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Full Length Research Paper

Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia

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A total of 500 soil samples were collected from different localities across the kingdom to isolate native *Bacillus thuringiensis* (Bt) strains. Sodium acetate- (0.25 M) -selection heat-pasteurization, and 50% ethanol treatment methods were used for Bt isolation. Phenotypic characterization and identification of recovered Bt isolates was accomplished on the basis of morphological characteristics of colonies, parasporal crystal, and spores, as well as hemolytic activity, motility, susceptibility to antibiotics, and carbohydrate utilization (API 50CH system). Analysis of 16S rRNA gene was performed to further confirm Bt species identity and differentiate the spore-forming bacilli. Bioassay tests were conducted on larvae of *Aedes caspius* and *Culex pipiens*. Based on results, a total of 64 Bt isolates were recovered and the overall Bt index corresponding to the whole sampling areas was 0.25. Most of Bt isolates showed spherical crystals (56%), while, irregular, bipyramidal, and attached crystal to the spores constituted 14, 10 and 6% respectively. The most common biochemical type (31%) was as that of the reference Bt subsp. *kurstaki* (Btk). The 16S rRNA gene analysis, using the universal primer, provided an efficient identification of Bt isolates and their discrimination from the closely related *Bacillus cereus*, as well as from other spore forming bacilli. Bioassay showed that only one Bt isolate exhibited anti-diptera activity similar to Btk strain while most of the native Bt isolates exhibited weak (<30% mortality) or non insecticidal activity. However, results of motility, hemolytic activity, antibiotic-susceptibility patterns, and crystal shape, seem to suggest that many of our Bt isolates may exhibit parasporins activity.

Key words: *Bacillus thuringiensis*, parasporal crystal, isolation, biochemical type, insecticidal, cry gene, mosquitoes.

INTRODUCTION

Recently, uncontrolled use of chemical insecticides has resulted in irreparable damage to the environment. Continuous use of chemical insecticides has led to the emergence and spread of resistance in vectors of human diseases and agricultural pests (Georghiou, 1990). A major alternative to chemical control is biological control, which is a crucial part of integrated pest management (Aramideh et al., 2010). Interestingly, *Bacillus thuringiensis* (Bt) is a Gram-positive spore forming saprophyte soil bacterium that was first isolated from diseased larvae of *Bombyx mori* (an economically important insect, being a primary producer of silk, called the silkworm) in Japan (Ishiwata, 1901). Bt during the sporulation produces one or more proteinaceous parasporal crystals (Cry), recognized as delta-endotoxin. This crystal protein under alkaline conditions of midgut of insects, gets solubilized, and then activated by intrinsic proteases into an active toxin that selectively binds specific receptor in the cell membrane, leading to pore-formation and consequent insect death (Soberón et al., 2000; Eswarapriya et al., 2010). The demonstration of parasporal crystals is the only phenotypic character that differentiates the two taxonomically closely related species, Bt and *Bacillus cereus* (Bravo et al., 1998). Bt has been used successfully as biological insecticide over the last 60 years and constitutes 90% of all commercial bio-insecticides, due to its high specificity, safety and effectiveness in the control of wide spectrum of human

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Table 1. Distribution of Bt isolates in samples collected from different localities of KSA.

Location	Processed samples	Number of Bt isolates	Mean Bt index *
Hafr Albaten	33	3	0.9
Yanbu	59	11	0.54
Jezan	65	23	0.22
Taif	43	2	0.18
Madina	50	4	0.15
Qassim	45	4	0.13
Assir	56	2	0.13
Riyadh	44	9	0.08
Eastern R	43	3	0.08
Jeddah	66	3	0.07
Total	500	64	0.25

*****The ratio of Bt isolates producing crystal to other non-Bt spore-forming bacilli.

disease vectors and agriculture-pests (Schnepf et al., 1998; Naster et al., 2002). Noteworthy, Bt var. *Israelensis* (H14) proved efficient in the control of the population levels of medically important mosquito vectors of malaria, dengue virus, rift valley virus, lymphatic filariasis, and the black fly that transmits onchocerciasis (Ohba et al., 2009). So far Bt Cry toxins have been classified into 59 families (ithat is, Cry1 to Cry59) and two groups of Cry proteins, based on their amino acid sequence homology (Crickmore et al., 1998; Liang et al., 2011). Several reports, however, indicated that non insecticidal Bt strains are more widely distributed (more than 90%) than insecticidal strains in natural environment (Yasutake et al., 2007; Ohba et al., 2009). Hence, novel biological activity other than insecticidal one has been considered. This has led to the discovery of a unique family of Cry proteins, designated parasporins (PS 1 to 4), which exhibit preferential cytocidal activity for human cancer cells (Mizuki et al., 1999b; Mizuki et al., 2000; Nadarajah et al., 2008; Ohba et al., 2009). These research findings have resurged the interest in Bt with special focus on the non insecticidal strains (Poornima et al., 2010; El-Hag and Safhi, 2011). To date, native Bt isolates were not isolated from Saudi Arabia, yet the organism is well recognized among Saudi researchers (Amin et al., 2008; Al-Roba et al., 2011; El-Hag and Safhi, 2011).

Therefore, the present study deals with the isolation of native Bt isolates from its natural habitats in Saudi Arabia, confirmation of Bt identity, phenotypic characterization and evaluation of Bt larval mosquitocidal activity.

MATERIALS AND METHODS

Soil sample collection and Bt isolation

Five hundred soil samples were collected from different regions across the country (Table 1). The soil surface was scraped with sterile spatula; about 10 g were taken from a depth of 2 to 5 cm, and stored in sterile cups. Samples were kept in the laboratory at 4°C until their processing (Bozlağan et al., 2010). Bt isolation from soil samples was carried out by the selective sodium acetate heat-pasteurization method as previously described (Martin and Travers, 1989; Xavier et al., 2007b). Briefly, 5 g of the sample was added to 10 ml of Luria Bertani (LB) broth containing 0.25 M sodium acetate. Presumably, sodium acetate selectively inhibits the germination of *B. thuringiensis* spores.

Inoculated flasks were then incubated on a rotary shaker (200 rpm) for 4 h at 30°C. After incubation, 1 ml aliquot of thoroughly mixed culture broth was transferred to a pre-warmed 6 ml glass tube and heated at 80°C for 10 min, and then serially diluted aliquots were placed on LB agar and incubated overnight at 30°C. Bt colonies that were white, spread out and seems to fried egg on plate (Travers et al., 1987; Rampersad and Ammons, 2005) were examined by phase contrast microscopy. Additionally, the 50% ethanol method was also carried out essentially as previously described (Koransky et al., 1978; Hong et al., 2009). For comparison, the Bt index was calculated for each positive sample as the number of Bt isolates/ total number of isolates of sporulated bacilli (Thaphan et al., 2008). The two reference Bt strains used were *B. thuringiensis* subs. *Kurstaki* (Btk), kindly provided by Prof G. Amin, Department of Biotechnology, Faculty of Sciences, Taif University, K.S.A., and *B. thuringiensis* var. *israelensis* (H14), kindly provided by Prof A. Abdel-Hameed (now deceased), Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Phase contrast microscopy

Bt colonies were suspended in sterile distilled water as a wet mount and examined with phase contrast microscope (100X oil immersion objective) for the presence of parasporal crystals. Bt colonies were then classified into different groups based on their crystal shape (Lopez-Pazos et al., 2009). Potential Bt candidates were sub-cultured and purified on nutrient agar plates and then stored as stock culture in sterile liquid Nutrient Broth containing 50% glycerol at −20°C (Hernandez et al., 2005).

