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Original Research Paper

Diversity, Identification and Biotyping of *Bacillus thuringiensis* Strains from Soil Samples in Iran

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ABSTRACT

Bacillus thuringiensis is a bacterium known for producing insecticidal and cytocidal crystal protein during sporulation. One hundred soil samples were analysed which were introduced by seven methods, out of which sixty *B. thuringiensis* strains were collected to study the distribution and diversity of crystal proteins. The strains were characterized on the basis of microscopic examination of parasporal crystal morphology, biochemical tests, and analysis of parasporal inclusions protein profiles by SDS-PAGE. Majority of strains had bipyramidal crystals. *B. thuringiensis* subsp. *thuringiensis* was the most common biochemical type. Overall, according to our finding, it could be concluded that genetic diversity of *B. thuringiensis* strains is dependant on the geographical areas. It means that the production of crystal protein by different strains of *B. thuringiensis* probably dependant on the geographical area.

INTRODUCTION

B. thuringiensis is a Gram positive bacterium belonging to family Bacillaceae. This bacterium is indigenous of many environment, including soil (Bernhard et al. 1997), stored product dust (Hongyu et al. 2000), grains (Apaydin et al. 2005), insect cadavers (Cadavos et al. 2001), leaves of plants (Mizuki et al. 1999) and water environments (Iriarte et al. 2000). The bacterium produces parasporal proteins which are highly toxic to a wide variety of insect pests, some invertebrates and cytocidal activity against some human cancer cells (Nagamatsu et al. 2010). This property makes the organism an environmentally safe microbial agent in controlling insect pests of agricultural and medical importance (Ichikawa et al. 2008, Poopathi et al. 2012). The brush border membrane receptors in insects play an important role in the specificity of parasporal insecticidal activity (Poornima et al. 2010, Krishnan et al. 2010). Hence, on the basis of host range and sequence homologies, the insecticidal proteins (ICP) have been grouped into four classes: Cry I, Cry II, Cry III and Cry IV proteins (Lereclus et al. 1993). On the other hand, recent studies showed that there are non insecticidal *B. thuringiensis* strains in the environment that are able to kill cancer cells without any insecticidal activity (Ohaba et al. 2009). Indeed the bacterium shows great variability and diversity which could be due to the plasmid exchange between the strains (Thomas et al. 2001). Therefore, the present study was conducted to evaluate the existence of this bacterium in the soil samples from different provinces in Iran and accordingly biotype the isolates.

MATERIALS AND METHODS

Sample collection of *B. thuringiensis*: In total one hundred soil samples were collected from 21 different sites of 7 provinces of Iran (Fig. 1). For collection of each sample, 200 g soil was placed in a plastic bag and held at room temperature. Sample processing was done on all of the samples using seven different methods (A-G) as mentioned in Table 1.

Isolation of *B. thuringiensis:* In pilot experiment *B. thuringiensis* strains were isolated by all the methods in Table 1. However, according to our results G method was able to detect *B. thuringiensis* strains relatively with high frequency. *B. thuringiensis* isolation using G method was carried out by wrapping 5 g of soil in aluminium foil and incubated for 3 h at 80°C in a dry oven. Then 1 g of soil sample was suspended in 9 mL of saline and incubated at 80°C for 12 min. The suspension was cooled on ice immediately for 5 min and diluted (10^{-1}-10^{-3}). 0.1 mL of each dilution was spread on LB agar and the plates were kept at 30°C for 5 days (Santana et al. 2008). The colonies similar to *B. thuringiensis* colonies were picked up and subjected to ob-

Table 1: Summary of all methods for *B. thuringiensis* isolation.

Method	Key point
A	0.25 g sample cultured in Nutrient Broth with 0.25 M sodium acetate at 37°C for 24 h, then heated for 5 min at 80°C then plated on Sporulation plates (Travers et al. 1987).
B	1 g sample in 10 ml L-broth with 0.25 M sodium acetate (30°C shaking at 250 rpm for 3 h) then heated for 15 min at 65°C then plated on Sporulation plates (Rampersad et al. 2005).
C	200 g sample was suspended in sterile physiological saline (1:5, w/v), mixed vigorously, and left to stand without any disturbance. A total of 2 mL of the clear supernatant was heated at 80°C for 5 min, then plated on nutrient agar at 31°C for up to 5 days (Salehi Jouzani et al. 2008).
D	2 g sample was suspended in distilled water then heated for 5 min at 90°C, filtered, plated on Nutrient agar plates (Bel et al. 1997).
E	1g sample was suspended in 9 ml distilled water, diluted up to 10^{-3} level and heated at 60°C for 30 min. An aliquot (100 µL, 10^{-3}dilution) of the suspension was mixed with 100 mL nutrient agar and plated on 5 plates (Chatterjee et al. 2007).
F	0.25 g sample heated for 15 min in N-broth at 65°C then plated on Sporulation plates Rampersad 2005).
G	5 g of sample were wrapped in aluminum foil and incubated for 3 h at 80 °C in a dry oven. Samples were then processed as described by WHO methodology (Santana et al. 2008).

