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Isolation and Characterization of *Bacillus thuringiensis* Isolated from Soil and their Possible Impact on *Culex pipiens* Larvae

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ABSTRACT

Use of microbial agents is an important tool for insect control because of their virtual specificity and lower environmental impacts. A total of four soil samples were collected from different localities across the black sea region in Turkey to isolate native *Bacillus thuringiensis* (*Bt*) strains. Sodium acetate-(0.25 M)-selection heat-pasteurization, and 50% ethanol treatment methods were used for *Bt* isolation**.** Characterization of *Bt* isolates based on their morphological, physiological and biochemical parameters. PCR analysis was performed, using novel general and specific primers for *cry* genes (*cry4A* and *cry10* genes) that encoding proteins active against mosquitoes. Based on results, the isolate *Bt*22.19 showed the highest larvicidal effect against 3rd instar *C. pipiens* larvae. *Bt* 22.19 was isolated from soil samples in hazelnut orchards. The isolated strains had *cry4A* and *cry10* genes. *Bt* isolates displayed highest similarities with the *Bacillus thuringiensis* subsp. *israelensis* regarding to the presence of *cry* genes. Obtained results are a promising introduction for further studies on evaluation of the potential usefulness of isolate *Bt*22.19 crystals for mosquito's control.

Key words: *Bacillus thuringiensis, Culex pipiens,* larvicidal effects.

INTRODUCTION

The desire for mosquito control increased significantly. Mosquitoes have the potential and lethal capacity to kill more than 1 million victims a year around the world. *Culex* spp. is a common mosquito species throughout the world including Turkey (Harbach, 2012). *Culex* spp. transmits pathogens causing human diseases throughout the world that include dengue fever, malaria and yellow fever (Almeida *et al.,* 2008). Common insecticides have been applied for mosquitos' elimination has given rise to problems for human and environment. The most useful methods for controlling these diseases based on vector control that is mainly accomplished by using synthetic insecticides. Use of entomopathogenic bacteria as biolarvicides is a favor alternative for insect controls (Regis *et al.,* 2001). It has been safely used against species of the orders Lepidoptera, Coleoptera, and Diptera for the last 50 years (Roh *et al.,* 2007). *Bacillus thuringiensis (Bt)* is a rod-shaped, aerobic, gram positive and spore-forming bacterium. It forms a parasporal crystal during sporulation (Höfte and Whiteley, 1989). These parasporal crystals consist of insecticidal-endotoxins with specific toxicity towards a variety of lepidopteran, dipteran and coleopteran larvae (Gill *et al.,* 1992) and called Cry proteins, expressed by the *cry* genes (Schnef *et al.,* 1998). Number of known *Bt* strains active on Diptera is growing (Guerchicoff *et al.,* 1997). *B. thuringiensis* subsp. *israelensis cry* and *cyt* genes encode dipteran-active toxins: Cry4A, Cry4B, Cry10A, and Cry11A, Cyt1A, and Cyt2B (Guerchicoff *et al.,* 1997 and Salehi *et al.,* 2008). To date, *Bt* strains have been isolated from many bacterial habitats, including plant tissue, soil, insects, and water (Ichimatsu *et al.,* 2000 and Iriarte *et al.,* 2000), free-living animals (Swiecicka *et al.,* 2002).

The aim of this study was, to find *Bt* isolates from different soils in Samsun Province, Turkey, to characterize these strains by molecular methods and to determine their larvicidal activity against *C. pipiens* larvae.

MATERIALS AND METHODS

Soil samples

Soil samples were collected in glass tube from surface to a depth of 10 cm in the hazelnut orchards. A total of 37 soil samples were collected from the black sea region of Turkey from April, 2013 to June, 2013. This origin of samples had not been previously treated with any *Bt* biopesticides. The collected samples were kept at about 4°C in an incubator till been used for bacterial isolation.

Isolation of bacteria

The soil samples of 1 g were suspended each in 10ml 0.85% NaCl and heated with shaking at 70°C for 10 min. Aliquots of 100µl of suspension were plated onto *nutrient agar* (Difco nutrient broth solidified with agar, 10 g l^{-1}). The plates were then incubated at 30±2°C for 48h, then the culture stained with amino black and Ziehl's carbol fuchsin and examined under a standard light microscope. *Bt* isolates were selected when black crystals dyed black were noticed (Yu *et al.,* 1991).

