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DETECTION OF TOXIN GENES AND RAPD ANALYSIS OF *Bacillus cereus* ISOLATES FROM DIFFERENT SOIL TYPES

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The aim of this study was to detect genes for enterotoxins (*hbl*a, *entFM* and *bceT*) and for emetic toxin (*cer*), to determine antibiotic resistance, and to estimate intraspecies diversity in *B. cereus* isolates by RAPD analysis. *B. cereus* was identified in 12 out of 117 indigenous *Bacillus* spp. using the classical microbiological methods and PCR. All isolates were resistant to penicillin and ampicillin, two to tetracyclin and four to trimethoprim-sulphamethoxazole. Also, all isolates produced inducible penicillinases and β-lactamase. Toxin genes were detected with PCR. *EntFM* and *cer* genes were present in all isolates, *hbla* in all, but two, and *bceT* in none. RAPD analysis was performed with four different primers, two of them designed for this study. The intraspecies diversity revealed 10 different patterns at the 90% similarity level. Two separate clusters were formed regardless of a soil type or utilization. The detection of genes encoding toxins in all *B. cereus* isolates indicated these bacteria as potentially pathogenic and seriously for human health. Regardless of a soil type or utilization, the RAPD analysis showed high intraspecies heterogeneity in *B. cereus* isolates. To the best of our knowledge, this is the first study to analyse the presence of entero- and emetic toxin genes and genetic heterogeneity in *B. cereus* isolates from different soil types and different soil utilization in Serbia.

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INTRODUCTION

Bacillus cereus, the gram-positive, spore-forming opportunistic human pathogen is widely spread in nature and frequently isolated from many types of soil, sediments, dust, plants and from intestinal tract of invertebrates and mammals (STENFORS ARNESEN *et al*., 2008; SAVIC *et al*., 2014). Forming endospores that are resistant to heat, dehydration and other physical stresses, a spore has the ability to survive in harsh environments over a long period of time (JÄÄSKELÄINEN, 2008). *B. cereus* has a saprophytic life cycle. Spores and vegetative cells have also been found in the gut of insects (MARGULIA *et al*., 1998), and different food and food ingredients, including rice, dairy products, spices, dried food, vegetables (LOGAN, 2011). This bacterium could germinate, grow and sporulate in soil (VILAIN *et a*l., 2006). *B. cereus* has also been isolated from stools of healthy humans (JENSEN *et al.,* 2003; STENFORS ARNESEN *et al*., 2008).

B. cereus causes two distinct types of food poisoning in human: the diarrhoeal (termolabile) and emetic (termostabile) type. Both types have serious effect on human health (EHLING-SCHULZ *et al*., 2006). Diarrhoeal diseases resulted from the ingestion of *B. cereus* spores are characterized by abdominal pain and diarrhoea, and occur 8-16 h after the ingestion of contaminated food (KONEMAN *et al.,* 1997). Emetic (cereulide) toxin is created in the gut. Its active toxic form is characterized by nausea and vomiting which occur 1-5 h after the ingestion of contaminated food (RAJKOVIC *et al*., 2006a, 2006b; TAYLOR *et al*., 2005; MAKARASEN *et al.,* 2009). The presence of toxin genes can be detected by polymerase chain reaction (PCR), targeting entero- (*hbl*a, *ent*FM and *bce*T) and emetic toxin (*cer*) genes. However, the presence of a toxin gene does not necessarily prove that the bacterium has produced the diarrhoeal and emetic toxin in the human gut.

The diversity of *B. cereus* has been assessed by phenotypic methods, such as biochemical and protein profiling and Fourier Transform Infra-Red (FTIR) typing. It has also been assessed by genotypic methods, such as repetitive PCR (rep-PCR), random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST) and ribotyping (SHANGKUAN *et al.*, 2001; EHLING-SCHULZ *et al*., 2005; VASSILEVA *et al.,* 2007; HELGASON *et al*., 2004; PRIEST *et al.,* 2004; GUINEBRETIÈRE *et al.,* 2008; MIETKE *et al.,* 2010). *B. cereus* isolates from the environments (soil, food, the dairy production chain) have a higher degree of heterogeneity as compared to clinical isolates (HELGASON *et al*., 2000; EHLING-SCHULZ *et al*., 2005).

