

## Isolation and Molecular Characterization of *cry4* Harboring *Bacillus thuringiensis* Isolates from Pakistan and Mosquitocidal Activity of their Spores and Total Proteins

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**Abstract.** The present study was aimed at screening the soil samples from the local environment for a potent mosquitocidal *cry4* positive *Bacillus thuringiensis* (*B.t.*). A total of 50 *B.t.* strains were isolated, of which 72% were from soil samples, 18% from animal waste and 10 % from dry wheat straw. Twenty two of these isolates were found positive for *cry4* genes. The toxicity bioassays with *B.t.* spores and the total cell proteins showed that six *B.t.* isolates harboring *cry4* genes (*viz.*, SBS *Bt* 23, 29, 34, 37, 45 and 47) were most toxic to 3<sup>rd</sup> instar larvae of mosquito, *Anopheles stephensi*. Shorter fragment of *cry4* gene of six *B.t.* isolates was cloned and sequenced. The 16S rDNA study revealed that these isolates showed maximum homology with *B.t.* serovar *tolworthi*, *B.t.* str. *Al Hakam*, *B.t.* serovar *thompsoni*, *B.t.* serovar *konkukian*, and *B.t.* serovar *fukuokaensis*. Among six *B.t.* isolates, SBS *Bt* 45 was found the most toxic and was isolated from dry soil containing decaying cattle waste.

**Key word:** *Bacillus thuringiensis*, insecticidal activity, *cry4* gene, total protein profile of *B.t.* isolates, bioinsecticide, *Anopheles stephensi*.

### INTRODUCTION

Bioinsecticides based on *Bacillus thuringiensis* (*B.t.*) are the most widely used environment-friendly alternative to synthetic pesticides for control of agricultural and forest pests and vectors of human diseases (Lambert and Peferoen 1992; Schnepf *et al.*, 1998). Mosquitoes belonging to different genera, responsible for various diseases such as dengue (*Aedes* spp.), malaria (*Anopheles* spp.), bancroftian filariasis and west Nile Virus disease (*Culex* spp.) must be eliminated, to weed out these malaise. Since mosquitoes have developed resistance to several of the synthetic insecticides, some alternate, environment friendly mosquitocidal agents need to be searched (Baird, 2000; Regis *et al.*, 2001).

*B.t.* is an aerobic, spore-forming, Gram-positive bacterium that synthesizes a crystalline parasporal inclusion composed of one or several proteins known as insecticidal crystal proteins (ICP) or  $\delta$ -endotoxins, during sporulation (Bechtel and Bulla, 1976). These proteins are specifically toxic to insect larvae and are widely used as bioinsecticides

against lepidopteran (Aronson *et al.*, 1986; Höfte and Whiteley, 1989; MacIntosh *et al.*, 1990), dipteran (Goldberg and Margalit, 1977) and coleopteran pests (Krieg *et al.*, 1983), and certain nematodes and protozoan pathogens (Feitelson *et al.*, 1992). Upon ingestion by susceptible insect larvae, crystalline inclusions are solubilized in the midgut lumen and converted to active toxins by trypsin-like proteases. The activated toxins bind to specific receptors on the brush border apical membrane of midgut columnar cells and insert into the membrane, where they form pores. As a consequence, epithelial midgut cells swell and lyse, causing the larvae to stop feeding and eventually die by septicemia or starvation (Knowles, 1994).

It has been well established that *B.t.* is globally distributed in a wide range of natural environments (Feitelson *et al.*, 1992; Damgaard, 2000). Many habitats such as soil (Chang *et al.*, 1998, 1999; Martin and Travers, 1989; Park *et al.*, 1998; Roh *et al.*, 1996), settled dust (DeLucca *et al.*, 1982), insects (Heimpel and Angus, 1959; Heimpel, 1967), phylloplane (Sneath, 1986), and water (Goldberg and Margalit, 1977) have been reported to be sources of *B.t.*

To date more than 231 receptor specific Cry proteins, classified in to 28 families and seven cytolytic proteins have been identified which directly bind to phospholipids

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([http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt) 2007), and have been proven to be toxic to specific pests. The *cry4* genes are dipteran specific and are known to control mosquito and black flies worldwide (Margalith and Ben-Dov, 2000). The insect pathogenicity *B.t.* harbouring *cry4* genes depends on the presence of pBtoxis megaplasmid (Faust *et al.*, 1983; Gonzalez and Carlton 1984; Ben-Dov *et al.*, 1996; Andrup *et al.*, 1998) which encodes four genes *cry4A*, *cry4B*, *cry4C* and *cry4D* of the dipteran specific proteins with molecular masses of 134, 128, 78, and 72 kDa, respectively. All Cry4 toxins Cry4A, Cry4B, Cry10 and Cry11, are synthesized at different times during sporulation and are added step-wise to the developing ovoid inclusion (Lee *et al.*, 1985). Cry4A and Cry4B proteins are structurally similar to Cry1 proteins and are proteolytically cleaved to smaller toxic fragments. The C-terminal halves of these proteins are similar to C-terminal halves of Cry1 proteins suggesting the localization of toxicity in the N-terminal halves (Chungjatupornchai *et al.*, 1988; Delecluse *et al.*, 1988; Pao-Intara *et al.*, 1988).

The main objective of this study was to isolate and characterize *B.t.* isolates from soil samples of different habitats of Lahore and Sheikhpura, Pakistan, and also characterize these isolates on the basis of ribotyping, total protein profile, and nucleotide sequence of *cry4* shorter fragment. The toxicity of spores and total proteins of *B.t.* isolates harbouring *cry4* genes was evaluated against third instar larvae of mosquito *Anopheles stephensi* for their potential use as bioinsecticide.

## MATERIALS AND METHODS

### *Sample collection*

Samples of organically rich soil, animal dung, bird droppings and grain dust were collected from different areas of Lahore and Sheikhpura in sterile and properly labeled glass jars. Soil samples were taken from one inch underneath the ground level. All the samples were brought to the laboratory and stored at 4°C for further analysis.

### *Isolation and biochemical characterization of B.t.*

Samples collected from different habitats of

Lahore and Sheikhpura were processed for the isolation of *B.t.* according to Martin and Travers (1989). Briefly, samples (0.5g) were suspended in 10ml of LB medium (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L) containing 0.2M sodium acetate, shaken well and incubated at 30°C for 4 hours. The incubated samples were filtered using filter paper (0.25µm) and heated at 80°C for 15 minutes to isolate spore formers. The above treated samples were diluted 1:2 and then spread on LB agar plates and incubated overnight at 30°C. Colonies with *B.t.* like morphology (entire margin, off white color, dry and rich growth of colony) were picked and streaked on LB agar plates and incubated overnight at 30°C for 24 hours.

For microscopic examination the *B.t.* strains were grown on LB agar medium at 30°C for 18 hours for vegetative culture and for 3 days for sporulated cultures. The cultures were observed under light microscope for determining the position of endospore and extra cellular protein particles. Gram staining and endospore staining with Malachite green and acid fuchsin was done. The Gram-positive rods and spore formers were characterized biochemically according to Sneath (1986).