Biochemical characteristics

Morphological, physiological and biochemical

Table (1): Characteristics of general and specific primers for *cry1A, cry2, cry3A, cry4A, cry6A, cry8, cry9, cry10, cry11, cry19, cry21, cry22, cry30, cry40, cry54, cry62A*, and *cyt1A* genes

Primer	Sequence*	Positions	Gene(s) recognized	Product size (bp)
Cry1A	F 5'-GAGGGAATGGCACGGGTTTA-3'	893-912	*cry1A*	689
	R 5'-CCCGAAAAACCGACAGGAGA-3'	1.581-1.562		
Cry2	*F 5'*-GTAGTGGACCACAGCAGACC-3'	836-855	*cry2*	336
	R 5'-TAGAGGTAGCAACGCCCTCT-3'	1.771-1.152		
Cry3A	*F 5'*-GTGGAGCGCTTGTTTCGTTT-3'	54-273	*cry3A*	528
	R 5'-AAACAACAGATGCCCAGCCT-3'	781-762		
Cry4A	*F 5'*- ACGGGGATTTTGAATCGGCT -3'	2.276-2.295	*cry4A*	940
	R 5'-CCAGTTACATGCCACCCCAT-3'	3.215-3.196		
Cry6A	*F 5'*-GGGGAAAGTAGTCCAGCTCA-3'	888-907	*cry6A*	465
	R 5'-CCAAGCATCAGAAGCGTCCT-3'	1.352-1.333		
Cry8	*F 5'*-GCGTTAATCCAGCTGCGATT-3'	101-120	*cry8*	1294
	R 5'-GTCCAAGCAAATGAAACCCTGT-3'	1.394-1.373		
Cry9	*F 5'*-TCTATGGGGCAAGATGGGGA-3'	656-675	*cry9*	1656
	R 5'-AATTTTCACGTGCGCTTGCT-3'	2.231-2.392		
Cry10	*F 5'*-ATAAATGGGAGCCAGCACGT-3'	470-489	*cry10*	1142
	R 5'-GTCTCCACCTGTGTGACCAG-3'	1.611-1.592		
Cry11	*F 5'*-ATAGGGAAATGGGCGGCAAA-3'	145-164	*cry11*	1406
	R 5'-TCTGTTGCTTGATCTGGCGT-3'	1.550-1.531		
Cry19	*F 5'*-CCACAAATGCCCATGCGAAA-3'	103-122	*cry19*	1422
	R 5'-TCCGTGTGTGCCTATTCCAC-3'	1.524-1.505		
Cry21	*F 5'*-ACACCCTGCTGAACGATCTG-3'	83-102	*cry21*	287
	R 5'-GGTGGTATACATCGGCAGGG-3'	369-350		
Cry22	*F 5*'-CAAGCAGGAGCAATTGCAGG-3'	151-170	*cry22*	364
	R 5'-TTCGCTGCATCTGAGCTAGG -3'	514-595		
Cry30	*F 5'*-TCCAGGAGCAGCTGTAGGAT-3'	252-271	*cry30*	1394
	R 5'-GGCCAGGACCTGCAATTACT-3'	1.645-1.626		
Cry40	*F 5'*-TGTGGGAATCAACCTCGAGC-3'	149-168	*cry40*	946
	R 5'-CCTACCCAGCCGGCAAAATA-3'	1.094-1.075		
Cry54	*F 5'*-CCGGAGTTAGTGCAGGTGTT-3'	197-216	*cry54*	1488
	R 5'-CTGTATGACCAGGACCAGGC-3'	1.684-1.665		
Cry62A	F 5'-GGACCCTGCCACGATTAACA-3'	687-706	*cry62A*	1301
	R 5'-TCACGAACCAGTGATTCGCA-3'	1.987-1.968		
Cyt1A	*F 5'*-CCCTCAATCAACAGCAAGGG-3'	57-76	*cyt1A*	593
	R 5'-AGCTCGCAGAATCTTGAATTGTG-3'	649-627		

* Position at 5' end of direct and reverse primers for each PCR primer pair. F and R forward and reverse primers, respectively.

characteristics of the *Bt* isolates were determined according to the standard methods recommended in Bergey's Manual of Systematic Bacteriology (Sneath *et al.,* 1984).