Table 2: Biochemical types of *B. thuringiensis.*

Biochemical type (described subspecies)	Biochemica test result				Number
	Aesculin	Salicin	Lecithinase	Sucrose	
1 (*thuringiensis*)	+	+	+	+	35
2 (*kurstaki*)	+	+	+	-	12
3 (*sotto*)	+	-	+	+	11
4 (*dendrolimus*)	+	-	+	-	2

serving crystal morphology using light and phase contrast microscope.

***B. thuringiensis* reference strain:** Reference strain *B. thuringiensis* used in this study was received from National Collection of Industrial Food and Marin Bacteria (NCIMB), UK, Bucksburn.

Crystal morphology: To observe crystal morphology, all the strains were fixed on slides by heat and stained using Coomassie Brilliant Blue solution (0.25% coomassie brilliant blue, 50% ethanol and 7% acetic acid) for 3 min. The smears were washed with tap water and examined under light as well as phase contrast microscope.

Biotyping of *B. thuringiensis* isolates: *B. thuringiensis* isolates were biotyped using biochemical tests viz., aesculin utilization, lecithinase production and acid formation from salicin and sucrose (Martin et al. 1985).

Parasporal inclusion proteins: Sporulated cultures were harvested and washed in 1 M NaCl + 0.01% Triton X-100 by centrifugation. Then this was washed three times in distilled water by centrifugation at 15000 rpm for 10 min. The supernatant containing spore and parasporal inclusion was separated from the pellet and kept at -20°C for further study. To extract proteins, the mixture was suspended in 50 mM Na_2CO_3 (pH 10.0) + 10 mM dithiothreitol + 1 mM EDTA at 37°C for 1 h. Protein solutions (pH 10.0) were treated with proteinase K (60 µg/mL and 300 µg/mL concentration) for 90 min at 37°C. After protease treatment, phenyl methylsulphonyl fluoride was added to the solution to stop the proteolytic reaction (Mizuki et al. 1999, 2000).

Analysis of parasporal inclusion protein by SDS-PAGE: The experiment was carried out as previously described by Laemmli (1970). Electrophoresis of the samples was done using 12% separation and 4% stacking gel. The gel stained using 0.1% Commassie Brilliant Blue R-250. The molecular weights of proteins were determined by molecular mass standards (Fermentas #SMO431) (Ozturk et al. 2008).

RESULTS

Sample collection and crystal morphology: In total sixty strain of the isolates have shown typical morphology character of crystal proteins such as bipyramidal crystals, often heterogenous in size, spherical and cube shape. The bipyramidal crystals morphology from the selected *B. thuringiensis* isolates are shown in Fig. 2.

Biochemical typing: As mentioned above biotyping of the *B. thuringiensis* isolates was performed based on four biochemical tests as recommended by Martin et al. (1985). The results obtained from this study indicated that all the isolates biotyped into four groups viz., *thuringiensis, kurstaki, sotto* and *dendrolimus*. Among the biotype frequency of occurrence of *thuringiensis* was more, however the frequency of existence of *dendrolimus* was less (Table 2).

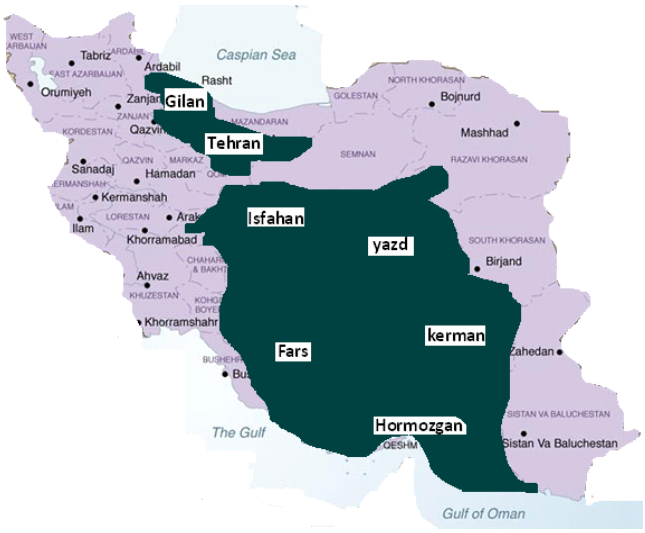

Fig. 1: The geographic locations of sample collection in Iran.