Scanning electron microscopy

In order to obtain the spore-crystal mixture, Bt isolates were grown in nutrient agar medium for 5 days at 30°C, until lysis. The spore-crystal mixtures were suspended in 1 ml of ice-cold 1 M NaCl and

centrifuged for 5 min at 13,000 ×g then washed 3 times with cold sterile distilled water centrifugation. The pellets were suspended in distilled water. Diluted suspensions of spore-crystal complexes were placed on aluminum stubs or cover- glasses and air dried. Samples were examined and photographed with a FEI-Inspect S50, scanning electron microscope operating at a voltage of 1500 kV at 24,000× magnifications (Ibarra et al., 2003; Kati et al., 2007).

Biochemical typing

Bt isolates were tested by API 50CH and API 20E systems (BioMerieux, Marcyle Etoile, France) according to the manufacturer instructions. Bt isolates were divided into biochemical types based on hydrolysis of esculin, urea or lecithin, and acid production from sucrose, or salicin. Lecithinase activity of Bt isolates was tested on nutrient agar containing 10% egg yolk (Oxoid, Uk) and then incubated at 37°C overnight (Logan and Berkeley, 1984; Martin and Travers, 1989; Lecadet et al., 1999; Keshavarzi, 2008; Aramideh et al., 2010; Martin et al., 2010).

Hemolytic activity

The production of extracellular hemolysins by Bt isolates was tested on blood agar (nutrient agar containing 2% (v/v) sheep erythrocytes). Bt isolates were pre-cultured on nutrient agar overnight at 27°C. Fresh cultures were then pin-spot inoculated onto blood agar plates. The formation of zone of hemolysis surrounding Bt colonies was examined after incubation at 27°C for 24 h (Ichikawa et al., 2008).

Motility testing

To evaluate Bt motility, culture plates were prepared using 1% (w/v) tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) agar. Bt isolates were then inoculated onto the middle of the plate from top to bottom by streaking two closely drawn parallel lines with 2 mm width along the diameter of the plates. The plates were then incubated at 30°C overnight. When Bt strain spreads out from the inoculation site, positive motility test was scored (Maheswaran et al., 2010). Bt reference strains (H14 and Btk) were used as positive control for motility.

Antibiotic susceptibility

Susceptibility of Bt isolates and the reference *Staphylococcus aureus* and *Escherichia coli* strains to antibiotics was tested by the disk diffusion method on Muller Hinton Agar, with Bio-disc® (Gokhan Laboratory Company, Izmir, Turkey). The used antibiotics were: Tetracycline (TE-30 mcg), ampicillin (AM-10 mcg), chloramphenicol (C-30 mcg), streptomycin (S-10 mcg), erythromycin (E-15 mcg), and bacitracin (B-10 mcg). The tests were done according to the manufacturers' instructions (Ichikawa et al., 2008).

DNA extraction

To extract the DNA, the test Bt strain and the reference H14 and Btk strains were cultured in nutrient broth over night at 37°C. Cells were harvested by centrifugation at 4000x rpm for 15 min, washed 3 times with sterile distilled water centrifugation, and finally, the supernatant was discarded. An aliquot of 100 ul of cell-pellet was processed for cell lysis with lysis buffer, and protease K at 65°C for 10 min, thereafter heating at 95°C for 5 min to denature the proteases and stop the reaction. The treated samples were then processed using ROCHE MagNA Pure automated DNA isolation system according to the manufacturer's instructions (MagNA Pure LC DNA Isolation Kit III, Bacteria & Fungi).

16S rRNA gene analysis

PCR amplification of 16S rRNA gene from tested Bt isolates was performed using the universal primers: forward (518F); 5'-CCAGCAGCCGCGGTAATACG-3', reverse (800R); 5'-TACCAG-GGTATCTAATCC-3'. Thirty five amplification cycles performed at 94°C for 45 s, 55°C for 60 s and 72°C for 60 s. DNA amplicons of about 1500 bp was obtained, purified, and sequenced (Macrogen, Seoul, Korea). Sequences were searched against the database (www.ncbi.nlm.com) using BLAST tool with a similarity cut off of 99.5% to identify the bacterium based on sequence similarity. The multiple alignments and phylogenetic analysis were carried out to discriminate and compare those sequences among each other and to determine the evolutionary DNA relatedness and the genetic distance.

Bioassay to insect species

The spores and crystals of each tested Bt isolates on nutrient agar plates (72 hrs) were scrapped into 10 ml of sterile water, to give an average count of 10^9 cfu/ml. At the beginning, a high crystal-spore mixture was used in parallel with that of similar Btk reference strain. The activity of isolates against mosquitoes was tested using *A. caspius* and *C. pipiens*. Ten of 3rd to 4th instars tested larvae were transferred by a Pasteur pipette to 20 ml of chlorine-free tap water in 30 ml plastic cups. As compared to corrected mortality of the negative control, larval mortality of tested isolate was scored 48 h after incubation at 22 ± 1°C (Aramideh et al., 2010).

RESULTS

Out of several hundred (> 900) examined Bt-like colonies, 64 Bt isolates were isolated. The average Bt index (the ratio of Bt isolates producing crystal to other non-Bt spore forming bacilli) was 0.25 with the highest Bt index recorded for samples of Hafr Albaten (0.9) while the lowest was from Jeddah (0.07). Most of our Bt isolates were isolated from Jezan samples. Bt index reflects the abundance of Bt strains but not necessarily their diversity (Table 1).

Colonial morphology of Bt isolates

Based on colonial morphology on nutrient agar plate, the recovered 64 Bt isolates were classified into 4 (A to D) groups as illustrated in Figure 1: A (white, round, flat, and with wavy/scalloped edged margin), B (white, round, slightly raised centre, and with fried egg appearance), C (white, shiny, round, little raised centre, and with irregular but entire margin), and D (white, round, mucoid, slightly raised centre, and spread out with irregular spike-like margin). Colonial appearance of the used Bt and H14 reference strains conform within group A where the majority of Bt isolates (85%) were falling. Whereas native

