Isolation, Identification and RAPD-PCR analysis of New Isolated *Bacillus thuringensis*

[1]Jamal S. Sabir, [1,2] Salah.E.M. Abo-Aba, [3]Manal M. Said, [1,4]Refaei M. Hussein, [1] Najla Al-Saud and [1]Mohammed Mutwakil

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia,
[2]Microbial Genetics Department, Genetic Engineering and Biotechnology Division, National Research Centre, Giza, Egypt.
[3]Department of Biotechnology, College of Science, Taif University, Kingdom of Saudi Arabia,
[4]Genetics and Cytology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Giza, Egypt
salah_aboaba@yahoo.com

Abstract: *Bacillus thuringiensis* (*B.t*) are important bacteria in the field of pest's biological control due to their insecticidal properties and its importance in agriculture. Therefore, the aim of the present study is to investigate and identify new12 native *Bacillus thuringiensis* strains using morphological, physiological, biochemical and molecular approaches. Morphological identification of studied isolates was carried out via dark field, phase contrast, electron microscopic scanning. Physiological, biochemical reactions and RAPD-PCR molecular techniques were done on native isolates. The electrophoretic analysis of RAPD band profiles showed the presence of polymorphism among the studied isolates. RAPD obtained data could be used to characterize, identify and discriminate the studied *Bacillus thuringiensis* isolates. The generated RAPD specific markers might be utilized in the tracking of these isolates.

[Jamal S. Sabir, Salah.E.M Abo-Aba, Manal M. Said, Reffay M. Hussein, Najla Al-Saud and Mohammed Mutwakil. **Isolation, Identification and RAPD-PCR analysis of New Isolated *Bacillus thuringensis***. *Life Sci J* 2013; 10 (2): 1352-1361]. (ISSN:1097-8135). http://www.lifesciencesite.com.

Key words: *Bacillus thuringensis*, DNA, RAPD-PCR, Isolation, Identification, Electrophoresis

1. Introduction

The use of chemical substances to control pests was started in the mid -1800s. Many of these chemicals are also being today. Chemical pesticides applications have caused many environmental problems including insect resistance, toxicity to humans and to beneficial insects (1).

The observation led to the development of bio-insecticides based on *Bacillus thuringensis* for the control of certain insect species among the order Lepidoptera, and Coleopteran and also active against other insect order Hymenoptera, Homoptra, Orthoptra, and Malloptra, also against nematodes, mites, and protozoa (2). This feature makes *Bacillus thuringensis* most important biopesticide on the world market (3).

The greatest successes in microbial pesticides have come from the use of commercial preparations of *Bacillus thuringiensis* (*B.t*). These have been the most successful biological pest control products worldwide, and 95% of microbial pesticides sold are of this bacterial agent, with annual sales estimated at $100 million (4,5).

The commercial interest in biological control of insects stimulated intensive screening programs to search for new strains in different countries allowing the discovery of new serovares presenting different spectrum of entomopathogenic activity (6).

Bacillus thuringensis is Gram positive spore forming bacilli able to synthesize parasporal crystalline inclusions during the sporulation at stationary phase of its growth cycle, the shape of the cell is rod. The wide of the rod is 3-5µm in size when grown in liquid media, the most distinguishing features between *Bacillus thuringensis* from closely related bacillus species (e.g. *Bacillus cereus* and *Bacillus anthraces*) is the presence of crystal body that is near to the spore, outside the exosporangium during the endospore formation (7-11).

Screening the environment for new and highly potent strains of *Bacillus thuringiensis* (*Bt*) has become inevitable as one of the strategies for insect resistance management (12).

Bacillus thuringiensis Isolates were characterized by SDS-PAGE, light microscopy, PCR, probe hybridization, and with selected isolates (13), DNA sequencing, bioassay or Electron Microscopy. Phase Contrast Microscopy as the backbone of the screening strategy (14, 15).

The random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been identified as one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus

do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable (16).

Several recent studies supported the utilization of the RAPD technique to characterize individuals among the same species (17, 18), to conduct phylogenetic relationships (19, 20), to detect genetic variability among closely related species (21, 22), to reveal genetic markers for certain trait (23).

The genetic diversity of *Bacillus thuringensis* strains shows different according to the region where they were isolated (24- 28).

Bacillus thuringiensis recovered from different habitats were compared using random amplification of polymorphic DNA (RAPD) to determine whether they could be differentiated at the molecular level. Total genomic DNA from each isolate and three reference strains were amplified using 10-mer primers. Electrophoreses analysis of the amplification products revealed the incidence of polymorphism among the isolates. Pair-wise comparisons of polymorphic products were used to construct a dendrogram applying the cluster analysis. Such analysis showed some regional variation among the isolates, but did not indicate a clearly defined habitat location pattern of the DNA polymorphism (29).

One hundred and twenty-six strains of *Bacillus thuringiensis* representing 57 serovars were allocated to 58 genomic types using random amplified polymorphic DNA (RAPD)-PCR patterns. Results showed that each serovars encompassed identical or closely related strains. Despite this genomic homogeneity, most of these serovars also included at least one variant strain. About 57 serovars examined, 31 contained at least one strain with a closely related or identical. RAPD pattern concluded that while the species is genomically diverse, the homogeneous serovars represent clonal lineages of successful insect pathogens (30).

