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ORIGINAL ARTICLE

Prevalence of toxicogenic bacteria in some foods and detection of *Bacillus cereus* and *Staphylococcus aureus* enterotoxin genes using multiplex PCR

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Abstract Thirty-three food samples representing seven different food products were collected from the market in Sharkia Governorate (Egypt) and analyzed for their bacterial burden, including total mesophilic bacteria, spore formers, *Staphylococcus aureus*, and *Bacillus cereus*, using specific and selective nutrient media. The identified strains were screened for their virulence factors using the agar diffusion method. *B. cereus* strains CH, GT1, LB3, and G8 were found to be the most potent isolates, with four *S. aureus* showing nearly equal potency in terms of the virulence factors investigated. Separation of the extracellular proteins of the four most potent *B. cereus* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of protein bands with molecular weights ranging from 30 to 53 kDa that were suspected to be hemolytic enterotoxins. Protein bands having molecular weights between 22 and 33 kDa were also observed in three strains (S1, S2, and S3) of the *S. aureus* strains examined. Applying the multiplex PCR technique, we used two pairs of primers (FHblC and RHblC; FCytK and R2Cytk) to detect the toxin genes (*hbl*C and *cyt*K) in the suspected toxic *B. cereus* strains and five pairs of primers (SEA-3 and SEA-4; SEB-1 and SEB-2; SEC-5 and SEC-6; SED-1 and SED-2; SEE-1and SEE-2) to detect the five enterotoxins in the *S. aureus* strains. Our results indicate that the multiplex PCR amplification enabled the rapid detection and identification of enterotoxin genes in food-borne bacteria.

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Introduction

Extracellular proteins of pathogenic bacteria are main the contributors to their pathogenesis and are indisputably involved in bacterial virulence. These proteins are involved in a variety of biological functions, ranging from host cell toxicity to more subtle alterations of the host cell for the benefit of the invader (Wooldridge 2009).

The virulence factors of pathogenic bacteria are divided into several groups on the basis of the mechanism of virulence and function. secretary proteins, such as toxins and enzymes, are considered to be important virulence factors (Wu et al. 2008). Members of the genus *Bacillus* and *Staphylococcus* are among the pathogenic bacteria, causing a wide variety of diseases through the production of toxins on several substrates, including "food", resulting in "food poisoning".

Bacillus cereus is a spore former and Gram-positive bacillus that is able to survive and proliferate in a wide range of environments, including soil, water, and many types of processed food, such as herbs, spices, milk, meat, raw and cooked vegetables, boiled or fried rice, vanilla sauce, custards, soups, ice cream, and cereals (Mckillip 2000; Granum 2001; Kotiranta et al. 2000; Lindback et al. 2004). Numerous studies performed worldwide have reported on the importance of *B. cereus* as a cause of food poisoning outbreaks, but as a less causative agent of diarrhea (Kotiranta et al. 2000).

Bacillus cereus causes diarrheal and emetic food poisoning and a variety of typically necrotic non-gastro-

Table 1 Oligonucleotides used for multiplex PCR amplification of *Staphylococcus aureus* enterotoxin genes

Target gene	Primer	Size (bp)	Primer sequence (5′→3′)	T (°C)	Location within genes	Product size (bp)
Sea	SEA-3	21	CCTTTGGAAACGGTTAAAACG	60	487–507	127
	SEA-4	22	TCTGAACCTTCCCATCAAAAAC	62	592–613	
Seb	SEB-1	20	TCGCATCAAACTGACAAACG	58	634–653	478
	SEB-2	20	GCAGGTACTCTATAAGTGCC	60	1088–1110	
Sec	SEC-5	21	GAACTAGACATAAAAGCTAGG	58	670–690	244
	SEC-6	20	CATTCTTTGTTGTAAGGTGG	56	913–894	
Sed	SED-1	20	CTAGTTTGGTAATATCTCCT	54	354–373	317
	SED-2	20	TAATGCTATATCTTATAGGG	52	652–671	
See	SEE-1	20	TAGATAAAGTTAAAAAACAAGC	54	491–510	170
	SEE-2	20	TAACTTACCGTGGACCCTTC	60	640–659	

intestinal infections (Beecher and MacMillan 1990; Drobniewski 1993; Beecher and Wong 2000; Granum and Baird-Parker 2000; Mckillip 2000; Callegan et al. 2002; Schoeni and Wong 2005). Among the many potential virulence factors of *B. cereus*, Hemolysin BL (HBL) is a unique and potent three-component pore-forming toxin consisting of three distinct proteins, namely the binding component (B), lytic component (L_1), and lytic component (L_2) (Beecher and MacMillan 1991; Beecher and Wong 1994).

Staphylococcus aureus is a Gram-positive facultative anaerobic, non-spore former, coagulase-positive coccus. Staphylococcal food poisoning is caused by the ingestion of food containing pre-formed toxins secreted by the bacteria. These are known as staphylococcal enterotoxins (SEs), and eight serologically distinct types (A, B, C1, C2, C3, D, E, and F) have been recognized to date. Enterotoxin F has been shown to be identical biochemically to toxic shock syndrome toxin 1 (TSST-1) that produces toxic shock syndrome commonly associated with the use of tampons during menstruation (Doyle 1989). SEs are responsible for the symptoms associated with staphylococcal food poisoning (Kenny et al. 1993; Matsunaga et al. 1993; Llewelyn and Cohen 2002). The disease is characterized by such symptoms as nausea, vomiting, abdominal cramps, and diarrhea lasting from 24 to 48 h, with complete recovery usually occurring within 1–3 days. The enterotoxin genes, however, are not uniformly distributed among all *S. aureus* strains. Dinges et al. (2000) and Boerema et al. (2006) reported on the potency of SEs.

