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Original Article

Imidacloprid and Thiamethoxam Induced Mutations in Internal Transcribed Spacer 2 (ITS2) of *Anopheles stephensi*

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ABSTRACT

The present article deals with the polymerase chain reaction (PCR)-based genotoxicity evaluation of neonicotinoid pesticides, imidacloprid and thiamethoxam, by using the genome of a mosquito *Anopheles stephensi* taken as an experimental model. After treatment of the second instar larvae with LC_{20} of the pesticides for 24 h, the induced nucleotide sequence variations in the internal transcribed spacer 2 (ITS2) of freshly hatched unfed control and treated individuals was studied from the sequence alignment data and the mutations in the form of insertion, deletion and substitution of bases were recorded. Measurable differences, indicative of the genetic damage due to imidacloprid and thiamethoxam were observed when ITS2 sequences of control and treated individuals were compared. It was found that imidacloprid-treated individual had 8 deletions, 29 insertions, 18 transitions and 33 transversions, whereas thiamethoxam-treated individual had 10 deletions, 8 insertions, 47 transitions and 68 transversions.

Keywords: *Anopheles stephensi*, Genotoxicity, imidacloprid, thiamethoxam

INTRODUCTION

In the past few years, the agricultural production has been enormously enhanced by the use of many synthetic pesticides. Although, their application is based on selective toxicity for certain organisms yet it has resulted in serious effects on many non-target organisms as well. The use of pesticides has created a type of chemical environment which is proving harmful to the living systems. As a consequence of this, the environmental monitoring and their impact assessment have become the priority areas of research. For the evaluation of genotoxic action of pesticides on the genetic material, a number of tests or protocols such as comet assay, chromosomal aberrations and DNA fingerprinting have been developed by using bacteria, yeast, insects and mammals as experimental models. With reference to the structure and functions of DNA, all experimental organisms are similar; therefore, 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. Recent developments in molecular biology have offered new possibilities for detecting DNA damage at the nucleotide level by the application of polymerase chain reaction (PCR) technique.[1-3] In relevance to this, the present PCR-based genotoxicity studies were undertaken to evaluate the genotoxic potential of neonicotinoid pesticides, imidacloprid and thiamethoxam, by using the genome of a mosquito *Anopheles stephensi* taken as an experimental model. Although this experimental test system differs from the rest in terms of metabolism, DNA repair and physiological process effecting chemical mutagenesis, yet the universality of DNA and the genetic code provides a rationale to predict

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the intrinsic mutagenicity of mutagens. In order to meet these objectives, internal transcribed spacer 2 (ITS2) was studied. For this the individuals were treated with LC_{20} of the pesticides and the nucleotide sequence changes in the DNA of control and treated stocks were studied from the sequence alignment data in which the mutations in the form of insertions, deletions and substitutions were recorded.

MATERIALS AND METHODS

Anopheles stephensi Liston, used as an experimental insect for the present investigations was collected from their resting sites from the cattle sheds in the early morning from the village inhabitations near Chandigarh. The gravid females were held in the test tubes where they were allowed to oviposit on a strip of wet filter paper. The eggs procured in this way were allowed to hatch and grow through all the larval stages on a protein-rich diet of finely powdered dog biscuits and yeast tablets mixed in the ratio of 6:4.[4-6] Freshly hatched unfed adults were stored in Eppendorf tubes at -20°C and the dried samples were individually homogenized for DNA extraction. Imidacloprid and thiamethoxam belong to a new class of pesticides called neonicotinoid, which have been developed to improve their insecticidal activity against a variety of sucking pests of plants and animals. These are the most important class of modern synthetic insecticides as they are modeled after basic nicotine molecule. As for their technical specifications, imidacloprid has a chemical formula $C_9H_{10}ClN_5O_2$ and CAS No. 138261-41-3, while thiamethoxam has formula $C_9H_{10}ClN_5O_2$ and CAS No. 153719-23-4. 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.[7] The second instar larvae of *A. stephensi* were treated with 2.3×10^{-5} µl/ml imidacloprid and 5×10^{-3} µl/ml thiamethoxam for 24 h after which they were transferred to chemical-free distilled water and allowed to become adults. The treated and parallel controls were maintained in the BOD incubator. The DNA extraction was carried out by following the phenol–chloroform extraction method of Ausubel *et al.*,[8] while the integrity of the samples was tested by following the procedure of Sambrook *et al.*[9] The amplification of DNA samples was carried out by using ITS2 specific forward and reverse primers *viz*: 5′-TGTGAACTGCAGGACACAT-3′and 5′-TATGCTTAAATTCAGGGGGT-3′ and the PCR reactions were performed as per the protocol of Williams *et al.*[10] The amplified PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and the DNA bands generated in this way were visualized over UV transilluminator. A 100 base pair DNA ladder was also run along with the amplicon for calculating the base pairs length of each DNA band. These amplified DNA fragments were sequenced and the DNA sequences were aligned by ClustalW.

