C content was 52% and 47% in control and treated sequences respectively. While an average A: T content was found to be 48% and 53% in the control and treated sequences respectively. Additionally, observed deletion of 26 bases and insertion of as many as 141 bases, with a high frequency of substitutions comprising of 138 base transitions and 98 base transversion (Table 3).

Similarly, a comparison between normal and induced ITS 2 sequences had a total base-pair length of 414 bases and 420 bases respectively. The percentage of G: C content was 57% and 58 % from the control and treated individuals respectively. While the A: T content was 43% for control and 42% for the treated stock. Further the base comparison revealed deletion of 48 bases and insertion of 54. Moreover noticed 71 transition and 46 transversion, instances of making it a total of 117 base substitutions (Table 3). The data deciphered significantly higher incidences of the point mutation in the ITS 1 sequence, with a total number of 403 base mutation compared to 219 bases of ITS 2 sequence. Thus, inferring disturbed splicing mechanism and ribosome biogenesis.

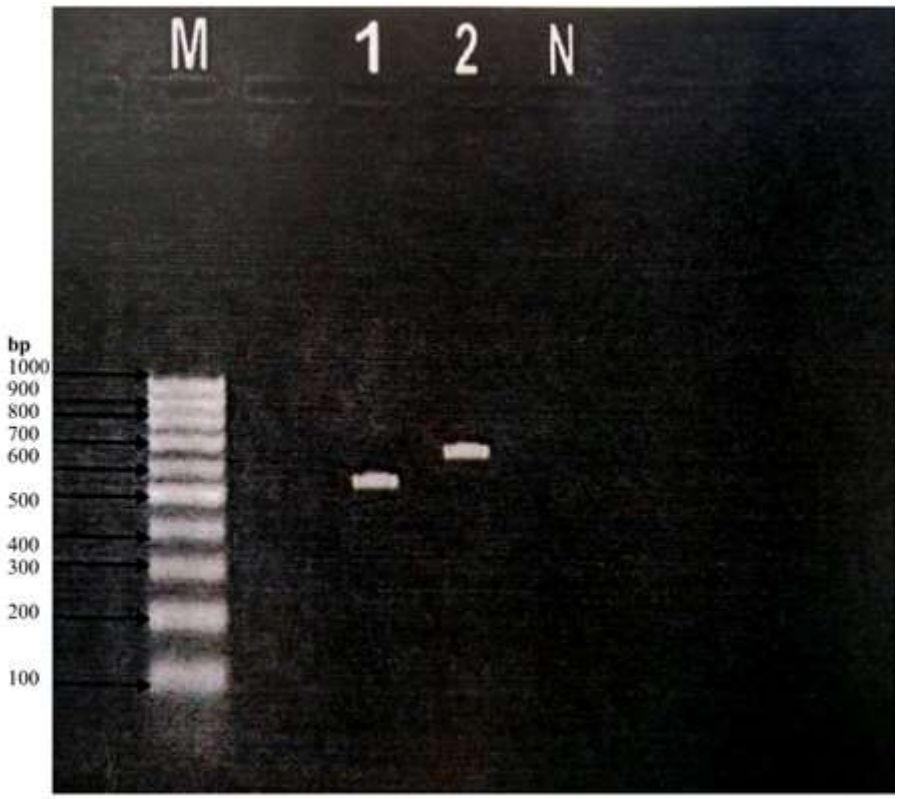

Fig. 1. PCR generated DNA bands from ITS1 of control and treated individuals.

Lane M-DNA ladder; Lane 1– DNA band from control; Lane 2– DNA band from treated; Lane N– Negative control.