Randomly amplified polymorphic DNA fingerprinting assay has been optimized that discriminate different *B. thuringiensis* isolates and serotypes. Due to advance in molecular biology technique, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism (31).

Genomic DNA fingerprints of *Bacillus thuringiensis* serovars representing different serotypes were obtained by Randomic Amplification of Polymorphic DNA (RAPD- PCR) using three different random primers. The electrophoretic analysis of RAPD band profiles showed the presence of polymorphism among the serovars and serotypes between the primers used in this study. Results suggested that genomic fingerprints obtained by RAPD can be used for the genotypic characterization and identification of *B. thuringiensis* serovars as a complement to flagellar serology (6).

Isolates of *Bacillus thuringiensis* were isolated from soil samples, were characterized by randomly amplified poylmorphic DNA (RAPD) markers to determine their genetic diversity pattern based on their source of origin. Different random primers were used for RAPD amplification. Some primers produced polymorphic fragments, whereas others produced monomorphic fragments. When the RAPD banding pattern data was subjected to dendrogram construction, isolates fell into two separate clusters. All primers showed amplification and indicated the good diversity of *B. thuringiensis* isolates (32).

The aims of the present work are to 1) isolation and characterization of *Bacillus thuringensis* from different Egyptian localities; also study their morphological, physiological and chemical characters 2) assessment of genetic diversity among isolated *Bacillus thuringensis*. Also, to isolate and characterize new and highly potent strains of *B. thuringiensis* (Bt) isolates which might be have insecticidal potentialities.

2. Materials and Methods

Sample Collections:

Soil samples were collected from different governorates of Egypt. The collected samples are summarized in Table 1. Scraping the surface of soil samples (fewer than 3cm below the surface) with sterile spatula, collected and stored in sterile plastic pages at 4^{o}C.

Isolation of *Bacillus thuringensis*:

Collected samples suspended in 5 mL test tubes containing nutrient broth (15, 40) with0.12M sodium acetate at pH 6.8. Suspensions were incubated overnight at 37^{o}C with shaking. Samples pasteurized for 5min at 80^{o}C to kill vegetative cells of bacteria and only remain is spore forming cells, plated on nutrient agar and incubated at 35^{o}C overnight. *Bacillus thuringensis* like colonies speeded over nutrient agar plates and single colonies were picked up and streaked in the same plates. Colony shape and the positions of the spore were checked by light microscope Table (1), the endospore appeared edged in black and are very bright and retractile. Endospores resist simple stains or dyes and hence appear as non-staining entities in Gram-stain preparations.

Microscopic studies

Phase contrast microscopy:

Cells were harvested at the mid log phase and 10 hrs after the attainment of the stationary phase. The cells were washed and suspended in 0.1 M

potassium phosphate buffer pH 7.2. A wet smear of the suspension was observed immediately under a phase contrast microscope (3).

Dark field microscopic examination:

Sporulation of the isolates were done by incubating *B. thuringiensis* in an orbital shaker at 250 rpm for 90 hrs . Visualization the formation of the endospore and the parasporal bodies under the phase contrast microscope were don as shown in Fig.(2). However, phase contrast microscopy could not clearly distinguish the capped spores.

Scanning Electron Microscopic analysis:

Axenic cultures of *Bacillus thuringensis* were obtained by streak plate method. The sporulated (72hrs), cells were washed thrice with 0.5M NaCl and fixed in gradients of aqueous ethanol (30%, 50%, 70%, 90%) and finally with absolute ethanol. The SEM examination was performed. All samples were sputtered with 150A thick gold layer (JEOL JFC-1600 Auto Fine Coater). The *B.T* cells were examined with a scanning electron microscope Quanta FEG 450, FEI, Amsterdam, Netherland. The microscope was operated at an accelerating voltage of 20 KV.

Physiological and biochemical reactions of isolated strains:

The Physiological and biochemical characteristics of isolates were done as shown in Table 2 according to standardized methods of Bergy's Manual of Systematic Bacteriology (34).

Molecular characterization

Genomic DNA extraction

Genomic DNA was extracted from five studied isolates using Easy Quick DNA extraction kit (Genomix) following the manufacturer's instructions.

Random amplified polymorphic DNA (RAPD-PCR)

PCR reactions were carried out using sixe arbitrary 10-mer primers (Operon Tech., Inc.). PCR reactions were conducted using 2x superhot PCR Master Mix (Bioron; Germany) with 10 Pmol of each 6 different arbitrary 10-mer primers. The codes and sequences of these primers are listed in Table 2. The 25 µl reaction mixture was (10 Pmol. Of each primer, 30-50 ng of DNA template and 12.5 µl of 2x superhot PCR Master Mix). The PCR protocol was initial denaturation, 94°C for 2.5 min and 35 cycles of subsequent denaturation, 94°C for 45 s; annealing temperature, 37°C for 30 s; extension temperature, 72°C for 2 min and final extension, 72°C for 10 min. PCR products were analyzed on 10 x 14 cm 1% agarose gel electrophoresis with DNA ladder standard 100 bp (Jena Bioscience, Germany)for 30 min using Tris-borate- EDTA Buffer and visualized by ultraviolet illumination after staining with 0.5 µg/ml ethidium bromide.

