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Mosquitocidal Activity of *cyt* Positive *Bacillus thuringiensis* Isolated from Soil Samples

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ABSTRACT

Bacillus thuringiensis (*B.t.*) produces parasporal inclusion bodies Cry and Cyt proteins. The present study focuses on determining the insecticidal activity of *cyt* positive *B.t.* strains against 3rd instar larvae of mosquito. Larval mortality was noted after 24 h and GCU *B.t.* 4 (400± 1.15) was found to be the most toxic against 3rd instar larvae of mosquitoes. Spores LC_{50} values of other isolates were 451± 0.90 (GCU *B.t.* 1), 511± 0.85 (GCU *B.t.* 2), 525± .013 (GCU *B.t.* 5), 582± 0.66 (GCU *B.t.* 3) and 850± 0.34 (GCU *B.t.* 6). GCU *B.t.*4, with LC_{50} value of 68± 0.46 µg/ml was the most toxic *B.t.* isolate while other *B.t.* strains GCU *B.t.* 1 (75± 0.95), GCU *B.t.* 2, (93± 0.88), GCU *B.t.* 5, (106± 1.32), GCU *B.t.* 3 (106± 0.95) and GCU *B.t.*6 (118± 1.55) showed LC_{50} values in decreasing order, respectively. These bacterial isolates have a great potential to grow into a bioinsecticidal formulation for the control of mosquitoes.

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DAB designed the study, supervised the work and wrote the article. RF performed the experiments, analyzed the results and wrote the article.

Key words
***Bacillus thuringiensis*, insecticidal activity, inclusion bodies, mosquitoes**

INTRODUCTION

Toxins produced by different strains of *B.t.* are environment friendly. During sporulation phase *B.t.* produces insecticidal proteins Cry and *Cyt* toxin (Bravo *et al*., 2011) which are also known as pore forming toxins (PFTs). PFTs are the proteins which can only be inserted into their host membrane through pores when these undergo conformational changes, and destroy host cells by disrupting their ion balance (Parker and Feil, 2005). Cry toxins are active against dipterans, coleopterans and lepidopteran insects. Cry toxins have also been used for the control of mosquitoes and several crop pests while *cyt* toxins are specifically active against dipteran insects, mosquitoes and black flies (de Maagd *et al*., 2003) and some Cyt toxin are also found active against coleopteran larvae (Rupar *et al*., 2000). The Cyt toxins produced by *B.t.* not only exhibit insecticidal activity but also exhibited cytolytic activity *in vitro* against different cell lines and these toxins also showed hemolytic activity (Thomas and Ellar, 1983; de Maagd *et al*., 2003).

The three Cyt proteins namely *cyt* 1Aa, *cyt* 2Aa and *cyt* 2Ba share almost a similar structure having α and β domain arranged in such a way that β-sheet being wrapped up by two layers of α-helix hair pins (Li *et al*., 1996; Cohen *et al*., 2008, 2011).

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B.t. synthesizes Cyt proteins as inactive toxins or protoxins which are converted into active toxins of 25 kDa by mid gut proteases in a process called as proteolytic activation. Binding of activated Cyt toxins to the mid gut epithelium of susceptible insect does not require midgut proteases rather these toxins bind to the mid gut epithelium by interacting with unsaturated membrane lipids such as sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (Ward and Ellar, 1983).

As far as the mechanism of action of *cyt* toxins is concerned two models have been proposed for membrane insertion of these toxins into the midgut of dipteran insects. First model is the pore formation model and according to this model the binding of Cyt toxin to the membrane of mid gut cells induces the formation of cation selective channels there. The formation of these channels basically occurs in the membrane vesicles that ultimately lead to colloid osmotic lysis of cells (Knowles *et al*., 1989, 1992; Promdonkoy and Ellar, 2003). Second model is the detergent effect model according to which membrane of the mid gut cells disrupt that ultimately results in cell death, due to the nonspecific aggregation of toxins on the surface of membrane lipid bilayer (Butko, 2003; Manceva *et al*., 2004).

