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Isolation and Molecular Characterization of *Cry* Gene for *Bacillus thuringiensis* Isolated from Soil of Gaza Strip

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Gaza strip is a narrow piece of land lying in the coast of the Mediterranean Sea. Its position on the cross roads from Africa to Asia made it a target for occupiers and conquerors over the centuries. The Gaza Strip is situated in the south part of historical Palestine and southeast of the mediterranean. Ten soil samples were collected from different agricultural and non-agricultural soils from different locations in Gaza strip, and all samples of *Bacillus thuringiensis* strains were isolated from soil. *B. thuringiensis* is a gram positive and spore forming bacterium. The most of the natural habitat for this bacterium is soil and it is capable of producing the diversified varieties of crystal proteins (*cry* proteins) with insecticide property. Polymerase Chain reaction (PCR) is a molecular tool widely used to characterize the insecticidal bacterium *B. thuringiensis*. This technique can be used to amplify specific DNA fragments and thus to determine the presence or absence of a target gene. The molecular identification of Bt cells through the PCR analysis of the delta-endotoxins genes coupled to ribotyping, is an innovative method, that has enabled the identification of this organism into wetland environments.

Introduction

Bacillus thuringiensis, insecticide, polymerase chain reactions (PCR), crystalline protein, *Cry gene*, Gaza strip.

Bacillus thuringiensis (Bt) is a rod-shaped, gram-positive, facultative anaerobic, and spore-forming bacterium (Konecka *et al.*, 2007). During sporulation, it produces insecticidal proteins, which are deposited within the sporangium as crystalline aggregates (Crickomre *et al.*, 1998;

Helgason *et al.,* 1998). Parasporal crystals known as δ-endotoxins, which are not produced by other species it resembles, namely *B. cereus, B. mycoides, and B. anthracis* (Koneman 1997; De Respinis *et al.,* 2006; Soberon *et al.,* 2007; Bizzarri and Bishop, 2008). These d-endotoxins are toxic to a great number of insects and turns Bt into a valuable tool to be used in the Insect Pest Management (IPM) (Valicente & Lana, 2010). Some of which are toxic to a high number of insect species of the orders lepidoptera, Diptera and Coleoptera, in addition to a few Hemiptera (MacIntosh *et al.,* 1990; Bravo *et al.,* 2007; Porcar *et al.,* 2009; Palma *et al.,* 2014), and Nematoda (Wei *et al.,* 2003). When orally ingested by insects, this crystal protein is solubilized in the midgut, forming proteins called delta-endotoxins. The toxicity of these crystals to the insects is determined by the presence of the specific receptors in the midgut epithelium (Bravo *et al.,* 2007). There are two types of N-endotoxins: the highly specific Cry (from crystal) toxins which act via specific receptors and the non-specific Cyt (cytolytic) toxins, with no known receptors. Both families of toxins are classified exclusively on the basis of their amino acid sequence identity. Genes encoding these proteins (*cry genes*) were among the first to be used in genetic engineering of plants for enhanced insect resistance (Roh *et al.,* 2007). *Cry* proteins have been used as bio-pesticide sprays on a significant scale against agricultural pests for more than 30 years, and their safety has been demonstrated, and he *cry gene* content of *B. thuringiensis* strains is known to be related to the toxicity (Federici *et al.,* 2006), and the identification of *cry genes* by means of PCR has been exploited to predict insecticidal activities of the strains and to determine the distribution of *cry genes* within a collection of *B. thuringiensis* strains (Porcar & Juárez-Pérez, 2003; Bozlağan *et al.,* 2010).

Different methods have presented to isolate the *B. thuringiensis* which are as following: Polymerase Chain Reaction (PCR), Southern blotting, serotyping and Bioassay method (Porcar & Juárez-Pérez, 2003).

