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Molecular Characterization and Enterotoxin Genes Typing of Local Strains of *Bacillus Cereus*

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Abstract: Two virulent strains of *Bacillus cereus* coded as BC13 and BC37 isolated from corn snacks collected from Egyptian market were identified using biochemical and staining methods. Further confirmation was done by determining their cellular protein pattern compared to a standard culture of *B. cereus* NRRL 569. The cellular protein profile revealed 97.55 to 99% similarity between NRRL 569 reference strain and local strains (BC13, BC37**)**. Random amplification polymerase DNA analysis of the two tested isolates compared to standard culture using eleven arbitrary primers showed similarities ranging from 75.5% to 77.09% between the tested isolates. Separation of extracellular proteins of both tested isolates using SDS-PAGE revealed the presence of protein bands with molecular weights between 34 and 54 kDa in both tested isolates, suspected as enterotoxins. To ensure the presence of suspected enterotoxins, two pairs of primers newly designed and reported during year 2008 (FHblC and RHblC) and (FCytK and R2Cytk) were used to detect the toxin genes in both tested isolates using multiplex PCR technique. The two primers were designed as (CCTATCAATACTCTCGCAA & TTTCCTTTGTTATACGCTGC) and (CGACGTCACAAGTTGTAACA& CGTGTGTAAATACCCCAGTT). The multiplex PCR amplification allowed rapid detection and identification of the toxin genes (*hbl**C*** and *cyt*K) in both isolates.

Key words: *Bacillus cereus*, molecular biology, protein pattern, enterotoxin genes, multiplex PCR.

INTRODUCTION

Bacillus cereus is often present in a variety of food such as milk, dairy products, spices, cereals, meat, cakes, deserts….etc. (Larsen and Jorgensen, 1997; Duc *et al.***,** 2005 and Svensson *et al*.,2007). *B. cereus* has been extensively reported to be involved in outbreaks of gastrointestinal diseases (emetic and diarrheal).The emetic toxin is a heat stable small ring forming peptide (Lund *et al*., 2000**)** and enterotoxin FM **(**Asano *et al*., 1997). Meanwhile the diarrheal disease is the most common form and is caused by at least four heat labile enterotoxins; hemolysin HBL **(**Beecher and Wong, 1997), nonhemolytic NHE (Lindback *et al.*, 2004), cytotoxin CytK (Lund *et al.*, 2000). Cytotoxin CytK was reported as the primary virulence factors in *B.cereus* diarrhea toxic syndrome (Lund *et al.*, 2000; Guinebretiere *et al*., 2002 and Brillard and Lereclus, 2007). HBL and NHE toxins are both three protein components complexes. HBL contains two lytic components [L1 and L2] and a binding component B encoded HBLC, HBLD& HBLA. NHE also contains two lytic elements NHEA & NHEB and a third protein encoded NHEC with unknown function (Granum *et al*., 1999).

Detection of toxin genes of *B. cereus* has been proposed by several authors in terms of polymerase chain reaction PCR primers of toxin subunits since the toxins were cloned and sequenced (Rayan *et al.,* 1997; Lund *et al*., 2000; Duc *et al*., 2005; Svensson *et al*., 2007 and Ngamwongsatit *et al*., 2008).

In this study, it was attempted to detect the hemolytic enterotoxin genes in two identified and molecularly confirmed local isolates of *B. cereus* (BC37& BC13) by rapid multiplex PCR technique using specific novel primers for the subunits HBLC and CytK in a single reaction.

MATERIALS AND METHODS

Selection of bacterial strains:

The two tested bacterial strains were chosen from a collection of bacterial cultures which has been isolated from corn snack packets collected from the market in Zagazig city, Egypt. Both strains were highly potent concerning their virulence factors namely, phospholipase, hemolysins and protease (data not shown). The isolates were plated on *B. cereus* selective agar supplemented by 8%egg yolk suspension and polymxin B (100

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IU; Oxoid). The growing pink colonies were stained for microscopic description and then subjected to biochemical tests according to Krieg and Holt (1984) and Holt *et al*. (1994) for characterization and identification.

