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RESEARCH

ISOLATION, IDENTIFICATION AND BIOASSAY OF NATIVE *BACILLUS THURINGIENSIS* ISOLATES AGAINST *SPODOPTERA FRUGIPERDA*

Suvathi Balu[1], Loveson Allwin[2]*, K. Elanchezhyan[3], Vaithiyanathan Radhakrishnan[4] Chandrasekaran Harisudan[5], Manivannan MI[6] and Mariappan Paramasivan[7]

[1]*Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India*
[2,3,6,7]*Agricultural College and Research Institute, Killikulam, TNAU, Tamil Nadu, India*
[4]*Krishi Vigyan Kendra, Needamangalam,TNAU, Tamil Nadu, India*
[5]*Regional Research Station, Vridhachalam,TNAU, Tamil Nadu, India*
Email: allwin.dr@gmail.com

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Abstract: Totally nineteen *B. thuringiensis* strains were isolated from 50 soil samples which were collected from various locations of Tirunelveli and Tuticorin districts. Out of 583 *Bacillus* like colonies 288 colonies were produced crystals and nineteen strains were identified as *Bt*based on crystal morphology. Bipyramidal, cuboidal, spherical shape of crystals and spores attached with crystals were observed in the isolates. All strains showed positive results to gram staining, motility test, Methyl Red (MR) test, catalase test. Some of the strains were negative for Voges Proskauer and Starch hydrolysis test.*cry1*, *cry2Aa* and *cry2Ab*genes were observed in these isolates through PCR analysis. KKM 2 and KKM 14 were caused above 60% mortality. KKM 5, KKM 17 and KKM 18 were showed lowest per cent mortality (16.67%) against *S. frugiperda.*

Keywords: *B. thuringiensis*, Isolation, *Cry* genes, Fall armyworm

INTRODUCTION

The use of entomopathogenic bacteria, such as *Bacillus thuringiensis* (Berliner, 1911), is one of the alternatives to reduce the use of chemical insecticides for pest control.The insecticidal properties of *B. thuringiensis* are mainly attributed to the synthesis of insecticidal crystal proteins (Cry proteins) and/or vegetative insecticidal proteins (Vipproteins), which are synthesized during sporulation or vegetative growth, respectively. More than 700 *cry* and 130*vip* genes have been identified and classified into classes and subclasses based on the percent of pairwise aminoacid identity of their corresponding proteins. It has been established that genes within the *cry1*, *cry2*, *cry9* and *vip3*groups encode proteins that are toxic against lepidopteranlarvae. Recently, a *cry8* gene has been included into this selected group of encoding lepidopteran active proteins. Generally, Cry producer strains synthesize 130---140 kDa proteins contained in bipyramidal crystals and also synthesize65 kDa proteins contained in smaller cuboidal crystals which have a somewhat extended toxicity spectrum, as some are also mildly toxic to mosquito larvae. Still, some lepidopteran-active *B. thuringiensis* strains can produce130 kDa proteins which occur as spherical inclusions. Most *B. thuringiensis* strains harbor complex insecticidal genecombinationssuch as the well-known HD-1 strain,whereas some others can harbor a single *cry* gene, such asstrain HD-73 strain. FAO reported that, 20 to 40% of yield loss is caused by the attack of insect pests and pathogenic organisms (Zhou *et al*., 2002). Most of the damaging pests belong to Lepidoptera (Pimentel 2009). Recently *Spodoptera frugiperda* has been reported in India as an invasive pest.*S. frugiperda* feed on maize cob or kernel and it reduced the grain yield of maize upto 34%. Currently, synthetic insecticides such as methomyl, carbaryl, and cypermethrin are being used against *S. frugiperda* which adversely affect the environment and non-targeted organisms. But, it has developed resistance to both insecticides and *Bt* toxins (Flagel*et al*., 2018).The selection and morphological, molecular, and protein characterizations of *B. thuringiensis* strains are extremely important for the identification of potential pest-control agents. The aim of the current study was to isolate and characterize native isolates of *B. thuringiensis*toxic to *S. frugiperda*as a source of genes that can be used infuture studies focused on the development of new bioinsecticide formulations and of genetically modified plants.

MATERIALS AND METHODS

Isolation of *B. thuringiensis* isolates from native soil

*Corresponding Author

Soil samples were collected from different locations of Tirunelveli and Tuticorin districts and used for the isolation of native *B. thuringiensis* strains. Details of collection of soil samples were represented in Table 1. Isolation of *B. thuringiensis* strains was done by following sodium acetate selection method as described by Travers *et al*. (1987). One gram of soil sample was taken in a 250 ml conical flask and it was mixed with 20 ml of LB broth buffered with 0.25 M Sodium acetate. Colonies obtained by sodium acetate selection method are plated in T_3 medium and incubated at 30°C for 48 hrs. Selected *B. thuringiensis* colonies were examined for the presence of crystal proteins using phase contrast microscope. *B. thuringiensis* like colonies were counted for determining *Bt* index (Baig *et al*., 2010).

Table 1. Details of collection of soil samples

S. No.	Area	Type of soil	Field
1.	Alangulam	Sandy clay loam	Tomato
2.	Vallanad series	Sandy clay loam	Rice
3.	Thenkasi	Sandy clay loam	Bhendi
4.	Melagaram	Sandy loam	Chilli
5.	Ezhuthani oothu series	Sandy loam	Rice
6.	Manakkarai series	Sandy loam	Banana
7.	Vallanad reserve forest	Loamy clay	Forest
8.	Manur	Sandy clay loam	Mulberry
9.	Karungulam	Sandy clay loam	Cotton
10.	AC& RI 'A' block	Sandy clay loam	Green gram
11.	Seranmahadevi	Sandy clay loam	Marigold
12.	Murappanad	Sandy clay loam	Banana
13.	Tirunelveli	Sandy clay loam	Mulberry
14.	Ambasamudram	clay loam	Rice
15.	Sankarankovil	Sandy clay loam	Marigold
16.	Mel Nilidanallur	Sandy clay	Jasmine
17.	Courtalam	Sandy loam	Pumpkin
18.	AC& RI 'D' block	Sandy clay loam	Guava
19.	AC& RI 'F' block	Sandy clay loam	Ashgourd

Identification of the *B. thuringiensis* isolates

Colony morphology

Morphological characters of all the *B. thuringiensis* isolates were observed and compared with the reference strain *Bt* - HD1.

