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RESEARCH ARTICLE

Determination of Enterotoxigenic Gene Profiles of *Bacillus cereus* Strains Isolated from Dairy Desserts by Multiplex PCR

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Summary

The aim of this study was to investigate the presence of *Bacillus cereus* and to detect enterotoxigenic genes in dairy dessert samples utilising a multiplex PCR technique. A total of 100 samples, 25 keskul, 12 tavuk gogsu, 12 kazandibi, 30 supangle, 4 profiteroles and 17 sutlac were analysed. *B. cereus* contamination was found in 7 of 100 (7.0%) samples including 3 (25%) tavuk gogsu, 2 (6.6%) supangle, 1 (4%) keskul and 1 (5.8%) sutlac. The average number of *B. cereus* was between 2.0×10^1 - 5.0×10^2 cfu/g in these dairy dessert samples. A total of 20 isolates were collected from the 7 positive samples. The results indicated that 30% (6/20) of *B. cereus* isolates contain three enterotoxic HBL complex encoding genes *hblA, hblC* and *hblD,* whereas 70% (14/20) had no *hbl* genes. In addition, all three enterotoxic NHE complex encoding genes, *nheA, nheB* and *nheC,* were detected in 40% (8/20), two *nhe* genes (*nheA* and *nheB*) were found in 45% (9/20) and one *nhe* gene (two *nheA* and one *nheB*) was found in 15% (3/20) of the isolates. The *ctyK1* gene was not detected in any sample. The presence of *B. cereus* and their enterotoxigenic genes in dairy desserts may be a potential risk for public health.

Keywords: *B.cereus, Dairy dessert, Enterotoxin genes*

Sütlü Tatlılardan İzole Edilen *Bacillus cereus* Suşlarının Enterotoksijenik Gen Profillerinin Multipleks PCR ile Belirlenmesi

Özet

Bu çalışmanın amacı sütlü tatlılarda *Bacillus cereus* varlığını araştırmak ve enterotoksijenik genleri multipleks PCR tekniğiyle belirlemektir. Bu amaçla 25 keşkül, 12 tavuk göğsü, 12 kazandibi, 30 supangle, 4 profiterol ve 17 sütlaç olmak üzere toplamda 100 örnek analiz edilmiştir. 3'ü tavuk göğsü (%25), 2'si supangle (%6.6), 1'i keşkül (%4) ve 1'i sütlaç (%5.8) olmak üzere toplamda 7 örnek (%7.0) *B. cereus* varlığı açısından pozitif bulunmuştur. Analiz edilen sütlü tatlı örneklerinde ortalama *B. cereus* sayısı 2.0×10^1 - 5.0×10^2 kob/g olarak tespit edilmiştir. 7 pozitif örnekten toplamda 20 izolat elde edilmiştir. Sonuçta, *B. cereus* izolatlarının %70'inin (14/20) *hbl* geni taşımadığı, %30'unun (6/20) üç enterotoksik HBL kompleksi kodlayıcı geni (*hblA, hblC* ve *hblD*) bulundurduğu saptanmıştır. Ayrıca yapılan incelemede, izolatların %40'ının (8/20) üç NHE kompleksi kodlayıcı geni (*nheA, nheB* ve *nheC*), %45'inin (9/20) iki *nhe* geni (*nheA* ve *nheB*) ve %15'inin (3/20) bir adet *nhe* geni (ikisi *nheA* ve biri *nheB*) içerdiği saptanmıştır. *ctyK1* geni hiçbir örnekte belirlenememiştir. Sütlü tatlılarda *B. cereus* varlığının gıda zehirlenmelerine yol açarak halk sağlığı açısından potansiyel bir tehlike olabileceği düşünülmektedir.

Anahtar sözcükler: *B. cereus, Sütlü tatlı, Enterotoksin genleri*

INTRODUCTION

The *B. cereus* group consists of six different species: *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis, B. weihenstephanensis* and *B. pseudomycoides* [1,2]. *B. cereus* is a Gram-positive bacteria inhabiting numerous environments, including soil, plant materials and many types of foods, especially those of plant origin; however, it is also frequently isolated from meat, eggs and dairy products [3]. An ubiquitous, spore-forming bacterium, *B. cereus* causes food spoilage and can produce two distinct types of toxins that differ in the main symptoms induced in humans. *B. cereus* is the etiologic agent of two types of food-borne disease, resulting in an infection causing vomiting and

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diarrhoea as the major clinical symptoms.