B.cereus strain type NRRL 569 was used as reference control in identification brought from (MIRCIN) at Faculty of Agriculture, Ain Shams University.

Random amplified polymorphic DNA Polymerase Chain Reaction (RAPD-PCR):

Chromosomal DNA of the two local bacterial strains coded as[BC13 and BC37] and *B. cereus* reference strain type NRRl569 were prepared by boiling and freezing the cells as described by Nilsson *et al.*(1998). Eleven arbitrary oligonucleotides primers (Table1) were used each in a separate reaction for the RAPD amplification.1Kbp Plus DNA ladder (Invitrogen©) was used as marker in agarose gel electrophoresis.

Table1: Arbitrary primers used for RAPD - PCR

Primer	Oligonucleotide sequence (5'-3')	Primer	Oligonucleotide sequence (5'-3')
A6	GGTCCCTGAC	B6	TGCTCTGCCC
A11	CAATCGCCGT	B15	GGAGGGTGTT
A13	CAGCACCCAC	B16	TTTGCCCGGA
A14	TCTGTGCTGG	B17	AGGGAACGAG
A17	GACCGCTTGT	C20	ACTTCGCCAC
B4	GGACTGGAGT		

Protein profile of bacterial strains:

Determination of cellular and extracellular protein patterns using SDS-PAGE technique according to Laemmli (1970) and LKB Application note (1977).

The two bacterial strains encoded BC13 and BC37 and B . *cereus* NRRL 569 were grown in 50ml Tryptone Soya Broth (TSB, Oxoid) at 30°C for 24 hours. Bacterial cells were harvested by centrifugation and the filtrate was separated for further extraction of extracellular proteins. Bacterial pellets were washed twice using sterile bi-distilled water. The bacterial pellets were sonicated, re-suspended in sterile distilled water and centrifuged. The precipitated cellular proteins were then separated and re-suspended in phosphate buffer pH 7.

The supernatants separated by centrifugation were concentrated100 times using 70%saturated $(NH_4)_2SO_4$. The precipitated extracellular proteins were re-suspended in phosphate buffer pH 7.

100µl of each cellular and extracellular protein preparation was mixed with 50µl of treatment buffer separately and boiled in a water bath for 5 minutes then injected into the wells of the prepared polyacrylamide gel.

The molecular weights of separated proteins were determined by electrophoresis compared with marker proteins having molecular weights ranging between 14 to 116 kDa after staining with commasie blue.

DNA extraction for multiplex- PCR:

DNA templates of the tested bacterial cultures (BC13, BC37 and NRRL569) were prepared from 4-hours cultures grown in TSB at 30°C separately according to the method described by Ngamwongsatit *et al.*(2008).

Multiplex PCR amplification for detection of enterotoxins genes hblC and cytK:

The multiplex PCR amplification was performed in a final volume of 20ul containing 5µl of DNA templates with final concentration 1X PCR buffer (10mM Tris-HCl pH 8.3 and 50 mM KCl), 1.5mM MgCl2, 200µM of each dNTP, 5U *Taq* DNA polymerase and 0.4µM *hlb*C primer & 0.2µM *cyt*K primer. Primers were imported from Metabion International AG. Lena-Christ-Strasse 44/I Deutschland. Reactions were carried out with the following cycling conditions: initial denaturing at 95°C for 5 min, followed by30 cycles of 94°C for 45 sec, annealing at 54&56°C for 1 min in case of *hbl*C and at 58°C in case of *cyt*K, elongation at 72°C for 2 min and final extension at72°C for 5 min (Ngamwongsatit,2008). Amplicons were separated on 1.5% agarose gel and sizes were estimated using 100bp DNA ladder (Amersham, USA) run on the same gel.

RESULTS AND DISCUSSION

B. cereus is considered a frequent cause of food poisoning due to its ability to produce several enterotoxins and virulence factors in food and probably in intestines (Granum *et al.,* 1996). Strains of *B. cereus* can be differentiated using different methods such as biotyping using API, serotyping or phage typing. Molecular typing and PCR techniques have proved quick and precise results in detecting *B. cereus* toxin genes in epidemiological investigations of clinical and/or food specimens.