Current detection and identification methods of crystal genes (*Cry*) of *B. thuringiensis* which isolated form soil of Gaza strip based on PCR was executed for the first time by (Porcar & Juárez-Pérez, 2003; Bozlağan *et al.,* 2010). PCR is a fast and accurate method for identification of the unknown *cry* genes with new insecticidal activity. In the recent decades, PCR has been used extensively in order to determine the content of *cry gene* from the *B. thuringiensis* strains. As yet, more than 100 pairs of specific and different primers have been designed to identify the cry gene subsets (Porcar & Juárez-Pérez, 2003). The aim of the current study was to develop a standardized and universally applicable molecular method for the detection of Bt, which could be directly applied to colonies grown on agar medium.

Materials and Methods

Materials

All media, Primers, Kits, chemicals and reagents were purchased from various suppliers and are prepared according to manufacturer`s recommendations shown in table 2.1.

Methodology

Soil Sample Collection

Ten soil samples were collected from different agricultural and non-agricultural soils from Gaza strip (Rafah, wadi Gaza, Khanyunes, Abbasan, Gaza – Alshikh ejleen, Gaza – Islamic university, chicken farm soil, mint farm soil, Citrus farm soil). About 25 grams of top soil (after removed 2 cm of soil surface) were collected in a sterile cup, labeled with date and source of collection. Transported to the laboratory and processed within 2 hours of collection.

Cultural Characterization

The selected isolate was plated onto the surface of *B. thuringiensis* chromogenic agar and incubated for 24 hours at 30 ^{o}C. Plates were inspected for growth and colony morphology (size, color and texture) were noted.

Microscopic Examination

For Gram Stain: A smear was prepared and air-dried, fixed and stained with gram staining reagents. In short, smears were flooded with crystal violet for one minutes and rinsed with water. Gram`s Iodine was added for one minutes and rinsed with water. Ethanol was used to decolorize smears for not more than 20 seconds and washed with water. Finally, safranin was added for 30 seconds. Slides were rinsed and plotted onto absorbent tissue and examined under the high and oil immersion objectives. Gram stain was used to determine gram reaction and the shape of the bacterial isolates under microscope.

**For Spore Staining (Schaeffer & Fulton`s):** A smear was prepared as previously described. The entire slide was flooded with Schaeffer & Fulton`s Spore Stain A solution (malachite green). The slide was steamed for 5 minutes and rinsed under running tap water. The slide was counterstained with Schaeffer & Fulton`s Spore Stain B solution (safranin) for 30 seconds. Slides were rinsed and plotted onto absorbent tissue and examined under the oil immersion objectives.

Enrichment on Selective Medium

One gram of soil sample placed in 9 ml of sterile saline, vortexed for one minute, and heated at 80^{o}C for five minutes to eliminate all vegetative bacterial and fungal spores. A loopful from the heated vortexed soil is streaked on R & F Bacillus cereus / *Bacillus thuringiensis* Chromogenic Plating Medium. Plates are incubated at 37 °C for 48 hours. Suspected colonies characterized by pale blue colored on chromogenic media are then sub cultured for testing their larvicidal activity before an identification process is initiated.

DNA Isolation from Bt

Bacillus thuringiensis strains were activated in NA at 37^{0}C overnight. The total DNA isolation by using Patho Gene-Spin ™ DNA/RNA Extraction Kit (iNtRON Biotechnology, Korea) is designed for rapid and sensitive isolation of DNA or RNA from a variety of pathogen such as virus, bacterium and etc.

Oligonucleotide PCR Primers

Semi-conserve PCR reaction with CU-F and CU-R primers (synthesis by Hy Laboratories Ltd.) (Table 2.1) was carried out in the volume of 25 µl containing 2 units of TaqDNA polymerase, enzyme buffer (50 mMof NaCl, 10 mM of Tris, pH 8.3), 2 mMof MgCl2, 0.25 mMdNTP, 10 pmol of each one of primers and 200 ng of DNA. After an initial 5 min denaturation at 94°, 35 cycles of 95°C for 40sec, 52°C for 60 sec, 72°C for 40 sec was carried out, followed by a 5 min extension at 72°C using a thermalcycler (Biometra, Germany).The 180-200 bp amplified product was electrophoresed on 2% agarose gel.