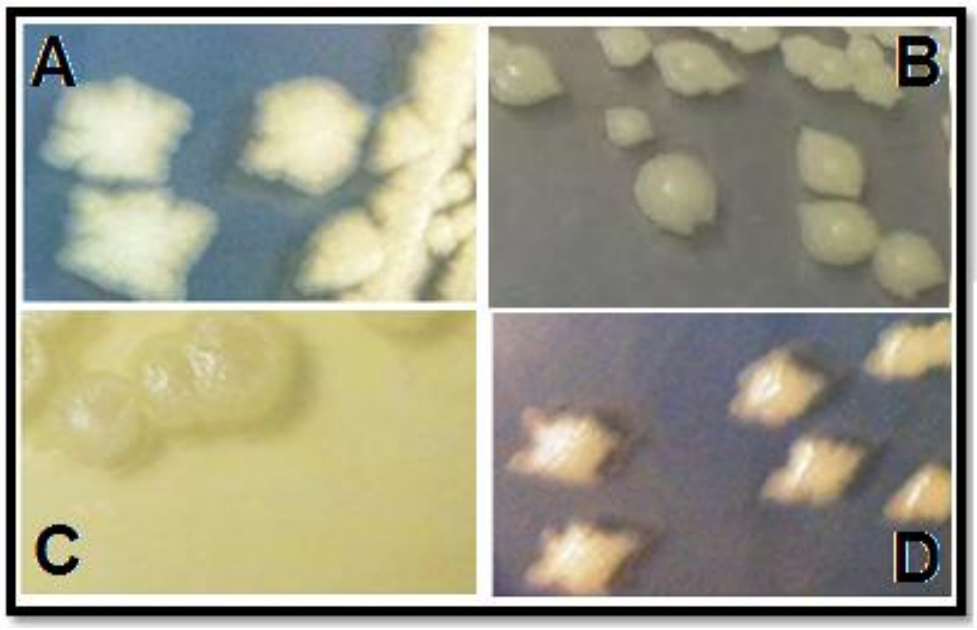

Figure 1. Photographs for different types of Bt clonial morphology. A: Flat and wavy margin (scalloped-edged); B: slightly raised centre with fried egg appearance; C: shinny entire margin; D: mucoid spread out with irregular spike-like margin.

Table 2. Microscopic- morphological characteristics of Bt isolates.

Microscopic-morphological characteristics		Bt isolates (%)
Cells in chains		100
Motility		95
Spore shape	Round	0
	Ellipsoidal	100
	Cylindrical	17
Spore position	Terminal	0
	Subterminal	100
	Para-central	5
	Central	0
Swollen sporangium		0
Presence of Parasporal crystals		100

Bt isolates that exhibited type B, C and D were rarely encountered constituting, 9, 6 and 6%, respectively.

Phase contrast and scanning electron microscopy

After preliminary screening with phase contrast microscopy, all Bt isolates shared many morphological characteristics in cell chains arrangement, ellipsoidal shape of spores, and non-swollen sporangia (Table 2). Out of these ellipsoidal spores, 11 (17%) Bt isolates tended to have a cylindrical shape. Also, majority (95%) of the isolates showed sub-terminal spores, while only 3 isolates (5%) showed para-central spores.

Based on the diversity of crystal morphology, the 64 Bt isolates were divided into eight classes (Figure 2); S (spherical: 56%), G (irregular: 14%), SG (spherical and irregular: 12.5%), B (bipyramidal: 5%), BS (bipyramidal

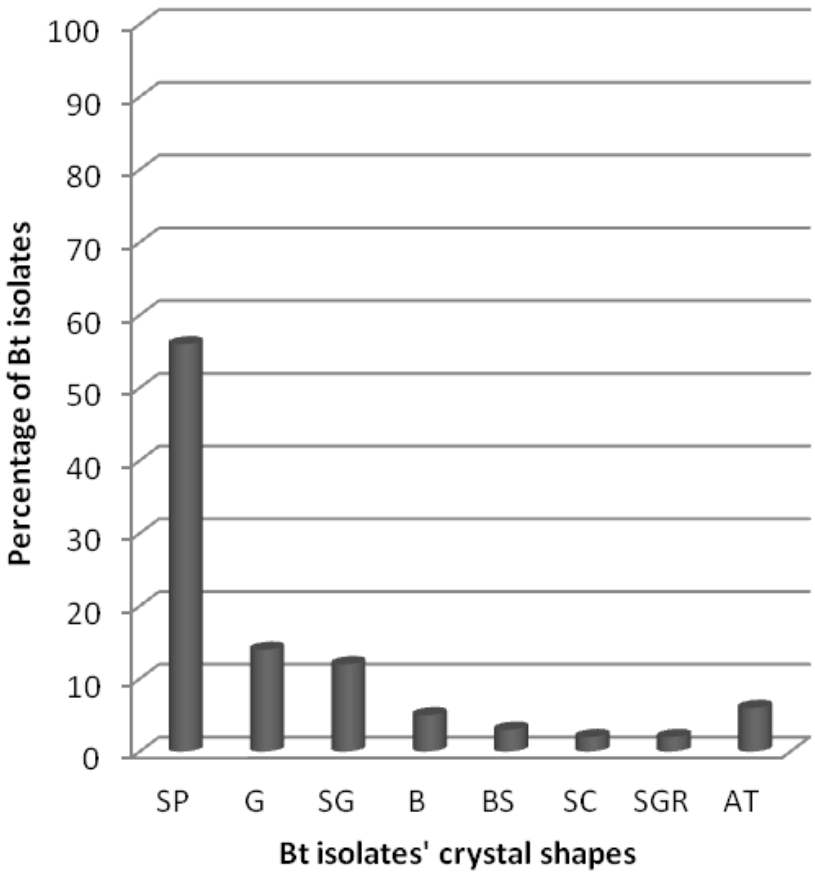

Figure 2. Distribution of different morphologies of para-sporal crystal produced by Bt isolates; S (spherical), G (irregular), SG (spherical and irregular), B (bipyramidal), BS (bipyramidal and spherical), SC (spherical and cubic), SGR (spherical, irregular, and rhomboidal) and AT (attached).

and spherical 3%), SC (spherical and cubic: 1.6%), SGR (spherical, irregular, and rhomboidal:1.6%) , and finally, AT (attached to the spores: 6%). Diversity of crystal morphologies are illustrated in Figure 3. All Bt isolates were positive for Gram, spore, and crystal staining.

Electron microscopy apparently confirmed the results of both phase microscopy and staining preparation by revealing the same crystal architectures seen with those methods. As presented in Figure 4, Bt isolate number 219 showed typical spherical crystal shape, thus confirming previous examination methods.

Motility, hemolysis, and antibiotic susceptibility

Motility testing indicated that 95% of Bt isolates were motile. Likewise, hemolytic assays indicated broad β–hemolysis zones with 94% of the 64 native Bt isolates. As expected, only 6% of Bt isolates were none-hemolytic. Antibiotic susceptibility testing indicated that all Bt isolates were sensitive to tetracycline, streptomycin, and erythromycin. Whereas 70 and 13% of Bt isolates were sensitive to chloramphenicol and bacitracin, respectively. In contrast, all Bt isolates were resistant to ampicillin with the exception of only 3 (4.6%) Bt isolates. Interestingly, these 3 isolates were also sensitive to bacitracin. Based on sheep RBCs hemolysis, motility testing, and susceptibility to ampicillin, the total 64 native Bt isolates were categorized into 3 groups as presented in Table 3. As expected, as much as 92% of the Bt isolates were motile, hemolytic and resistant to ampicillin (gp: III). Rarely, only 2 isolates (3%) were non motile, non hemolytic, and resistant to ampicillin (gp: I). It is also observed that these two isolates were sensitive for all tested antibiotics except ampicillin. Surprisingly, 3 Bt isolates (5%) exhibited a pattern similar to *Bacillus anthracis*, that is non motile, non hemolytic, and sensitive to ampicillin (gp: II).