RAPD data analysis

The presence / absence RAPD data were analyzed using the SPSS-PC programs of (35). Pair-wise comparisons between strains were used to calculate the genetic similarity values (F) derived from Dice similarity coefficient.

Oligonucleotide (RAPD) Primers used in this study:

The RAPD primers have been used in this study are listed in Table 2. A pair of RAPD for each 6 primers was applied to amplify isolated DNA from *Bacillus thuringensis* strains under study. Their sequences and total scored fragments produced by each primer among 12 studied isolates are shown in Table 2.

Table 1: Samples, nomination, location and characteristics of Bt isolates

Samples	Isolates Name	Governorates Locations	Isolate characteristics								
			Gram stain	Cell shape	Endospore formation	Growth at 5% NaCl	Growth at pH 5.6-6.7	Glucose	Anaerobic Growth	Catalase reduction	Nitrate reduction
1	1	Kafr Elsheekh	G^+	Rod	+	+	+	+	+	+	+
2	2	Kafr Elsheekh	G^+	Rod	+	+	+	+	+	+	+
3	3	Al-Behira	G^+	Rod	+	+	+	+	+	+	+
4	5	Alexandria	G^+	Rod	+	+	+	+	+	+	+
5	7	Menofia	G^+	Rod	+	+	+	+	+	+	+
6	8	Cairo	G^+	Rod	+	+	+	+	+	+	+
7	9	Al-Fayoum	G^+	Rod	+	+	+	+	+	+	+
8	14	Al-Menia	G^+	Rod	+	+	+	+	+	+	+
9	15	Sohag	G^+	Rod	+	+	+	+	+	+	+
10	16	Sohag	G^+	Rod	+	+	+	+	+	+	+
11	D	El-Gharbia	G^+	Rod	+	+	+	+	+	+	+
12	D2	El-Gharbia	G^+	Rod	+	+	+	+	+	+	+

3. Results and Discussions

Isolation of *Bacillus thuringensis:*

Soil samples were collected from different governorates of Egypt. Twelve *Bacillus thuringensis* isolated from collected soil sampled. Physiological and biochemical characteristics are studied as shown in Table (1).

All *Bacillus thuringensis* isolates were gram-positive and spore forming it showed rod shaped and endospores of living bacilli appear edged in black and are very bright and retractile. Endospores strongly resist application of simple stains or dyes and hence appear as non-staining entities in Gram-stain preparations. However, once stained, endospores are quite resistant to decolorization. Endospores do not form normally during active growth and cell division. Rather, their differentiation begins when a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion. Typically one endospore is formed per vegetative cell. The mature spore is liberated by lyses of the mother cell (sporangium) in which it was formed. The isolated *Bacillus thuringensis* could grow in sodium chloride solutions with concentration of 5% it also grow at pH 5.6-6.7. A positive reaction in anaerobic and positive reaction in fermentation of glucose without gas production was obtained with all isolates. All isolates showed catalase and nitrate reducion.

Morphological characters:

B.T isolated strain was observed using light, dark field, phase contrast and under transmition electron microscopy, (Figs. 1-3).

Dark field (Fig. 1) micrographs from the same field of view show that the vegetated cells present, including both phase-bright spores. These results are in agreement with Cheng and neckerson (36).

Screenings of *B. thuringiensis* by phase-contrast microscope were done to screen for the presence of endospore and parasporal body in order to distinguish *B. thuringiensis* from other *Bacillus* groups (Figure 2). Phase contrast microscopy was done after 90 hrs to ensure the presence of rod-shaped vegetative cells and to screen for the presence of parasporal bodies, respectively.

As shown in Fig.2 Phase-contrast indicate the presence of both a parasporal body and a long filament which are retained after sporulation. The filament is shown to consistently arise from the end of the exosporium and next to the parasporal body. Upon spore germination, the parasporal body/filament complex is retained on the cell wall of the resulting bacterium. The colonies were microscopically examined and those having visible parasporal inclusions were classified as *B. thuringiensis* this result is in agreement with Bernhard *et al*. (37).

Electron microscope was performed on cells stained with uranyl acetate and viewed with Quanta FEG 450, FEI, Amsterdam transmission scanning electron microscope. Measurements were made for filament length with width from the micrograph was taken by using the magnification factor for the instrument setting. However, transmission electron microscopy revealed some electron-dense areas within the cells, similar to inclusions, which may suggest the formation of aggregations of cells, unable to be purified by differential centrifugation Fig. 3.

These results, as well as other evidence, indicate that the parasporal crystal formation in *B. thuringiensis* follows a complex process, which requires more study. It is clear that crystallization starts with the accumulation of molecules, which may interact through hydrogen bridges and/or disulphide bonds in order to regulate the crystalline arrangement (38, 39).

Molecular genetics characterization

In the last decade, RAPD technique has been one of the most commonly used PCR based molecular markers. Relationships between species may be determined by comparing their unique fingerprint information, which are expected to be identical among related species. The optimization of certain parameters like PCR buffer, magnesium concentration and suitable primer, which produces discriminatory a reproducible fingerprints of *B. thuringiensis* isolates, was achieved. In addition an alkaline lysate method was optimized which give reproducible results.

