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Antimicrobial susceptibility and β-lactamase production in *Bacillus cereus* isolates from stool of patients, food and environment samples

Osetljivost na antibiotike i proizvodnja β-laktamaza kod *Bacillus cereus* izolata iz stolice pacijenata, hrane i okoline

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Abstract

Background/Aim. *Bacillus cereus* (*B. cereus*) usually ingested by food can cause two types of diseases: vomiting due to the presence of emetic toxin and diarrheal syndrome, due to the presence of diarrheal toxins. Systemic manifestations can also occur. The severe forms of disease demand antibiotic treatmant. The aim of this study was to determine the differences in antibiotic susceptibility and β-lactamase activity of *B. cereus* isolates from stools of humans, food and environment. **Methods.** Identification of *B. cereus* was performed with selective medium, classical biochemical test and polymerase chain reaction (PCR) with primers specific for *bal* gene. Thirty isolates from each group were analysed for antibiotic susceptibility using the disk-diffusion assay. Production of β-lactamase was determined by cefinase test, and double-disc method. **Results.** All strains identified as *B. cereus* using classical biochemical test, yielded 533 bp fragment with PCR. Isolates from all the three groups were susceptible to imipenem, vancomycin, and erythromycin. All isolates were susceptible to ciprofloxacin but one from the environment. A statistically significant difference between the groups was confirmed to tetracycline and trimethoprim-sulphamethoxazole sensitivity. A total of 28/30 (93.33%) samples from the foods and 25/30 (83.33%) samples from environment were approved sensitive to tetracycline, while 10/30 (33.33%) isolates from stools were sensitive. Opposite to this result, high susceptibility to trimethoprim-sulphamethoxazole was shown in samples from stools (100%), while isolates from foods (63.33%) and from environment (70%) had low susceptibility. All samples produced β-lactamases. **Conclusion.** The strains of *B. cereus* from all the three groups showed high rate of sensitivity to most tested antibiotics, except to tetracycline in samples from human stool and to trimethoprim-sulphamethoxazole in samples from food and environment. The production of β-lactamases was confirmed in all the strains.

Key words:
bacillus cereus; anti-bacterial agents; drug resistance, microbial; beta-lactamases.

Apstrakt

Uvod/Cilj. *Bacillus cereus* (*B. cereus*) koji se u organizam čoveka unosi uglavnom putem hrane, može izazvati dva tipa oboljenja: povraćanje usled prisustva emetičkog toksina i dijarealni sindrom, usled prisustva dijarejnih toksina. Moguće su i sistemske manifestacije. Teže forme bolesti zahtevaju lečenje antibioticima. Cilj ove studije bio je da se ispitata osetljivost na antibiotike i utvrdi proizvodnja β-laktamaza kod sojeva *B. cereus* izolovanih iz stolice ljudi, hrane i okoline. **Metode.** *B.cereus* je identifikovan primenom selektivne podloge, klasičnog biohemijskog testa i metodom lančane reakcije polimeraze (PCR) pomoću prajmera specifičnih za *bal* gen. Iz svake grupe analizirana je osetljivost na antibiotike kod 30 izolata, disk-difuzionom metodom. Proizvodnja β-laktamaza rađena je Cefinaza testom i duplom disk metodom. **Rezultati.** Kod svih sojeva identifikovanih kao *B. cereus* primenom biohemijskog testa, metodom PCR umnožen je fragment od 533 bp. Izolati iz sve tri grupe bili su osetljivi na imipenem, vankomicin i eritromicin. Na ciprofloksacin su bili osetljivi svi sojevi osim jednog iz okoline. Statistički značajna razlika između grupa utvrđena je za osetljivosti na tetraciklin i trimetoprim-sulfametoksazol. 28/30 (93,33%) uzoraka iz hrane i 25/30 (83,33%) uzoraka iz okoline bili su osetljivi na tetraciklin, dok je samo 10/30 (33,33%) uzoraka stolice bilo osetljivo. Nasuprot ovim rezultatima, visoka osetljivost na trimetoprim-sulfametoksazol