Biochemical typing

The obtained data of sugar utilization using the API CH50 system for each of the Bt isolates were processed by the

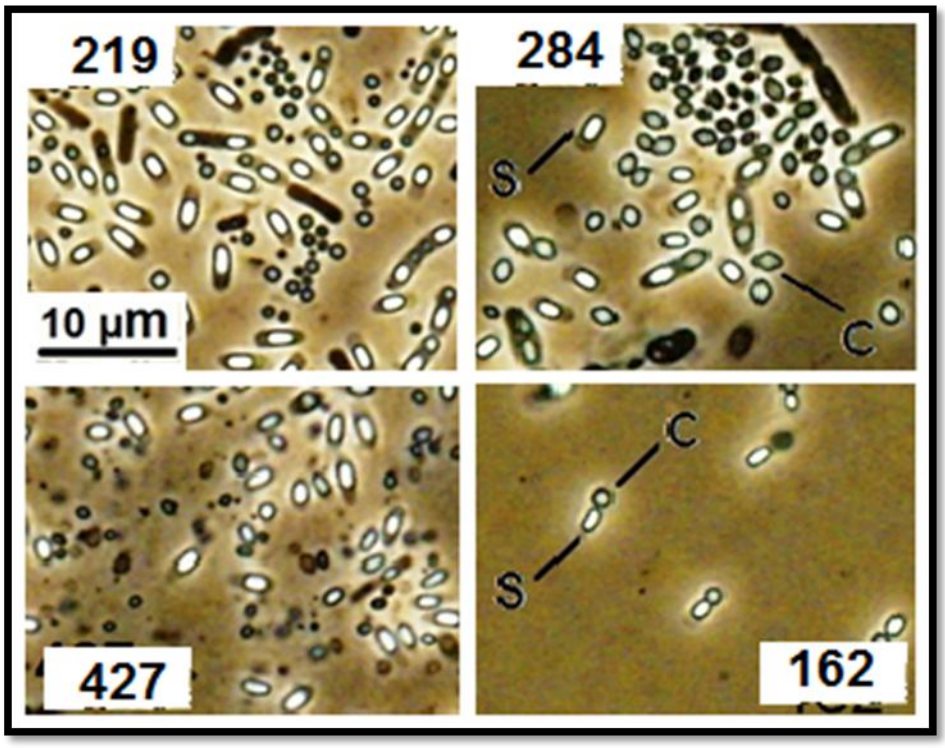

Figure 3. Phase contrast micrograpfs of Bt isolates showing spore (S) and diversity on crystal shapes (C); isolates number 219 (spherical), 284 (bipyramidal), 427 (irregular) and 162 (attached).

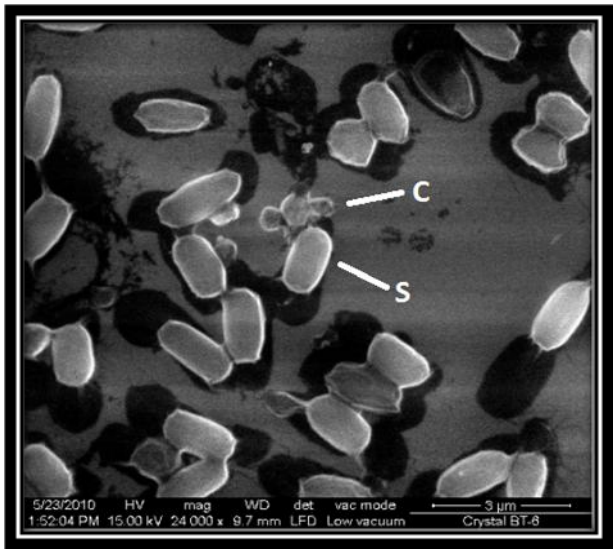

Figure 4. Scanning electron micrograph of the spores (S) and crystal proteins (C) from Bt isolate No: 219 (9.7 mm × 1500 kV; 24000x, low vacuum).

provided kit's software. The software results revealed that all tested isolates were identified with a possibility of over > 90% as *B. thuringiensis*, but only after the final input results of 48 h. Furthermore, each of the 64 Bt isolates was also examined by API E20 system for relevant biochemical reactions to shed more light on the phenotypic characteristics and to help determining possible biochemical types. The results obtained by API

Table 3. The occurrence of Bt isolates according to their motility, hemolysis and ampicillin susceptibility.

Group	Motility	Hemolysis	Ampicillin	Bt isolates (%)
I	Non-motile	Non-hemolytic	Resistant	3
II	Non-motile	Non-hemolytic	Sensitive	5
III	Motile	Hemolytic	Resistant	92

Table 4. Distribution of different BRs with Bt isolates.

Positive BRs >90%	Negative BRs			Discriminating BRs
	RHAmnose	β-galactosidase (ONPG test)	L-ARbitoL	
GELatinase[1]	MELibiose	H2S	Potassium 2-KetoGluconate	Lecithinase[4]
D-FRUctose	ERYthritol	D-ADOnitol	Potassium 5-KetoGluconate	Esculin[5]
N-Acetyl -	D-ARAbinose	L-SerBoseE	D-LACtose (bovine origin)	Urease[6]
Glucoseamine	D-XYLose	L-RHAmnose	D-FUCose	Salicin[7]
Trehalose	L-XYLose	DULcitol	L-FUCose	Sucrose[8]
ARButin	D-MELibiose	D-ARbitoL	D-TAGatose	
GLYcoGen[2]	INUlin	Methyl-aD-mannopyranoside	D-TURanose	
D-RIBose[3]	D-MrLeZitose	Methyl-aD-glucopyranoside	D-LYXose	
	D-RAFfinose	Methyl-BD-Xylopyranoside	GENtiobiose	

1: (92%), 2: (95%), 3: (90%), 4: (87%), 5: (32%), 6: (20%): 7 (37%) and 8: (34%) positive result with all Bt isolates.

Table 5. Biochemical tests used to generate the seven biochemical types for the 64 Bt isolates.

Biochemical types (%)	Biochemical pattern				
	E	U	S	A	L
EUSA (8)	+	+	+	+	V
EUS (11)	+	+	+	-	V
ELA (31)	+	V	-	+	+
EU (25)	+	+	-	-	V
ES (13)	+	-	+	-	V
L (6)	-	V	-	-	+
Ø (6)	-	-	-	-	V

+: Positive, -: negative, V: variable, E: esculin, U: urease, S: sucrose, A: salicin, and L: licithinase.

CH50 and API E20 biochemical systems revealed that some biochemical reactions (BRs) were found to be positive in general; production of gelatinase and assimilation of fructose, glycogen, trehalose, ribose, and N-acetyle glucoseamine. Contrastingly, ONPG test, H2S production, and assimilation of xylose, fucose, lactose, and galactose were negative in general with all Bt isolates (Table 4). Citrate utilization, VP test and indole production revealed variable results of 39, 64 and 23%, respectively.