RESULTS AND DISCUSSION

PCR amplification of ITS2 region of *A. stephensi* generated a single prominent band of approximately 550 bp length from control and individuals treated with imidacloprid and thiamethoxam. In Figure 1, lane M shows the bands of standard DNA ladder, while lanes 1 and 2 contain the amplified products from ITS2 of control- and imidacloprid-treated samples, respectively. Similarly, in Figure 2, lane M shows the bands of standard DNA gene ruler, while lanes 1 and 2 contain the amplified products from ITS2 of control- and thiamethoxam-treated samples, respectively. These amplified products were sequenced and read from the sequence alignment using ClustalW program. In the sequence alignment of control and treated individuals

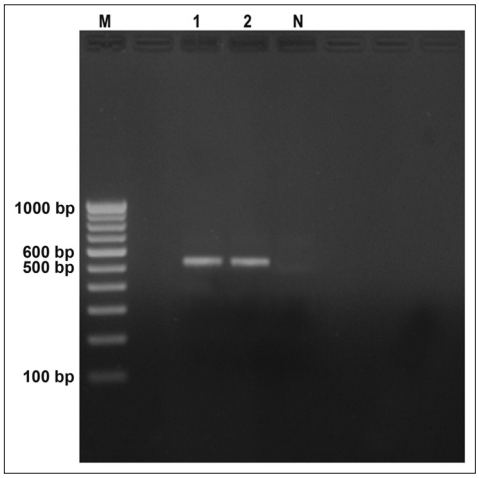


Figure 1: PCR-generated DNA bands from ITS2 of control- and imidacloprid-treated *Anopheles stephensi*. Lane M - DNA ladder, lane 1 - DNA band from control individuals, lane 2 - DNA band from treated individuals, lane N - Negative control

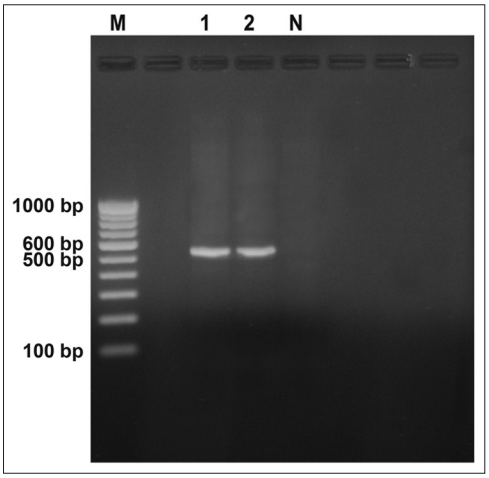


Figure 2: PCR-generated DNA bands from ITS2 of control- and thiamethoxam-treated *Anopheles stephensi*. Lane M - DNA ladder, lane 1 - DNA band from control individuals, lane 2 - DNA band from treated individuals, lane N - Negative control

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Control         ----------------------TGGCGCATCGGACGACTCAACCCGACCGATGCACACA  37
Treated         GTACATCAACACGTTGACGCATATGGCGCATCGGACGACTCA-CCCGACCGA-GCACACA  58
                                      ******************  ********* *******

Control         TCCTTGAGTGCCTACCAAGTTATCTCAACACTCTAACCAAACTGACCGTCCTGACCCCAT  97
Treated         TCCT-GAGTCCCTTCCAAGTTATCT--ACACTCAAACCAAACTGACCGACCTGACCCTAT  115
                **** **** *** ***********  ****** ************** ******** **

Control         CATAGGGGGGTAGGCTGTCGCAGCATGGCGTGCTCGGATCCGCATCCTTGTCGGGACCGT  157
Treated         CGTAGCGGGGTAGGGGGTCGCAGCCTGGCGTGCTCG-ATCCGCATCCTTGTCGGGCCCGT  174
                * *** ********  ******** *********** ****************** ****

