Research article

ENTEROTOXIN AND EMETIC TOXIN GENES PROFILES AND GENETIC DIVERSITY OF *BACILLUS CEREUS* ISOLATED FROM FOOD, ENVIRONMENTAL AND CLINICAL SAMPLES IN SERBIA

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Bacillus cereus, usually ingested by food, can cause two types of disease due to the presence of toxins: vomiting and diarrhea syndrome. Systemic infections can also occur. The aim was to detect genes for enterotoxins (*hbl*A, *entFM*) and emetic toxin (*cer*) and to investigate the genetic heterogeneity of *B. cereus* isolates from food, environment and human stool. Identification of *B. cereus* was performed by means of selective medium, classical biochemical test and polymerase chain reaction (PCR). Toxin genes were detected by PCR. Typing was performed by random amplified polymorphic DNA (RAPD). *EntFM* gene was present in all stool and food samples and in 28/30 environmental isolates. *Hbl*A gene was present in 29/30 stool, 23/30 food and 24/30 environmental isolates. *Cer* gene was present in 30/30 stool, 28/30 food and 25/30 environmental isolates. The RAPD results show high heterogeneity among the isolates from each group. In the cumulative dendrogram, representative isolates from all three groups formed two clusters with a difference of 53%. The detection of toxin genes in all *B. cereus* isolates indicated these bacteria as potentially pathogenic and a serious threat for human health. The presence of isolates from all three groups in the same cluster suggests the existence of similar strains in the environment, food and patients, which is in line with the circulation of strains in nature through the food chain.

Key words: *Bacillus cereus*, enterotoxin genes, emetic toxin gene, PCR, RAPD typing

INTRODUCTION

Bacillus cereus (*B.cereus*) is a Gram-positive spore forming food pathogen commonly found in a wide variety of different foods and environments (soil, water, air), as well as in the feces of humans and animals [1]. The vegetative cell or spores of *B. cereus* into

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the environment enter the food chain, from soil to plants, animals and humans, leading to a major problem in the agricultural and food industry, as well as in veterinary and human medicine [2].

In recent decades, the incidence of infections after consuming microbiologically contaminated food has increased, despite microbiological food control. The reasons are associated with the latest trends in global food production in industrialized countries, expansion in production of semi-processed, ready to eat food and storage of food at an inadequate temperature. Unfortunately, not all countries investigated stool samples for the presence of *B. cereus*, neither report cases nor outbreaks.

Although the presence of pathogenic strains of *B. cereus* is most often associated with intoxication and toxicoinfection of the gastrointestinal system, recent studies have linked this bacterium with infections in neonatology, surgical and traumatic wounds, intravenous drug delivery, catheter placement, and invasive bacteremia of the central nervous system (meningitis and brain abscess), endophthalmitis, pneumonia and gas gangrene [3]. The registration of fatalities [3-5] caused by strains of *B. cereus*, due to delayed diagnosis or inadequate response to antibiotic therapy, has highlighted the great importance of introducing methods for the rapid detection of *B. cereus*.

At appropriate physical - chemical conditions, *B. cereus* can cause two types of intoxications: vomiting, due to the presence of heat and acid stable emetic toxin and diarrheal syndrome, due to the presence of heat-labile enterotoxins. Thereby, gene detection gives us proper information about the possible production of enterotoxins and emetic toxins [6,7].

Due to the wide distribution of *B. cereus* strains in the soil, water, air, food, as well as in the feces of humans and animals [1], there is a need to investigate the genetic heterogeneity of isolates from different sources [8,9].

In this study, we detected the presence of genes responsible for diarrhegenic and emetic toxin and fortified the genetic diversity of *B. cereus* isolates from investigated groups (food, environment and patients' stool) by RAPD analysis (Random Amplified Polymorphic) DNA.

MATERIAL AND METHODS

Samples

The samples were classified into three groups, each of them comprising 30 isolates: different types of food (F1-F30), isolates from patients (P1-P30), and from the environment (E1-E30). *B. cereus* ATCC 11778 was used as the positive control.