### *PCR based detection of cry4 gene*

The *B.t.* isolates were scanned for *cry4* gene by polymerase chain reaction (PCR) using the following primers.

Forward 5'-GCATATGATGTAGCGAAACAAGC'-3  
Reverse 5'-GCGTGACATACCCATTTCAGGTCC-3'

PCRs were done with crude DNA as described by Carozzi *et al.* (1991). A loop full of *B.t.* colony from an overnight LB agar plate was transferred to 100 µl distilled water in 0.5ml Eppendorf tube. The tube was placed in boiling water bath for 10 minutes for cell lysis and then kept at -20°C for 10 minutes. The heat shock process was repeated thrice for complete lysis of cells. The resulting cell lysate was spun down at 10,000 rpm (5,500xg), for 10 minutes at 4°C. This supernatant comprising crude DNA was used for PCR amplification according to Saiki *et al.* (1988). A total volume of 50µl of reaction mixture contained 2.5 mM MgCl<sub>2</sub>, 10mM Tris-HCl pH8.0, 200mM of

deoxyribonucleoside triphosphate (dNTPs), 50 pmoles of each forward and reverse primer, 1 unit of *Taq* DNA polymerase and 15  $\mu$ l of the cell lysate. The reaction cycle comprised initial denaturation at 94°C for 5 minutes followed by 35 cycles, each of denaturation at 94°C for two minutes, annealing at 51°C for 45 seconds and extension at 72°C for 2 minutes, with final extension of 7 minutes in an Applied Biosystem 2720 thermocycler. The PCR product was analyzed by 1% agarose gel electrophoresis.

#### *Biotoxicity assays of B.t. isolates harbouring cry4 gene*

The toxicity of spores as well as total cell protein of *B.t.* isolates found positive for *cry4* gene was evaluated against third instar larvae of mosquito, *Anopheles stephensi*.

#### *Preparation of B.t. spores*

Spores of bacterial isolates were prepared according to Makino *et al.* (1994), for which single isolated *B.t.* colony was inoculated in LB broth and incubated at 37°C. After 24 hours, the above inoculum was streaked on sporulation medium T3 (Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, MnCl<sub>2</sub> 0.005g/L, NaH<sub>2</sub>PO<sub>4</sub> 6.9g/L, Na<sub>2</sub>HPO<sub>4</sub> 8.9g/L and agar 15g/L) and incubated at 30°C for 72 hours. After 3 days, the spores were harvested from the medium and washed five times with autoclaved distilled water. The pellet was incubated at 37°C for 40 minutes in 10 ml of the solution I - 2M KCl : 10mM Na<sub>3</sub>PO<sub>4</sub> (1:1) pH 7.5. The spores were washed twice with the autoclaved distilled water. The pellet was incubated at 37°C for 30 minutes in 10ml of the solution II - 5 mM urea : 5mM CHES (2-Cyclohexylamino-ethanesulfonic acid, monosodium salt) (1:1), pH 9.3. This was followed by addition of 25 mM of 2-mercaptoethanol. The spores were then washed five times with autoclaved distilled water and stored at 4°C.

The spores (0.5 mg) were dried at 70°C for 24 hours and then transferred to desiccators until dried. Dried material was then weighed up to fourth decimal place. This was replicated thrice to get mean dry weight. Spore concentration was determined according to Cavados *et al.* (2005). The serially diluted dried spore suspension in autoclaved

distilled water (1 $\mu$ g/ $\mu$ l), heat shocked at 80°C for 12 minutes in water bath, was spread on spore forming T3 medium and incubated at 32°C for 3 days. The viable cell count was taken. The number of spores/ $\mu$ g was determined from the mean of number of colonies from three plates.

#### *Preparation of total B.t. cell protein*

The single isolated *B.t.* colony from 24 hours culture on LB agar plate was streaked on T3 plates and kept at 30°C for 72 hours. *B.t.* culture was scraped off the plate by adding 3ml of autoclaved distilled water. The culture was centrifuged at 7000rpm (4500 x g) at 4°C for 15 minutes, and the pellet was suspended in autoclaved distilled water. The suspension was centrifuged and washing was repeated twice with cold autoclaved distilled water. The pellet was suspended in alkaline buffer (Na<sub>2</sub>CO<sub>3</sub> 0.265g, DTT 0.08g, autoclaved distilled water 50ml, pH 10.5-11) incubated for 3 hours in shaking incubator at 37°C, spun at 7000rpm (4500 x g) at 4°C for 20-25 minutes. The protoxin in supernatant was activated by trypsin digestion. Since trypsin is optimally active at neutral pH, the pH of solubilized protoxin (pH 10) was adjusted to 7-8 by adding 1N HCl. One  $\mu$ g of trypsin (stock: 1mg/ml in autoclaved distilled water) for each 20  $\mu$ g of protoxin was mixed well in samples followed by incubation at 37°C for 3 hours.

#### *Procedure adopted*

The bioinsecticidal activity of sporulated form and that of activated solubilized prototoxin of *B.t.* isolates as well as those of positive controls IPS 78 and HD 500 obtained from Daniel R. Zeigler of Bacillus Genetic Stock Centre, Columbus, Ohio, USA were measured in triplicates. The third instar larvae of *Anopheles stephensi* were obtained from Malarial Research Center, Birdwood Road, Lahore. Twenty larvae were placed in wide mouthed cups containing 20 ml of autoclaved distilled water. Three cups were used for each concentration. Then required concentration of spores, ranging from 100 to 1000  $\mu$ g/ml of spores, and that of solubilized protein ranging from 25 to 300  $\mu$ g/ml, was added to these cups and incubated at 25°C for 24 hours. The larvae, which were knocked down at the bottom of cup and failed to swim to the surface of water, were counted dead.

### *Ribotyping of B.t. isolates*

Ribotyping of six most toxic *B.t.* isolates was done to confirm their identification upto sub species level. Specific primers for 16s rDNA 5F 5'- TGAAAACCTGAACGAAACAAAC 3' 3R 5'- CTCTCAAAAACCTGAACAAAACGAAA 3' of full-length 1.6kb gene were used. PCR was done according to Saiki *et al.* (1988) using Fermentas PCR reagents (#EP0402). A total volume of 50µl contained 2.5 mM MgCl<sub>2</sub>, 1 µl of 10X Taq buffer with KCl (100mM Tris-HCl pH 8.8, and 500 mM KCl), 200mM deoxyribonucleoside triphosphate (dNTPs), 50 pmoles of each forward and reverse primers, 1unit of Taq DNA polymerase and 15 µl of DNA., 20ng/ µl DNA). The 16S rDNA full-length gene was amplified in a thermal cycler (Applied Biosystem 2720) by initial denaturation at 94°C for 5min followed by 30 cycles, each of denaturation at 94°C for 2 min, 52°C for 1.30min, and 72°C for 2min, with final extension at 72°C for 7min. Each of the amplified products was run on 1% agarose gel, cut and purified using Fermentas gel elution kit (#K0513) and then cloned in pTZ57R/T (T/A cloning vector) by using DNA ligation kit (#K1214). The gene cloning was later confirmed by colony PCR, and restriction digestion with *EcoRI* and *HindIII*,

The 16S rDNA gene was sequenced to confirm the identification, determine the evolutionary relationships among the isolates and to evaluate the mutations in the conserved region. For this purpose sequences of full length 16S rDNA gene from local *B.t.* isolates were aligned and BLASTed to find out the homology with the already sequenced and deposited genes in the GenBank sequence database. 4Q281 *Bacillus thuringiensis* 16S ribosomal RNA gene was used as a reference gene. The nucleotide sequences of full-length 16S rDNA gene of local *B.t.* isolates were later deposited in the NCBI database.

