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DETERMINATION AND DISTRIBUTION OF CRY1-TYPE GENES IN *Bacillus thuringiensis* ISOLATED FROM NORTH INDIA

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Abstract

Bacillus thuringiensis (*Bt*) is a spore-forming bacterium which produces insecticidal crystal protein in the sporulation phase. Polymerase chain reaction (PCR)-based identification of *Bacillus thuringiensis* toxin genes has become a routine step in most *B. thuringiensis* isolation and characterization initiatives. In the present study, eighteen native *Bt* isolates from diverse habitats of North India were taken for the presence of *cry1* type genes. The distribution of *cry1* gene families in native *Bt* isolates was examined by PCR amplification of genes with three sets of corresponding PCR primers. In native *Bt* isolates many variant bands were also observed in addition to expected bands on PCR amplification. Different sets of primers for the same gene give different results due to different sites of primer binding. Maximum number of isolates showed expected bands when primers designed by Ceron were used compared to other primers. However, with other primers, the numbers of variant bands was larger. The isolates, SK-13, 63 and 105 showed the maximum number of *cry1*-type genes, followed by SK-20, 28, 48, 88, 94, 301, 304 and 307, whereas SK-3 showed the presence of only 3 *cry1*-type genes. RFLP analysis of 1.6 kb fragment indicated the presence of variant bands from the reference strains. Several promising isolates with predicted toxicity towards lepidoptera have been observed in this study.

Key words: Bt isolates, *cry* gene, molecular characterization, PCR analysis

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1. Introduction

Bacillus thuringiensis (Bt) is a spore-forming, gram-positive, facultative anaerobic bacterium, which produces crystals comprised of Cry and Cyt proteins active against insect pest and other organisms like nematodes and protozoa (Ibrahim et al., 2010; Konecka et al., 2006). The diversity of toxins produced by *Bt* allows the use of *Bt* as bioinsecticides formulation or expressing the toxin genes in transgenic plants. Among biopesticides, microorganism-based products represent about 30% of total sales and have a variety of applications (Cawoy et al., 2011). *Bt* is a biocontrol agent that has been used for pest control since 1938 (Sanahuja et al., 2011). The lack of toxicity of Cry proteins in mammalian and environment has resulted in an increased use of *B. thuringiensis* as an insecticide and intensified the search for new strains (Cawoy et al., 2011). The genes coding for Cry proteins are plasmid-borne and the size of plasmids ranges from 3 to 150 Mda (Aronson, 2002).

From *Bt*, 72 groups of Cry and 3 groups of Cyt toxins have been known (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html). The toxicity of most of the Cry and Cyt proteins are already determined (Frankenhuyzen, 2009). Baig and Mehnaz, (2010) proposed that the characterization of strains provide information about the ecological distribution of *Bt.* An effective tool in estimating the utility of crystal proteins against pests is identification of genes coding

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for insecticidal toxins (Nazarian et al., 2009). For example, genes *cry1*, *cry2*, *cry7*, *cry8*, *cry9*, *cry15*, *cry22,* and *cry51* code for proteins active against *Lepidoptera,* however, *cyt1A was possibly active* (Frankenhuyzen, 2009). Similarly, *cry54* codes for a protein that is harmful to moths (Tan et al., 2009). Other *cry* genes codes for proteins found to be toxic for Diptera (Ibarra, 2003), Coleoptera*,* Hemiptera, Hymenoptera (Frankenhuyzen, 2009), Homoptera, Orthoptera, and Mallophaga orders (Schnepf et al., 1998).

However, there is a threat of eventual development of resistance in insects upon large-scale cultivation of transgenic crops, therefore, the isolation of novel *Bt* strains and characterizing them for their insecticidal gene content may lead to the discovery of novel genes with higher toxicity which can provide an alternative to cope up with emergence of insect-resistant population against currently deployed limited number of *cry*-type genes (Meena and Kaur, 2014). The diversity of *Bt* strains facilitates isolation of new types of *cry* genes. New variants of already known *cry* gene subgroups could encode crystal proteins with a significant difference in the level and spectrum of toxicity due to variation in their sequences (Ramalakshmi and Udayasuriyan, 2010). Furthermore, detection of *cry* genes by PCR method enables discovering genes of novel crystalline toxins (Nazarian et al., 2009).

With this perspective, the present study was undertaken to address the molecular characterization of *Bt* isolates for the presence of *cry1* type genes profile with three different sets of primers designed by Ceron et al., (1994); Kalman et al., (1993) and Juarez-Perez et al., (1997).

2. Material and methods

Eighteen native *Bt* isolates SK- 3, 4, 9, 13, 20, 28, 48, 51, 63, 82, 88, 94, 105, 110, 301, 302, 304 and 307) from diverse habitats of north India and fifteen known *Bt* reference strains with BGSC code (*Bt* subsp. thuringiensis, 4A6; *Bt* subsp. finitimus, 4B1; *Bt* subsp. finitimus, 4B2; *Bt* subsp. kenyae, 4F3; *Bt* subsp. galleriae, 4G6; *Bt* subsp. kurstaki, HD1; *Bt* subsp. aizawai, 4J2; *Bt* subsp. aizawai, 4J3; *Bt* subsp. aizawai, 4J4; *Bt* subsp. morrisoni, 4K1; *Bt* subsp. tolworthi, 4L3; *Bt* subsp. israelensis, 4Q5; *Bt* subsp. indiana, 4S2; *Bt* subsp. wuhanensis, 4T1 and *Bt* subsp. kumamotoensis, 4W1) and six *E. coli* strains (*E. coli* strain ECE-52, ECE-53, ECE-54, ECE-127, ECE-128 and ECE-129) were used in the present study. *Bt* strains used as a reference in this study were obtained from the Bacillus Genetic Stock Centre, Department of Biochemistry, Ohio State University, Columbus, Ohio 43210, USA.

2.1. Growth of microorganism

Luria Bertani agar (LA) and Luria Bertani broth (LB) were used for the growth of both, *E. coli* as well as *Bt* strains. The medium was prepared by adding bacto tryptone - 10 g; yeast extract - 5 g and sodium chloride - 10 g in 1 l of distilled water. For LA, 20 g of agar was also added.

2.2. DNA preparation

A single colony of *Bt* isolates was inoculated in 2 ml LB with Penicillin (10 µg/ml) and incubated at 30°C for 16 h at 150 rpm. The cells were pelleted by centrifugation and resuspended in 200 µl sterile distilled water. The cell suspension was boiled for 5 min and after that kept at -70°C for 5 min (cycles were repeated for 3 times) followed by centrifugation for 15 min and the supernatant was directly used for PCR reaction. For *E. coli* strains, plasmid DNA was isolated by Qiagen Midiprep plasmid isolation kit according to manufacturer protocols.