Control         GGGCGCTGAAAGTGAGAGTGCTAACACAGAGAGACATACGAGGTATGGTACACAAACCTA  217
Treated         GGGCGCTGAAAGTGAG-GTGCTAACACAGAGAGATATTCGTGGTATGGTACACAAACCGA  233
                *************** ***************** ** ** ***************** *

Control         AACACACACACACATGTGTGAGTGTGGGAGAAGAGAGAGCGCGCGTCAAGTCACAGGGCT  277
Treated         AACACACACACAAATGTGTGAATGTGGGTAAAGAGAGAGCGCGCGTCAAGTCACAGGTCT  293
                *********** ******** ******  ***************************** **

Control         ACACCTCTAATATCCCCCCAGATATGTATCCCCCACAATATATAGGGATATCACCTTGTG  337
Treated         CCACCTCTAATATCCCCCAGGAGGTGTATCCCCCACAATATATAGAAATATCCCCTTGTG  353
                 ***************  **  ********************  ***** *******

Control         CGGGGACACTTCCCCCGGGTGTCTCGGGTAGAGAAACATGTGCGGCCCAACGCTCTCATA  397
Treated         CGGGGACATCTCCCGCGGGTGTCTCGGCTAGAGAAACATGTGGGCCCCAACGCTCTCATA  413
                ********  **** ************ ************** * ***************

Control         TTTTTCCTCATATCCTTGTGCACTCGAGAGATGTGCTCACGCCGTGTGTGAGAGAGATGA  457
Treated         TTTTTC-TCATATCCTTGCGCACTCGAGAGATGTGCTCACGCCTTGTGTGAGAGAGATTA  472
                ****** *********** ************************ ************** *

Control         TAACAACACAGG-TGAGATATATCAGCGCCTCTCGTCTCACATCTGTGAGACAGGAGACT  516
Treated         TAACAACAGAGGGTGAGATATATCAGCGCCTCTCCCCTCACATCTGTGAGACAGTGGACT  532
                ******** *** ********************  ******************  ****

Control         CTGTACGTCTCATGAGATGTGAGAACACCCTGTGA-TTTACACAT--  560
Treated         GTGTGCGCCACGTGAGATGTGACAACACCCCTTGAATTTAAATATAT  579
                 *** ** * * **********  *******  *** **** * **
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Figure 3: Sequence comparison of ITS2 of control- and imidacloprid-treated *Anopheles stephensi*

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Control     --------TGGCGCATCGGACGACTCAACCCGACCGATGCACACATCCTTGAGTGCCTAC 52
Treated     AGAACGTATGTGGCATCGGGGGGCTCAACCCGCGAGAGGCACACATCTCTGAGTGCCCCC 60
                   **  *******  * *********   ** *********  ********  *

Control     CAAGTTATCTCAACACTCTAACCAAACTGACCGTCCTGACCCCATCATAGGGGGGTAGGC 112
Treated     CAAGTTATATCTACACTCTAAACAAAATGACCGTCTTGTCCCCATCATAGGGGGGTAGGG 120
            ******** ** ********* **** ******** ** ********************

Control     TGTCGCAGCATGGCGTGCTCGGATCCGCATCCTTGTCGGGACCGTGGGCGCTGAAAGTGA 172
Treated     GGTCTCAGCACAGCGTGTTCGGATCTGCATCCTTTTGGGGACCGTGGGGGCTGAAAGTGA 180
             *** *****  ***** ******* ******** * *********** ***********

Control     GAGTGCTAACACAGAGAGACATACGAGGTATGGTACACAAACCTAAACACACACACACAT 232
Treated     GAGAGTTCTCACAGAGAGACATATGAGGTATGGTACACAAACCTAAACACACACACACAC 240
            *** * *  ************** ************************************

Control     GTGTGAGTGTGGGAGAAGAGAGAGCGCGCGTCAAGTCACAGGGCTACACCTCTAATATCC 292
Treated     ATGTGAGCATGGGTGAAGAGCGAGCGCGCGTCAAGTCGCACGGTTCGACCTCTAGTATCA 300
             ******  **** ****** ***************** ** ** *  ******* ****