Staining analysis

Isolates were subjected to Gram staining (Lalitha *et al*., 2012) and Crystal staining to study the characters of *Bacillus* colonies and for observing crystal proteins (Ammons *et al*., 2005).

Biochemical analysis

Further characterization of *B. thuringiensis*isolates was done by Biochemical tests includesMotility test, Starch hydrolysis test, Methyl Red (MR) test, Voges Proskauer (VP) test and Catalase Test.

PCR screening for *cry* genes

Universal primer and specific primer were used in PCR screening to characterize the isolated *B. thuringiensis* strains. Details about primer sequences and amplicons size were given in Table 2. PCR screening was done by following the method described by Ramalakshmi *et al*. (2018). PCR amplification of total genomic DNA was carried out in Eppendorf thermal cycler for 30 reaction cycles each. PCR reactions were accomplished by using 25 µl reaction volume containing 30 ng of template DNA of *Bt* mixed with 2.5 µl of 10X PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM MgCl2), 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase. The condition for the PCRs done with *cry1, cry 2A* and *cry2Ab* primers were as follows: denaturation step of 2 minutes at 94°C, annealing at 62°C for 40 seconds, and extension at 72°C for 1 minute and an extra step of extension at 72°C for 1 minute. Following amplification, a 15µl sample of each PCR products were resolved by electrophoresis in 2% agarose gel in Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) in that 2 µl of ethidium bromide was added and then electrophoresed at 120 V for 30 to 45 minutes.

Table 2. Primer sequences and amplicons size

S. No	Primer sequences	*cry* genes	Product size (bp)	References
1.	FP: 5'CATGATTCATGCGGCAGATAAAC3'	*cry1*	277	Ben-dov *et al*. (1997)

	RP: 5'TTGTGACACTTCTGCTTCCCATT			
2.	FP: 5'GTTATTCTTAATGCAGATGAATGGG3' RP: 5'CGGATAAAATAATCTGGGAAATAGT3'	*cry2A*	700	Ben-dov *et al*. (1997)
3.	FP: GTTATTCTtAATGCAGATGAATGGG RP: TGGCGTTAACAATGGGGGGAGAAAT	*cry2Ab*	546	Ben-dov *et al*. (1997)

Toxicity studies

Preliminary bioassays were carried out to screen the virulent strains of isolated *B. thuringiensis.*Young maize leaves were collected freshly from the greenhouse, thoroughly washed with distilled water, dried and cut into rectangular leaf discs (approximately 2x1 cm). Each leaf disc was surface coated with 20 µl of crude spore-crystal mixture and allowed to dry in laminar air flow chamber. Ten neonate larvae were released on the leaf disc using camel hair brush without any physical injury. The leaf disc was placed in a Petridisc with a diameter of 9 cm. One larva was released per leaf disc. Ten larvae were used per replication and three replications were maintained for each treatment. *Bt*-HD1 strain was used as a positive control. Larval mortality was observed periodically upto seven days. Per cent mortality in the treatments was corrected by using Abbott's (1925) formula.

RESULTS

Isolation of native *B. thuringiensis*isolates

Nineteen strains were isolated from 50 soil samples which were sub-cultured in T_3 medium for single colony isolation. Based on the presence of crystal protein, 288 colonies were identified as crystal formers from 583 colonies, out of which 19 isolates were *B. thuringiensis* strains and named as KKM 1, KKM 2, KKM 3, KKM 4, KKM 5, KKM 6, KKM 7, KKM 8, KKM 9, KKM 10, KKM 11, KKM 12, KKM 13, KKM 14, KKM 15, KKM 16, KKM 17, KKM 18 and KKM 19. Recovery of *B. thuringiensis* from native soil samples was highest in isolate KKM 16 (0.75%) followed by isolate KKM 1 and KKM 4 (0.58%) and lowest in isolates KKM 14 and KKM 19 (0.33%). The estimated average *Bt* index of native soil sample was 0.49%.

Identification of *B. thuringiensis*isolates

Colony morphology

A total of 583 colonies were isolated based on the morphological characters like colony color, colony shape, colony margin, colony elevation and colony surface and sub-cultured in T_3 medium for single colony isolation. Colonies of all isolates were shown creamy white to off white colonies with regular or irregular shape, entire or errose or undulated margin, slightly raised elevation and glistening surface (Table 3, Plate 1).

Table 3. Colony morphology of *B. thuringiensis* strains

S.No.	Isolates	Colony Colour	Colony shape	Colony margin	Colony Elevation	Colony Texture
1.	KKM 1	Creamy white	Irregular	Entire	Raised elevation	Smooth
2.	KKM 2	Creamy white	Irregular	Errose	Raised elevation	Smooth
3.	KKM 3	Creamy white	Irregular	Undulate	Raised elevation	Smooth
4.	KKM 4	Creamy white	Irregular	Errose	Raised elevation	Smooth
5.	KKM 5	Creamy white	Irregular	Errose	Raised elevation	Smooth
6.	KKM 6	Creamy white	Irregular	Errose	Raised elevation	Smooth
7.	KKM 7	Creamy white	Round	Errose	Raised elevation	Smooth
8.	KKM 8	Creamy white	Irregular	Errose	Raised elevation	Smooth
9.	KKM 9	Creamy white	Round	Errose	Raised elevation	Smooth
10.	KKM 10	Creamy white	Irregular	Errose	Raised elevation	Smooth
11.	KKM 11	Creamy white	Irregular	Errose	Raised elevation	Smooth
12.	KKM 12	Creamy white	Irregular	Errose	Raised elevation	Smooth