So that RAPD technique was used to characterize the isolates, conduct the genetic fingerprinting, construct the genetic relationship and determine genetic distance between the studied isolates and identify specific molecular markers. Total genomic DNA from each isolate and three reference strains were amplified using RAPD-PCR primers. Electrophoreses analysis of the amplification products revealed the incidence of polymorphism among the isolates. An informative profile was obtained (Figures 4 and 5). The six used primers produced multiple band profiles with a variable number.

The six used primers amplified 119 bands and produced 499 total numbers of bands with all 12 studied Bt isolates as shown in Table 2. Out of 119 amplified bans, 102 were polymorphic bands (85.7%) and 17 were monomorphic bands (14.3%) Table 3.The highest number of bands was 114 and produced by C5 primer while the lowest number was 55 and produced by C3 Primer. The obtained banding pattern revealed high percentage of genetic variation

(85.7) among studied isolates and introduced great differentiation of all examined isolates (Table 3).

Table 4 shows the specific markers obtained across RAPD-PCR analysis. Primers A5 and C5 generated the highest number of specific markers. Six markers were generated from each primer. In contrast, A1 produced the least number of markers (2 markers) .B5 and C3 primers produced 4 markers for each primer.C2 was the only primer which produces five distinct.

Based on the RAPD obtained data phylogenetic tree was constructed (Figure 6). The 12 isolates were conduct dendrogram and grouped into two clusters. Pair-wise comparisons of polymorphic products were used to construct a dendrogram applying the cluster analysis of the isolates was all in two clusters (Fig. 6). The D2 isolate formed a distinct isolate, separated from all of the others studied. The first cluster includes six isolates (1, 2, 3, 5, 7 and 8). This cluster was further divided into two groups. The first group included four isolates (1, 2, 3 and 5). In this group, the lowest genetic distance was detected between 1and 2 isolates, but the 3 isolate was closely related. The second group included two isolates (7 and 8). The second cluster included five isolates (9, 14, 15, 1and D) and it was divided into two groups. The first group included three isolates (15, 16, and D). The closest genetic distance was found between the 15 and 16 isolates, which were closely related to that of D. The second group included two isolates (14 and 9). The generated DNA fragments from RAPD -PCR experiments with different primers among all 12 studied Bt isolates were used to estimate the genetic similarity coefficient and illustrated in table 5. The highest genetic similarity (0.86) was recorded between 1 and 2 isolates, while the lowest similarity coefficient was 0.15 and observed between 2 and 9 studied isolates. Such analysis showed some regional variation among the isolates, but did not indicate a clearly defined habitat location pattern of the DNA polymorphism.

B.t isolates 8 and 9 were the highest isolate producing specific RAPD markers. Isolate 8 produced 8 markers and isolate 9 produced 7 markers. Isolates 1,2,3,5 and 16 were failed to produce any specific markers.

B. thuringiensis constitutes a genetically diverse species; the great number of strains known today may form distinctive groups, according to their Physiological, biochemical reactions and genetic traits of isolated strains.

Molecular tools have been developed in recent years, trying to offer a new typing alternative for *B. thuringiensis* strains and to recognize the actual phylogenetic relationships between subspecific groups. RAPD-PCR offers a good tool to identify these groups, based on the use random designed. The potential of this technique was tested in this work and proved to be sensitive, specific, reproducible, and fast; it may become a standardized characterization procedure. It may also help in the establishment of a new subspecies-level classification of *B. thuringiensis*.

Primer (C2) was the most discriminatory and, therefore, was used to build the dendrogram. Results suggested that genomic fingerprints obtained by RAPD can be used for the genotypic characterization and identification of our isolated *B. thuringiensis*.

PAPD-PCR fingerprinting of the 12 used strains showed that practically all displayed a distinct pattern. It also shows a phylogenetic relationship between the 12 used strains in the analysis On the other hand; RAPD-PCR patterns from the used strains showed slightly differ from each other.

Results suggested that genomic fingerprints obtained by RAPD can be used for the genotypic characterization and identification of our isolated *B. thuringiensis* as a complement to physiological and biochemical reactions.

Table 2. List of used primers, their nucleotide sequences, total number of produced bands

Primer code	sequence	Isolates												Total
		1	2	3	5	7	8	9	14	15	16	D1	D2	
A1	5'-CAGGCCCTTC-3'	7	7	7	6	8	7	5	7	6	6	4	6	76
A5	5'-AGGGGTCTTG-3	6	5	6	3	6	6	5	5	12	8	5	4	71
B5	5'-TGCGCCCTTC-3'	4	5	8	7	6	6	8	10	9	7	7	9	86
C2	5'-GTGAGGCGTC-3'	6	8	8	8	6	9	8	9	9	9	13	4	97
C3	5'-GGGGGTCTTT-3'	4	4	3	3	6	5	3	4	6	6	6	5	55
C5	5'-TGCGCCCTTC-3'	11	12	15	14	10	10	6	7	6	6	6	11	114
Total		38	41	47	41	42	43	35	42	48	42	41	39	499

Table 3. Monomorphic and polymorphic bands produced by six used primers

Primer code	Amplified bands	Polymorphic bands	Monomorphic bands
A1	19	16	3
A5	20	19	1
B5	20	17	3
C2	23	20	3
C3	15	13	2
C5	22	17	5
Total	119	102	17

Table 4. Specific markers for Bt isolates across RAPD-PCR analysis.