SEA is the most common enterotoxin recovered from food poisoning outbreaks (Balaban and Rasooly 2000), and it is known that 59% of staphylococcal food poisoning outbreaks are caused by SEA to SEE (Bergdoll 1989). Argudin et al. (2010) reported that *S. aureus* produces a wide variety of toxins, including SEA to SEE, SEG to SEI, and SER to SET), as well as and staphylococcal-like (SEl) proteins, which are not emetic in a primate model (SElL and SElQ) or have yet to be tested (SElJ, SElK, SElM to SElP, SElU, SElU2, and SElV). SEs and SEls have been traditionally subdivided into classical (SEA to SEE) and new (SEG to SElU2) types.

Food-borne diseases are mainly caused by pathogenic bacteria which are either transmitted to humans from the animal reservoir or which contaminate the food processing chain. The detection and isolation of pathogenic bacteria from food are often difficult due to the high number of contaminating and indigenous bacteria and the low number of the pathogenic bacteria of concern. In order to obtain even a modest sensitivity, most traditional isolation methods include a selective enrichment and, occasionally, a pre-enrichment step, both of which are labor-intensive and time-consuming.

There is, consequently, scope for improvement in terms of detection and isolation methods, especially with respect to the time needed to produce a diagnosis. The last 20–30 years have seen many developments in techniques and also the dawning of technologies, which were predicted to change the approaches used to detect pathogenic bacteria in food. Several reviews have dealt with the use of DNA probes and the PCR technique in food microbiology (Hill and Keasler 1991; Wolcott 1991; Olsen et al. 1995; Hill 1996). Based on the both the speed and the scale that these techniques have been

Table 2 Primers used for multiplex PCR amplification of *Bacillus cereus* entrotoxin genes

Target gene	Primer	Size (bp)	Primer sequence (5→3′)	T (°C)	Product size (bp)	Concentration (μM)
*hbl*C	FHblC	19	CCTATCAATACTCTCGCAA	54	695	0.4
	RHblC	20	TTTCCTTTGTTATACGCTGC	56		
*cyt*K	FCytK	20	CGACGTCACAAGTTGTAACA	58	565	0.2
	R2CytK	20	CGTGTGTAAATACCCCAGTT	58		

implemented for research purposes, the reviews unanimously praise the potential of these techniques to overcome some of the inherent problems in detecting and isolating bacterial pathogens from food (Olsen 2000).

PCR-based techniques are used increasingly in food microbiology research as they are well developed and, when applied as culture confirmation tests, reliable, rapid, and sensitive. PCR methods offer a sensitive and specific detection of pathogens and can also discriminate virulent bacteria from avirulent members of the same speciesl (Olsen 2000). In the last 10 years, many authors have proposed the use of PCR methods for the detection of food-borne pathogens to replace the time-consuming culture-based classical techniques (Miethke et al. 1992; Gravet et al. 1999); they are rapid, easy to perform, sensitive, and specific and, therefore, constitute very valuable tools for routine applications.

Several pathogens can be detected simultaneously in one step by multiplex PCR. Various multiplex methods relevant in the field of food microbiology have been used to detect variants of food-borne pathogens with special focus on the enterotoxic strains of *S. aureus* and *B. cereus* (Becker et al. 1998; Pinto et al. 2005; Guinebretiere et al. 2006; Ngamwongsatit et al. 2008). Such methods demonstrate the potential for the practical everyday use of PCR methods in food microbiology.

Table 3 Distribution of bacterial load among food samples

Food product	No. of samples	Total no. mesophilic (37°C) (CFU/g)	Total no. spore formers (75°C) (CFU/g)	*Bacillus cereus* group MYP agar (CFU/g)	*Staphylococcus aureus* Baird Parker agar (CFU/g)
1. Beef luncheon	1	2.4×10^8	1.7×10^3	1.4×10^2	–
	2	4×10^8	3×10^3	1.2×10^2	–
	3	6.5×10^8	2.8×10^3	1×10^2	–
	4	7×10^7	1.4×10^3	3×10^2	–
2. Karish cheese (defatted cheese)	1	2.3×10^8	1.1×10^3	1.6×10^2	–
	2	9×10^8	6.4×10^3	1×10^2	–
	3	6.8×10^8	2.1×10^3	1.3×10^2	–
	4	1×10^9	7.3×10^3	3.3×10^2	–
	5	6×10^8	3.9×10^3	2×10^2	–
3. Koshary	1	2.6×10^6	3.2×10^3	5×10	–
	2	3.7×10^6	1.5×10^3	7×10	–
	3	2×10^7	4.7×10^3	3×10^2	–
	4	1.2×10^7	3.3×10^3	2.7×10^2	–
	5	1×10^8	1.4×10^4	1.4×10^2	–
4. Raw milk	1	2.8×10^6	5.6×10^2	4×10	–
	2	4×10^8	2.8×10^3	6×10^2	–
	3	5.7×10^7	1.6×10^3	4.2×10^2	–
	4	6.3×10^6	3.3×10^2	3×10	–
	5	2.2×10^6	$1{,}3\times10^2$	5×10	–
5. Double cream cheese	1	3.3×10^7	1.6×10^4	2.7×10^2	2×10^2
	2	5×10^6	6.4×10^3	1.2×10^2	3×10^2
	3	5.8×10^6	1.5×10^3	3×10^2	–
	4	3×10^7	2×10^3	1.1×10^2	–
6. Turkish cheese	1	5.2×10^7	8.8×10^3	4×10^2	4×10^2
	2	9.7×10^6	2.2×10^3	1.8×10^2	2×10^2
	3	8.1×10^6	3×10^3	2×10^2	–
	4	3.9×10^7	1.7×10^3	1.1×10^2	–
	5	7.4×10^7	4×10^3	5×10^2	–
7. Raw meat	1	2.8×10^8	2×10^4	6×10^2	–
	2	6.6×10^8	9.3×10^3	4.8×10^2	–
	3	4.6×10^8	1.8×10^4	3.3×10^3	–
	4	5.8×10^8	5.9×10^3	2×10^2	–
	5	3.2×10^8	3.8×10^3	1.7×10^2	–
Total (%)	33	100%	100%	100%	12.12%

