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Distribution of *cry* and *cyt* Genes among Indigenous *Bacillus thuringiensis* Isolates with Mosquitocidal Activity

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ABSTRACT

Bacillus thuringiensis strains isolated from Madurai, TamilNadu, India were evaluated for their mosquitocidal activity, as well as *cry* and *cyt* genes diversity. It revealed that 99% of the parasporal crystal morphology of these isolates was spherical in nature and a variable percentage (0% - 100%) of toxicity was observed against *Culex quinquefasciatus* and *Aedes aegypti.* PCR analysis revealed that 53% of the isolates were positive for the various *cry* and *cyt* genes tested, whereas 47% did not produce any PCR product for the *cry* gene analyzed. Diverse pattern of *cry* and *cyt* genes distribution was observed even in the isolates from the same sample. *B. thuringienis* subsp. LDC-9 showed three-fold higher toxicity against *Culex quinquefasciatus* than that of *B. thuringiensis var israelensis* which might be used as a potential strain to control mosquitoes in near future after field evaluation.

Keywords: *Bacillus thuringiensis*; Crystal Endotoxin; *Culex quinquefasciatus*; *Aedes aegypti*

1. Introduction

The search for native strains to control dipteran species have an impact on the control of mosquitoes worldwide, as vector borne diseases are major public health problems and their prevalence has dramatically increased worldwide [1]. Chemical insecticides have been proved to be very effective in vector control programme, but their adverse environmental effects, insecticidal resistance and resurgence have prompted the search for alternative strategies for insect pest control [2]. Among the various alternatives, *B. thuringiensis* and *B. sphaericus* are the most potent and successful group of organisms for effective control of pests among the microorganisms [3]. The environmental safety of *Bt.* based products employed in pest control methods are well documented [4]. *B. thuringiensis* is a gram positive organism that synthesizes crystalline inclusions (*δ*-endotoxins) during sporulation. These toxins are highly specific, completely degradable and harmless to humans, vertebrates and plants. Hence, researchers across the world are interested for screening new strains with increased levels of insecticidal toxicity with a broader spectrum of activity [5]. The various screening programmes resulted in the number of *B. thuringiensis* strains not only active against Lepidoptera, Diptera, Coleoptera but also against Hymenoptera, Phthiraptera or Mallophaga, Acari, Nematheliminthes, Platyhelminthes and Sarcomastigophora [6-8].

The insecticidal activity of *B. thuringiensis* strains against Dipterans is attributed to the presence of Cry and Cyt proteins [9]. Cry toxins are activated by host proteases, which interact with specific receptors located on the host cell surface, resulted in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast, Cyt toxins directly interact with membrane lipids and insert into the membrane [10]. The mosquitocidal activity of a *B. thuringiensis* strain is due to the additive effect of each toxin and a complex synergistic interaction among them. *B. thuringiensis* subsp. *israelensis* produces four Cry toxins such as Cry4Aa, Cry4Ba, Cry10Aa, and Cry11Aa, Cyt1Aa and Cyt2Ba respectively [11,12]. The presence of the Cyt toxin synergizes and delays or prevents the development of resistance to Cry toxins by functioning as a Cry-membrane bound receptor [10]. However, information on the diversity and distribution of *cry* and *cyt* genes among mosquitocidal *B. thuringiensis* isolates from southern part of India is negligible [13,14]. Hence, the present study was envisaged to analyze the distribution of the *cry* and *cyt* genes of indigenous mosquitocidal *B. thuringiensis* isolates.

2. Materials and Methods

2.1. Sampling Procedure

Triplicate samples were collected with an internal di-

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ameter of 2.5 cm from different locations of Madurai district, Tamilnadu, India situated at an altitude of 100.58 meters above mean sea level, 9.3˚N latitude and 77˚E longitude with annual rainfall of 85 cm, relative humidity of 40% - 70% with mean maximum and minimum temperature of 37.5˚C and 20.9˚C respectively. A total of 360 soil samples were collected which included 60 samples from the various ecological niches such as River bank, Subterranean, Urban, Agricultural land, animal contaminated soils and mountain regions as indicated in **Table 1**. Approximately, 10 g of soil samples were collected from four different sites of each location and pooled into one sample, homogenized by thorough mixing, sieved, air-dried up to 20% moisture content [15] and stored in sealed polythene bags within desiccators. A subsample of 1 g was used for further analysis. The storage time ranges from few days to three weeks with moisture level of 20%.

Leaf samples such as *Murraya koengii* and *Ricinis communis* were obtained from 2.0 to 2.5 m above the ground, 0.3 m inside the outer leaf canopy and from the east side of each tree or shrub. Cross contamination between samples was prevented by detaching leaves or needles while they were enclosed in standard plastic sandwich bags, which were immediately sealed for storage.

Samples containing leaf litters, leaf dust and animal droppings (contaminated samples) were collected and stored at 4˚C until use. A total of 60 samples of leaf litters were collected from Alagarkovil. Approximately 10 number of dead insect includes centipede, millipede, *Spodoptera* larva, pupa and *Culex quinquefasciatus* larva were assumed to be infected by *B. thuringensis* were collected from soil (sandy soil), plant surface (*Ricinis communis* leaf) and water bodies (stagnant water) using sterile forceps and was transferred in sterile polythene bags to the laboratory for analysis.