### *Total cell protein profile of B.t. isolates*

Crystal proteins were isolated from *B.t.* according to Alberola *et al.* (1999) and according to Sayyed *et al.* (2000). These crystal proteins were also isolated according to the method developed in the laboratory, and already described above under Bioassays, except for the trypsin digestion of the

protein in the supernatant. The protein content of the supernatant was estimated by Lowry method (Lowry *et al.*, 1951) and stored at -20°C for SDS-PAGE analysis and further studies.

### *Analysis of shorter fragment of cry4 gene (439bp)*

The conserved regions of *cry4* gene from the six most toxic *B.t.* isolates were amplified using universal primers for *cry4* gene and cloned. Cloning was done in pTZ57R/T (T/A cloning vector). Transformation was done in *E. coli* DH5α. Positive transformants were primarily screened for the presence of shorter fragment of *cry4* gene (439bp) on X-gal (270µg/ml) IPTG (120µg/ml) and Ampicillin (100µg/ml) plates. The positive transformants were later on confirmed by colony PCR amplification using the same universal primers of *cry4* gene. The clone was further confirmed by restriction analysis of recombinant plasmid DNA containing ligated *cry4* gene in pTZ57R with *EcoRI* and *Hind III* to separate the ligated 439bp *cry4* gene fragment. The conserved region of *cry4* gene was sequenced. The sequences were aligned with already reported *cry4* gene and deposited in NCBI GenBank DNA database.

## RESULTS

### *Characteristics of B.t. isolates*

A total of 54 samples have been collected from different ecological environments of Lahore and Sheikhpura under sterile conditions. It was found that 78% *B.t.* isolates were isolated from soil samples including saline soil (8%), sandy soil (12%), dry soil (38%) and moist soil (20%), and other 22% were isolated from cattle waste (14%) and wheat straw (8%), respectively.

After heat shock, a total of 470 *B.t.* like colonies appeared on the LB medium, out of which 250 isolates were identified as *B.t.* on the basis of purple colour after Gram staining (Fig. 1). Fifty *B.t.* isolates were selected for further study, on the basis of various biochemical tests. *B.t.* was positive for catalase activity and Voges Proskauer test, could utilize citrate and reduce nitrate, could decompose tyrosine and hydrolyze casein and starch. It could grow on media containing 7% NaCl, Sabouraud Dextrose agar and 0.001% lysozyme. Acid was

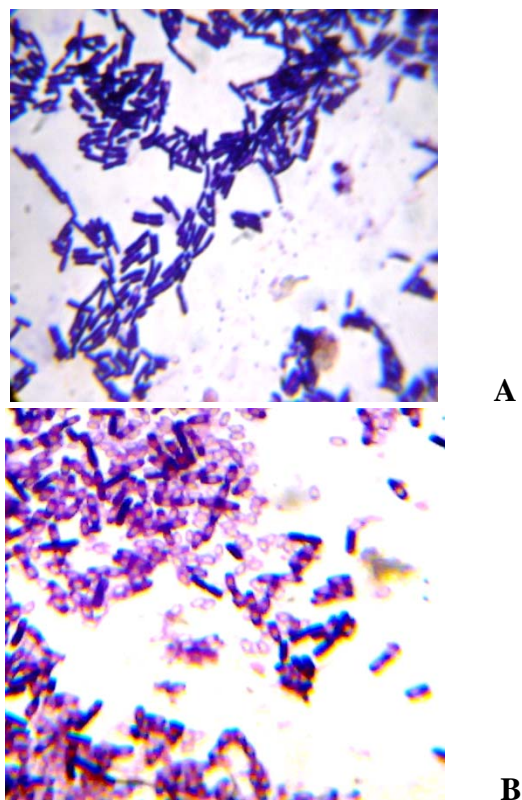


Fig. 1. Sporangia and vegetative cells of *B.t.* isolate SBS *Bt* -23 after 18 hours (A) and 60 hours (B) of sporulation. Note that unswollen sporangia bear parasporal crystals, and endospore lie at paracentral and subterminal positions.

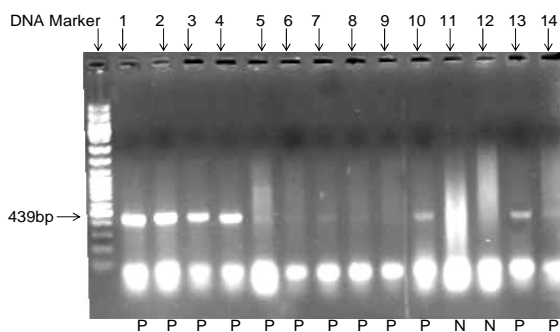


Fig. 2. Agarose gel electrophoresis of PCR product of 439bp fragment representing conserved region of *cry4* gene of *B.t.* isolates - *Bt* 11 (lane 1), *Bt* 13 (lane 2); *Bt* 19 (lane 3), *Bt* 23 (LANE 4), *Bt* 26 (lane 5), *Bt* 29 (lane 6), *Bt* 32 (lane 7), *Bt* 34 (lane 8), *Bt* 35 (lane 9), *Bt* 37 (lane 10), *Bt* 1 (lane 11), *Bt* 3 (lane 12), *Bt* 38 (lane 13), and *Bt* 40 (lane 14). P indicates *cry4* positive lanes and N *cry4* negative lanes. The first lane on left is 1Kb DNA marker.

produced after utilization of glucose whereas no acid production took place after xylose, arabinose and manitol utilization. The bacterium did not grow at 65°C. They produced intracellular protein crystals, which were visible deep pink in colour after acid fuchsin staining.

#### Prevalence of *cry4* gene in the *B.t.* isolates

A 439bp fragment representing conserved region of *cry4*  $\delta$ -endotoxin gene on the basis of PCR was amplified (Fig. 2). After PCR based detection, 44% of *B.t.* isolates was found positive for *cry4* gene. Among them 24% were from dry soil, 6% from moist soil, 8% from cattle waste and 6% from wheat straw.

