Pakistan J. Zool., vol. 45(3), pp. 759-767, 2013

Cloning and Molecular Characterization of *cry1Ca* and *cry1Cb* Genes from Locally Isolated *Bacillus thuringiensis* Strains

Munawar Saleem Ahmad* and Abdul Rauf Shakoori**
School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan

Abstract.- *Bacillus thuringiensis (Bt)* is endospore former, Gram positive bacterium and makes parasporal crystals (Cry proteins), which kill particular target pests of different crops. *Bt* isolated from different localities of Pakistan were screened for *cry1Ca and cry1Cb* domain III genes by polymerase chain reaction. Among all the screened *Bt* strains, five were positive for *cry1C* domain III gene, 3 of which were positive for *cry1Ca* and 2 for *cry1Cb*. Confirmation was done by colony PCR, restriction analysis, nucleotide sequencing and alignment on BLAST. The complete gene (3.6kb) encoding *cry1Ca* endotoxin of one of the *Bt* isolate (MS-SBS Bt1) was amplified and analyzed for the nucleotide sequence. The nucleotide and deduced amino acid sequences of endotoxin gene was compared with that of *Bt* strains available in the literature. The genes amplified from the positive strains had 99% similarity and had 100% same deduced amino acid with that of *Bt* strains reported in the Gene Bank. Full gene sequence was submitted in Gene Bank.

Keywords: Bioinsectide, *cry1Ca* gene, *cry1Cb* gene, *Bacillus thuringiensis*, PCR, Colony PCR, Restriction analysis

INTRODUCTION

Bacillus thuringiensis (*Bt***)** endotoxins are glycoproteins, encoded by a single gene, which interact with the membrane of epithelial cells of midgut of target organisms and rupture them by osmotic lyses (Jurat-Fuentes, 2006).

Bt. δ endotoxins are made up of three domains and are globular proteins. Domain I is highly conserved whereas domain II is highly variable in all *δ* endotoxins (Bravo, 1997). X-ray crystallography has helped in determining the three dimensional structure of δ. endotoxins. Domain I consists of seven α-helices out of which six are (ά-1, 2, 3, 4, 6, 7) amphipathic helices and one (α-5) is middle hydrophobic helix. Domain II is highly variable and is for the specificity of a toxin to specific insect. Domain II is made up of three antiparallel β-sheets. This specificity is due to exposed loops of domain II (Grochulski *et al*., 1995). Domain III consists of β -sandwich structure which are formed by two antiparallel β-sheets. Domain III gives the strength to the protein, receptor binding, specificity determination and ion channel gating (Schnepf *et al*., 1998).

Among all the *cry1* proteins, *cry1C* has dual toxicity against lepidopteran such as *Spodoptera* species as well as dipteran insects such as mosquito (Ahmad and Shakoori, 2013; Smith *et al*., 1996; Moar *et al*., 1989). Cry1C protein has three-domains at N-terminal region having toxicity and the other half of toxin at C-terminus is responsible for crystallization of protein during stationary phase of *Bt* life (de Maagd *et al.,* 2003; Whiteley and Schnepf 1986; Aronson, 2002). When the spores along with parasporal crystals are eaten by insects then 60–70 kDa carboxyl end fraction of the protein and 2-3 kDa of N-terminus protein is chopped. Portion of protein which is left behind takes its final structure and its three domains do their proper functions i.e. insertion and lysis of the cells of larval gut epithelium (Bravo *et al*., 2004, 2008).

The *cry1C* cluster of *Bt* proteins has 10 extremely homologous proteins of the *cry1Ca* sub-set and three proteins of the *cry1Cb* sub-set which vary in domain III sequence. The insects which show complete or partial resistance to *Bt* crops can be controlled by using *cry1Ca* toxin. Genetically modified plants harbouring *cry1Ca* gene may effectively manage particular lepidopteran pests. Attempt is being made to introduce *cry1Ca* gene for its application in the field. The amino acid sequence alignments showed that *cry1Ca* N-terminal portion of the protein has fewer conserved amino acids than the C-terminal fraction. Bioinformatics analyses shows more variable sequence in those parts of

