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Nimbecidine induced gene mutations in internal transcribed spacers 1 and 2 of *Anopheles stephensi*

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Abstract: The genotoxic effects of nimbecidine, a commercial botanical pesticide derived from the neem tree were assessed by PCR assay on a mosquito *Anopheles stephensi* taken as an experimental model. After treatment with LC_{20} of the nimbecidine, the sequence variations in the internal transcribed spacers 1 and 2 (ITS1 and ITS2) of control and treated individuals were studied from their sequence alignment data and the mutations in the form of insertions, deletions, and substitutions were analyzed. Nimbecidine treatment induced 16 deletions, 13 insertions, 93 transitions and 140 transversions in the ITS1 sequence. Similarly, in the ITS2 sequence of treated individual there were 2 deletions, 4 insertions, 15 transitions and 39 transversions. Present study suggests that plant based pesticides also effect the integrity of normal DNA sequences.

Keywords: *Anopheles stephensi*, Nimbecidine genotoxicity, ITS1 and 2

INTRODUCTION

As an alternative to synthetic pesticides, nowadays chemical derivatives isolated from neem seed extract are currently being used in agriculture (Anon, 1992; Liang *et al*., 2003; Saber *et al*., 2004; Shoaib *et al*., 2010). Nimbecidine is one such commercial compound in which azadirachtin is the principal active ingredient. Azadirachtin, a steroid akin to tetranortriterpenoid (limonoid), is the most active principle component present in the extract of neem (Singh *et al*., 1993). The products of neem in the form of different formulations are being extensively used for their supposedly non-pollutant and environment friendly nature and are also being used for the treatment of a number of diseases in man (Van Der Nat *et al*., 1991). However, some of these neem derivatives have been experimentally shown to induce chromosomal aberrations in the bone marrow cells of rats (Awasthy *et al*., 1995, 1999). Rojanapo and Tepsuwan (1992) also reported a certain level of mutagenicity of flower extract of neem in TA 98 strain of *Salmonella typhimurium*. As a contraceptive, various neem formulations were found to adversely affect the reproductive performance in males (Sinha *et al*., 1984; Upadhyay and Talwar, 1993) clearly depicting the biological hazards of the neem products. In the recent years large scale use of nimbecidine in agricultural practices, the exposure of human population in general and the vulnerable groups such as virgin or pregnant women in particular has become a subject of concern (Srivastava and Raizada, 2007). Apart from a number of physiological complications expected from such formulations, the assessment of genotoxic potential of nimbecidine at various levels of its action on the genetic material has become crucial. In reference to this, the present study was carried out to evaluate the mutational index of this pesticide at the molecular level of nuclear DNA. For this, internal transcribed spacers 1 and 2 (ITS1 and ITS2) were PCR amplified to study the incidence of induced point mutations in a mosquito, *Anopheles stephensi* taken as an experimental model.

MATERIALS AND METHODS

Anopheles stephensi Liston, taken as an experimental insect for the present set of investigations was procured from the cattle sheds in the early morning collections 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 grow through all the larval stages on a protein rich diet of finely powdered dog biscuits and yeast tablets (Singh *et al*., 1975; Rao, 1984; Clements, 1996). Freshly hatched unfed adults were stored in eppendorf tubes at -20°C and the dried samples were individually homogenized for DNA extraction. Nimbecidine, used in the present experiments, is a commercial botanical pesticide derived from the seed kernels of neem plant. The extract contains azadirachtin which is the principal constituent of this chemical. Nimbecidine is commercially available as a white liquid with a strong smell. Its technical specifications are: CAS No. 11141-17-6, chemical formula $C_{35}H_{44}O_{16}$ and molecular weight 720.71. In the experimental studies aimed at the evaluation of genotoxicity of any such chemical formulation it is important to determine a suitable dose

