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Research Article

Cry gene and plasmid profiling of *Bacillus thuringiensis* isolated from Indian soils

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ABSTRACT: HCry gene and plasmid profiling of indigenous *Bacillus thuringiensis* isolates from North East India and Andaman were carried out. A total of 29 isolates were screened and HD-1 was used as reference. Plasmid profiling showed distinct bands of different sizes with unique patterns for each strain. Twenty one isolates had plasmids above 33500 bp and only 14 had plasmids of the same size. One isolate NBAII-TRBT17 showed presence of four plasmids having sizes of 2500bp, 7000bp, 7500bp and 33500bp.Six of them had three plasmids of different sizes. The isolates NBAII-BT5, NBAII-TRBT9, NBAII-TRBT18, NBAII-ASBT15, NBAII-ASBT11 andNBAII-AGBT5 showed similar band migration with three plasmids between 7000 to 9000 bp and 33500 megaplasmid each. Strain NBAII-BTN3 showed two different plasmids but plasmid size ranged as 9000 bp and 33, 500 bp which had similar pattern with NBAII-ASBT1, but NBAII-ASBT1 also harbored plasmid above 33500 bp. The isolates NBAII-TRBT10, NBAII-TRBT16, NBAII-TRBT8, NBAII-BTAN$,NBAII-BTAN5, NBAII-ASBT20, NBAII-ASBT2, NBAII-ASBTN1, NBAII-BTEG1, NBAII-AGBT13,NBAII-AGBT6, NBAII-AGBT1 and NBAII-ASBT21 showed presence of a single plasmid above 33500bp. The isolates NBAII-ASBT24, NBAII-BT3 and NBAII-AGBT25 showed similar migration of plasmids ranging between 15000-33500 bp. The plasmids were probed for Cry1, Cry2 and Cry3 genes with universal primers and isolates showed differential expression of the genes. Six of the isolates namely NBAII-ASBT20, NBAII-ASBT24, NBAII-TRBT10, NBAII-TRBT17, NBAII-BTAN4 and NBAII-BTAN5 showed presence of Cry3 gene also apart from Cry1 which could indicate that it could be active against coleopterans also. Plasmid DNA profiles and cry protein characterization provided information on diversity among the isolates and the cry gene diversity.

KEY WORDS: Plasmid profile, *Bacillus thuringiensis*, Cry gene, Indigenous.

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INTRODUCTION

Bacillus thuringiensis (*Bt*) is present in varying habitats like soil, surface of plants, insect cadaver, stored grains andwaterbodies (Travers *et al*., 1987; Medows *et al*., 1992). The Gram positive spore producing *Bt* is mainly exploited for its insecticidal activity for over more than 50 years and is considered as a substitute to chemical insecticides (Ramirez, 2008). *Bacillus thuringiensis* secretes highly toxic insecticidal crystal proteins that are encoded by different *cry* genes. These *cry* genes are expressed mainly during late exponential and stationary phases (Bravo *et al*., 1998; Yamamoto and Dean, 2000). More than 500 cry gene sequenced and classified into 67 groups (Cry1 to Cry67) (Crickmore *et al.,* 2010).

Earlier it was shown that the insecticidal crystal proteins were mainly encoded in plasmid but later it was proved that the cry genes are present in both genomic DNA (chromosomal) and in plasmids (Lereclus *et al*., 1993). It is reported that there are two groups of plasmid patterns that can be detected: i.e. ≤30 MDa (smaller plasmids) and those that are ≥30 MDa ormega plasmids. Smaller plasmids invariably occur as high copy numbers whereas the mega plasmids occur in low copy numbers (Ramirez and Ibarra, 2008). The *cry* genes are present in both genomic DNA as well as in plasmids of varying sizes (4-150 MDa). The *cry* genes can be present in varying combinations and also asmultiple copies within a plasmid in different isolates of *Bt* (Lereclus *et al*., 1993). Smaller *Bt* plasmids can be cryptic since their specific functions are not clear. Mega plasmids are known to code for the c*ry* genes (Roh *et al*., 2007). Since plasmids can be exploited using molecular tools, protocols for extraction and purification have been standardized (Gitahy *et al*., 2005). Widely used protocol is based on extraction using alkaline lysis and purification under

gradient ultracentrifugation in cesium chloride (Sambrook *et al*., 1989). Ramirez and Ibarra (2008) later standardized a quicker method.