During 2013, at the Center for Microbiology, Institute for Public Health, Nis (IPHN), 30 clinical stool samples (P 1-30) were collected. At the same time, 30 specimens from different food types (F) were obtained from retail and wholesale facilities and processed in routine work in the Laboratory for Sanitary Microbiology of the IPHN:

spices of herbal origin (ginger F8, coriander F9, cinnamon F10, sesame F11 rosemary F12, and oregano F13). Other food samples were processed at the Military Medical Academy, Belgrade (MMAB) dietary products (F2, F4, F5, F17-30), milk powder (F1 and F 6), beet salad (F3), cheese (F15 and F16), food for pets (F7), and ham (F14). Also, 30 specimens from the environment (E) were analyzed, that consisted of soil samples (12), and hospital environment samples (18). Soil samples, originated from broader surrounding of Belgrade city area (arable land, pastures and contaminated soil), were processed at the Department of Microbiology, Genetic Laboratory, Institute of Soil Science, Belgrade. Hospital environment samples, processed at the MMAB, were swabs from the staff hands (E1, E2, E6, E7), working surfaces (E3, E4, E5), air conditioners (E9, E13), floors (E10, E11), a stairway (E12), a room lavatory (E14), kitchen ventilation (E15), room ventilation (E16), dining room wall (E17), kitchen knife (E 18), and one air sample from the syringe production facility (E8).

Identification of *B. cereus* isolates

All types of samples were cultured on selective mannitol egg yolk polymyxin agar (MYP) (HiMedia, India), and incubated for 18-24 h at 37⁰C. Pink colonies with a lecithinase reaction were transferred on 5% sheep blood agar (HiMedia, India), and thereon incubated. Presence of β-hemolysis was screened on 5% sheep blood agar following the procedure of Collins et al. [10]. In Gram-staining preparations, we confirmed the presence of terminal to subterminal spores, and existence of parasporal crystalline inclusions [11]. Morphological properties such as β-hemolysis, spores and inclusions were used to identify *Bacillus* spp. Finally, *B. cereus* was identified with an interactive database by using BBL Crystal GP ID Biochemical profiles (Becton, Dickinson and Company, USA). *B. cereus* group was confirmed by PCR, yielded 533 bp amplified fragments with primer pair BalF/BalR.

Preparation of DNA template and polymerase chain reaction (PCR)

A single colony of each isolate of *B. cereus* was incubated in the Brain-heart infusion broth (HiMedia, India) at 37 °C for 18-24 h. A pellet of 1 ml of culture was rinsed in saline solutions overnight, resuspended in 500 μl of distilled water, and boiled for 10 min. The prepared DNA was used directly for PCR or stored at –20 °C until use.

PCR was used for the identification of *B. cereus* (*balFR* gene), as well as for the detection of enterotoxin genes (*hbla, entFM* and *bceT*), and emetic toxin gene (*cer*) using specific primers (Invitrogen, Vivogen D.O.O.) (Table 1). The PCR mixture was prepared in a volume of 25 μl, with DreamTaqGreen Master Mix (ThermoScientific, Lithuania), 0.2 μmol l^{-1} final concentration of each primer, and 2.5 μl of prepared DNA template. The primer sequences and PCR conditions were the same as described earlier [12,13]. PCR was performed on thermocycler Eppeddorf MasterCycler (Eppendorf, Germany).

Table 1. Sequence of primers and PCR reaction conditions used in this study

Primers	Sequence (5''3')	Reaction condition				Gene	Amplicon size	Source
		Denaturation[a]	Primer binding	Polymerization[b]	Cycle			
BalF BalR	TGCAACTGTATTAGCACAAGCT TACCACGAAGTTTGTTCACTTACT	94 °C 45 s	55 °C 45 s	72 °C 45 s	30	*bal*	533 bp	Das at al. 2009.[12]
HblA1 HblA2	GCTAATGTAGTTTCACCTGTAGCAAC AATCATGCCACTGCGTGGACATATAA	94 °C 30 s	58 °C 45 s	72 °C 60 s	30	*hbla*	834 bp	Das at al. 2009.[12]
EntA EntB	ATGAAAAAAGTAATTTGCAGG TTAGTATGCTTTTGTGTAACC	94 °C 45 s	52 °C 45 s	72 °C 60 s	30	*entFM*	1300 bp	Das at al. 2009.[12]
CerF-5 CerR-5	CAAGTCAAGATAAGAGGCTTC AAAGCTCTTGCCAAATAACC	94 °C 60 s	52 °C 60 s	72 °C 60 s	35	*cer*	188 bp	Kim at al.2010b[13].