2.2. *B. thuringiensis* Isolation

The insect samples except *Culex quinquefasciatus* were surface sterilized following the methodology described by Alves [16] which eliminated external contamination. The larvae were passed first in 70% alcohol for 2 sec, followed in 5% sodium hypochlorite for 3 min and finally in sterile 10% sodium thiosulfate for 5 min. The specimens were then washed three times in sterile distilled water and transferred aseptically into a sterile mortar and macerated with a sterile pestle. The macerate was resuspended in 10 mL sterile distilled water and 5 mL was heated at 80˚C for 3 min followed by cooling on ice for 5 - 10 min. The diluted samples (0.1 mL) were plated on nutrient agar and plates were incubated at 33˚C ± 2˚C for 24 h. In case of leaves, the leaf sections with an area of approximately 2 - 3 cm^2 were trimmed and ground with a small mortar with 1 mL sterilized distilled water. Soil samples, animal contaminated soil samples, leaf litter samples, leaf dust, effluent samples were prepared as suspensions in 10 mL sterile distilled water. 5 mL of the resulting suspensions were transferred in a fresh tube and incubated in the water bath at 80˚C for 3 min followed by cooling on ice for 5 - 10 min. The aliquots (0.2 ml) were spread on plates of nutrient agar and incubated at 33˚C ± 2˚C for 24 h.

2.3. Genomic DNA Isolation

Total genomic DNA of *B. thuringiensis* was isolated by the method of Kalman *et al*., [17] with some modifications. Cultures were grown in Luria-Bertani broth (10 g tryptone, 5 g yeast extract, 10 g $NaCl \cdot L^{-1}$) to an optical density of 0.8 at 600 nm. The cells were washed once in 0.5 mL of TES (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl) and resuspended in 2 mL of SET (25% sucrose, 25 mM EDTA, 25 mM Tris-HCl (pH 8.0) with 2 mg of lysozyme$\cdot mL^{-1}$ and were incubated at 37˚C for 1 h. Then SDS was added to a final concentration of 1% and incubated at 50˚C for 5 min and 4˚C for overnight. The supernatant was then extracted thrice with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with ethanol. Following centrifugation, the washed DNA pellets were resuspended in 100 µl of 1X TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

2.4. PCR Amplification

The family-specific primers (**Table 1**) for *cry* (*cry*2, *cry* 4-spe, *cry*10-spe and *cry*11-gen) and *cyt* (*cyt*1 *gra1*, *cyt*2 *gra*1) genes were used for screening *cry* and *cyt* genes through Polymerase Chain Reaction (PCR) following the method of Ibarra *et al.*, [18]. The PCR mix (25 µL) consisted of 30 ng of total genomic DNA, 1X buffer, 3 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, I U proofreading Taq DNA polymerase, 1.0 µM each primer set and 3% DMSO as PCR additive. Amplification was done in an Eppendorf PCR system (Master cycler Personal 5332, Eppendorf, Hamburg, Germany) with a 3 min denaturation step at 95˚C, followed by 30 amplifications cycles of 95˚C for 1 min, 52˚C for 1 min, 72˚C for 1 min and an extra extension step of 10 min at 72˚C. The amplified products were electrophoresed on agarose gel [19] and filed using Gel-Doc photodocumentor device (Geneline, Spectronics, India). *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *israelensis* (gift from *Bacillus* Genetic Stock Center, Ohio) were used as positive controls and *B. subtilis* as a negative control. The distribution frequency of *cry* gene in *B. thuringiensis* strains from a certain origin is defined as the percentage of *B. thuringiensis* isolates containing this gene among all the isolates from that origin.

Table 1. Characteristics of primers employed for screening *cry*1, c*ry*2, *cry*4, *cry*10, *cry*11 and *cyt* genes.

Primer pair	Sequence[a]	Positions[b]	Gene(s) recognised	Pdt size (bp)	Genbank Accession no.	Annealing Temp ˚C
*cry*1*gra1*	5'CTGGATTTACAGG-TGGGGATAT(d) 3'TGAGTCGCTTCGC-ATATTTGACT(r)	1472 - 2029	*cry*1*Aa*, *cry*1*Ab*, *cry*1*Ac*, *cry*1*Ae*, *cry*1*Af*, *cry*1*Ba*, *cry*1*Bb*, *cry*1*Bc*, *cry*1*Ca*, *cry*1*Cb*, *cry*1*Da*, *cry*1*Db*, *cry*1*Ea*, *cry*1*Eb*, *cry*1*Fb*, *cry*1*G*, *cry*1*Ha*, *cry*1*Hb*, *cry*1*Ia*, *cry*1*Ib*, *cry*1*Ja*, *cry*1*Jb*, *cry*1*K*	543 - 594	M11250 M73250	52
*cry*4*Aspe*	5'TCAAAGATCATTTC-AAAATTACATG(d) 5'CGGCTTGATCTATG-TCATAATCTGT(r)	1706 - 2165	*cry*4*Aa*	459	Y00423	55
*cry*4*Bspe*	5'CGTTTTCAAGACCTA-ATAATATA(d) 5'CGGCTTGATCTATGT-CATAATCTGT(r)	1868 - 2189	*cry*4*Ba*	321	X07423	55
*cry*10*spe*	5'TCAATGCTCCATCCA-ATG(d) 5'CTTGTATAGGCCTT-CCTCCG (r)	978 - 1326	*cry*10	348	M12662	55
*cry*11*gra1*	5'CGCTTACAGGATGG-ATAGG(d) 5'GCTGAAACGGCACG-AATATAAAT(r)	990 - 1332 1025 - 1368 1048 - 1400	*cry*11*Aa* *cry*11*Ba* *cry*11*Bb*	342 343 352	M31737 X86902 AF017416	55
*cyt*1*gra1*	5'CCTCAATCAACAGCA-AGGGTTATT(d) 5'TGCAAACAGGACATT-GTATGTGTAATT(r)	197 - 674 85 - 565	*cyt*1*Aa* *cyt*1*Ab*	477 480	X03182 X98793	55
*cyt*2*gra1*	5'ATTACAAATTGCAAA-TGGTATTCC(d) 5'TTCAACATCCACAGTA-ATTTCAAATGC(r)	509 - 865 529 - 884 649 - 1004 196 - 551	*cyt*2*Aa* *cyt*2*Ba* *cyt*2*Bb* *cyt*2*Ca*	356 355 355 355	Z14147 U52043 U82519 AAK50455	55

[a]Position at 5' end of direct and reverse primers for each PCR primer pair. [b]d and r, direct and reverse primers, respectively.