Control     CCCCAGATATGTATCCCCCACAATATATAGGGATATCACCTTGTGCGGGGACACTTCCCC 352
Treated     ACCAACGGATGTATCCACCACAGCATATAAGGTTATCACCAATTGCACGGGGACTTCCAC 360
             ** *   ******** *****  ***** ** *******   ***  **  ****** *

Control     CGGGTGTCTCGGGTAGAGAAACATGTGCGGCCCAACGCTCTCATATTTTTCCTCATATCC 412
Treated     CGGTTGGCTCGGGTCGAGTAACACTTGCGGCCCAACGCGCTCGTATCTTTCCTCGCATCC 420
            *** ** ******* *** ****  ************** *** *** *******  ****

Control     TTGTGCACTCGAGAGATGTGCTCACGCCGTGTGTGAGAGAGATGATAACAACACAGGTGA 472
Treated     AGTTGCAGTCGCGAGACGTGCTCACGCCGTGTG---GTGAGGAGGTCGCACGACAGG-GG 476
               **** *** **** *****************   * ***  * *  **  ***** *

Control     GATATATCAGCGCCTCTCGTCTCACATCTGTGAGACAGGAGACTCTGTACGTCTCATGAG 532
Treated     TATTTATCACCGCTCTCC--CGCGAATCCTTTGTA---GGGACTTT-TAGGCCCCGGGCC 530
             ** ***** ***    *  * *  ***  *   *   * **** * ** * * *  *

Control     A 533
Treated     G 531
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Figure 4: Sequence comparison of ITS2 of control- and thiamethoxam-treated *Anopheles stephensi*

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of *A. stephensi*, the loci marked with asterisk (*) are the regions where base sequences are identical in both type of individuals while dashes (-) indicate the loci differing due to insertion or deletion of bases [Figures 3 and 4]. Those regions which are not indicated by asterisk or dash show transitions and transversions. Measurable differences indicative of genetic damage due to imidacloprid and thiamethoxam were observed when control and treated sequences were compared. It was found that imidacloprid-treated ITS2 sequence had 8 deletions, 29 insertions, 18 transitions and 33 transversions. Bases that got deleted were 20, 30, 42, 63, 64, 134, 174 and 404. Out of the total 27 insertions, there was a stretch of 23 bases inserted before the first base and 2 were after the last base of control sequence and 1 each from bases 469–470 and 551–552. In case of transitions the maximum mutations were detected in the form of substitution of adenine with guanine (A→G) which resulted in a total of six such substitutions. In case of transversions, six substitutions occurred from guanine to cytosine (G→C). The average GC and AT content was the same in control- and imidacloprid-treated sequences. In the same way, thiamethoxam-treated sequence had 10 deletions, 8 insertions, 47 transitions and 68 transversions. There were no deletions from base 1 to 445 while there were a total of 10 deletions of bases from 446 to 519. All the 8 bases were inserted at a stretch before the first base. Similar to the effect of imidacloprid, thiamethoxam also induced maximum adenine to guanine (A→G) transitions at 17 loci, while 6 cases of transversions were detected in the form of guanine to cytosine (G→C). The average GC content of ITS2 was 53% in the control as against 55% in thiamethoxam-treated sequence with an average AT content of 47% and 45% in the control and treated sequences, respectively [Tables 1-5].

Table 1: Deletions and insertions in ITS2 sequence of imidacloprid-treated *Anopheles stephensi*

Type of mutation	Total number of mutations	Bases involved	Number of base/s	Type of base/s
Deletion	8	20	1	A
		30	1	T
		42	1	T
		63-64	2	CA
		134	1	G
		174	1	A
		404	1	C
Insertion	27	Before 1	23	8A, 5T, 6C, 4G
		469-470	1	C
		551-552	1	A
		After 560	2	A, T

Table 2: Substitutions in ITS2 sequence of imidacloprid-treated *Anopheles stephensi*

Type of substitution	Total number of substitutions	Type of bases substituted	Total number of bases substituted	Position of bases in the sequence
Transition	18	A→G	6	99, 297, 301, 512, 521, 528
		G→A	4	239, 247, 323, 324
		T→C	5	347, 416, 492, 524, 547
		C→T	3	95, 192, 346
Transversion	33	A→T	4	51, 195, 198, 246
		T→A	3	71, 86, 526
		A→C	4	122, 153, 278, 330
		C→A	3	230, 299, 556
		G→T	5	275, 441, 456, 511, 548
		T→G	3	113, 216, 300
		G→C	6	47, 103, 365, 382, 491, 539
		C→G	5	112, 352, 380, 466, 517

