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Full Length Research Paper

Characterization and pathogenic evaluation of *Bacillus thuringiensis* isolates from West Azerbaijan province-Iran

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In order to find native strains of *Bacillus thuringiensis*, toxic to some agricultural pests, a survey was conducted in West Azerbaijan province. *B. thuringiensis* strains were isolated using acetate selection method with different concentrations. The morphology of crystals was studied using light microscopy. Bioassay tests were conducted on *Culex pipiens* (L.) as well as *Pieris brassica* (L.). Biochemical tests performed to identify the isolated strains. Based on the results, 48 *B. thuringiensis* strains were isolated from 740 samples. The best acetate concentration was determined as 0.25M (56.25%). Soil samples were the main source of *B. thuringiensis* (66%). Majority of strains (58%) had bipyramidal crystals. There was significant difference in toxicity to insects among *B. thuringiensis* isolates, 18.74 and 35.41% of the isolates were toxic to larvae of *C. pipiens* and *P. brassicae*, respectively, causing more than 50% mortality. *B. thuringiensis* subsp. *kurstaki* was the most common biochemical type (12 isolates = 25%). Results indicated that B_t isolates with insecticide activity could be used in integrated pest management to control agricultural and medical pests.

Key words: *Bacillus thuringiensis* isolate, *Culex pipiens*, *Pieris brassica*, Insecticidal activity

INTRODUCTION

Insect pests are major limiting factors in successful crop production (Boulter et al., 1989). Over dependence, indiscriminate and uncontrolled use of chemical pesticides has resulted in irreparable damage to environment. Continuous use of chemical insecticides has led to the emergence and spread of resistance in agricultural pests and vectors of human diseases (Georghiou, 1990). A major alternative to chemical control is biological control, which is an integral part of integrated pest management (IPM). Of all the microbial agents, *Bacillus thuringiensis* has been successfully used as a biocontrol agent. This bacterium is widely distributed in the environment and can be isolated from soil, insects, sericulture environments and leaves of certain deciduous and coniferous trees (Bernhard et al., 1997; Mizuki et al., 1999; Swiecicka and Devos, 2003; Jara et al., 2006).

B. thuringiensis is a spore-forming, gram-positive bacterium which could be distinguished by production of one or more proteinaceous parasporal crystals (-endotoxin) during sporulation (Lacey et al., 2001; Kumar, 2002). In certain strains, the delta-endotoxin proteins are toxic to members of specific insect genera and this has led to commercial development and use of

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some strains as microbial insecticides. Morphological and biochemical techniques have been used to differentiate newly isolated *B. thuringiensis* strains obtained from a variety of sources (Schnepf et al., 2005).

In this study, the distribution, frequency and diversity of *B. thuringiensis* were assessed in different environments of West Azerbaijan province of Iran. *B. thunrngiensis* isolates were differentiated primarily on the basis of crystal morphology, biochemical tests and toxicity to insect species.

MATERIALS AND METHODS

Sample collection

Totally, 740 samples were collected from 11 locations in West Azerbaijan province. *B. thuringiensis* subspecies were isolated from uncultivated site that have no history of treatment with *B. thuringiensis* products include soil, beaches, forests, stored product, agricultural fields, insect cadavers and grasslands. Soil samples were collected by scraping off surface material with spatula and then obtaining a10 g sample from 5 – 15 cm below the surface. All samples were stored in sterile plastic bags at ambient temperature.

B. thuringiensis isolation

The samples were processed by acetate selective method (Travers et al ., 1987) in four concentrations of acetate sodium (0.2, 0.25, 0.3 and 0.35 M.) (pH = 6.8). Each concentration was applied for 186 samples. In this procedure, acetate inhibits germination of *B. thuringiensis* spores, so other spore germinates and non-spore forming bacteria eliminated by heat treatment (7 min at 80°). The surviving spores were plated and grown on nutrient agar and incubated at 30°C for 24 h to obtain colonies. Anywhere from 5 to 20 different colony types were usually obtained. The colonies were cultured onto T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate [pH = 6.8], 0.005 g of $MnCl_2$ per liter) for 4 - 5 days and studied for crystal morphology and bioassay tests.