* Present address: Department of Zoology, Hazara University, Mansehra
** Corresponding author: arshaksbs@yahoo.com
0030-9923/2013/0003-0759 $ 8.00/0

protein which interact with membrane receptors. Domains I, II and III of *cry1Ca* intermingle *in vitro* among membrane vesicles created from gut. This contact is due to the typical assemblage of membrane lipid raft domains. *cry1Ca* interface among the cells is with the particular aminopeptidase-N receptors which differ from other *cry1* active proteins. The participation of additional membrane apparatus in the dealings is not yet understood. Even those insects which are not sensitive to *cry1*A like *cry1Ac* insensitive mutants of diamond backmoth are killed by *cry1Ca*, owing to the participation of diverse hereditary loci. Therefore *cry1Ca* and additional Cry toxins can expand the bioinsecticidal range of *Bt* crops and at the same time hinder the development of Cry-resistant insect (Avisar *et al*., 2009). Well known *cry1Cb* protein genes were identified in *Bt.* 087 strains and *Bt. galleriae* while *cry1Ca* protein genes available now were identified in HD110, *Bt. entomocidus* 60.5, *Bt. aizawai* 729, G10-01A, AF- 2 and K26-21 (Crickmore *et al.*, 2008).

Present study focuses on screening of local isolates for *cry1Ca* and c*ry1Cb* genes by PCR amplification of domain III. Primers were designed to amplify full length gene of *cry1Ca* (3.6kb) by PCR. Conditions were optimized for PCR amplification of *cry1Ca* (3.6kb) gene. This study also encompasses the cloning and sequencing of all PCR amplified *cry1Ca* and c*ry1Cb* gene products. Gene sequences were BLASTed to compare them with the already reported sequences of *cry* genes.

MATERIALS AND METHODS

Bacterial growth and isolation of DNA

One loop full of overnight grown *Bt* culture from LB agar plate was taken and suspended in 1500µl of germfree distilled water, centrifuged at 9,000 x g for 2 minutes at 4°C. The detergent solution (300 µl) (10mM Tris- HCl pH8.0, 3mM MgCl2, 200 µl Triton X100, 6.6ml autoclaved distilled water) and 6 µl of proteinase K (20mg/ml) was added to the pellet vortexed and placed at 45°C for 30 minutes. Phenol: chloroform extraction was done to purify the DNA. DNA was precipitated with two volumes of absolute ethanol, centrifuged at 12000 x g and the pellet washed with 70% ethanol, dried and dissolved in 100µl sterile water and stored at-20°C. DNA was run on 1% agarose gel and observed in UV light.

PCR amplification of cry1Ca and cry1Cb

DNA was amplified by using thermocycler (Applied Biosystem 2720). Shorter fragments of *cry1Ca* and *cry1Cb* were amplified by using isolated DNA and specific universal primers (Table I). PCR was done by a modified procedure described by Saiki *et al*. (1988). In PCR mixture total volume of 50µl contained 2.0mM MgCl2, 10mM Tris-HCl pH 8.0, 200µM of deoxyribonucleoside triphosphate (dNTPs), 100 pmoles of each forward and reverse primer, 2.5 units of Taq DNA polymerase and 100-150 ng of crude DNA. Total volume of reaction mixture was made 50µl by adding sterile water and dispensed in 200µl tube. PCR was done with an initial denaturation only once at 94° for 5 min and then 40 cycles each of 45 sec denaturation at 96°C, 1 min annealing at 57°C for *cry1Ca* and 52°C for *cry1Cb* and 1 min extension at 72°C. Final extension was at 72°C for 10 mins. The amplified shorter fragments of *cry1Ca* and *cry1Cb* genes were run on 1% agarose gel.

Cloning of cry1Ca and cry1Cb

The relevant PCR product bands were extracted according to Sambrook *et al.* (1989), and gene cleaned with Fermentas kit. Purified DNA fragment was ligated in a cloning vector pTZ57R/T (T/A cloning vector) using Fermentas DNA ligation kit. Ligation mixture comprised PTZ57R/T 1 µl, 5X ligation buffer 4 µl, ligase 14 µl, 6 µl DNA fragment (100ng) and 8 µl sterile water to make final volume up to 20 µl. Whole ligation mixture was incubated at 22°C overnight.

Competent cells of DH5α (200µl) were transformed with 10 µl of ligation mixture. Cells were left on ice for 10 mins after mixing with ligation mixture, shifted to water bath set at 42°C for 90 sec to give heat shock and then shifted to ice for 5 min. Sterilized LB broth (800 µl) was added in the microfuge tube and was placed in a shaking incubator at 37°C and 100rpm for 3 h. The tubes were centrifuged and 600 µl of supernatant was discarded, remaining 400 µl was mixed well with

Table I.- Primers of cry1Ca and cry1Cb used in this study.