Results and Discussion

Isolation of *Bt* from Soil of Gaza Strip

After removing the surface layer of soil, collection of 100 gram has been done from the aforementioned areas in sterile bottles of polyethylene then proper storage in refrigerator were done and these samples shown in Figure 3.1

Cultural Characterization

The cultural characteristics of the suspected *Bacillus thuringiensis* isolates were examined. Generally, colonies were white to cream in color, tend to have large frosted glass appearance, initially, but may become opaque. Some colonies were mucoid in nature, others brittle. The isolates are Gram-positive, spore formers and motile. The spore is found in the center of the cell. The shape of spores is ellipsoidal. All isolates produced crystal proteins with various forms and size**,** this result presented in Figure 3.2

Microscopic Examination

For Gram Stain: Slides were treated by gram stain reagents, and examined under the high and oil immersion objectives. *Bt* appeared as gram positive bacilli and presented in Figure 3.3.

For Spore Stain: Slides were treated by Schaeffer & Fulton's reagents, and examined under the oil immersion objectives. Green free spores as well as centrally located spores were detected, and illustrated in Figure 3.4.

Enrichment on Selective Medium

This selective media that contains nutrient broth and 0.25 gram of sodium acetate used as effective media for enrichment of *Bt* bacteria. Selective media of the *Bt* was made using (Sigma-HiCrome *Bacillus* Agar) as effective media, and the enrichment of Bt shown in Figure 3.5

Table.2.1 List of Media, Chemicals and Reagents used in this Study

#	Item Name	Manufactures	Country
1	Patho Gene-Spin ™ DNA/RNA Extraction Kit	iNtRON Biotechnology	KOREA
2	Master Mix 2X	Thermo Fisher Scientific Inc	USA
3	Ladder 100 bp	Thermo Fisher Scientific Inc	USA
4	Agarose	SIGMA- Aldrish	USA
5	Primer **CU-F** **5’- GGA TTG GAA TGG GAA ACA -‘3**	Hy Laboratories Ltd.	Israel
6	Primer **CU-R** **5’- AAA TAG CCG CAT TGA CAC-‘3**		
7	Nutrient Agar	HiMedia	India
8	Broth Agar	HiMedia	India

Table.2.2 Sequences of the *Cry genes* Primers used for PCR Amplification

#	Primer Sequences (5 to 3)	Annealing (C^0)	Size (bp)	References
1	Primer **CU-F** **5'- GGA TTG GAA TGG GAA ACA -'3**	**$52C^0$**	**180-200 bp**	**(Porcar & Juárez-Pérez, 2003; Bozlağan *et al.*, 2010)**
2	Primer **CU-R** **5'- AAA TAG CCG CAT TGA CAC-'3**			

Figure.3.1 Soil Samples that Collected for *Bacillus thuringiensis* Isolation

Figure.3.2 Colonies of *Bacillus spp*. on Nutrient Agar Medium

Figure.3.3 Bt. Stained by Gram Positive (Purple) 1000X

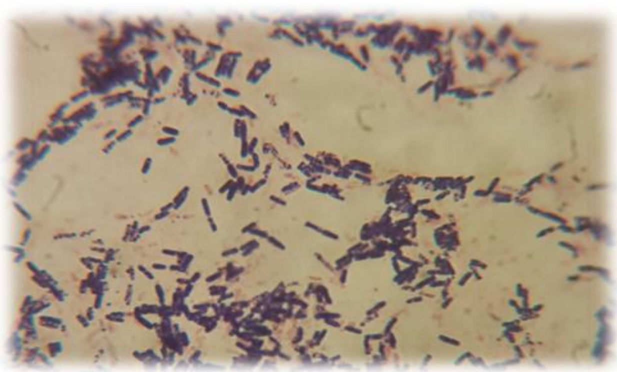


Figure.3.4 Bt. Spore Staining (Schaeffer & Fulton`s)