However, some BRs were found to be good at discriminating our native Bt isolates; production of urease (U, 20%), acid production from sucrose (S, 34%), acid production from salicin (A, 37%), hydrolysis of esculin (E, 32%) and lecithinase production (L, 87%). These 5 traits allowed a total of 7 possible biochemical types; ELA, EU, ES, EUS, EUSA, UL and finally Ø, as shown in Table 5. The biochemical types ELA and EU constituted 31 and 25% of the total Bt isolates respectively whereas ES, EUS, EUSA, L, and Ø constituted 13, 11, 8, 6 and 6% respectively. Btk and H14 reference strains occurred within ELA and U biochemical types respectively. 50% of Bt isolates that produced bipyramidal crystals were found within ELA biochemical type. 27% of Bt isolates that produced irregular crystals were within the L & Ø biochemical types. 50% of the attached crystals occurred within the Ø biochemical type. Finally, Bt isolates producing other crystal types were found to be distributed

Table 6. Sequence similarity search (BLAST) of the 20 sequenced 16S rRNA genes from Bt isolates.

Isolates	Hit	Score	Identity (%)	e.value
H14	*Bacillus thuringiensis* gene for 16S rRNA, partial sequence, strain: JAM-GG01	2667	99	0.0
Btk	*Bacillus thuringiensis* partial 16S rRNA gene and ITS1, strain IEBC T12001	2682	99	0.0
N1	*Bacillus cereus* 16S rRNA gene, isolate AB1A	2651	99	0.0
N2	*Bacillus cereus* gene for 16S rRNA, partial sequence, strain:PDa-1	2736	100	0.0
N3	*Bacillus endophyticus* strain EH6 16S ribosomal RNA gene, partial sequence	2571	98	0.0
3	*Bacillus thuringiensis* strain P22 16S ribosomal RNA gene, partial sequence	2680	99	0.0
6	*Bacillus thuringiensis* strain P22 16S ribosomal RNA gene, partial sequence	2697	99	0.0
11	*Bacillus thuringiensis* strain Probio-33 16S	2651	99	0.0
125	*Bacillus thuringiensis* 16S rRNA gene	2665	99	0.0
130	*Bacillus thuringiensis* BMB171, complete genome	2641	99	0.0
131	*Bacillus thuringiensis* partial 16S rRNA gene and ITS1, strain IEBC T24001	2715	99	0.0
159	*Bacillus thuringiensis* partial 16S rRNA gene	2719	99	0.0
162	*Bacillus thuringiensis* BMB171, complete genome	2721	99	0.0
216	*Bacillus thuringiensis* BMB171, complete genome	2693	99	0.0
231	*Bacillus thuringiensis* 16S rRNA gene	2680	100	0.0
248	*Bacillus thuringiensis* strain P22 16S ribosomal RNA gene	2663	99	0.0
381	*Bacillus thuringiensis* 16S rRNA gene and 16S-23S IGS, strain CMBLBT-5	2678	99	0.0
388	*Bacillus thuringiensis* gene for 16S rRNA	2678	99	0.0
283	*Bacillus thuringiensis* 16S rRNA gene and 16S	2643	99	0.0
461	*Bacillus thuringiensis* partial 16S rRNA gene, isolate CCM11B	2687	99	0.0

Statistical analysis of the 20 sequenced 16S rRNA genes from Bt isolates

Number of Sequences	Mean length	Minimum length	Maximum length	Std Dev.
20 sequences	1460.3[b]	1446[b]	1481[b]	10.6

randomly within different biochemical types in general.

16S

rRNA gene analysis

A total of 15 randomly selected Bt isolates representative for the different assigned biochemical type and abundant crystal shape morphology were selected for this experiment, along with the reference strains BTK and H14. Additionally, three non-Bt isolates (N1, N2 and N3) that are spore forming and Gram positive bacilli were used for comparative purposes. The analysis of PCR products by agarose gel electrophoresis revealed amplified target bands ~1550 bp (Figure 5). The BLAST analysis of the nucleotide sequence returned confident results and confirmed that the 15 tested local Bt isolates and the two Bt reference strains (H14 & Btk) all belonged to *B. thuringiensis*. As expected, N1and N2 negative controls belonged to *Bacillus cereus*, while N3 belonged to *Bacillus endophyticus* (Table 6). Furthermore, as illustrated in Figure 6, the multiple alignment graph generated was reflecting a high degree of DNA relatedness among native Bt isolates and Bt reference strains, while N3 (*Bacillus endophyticus*) showed low level of DNA relatedness among the entire group. The phylogenetic tree created by the GEN analysis showed 3 major distinct phylogenetic groups consisting of clusters A, B, and C comprising the 15 native representative Bt isolates, 2 reference strains, fand 3 non-Bt spore forming bacilli (N:1 to 3) as illustrated in Figure 7. Cluster A contained 4 native Bt strains and the N1 (*B. cereus*). Cluster B contained only the N3 (*B. endophyticus*) which is evolutionary in distance among the entire tested bacilli. The largest cluster, designated as group C, included the remaining 11 native Bt strains, the two Bt reference

Table 7. Classification of the Bt isolates according to their toxicity levels against larvae of *Culex pipiens* and *Aedes caspius*.

Morality (%)	*Culex pipiens*		*Aedes caspius*	
	Number of isolates	Isolates (%)	Number of isolates	Isolates (%)
0-25	41	64	53	83
25-50	12	19	6	9
50-75	7	11	3	4.7
75-100	4	6	1	3.3

strains and N2 (*B. cereus*) and showed a high degree of DNA relatedness. Moreover, Figure 7 also shows all tested Bt isolates as correlated to their respective biochemical types and crystals shape.

Bioassay

Bioassay with highly concentrated spore-crystal suspensions was carried out on larvae of *C. pipiens* and *A. caspius*. The percentage of insect mortalities obtained with the 64 Bt isolates are shown in Table (7). Most strains (64 and 83%) have mortality in the range of 0 to 25% on *C. pipiens* and *A. caspius* respectively. However, there were 4 isolates (6%) that produced more than 75% mortality on *C. pipiens* while only 1 isolate (3.3%) showed a mortality of >75% on *Aedes caspius* (Table 6).

DISCUSSION

The objective of this study was the isolation and introduction of native Bt isolates from different environments in Saudi Arabia. It is hoped that the overall results of this research would prove usefulness in local large-scale production of the isolated Bt strains to control insects as well as the management of emerged insect resistance. Hence, reliable integrated prophylactic and eradication program would be constructed to control local spreading of Dengue viruses and/or their future possible reactivation and rearing. Beside the fact that the initiation of a nucleus of local Bt isolates collection may also help in the exploration of novel activity of Bt crystal proteins as antihuman cancer (parasporins group).

Several investigators have used the sodium acetate (0.25 M) selection heat-pasteurization method for this purpose (Travers et al., 1987; Martin and Travers, 1989; Xavier et al., 2007a, b; Santana et al., 2008; Patel et al., 2009; Maheswaran et al., 2010). However, in agreement with Santana et al. (2008), this method did not efficiently allow for the isolation of Bt strains from soils. This would explain the low Bt index (0.07) obtained with this method. Furthermore, Xavier et al. (2007a) reported a problem of high background of other microorganisms as confirmed by Patel (2009), as well as the present study. This led us to attempt the 50% ethanol method (Koransky et al., 1978; Hong et al., 2009), which revealed 4 fold increases in Bt index (0.28).

The colonial morphology of almost all the native Bt isolates were highly in agreement with the colonial characterizations described by many authors (Hoti and Balaraman, 1993; Rampersad and Ammons, 2005; Chatterjee et al., 2007; Thaphan et al., 2008; Gobatto et al., 2010; Maheswaran et al., 2010; Kavitha et al., 2011).