In this study, indigenous *Bacillus* spp. was isolated from four types of soil in Serbia - stagnosol, chernozem, serpentine and contaminated soil. The aim was to prove the presence of genes for enterotoxins (*hbl*a, *entFM* and *bceT*) and emetic toxin (*cer*), to determine antibiotic resistance, and to assess intraspecies diversity in *B. cereus* isolates by RAPD analysis.

MATERIALS AND METHODS

Isolation of Bacillus spp., identification of B. cereus isolates and antibiotic sensitivity test

The soil samples were collected in Serbia from four soil types: stagnosol, chernozem, serpentine (all with different utilizations – agriculture or pasture), and contaminated soil. The bacteria were isolated from soils following a serial-dilution method and plating technique. Dilutions of the tested soil samples were heated for 10 min at 80ºC in a water bath to kill

vegetative bacteria and recover bacterial spores. The aliquots of 200 µl were spread plated onto nutrient agar plates and incubated at 28ºC for 24–48 h. Total of 117 *Bacillus* spp. were collected and subjected to the morphological and biochemical tests (Gram staining, spore formation, catalase production, glucose fermentation, starch hydrolysis and growth on 5% NaCl).
In the process of the identification of *B. cereus*, the first step was screening for the presence of β hemolyisis on 5% sheep blood agar, following the procedure of COLLINS (2001). After that, positive isolates were tested on the selective Mannitol egg yolk polymyxin agar (HiMedia, India) for *B. cereus*. At the final stage, BBL Crystal GP ID Biochemical profiles of *B. cereus* were determined with Interactive Database.

Antibiotic susceptibility was determined by the disk diffusion method on Mueller Hinton agar (HiMedia, India), as recommended by the Clinical and Laboratory Standards Institute (CLSI 2006; 2013). All discs were manufactured by Bionalyse: Ampicillin (10µg), Penicillin G (10 U), Tetracycline (30 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Vankomycin (30 µg), Imipenem (10 U) and Trimethoprim-sulphamethoxazole (1.25/23.75 µg).

The production of β-laktamases-penicilinase was determined by Cefinase test (Cef-F, *bioMérieux*), while cephalosporinases were detected using the double disc method (Ampicillin-clavulonic acid (20 µg /19 µg), Ceftazidim (30 µg), Cefotaxim (30 µg) (CLSI 2006; 2013).
B. cereus ATCC 11778 was used as positive control.

Detection of bal gene, entero- and emetic-toxin-genes

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

Polymerase chain reaction assay was used for the identification of *B. cereus* group (*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.).

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 (SANJOY et al., 2009; KIM et al., 2010). PCRs were performed on thermocycler Eppeddorf MasterCycler (Eppendorf, Germany).

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

RAPD analysis

RAPD analysis was used for the molecular comparison of selected isolates. The four primers were applied: DJ15 (5´-AGCCGTATGGAGCTG-3´) and DJ16 (5´-GTGCGCATCAGGCCGT-3´) designed for this investigation, in addition to AG15 and AX16 (MLIKI *et al.,* 2001).

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 amplification conditions for primers AG15, and

AX16 were as described earlier (MLIKI et al., 2001), and for DJ15 and DJ16: 4 min at 95°C for initial denaturation, (1 min at 95°C, 1 min at 38°C, 2 min at 72°C) for 35 cycles and 7 min at 72°C for final extension. The amplified DNA fragments were analysed as in 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 7 program.