2.5. Preparation of Spore-Crystal Suspensions and Morphological Characterization

B. thuringiensis isolates were transferred to 50 ml SCG media [20] in 250 ml Erlenmeyer flasks and incubated for 3 days at 33˚C ± 2˚C with shaking at 250 r·min^{-1} [21]. Spore-crystal mixtures were harvested after complete autolysis of the cells and were centrifuged at 12,000 g (4˚C) for 20 min. The pellet was washed three times in 0.5 M NaCl, thrice in distilled water to eliminate extra cellular components, including proteases and β-exotoxins, known to accumulate in the cell culture supernatant and finally resuspended in sterile distilled water as 50-fold concentrate [22]. The morphology of parasporal body was analyzed as described [23] by Phase Contrast microscopy (Olympus DP-12, CX46, Tokyo, Japan) and Scanning electron microscopy for the spore-crystal complex as explained [24].

2.6. Bioassay

The 50-fold concentrated sporulated cultures of *B. thuringiensis* isolates were examined for qualitative toxicity against early fourth instar larvae of *Culex quinquefasciatus* and *Aedes aegypti* by one-dose assays, according to the method described previously [25]. Twenty larvae as triplicates were maintained in distilled water for the control experiment. Bioassays were repeated thrice with the 30 isolates that have shown mortality above 50%. Lethal concentrations (LC_{50}) were determined by probit analysis [26]. *B. thuringiensis* var. *israelensis* was employed as the positive control. The data were expressed as arithmetic mean ± standard error. Statistical analysis involved one-way analysis of variance (ANOVA) followed by Tukey's multiple pair wise comparison test. The levels of significance were expressed as *P*-value less than 0.05.

2.7. SDS-PAGE Analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously [27].

3. Results and Discussion

The results of the experiments performed in this study

are presented and discussed in the following sections.

3.1. Environmental Distribution of *B. thuringiensis*

The strains with typical *B. thuringiensis* colony morphology (fried egg appearance) were noted in 495 of the 540 samples collected from various ecological niches as shown in **Figure 1**. *B. thuringiensis* occurrence was highest in the agricultural soil (93%). Similar observation of the ubiquitous distribution of *B. thuringiensis* was previously reported [21]. *B. thuringiensis* abundance in soil might be due to the high levels of insect activity and a large amount of nutrients in the soil, allowing optimum survival and enrichment as previously reported [28]. While comparing *B.t* diversity from various samples, many isolates were selected from soil samples (89%) than from insect and leaf samples (67%) with respect to the biological origin as noted in **Table 2**. This observation deviated from studies of Wang *et al.* [29] wherein they have reported that the average frequency of *B. thuringiensis* isolates from soil samples were 29.8% only.

As *B. thuringiensis* is an insect pathogen, the soil samples were further analyzed based on the presence and absence of plants and insects, in order to correlate how environment influenced the densities of *B. thuringiensis.* The analysis of samples sites based on the presence and absence of plant communities revealed that *B. thuringiensis* index of 0.5 was observed in agricultural fields, which included plants such as *Oryza sativa* (Rice), *Brassica oleracea* (Cabbage), *Zea mays* (Corn), *Pennisetum glaucum* (pearl millet), *Pisum sativum var. sativum* (Garden pea), *Vigna mungo* (Black gram) and *Brassica napus* (Rapeseed) (**Table 2**). On the other hand, analysis of soil samples based on the presence and absence of intense insect activity revealed that *B. thuringiensis* index of 0.89 was isolated from soil samples without insects (**Table 3**). This observation did not correlate with the relationship between insect environments and densities of *B. thuringiensis* as indicated in the earlier studies [30,31].

The isolated *B. thuringiensis* strains were screened for crystal inclusions using Phase Contrast Microscopy. A total of 417 strains were selected among 1956 spore formers based on the crystal inclusions after Phase Contrast Microscopic observation. 99% of these isolates showed spherical parasporal inclusions (**Figure 2**). This observation was different from the earlier reports of

