Published Online on 9 July 2018

Proc Indian Natn Sci Acad **84** No. 3 September 2018 pp. 723-729

DOI: 10.16943/ptinsa/2018/49400

Research Paper

Toxicity Analysis and *cry* Gene Profiling of *Bacillus thuringiensis* Isolated from Western Ghats of Tamil Nadu State, India

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(Received on 11 December 2017; Revised on 16 April 2018; Accepted on 08 May 2018)

Insect bioassays and PCR screening with specific primers are the two techniques widely followed for identification of novel *cry* genes from new isolates of *Bacillus thuringiensis* (Bt). In the present study, seventy new isolates of Bt isolated from the Western Ghats were evaluated for its toxicity against *Helicoverpa armigera* (Hubner) and for the presence of *cry* genes through PCR screening with specific primers. The isolates showing 90 to 100, 50 to 89 and less than 50 per cent mortality of *H. armigera* were categorised into group I, II and III respectively. Eight and fourteen new isolates of Bt were grouped under I and II, respectively. Whereas 48 new isolates of Bt were clustered under the group III. The group I isolates were positive for *cry1*, *cry2* and *cry9* and negative for *cry3* and *cry4* genes. Five different *cry* genes *viz*., *cry1*, *cry2*, *cry3*, *cry4* and *cry9* were found in group II isolates. All 48 isolates under group III were not positive for *cry3* gene. Similarly, 20 of 48 isolates of group III were negative for all the five *cry* genes tested. The remaining 28 isolates were positive for one or more of the following *cry* genes: *viz*., *cry1*, *cry2, cry4* and *cry9*. Of the 70 new isolates screened for cry genes, *cry1*, *cry2*, *cry4* and *cry9* genes were observed in 41.4 and 35.7, 12.8 and 15.7 per cent of the isolates, respectively.

Key words: *B. thuringiensis*; Western Ghats; *H. armigera*; *cry* Gene Profiles

Introduction

Indiscriminate use of chemical pesticides has long-term detrimental effects on soil crop and environment in total. It is estimated that globally around 500 pesticides are in use, India alone uses 250 different pesticides. However, studies reveal that only 1% of the pesticide reaches the target and remaining 99% are released in to the environment. This leads to environmental degradation, elimination of natural parasitoids and predators; and the residues are entering into human food chain. Besides that, continuous exposure of several pesticides to insect pests results in development of resistance to one or more chemical insecticides. Biological control of pests using *B. thuringiensis* (Bt) products has advantages over chemical pesticides due to its specific toxicity against target insects, lack of polluting residues and safety to non-target organisms such as mammals, birds, amphibians and reptiles. Bt is a Gram positive, sporulating soil bacterium that forms insecticidal crystal proteins during sporulation phase of its growth. Bt is the major source for development of insect-resistant transgenic plants. Several isolates have been tested and characterized against insect pests and disease for production of biopesticides. The development of such bio pesticides leads to reduction in chemical pesticide use. The use of biotech crops has reduced pesticide spraying by 352 million kg (-8.4%) and, as a result, decreased the environmental impact associated with herbicide and insecticide use on these crops (as measured by the indicator the environmental impact quotient) by 16.3% (Brookes and Barfoot, 2010). In this regard, intensive screening programmes are carried out worldwide to isolate large number of Bt, in order to identify new strains of Bt with increased levels of insecticidal activity against a broader spectrum of insect pests for development of Biotech crops. It is also reported that new variants of the already known *cry* gene subgroups could encode crystal proteins with significant difference in the level

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and spectrum of toxicity due to variation in their sequences (Xue *et al*., 2008). In our previous study, variation in morphology of crystalline inclusions and crystal protein profile were observed for 70 of 316 Bt isolates from Western Ghats (Ramalakshmi and Udyasuriyan, 2010). Further screening of the new isolates for its toxicity against lepidopteran and for the presence of cry gene may result in novel cry genes. Hence, the present study was aimed to screen the 70 new isolates of Bt for its toxicity against neonatal larvae of *H. armigera* by artificial diet- based-bioassay and for the presence of cry genes by PCR screening.

Materials and Methods

Sources of Bt Strains

Seventy new isolates of Bt isolated from the Western Ghats and identified based on the crystal morphology were used for the present study (Ramalakshmi, and Udayasuriyan, 2010). The reference strains of Bt available with corresponding author were used for the present study.

