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PCR pathotyping of native *Bacillus thuringiensis* from Mizoram, India

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ABSTRACT

Bacillus thuringiensis is a ubiquitous, gram-positive and spore-forming bacterium. During sporulation, it produces intracellular crystal (cry) proteins, which are toxic to insects. The genetic diversity of *B. thuringiensis* strains shows regional differences. Thus, each habitat may contain novel strains with new insecticide. The aim of this study was to isolate *B. thuringiensis* strains from different environments of Mizoram, India, and to identify the *cry* gene content of the isolates using PCR. The universal primers specific to *cry1, cry2, cry3, cry4* and *cry9* genes were used to detect the type of *cry* gene carried by each environmental isolate. Altogether, a total of 42 *cry* genes were detected out of which 12 were *cry1*, 5 were *cry2*, 3 were *cry3*, 18 were *cry4* and 22 were *cry9* out of 45 selected strains.

Key words: *Bacillus thuringiensis*; *cry*; PCR; insecticidal prediction .

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive, soil dwelling, insect, stored-product dust, and deciduous and coniferous leaves bacterium, having the genome size of about 2.4-5.7 million basepairs that forms characteristic protein inclusions adjacent to the endospore.[1-4] *Bt* makes a dormant spore with one or more large crystalline parasporal inclusion proteins, called δ-endotoxins which have a toxic and deadly effect on insects and are formed by different insecticidal crystal proteins (ICP). *Bt* subspecies can synthesize more than one parasporal inclusion. Crystalline inclusion bodies account for up to 30% of the total protein content of the bacterium.[5]

The basic phenotypic taxon is the subspecies, identified by the flagellar (H) serotype.[6] By 1998, 67 subspecies had been described. The genes that encode the ICPs are mostly on conjugative plasmids. Each ICP is the product of a single gene. Two kinds of insecticidal crystal proteins genes were identified, the *cry* (for "crystal") genes, and the *cyt* (for "cytolytic") genes. 168 *cry* and *cyt* genes and 28 groups of *cry* genes are currently know.[5,7]

It is now realized that resistance to *Bt* can evolve rapidly under situations of selection pressure. However, the insect lines which developed resistance to a particular kind of *Bt*

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crystal proteins were found to be sensitive to a different kind of *Bt* crystal protein. Therefore, searches are continued throughout the world to isolate novel *Bt* crystal proteins, which might be effective killing the *Bt*-resistant insect lines. It has been well established that *Bt* is globally distributed in a wide range of natural environments. *Bt* accounts for about 5-8% of *Bacillus* spp. population in the environment.[8] The distribution of this bacterium in Mizoram soil remains unexplored and hence, *Bt* are isolated from different soils to know the geographical diversity with the prediction of their insecticidal activity through polymerase chain reaction (PCR).

MATERIALS AND METHODS

Isolation of Bt *from soil samples*

A total of 428 soil samples from 39 spots with 11 different habitats in Mizoram, India, were used for isolation of *Bt* (Table 1). Soil samples were collected from top to a depth of 10 cm. *Bt* strains were isolated following the technique of Travers *et al*.[9] Soil samples (1 g) were incubated in a shaker for 4 h at 37°C in 10 ml of Luria Broth (LB) buffered with 0.25 M sodium acetate (pH 6.8). 1 ml of the sample was serially diluted to 10^3 times, heat shocked at 80°C for 3-5 min. Then the samples were spread on LB agar plates. (Fig. 1).

DNA isolation

A total of 28-45 isolates were selected after identification and biochemical characterization. DNA was extracted according to Bobrowski *et al.*[10] and was used as a template for PCR. The cultures were incubated overnight at 30°C in LB agar at 37°C. After 16-20 h one loop full of culture was transferred to 300 µl of milliQ water and vortexed. It was then kept at -80°C for 15 min. The frozen DNA was immediately transferred to boiling water and kept for 10 min. The resulting cell lysate was

Table 1. *Bt* isolates and their *cry* gene profiling.