Determination of specific *cry* genes

To identify the larvicidal genotypes of *Bt* isolates, seventeen pairs of primers were designed to detect the presence of the *cry* and *cyt* genes through PCR of their conserved regions. All primers were designed using Geneious 7 software (Biomatters, Auckland, New Zealand) based on manual identification of specific cry and cyt genes regions of the sequence alignment. BLAST analysis was also performed among chosen *cry* and *cyt* genes sequences in NCBI database to confirm the specificities of the primer sets. The full list of primers is provided in table (1). The PCR mixture contained 1 µl of DNA in a total volume of 10 µl containing 200 nM concentrations of each primer constituted in 1x BioMix Red (Bioline, Boston,F Massachusetts, United States) PCR reaction buffer. The PCR mixture contained 1 µl of DNA in a total volume of 20 µl containing 200 nM of each primer in 1× reaction buffer. PCR amplifications were performed in a *T100 thermal cycler* (*Bio-Rad*) with the following touchdown cycling conditions: 4 min at 94°C, 24 cycles of 0.30 min at 94°C, 0.30 min at 65°C with a 1°C decrease per cycle, and 1.30 min at 72°C, followed by 10 cycles of 0.30 min at 94°C, 0.30 min at 56°C, and 1.30 min at 72°C, ending with a 16°C hold. PCR products were monitored on a 1.0% agarose gel in 1x TAE buffer at 100V for about 60 min and checked their quality, size and yield.

Bioassays for mosquito larvae

The treatments consisted of:

(i) *B. thuringiensis* isolates 22.19;
(ii) *B. thuringiensis* isolates hma 5;
(iii) *B. thuringiensis* isolate hma7;
(iv) *B. thuringiensis* isolates bmeg;
(v) Positive control - commercial biopesticide of *B. thuringiensis* (VectoBac) WG; and
(vii) Negative control.

Bt isolates were grown onto nutrient agar (Difco) at 30±2°C for 24 h. The cells were then harvested and suspended in sterile distilled water at concentrations of [10^1, 103, 10^6 and 10^9 cfu/ml, colony-forming units/ml] and heat shocked (10 min, 70°C). Different doses of 8,000; 16,000; and 32,000 international toxic units (ITU)/mg of commercial biopesticide VectoBac WG was used as positive control. Untreated box was negative control. Third-instar larvae were used for all bioassays in 50ml of tap water in plastic cups according to the standard bioassay procedure (WHO, 2005). Each bioassay was independently performed three times in duplicate. *C. pipiens* larvae were provided by Ondokuz Mayis University, Agricultural Faculty, Plant Protection Department, Entomology Lab. (Samsun, Turkey). Mortality was recorded after 6, 12, 24 and 48h.

Data analysis

Kolmogorov-Smirnov one sample test used to examine normal distribution and Levene test for equal variance (homosceasticity) assumption (Onder, 2007).

RESULTS AND DISCUSSION

Biochemical characteristics

Four of gram-positive and spore forming bacilli were isolated from soil. Morphological, physiological and biochemical characteristics of isolates are shown in table (2). All isolates showed a positive reaction in aerobic growth and gelatin liquefaction tests. Biochemical data revealed that a negative reaction in arabinose and mannitol. The isolates of Bt22.19 and Btbmeg could grow in sodium chloride solutions at concentrations ranged 5-10%, while the isolates of Bthma5 and Bthma7 that couldn't grow in NaCl solution at concentration of 10%.

Determination of specific *cry* genes

The mosquitocidal genotypes of *Bt* were determined through PCR designed primers by using Geneious 7 software (Biomatters, Auckland, New Zealand) for the *cry1A, cry2, cry3A, cry4A, cry6A, cry8, cry9, cry10, cry11, cry19, cry21, cry22, cry30, cry40, cry54, cry62A,* and *cyt1A* genes. Four *Bt* isolates obtained from soil samples carried *cry4A,* and *cry10* genes. The isolates exhibited the 940 bp and 1142 bp fragments of the *cry4A* and *cry10* genes, respectively, that encode the crystal protein toxic to mosquitoes. None of the isolates had *cry5, cry6A, cry8, cry9, cry19, cry21, cry22, cry30, cry40, cry54, cry 62A* and *cyt1* genes. BLAST analysis indicated that it corresponded to the *cry4A* and *cry10* genes (100% identity). The *Bt* serovar strain BRC-LLP29 was used as a control for *cry4A,* and *cry10* genes (data not shown).