Toxicity Analysis of Bt Against Helicoverpa armigera

A bioassay was performed to analyse the toxicity of Bt against lab grown culture of *H. armigera* (originally initiated from field-collected larvae), *H. armigera* was reared on a semi synthetic diet (Patel *et al*., 1968). The Bt isolates were grown in T3 broth till more than 90 per cent cell lysis occurred and then OD was measured at 600nm. The Bt cultures were subsequently equalized to 0.5 OD and used for bioassay. Approximately one ml of the semi- synthetic diet was dispensed into 1.8 ml cryovial (Tarson®; 1cm dia.) and allowed to dry for an hour. After solidification of the diet, the Bt culture broth was coated on the diet surface @ 10µl per vial using a sterile glass rod. The vials were allowed to dry for 30 min and to each vial a neonate larva of *H. armigera* was released using a soft hairbrush. They were closed halfway with a screw cap, leaving space for gas exchange. All the above steps were carried out under sterile conditions and controlled atmosphere. A treatment without Bt culture served as control and ten vials were maintained for each treatment with three replications. Larval mortality was recorded periodically for seven days. A larva was marked dead when they did not move even when prodded with hairbrush. All the experiments were carried out in a room with a photoperiod of 14:10 (L: D); and optimum temperature of 27°C and 60 per cent RH.

Genomic DNA Isolation

Total genomic DNA was extracted from all 70 new isolates and four reference strains of Bt, as described by Kalman *et al.* (1993). Genomic DNA of Bt was quantified by loading on 0.8 per cent agarose gel with reference to known quantity of λ/*Hind* III. The DNA samples were analysed by agarose gel electrophoresis as described by Sambrook *et al*. (1989).

PCR Screening for cry Gene Content

The new isolates of Bt were screened through PCR for the presence of *cry1*, *cry2*, *cry3*, *cry4* and *cry9* family gene(s). Details regarding primer sequences and amplicons size are given in Table 1. Total genomic DNA isolated from Bt strains was used as template for PCR screening with *cry* gene family primers. The PCR was accomplished using an Eppendorf thermal cycler in 25 µl reaction volume containing 30 ng of total genomic DNA of Bt, 2.5µl of 10X PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles. Temperature profile for PCR screening of *cry1*, *cry2*, *cry3*, *cry4* and *cry9* genes were as per reference cited in Table 1. An aliquot of amplified PCR product was analyzed on 1.2 per cent agarose gel.

Results

Results from the previous study (Ramalakshmi and Udayasuriyan, 2010) show that out of 70 new isolates of Bt, six different types of crystal protein profile *viz*., 135 and 65, 135, 95, 65, 43 and 30kDa were observed in 17 (24.2%), 15 (21.4%), 12 (17.1%), 7 (10%), 4 (5.7%), and 7 (10%) Bt isolates, respectively and the remaining eight Bt isolates did not show any distinct band of crystal protein. The results of the present study on toxicity analysis of 70 new isolates of Bt are presented in Fig. 1. The isolates Nn1, Ns1and Nn10 recorded 100 per cent mortality of *H. armigera,* similar to reference strain Bt, HD1. The 17 new isolates of Bt having crystal proteins of 135 and 65 kDa recorded 16.7 to 100 per cent mortality in *H.*

Table 1: Primers used for screening of *cry* genes

S.No.	Primer sequences	*cry* genes	Amplicon size (bp)	References
1.	FP: 5'CATGATTCATGCGGCAGATAAAC 3' RP: 5'TTGTGACACTTCTGCTTCCCATT 3'	*cry1*	277	Ben-dov *et al*. (1997)
2.	FP: 5'GTTATTCTTAATGCAGATGAATGGG 3' RP: 5'CGGATAAAATAATCTGGGAAATAGT 3'	*cry2A*	700	Ben-dov *et al*. (1997)
3.	FP: 5'CGTTATCGCAGAGAGATGACATTAAC 3' RP: 5'CATCTGTTGTTTCTGGAGGCAAT 3'	*cry3*	590	Ben-dov *et al*. (1997)
4.	FP: 5'GCATATGATGTAGCGAAACAAGCC 3' RP: 5'GCGTGACATACCCATTTCCAGGTCC 3'	*cry4*	440	Ben-dov *et al*. (1997)
5.	FP: 5'CGGTGTTACTATTAGCGAGGGCGG 3' RP: 5'GTTTGAGCCGCTTCACAGCAATCC 3'	*cry9*	350	Ben-dov *et al*. (1999)

