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# Molecular Characterization of *Cry11* Crystal Protein Gene from *Bacillus thuringiensis* Isolated from Different Soil Samples

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## ABSTRACT

The present study was aimed at screening the local *Bacilllus thuringiensis (B.t.)* isolates for Cry11 and assess their potential use for mosquito control. A total of 15 *B.t.* isolates were collected, of which 75% were from dry, leaf litter, garden soil samples, 15% were from animal waste and 10 % were from moist soil of the crop area. A 650bp of *cry11* gene was amplified by PCR and seven isolates were found positive for *cry11* gene. The 16S rDNA study exposed that these screened *B.t.* confirmed 99% homology with *B.t.* serovar *tolworthi*, *B.t.* str. *Al Hakam*, *B.t.* serover *thuringiensis*, *B.t.* serovar *konkukian*, *B.t.* serovar *Chinensis*. *B.t.* serover *Indiana,* and *B.t.* serover *kurstuki.* The toxicity bioassays with *B.t.* spores proved that seven *B.t.* isolates harboring *cry11* gene (*viz.,* NF *B.t.*,1,2,3,4,5,6,7) were toxic to 3rd instar larvae of mosquito, *Aedes aegypti*. Among seven *B.t.* isolates, three isolates NF5 *B.t.* 7.2, NF1 *B.t.* 1.1 and NF2 *B.t.* 4.2 were found most toxic which were isolated from moist soil containing decaying cattle waste, dry waste animal dung and leaf litter soil, respectively. So, these isolates have a great potential to grow into a biopesticidal formulation for control of mosquitoes.

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**Authors' Contribution**
**DAB designed the study and supervised the work. NF performed experiments and analyzed the results. AR helped in manuscript preparation and revision.**

**Key words**
***Bacillus thuringiensis*, Insecticidal activity, *cry11* gene, Bioinsecticide *Aedes aegypti*.**

## INTRODUCTION

Synthetic pesticides have been successfully used during past several decades to kill variety of insects and crop pests. Pest controlled by chemicals has caused serious damages to wild life. Over use of pesticides develop confrontation in pests, making the chemicals ineffective. Insecticides have also caused toxic effects on non-target pest and also cause the pollution of bottom water table. The requirement of most suitable, alternate, more effectual and environment-friendly control agents became imperative (Lacey *et al*., 2001). Bio-insecticides were continuously in front position. Cry protein invention was an influential approach for enlightening the use of *B.t*-based biopesticides. 80-90% control of pest is through *Bacillus thuringiensis* (Schnepf *et al*., 1998; Glare and O'Callaghan, 2000).

*B.t.* was first discovered in 1901 by Japenese Shigetane Ishiwatta, from diseased silkworm larvae (Burges, 1967; Dulmage *et al*., 1971; Krieg *et al*., 1983). *B.t.* is an aerobic ubiquitous, gram-positive, spore former and produced δ-endotoxins (cry protein). It forms various shapes of insecticidal crystal proteins. Specificity of *B.t.* is their toxicity against specific insect. Magnesium, calcium and inorganic phosphate enhance ICPs (Kumar and Bambawale, 2002).

It grows aerobically and forms heat resistant spores. They have large filamentous and make parasporal crystals (Cry) proteins with molecular masses of 135, 125, 68 and 28 kDa during sporulation which kill particular target pests of different crops (Ahmad and Shakoori, 2013).

During sporulation many *B.t.* strains produce ICPs (proteinaceous Cry protein encoded by *cry* genes and *cyt* gene to be found on the plasmid). *B.t.* δ-endotoxin is a globular protein molecule mount up as a protoxin and released in the gut of a pest in alkaline medium. The specific enzyme proteases convert the protoxin into active toxin. This active toxin then binds to its specific receptors (lacking aminopeptidase activity) inside the insect's gut and causes pore formation in epithelial cell surface, which ultimately leads to disturbance in electric potential across the membrane due to leakage of ions and finally death of insect larvae due to swelling of epithelial cells of midgut. The *cry11* genes are dipteran specific and are known to control mosquito and black flies worldwide (Abdullah *et al*., 2006).