–, Negative result

Materials and Methods

Collection of samples

Replicates of seven different food samples [beef luncheon meat, defatted Karish cheese (Koshary), raw cow milk, double cream cheese, Turkish cheese (kasar cheese), raw beef] were collected randomly from different localities in Sharkia Governorate (Egypt).

The food samples were transported in sterile containers within a few hours of collection to the Bacteriology laboratory of the Botany Department, Faculty of Science, Zagazig University and kept under sterile conditions.

Microbiological analysis

A 25-g sample of each food sample was homogenized in 225 ml of sterile saline solution (0.85% NaCl). Decimal dilutions up to 10^{-8} were prepared to enumerate total mesophilic bacteria (CFU/g) using the pour plate technique and tryptone soya agar (TSA) medium and to enumerate total spore-forming bacteria (CFU/g) (homogenized food samples placed in water bath at 75°C for 20 min) using the pour plate technique and TSA medium. *B. cereus* and *S. aureus* were enumerated and isolated by surface spread plating of 0.1 ml aliquots on the surface of mannitol yolk polymyxin (MYP) agar and Baird Parker agar media, respectively. All media used in our investigation were prepared as described in the manuals of Difco (1994) and Oxoid (1990).

Table 4 Test for virulence factors of toxic bacterial isolates

Number of virulence factor	Code	Identification	Zone diameter (in mm)		
			Lecithinase	Hemolysin (β)	Proteinase (casein)
1	G_8	*B. cereus*	29	40	31
2	LB_3	*B. cereus*	40	37	31
3	K_2	*B. cereus*	31	25	28
4	L_4	*B. cereus*	30	28	28
5	LB_5	*B. cereus*	22	19	18
6	LB_6	*B. cereus*	28	23	21
7	GT_1	*B. cereus*	31	27	42
8	GT_3	*B. cereus*	25	28	21
9	M_5	*B. cereus*	23	31	28
10	G_4	*B. cereus*	26	32	27
11	M_2	*B. cereus*	32	21	32
12	M_6	*B. cereus*	27	23	34
13	M_8	*B. cereus*	26	28	26
14	L_3	*B. cereus*	12	31	18
15	G_5	*B. cereus*	13	33	21
16	CH	*B. cereus*	36	31	38
17	M_7	*B. cereus*	33	30	33
18	L_6	*B. cereus*	30	25	21
19	G_2	*B. cereus*	13	11	13
20	S	*S. aureus*	30	33	28
21	S1	*S. aureus*	21	30	23
22	S3	*S. aureus*	34	23	24
23	S3	*S. aureus*	31	28	31

Fig. 1 Pro-analysis of separated cellular protein bands of the eight toxigenic strains on acrylamide gel showing their intensities and molecular weights compared with marker protein (6.8-214 kDa)

Identification of bacteria

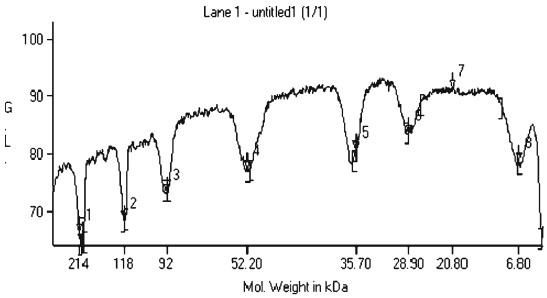
Suspected colonies growing on specific media (MYP and Baird Parker agar media, respectively) were picked, purified, and identified according to procedures recommended by the American Public Health Association (1992) and Federal Drug Administration (2001) using Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984), Rhodehamel and Harmon (2001), and Todar (2005).

Determination of virulence factors

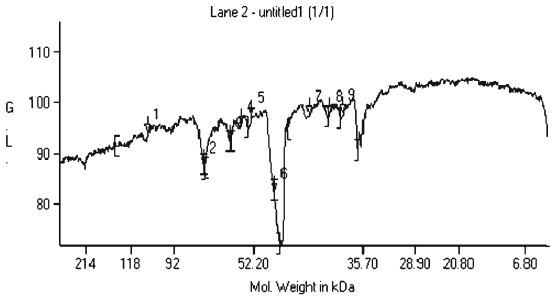
Hemolysin, lecithinase, and protease enzymes were determined using an agar well diffusion assay according to Reinheimer et al. (1990) and Misra and Kuila (1992). Wells in blood agar, egg yolk agar, and casein agar plates were filled with 40-μl aliquots of filter-sterilized (pore size 0.45 μm) bacterial cultures filtrates. Plates were incubated at the desired temperature for 24 h.

Cellular and extracellular protein patterns were determined using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique according to Laemmli (1970) and LKB application instructions (1997).