13.	KKM 13	Creamy white	Round	Errose	Raised elevation	Smooth
14.	KKM 14	Creamy white	Irregular	Undulate	Raised elevation	Smooth
15.	KKM 15	Creamy white	Irregular	Errose	Raised elevation	Smooth
16.	KKM 16	Creamy white	Irregular	Errose	Raised elevation	Smooth
17.	KKM 17	Creamy white	Irregular	Errose	Raised elevation	Smooth
18.	KKM 18	Creamy white	Irregular	Undulate	Raised elevation	Smooth
19.	KKM 19	Creamy white	Irregular	Undulate	Raised elevation	Smooth

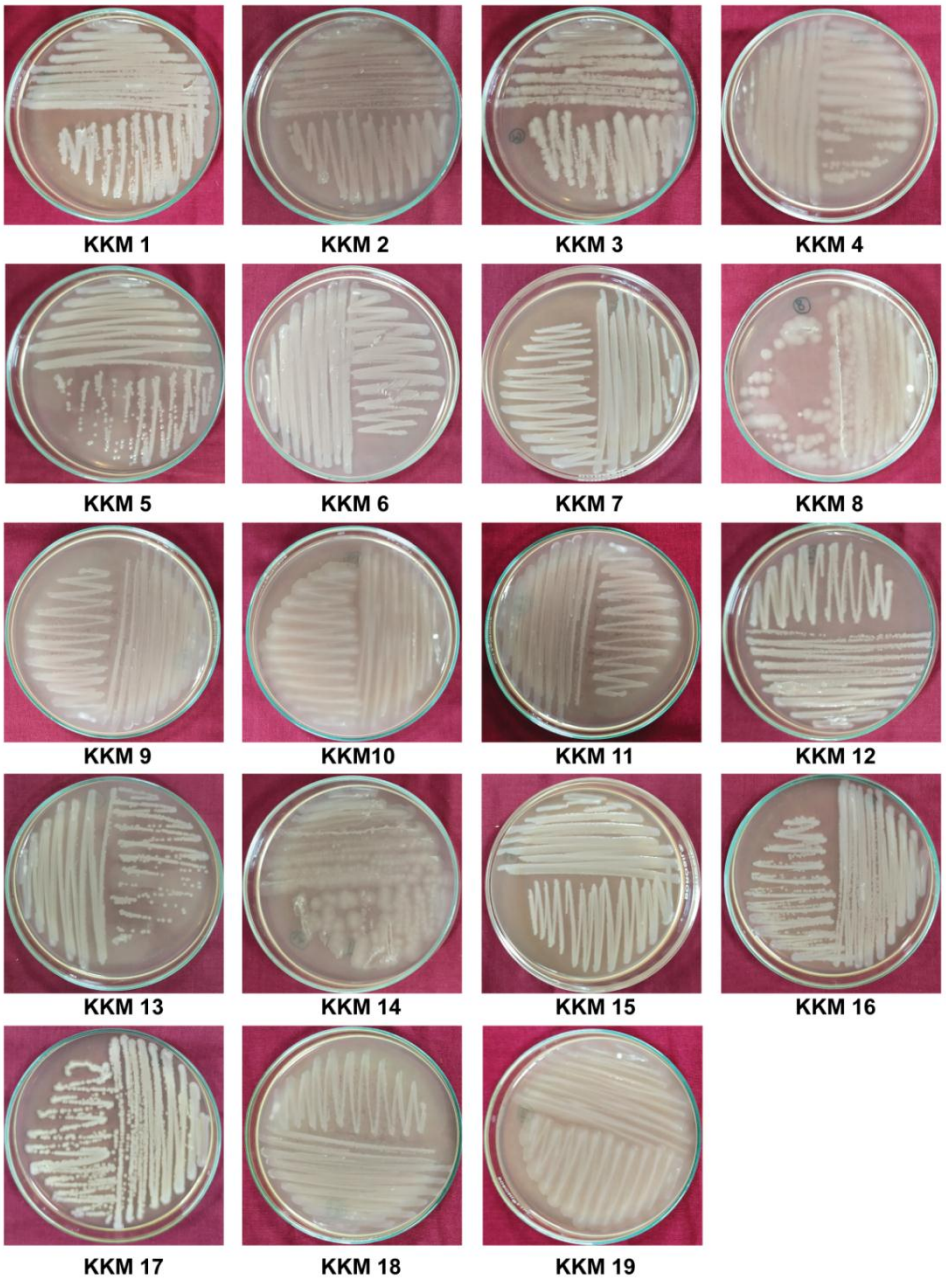


Plate 1. Colony morphology of *B. thuringiensis* strains

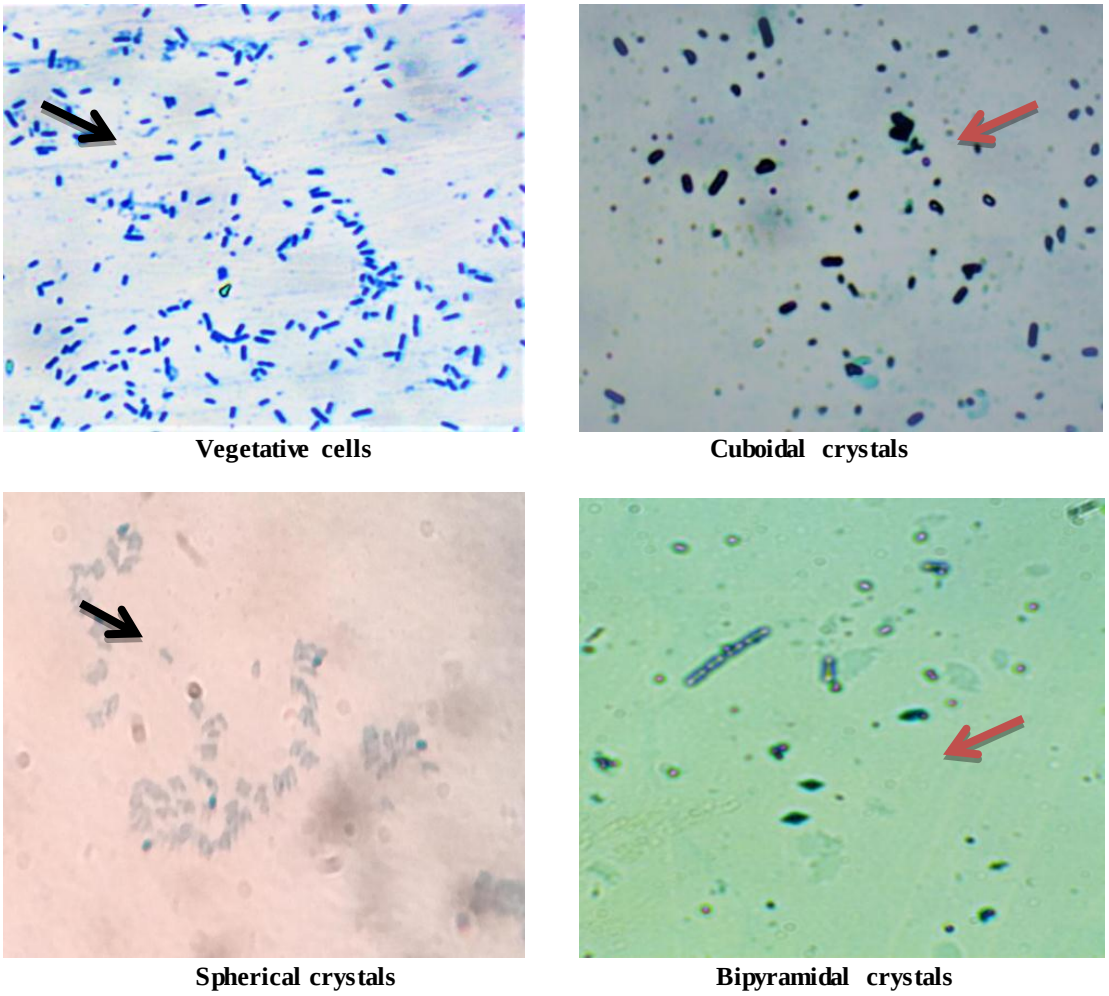
Staining analysis

Gram staining

Gram staining method was used to distinguish whether it was a gram positive or gram negative bacteria. Rod shaped and violet color cells indicated the presence of gram positive *Bacillus* species. All isolates were shown positive result for gram staining.

Crystal staining

All isolates were subjected to coomassie brilliant blue staining for further characterization based on crystal morphology. *Bacillus* like colonies were examined under light microscope and 288 colonies out of 583 colonies were shown the presence of crystal proteins. Nineteen isolated strains possessed three types of crystals *i.e.