*Cry* proteins have been classified into 56 groups divided into classes and subclasses according to their amino acid similarity. Genes coding for the Cry proteins (*cry* genes) follow the protein classification. The *cry11* genes encode 67-94 kDa proteins highly active against different species of mosquito larvae. Cry11Aa, Cry11Ba

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and Cry11Bb proteins are very active toxins found in the *Bti, jegathesan* and *Medellin,* respectively (Crickmore *et al*., 1998). *B.t.* subsp. *chinensis* strain CT-43 has been widely used as an agricultural biopesticide for a long time. As a pest producing strain, *B.t.* subsp. *chinensis* strain CT-43 is highly toxic to lepidopterous and dipterous insects. It forms various ICPs consisting of Cry1Aa3, Cry1Ba1, Cry1Ia14, Cry2Aa9, and Cry2Ab1 which is very toxic to diptarian insect (Jieping *et al*., 2011).

The present study was aimed at isolating and characterizing *Bacilllus thuringiensis* strains from different soil samples from Lahore. The toxicity of spores of *B.t.* strains harbouring *cry11* genes was evaluated against 3rd instar larvae of mosquito *A. aegypti* for their potential use as bioinsecticide.

## MATERIALS AND METHODS

*Isolation and biochemical characterization of* B.t.

Soil samples were collected from different localities of Lahore and were further processed for the isolation of the *B. thuringiensis* according to Martin and Travers (1989) and Bukhari and Shakoori (2010). Gram staining, crystal staining and endospore staining (Sneath, 1986) were performed for morphological characterization. The Gram-positive rods, spore and crystal formers were characterized biochemically according to Cheesbrough (1993), Collee and Miles (1989) and Benson (1994).

*Molecular characterization of bacterial isolates*

DNA was isolated from *B.t.* according to Kronstad *et al*. (1983). Specific primers for 16S rDNA of full-length 1.6kb gene were used (Bukhari and Shakoori, 2010). PCR amplification was done according to Saiki *et al*. (1988) using Fermentas PCR reagents (#EP0402). 4Q281 *B. thuringiensis* 16S ribosomal RNA gene was used as a reference gene. The nucleotide sequences of 16S rDNA gene of local *B.t.* isolates were later deposited in the NCBI database. Moreover, dendrograms were constructed on the basis of homology using MEGA7 program.

*PCR based detection of* cry11 *gene*

Detection of *cry* 11 gene in *B.t.* isolates was done by amplification of Cry 11 gene using the following primers; Forward: 5′ATGGAAGATAGTTCTTTAGAT3′ Reverse: 5′ CTACTTTAGTAACGGATT 3′, in a thermal cycler (Progene, Techne) (Bukhari and Shakoori, 2009).

*Biotoxicity assays of bacterial spores*

Bacterial spores were prepared according to Makino *et al*. (1994). The toxicity of spores of *B.t.* isolates found positive for *cry11* gene was evaluated against 3rd instar larvae of *Aedes aegypti* according to procedure described by Bukhari and Shakoori (2010). Bioinsecticidal activity of *B.t.* isolates was checked and compared with the control HD500 strain. The toxicity assessment was determined through Log- Probit analysis (Finney, 1952).