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for its effective action in the test system. Therefore, for the present motive of research LC_{20} was found to be an ideal concentration in water, which was standardized by probit analysis (Finney, 1971). The second instar larvae of *An. stephensi* were treated by rearing them in water having $4.6X10^{-2}$ µl of nimbecidine for 24 h after which they were transferred to chemical free distilled water in order to complete their metamorphosis upto the stage of adult. The treated and parallel controls were maintained in a BOD incubator. The extraction of DNA, its integrity testing and amplification of ITS1 and 2 were carried out as per the standard protocols of Sambrook *et al.* (1989), Williams *et al*. (1990) and Ausubel *et al*. (1999), respectively. The specific forward and reverse primers: 5'-CCTTTGTACACACCGCCCGT-3' and 5'-GTTCATGTGTCCTGCAGTTCAC-3' for ITS1 and 5'-TGTGAACTGCAGGACACAT-3' and 5'-TATGCTTAAATTCAGGGGGT-3' for ITS2 were used for amplifying both the sequences of control and treated stocks of *An. stephensi* and the PCR end products were electrophoesed through 2% agarose gel stained with ethidium bromide. The DNA bands generated in this way were visualized over UV Transilluminator and photographed using Polaroid camera. A 100 base pair DNA ladder was also run along with the amplification products for calculating the number of base pairs in each band. The amplified products were sequenced by outsourcing the DNA samples to Chromus Biotech Pvt. Ltd., Bangalore and the data so obtained were aligned and analyzed using ClustalW software.

RESULTS AND DISCUSSION

The PCR amplification of ITS1 region of *An. stephensi* generated a single prominent band of approximately 800 bp length from the non-treated controls and nimbecidine treated individuals while a band of approximately 400 bp was generated from ITS2. In Fig. 1, lane M shows the standard DNA gene ruler while lanes 1 and 2 contain the amplified products from ITS1 of control and nimbecidine treated samples respectively and lane N represents the negative control. Similarly, in figure 2, lane M shows the standard DNA gene ruler while lanes 1 and 2 contain the amplified products from ITS2 of control and nimbecidine treated samples respectively and lane N represents the negative control. In the sequence alignment of control and treated individuals of *An. stephensi* (Figs. 3, 4) the loci marked with asterisk (*) are the regions where bases are identical in both type of individuals while dashes (-) indicate the loci differing due to insertion or deletion of bases. Those regions which are not indicated by either asterisk or dash are the loci effected by substitution i.e. transitions and transversions. Measurable differences indicative of genetic damage due to nimbecidine were observed when control and treated sequences were compared. It was found that nimbecidine treated ITS1 sequence had 16 deletions, 13 insertions, 93 transitions

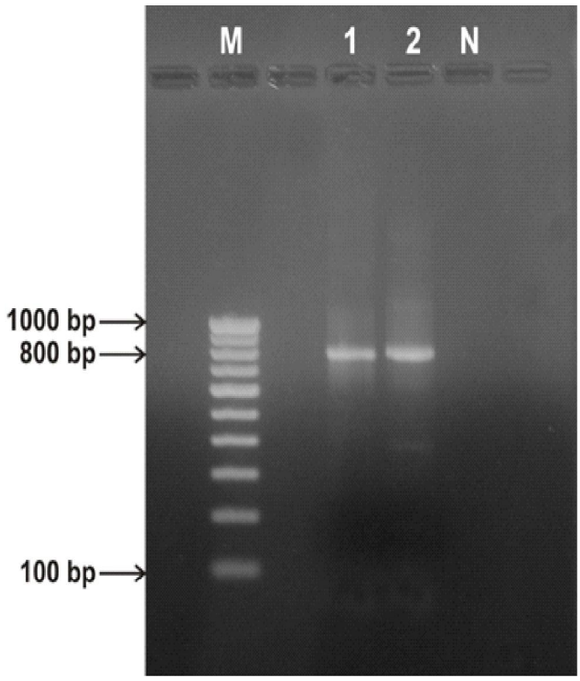

Fig. 1. *PCR generated DNA bands from ITS1 of nontreated and treated An. stephensi. Lane M-DNA ladder, lane 1-band generated from nontreated individual, lane 2- band generated from treated individual, lane N- negative control.*