The mosquitocidal strains of *B.t.* subspecies *israelensis* (*B.t.i*) have been observed to have synergistic effect of Cyt and Cry toxins (Chang *et al*., 1993; Wu *et al*., 1994). *B.t.i* produces parasporal crystals of two types namely Cry and Cyt proteins. Cry proteins include Cry 4Aa, Cry Ba, Cry 10Aa and Cry 11Aa while Cyt proteins

include Cyt 1Aa and Cyt 2Ba (Berry *et al*., 2002). The individual toxicities of these toxins are lower but the combined effect of their toxicity against mosquito larvae is higher than it was expected on the basis of their individual toxicities (Crickmore *et al*., 1995; Perez *et al*., 2005).

The main objective of the present study was to isolate and characterize local strains of *B.t.* on the basis of ribotyping. Biotoxicity of *cyt* positive *B.t.* spores and total cell protein (mixture of Cry and Cyt proteins) against 3rd instar larvae of mosquito was also determined.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from different areas of Lahore, Faisalabad, and Kasur. For sampling sterilized spatula was used and soil from 1.5 to 2 cm below the surface is used and saved in sterilized zipper bags with proper labeling. Samples were then brought to laboratory and stored at 4°C for processing.

Isolation and biochemical characterization of bacteria

Bacteria were isolated according to method described by Martin and Travers (1989) using sodium acetate selective media. One gram soil sample was added to 10 ml of LB medium buffered with 0.3 M sodium acetate in a conical flask and incubated at 30°C for 4 h in shaking incubator at 150 rpm. The broth was filtered using syringe filter of 0.25 um pore size. Filtrate was then heat shocked at 80°C for 10 min and 200 microliter of each heat shocked filtrate was then spread on T3 agar plates and incubated at 37°C for 24-48 h. Similar colonies were then picked and streaked on LB agar plates and incubated at 30°C for one day.

In order to observe the vegetative cells under microscope 18 h grown cultures were used while to observe endospores 72 h old cultures were used. Gram staining of vegetative cells was done using safranin while endospore staining was done by using malachite green and acid fuchsin. Gram positive rods were then biochemically characterized according to methods described by Sneath (1986).

Ribotyping of B.t. isolates

PCR amplification was performed for the conserved region of 16S rRNA gene (appendix). Universal primers used for the amplification of 16S rRNA gene were as follows:

Forward primer: TGAAAACTGAACGAAACAAAC;

Reverse primer: 3′,CTCTCAAAACTGAACAAAACGAAA 3′

PCR was done according to Saiki *et al*. (1988). For this purpose PCR master mix (Fermentas) was used. Full length gene was amplified in thermal cycler under following conditions: first denaturation step for 5 min at 94 following 30 cycles, and each cycle with denaturation for 2 min at 94°C, annealing for 1.30 min at 52°C and elongation for 2 min at 72°C. At the end of 30 cycles a final elongation step at 72°C for 7 min. Amplified PCR products were run on 1% agarose gel. Bands were cut from the gel and Fermentas gel elution kit (K0513) was used for gene purification. The amplified and purified 16S rRNA genes were then sequenced.

In order to check the homology of isolated *B.t.* strains with already sequenced genes in Gene Bank sequence database, the sequences of 16S rRNA gene sequence of isolated *B.t.* strains were aligned and blasted. The gene sequences of isolated *B.t.* strains were then deposited in the NCBI DNA database.

PCR based detection of cyt 2B gene

Polymerase chain reaction was used in order to screen *B.t.* isolates for the presence of *cyt* 2B gene. For this purpose following primers were used (Guerchicoff *et al*., 2001). Forward, 5'- AATACATTTCAAGGAGCTA-3', and Reverse, 5'- TTTCATTTTAACTTCATATC-3'.