Identification of *cry* genes using PCR techniques has proven to be very effective in screening large native *Bt* isolates which helps in predicting toxicity of *Bt* isolates or strains. While searching for novel strains other characters like, enhanced insecticidal activity as evinced through bioassay, number of plasmids, genomic DNA, crystal morphologyand protein structure arealso used. In the present study, plasmids were isolated from *B. thuringiensis* strains isolated from Indian soils sampled in North East and Andamans. Plasmid profiling was done to study the number of plasmids in each isolate. The isolates were further screened for genes coding for *cry*1, *cry*2 and *cry*3 using universal primers.

MATERIAL AND METHODS

Bacillus thuringiensis isolates

Twenty nine native strains of *Bacillus thuringiensis* were isolated from different soil samples collected from North East and Andaman as per the method of Travers *et al*. (1987) and Obeidat *et al*. (2004) and maintained as NBAII culture collection. The isolates were freshly cultured on 50 ml of T3 agar (Tryptose;2 g, tryptone; 3 g, MnCl2; 0.005g; Yeast extract; 1.5 g, 0.05M sodium phosphate;) taken in 150 ml Erlenmeyer flasks and incubated at 28°C overnight. Plasmid DNA was extracted as per protocols described by Sambrook *et al*., 1989 (based on methodology of Birnboima and Doly, 1979).

Detection of *cry* genes

PCR detection of *cry*1, *cry*2 and *cry*3 genes was carried out using specific primers. The primers were designed as per earlier studies(Ben-Dov *et al*., 1997; Bravo *et al*., 1998; Porcar and Juaez-Perez., 2003) (Table 1.). PCR amplification was carried out using thermal cycler (Qantarus, UK). The reactions mixture consisted of 50µl volume that contained 1X PCR buffer, 200 µM of each deoxynucleotide triphosphates, 0.5 µM load of each primers, 2 µl of template DNAand 0.5 U of Taq DNA polymerase). The PCR conditions were programmed for 35 cycles that consisted of single denaturation step for 3 min at 94°C, denaturation for 1 min at 94°C, the annealing temperature was set at 52-59°C for 30 seconds (as per each pair of primers used), extension time was 1 to 3 minat 72°C. The PCR experiment also consisted of a positive control (DNA template of *B. thuringiensis*) and negative controls (without DNA template). The amplified PCR product was run on 1.5 X agarose gels at 100V and presence of cry genes were confirmed based on relative molecular sizes. Gel images were recorded in DNRMiniLumi (Israel)Bio- Imaging System.

RESULTS AND DISCUSSION

Plasmid profiling

Plasmid profiling of 29 indigenous *Bacillus thuringiensis* (*Bt*) isolates was carried out and the isolates showed distinct bands ofvarying sizes (Table 1). Among them 21 isolates had plasmids above 33500 bp and 14 isolates had plasmids of same size. The isolate NBAII-TRBT17 harbored four plasmids with sizes of 2500bp, 7000bp, 7500bp and 33500bp and could be differentiated from one another.