Both *B. cereus*-derived diseases are caused by toxins: the diarrhoeal type occurs due to protein toxins formed in the intestinal tract by growing organisms (enterotoxins) and the emetic toxin type results from a peptide that is preformed in the food (emetic toxin or cereulide) [4,5]. Within 12 h of food consumption, the enterotoxin disease type is characterised by diarrhoea. The emetic disease type is characterised by vomiting and nausea within two hours after consumption of the suspected food. For both types of the disease, clinical signs last approximately 24 h [3].

B. cereus produces at least three different proteins, referred to as enterotoxins, that may cause foodborne illness. The haemolysin BL (Hbl), nonhaemolytic enterotoxin (NHE) and cytotoxin K (CytK) proteins are considered the primary virulence factors in *B. cereus*. The HBL complex is composed of three proteins, B, L1 and L2 [6,7], transcribed from the genes *hblC* (encoding L2), *hblD* (encoding L1), and *hblA* (encoding B). The NHE complex is composed of three proteins, NheA, NheB and NheC encoded by the three genes *nheA*, *nheB* and *nheC*, respectively [8].

B. cereus is a common contaminant of milk [9]. Some investigations have examined the occurrence of toxin genes and toxin producing strains in milk and dairy products [10,11]. Exclusion of *Bacillus* spp. from the food chain is not possible, and the only recognised control method involves maintaining a cold temperature throughout the processing, manufacture and storage stages. It had been reported that toxogenic *B. cereus* strains could affect the constituents in dairy desserts during toxin formation [12]. *B. cereus* causes problems by degrading dairy products, creating off-flavours, sweet curdling and bitter cream because of the production of proteinase, lipase and phospholipases, and it endangers public health through food contamination [13,14].

In dairy products, the presence of *Bacillus* spp. is inevitable, and the spore-formation ability of this organism allows it to survive pasteurisation easily [15]. In milk and dairy products, the presence of *B. cereus* decomposes casein peptides into amino acids and free fatty acids into milk fat, thus degrading the quality of milk products and shortening shelf life [16]. The occurrence of *B. cereus* was previously reported as a contaminant of cheese [17,18].

The objective of this study was to investigate the presence of *B. cereus* and its associated toxins in dairy dessert samples utilising an mPCR technique for special emphasis on their enterotoxigenic potential.

MATERIAL and METHODS

Materials

From June through August 2010, 100 dairy dessert samples were analysed, consisting of 25 keskul (a milk pudding containing coconut), 12 tavuk gogsu (a milk pudding containing chicken breast meat), 12 kazandibi (a milk pudding slightly burned on the bottom), 30 supangle (a milk pudding containing chocolate), 4 profiteroles and 17 sutlac (milk puddings containing rice). The samples were collected from 16 different small patisseries in Samsun. Dairy dessert samples were immediately transported to the laboratory in a refrigerated box and examined within 1-2 h of sampling for the presence of *B. cereus*.

Method

- Isolation and Identification of B. cereus

B. cereus count was determined according to ISO 7932/2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxoid CM0929), and the plates were incubated at 30°C for 24 h. Rough and bright pink colonies with a zone of egg yolk precipitation were then transferred to nutrient agar slants, and identification was confirmed by microscopic and biochemical characterisation that included Gram stain, anaerobic utilisation of glucose, reduction of nitrate, Voges-Proskauer test, motility, oxidase production, catalase production, endospore formation and haemolysis, as suggested by other authors [19-21].

- Detection of B. cereus Enterotoxins Production and Toxin Type

The multiplex PCR technique was used to detect the presence of the *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC* and *ctyK1* genes of *B. cereus* according to the procedure provided by Wehrle *et al*.[22]. For our study, the analysis was a combination of two multiplex PCRs and a general PCR (PCR 1-3). PCR 1 and 2 each included three primer pairs for *hblA*, *hblC*, *hblD* and *nheA*, *nheB* and *nheC*. PCR 3 included a primer pair for *ctyK1* alone. All primer pairs used in this study and corresponding multiplex PCR systems (PCR 1–3) are listed in *Table 1*.

The strains NTCC 11145 *(Hbl, Nhe)*, and NVH 391/98 (*ctyK1*) were used as reference strains.

The template DNA was initially obtained using the boiling method. Two colonies were chosen and inoculated into a microcentrifuge tube containing 500 µl sterile distilled water, suspended in a water bath (Memert, Germany), and boiled for 10 min at 95°C. The tubes were centrifuged at 9500 × *g* for 10 min at 4°C (Hettich-Universal-320R, Germany), after which the supernatant containing DNA was transferred into Dnase/Rnase-free microcentrifuge tubes and stored at -20°C for use as the template DNA.