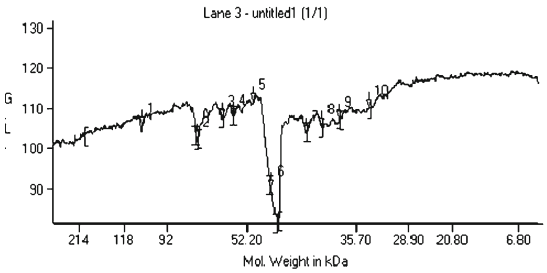
The examined bacterial strains were grown in 50 ml tryptone soya broth (TSB) at 30°C for 24 h. Bacterial cells were harvested by centrifugation, and the filtrates were separated for further extraction of the extracellular proteins. Bacterial pellets were washed twice using sterile double-distilled water. 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 concentrated 100 times using 70% saturated $(NH_4)_2{\cdot}SO_4$. The precipitated extracellular proteins were re-suspended in phosphate buffer, pH 7, and 100 μl of each cellular and extracellular protein preparation was then mixed with 50 μl treatment buffer, boiled in a water bath for 5 min, and injected into the well of the prepared polyacrylamide gel. The separated proteins were first stained with commasie blue, and their molecular weights were determined by comparison with marker proteins having molecular weights ranging between 14 to 116 kDa.



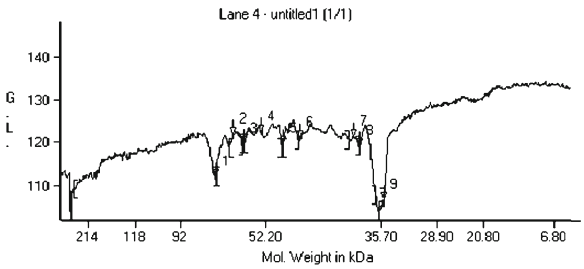
Marker



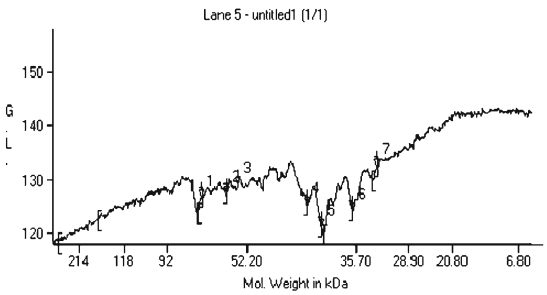
B. cereus **(CH)**



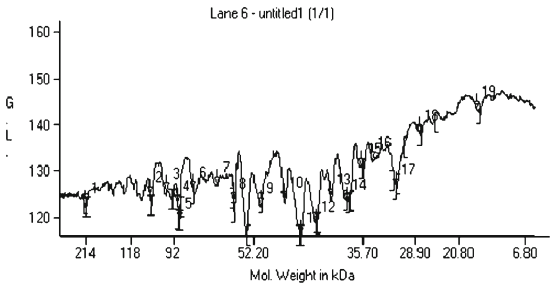
B. cereus **(GT1)**



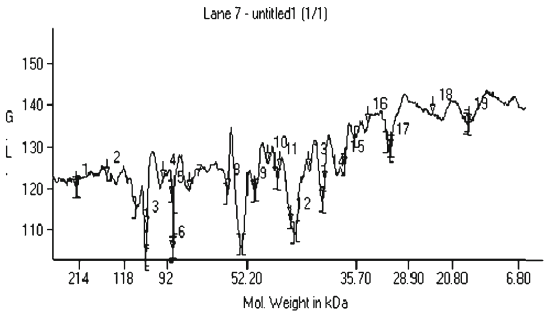
B. cereus **(LB3)**



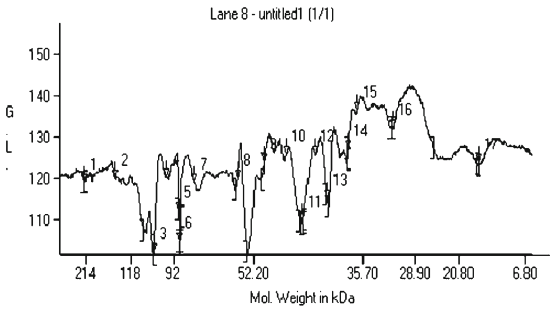
B. cereus **(G8)**



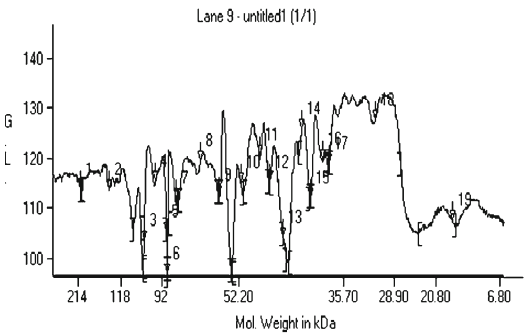
S. aureus **(S)**



S. aureus **(S1)**



S. aureus **(S2)**



S. aureus **(S3)**

Table 5 Similarity percentage between selected *B. cereus* strains for cellular protein

Strains	CH	GT1	LB3	G8
CH	100	89	33.3	44.4
GT1	90	100	20	40
LB3	30	20	100	20
G8	57.1	57.1	28.6	100

DNA extraction for multiplex-PCR

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

Specific multiplex PCR amplification conditions for *S. aureus* enterotoxin genes *sea*, *seb*, *sec*, *sed*, and *see* were as described by Pinto et al. (2005).

