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Isolation and distribution of mosquito-larvicidal *cry* genes in *Bacillus thuringiensis* strains native to Saudi Arabia

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Abstract. A total of 157 environmental samples were collected from 11 ecological regions across Saudi Arabia to isolate native *Bacillus thuringiensis* (Bt) strains. Bt isolates (n=103) were recovered by the 50% (v/v) ethanol treatment method with Bt index range of 0.01 to 0.4. Most of Bt isolates showed spherical crystals (54%), while, irregular, bi-pyramidal, and spore-attached crystal constituted 27, 16 and 3% respectively. PCR analysis with eight general and specific dipteran primers of Cry and Cyt genes, revealed positive amplification for *cry4 & cyt1*, and *cry4A*, *cry4B* and *cyt2*, and *cry 10* and cry 11 genes in 28%, 26%, 22%, and 25% of tested strains respectively; whereas *cry2* gene was not detected except with the reference *Bt kurstaki* HD-1 strain. Bioassays against *Aedes caspuis* and *Culex pipiens* larvae indicated that 11 strains displayed better larvicidal activity compared with *Bacillus thuringiensis* H14 (Bti) reference (LC50 0.6 µg/ml) strain against *Ae. caspuis*, but only two strains (620A & 633R1, LC50 of 0.09 µg/ml & 0.064 µg/ml) that gave significant enhancement. Additionally, one strain (633R1) showed LC50 similar to that of Bti H14 (LC50 0.064 µg/ml) against *Cx. pipiens*. With the exception of *cyt* primers, sequenced DNA of all positive primers amplicons revealed 95 to 99% identity in GenBank with *Bacillus thuringiensis* subsp. *israelensis* plasmid pBtoxis and also correlated with its SDS-PAGE expressed protein profiles analysis. It is hoped that our wild bio-insecticide Bt strains can be explored in future in the control of mosquito-vector borne diseases in Saudi Arabia.

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive spore forming saprophyte soil bacterium. During the sporulation process Bt produces one or more proteinaceous parasporal crystals (Cry), recognized as delta-endotoxin. This crystal protein under alkaline conditions of mid-gut of susceptible insects, gets solubilized, and then activated by intrinsic proteases into an active toxin that selectively binds specific receptor in the epithelial cell membrane, leading to swelling, pore-formation, and/or lysis of epithelial cells that is followed by larval death from starvation (Eswarapriya *et al.*, 2010). The demonstration of parasporal crystals is the most feasible 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 disease vectors and agriculture-pests (Nester *et al.*, 2002). Additionally, 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). Since genetic diversity and toxic potential of Bt strains differ from a country-region to another as well as from one country

to others, hundreds of Bt strains have been isolated and characterized all over the world, with the aim to find novel Cry genes and/or to combat the emergence of resistant insects. So far Bt Cry toxins have been classified into 72 families (that is, Cry1 to Cry72) and three groups of Cry proteins targeting specific insects, based on their amino acid sequence homology (Crickmore *et al.*, 2013). To date, native Bt isolates were reported from Saudi Arabia, by only two groups of researches, Umm Al-Qura University and King Saud University (Assaeedi *et al.*, 2011; El-kersh *et al.*, 2012) respectively. In our previous report (El-kersh *et al.*,2012) we did not succeed to find native Bt with mosquito larvicidal activity at that time, hence we rather focused on native Bt strains identity, morphologically, biochemically and by 16S rRNA genes analysis. This study deals with further isolation of native Bt strains from different ecological regions across Saudi Arabia, confirm their identity, testing their Dipteran Cry and Cyt genes content by PCR, DNA sequencing, as well as bioassay-toxicity against *Aedes caspuis* and *Culex pipiens* larvae in parallel with two references, *Bacillus thuringiensis kurstaki* HD1and *Bt israelensis* H14 strains.

MATERIALS AND METHODS

Sample collection and Bt isolation

One hundred fifty seven (157) samples were recently collected (Table 1 & Figure 1) from different regions across the country. Bt isolation from samples was carried out using the 50% (v/v) ethanol–treatment method (Hong *et al.*, 2009; El-kersh *et al.*, 2012), whereby specimen of dead insects were dispatched and then macerated in sterile saline using a sterilized mortar and pestle, thereafter 1 ml aliquots were transferred to sterilized test-tubes (Gobatto *et al.*, 2010) and treated by the 50% ethanol method. After ethanol-treatment for 1h at room temperature, 1/10 serial dilutions in sterile distilled water were carried out, then appropriate dilutions were spread on nutrient agar (NAS medium) supplemented with 0.2% yeast extract (Sisco research laboratories, Mumbai, India); and 0.0005% of manganese chloride ($MnCl_2$), and incubated at 30 C for 2 to 3 days depending on spore maturation. After incubation, Bt like colonies (Gobatto *et al.*, 2010) that appeared circular, scalloped-edged, non-pigmented, off-white with irregular margin, but not flat regular or elevated center were examined. For comparison, the Bt index was calculated

Table 1. Distribution of Bt isolates in tested samples from different localities in Saudi Arabia

City-location	Key map	No of samples analyzed	No of positive samples	No of *Bt* isolates	*Bt* index*
A-Makkah	A	15	12	24	0.2
A-Taif		3	2	3	0.4
B-Medina	B	14	6	10	0.01
B-Yanbu		8	4	6	0.05
C-Albaha	C	27	11	15	0.25
D-Abaha	D	6	5	8	0.01
D-Khamis mushait		1	1	1	0.03
D-Assir		2	1	1	0.03
E-Najran	E	4	1	2	0.08
F-Jizan	F	19	10	15	0.14
G-Tabouk	G	6	3	5	0.01
H-Hafer albaten	H	8	3	4	0.2
I-Qassim	I	25	5	5	0.4
J-Riyadh	J	15	3	3	0.1
K-Eastern region	K	4	1	1	0.14
Total		157	68	103	0.13*

* The number of identified *Bt* colonies divided by the total number of spore-forming colonies

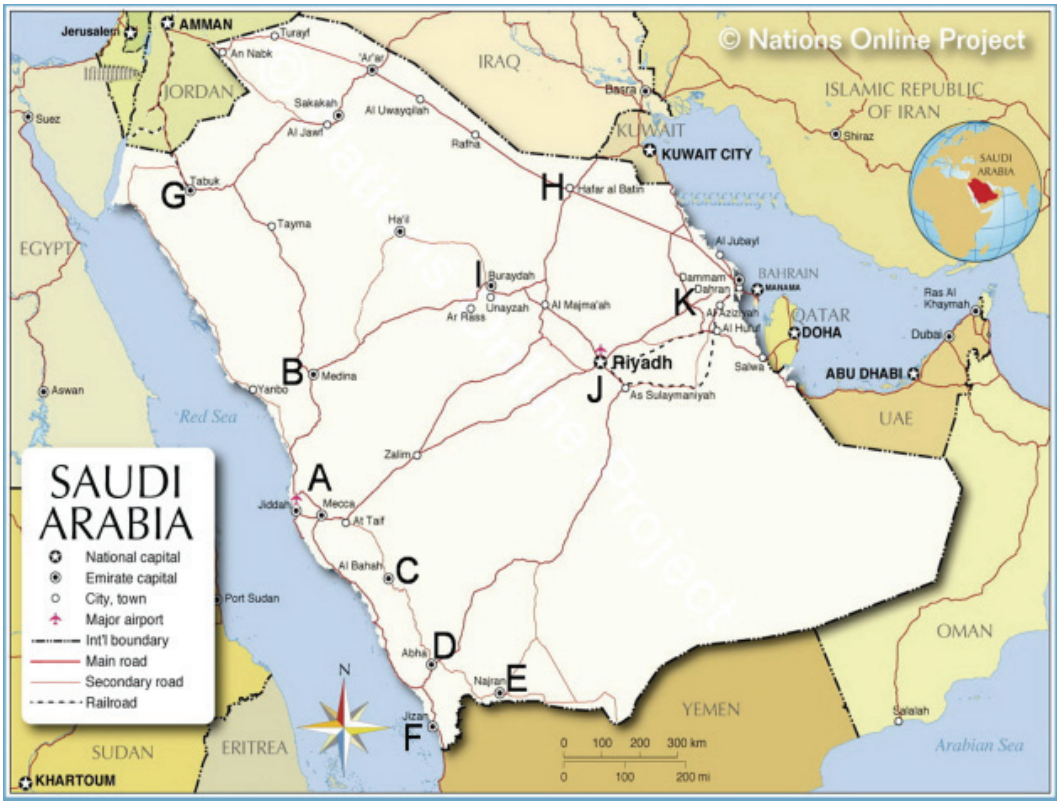

Figure 1. Map showing sample collected sites as indicated by letters A to K, where: A-Makkah-Al Taif, B-Medina-Yanbu, C-Albaha,-Abha, D-Khamis mushait-Assir, E-Najran-Jizan ,G-Tabouk, H-Hafer albaten, I-Qassim, J-Riyadh, and K-Eastern region.(http://www.nationsonline.org)