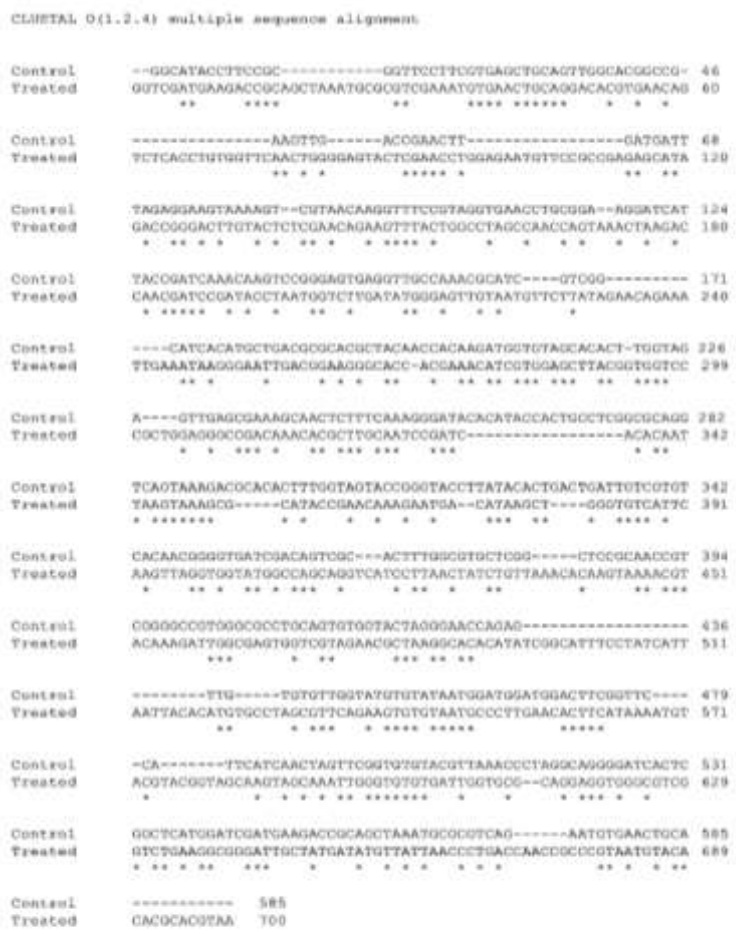

Fig. 2. Sequence comparison of Internal Transcribed Spacer 1 (ITS 1) of control and treated stocks of *Anopheles stephensi*.

Loci marked with the asterisk (*) indicates identical bases in both the sequences.
Loci marked with dashes (- -) in control sequence indicates insertion and dashes in treated sequences indicates deletion. The region of base sequences not indicated by the asterisk or dash indicates transition and transversion

Table 3. Sequence characteristic of ITS 1 and 2 sequences of control and treated *Anopheles stephensi*

S. No.	Parameter	ITS 1		ITS 2	
		Control	Treated	Control	Treated
1.	Total length of sequence	585	700	414	420
2.	Total no. of bases mutated	-	393	-	219
3.	G:C content	52%	47%	57%	58%
4.	A:T content	48%	53%	43%	42%
5.	No. of deletions	-	26	-	48
6.	No. of insertions	-	141	-	54
7.	No. of transition	-	138	-	71
8.	No. of transversion	-	98	-	46

Upon metabolization in animal body malathion degrades into malaoxon, potentially more toxic analogue inhibiting acetyl-cholinesterase thus eliciting hyperactivity, restlessness, and finally death [23, 24]. Studies have detailed its toxicity with varying intensity in insects, invertebrates, and fish [3, 25, 26, 27, 28]. It has been documented to persists for 11 to 14 days, ranging 52 - 21% level in river water [29]. Lien *et al.* [30] have reported developmental deformities in the form of the deformed notochord and pericardial oedema in *C. gariepinus* larvae exposed to malathion . Bhinder and Chaudhry [31, 32] elucidated altered restriction enzyme digestion sites upon organophosphate treatment in *Culex quinquefasciatus.* Temperature acts as important factor in enhancing malathion toxicity [27, 33,34]. The insecticide has been reported to affect various tissue such as liver, kidney, and testicles in rats [35, 36, 37, 38].

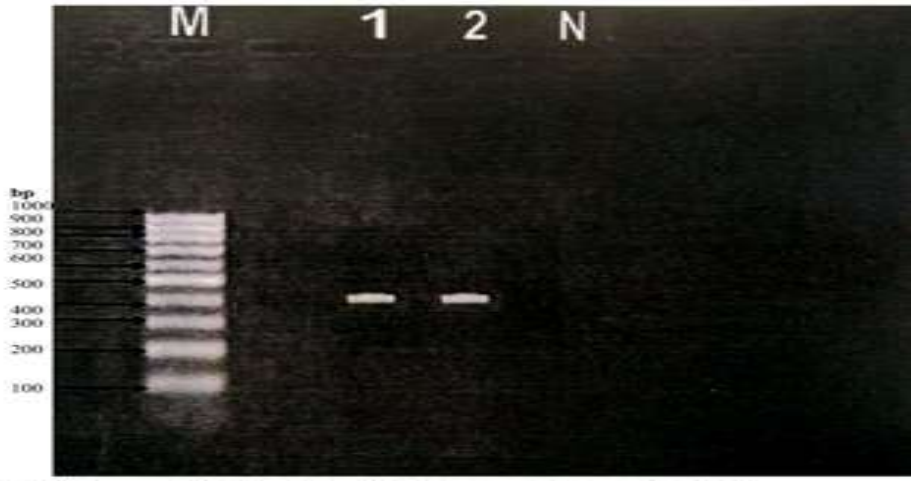

Fig. 3. PCR generated DNA bands from ITS 2 of control and treated individuals.