Our preliminary screening with phase contrast microscopy examining the cellular morphological characteristics of Bt isolates including the presence of cell in chains, ellipsoidal shape of spores, absence of swollen sporangia, and subterminal spore position were highly in agreement with several previous reports (Logan and Berkeley, 1984; Rampersad and Ammons, 2005; Chatterjee et al., 2007). On the other hand, the primary identification of Bt is based on the presence of crystalline inclusions (Rampersad and Ammons, 2005). In this study, the majority of the Bt isolates (56%) showed spherical crystals and the irregular, bipyramidal, and attached crystal to the spores constituted 14, 10, and 6% respectively. These findings were nearly consistent with those of Poornima et al. (2010) in India, who found that 91 and 9% of their 82 Bt isolates were spherical and irregular respectively. In a worldwide study, Martin and Travers (1989) found that Bt isolates with bipyramidal and spherical crystals were the most common. Martin et al. (2010) studied 3639 Bt isolates from 34 countries and reported that the majority (44%) showed bipyramidal crystals while 27 and 12% were irregular and attached to the spores respectively. Similarly, Aramideh et al. (2010) found that the bipyramidal crystals constituted the majority (58.3%) of their 46 Bt isolates from West Azerbaijan province. As indicated in this study, divers Bt crystal shapes were recovered. In agreement with Aramideh et al. (2010), we attribute the diversity in the dominancy of crystal shapes among habitats in Saudi Arabia in this study to the difference in sample location, habitat, and genetic variation. The enormously varied crystal shapes among Bt isolates have also been reported by several investigators (Chatterjee et al., 2007; Martin et al., 2010; Nisnevitch et al., 2010; Ramalakshmi and Udayasuriyan, 2010; Sauka et al., 2010; Anitha et al., 2011).

However, it is interesting to notice that while most of the Bt strains produce free crystals in the mother cell compartment, it was observed that some Bt strains presented the crystal adhered to the spore. It is well

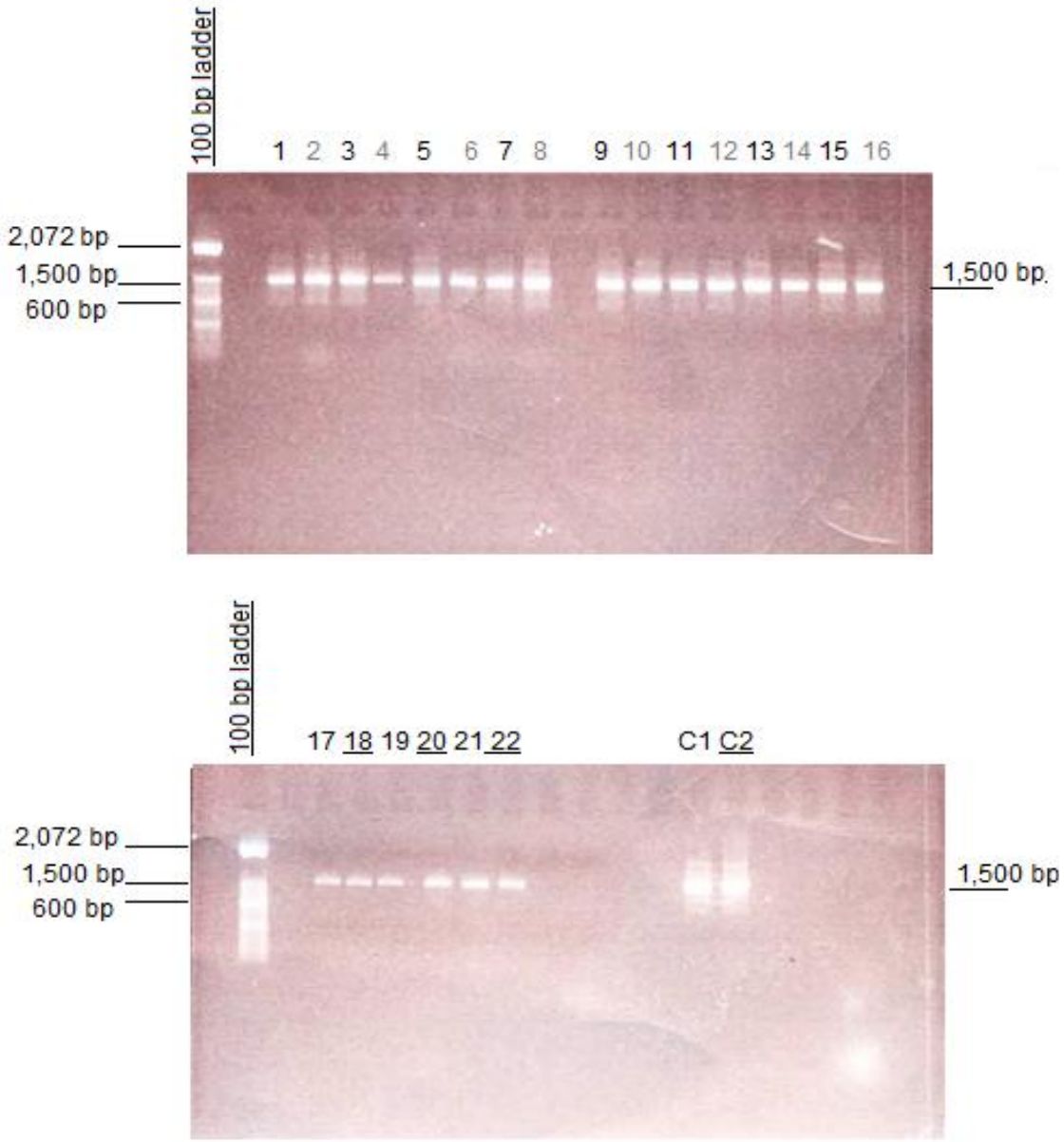

Figure 5. Agarose gel electrophoresis of PCR amplified product of 16s rRNA gene. A ladder of 100 bp was used as a standard size maker along with 2 positive control (C1 and C2) of known size.

known that the spore and crystal are liberated to the environment by the action of lytic enzymes that destroy the membrane that contains them (Bechtel and Bulla, 1976). The absence of this step in some strains of this collection is similar to the one observed in *Bacillus sphaericus*, were the binary protein crystal is adhered to the spore and covered by the exosporium membrane (Arrieta et al., 2004). Interestingly, a previous study showed that Bt strain was isolated with crystal remained attached to spores after autolysis and was with distinctive crystal shape that appeared square and flat (Lopez-Meza and Ibarra, 1996). Surprisingly, in our hands, one isolate showed the same peculiar crystal morphological feature that attribute to its uniqueness among other Bt isolates. However, additional 3 (4.6%) Bt isolates also showed attached crystals but with different shapes.

As stated by Logan (2005), motility testing, hemolytic activity, and ampicillin susceptibility testing, is taxonomically important classic key types for identification of BT, *Bacillus cereus*, and *Bacillus anthracis* group. In general, Bt strains are motile by peritrichous flagellum (Bouillaut et al., 2005; Logan, 2005; Maheswaran et al., 2010).