2.3. PCR analysis of cry1 gene families

A set of primers designed as per Ceron et al., (1994) were used to identify the presence of different *cry1*-type genes (*cry1Aa*, *cry1Ab, cry1Ac*, *cry1Ad*, *cry1B, cry1C* and *cry1D*) in native *Bt* isolates. The set of primers for respective genes were; CJ1, reg5' and CJ2, reg3' (*cry1Aa* and *cry1Ad*); CJ3, reg5' and CJ2, reg3' (*cry1Ad*); CJ4, reg5' and CJ5, reg3' (*cry1Ab* and *cry1Ac*); CJ6, reg5' and CJ7, reg3' (*cry1Ac*); CJ8, reg5' and CJ9, reg3' (*cry1B*); CJ10, reg5' and CJ11, reg3' (*cry1C*); CJ12, reg5' and CJ13, reg3' (*cry1D*). Another set of primers designed as per Kalman et al. (1993) were used to identify the presence of *cry1Aa* (TY1AA and TY1UN12)*, cry1Ab* (TY6 and TY14)*, cry1Ac* (TY1AC and TY1UN12)*, cry1B* (TY1B and TY1UN12)*, cry1C* (TY1C and TY1UN12)*, cry1D* (TY1D and TY1UN12)*, cry1E* (TY1E and TY1UN12) and *cry1F* (TY1F and TY1UN12), whereas, the sets of primers as per Juarez-Perez et al. (1997) were used for the amplification of *cry1* (I(-) and I(+)) , *cry1A* (I(-) and IA's)*, cry1Aa* (I(-) and IAa)*, cry1Ab* (I(-) and IAb)*, cry1Ac* (I(-) and IAc)*, cry1Ad* (I(-) and IAd)*, cry1B* (I(-) and IB)*, cry1C* (I(-) and IC)*, cry1D* (I(-) and ID)*, cry1E* (I(-) and IE)*, cry1F* (I(-) and IF) and *cry1G* (I(-) and IG) in native *Bt* isolates. The PCR reaction mixture of 50 µl volume contained DNA template 50 ng (5 µl), PCR buffer (10X) 5 µl, dNTPs (2mM) 5 µl, $MgCl_2$ (25mM) 5 µl, primers 0.1 µM each (1 µl each), Taq DNA polymerase 1.0 U (1 µl) and sterile distilled water. The DNA was amplified in a thermal cycler (Gene Amp) for 30 cycles with denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min and primer extension at 72°C for 2 min. PCR products were analyzed on 1.5 % agarose gel electrophoresis.

2.4. Restriction analysis

PCR amplification of *cry1A* genes was carried out by using primer pair (K5Un2 and K3Un2) designed as per Kuo and Chak, (1996). The reaction mixture contained PCR buffer (10X) 10 µL, $MgCl_2$ (25 mM) 10 µL, dNTPs (2 mM) 10 µL, forward and

reverse primers 0.1 µM each (1 µl each), Taq DNA Polymerase 2 µl (1 U/µL), template DNA 50 ng (5 µl) and sterile distilled water to make the total volume 100 µl. The reaction was carried out in a thermal cycler (Gene Amp) for 35 cycles with denaturation at 94°C for 1 min, primer annealing at 42°C for 1 min and primer extension and polymerization at 72°C for 2 min.

Denaturation was carried out for 2 min and extension at 72°C was carried out for 10 min in the first and the last cycle respectively. The PCR products were run on 1% low melting agarose gel and DNA fragments were extracted and purified from the gel by Qiagen MinElute Gel extraction kit as per the supplier protocol. The DNA fragment eluted was digested with restriction enzymes in two sets: (i) with Pst1 and (ii) combination of Xba1 and Pst1. The restriction pattern was analyzed on 2% agarose gel electrophoresis.

3. Results

Eighteen native *Bt* isolates SK-3,4, 9, 13, 20, 28, 48, 51, 63, 82, 88, 94, 105, 110, 301, 302, 304 and 307 were selected for PCR analysis for the presence of different *cry*1-type genes. Four isolates SK-1, 5, 84 and 305 were omitted as they did not yield the expected PCR product of 277 bp in screening for *cry1* gene family using primers as per BenDov et al., (1997), as has been reported previously (Shankar et al., 2010).

3.1. Presence of cry1-type genes with primer sets

The presence of *cry1* type genes in native *Bt* isolates using primer sets designed as per Ceron et al., (1994) is depicted in Table 1. Amplification of the expected 246 bp band, corresponding to *cry1Aa* and *1Ad* was observed in all native *Bt* isolates, except SK-3 and 110. Additional bands of *ca.* 350 bp and *ca*. 800 bp were seen in SK-20, 28, 105, 302 and 307, whereas only one additional band of *ca.* 350 bp was observed in SK-48 and 63. The expected band of 246 bp was observed in *Bt* subsp. *kurstaki* (HD1) and *Bt* subsp. *finitimus* (4B2) and the *E. coli* strain ECE-52 harboring a cloned *cry1Aa* gene.

The expected band of 171 bp, corresponding to *cry1Ad* gene was observed in all isolates except SK-82, 88, 110, 302 and 307. Variant band of *ca.* 350 bp was found in SK-82, 88, 105, 110, 302 and 307. Another variant band of *ca.* 800 bp was seen in SK-20, 63, and 307. *Bt* subsp. *aizawai* (4J4) used as a positive reference showed the presence of 171 bp band, whereas *E. coli* strain ECE-52 containing *cry1Aa* gene, used as a negative control, did not amplify this band confirming the specificity of the reaction. The expected band of 216 bp, corresponding to *cry1Ab* and *1Ac* genes was observed in all isolates except SK-3, 9 and 51. This band was also present in *Bt* subsp. *finitimus* (4B2), *aizawai* (4J3) and *thuringiensis* (4A6) used as positive controls.

Using another set of primer corresponding to *cry1Ac* gene, the expected band of 180 bp was seen only in six isolates, namely SK-105, 110, 301, 302, 304 and 307. An additional band of *ca.* 300 bp was also observed in SK-304. The positive reference *Bt* subsp. *kurstaki* (HD1) and *kenyae* (4F3) also yielded the amplification of the 180 bp band, as expected.

Nine out 18 native isolates showed the presence of *cry1B* gene as indicated by the expected band of 367 bp.