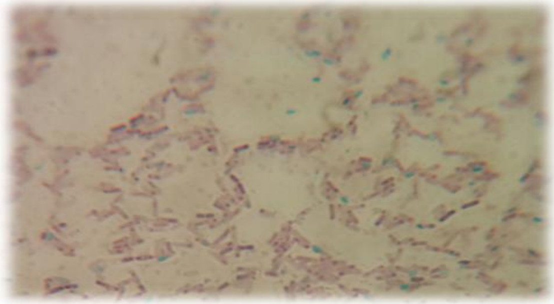
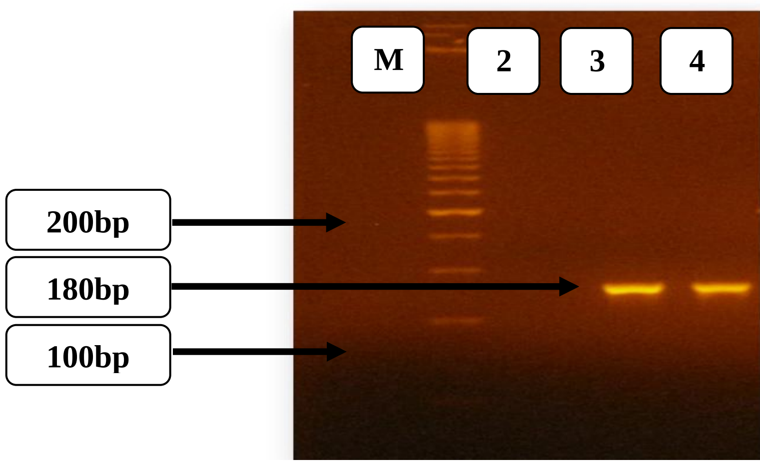


Figure.3.5 Subculturing on Selective Media Containing on Sodium Acetate

Figure.3.6 Result of Amplification of *Cry Gene* on the Bacterium Sample Isolated from the Soil of Gaza Strip



Line 1, size Marker 100 to 3000 bp, Line 2, as negative control by using *E. coli.* fermentase. Line 3, *Cry* gene positive amplification by semi conserve PCR for *B. thuringiensis* *israelensis*. Line 4, *Cry* gene positive amplification by semi-conserve PCR for samples of *B. thuringiensis* isolated from Gaza strip

Molecular Identification of Cry Gene

In all, 10 *Bacillus spp*. isolates were obtained from the soil samples from different regions of Gaza strip using selective media. Total DNA was isolated and analyzed by PCR using *cry1* general primers. Of the 10 isolates, 2 carried the *cry1* gene. Plasmids of those strains were isolated and their insecticidal activity and the result of amplification of Cry gene presented in figure 3.6

In the present study 10 different putative *Bacillus spp*. isolates were obtained from soil in different regions of Gaza strip, and the presence of *B. thuringiensis* strains. Morphological characterization and Gram staining of the isolates were carried out. Although colony morphology of the isolates was similar to that of the reference strain (BtI), morphological characterization only is not reliable for identification of isolates. As different Bt isolates might have a similar colony morphology, molecular characterization of the isolates is better than either serological or morphological characterization (Iriarte *et al.*, 2000).

Cry proteins are encoded by cry genes that are frequently carried on plasmids; to date, nearly 300 cry genes have been identified and classified into 51 groups and subgroups on the basis of amino acid sequence similarity (Ye *et al.*, 2012).

B. thuringiensis, which tested in this work, were also analyzed for the presence of *cry* gene sequences. The designed primers are specific to *Cry gene*, resulting in the amplification of a DNA fragment of around 180-200bp as PCR product, and the results for amplification of cry gene for molecular identification of Bt in (Porcar & Juárez-Pérez, 2003) support the result in the presence study.

Comparative analyses of cry genes, amplification with CU primers, plasmid pattern and protein profile allowed the clear differentiation of the evaluated strains. It may also help in the establishment of a new subspecies-level classification of Bt. Due to this discrimination, these analysis can be an useful tool in the characterization of Bt strains, something highly valuable in intellectual property claims.

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