The PCR reaction was performed in a reaction mixture of 50 µl final volume containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 µM of forward and reverse primers, 1.5 U Taq polymerase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5

Table 1. *Characteristics of PCR primers used for multiplex detection of B. cereus toxin genes*

Tablo 1. *B. cereus toksin genlerinin tespiti için kullanılan PCR primerlerinin özellikleri*

Primer	Sequence (5'-3')	Product Size (bp)	Reference
hblC F hblC R	CGAAAATTAGGTGCGCAATC TAATATGCCTTGCGCAGTTG	411	[39]
hblD F hblD R	AGGTCAACAGGCAACGATTC CGAGAGTCCACCAACAACAG	205	[39]
hblA F hblA R	ATTAATACAGGGGATGGAGAAACTT TGATCCTAATACTTCTTCTAGACGCTT	237	[40]
nheA F nheA R	GAGGGGCAAACAGAAGTGAA TGCGAACTTTTGATGATTCG	186	[39]
nheB F nheB R	CCGCTTCTGCAAAATCAAAT TGCGCAGTTGTAACTTGTCC	281	[39]
nheC F nheC R	ACATCCTTTTGCAGCAGAAC CCACCAGCAATGACCATATC	618	[41]
cytK1 F cytK1 R	AACAGATATCGGTCAAAATGC CGTGCATCTGTTTCATGAGG	623	[42]

µL 10× reaction buffer and 2 µL template DNA for PCR 1 and 2. The final reaction mixture for PCR 3 contained 0.1 mM of each dNTP, 1 mM $MgCl_2$, 0.3 µM of forward and reverse primers, 1 U Taq polymerase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 µL 10× polymerase buffer and 2 µL of template DNA. A negative control without template DNA was included in each experiment. The mixture was then processed in a thermocycler as explained below (Bio Rad-MJ Mini-PTC-1148, Singapore).

The PCR samples were subjected to amplification according to the following program: initial denaturation at 95°C for 5 min, followed by 30 cycles comprising denaturation at 94°C for 1 min, annealing at 55°C for 1 min (PCR 1) and 49°C for 1 min (PCR 2 and 3) and an extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min.

- Gel Electrophoresis

The PCR products were detected from a 20 µl volume of the amplification mixture (supplemented with 4 µl loading dye), loaded onto a 2.0% agarose gel containing ethidium bromide (Gene Choice), and electrophoresis (BioRad, Power Pac-Basic, Singapore, BioRAD, electrophoresis tank, WideMini, Singapore) was carried out at 90 V for 1.5 h. A 1000-100 bp DNA ladder molecular weight marker was used to identify the amplified products. The PCR products were visualised under UV illumination (Wise-UV-Wuv-L50, Korea).

RESULTS

The numbers of *B. cereus*, enterotoxigenic *B. cereus*, and enterotoxin types isolated from 100 dairy dessert samples are presented in *Table 2*.

B. cereus were detected in 7 (7.0%) of the total 100 dairy dessert samples. Three of tavuk gogsu (25%), 2 of supangle (6.6%), 1 of keskul (4%) and 1 of sutlac (5.8%) samples were positive. The average number of *B. cereus* isolated from dairy dessert samples was $5.0x10^2$ and $2.0x10^1$cfu/g.

A total of 20 *B. cereus* isolates from 7 positive dairy desserts samples were analyzed for the presence of seven enterotoxic gens by PCR. The results showed that 30% (6/20) of *B. cereus* isolates contained three enterotoxic HBL complex encoding genes *hblA*, *hblC* and *hblD*, whereas 70% (14/20) had no *hbl* genes. All three enterotoxic NHE complex encoding genes *nheA*, *nheB* and *nheC* were detected in 40% of isolates (8/20), whereas two nhe genes (*nheA* and *nheB*) were found in 45% (9/20) of the isolates