Table 1. Distribution of *cry1*-type genes in native *Bt* isolates using primers designed as per Ceron et al. (1994)

Isolates	***cry1Aa, cry1Ad***	***cry1Ad***	***cry1Ab, cry1Ac***	***cry1Ac***	***cry1B***	***cry1C***	***cry1D***
SK-3	-	+	-	-	-	+ #^{900}	-
SK-4	+	+	+	-	+	-	+
SK-9	+	+	-	-	-	+ #^{900}	+
SK-13	+	+	+	-	+	+	+
SK-20	+ #^{350,800}	+ #^{800}	+	-	-	+	+
SK-28	+ #^{350,800}	+	+	-	-	+	+#^{900}
SK-48	+ #^{350}	+	+	-	-	+	+
SK-51	+	+	-	-	-	+	+
SK-63	+#^{350}	+ #^{800}	+	-	+	-	+#^{900}
SK-82	+	- #^{350}	+	-	-	+	+
SK-88	+	- #^{350}	+	-	-	+	+
SK-94	+	+	+	-	+	+ #^{700}	+
SK-105	+ #^{350,800}	+ #^{350}	+	+	+	+#^{900}	+#^{900}
SK-110	-	- #^{350}	+	+	+	+	+
SK-301	+	+	+	+	+	+ #^{700}	+
SK-302	+ #^{350,800}	- #^{350}	+	+	-	+	+#^{900}
SK-304	+	+	+	+ #^{300}	+	+ #^{900,700}	+
SK-307	+#^{350,800}	- #$^{350,\ 800}$	+	+	+	+	+#^{900}
Ref. strains	HD1+	4J4+	4B2+	HD1+	4A6+	4G6+	4J2+
	4B2+	ECE52-	4J3+	4F3+	4T1-	4J3+	4J3+
	ECE52+		4A6+			4T1+#^{700,900}	4J4+

(+) Represents the presence of expected band; (-) represents the absence of expected band; (#) represents the presence of variant band and numbers represent the size (bp) of variant bands

This band was observed in SK-4, 13, 63, 94, 105, 110, 301, 304, and 307 and the *Bt* positive control strain *Bt* subsp. *thuringiensis* (4A6) but was absent in *Bt wuhanensis* (4T1).

The *cry1C* gene was found to be present in all the isolates except SK-4 and 63 as indicated by the expected PCR product of 130 bp. A variant band of *ca.* 900 bp was seen to be present in SK-3, 9, 105, and 304. Another variant band of *ca.* 700 bp was seen in SK-94, 301, and 304.

These two variant bands are also seen in *Bt* subsp. *wuhanensis* (4T1) in addition to the expected band of 130 bp. However, *Bt* subsp. *galleriae* (4G6) and *aizawai* (4J3) showed the presence of only 130 bp band. The expected band of 290 bp corresponding to the presence of *cry1D* gene was seen in all isolates with varying intensity except SK-3. *Bt* subsp. *aizawai* (4J2, 4J3, 4J4) also showed amplification of that band. A variant band of *ca.* 900 bp was seen in SK-28, 63, 105, 302 and 307.

3.2. Presence of cry 1 genes with primer sets designed as per Kalman et al., (1993)

The presence of *cry1* type genes using PCR primers as per Kalman et al., (1993) is depicted in Table 2. All eighteen *Bt* isolates were taken for the PCR screening with this set of primers. Amplification of the expected band of 724 bp corresponding to *cry1Aa* was seen in only five *Bt* isolates SK-3, 4, 9, 302 and 304. The variant band of *ca.* 500 bp was seen in all the isolates except SK-20, 28, 63, 82, 105, 301, 304 and 307. Another variant band of *ca.* 250 bp was seen in SK-3, 9, 13, 51, 82, 88, 110, and 302. Neither the expected nor any variant bands were seen in *Bt* subsp. *kurstaki* (HD1) and *Bt* subsp. *finitimus* (4B2) used as reference strains.

Amplification of expected band of 274 bp, corresponding to *cry1Ab* was seen in only SK-63, SK-105, and SK-302. A variant band of *ca.* 2 kb was seen in SK-20, 28, SK-105 and SK-302. Another variant band of 1500 bp was observed in SK-28, 105, and 302. No amplification was observed in the positive reference *Bt* subsp. *thuringiensis* (4A6). For *cry1Ac,* the expected band of 619 bp was observed only in SK-28 and 63, but not observed in reference strains. A variant band of *ca.* 500 bp was observed in SK-13, 28, *Bt* subsp. *kurstaki* (HD1) and *E. coli* strain ECE-53. Another variant band of ca. 250 bp was observed in all the native *Bt* isolates except SK-63, 88, 105, 301, 302, 304 and 307. *Bt* subsp. *kenyae* (4F3) did not show any amplification.

For *cry1B,* expected band of 1268 bp was not observed in any of the native *Bt* isolates as well as positive reference strains *Bt* subsp. *wuhanensis* (4T1). A variant band of *ca.* 900 bp was observed in SK-4, 13 and 94. Another variant band of *ca.* 250 bp was observed in SK-88 and *Bt* subsp. *wuhanensis* (4T1). For *cry1C*, neither native *Bt* isolates nor reference *Bt* strains showed the expected band of 740 bp. A variant band of *ca.* 250 bp was seen in SK-3, 4, 48, 63, and *Bt* subsp. *aizawai* (4J3). Another variant band of *ca.* 500 bp was seen in SK-63 and 82. No amplification was observed in *Bt* subsp. *wuhanensis* (4T1) used as a positive reference.

Amplification of expected band of 721 bp, corresponding to *cry1D* was observed in only 2 native *Bt* isolates SK-9 and SK-13.