[a]The initial denaturation took place at 95°C and lasted for 5 minutes
[b]The final polymerization lasted 5 minutes

Table 2. Sequence of primers and PCR reaction conditions used in the RAPD analysis

Primers	Sequence (5''3')	Reaction condition				Source
		Denaturation[a]	Primer binding	Polymerization[b]	Cycle	
SPH1	GTG GTG GTG GTG GTG	94°C 5 min	32 °C 60 s	72 °C 120 s	40	Dolley at al. 1993.[14]
AG15	CCC ACA CGCA	94 °C 5 min	32 °C 60 s	72 °C 120 s	40	Mliki at al. 2001.[15]
AX16	GTC TGT GCGG	94 °C 5 min	32 °C 60 s	72 °C 120 s	40	Mliki at al. 2001.[15]
DJ16	GTG CGC ATC AGG CCGT	94°C 5 min	38 °C 60 s	72 °C 120 s	35	Savic at al. 2015.[16]

[a]The initial denaturation took place at 95°C and lasted for 5 minutes
[b]The final polymerization lasted 5 minutes

185

The PCR products were separated on 1.5% agarose gel (ICN Biomedicals) using electrophoresis (Pharmacia LKB), stained with ethidium bromide, visualized on a UV transilluminator (Shimadzu 160UV-Vis) and photographed by gel documentation system.

Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis was used for the molecular comparison of selected isolates. The four primers were used and the amplification conditions for them were described in the papers of the above authors: DJ16 [14], SPH1 [15], AG15 and AX16 [16] (Table 2). PCR was carried out in a 25 µl volume with DreamTaqGreen Master Mix (ThermoScientific, Lithuania), 2.5 µl of the prepared bacterial DNA as template, and 0.2 µmol l^{-1} final concentration of an appropriate primer.

The amplified DNA fragments were analyzed as in the PCR reaction described above. The sizes of fragments were determined by comparison with DNA molecular weight markers GeneRuler DNA Ladder mix SM0331 (Thermo Scientific, Lithuania) and HyperLadder 50bp, Bioline, Germany.

The cluster analysis of the RAPD patterns was performed using STATISTICA 8 program.

RESULTS

Detection of enterotoxin and emetic toxin genes

The presence of enterotoxigenic genes *hbl*A, and *entFM* was detected by PCR. The *hbl*A gene specific PCR yielded an amplified product of 834 bp in 29/30 *B. cereus* isolates from stools, 23/30 isolates from food and in 24/30 isolates from the environment (Figure 1 A, B, C).
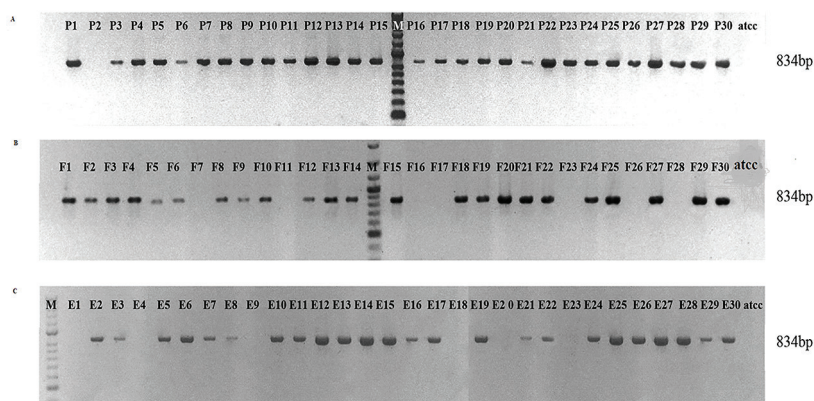

Figure 1. Detection of hblA gene by PCR method
Lane M: marker DNK 100 bp "DNA ladder"; Lane attc: Control strain B. cereus ATTC 11778; **a)** Lane P1-P30 B. cereus isolates from patient stool; **b)** Lane F1-F30 B. cereus isolates from food; **c)** Lane E1-E30 B. cereus isolates from environment