for each positive sample as colony number of Bt isolates divided by total colony-number of spore forming bacilli (El-kersh *et al.*, 2012). Potential Bt candidates were examined by phase contrast microscopy for the presence of parasporal crystals and their shapes (Figure 2). To further characterize the crystal shape precisely Bt isolates were also examined by scanning electron microscopy (Figure 3) essentially as previously described by El-Kersh *et al.* (2012). The pure Bt isolates coded by Lab-number for geographical regions were harvested aseptically from surfaces of NAS plates (mature spores) in sterile nutrient broth containing 50% glycerol as heavy suspensions and then stored as stock culture at -20ºC (Hernandez *et al.*, 2005).

DNA extraction

Bt isolates were sub-cultured on Luria Bertani agar (contains per liter: tryptone, 10g; yeast extract, 5g; sodium chloride (NaCl), 5g; pH 7.5; bacto-agar, 15g) and incubated at 30ºC overnight. After incubation, a single colony was suspended into 100 µl sterile distilled water in an Eppendorf tube and placed in a shaking boiling water-bath for 10 min followed by an immediate cool shock at -20ºC. This heat-shock process was repeated three times to allow complete cell-lysis before centrifugation (Multifuge 3SR+ Centrifuge, Thermo Scientific Electron Corporation, Germany) at 10,000 rpm for 10 min at 4 C. The resultant supernatant containing the crude DNA was used for PCR amplification (Bukhari & Shakoori, 2010), using Hot Star Taq DNA polymerase SE master mix Kit (QIAGEN Technologies, Germany).

PCR analysis

Each of the 103 native Bt isolates and the Bt-H14 & Bt-HD1 references strains were

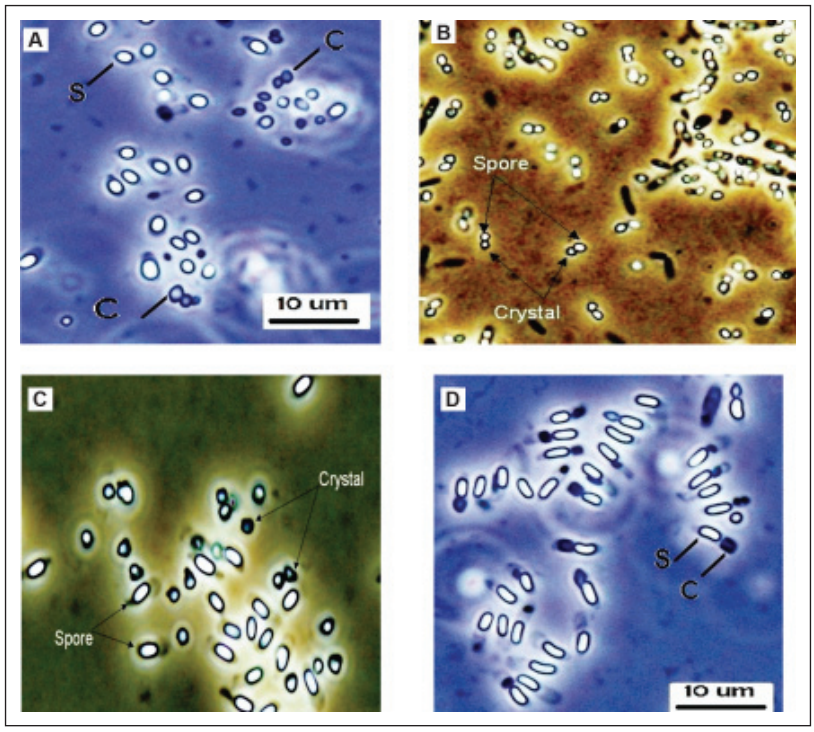

Figure 2. Phase Contrast micrographs of native Bt isolates A, bi-pyramidal and/or cubic crystals; B, spherical to cubic attached crystals; C, irregular small spherical, triangular merged, cubic to rhomboid crystals resembles Bti H14; D, bi-pyramidal to square spore-attached crystals

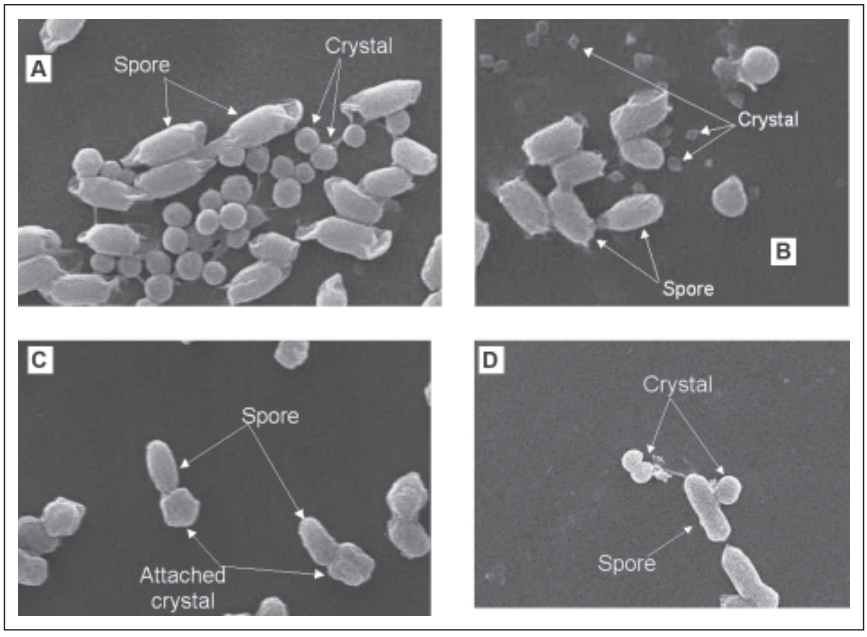

Figure 3. Scanning electron micrograph of : A, spore and spherical crystal proteins from Bt native isolate No: 586B (X 7,000); B, spores and small bi-pyramidal crystal proteins from Bt native isolate No: 627 (X 8,000); C, spores attached cubic crystal proteins from Bt native isolate No: 632R (X 6,500); and D, spore and irregular crystal proteins from Bt native isolate No: 641AT (X 8,000)

screened for 5 universal primers namely *cry 2*, *cry 4*, *cry 11*, *cyt 1*, *and cyt 2* designed from conservative regions of related genes as reported by Ibarra *et al.* (2003) and Jouzani *et al.* (2008), as well as the 3 specific primers from highly variable region (Ibarra *et al.*, 2003) namely *cry*4 Aa, *cry*4 Ba and *cry10*. The overall oligonucleotide-primers (Table 2) were synthesized in a DNA synthesizer (Applied Bio systems, UK) as the specified manufacturer.