Table 2. Distribution of *cry1*-type genes in native *Bt* isolates using primers designed as per Kalman et al. (1993)

Isolates	*cry1Aa*	*cry1Ab*	*cry1Ac*	*cry1B*	*cry1C*	*cry1D*	*cry1E*
SK-3	+ #^{250,500}	-	- #^{250}	-	- #^{250}	- #^{450}	- #^{900}
SK-4	+ #^{500}	-	- #^{250}	- #^{900}	- #^{250}	- #^{450}	- #^{900}
SK-9	+ #^{250,500}	-	- #^{250}	-	-	+	-
SK-13	- #^{250,500}	-	- #^{250,500}	- #^{900}	-	+ #^{200}	-
SK-20	-	- #^{2000}	- #^{250}	-	-	-	-
SK-28	-	- #^{1500,2000}	+ #^{250,500}	-	-	-	-
SK-48	- #^{500}	-	- #^{250}	-	- #^{250}	- #^{450}	-
SK-51	- #^{250,500}	-	- #^{250}	-	-	-	-
SK-63	-	+	+	-	-#^{250,500}	-	-
SK-82	- #^{250}	-	- #^{250}	-	- #^{500}	- #^{200}	-
SK-88	- #^{250,500}	-	-	- #^{250}	-	-	- #^{300,900}
SK-94	- #^{500}	-	- #^{250}	- #^{900}	-	-	-
SK-105	-	+#^{1500,2000}	-	-	-	-	-
SK-110	- #^{250,500}	-	- #^{250}	-	-	-	-
SK-301	-	-	-	-	-	-	-
SK-302	+#^{250,500,900}	+#^{1500,2000}	-	-	-	-	-
SK-304	+	-	-	- #	-	-	-
SK-307	-	-	-	-	-	- #^{450}	- #^{300}
Ref. Strain	HD1-	4A6-	HD1-#^{500}	4T1-#^{250}	4J3-#^{250}	4J3-#^{450}	4L3-
	4B2-		4F3-		4T1-	4J4-#^{450}	4F3+#^{950}
			ECE53 -#^{500}				ECE127+#^{950}

(+) Represents the presence of expected band; (-) represents the absence of expected band; (#) represents the presence of variant band and numbers represent the size (bp) of variant bands

Whereas, in SK-3, SK-4, SK-48, SK-307 and *Bt* subsp. *aizawai* (4J3, 4J4) a variant band of *ca.* 450 bp was seen. Another variant band of *ca.* 200 bp was seen in SK-13 and 82. Amplification of expected band of 1297 bp corresponding to *cry1E* was absent in all the native *Bt* isolates. The expected band as well as a variant band of *ca.* 950 bp was observed in *Bt* subsp. *kenyae* (4F3) and *E. coli* strain ECE-127. *Bt* subsp. *tolworthi* (4L3) did not show any amplification. A variant band of *ca.* 900 bp was found in SK-3, 4 and 88, whereas, another variant band of *ca.* 300 bp was found in SK-88 and SK-307.

3.3. Presence of cry1-type genes with primer set designed as per Juarez-Perez et al., (1997)

Seven native *Bt* isolates, namely SK-13, 20, 28, 48, 63, 88 and 105 were selected for PCR amplification of *cry1*-type genes using primers designed as per Juarez-Perez et al. (1997). The results are presented in Table 3. Amplification of the expected 1.6 kb *cry1* gene family band was observed in 4 isolates, namely SK-20, 28, 48 and 105. This band was of strong intensity in *Bt* subsp. *kurstaki* (HD1). *Bt* subsp. *aizawai* (4J4) did not show amplification of this band, although is known to contain genes of *cry1* family. Instead, a variant band of *ca.* 300 bp was observed in this strain. The 300 bp band was also observed in *Bt* subsp. *kurstaki* (HD1). Two bands of 1.6 kb and *ca.* 1.4 kb with this set of primers were observed in *Bt* subsp. *morrisoni* (4K1), whereas *E. coli* strains ECE-52 containing *cry1Aa* gene did not show any amplification. This suggested that the set of degenerate primers used in this screening may not always recognize the *cry1* gene present in a particular strain.

Amplification of the expected 1.2 kb band corresponding to *cry1A* was not observed in any native as well as reference *Bt* isolates, while a variant band of 1.7 kb was observed in SK-13, 48 and reference strains *Bt* subsp *kurstaki* (HD1), 4K1 and 4J4. Amplification of the expected 1.3 kb band using primers specific for *cry1Ab* was observed only in SK-28, HD1, and *E. coli* strain ECE-54. The presence of *cry1Ac* gene was indicated in SK-13 and 48 which showed an additional band of *ca*. 300 bp apart from the expected 844 bp band. In SK-20 and SK-28, the amplicon observed was *ca.* 900 bp. *Bt* subsp. *kurstaki* (HD1) and *E. coli* strain ECE-53 containing *cry1Ac* gene used as positive reference yield the expected PCR product of 844 bp. Out of seven native *Bt* isolates only SK-63 showed the PCR amplification of the expected 1.3 kb band corresponding to *cry1B*. The expected band was observed in *Bt* subsp. *thuringiensis* (4A6) and *E. coli* strain ECE-128 used as positive references for *cry1B*.

Amplification of expected 1.1 kb band using primers specific for *cry1C* was observed in all the native *Bt* strains SK-13, 20, 28, 48, 63, 88 and 105. However, *Bt* subsp. *galleriae* and *E. coli* strain ECE-125 did not show any amplification. In SK-13, 20, 48 and 63, an additional band of *ca.* 450 bp was present. In *Bt* strain SK-20, 28, 48, 63, 88 and 105, a variant band *ca*. 700 bp of high intensity was observed. In all native *Bt* isolates except SK-13, a variant band of size *ca.* 1.8 kb was present. The expected PCR product of 1.1 kb corresponding to *cry1D* was observed in all native *Bt* isolates as well as positive references *Bt* subsp. *aizawai* (4J4) and *E. coli* strain ECE-129.

All native Bt isolated showed the expected PCR product of size 1.1 kb corresponding to *cry1E*, whereas, *Bt* subsp. *kenyae* (4F3) which was used as a positive reference did no show expected band. The expected band was present in *E. coli* strain ECE-127. In the reference strain *Bt* subsp. *kenyae,* two variant bands- *ca.* 1.0 kb and 250 bp were observed, whereas a variant band of *ca.* 250 bp was observed in two native *Bt* isolates SK-13 and 48 also.

Table 3. Distribution of *cry*1-type genes in native *Bt* isolates using primers designed as per Juarez-Perez et al., (1997)

Isolates	***cry1***	***cry1A***	***cry1Ab***	***cry1Ac***	***cry1B***	***cry1C***	***cry1D***	***cry1E***	***cry1F***	***cry1G***
SK-13	-	- #1700	-	+#300	-	+ #450	+	+ #250	-	-
SK-20	+	-	-	- #900	-	+#450,700, 1800	+	+	-	-
SK-28	+	-	+	- #900	-	+ #700, 1800	+	+	-	-
SK-48	+	- #1700	-	+#300	-	+#450,700, 1800	+	+ #250	-	-
SK63	-	-	-	-	+	+#450,500,700, 1800	+	+	-	-
SK-88	-	-	-	-	-	+#500,700, 1800	+	+	-	-
SK-105	+	-	-	-	-	+#300,500,700, 1800	+	+	-	-
Ref. strains	HD1+#300	HD1- #1700	HD1+	HD1+	4A6+	4G6-	4J4+	4F3- #1000,250	4J4+	4G6+
	4J4- #300	4J4- #1700	ECE54+	ECE53+	ECE 128+	ECE125-	ECE 129+	ECE 127+		
	4K1+#1600,1400	4K1 #1700								
	ECE52-	ECE 52-								

(+) Represents the presence of expected band; (-) represents the absence of expected band; (#) represents the presence of variant band and numbers represent the size (bp) of variant bands

Among the seven *Bt* isolates, none showed any amplification for *cry1F* and *cry1G* by the respective sets of primer. The *Bt* subsp. *aizawai* (4J4) and *Bt* subsp. *galleriae* (4G6) showed amplification of an expected band of 900 bp and 1.1 kb for *cry1F* and *cry1G*, respectively.