Lane M- DNA ladder; Lane 1– DNA band from control; Lane 2– DNA band from treated; Lane N– Negative control

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CLUSTAL O(1.2.4) multiple sequence alignment

Control     ----------GTTTCACCCGACCGATGCACACATCCTTGAGTGCCTACTAGGTACTGA 48
Treated     TGCGCATCGGACGTTTCACCCGACCGATGCACACATCCTTGAGTGCCTACCAAGTTATCT 60
                      ****************************************** * **  *

Control     AAGATTCCTATAACTTGACTACAGACGGCGCCACAAACGGGCTGACGGCCATCCGTCGT 108
Treated     ATATGCTCCTACCAGACTGACCGTCCCATCCCCGTGATGGGCT----------------- 103
            *     *             *  *    **    * *****

Control     CCGGCGTGCGACTGTGCAGCATGGCGTGCTCGGGTCTCG--GCGTGGACCCTTGGGCGCT 166
Treated     ----------GTCGCAGAATGGCGTGCTCGGACCCGCTTGCGCGGGACCGTGGGCGCT 151
                        **** ************  *     *** **  ** ********

Control     GAAAGTGGACACTGTTTGGCGGCACCTGCGCGTGTGCTCTCAGTGTTGACGTATGGTGAG 226
Treated     GAAAGTGAGAGTGCTATTAGAC-AGG----TATGGTACACGCAAGGCGAGAGATGAACGG 206
            *******       * *      *         **           *  **   ***    *

Control     GGTAGTGTCAAATCGCACGGTTCGACAACAAGCGTACCGTCGAGTTT------------- 273
Treated     GCGCGCGTCAAGTCGCACGGTTCGACCTCCAGTATCAACTAGGGATGAAACCCCCGCAGC 266
            *   * ***** **************  * **  *    * * * *

Control     -------GGTGCAATCGGATGCCTACTACCAGGGGCG----GAGCCGGCGTGCATTCAAC 322
Treated     CTAATGTATTAACACCGGGCGCTAGCAAAGGGGTCCTTGGTTGGCTCGGGTCGAGTAAAC 326
                  *   * *** **   * *   **  *       **  * **  * * ***

Control     ACTCGACGTCCTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCATACAGGAC 382
Treated     ACTTGCGGGCCCAACGCGCCCGTCT--TCGTCTGCTCGCTGGTCTCGCTCAAAGTAGG-- 382
            *** *  * **     *  *** *       ** * * ****  **    *   ***

Control     ACTGAATCGATCTTGGTGGTACAACCAACATG------  414
Treated     CCTCAAGTGATGTGTGACGACCCCCCGAATTTTAACAT  420
             ** **  *** *  *  *  *  ** *  *
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Fig. 4. Sequence comparison of Internal Transcribed Spacer 2 (ITS 2) of control and treated stocks of *Anopheles stephensi*.

Loci marked with the asterisk (*) indicates identical bases in both the sequences.
Loci marked with dashes (- -) in control sequence indicates insertion and dashes in treated sequences indicates deletion. The region of base sequences not indicated by the asterisk or dash indicates transition and transversion

Additionally, Al-Attar [39] reported damaged histo-architecture and elevated activities of serum enzymes GOT, GPT, ALP, ACP, and levels of creatinine, urea, and uric acid, while depressed levels of total protein and total albumin in malathion exposed rats. Kumar *et al*. [40] demonstrated damaged DNA in gill, kidney, and lymphocytes of *Channa punctatus.* A linear relation between exposure time and DNA damage has been elucidated in *Oncorhynchus mykiss [5, 41].*

4. Conclusion

Malathion exposure induced mutations in ITS 1 and ITS 2 sequences of rDNA in the form of deletion, insertion, transition, and transversion in *Anopheles stephensi*. Comparison of the non- treated and treated sequences through Clustal W reflected ITS 1 as more affected. Thus, it can be concluded that the PCR technique in combination with ITS sequences

developed pathways for genotoxicity evaluation in a reliable way.

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