EntFM gene was detected by the primer pair ENTA/ENTB. Amplified product of 1.3 kb was present in all isolates from stools and food, and 28/30 isolates from the environment (Figure 2 A, B, C).
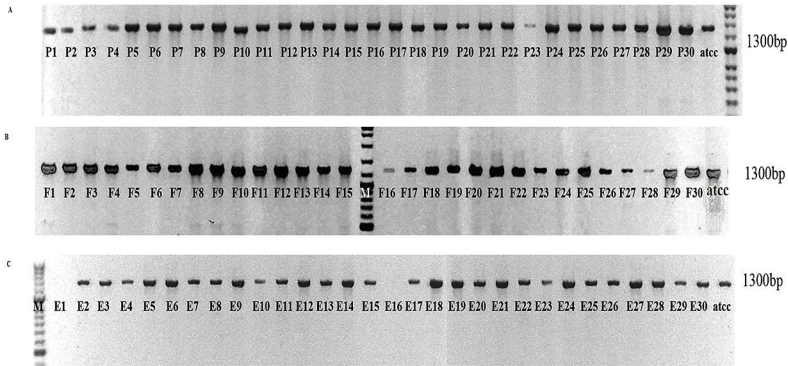

Figure 2. Detection of EntFM gene by PCR method
Lane M: marker DNK 100 bp "DNA ladder"; Lane attc: control strain B. cereus ATTC 11778; **a)** Lane P1-P30 B. cereus isolates from patient stool; **b)** Lane F1-F30 B. cereus isolates from food; **c)** Lane E1-E30 B. cereus isolates from environment

CER primer pair was used to detect the presence of the emetic toxin gene. The amplified product of 188 bp was detected in all 30 *B. cereus* isolates from stools, in 28/30 isolates from food and 25/30 isolates from the environment (Figure 3 A, B, C; Figure 4).
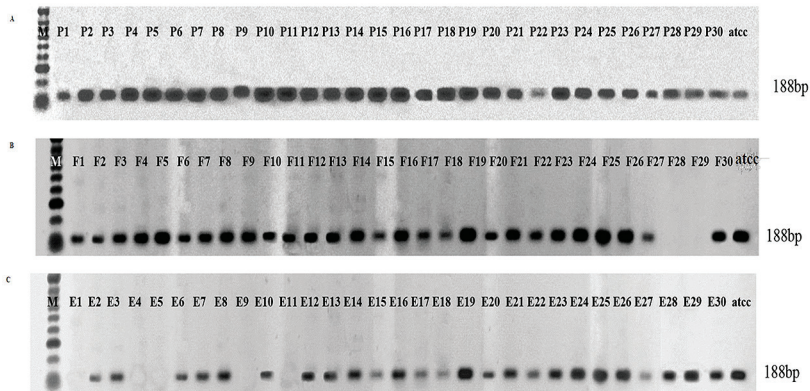

Figure 3. Detection of Cer gene by PCR method
Lane M: marker DNK 100 bp "DNA ladder"; Lane attc: control strain B. cereus ATTC 11778; **a)** Lane P1-P30 B. cereus isolates from patient stool; **b)** Lane F1-F30 B. cereus isolates from food; **c)** Lane E1-E30 B. cereus isolates from environment
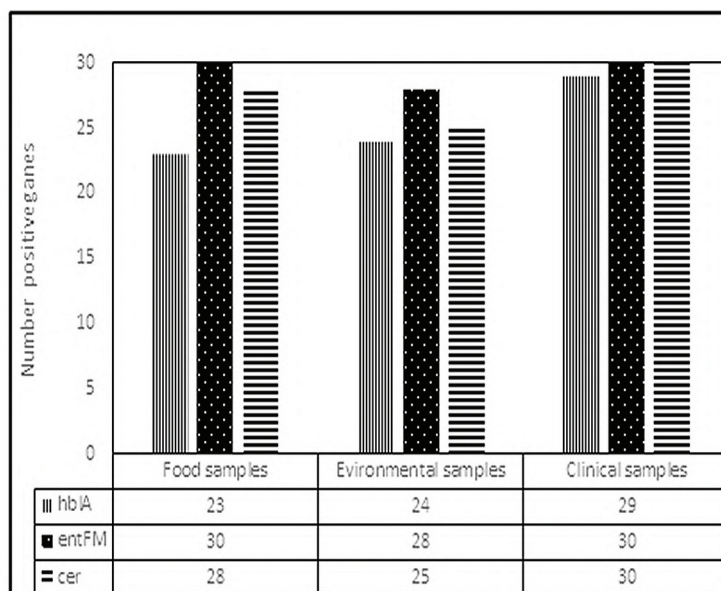
Figure 4. Presence of gene for enterotoxin and emetic toxin from tested group