Table (2): Morphological, physiological and biochemical characteristics of *Bt* isolates

Parameters	*Bacillus thuringiensis* isolates			
	Bt2219	Bthma5	Bthma7	Btbmeg
Shape	rod	rod	rod	Rod
Gram staining	G^+	G^+	G^+	G^+
Aerobic growth	+	+	+	+
Gelatin liquefaction	-	-	-	-
Acid from Arabinose	-	-	-	-
Mannitol	-	-	-	-
Growth at 5%	+	+	+	+
10%	+	-	-	+
Growth at 30°C	+	+	+	+
40°C	+	+	+	+
50°C	-	-	-	-

*****(-) negative reaction, (+) positive reaction, (G^+) Gram positive.

Bioassays

Bioassay results indicated that the *Bt* isolates were toxic against the 3rd instar larvae of *C. pipiens*. Percentage mortality of *Bt* isolates are shown in table (3). At bacterial concentration of 10^6 *cfu/ml* within 48h, percentages of mortality ranged 26.67 - 98.33%, where they were 98.33, 36.67, 31.67 and 26.67% for the isolates Bt22.19, Btbmeg, Bthma7 and Bthma5, respectively (Figure 1). Insignificant differences were recorded among mortality rates in the isolates of Btbmeg, Bthma7 and Bthma5. At concentration of *10^9 cfu/ml*, the mortality percentage ranged between 33.33 - 100.0%, where the highest mortality (100.0%) was recorded at the isolate Bt22.19. Btbmeg, Bthma7 and Bthma5 isolates caused the least larval death, where the percentage mortality ranged of 33.33 - 40.0% (Figure 2). Highest mortality % were 100, 88.33, 78.33, and 50.0%, obtained at the treatment of Bt22.19 isolates at the concentrations of *10^9 cfu/ml*, while treatment with the commercial biopesticide Wertobag gave highest mortality by 100, 100, 93.33 and 66.67% at the concentration of 32000, ITU/mg within 48, 24, 12 and 6h, respectively (Figure 3).

Synthetic insecticides have been associated with human health problems such as cancer, liver damage and birth defects beside environmental problems. Some of the microorganisms like, *Bacillus* spp. in controlling insects that transmit human diseases, is well established. Accordingly, the present work was proposed to isolate and characterize *Bt* isolates from black sea region habitat and to test their effect against

Table (3): Effects of *Bacillus thuringiensis* isolates on mean percentage mortality of the 3rd instar larvae

Isolates	CFU/ml	Mean percentage mortality rate (%) ± SD			
		Time (h)			
		6	12	24	48
Bt2219	10^1	0.00 ± 0.00g*	28,33 ± 1,67e	63,33 ± 4,41d	78,33 ± 1,67c
	10^3	0.00 ± 0.00g	46,67 ± 1,67d	83,33 ± 6,01bc	88,33 ± 6,01bc
	10^6	41,67 ± 4,41c	65.00 ± 5,77c	86,67 ± 1,67b	98,33 ± 1,67ab
	10^9	50.00 ± 5,77b	78,33 ± 1,67b	88,33 ± 1,67b	100.00 ± 0.00a
Bthma5	10^1	0.00 ± 0.00g	5.00 ± 0.00ij	5.00 ± 0.00gh	10.00 ± 2,89hi
	10^3	0.00 ± 0.00g	8,33 ± 3,33hij	10.00 ± 2,89fgh	16,67 ± 4,41gh
	10^6	8,33 ± 1,67def	15.00 ± 0.00fg	21,67 ± 4,41ef	26,67 ± 4,41efg
	10^9	13,33 ± 1,67d	20.00 ± 5,77ef	28,33 ± 6,01e	33,33 ± 1,67def
Bthma7	10^1	0.00 ± 0.00g	6,67 ± 1,67hij	11,67 ± 1,67fg	16,67 ± 1,67gh
	10^3	0.00 ± 0.00g	13,33 ± 3,33fgh	16,67 ± 6,67efg	20.00 ± 7,64gh
	10^6	5.00 ± 2,89fg	13,33 ± 1,67fgh	25.00 ± 2,89e	31,67 ± 4,41def
	10^9	6,67 ± 1,67efg	26,67 ± 1,67e	28,33 ± 1,67e	40.00 ± 2,89d
Btbmeg	10^1	0.00 ± 0.00g	6,67 ± 1,67hij	10.00 ± 2,89fgh	10.00 ± 2,89hi
	10^3	0.00 ± 0.00g	10.00 ± 2,89hij	16,67 ± 4,41efg	23,33 ± 6,01fg
	10^6	5.00 ± 0.00fg	15.00 ± 2,89fg	25.00 ± 2,89e	36,67 ± 3,33de
	10^9	11,67 ± 1,67de	25.00 ± 2,89e	28,33 ± 6,01e	38,33 ± 4,41d
Wertobag (International toxic unit-ITU)	8000	38,33 ± 1,67c	63,33 ± 1,67c	75.00 ± 2,89c	96,67 ± 1,67ab
	16000	53,33 ± 1,67b	81,67 ± 1,67b	91,67 ± 1,67ab	100.00 ± 0.00a
	32000	66,67 ± 1,67a	93,33 ± 1,67a	100.00 ± 0.00a	100.00 ± 0.00a
Control	0	0.00 ± 0.00g	3,33 ± 1,67j	0.00 ± 0.00h	0.00 ± 0.00i
P	1.000	<0.001	<0.001	<0.001	<0.001