No	Site	Vegetation	District	*cry*
1	Khengkhawng	Shrub	Saiha	nil
2	Chhippui	Jhum	Mamit	2,9
3	Champhai	Paddy	Champhai	3,9
4	Campus	Shrub	Aizawl	4
5	Chhimtuipui	River banks	Lawngtlai	2,3,9
6	Ramrikawn	Fish pond	Aizawl	2,3,9
7	West Phaileng	Jhum	Mamit	4
8	Sailam	Paddy field	Aizawl	4,9
9	Hmunpui	Jhum	Mamit	9
10	Sailam	Stream banks	Aizawl	9
11	Serkawr	Grass	Saiha	1,4,9
12	Sihhmui	Jhum	Aizawl	4,
13	Serchhip	Jhum	Serchhip	9
14	Seling	Jhum	Aizawl	4,9
15	Sairang	Jhum	Aizawl	9
16	Champhai	Paddy side	Champhai	9
17	Lengte	Forest	Mamit	nil
18	Lengpui	Teak plantation	Mamit	2,9
19	Lengpui	Fish pond	Mamit	9
20	Lengpui	Papaya	Mamit	nil
21	Lengpui	Bamboo	Mamit	9
22	Rawpuichhip	Jhum	Mamit	nil
23	Champhai	Grass	Champhai	1,2,9
24	Thenzawl	Fish pond	Serchhip	4
25	Lunglei	Flower garden	Lunglei	1,4,9
26	Chhippui	Roadside	Mamit	1,9
27	Saiha	shrub	Saiha	1,4
28	Serkawr	Forest	Saiha	9
29	Serkawr	barren	Saiha	1,4,9
30	Lawngtlai	Roadside	Lawngtlai	1,4,9
31	Lawipu	Trees	Aizawl	4,9
32	Lawipu	Jhum	Aizawl	1,4
33	Tuivamit	Teak	Aizawl	nil
34	Ropaiabawk	Grass	Aizawl	nil
35	Tanhril	Banana	Aizawl	4
36	Ropaibawk	Bamboo	Aizawl	4
37	MZU campus	Grass	Aizawl	1,4
38	MZU clinic	Barren	Aizawl	4
39	Zawrkawt	Flower garden	Aizawl	1
40	Tanhril	Banana	Aizawl	1
41	Tuivamit	Turmeric	Aizawl	4
42	Zonuam	Water hole	Aizawl	nil
43	Chite	Shrub	Aizawl	nil
44	Dampa	Mixed	Mamit	nil
45	Zawlnuam	Jhum	Mamit	1

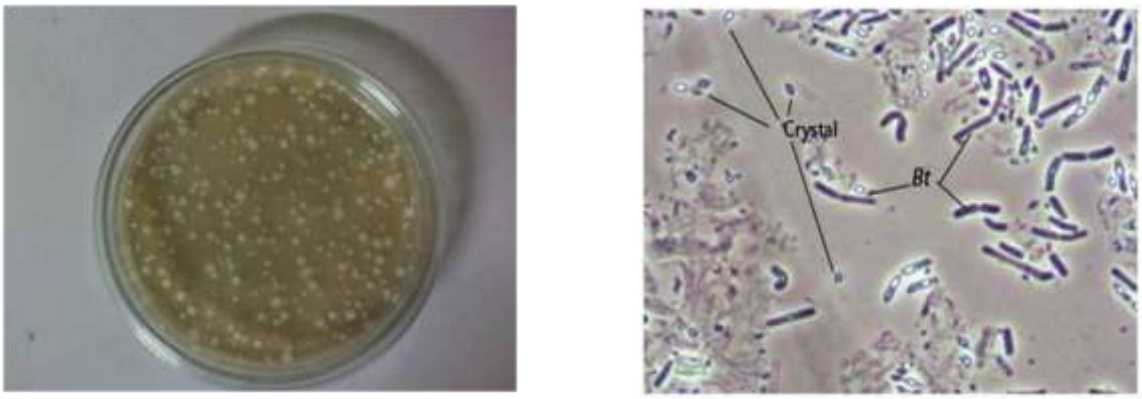


Figure1. *Bt* on nutrient agar and *Bt* with crystals (Phase contrast).

briefly spun at 6000 rpm for 3-4 sec. The supernatant was used as the DNA template.