RAPD analysis

RAPD patterns, obtained by amplification using four primers, were used to estimate the relationships among the *B. cereus* isolates. Within each group of isolates, two clusters (groups) were formed with a 68% difference in the patients stool, 55% in food samples and 54% in environmental samples. For the comparison of isolates between groups, on the level of mutual similarity to values of 80%, one sample for further analysis was selected from each subcluster, so that the cumulative dendrogram was obtained by RAPD analysis of 9 representative *B. cereus* isolates from stool samples (P1, P9, P8, P28, P30, P18, P10, P11, P19), 14 representative *B. cereus* food isolates (F18, F13, F6, F9, F3, F10, F21, F22, F15, F17, F24, F29, F11, F30), and 13 representative *B. cereus* isolates from the environment (E1, E5, E2, E21, E23, E9, E26, E14, E12, E28, E8, E13, E17).

The isolates were formed by two clusters, with a difference of 53%. Cluster I contained two subclasses, with a difference of 34%, and cluster II contained two subclasses with a 47% difference. Five isolates, i.e. two isolates from patients and three environmental isolates grouped in one cluster. All other isolates were grouped in another cluster. Between them, two isolates from food (F6 milk powder and F18 dietetic product) showed the highest degree of similarity: 97.5%. Another seven isolates from food (F13 beet salad, F3 oregano F10 cinnamon, F15 cheese, F29, F17 and F24 dietetic products) grouped in a branch with 83% similarity. All other subclusters and branches grouped isolates from different sources. (Figure 5).

Figure 5. RAPD analysis for the comparison of selected isolates between groups

DISCUSSION

The aim of our study was to detect genes for enterotoxins (*hbl*A, *entFM*) and emetic toxin (*cer*) and to investigate the genetic heterogeneity/similarity of *B. cereus* isolates from food, environment and human stool. The high prevalence of gene for diarrheal and emetic toxins in samples of different origin [17-19] which indicates a potential risk to human health has been confirmed by our results from all three groups of isolates tested. Similar results were obtained in several studies [20-22] confirming the presence of both genes in all tested food samples, especially in fast food [23,24]. On the other hand, there are studies [7,21] which confirmed only the presence of *entFM* gene in all food samples tested, or only significant presence of *hbl*A gene in milk and milk products samples [22]. Also there are reports [24] which confirmed a high percentage of these genes in samples from patients and food. Similar to our results a high percentage (68%) of the *hbl*A gene detection in *B. cereus* strains from the soil was confirmed, too [25]. It is of most importance to point out that all three tested genes were detected in most of our *B. cereus* isolates. Moreover, we noted that in the isolates in which the *hbl*A gene was not detected, *entFM* or *entFM* was revealed. Thus, the obtained results are consistent with the authors whose results confirm the presence of the *entFM* gene in emetic strains. We also obtained higher percentage of *cer* gene in food and clinical samples then other authors [26]. Based on the data we recorded, strains we investigated possess a substantial virulence potential, or due to *hbl*A and *entFM* genes, or due to *cer* gene. It cannot be excluded that in the same time when

189

enterotoxins are produced, cereulin is synthesized, too. We could notice that there are more toxinogenic strains in Serbia in food, environmental and clinical samples than in other countries [12,27].