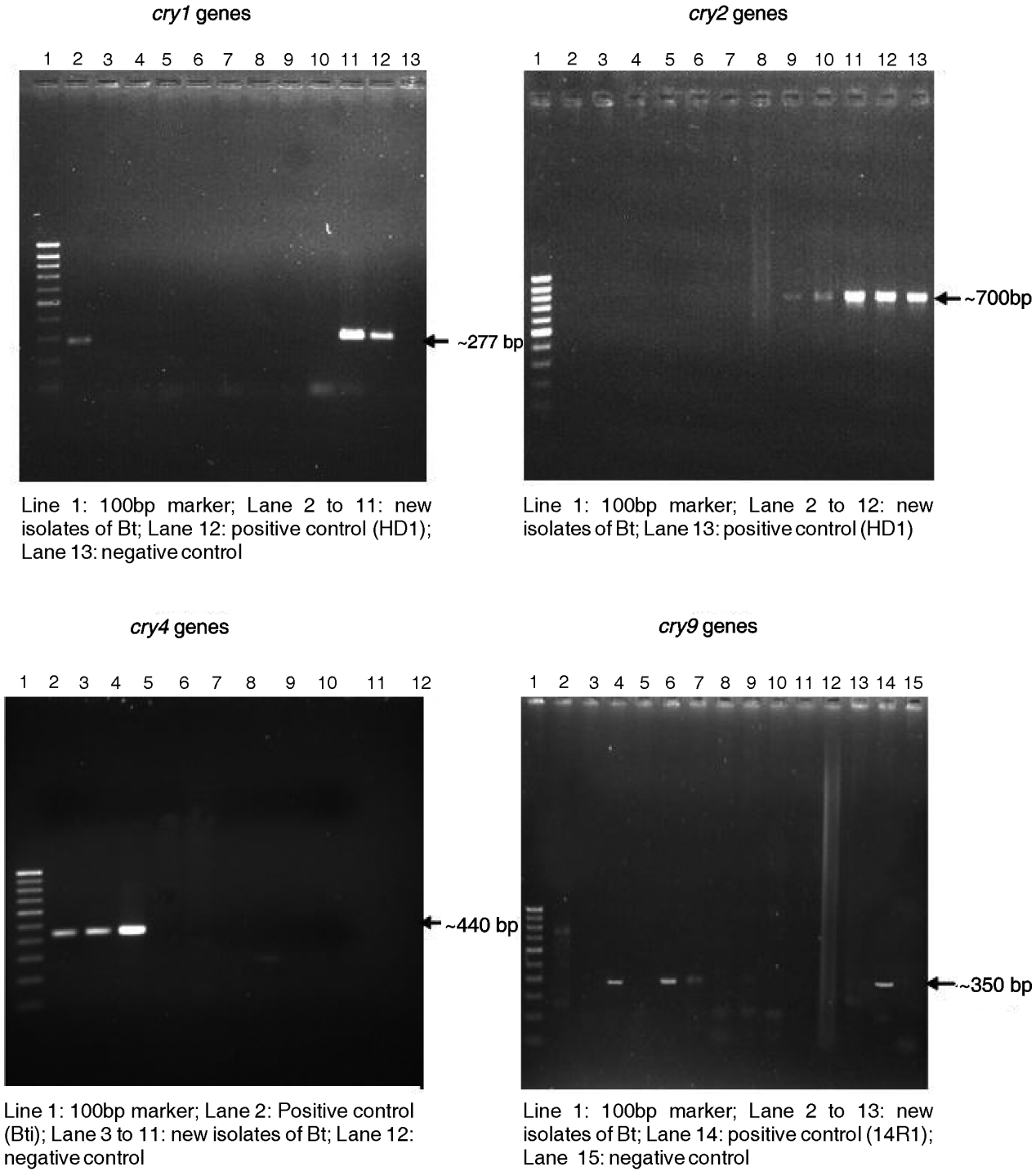
FP: forward primer; RP: reverse primer

armigera. The Bt isolates, Po29 and Co3 showed 93.3 and 90 per cent mortality, respectively. The remaining 12 isolates showed different levels of toxicity (10 to 80 per cent) at 7 DAT. All the isolates of 95 kDa showed less than 50 per cent mortality except the isolate, Ko19 which showed fifty per cent mortality at 7 DAT. All the seven isolates of 65 kDa crystal proteins showed mortality to *H. armigera* in the range of 30 to 100 per cent. The isolates, Gu2 and Mu12 recorded 96.7 and 90.0 per cent mortality respectively, whereas the Bt isolate, Co37 recorded lowest mortality (30 per cent) at 7 DAT. The Four new isolates of Bt having ~43 kDa crystal protein(s) showed less than fifty per cent mortality at 7 DAT. The Bt isolate, Di11 having crystal protein of ~30 kDa showed 76.7 per cent mortality, whereas the remaining six isolates showed less than 50 per cent mortality at 7 DAT. The eight new isolates of Bt showing no discrete bands of crystal protein showed less than 50 per cent mortality at 7 DAT. Based on level of toxicity against *H. armigera*, the 70 new isolates of Bt were classified into three groups. The isolates showing 90 to 100 were grouped as I, 50 to 89 are II and less than 50 per cent were grouped as III respectively. Eight and fourteen new isolates of Bt were of group I and II, respectively, whereas 48 new isolates of Bt were clustered under the group III (Table 2). All the 70 new isolates of Bt were screened by PCR for the presence of five different *cry* genes *viz*., *cry1, cry2, cry3, cry4* and *cry9* using gene specific primers (Fig. 2). The reference strain of Bt, HD1 was used as a positive control for *cry1* and *cry2* genes and the reference strains, Btt, Bti and 14R1 were used as a positive control for *cry3*, *cry4* and *cry9*, respectively. All the eight isolates under group I gave amplification for one or more of the following *cry* genes viz., *cry1*, *cry2* and *cry9* (Table 3) whereas