RESULTS

Isolation of Bacillus spp., detection of B. cereus isolates and antibiotic sensitivity test

From different locations of agriculture and pasture soils in Serbia, belonging to stagnosol, chernozem or serpentine types, and contaminated soil, 117 isolates of *Bacillus* spp. were obtained. The β hemolyisis on sheep agar was observed in 34 out of 117 isolates. Using MYP agar medium, 18 samples were selected as positive and 12 of them were identified as *B. cereus* based on BBL Crystal. It was confirmed by PCR that all *B. cereus* isolates yielded 533 bp amplified fragments with primer pair BalF/BalR specific for *B. cereus* group (Fig. 1). Further analysis was carried out on twelve *B. cereus* isolates.

Disk diffusion susceptibility testing revealed that all *B. cereus* isolates were susceptible to imipenem, vancomycin, eritromicyn and ciprofloxacin (Table 1). Two (16.66%) isolates showed resistance to tetracyclin and 33.33% (4/12) were resistant to trimethoprim-sulphamethoxazole. All samples were resistant to penicillin and ampicillin. Using the Cefinase test, all isolates produced inducible penicillinases. The presence of cephalosporinases was approved with the double disc method and the production of the β-lactamase was detected in all *B. cereus* isolates.

Detection of enterotoxin- and emetic-toxin-genes

The presence of enterotoxigenic genes *hbla, bceT* and *entFM* was confirmed by use of PCR. The *hbla* gene specific PCR yielded an amplified product of 834 bp in 10 out of 12 *B. cereus* isolates (Fig. 1). Only one isolate from contaminated soil collected in Belgrade, and another isolate from serpentine soil (pasture) near Sevojno were negative (Table 1).

EntFM gene was detected by primer pair ENTA/ENTB, amplified products of 1.3 kb was present in all isolates (Fig. 1).

None of *B. cereus* isolates gave PCR products using primers BceT1/BceT2, which is specific for *bceT* gene (not shown).

CER primer pair was used to confirm the presence of emetic toxin gene. The amplified product of 188 bp was detected in all of 12 *B. cereus* isolates (Fig. 1).

Table 1. Susceptibility to antibiotics of B. cereus isolates and detection of genes for enterotoxins and emetic toxin

a

Isolate	Locality	Type of soil/ utilization	Susceptibility to selected antibiotics*		Enterotoxin genes			Emetic toxin gene
			trimethoprim-sulphame-thoxazole	tetracyclin	*hbla*	*bceT*	*entFM*	*cer*
Z1	Belgrade	contaminated	S	S	+	-	+	+
Z5	Belgrade	contaminated	S	R	-	-	+	+
Z11	Arandjelovac	contaminated	S	S	+	-	+	+
Z17	Arandjelovac	contaminated	S	R	+	-	+	+
Z19	Sevojno	serpentine /pasture	R	S	-	-	+	+
Z22	Sevojno	contaminated	S	S	+	-	+	+

* all sensitive to imipenem, vancomycin, eritromicyn, ciprofloxacin and resistant to penicillins and ampicillin

b

Isolate	Locality	Type of soil/ utilization	Susceptibility to selected antibiotics*		Enterotoxin genes			Emetic toxin gene
			trimethoprim-sulphame-thoxazole	tetracyclin	*hbla*	*bceT*	*entFM*	*cer*
Z24	Topola	stagnosol /agriculture (maize)	S	S	+	-	+	+
Z26	Pancevo	contaminated	S	S	+	-	+	+
Z29	Pancevo	chernozem/ agriculture (maize)	S	S	+	-	+	+
Z30	Pancevo	chernozem/ agriculture (red clover)	R	S	+	-	+	+
Z31	Pancevo	chernozem/ pasture	R	S	+	-	+	+
Z34	Kovin	chernozem/ pasture	R	S	+	-	+	+

* all sensitive to imipenem, vancomycin, eritromicyn, ciprofloxacin and resistant to penicillins and ampicillin