3.4. Restriction fragment length polymorphism (RFLP) of PCR amplified cry1-type gene in native Bt isolate

For RFLP analysis, native *Bt* isolates and references strains were taken for PCR amplification of *cry1*-type genes with a primer pair designed as per Kuo and Chak (1996). Fig. 1 shows the amplification of expected band of 1.6 kb corresponding to *cry1* type gene in SK-20 (lane 7), 28 (lane 8), 48 (lane 9), 63 (lane 11), 88 (lane 14), 105 (lane 16), 307 (lane 22) and all reference strains except *Bt* subsp. *finitimus* (4B1-lane 24 and 4B2-lane 25), *Bt* subsp. *morrisoni* (4K1-lane 31) and *Bt* subsp. *israelensis* (4Q5-lane 33). In this, 4Q5 is a negative control for *cry1* type gene. A variant band of *ca.* 300 bp was observed in SK-20 (lane 7), 28 (lane 8), 48 (lane 9), 63 (lane 11), 88 (lane 14), 94 (lane 15), 105 (lane 16), 301 (lane 18), 302 (lane 19), 304 (lane 20), 307 (lane 22), 4B1 (lane 24), 4B2 (lane 25) 4S2 (lane 34), and 4W1 (lane 36). Another variant band of *ca.* 500 bp and *ca.* 800 bp were observed in SK-110 (lane 17) and *Bt* subsp. *morrisoni* (4K1-lane 31). However, reference *Bt* strains, 4K1 (lane 31), 4L3 (lane 32), 4Q5 (lane 33), 4S2 (lane 34), 4W1 (lane 36), and 4A6 (lane 37) showed multiple variant bands. The expected 1.6 Kb PCR product observed in native *Bt* isolates and *Bt* reference strains was eluted from the gel. The variant bands of *ca.* 800 and *ca.* 500 bp in SK-110 was also eluted from the gel. All the eluted PCR products were taken for further analysis.

3.5. Restriction analysis of gel eluted PCR product

In this set of restriction by a single enzyme, *Pst1*, no restriction was seen in 1.6 kb band of SK-20 (lane 5), 28 (lane 6), 48 (lane 7), 63 (lane 8), 88 (lane 9), and 307 (lane 10) (Fig. 2a). In this set of double restriction by *Pst1* and *Xba1*, no restriction was observed in 1.6 kb PCR product of native *Bt* isolates SK-20 (lane 5), 28 (lane 6), 48 (lane 7), 63 (lane 8), 88 (lane 9), and 307 (lane 10) (Fig. 2b). The 800 bp PCR product of SK-110 (lane 11) showed two bands of 800 bp and *ca.* 700 bp upon restriction. No restriction was seen in 500 bp PCR product amplified from SK-110 (lane 12). Among reference, *Bt* strains three bands in *Bt* subsp. *kurstaki* (HD1-lane 13) of 1.6 kb, *ca.* 1.4 kb and *ca.* 500 bp, in *Bt* subsp. *kenyae* (4F3-lane 14) four bands of 1.6 kb, *ca.* 1.4 kb, 900 bp and 500 bp, in *Bt* subsp. *galleriae* (4G6-lane 15) four bands of 1.6 kb, *ca.* 700 bp, 500 bp and *ca.* 350 bp, in *Bt* subsp. *aizawai* (4J2-lane 16) two bands of 1.6 kb and 500 bp, in *Bt* subsp. *israelensis* (4L3-lane 17) five bands *ca.* 1.2 kb, *ca.* 800 bp, *ca.* 700 bp, 500 and *ca.* 200 bp, in *Bt* subsp. *thuringiensis* (4A6-lane 18) five bands of 1.6 kb, *ca.* 1.3 kb, *ca.* 1.2 kb, *ca.* 700 bp and 500 bp were seen. In *E. coli* strain ECE-52 (lane 19) containing *cry1Aa* gene, three bands of size 1.6 kb, *ca.* 1.3 kb and 500 bp and in ECE-53 (lane 20) containing *cry1Ac* gene, three bands of 1.6 kb, *ca.*1.4 kb and *ca.* 300 bp were seen.

Four sets of primers have mapped the possibility of the presence of *cry1*-type genes in native *Bt* isolates (Fig. 3).