Table 2 : Primers used for multiplex PCR detection of enterotoxin genes(Ngamwongsatit,2008):

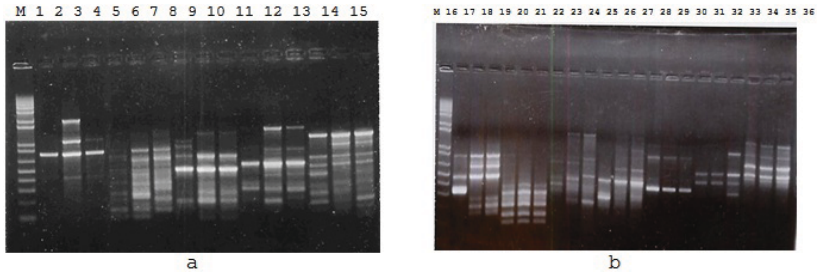
Target gene	Primer[a]	Primer sequence (5'—3')	Size in bp	T_m °C	Product size in bp	Primer conc(uM)
*hbl*C	FHblC	CCTATCAATACTCTCGCAA	19	54	695	0.4
	RHblC	TTTCCTTTGTTATACGCTGC	20	56		
*cyt*K	FCytK	CGACGTCACAAGTTGTAACA	20	58	565	0.2
	R2CytK	CGTGTGTAAATACCCCAGTT	20	58		

[a] F= forward primer R= reverse primer

In this study, two of most potent bacterial strains encoded BC13 and BC37 previously isolated from corn snack packets collected from Egyptian market were preliminarily identified as *B. cereus* using biochemical tests according to Bergy's manual. RAPD-PCR method was used to compare both local strains to *B. cereus* NRRl569 reference strain**.** Eleven arbitrary short primers (10bp) (table1) were used in the RAPD technique to amplify different DNA segments to obtain finger prints of the tested isolates. RAPD-PCR has been extensively reported to be a quick and reliable method for direct detection and typing of toxic strains of *B.cereus* contaminating different food products (Nilsson *et al.*,1998; Priest *et al.*, 2004; Ehling-Schulz *et al.*, 2005;Thorsen *et al.*, 2006 and Vassileva *et al.*, 2007).

Table3: Similarity matrix of DNA segments amplified by RAPD-PCR

	B.cereusNRRl 569	BC13	BC37
B.cereusNRRL569	100	75.5	77.09
BC13	75.5	100	76
BC37	77.09	76	100



Photo(1a&b): Agarose gel stained with ethidium bromide in which 0.7µg/lane1Kbp DNA plus ladder in lanes M; lanes1,2,3 products of primer A11; lanes4,5,6 products of primer A6; lanes7,8,9 products of primersB6; lanes10,11,12 products of primerB4; lanes13,14,15 products of primerB15; lanes116,17,18 products of primerC20; lanes 22,23,24 products of primerA14; lanes 25,26,27 products of primerA13; lanes 28,29,30 products of primerB17; lanes 31,32,33 products of primerB16 and lanes 34,35,36 products of primerA17.

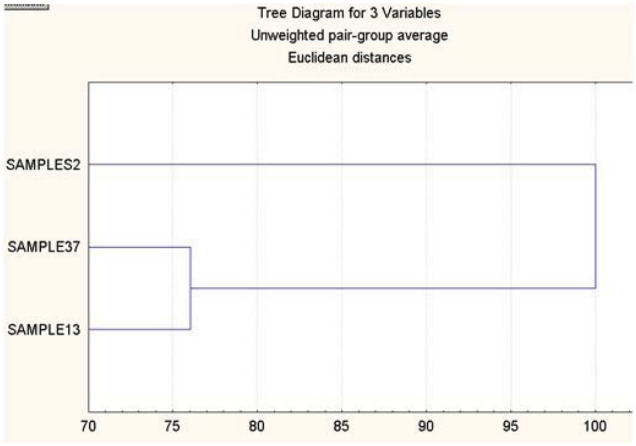


Fig1: Dendrogram constructed with UPGMA cluster analysis of RAPD-PCR data showing relationship among the two tested strains (BC13 & BC37) and reference strain (S2)NRRL569 to determine the phylogenetic relation between them.