Marker		Bt isolates												Total
Primer	**Band MW (bp)**	**1**	**2**	**3**	**5**	**7**	**8**	**9**	**14**	**15**	**16**	**D**	**D2**	
A1	**500**												+	**2**
	650							+						
A5	**220**												+	**6**
	650						+							
	700							+						
	800							+						
	900						+							
	1100						+							
B5	**400**						+							**4**
	500									+				
	700							+						
	850									+				
C2	**130**									+				**5**
	300						+							
	350											+		
	420						+							
	600					+								
C3	**350**							+						**4**
	550								+					
	700								+					
	950												+	
C5	**350**											+		**6**
	460											+		
	750							+						
	900							+						
	1000						+							
	1200						+							
Total		**0**	**0**	**0**	**0**	**1**	**8**	**7**	**2**	**3**	**0**	**3**	**3**	**27**

Table 5: Genetic similarity and distance values calculated from amplified DNA bands across six used RAPD primers

	1	2	3	5	7	8	9	14	15	16	D	D2
1	1.00											
2	0.86	1.00										
3	0.72	0.79	1.00									
5	0.67	0.70	0.75	1.00								
7	0.64	0.64	0.55	0.56	1.00							
8	0.53	0.59	0.48	0.67	0.59	1.00						
9	0.22	0.15	0.19	0.19	0.19	0.17	1.00					
14	0.26	0.26	0.31	0.23	0.23	0.27	0.29	1.00				
15	0.26	0.26	0.26	0.21	0.21	0.32	0.42	0.53	1.00			
16	0.27	0.27	0.29	0.27	0.27	0.33	0.53	0.47	0.77	1.00		
D	0.25	0.24	0.25	0.22	0.19	0.36	0.48	0.48	0.73	0.73	1.00	
D2	0.36	0.27	0.32	0.24	0.30	0.25	0.30	0.40	0.38	0.40	0.41	1.00

Fig.(1): Dark field microscopic observation of *Bacillus thuringensis* isolates.

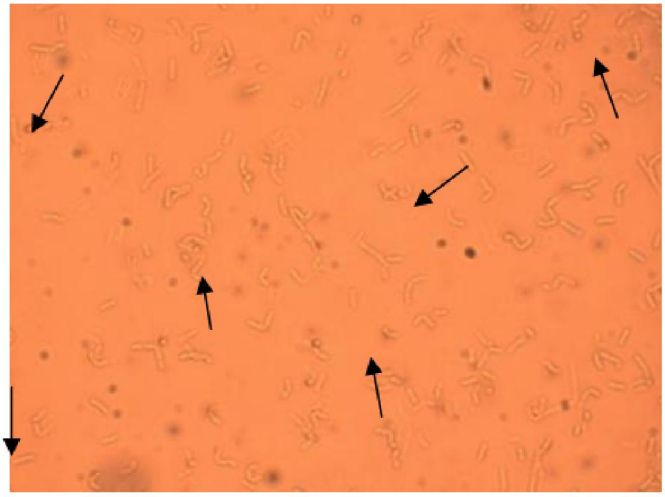

Fig.(2): Phase contrast microscopic observation of Bt strains, the arrows shown the refractile unlysed sporulated cells and their linked crystals located at one end pole of the cells appears.