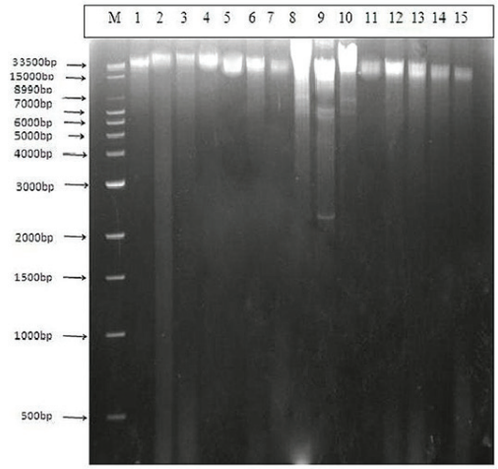

Fig. 1a. Agarose gel showing plasmid profiles of native *Bacillus thuringiensis* isolates;M= Supermix DNA ladder (Genei™), Lane 1 = NBAII-TRBT10, Lane 2 =NBAII-TRBT16,Lane 3 = NBAII-TRBT8, Lane 4 =NBAII-BTAN4,Lane 5 =NBAII-BTAN5, Lane 6 = NBAII-ASBT20,Lane 7 =NBAII-ASBT2, Lane 8 = NBAII-ASBT1,Lane 9 =NBAII-TRBT19, Lane 10 = NBAII-ASBT12,Lane 11 =NBAII-BTN1, Lane 12 = NBAII-BTEG1,Lane 13 = NBAII-ASBT24, Lane 14 =NBAII-BT3,Lane 15 =NBAII-AGBT25.

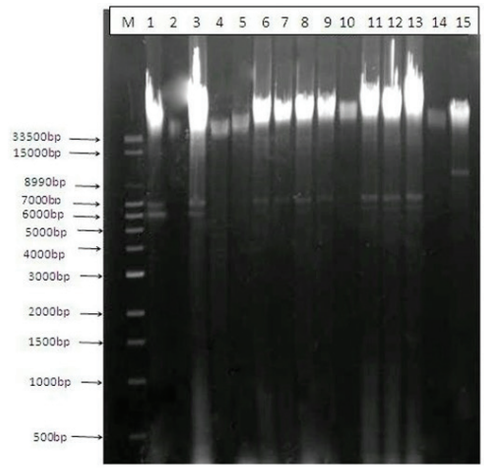

Fig. 1b. Agarose gel showing plasmid profiles of *Bacillus thuringiensis*, Lane 1, Supermix DNA ladder (Genei™), Lane 1 =NBAII-BT5, Lane 2 =NBAII-AGBT13, Lane 3 = NBAII-TRBT9, Lane 4 =NBAII-AGBT6, Lane 5 =NBAII-AGBT1, Lane 6 = NBAII-TRBT18, Lane 7 =NBAII-ASBT15, Lane 8 =NBAIIASBT11, Lane 9 =NBAII-AGBT5, Lane 10 =NBAII-ASBT21,Lane 11 =HD-1,Lane 12 =NBAII-TRBT7, Lane 13 = NBAII-BTC2, Lane 14 =NBAII-BTAN2, Lane 15 =NBAII-BTN3

Table 1. Plasmid profiles of native *Bacillus thuringiensis* isolates tested showing different band patterns

Isolate	Size (bp)	Isolate	Size (bp)
NBAII-AGBT1	Above 33500bp	NBAII-TRBT 9	Above 33500bp, 7000bp and 6000bp
NBAII-AGBT5	33500bp and 7000bp	NBAII-TRBT10	Above 33500bp
NBAII-AGBT6	Above 33500bp	NBAII-TRBT16	Above 33500bp
NBAII-AGBT13	Above 33500bp	NBAII-TRBT17	33500bp, 7000bp,6000bp and 2500bp
NBAII-AGBT25	Between 15000-33500 bp	NBAII-TRBT18	Above 33500bp and 7000bp
NBAII-ASBT1	Above 33500bp, 33500bp,9000bp	NBAIIBTEG1	Above 33500bp
NBAII-ASBT2	Above 33500bp	NBAII-BTAN2	Above 33500bp
NBAII-ASBT11	Above 33500bp and 7000bp	NBAII-BTAN 4	Above 33500bp
NBAII-ASBT 12	Above 33500bp, 8990bp, 7000bp	NBAII-BTAN5	Above 33500bp
NBAII-ASBT15	Above 33500bp and 7000bp	NBAII-BT3	Between 15000-33500 bp
NBAII-ASBT 20	Above 33500bp	NBAII-BT5	Above 33500bp, 7000bp, 6000bp
NBAII-ASBT21	Above 33500bp	NBAII-BTN1	Above 33500bp
NBAII-ASBT24	Between 15000-33500 bp	NBAII-BTC2	Above 33500bp, 7000bp and 6000bp
NBAII-TRBT7	Above 33500bp, 7000bp and 6000	NBAII-BTN3	33500bp and 9000bp
NBAII-TRBT8	Above 33500bp	HD-1 (Reference)	Above 33500bp, 7000bp and 6000bp