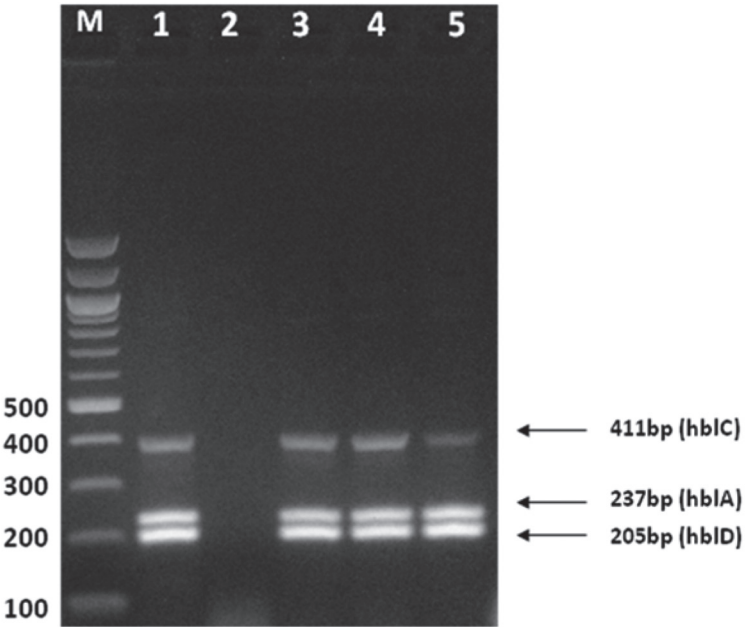

Fig 1. Demonstration of *hblA, hblC,* and *hblD* genes of *B. cereus* strains by multiplex PCR technique. M: Marker; 1: positive control; 2: negative control; 3–5: *B. cereus* isolates

Şekil 1. *B. cereus* izolatlarında *hblA, hblC* ve *hblD* genlerinin multipleks PCR tekniği ile gösterilmesi. M: Marker; 1: pozitif control; 2: negative control; 3-5: *B. cereus* izolatları

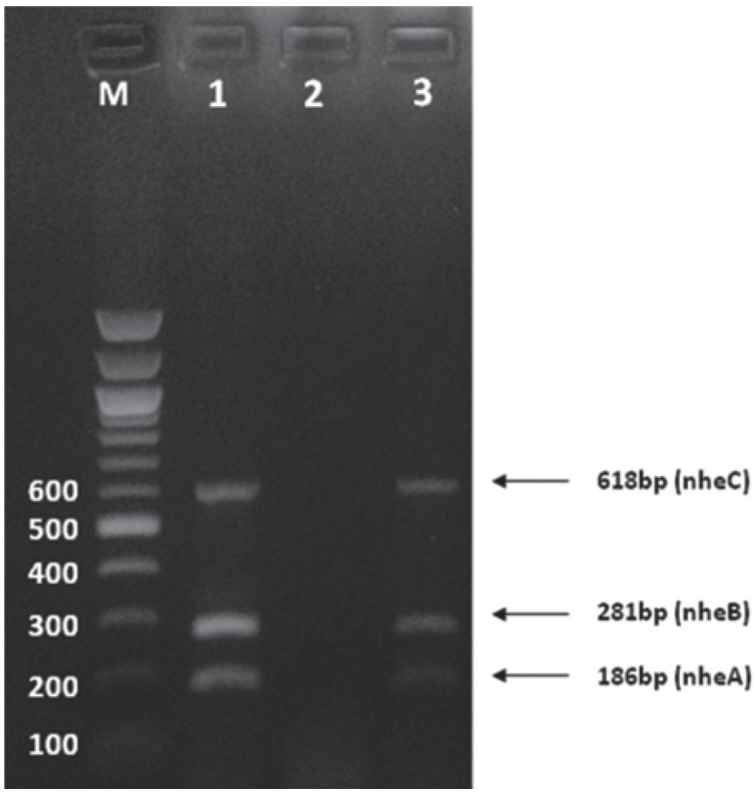

Fig 2. Demonstration of *nheA, nheB,* and *nheC* genes of *B. cereus* strains by multiplex PCR technique. M: Marker; 1: positive control; 2: negative control; 3: *B. cereus* isolate

Şekil 2. *B. cereus* izolatlarında *nheA, nheB,* and *nheC* genlerinin multipleks PCR tekniği ile gösterilmesi. M: Marker; 1: pozitif control; 2: negative control; 3: *B. cereus* izolatı

Table 2. *Prevalence of enterotoxin genes of B. cereus isolates*
Tablo 2. *B. cereus izolatlarının enterotoksin gen dağılımı*