PCR amplifications were conducted in a solution containing 1× PCR buffer (10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 100 mM of each dNTP, 1 mM of each primer, and 0.5 U of thermostable DNA polymerase (DyNAzyme II DNA polymerase; Finnzymes Oy, Vantaa, Finland) and 5 μl of DNA template, in a final volume of 50 μl. The amplification conditions consisted of 5 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at the corresponding annealing temperature, and 45 s at 72°C, with a final extension of 10 min at 72°C. PCR products (15 ml) were electrophoresed in a 2% agarose gel in TAE buffer (40 mM Tris–acetate, pH 8.0; 1 mM Na_2EDTA). The sizes of the amplicons were estimated using a 100-bp DNA ladder (Amersham Pharmacia Biotech, Piscataway, NJ) run on the same gel. The oligonucleotides used in this work, their sequences, target positions, and size of amplification fragments are summarized in Table 1 according to Pinto et al. (2005).

Specific multiplex PCR amplification conditions for *B. cereus* enterotoxin genes *hblC* and *cytK* were according to Ngamwongsatit et al. (2008).

The multiplex PCR amplification was performed in a final volume of 20 μl containing 5 μl of DNA template in a final concentration 1×PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5 U *Taq* DNA polymerase, 0.4 μM *hlb*C primer, and 0.2 μM *cyt*K primer. The oligonucleotides used in our study work, their sequences, target positions, and size of amplification fragments are summarized in Table 2 according to Ngamwongsatit et al. (2008).

Table 6 Similarity percentage between selected *S. aureus* for cellular protein

Strains	S	S1	S2	S3
S	100	80	73.7	63.2
S1	80	100	84.2	68.4
S2	82.4	82.4	100	70.1
S3	63.2	68.2	63.2	100

Fig. 2 Pro-analysis of separated extra-cellular protein bands of the eight toxigenic strains on acrylamide gel showing their intensities and molecular weights compared with marker protein (6.8-214 kDa)

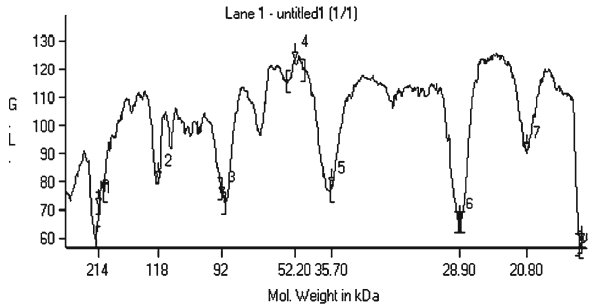
The amplification consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, annealing at 54 and 56°C for 1 min for *hbl*C and at 58°C for *cyt*K, and elongation at 72°C for 2 min, with a final extension at 72°C for 5 min. Amplicons were separated in a 1.5% agarose gel and sizes were estimated using a 100 bp DNA ladder (Amersham Pharmacia Biotech) run on the same gel. All multiplex PCR reactions were carried out in a GeneAmp PCR System 9700 Thermal Cycler (PE Applied Biosystems, Norwalk, CT).

Primers used in this study were synthesized by Metabion International AG (Martinsried, Germany).

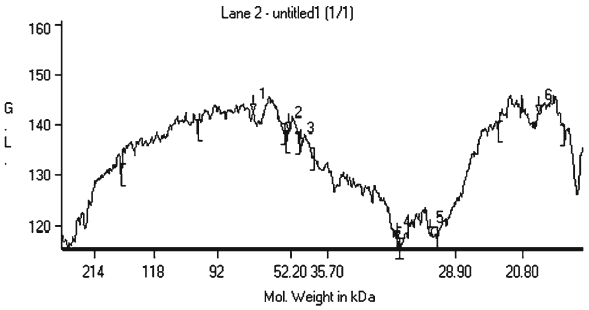
Results and discussion

Contaminated food is a real threat to human public health and welfare. Food-borne diseases are mainly caused by pathogenic bacteria which are either transmitted to humans from the animal reservoir or which contaminate the food processing chain. *B. cereus* and *S. aureus* are currently the focus of increasing attention due to their capability to produce a range of enterotoxins and tissue-degrading enzymes (Lund and Granum 1997; Klotz et al. 2003; Do Carmo et al. 2004; Schoeni and Wong 2005). The prevalence of toxigenic stains of *B. cereus* and *S. aureus* has been extensively documented in different starchy foods, such as vegetables, puddings, sauces, milk, dairy products, cereals, infant cereal formulas, fried and cooked rice, salads, meat products, salmon, meatballs, pork entrails, pasteurized eggs, ready-to-serve dishes, and cakes (Agata et al. 2002; Schneider et al. 2004; Duc et al. 2005; Pinto et al. 2005; Shaheen et al. 2006; King et al. 2007; Svensson et al. 2007; US FDA/CFSAN 2007). Thus, it is important to evaluate the food safety of different food products widely distributed and sold in Egypt.

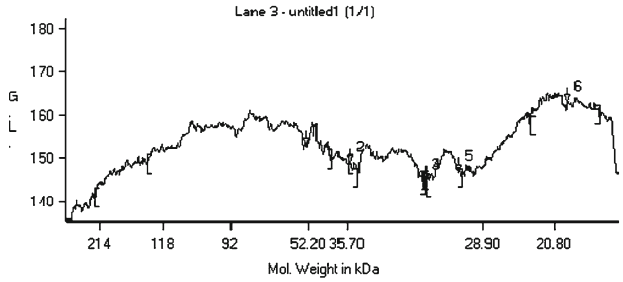
The data presented in Table 3 show the prevalence and frequency of *B. cereus* and *S. aureus* among the tested food samples. Suspected colonies were counted and isolated using MYP agar and Baird Parker agar media, respectively. In total, 19 isolates related to the *B. cereus* group and four