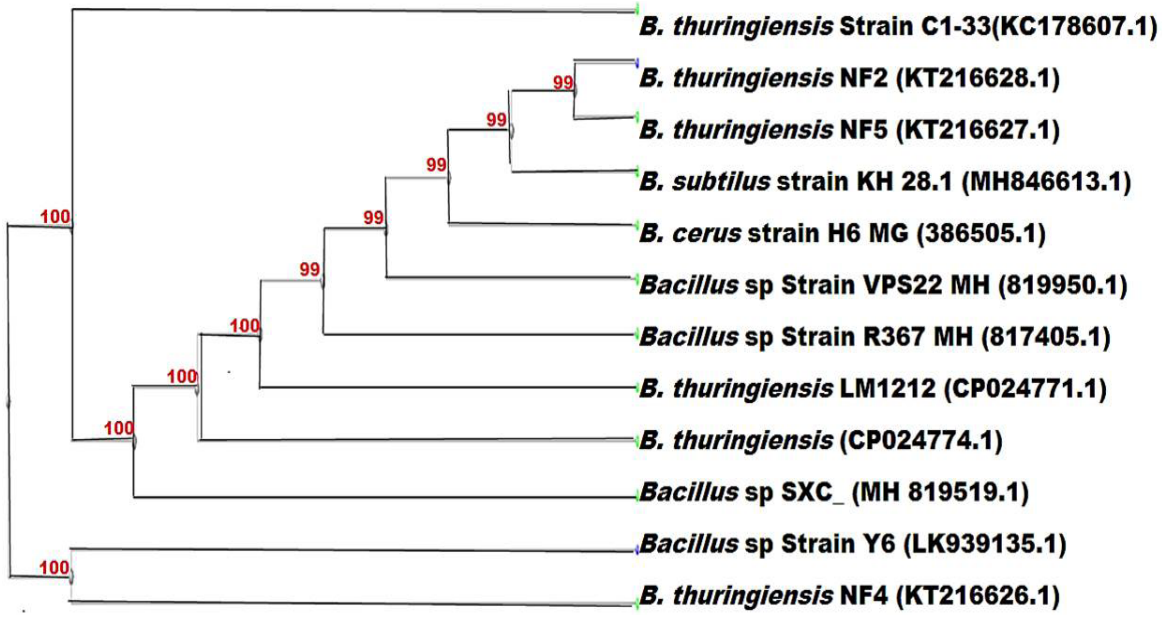


Fig. 1. Neighbor-joining method based tree of selected *B. thuringiensis* strains. *B. thuringiensis* strain NF4, *B. thuringiensis* strain NF5, *Bacillus* sp. strain R367, *Bacillus* sp. strain SXC-M, *Bacillus* sp. strain VPS22, *B. thuringiensis* strain LM1212 with 500 bootstrap test value in MEGA7 software.

## RESULTS AND DISCUSSION

*Characteristics of* B.t. *isolates*

Fifteen *B.t.* isolates, collected from different ecological environment, were selected on the basis of various biochemical tests. *B.t.* was positive for catalase activity and Voges-Proskauer test, could decompose tyrosine and hydrolyze casein and starch. It could grow on media containing 0.001% lysozyme and showed strong lecithinase and hemolytic activity with blood agar test. The bacterium did not grow at 65°C. They produced spores and intracellular protein crystals which were visible green and deep pink in colour after malachite green and acid fucshin staining.

*Molecular characterization* B.t. *isolates*

The genomic DNA of *B.t.* was isolated and PCR products of 16S rDNA were visualized through agarose gel electrophoresis. The sequence alignment of 16S rDNA gene from NF.*B.t* 2, 4, 5, respectively showed 99% homology with *B.t.* str. *Al Hakam*, *B.t.* serovar *konkukian*, and *B.t.* serovar *Chinensis,* respectively. All the sequences of 16S rDNA gene from *B.t.* isolates were submitted to GenBank and their accession numbers are KT216626, KT216627 and KT216628, respectively. Dendrogram is showing the relatedness of 16S rDNA of these *B.t.* isolates (Fig. 1).

*Prevalence of* cry11 *gene in the* B.t. *isolates*

PCR is an effective tool for characterization of gene coding for Cry protein and also used to analyze *B.t.* collections. At first time, it was introduced by Carrozi *et al.* (1991) who identified *cry* gene to predict its insecticidal activity. In this study *B.t.* specific primer and *cry11*gene specific primers were designed to amplify the conserved region of *cry* gene family. The use of degenerate oligonucleotide primer increases the probability of amplification of novel gene and limited the detection of closely related gene Kuo and Chak (2000).

In the present study, five *B.t.* isolates were positive for *cry11* gene. In which 48% *B.t.* strain were screened from dry soil samples and 14% from leaf litter, moist and garden soil. *B.t.* is effective against larval stage as compared to the adult stage and forms parasporal crystalline inclusion during sporulation.