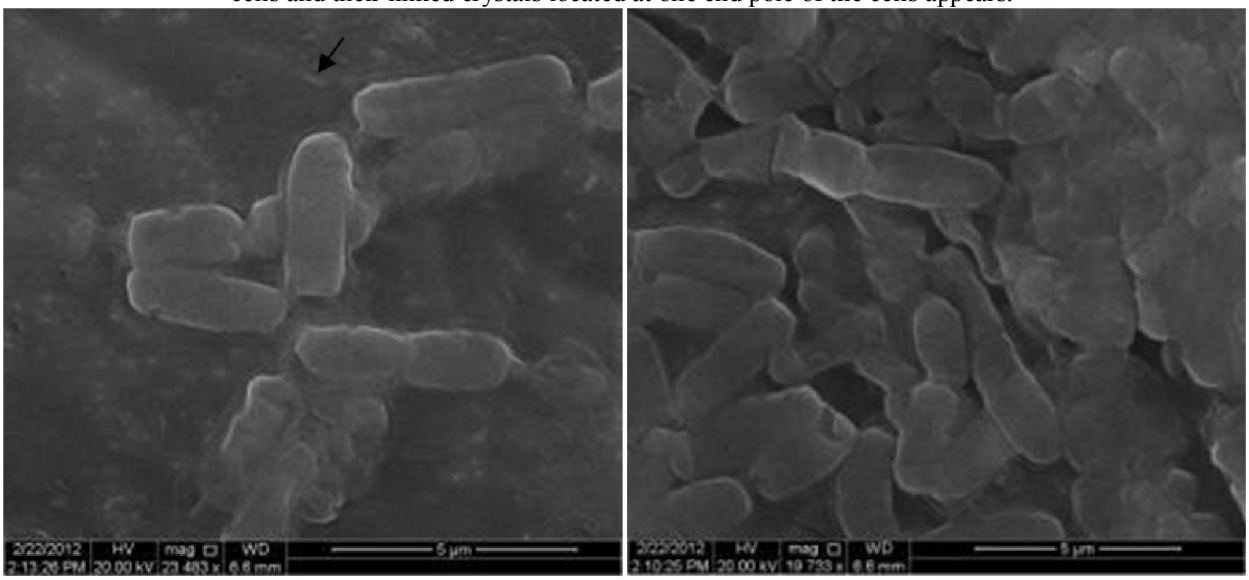

Fig. (3). Scanning transmition of electron micrographs of isolated *B. thuringinesis*. The result is a combination image of the surface of the sample.

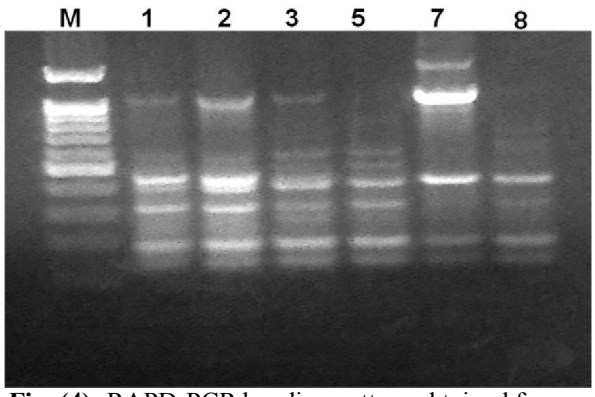

Fig. (4): RAPD-PCR banding pattern obtained from different Bt isolates (1,2,3,5,7 and 8) generated by random primer A1 with 100bp standard DNA marker

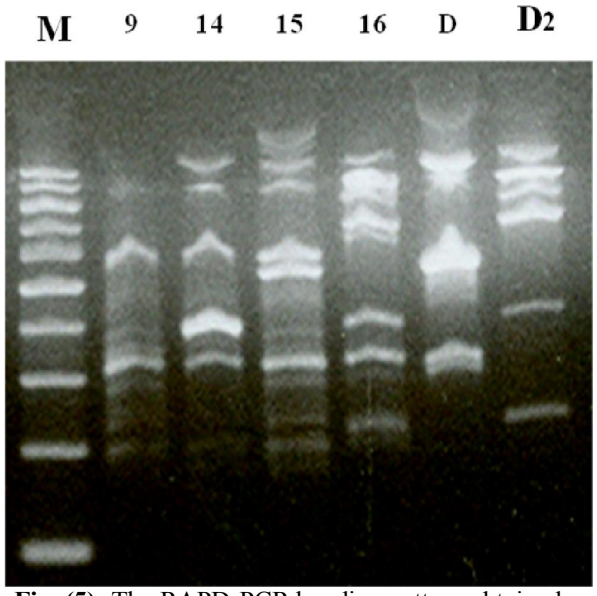

Fig. (5): The RAPD-PCR banding pattern obtained from different (9, 14, 15, 16, D, and D2) Bt isolates generated by random primer A1 with 100bp standard DNA marker

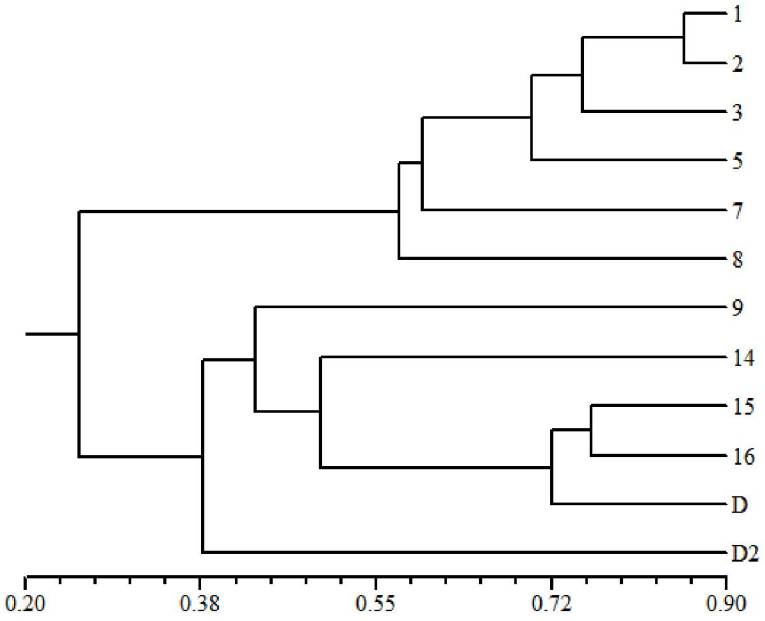

Fig. (6): Phylogenetic tree of the 12 Bt isolates usingRAPD-PCR analysis.