Crystal morphology

Smears of bacterial strains were stained with coomassie brilliant blue solution (0.25% coomassie brilliant blue, 50% ethanol and 7% acetic acid) for 3 min, washed with tap water, dried and observed under a light microscope without cover and oil emersion (Fadel et al., 1988).

Biochemical test

To divide the *B. thuringiensis* isolates into biochemical types, aesculin utilization, lecithinase production and acid formation from salicin and sucrose was performed (Martin et al., 1985).

Bioassays

The activity of *B. thuringiensis* isolates against insects of order lepidoptera and diptera were tested using *Piers brassica* (an economically relevant pest of vegetable patch and relish brassicas) and *Culex pipiens* (a species of mosquito is important in as vectors of important diseases). For toxicity testing, spore-crystal preparations were grown on T3 plates. The spores and crystals from the agar were floated on 10 ml of sterile water and suspension was stored in sterile vials until it was tested.

Culex pipiens

The activity of isolates against mosquitoes was tested using *C. pipiens*. Mosquito larvae were collected by net trap from ponds in Nazlo area. Ten 2nd instars larvae of *C. pipiens* were added to 20 ml pound water in 30 ml plastic cups in 3 replicate, then 3 ml of each bacterial suspension (from 48 isolate) was added. For this bioassay, 1500 2nd instars larvae of *C. pipiens* were used. *B. thuringiensis* subsp. *israelensis* and distilled water were used as positive and negative controls. Larval mortality was scored 48 h after incubation at 22 ± 1°C.

Pieris brassica

P. brassicae larvae were collected from cabbage farm near Urmia University. From each isolate five ml spore-crystal suspension containing 0.1% Triton X-100 as a wetting agent was smeared on the cabbage leaves and air dried at room temperature. Ten 3rd instars larvae of *P. brassica* were fed on this contaminated leaves. A standard strain *B. thuringiensis* subsp. *kurstaki* was used as positive and sterile distilled water containing Triton X-100 as negative control. Each isolate was tested on 30 larvae in three replicates and mortality recorded after incubation at 20 ± 2°C for 48 h.

RESULTS

B. thuringiensis strain collection

Soil samples were the most abundant and diverse sources of *B. thuringiensis* (Figure 1). From 3010 different colonies of spore-forming bacteria, 48 *B. thuringiensis* isolates were obtained after microscopic observation (Table1) . The average of *Bt* index was 6.4% (48 isolate from 740 sample). Most strains were isolated from Urmia soil but the highest *Bt* index was obtained in Khoy (Table 1).

The inability of *B. thuringiensis* strains to germinate in the presence of acetate buffer allows screening of organism from samples. One hundred eighty six samples were tested for their ability to germinate in four concentrations acetate-buffered medium. More *Bt* isolate obtain from samples which incubated in 0.25 M acetate for 4 h (56.25%) (Table 2).

After screening of the isolates for the presence of parasporal bodies, all 48 isolates showed the *B. thuringiensis* parasporal bodies and were divided into eight classes based on crystal morphology: Spherical (S), Bipyramidal (BP), Cubical (Cu), Irregular (I), Cubical Plus Bipyramidal (Cu+BP), Spherical Plus Bipyramidal (S+BP), Cubical Plus Spherical (CU+S) and (Unknown)(UN). The results indicated that crystals produced by *B. thuringiensis*