#### Characteristics of *cry4* positive isolates

##### Colony morphology and physical characteristics

All *cry4* positive isolates were Gram positive rods found in aggregates as chains of rods that stem from the aggregates. The vegetative cells containing spores become visible after 18 hours of incubation (Fig. 3A). Subsequent to that sporulation is enhanced and the endospores, stained green with malachite green, are gradually released in the medium after 30 h (Fig. 3B). Sporulation is completed after 48 h, when most of the *B.t.* cells contained spores and protein crystals (Fig. 3D). Almost 75% culture got sporulated after 36 h of incubation (Fig. 3C). The endospores in all the *B.t.* isolates were either paracentral or sub terminal.

#### Biotoxicity assay with *B.t.* isolates

Bioassay was done with *B.t.* spores to screen the most toxic local isolates positive for *cry4* gene against 3<sup>rd</sup> instar larvae of *Anopheles stephensi*. Table I shows the result of such bioassays. SBS *Bt*-45 showed 100% mortality at 800 $\mu$ g of spores/ml. At this dose, SBS *Bt* -23, 29, 34, 37 and 47 showed 58%, 86%, 79%, 87%, and 95% mortality, respectively. The positive controls IPS 78 and HD-500 showed 90% and 60% mortality respectively. Figure 4 shows LC<sub>50</sub> values of *B.t.* spores against third instar larvae of mosquito. Among these the most toxic *B.t.* isolate SBS *Bt* 45 was isolated from dry soil containing decaying cattle waste and had LC<sub>50</sub> 366 $\pm$ 0.7  $\mu$ g /ml against third instar larvae of

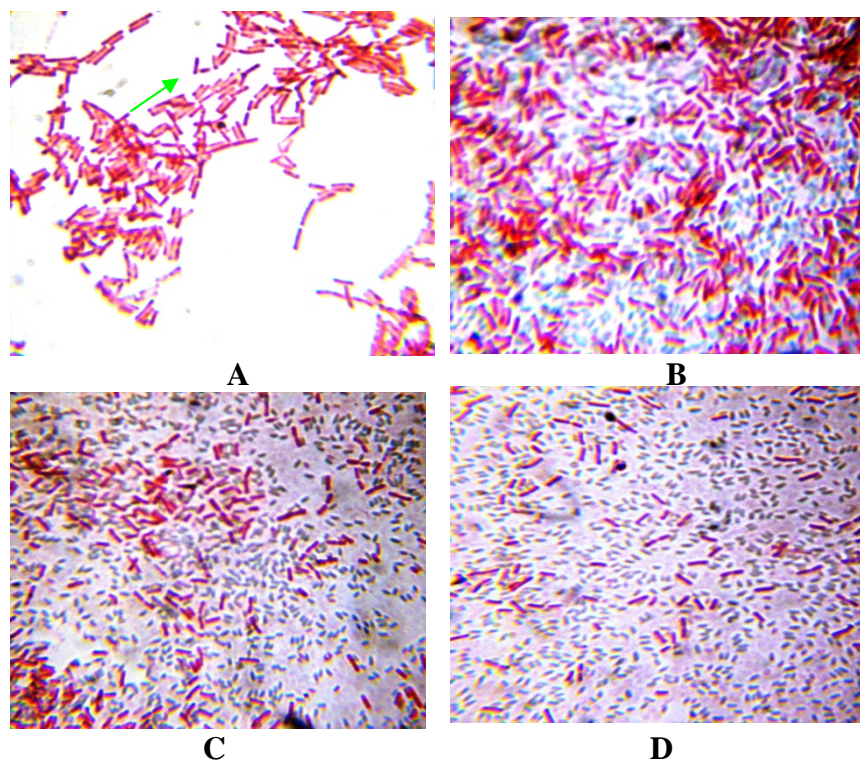


Fig. 3. Spore staining of SBS *Bt* -45 after 18 hours(A), 30 hours (B), 36 hours (C), and 48 hours (D) of sporulation. The vegetative cells containing spores are visible inside the cell in A. Some endospores green in colour have been released in the medium, while some vegetative cells still containing spores are visible inside the cells in B and C. In D the green coloured endospores have been released in the medium and almost the entire culture has sporulated.

**Table I.** The six most toxic *B.t.* isolates, isolated from different areas and soil textures, against 3<sup>rd</sup> instar larvae of mosquito *Anopheles stephensi* which were finally selected for detailed molecular analysis.

S.No.	<i>B.t.</i> isolates	Renamed as	Soil texture	LC <sub>50</sub> of spore(µg/ml)	LC <sub>50</sub> of total cell protein (µg/ml)
1	SBS <i>Bt</i> -23	DAB <i>Bt</i> 1	Dry soil	755 ± 1.1	104± 0.17
2	SBS <i>Bt</i> -29	DAB <i>Bt</i> 2	Decaying cattle waste	524±0.19	81 ± 0.54
3	SBS <i>Bt</i> -34	DAB <i>Bt</i> 3	Cattle waste	571±0.95	84± 1.23
4	SBS <i>Bt</i> -37	DAB <i>Bt</i> 4	Dry soil	483± 1.25	55± 1.19
5	SBS <i>Bt</i> -45	DAB <i>Bt</i> 5	Dry soil containing decaying cattle waste	366 ± 0.7	50± 1.68
6	SBS <i>Bt</i> -47	DAB <i>Bt</i> 6	Moist soil	458± 1.55	80± 0.59

*Anopheles stephensi* with LC<sub>50</sub> 483±1.33 µg/ml and with LC<sub>50</sub> 458±1.55µg/ml were isolated from dry soil and moist soil, respectively, while with LC<sub>50</sub> 524±0.19 µg/ml and with LC<sub>50</sub> 571±0.95 µg/ml were isolated from decaying cattle waste. with LC<sub>50</sub> 755±1.1 µg /ml was the least toxic amongst the six

*B.t.* isolates selected for further study.

#### Bioassays with total cell protein

Table I shows LC<sub>50</sub> of total proteins of local isolates. Sodium carbonate buffer (alkaline lysis buffer) was used as negative control and IPS78 and HD500 were used as positive controls of *cry4* gene.