Primers for *cry1Ca* (670bp)	PRIMER F	5 ` ATTGGGGAGGACATCGAGTAATATCTAGCCTTA 3 `
	PRIMER R	5` ACTCCTGTGGATGCCGCTCCTGTTAATACTAT 3 `
Primers for *cry1Cb* (730bp)	PRIMER F	5 `GGACTTTATCAAATCCTACTTTTAGACCT 3 `
	PRIMER R	5 `TCCTCAAATGTAGAAGTAACTGGGAGAA 3 `
Primers for Full length *cry1Ca* (3.6kb)	PRIMER F	5`GGTAGTTTAATAAAAAAACGGAGG **3`**
	PRIMER R	**5`** CGTATCTTATTCCTCCATAAGG **3 `**

pellet and spread on prewarmed (37°C) LB agar plates (X-gal (270 µg/ml) IPTG (120 µg/ml) and ampicillin (100 µg/ml). Agar plates were incubated at 37°C for 20 hours. Next day white colonies were picked up and used for confirmation of clone by colony PCR and recombinant DNA double restriction.

Confirmation of clones

For colony PCR, many white colonies were picked and streaked on new X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml) plates and incubated at 37°C for 20 hours. Isolated colony was resuspended in 20 µl autoclaved distilled water in PCR tube. Thirty µl of PCR master mix (2.5 mM MgCl2, 10mM Tris- HCl pH8.0, 0.3mM of deoxyribonucleoside triphosphate (dNTPs), 50 pmoles of each forward and reverse primers and 1 unit of Taq DNA polymerase in final volume of 50 µl) was mixed in PCR tube. Forty PCR cycles were performed as described above, run on 1% agarose gel and bands were visualized under UV light. Positive clones should give bands at positions identical to PCR amplified bands for *cry1Ca* and *cry1Cb*.

For restriction analysis plasmid DNA was isolated by modified alkaline lysis method described by Birnboim and Doly (1970), for which ten ml LB medium in 100ml flask was inoculated with isolated white colony and 10 µl Ampicillin (100 µg/ml) was also added. Cells were grown overnight at 37°C at100 rpm. Cells of 3 ml of the culture were harvested in microcentrifuge tube at 13,000 x g for 2 minutes.

One hundred µl of ice cold solution I (25mM Tris-HCl, pH8.0, 10 mM EDTA, 50 mM glucose) was added to pellet and vortexed to mix the cells in solution, placed on ice for 5 minutes. Freshly prepared 200 µl solution II (1%SDS, 0.2M NaOH) was dispensed in microfuge tube, kept on ice for 4 minutes, mixed by inverting the tubes three to four times. Ice-cold solution III 150 µl (5M potassium acetate 30ml, glacial acetic acid 6.75ml, autoclaved distilled H2O 14.25ml, pH4.8) was dispensed, mixed immediately by vortexing, placed on ice for 5-10 minutes. Tube was spun at 13,000 x g for 5 minutes at 4°C. Two volumes of chilled absolute ethanol was added after the supernatant was carefully shifted to fresh eppendorf tubes, placed at -20°C for 20 minutes. The tubes were centrifuged at 13,000 x g, the pellet was rinsed with 70% ethanol, air dried and dissolved in 60 µl of sterile water. One µl of DNAase free RNAase A (10mg/ml) was added to digest RNA. Approximate concentration of plasmid DNA was determined by running it on 1% agarose gel and comparing the bands of DNA with marker. Confirmation of positive colonies was done by restriction of the plasmid as described below.

The purified plasmids were checked for the presence of insert by double restriction digest using *Eco*RI (Fermentas, cat# ER 0271) and *Hind*III (Fermentas, cat# ER 0501) (10 units each). For double restriction a 50 µl reaction mixture comprised 1 µg of the recombinant plasmid, 10 units of *Eco*RI and 10 units of *Hind*III in 1x tango buffer. The reaction mixture was incubated at 37 ^{0}C for 14 hours. Restricted plasmid DNA was run on 1%agarose gel for confirmation of clone. Positive clones should give bands at positions identical to PCR amplified bands for *cry1Ca* and *cry1Cb*.

Cloning of full-length cry1Ca gene

Full-length *cry1Ca* gene was amplified from

Table II.- Biochemical characteristics of rod-shaped, endospore forming bacteria.