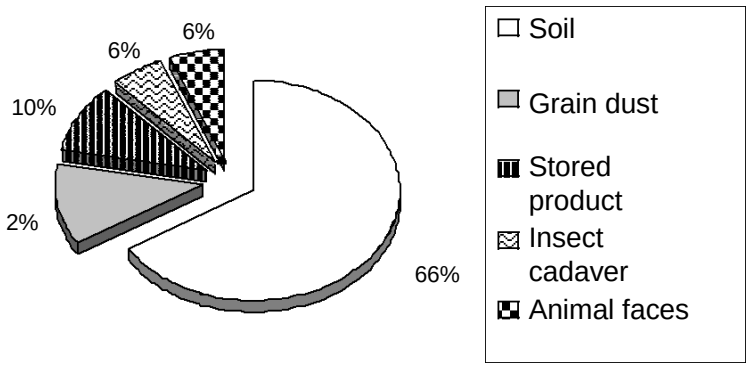

Figure 1. Frequency of *B. thuringiensis* isolates in diverse sources.

Table 1. Distribution of *B. thuringiensis* isolates in samples collected from different localities of WA province.

Location	No of samples	No of colonies	No of isolates	*Bt* index (%)[a]
Orumie	337	2120	14	0.041
Mahabad	49	170	4	0.081
Khoy	34	80	5	0.147
Salmas	39	85	2	0.051
Shahindezh	39	77	4	0.102
Miyandoab	36	84	2	0.055
Bokan	40	70	2	0.05
Naghadeh	46	90	4	0.08
Oshnaviyeh	46	88	5	0.108
Mako	34	72	3	0.088
Sardasht	40	74	3	0.075
Total	740	3010	48	0.064

[a]The ratio of *Bt* isolates producing crystal to all isolates.

isolates from West Azerbaijan habitat were Bipyramidal crystals with 17 isolates (35.41%), Spherical and Bipyramidal + Spherical classes with 8 isolates (16.66%) and others classes with 3 isolates (6.25%) (Figure 2).

Bioassay with normal concentrated spore-crystal suspensions was carried out on larvae of *P. brassica* and *C. pipiens.* The percentage of insect mortalities obtained with the 48 *B. thuringiensis* isolates are shown in Table 3. Most strains have mortality in the range of 0 - 25% (78.2%) on *C. pipiens*. However, there were 4 isolates that produced more than 75% mortality and 12 isolates have mortality more than 75% on *P. brassica*. The most toxic isolate came from sample of soil. Approximately 35.41% of the isolates showed toxicity (more than 50%) against *P. brassica* and only 18.74% of the isolates produced mortality (more than 50%) in *C. pipiens* (Table 3).

One important and rapid method for identifying *B. thuringiensis* isolates is biochemical tests based on Martin et al. (1985) method (Table 4). The results of biochemical tests provided useful taxonomic information about isolates. After biochemical test, we arrange and classified all isolates in 14 subspecies (Table 5). The most abundant biochemical subspecies was kurstaki 12 (25%).

DISCUSSION

Primary identification of *Bt* is based on the presence of crystalline inclusions (Rampersad and Ammons, 2005). In the present study, from 3010 stained bacterial colo-nies, crystalline inclusions were observed in 48 isolates under light microscope and were identified as *Bt* Based on the shape and size. The 48 new isolates of *Bt* were characterized into seven groups without any identification (unknown) (Figure 2). Martin and Travers (1989) have isolated *B. thuringiensis* from several locations in Eastern Asia. They found that isolates with bipyramidal and

Table 2. *B. thuringiensis* isolation analysis according to four different sodium acetate concentrations.

Acetate sodium concentrations (M)	isolate code	No. of isolation	Percent of isolation
0.2	Wz-130, Wz-141, Wz-149, Wz-154, Wz-155, Wz-157, Wz-159, Wz-160, Wz-166, Wz-172, Wz-176, Wz-178, Wz-179	13	27.08
0.25	Wz-181, Wz-182, Wz-183, Wz-184, Wz-186, Wz-187, Wz-188, Wz-189, Wz-190, Wz-192, Wz-193, Wz-200, Wz-216, Wz-270, Wz-300, Wz-352, Wz-400, Wz-401, Wz-500, Wz-555, Wz-600, Wz-666, Wz-690, Wz-734, Wz-77, Wz-101, Wz-102	27	56.25
0.3	Wz-105, Wz-107, Wz-108, Wz-111, Wz-116, Wz-117	6	12.5
0.35	Wz-120, Wz-122, Wz-125	3	6.51

