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Isolation, toxicity and detection of *cry* genes of *Bacillus thuringiensis* B. isolates from West-Azerbaijan province, Iran

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Abstract
Distribution and diversity of *cry* genes in *Bacillus thuringiensis* isolates from different ecological regions of West-Azerbaijan province of Iran were studied. Determination of *cry* (*cry1*, *cry2*, *cry3*, *cry4*, *cry7*, *cry9* and *cry11*) genes was based on polymerase chain reaction (PCR). Forty-eight strains were isolated from 744 different samples. Strains containing *cry1* genes were the most abundant, 93.75%. *B. thuringiensis* strains harboring *cry7* and *cry2* genes represented 4.16% and 76.16% of the strains. *Cry3*, *cry4*, *cry9*, and *cry11* genes were found in 50%, 45.83%, 12.68% and 18.75% of the strains, respectively. The 48 isolates were tested against *Culex pipiens* L., *Plodia interpunctella* (Hübner) and *Tribolium confusum* (Jacquelin du Val) larvae. Four isolates (8.33%) exhibited high toxicity against *C. pipiens*, 16 strains (30%) against *P. interpunctella* and 4 strains (8.33%) against *T. confusem* larvae.

Keywords: *Bacillus thuringiensis*, *Cry* genes, *Culex pipiens, Plodia interpunctella*, *Tribolium confusum*

1. Introduction
Chemical insecticides may be toxic and may cause environmental and health problems when used improperly. This problem is increasing due to the selection of insect resistance to some pesticides. Consequently, interest has developed in the use of alternative strategies for insect control, such as *Bacillus thuringiensis* (*Bt*) toxins (Waage, 1997) [25]. *B. thuringiensis is* an aerobic, gram-positive and endospore-forming bacterium which produces parasporal crystal proteins (*cry* proteins or δ-endotoxins) during sporulation. The insecticidal activity of *B. thuringiensis* is attributed to the parasporal crystals, also commonly known as delta endotoxins or insecticidal crystal proteins (ICP). The crystals are toxic to many insect pests from lepidoptera, diptera, and coleoptera orders (Schneph *et al.,* 1998) [8, 21]; but are harmless to most other organisms, including wildlife and beneficial insects (De Maagd *et al.,* 2001) [9]. The insecticidal crystal proteins are codified by *cry* genes and more than 200 *cry* genes have been described and classified into a large number of groups and subgroups based on their insecticidal activity and amino acid homology (Hernandez and Ferre, 2005) [11]. The proteins toxic for lepidopteran insects belong to *cry1*, *cry2*, and *cry9* groups; toxins active against Coleopteran insects are the *cry3*, *cry7* and *cry8* proteins, which have dual activity. The *cry5*, *cry12*, *cry13*, and *cry14* proteins are nematicidal and the *cry2*, *cry4*, *cry10*, *cry11*, *cry16*, *cry17*, *cry19*, and *cyt* proteins are toxic for Dipteran insects (Crickmore *et al.,* 1998) [8, 21]. Identification of *Bt cry* genes by PCR has proven to be a useful method for strain characterization and selection (Porcar and Juárez-Pérez, 2003) [18]. In addition Carozzi *et al.* (1991) [5] found correspondence of toxicity with the amplification of particular *cry* genes profiles, introducing PCR as a tool to predict *Bt* insecticidal activity.
In this study, *Bt* strains were isolated from West-Azerbaijan province, highly toxic isolates on *C. pipiens*, *P. interpunctella* and *T. confusum* larvae were selected based on *cry* gene profiles and confirmed by bioassay.

2. Materials and methods
2.1 Sample collection
A total of 744 samples were collected from 11 locations of West-Azerbaijan province of Iran. *B. thuringiensis* were isolated from soil, beaches, forests, stored product, agricultural fields, insect cadavers, grasslands and urban locations that have no history of treatment with *Bt*

products. Samples were collected by scraping off surface material with spatula and soil from 5-15cm below the surface. All samples were stored in sterile plastic bags at ambient temperature.