*, bipyramidal, cuboidal, spherical shape of crystals and spores attached with crystals (Plate 2). Among four types of crystal, cuboidal shape of crystal showed highest frequency (42.36%) followed by bipyramidal crystal (27.43%) and lowest frequency was observed in spherical shape of crystal (10.41%).



Vegetative cells **Cuboidal crystals**

Spherical crystals **Bipyramidal crystals**

Plate 2. Crystal morphology of *B. thuringiensis* strains

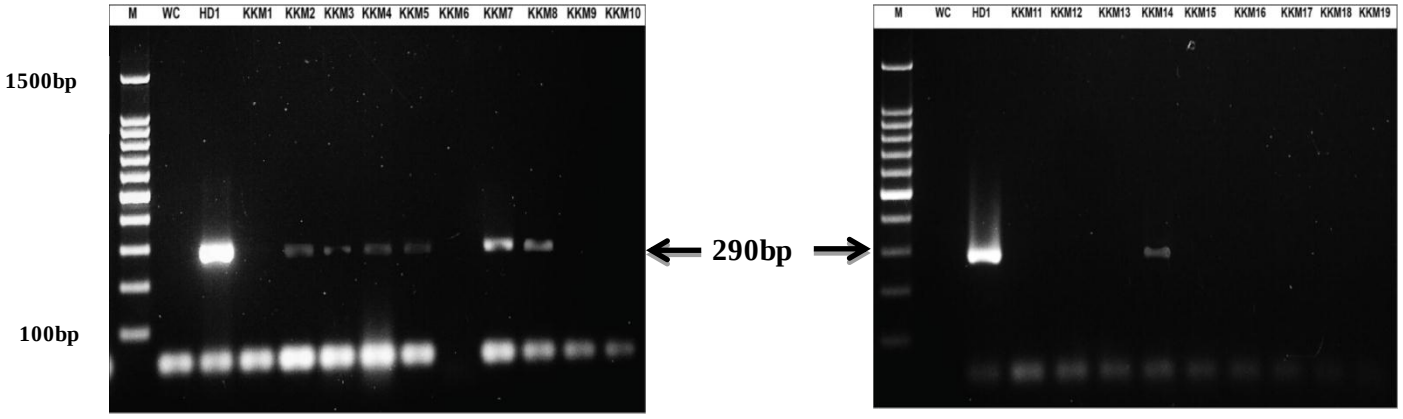
Biochemical analysis

All strains showed positive results to motility test, methyl red (MR) test, catalase test. All isolates were negative for Voges Proskauer (VP) test and isolates KKM 3, KKM 4, KKM 8, KKM 11, KKM 13, KKM 15, KKM 16, KKM 17 and KKM 18 showed negative reaction to starch hydrolysis test.

Screening of *cry* genes for *cry1, cry2Aa, cry2Ab*

Nineteen isolates and one reference strain HD1 were further characterized by PCR method to identify *cry* gene spectrum of native *B. thuringiensis* strains.PCR analysis was performed with three specific primers *i.e., cry1, cry2Aa, cry2Ab* to screen toxic *cry* genes. Three primers were successfully amplified in the isolates (Plate 3).