none of the Bt isolates of group I gave amplification for *cry3* and *cry4* genes. Among the fourteen isolates of group II tested, four failed to give amplification for all the five *cry* genes tested. The remaining ten isolates had one or more of the following the *cry* genes viz., *cry1*, *cry2* and *cry9*. All the fourteen isolates were not positive for *cry3* and *cry4* genes. Twenty out of 48 isolates of group III failed to give amplification for all the five *cry* genes tested. The remaining 28 isolates had one or more of the following *cry* genes *viz*., *cry1*, *cry2, cry4* and *cry9*. All the 48 isolates did not show presence of *cry3* gene (Table 4). Of the 70 isolates of Bt, 24 isolates (34.3 per cent) failed to give amplification for all the five *cry* genes tested. The remaining 46 isolates gave amplification for one or more of the following four *cry* genes *viz*., *cry1, cry2, cry4* and *cry9* and

Table 2: Grouping of *B. thuringiensis* isolates of Western Ghats based on toxicity level against *H. armigera*

S.No.	Grouping of Bt isolates based on toxicity against *H. armigera*		Bt isolates	
	Group number	Mortality on 7th day (%)	Number	Per cent
1	I	90-100	8	11.4
2	II	89-50	14	20.0
3	III	0-50	48	68.6
	Total	**70**		

Fig. 1: Analysis of new isolates of *B. thuringiensis* for toxicity against *H. armigera*



Line 1: 100bp marker; Lane 2 to 11: new isolates of Bt; Lane 12: positive control (HD1); Lane 13: negative control

Line 1: 100bp marker; Lane 2 to 12: new isolates of Bt; Lane 13: positive control (HD1)

Line 1: 100bp marker; Lane 2: Positive control (Bti); Lane 3 to 11: new isolates of Bt; Lane 12: negative control

Line 1: 100bp marker; Lane 2 to 13: new isolates of Bt; Lane 14: positive control (14R1); Lane 15: negative control

Fig. 2: Analysis of *cry* gene profiles in new isolates of *Bacillus thuringiensis* by PCR

showed six different *cry* gene profile(s) *viz*., *cry1*; *cry2*; *cry4*; *cry1* & *cry2*; *cry2* & *cry9*; and *cry1*, *cry2* & *cry9* genes (Table 4).