With the knowledge that plasmids carry *cry* genes, efforts were made to transfer plasmids between different strains of *B. thuringiensis* and from *B. thuringiensis* to *B. cereus* (González and Carlton, 1980; Kronstad *et al.,* 1983; Hu *et al.,* 2004). Studies on *Bt* plasmids are rare and very few articles relating its importance in characterization of *Bt* strains are present. In this study plasmid patterns were unique to each strain (Fig. 1 and Fig. 2). Sorgo *et al.* (2011) used plasmid profiling to differentiate specific strains of *B. thuringiensis* harboring different *cry* genes. Plasmids of different sizes (4-150 MDa) harbor the *cry* protein genes, the cry genes are also present in the bacterial chromosomes. The *cry* genes can occur in different combinations; the plasmids also harbor multiple copies of these *cry* genes(Lereclus *et al*., 1993; Loeza*et al*., 2005).

The present study revealed that six isolates harbored three plasmids with varying sizes and among them NBAII-BT5,NBAII-TRBT9, NBAII-TRBT18, NBAII-ASBT15, NBAII-ASBT11 and NBAII-AGBT5 showed similar band migration with three plasmids between 7000 to 9000 bp and 33500 megaplasmid each. In subspecies *kenyae* it is reported that variations in migration pattern can occur among strains of similar serovars (Ramirez, 2008). Likewise, these authors observed pattern diversity in ten strains belonging to serovars *sotto* and *dendrolimus*. The strain NBAII-

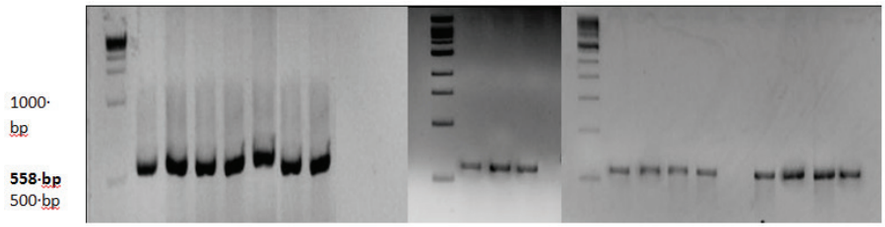

Fig. 2. Agarose gel showing electrophoresis PCR products amplified from plasmids of native *Bacillus thuringiensis* isolates with universal primers for cry1 (558bp).

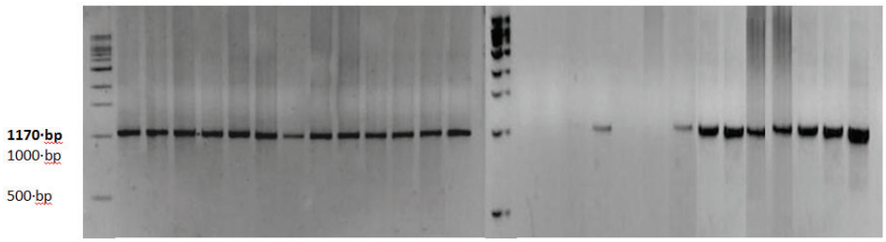

Fig. 3. Amplification of cry2 gene (1170bp) from plasmids of Indian *Bacillus thuringiensis* isolates