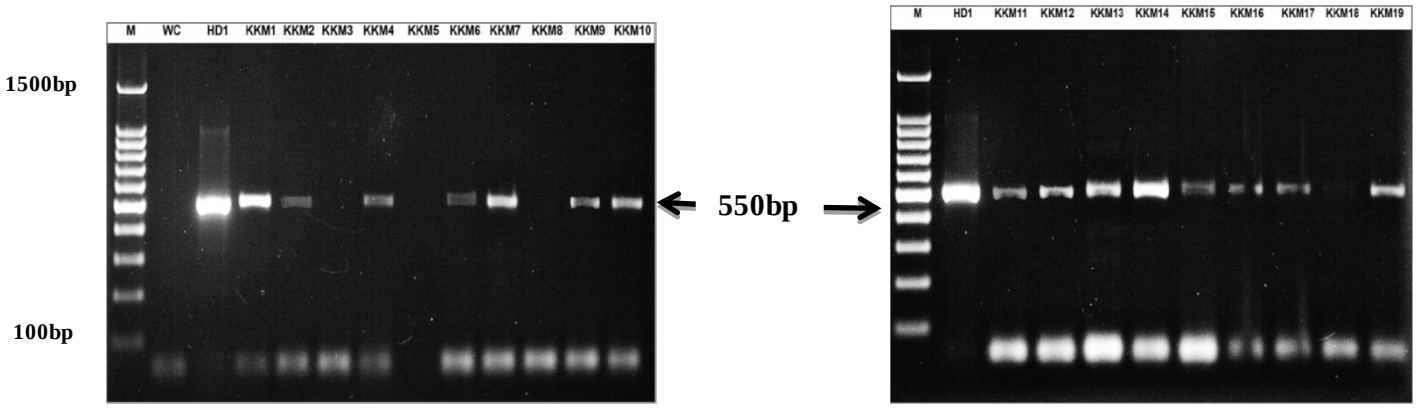
cry 1 genes



M: 100bp marker; WC: negative control; HD1: Positive control; KKM 1 to 10: Isolated *Bt* strains

M: 100bp marker; WC: negative control; HD1: Positive control; KKM 11 to 19: Isolated *Bt* strains

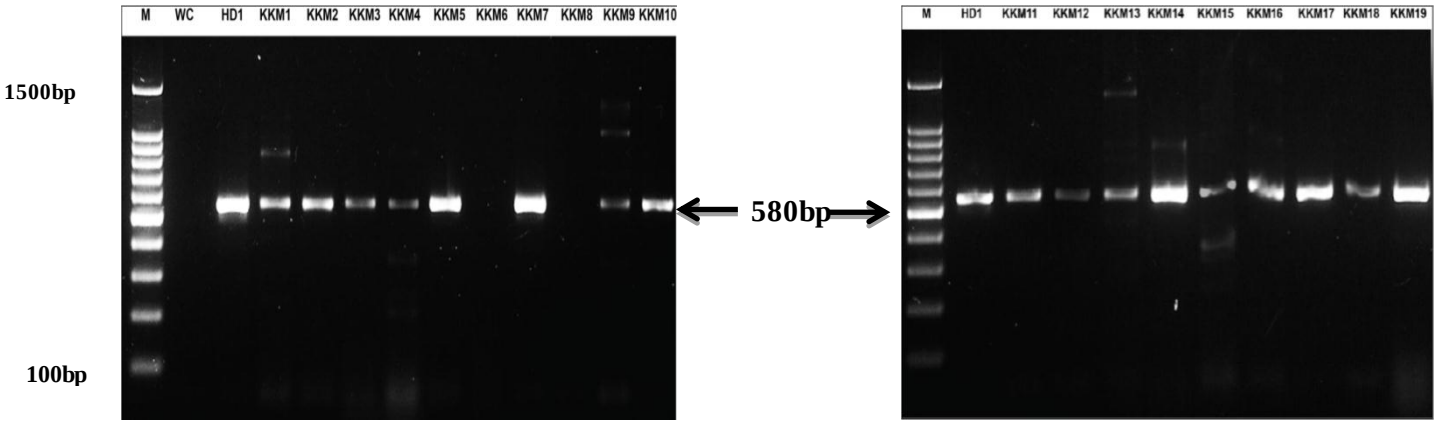
***cry 2Aa* genes**



bp marker; WC: negative control; HD1: Positive control; KKM 1 to 10: Isolated *Bt* strains

M: 100bp marker; HD1: Positive control; KKM 11 to 19: Isolated *Bt* strains

***cry 2Ab* genes**



bp marker; WC: Negative control; HD1: e control; KKM 1 to 10: Isolated *Bt* strains

M: 100bp marker; HD1: Positive control; KKM 11 to 19: Isolated *Bt* strains

Plate 3. Screening of lepidopteran toxic *cry* genes

Protein estimation

PCR screened nineteen *B. thuringiensis* isolates and reference strain *Bt*-HD1was subjected to protein estimation. Protein concentration was estimated by Lowry's method. KKM 14 had contained highest protein concentration (17.88 mg) followed by KKM 2 (13.82 mg). KKM 1 had lowest protein concentration (0.60 mg). Reference strain *Bt*-HD1 had 15.85 mg protein concentration which was comparatively lower than KKM 14.

Toxicity of *B. thuringiensis* strains against *S. frugiperda*

Entomopathogenic potential of nineteen strains was found by conducting preliminary bioassay against neonate larvae of *S. frugiperda*. 20 µl of Crude crystal protein was coated on the maize leaf disc which was fed up by neonate larvae. Among nineteen isolates, KKM 14 (62.33%), KKM 2 (60.67%), KKM 4 (58.67%) and KKM 7 (56.67%) strains were on par with each other with highest per cent mortality. The per cent mortality recorded by *B. thuringienis* strains was inferior to reference strain *Bt*-HD1 (90.00%). KKM 5, KKM 17 and KKM 18 isolates were on par with each other which showed lowest per cent mortality (16.67%) (Table 8).