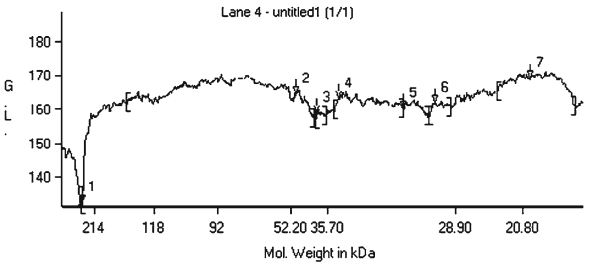
Marker



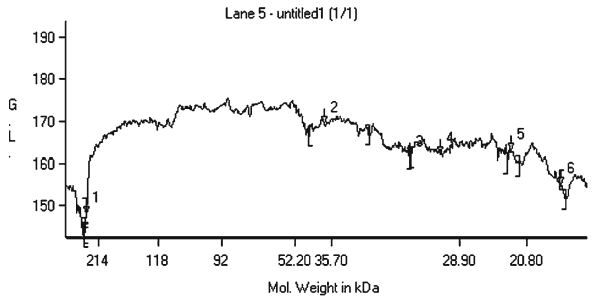
***B. cereus* (CH)**



***B. cereus* (GT1)**



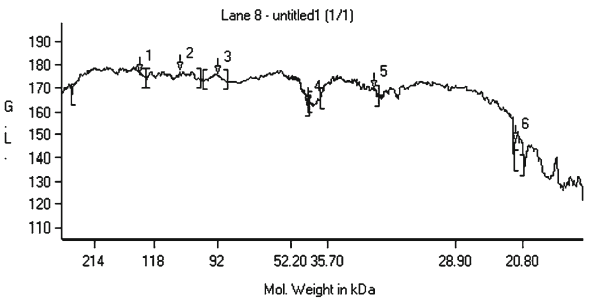
***B. cereus* (LB3)**



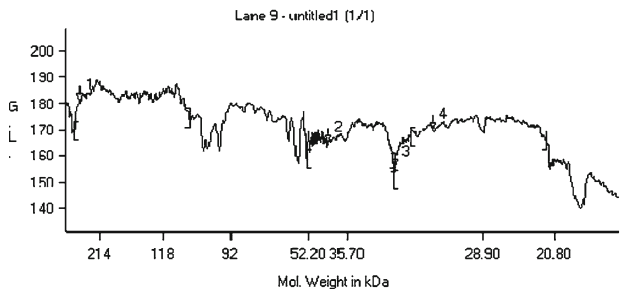
***B. cereus* (G8)**

***S. aureus* (S)**

***S. aureus* (S1)**



***S. aureus* (S2)**



***S. aureus* (S3)**

isolates related to S. *aureus* were isolated and their identify confirmed according to key of Bergey (Krieg and Holt 1984). Thereafter, the identified isolates were screened for their capability to produce specific virulence factors, namely, hemolysin, licithinase, and protease enzymes (Table 4), using zone diameters (mm) based on a well agar diffusion assay on blood agar, egg yolk agar, and casein agar plates, respectively. *B. cereus* strains 1, 2, 3, and 4 and *S. aureus* strains 5, 6, 7, and 8 were chosen as the most potent strains in terms of their virulence factors.

Bacillus cereus strains were observed to produce both an emetic and diarrhoeal enterotoxin, respectively, in addition to other virulence factors, including phospholipase protease and hemolysins, one of which, cereolysin, is a thiol-activated hemolysin. These virulence factors may contribute to enteric and non enteric diseases (Drobniewski 1993). *B. cereus* emetic toxin has been associated with life-threatening acute conditions, such as fulminant liver failure and rhabdomylosis (Mahler et al. 1997; Yokoyama et al. 1999). This toxin is unique among enterotoxins since it is resistant to proteolytic degradation, extreme pH, and elevated temperatures, being able to survive at 121°C for 90 min (Granum and Lund 1997). Lund et al. (2000) were the first to report that the cytotoxic gene *cyt*K of *B. cereus* (a clinical isolate) was the only cause of a severe food poisoning outbreak that killed three people. They also reported that CytK toxin had necrotic and hemolytic actions and was completely different from other *B. cereus* enterotoxins.

Staphylococcus aureus produces one or more toxins simultaneously. Classically, SEs have been divided into five major serological types (SEA, SEB, SEC, SED, and SEE) on the basis of their antigenic properties (Su and Wong 1997). SEA is the most common enterotoxin recovered from food poisoning outbreaks (Balaban and Rasooly 2000), and 59% of all staphylococcal food poisoning outbreaks are caused by SEA to SEE (Bergdoll 1989). Staphylococcal food poisoning (SFP), a form of enteritis, is an intoxication rather than a disease, and results from the ingestion of food contaminated with preformed staphylococcal enterotoxins (Bergdoll et al. 1974). Symptoms of SFP usually occur within 1–6 h after food intake and are characterized by nausea, vomiting, abdominal cramps, and diarrhoea. These symptoms usually subside within 1–3 days, but the patient remains sick for 7–10 days due to effects of toxic shock (Jett et al. 1994; Do Carmo et al. 2004).