The SBS *Bt*-45 with  $LC_{50}$   $50 \pm 1.68$   $\mu\text{g/ml}$  was found to be the most toxic isolate, while SBS *Bt*-29 with

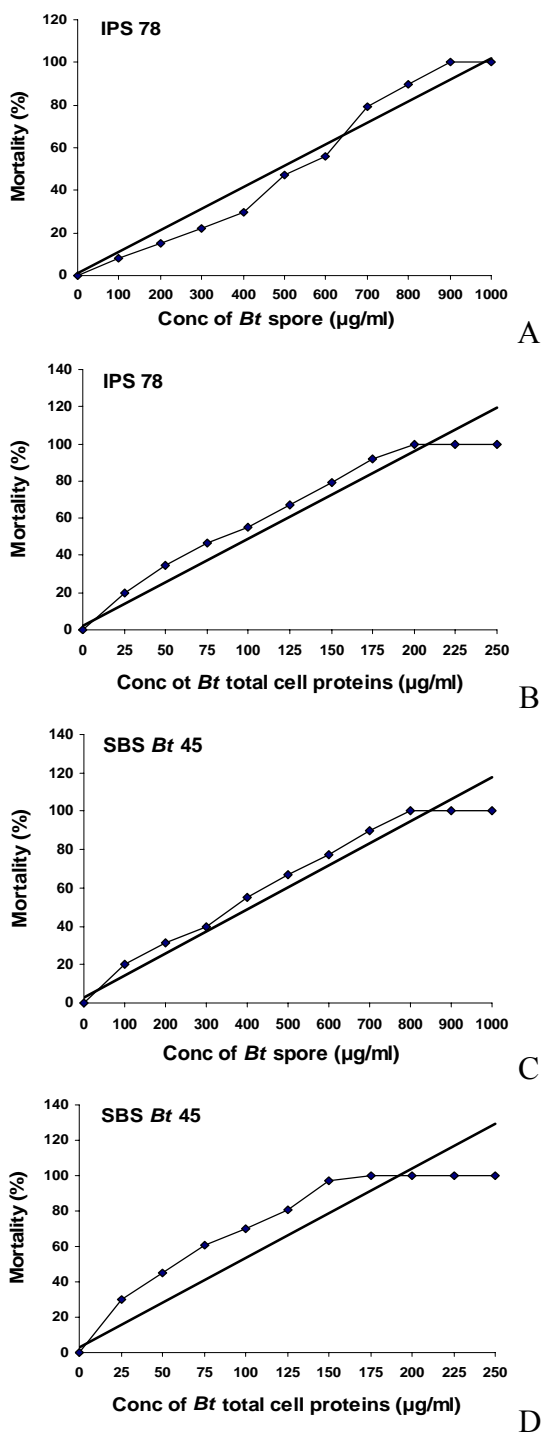


Fig.4. Toxicity (% Mortality) of spores of SBS *Bt* 45 (C) and positive control IPS 78 (A), and that of total cell proteins of SBS *Bt* 45 (D) and positive control IPS 78 (B) against third

instar larvae of *Anopheles stephensi*. Straight lines represent regression lines based on data shown in the form of curve for each bioassay.

$LC_{50}$   $81 \pm 0.54$   $\mu\text{g/ml}$ , SBS *Bt*-34 with  $LC_{50}$   $84 \pm 1.23$   $\mu\text{g/ml}$  and SBS *Bt*-47 with  $LC_{50}$   $80 \pm 0.59$   $\mu\text{g/ml}$  showed the same level of toxicity, almost similar to that of IPS 78 ( $LC_{50}$   $84 \pm 1.1$   $\mu\text{g/ml}$ ). SBS *Bt*-23 and HD500 showed the same  $LC_{50}$  values viz.,  $104 \pm 0.17$   $\mu\text{g/ml}$  and  $100 \pm 1.81$   $\mu\text{g/ml}$  of  $LC_{50}$ , respectively (Fig. 4).

#### Ribotyping of *B.t. isolates*

The sequence alignment of 16S rDNA gene from SBS *Bt*- 23, 29, 34, 37, 45 and 47, respectively showed maximum homology with *B.t.* serovar *tolworthi*, *B.t.* str. *Al Hakam*, *B.t.* serovar *thompsoni*, *B.t.* serovar *konkukian*, and *B.t.* serovar *fukuokaensis*, respectively. All the sequences of 16S rDNA gene from *B.t.* isolates SBS *Bt* 23, 29, 34, 37, 45 and 47, respectively) were submitted to GenBank database as DAB *Bt*1-DAB *Bt*6 under accession numbers EU124378, AM293345, EU124379, AM29334, EU124380 and AM293344, respectively.

The 16S rDNA gene sequences of six *B.t.* isolates (DAB-*Bt* 1-6) were 99% homologous to *Bacillus thuringiensis* strain 4Q281 16S ribosomal RNA gene, which was used as reference sequence. Two common mutations at position no 463 A→C and 561 T→C were found in 16S rDNA of DAB *Bt*-4 (SBS *Bt* 37) and 6 (SBS *Bt* 47), while no mutation was found in 16S rDNA gene of DAB *Bt* 5 (SBS *Bt* 45). Seven mutations were recorded at positions 253 (C →T), 313 (C→G), 366 (C→ G), 555 (A→ G), 1092 (C→ T), 1161 (A→ T), and 1453 ( A→ T) in DAB *Bt* I (SBS *Bt* 23), and at positions 317 (G→ T), 439 (T→ G), 738 (C→ G), 1218 (C→ G), 1414 (G→ T), 1425 ( C→ G), and 1429 (A→G) in DAB *Bt* 3 (SBS *Bt* 34). Out of a total of 20 mutations recorded, there were only six which were transitions (Pu→Pu, Py→Py), whereas remaining 14 were tranversions : eight Py→ Pu and six Pu→ Py.

#### Total cell proteins of *B.t. isolates*

Figure 5 shows the total cell protein profile of the *B.t.* isolates, extracted and run on SDS-PAGE by three different methods. Figure 5A shows protein profile after isolation according to Alberola *et al.* (1999). A large number of bands are present in all

the six *B.t.* isolates, but the prominent ones are 130, 70, and 40kDa. After sporulation the prominent