Table 8. Per cent mortality caused by *B. thuringiensis*strains against neonate larvae of *S. frugiperda*

S.No.	Strains	Per cent Mortality
1	KKM 1	33.33 (35.22)^{def}
2	KKM 2	60.67 (51.16)^{b}
3	KKM 3	26.67 (31.00)^{fgh}
4	KKM 4	58.67 (50.00)^{b}
5	KKM 5	16.67 (23.86)^{i}
6	KKM 6	36.67 (37.22)^{de}
7	KKM 7	56.67 (48.83)^{b}
8	KKM 8	20.00 (26.57)^{hi}
9	KKM 9	23.33 (28.78)^{ghi}
10	KKM 10	30.00 (33.21)^{efg}
11	KKM 11	43.33 (41.15)^{c}
12	KKM 12	33.33 (35.22)^{def}
13	KKM 13	40.00 (39.23)^{cd}
14	KKM 14	62.33 (52.15)^{b}
15	KKM 15	33.33 (35.22)^{def}
16	KKM 16	23.33 (28.78)^{ghi}
17	KKM 17	16.67 (23.86)^{i}
18	KKM 18	16.67 (23.86)^{i}
19	KKM 19	43.33 (41.15)^{c}
20	HD-1	90.00 (71.57)^{a}
21	Control	0.00 (1.65)^{j}

DISCUSSION

The present investigation was made on isolation, characterization and bioassay against *S. frugiperda*. The selection of newer native strains help in building up of genetic pool, which is always necessary to keep in check the pests that are fast adapting themselves to biological pest control agents.

Aim of the study was to obtain novel native strain and also to study the distribution of *B. thuringiensis* in various locations of Tirunelveli and Tuticorin district (Fig. 1). Atotal of 50 soil samples were processed for isolation based on the 0.25 M sodium acetate selection and heat shock treatmemt methodology given by Travers *et al.*(1987). Several authors previously reported that *B. thuringiensis* has been broadly present in soil (Ohba and Aizawa, 1986;Martin and Travers, 1989; Chilcott and Wigley, 1994). Nineteen strains were isolated and identified as *B. thuringiensis* based on the crystal morphology. Distribution frequency of *B. thuringiensis* in native soil samples was 0.33 – 0.75% (Fig. 2). Distribution frequency of *B. thuringiensis* differs from soil to soil which was reported by 9.67%(Martin and Travers, 1989) to 70% (Chilcott and Wigley, 1994).

Colonies of all the isolates were creamy white to off white colonies with regular or irregular shape, entire or errose or undulated margin, slightly raised elevation and glistening surface. The results are in accordance with the findings of Chatterjee *et al.*(2007) who reported that circular, white, flat, and undulate colonies were observed in their studies. Similar colonial morphology results were also reported by El-kersh *et al.* (2012); Mukhija and Khanna, (2018).

All the nineteen isolates produced rod shaped and violet color cells which were indicated the presence of gram positive *Bacillus* species and similar resultsalso reported by Das *et al.*(2015). In the present study three types of crystals were observed in all the nineteen strains *i.e.,* bipyramidal, cuboidal, spherical shape of crystals and spores attached with crystals. Cuboidal shape of crystal showed highest frequency (42.36%) followed by bipyramidal crystal (27.43%). Bipyramidal shape of crystals were observed in the findings of Hofte and Whiteley (1989). Li *et al.* (2015)reported that *Bt*-HD521 strain had bipyramidal parasporal crystals. Ammouneh *et al.* (2011)observed bipyramidal and cuboidal parasporal bodies in their studies.

All strains were positive for motility test, Methyl Red (MR) test, and catalase test and negative for Voges Proskauer (VP) test. Similar results were observed by Gorashi *et al.* (2014) and they reported that all strains showed positive reaction for catalase and nitrate reduction and showed negative results for Malonate and Voges Proskauer test. These findings are similar with the findings of Das *et al.* (2015).

In the present investigation three specific primers *i.e., cry1, cry2Aa, cry2Ab* were used to screen lepidopteran toxic *cry* genes and successfully amplified. Pinto *et al.* (2003) also found that the amplification of lepidopteran toxic *cry* genes *viz., cry1, cry2, cry9* genes (56.51 %) in their study. Among nineteen isolates, 7 isolates were amplified for *cry1* gene with the frequency of 36.84% and 15 of them were amplified for *cry2Aa* gene which showed 78.94% frequency. Udayasuriyan and Balasubramani (2018) also found similar variation in the amplification of *cry* genes and reported that most of the isolates (41.4%) possessed *cry1* gene followed by *cry2* gene (35.7%). About 17 isolates possessed *cry2Ab* gene which showed high frequency (89.47%) compared to others (Fig. 3). These findings are similar to the findings of (Reyaz *et al.*, 2017) and they reported that *cry1, cry2Aa, cry2Ab* genes were amplified in six strains. Djenane *et al.* (2017) also found similar results in PCR amplification and reported that 50% of the isolates harbored *cry1, cry2 or cry9* genes and 69.3% contained a vip3 gene. Wang *et al.* (2003) also found that 70% of the isolates harbouring *cry2* genes.

Combination of different *cry* genes in same isolate was reported in several findings (Martinez and Caballero (2002); Ammouneh *et al.* (2011). In this study *cry1* and *cry2Aa* combination was existed in 4 isolates which showed 21.05% frequency and these results were related with Ben-Dov *et al.* (1997), Bravo *et al.* (1998). Combination of *cry1* and *cry 2Ab* gene was present in 6 isolates with frequency of 31.57%. In this study, 15 isolates had combination of *cry2Aa* and *cry2Ab* genes with the frequency of 73.68% and the results are in accordance with the findings of Liang *et al.* (2011) they revealed that the combination of *cry2Aa/cry2Ab* genes were the most frequently observed in their studies. Combination of *cry1, cry2Aa, cry2Ab* geneswere observed in 4 isolates (KKM 2, KKM 4, KKM 7 and KKM 14) and 21.05% of frequency was observed in this combination. Most of the isolates possessed *cry2Ab* gene and this findings are similar with the findings of Mendoza *et al.* (2012). Reyaz *et al.* (2017) also reported that 90% isolates harbouring *cry2Ab* gene. Lone *et al.* (2017) also found different combinations of *cry* genes in their studies. Reference strain *Bt*- HD1 was amplified for three genes *viz.,cry1, cry2Aa, cry2Ab*. Protein concentration was estimated by Lowry's method (Fig. 4). KKM 14 was contained highest protein concentration (17.88 mg) followed by KKM 2 (13.82 mg). Reference strain *Bt*-HD1 had 15.85 mg protein concentration which was comparatively lower than KKM 14. Similarily Barreto *et al.* (1999) estimated the total protein content and also concluded that toxicity of *B. thuringiensis*strains which were not associated with the effect produced by the parasporal crystal inclusions. Alves *et al.* (2000) also estimated protein content of the isolates.