2.2 *B. thuringiensis* isolation

The samples were processed by acetate-selection method (Travers *et al.,* 1987) [23]. One gram of sample was subjected to acetate selection using 0.25 M. sodium acetate [pH 6.8]. Acetate inhibited the germination of *Bt* spores; so other spores germinated, and then the growing cells, non-spore-forming bacteria were eliminated by heat treatment (7 min at 80 ºC). The surviving spores were then plated onto nutrient agar cultures and incubated at 30 °C for 24hrs. Anywhere from 5-20 different colony types were usually obtained. By using the agar dot method for further identification, we picked a sample of colonies from these plates and placed onto T3 medium (3g of tryptone, 2g of tryptose, 1.5g of yeast extract, 0.05 M. sodium phosphate [pH 6.8], 0.005g of $MnCl_2$ per liter) for crystal production and use in crystal morphology and bioassay testing after 72 hr of incubation at 30 °C.

2.3 Bioassays

The activity of 48 *B. thuringiensis* isolates against insects of order lepidoptera, diptera and coleptera were tested using *P. interpunctella, C. pipiens* and *T. confusum,* respectively. For toxicity testing, spore-crystal preparations were grown onto T3 plates. The spores and crystals were then floated in 20 ml of sterile water and suspension was stored in sterile vials until was tested. Mosquito (*C. pipiens*) larvae were collected by net trap from ponds in Nazlu area (Urmia, West-Azerbaijan province). Ten 2nd instar larvae were added to 30ml pond water in 40ml plastic cups in 3 replicate. Then 5ml of each isolate suspension were added to the pound water. *B. t.* subsp. *israelensis* (HD500) and distilled water were used as positive and negative controls. Larval mortality was scored 48hrs after incubation at 22±1 °C. *P. interpunctella* was reared on artificial diet (Ozkan, 2006) [16]. From each isolate five ml spore-crystal suspension was sprayed over 1.5 g artificial diet on Petri dishes and air dried at room temperature. Ten 2nd instars larvae of *P. interpunctella* were released on this treated diet. A standard strain *B. t.* subsp. *kurstaki* (HD1) was used as positive and sterile distilled water as negative control. Each isolate was tested on 30 larvae in three replicates and mortality recorded after incubation at 20±2 °C for 48hrs. The bioassay on *T. confusum* larvae were carried out by rearing larvae on artificial diets (wheat flour plus 5% yeast) treated with *B. thuringiensis* isolate. *B. t.* subsp. *tenebrionis* (4AA1) was used as positive and sterile distilled water as negative control. Each isolate was tested on 30 larvae in three replicates and mortality recorded after incubation at 20±2 °C for 48hr.

2.4 DNA Isolation

Genomic DNA was extracted from 48 local *B. thuringiensis* isolates and four strains including *B. t.* subsp. *kurstaki*, *B. t.* subs p. *aizawai*, *B. t.* subsp. *tenebrionis*, *B. t.* subs p *israelensis* as reference *Bt* (kindly provided by Zihni Demirbag Karadeniz Technical University, Trabzon, Turkey) by using a modified version of the Dellaporta protocol (Dellaporta *et al.,* 1983) [10]. The isolates were grown overnight in LB broth in a shaker-incubator with 37 ºC temperature and 200 RPM speed. Each isolates were harvested by centrifugation at 3600 rpm for 10 min and suspended in water than incubated in a DNA extraction buffer (0.1M Tris [pH 8], 0.05M EDTA (pH 8), 0.1M NaCl, 0.5% SDS) for 30min at 65 °C. To eliminate residual carbohydrates, potassium acetate was added to a final concentration of 0.25M. and the solutions were incubated on ice for 10 min. Then 200 µl chloroform: isoamyl alcohol (24:1) was added to the solution and centrifuged at 10 000 rpm for 10 min. Then 200 µl isopropanol was added to the supernatant and incubated for 20 min at -20 °C. After further centrifugation (10000 RPM for 10 min), the supernatant was discarded and the genomic DNA pellet was washed at least once with 80% ethanol, air dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0) and stored at -4 ºC until use.