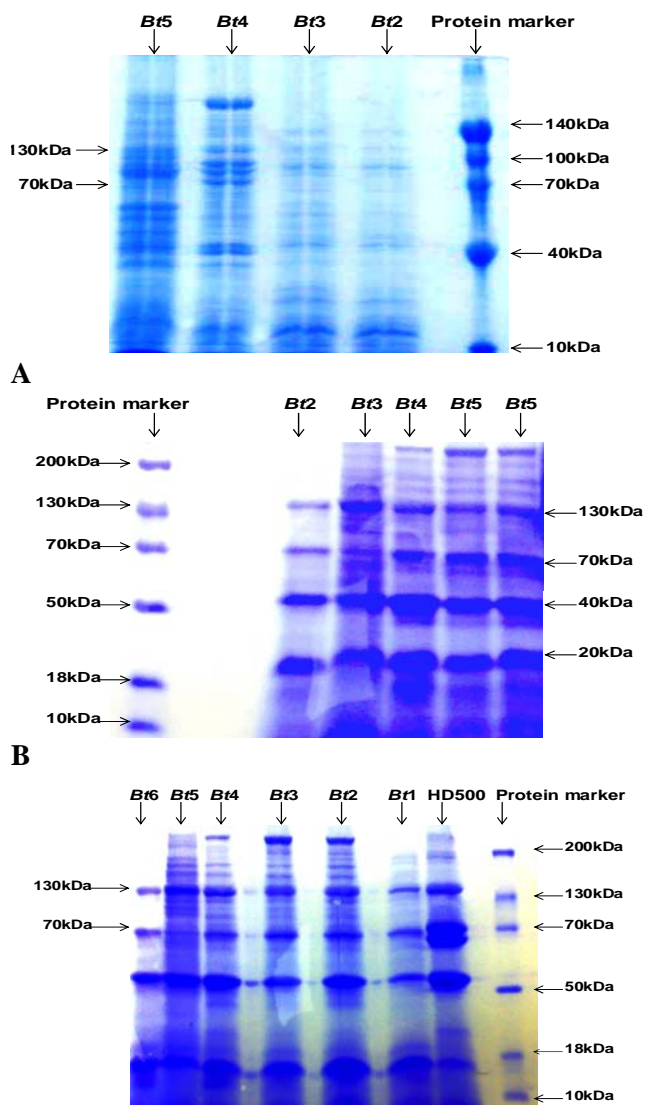


Fig.5. (A) Polyacrylamide gel electrophoretic pattern of total proteins of sporulated *B.t.* isolates DAB *Bt* 2 (SBS *Bt* 29), 3 (SBS *Bt* 34), 4 (SBS *Bt* 37), and 5 (SBS *Bt* 45), isolated according to Alberola *et al.* (1999). Variety of protein bands are visible. Two proteins of 130 kDa and 70 kDa are shown with the help of arrows on the left side.

(B) Polyacrylamide gel electrophoretic pattern of total proteins of sporulated *B.t.* isolates DAB *Bt* 2, 3, 4, and 5, isolated according to Sayyed *et al.* (2000). The protein bands obtained by this method are comparatively more prominent than obtained by Alberola *et al.* method of 1999. The proteins of

130, 70, 40 and 20kDa size are shown with the help of arrows on the right side of the gel.

bands presumably belong to Cry proteins. The protein bands obtained by this method were however, not as robust as in the other two methods.

Fig. 5B shows protein profile after isolation according to Sayyed *et al.* (2000). In this method, alkaline buffer was used to lyse the cells, which immediately dissolved the protein after being released from the cells, which explains robust bands of 130, 68, 40 and 20 kDa. Figure 5C shows protein profile of crystal protein isolated from the 72 hour sporulated *B.t.* culture, lysed in alkaline buffer (pH 10.5-11.00) and incubated for three hours at 37°C. The intensity of protein bands of 130, 70, 50 and 20 kDa is very high as crystal proteins are highly soluble in high basic pH. This method was found the best method for isolation of crystal proteins from *B.t.* isolates.

#### Nucleotide sequencing of shorter fragment of cry4 gene

The amplified 439 bp (Fig. 6A) conserved region of *cry4* gene containing clones were confirmed by colony PCR and restriction analysis of plasmid DNA containing shorter fragment of *cry4* gene in pTZ57R, with *Eco*R1 and *Hind*III (Fig. 6B). The nucleotide sequencing of amplified 439bp conserved region of *cry4* genes of all the 6 *B.t.* isolates were BLASTed using NCBI data base showed 99% homology with [gb|AY847707.1|](#) *B.t.* strain PBT602 130 kDa crystal protein (*cry*) gene, complete cds Length 3540.

The sequences of *cry4* conserved region of *B.t.* isolates SBS *Bt*-23, 29, 34, 37, 45 and 47 were submitted to GenBank database under accession numbers DQ788667, DQ788668, DQ788669, DQ788670, DQ788671 and DQ788672, respectively.

(C) Polyacrylamide gel electrophoretic pattern of total proteins of sporulated *B.t.* isolates DAB *Bt* 2, 3, 4, and 5, and that of control HD 500 isolated after lysis in alkaline buffer (pH 10.5-11.00) and incubation at 37°C for 3 hours according to the method developed in this laboratory (For details, see Materials & Methods). Robust protein bands are visible,



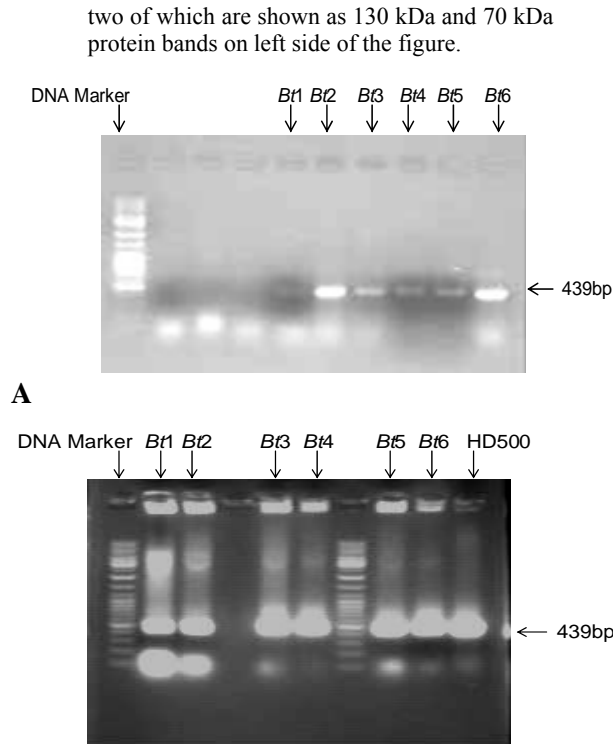


Fig.6. Amplified *cry4* conserved region (439bp) of *B.t.* isolates DAB *Bt*1-6 (SBS *Bt* 23, 29, 34, 37, 45 and 47, respectively) (A) and Colony PCR of positive transformants of clones of short fragment (439bp) of these *B.t.* isolates and positive control (HD500). The amplified *cry4* conserved region (439bp) is indicated with an arrow on right side. One Kb DNA marker is also shown.

**DISCUSSION**

*Habitat*

In this study, *B.t.* occurrence was examined in different habitats like soil, animal dung, bird droppings and grain dust where no *B.t.* products have been applied before. Although *B.t.* was found in all the samples but its occurrence in the soil samples was found to be relatively high compared to other types of samples. This observation is in disagreement with those of DecLucca *et al.* (1981), Theunis *et al.* (1998) and Liu *et al.* (1991) who reported 5%, 14% and 16% incidence of *B.t.* positive soil samples, respectively. It appears, the isolation of *B.t.* from soil is variably successful with

the rates ranging from 3-85% (Martin and Travers 1989) and from 22-50% (Chilcott and Wigley 1993,