S. frugiperda has been reported as a serious invasive pest in India and 9.0 to 62.5 % of incidence was

observed (Kalleshwaraswamy *et al.*, 2018). PCR screened *Bt* isolates were selected for preliminary bioassay against *S. frugiperda* and insecticidal activity of these isolates were also assessed (Fig. 5). Several authors studied the susceptibility of *S. frugiperda* for different *B. thuringiensis*strains (Bernardi *et al.*, 2016 and Burtet *et al.*, 2017). The results of the present investigation revealed that KKM 14 (62.33%) and KKM 2 (60.6%) were caused more than 60 per cent mortality whereas KKM 5, KKM 17 and KKM 18 were shown lowest per cent mortality (16.67%). Bohorova *et al.* (1997) studied the performance of *cry1*gene on S. *frugiperda* larvae and found that *cry1D* and *cry1F* gene had highest insecticidal activity.

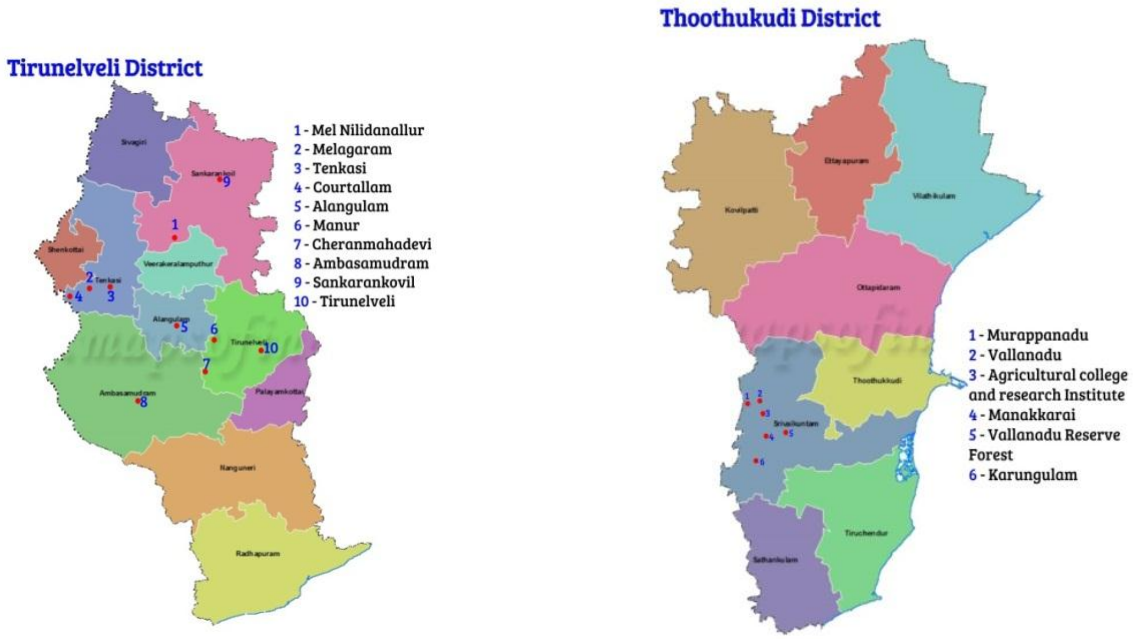


Figure 1. Soil samples collected from various locations of Tirunelveli and Tuticorin district