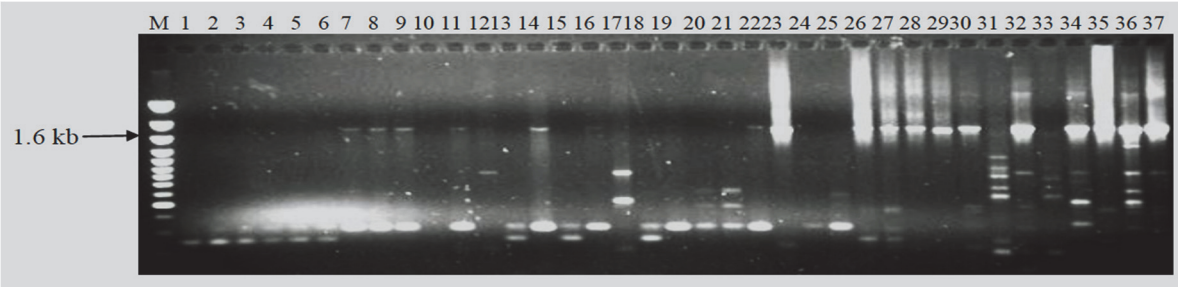
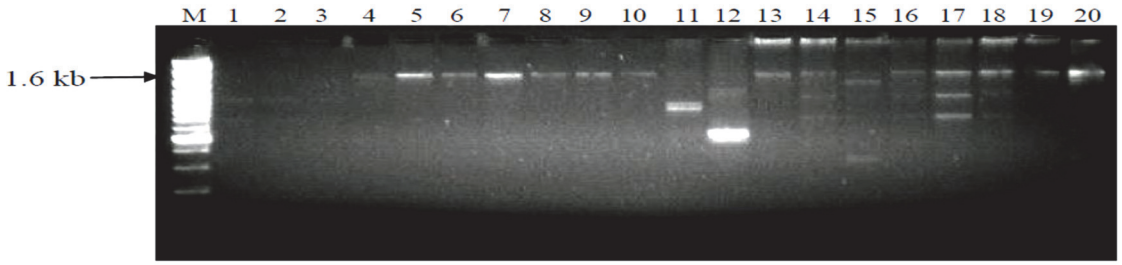
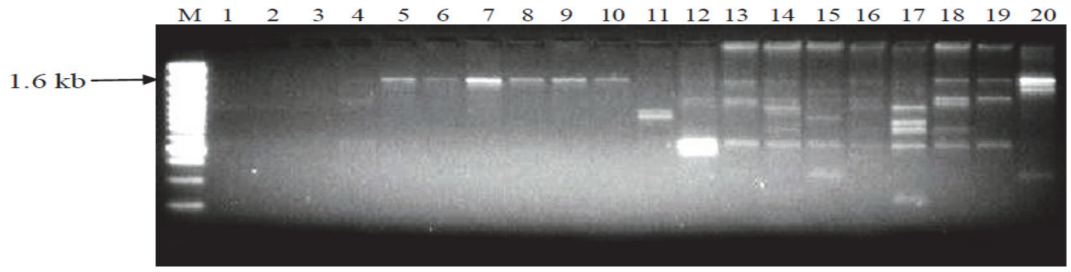


Fig. 1. PCR amplification of *cry1* gene in native *Bt* isolates and reference strains. Lane M-molecular marker (100 bp ladder). Lanes number for *Bt* isolates are in bracket: SK- 3 (lane 2), 4 (lane 3), 9 (lane 5), 13 (lane 6), 20 (lane 7), 28 (lane 8), 48 (lane 9), 51 (lane 10), 63 (lane 11), 82 (lane 12), 88 (lane 14), 94 (lane 15), 105 (lane 16), 110 (lane 17), 301 (lane 18), 302 (lane 19), 304 (lane 20), and 307 (lane 22), HD1 (lane 23), 4B1 (lane 24), 4B2 (lane 25), 4F3 (lane 26), 4G6 (lane 27), 4J2 (lane 28), 4J3 (lane 29), 4J4 (lane 30), 4K1 (lane 31), 4L3 (lane 32), 4Q5 (lane 33), 4S2 (lane 34), 4T1 (lane 35), 4W1 (lane 36)and 4A6 (lane 37)



(a)



(b)

Fig. 2. (a) Restriction pattern of gel eluted PCR products after restriction digestion by *Pst1.* Lane M –molecular marker (100 bp ladder). Lanes 5-12 are native *Bt* isolates SK-20 (lane 5), 28 (lane 6), 48 (lane 7), 63 (lane 8), 88 (lane 9), 307 (lane 10), 110 (800 bp) (lane 11) and 110 (500 bp) (lane 12), respectively. Lanes 13-20 are reference strains HD1 (lane 13), 4F3 (lane 14), 4G6 (lane 15), 4J2 (lane 16), 4L3 (lane 17), 4A6 (lane 18), ECE-52 (lane 19), and ECE-53 (lane 20), respectively; (b) Restriction pattern of gel eluted PCR products after double restriction digestion by *Pst1* and *Xba1.* Lane M- molecular marker (100 bp ladder). Lanes 5-12 are native *Bt* isolates SK-20 (lane 5), 28 (lane 6), 48 (lane 7), 63 (lane 8), 88 (lane 9), 307 (lane 10), 110 (800 bp-lane 11) and 110 (500 bp-lane 12), respectively. Lanes 13-20 are reference strains HD1 (lane 13), 4F3 (lane 14), 4G6 (lane 15), 4J2 (lane 16), 4L3 (lane 17), 4A6 (lane 18), ECE-52 (lane 19), and ECE-53 (lane 20), respectively

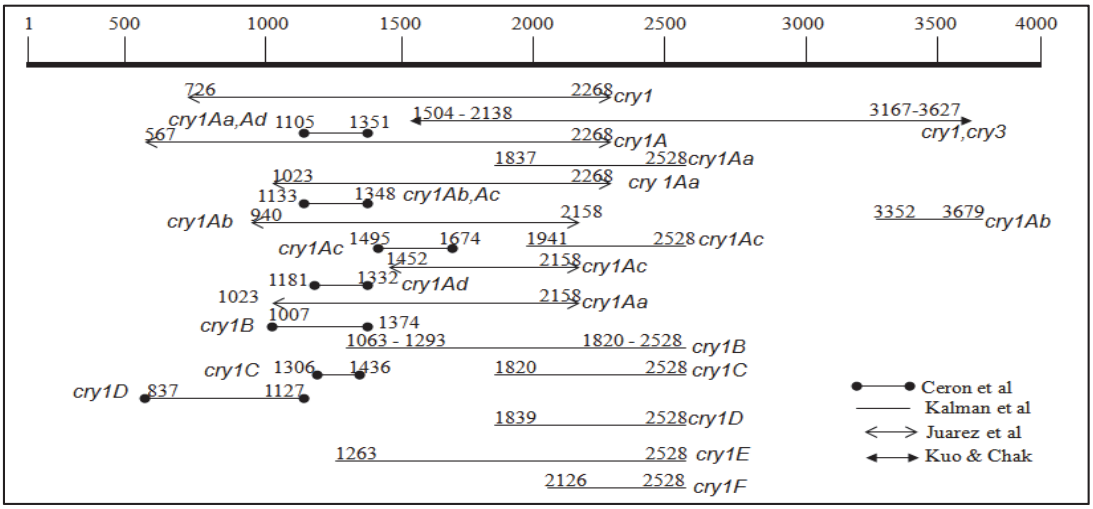


Fig. 3. Position of hybridization of different sets of primers used for detection of *cry1*-type genes

Several native isolates showed the presence of various combinations of *cry1*-type genes (Table 4). Isolates, SK-13, 63 and 105 showed the maximum number of *cry*1-type genes, followed by SK-20, 28, 48, 88, 94, 301, 304 and 307 whereas SK-3 showed the presence of only 3 *cry1*-type genes. The groups of isolates, SK-13, 63, and 105; SK-9 and 51; SK-20, 28, 48, and 88; SK- 94, 301, 304, and 307 showed a similar profile.