	Voges Proskauer	Motility	Acid production	Citrate utilization	Growth at 65°C	Growth at 7% NaCl	Growth at Sabouraud Dextrose Agar	Starch hydrolysis	Phenylalanine deamination test	Tyrosine decomposition test	Indole test	Casein hydro-lysis	Growth in 0.001% lysozyme	Nitrate reduction test	Catalase test
A1	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A2	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A3	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A4	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A5	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A6	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A7	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A8	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A9	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A10	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A11	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A12	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A13	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A14	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A15	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A16	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A17	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A18	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
A19	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A20	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A21	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A22	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A23	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+
A24	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A25	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A26	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A27	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A28	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A29	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A30	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A31	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A32	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A33	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A34	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A35	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A36	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A37	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A38	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A39	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A40	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A41	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A42	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A43	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A44	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A45	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A46	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
A47	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
A48	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
HD-29	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+

local *Bt.* isolate (MS-SBS-*Bt*1) and also from positive control HD137 and cloned in pTZ57R/T (T/A cloning vector). Primers were designed to amplify full-length 3.6 Kb *cry1Ca* genes from local strains of *B. thuringiensis* from the already known sequences of *cry1Ca* gene **(**Table I).

Different conditions were optimized for the amplification of *cry1Ca* full-length gene from local strains of *B. thuringiensis*. Conditions optimized were DNA concentration (50ng/µl, 100ng/ µl, 150ng µl…300 ng µl), dNTPs concentration (100 µM, 150 µM, 200 µM…300 µM), MgCl2 concentration (1.5mM, 2mM, 2.5mM, 3mM, 3.5mM and 4mM), Primers concentration (50pmoles, 100pmoles, 150pmoles and 200pmoles), Taq DNA polymerase concentration (0.5 units, 1 unit, 1.5 units, 2.0 units and 2.5 units) and annealing temperature (48°C, 50°C, 52°C, 54°C, 55°C, 56°C, and 58°C). Final volume of reaction mixture was always kept 50µl. DNA was amplified by using thermocycler (Applied Biosystem 2720). PCR was done with an initial denaturation only once at 94° for 6 mins and a set of 35 cycles in which each cycle comprises of 1 min denaturation at 96°C, 1min annealing at 51°C and 3.30 min extension at 72°C. Final extension was done at 72°C for 10 mins. Cloning was confirmed by double digestion with restriction endonucleases *EcoRI* and *HindIII* and colony PCR as described above for shorter fragments.

DNA sequencing

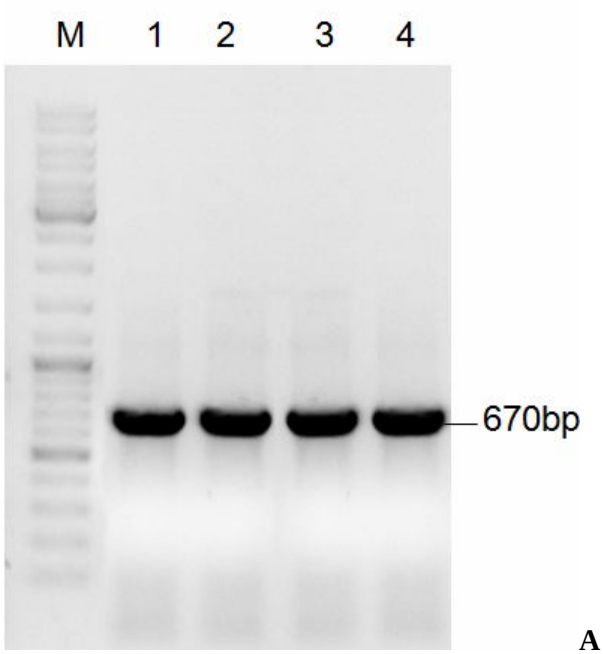
cry1Ca full length gene and *cry1Ca* and *cry1Cb* shorter fragments were sequenced by using DNA sequencing system (CEQ System, Ver 9.0.25). The sequences were BLASTed to compare them with sequences from NCBI.

RESULTS AND DISCUSSION

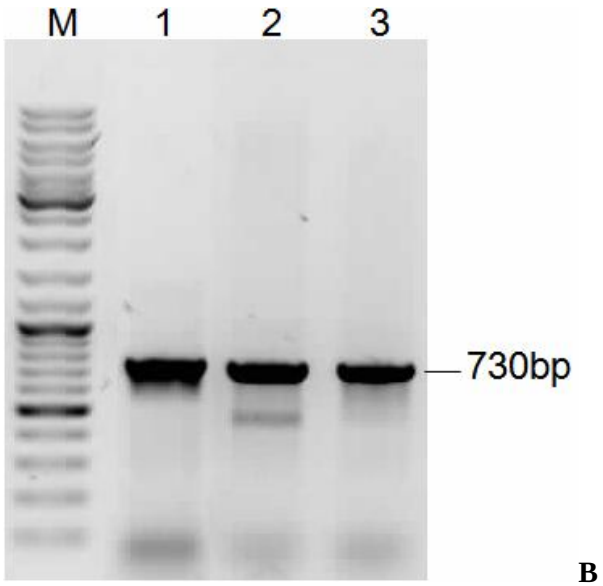
Domain III of active toxin of cry1Ca and cry1Cb

Domain III was amplified by using universal primers which gave amplification at 670bp for *cry1Ca* and at 730bp for *cry1Cb* (Fig. 1).