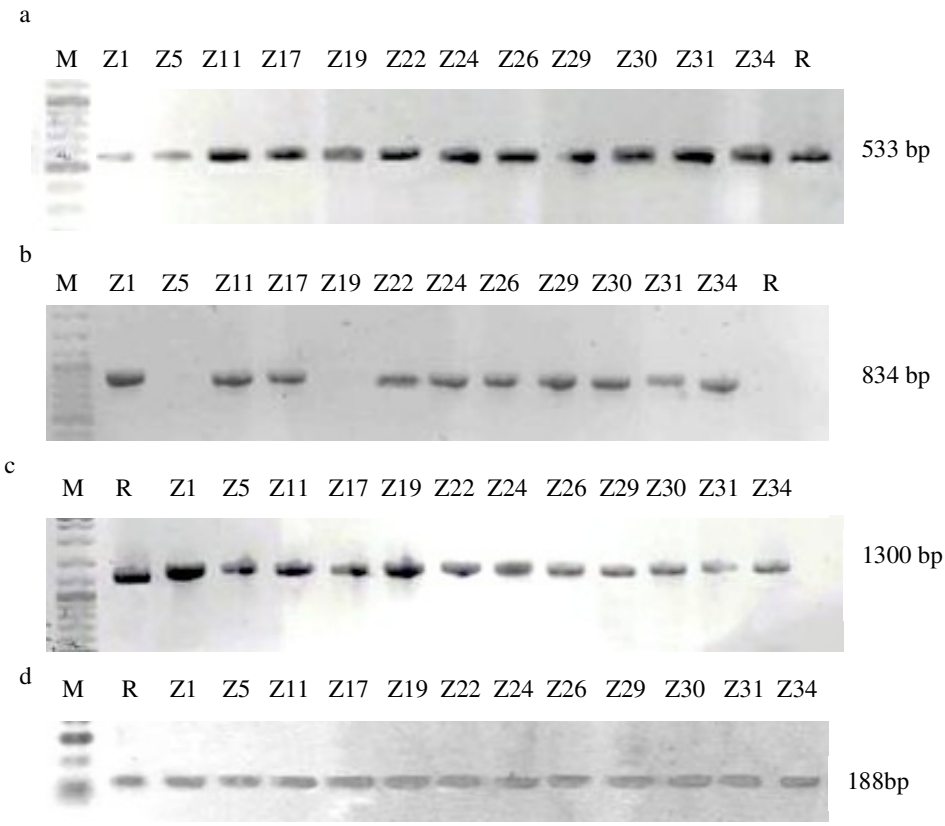

Figure 1. a: *B. cereus* group specific PCR; b: *hbla* gene specific PCR; c: *entFM* gene specific PCR; d: *cer* gene specific PCR; Line M (a,b,c): 100 bp DNA ladder; Line M (d): 50 bp DNA ladder; Lines Z1-Z34: *B. cereus* isolates; Line R: reference strain *B. cereus* ATTC 11778

RAPD

RAPD amplify genomic DNA using non-specific primers that are complementary to a number of sites within the genome. RAPD patterns, resulting from the amplification, using the four primers, two of them designed for this study, were used to estimate the relationships among the 12 studied *B. cereus* isolates (Fig. 2). Data presented in dendrogram (Fig. 3) was obtained from all amplified fragments in cumulative analysis, represented by amplicons of 57 different sizes. All isolates were clustered into two major clusters. The data showed the maximal similarity of *B. cereus* Z30 and Z31 isolates, and their closer proximity to Z34. The difference between Z22 and Z24 were less to 10% (9.8%).