The PCR reaction mixture included 1 µl forward primer, 1 µl reverse primer, 5 µl of the prepared crude DNA (cell-lysate), 12.5 µl master mix (Hot Star Taq DNA polymerase SE master mix Kit (QIAGEN Technologies, Germany)), 5.5 µl nuclease free sterile water (QIAGEN Technologies, Germany) to give a total volume 25 µl. The reaction cycle comprised initial denatrution at 96ºC for 10 min, followed by 35 cycles each cycle consisted of denaturation at 96ºC for 1 min, annealing temperature(s) (Table 2) for 1 min and extension at 72ºC for 1 min then final extension for 7 min (Veriti® 96-Well Thermal Cycler, Applied Biosystems, USA) at 72ºC. The PCR product (15 µl) was analyzed by 2% agarose gel electrophoresis (Mahalakshmi *et al.*, 2012). The distribution frequency of *cry* genes in Bt strains was calculated as the percentage of *cry* (+) Bt strains to the total 103 examined native Bt isolates.

DNA Sequencing

Polymerase chain reaction (PCR) products of Cry, Cyt genes in potential Bt isolates, were subjected to automated purification and DNA sequencing as conducted in the Sequencing Core Facility at King Faisal Specialized Hospital & Research Centre, Riyadh, Saudi Arabia. The sequencing based Sanger Sequencing Technology on ABI Prism 3730XL (Applied Biosystems/Sanger, USA), was carried out according to the dideoxy chain-termination method (Sanger *et al.*, 1977). The obtained sequences were manually-cleaned and edited by use of BioEdit Sequence Alignment Editor (Hall, 1999). Database

Table 2. Characteristics of general and specific primers for cry2, cry4, cry 4A, cry 4B, cry10, cry11, cyt1 & cyt2

Primer	Sequence	Gene(s) recognized	Product Size (bp)	Condition of Annealing	GenBank Accession No	References
Cry2 (UN)	(F) 5'-GAGTTTAATCGACAAGTAGATAATTT-3'	*cry2Aa*	526	50 C	M31738	Ibarra *et al.*,
	(R) 5'-GGAAAAGAGAATATAAAAATGGCCAG-3'	*cry2Ab*	526		M23724	2003
		cry2Ac	520		X57252	
		cry2Ad	500		AF200816	
Cry 4 (UN)	(F) 5'-GCATATGATGTAGCGAAACAAGCC-3'	*cry 4A2*	439	58 C	D00248	Jouzani *et al.*,
	(R) 5'-GCGTGACATACCCATTTCCAGGTCC-3'	*cry 4B4*	439		D00247	2008
Cry 4A (spe)	(F) 5'-TCAAAGATCATTTCAAAATTACATG-3'	*cry 4Aa*	459	50 C	Y00423	Jouzani *et al.*,
	(R) 5'-CGGCTTGATCTATGTCATAATCTGT-3'				Y00423	2008
Cry 4B (spe)	(F) 5'-CGTTTTCAAGACCTAATAATATAATACC-3'	*cry4Ba*	321	50 C	X07423	Jouzani *et al.*,
	(R) 5'-CGGCTTGATCTATGTCATAATCTGT-3'					2008
Cry 10 (spe)	(F) 5'-TCAATGCTCCATCCAATG-3'	*cry 10*	348	51 C	M12662	Jouzani *et al.*,
	(R) 5'-CTTGTATAGGCCTTCCTCCG-3'					2008
Cry11 (UN)	(F) 5'-CGCTTACAGGATGGATAGG-3'	*cry11Aa*	342	50 C	M31737	Ibarra *et al.*,
	(R) 5'-GCTGAAACGGCACGAATATAATA-3'	*cry11Ba*	342		X86902	2003
		cry11Bb	452		AF017416	
Cyt1 (UN)	(F) 5'-CCTCAATCAACAGCAAGGGTTATT-3'	*cyt1Aa*	477	52 C	X03182	Ibarra *et al.*,
	(R) 5'-TGCAAACAGGACATTGTATGTGTAATT-3'	*cyt1Ab*	480		X98793	2003
		cyt1Ba	477		U37196	
Cyt2 (UN)	(F) 5'-ATTACAAATTGCAAATGGTATTCC-3'	*cyt2Aa*	356	50 C	Z14147	Ibarra *et al.*,
	(R) 5'-TTTCAACATCCACAGTAATTTCAAATGC-3'	*cyt2Ba*	355		U52043	2003
		cyt2Bb	355		U82519	
		cyt2Ca	355		AAK50455	

* UN: universal; spe: specific

search for sequences (other than human and mouse genomic + transcripts) for annotated genes corresponding to obtained sequences was carried out using the NCBI nucleotide Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome. The NCBI Blast search was based on the nucleotide collection (nr/nt) of "others" database which was optimized for highly similar sequences (megablast). Sequence multiple alignments were carried out using the Multalin interface (Corpet, 1988). (http://multalin.toulouse.inra.fr/multalin/)

SDS-PAGE analysis

The SDS-PAGE analysis for protein profile of Bt isolates was carried out according to Bukhari & Shakoori (2010). Briefly, a pure colony of each of Bt isolates as well as the Bt-H14 & Bt-HD1 references strains from an overnight culture on LB agar was streaked on T3 agar medium (contains per liter: tryptone, 3g; tryptose, 2g; yeast extract, 1.5 g; sodium dihydrogen phosphate (NaH_2PO_4), 6 g; disodium hydrogen phosphate (Na_2HPO_4), 7.1 g [pH 6.8]; manganese chloride ($MnCl_2$), 0.005g and 15g Bacto-agar (Bozlagan *et al.*, 2010)) and incubated at 30 C for 72 hours. After incubation, Bt growth was harvested in sterile distilled water followed by centrifugation in a cooled (4 C) centrifuge at 7000 rpm for 15 min. The pellet was suspended in 3mL sterile distilled water and washed twice with cold sterile distilled water by centrifugation. The washed pellet was then dissolved in alkaline buffer (sodium carbonate (Na_2CO_3), 0.256 g; and dithiothreitol (DTT), 0.08 g in 50 ml sterile distilled water, pH 10.5-11) for 3 hours in a shaking incubator at 37°C, followed by centrifugation at 7000 rpm for 20-25 min at 4°C. The resultant supernatant (containing protoxin) was subjected to pH adjustment with 1N HCl to around 7-8, followed by Lowry-protein determination (Lowry *et al.*, 1951) and stored at -20°C before later trypsin activation. One µg of trypsin (stock: 1mg/ml) for each 20 µg of protoxin was mixed well in tested samples followed by 3 hours trypsin-digestion at 37 C. Equal volume of the protoxin – activated sample and 2X Laemmli buffer (125 mM tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% B-mercaptophenol, 50% glycerol, 0.02% bromophenol blue (tracking marker)) were thoroughly mixed and immediately immersed the mixture in a boiling water bath for 10 min, then processed for SDS-PAGE (10%) analysis (Laemmli, 1970). For bands - visualization, gels were stained in Coomasie blue solution for 15-20 min followed by overnight destaining by distilled water (containing v/v 25% methanol and v/v 10% acetic acid). Protein molecular mass standards (Protein marker II (6.5 – 200) pre-stained) were applied from Appli-Chem GmbH, Ottoweg, Darmstadt, Germany. The proteins used as molecular mass markers were; myosin (200 kDa), beta-galactosidase (116 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa).

Preparation of the spore-crystal mixture for the bioassay

The PCR-positive Bt isolates were cultured from single colony in liquid T3 medium (100 ml broth in 500 ml flask) and incubated on a rotary shaker (200 rpm) at 30°C for 5 days (> 90% sporulation). After incubation, whole spores and crystals were harvested in one M NaCl solution, then pelleted by centrifugation and washed thrice with sterile distilled water. The washed pellets were then suspended in a small volume of sterile distilled water then processed through a freeze drier (temperature: -55°C, vacuum pressure: 0.1 mbar), and the resulting dry powders were assayed for biological activity (Ammouneh *et al.*, 2011).