The PCR amplified 670 bp and 730bp fragments were T/A cloned in pTZ57R/T plasmid vector. The colony PCR results gave the DNA bands at 670bp for *cry1Ca* and 730bp for *cry1Cb* (Fig. 2) and double restriction of vector gave a DNA



A



B

Fig.1. A, PCR amplification of domain 3 of *cry1Ca* gene of *Bt.* isolates. Lane#1, MS-SBS-*Bt.* 1; Lane#2, MS-SBS-*Bt.* 2; Lane#3, MS-SBS-*Bt.* 3; Lane#4, HD137 (Positive control). M, DNA marker (Fermentas cat# SM0403).
B, PCR amplification of domain 3 of *cry1Cb* gene of *Bt.* isolates. Lane#1, MS-SBS-*Bt.* 4; Lane#2, MS-SBS-*Bt.* 5; Lane#3, HD29 (Positive control). M, DNA marker (Fermentas cat# SM0403).

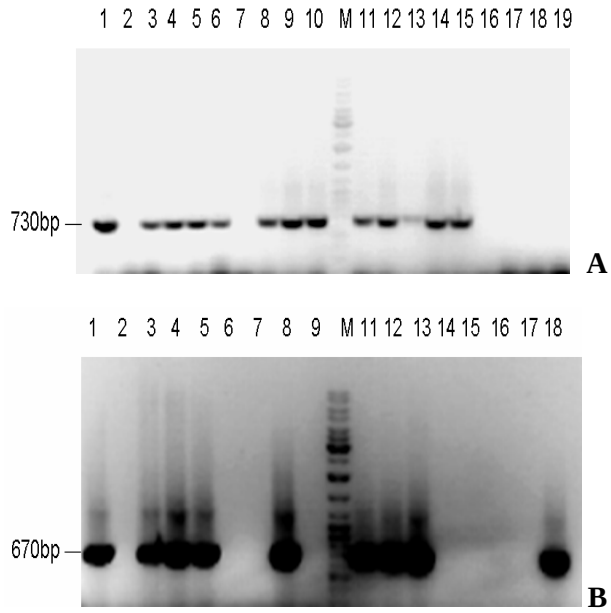


Fig.2. A, Colony PCR of transformants of clones of 730bp for *cry1Cb* of B.t isolates MS-SBS-*Bt* 4 and 5. The amplified 730bp for *cry1Cb*.is indicated with an arrow. Lanes#1, 3-6, 8-10 and 11-15 are positive colonies. Lanes# 2, 7 and 16-19 are without amplified product. M, DNA marker (Fermentas cat# SM0403).
B,Colony PCR of transformants of clones of 670bp for *cry1Ca*.of B.t isolates MS-SBS-*Bt* 1-3. The amplified 670bp for *cry1Ca*.is indicated with an arrow. Lanes#1, 3-5, 8, 11-13 and 18 are positive colonies. Lanes# 2, 6, 7, 9 and 14-17 colonies are without insert. M, DNA marker (Fermentas cat# SM0403).