(*) Mean followed by the same letters in each column are not significant.

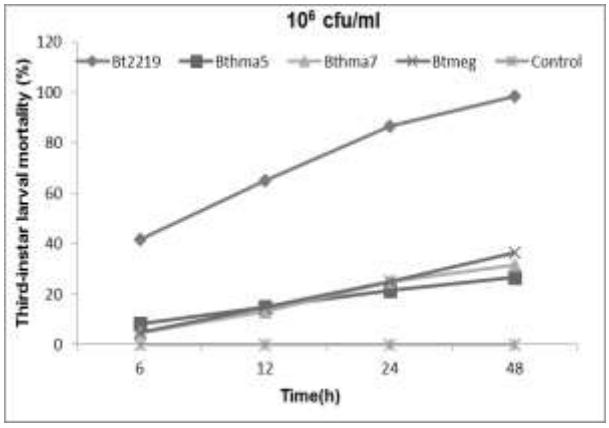

Fig. (1): Mortality of *Culex pipiens* 3rd instar larvae treated with *Bacillus thuringiensis* isolates for different times at the concentration of 10^6 cfu/ml.

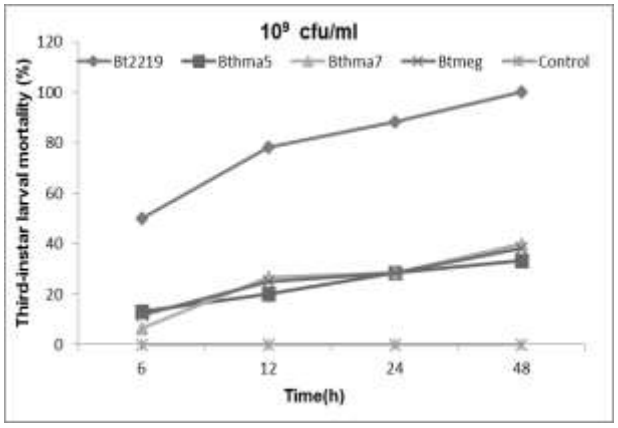

Fig. (2): Mortality of *Culex pipiens* 3rd instar larvae treated with *Bacillus thuringiensis* isolates for different times at the concentration of 10^9 cfu/ml.

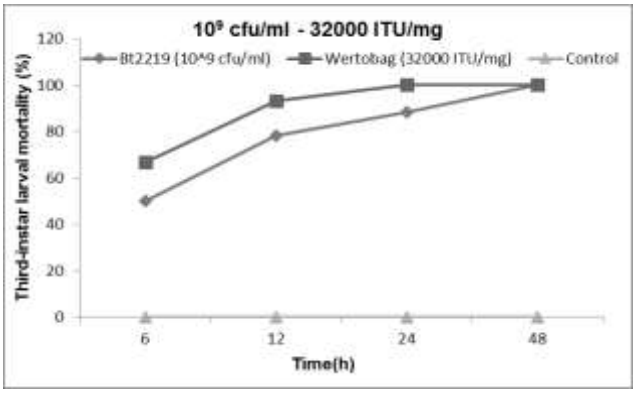

Fig. (3): Mortality of *Culex pipiens* 3rd instar larvae treated with *Bacillus thuringiensis* isolates and the commercial biopesticide Wertobag for different times at the concentrations of 10^9 cfu/ml and 32000, ITU/mg.

to the larvae of the *C. pipiens*. *Bt* can be isolated from soil, leaves, dead larvae or water (Armengol *et al.*, 2007; Hernández-Soto *et al.*, 2009; Liang *et al.*, 2011 and Valicente *et al.*, 2010). The *Bt* strain produces crystal proteins that have been successfully used for controlling the mosquito population (Liang *et al.*, 2011).

