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MOLECULAR CHARACTERIZATION OF *BACILLUS THURINGIENSIS* STRAINS ISOLATED FROM A SELECTED SITE IN NOCHCHIYAGAMA, ANURADHAPURA IN SRI LANKA

MCM Zakeel*, DMD Dissanayake and PA Weerasinghe
Faculty of Agriculture, Rajarata University of Sri Lanka, Puliyankulama, Anuradhapura

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ABSTRACT

The protein toxins produced by *Bacillus thuringiensis* are the most widely used natural insecticides in vector and pest control in agriculture. *B. thuringiensis* strains present in surface and sub-surface soil samples collected from Nochchiyagama were isolated by 0.25M sodium acetate selection method. Isolated *B. thuringiensis* was grown on Luria Bertani agar medium and stained by Gram staining procedures. Sixty isolates of *B. thuringiensis* were identified by Coomassie Blue staining procedure and characterized based on colony morphology, crystal shape, plasmid profile and bioassay. Results revealed that sub-surface samples had more *B. thuringiensis* counts than surface soils. This study also indicated that *B. thuringiensis* was abundant in soils contaminated with animal wastes. All the isolates formed 'pan cake' shape circular colonies with smooth or serrate margins with varying diameter. Fifty five isolates were found to have rod shape crystals, 4 were spherical shape and only one isolate had rhomboidal shape crystal. Thirty six isolates were toxic to the third instar larvae of *Aedes aegypti* including the isolate which contained rhomboidal shape crystal. All the other isolates found toxic to the mosquito larvae consisted with rod shape crystal inclusion bodies. There were eight different *B. thuringiensis* strains among the isolates and 55% of these were *B. thuringiensis israelensis*.

***Key words**: Bacillus thuringiensis*, Colony shape, Molecular characterization, Parasporal crystal inclusion, Plasmid

INTRODUCTION

The use of chemical pesticides for pest and vector management results in death of natural enemies and thereby necessitates repeated sprays of the insecticides leading to the development of pest and vector resistance and resurgence. Further, the chemical pesticides pollute the environment as well. Biological pesticides are therefore, becoming key components of integrated pest management strategies (Obeidat *et al*. 2004). The tremendous success in microbial pesticides has come from the uses of *B. thuringiensis* (Obeidat *et al*. 2004). *B. thuringiensis* strains show specific insecticidal activity against insects of different orders such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga (Schnepf *et al*. 1998). However, no adequate studies have been conducted to characterize *B. thuringiensis* strains in Sri Lanka. Therefore, this study was initiated to isolate and characterize *B. thuringiensis* strains from a selected site in Nochchiyagama, Anuradhapura in Sri Lanka.

MATERIALS AND METHODS

Soil sample collection

B. thuringiensis strains were isolated from surface and sub-surface (5cm below soil surface) soil samples collected from different locations in a private livestock farm at Nochchiyagama town area, Anuradhapura in Sri Lanka.

Isolation

The bacteria were isolated from the soil samples according to Ohba and Aizawa (1986) by heating the sample suspensions at 80^{o}C for 30min. The suspensions were then enriched in 0.25M sodium acetate buffered Luria Bertani (LB) broth (Travers *et al*. 1987). Serial dilution was made and at appropriate dilution 30µl of suspension was plated on Luria Bertani (LB) agar medium. Isolated *B. thuringiensis* was grown on the medium for overnight at 25^{o}C in a shaking incubator. Then bacterium from each colony was stained by Gram staining procedures to confirm the presence of bacteria. Then each colony was examined under light microscope for the presence of endospore and the parasporal bodies after staining with Coomassie Brilliant Blue R-250.

Plasmid isolation and agarose gel electrophoresis

Sixty isolates of *B. thuringiensis* were identified by Coomassie Blue staining procedure described by Ammons *et al*. (2002). An identification code of S_xI_y where $S_{x;x:}$ sample number and $I_{y;y:}$ isolate number was assigned to isolates of *B. thuringiensis.* Plasmid DNA was isolated and prepared by alka-

*Corresponding author

line lysis with sodium dodecyl sulfate (SDS): mini preparation method described by Sambrook and Russell (2001) and subjected to agarose gel electrophoresis. 20µl of plasmid DNA was loaded into each well of a 0.8% agarose gel. Electrophoresis was conducted at a constant current at 80V for 2.5h. Gels were viewed using an ultraviolet Chromato-Vue transilluminator model [TM-20, San Gabriel, CA 91778 U.S.A]. The gels were then photographed using an instant Polaroid camera. Plasmid DNA profiles were obtained by running DNAs in comparison with *B. thuringiensis israelensis* as a standard. Number of colonies recovered from soil samples was analyzed using SAS computer package and Duncan mean separation procedure. The significance of the variation of each variable was tested using a one-way analysis of variance (ANOVA).

