

## **Isolation and molecular identification of cry gene in *Bacillus thuringiensis* isolated from soils by semi-conserved PCR**

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### **ABSTRACT**

*Bacillus thuringiensis* is a gram positive and spore forming bacterium. The most of the natural habitat this bacterium is soil and it is capable of producing the diversified varieties of crystalline proteins with insecticide property. The aim of this study has been isolation of strains of *Bacillus thuringiensis* carrying cry gene from soils of the west Mazandaran provinces. After screening the soil of 35 regions, 12 strains of *Bacillus thuringiensis* were isolated by the selective minimal medium contain sodium acetate and L-serine. After DNA extraction, *Bacillus thuringiensis* strains were confirmed using 16srDNA PCR sequencing. Also, relative molecular weight of crystalline protein of the strain containing the cry gene was evaluated through SDS-PAGE. Out of 12 isolated strains, cry gene from 1Aa class was identified only in one strain by Semi-conserved PCR. Study of rDNA sequence of the isolated strain showed a 99% homology with IBL200 strain of the *Bacillus thuringiensis*. Also approximate weight of crystalline protein was measured in the range of 90 to 100 KDa. With regard to very much diversity of crystalline gene and lack of existence of accurate phenotypic methods to identify the presence of the crystalline protein using of this semi-conserved PCR method can identify the bacilli containing the crystal gene with low cost and fast method.

**Keywords:** *Bacillus thuringiensis*, crystalline protein, Semi-conserved PCR.

### **INTRODUCTION**

Since 1901 when Ishiwata, the Japanese bacteriologist, isolated the bacterium *Bacillus thuringiensis* from the body of dead larvae of silk worm, it has been approximately for one century which the extensive studies are performed regarding the effects and manner of performance and effect of this bacterium [1]. *Bacillus thuringiensis* is a familiar name in controlling the pests and insects which applies in production of more than 90% of the microbial pesticides and a large number of the transgenic plants being resistant to insects as a factor for biologic fights [1]. This bacterium is a kind of gram-positive and spore-forming which is able to act against the agricultural pests like a toxin and to be used in the biologic control of pests [2]. This bacterium through production of the protein crystals in spore known as Gammaendotoxin (Internal Crystal Protein) (ICP) is toxic for varieties of larvae of insects [3]. The genes expressing crystal proteins can be into plasmid or chromosome. Insecticidal activity of *Bacillus thuringiensis* relates to its ability in the synthesis of a great quantity of ICP. When bacterium's spore is ingested by the susceptible insects, these proteins dissolve in the intestine of insect and turn into the active toxins by intestine's proteases after proteolytic digestion. These toxins connect to the surface protein receptors of epithelial cells of insect's intestine specifically and create the pores in their intestine. Then, epithelial cells are torn and feeding activity of insect is destroyed and finally, insect dies of starvation. There are two types of Gamma endotoxin. Type of cry which, acts full specifically and type of cyt which does not require the specific receptors. These two families of toxins have been

classified on the basis of sequence of their amino acids. Degree of similarity between cry proteins is variable severely and ranges from 45% to 95% differently[4].

Various methods have presented to isolate the *Bacillus thuringiensis* which are as following: Polymerase Chain Reaction (PCR), Southern blotting, serotyping and Bioassay method[5].

Identification of crystal genes of *Bacillus thuringiensis* based on PCR was carried out for the first time by Perez et al[6]. They showed that 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 employed extensively in order to determine the content of cry gene from the *Bacillus thuringiensis* strains. As yet, more than 100 pairs of specific and different primers have been designed to identify the cry gene subsets[7]. In this study, one pair of semi-universal primer, on the basis of sequence alignment of a few cry genes, including class of 1,2,3 and 4 was designed and it was used for molecular screening of the bacilli isolated in the selective media. The aim of this study has been isolation of strains of *Bacillus thuringiensis* carrying the new cry gene from the soils of different parts of the west Mazandaran provinces.