2.5 Identification of *Cry* genes by PCR

Presence of *cry*1, *cry2*, *cry3*, *cry4*, *cry7*, *cry9* and *cry11* genes in 48 native isolates were investigated according to Ceron *et al.,* (1995) [6], Ben-Dov *et al*., (1997) [2] and Alberola *et al.,* (1999) [1] (Table 1). All PCR reactions were carried out in 25 µl reaction volume containing 2 µl (reverse and direct) primers (Takapozist, Co., Ltd., Iran), 12.5µl master mix kit (Cinnagen, Co., Ltd., Iran), 7µl deionized water and 1µl DNA template in a DNA thermal cycler gradient (Eppendorf Mastercycler®). PCR cycles were programmed as: an initial denaturation step for 1 min at 94 °C, 38 cycles including: annealing for 1min (Table 1) and extension for 1min at 72 °C, followed by the final extension step for 5min at 72 °C. Each experiment was performed with positive (DNA template of the reference strain) and negative control (deionized distilled water). Finally, 12 µl PCR products was mixed with 5µl loading buffer and separated in a 1.2% agarose-ethidium bromide gel in TBE buffer (89mM Tris, 89mM Boric acid, 2.0mM EDTA, pH8.0) at 80V for 2hrs by electrophoresis (Biorad Powerpac®) and the gels were visualized using a gel documentation system.

Table 1: PCR primers used for identification of *cry* genes in *B. thuringiensis* isolates.

Target gene		Primer sequences (5' -> 3')	Annealing ºC	Size (bp)	Reference
Cry1	(d)	catgattcatgcggcagataaac	57	274-277	Ben-Dov *et al.*, (1997) [2]
	(r)	ttgtgacacttctgcttcccatt			
Cry2	(d)	gttattcttaatgcagatgaatggg	53	689-701	″
	(r)	cggataaaataatctgggaaatagt			
Cry3	(d)	cgttatcgcagagagatgacattaac	56	589-604	"
	(r)	catctgttgtttctggaggcaat			
Cry4	(d)	gcatatgatgtagcgaaacaagcc	58	439	"
	(r)	gcgtgacatacccatttccaggtcc			
Cry7	(d)	aagcagtgaatgccttgtttac	50	264	Alberola *et al.*,(1999) [1]
	(r)	Cttctaaaccttgactactt			
Cry9	(d)	Cggtgttactattagcgagggcgg	68	351-354	Ben-Dov *et al.,* (1997) [2]
	(r)	Gtttgagccgcttcacagcaatcc			
Cry11	(d)	Ttagaagatacgccagatcaagc	52	305	"
	(r)	Catttgtacttgaagttgtaatccc			

3. Results

3.1 *B. thuringiensis* strain collection

From 3010 different colonies of spore-forming bacteria, 48 *Bt* isolates were obtained after microscopic observation. *Bt* index corresponding to the ratio of *Bt* isolates to total number of isolates was 6.4% (48 isolates from 744 samples). This index is an important measure of success in isolating *B. thuringiensis*. Most strains were isolated from Urmia city samples but the highest *Bt* index was belonged to Khoy town samples. Soil samples were the most abundant and diverse source of strains.

3.2 Activity of *B. thuringiensis* isolates against pests

Activity of 48 *Bt* isolates on three different group of agricultural pests indicated that 4 isolates (representing 8.33%) exhibited high activity (mortality percentage over 75%) against *C. pipiens*, 16(30%) against *P. interpunctella* and 4(8.33%) against *T. confusum* larvae (Table 2). Wz-105, wz-111, wz-120, wz-122, wz-125, wz-149, wz-155, wz-157, wz-184, wz-187 and wz-189 isolates had mortality more than 90% against *P. interpunctella*.

Table 2: Classification of the *B. thuringiensis* isolates according to their toxicity levels against *P. interpunctella, T. confusum* and *C. pipiens* after 48 h.

Percentage of Mortality	*P. interpunctella*		*T. confusum*		*C. pipiens*	
	No. of isolates	% of isolates	No. of isolates	% of isolates	No. of isolates	% of isolates
Isolates causing a mortality of 0-25%	12	25	22	45.83	35	72.91
Isolates causing a mortality of 25-50%	12	25	13	27.08	4	8.33
Isolates causing a mortality of 50-75%	8	20	9	18.75	5	10.41
Isolates causing a mortality of >75%	16	30.33	4	8.33	4	8.33