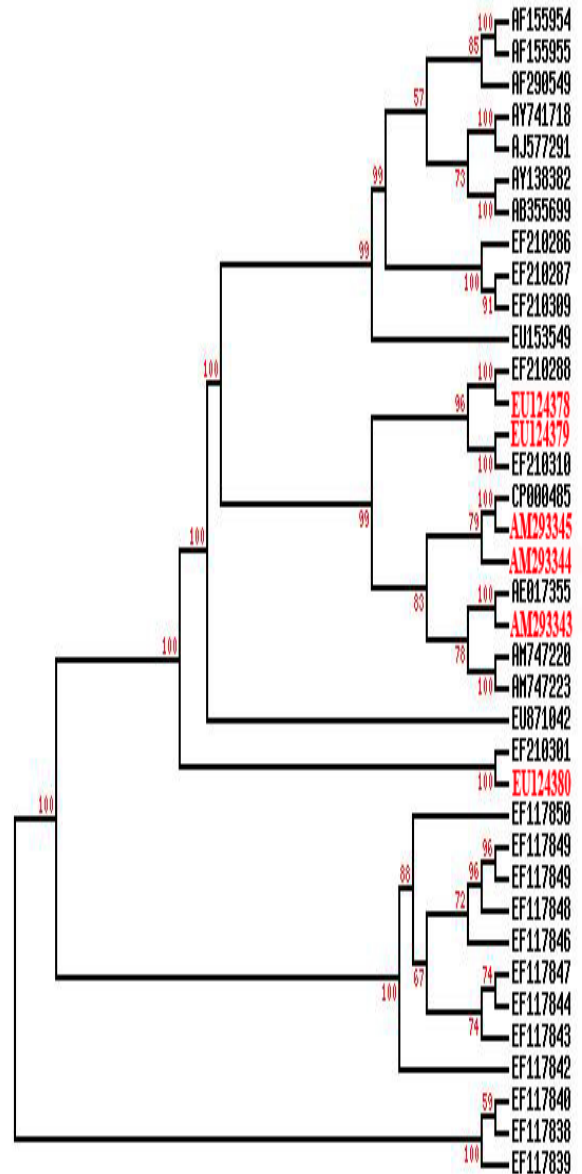


Fig.7. Dendrogram showing the relatedness of 16SrDNA of six *B.t.* isolates, with accession no EU124378 (SBS *Bt* 23) , AM293345 (SBS *Bt* 29), EU124379 (SBS *Bt* 34), AM293343 (SBS *Bt* 37), EU124380 (SBS *Bt* 45) and AM293344 (SBS *Bt* 47) with already reported genes in the GENbank data base. The values shown at the base of divergent lines are bootstrap values.

Ohba *et al.*, 2000). *B.t.* is a common member of soil

microflora. After soil, a particularly good source of *B.t.* has been found to be stored product material. Theunis *et al.* (1998) have reported the grain dust to be the richest source with 63% samples containing *B.t.* due to low level of humidity and ultra violet rays in grain mills favour the *B.t.* spores (Petras and Casida 1985, Ignoffo *et al.*, 1977). However, in this study 80% local isolates of *B.t.* were isolated from soil samples, 12% were from animal waste, while 8% were from wheat straw sample. Obeidat *et al.* (2004) reported *B.t.* in grain dust, olive-cultivated soils, waste and industrial-byproducts contaminated soils, and animal byproducts-contaminated soils. Balaraman (2005) reported the occurrence of mosquito-toxic strains of *B.t.* from different continents except, the America and Australia and the sources include soils/sediments, plants (rhizoplane of aquatic plants, phylloplanes, etc.), insects (mosquito larvae, stem borer, etc.), animal feces (wild mammals, zoo-animals and deer) and water. These *B.t.* strains belonged to different subspecies/serotypes and the highly toxic strains are restricted not to the first recognized subspecies, *B.t.* var *israelensis*.

All *B.t.* strains tested were characterized by microscopic observations after staining with Gram stain, acid fuchsin and malachite green, biochemical tests and PCR amplification of *cry4* gene sequences. During sporulation endospore formation which matured after 30 hours and started to be released in the surrounding medium after 36 hours. After 48 hours of growth 99% endospores matured and were released into the medium. These findings were according to Bechtel and Bulla (1976).

Twenty two out of 50 *B.t.* isolates were found positive for *cry4* genes, 72% of which were isolated from soil sample, 18% from animal waste and 10 % from dry wheat straw. Chak *et al.* (1994) and Bravo *et al.* (1998) reported relatively low proportion of strains with *cry4* in contrast to Ben-Dov *et al.* (1997) who reported frequent occurrence.

In the present study, six most toxic *B.t.* isolates harboring *cry4* genes namely SBS *Bt*-23, 29, 34, 37, 45 and 47, respectively were found to be toxic to 3<sup>rd</sup> instar larvae of mosquito, *Anopheles stephensi*. SBS *Bt* 45 was found to be the most toxic isolate which was demonstrated by the protein bioassay. It was isolated from the semi-damp soil

containing decaying cattle wastes. It also suggests that cattle (herbivores) are in contact with naturally occurring *B.t.* at high frequencies through the daily food intake of plants (Lee *et al.*, 2003).

#### Ribotyping

In this study 16S rDNA gene based primers were modified and used for the PCR based detection of *B.t.* strains. The sequence of the 16S rDNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The 16S rDNA gene sequences of *B. anthracis*, *B. cereus*, and *B.t.* have high levels of sequence similarity (>99%).

*B. cereus* is one of the common food pathogens, while *B.t.* is able to produce bioinsecticide, which is toxic to insect larvae (Höfte and Whiteley, 1989). On the other hand, *B.t.* can be distinguished from *B. cereus* by its ability to produce an insecticidal crystal inclusion inside the cell during sporulation (Henderson *et al.*, 1995). However, other workers have analysed the 16S rDNA sequences of the *B. cereus* group species and shown that they are virtually identical and within the variation expected for a single species (Seki *et al.*, 1978; Priest *et al.*, 1994; Giffel *et al.*, 1997; Yamada *et al.*, 1999).

A limited number of 16S rDNA sequences of *B. anthracis* (7 sequences), *B. cereus* (34 sequences), and *B.t.* (16 sequences) have been available at GenBank. Although these sequences are of different lengths and qualities, they differ from each other by no more than a few nucleotides in complementary regions. Therefore, this minimal level of diversity seen in the 16S rDNA of *B. anthracis*, *B. cereus*, and *B.t.* was thought to be an obstacle for using 16S rDNA gene sequencing to identify and differentiate these three species. The goal of this study was to evaluate the potential of 16S rDNA sequencing to rapidly identify local *B.t.* isolates up to sub species level and for this purpose full length (1.6 Kb) 16S rDNA sequencing of *B.t.* strains was done to rapidly identify the isolates up to species level. The sequence alignment of 16S rDNA gene from SBS *Bt*- 23, 29, 34, 37, 45 and 47) showed the maximum homology with *B.t.* serovar

*tolworthi*, *B.t.* str. *Al Hakam*, *B.t.* serovar *thompsoni*, *B.t.* serovar *konkukian*, *B.t.* serovar *fukuokaensis* and *B.t.* str. *Al*, respectively. So sequencing of 16S rDNA gene is a rapid tool for identification of *B.t.* at sub-species level.