Toxicity tests and bioassays

Following the law in Saudi Arabia, it is prohibited to transfer *Aedes aegypti*, the dengue mosquito vector, from infested areas, Jeddah, Makah and Jizan, to a non- infested area the Riyadh-capital, to avoid local spreading of dengue fever. Thus, mosquito-larvicidal activity of native Bt isolates was tested against larvae of *Ae. caspuis* and *Cx. pipiens* (kindly provided by Dr. Ashraf Mohamed Ahmed, Zoology Department, College of Sciences, KSU). Twenty of 3rd to

4^{th} instars tested larvae were added to 15 ml tap water in 30 ml plastic cups in 3 replicates, then 5 ml of calculated ($ug.mL^{-1}$) lyophilized powder (spore-crystal mixture) of each isolate was added and incubated at room temperature for 24h and 48h scoring mortality (Aramideh *et al.*, 2010). Meanwhile appropriate serial dilutions were concomitantly cultured on GYS medium (contains per liter: glucose, 5g; yeast extract, 5g; magnesium sulphate ($MgSO_4$), 0.82g; calcium chloride ($CaCl_2$), 0.08g; manganese chloride ($MnCl_2$), 0.084g; ammonium sulphate ($(NH_4)_2SO_4$), 4g; dipotassium phosphate (K_2HPO_4), 1g (pH 7.2) and 15 g bacto-agar) for determination of spore count. As a positive control for larvicidal activity, *Bti* (H14) was processed and used with each run. Corrected mortality percentage which regarded mortality or dead larvae in negative control, were calculated by using Abbotts formula (Abbott, 1925; Vidal-Quist *et al.*, 2010). The corrected mortality = [(% mortality in treatment – % mortality in control) / (100 – % mortality in control)] x 100.

Statistical analysis

The scored mortality and respective spore-count were analyzed by generalized linear models for binomial distribution and logit link function with Post Hoc test (Bonferroni and Dunnett T3). For means comparisons to find the significant difference (P-value < 0.05 was considered as significant) using Statistical Package for Social Science (SPSS); release 19.0. The LC50 and LC90 (lethal concentration required to kill 50% and 90% of larvae) were calculated by probit analysis using SPSS 19.0 (Vidal-Quist *et al.*, 2010).

RESULTS

Geographical distribution of *Bacillus thuringiensis* isolates

A total of 157 samples (11 localities) were processed for Bt isolation. Of these 89 samples yielded no Bt isolates, whereas 103 Bt isolates were recovered from 68 samples. As presented in Table 1 and Figure 1 certain regions (A, B, C, D, E, F, G, H) proved very rich in Bt contents and yielded Bt isolates with different diversities in crystal shape and colonial morphology, whereas in regions of J, K, and I (Figure 1) most of their samples yielded one single Bt isolate. This distribution however does not correlate well with the observed overall Bt index for each regions. For instance samples from Makkah region (Figure 1, A) showed the highest diversity of Bt isolates yet its Bt index was only 0.2, whereas samples of Qassim region (Figure 1, I) exhibited (Table 1) the highest Bt index (0.4) but with very low diversity of Bt isolates. Results suggest that such variation in Bt diversity may correlate with the respective geographical habitat variation with urbanization and/or rural regions.

Phase contrast and electron microscopy

All Bt isolates shared many morphological characteristics in cell chains arrangement, ellipsoidal shape of spores, and non-swollen sporangia. Based on the diversity of crystal morphology, the 103 Bt isolates were divided into four classes (Figure 2 A to D); S (spherical: 54%), G (irregular: 27%), B (bipyramidal: 16%) and finally, AT (attached to the spores: 3%). As illustrated in (Figure 2 B & 3 C) the spores of Bt strain number 632R were strikingly morphometrically distinct. Thus the spores of this strain showed an appearance of shortened ellipsoid, more round than those of the other strains.

Prevalence of *cry* and *cyt* genes in native Bt isolates

Agarose gel electrophoresis of the PCR products obtained with H14 reference strain and some represented 636R and 662RN native strains are shown in Figure 4 and Table 3. The positive control Bt H14 gave positive amplified respective fragments to all tested Cry and Cyto primers. Thus a 439bp fragment representing conserved (universal) region of *cry*4 delta-endotoxin gene on the basis of PCR was amplified as well as that of 477 bp fragment of cyt1 in 28 isolates (28%) out of 103 tested native isolates. In contrast, 526 bp fragment of cry2 (Table 2) was not detected in any of the tested strains, but the Bt kH1 reference strains yielded positive PCR fragment, while 459 bp fragment of *cry*4A, 321 bp fragment of *cry*4B and 355 bp fragment

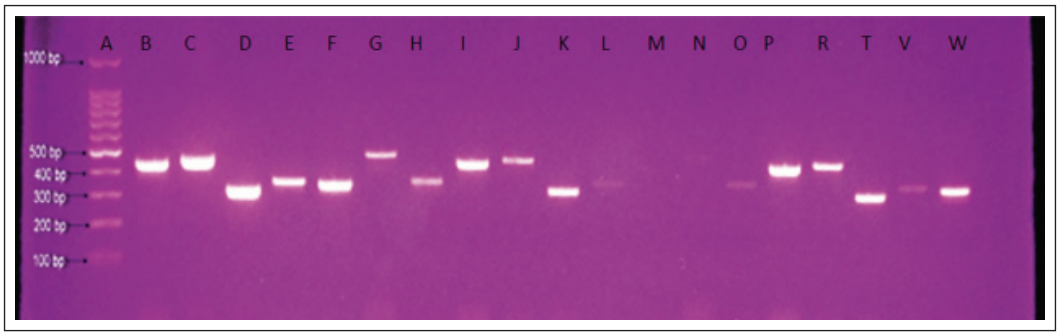

Figure 4. Agarose gel (2%) electrophoresis of PCR-Positive fragments amplification of Cry and Cyt genes primers with reference Bti H14 and represented 636R and 662RN native strains. Lane A, 100-1000 bp ladder; Lanes B to H, Cry4UN, Cry4A, Cry4B, Cry10, Cry11, Cyt1 & Cyt2 of H14 respectively; Lanes I to O, same those primers genes of native strain 636R (missing only Cry 11 at lane M) and Lanes P to W, Cry4UN, Cry4A, Cry4B, Cry10 & Cry11 for native strain 662RN respectively

Table 3. General characteristics of 28 native Bt strains tested against 3rd to 4th in stars larvae of *Ae. caspuis and Cx. pipiens*

Isolates	Geographical source (key word in Fig.1)	Biochemical reactions			*cry* and *cyt* genes amplicons detected	Protein bands (kDa)
		Citrate	VP	Nitrate		
H14		+	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 130, 116, 89, 76, 70, 60, 45, 25, 21, 19, 6.5
616A	A (Soil)	–	+	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 130, 116, 89, 70, 60, 45, 25, 21, 14.5, 9
616B		–	++	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 116, 67, 28, 24, 22, 21, 20, 18, 14.5, 14
616 2T		–	–	+	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 186, 162, 116, 63, 43, 28, 24, 23, 21, 20, 18, 14.5, 14
618A		+	+	–	*cry4*, *cry4A*, *cry4B*, *cry11*, *cyt1*, *cyt2*	76, 25, 21, 19, 14.5
618B		–	+	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	28, 21, 20, 14
618 3A		++	+	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 186, 158, 28, 21, 20, 18, 14.5, 14
618 4A		–	–	–	*cry4*, *cry4A*, *cry4B*, *cry11*, *cyt1*, *cyt2*	45, 25, 21, 14.5
620A	A (Leaf)	++	++	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	76, 45, 25, 19, 14.5
620B		–	–	++	*cry4*, *cry4A*, *cry4B*, *cry11*, *cyt1*	76, 45, 25, 21, 19, 14.5
621A	A (Soil)	+	–	–	*cry4*, *cry4A*, *cry4B*, *cry11*, *cyt1*, *cyt2*	45, 21, 19, 14.5
621B		–	–	++	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 186, 178, 160, 28, 21, 20, 18, 14.5, 14
624R	D (Soil)	–	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	116, 89, 45, 25, 19, 14.5
625R		+	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	158, 63, 28, 20,18, 14.5, 14
626R		+	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 158, 28, 20, 18, 14.5, 14
628RN	D (Leaf)	–	–	++	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 141, 120, 60, 29, 22, 20, 18, 14, 12
628RS		+	++	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	162, 158, 28, 22, 21, 20, 18, 14.5, 14
632R	C Animal dung	–	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	158, 28
633R1	B (Soil)	–	++	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 130, 116, 89, 76, 45, 21, 19, 14.5
633R2		+	++	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	200, 186, 178, 162, 78, 28, 21, 20, 18, 14.5, 14
635R	B Animal dung	–	+	++	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	120, 60, 50, 23, 22, 20, 18, 14, 12
636R	C (Leaf)	+	+	+	*cry4*, *cry4A*, *cry4B*, *cry10*, *cyt1*, *cyt2*	200, 89, 76, 60, 50, 45, 25, 21, 19, 14.5
641R	E Animal dung	–	+	+	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	120, 60, 18, 14, 12
648R	G (Leaf)	–	+	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	141, 120, 60, 28, 22, 20, 14, 12
649R		+	+	–	*cry4*, *cry11*, *cyt1*, *cyt2*	200, 89, 76, 70, 50, 45, 40, 14.5, 11
660R	B (Leaf)	+	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	60, 12
662RN	A (Soil)	–	–	–	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	60
660RS		–	+	+	*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*, *cyt2*	168, 141, 60, 28, 23, 22, 20, 18, 14, 12
663R	F Animal dung	+	++	++	*cry4*, *cry4A*, *cry4B*, *cry11*, *cyt1*, *cyt2*	ND