Aronson *et al*. (1986) reported that *cry11* gene which is toxic absolutely to dipteran species (*A. aeygypti*). In this study, the suitable conditions for amplification of full length gene 1.9kb were ascertained but in our *B.t.* strain the PCR product size is above 650bp. So, partial type *cry11* gene was amplified**.** Expected restriction fragment sizes of digested *cry11* genes fragment size (bp) is in between 550 to 750bp because *cry11* specific primers were used to detect *cry11* gene group. Therefore, the band at 650 bp could be corresponding to a different sub-type of *cry11* gene Corazzi *et al*. (1991). In this study, the nucleotide sequence of conserved region of *cry11* has a very high similarity (99%) with other reported strain *B.t.* BMB171, *B.t.* serovar *thuringiensis, gallerie, chinensis CT-43*, *kurstaki*, *tolworthi*, *Bacillus thuringiensis B.t.* 407, *Bacillus thuringiensis* subspecies *morrisoni*, *Bacillus thuringiensis* IGS strain *SBS B.t.* 4-6*,* having mosquitocidal toxin genes (Sauka *et al*., 2010).

Various species of *B. thuringiensis* serotypes reported to be toxic against mosquitoes, black flies and other flies. The conserved regions of *cry11* gene of PCR products of 650 bp have a very high similarity (99%) with other reported mosquitocidal toxin genes. *B.t.* subsp. CT-43 chinensis and strongly suggest that their encoding genes are restricted to this *B. thuringiensis* svar and showed maximum homology with *B.t.* serovar *konkukian* serovar *tolworthi*, *B.t.* serovar. *Al Hakam*, *B.t.* serover *thompsoni*, *B.t.* serovar *konkukian* and *B.t.* serovar *fukuokaensis, gallerie, chinensis, Indiana, kurstaki* (Bukhari and Shakoori, 2010).

**Table I.- The toxic *B.t.* isolates, screened from various areas and soil textures, against 3rd instar larvae of mosquito (*Aedes aegypti*). Out of six, the most toxic *B.t.* isolate was NF5.**

| Strain ID | Area of collection | Soil texture | $LC_{50}$ (µg/ml) |
|---|---|---|---|
| NF1 | Faisal Town (cow rearing area) | Dry waste, Animal dung | 645.211±1.30 |
| NF2 | Faisal Town near AMC | Leaf litter soil | 570.262±0.95 |
| NF3 | Wheat crop area (PU) | Dry soil | 763.731±1.2 |
| NF4 | Garden soil | Model Town park (H Block) | 754.5960±1.1 |
| NF5 | Moist soil | Dirty, sewage water Pico road, Township | 522.027±0.17 |
| NF6 | Dry soil | Soil drain area | 914.634±0.99 |
| NF7 | Dry soil | Soil drain area | 1007.33±1.3 |
| HD 500 | - | Reference strain | 673± 1.34 |