The homology of 16S rDNA gene sequences of local *B.t.* isolates were determined with those of other 16S rDNA genes reported in literature and a rectangular phylogenetic tree based on topological algorithm was prepared (Fig.7). The distance matrices showed that 16S rDNA gene of DAB *Bt* 1 (EU124378) had maximum homology with *Bacillus thuringiensis* serovar *tolworthi* (EF210288), DAB *Bt* 2 (AM293345) with *Bacillus thuringiensis* str. *Al Hakam* (CP000485), DAB *Bt* 3 (EU124379) with *Bacillus thuringiensis* serovar *thompsoni* (EF210310), DAB *Bt* 4 (AM29334) with *Bacillus thuringiensis* serovar *konkukian* (AE017355), DAB *Bt* 5 (EU124380) with *Bacillus thuringiensis* serovar *fukuokaensis* (EF210301), and DAB *Bt* 6 (AM293344) showed homologies with two genes DAB *Bt* 2 (AM293345) and *Bacillus thuringiensis* str. *Al Hakam* (CP000485). The isolate DAB *Bt* 1 (EU124378) with maximum homology with *Bacillus thuringiensis* serovar *tolworthi* (EF210288), appears to be very much divergent from the remaining 5 isolates, which are fairly closely related *B.t.* isolates (Fig. 7).

#### *Toxic protein*

*B.t.* synthesizes highly specific larvicidal proteins in large quantities as different forms of parasporal crystalline inclusions during sporulation (Aronson *et al.*, 1986; Höfte and Whiteley, 1989). These cytoplasmic inclusions are composed of one or several insecticidal proteins ( $\delta$ -endotoxins) that have been classified into two major families - Cry and Cyt toxins - based on the similarity of their deduced amino acid sequences (Höfte and Whiteley, 1989; Crickmore *et al.*, 1998). Most of the native *B.t.*  $\delta$ -endotoxins are synthesized as inactive protoxins that are found within inclusion bodies (Aronson *et al.*, 1986). After ingestion by the susceptible insect larvae and dissolution in the midgut lumens (generally alkaline pH), the dissolved protoxins are activated by larval gut proteases to yield toxic fragments that are relatively resistant to further proteolysis (Aronson *et al.*, 1986;

Höfte and Whiteley, 1989).

*B.t.* subsp. *israelensis* produces at least three other proteins with molecular masses of 125kDa (Cry4A), 130kDa (Cry4B), and 68kDa (Cry4D) (Donovan *et al.*, 1988; Höfte and Whiteley, 1989; Federici *et al.*, 1990), which are toxic exclusively for dipteran species belonging to the suborder Nematocera. Among members of this suborder are insect vectors of important tropical diseases such as malaria, as well as some insect species of agricultural importance. *Anopheles stephensi*, an important vector of malaria, is resistant to a large group of inexpensive conventional insecticides. *B.t.* subsp. *israelensis* is a good alternative larvicide for anopheline species.

#### *Protein profile*

Protein profiles from six different *B.t.* isolates were analyzed by SDS-PAGE. Three methods were employed to isolate *B.t.* proteins. In one method 130, 70 and 40kDa protein bands were obtained, which probably represented Cry4A and B, Cry4C (Cry 10 and 4D (Cry11), and Cyt proteins, respectively. This method of protein isolation from *B.t.* did not give a strong total protein profile, whereas the second method gave much stronger crystal protein bands of 130, 68, 70 and 20-40 kDa. In the third method, however, the crystal protein bands were much robustly represented as compared with the ones isolated by the first two methods. The 130 and 70kDa bands were present in all the *B.t.* isolates as well as in the positive control HD500. The robustness of the proteins is apparent because of their high solubility in basic pH. Different methods of protein isolation from *B.t.* have been reported in literature. Kalman *et al.* (1995) isolated crystal protein using 1 volume of 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid)-NaOH (pH 10.5), 0.5 M NaCl, 1 mM EDTA and resuspended in 1 volume of 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA. Benintende *et al.* (1999) used 50 mmol Na<sub>2</sub>CO<sub>3</sub>, pH 10.5, 25 mmol 1,1 dithiothreitol (DTT) as lysis buffer and solubilized crystal proteins were subjected to trypsin treatment by incubating the samples at 37°C for 2 h in a 1:10 (v/v) trypsin solution (1mg/ml)/protein ratio, at a pH of 8.3. Orduz *et al.* 1996) reported the pH-mediated solubilization of crystals by using pH solutions of

the buffer system, ranging from 2.55 to 11.22, during 24 hr, at 30°C at 200 rpm. The solubilized proteins were dialyzed against phosphate buffer saline (0.32g NaH<sub>2</sub>PO<sub>4</sub>, 1.18g Na<sub>2</sub>HPO<sub>4</sub>, 8.58g NaCl, per liter, pH 7.2). The optimal solubilization was found at pH 11.3 when tested on a bioassay challenging 1st instar larvae of *C. quinquefasciatus*, larval mortality was 100% only when crystals were solubilized at pH 9.05 to 11.3. So crystal protein was isolated from *B.t.* isolates using highly alkaline pH buffer.

The interpretation of 130 and 70kDa protein bands as Cry4 protein bands is based on the fact that the six *cry4* harbouring *B.t.* isolates were found negative for *cry* 1,2,3,5,6 and 9 genes, except for two isolates SBS *B.t.*23 (DAB *Bt* 1) and SBS *B.t.*37 (DAB *Bt* 4), which had both *cry4* as well as *cry9* genes.

#### Conserved region of *cry4* gene

The sequences of conserved regions of *cry4* genes of the *B.t.* isolates were BLASTed using NCBI database to check the maximum homology of this region with already known sequences. All the sequences gave maximum homology (99%) to [gb|AY847707.1|](#) *B.t.* strain PBT602 130 kDa crystal protein. Cloning and sequencing of conserved region of *cry4* genes from all *B.t.* isolates is actually a confirmation of PCR based detection of *cry4* genes positive *B.t.* isolates. In PCR these conserved region from *B.t.* isolates were only amplified using universal primers but cloning, sequencing and alignment of amplified gene to already sequences confirmed that the amplified 439bp fragment in six local *B.t.* isolate was conserved region of *cry4* gene. These findings are according to the strategy of Jua'Rez-Pe'Rez *et al.* (1997) which allows to detect and further clone and sequence genes for which no specific primers are available and in which a variable region exists between conserved regions.

In conclusion, we have isolated and characterized the mosquitocidal *B.t.* from the local environment, 80% of which were from soil samples and out of which 44% were positive for *cry4* gene. Six of these *cry4* positive isolates were found comparatively more toxic to larvae of *Anopheles stephensi*, SBS-*Bt* 45 from the cattle waste being the most mosquitocidal. SBS-*Bt* 45

showed paracentral endospore, stainable with Malachite green in the vegetative cell, first after 18 hours, which were then released in the surrounding medium after 36 hours. Sporulation was completed after 48 hours. SDS-PAGE analysis showed that 130kDa (probably Cry4A, B), 70kDa (Cry4C, D) and 20kDa (Cyt enhancer protein of Cry4) proteins were present in all *B.t.* isolates as well as in positive control IPS 78. These important characteristics of mosquitocidal *B.t.* isolates will not only provide baselines data for further studies on *cry* genes and their expression, but would also be helpful in isolating and screening of mosquitocidal *B.t.*s in other parts of the world..

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