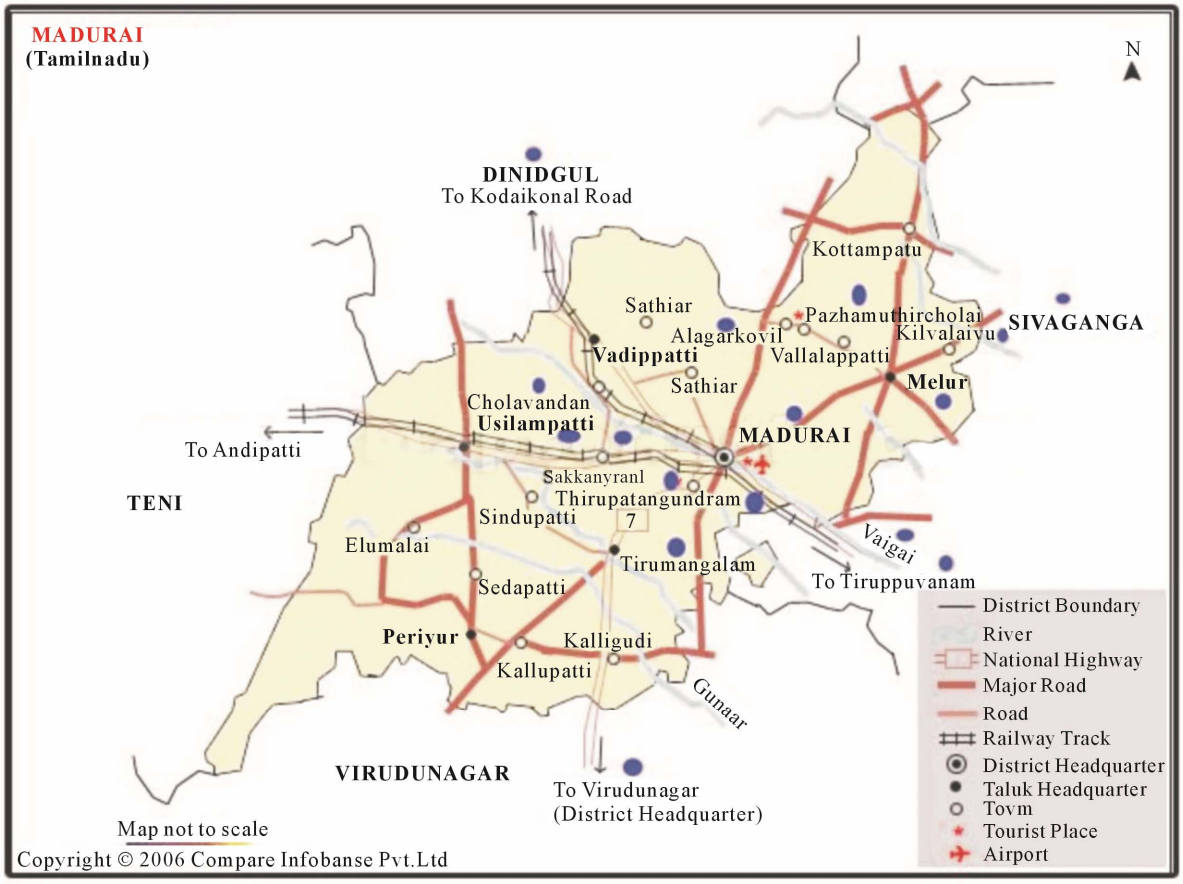


Figure 1. Map showing sample collected sites; closed circle blue dots indicates sample collected sites.

Table 2. Distribution of *B. thuringiensis* in different habitats of Madurai.

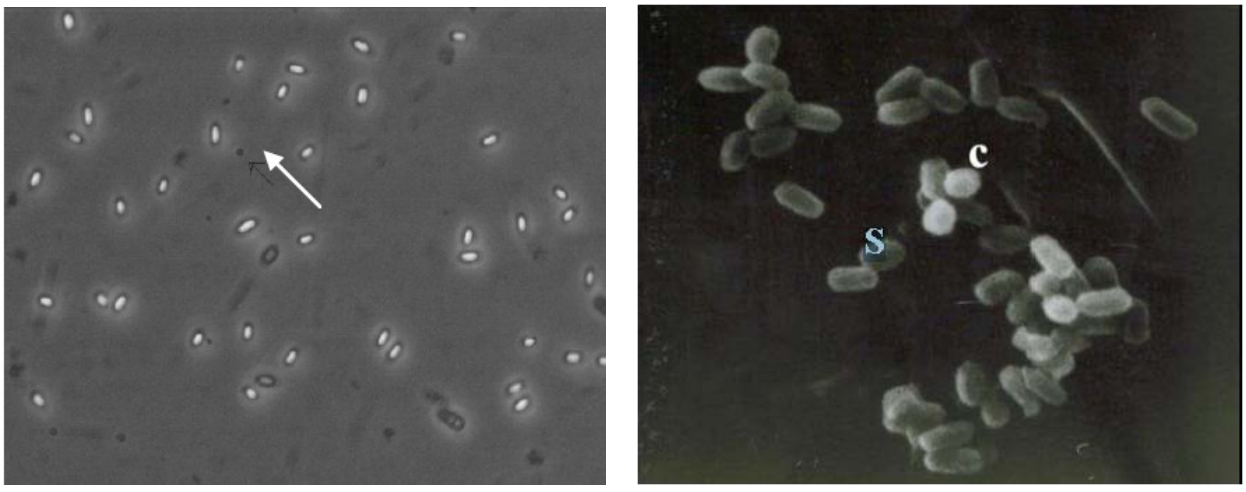
Habitat	No. of samples examined	No. of samples with atleast one *B. thuringiensis*	Percentage of samples with atleast one *B. thuringiensis*	*B. thuringiensis* Index*
River bank[a]	60	15	25	0.07 (50/642)
Subterranean[b]	60	15	24	0.06 (16/266)
Urban[c]	60	48	80	0.3 (75/250)
Agricultural land[d]	60	56	93	0.5 (160/320)
Animal Contaminated soils[e]	60	54	89	0.4 (75/188)
Waste and Industrial byproducts[f]	60	4	7	0.05 (5/100)
Mountain regions[g]	60	36	60	0.1 (6/60)
Insects[h]	60	30	50	0.2 (10/50)
Leaves[i]	60	40	67	0.25 (20/80)
Total	540	298	-----	417/1956

[a]River bank refers to the margin of the land along lakes and rivers where sediments are reworked or deposited. It includes regions from Tiruparankundram, Sholavandan; [b]Subterranean—underground samples (below 10 cm) from Ladan Cave Temple, Tiruparankundram and Jain cave temples; [c]Urban—Samples collected from sites wherein there is no human intervention or human interference; Mountain region—samples collected from mountains of Tiruparankundram; [d]Agricultural fields—samples were collected included. rice, corn, cabbage, millet, pulses, cotton, oilseed, sugarcane, cotton, maize, green gram, sorghum and tomato; [e]Animal contaminated soils-soils with animal feces such as cow, cat, dog, goat; [f]Waste products—tannery effluent, diary effluent, molasses and sewage sludge; [g]Mountain—samples collected from Tiruparankundram and Alagarkovil; [h]Insects—centipede, millipede, *Spodoptera* larva & pupa and *Culex quinquefasciatus* larva; [i]leaves—*Murraya koengii*, *Ricinis communis*; *The *B. thuringiensis index* indicates the number of *B. thuringiensis* isolates recovered divided by the total number of sporulated *bacilli.*

Table 3. *B. thuringiensis* and its association with insect habitats.