3.3 PCR-based detection of *cry* genes in *B. thuringiensis* isolates

Each set of primers produced a PCR product with a unique molecular weight. Comparing amplification products of local isolates with those of *B. t.* subsp. *kurstaki* and *B. t.* subsp. *aizawai* (lepidopteran active), *B. t.* subsp. *tenebrionis* (coleopteran active) and *B. t.* subsp. *israelensis (*dipteran active) reference strains indicated the similarity of bonding patterns. *B. t.* subsp. *kurstaki* and *B. t.* subsp. *aizawai* contain *cry1, cry1, cry2* and *cry9*, *B. t.* subsp. *tenebrionis* contain *cry3, cry7* and *B. t.* subsp. *israelensis, cry4* and *cry11 genes* (Wang *et al.,* 2003) [26]. Strains harboring *cry1*, *cry2* and *cry9* genes were found at 97.9%, 79% and 12.5% frequencies, respectively (Figure 1 and 4). Frequency of strains harboring *cry3*, *cry7, cry4* and *cry11* genes were 50% and 4.1%, 45.8% and 18.75%, respectively (Figure 1, 3 and 4). As a result, isolates containing *cry1* and *cry2* genes were the most abundant compared with isolates containing other *cry* genes and the least common *cry* gene was *cry7*. In addition, it was observed that some of the isolates harbored two or more *cry* genes with different combinations (Figure 1). The most frequent combination was observed in *cry1 +cry2* (77.08%) and the least in *cry3 +cry7* (0%) (Figure 1, 2 and 4).

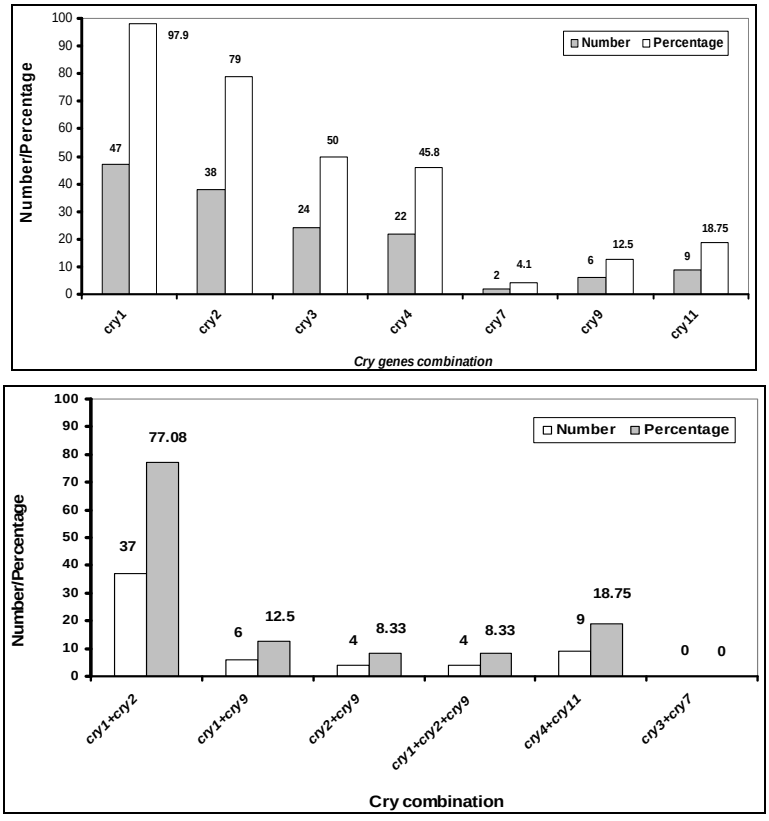

Fig 1: Frequency of combination and individual *cry* genes Lepidoptera. group (*cry1*, *cry2* and *cry3*), Diptera. group (*cry4* and *cry11*) and Coleoptera. group (*cry3* and *cry7*) genes among the 48 *B. thuringiensis* isolates from the WA province collection**.**