For PCR amplification DNA was used according to Carozzi *et al*. (1991). Strains were grown in LB broth medium and isolated DNA was used for PCR amplification. PCR conditions were initial denaturation step at 94°C for 3 min followed by 35 cycles each of, denaturation for 45 sec at 94°C, annealing for 45 sec at 45°C and extension for 1 minute at 72°C. At the end of 35 cycles a final elongation step was added for 5 min at 72°C. Amplified PCR products were analyzed using 1% agarose gel electrophoresis.

Biotoxicity assay of B.t. isolates positive for cyt 2B gene

Bacterial spore dose preparation

B.t. spore dose was prepared by using method described by Makino *et al*. (1994). LB broth was inoculated with single isolated colony of *B.t* and incubated at 37°C for 24 h. T3 sporulation medium was then streaked with this inoculum and incubated at 30°C for 3 days. Plates were then scraped off with the help of autoclaved distilled water and centrifuged. Pellets were washed twice with autoclaved distilled water and incubated for 40 min at 37°C with 10 ml of KCl sodium phosphate buffer and centrifuged. Two washes were given with autoclaved distilled water and spore pallet was incubated with 10 ml of urea buffer along with 25 mM of 2-mercaptoethanol at 37°C for 30 min and then centrifuged. Five washes were given with autoclaved distilled water and spore pallet was stored at 4°C.

The method as described by Cavados *et al*. (2005)

was used in order to determine the spore concentration in spore pellets. For each sample 0.5 mg of spore pellet was taken and oven dried at 70°C for one day. Samples were then transferred to desiccator until dried. It was then weighed up to fourth decimal and mean weight of biomass was determined. All these experiments were run in triplicates. Serial dilution of dried spores was used with a final concentration of 1 µg/ml by using autoclaved distilled water. Heat shock was given to the samples in water bath at 80°C for 12 min and plated on T3 agar medium (appendix). Incubated at 30°C for 72 h and number of spores per µg was counted as number of colonies per plate.

Extraction of B.t. cell protein

B.t. cell pellets were obtained as mentioned above in spore diet preparation. Pellets were then resuspended in alkaline buffer of high pH (appendix) and incubated at 37°C for 3 h with shaking. After centrifugation pellet was discarded and supernatant was saved. Trypsin activation of protoxin was done. The pH of solubilized protoxin was adjusted between 7-8 because trypsin is optimally active at neutral pH and was done by adding 1N HCl.

Experimental setup for bioassays

Third instar larvae of *Aedes aegypti* were used kindly provided by Malarial Research Center, BirdWood Road, Lahore. Ten different doses for each of the bacterial strains and also for positive control HD 500 kindly provided by Bacillus Genetic Stock Centre, Columbus, Ohio State, USA, were prepared viz., 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/ml of *B.t.* spores and different doses of *B.t.* total cell proteins (activated) ranging from 25 to 250 µg/ml in 20 ml of distilled water and 20 larvae were added to each cup.

These doses were prepared in wide mouthed plastic cups and 20 third instar larvae were added in each of the eleven cups which were then covered with fine net. Room temperature was maintained at 25°C. Larval mortality, larvae which were knocked down at the bottom of the cup were considered dead, was recorded after 24 h. Biotoxicity was determined by using probit analysis (Finney, 1971).

RESULTS

Characteristics of B.t. isolates

Total 50 samples were collected from different areas of Lahore, Faisalabad, and Kasur (Pakistan). During initial isolation a total of 200 *B.t.* like colonies were picked out, of which 70 were selected after Gram staining on the basis of purple colored rods (Fig. 1). These strains were then subjected to biochemical characterization. *B.t.* was found positive for indole test, Voges-Proskauer test, catalase activity, can utilize citrate, hydrolyze starch and casein and can decompose tyrosine. It can also grow on medium containing Sabouraud Dextrose agar, 7% NaCl and on medium containing 0.001% lysozyme. The presence of ICPs (insecticidal crystal proteins) produced by these bacteria which appear deep pink after acid fuchsin staining.