Local (infestation)	No. of samples	No. of samples with at least one *B. thuringiensis* isolate	*B. thuringiensis* index*	% of isolates in Diptera toxicity		
				Culex quinquefasciatus	*Aedes aegypti*	Non-toxic
Insect infested soils	10	5	0.16 (73/456)	20	20	80
Soil without insects	5	5	0.89 (170/191)	15	15	85

*The *B. thuringiensis index* indicates the number of *B. thuringiensis* isolates recovered divided by the total number of sporulated *Bacilli* examined.



(a) (b)

Figure 2. (a) Phase Contrast Microscopic view of *B.t* LDC-9 showing spore-crystal complex; Arrow indicates crystals and refractile bodies indicate spores; (b) Scanning electron microscopic view of *B.t* LDC-9 showing spore-crystal complex; c indicates crystals and s indicates spores; magnification for micrograph is ×1000 (Bar = 5 µm).

Bernhard *et al.*, [32] wherein strains with bipyramidal crystals were predominant in Eastern Asia, except South-east habitats. The differences in the distribution of morphology of parasporal body might be due to the genetic variation caused by the differences in the environmental conditions or to habitat effects [33].

3.2. Identification of *cry* and *cyt* Gene Composition of *B. thuringiensis* Isolates and Their Distribution in Different Sources

Identification of *B.t cry* and *cyt* genes by PCR has proven to be a very useful method for strain characterization, offering several advantages in terms of rapidity and reproducibility [34]. Selected 417 strains were characterized for the presence or absence of the specific *cry*2, *cry*4, *cry*10, *cry*11, *cyt*1 and *cyt*2 genes by PCR. The most frequent *cry* genes were *cry*4 (50%) [53% *cry*4*a* + 47% *cry*4*b*] and *cry*2 (25%) followed by *cry*11 (10%) and *cry*10 (15%) genes. Similar occurrence of *cry*4 and *cry*2 gene diversity was reported by Ibanez *et al.* [35]. Our study revealed that *cry*11 (10%) whereas Bravo *et al.*, [36] indicated that 38% of the tropical strains were with *cry*11 and *cyt* genes. Cytolytic genes, *cyt*1 (9%) and *cyt*2 (7%) genes were the least identified. Strains with multiple *cry* genes were found at a lesser frequency (less than 3%) than strains with single *cry* genes. Isolates with a *cry* gene harbouring *cyt*1 and or *cyt*2 gene were less than 2% (**Figure 3**). Interestingly, few strains such as *B. thuringiensis* LDC-9, LDC-14 and LDC-21 in our *B. thuringiensis* collection harbored more than one *cry* gene as reported earlier [37]. On the other hand, 47% of the strains with crystal inclusions failed to give PCR product when assayed with the primers used in this study. It did not necessarily imply that these strains were devoid of genes coding for insecticidal properties, as all of them did produce crystals [36]. These strains might contain other *cry*, *cyt* or non insecticidal parasporal inclusions as suggested by Uemori *et al.* [37]. Further, the *cry* gene frequency of these isolates were correlated to the source of the sample and classified into three groups. The first group contained the most common *cry* genes (*cry*2, *cry*4 and *cry*11) with high frequencies noted in the soils, compared to other sources. The second group included *cry*10 gene, found only in the samples derived from the insects, while absent in other samples. The third group contained the cytolytic *cyt*1 and *cyt*2 genes, present only in soil and insect samples (**Figures 4** and **5**). These results are in close agreement with Wang *et al.*, [29], where the strains from different sources differed in their *cry* gene content.

3.3. Correlation with Insect Toxicity

All the selected *B. thuringiensis* isolates with dipteran specific *cry* genes such as *cry*2, *cry*4, *cry*10 and *cry*11 were tested for its mean toxic mortalities using three replicates against *Culex quinquefasciatus* and *Aedes aegypti* as noted (**Table 4**). The spore-crystal complex of *B. thuringiensis* isolates, which promoted 50% or more larval mortality, were considered as active strains as reported by Hossain *et al.*, [30]. Toxicity tests revealed that only 7% of the *B. thuringiensis* isolates were pathogenic (>50%) to dipteran larvae. Nontoxic *B. thuringiensis* is more common than toxic *B. thuringiensis* as reported by earlier studies of Ohba [38]. The variation in toxicity was not related to *cry* gene content in all cases, as some strains sharing the same *cry* gene but significantly differed in their insecticidal potency. In this study, for ex-