4. Discussions

Bt is ubiquitous in soil, dead larvae, leaves or dust from stored grains. India covers a vast area with many complex ecological environments. As a soil-harboring bacterium, *B. thuringiensis* is widely distributed throughout the country. In our previous study, we have used two sets of published primers (Ben-Dov et al., 1997; Carozzi et al., 1999) corresponding to the highly conserved region of *cry* gene families to identify the presence of *cry* genes in 22 native *Bt* isolates (Shankar et al., 2010). Out of 22 isolates, 18 native Bt isolates showed the presence of cry1 genes (Shankar et al., 2010). In this study, we have chosen 18 native *Bt* isolates which had tested positive for the presence of *cry1* type specific genes for further characterization for the presence of *cry1*-type genes using PCR amplification. Four sets of published primers (Ceron et al., 1994; Juarez-Perez et al., 1997; Kalman et al., 1993; Kuo and Chak 1996) were used, in order to attempt to cover the different regions of the *cry1* specific genes that these primers correspond to (Fig. 3). With primers designed as per Ceron et al., (1994), *cry1Ad* and *cry1D* were found to be the most abundant gene, being present in 17 native *Bt* isolates followed by *cry1C* and *cry1Ac* genes which were detected in 16 and 15 native *Bt* isolates, respectively. \Out of 18 isolates, and *cry1B* was present in only 7 isolates. Within the *cry1A* group, *cry1Aa* and *cry1Ad* genes were more frequent as compared to *cry1Ab* and *cry1Ac* genes in native *Bt* isolates. Using the strategy of Juarez-Perez et al. (1997) wherein a common reverse primer and specific forward primers are used for identification of genes of *cry1* family, although four out of seven isolates were positive for the presence of *cry1* gene, the presence of *cry1A* and *cry1Ab* genes was not detected in any isolates.

Table 4. The *cry* gene profile of native *Bt* isolates detected by 4 sets of primers

Isolates	*Ceron et al., (1994)*	*Kalman et al., (1993)*	*Juarez-Perez et al., (1997)*	*Kuo and Chak, (1996)*
SK-3	*cry1Ad, cry1C*	cry1Aa,	NA	ND
SK-4	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1D	cry1Aa,	NA	ND
SK-9	cry1Aa, cry1Ad, cry1C, cry1D	cry1Aa, cry1D	NA	ND
SK-13	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D,	cry1D	cry1Ac, cry1C, cry1D, cry1E	ND
SK-20	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	ND	cry1, cry1C, cry1D, cry1E	cry1,
SK-28	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	cry1Ac,	cry1, cry1Ab, cry1C, cry1D, cry1E	cry1,
SK-48	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	ND	cry1, cry1Ac, cry1C, cry1D, cry1E	cry1,
SK-51	cry1Aa, cry1Ad, cry1C, cry1D	ND	NA	ND
SK-63	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1D	cry1Ab, cry1Ac	cry1B, cry1C, cry1D, cry1E	cry1,
SK-82	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	ND	NA	ND
SK-88	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	ND	cry1C, cry1D, cry1E	cry1,
SK-94	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D	ND	NA	ND
SK-105	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D	cry1Ab,	cry1, cry1C, cry1D, cry1E	cry1,
SK-110	cry1Ab, cry1Ac, cry1B, cry1C, cry1D	ND	NA	ND
SK-301	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D	ND	NA	ND
SK-302	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1C, cry1D	cry1Aa, cry1Ab,	NA	ND
SK-304	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D	cry1Aa,	NA	ND
SK-307	cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D	ND	NA	cry1,

NA: These isolates were not taken for the detection of cry gene; ND: The isolated did not show the expected band for any gene with the primer set; Ceron et al. (1994): cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D; Kalman et al. (1993): cry1Aa, cry1Ab, cry1Ac, cry1B, cry1C, cry1D, cry1E; Juarez-Perez et al. (1997): cry1, cry1A, cry1Ab, cry1Ac, cry1B, cry1C, cry1D, cry1E, cry1F, cry1G; Kuo and Chak (1996): cry1

Similarly, *cry1Ac* was detected in two and *cry1B* in only one isolate. The *cry1C* and *cry1D* genes were found to be present in all the isolates. Wang et al., (2003) have also found the prevalence of *cry1A*, *cry1C* and *cry1D* genes over other types of *cry* genes in their *Bt* isolates from China. The *cry1E* gene was also found to be present in all the isolates, while *cry1F* and *cry1G* were not found in any isolate.

Interestingly, using the set of primers designed as per Kalman et al., (1993) used for screening for the presence of genes of the *cry1* family in native *Bt* isolates, *cry1C* and *cry1E* were detected in none of the isolates. This result is in contradiction with that obtained with primers based on Juarez-Perez et al., (1997) and Ceron et al., (1994). The primers of Juarez-Perez et al., (1997) explore a region between 1160 bp to 2268 bp and 1155 bp to 2268 bp for *cry1C* and *cry1E*, respectively. Whereas, the primers of Kalman et al., (1993) hybridize to a region between 1820 bp to 2528 bp and 1263 bp to 2528 bp for cry1C and cry1E, respectively (Fig. 3). When comparing the primer regions according to Juarez-Perez et al., (1997) and Kalman et al., (1993), the regions between 1820 – 2268 and 1263-2268 overlaps for *cry1C* and *cry1E*, respectively. This discrepancy in results could be solved by using additional sets of primers such as the use of forward primer of Juarez-Perez et al., (1997) and reverse primer of Kalman et al., (1993) and vice versa. The primers based on Kalman et al., (1993) have also not detected the presence of other *cry1* type genes as per the expected bands, except for *cry1Aa* in four isolates and *cry1Ab* in three isolates. However, a large number of variant sized bands have been observed with these primers. The reverse primer is common for all genes except *cry1Ab* in this set and a lower homology in this region could lead to the observed non-amplification of the genes tested. Furthermore, Kalman et al., (1993) have used genomic DNA for PCR amplification with their primers, whereas plasmid DNA has been used as a template for PCR amplification in this study since *cry* genes are generally plasmid-borne (Gonzalez et al., 1984).