Table 7 Similarity percentage between selected *B. cereus* strains for extracellular protein

Strains	CH	GT1	LB3	G8
CH	100	80	60	60
GT1	80	100	80	60
LB3	43	57	100	71.4
G8	50	50	83.3	100

Table 8 Similarity percentage between selected *S. aureus* strains for extracellular protein

Strains	S	S1	S2	S3
S	100	100	66.7	66.7
S1	75	100	50	50
S2	50	50	100	50
S3	50	50	50	100

SDS-PAGE, which is a common technique used to analyze complex mixtures of proteins, is considered to be a suitable tool for studying gene expression and for fingerprinting living organisms (Smith 1997; Wong and Hancock 2000). In our study, total cellular proteins of selected virulent strains of *B. cereus* (GT1, CH, LB3, and G8) and *S. aureus* (S, S1, S2, and S3) were extracted and then fractionated by PAGE. The electrophoretic separation verified the protein patterns of the selected strains and revealed protein bands with molecular weights ranging between 33 to 108 kDa (*B. cereus* strains) and between 17 to 220 kDa (*S. aureus*) (Fig. 3). Analysis of the gel photograph by Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD) revealed similarity percentages within *B. cereus* strains and within *S. aureus* strains that varied between 20 and 90% and between 63.2 and 82.4%, respectively. Differences were also observed in the number of separated

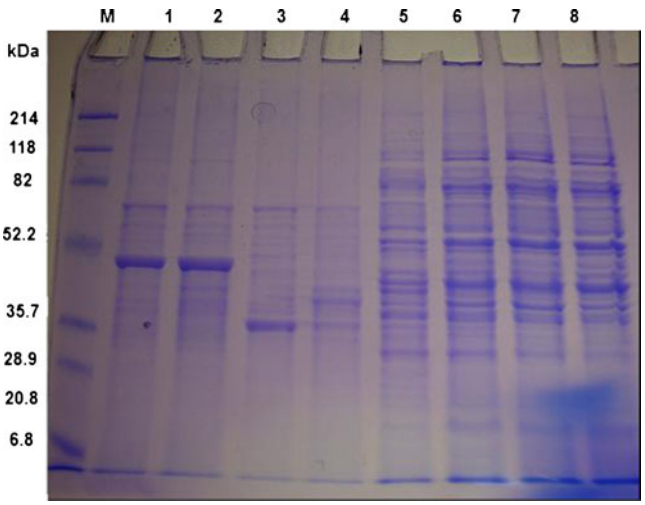


Fig. 3 Polyacrylamide gel electrophoresis showing the cellular protein pattern of virulent isolates. *M* Protein marker (mixture of 7 purified proteins with molecular weights of 214, 118, 92, 52.2, 35.7, 28.8, 20.8, and 6.8 kDa.), *Lanes 1*, *2*, *3* and *4 B. cereus* strains CH, GT1, LB3, and G8. *Lanes 5*, *6*, *7* and *8 S. aureus strains* S, S1, S2, S3

cellular protein bands in the investigated strains (Tables 5, 6; Fig. 1). *B. cereus* GT1 showed nine protein bands, while *B. cereus* G8 showed only seven bands; *S. aureus* strains S and S3 showed 19 bands and strains S1 and S2 showed 17 bands.

The Gel-Pro Analyzer analysis of the extracellular protein bands of the tested isolates also revealed varying degres of similarity between the virulent strains, with a range of 50 to 83.3% in *B. cereus* and 50 to 75% in *S. aureus* (Tables 7, 8; Fig. 2). Figure 4 shows distinct protein bands in lanes 1, 2, 3 and 4 (*B. cereus* strains CH, GT1, LB3, and G8, respectively), with molecular weights of 19.26, 30.24, 33.98, 53.44, and 72.59 kDa; 19.6, 30.12, 33.75, 35.53, and 53.19 kDa; (22.11, 30.03, 33.67, 35.08, 43.24, 53.14, and 234.36 kDa, and 19.63, 22.77, 30.93, 33.53, 43.23, and 234.36 kDa, respectively. *S. aureus strains* S, S1, S2, and S3 possessed extracellular proteins with molecular weights of 43.23, 93.486, and 234.36 kDa; 22.82, 43.54, 53.676, and 234.36 kDa; 22.67, 33.21, 43.57, and 92.19 kDa, and 30.39, 33.31, 43.74, and 234.36 kDa, 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) reported that the molecular weights of the B, L1, and L2 components were 37.8 38.5, and 43.2 kDa, respectively. Schoeni and Wong (2005) 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 and

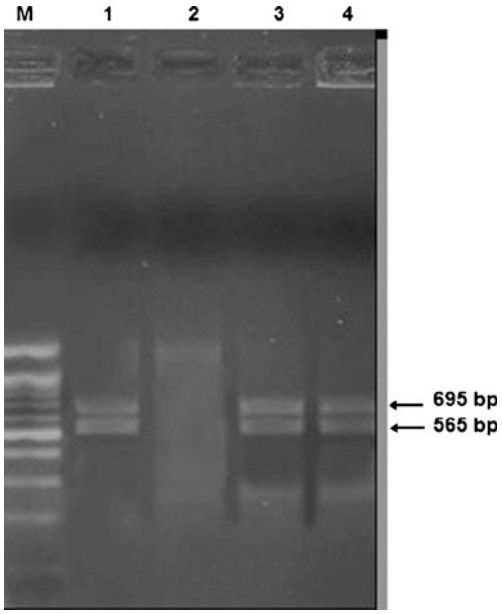


Fig. 5 Agarose gel showing the PCR amplicons resulting from amplification of enterotoxins genes *hbl*C and *cyt*K using the FHblC and RHblC and FCytk and R2Cytk primer pairs, respectively. *Lanes*: *M*100-bp DNA ladder marker, *1*, *2*, *3*, *4* DNA amplicons of *B. cereus* G8, CH, GT1 and LB3, respectively. The gel reveals the presence of both enterotoxin genes (hblC and cytK) in strains GT1, LB3, and G8