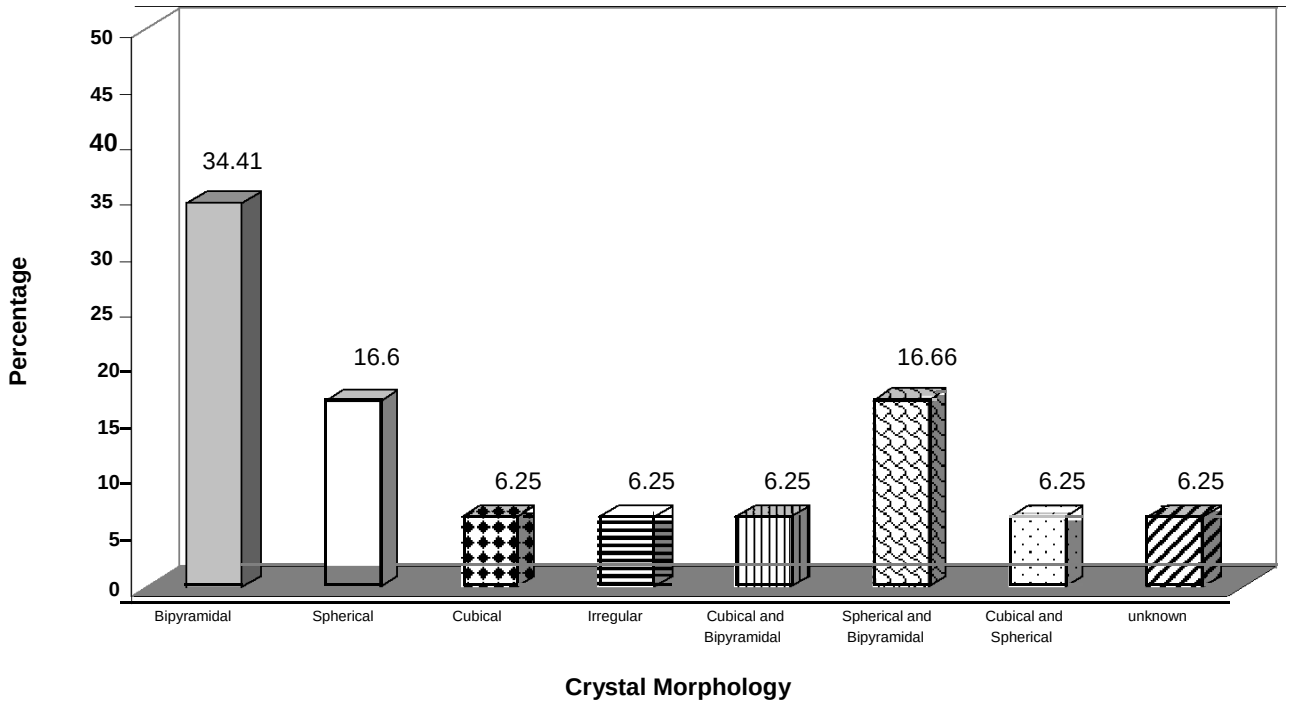

Figure 2. Percentage distribution of crystal morphologies of *B. thuringiensis.*

Table 3. Classification of the *B. thuringiensis* isolates according to their toxicity levels against *P. brassica* and *Culex papiens* after 48 h.