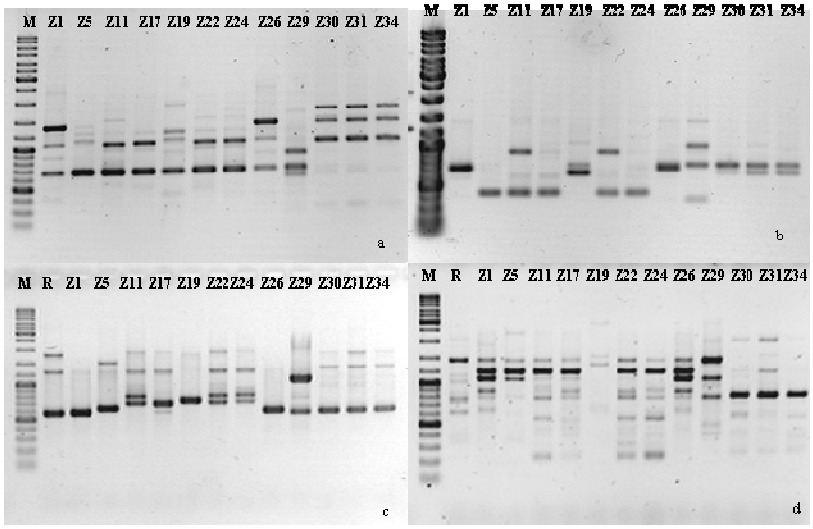

Figure 2. RAPD analysis of *B. cereus* isolates from Serbian soils using a) AG15; b) AX16; c) DJ15 and d) DJ16 primers. M- GeneRuler DNA Ladder mix SM0331 (Thermo Scientific, Lithuania); *B. cereus* isolates Z1-Z34; R- reference strain *B. cereus* ATTC 11778

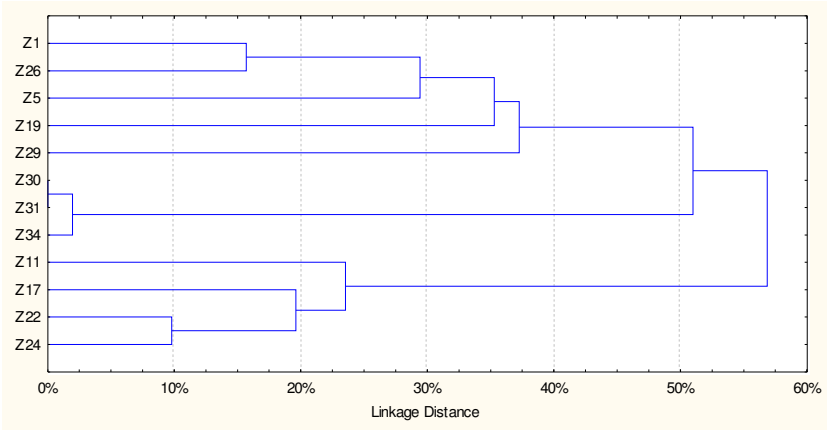

Figure 3. Dendrogram of *B. cereus* isolates similarity based on RAPD

DISCUSSION

Among 117 *Bacillus* spp. isolated from agriculture, pasture and contaminated soils in Serbia, only 12 (10.26%) of them were detected as *B. cereus* based on BBL Crystal and PCR.

All *B. cereus* isolates were resistant to penicillin and ampicillin. The total resistance to this antibiotics and cephalosporins was consequence of the production of β-lactamases which were detected by commercial method – Nitrocefin, for the production of penicilinase and double disk method for the production of cephalosporinase. Many other studies confirmed the existence of β-lactamases and therefore, the resistance to penicillin, ampicillin and cephalosporinase in different types of samples (ASLIM, 2002; SCHLEGELOVA *et al*., 2003; ABDEL-SHAKOUR and ROUSHDY, 2010). All our *B. cereus* isolates were susceptible to imipenem, vancomycin, eritromicyn and ciprofloxacin. The sensitivity to vancomicin and ciprofloxacin was confirmed by JENSEN *et al*., (2001) in *B. cereus* agricultural soil isolates from Denmark.