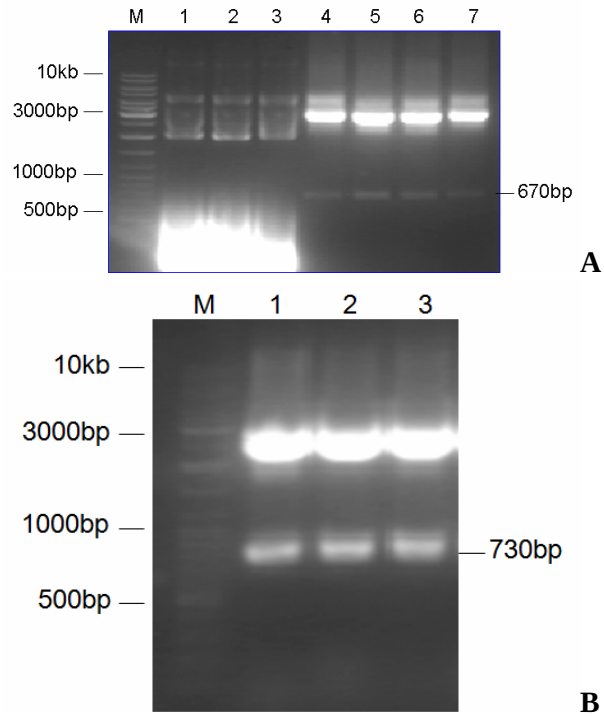


Fig. 3. A, Digestion of recombinant plasmid DNA pTZ57R/T having amplified product of 670bp of *cry1Ca* gene of *Bt.* isolates with *Eco*RI and *Hind*III. Lanes#1-3, MS-SBS-*Bt*1-3 undigested; Lane#4-6, MS-SBS-*Bt*1-3 digested showing 670bp insert and 2.88kb vector; Lane#7, HD137 (Positive control). M, DNA marker (Fermentas cat# SM0403).
B, Digestion of recombinant plasmid DNA pTZ57R/T having amplified product of 730bp of *cry1Cb* gene of *Bt.* isolates with *Eco*RI and *Hind*III. Lanes#1-2, MS-SBS-*Bt*4-5, digested showing 730bp insert and 2.88kb vector; Lane#3, HD29 (Positive control). M, DNA marker (Fermentas cat# SM0403)

band at 2.88kb and DNA bands at positions identical to the colony PCR products (Fig.3). Domain 3 of *cry1Ca* and *cry1Cb* were further confirmed by the nucleotide sequence analysis. Nucleotide sequence was 99% similar to sequences of *cry1Ca* (accession number X07518) and *cry1Cb* accession number M97880) on Basic local alignment tool (BLAST). Deduced amino acid sequence was 100% similar with that available in literature.

cry1Ca gene

The amplified *cry1Ca* gene of 3.6 kb (Fig.4A) at 51 ^{o}C (annealing temperature) was T/A cloned in pTZ57R/T plasmid vector. Colony PCR was performed to confirm the positive white colonies. The colony PCR showed 3.6kb DNA band (Fig.4B) in accordance with the size of insert. Further confirmation was done by single and double restriction of final construct. Single restriction of recombinant plasmid with *Eco*RI gave a single band at 6.5kb and two bands at 3.6kb and 2.88kb with *Eco*RI and *Hind*III digestion (Fig.5). Full length gene was sequenced and compared with other *cry1Ca* genes available in literature which showed 99% similarity to already reported sequences of *cry1Ca*. Deduced Amino acid sequence was same as already reported in literature.

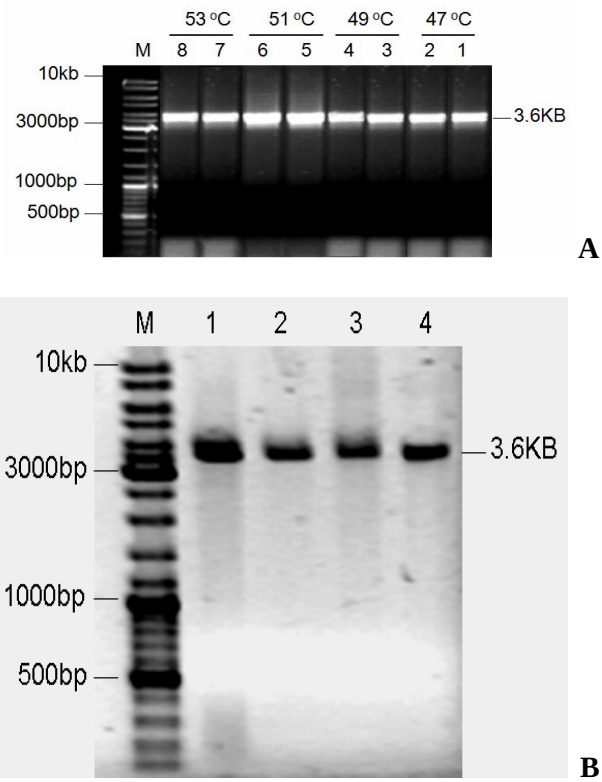


Fig.4. A, PCR amplification of *cry1Ca* full gene (3.6kb) of *Bt*. isolate MS-SBS-*Bt*.1 and HD 137 at different temperatures. Lanes# 1 and 2, 47 °C Lanes# 3 and 4, 49 °C; Lanes# 5 and 6, 51 °C; Lanes# 7 and 8, 53 °C. M, DNA marker (Fermentas cat# SM0403).
B, Colony PCR of recombinant white colonies containing pTZ-*cry1Ca* analyzed by 1% agarose gel electrophoresis. Lanes#1-4, Colony PCR of white colonies containing pTZ- *cry1Ca* constructs. M, DNA marker (Fermentas cat# SM0403).