Bioassay

The toxicity of *B. thuringiensis* isolates against third instar larvae of *Aedes aegypti* was determined according to the method described by Karamanlidou *et al*. (1991).

RESULTS AND DISCUSSION

Sixty colonies were recovered after heat treatment of soil samples collected from different locations in a livestock farm in Nochchiyagama, Anuradhapura in Sri Lanka and were characterized based on colony morphology, crystal shape, plasmid profile and bioassay.

On average subsurface soil had 69 colonies and surface soil had 50 colonies per plate spread with 50µL of isolated bacterial suspension. The results revealed that sub-surface samples had more *B. thuringiensis* counts than surface soils. As stated by Braun (2000), this may be due to the fact that *B. thuringiensis* spores are readily inactivated by exposure to UV light of the sun and as a result less count of *B. thuringiensis* in surface soil could have been recorded.

Comparatively more number of colonies was recovered from soil samples that contained more organic matter or livestock farm wastes. Although *B. thuringiensis* is ubiquitous, the results showed that *B. thuringiensis* was highly abundant in soils contaminated with animal wastes. This is in agreement with the study done by Obeidat *et al*. (2004).

Purple staining bacterial candidates were observed through light microscope after gram staining (Fig 1). It assured that the isolated bacteria were gram-positive. This method can be used to tentatively identify and differentiate *B. thuringiensis* from morphologically indistinguishable yet of different species after sodium acetate selection (Obeidat *et al*. 2004).

Rod, spherical and rhomboidal shape blue-staining crystal inclusions were observed after Coomassie Brilliant Blue staining. It shows that the isolated organisms belong to different *B. thuringiensis* strains. Fifty five isolates were found to have rod shape crystals, the isolates S_1I_4, S_1I_7, S_1I_8 and S_1I_9 were spherical shape and only one isolate (S_6I_1) had rhomboidal shape. All the isolates formed 'pan cake' like circular colonies with smooth or serrate margins with varying diameter (Fig 2). The variation in the dominancy of parasporal crystal shapes among isolates might be related to the difference in sample location and also due to

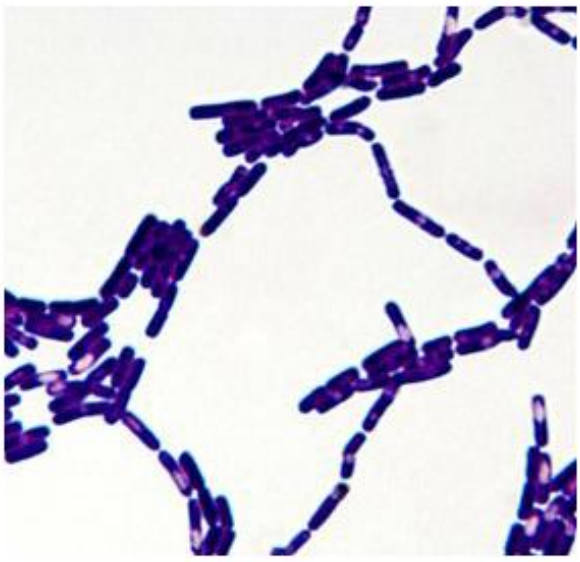

Figure 1: Microscopic Observations of Germ Stained Candidates Magnification X 1000

Figure 2: Colony morphology of Bacillus thuringiensis

genetic variation. In this investigation, the reference strain *B. thuringiensis israelensis* was found to produce rod shape crystal inclusions as recorded by Karamanlidou *et al*. (1991).

Two intense plasmid bands were observed (lane number 7) in the gels run with *B. thuringiensis israelensis* (Fig 3). Four plasmid DNA bands and a smear were observed in one isolate (S_6I_{10}) whereas four isolates (S_1I_7, S_1I_8, S_1I_9 and S_5I_5) showed three bands of 20, 15 and 6kb (gel photo is not included). Rest of the isolates displayed two clear bands (as in lane number 2, 4 and 5) as observed in *B. thuringiensis israelensis.*

Out of 60 isolates, 36 isolates were toxic to the third instar larvae of *Aedes aegypti* (Table 1). An isolate (S_6I_1) which contained rhomboidal shape crystal was one in the toxic isolates. All the other isolates toxic (140µg/L of LC50) to the mosquito larvae consisted with rod shape crystal inclusion bodies.

Based on colony morphology, crystal shape plasmid profile and bioassay, it was evident (Table 2) that there were eight different *B. thuringiensis* strains available among the isolates and 55% of those were *B. thuringiensis israelensis* which gave two prominent plasmid DNA bands of 15 and 20kb as shown in Fig 3. Some of the DNA was not well resolved and remained in the wells (Fig 3) and it indicates the inability of conventional electrophoresis for resolving high molecular weight plasmid DNA.