The profile pattern of PCR products of the two local strains and reference strain (Photos 1a&b) revealed meaningful results in almost all used primers that gave rise to strong and reproducible band patterns especially primers(A6, B6, B4, B15, B16&A17). The occurring genes sequences suggested the close relation between the bacterial strains BC13 and BC37 showing 76% similarity; 75.5% between BC13 and reference strain NRRL569 and 77.09% between BC37 and HRRL569 strain (table3). Ehling-Schulz *et al.* (2005) and Thorsen *et al.* (2006) observed similarity reaching 92% between *B. cereus* strains. The results still revealed slight genetic heterogeneity between tested strains which may be related to variation of toxins genes within different strains of *B.cereus.*Schoeni and Wong (1999) reported that the multiple toxins genes are responsible for HBL heterogeneity among *B. cereus* strains.

Since genes are expressed in the form of proteins, thus determining the cellular and extracellular protein profile of tested isolates by means of SDS-PAGE technique is considered a suitable tool for studying gene expression, typing and differentiating bacterial strains (Smith, 1997 and Wong and Hancock, 2000).

Total cellular proteins of BC13 and BC37 and reference strain NRRL569 were extracted and then fractionated by means of polyacrylamide gel electrophoresis. The produced protein bands (photo2) were analyzed by gel pro-analyzer and IBM compatible personal computer.

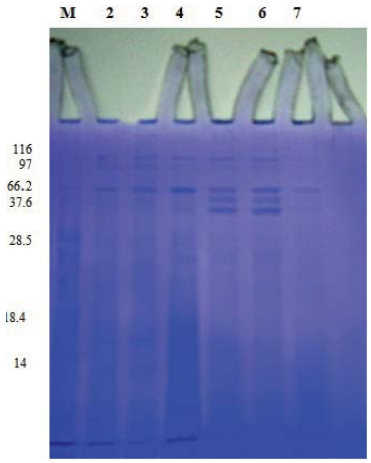


Photo 2: SDS-PAGE showing total cellular proteins of reference strain BC37,BC13 and NRRL569; lanes 2, 3, 4; respectively. Lanes 5, 6, 7 show protein bands (mol. wt. ranging between 34 to 54kDa) in cell free filtrates of BC37, BC13and NRRL569, respectively. M protein molecular weight marker having a mixture of 7 purified proteins with mol. wt.116,97.4,66.2,37.6,28.5,18.4 and 14 kDa.

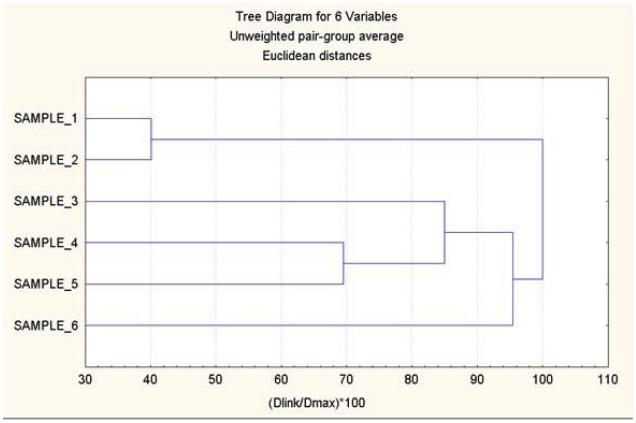


Fig. 2: Dendrogram of protein gel showing relation between tested isolates and the reference strain.

From the constructed dendogram in (fig 2) it is clear that the separated cellular protein bands of the three tested strains showed 99% similarity between BC37 and BC13 local strains. Meanwhile, similarity between reference strain NRRL569 and the two local isolates were 97.76 and 97.55% respectively. Also, the pro-analysis of the extracellular protein bands of the three tested isolates revealed 98.27% similarity between the two local strains and about 97% between both strains and reference strain.