Nucleotide sequence

The nucleotide sequences are given in the Fig.6. The gene sequence for *cry1Ca* (670bp) was 99% similar to *cry1Ca*1 gene (accession# X07518.1) and gene sequence for *cry1Cb* (730bp) was also 99% homologous to *cry1Cb*1 gene (accession# M97880.1), when aligned on Basic Local Alignment Tool (BLAST). Full length gene (submitted online, accession# AB762699.1) was also 99% homologous to *cry1Ca*1 gene (accession# X07518.1 and Figure 6).

Three isolates (MS-SBS-*Bt*1-3) gave positive amplification for domain 3 of *cry1Ca* and another two isolates (MS-SBS-*Bt* 4 and 5) gave domain 3 amplification for *cry1Cb*.

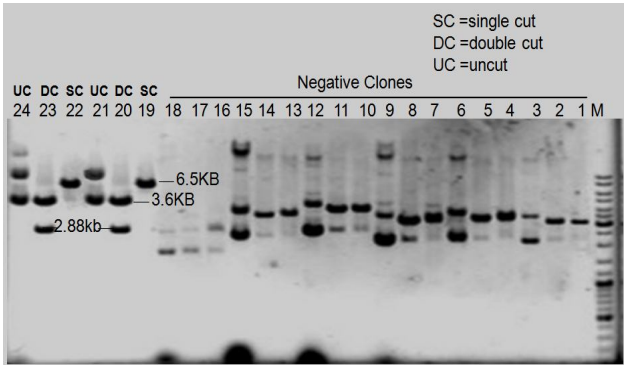


Fig. 5. Restriction digestion of pTZ-*cry1Ca*3.6kb with *Eco*RI and *Hind*III. Lane#1-18, Negative constructs, Lane#19-Single restriction of pTZ-*cry1Ca*3.6kb (MS-SBS-*Bt*1) with *Eco*RI; Lane#20- Double restriction of pTZ-*cry1Ca*3.6kb (MS-SBS-*Bt*1) with *Eco*RI and *Hind*III; Lane#21- Unresticted pTZ-*cry1Ca*3.6kb (MS-SBS-*Bt*1); Lane#22-Single restriction of pTZ-*cry1Ca*3.6kb (HD137) with *Eco*RI; Lane#23-Double restriction of pTZ-*cry1Ca* 3.6kb (HD137) with *Eco*RI and *Hind*III Lane#24- Unresticted pTZ-*cry1Ca*3.6kb (HD137). M, DNA marker (Fermentas cat# SM0403). SC, single cut; DC, double cut; UC; uncut.

It shows that *cry1C* gene frequency in local isolates from Pakistan is very low as compared to other genes such as *cry2* and *cry4* genes (Ahmad and Shakoori, 2013; Saleem, 2008; Bukhari, 2008). It was found easy to screen the strains for different *cry1C* genes by PCR rather than other methods. It was in accordance with the literature that PCR amplification is a good technique to identify *Bt* and *cry* genes (Xavier *et al.*, 2003).

cry1Ca is a 3.6kb protein gene comprising of three major domains at N-terminal region plus C-Terminal portion for crystallization of protein. Four components of N-terminal region encoded by 1.86kb comprise the active region of *cry1Ca* protein and 28 amino acids at N-Terminal end of active toxin while 1.82kb region encodes for active toxin three domains (Kouskoura *et al.*, 2001). These all portions of *cry1Ca* genes were cloned and sequenced. Sequence analysis showed that full gene of MS-SBS-*Bt*1 was 99% similar and deduced

Fig.6. The nucleotide sequence encoding complete polypeptide of cry1Ca with the amino acid sequence (submitted online, accession# AB762699.1).

amino acid sequence was 100% similar as described in literature. It proves that *cry1Ca* genes have very specific gene sequences and amino acid sequence to kill specific insects. It yet remains to be explored that how this gene sequence is responsible to translate a protein which can kill lepidoteran as well as dipteran insects. This study is first attempt in Pakistan to characterize *cry1Ca* gene in local isolates.

REFERENCES

AHMAD, M.S AND SHAKOORI, A.R., 2013. Isolation and molecular characterization of *cry1C* harboring *Bacillus thuringiensis* from different habitats and localities of Pakistan. *Pakistan J. Zool.*, **45:** 261-271.

ARONSON, A., 2002. Sporulation and delta-endotoxin synthesis by *Bacillus thuringiensis. Cell Mol. Life Sci*., **59**: 417–425.