The resistance to tetracyclin was confirmed in two (16.66%) *B. cereus* isolates – Z5 and Z17, both originated from contaminated soil from different locations. Similar resistance to tetracyclin in few strains from soil was detected by Aslim (2002). Three our isolates from different localities under pasture soil, belonging to serpentine (Sevojno - Z19) and chernozem soil (Pancevo - Z31, and Kovin - Z34), and Z30 isolate from rhizosphere of red clover grown on chernozem, were resistant to trimethoprim-sulphamethoxazole, representing 33.33% of *B. cereus* isolates.

The detection of toxin genes made possible to discriminate between pathogenic and non-pathogenic strains of *B. cereus*. Our results suggest that the 10.26% (12/117) of the *Bacillus* spp. isolated from different soils, and having the high prevalence of toxin genes, represent a potential risk to the human health.

In our study, all *B. cereus* isolates were positive to *entFM* gene specific for the production of enterotoxin, but 2 of 12 did not have *hbla* gene. NOORTATINY and SAHILAH (2013), VYLETELOVA and BANYKO (2010) have shown similar result for the detection of *entFM* and *hbla* gene. Noortatiny and Sahilah confirmed presence of both genes in all testing samples. On the other side, in the study by Vyletelova and Banyko *ent* gene was found in all samples, while *hbla* was confirmed in 29 out of 41 samples. The presence and importance of *hbla* gene were confirmed by many other authors suggesting that its presence can indicate enterotoxic potential (MÄNTYNEN and LINDSSTRÖM, 1998). On the basis of their presence or absence in *B. cereus* isolates, these isolates can be divided into enterotoxigenic and non-enterotoxigenic (SONJOY DAS et al., 2009). However, the same authors could not found a relationship between enterotoxin diarrheal production and the presence of e*ntFM* gene, although this gene is specific for enterotoxin. The presence of the third gene specific for the production of enterotoxin (*bceT* gene) was not found in any of our isolates, just as in the results of MÄNTYNEN and LINDSSTRÖM (1998). As opposed to their and our results, AGATA *et al.,* (1995) had detected this gene in all strains they tested, and VYLETELOVA and BANYKO (2010) only in 8 of 41 isolates.

The presence of emetic toxin gene was detected by *cer* gene. All our *B. cereus* isolates confirmed the existence of this gene. KIM *et al.,* (2010) showed a higher sensitivity (100%) of CER primers than all the other primers tested in their study. They also recommended this primer pair for accurate screening of Korean emetic toxin producing *B. cereus* strains. In human medicine, a rapid detection of *B. cereus* emetic toxin gene is important for preventing potential morbidity.

RAPD method was applied to characterize *B. cereus* isolates occurring in Serbian soils and to estimate their intraspecies diversity. Using four primers in the analysis, *B. cereus* isolates

yielded 10 different patterns on the basis of the 90% similarity. Two major clusters comprising eight and four isolates with 56.86% of dissimilarity were found. The cluster 1 was predominant with eight isolates - two of them showing 100% and the third 98% similarity. Those isolates (Z30, Z31 and Z34) formed one subcluster and were found in pasture soils in two close locations (Pancevo and Kovin) and in agricultural soil when red clover was cultivated. The second subcluster formed three isolates from contaminated soils near Belgrade and Pancevo (70.59% similarity), isolate Z19 from pasture soil (Sevojno) and Z29 from maize rhizosphere with 64.71 and 62.75% similarity, respectively. The differences between two subclusters were 50.98%, which is slightly lower than the difference between two clusters (56.86%). The four isolates showing three different patterns were grouped in cluster 2 comprising three isolates from contaminated soils of different location (Arandjelovac and Sevojno). Isolates Z24 originated from agriculture soil under maize cultivation (Topola) and Z22 from contaminated soils (Sevojno) showed 90.2% similarity.