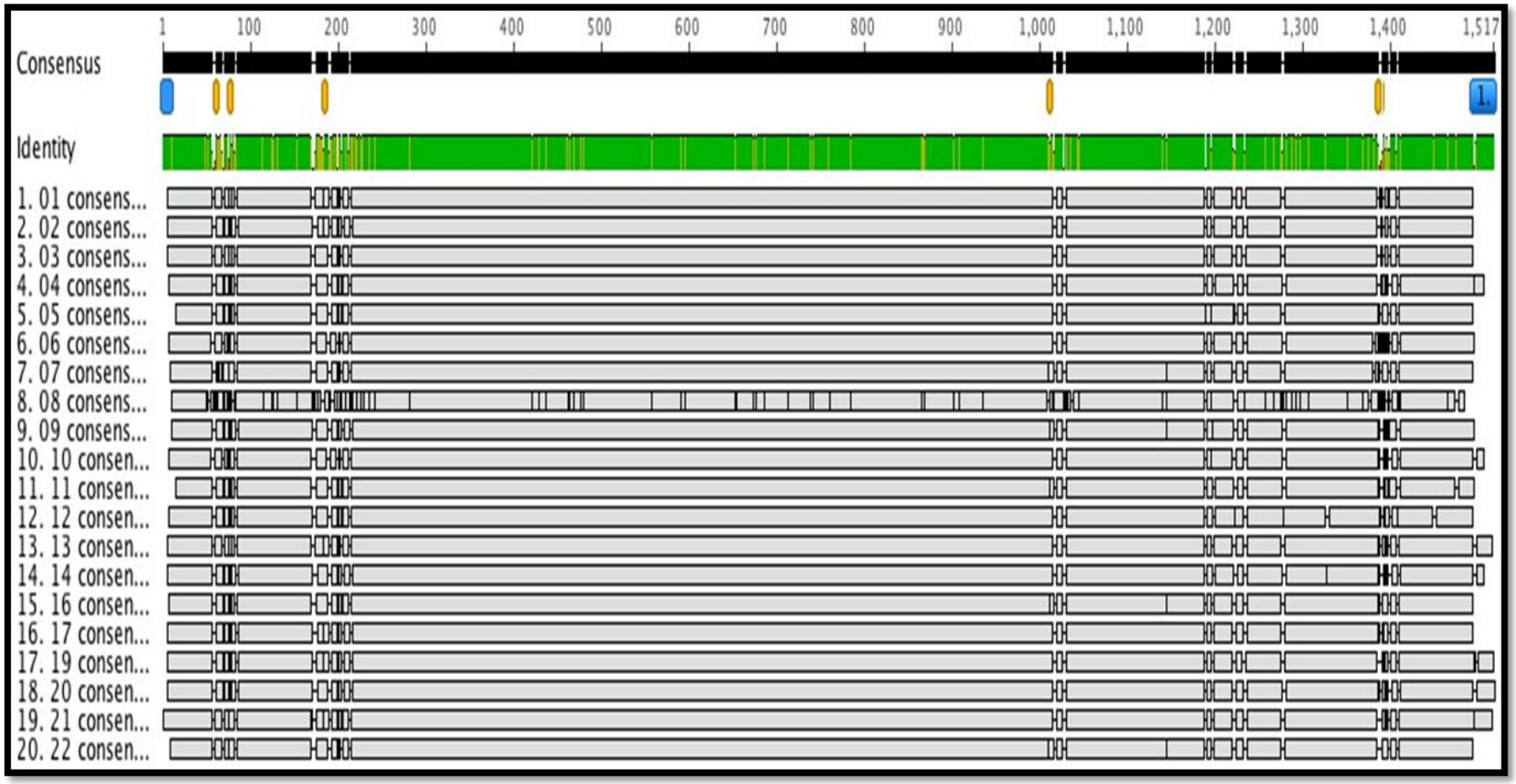

Figure 6. Multiple alignment graph generated form (www.ncbi.nlm.com).

Nevertheless, non motile Bt isolates had also been reported (Damgaard et al., 1997; Maheswaran et al., 2010; Kavitha et al., 2011). According to Bouillaut et al. (2005), positive motility is an indirect indicator of virulence and biological activity of Bt strains. Results of the present study revealed that 92% of the native 64 Bt isolates were motile and resistant to ampicillin but all isolates were sensitive to streptomycin. These results are compatible with those previously described by Chatterjee et al. (2007). However, the fact that in natural environment, non-insecticidal Bt isolates are often more prevalent than their insecticidal counterparts by > 90% (Mizuki et al., 1999a; Ohba et al., 2000; Yasutake et al., 2007) led Ohba et al. (2009) to suggest that Bt as a species is merely an environmental saprophyte and should not be considered only as an obligate pathogen of insects. This would explain the discovered novel biological activity of Bt crystal proteins that target protozoa (Kondo et al., 2002; Xu et al., 2004; El-Sadawi et al., 2008) and human cancer cells (Mizuki et al., 1999b, 2000; Nadarajah et al.,