## MATERIALS AND METHODS

### Samples Collecting

65 samples of the mountain, forest, grain farm, citrus garden and small garden soils were collected from regions of Ramsar, Tonekabon and Kelardasht located in northern of Iran. Sampling was accomplished from depth of 30 to 40 cm of the soil's surface and, then, samples were placed into a clean plastic bag and transferred to laboratory.

### Culture

At Beginning, of spore-bearing bacteria germination present in the soil samples was prevented by sodium acetate and non-spore bacteria were killed by heat treatment. Sodium acetate, as an inhibitor, prohibits from budding of the bacterial spores[8]. 0.25 g of the soil's sample was added into the tubes containing 10 ml of Luria and Bertani liquid medium containing the 0.25 molar sodium acetate and placed into the shaker incubator with 150 rpm in 37°C for overnight. Then, tubes placed into the hot water batch were treated heatedly under 80°C for 5 minutes. After heat treatment, 150 µl of the supernatant was cultured into the luria and bertani agar and the plates were placed into incubator under 37°C for overnight.

### *Bacillus thuringiensis* isolation using selective medium

Considering that L-Serine is able to prevent from the growth of bacillus species, except for *Bacillus thuringiensis*[9], M9 minimum Medium containing 0.2 mM per liter of L-Serine was used as a selective medium. Then, bacterial colonies grown on the Luria and Bertani medium were isolated and cultured on the selective medium of L-Serine for 2 days. In the next step, white single colony was selected and their morphological characterizations were studied after gram staining under the microscope.

### Molecular identification

After alignment of the complete nucleotide sequence, a few cry genes, including 1,2,3 and 4 classes with DNAMAN (LynnonBioSoft, version 5.2.9) software, a pair of primer was designed for their conserved region. After of the total DNA extraction of bacterial colony by RTP® Bacteria DNA Mini Kit (Invitex, Germany), Semi-conserved PCR reaction with CU-F and CU-R primers (synthesis by Metabion Co.) (table 2) was carried out in the volume of 25 µl containing 2 units of TaqDNA polymerase, enzyme buffer (50 mM of NaCl, 10 mM of Tris, pH 8.3), 2 mM of MgCl<sub>2</sub>, 0.25 mM dNTP, 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 thermocycler (Eppendorf, Germany). The 200bp amplified product was electrophoresed on 2% agarose gel and was confirmed to PCR sequencing (Macrogen, South Korea). Then, the positive bacteria for cry gene to the Semi-conserved PCR, was identified through 16S rDNA PCR[10] and PCR sequencing.

Table 1. Sequences of the Cry and 16S rRNA Genes Primers used for PCR Amplification

Primers	sequence (5' to 3')	Annealing (°C)	Size (bp)	Reference
CU-F	GGA TTG GAA TGG GAA ACA	52	200	This study
CU-R	AAA TAG CCG CAT TGA CAC			
16S rDNA-F	AAC TGGAGG AAG GTG GGG AT	56	380	10
16S rDNA-R	AGGAGG TGA TCC AAC CGC A			

### Purification and SDS- PAGE of crystal protein

In this study, purification of crystal protein was performed according to the method of Bell et al[4]. After culture of isolates of *Bacillus thuringiensis* on the sporulation conditions, two loops of the desired colony were transferred to tube containing 1 ml of 1% cold NaOH. After dissolving the bacterium, it was centrifuged in 13000 rpm for 5 minutes and was discarded supernatant. The deposition was dissolved in 140 µl of 0.01% BetaMercaptoethanol and 1% SDS solution. Then, it was placed in the hot water bath for 10 minutes. In the next step, it was centrifuged by 13000 rpm for 10 minutes and the supernatant was discarded and the deposition was mixed with 140 µl of 20% Trichloroacetic acid, and after 10 minutes of incubation on the ice, it was centrifuged in 10000 rpm for 15 minutes and the supernatant was discarded accurately. Then, 140 µl of cold acetone were added to sediment, and the centrifuge was carried out in 12000 rpm for 5 minutes. We discarded the supernatant and dissolved the sediment in the 100 µl of the phosphate buffer, and 10 µl of it were prepared and loaded for electrophoresis on 10% polyacrylamide gel along with marker of protein's molecular weight (Fermentas, USA). After electrophoresis and stabilization, gel was stained by Coomassie Brilliant Blue.