of *cyt2*, were also detected in 27 isolates (26%). The 348 bp fragment of cry10 and 342 bp fragment of cry11 were amplified in 23 (22%) and 26 (25%) of the tested native Bt isolates respectively (Table 3).

DNA sequencing

A total of 35 Dipteran Cry genes of represented native Bt strains were also sequenced and their sequence aligned are illustrated in Table 4 and Figure 5. Nucleotide sequence homology for the cry genes were compared with *B. thuringiensis* using BLAST search tool (www.ncbi.gov). With the exception of Cyt 1 and Cyt 2 genes, all examined cry genes showed close homology and similarities that ranged from 95% to 99% as that of the tested *Bt israelensis* H14 positive control. This finding was also reflected with expressed protein profiles in SDA-PAGE analysis (Figure 6), whereas as Cyt 1 and Cyt 2 sometimes exhibited (strain 618 A) close similarities to that of *Bti* H14 that ranged from 95% to 97% but in other cases varied and/or even no detected significant similarity (Table 4 and Figure 5).

Bioassays

The overall native Bt strains which showed positive amplicon of the examined *cry* and *cyt* genes as well as their geographical sources, and three discriminative bio-chemical reactions are presented in Table 3. Each of these 28 strains was subjected for its bio-insecticidal activity (LC_{50}) against larvae of *Ae. caspuis* and *Cx. pipiens* in terms of $\mu g/mL^{-1}$ and spore count (CFU/mL). Figure 7 showed some represented Bt strains and Table 5 showed that out of the 28 Bt strains, 11 strains with LC_{50} (24 h) values concentration ranging (from 0.6 to < 2 $\mu g.mL^{-1}$) showed higher toxicity against *Ae. caspuis* larvae than that of H14 (LC_{50} 24 h: 2 $\mu g.mL^{-1}$). However, only 3 strains were more toxic when compared with H14 based on LC_{50} spore count (628RN: 0.26 $*10^8$ spore/ml, 632R: 1.3 $*10^8$ spore/ml and 641R: 1.13 $*10^8$ spore/ml), yet this difference (Table 5) was not significant (P_{value} ranged from 0.059 to 1.000). On other hand, toxicity against *Cx. pipiens* showed one strain (633R1) similar to H14, while 23 strains showed LC_{50} concentration ranged from 0.09 to 2.6 $\mu g.mL^{-1}$, again this difference (Table 5) was not significant (P_{value} ranged from 0.059 to 1.000).

DISCUSSION

To our knowledge and as stated by Assaeedi *et al.* (2011) Bt commercial products were not used to control insects and/or pests in Saudi Arabia. Therefore all of our 103 *Bt* isolates are considered as part of the indigenous microflora of the areas which have been explored. Additionally, results indicated that the Bt isolates exhibited crystal morphology of spherical (54%), irregular (27%), bi-pyramidal (16%) and attached to the spores (3%). These findings are almost compatible with those previously reported by Poornima *et al.* (2010), while from Colombia, Hernandez-Fernandez *et al.* (2011) isolated

Table 4. Nucleotide sequence homology-similarity percentage of the Cry &Cyt genes for five represented native Bt isolates as compared to GenBank number of Bti H14 *Bacillus thuringiensis* using BLAST search tool (www.ncbi.gov)

Strain/cry-cyt	GeneBank Number of Bti H14 & native Bt strains matching similarity percentage %						
	Cry 4	Cry 4A	Cry 4B	Cry 10	Cry 11	Cyt 1	Cyt 2
618 3A	99	99	96	99	99	96	95
624 R	99	96	92	98	97	89	<45
660 R	96	97	98	98	98	<45	<45
Bti-H14 reference strain	100 AL731825.1 D00248.1	100 AL731825.1 D00248.1	100 AL731825.1 D00247.1 AY729887.1	100 JQ228566.1 AL731825.1	100 JQ228567.1 AL731825.1	100 EF656359.1 AJ296639.1	100 GQ919039.1 FJ205866.1 U52043.1
662RN	99	96	99	98	99	<45	<45
662RS	99	98	97	98	99	<45	92

99 Bt isolates and the predominant crystal shape was amorphous (38%), followed by bi-pyramidal (27%), square (22%), round (8%) and triangular (5%). In Bangladesh, Shishir *et al.* (2012) reported that out of 26 Bt isolates 33%, 20%, 20% and 7% revealed spherical, bi-pyramidal, bi-pyramidal & spherical and spherical & irregular pointed, cubic, bi-pyramidal & irregular pointed or irregular pointed & irregular crystal shape