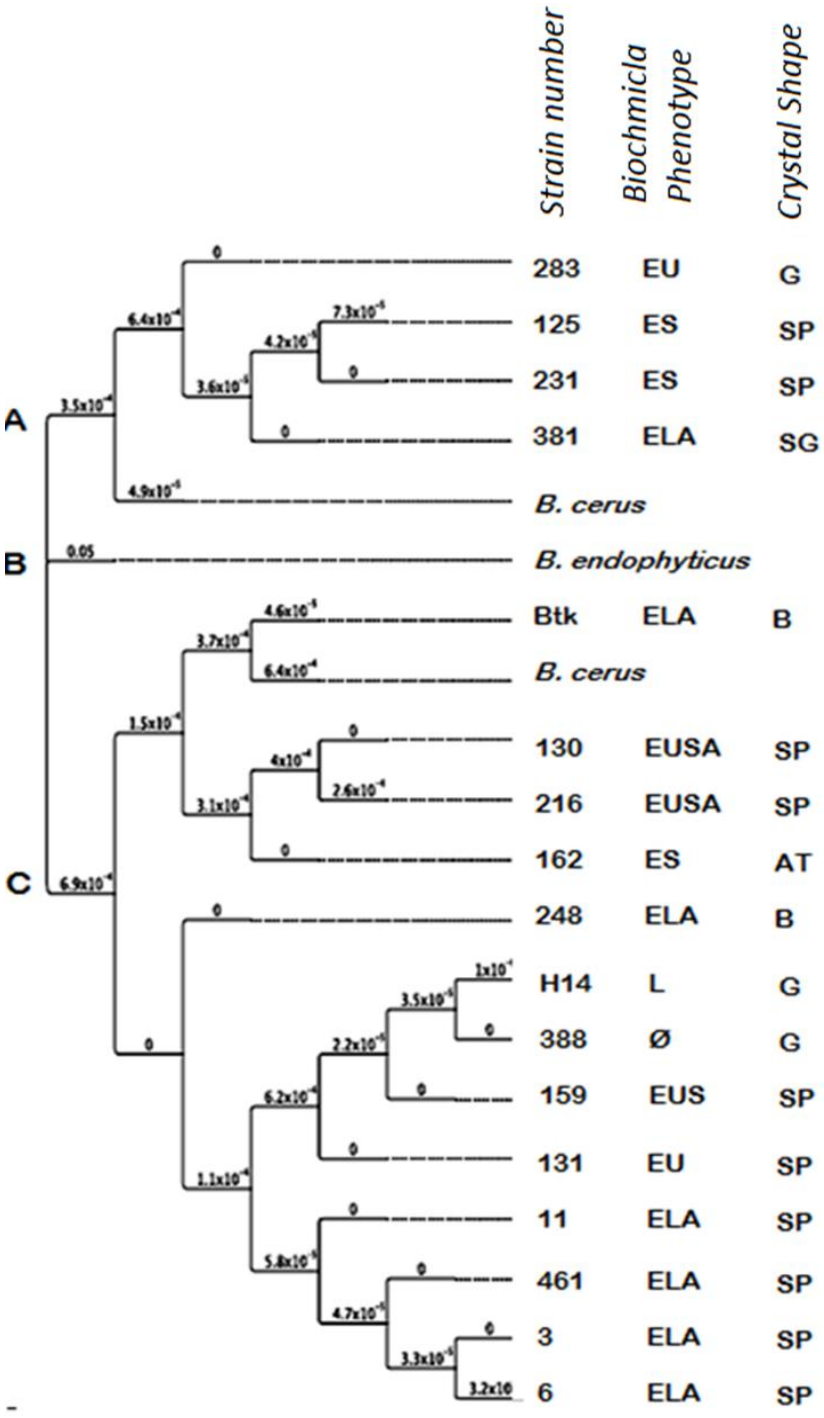

Figure 7. Phylogenetic tree dendrogram generated by GEN analysis. S (spherical), G (irregular), SG (spherical and irregular), B (bipyramidal), BS (bipyramidal and spherical), SC (spherical and cubic), SGR (spherical, irregular, and rhomboidal) and AT (attached). Biochemical types are described in legend of Table 5.

2008; Ohba et al., 2009). Bt isolate with crystal proteins that exhibit strong cytocidal activity preferential for human cancer cells of various origins have been termed parasporins (PS) 1 to 4 (Ohba et al., 2009). The present study showed that only 2 (3%) isolates of the 64 studied Bt isolates exhibited phenotypic traits that simulates those previously described for the anticancer PS1. According to Ichikawa et al. (2008), PS1 producers, differ

from Bt insecticidal isolates in being non- motile and non-hemolytic, hence sharing these characters with *B. anthracis*, but differ from it via its resistance to ampicillin. Whereas those that resembles PS2, 3, or 4 cannot be differentiated from insecticidal Bt isolates, thus sharing the common character of being actively motile, hemolytic for sheep RBCs, and also resistant to ampicillin. In consistent with our results, these authors also stated that parasporins producers in general are bacitracin sensitive, whereas insecticidal Bt isolates are usually resistant.

The present study revealed that most of 64 Bt isolates showed positive activity for gelatinase liquefaction, and assimilation of fructose, glycogen, trehalose, ribose, and N-acetyle glucoseamine. In contrast, all isolates exhibited negative reactivity for ONPG, H2S, xylose, fucose, lactose, galactose, and manitol. These findings are consistent with those previously reported by Logan and Berkeley (1984), Lecadet et al. (1999) and Martin et al. (2010). While these common traits are helpful in defining the core type of the species, they are less useful for differentiating strains (Martin et al., 2010). In agreement with several researchers (Lecadet et al., 1999; Keshavarzi, 2008; Aramideh et al., 2010; Martin et al., 2010), hydrolysis of esculin, urea, or lecithin, and acid production from sucrose, or salicin were highly discriminative BRs's for the 64 native Bt isolates. Taking in consideration the results of these 5 BRs's, the 64 native Bt isolates were classified into 7 types as mentioned before. As expected, the distribution of such biotypes varies from one geographical region to another. In this study, the biotype ELA was the most common (31%), and in Iran ranged from 25 to 38% as reported by Aramideh et al. (2010) and Keshavarzi (2008), respectively whereas, type L constituted only 6% of our isolates, and around 14% in Iran isolates (Keshavarzi, 2008; Aramideh et al., 2010). The respective prevalence in USA (including other countries) was 4.6 and 23.6 % for ELA and L biotypes, respectively (Martin et al., 2010).

The present study also revealed and confirms previous finding (Martin et al., 2010) that the distribution of crystal types within biochemical types was generally different than the distribution of crystal types separately. Thus, our results indicated that 50% of bipyramidal crystal-producers occurred within ELA biochemical type and those irregular crystal-producers occurred 27% within L & Ø biochemical types out of the total studied 64 native Bt isolates. Working on a total of 3639 Bt isolates, those types of crystals occurred in 84.5 and 55% respectively (Martin et al., 2010). Martin et al. (2010) stated that a full phylogenetic analysis of Bt strains selected by biochemical type will be an interesting addition and may help to clarify the population structure of this complex group of bacteria.

Several researchers (Joung and Cote, 2002; Soufiane and Cote, 2009; Poornima et al., 2010) have used 16S rDNA gene analysis as a molecular identification tool for Bt, while Soufiane and Cote (2009) not only used this tool for the identification of Bt species but also claimed its ability to discriminate Bt different H serotypes. In the present study, 16S rRNA gene analysis proved useful in the identity of the tested local Bt isolates and the two Bt reference strains as belonged to *B. thuringiensis*. Hence, it unambiguously confirmed the biochemical phenotypic identity. At the same time, it provided efficient discrimination between Bt, closely related *B. cereus*, and *B. endophyticus*. Phylogenetic analysis revealed that two of the non-Bt spore-forming bacilli belonging to *B. cereus* were aligned evolutionary close to Bt strains while one belonged to *B. endophyticus* was aligned alone in an evolutionary distant cluster from the entire group. In other words, all tested strains, except *B. endophyticus*, were found to be relatively homogenous and to share a high degree of DNA relatedness.

A noteworthy significant correlation between the biochemical types and the relevant clusters of phylogenetic tree was observed (Figure 5). For instance, strains number 3, 6, 11, and 461 having the same biochemical type (ELA) and the crystal morphology (spherical) were found to be closely clustered at the same scope. Likewise, the stains; 125 and 231, 130 and 216, and H14 and 388, fall within same cluster and exhibited similar biochemical type and crystal shape.

Bioassay results showed that only one Bt isolate exhibited similar anti-diptera activity as that of Btk strain while most of our native Bt isolates exhibited weak or non-insecticidal activity. The observed lack of insecticidal activity might be explained by the fact that most of these strains exhibited spherical crystal shape. These findings were in agreement with previous reports that the majority of Bt isolates with spherical crystals are non insecticidal (Momani et al., 2002; Poornima et al., 2010). However, our conclusion is only limited to the tested larval mosquito-species, and the possibility to find insects susceptible to our native Bt isolates still exist. The periodic isolation and introduction of native Bt isolates, from different environments, may be helpful in the control of insects as well as the management of emerged insect resistance (Xavier et al., 2007a). In the present study, results of motility, hemolytic activity, antibiotic-susceptibility pattern, crystal shape and mosquito-larvicidal activity, seem to suggest that most of our Bt isolates may exhibit parasporins activity.

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