Oligonucleotide primers for PCR

The primers used in this study were described by Ben-Dov *et al*.[11,12] One pair of universal primers (e.g. Un1 direct and reverse) for each four homology groups was applied to amplify a specific fragment. Their sequences and the expected sizes of their PCR products are shown in Table 2.

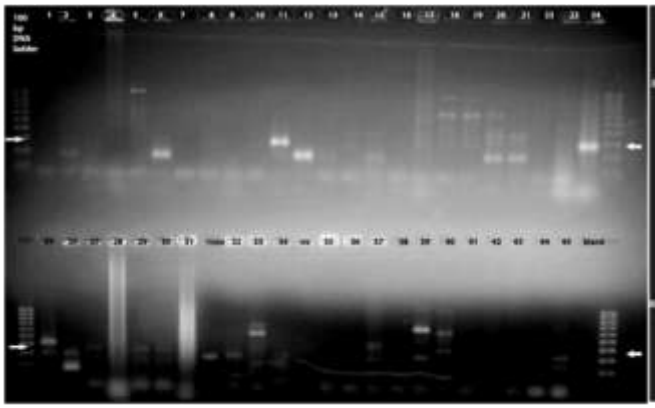
Cry *gene profiling and PCR pathotyping of the isolates*

All PCR reactions were carried out in 50 µl reaction tubes. For 10 µl reaction, 2 µl of template DNA was mixed with reaction buffer containing 200 µM dNTP mix, 0.5 µM (reverse and direct) primers, 3 mM magnesium chloride, and 1.5 U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Eppendorf). The conditions for PCR were as follows: a single denaturation step for 3 min at 94°C, a step cycle program set for 34 cycles with a cycle of denaturation step for 1 min at 94°C, annealing for 45 seconds at 54°C for *cry1* and 60-62°C for *cry2*, *3*, *4* and *9* with extension for 30 min at 72°C. Finally, an extra extension step of 5 min at 72°C was used. Each experiment was performed with positive (a standard template) control and negative control.[10-12]

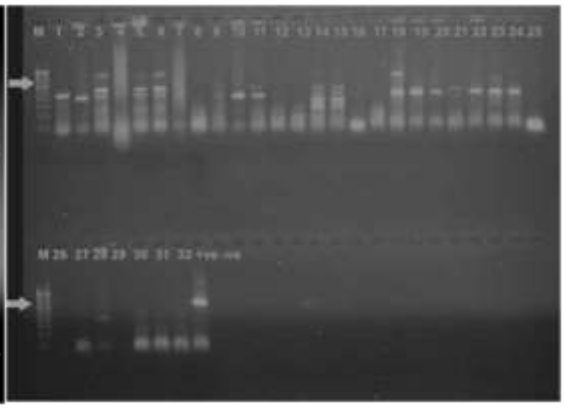
Table 2. Primers used in the present study.