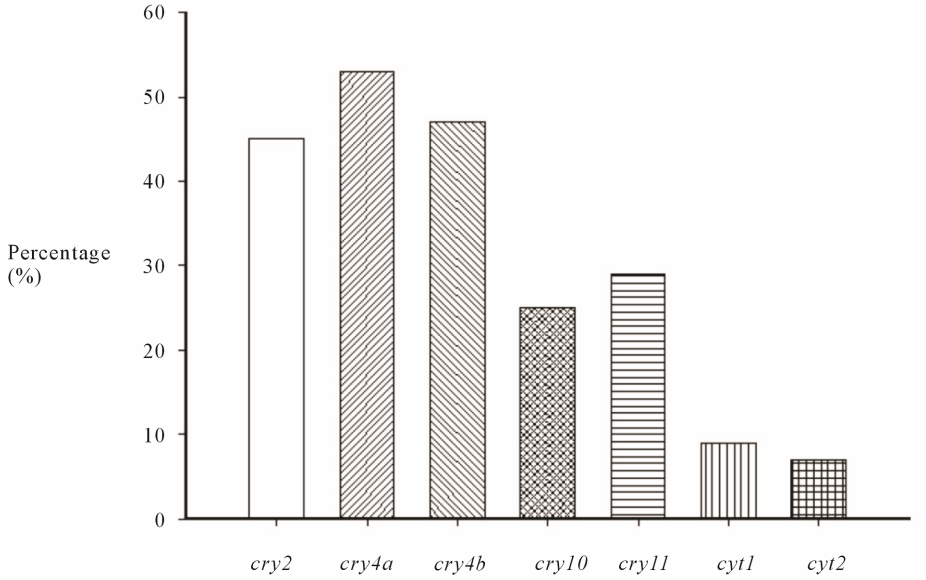


Figure 3. Frequency of single mosquitocidal *cry* or *cyt* genes in indigenous *Bacillus thuringiensis* isolates.

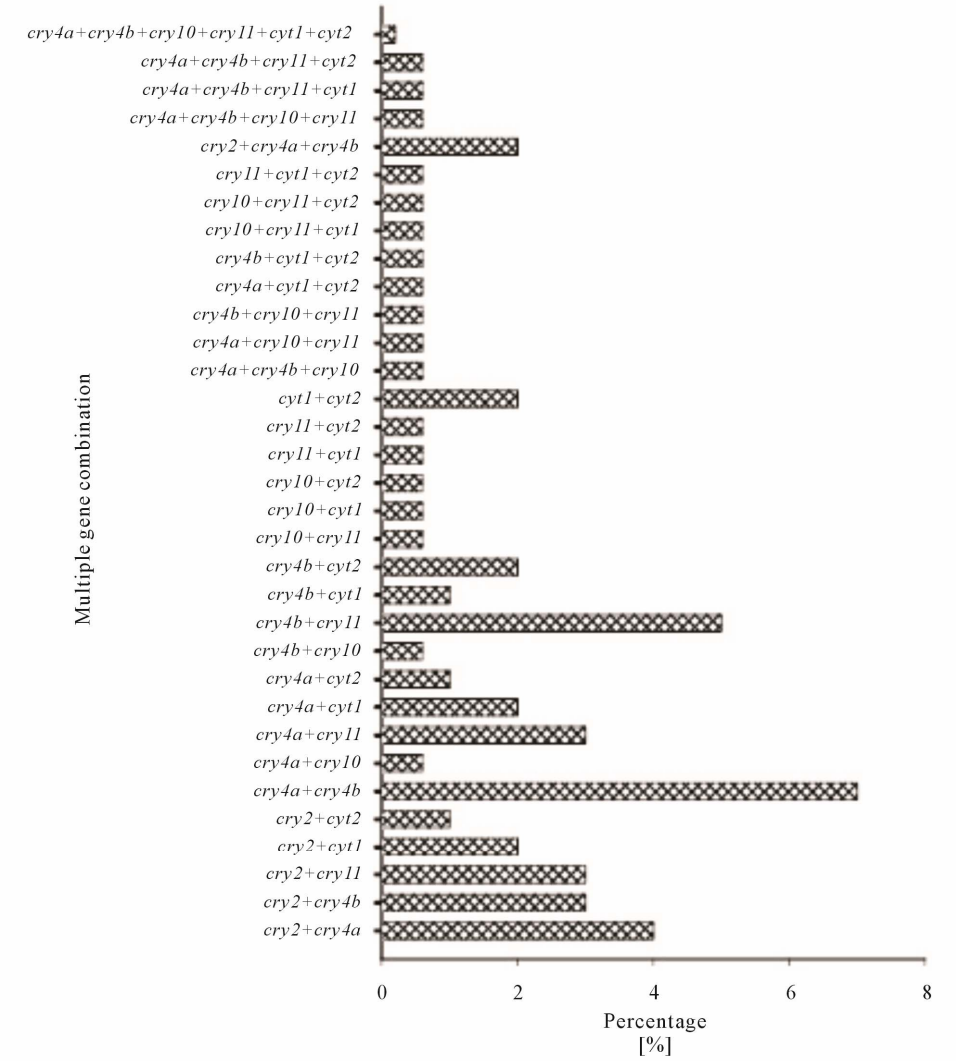


Figure 4. Frequency of multiple mosquitocidal *cry* or *cyt* gene in indigenous *B. thuringiensis* isolates.