Percent of morality	*Pieris brassica*		*Culex pipiens*	
	No. of isolates	% of isolates	No. of isolates	% of isolates
Isolates causing mortality of 0-25% a	4	8.33	35	72.91
Isolates causing mortality of 25-50% a	27	56.25	4	8.33
Isolates causing mortality of 50-75% a	5	10.41	5	10.41
Isolates causing mortality of >75% a	12	25	4	8.33

Table 4. Biochemical types of *B. thuringiensis.*

Biochemical type (described subspecies)	Biochemical test result			
	Aesculin	Salicin	Lactinaase	Sucrose
1 (thuringiensis)	+	+	+	+
2 (kurstaki)	+	+	+	-
3 (indiana)	+	+	-	+
4 (galleriae)	+	+	-	-
5 (sotto)	+	-	+	+
6 (dendrolimus)	+	-	+	-
7 (morrisoni)	+	-	-	+
8 (darmstadiensis)	+	-	-	-
9	-	+	+	+
10	-	+	+	-
11	-	+	-	+
12 (ostiinae)	-	+	-	-
13	-	-	+	+
14 (israelensis)	-	-	+	-
15	-	-	-	+
16	-	-	-	-

The + sign indicated a positive reaction that is, utilization of Aesculin acid production from salicin and sucrose, and production of lacithinase.

Table 5. Frequencies of *B. thuringiensis* biochemical types.

Biochemical types	Number (%) of isolates	Biochemical types (described subsp)	Number (%) of isolates
thuringiensis	5(10.41)	*dendrolimus*	2(4.16)
israeliensis	7(14.58)	*Indiana*	2(4.16)
kurstaki	12(25)	*Sotto*	2(4.16)
morrisori	2(4.16)	9	2(4.16)
galleriae	7(14.58)	10	2(4.16)
ostriniae	1(2.08)	11	1(2.08)
darmstadiensis	2(4.16)	15	1(2.08)

spherical crystals were the most common. In this study, majority of the isolates (58.32%) had bipyramidal crystals. The diversity in the dominancy of parasporal shapes among habitats in West Azerbaijan of Iran and Eastern Asia might be related to the difference in sample location, habitat and genetic variation.

Travers et al*.* (1987) tested 37 strains of spore-forming bacteria in four sodium acetate concentrations (0.06, 0.12, 0.25 and 0.5 M) in order to determine their ability to germinate in acetate buffered medium. They reported that the germination of *B. thuringiensis* strains was usually inhibited by 0.25 M sodium acetate concentration. In our study, four different sodium acetate concentrations (0.2, 0.25, 0.3 and 0.35 M) were used to increase the rate of *B. thuringiensis* isolation and to eliminate other spore-formers. According to Travers results, number of isolates in this study showed that the most isolates obtained in 0.25 M concentration (56.25%).

An average *Bt* index (the ratio of crystal producing isolates of *Bt* to all isolates) was found to be 0.064for all samples (48 isolates from 740 samples) but the index changed according to sample types and origins. However, Martin and Travers (1989) found the highest *Bt* index (0.85) in the soil samples collected from Asia. This may be related to climate and geographic conditions. The abundance of *B. thuringiensis* was the highest in soil samples. Unlike this study, Hongyu et al. (2000) and Bernhard et al*.* (1997) reported that *B. thuringiensis* is more abundant in stored product environments than soil. However, in our study, among the stored product samples, only 6% of *B. thuringiensis* strains were isolated, but soil samples were the most abundant and

diverse sources of *B. thuringiensis* (66%).

The bioassay results showed that 35.41% isolates were toxic to *P. brassica* larvae (toxicity > 50) and *C. pipiens* active isolates were 18.74% (toxicity > 50). The results indicated that the most of the isolates are toxic to *P. brassica* larvae.

In biochemical tests, 48 isolates were characterized in 14 groups which *B*. *thuringiensis* subsp. *kurstaki* (Es^+ Sa^+ Le^+ Su^-) was the most prevalent type in West Azerbaijan (25%). Same results obtained by Keshavarzi (2008); Martin and Travers (1989).

This study showed that *Bt* isolates could be changed in different areas and different types of samples. In this study, the soil is an ideal source of *B. thuringiensis*. Further studies on cloning and characterization of different cry genes from these new isolates of *Bt* will be useful to use in integrated pest management for sustainable agriculture. These isolates could be utilized for bioinsecticide production, aiming to reduce the use of chemical insecticides.

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