Cry genes		Primer sequence	Expected size
cry1	Un1, D1	5'- CATGATTCATGCGGCAGATAAAC -3'	270-320 bp
	R1	5'- TTGTGACACTTCTGCTTCCCATT -3'	
cry2	Un2, D2	5'-GTTATTCTTAATGCAGATGAATGGG -3'	680-720 bp
	R2	5'- CGGATAAAATAATCTGGGAAATAGT -3'	
cry3	Un3, D3	5'- CGTTATCGCAGAGAGATGACATTAAC -3'	580-620 bp
	R3	5'- CATCTGTTGTTTCTGGAGGCAAT -3'	
cry4	Un4, D4	5'- GCATATGATGTAGCGAAACAAGCC -3'	420-450 bp
	R4	5'- GCGTGACATACCCATTTCCAGGTCC -3'	
cry9	Un9, D6	5'- CGGTGTTACTATTAGCGAGGGCGG -3'	351-354 bp
	R6	5'- GTTTGAGCCGCTTCACAGCAATCC -3'	

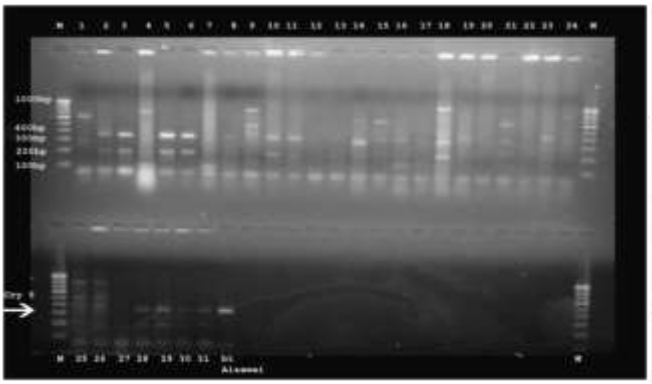
cry1 gene analysis. Arrow mark set at to 300 bp.



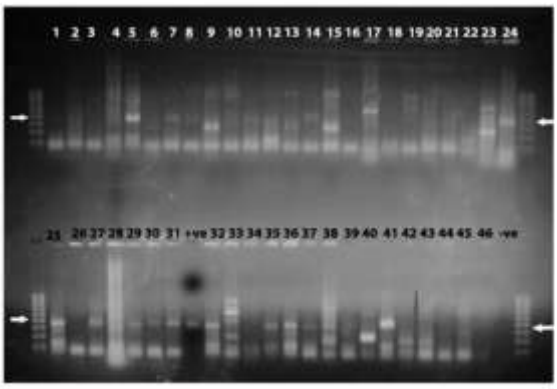
cry2 gene analysis. Arrow mark set at to 700 bp.



cry9 gene analysis. Arrow mark set at to 350 bp.



cry4 gene analysis. Arrow mark set at to 400 bp.



cry3 gene analysis. Arrow mark set at to 600 bp.

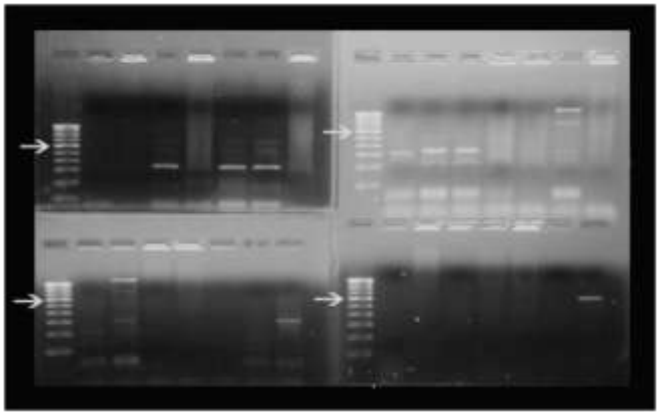


Figure 2. *cry* gene profiling of *Bt* isolates.