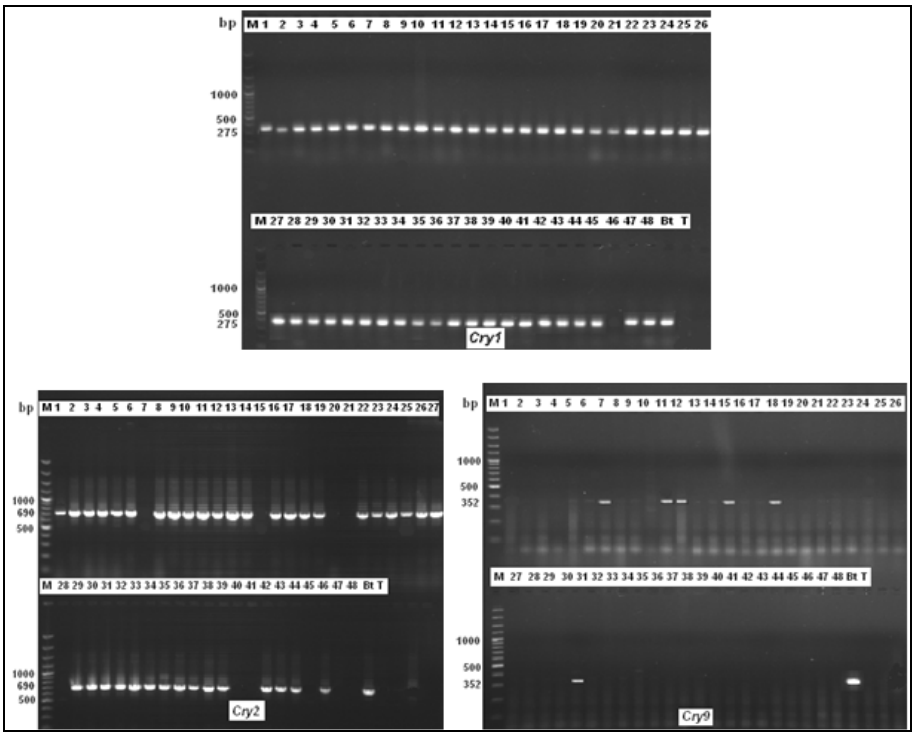

Fig 2: Agarose gel (1.2%) electrophoresis of PCR products for *cry1*, *cry2* and *cry9* genes: Lane M (100 bp DNA ladder), Lane 1-48 (48 isolates), Lane 49 (*B. thuringiensis*): *B. t.* subsp. *kurstaki* as *cry1*, *cry2* and *B. t.* subsp. *aizawai* for *cry9* positive control and lane 50(T): negative control (without DNA template).

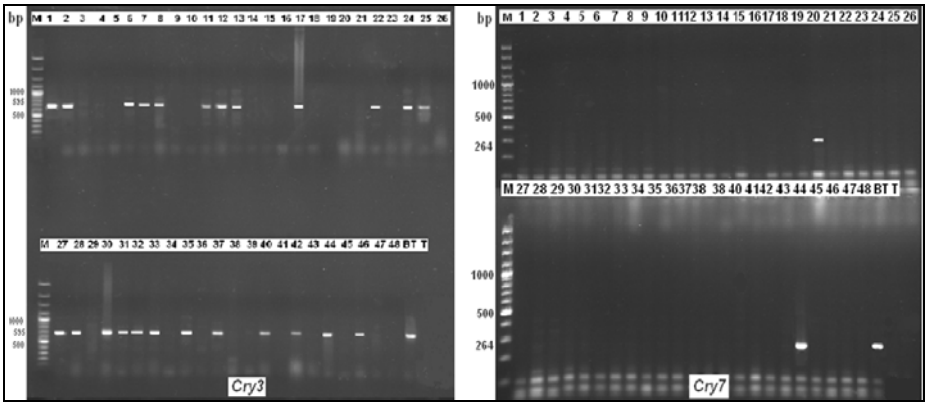

Fig 3: Agarose gel (1.2%) electrophoresis of PCR products for *cry3* and *cry7* genes: Lane M (100 bp DNA ladder), Lane 1-48 (48 isolates), Lane 49 (*B. thuringiensis*): *B. t.* subsp. *tenebrionis* as *cry3 B. t.* subsp. *aizawai* for *cry7* positive control and lane 50 (T): negative control (without DNA template)**.**