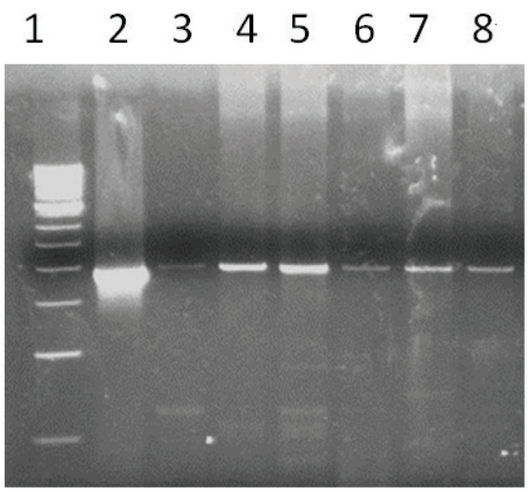

Fig. 4. PCR amplification of cry3 (951 bp) from plasmids of native *Bacillus thuringiensis* with universal primer, Lane1 = 1 Kb marker, 2=4AA1 (Standard Bt subsp. *tenebrionis*, 3 =NBAII-TRBT17, 4 =NBAII-BTAN4, 5 =NBAII-BTAN5, 6=NBAII-ASBT20, 7 = NBAII-ASBT21, 8 = NBAII-ASBT24

BTN3 showed two different plasmids but plasmid size ranged as 9000 bp and 33, 500 bp which had similar pattern with NBAII-ASBT1, and this isolate also harbored plasmid above 33500 bp.NBAII-TRBT10, NBAII-TRBT16, NBAII-TRBT8, NBAII-BTAN4. NBAII-BTAN5, NBAII-ASBT20, NBAII-ASBT2, NBAII-BTN1, NBAII-BTEG1, NBAII-AGBT13, NBAII-AGBT6, NBAII-AGBT1 and NBAII-ASBT21 showed presence of single plasmid above 33500pb. NBAII-ASBT24, NBAII-BT3 and NBAII-AGBT25 showed similar migration of plasmids ranging between 15000-33500 bp. Plasmids showing differences in their sizes may have variations in the DNA sequence and could also indicate differences in *cry* gene expression.

Characterization of plasmids for its insecticidal *cry* gene specificity

PCR studies were carried out to detect the cry genes present in the plasmids of the indigenous *Bt* isolates. Universal primers for *cry*1, *cry*2 and *cry*3 were used (Table 2). The reference strains used were HD-1 (for *cry*1 and *cry*2) (Porcar and Juarez-Perez, 2003) and *Bt* subsp. *tenebrionis* (for *cry*3) (Thammasittirong and Attathom, 2008; Bourque *et al*., 1993). It was observed that 18 isolates harbored *cry*1 gene, among them NBAII-TRBT8, NBAII-ASBT1 and NBAII-AGBT1 had *cry*1 gene alone and *cry*2 was detected in 15 of the other isolates (Fig. 2 and 3). The study also showed that 6 isolates harbored the *cry*3 gene (Fig. 4 and Table 3). Reports indicate that *cry*2 can be toxic to both lepidopteran and dipteran pests(Ozturk *et al*., 2009). It is also shown that *cry*1 and *cry*2 genes can occur together and are common in many *Bacillus thuringiensis* isolates (Lopez-Pazos *et al*., 2009). In our study *cry*1 and *cry*2 together occurred in 10 isolates (NBAII-AGBT5, NBAII-ASBT12, NBAII-ASBT20, NBAII-ASBT24, NBAII-TRBT10, NBAII-TRBT16, NBAII-TRBT17, NBAII-BTEG1, NBAII-BT3 and NBAII-BTN1). HD-1 also harbored *cry*1 and *cry*2 together. The plasmids of six of the isolates (NBAII-TRBT17, NBAII-BTAN4, NBAII-BTAN5, NBAII-ASBT20, NBAII-ASBT21 and NBAII-ASBT24) also harbored the *cry*3 gene that is reported to be active against coleopterans. The *cry*3 isolates also harbored *cry*1, *cry*2 or both together in their plasmids. None of the isolates showed presence of *cry*3 gene alone (Table 3). Pinto and Fiuza (2003) obtained 46 *Bt* isolates collected from rice fields in Rio Grande do Sul, among which one *Bt* isolate showed presence of *cry*1 and *cry*3 gene and exhibited 100% mortality against *Oryzhophagus oryzae.* Since

NBAII-AGBT25, NBAII-BT5, NBAII-AGBT13, NBAII-TRBT9, NBAII-AGBT1 andNBAII-TRBT18 showed amplification of both *cry*1 and *cry*3, they have potential for use against both lepidopteran and coleopteran pests. *Bt* isolates with wide insecticidal toxicity are rarely found. Aly (2007) isolated *Bt* having lepidopteran, lepidopteran-dipteran active and coleopteran active *cry* genes using PCR technique. Thaphan *et al*. (2008) isolated 91 *Bacillus thuringiensis*