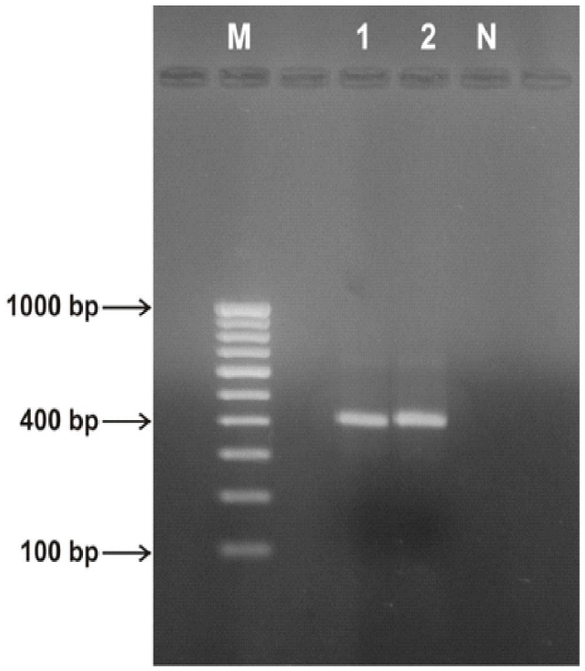

Fig. 2. *PCR generated DNA bands from ITS2 of nontreated and treated An. stephensi. Lane M-DNA ladder, lane 1-band generated from nontreated individual, lane 2- band generated from treated individual, lane N- negative control.*

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Control         CCTTTGTTCCCCGCCTGTCTATTGTACGTCCTGCCGGTGAACTAGTCGACTACTCCTCCT 60
Treated         -CCTTGT-CCACGCTT-TTTAGTGTCGTCTCTGAAGGTGAACTATTTG-CTAGTCCTCCC 56
                 * **** ** *** * * ** ***     ***  ********* * * *** ******

Control         GGTATTACATTTGAATCGCTAAAATTGACCGAACTTGATGATTTAAGAGGAAGTAAAAGT 120
Treated         GGTATTACATTTGAATCGCTGAAATTGACCGAACTTGATGATTTAGGAGGAAGTAAAAGT 116
                ******************* ********************** ************

Control         CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGTACTTCATACCCATG 180
Treated         CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGTACTTCATACCCCTG 176
                ********************************************************** **

Control         GAGCGCATAAATGAA--CAACACCCCCCGCAATGTGCCAGCGTGCGCGCCCGAGTCTCCT 238
Treated         GGGCGCATAAATGAAAACAACCCCCCCAGTTGCGTACCCGTGTGCGCGCCCGACTCATCG 236
                * ************** **** ***** *    ** ** * ************ **  *

Control         AGTCTCGTTGAGTACGTTCGCCCACTCGCCCCTCGAGACTGCTCGGGCCCGTTGCACGGG 298
Treated         AGTCTCTTTGAGTACTCTCCCCTACTCACCCCTCGAGACTGCGCGG-CGCGTTACGGACG 295
                ****** ********  ** ** **** ************** *** * **** *    *

Control         TGAGAATGGGAAATATAGGCTACATCTCACTACACCG--AGCGGGGCTGGCCATATTATA 356
Treated         TGAGAATGGAATATAAGATCCAACTCTCACTCCACCGCCGGACAGGCTGTTTAGTCTATC 355
                ********* * ***    * ** ******* *****   *   *****   *   ***

Control         TTACCATCCCAACGGGGCCGGGTGGGTCGGTCTATGTTCTGCCCGGCAAACCAAATGCGT 416
Treated         TCACCATGCCGAGCGAGCGGGGTCGGCCTGTTCGTGCTATGCCCAGCACACCGAACTCCT 415
                * ***** ** *  * ** **** ** * **   ** * ***** *** *** **  * *