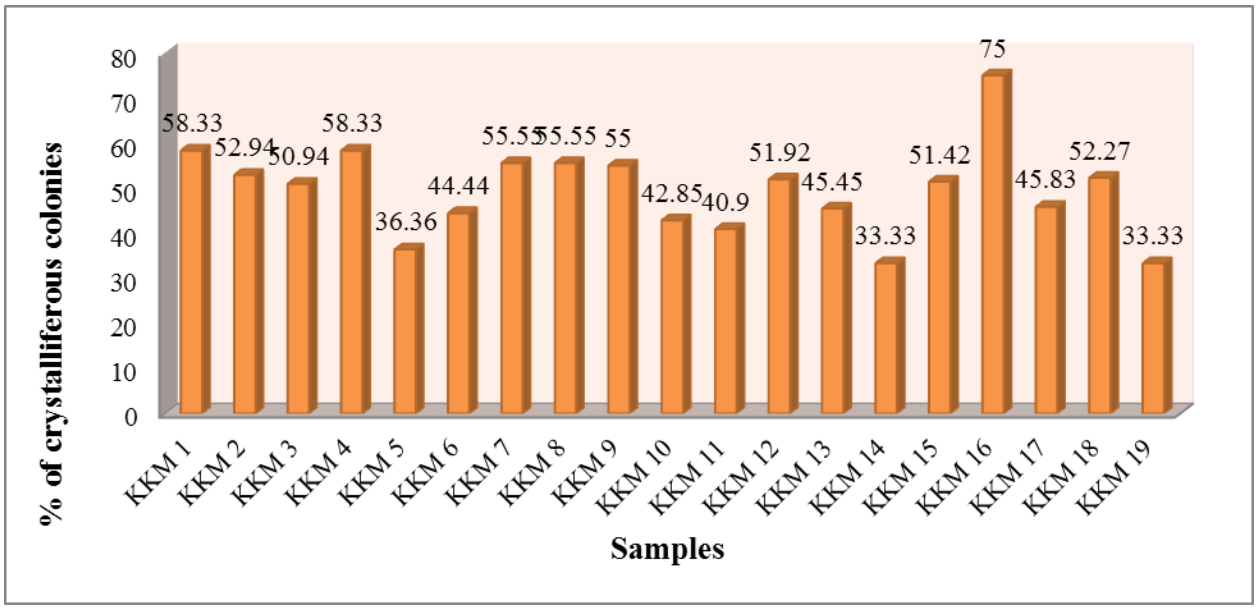


Figure 2. Distribution of *B. thuringiensis* in soil samples

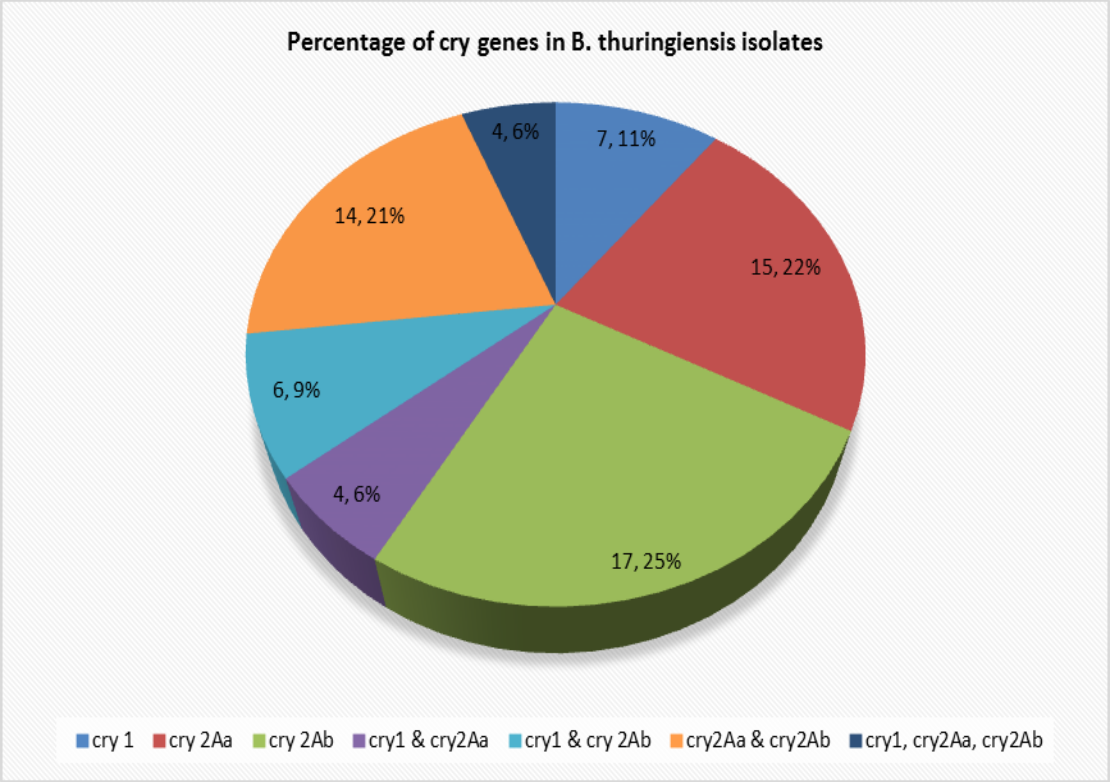


Figure 3. Percentage of *cry* genes in *B. thuringiensis* strains

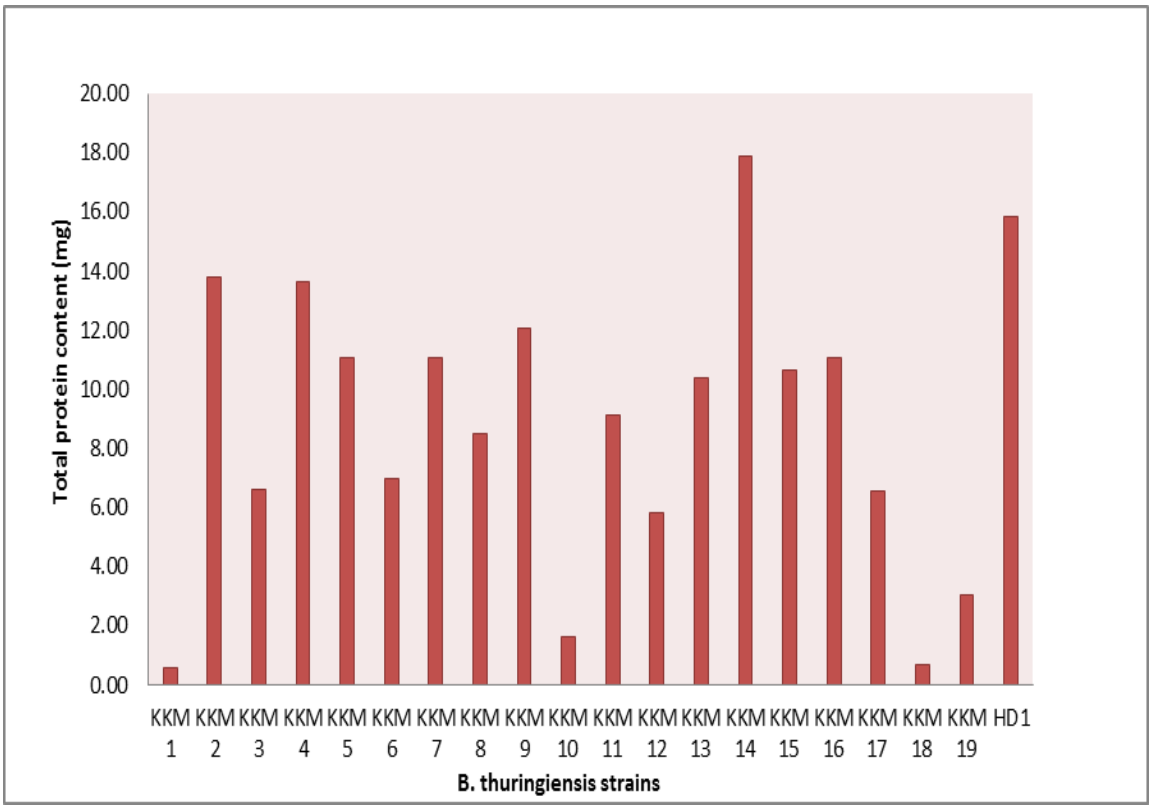


Figure 4. Total protein content of *B. thuringiensis* strains

Figure 5. Per cent mortality caused by *B. thuringiensis* strains against neonate larvae of *S. frugiperda*

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