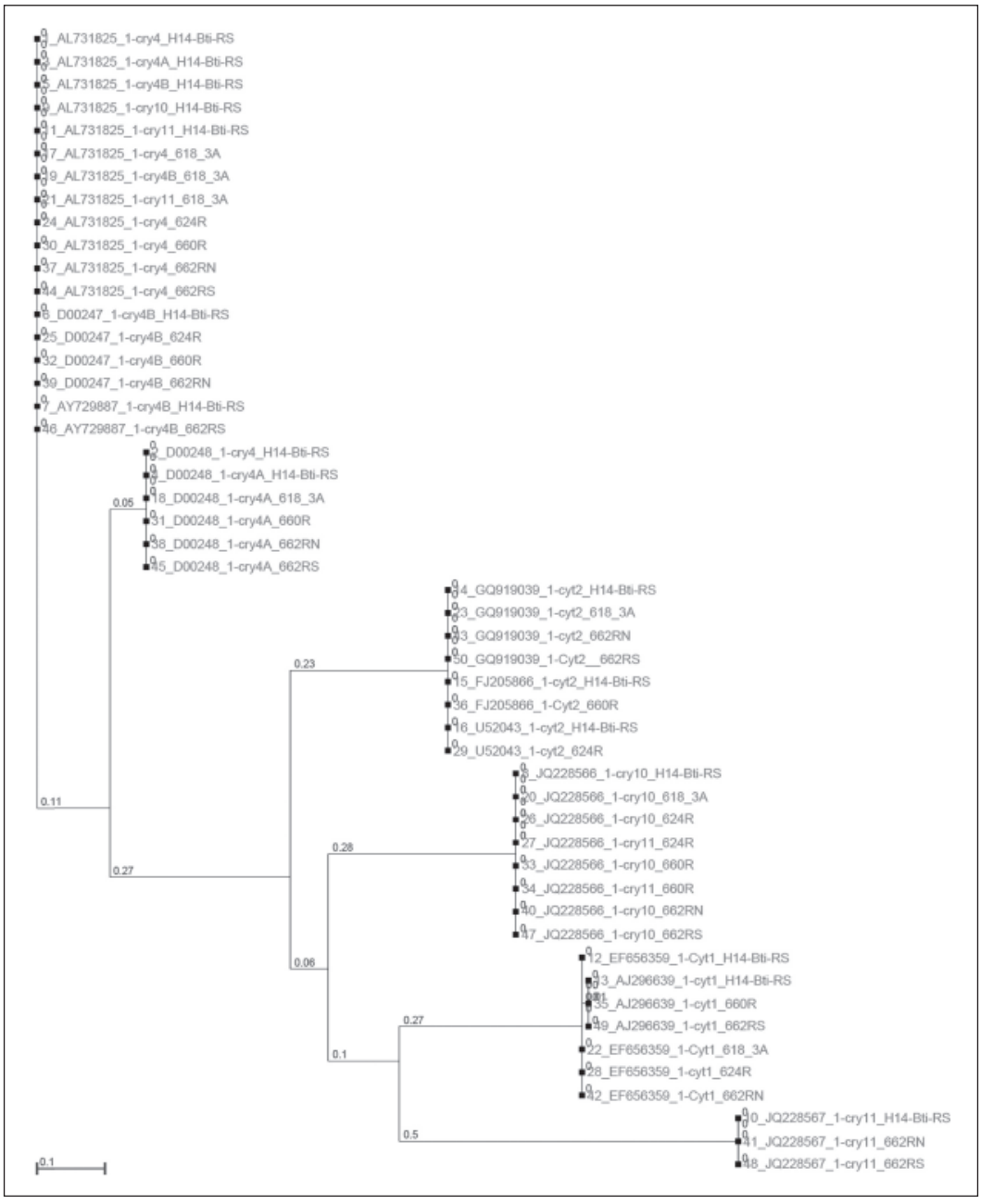

Figure 5. The phylogenetic tree represents the GeneBank accession number ID's of cry and cyt genes of reference Bti H14 as compared to 5 native Bt strains. The phylogram was constructed using NJ/UPGMA Phylogeny version 7.110. The scale on the tree shows genetic change value 0.1, the branches represents bootstrap and branch length values; the horizontal distances are proportional to genetic distances among the different isolated strains

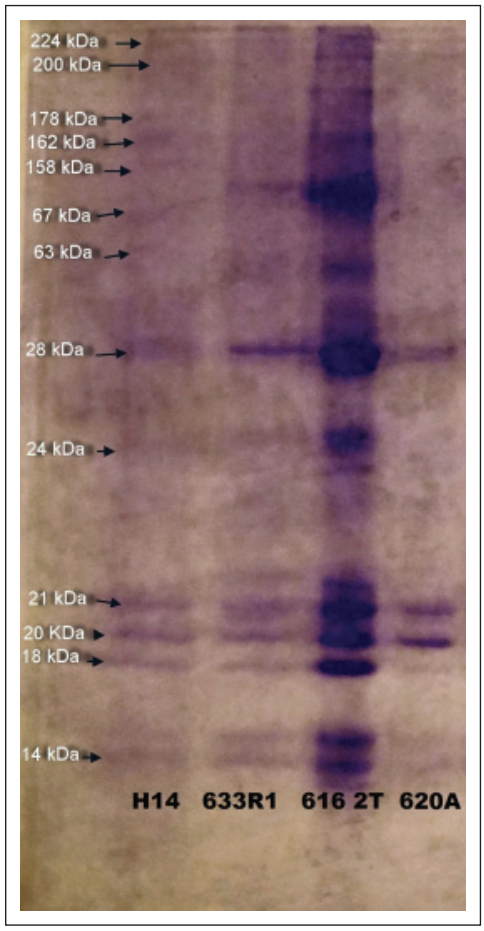

Figure 6. SDS –PAGE analysis of trypsin treated parasporal crystal protein profiles of reference Bt H14 and 3 native Bt isolates

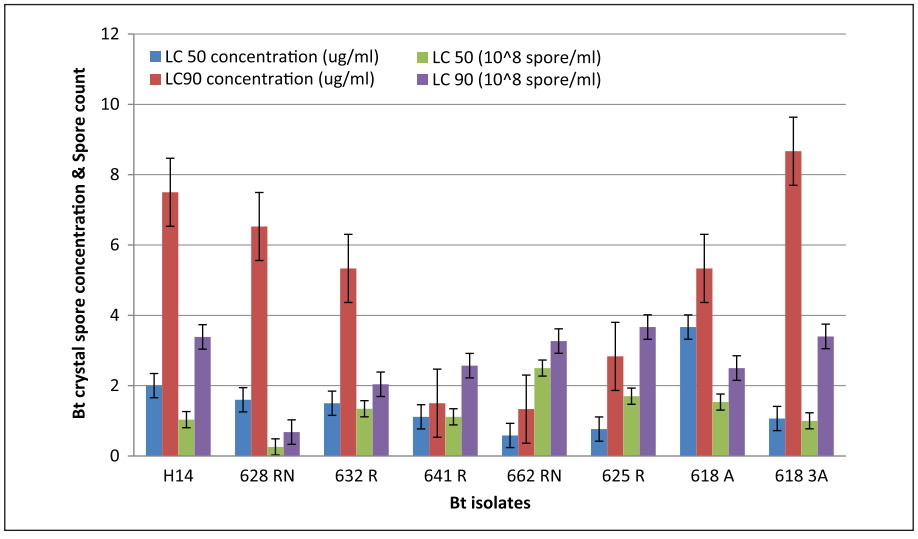

Figure 7. LC50 and LC90 values (concentration, ug/ml and *X 10^8 spore/ml) of some native Bt strains against *Aedes caspuis* larvae. Bti H14 (control)

Table 5. Toxicity bioassay of 28 native *Bt* isolates against 3rd to 4th instar larvae of *Adeas caspuis* and *Culex pipiens* as compared to H14 reference control