Control         TGCCAATCCCGCGGGGGGTGGGTGCACTGACCTCCCGCACGGCAGGACGGCCCATCCGAA 476
Treated         TTCCGCTACCGCGGTGCGTGCGGTCCCTG-CCTCACGACCGGTACTACTGGTCGTACGAC 474
                * **  * ****** * *** *  * *** **** **  *** *  ** *  * * ***

Control         CTGACCACCCCCCAGGACTTCGTTTCCGTTTTCTAGACTATATAACATATTA----TCAA 532
Treated         CTGCACAACCGCCATGACATTGTTTTCATTTTGTTGAATATGAGAGATATCACCAATCAA 534
                ***  ** ** *** **  * **** * **** * ** *** * * ** * *    ***

Control         ACCCCACGCCGGGGGTCGGTCATCTCTCGGCTCGATGATCAACGAAGACCACTGCTCGTT 592
Treated         AGGCTAGGAATGGGATCGCTCGTCTCTTGAAACCATAAATACCTCAGCTTAATGCTGGTC 594
                *  * * *   *** *** ** ***** *   * ** *  * *  **   * **** **

Control         GCGCTGTGTATGTGGCCCGCATGACA----AACAAAAAAACAAAATGTGCCATGCGCATG 648
Treated         ACAATGGGAAC-TGCTCAGAAAACCATGGGACCAAAAAAAAAAGTCGTAACAAG-GTTCG 652
                 *  ** * *  **  *   *   **    * ******** **   **  ** * *   *

Control         TAGCGCGCGCATGAGGAAGTGCGCACGAGAGTGTGCGTCAGACACGTGACGC-CGCGACT 707
Treated         TAG-GTGAACCTGCGGAAG---GATCATTACTGTACTTCATACCCATGGAGCGCATAAAT 708
                *** * *  * ** *****   *  *   * *** * *** ** * **  ** *   * *

Control         GAGAGATGACATGAGAGAGAGAGATGCGCTAAGCGCTGAGGGCGCATGACAGCGCTGCAG 767
Treated         GAACCACACCCTCCCCCATGTGCCCGTGC-GAGCGCCCGACTCAGCGAGTCTCTTTG--- 764
                **   *     *     *       * *  ******      *         *   *
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Fig. 3. *Sequence alignment of ITS1 sequences of control and treated An. stephensi. (*identical bases, - inserted/ deleted bases).*

and as many as 140 transversions. Similarly, in nimbecidine treated ITS2 sequence there were only 2 deletions, 4 insertions, 15 transitions and 39 transversions (Tables 1-5).

The present results have clearly demonstrated gene mutations in *An. stephensi* DNA. Most of the toxic chemicals which produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes in the form of breaks and other related damage in the double helical organization of nucleotides (Klopman *et al.*, 1985). Azadirachtin, which is a principle component of nimbecidine, has been reported to have a mitotic poisoning effect on mouse chromosomes (Awasthy *et al.*, 1995; Awasthy, 2001) while its enzymatic biotransformation has been suspected to produce metabolites and oxygen free radicals (Sies, 1993). These

Table 1. Deletions and insertions in ITS1 of nimbecidine treated *An. stephensi.*

Type of mutation	Total number of mutations	Bases involved	Number of base/s	Type of base/s
Deletion	16	1	1	C
		8	1	T
		17	1	G
		49	1	A
		285	1	G
		446	1	A
		604	1	G
		643	1	C
		652	1	C
		668-670	3	T, G, C
		737	1	T
		765-767	3	C, A, G
Insertion	13	195-196	2	A, A
		335-336	2	C, C
		528-529	4	C, C, A, A
		618-619	4	T, G, G, G
		700-701	1	G