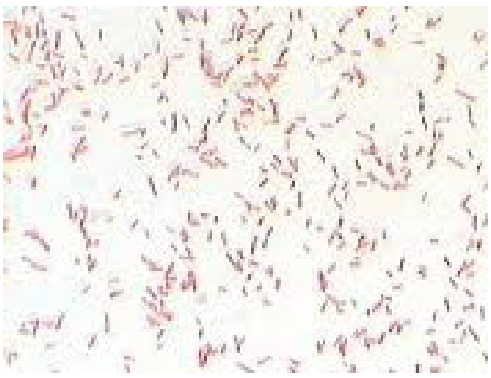

Fig. 1. Gram staining of GCU Bt 4 isolate.

Characteristics of cyt 2B positive isolates

Colony morphology and physical characteristics

All the *B.t.* isolates positive for *cyt* 2B gene appeared as Gram positive rods. Endospores produced by these isolates become visible inside the vegetative cell after 18 h of incubation (Fig. 2a). After 30 h of incubation endospores are released into the medium and are stained green with malachite green (Fig. 2b) and after 48 h of incubation sporulation is completed and at this stage most of the *B.t.* isolates also contained ICPs (Fig. 2c).

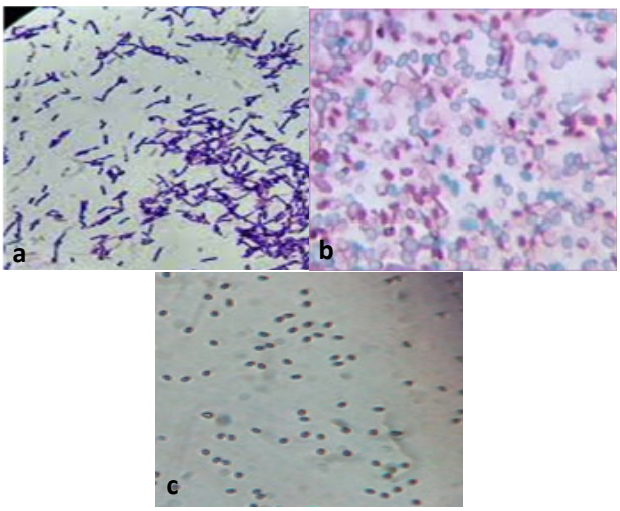

Fig. 2. Endospore staining of GCU Bt 4 (a) 18 h, (b) 30 h and (c) 48 h of sporulation.

Table I: Biotoxicity of six most toxic *Bt* isolates against 3rd instar larvae of *Ae. aegypti*.

Sr.No	B.t. isolates	Renamed as	Soil texture	LC_{50} of spores (µg/ml)	LC_{50} of total cell protein (mixture of Cry and Cyt proteins) (µg/ml)
1	GCU *Bt*1	NBBt1	Dry soil containing decaying cattle waste	451± 0.90	75± 0.95
2	GCU *Bt*2	NBBt2	Dry soil containing decaying cattle waste	511± 0.85	93± 0.88
3	GCU *Bt*3	NBBt	Dry soil	582± 0.66	106± 0.95
4	GCU *Bt*4	NBBt4	Moist soil rich in organ-ic manure	400± 1.15	68± 0.46
5	GCU *Bt*5	NBBt5	Moist soil	525± .013	106± 1.32
6	GCU *Bt*6	NBBt6	Moist soil	850± 0.34	118± 1.55
7	Commercial Bti	HD 500	Reference strain	700± 1.34	100± 1.81

Toxicity of purified Cyt protein was compared with total cell protein (mixture of Cyt and Cry proteins).

Ribotyping

After ribotyping, the sequence alignment of 16S rRNA gene of 6 *B.t.* isolates showed maximum homology with already reported strains and their gene sequences were submitted to Genbank database under following accession numbers GCU Bt 1 to GCU Bt 6 as KX611122, KX611120, KX611121, KY611803, KY612210 and KY611804.