Conclusion:

Morphological, physiological, molecular characteristics of *Bacillus thuringiensis* new isolates were investigated, Also molecular study reviled that a high level of genetic divergence both intra and inter-group. Results suggested that genomic fingerprints obtained by RAPD can be used for the genotypic characterization and identification of our isolated *B. thuringiensis*. These new and highly potent strains of *B. thuringiensis* (Bt) isolates might be have insecticidal potentialities.

References

1- Glazer A. H., and H. Nikaido, "Microbial insecicides". In Microbial Biotechnology Fundamentals of Applied Microbiology, (W. H.

Freeman and Company, New York, pp.209-229. 1995.
2- Schnepf E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, and D.H. Dean, "*Bacillus thuringensis* and its pesticidal crystal proteins" Microbiol. Mol. Biol. Rev, vol. 62, no.3, pp. 775- 806, 1998.
3- Bernhard K., P. Jarret, M. Meadows, J. Butt, D.J. Ellis, G.M, Roberts, S. Pauli, P. Rodgers and H.G. Burges "Natural isolates of *Bacillus thuringiensis*: Worlwide Distribution, Characterization, and Activity against Insects Pests" J. Invertebr. Pathol., vol.70, pp.59-68. 1997.
4- Kaur S., "Potential for developing novel *Bacillus thuringiensis* strains and transgenic crops and their implications for Indian agriculture" Agr. Biotech Let vol. 4, pp. 1-10, 2002.
5- Federici B. A., H.W. Park, Y. Sakano "Insecticidal protein crystals of *Bacillus thuringiensis*. In: Inclusions in Prokaryotes (Ed. JM Shively), Springer-Verlag, Berlin-Heidelberg, pp. 195-235. 2006.
6- Chaves J., F. Stringuini, E. Pires and F.G.C. Cavados "Genotypic analysis of *Bacillus thuringiensis* serovars by RAPD-PCR". Neotropical Biology and Conservation. Vol. 5, no.2, pp. 106-112, 2010.
7- Stahly D.P., R.E. Andrews and A.A. Yousten, "The genus of *Bacillus*-insect pathogens, in the prokaryots" 2nd edition, edited by A. Ballows, (Springer Verlag, New York, pp.1097- 1745, 1991.
8- Andrews R.E., M.M. Bibilops, and L.A. Bulla "protease activation of the entomocidalprotoxin of *Bacillus thurengensis* subsp. *krostaki*" Appl. Environ.Microbiol., no. 50, pp.737-742, 1985.
9- Andrews R.E., R.M. Faust, H.Wabiko, K.C. Raymond, and L.B. Bulla "The biotechnology of *Bacillus thuringensis"* CRC Critical Reviews of biotechnology, no.6, pp.163-232, 1987.
10- Bulla L. A., R.M. Faust, R.E. Andrews and N. Goodman, " Insecicidal Bacilli" In the Molecular Biology of Bacilli, Vol 2, (Academic press, Inc., New York), pp. 185-209, 1985.
11- Ohba M., and K. Aizwa, "Distribution of *Bacillus thuringensis* in soil of Japan. J. Invertibrate" Pathol, vol. 47, pp. 277-282, 1986.
12- Xavier R., P. Nagarathinam, Gopalakrishnan, V. Murugan, K. Jayaraman "Isolation of Lepidopteran Active Native *Bacillus thuringiensis S*trains Through PCR Panning" Asia Pacific Journal of Molecular Biology and Biotechnology, Vol. 15 no. 2, pp. 61-67, 2007.
13- Rampersad J. and D. Ammons, "A *Bacillus thuringiensis* isolation method utilizing a novel stain, low selection and high throughput produced atypical results" BMC Microbiology. Pp. 5:52, 2005.
14- Martin P. A. W. and R. S. Travers, "Worldwide abundance and distribution of *Bacillus thuringiensis* isolates", Appl Environ Microbiol. Vol. 55, pp. 2437-2442, 1989
15- Johnson C. and A.H. Bishop, "A technique for the effective enrichment and isolation of *Bacillus thuringiensis*" FEMS Microbiology Letters. Vol. pp. 142:173-177, 1996.
16- Bardakci F. "Random Amplified PolymorphicDNA (RAPD) Markers" Turk. J. Biol. vol. 25, pp. 185-196, 2001.
17- Awad N. S., H. H. Sabit, S. E. M. Abo-Aba and R. A. Bayoumi, "Isolation, characterization and fingerprinting of some chlorpyrifos- degrading bacterial strains isolated from Egyptian pesticides-polluted soils" African Journal of Microbiology Research Vol. 5, no.18, pp. 2855-2862, 2011.
18- Powed-chagun P., H. Suzuk and S. Rengpipat, "Characterization of a probiotic *Bacillus* S11 bacterium of black tiger shrimp *Penaeusmonodon. Songklanakarin*" J. Sci. Technol.vol.33, no.1, pp. 1-8, 2011.
19- ThangarajM., V, Prem, T, Ramesh and A.P. Lipton, "RAPD Fingerprinting and Demonstration of Genetic Variation in Three Pathogens Isolated from Mangrove Environment" Asian Journal of Biotechnology, vol. 3, no.3, pp. 269-274, 2011.
20- Khayamie S., K. N. Niknejad, B. Rabie, S. Sassanie and A.A. Ebadie, "Genetic characterization of *pseudomonas syringaep* v. *syringae* strains from stone fruits based on RAPD analysis in iran" AgriculturaTropicaetSubtropica, vol. 42, 4,pp. 162-166, 2009.
21- Awad N. S., S. F. M.. Allam, M. A. Rizk, M. F. Hassan and A.Y. Zaki, "Fingerprinting and assessment of genetic variability of Varroa destructor in Egypt", vol. 49, no.3. pp. 251-256, 2010.
22- Procópio R. E. L. W. L. Araújo, Jr. W. Maccheroni and J. L. Azevedo. "Characterization of an endophytic bacterial community associated with *Eucalyptus spp*" Genet. Mol. Res. Vol. 8, no.4, pp. 1408-1422, 2009.
23- Bayoumi R.A., N.S. Awad and M.M.M. Ibrahim, "Molecular geneticcharacterization of some Biosurfactant producing bacteria isolated from Egyptian red sea mangrove forests" Arab J. Biotech., vol.13,no. 2, pp. 209-222, 2010.
24- Hongyu Z., Y., D. Ziniu, and X.I. Wang, "Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehauses in China" Crop Protection, vol.19, pp. 449- 454, 2000.