Gel electrophoresis

Following the amplification, 10 µl of each PCR sample was electrophoresed on 1.5% agarose-ethidium bromide gel in Tris-Actate/ EDTA (TAE) electrophoresis buffer (0.04 M Tris-Acetate, 0.001 M EDTA; pH 8) at 100V for 40 min.[10]

RESULTS AND DISCUSSION

Total DNA, i.e. genomic and plasmid, from each *Bt* isolate served as template in PCR reactions. Reactions without any DNA template served as negative control in each PCR experiment. Reactions with Un1 (direct and reverse), Un2, Un3, Un4, Un9 primers were carried out to detect *cry1*, *cry2*, *cry3*, *cry4* and *cry9* genes, respectively. A similar PCR analysis for *cry*-type genes was previously reported by Ben-Dov *et al.*[11,12] Each PCR analysis was checked with the appropriate positive control strains of *Bt* such as *Bt* serovar. *Alesti, Bt* subsp. *kurstaki* for *cry1* and *cry2* gene groups, *Bt* biovar. *tenebrionis* for *cry3* gene groups, *Bt* serovar. *israelensis* for *cry4* group and *Bt* subsp. *aizawai* for *cry9* gene groups. All positive controls gave the expected PCR products. All the DNA ladder used was 100 bp DNA ladder (Bangalore Genei).

When the template DNA from environmental isolates of *Bt* samples was amplified with PCR in the presence of primers for *cry1* gene, twelve isolate were shown to contain *cry1* gene (lanes 11, 23, 25, 26, 27, 29, 30, 32, 37, 39, 40, 45) which is lepidopteran larvae specific as in the positive control (Fig. 2 & Table 1). Universal primers produced PCR products of expected size at around 270-320 bp. However, some isolate produced a strong band at 250-350 bp, which was larger than the expected size (lane 12, 20, 21, etc). Because 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. Therefore, the band at 250-350 bp could correspond to a different sub-type of *cry1* gene. Carozzi *et al.*[1] suggested that novel isolates containing novel *cry* genes may give PCR products different in size relative to the standard or may completely lack PCR products. Therefore, homology to known *cry* genes for these strains can be analyzed by using additional primers. In this present study, some *Bt* isolates (8-0.25-C) containing *cry1* gene produced PCR product profile that was different from that of reference strain. This *Bt* isolate most probably contains subgroups of *cry1* gene. Future experiments with specific primers for *cry1* gene subtypes is expected to reveal the type of *cry* gene subgroup and also new *cry* gene carried by this isolate.

30 isolates were screened for *cry2* with one blank (-ve control) along with a +ve control (*Bt. kurstaki*). The gel did not clearly separate around 700 bp, as such bands are purified and sequenced and then BLAST. Five isolates (lane 3, 5, 6, 18, 23) are found to be having *cry2* gene. *cry3* gene is rare among the isolates, only three (lane 3, 5, 6) are found to be having *cry3* gene. But, the number of isolates that has been profiled are of only 28 in numbers, future study will only reveal the real status of being considered as infrequent. The environmental isolates which produced 450 bp PCR product may contain a few *cry* gene subgroups, which might have homology with the *cry3* gene universal primer-binding region. *cry4* gene which code for the insecticidal protein that is toxic to the dipteran larvae. Out of 45 isolates 18 strains have *cry4* gene (lane 5, 7, 8, 11, 12, 14, 24, 25, 27, 29, 30, 31, 32, 35, 36, 37, 38, 41) (Fig. 2 & Table 2). Since this is one of the most abundant genes among the isolates, it might account for the low incidence of mosquito and other dipteran-borne diseases with respect to neighboring states and countries.

20 of the 31 isolates produced positive results with Un9 universal primers for *cry9* gene. Although, a single band at 300 bp was obtained with *Bt* subsp. *aizawai*, which served as positive control, the field-collected strains

of *Bt,* except isolates produced an extra band. This could due to the genetic diversity of *Bt* strains isolated from environmental samples. These strains may contain a few *cry* gene subgroups, which may have homology with the *cry9* gene universal primer-binding region. Altogether, the number of *cry1* detected clearly is 12, *cry2* is 5, *cry3* is 3, *cry4* is 18 and *cry9* is 22 (Fig. 2 & Table 2). The strain in which the *cry* gene was not detected could be due to different type of *cry* gene other than the primer used, or it could be a *Bt* strain which do not produce parasporal crystal protein.

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