RFLP patterns of the 1.6 kb PCR product amplified by primers corresponding to *cry1* gene family (primer pair K5Un2 and K3Un2) were compared to detect any unexpected and novel restriction profiles as presented by Kuo and Chak (1996). The expected PCR product of 1.6 kb was

observed in six out of eighteen isolates. In SK-110, 2 variant bands of *ca.* 500 bp and *ca.* 800 bp were seen, but the expected 1.6 kb band was absent. The restriction pattern of these six isolates differed from that of the reference *Bt* strains. In all six isolates, no restriction site was observed as compared with the restriction pattern of reference *Bt* strains with *Pst1* alone and the combination of *Pst1* and *Xba1* enzymes in a double digestion reaction. Since all samples were processed simultaneously, inhibition of restriction due to experimental artifacts seems unlikely. However, this experiment needs to be further carried on with other restriction enzymes to determine if *Pst1* and *Xba1* sites, as expected in the known *cry1* genes do not occur in the 1.6 kb PCR product amplified in the native *Bt* isolates. Further analysis will indicate if the observed 1.6 kb could be a variant *cry1* gene sequence. This may be a very useful preliminary study of strain characterization for *cry* gene content in their genome before isolation of specific full-length genes.

5. Conclusions

A collection of 18 native *Bt* isolates were characterized to reveal their insecticidal *cry* gene content. The amplification of a particular *cry* gene was not the same for all sets of primers because of different sites for the binding of primers. Thus, four sets of primers were used to map the presence of *cry1*-type genes in native *Bt* isolates.

Several native isolates showed the presence of various combinations of *cry1*-type genes. SK-13, 63 and 105 showed the maximum number of *cry1*-type genes followed by SK-20, 28, 48, 88, 94, 301, 304 and 307, whereas SK-3 showed the presence of only 3 *cry1* type genes.

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References

Aronson A., (2002), Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis*, *Cellular and Molecular Life Sciences*, **59**, 417–425.

Baig D.N., Mehnaz S., (2010), Determination and distribution of cry-type genes in halophilc *Bacillus thuringiensis* isolates of Arabian Sea sedimentary rocks, *Microbiological Research*, **165**, 376–383.

Ben-Dov E., Zaritsky A., Dahan E., Barak Z., Sinai R., Manasherob R., Khamraev A., Troitskaya E., Dubitsky A., Berezina N., Margalith Y., (1997), Extended screening by PCR for seven *cry*-group genes from field collected strains of *Bacillus thuringiensis*, *Applied and Environmental Microbiology*, **63**, 4883-4890.

Carozzi N.B., Kramer V.C., Warren G.W., Evola S., Koziel M.G., (1999), Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles, *Applied and Environmental Microbiology*, **57**, 3057-3061.

Cawoy H., Bettiol W., Fickers P., Ongena M., (2011), Bacillus-based biological control of plant diseases, In: Pesticides in the modern world-Pesticides use and management, Stoytcheva, M. (Ed.), ISBN: 978-953-307-459-7, 273-302.

Ceron J., Covarrubias L., Quintero R., Ortiz A., Ortiz M., Aranda E., Lina L., Bravo A**.** (1994), PCR analysis of the *cry1* insecticidal crystal family genes from *Bacillus thuringiensis*, *Applied and Environmental Microbiology*, **60**, 353-356.

Frankenhuyzen K.V., (2009), Insecticidal activity of *Bacillus thuringiensis* crystal proteins, *Journal of Invertebrate Pathology*, **101**, 1–16.

Gonzalez J.M., Dulmage H. T., Carlton B. C., (1984), Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*, *Plasmid*, **5**, 351-365.

Ibarra J.E., Del Rincón M. C., Ordúz S., Noriega D., Benintende G., Monnerat R., Regis L., de Oliveira C. M. F., Lanz H., Rodriguez M. H., Sanchez J., Peria G., Bravo A., (2003), Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species, *Applied and Environmental Microbiology*, **69**, 5269–5274.

Ibrahim M.A., Griko N., Junker M., Bulla L.A., (2010), *Bacillus thuringiensis*: A genomics and proteomics perspective, *Bioengineered Bugs*, **1**, 31–50.

Juarez-Perez V.M., Ferrandis M.D., Frutos R., (1997), PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes, *Applied and Environmental Microbiology*, **63**, 2997–3002.

Kalman S., Kiehne K.L., Libs J.L., Yamamoto T., (1993), Cloning of a Novel *cryIC*-Type Gene from a Strain of *Bacillus thuringiensis* subsp. Galleriae, *Applied and Environmental Microbiology*, **59**, 1131-1137.

Konecka E., Kaznowski A., Ziemnicka J., (2006), Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L., *Journal of Invertebrate Pathology*, **94**, 56–63.

Kuo W.S., Chak K.F., (1996), Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA, *Applied and Environmental Microbiology*, **62**, 1369-1377.

Meena R.K., Kaur S., (2014), Screening of *Bacillus thuringensis* isolates recovered from diverse habitats in India for the presence of insect and nematode-active *cry* genes, *International Journal of Agriculture, Environment and Biotechnology*, **7**, 55-62.

Nazarian A., Jahangiri R., Jouzani G.S., Seifinejad A., Soheiliv S., Bagheri O., Keshavarzi M., Alamisaeid K. (2009), Coleopteran-specific and putative novel *cry* genes in Iranian native *Bacillus thuringiensis* collection, *Journal of Invertebrate Pathology*, **102**, 101–109.

Ramalakshmi A., Udayasuriyan V., (2010), Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamilnadu state, India, *Current Microbiology*, **61**, 13-18.

Sanahuja G., Banakar R., Twyman R.M., Capell T., Christou P., (2011), *Bacillus thuringiensis*: a century of research, development and commercial applications, *Plant Biotechnology Journal*, **9**, 283-300.

Schnepf E., Crickmore N., Van Rie J., Lereclus D., (1998), *Bacillus thuringiensis* and its pesticidal crystal proteins, *Microbiology and Molecular Biology Reviews*, **62**, 775-806.

Shankar S., Jaiswal L., Kaur S., (2010), Diversity of *Bacillus thuringiensis* isolated from diverse habitats of North India for the presence of *cry* gene profile, *Journal of Applied Bioscience*, **36**, 124-129.

Tan F., Zhu J., Tang J., Wang S., Zheng A., Li P., (2009), Cloning and characterization of two novel crystal protein genes, *cry54Aa1* and *cry30Fa1*, from *Bacillus thuringiensis* strain BtMC28, *Current Microbiology*, **58**, 654-659.

Wang J., Boets A., Rie J.V., Ren G., (2003), Characterization of *cry*1, *cry*2 and *cry*9 genes in *Bacillus thuringiensis* isolates from China, *Journal of Invertebrate Pathology*, **82**, 63-71.

Web sites

http://www.lifesci.sussex.ac.uk/home/NeilCrickmore/Bt/toxins2.html.