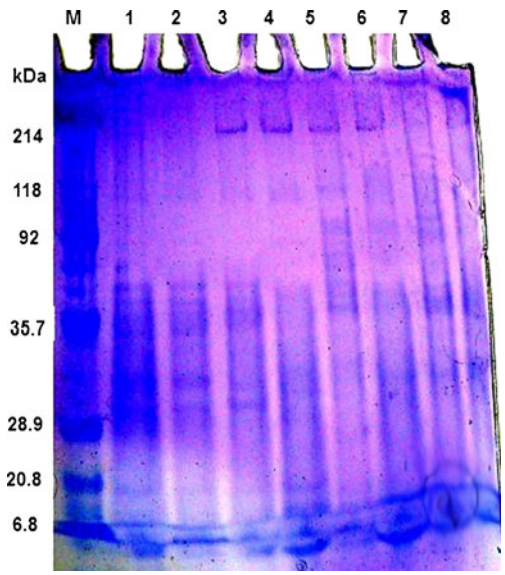


Fig. 4 PAGE showing the extracellular protein pattern of the selected toxigenic isolates. *Lanes*: *M* Protein marker (mixture of 7 purified proteins with molecular weights 116, 97.4, 66.2, 37.6, 28.5, 18.4, and 14 kDa, respectively, *1*, *2*, *3*, *4 B. cereus* strains CH, GT1, LB3, G8, respectively, *5*, *6*, *7*, *8 S. aureus* strains S, S1, S2 and S3, respectively

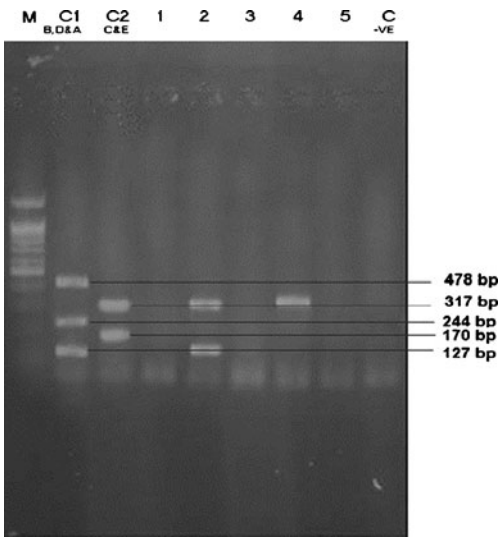


Fig. 6 Agarose gel showing the PCR amplicons resulting from amplification of enterotoxin genes *Sea*, *seb*, *sec-1*, *sed*, and *see* using their specific primers. *Lanes*: *M* 100-bp DNA ladder marker, *1*, *2*, *3*, *4* DNA amplicons of *S. aureus* S, S1, S2, and S3, respectively. The gel reveals the presence of enterotoxins *Sea* and *sed* in *S. aureus* S1 and *sed* in *S. aureus* S3

42 kDa) for the B protein, two L1 proteins (38 and 41 kDa), and two L2 proteins (both 43 kDa) in a soil isolate encoded S1C strain.

In terms of *S. aureus*, the toxic shock causing SEs are single chain polypeptides having a molecular weight ranging from 27 to 29 kDa. SEs can withstand a boiling temperature for several minutes, extremes of pH (3–11), and protease digestion by gastric enzymes (Soriano et al. 2002). Twenty different types of SEs, including SEA through SEE, SEG through SER, and SEU have already been identified, however, only a few of the toxin serotypes are frequently associated with food poisoning outbreaks (Martin et al. 2004; Smyth et al. 2005; Fernandez et al. 2006).

Multiplex PCR technique has been recently used for the rapid detection and discrimination of enterotoxin genes in *B. cereus* (Guinebretiere et al. 2006; Ngamwongsatit et al. 2008) and also for the direct detection of food contamination with enterotoxigenic *B. cereus* (Ombui et al. 2008). Ngamwongsatit et al. (2008) have developed and evaluated a group of new primers and found these to be highly efficient in detecting the toxin genes in 100% of their tested *B. cereus* and *B. thuringensis* strains. Thus, it can be expected that the presence of either gene is an indication for the presence of the whole operon.

In this study, we used the primers designed by Ngamwongsatit et al. (2008) under specific multiplex PCR conditions (see Materials and methods) to detect the presence of the enterotoxin genes (*hbl*C and *cyt*K) in the tested strains. Figure 5 shows that the presence of amplified DNA fragments of these two toxin genes in three strains of *B. cereus* (CH, GT1, and G8) can be determined in one quick step. The toxin genes *hbl*C and *cyt*K predicted molecular sizes of 695 and 565 bp, respectively.

Regarding the enterotoxin genotype, previous studies on *S. aureus* proved that enterotoxin PCR determinations are in a high agreement (97–100%) with toxin production, as defined by immunoassays (Fueyo et al. 2001, Letertre et al. 2003; McLauchlin et al. 2000).

Enterotoxin genotyping of the tested strains revealed the presence of the *sed* gene in two strains of *S. aureus* (S1 and S3) and the *sea* gene in strain S1 only (Fig. 6). Pinto et al. (2005) found a total of 40 (30%) *S. aureus* food isolates positive for *se* genes. Among these, the *sec* genotype was the most frequent (22 strains, 20% of total *se*-positive strains), followed by the *sea* genotype (14 strains, 13%), which is in accordance with the results obtained by Fueyo et al. (2001). Data on enterotoxin genotyping confirmed the grouping of strains according to *nuc* PCR-positivity and *se* PCR-positivity in *S. aureus* clusters. On the basis of these results, we suggest the amplification of enterotoxin genes as target genes, using multiplex PCR test as a rapid and valuable technique that can be applied directly to single colonies growing on selective plates, for the rapid, accurate, and unequivocal identification of *B. cereus and S. aureus*. This approach could be implemented as an alternative to phenotypic and immunology-based tests in routine food microbiological analyses.

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