Strain	Mortality+ (*Aedes caspuis*)				Mortality+ (*Culex pipiens*)			
	LC_{50} (24h)		LC_{50} (48h)		LC_{50} (24h)		LC_{50} (48h)	
	Conce. ($\mu g.mL^{-1}$)	Spore /ml ($*10^8$)	Conce. ($\mu g.mL^{-1}$)	Spore /ml ($*10^8$)	Conce. ($\mu g.mL^{-1}$)	Spore /ml ($*10^8$)	Conce. ($\mu g.mL^{-1}$)	Spore /ml ($*10^8$)
H14	2 (±1)	1.03 (±0.15)	0. 6 (±0.01)	1.34 (±0.05)	0.06 (±0.01)	0.3 (±0.05)	0.02 (±0.01)	0.1 (±0.09)
616A	4.8 (±1)[a]	5.82 (±7.5)[a]	2.48 (±1.2)[a]	4.5 (±1.2)[a]	0.16 (±0.02)[a]	5.4 (±2.4)	0.05 (±0.01)	1.91 (±0.59)
616B	1.6 (±2)	7.6 (±4.08)	0.93 (± 0.5)	6.85 (±1)	0.55 (±0.04)[a]	6.5 (±0.5)[a]	0.25 (±0.02)[a]	4.1 (±0.85)
6162T	8 (±0.3)[a]	8.25 (±2.65)[a]	2.23 (±1)[a]	3.77 (±2)	1.22 (±0.01)[a]	6.3 (±0.85)[a]	0.73 (±0.03)[a]	3.5 (±0.4)[a]
618A	3.33 (±0.2)[a]	1.31 (±0.35)	1.49 (±0.1)	3.59 (±0.2)[a]	0.87 (±0.01)[a]	4.9 (±1.6)	0.25 (±0.05)[a]	1.6 (±0.19)[a]
618B	4.5 (±1.05)	9.1 (±1.02)	0.8 (±0.02)	0.77 (±0.25)	0.8 (±0.02)[a]	0.77 (±0.25)	0.14 (±0.03)	0.64 (±0.14)
6183A	1.06 (±1)	1.04 (±1.21)	0.93 (±0.1)	0.79 (±0.45)	0.73 (±0.03)[a]	5.97 (±1.95)	0.41 (±0.05)[a]	0.5 (±0.05)
6184A	1.06 (±2)	4.28 (±4.79)	0.93 (±0.2)	3.94 (±1.05)	0.73 (±0.1)[a]	4.06 (±0.9)	0.16 (±0.01)[a]	0.11 (±0.02)
620A	6.3 (±0.3)	16.83 (±021)[a]	0.09 (±0.02)[a]	0.27 (±0.12)	0.09 (±0.02)	0.27 (±0.12)	0.03 (±0.01)	0.073 (±0.04)
620B	5.6 (±2)[a]	25.5(±11.53)[a]	3.72 (±1)[a]	24 (± 1.07)[a]	0.35 (±0.02)[a]	2.3 (±0.2)	0.25 (±0.04)[a]	1.63 (±0.34)
621A	8.67 (±1)[a]	1.3 (±0.25)[a]	1.49 (±0.3)	0.59 (±0.4)	ND	ND	2.2 (±01)[a]	1.23 (±0.25)
621B	2 (±1)	10.14 (±4.61)	1.3 (0.76)	3.51 (±5)	0.14 (±0.02)[a]	1.39 (±0.56)	0.05 (±0.01)	0.59 (±0.03)
624R	0.93 (±0.01)	4.18 (±0.19)	0.12 (±0.2)[a]	0.095 (±0.005)	0.12 (±0.02)	0.1 (±0.005)	0.03 (±0.01)	0.03 (±0.01)
625R	0.8 (±1)	1.7 (±1.51)[a]	0.31 (±0.2)[a]	1.04 (±0.05)	0.14 (±0.01)[a]	0.62 (±0.29)	0.06 (±0.01)	0.17 (±0.14)
626R	4 (±1)[a]	0.4 (±0.19)[a]	1.24 (±0.1)	0.23 (±0.06)	0.35 (±0.03)[a]	0.96 (±0.05)[a]	0.13 (±0.01)[a]	0.39 (±0.27)
628RS	83.3 (±0.2)	40.6 (±0.9)[a]	1.56 (±0.045)[a]	5.37 (±2.37)	1.56 (±0.05)[a]	5.37 (±2.37)	1.47 (±0.45)	4.16 (±1.75)
628RN	1.6 (±1)	0.26 (±0.12)	0.31 (±0.03)	0.14 (±0.01)	0.12 (±0.02)	0.051 (±0.003)	0.059 (±0.005)	0.034 (±0.005)
632R	1.5 (±1)	1.3 (±1.16)	0.11 (±0.1)	0.59 (±0.2)	1.15 (±0.14)[a]	1.1 (±0.2)	0.52 (±0.06)[a]	0.4 (±0.05)
633R1	19.13 (±0.76)[a]	2.77 (±0.15)[a]	0.064(±0.004)[a]	0.14 (±0.032)	0.064 (±0.004)	0.14 (±0.03)	0.02 (±0.01)	0.03 (±0.02)
633R2	1.21 (±2)	8 (±3.7)	0.11 (±0.03)	1.5 (±0.04)	0.18 (±0.02)[a]	1.89 (±0.09)[a]	0.04 (±0.01)	0.37 (±0.24)
635R	2.24 (±0.02)	1.65 (±0.05)[a]	0.1 (±0.02)	0.106 (±0.012)	0.1 (±0.02)	0.1 (±0.01)	0.03 (±0.02)	0.036 (±0.006)
636R	8.34 (±0.01)	9.78 (±0.23)[a]	0.18 (±0.03)	1.08 (±0.49)	0.18 (±0.03)	1.1 (±0.49)	0.04 (±0.02)	0.13 (±0.02)
641R	1.11 (±0.76)	1.13 (±0.1)	0.19 (±0.05)	1.00 (±0.1)	2.6 (±0.1)[a]	3.3 (±0.3)[a]	0.52 (±0.04)[a]	0.82 (±0.28)[a]
648R	1.5 (±0.5)	4.5 (±1.05)	0.29 (±0.01)[a]	2.27 (±0.6)[a]	0.41 (±0.02)[a]	0.41 (±0.02)	0.25 (±0.050	0.32 (±0.01)
649R	ND	ND	1.04 (±0.04)[a]	0.44 (±0.59)	1.04 (±0.04)[a]	0.44 (±0.59)	0.41 (±0.01)[a]	0.073 (±0.006)
660R	3.33 (±0.2)[a]	1 (±0.12)	0.19 (±0.1)	0.79 (±0.2)	ND	ND	ND	ND
662RS	2.02 (±0.25)	2.11 (±0.45)	0.74 (0.2)	1.59 (0.2)	ND	ND	ND	ND
662RN	0.6 (±0.05)[a]	2.5 (±2.35)[a]	0.19 (±0.1)	1 (±0.35)	ND	ND	ND	ND
663R	6 (±0.2)[a]	1.6 (±3)[a]	1.12 (±0.1)	1.19 (±0.4)	ND	ND	ND	ND
Btk	160 (±5)[a]	570 (±1)[a]	3.12 (±0.02)[a]	14 (±1)[a]	3.12 (±0.02)[a]	14 (±1)[a]	0.87 (±0.01)[a]	3.66 (±0.6)

+: Mortality is expressed as average ± standard deviation; a, indicates significant difference at $P < 0.05$; ND means not determined

respectively. Also in a worldwide study, Martin *et al.* (2010) studied 3639 Bt isolates from 34 countries and reported that the majority (44%) showed bi-pyramidal crystals while 27 and 12% were irregular and attached to the spores respectively. In agreement with Aramideh *et al.* (2010), we attribute the diversity in the dominancy of crystal shapes among habitats in Saudi Arabia to the difference in sample location, habitat, and genetic variation. It is conceivable that such enormously varied crystal shapes among Bt isolates, is apparently correlated with different ecological systems in various countries. It is observed in this study that native Bt isolates with bi-pyramidal crystal shape (*cry2* gene active against Lepidopteran pests), are less frequently encountered among our isolates, probably use of agriculture fields are only recently introduced in this country while the majority of the country land are desert. Thus according to Hernandez-Fernandez *et al.* (2011), morphology and genetic composition of Bt is highly variable among different types of soils and places as it is influenced by several factors including soil humidity, organic matter, temperature, structure and pH, macro/micro-nutrients, richness and local insect distribution.

Furthermore, our 103 native Bt strains were characterized for the presence or absence of the general primers *cry*2, *cry*4, *cry*11, *cyt*1 & *cyt*2 and specific primers *cry4A*, *cry4B* & *cry*10 genes by PCR. In contrast to the tested BtkH1 positive strain, none of the examined 103 native Bt strains (0.0 %) revealed positive PCR results for *cry2* gene; suggesting that our native strains have no bio-insecticidal activity against