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Control         CCTTGTCACCGCCCGTCCATTGTACCCTCTGCCGGCCTTGTTACTACTCCC-CTGGATTA 59
Treated         -TTCGTAGCCGCCCGTCCTTTGTACAATG-GCCGTGTTGTAGACTACTCCCGCTCCTATG 58
                  * **  ********** ******  *  ****   *    ********* ***   *

Control         CTAGTTTGAATCGCTAACATTGACCGAACTTGATGATTTAGAG---GAAGTAAAAGTCGT 116
Treated         CACTATTGCATCGTTAACATTGTACGAACTTGATGATTATAAGAGAGAAGTAAAAGTCGT 118
                *    *** **** ********  **************   **   **************

Control         AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGTACTTCATACCCATGGAG 176
Treated         AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGTACTTCATACCCATGGAG 178
                ************************************************************

Control         CGCATAAATGAACCACACCCCCCCCCATGTGCCCGTGCGCGCGCCCGACTCAGCGAGTCT 236
Treated         CGCATAAATGAACCACACCCTCCCCCATGTGCCCGTGCGAGCGCCCGACTCAGCGAGTCT 238
                ******************** ****************** ********************

Control         CTTTGAGTACTTTCCCCTACTCACCCCTCGAGACTGCCCGGGGGCATTGCACACGTGAGA 296
Treated         CTTTGAGTACTTTCCCCTACTCACCCCTCGAGACTGCGCGGGGTCATTGGACACGTGAGA 298
                ************************************ ***** ***** **********

Control         ATGGTATAGCTTCGAGAAATCTCCCTCCAGCTGCCAACTCGATGTTCAGTCTCCATCCCA 356
Treated         GTGGTCTAGCTTCGAGAAATCTACCTCCAGCTGGCCAGTCGATGTTCAGTTTCTATCGGA 358
                 **** ***************** ********** * * ************ ** **** *

Control         ATGCCGACCGAGGGCTGGCCGTATGTCCGGCCTATGTCCCGCGCACCACTCCCATTTGCG 416
Treated         GTGCCGACGGAGGGCTGGCCGTATGTCCGGCCTACGTAACGCGTTGCACTCCCATTTGTG 418
                ******* ************************ **  ****   ************ *
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Fig. 4. *Sequence alignment of ITS2 sequences of control and treated An. stephensi. (*identical bases, - inserted/ deleted bases).*

effects were considered similar to other xenobiotics, including damage to spindle apparatus and unequal distribution of the chromosomes during anaphase, leading to mitotic breakdown. In relevance to the structure based toxicity of azadirachtin, Rosenkranz and Klopman (1995) identified the presence of atleast five copies of biophores in azadirachtin, which were considered as potent carcinogens. Akudugu *et al.* (2001) evaluated the cytotoxicity of azadirachtin in human glioblastoma cell line in which they found considerable reduction in the percentage of dividing cells, formation of micronuclei and decreased cell survival. While studying the sperm head assay, Khan and Awasthy (2003) observed that azadirachtin extract induced structural and numerical changes in the spermatocyte chromosomes as well as synaptic disturbances at the first metaphase with