To the best of our knowledge, there are no results on the *B. cereus* prevalence and genetic heterogeneity in different soil types and utilization in Serbia. Testing 126 soil isolates of *Bacillus* spp., regardless of type and utilization, BERIC et al., (2009) reported the presence of *B. cereus* group *(B. cereus/B. thuringiensis*) in 23%, *B. subtilis* in 65% and *B. pumilus* in 12%. As a result of RAPD analysis of the 60 isolates originating from soil, straw and manure that belonged to *B. cereus/B. thuringiensis*, the same authors obtained the five distinctive groups one subgroup.

KEIM *et al.,* (2000) found high diversity in *B. cereus* and *B. thuringiensis* soil isolates collected from different geographic regions. *B. cereus* or *B. thuringiensis* strains appeared to coexist in a French forest soil (VILAS-BOAS *et al.,* 2002), but were genetically distinct and diverged to a greater extent than the strains of the same species isolated from geographically different locations (KEIM *et al*., 2000).

In conclussion, *B. cereus* is the only pathogen from *B. cereus* group which produces entero- and emetic toxins, that, in humans, can cause disease with severe clinical symptoms. Therefore, the detection of genes for these toxins is of major importance for the timely determination of pathogenic strains. In all our *B. cereus* isolates, emetic toxin gene and some of the genes for enterotoxins were detected, so that all isolates were potentially pathogenic for man.

Regardless of a soil type or soil utilization, the RAPD analysis showed high intraspecies heterogeneity in *B. cereus* isolates, with 10 different patterns on the basis of the 90% similarity. Under different utilization - as pasture and agricultural soil, a low diversity was estimated in three *B. cereus* isolates originated from chernozem in two close locations. Moreover, the two isolates from distinct location and different soil types and soil utilization (contaminated and stagnosol under maize cultivation) were similar. To the best of our knowledge, the presence of entero- and emetic toxins genes and genetic heterogeneity in *B. cereus* isolates, taken from different soil types and soil utilizations, was analysed in Serbia for the first time.

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DETEKCIJA GENA ZA PRODUKCIJU TOKSINA I RAPD ANALIZA *Bacillus cereus* IZOLATA IZ RAZLIČITIH TIPOVA ZEMLJIŠTA

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Izvod

Cilj rada bio je da se detektuju geni za toksine *B. cereus* izolata: entero- (*hbla*, *entFM* i *bceT*) i emetični toksin (*cer*), da se utvrdi osetljivost na antibiotike i detektuje raznolikost genoma između izolata *B. cereus*, RAPD analizom. Primenom klasičnih mikrobioloških metoda i PCR, *B. cereus* je identifikovan kod 12 od 117 uzoraka *Bacillus* spp. Svi izolati su bili rezistentni na penicilin i ampicilin, dva na tetraciklin i četiri na trimetoprim-sulphamethokazole. Takođe, svi izolati su produkovali penicilinaze i β-lactamaze. Geni za produkciju toksina su detektovani PCR metodom. *EntFM* i *cer* geni bili su prisutni u svim izolatima, *hbla* nije detektovan kod dva, a *bceT* ni u jednom izolatu. RAPD analiza je izvedena upotrebom četiri prajmerima, od kojih su dva dizajnirana za ovu studiju. Dobijeno je 10 različitih profila na nivou sličnosti od 90%. Izolati su grupisani u dva klastera nezavisno od tipa zemljišta ili načina upotrebe zemljišta. Prisustvo gena koji kodiraju toksine u svim *B. cereus* izolatima ukazuje da su ove bakterije potencijalno patogene i opasne po zdravlje čoveka. RAPD analiza je pokazala visoku heterogenost unutar vrste *B. cereus* kod naših izolata, bez obzira na tip ili upotrebu zemljišta. Po našem saznanju, ovo je prva studija koja analizira prisustvo gena za toksine i heterogenost genoma kod *B. cereus* izolata iz različitih tipova zemljišta u Srbiji.

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