Table 1: Toxicity of Bacillus thuringiensis isolates against Aedes aegypti

Toxicity	*Bacillus thuringiensis* Isolates
Toxic isolates	S_1I_3, S_1I_4, S_1I_5, S_1I_7, S_2I_2, S_2I_5, S_2I_6, S_2I_7, S_2I_{10}, S_3I_1, S_3I_2, S_3I_3, S_3I_4, S_3I_5, S_3I_6, S_3I_7, S_3I_8, S_3I_9, S_3I_{10}, S_4I_3, S_4I_4, S_4I_5, S_4I_6, S_4I_8, S_5I_1, S_5I_4, S_5I_6, S_5I_7, S_5I_8, S_5I_{10}, S_6I_1, S_6I_2, S_6I_3, S_6I_4, S_6I_7, S_6I_8
Non-toxic isolates	S_1I_1, S_1I_2, S_1I_6, S_1I_8, S_1I_9, S_2I_1, S_2I_3, S_2I_4, S_2I_8, S_2I_9, S_2I_{11}, S_2I_{12}, S_4I_1, S_4I_2, S_4I_7, S_5I_2, S_5I_3, S_5I_5, S_5I_9, S_5I_{11}, S_6I_5, S_6I_6, S_6I_9, S_6I_{10}

$S_{x;\ x:}$ Sample Number, $I_{y;\ y:}$ Isolate Number

Table 2. Comparative toxicity against *Aedes aegypti*, crystal shapes and plasmid profile of *Bacillus thuringiensis* isolates

Toxicity	Crystal shape	Number of bands 2	3	4 & smear
Toxic	Rod	S_1I_3, S_1I_5, S_2I_2, S_2I_5, S_2I_6, S_2I_7, S_2I_{10}, S_3I_1, S_3I_2, S_3I_3, S_3I_4, S_3I_5, S_3I_6, S_3I_7, S_3I_8, S_3I_9, S_3I_{10}, S_4I_3, S_4I_4, S_4I_5, S_4I_6, S_4I_8, S_5I_1, S_5I_4, S_5I_6, S_5I_7, S_5I_8, S_5I_{10}, S_6I_2, S_6I_3, S_6I_4, S_6I_7, S_6I_8	-	-
	Spherical	S_1I_4	S_1I_7	-
	Rhomboidal	S_6I_1	-	-
Non-toxic	Rod	S_1I_1, S_1I_2, S_1I_6, S_2I_1, S_2I_3, S_2I_4, S_2I_8, S_2I_9, S_2I_{11}, S_2I_{12}, S_4I_1, S_4I_2, S_4I_7, S_5I_2, S_5I_3, S_5I_9, S_5I_{11}, S_6I_5, S_6I_6, S_6I_9	S_5I_5	S_6I_{10}
	Spherical	-	S_1I_8, S_1I_9	-
	Rhomboidal	-	-	-

Sx; x: Sample Number, Iy; y: Isolate Number

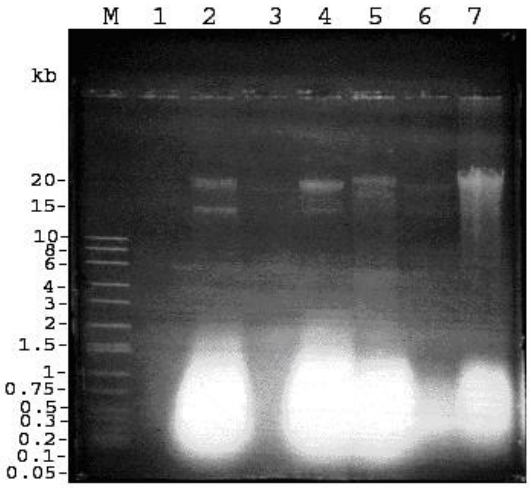

Figure 3: Comparative agarose gel electrophoresis of the plasmid profile of Bacillus thuringiensis isolates. Lanes: M, Marker in kilobases; 1, D3A4; 2, D3A5; 3, D3A6; 4, D3B1; 5, D3B2; 6, D3B3; 7, ST, Bacillus thuringiensis israelensis (Standard)
Dx; x: Sample number, Ay, By; y: Isolate number, A&B : Two replicates

CONCLUSIONS

Sub-surface soil samples collected from Nochchiyagama town area had more *B. thuringiensis* counts than surface soils. *B. thuringiensis* was abundant in soils contaminated with animal wastes. There were eight different *B. thuringiensis* strains among the isolates collected from Nochchiyagama town area and 55% of these were *B. thuringiensis israelensis*.

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