### RESULT AND DISCUSSION

After screening of 35 samples of soil by the sodium acetate method and, then, growing of the isolated colonies in M9 medium containing L-Serine, 12 strains of bacterium *Bacillus thuringiensis* were isolated (Figure 1). Among these 12 strains of *Bacillus thuringiensis*, only one positive case was shown by semi-universal PCR. (Figure 2)

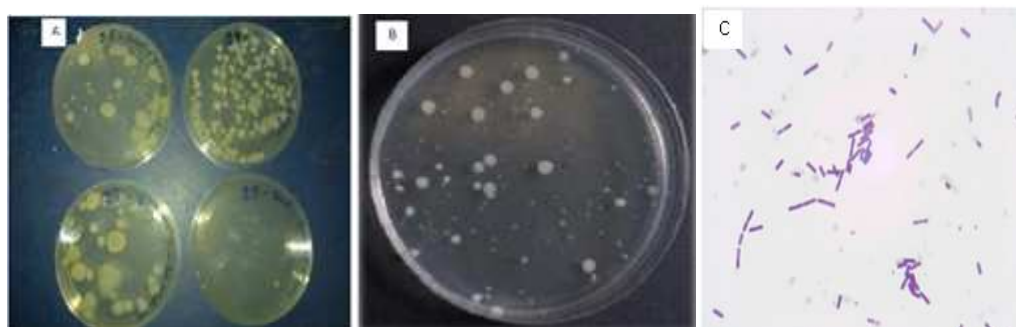


Figure (1). Screening of the soil samples in the sodium acetate (A), Medium containing L-Serine (B) and gram staining (C).

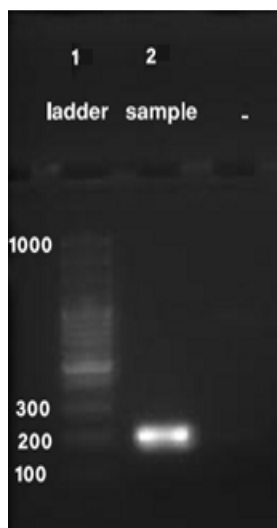
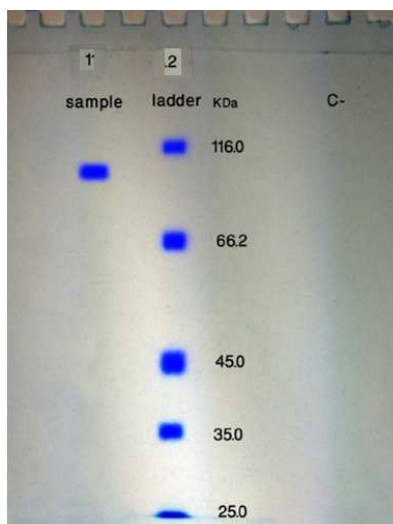


Figure (2). Result of amplification of cry gene on the bacterium sample isolated from the soil of Kelardasht region. Line 1, size Marker 100 to 3000 bp (fermentase). Line 2, Cry gene positive amplification by semi-conserved PCR. Line 3, negative control.

After sequencing 200 bp PCR product of the Kelardasht isolate and BLAST alignment was confirmed that it is part of cry gene. Result showed a 96% homology with cry 1Aa gene of LS- R-21 strain of *Bacillus thuringiensis*. On the other hand, result of BLAST of a part of rDNA gene (380 bp) the isolate containing cry gene showed a 99% similarity with the IBL200 strain of *Bacillus thuringiensis* (Accession NO: ACNK01000211.1). Also, result of extraction of crystal protein and its SDS- PAGE indicated a molecular weight ranged from 90 to 100 kD (Figure 3).