Table 2. Substitutions in ITS1 of nimbecidine treated *An. stephensi.*

Type of substitution	Total number of substitutions	Type of bases substituted	Total number of bases substituted	Position of bases in the sequence
Transition	93	A→G	22	81, 106, 182, 210, 294, 315, 336, 367, 400, 419, 421, 470, 518, 520, 554, 632, 697, 727, 729, 738, 756, 758
		G→A	26	214, 265, 292, 296, 308, 316, 340, 372, 411, 504, 547, 562, 569, 593, 595, 615, 637, 657, 675, 683, 694, 702, 704, 710, 715, 749
		T→C	20	3, 29, 60, 211, 255, 319, 352, 358, 383, 389, 403, 412, 527, 592, 603, 634, 647, 714, 732, 744
		C→T	25	15, 30, 47, 208, 219, 236, 261, 347, 348, 388, 459, 468, 497, 502, 537, 560, 572, 581, 582, 608, 645, 654, 703, 734, 765
Transversion	140	A→T	9	209, 310, 351, 511, 633, 646, 673, 676, 766
		T→A	9	235, 314, 495, 519, 564, 571, 601, 614, 641
		A→C	26	26, 178, 200, 217, 322, 330, 356, 422, 442, 455, 476, 480, 574, 577, 580, 616, 620, 659, 662, 692, 711, 716, 718, 721, 723, 731
		C→A	29	11, 34, 35, 206, 321, 338, 405, 424, 451, 454, 471, 481, 484, 514, 541, 542, 562, 563, 584, 596, 610, 612, 629, 638, 656, 672, 698, 706, 757
		G→T	24	28, 45, 245, 254, 317, 346, 385, 413, 418, 431, 440, 462, 465, 491, 543, 576, 677, 685, 689, 726, 728, 751, 759, 761
		T→G	7	22, 238, 281, 350, 439, 589, 754
		G→C	21	232, 258, 297, 339, 370, 380, 415, 433, 437, 461, 551, 566, 607, 679, 712, 720, 722, 724, 730, 745, 755
		C→G	15	27, 53, 287, 295, 364, 369, 375, 467, 487, 509, 522, 534, 535, 539, 589

Table 3. Deletions and insertions in ITS2 of nimbecidine treated *An. stephensi.*

Type of mutation	Total number of mutations	Bases involved	Number of base/s	Type of base/s
Deletion	2	1	1	C
		30	1	T
Insertion	4	53-54	1	G
		102-103	3	A, G, A

a significant increase in the frequency of sperm head abnormalities. Chandra and Khuda-Bukhsh (2004) also encountered an abnormal increase in the incident of chromosome aberrations, abnormal red cell nuclei and sperm morphology induced by azadirachtin in a fish, *Oreochromis mossambicus*. Recently, Cordeiro *et al*. (2010) have also detected insecticide repellence, irritability and 100% mortality of the larvae of *Chrysoperla externa* and *Ceraeochrysa cubana*. The experimental data generated so far on the toxicity of nimbecidine/ azadirachtin suggests that this pesticide has a considerable potential to cause irreparable damage to the biochemical, physiological and genetic components of the effected living systems. Therefore, bioinsecticides should not be exempted from risk assessment while preferring them for potential use in agriculture. To our knowledge this is the first report to describe the genotoxic effects of this nimbecidine at the molecular level of DNA, which may be considered as a valuable contribution to the data bank of genotoxicity assessment of pesticides.

Table 4. Substitutions in ITS2 of nimbecidine treated *An. stephensi.*

Type of substitution	Total number of substitutions	Type of bases substituted	Total number of bases substituted	Position of bases in the sequence
Transition	15	A→G	5	8, 9, 59, 297, 357
		G→A	1	100
		T→C	2	4, 391
		C→T	7	37, 73, 197, 347, 350, 400, 415
Transversion	39	A→T	5	19, 56, 82, 99, 401
		T→A	5	41, 57, 61, 64, 98
		A→C	4	62, 68, 302, 332
		C→A	8	7, 26, 27, 83, 216, 319, 394, 395
		G→T	4	35, 40, 63, 280
		T→G	1	39
		G→C	2	54, 55
		C→G	10	29, 36, 274, 286, 330, 334, 354, 355, 365, 402

Table 5. Sequence characteristics of ITS1 and 2 of control and nimbecidine treated *An. stephensi.*

S. No.	Parameter	ITS1		ITS2	
		Control	Treated	Control	Treated
1	Total length of sequence (no. of bases)	767	764	416	418
2	GC content (%)	55	51	55	53
3	AT content (%)	45	49	45	47
4	Deletions	-	16	-	2
5	Insertions	-	13	-	4
6.	Transitions	-	93	-	15
7	Transversions	-	140	-	39

Along with this, the results of present study also advocate the use of PCR which is an accurate, reliable and highly sensitive technique for detecting pesticides related DNA damage.

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