The extracellular protein profile of tested isolates in SDS-PAGE showed obvious bands in lanes 5&6 (photo1) having molecular weights (33.7, 41.29 & 54.044 kDa) and (34, 42.33 & 54.64 kDa) in case of BC37 and BC13, respectively. Beecher and MacMillan (1990&1991) reported that molecular weights of *B. cereus* enterotoxin proteins were 35, 36 and 45 kDa for binding protein B, lytic protein L1 (HBLD) and lytic protein L2 (HBLC), respectively. Beecher and Wong (1994) also found that the molecular weight of B component was 37.8kDa, L1 was 38.5kDa and L2 was 43.2kDa. Schoeni and Wong (1999) reported that the three components isolated from prototype strain of *B. cereus* F837/76 have molecular weights of 37.5, 38.2 and 43.5 kDa, respectively. They also added that an individual strain could produce single or multiple bands of each component. They observed two bands (38&42kDa) for B protein, two L1proteins (38&41kDa) and two L2 proteins (both 43kDa) in a soil isolate encoded S1C strain.

Lund *et al*. (2000) reported for the first time that the cytotoxic gene *cyt*K of *B. cereus* (a clinical isolate) was the only cause of severe food poisoning out break that killed three people. He also reported that CytK toxin had necrotic and hemolytic action and was completely different from other *B. cereus* enterotoxins with molecular weight 34 kDa. Guinebretiere *et al*. (2002) found that *cyt*K gene was frequently detected among 73% of the diarrheal *B. cereus* strains and prevalent in only 37% of food borne strains. Shadrin *et al*. (2007) reported that frequency of occurrence of *cyt*k gene among 40 strains of *B. cereus* group was 56%. Fagerlund *et al*. (2007) reported the presence of *cyt*k gene in a highly virulent strain of *B. cereus* isolated from a case of fatal enteritis.

Ngmwongsatit *et al*. (2008) recently developed and evaluated group of new primers which were highly efficient in detecting the toxin genes in 100% of their tested *B. cereus* and *B. thuringensis* strains. Thus, it could be expected that the presence of either genes is an indication for the presence of the whole operon. They also reported that 100% of the tested toxic strains of *B. cereus* harboured three genes of hemolytic toxin *hbl*CDA. Under multiplex PCR conditions, previously mentioned in materials and methods, the enterotoxin genes (*hbl*C &*cyt*K) were detected in the amplified DNA fragments of the two local strains (BC37 &BC13) in one quick step. The toxin genes *hbl*C & *cyt*K predicted molecular sizes of 695 & 565 bp, respectively (photo 3). It is also surprising that the reference strain NRRL569 is devoid of these toxins genes.

Multiplex PCR technique has been recently used for rapid detection and discrimination of enterotoxins genes in *B. cereus* (Guinebretiere *et al.,* 2006 and Nagmwongsatit *et al*., 2008); and for direct detection of food contamination with enterotoxigenic *B. cereus* as well (Ombui *et al*. 2008).

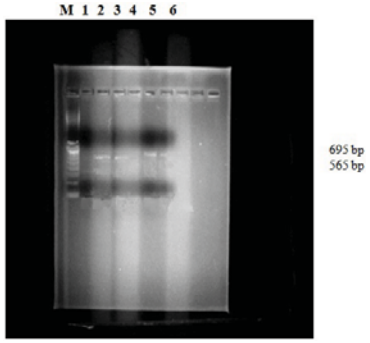


Photo 3: Agarose gel electrophoresis for separation and detection of toxins genes. Lane M is 100bp DNA ladder marker. DNA pattern of strain NRRL569, BC13 and BC37 in lanes 1,2 & 3, respectively treated with FCytk & R2Cytk primers show presence of *cyt*K gene in both local isolates (lanes2&3) only. DNA pattern of NRRL569, BC13 and BC37 treated with primers FHblC & RHblC in lanes 4,5 & 6 show presence of hblC *gene* in both local isolates (lanes 5&6) only.

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