Cumulative RAPD dendrogram of representative isolates of all three groups, the most food isolates grouped together at the level of 78%, indicating high genetic similarity among food isolates. The other food isolates were present in both clusters and subclasters together with patient and environmental isolates indicating the widespread distribution of genetically similar *B. cereus* strains nevertheless their different geographic distribution. Analyzing cumulative RAPD dendrograms within each group of *B. cereus* isolates, a large heterogeneity was determined. This data may indicate a greater similarity of RAPD profile between isolates within the group of patients compared to isolates from the food and environment. Similarly, Prasad et al. [8] confirmed high genetic heterogeneity among strains of *B. cereus* by RAPD in fish from tropical seas. According to Ghelardi et al. [28] who characterized *B. cereus* isolated from diseased and food samples, in two related epidemics, their findings indicated clonality between isolates from patients and from food, thus confirming the source of contamination. RAPD method with PCR for detection of toxin genes could be successfully used in the characterization of epidemic and non-epidemic strains and monitoring both clinical and environmental *B. cereus* isolates.

Generally, there are not enough data on epidemic and individual cases [29]. Recording clinical cases and monitoring of *B. cereus* infections and outbreaks in Serbia could give us relevant data on enteric and emetic diseases. Many conditions [3] impose the need to improve the diagnostics of these bacteria. As *B. cereus* is increasingly associated with non-gastrointestinal infections, controlling the presence of genes for toxin production should take a significant role in the control of intrahospital infections. Additionally, characterization of strains from food, environment and patients by RAPD analysis could provide more valuable information on the sources of infection.

In conclusion, the detection of toxins genes in all *B. cereus* isolates indicated these bacteria as potentially pathogenic and a serious threat for human health. To the best of our knowledge, this is the first study to on the presence of entero and emetic toxin genes and genetic diversity in *B. cereus* isolates from different sources in Serbia. The presence of isolates from all three groups in the same cluster suggests the existence of similar strains in the environment, food and stools of patients, which is in line with the circulation of strains in nature through the food chain.

Acknowledgment

Authors' contributions

SD collected test data, interpreted the results and drafted the manuscript. RE participated in the molecular genetic studies. MSB conceived of the study, participated in the study design, manuscript drafting and collected samples from a stool of patients. RS provided samples from hospital environment. JD collected samples from soil and typed the samples by molecular method using RAPD technique. LZ designed the study.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PRISUSTVO GENA ZA ENEROTOKSINE I EMETIČNI TOKSIN I GENETIČKA RAZNOLIKOST BAKTERIJE *BACILLUS CEREUS* IZOLOVANE IZ HRANE, OKOLINE I KLINIČKIH UZORAKA U SRBIJI

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Bacillus cereus se najčešće unosi hranom i usled prisustva toksina može izazvati povraćanje i dijareu. Sistemska infekcija je moguća. Cilj je bio da se utvrdi prisustvo gena za enterotoksine (*hbl*A, *ent*FM) i emetični toksin (*cer*) i istražiti genetsku heterogenost izolata *B. cereus* iz uzoraka hrane, životne sredine i stolice pacijenata. Identifikacija *B. cereus* je urađena upotrebom selektivne hranljive podloge, klasičnih biohemijskih testova i lančane reakcije polimeraze (PCR). Detekcija gena je rađena PCR-om. Tipizacija je urađena pomoću nasumičnog umnožavanja polimorfne DNA (RAPD). *Ent*FM gen je bio prisutan u svakom uzorku stolice i hrane, a u 28/30 izolata iz okoline. *Hbl*A gen je bio prisutan u 29/30 izolata stolice, 23/30 izolata hrane i u 24/30 izolata iz okoline. *Cer* gen je bio prisutan u svim izolatima stolice, 28/30 izolata hrane i 25/30 izolata iz okoline. RAPD rezultati pokazuju visoku heterogenost među izolatima iz svake grupe. Zbirni dendrogram dobijen od odabranih izolata iz sve tri ispitane grupe, pokazuje formiranje dva klastera sa međusobnom razlikom od 53%. Detekcija gena koji kodiraju prisustvo toksina u svim izolatima *B. cereus*, ukazuje da su ove bakterije potencijalno patogene i predstavljaju ozbiljnu pretnju po ljudsko zdravlje. Prisustvo izolata iz sve tri grupe u istom klasteru ukazuje na postojanje sličnih sojeva iz različitih izvora, što se dovodi u direktnu vezu sa kretanjem sojeva u prirodi kroz lanac ishrane.