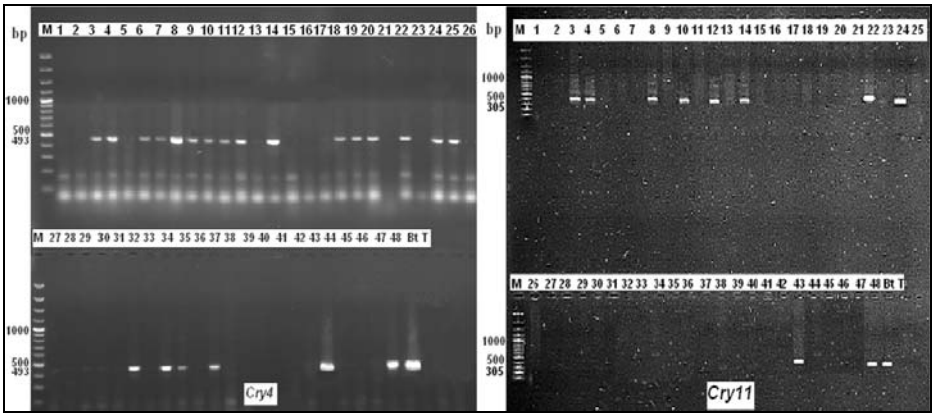

Fig 4: Agarose gel (1.2%) electrophoresis of PCR products for *cry4* and *cry11* genes: Lane M (100 bp DNA ladder), Lane 1-48 (48 isolates), Lane 49 (*B. t.* biovar. *israelensis* as *cry4* and *cry11* positive control) and Lane 50 (T): negative control (without DNA template).

4. Discussion

The *Bt* index average were found to be 0.064 for all samples (48 isolates from 744 samples) but the index changed according to the sample types and origins. Martin and Travers (1989) [14] found the highest *Bt* index (0.85) in the soil samples collected from Asia. This may be related to climate and geographic conditions. In present study abundance of *Bt* was the highest in soil samples while Hongyu *et al*. (2000) [12] and Bernhard *et al.* (1997) [3] reported that *Bt* is more abundant in stored products than soil. In recent study, only 6% of *Bt* strains were isolated from stored product samples and the most rich source of *Bt* was soli (66%).

In this study native strains showed a wide genetic diversity based on insecticide genes contents, and each isolate contained between different insecticide genes (Seifinejad *et al*., 2008) [13, 22]. In this research from 744 sample 49 isolate were detected that similar to result of Jouzani *et al*. (2008) [13, 22] (128 isolate from 2292 sample) and different from Salekjalali *et al*. (2012) (62 isolate from 150 sample).

It has been reported that *P. interpunctella* is resistant to *B.t.* subsp. *kurstaki* (Van Rie *et al.,* 1990) but in our research it was found that 11 isolates were highly toxic to this pest (90-100% mortality).

The most common *cry* gene found in nature belongs to *cry1* gene group (Porcar and Juarez-Perez, 2002) [17]. Ben-Dov *et al.* (1997) [2], Bravo *et al.* (1998) [4] and Wang *et al.* (2003) have reported *cry1* genes were the most frequent in their collections. Similarly, in this study isolates containing *cry1* gene were most abundant group (93.75%). Bravo *et al*. (1998) [4], have detected *cry4* gene as about 8%; however, Chack *et al*., (1994) [7] detected this gene only in 4 of 536 isolates but percentage of *cry4* gene in our *Bt* isolates was 45.83%. Many studies have reported that *cry1* and *cry2* genes were most often present together (Zhang *et al*., 2000 Ben-Dov *et al*., -1997; Wang *et al*., 2003) [27, 2, 26]. Wang *et al.,* (2003) [26,] have found that among the *cry1* gene containing isolates, 90.7% strains also harbored a *cry2* gene. Ben-Dov *et al.,* (1997) [2] have reported that most of the isolates containing *cry1* gene were also positive for *cry2* gene. The PCR results of the present study showed that about 79% of the *cry1* gene positive isolates contained *cry2* gene. About 4.16% of the isolates contained *cry7* gene less abundant.

There usually was a correlation between bioassay results and *cry* gene profiles but some *cry* gene-containing strains exhibited low toxicity indicating that biological activity of *Bt* is not always depend on *cry* gene content and some other factors including spore interaction with crystal proteins, other soluble toxins and the possible presence of undetected crystal proteins may also contribute to the toxicity (Porcar *et al*., 2000; Martinez *et al*., 2004) [19, 15].

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