Lepidoptera pests. Likewise, the tested *cry4*, *cry4A*, *cry4B*, *cry10*, *cry11* and the cytolytic *cyt1* and *cyt2* genes were not detected in 72% of 103 Bt strains. These findings did not necessarily imply that these strains were devoid of genes coding for insecticidal properties, as all of them did produce crystals (Bravo *et al.*, 1998). These strains might contain other *cry*, *cyt* or non-insecticidal parasporal inclusions as suggested by Uemori *et al.* (2007). For comparison, Thammasittirong & Attathom (2008) reported that strains containing *cry2* genes were the most abundant and represent more than 70% of the isolates. Liang *et al.* (2011) found 40.7% of 791 tested strains were PCR positive for *cry*2 genes. These findings seem to indicate that Bt bio-insecticidal activity varied from one country to another and even within the same country from ecological region to another. Thus according to Mahalakshmi *et al.* (2012), *cry*2, *cry*4 and *cry*11 genes were found with high frequencies in the soils, than other sources, but *cry*10 gene, was found only in the insects samples, and absent in others and cytolytic *cyt*1 and *cyt*2 genes, were present only in soil and insect samples. Current results showed that the frequency of cytolytic *cyt1* and *cyt2* genes among the 103 native Bt strains were 28% and 26% respectively, whereas Mahalakshmi *et al.* (2012) found that cytolytic genes were the least identified *cyt*1 (9%) and *cyt*2 (7%) out of 417 Bt tested isolates. Jouzani *et al.* (2008) reported that out of 18 Bt isolates examined the occurrence of *cyt1* and *cyt2* were 10% and 28.2% respectively. It is noteworthy that the mosquitocidal activity of Bt strain is not only the additive effect of each toxin but a complex synergistic interaction among them. Thus, the reference *B. thuringiensis* subsp. *israelensis* (H14) produces two *cyt* toxins (Cyt1Aa and Cyt2Ba) and four *cry* toxins (Cry4Aa, Cry4Ba, Cry10Aa, and Cry11Aa) and synergism between these toxins has been reported (Ibarra *et al.*, 2003). Results of the present study confirmed the occurrence of all these toxins genes in the reference *B. thuringiensis* subsp. *israelensis* (H14); thereby validate the adopted PCR procedure in this study. In addition, as for *cry4* gene 28% of 103 tested native Bt strains posed such genes and all of these strains contained also *cry4A* and *cry4B* with the exception of one Bt strain (649R). In contrast, Jouzani *et al.* (2008) reported that *cry4* occurred in 14.3% of 128 Bt strains and *cry4A*, *cry4B*, and *cry4C* or *cry4D* occurred only in 0.0%, 60% or 40% of these strains (14.3%) respectively. Mahalakshmi *et al.* (2012) detected *cry4* genes in 50% of 417 Bt strains and *cry4A* and *cry4B* occurred only in 53% and 47% of these strains (50%) respectively. Likewise, in this research *cry10* and *cry11* were also detected concomitantly with *cry4*, *cry4A*, *cry4B*, *cyt1* and *cyt2* in 22% and 25% of 103 Bt strains respectively. Mahalakshmi *et al.* (2012) and Jouzani *et al.* (2008) found 15% (417 Bt strains) and 33% (128 Bt strains) of *cry10* respectively and also 10% and 26% of their strains for *cry11* respectively. Obviously, the PCR detection of such combination of *cry* and *cyt* genes would predict that these isolates will show high toxicity against mosquitoes. However PCR based detection of *cry* genes should not replace bioassay against targeted insects. In this study, nucleotide sequence homology for the cry genes were compared with *B. thuringiensis* using BLAST search tool (www.ncbi.gov). Data showed high degree of identity compared with *Bacillus thuringiensis* in GenBank. According to Nemappa *et al.* (2012), variation of even a single nucleotide residue at certain positions of Cry proteins can remarkably influence the level of toxicity. During the course of this work, heavy concentration of spore-crystal mixtures of the 103 native Bt strains were tested for their larvicidal activity against 2 genera of dipterans larvae (3^{rd} to 4^{th} instar), namely *Ae. caspuis* and *Cx. pipiens*. In agreement with Ohba *et al.* (2009), our results revealed that as much as 72 % of the 103 native Bt strains exhibited non-insecticidal activity while only 28% of isolates showed 50% mortality or more, and therefore were considered as active strains as reported by Mahalakshmi *et al.* (2012). Interestingly, all of these isolates (28 strains) showed earlier positive PCR amplicons for all and/or some of the tested general and specific primers. Accordingly, these 28 Bt strains were furthered processed for

quantitative mortality determination of LC_{50} concentration and spore CFU/ml against the two tested dipterans larvae after 24 h and 48 h. The mortality-LC_{50} concentrations result showed significant toxicity – variation among the tested Bt strains. The variation in toxicity apparently was not related to *cry* gene content(s) in all cases, as some strains sharing the same *cry* gene but significantly differed in their insecticidal potency. For instance, strains coded 628 RN and 616 2T displayed all *cry* and *cyt* genes (*cry4*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1* and *cyt2)* but the 24 h LC_{50} against *A. caspuis* was 5 folds lower for the former (LC_{50}: 1.6 ug/mL) as compared to that of latter (LC_{50}: 8 ug/mL) strain. This difference in larvicidal activity was even more pronounced for their LC_{50} against *Cx. pipiens* where mortality of the former (24 h LC_{50}:0.12 ug/mL) was 10 folds lower than that of the latter (24 h LC_{50}: 1.22 ug/mL). In agreement with Martinez & Caballero (2002) and Mahalakshmi *et al.* (2012), this discrepancy may be attributed to variation in the level of gene expression in respective strains. Lakxmy *et al.* (2011), stated that periodical introduction of such native strains of Bt will add a new weapon in the armory to manage the vector borne diseases and also in the management of insect resistance in targeted pests. Thus, González *et al.* (2011) and Mahalakshmi *et al.* (2012) reported that their respective native Bt strains exhibited eleven-fold or three-fold higher mosquitocidal activity than the reference strain *B. thuringiensis israelensis*. Likewise, our results revealed that native strains (641R, 632R & 628 RN) all showed two-fold higher mosquito-cidal activity against *Ae. caspuis* as compared to the reference strain Bt H14 after 24 hours. Such increase in mosquitocidal activity was even pronounced in the range of two-fold to seven-fold after 48 hours with the 11 native Bt strains. However, such increase in mosquitocidal activity of these strains was not that pronounced against *Cx. pipiens*. The finding that LC_{50} mortality for the tested Bt strains varied with exposure time of 24h or 48h has also been reported by Gama *et al.* (2013). These authors found that the 72 h *Ae. aegypti* exposure time was more effective than respective 24 h and 48 h exposure times and explained that the increased time allows the toxin to accumulate, and the toxin's (delta-endotoxin) effects increase in accordance with exposure. It is noteworthy that the current study emphasized the importance of the presence of *cry4A* and *cry4B* (rather than mere *cry4*) for a Bt strain to exhibit larvicidal activity against *Ae. caspuis*. Thus based on PCR data Bt strain 649R does not contain the specific *cry4A* and *cry4B* genes but it does contain the general *cry4*, yet it showed larvicidal activity against *Ae. caspuis* only after 48h of exposure, thereby implying the necessity of *cry4A* and *cry4B* for larvicidal activity against *Ae. caspuis*. However, the same Bt strain showed that the presence of *cyt* genes would elevate such necessity for larvicidal activity against *Cx. pipiens*. Thus Promdonkoy *et al.* (2005) reported that *Cry4Ba* showed higher toxicity than *Cyt2Aa2* when tested against *Ae. aegypti* larvae, but was virtually nontoxic to *Cx. quinquefasciatus*. The necessity of *cyt1A* gene for high mosquito larvicidal activity has also been demonstrated between Bti and *B. sphaericus* against *Ae. aegypti* as well as resistant strains of *Anopheles stephensi* (Chenniappan & Ayyadurai, 2012).

On the other hand, Likitvivatanavong *et al.* (2011) reported that *cry11Ba* and *cry4Ba* apparently have common sites involved in binding *Ae. aegypti*. This may explain the fact that our native strain 636R did not harbour the *cry11* yet its (LC_{50}: 0.18 ug/mL) after 48 h was three-fold higher toxicity as compared that (LC_{50} 48 h: 0.6 ug/mL) of H14 reference strain. In contrast, the absence of *cyt2* and *cry10* in 620B native strain resulted almost six-fold decrease in LC_{50} as compared to that of the reference H14; but missing only *cry10* (618A, 618 4A & 621A) resulted in minor decrease in LC_{50} mortality.

In agreement with our result, Hernández-Soto *et al.* (2009) reported that *cry10A* exhibited very low toxicity against *Ae. aegypti* as compared to that of its combination with *cyt1A*. Nevertheless, the complex toxin arsenal of *B. thuringiensis* subsp. *israelensis* makes it difficult to assess

the contribution of each component by testing them separately (Hernández-Soto *et al.*, 2009).

Since the larvicidal activity of *cry* proteins is often correlated with the high affinity binding of the proteins to specific membrane-bound receptors in the larval midgut (Ketseoglou & Bouwe, 2012), the susceptibility differences among *Ae. caspuis* and *Cx. pipiens* could be due to inherent differences between the species in the structure or density of midgut receptors for *cry* and cyt proteins. The observed variation of bio-toxicity potency against tested dipteran larvae, despite the presence of dipteran Cry genes in certain native Bt strains is probably attributed to their level of phenotypic expression as evidence from variation in SDS-PAGE trypsin-treated protein profile patterns.

In summary, it is hoped that our discovered wild Bt strains against dipterans could have an impact on mosquito- vector borne diseases control programs in Saudi Arabia.

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