Discussion

Several attempts have been made to determine the insecticidal activity of Bt strains isolated from different environments against the target insect species belonging to orders Lepidoptera, Coleoptera and Diptera using spore-crystal preparations (Bravo *et al*., 1998). In the present study, the efficacy of new isolates of Bt compared with HD1 against the

Table 3: Crystal protein and cry gene profile of Group I isolates (90-100 % mortality)

Isolate name	Crystal protein(s) (KDa)	*cry* gene profile(s)
Mu4	135 & 65	*cry1, cry2, cry9*
Nn1	135 & 65	*cry1, cry2*
Mu12	65	*cry2*
Gu2	65	*cry2*
Nn10	65	*cry2*
Ns1	135	*cry1*
Co3	135	*cry1*
Po29	135	*cry1*

Table 4: Types of cry gene profile in *B. thuringiensis* isolates of Western Ghats

S.No.	Presence of *cry* gene(s)	Bt isolates	
		No. out of 70	Per cent
1.	*cry1*	12	17.1
2.	*cry2*	6	8.6
3.	*cry4*	9	12.8
4.	*cry1&2*	8	11.4
5.	*cry2&9*	2	2.9
6.	*cry1,2 & 9*	9	12.8
	Negative for all five *cry* genes screened	24	34.3
	Total	**70**	

lepidopteran pest of cotton, *H. armigera* revealed that high variability exist among the new isolates of Bt in terms of toxicity. The eight isolates having 135 & 65 or 135 or 65 kDa crystal proteins showed 100 per cent mortality; may be related to the Cry1 and Cry2 proteins groups which are known to be effective against lepidopteran insects. Forty eight of the 70 Bt isolates screened for toxicity recorded less than 50 per cent mortality. Among them, 21 isolates showed ~95 or 43 or 30 kDa crystal proteins. These isolates with uncommon sizes of crystal proteins may be more toxic to other lepidopteran insects or insects of other orders such as dipteran or coleopteran. Chilcott and Wigley, (1993) reported that 130, 65, and 28 kDa proteins are mosquitocidal and 73 kDa proteins are Coleopteran active. Information pertaining to toxicity of the new isolates presented in the present study is a preliminary screening of the isolates against one target insect, *H. armigera*. Further studies on toxicity analysis of purified crystal protein(s) against insects of different orders (groups) will throw more light on the insecticidal potency of the new isolates of Bt. PCR-based methods have been developed for identification of Bt isolates having novel *cry* gene profiles (Porcar and Juarez-Perez, 2003). PCR being a highly sensitive and relatively fast technique is especially suitable for rapid and large scale screening of Bt isolates. Bt strains harbouring novel *cry* genes and also the less frequently observed *cry* genes have been identified by PCR using specially designed primers corresponding to the highly conserved regions (Bravo *et al*., 1998).

In the present study, four of the five *cry* genes (*cry1*, *cry2*, *cry4* and *cry9*) were found in 46 of the 70 new isolates of Bt. Among them, the *cry1* gene was present in most of the isolates (41.4 per cent) followed by *cry2* gene (35.7 per cent). None of the Bt isolates showed positive result for *cry3* gene. The same trend has also been found in the previous studies conducted by Bravo *et al*. (1998); Kim (2000), who reported that the *cry1* gene has been found to be most abundant in every region and source followed by the *cry2* gene. On the other hand, 24 of the 70 isolates failed to generate a specific amplicon for any of the five *cry* genes tested (*cry1*, *cry2*, *cry3*, *cry4* and *cry9*). However out of 24 isolates, four *viz*., Ko19, Co14, Di3 and Di11 showed 50 to 89 per cent mortality of *H. armigera* and failed to give amplification of lepidopteran specific *cry1*, *cry2* and *cry9* genes. Therefore, the toxicity observed in these four isolates may be due to the presence of other than *cry1*, *cry2* and *cry9* lepidopteran specific *cry* genes such as *cry15* or due to proteins encoded by novel *cry* gene(s). The remaining 20 isolates which did not give for any of the five *cry* genes tested showed less than 50 per cent mortality of *H. armigera*. Hence, it may be suggested that *cry* genes specific to insects of other orders such as Diptera/Coleoptera may be present in these isolates. Further studies on these 24 isolates, especially the four which showed more than 50 per cent mortality of *H. armigera* with degenerate primers will be useful to identify novel *cry* genes.

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