Bacillus was confirmed morphologically and biochemically accordingly to Sneath (1986). The isolates were subjected to further biochemical characterization test according to Claus and Berkeley (1986) and resulted in four isolates closely resembling *B. thuringiensis* 22.19. Wild strains isolated form environmental samples can synthesize crystals that display higher activity against insect pests in comparison to *Bt* strains already used in pesticide production. Previous workers were not able to isolate this entomopathogenic bacterium from different habitats in black sea region including hazelnut orchards. Results showed that *Bt*22.19 is an important strain that produces secondary metabolites and active compounds. The strain plays an important role in biological control of *C. pipiens* larvae.

It is well known that the characteristic shape of the mosquitocidal crystals is the spherical-shaped crystals. *Bt* produces this type of crystals (Charles and de Barjac, 1982), to which *Culex* larvae are more susceptible (Boisvert, 2005). In the present study, the highest mortality percentage recorded was 100%, obtained by the isolate Bt22.19, as compared to other strains in the untreated control. Present results reported that successful pupation of *Culex* spp. was significantly delayed when exposed to Bt22.19. In addition, Lacy *et al.* (2004) confirmed that *Bt* strains affected *C. quinquefasciatus.*

Larvicidal effects of *Bt* isolates and its commercial biopesticide Wertobag were compared using percentages of mortality of *C. pipiens* larvae at 6, 12, 24 and 48h post treatment. The results showed a good performance for the isolate of *Bt*22.19 (100% mortality at a concentration of 10^9 cfu/ml within 48h) and the commercial biopesticide Wertobag (100% mortality at concentrations of 16000 and 32000 ITU/mg within 48h). Isolate *Bt*22.19 that had higher activity than the other isolates (Btbmeg, Bthma7 and Bthma5) against *C. pipiens* larvae were identified in spite of their high similarity *cry* genes. These data support the idea that although a great variability in *cry* genes codifying for different mosquitocidal toxins that exist in the natural strains of *B. thuringiensis* (Schnepf *et al.*, 1998). Similarly, the results suggest that insecticidal potency of the isolates was not directly related with their *cry* gene content as stated by Padidam (1992). In addition, Seifinejad *et al.* (2008) confirmed that presence of specific genes was not an accurate indicator of toxicity, because the genes could be inactive, under the control of an inefficient promoter or be expressed in a concentration too low to affect toxicity. However, the detection of these genes in most *Bt* isolates collected locally indicated that they can be effectively used to produce spore-crystal mixture for controlling mosquitoes. Similarly, many studies stated that *Cry4* and *Cry10* play a major role in mosquitocidal activity of *Bt* strains (Delecluse *et al.*, 1988; Guerchicoff *et al.*, 1997; Ben-Dov *et al.*, 1999; Armengol *et al.*, 2007; Hernández-Soto *et al.*, 2009 and Baig and Mehnaz, 2010). Misztel *et al.* (1996) reported that differences in potency, in general, could be attributed to the differences in susceptibility of the treated insects, where explained the connection between insect mortality and exposure time. This study reported that highly susceptible insects stopped feeding within 1 hour and died within 12-48h after ingestion of the toxin, less susceptible ones ceased feeding after 6 h and died after 2 days. While the slightly susceptible insects stopped feeding after 24 h and died after 2 weeks.

In conclusion, larvicidal potency of the two novel crystal protein genes, *cry4A* and *cry10*, was encoded at highly mosquitocidal *Bt* isolate *Bt*22.19. It is essential to perform additional bioassays with these *Cry* toxins against resistant mosquito colonies selected ith *B.thuringiensis* subsp. *israelensis* toxins. *Bt*22.19 exhibited an effect effortlessly against *C. pipiens* larvae. So, it can be used as an alternative insecticide because it is safe for the environment. Further studies are needed to identify the active compounds that can be used in broad spectrum for controlling insects and also to determine the mode of action of these compounds.

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