Prevalence of cyt 2B gene in B.t. isolates

A conserved region of 469 base pair fragment of *cyt* 2B gene was amplified using PCR (Fig. 3). The sequences were then submitted to Gene bank and were assigned following accession numbers, NBBt1-6, KY777430, KY777431, KY888138, KY888137, KY888139 and KY777429. Among the isolated *B.t.* strains 35% were found positive for *cyt* 2B gene. Among these positive strains 25% were isolated from soil of cattle rearing area which was rich in organic manure, 7% from garden soil where cattle waste was added as fertilizer and 3% from dry soil.

Biotoxicity assay with B.t. isolates

In order to screen the most toxic *B.t.* isolate bioassay was performed using spores of *B.t.* against 3rd instar larvae of mosquito. LC_{50} values of *B.t.* spores against 3rd instar larvae of mosquitoes are shown in Table 1. Bioassay indicated that GCU *B.t.*4 is the most toxic *B.t.* isolate and it was isolated from moist soil rich in organic manure and has LC_{50} value of 400± 1.15 µg/ml. With LC_{50} value of 451± 0.90 µg/ml GCU *B.t.* 1 was isolated from dry soil containing decaying cattle waste, with LC_{50} values of 511± 0.85 µg/ml and 525± .013 µg/ml GCU *B.t.* 2 and GCU *B.t.* 5 were isolated from soil containing decaying cattle waste and from moist soil respectively, with LC_{50} values of 582± 0.66 µg/ml and 850± 0.34 µg/ml GCU *B.t.* 3 and GCU *B.t.* 6 were isolated from dry and moist soil respectively (Table 1).

Bioassay with total cell proteins

For bioassays with total *B.t.* cell proteins HD 500 was used as positive control (It does not contain *cty* 2B gene). GCU *B.t.*4, with LC_{50} value of 68± 0.46 µg/ml was the most toxic *B.t.* isolate while GCU *B.t.* 1, showed LC_{50} value of 75± 0.95 µg/ml, GCU *B.t.* 2, 93± 0.88 µg/ml, GCU *B.t.* 5, 106± 1.32 µg/ml, GCU *B.t.* 3 106± 0.95 µg/ml and GCU *B.t.*6, 118± 1.55 µg/ml in decreasing order respectively (Table 1).

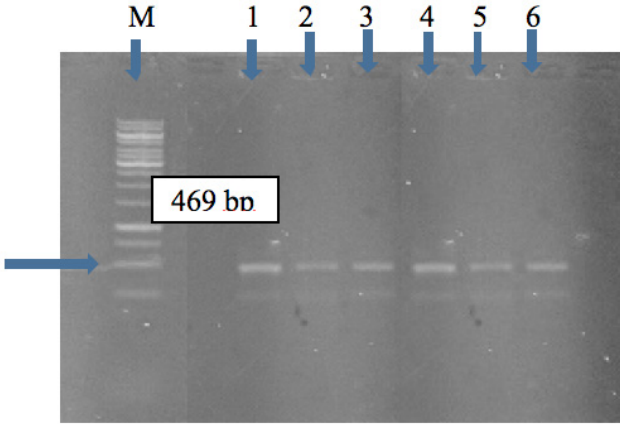

Fig. 3. Gel electrophoresis of 469 bp fragment of cyt 2B gene of the six most toxic Bt isolates, where 1, 2, 3, 4, 5 and 6 represent 6 Bt isolates viz GCU Bt 1, 2, 3, 4, 5 and GCU Bt 6 respectively while M stands for DNA marker.

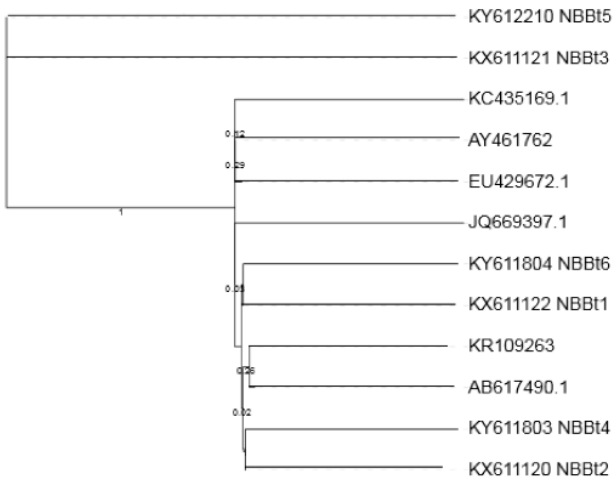

Fig. 4. Dengrogram representing 16S rRNA gene relatedness of six most toxic B.t. isolates NBBt 1,2,3,4,5 and 6 with accession numbers KX611122, KX611120, KX611121, KY611803, KY612210 and KY611804 with already reported genes in the Genbank DNA database.