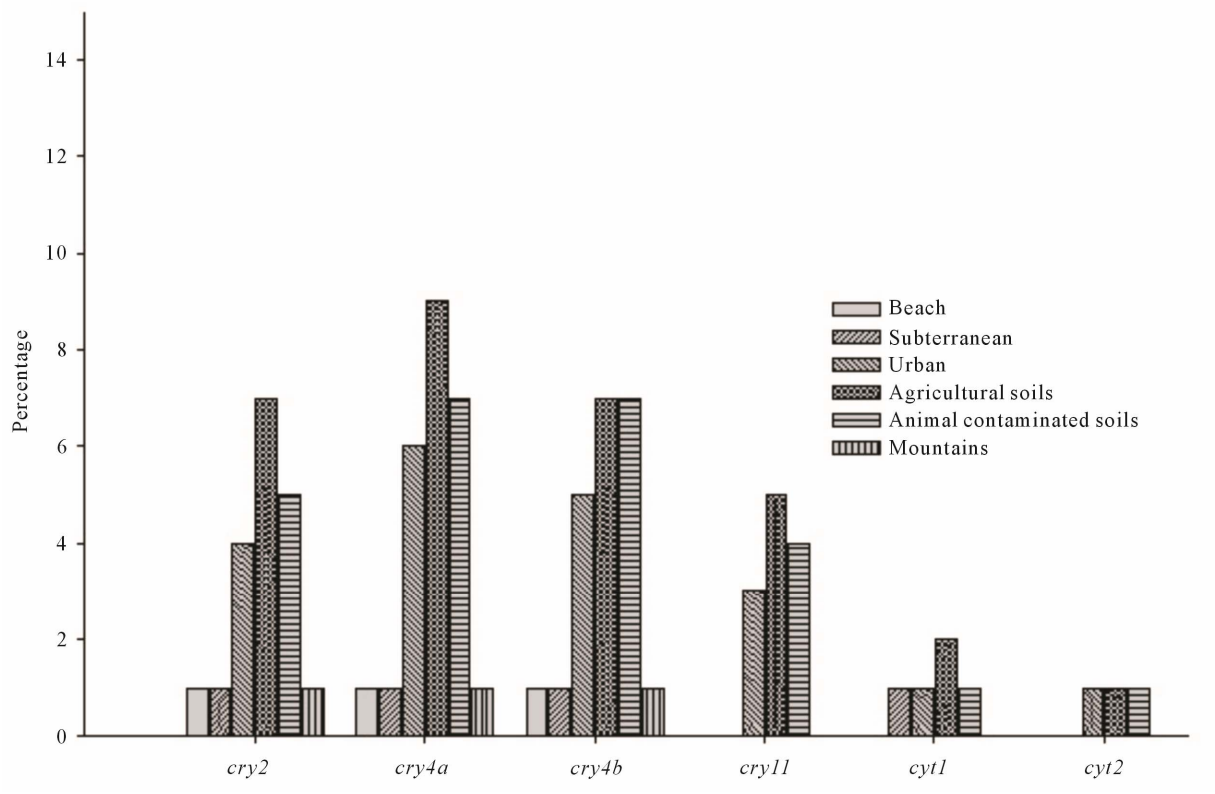
ample, strains (*B. thuringiensis* LDC-14 and *B. thuringiensis* LDC-21) displayed the *cry*4 gene but the mortalities produced by the spore-crytsal complex were 96% and 24% respectively. This might be explained by a variation in the level of gene expression, which can strongly influence the insect toxicity as reported by Martinez and Caballero [39]. Strain *B. thuringiensis* LDC-9 alone with unique combinations of *cry* (*cry*4*a*, *cry*4*b*, *cry*10, *cry*11) and *cyt* genes (*cyt*1 and *cyt*2) demonstrated three-fold higher mosquitocidal activity (6 ng·mL^{-1}) than *B. thuringiensis israelensis* (19 ng·mL^{-1}).

3.4. Protein Profiling

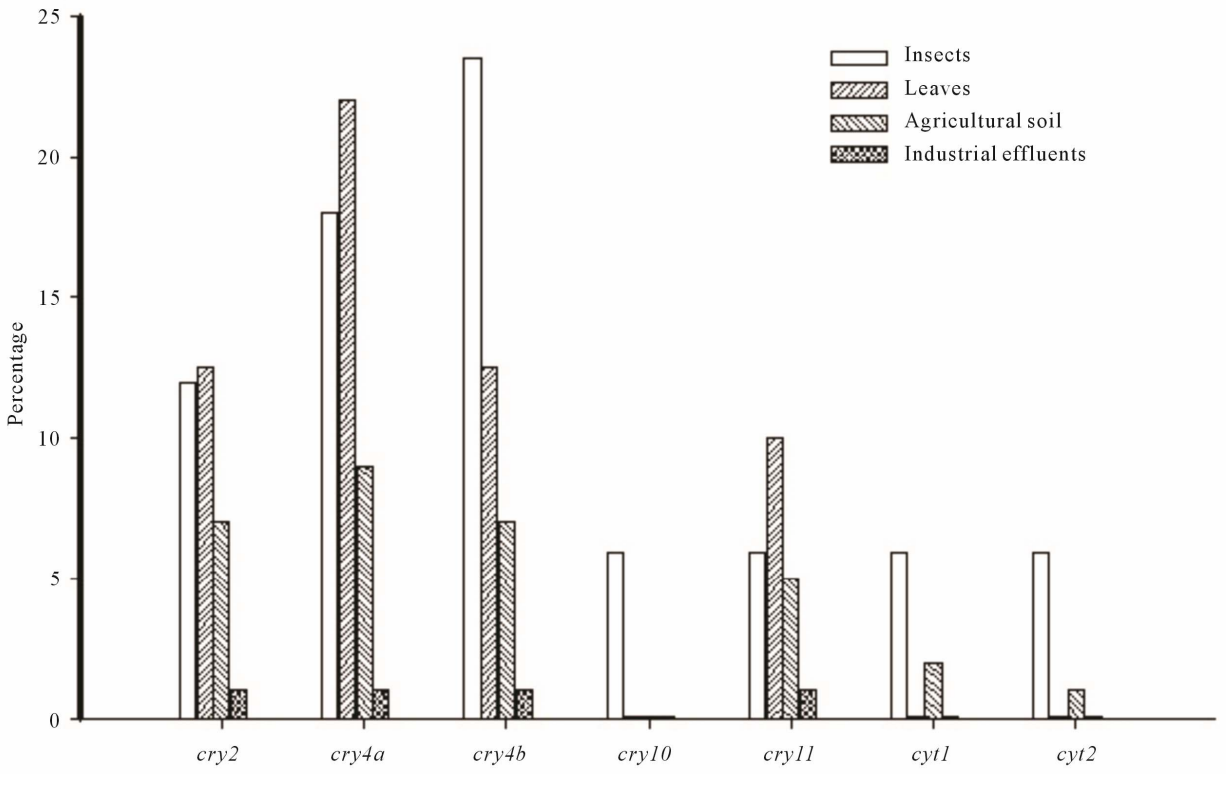
B. thuringiensis LDC-9 was noted to be the toxigenic strain based on the mosquitocidal activity and SDS-PAGE of spore-crystal suspensions of selected strain as shown (**Figure 6**). *B. thuringiensis* LDC-9 exhibited protein profile which is distinct from *B. thuringiensis* sp. *israelensis* as reported earlier [18]. The present study resulted in identification of a novel *B. thuringiensis* LDC-9 with higher mosquitocidal activity than the earlier reported *B. thuringiensis* strains. Hence, this strain is likely to be a viable mosquito control agent after field evaluation and toxicity analysis against other aquatic insects including dragonflies, damselflies, mayflies, stoneflies, caddisflies, water beetles or bugs and other invertebrates such as Daphnia, Cyclops, rotifers and crustaceans.