Table 2. Primers used to characterize *Bacillus thuringiensis* for presence of insecticidal *crygenes*

Gene	Primer sequence	Size (bp)	Annealing Temp.
Cry 1	5'-CTGGATTTACAGGTGGGGATAT-3' (f)	558	52°C
	5'-TGAGTCGCTTCGCATATTTGACT-3' (r)		
Cry 2	5'-CGATATGTTAGAATTTAGAAC-3'(f)	1170	50°C
	5'-TACCGTTTATAGTAACTCG-3'(r)		
Cry 3	5'-CGTTATCGCAGAGAGATGACATTAAC-3'(f)	951	59°C
	5'-TGGTGCCCCGTCTAAACTGAGTGT-3'(r)		

Table 3. Plasmid cry gene profile of the native *Bacillus thuringiensis* isolates.

S.No	Isolate	Insecticidal cry gene		
		Cry 1	Cry 2	Cry 3
1	NBAII-AGBT1	-	✓	-
2	NBAII-AGBT5	✓	✓	-
3	NBAII-AGBT6	-	✓	-
4	NBAII-AGBT13	-	✓	-
5	NBAII-AGBT25	-	✓	-
6	NBAII-ASBT1	✓	-	-
7	NBAII-ASBT2	✓	-	-
8	NBAII-ASBT11	-	✓	-
9	NBAII-ASBT 12	✓	✓	-
10	NBAII-ASBT15	-	✓	-
11	NBAII-ASBT 20	✓	✓	✓
12	NBAII-ASBT21	-	✓	✓
13	NBAII-ASBT24	✓	✓	✓
14	NBAII-TRBT7	-	-	-
15	NBAII-TRBT8	✓	-	-
16	NBAII-TRBT 9	-	✓	-
17	NBAII-TRBT10	✓	✓	-
18	NBAII-TRBT16	✓	✓	-
19	NBAII-TRBT17	✓	✓	✓
20	NBAII-TRBT18	-	✓	-
21	NBAIIBTEG1	✓	✓	-
22	NBAII-BTAN2	✓	-	-
23	NBAII-BTAN 4	✓	-	✓
24	NBAII-BTAN5	✓	-	✓
25	NBAII-BT3	✓	✓	-
26	NBAII-BT5	-	✓	-
27	NBAII-BTN1	✓	✓	-
28	NBAII-BTC2	-	✓	-
29	NBAII-BTN3	✓	-	-
30	HD-1	✓	✓	-

isolates from Krabi Province which harbored different sub classes of *cry*1 gene and *cry*2A and was found to occur both in chromosomal and plasmid DNA.

The advent of PCR techniques has been as boon in helping researchers to detect *cry* genes (Bourque *et al*., 1993; Ben-Dov *et al*., 1997, Bravo *et al*., 1998; Porcar and Juarez-Perez, 2003). Earlier reports suggest the occurrence of various *cry* genes in a single strain of *B. thuringiensis* (Aronson, 1994; Ben Dov *et al*., 1997). Their studies revealed the occurrence of *cry*1, *cry*3, *cry*8, or *cry*7 genes in a single strain of *B. thuringiensis*. In another study *cry*1 (lepidopteran active) and the coleopteran specific *cry*3A, *cry*3Ba and *cry*7A genes were detected in a single strain (Bravo *et al*., 1998). They opined increased frequency of genetic material transfer can occur in *B*. *thuringiensis* strains that carried multiple or more than one *cry* gene. Since, we used universal primers to detect each *cry* gene group it was not possible to determine the *cry* gene sub-type in this present study. *B*. *thuringiensis* isolates carrying novel *cry* genes will show PCR products varying in size when compared with to the standard. Also in these novel strains PCR products may not be detected (Carozzi *et al.*, 1991). Hence, use of additional primers is needed while studying the homology to known *cry* genes.

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