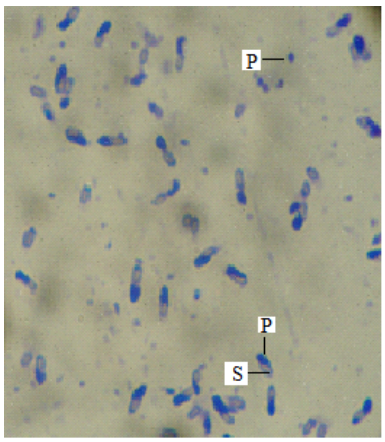

Fig. 2: Light micrograph of sporulating cells of *Bacillus thuringiensis* isolate. S spore, P parasporal inclusion.

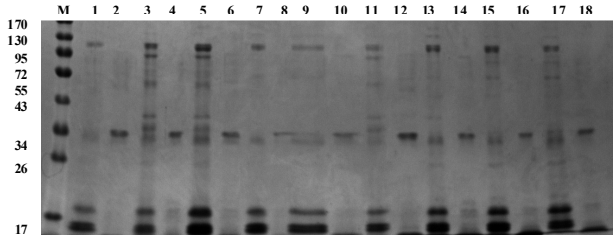

Fig. 3: SDS-PAGE of spore-crystal from some Iranian *B.thuringiensis* strains. Lane M, molecular standards. Lane1,3,5,7,9,11,13,15 and 17, alkali-solubilized inclusion protein of *B. thuringiensis* strains. Lane 2, 4, 6, 8, 10, 12, 14, 16 and 18, alkali- solubilized and protease K-treated inclusion protein of *B. thuringiensis* strains.

SDS-PAGE of parasporal inclusion protein: Although all *B. thuringiensis* isolates were characterized by SDS-PAGE, in this study nine isolates were randomly selected for evaluation of crystal protein (Fig. 3). The results obtained indicated that varied protein profile were observed with a molecular mass within 20-170 kDa. As seen in Fig. 3, most of the proteins showed more than one band. In addition Protease K digestion of the solubilized inclusion proteins generated two proteins: a major protein 43 kDa and minor protein of 32 kDa (lane 2, 4, 6, 8, 10, 12, 14, 16 and 18).

DISCUSSION

B. thuringiensis is a Gram positive bacterium with potential for producing crystalline parasporal body. This character could discriminate *B. thuringiensis* from the other species of this genus (Logan et al. 2005). Regarding to its function many scientists believed that this protein has larvicidal activity. Although insecticidal activity of this protein was first area of investigation, recently, a survey on its anti cancer property was carried out in wide ranging (Mizuki et al. 1999, Kitada et al. 2006, Ohaba et al. 2009). The present study was conducted to investigate on isolation, identification and biotyping of *B. thuringiensis* strains from soil samples in order to achieve information concerning to the frequency of occurrence of this bacterium in the area of investigation. In this study, 1887 suspected colonies were identified from one hundred soil samples in 21 sites of seven provinces in Iran. Out of all, 60 isolates showed typical crystal morphology; in addition the G method recommended by Santana (2008) was relatively the best method for *B. thuringiensis* isolation. In this study, characterization of the isolates was done using biochemical tests (Martin et al. 1985, Dow & Lonc 1999, De Barjac & Frachon 1990, Elubieta et al. 2001) and protein profile (Itoua-Apoyolu et al. 1995, Kitada et al. 2006, Ozturk et al. 2008). Our results indicated that four subspecies were identified from different sites with varied climatic habitat in Iran. These subspecies were characterized as *thuringiensis*, *kurstaki, sotto* and *dendrolimus*. Out of these subspecies, frequency of occurrence of the *thuringiensis* and *dendrolimus* was relatively more and less respectively. Itoua-Apoyolo et al. (1995) reported that the subspeices of *thuringiensis* and *kurstaki* isolated from a population of the European sunflower moth (*Homoeosoma nebulella*) was relatively more; this report was parallel with our study. Concerning to protein profile of crystal protein, it must be noted that most of the proteins have the molecular weight range from 20-170 kDa. This finding was supported by many scientists (Ozturk et al. 2008, Tamer et al. 2009). Hence, according to foregoing evidence, it can be concluded that *B. thuringiensis* species are abounded in our geographical area of investigations. This study showed that *B. thuringiensis* isolates could be changed in different areas and the soil is an

ideal source of *B. thuringiensis* (Aramideh et al. 2010). In addition, varied subspecies could be expected in different habitats worldwide.

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