4. Conclusion

B. thuringiensis presents great genetic and molecular diversity even in isolates from the same soil sample. Moreover, the diversity and activity of the isolates might have a relationship with the geographical origin of the samples. The results obtained here indicate that the *B. thuringiensis* LDC-9 may be a potential control agent



(a)



(b)

Figure 5. (a) Mosquitocidal gene distribution based on the nature of the soil samples; (b) Mosquitocidal gene distribution based on the origin of the samples.

Table 4. Larvicidal activity of indigenous *B. thuringiensis* strains against *Culex quinquefasciatus* and *Aedes aegypti.*

Source	Number of Strains	Mortality (%) of *Culex quinquefasciatus*	Mortality (%) of *Aedes aegypti*
River bank (21), Subterranean (1), Urban (33), Agricultural land (95), Animal contaminated soils (47), Waste and Industrial byproducts (2), Insects (3), Leaves (13)	215	0.0	0.0
River bank (20), Subterranean (9), Urban (36), Agricultural land (36), Animal contaminated soils (18), Waste and Industrial byproducts (2), Mountain regions (1), Insects (2), Leaves (2)	126	13.71 ± 3.59^{a}	13.71 ± 3.59
River bank (5), Subterranean (5), Urban (5), Agricultural land (21), Animal contaminated soils (4), Waste and Industrial byproducts (1), Insects (2), Mountain regions (2), Insects (2), Leaves (2)	49	30 ± 5.14^{b}	30 ± 5.14^{b}
River bank (2), Subterranean (1), Urban (1), Agricultural land (4), Animal contaminated soils (3), Insects (1), Mountain regions (2), Leaves (1)	15	50.6 ± 6.28^{c}	50.6 ± 6.28^{c}
River bank (2), Agricultural land (3), Animal contaminated soils (3), Insects (2), Mountain regions (1), Leaves (1)	12	69.5 ± 6.80^{d}	69.5 ± 6.80^{d}
Agricultural soil sample (1)	1	100 ± 0^{e}	100 ± 0^{e}

Mortality is expressed as average ± standard deviation. Different alphabets in superscripts indicates significant difference at $P < 0.05$. Number in parenthesis indicate the number of *B.t* strains employed in this study (To include strains employed in this study).

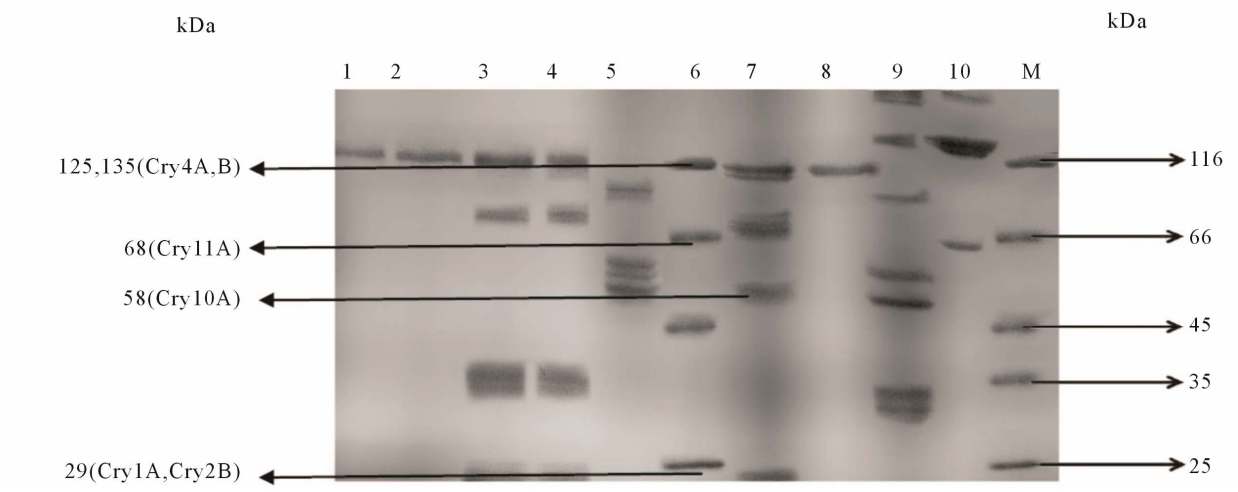


Figure 6. Protein profile of spore-crystal complex of selected *B. thuringiensis* isolates; Lane 1: *B.t* LDC-7; Lane 2: *B.t* LDC-21; Lane 3: *B.t* LDC-43; Lane 4: *B.t* LDC-52; Lane 5: *B.t* LDC-64; Lane 6: *B.t* LDC-9; Lane 7: *B.t* subsp. *israelensis*; Lane 8: *B.t* LDC-71; Lane 9: *B.t* LDC-127; Lane 10: *B.t* subsp. *kurstaki* HD-1; Lane M: Marker.

that could be used in control programmes against mosquitoes.

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