Samples Positive/Total (%)	No. of Isolates	Haemolytic BL Complex			Non-Haemolytic Enterotoxin Complex			Cytotoxin K
		hbl A	*hbl D*	*hbl C*	*nhe A*	*nhe B*	*nhe C*	*cyt K*
Keskul 1/25 (4%)	2	-	-	-	2 (100%)	2 (100%)	-	-
Tavuk gogsu3/12 (25%)	9	6 (66.6%)	6 (66.6%)	6 (66.6%)	9 (100%)	7 (77.7%)	-	-
Kazandibi 0/12	-	-	-	-	-	-	-	-
Supangle 2/30 (6.6%)	4	-	-	-	3 (75%)	4 (100%)	3 (75%)	-
Profiterole 0/4	-	-	-	-	-	-	-	-
Sutlac 1/17 (5.8%)	5	-	-	-	5 (100%)	5 (100%)	5 (100%)	-
Total7/100 (7%)	20	6 (30%)	6 (30%)	6 (30%)	19 (95%)	18 (90%)	8 (40%)	-

and one nhe gene (two *nheA* and one *nheB*) was found in 15% (3/20) of the isolates. The *ctyK1* gene was not detected in any sample (*Fig. 1, Fig. 2* and *Table 2*).

DISCUSSION

High numbers of *B. cereus* result in food poisoning due to the presence of toxins and their subsequent production of toxins, presenting a potential risk to the consumer [23]. In addition to causing food-borne illness, *B. cereus* is responsible for the spoilage of various food products. Borge *et al.*[24], reported that psychrotolerant micro-organisms such as *B. cereus*, contaminate refrigerated foods and continue to be a safety problem. Under normal circumstances, *B. cereus* concentrations found in food are $<10^3$/g and, for the most part, $<10^2$/g. The minimum level required to cause disease from food consumption is estimated at $>10^5$/g [25]; unfortunately, a small dose ($<10^3$/g) may cause disease in susceptible individuals [26].

In this study, *B. cereus* was detected in 7.0% of dairy dessert samples which is in agreement with that of Ahmed *et al.*[27], who examined the presence of *B. cereus* in 400 milk and dairy products and isolated *B. cereus* strains from 9%, 14%, 35%, and 48% of raw milk, cheese, pasteurised milk and ice cream samples, respectively. Te Giffel *et al.*[28], analyzed 334 pasteurised milk samples from household refrigerators in the Netherlands and isolated *B. cereus* from 133 (40%) of the samples. Larsen and Jørgensen [29], examined 458 pasteurised milk samples in Denmark, and the occurrence of *B. cereus* in Danish pasteurised milk was 56%. This rate of contamination is higher than pasteurised milk from Iran and the Netherlands. In Turkey,

B. cereus contamination has been determined previously by Ozdemir [30], in pasteurised milk samples and was isolated from 56 (46.6%) of the samples. Molva *et al.*[31], also detected *B. cereus* from 6% of cheese samples.

B. cereus produces four enterotoxins, called HBL, NHE, cytK and enterotoxin-T [3,8,9]. HBL is considered one of the most important virulence factor of *B. cereus* [6,7]. NHE is a three component (nheA, nheB and nheC), enterotoxin responsible for the diarrheal food poisoning syndrome [9,24]. Cytotoxin K is responsible of diarrheal syndrome with necrotic, hemolytic and cytotoxic effects on the intestinal ephithelium [10].

The presence of diarrhoeal enterotoxin genes in the isolated strains was screened by mPCR as described by Gaviria *et al.*[32], Hansen and Hendriksen [33] and Zahner *et al.*[34]. In the detection of enterotoxin genes, 30% of *B. cereus* strains were found to carry the three *hbl* genes. Molva *et al.*[31], found that all detected strains contained *hblD* gene. Hansen and Hendriksen [33], reported that 64% of *B. cereus* strains contained this gene. Mäntynen and Lindström [35], detected the *hblA* gene in 52% of their *B. cereus* strains. Conversely, Hansen and Hendriksen [33], reported the high prevalence of the *hblA* gene in 13/22 (59%) of the *B. cereus* strains. They also reported that 16 *B. cereus* (73%) strains contained the *hblC* gene. In our study, it was determined that nhe enterotoxins contained a gene distribution of 19 (95%) *nheA*, 18 (90%) *nheB* and 8 (40%) *nheC*. Nduhiu *et al.*[36], detected 3.9% *nheA*, 19.6% *nheB* and 3.9% *nheC* genes from milk and 3.9% *nheA*, 11.8% *nheB* and 3.9% *nheC* genes from cheese from their *B. cereus* strains. However, several authors have also reported that almost all *B. cereus* strains contain *nhe* genes [31,33,37,38].

In conclusion, *B. cereus* strains isolated from dairy dessert samples should be regarded as potential enterotoxin producers according to the PCR results. Therefore, improving product quality and safety should be achieved by applying good manufacturing practice and implementing the hazard analysis and critical control point system. To minimise risk, better hygiene practices are required in the production of the foods.

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