DISCUSSION

In the present study, different soil samples were collected for the isolation of *B.t.* these include soil from cattle rearing areas, garden soil, dry and moist soil and a large number of *B.t.* were isolates from soil rich in organic manure this is in agreement with Meadows *et al.* (1992) who reported omnipresent distribution of *B.t.* The abundance of *B.t.* in soil might be due to the presence of enormous amount of nutrients and an increased insect activity that leads to optimum survival (Al-Momani *et al*., 2004). *B.t.* strains have also been isolated from diverse habitats other than soil, these include aquatic environments (Ichimatsu *et al*., 2000), plants (Maduell *et al*., 2002), insects (Cavadoes *et al*., 2001), animal faeces (Lee *et al*., 2003) and arid environments (Assaeedi *et al*., 2011). In the Middle East, the natural occurrence of *B.t.* in soil environments was reported from Egypt (Merdan and Labib, 2003) and Jordan (Sadder *et al*., 2006).

All the *B.t.* strains were examined microscopically after Gram staining and endospore staining and it was observed that sporulation matured or completed after 30 h of incubation and after 36 h they are now released into the medium. Around 99% of the spores were released into the medium after 48 h of incubation. These findings are in accordance with the findings of Bechtel and Bulla (1976).

Fifteen out of fifty *B.t.* isolates were found positive for *cyt* 2B gene. Out of these 30% *cyt* 2B positive isolates 53% were isolated from soil rich in organic manure, 27% were isolated from garden soil containing animal waste and 20% were isolated from agricultural soil. These findings were in accordance with the findings of Mahalakshmi *et al*. (2012), who reported the occurrence of *cyt* positive strains of *B.t.* from soil and insects.

In the present study, six *cyt* 2B positive most toxic isolates of *B.t.* namely GCU *B.t.* 4, 1, 2, 5, 3 and 6, respectively showed toxicity against 3rd instar larvae of mosquito. Among these isolates GCU *B.t.* 4 was found to be most toxic as indicated by spore and protein bioassay. This is in accordance with the work of Juárez-Pérez *et al*. (2002), who reported that *cyt* 2B positive strains of *B.t.* are active against mosquito larvae i.e., *Aedes aegypti, Culex pipiens, Culex quinquefasciatus* and *Anopheles stephensi.*

In the present study, specific primers were used for the amplification of 16S rRNA gene. 16S rRNA gene sequence is widely used tool now a day to identify a bacterium up to species level. There is a great sequence homology in 16S rRNA gene sequence of some members of the genus Bacillus namely *B. thuringiensis, B. cereus,* and *B .anthracis*, and this homology is up to 99%. The major difference between *B.t.* and the other members of the genus Bacillus is the production of ICPs during sporulation phase. After sequence alignment the 16S rRNA gene of the *B.t.* isolates were assigned following accession numbers GCU Bt 1 to GCU Bt 6 as KX611122, KX611120, KX611121, KY611803, KY612210 and KY611804 and a dendrogram has been constructed on this similarity (Fig. 4). In the present investigation, it was found that *B.t.* strains showed maximum homology with *Bacillus thuringiensis* serover *israelensis* strains with accession numbers, AY461762, EU429672.1, KR109263, KC435169.1, AB617490.1 and JQ669397.1 respectively. This study provides a convenient method which is time saving and economical and also recommends that *B. thuringiensis* can be used to control mosquitoes efficiently at larval stage.

Statement of conflict of interest

Authors have declared no conflict of interest.

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