Table 3: Deletions and insertions in ITS2 sequence of thiamethoxam-treated *Anopheles stephensi*

Type of mutation	Total number of mutations	Bases involved	Number of base/s	Type of base/s
Deletion	10	446-448	3	T, G, A
		470	1	T
		491-492	2	G, T
		508-510	3	C, A, G
		519	1	G
Insertion	8	before 1	8	4A, 2G, C, T

Studies carried out so far on the mutational activity of imidacloprid and thiamethoxam have shown that these pesticides were able to induce a variety of changes in the genomic integrity of the affected individuals. For example, imidacloprid has been reported to increase the incidence of sister chromatid exchange, micronuclei formation and genetic damage in human lymphocytes.[11-13] Demsia *et al*.[14] performed *in vivo* micronucleus assay with rat bone marrow polychromatic erythrocytes and showed a statistically significant effect after treatment with imidacloprid. Significant increase in the DNA damage in human peripheral blood lymphocytes was observed with comet assay and micronucleus test.[15] The studies carried out on thiamethoxam showed that it induces liver tumor in mice[16-18] and contact toxicity and mortality of adult eye gnat.[19] It also caused the impairment of olfactory learning and abnormal responsiveness to water in *Apis mellifera*[20] and blocked the normal process of oviposition and feeding in brown cocoa mired.[21] Recently, Rodrigues *et al*.,[22] has reported a significant increase in high affinity choline uptake and acetylcholine activity in the brain of rats exposed to the thiamethoxam. In one of our recent studies to evaluate the genotoxicity of LC_{20} of cypermethrin on ITS1 and 2 of *Culex quinquefasciatus*, a

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Table 4: Substitutions in ITS2 sequence of thiamethoxam-treated *Anopheles stephensi*

Type of substitution	Total number of substitutions	Type of bases substituted	Total number of bases substituted	Position of bases in the sequence
Transition	47	A→G	17	12, 15, 270, 287, 299, 315, 343, 395, 407, 454, 457, 460, 472, 496, 505, 512, 528
		G→A	4	124, 233, 241, 322, 339
		T→C	5	41, 50, 123, 232, 240, 316, 376, 399, 408, 429, 487, 489, 501, 524, 526
		C→T	3	40, 88, 130, 138, 178, 196, 276, 486, 488, 517
Transversion	68	A→T	4	64, 91, 181, 246, 325, 371, 450, 476
		T→A	3	176, 333, 334, 413, 455
		A→C	4	25, 51, 180, 253, 278, 367, 424, 459, 463, 531
		C→A	3	27, 61, 74, 79, 292, 293, 296, 309, 351, 497
		G→T	5	3, 117, 147, 335, 356, 377, 415, 473, 502, 504, 506
		T→G	3	30, 113, 300, 359, 391, 414, 494, 529
		G→C	6	273, 298, 340, 482, 532
		C→G	5	4, 13, 26, 112, 149, 161, 279, 344, 420, 464, 522

significant increase in the incidence of induced nucleotide mutations were observed in the form of deletion, insertion and substitution of bases at several loci along the amplified

Table 5: Sequence characteristics of ITS2 of control and individuals of *Anopheles stephensi* treated with imidacloprid and thiamethoxam

Parameter	Imidacloprid		Thiamethoxam	
	Control	Treated	Control	Treated
Total length of sequence (no. of bases)	560	579	533	531
GC content (%)	52	52	53	55
AT content (%)	48	48	47	45
Deletions	-	8	-	10
Insertions	-	29	-	8
Transitions	-	18	-	47

sequences.[3] From the present study, it is concluded that both imidacloprid and thiamethoxam could induce mutations in living organisms.

PCR assay should be used in the process of regulatory approval to market the compound, along with the other tests used to screen the novel compound prior to its use. This information may be used to determine the potential of a chemical to induce carcinogenicity. PCR test does not replace other genetic toxicity assays and the results are both independent of others and also supplement results from other protocols. The present study advocate the use of PCR as an accurate, reliable and highly sensitive technique for detecting pesticides-related sequence specific DNA damage and also suggestive of the fact that a sufficient level of caution is desired in the reckless use of pesticides.

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