**Figure (3).** Result of SDS- PAGE of the crystal protein extracted of the Kelardasht isolate. Line 1, extracted protein, Line 2 Protein Size Marker(fermentas) and Line 3, negative control.

*Bacillus thuringiensis* is a gram positive, ubiquitous and spore-forming bacterium. This organism produced the crystal proteins which are toxic for insects[11]. Up to now, more than 300 crystal proteins have been isolated and determined sequentially classified into 53 various groups on the basis of similarity in their sequences[12]. With regard to possibility of existence of new species and strains, identification of new *Bacillus thuringiensis* in different regions of country through the fast and reliable methods that were evaluated in this study to an obviously something. As well as identify new bacteria and Cry genes can produce highly effective new pesticides and prevent resistance to these insecticides. In this study, 12 isolates of the *Bacillus thuringiensis* were isolated from 35 various soils located in northern of Iran. Due to the lack of precise phenotypic methods for the study of crystalline gene, the genotypic method is very important. As yet, of PCR technique was used only for detection certain class from the crystalline protein gene via of designed pair primer.

However, in this study, with sequence alignment of several Cry genes, including Class 1, 2, 3, 4, and 8, the homology regions in them were detected.

Identification of cry 1Aa crystal gene by means of this primer suggests the capability of this primer in the simultaneous identification of a few classes of crystal gene and it can be used for rapid screening of the *Bacillus* strains carrying the crystal gene.

In the continuation, it is suggested that, after studying the manner of toxic affecting of this gene on the insect pests, the damages created by these pests can be controlled by transfer of that gene to the soil bacteria or endophyte microorganism[13]. At the end, it must be reminded that usage of methods based on biological fights with pests instead of usage of chemical poisons is of high importance both from view point of environment protection and from view point of economical saving of farmers, in other words, it can be optimization in fighting with and controlling of pests.

## CONCLUSION

Achievement of this research is to gain access to a new primer which, in form of semi-conserved PCR, is able to identify 4 genes simultaneously and we were able to isolate the crystal gene in bacterium *Bacillus thuringiensis* isolated in the desired region.

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## REFERENCES

- [1] Tuba EF, Isolation of *Bacillus thuringiensis* and Investigation of Its Crystal Protein Genes, Izmir Institute of Technology İzmir, Turkey, **2002**.
- [2] Thaphan P, Keawsompong S, Chanpaisaeng J, Songklanakarin J *Sci Technol*, **2008**, 30 (5), 597-601.

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- [3] Schnepf EN, Crickmore J, Rife VD, Lereclus J et al, *Microbiology and Molecular Biology Reviews*, **1998**, 775-806.
- [4] Bell Y, Granedo TF, Alberola M, Martinez-Sebastian MJ, and Ferre J, *Systematic of Applied Microbiology*, **1997**, (20), 652-658.
- [5] Porcar M, Perez JR, *FEMS Microbiology*, **2003**, 26(5), 419-432.
- [6] Juarez-Perez VM, Ferrandis MD, Frutos R, *Appl Environ Microbiol*, **1997**, 63, 2997-3002.
- [7] Nariman AH, *Australian Journal of Basic and Applied Sciences*, **2007**, 1(4), 461-466, ISSN 1991-8178.
- [8] Keshavarzi M, *J AgricSciTechnol*, **2008**, 10, 493-499.
- [9] Andrzejczak S, Lonc E, *Polish Journal of Microbiology*, **2008**, 57(4), 333-335.
- [10] Greisen K, Loeffelholz M, Purohit A, Leong D, *J ClinMicrobiol*, **1994**, 32, 335-351.
- [11] Chen F, Tsai M, Peng C, Chak K, *Current microbiology*, **2004**, 48, 270-275.
- [12] Gene Bank of *Bacillus thuringiensis*, Agricultural Biotechnology Research Institute, Journal of Biotechnology Commission, **2003**.
- [13] Thammasittirong A, Attathom T, *Journal of Invertebrate Pathology*, **2008**, 98, 121-126.