AVISAR, D., EILENBERG, H., KELLER, M., REZNIK, N.,

SEGAL, M., SNEH, B. AND ZILBERSTEIN, A., 2009. The *Bacillus thuringiensis* delta-endotoxin *cry1C* as a potential bioinsecticide in plants. *Plant Sci*., **176:** 315-324.

AVISAR, D., KELLER, M., GAZIT, E., PRUDOVSKY, E., SNEH, B. AND ZILBERSTEIN, A., 2004. The role of *Bacillus thuringiensis cry1C* and *cry1*E separate structural domains in the interaction with Spodoptera littoralis gut epithelial cells. *J. biol. Chem.,* **279**: 15779–15786.

BRAVO, A. AND SOBERON, M., 2008. How to cope with insect resistance to Bt toxins? *Trends Biotechnol.* ,**26**: 573–579.

BRAVO, A., 1997. Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. *J. Bact.,* **179:** 27932801.

BRAVO, A., GOMEZ, I., CONDE, J., MUNOZ-GARAY, C., SANCHEZ, J., MIRANDA, R., ZHUANG, M., GILL, S. S. AND SOBERON, M., 2004. Oligomerization triggers binding of a *Bacillus thuringiensis cry1Ab* pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim. biophys. Acta***. 1667:** 38–46.

BUKHARI, D.A., 2008. *Cloning and Molecular characterization of cry4 genes from local isolates of Bacillus thuringiensis.* PhD thesis, University of the Punjab, Lahore, Pakistan.

CRICKMORE, N., ZEIGLER, D.R., SCHNEPF, E., VAN, J., RIE, D., LERECLUS, J., BAUM, A., BRAVO, D.H., AND DEAN, A., 2008. ''*Bacillus thuringiensis* toxin nomenclature'' http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/.

DE MAAGD, A., BRAVO, A., BERRY, C., CRICKMORE,N. AND SCHNEPF, E., 2003. Structure, diversity,and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annu. Rev. Genet*., **37**: 409–433.

GROCHULSKI, G., MASSON, L., BORISOVA, S., PUSZTAI-CAREY, M., SCHWARTZ, J.L., BROUSSEAU, R. AND CYGLER, M., 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. mol. Biol*., **254**:447–464.

HERRERO, S., GONZALEZ-CABRERA, J., FERRE, J., BAKKER, P.L., AND DE MAAGD, R.A., 2004. Mutations in the *Bacillus thuringiensis cry1Ca* toxin demonstrate the role of domains II and III in specificity towards *Spodoptera exigua* larvae. *Biochem. J*., **384**: 507–513.

JURAT-FUENTES, J.L. AND ADANG, M.J., 2006. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. *J. Invertebr. Pathol.,* **92**: 166–171.

MOAR, W.J., TRUMBLE, J.T. AND FEDERICI, B.A., 1989. Comparative toxicity of spores and crystals from the NRD-12 and HD-1 strains of *Bacillus thuringiensis* subsp. *kurstaki* to neonate beet armyworm (Lepidoptera: Noctuidae). *J. econ. Ent.,* **82**: 1593–1603.

SALEEM, F.S., 2008. *Cloning and Molecular characterization of cry2 genes from local isolates of Bacillus thuringiensis.* PhD thesis, University of the Punjab, Lahore, Pakistan.

SAMBROOK, J., FRITSCH, E.F. AND MANIATIS, T., 1989. Molecular cloning: a laboratory manual, third ed. Cold Spring Habor Laboratory Press, New York.

SCHNEPF, E., CRICKMORE, N., VAN RIE, J., LERECLUS, D. AND BAUM, J., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. mol. Biol. Rev*., **62:** 775–806.

SMITH, G.P., MERRICK, J. D., BONE, E. J. AND ELLAR, D. J., 1996. Mosquitocidal Activity of the CryIC δ-Endotoxin from *Bacillus thuringiensis* subsp. *Appl. Environ. Microbiol*., **62**: 680-684.

SOBERON, M., PARDO-LOPEZ,L., LOPEZ, I., GOMEZ, I., TABASHNIK, B.E. AND BRAVO, A., 2007. Engineering modified Bt toxins to counter insect resistance. *Science,* **318**: 1640–1642.

WHITELEY, H.R. AND SCHNEPF, H.E., 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annu. Rev. Microbiol*., **40**: 549–576.

XAVIER, R., NAGARATHINAM,P., MURUGAN, V. AND JAYARAMAN, K., 2007. Isolation of Lepidopteran Active Native *Bacillus thuringiensis* Strains through PCR Panning. *Appl. environ. Microbiol*., **53:** 1263-1266.

(Received 10 June 2012, revised 3 March 2013)