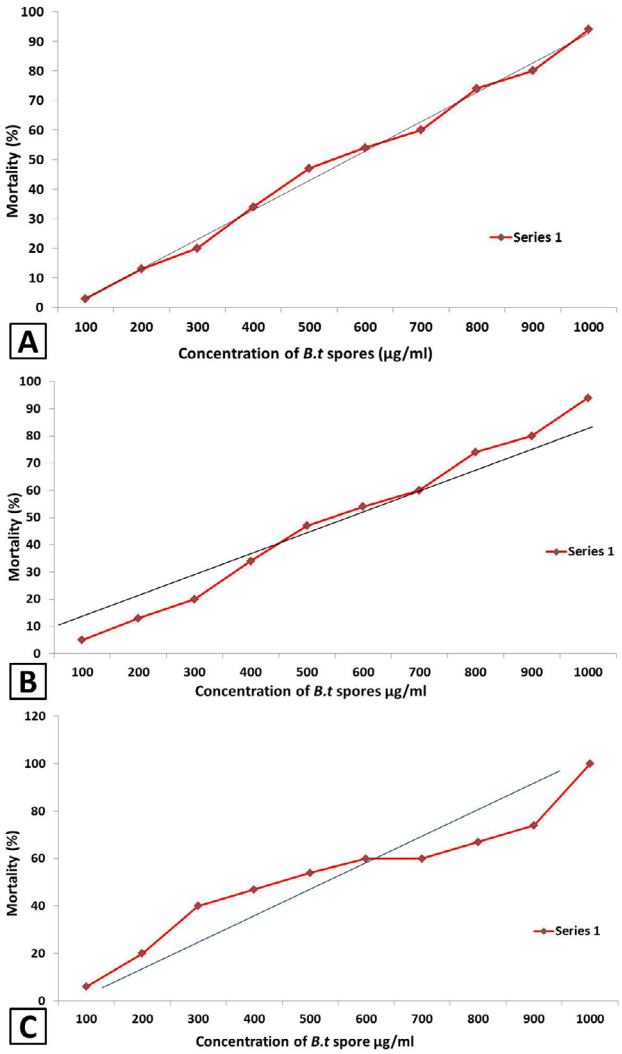


Fig. 2. Mortality (%) of larvae after 24 h applied dose of strain (A) NF1 (*B. thuringiensis* serovar *Kurstuk*i strain HD1011, (B) NF2 (*B. thuringiensis* strain *Al Hakam,* and (C) NF5 (*B. thuringiensis tolworthi, Chinensis* CT-43).

*Biotoxicity of* B.t. *isolates*

Among six *B.t.* isolates, NF5 was found the most toxic and was isolated from the moist soil containing a dirty sewage water. The $LC_{50}$ was 522.027±0.17 µg/ml against the 3rd third instar larvae of *A. aegypti* and showed 100% mortality at 1000µg of spores/ml (Fig. 2C). At this dose, NF1 and 2 showed 100% (Fig. 2A) and 94% (Fig. 2B), mortality. The positive control HD-500 showed mortality of 94%. The $LC_{50}$ (522 µg/ml) of NF5 was quite less than HD500 $LC_{50}$ (673µg/ml) (Table I). So, NF5 is more toxic as compared to the HD500. Shakoori *et al*. (2011) reported that the $LC_{50}$ of SBSB *B.t.* 48 spores, recombinant organisms and recombinant Cry 11B protein was 700 µg/ml, 525 µg/ml and 390 ng/ml, respectively, as against 850 µg/ml, 550 µg/ml and 470 ng/ml HD500 standard *B.t.* strain. Bukhari and Shakoori (2009) reported that the $LC_{50}$ of DAB *Bt* 5 (SBS *Bt* 45) recombinant organisms and crude Cry 11B protein was 350 ±1.76 µg/ml and 407 ± 0.69 ng/ml, respectively. All these *B.t.* isolates, with minor difference, showed high mortality percentage against mosquito larvaes.

Yamada and Agata (1999) mentioned the PCR based detection of *B.t.* strains, the 16S rDNA gene based primers were made to order and used to differentiate the *Bacillus* spp. The sequence of 16S rDNA gene is a molecular clock to estimate relationship among bacteria and helpful to identify bacterium up to genus and species level. Among six *B.t.* isolates, NF5 was found the most toxic and was isolated from moist soil containing sewage water, decaying dirty, cattle waste.

Malik *et al*. (2012) emphasized on the use of the pellet of the *B. thuringiensis* spores as a biopesticide. The present work was done by using the spore diet of *B. thuringiensis* instead of using the isolated crystal proteins; the spore forms in the pellet contain variety of crystal proteins. One of these proteins is the Cry11 protein which is really very toxic for the Dipterans. The formulation used in this study was safe, easy to use, and having long shelf time. The active ingredient in the formulation was the spore crystal complex, which is effective, cheaper to achieve than the crystal alone, and often used in test.

## CONCLUSIONS

In the present study a total of 15 local *B. thuringiensis* isolates were collected from different habitats. Out of 15, 7 isolates were found positive for *cry11* gene after amplification through PCR and the most toxic isolates were identified on 16S rDNA sequencing. The toxicity bioassays with *B.t.* spores were performed and NF5 with $LC_{50}$ (522 µg/ml) was found more toxic as compared to the positive control HD500. This study provides a convenient method which is time saving and economical. This study also recommends that *B. thuringiensis* at spore stage provides good mortality percentage. These *Bt* strains have promising potential to grow into a biopesticidal formulation for mosquitoes control.

*Statement of conflict of interest*

The authors declare no conflict of interest.

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