25- Mizuki E. T., S.-H. Ichimatsu, Y. S. Hwang, H. Park, K. Saitoh, and M. Ohba "Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants in Japan" Journal of Applied Microbiology, vol. 86, pp. 979- 984, 1999.
26- Ichimatsu T., E. Mizuki, K. Nishimura, T. Akao, and H. Saitoh, "Ocurance of *Bacillus thuringiensis* in fresh waters of Japan" Current Microbiology, vol. 40, pp. 217-220, 2000.
27- Maeda M. E., Y. Mizuki, T. Nakamura, and M. Ohba, "Recovery of *Bacillus thuringiensis* from marine sediments of Japan" Current Microbiology, vol. 40, pp. 418-422, 2000.
28- Forsty G., and N: A. Logan, "Isolation of *Bacillus thuringiensis* from Northern Victoria Land Antartica" Letters in Applied Microbiology, vol. 30, pp. 263-266, 2000.
29- Malkawi H. I , F. Al-Momani, M.M. Meqdam, I. Saadoun and M. J. Mohammad, "Detection of genetic polymorphism by RAPD-PCR among isolates of *Bacillus thuringiensis*. New Microbial. Vol. 22, no. 3, pp. 241-247, 1999.
30- Gaviria-Rivera A.M. , and F.G. Priest, "Molecular typing of *Bacillus thuringiensis* serovars by RAPD-PCR" Syst. Appl. Microbiol. Vol. 26,no. 2, pp. 254-61, 2003.
31- Alokr R., U. Sudhir, M. Meshra, and A.B. Dongre, "Optimization of RAPD-PCR for discrimination of different strains of *Bacillus thuringiensis*". Rom. Biotechnol. Lett, vol.14, no. 2, pp. 4307-4312, 2009.
32- KumarD., K. Chaudhary and K. S. Boora, "Characterization of native *Bacillus thuringiensis* strains by PCR-RAPD based fingerprinting" Indian Journal of Microbiology. Vol. 50, no. 1, pp. 27-32, 2010.
33- Rajalakshmi S. and Y. I. Shethna, "Spore and crystal formation in *Bacillus thuringiensis* var. *thuringiensis* during growth in cystine and cysteine" J. Biosci., vol. 2, no. 4, pp. 321–328, 1980.
34- Sneath P.A., N.S. Mair, M.E. Sharpe, and J.G. Holt, "Bergy's Manual of Systimatic Bacteriology" Vol. 2, pp,1132, Wlliams and Wilkins, Balimore. 1984.
35- Nie N. H., C.H. Hull, J.G. Jenkins, Steinbremmer, D.H. Bent, "Statistical package for the social sciences" 2nd edn. Megraw Hill, New York, 1975.
36- Cheng D. and K. W. Nickerson "*Bacillus thuringiensis* HD-73 Spores Have Surface-Localized*Cry*1Ac Toxin: Physiological and Pathogenic Consequences" Applied and Environmental Microbiology, vol. 62, no. 10, pp. 3722–3726, 1996
37- Bernhard K., P. Jarrett, M. Meadows, J. Butt, D. J. Ellis, G.M. Roberts, S. Pauli, P. Rodgers and H.D. Burges, "Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests" J. Invertebr. Pathol. Vol.70, pp. 59-68, 1997.
38- Agaisse H. and D. Lereclus "How does *Bacillus thuringiensis* produce so much insecticidal crystal protein?" J. Bacteriol. Vol.177, no. 21, pp. 6027–6032, 1995.
39- Baum J. A. and T. Malvar, "Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*" Mol. Microbiol. Vol.18, no. 1, pp. 1-12, 1995.
40- Loboffe M. J., and B. E. Pierce. "A photographic atlas for the microbiology laboratory" 3rd ed. Morton Publishing Co., Englewood, CO. 2005.