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utvrđena je kod uzoraka iz stolice i iznosila je 100%, dok je kod izolata iz hrane i okoline bila niža i iznosila je 63,33% i 70%. Svi izolati proizvodili su β-laktamaze. **Zaključak.** Izolati *B. cereus* iz sve tri grupe pokazali su visoku osetljivost na većinu testiranih antibiotika, osim na tetraciklin iz uzoraka poreklom iz stolice i na trimetoprim/sulfametoksazol iz uzoraka hrane i okoline. Produkcija β-laktamaza potvrđena je za sve izolate.

Ključne reči:
bacillus cereus; antibiotici; lekovi, rezistencija mikroorganizama; beta laktamaze.

Introduction

Bacillus cereus, the Gram-positive, spore-forming opportunistic human pathogen, is found frequently as a saprophyte in the environment: many types of soils, sediment, dust and plants [1]. From all these habitats it is easily transferred to food, and to intestinal tract of invertebrates and mammals. *B. cereus* can be found in different foods and food ingredients (rice, dairy products, spices, dried foods, vegetables) and cross-contamination can distribute spores or vegetative cells to other foods (meat, milk). Spores of *B. cereus* are resistant to harsh environments, heat, dehydration, gastric acid and other physical stresses [2]. Regardless of thermal and other types of food processing, a human can be infected by spores that germinate and grow in the intestinal tract. But, disease can be caused by toxins already present in food performed by bacteria *B. cereus* that has also been isolated from stools of healthy humans [1, 3, 4].

B. cereus causes two distinct types of food poisoning in humans: the diarrhoeal (termolabile toxin) and emetic (termostabile toxin) type. Both types can seriously ruin human health [5], causing severe infections including sepsis, meningitis, endocarditis, endophthalmitis, respiratory and surgical wound infections [6]. Recently *B. cereus* was connected to hospital infection [7]. In some countries, diarrheal disease has been a major public health problem causing high morbidity and mortality among children [8].

Resistance to antibiotics is an increasing problem today. It is known that *B. cereus* has developed innate mechanisms of resistance through production of β-lactamases [9, 10]. In *B. cereus*, the production of β-lactamases can lead to resistance even up to the third generation of cephalosporins [9, 11]. Excessive use of antibiotics has led to increased antimicrobial resistance in various bacterial species [12]. Bearing in mind the circulation of *B. cereus* in nature, from soil to plants and different animals (insects, arthropods, others invertebrate and mammalian) to humans, resistance to antibiotics, under certain conditions, can be linked to transfer of resistance genes [9, 13, 14]. It is known that genes for resistance are transferred between the strains in *Bacillus* groups, and between different species [15–17]. However, wild types of strains isolated in nature and in patients, previously not exposed to effects of antibiotics and disinfectants, usually, are more sensitive to antibiotics.

Therefore, this study was conducted to determine the differences of *B. cereus* isolates from stools of patients, food and environment in antibiotic susceptibility and β-lactamase activity.

Methods

Samples

During 2013, 62 diarrhoeal stool specimens collected from outpatients and inpatients, were obtained at the Center for Microbiology, Institute of Public Health, Niš. At the same period, 40 specimens from different types of food (tea, dietary products, spices, milk powder, and ham) and 146 specimens from the environment (110 from soil, 36 from hospital environment) were collected in routine work at the Department of Sanitary Microbiology, Institute of Hygiene, Military Medical Academy, Belgrade and Department of Microbiology, Genetic Laboratory, Institute of Soil Science, Belgrade.

The samples were classified into three groups: isolates from patients, different types of food and from the environment. *B. cereus* ATCC 11778 was used as positive control.

Identification of B. cereus isolates

For identification of *B. cereus*, the first step was screening for the presence of β-hemolysis on 5% sheep blood agar, following the procedure of Collins et al. [18]. After that, positive isolates were tested on the selective Mannitol egg yolk polymyxin agar (MYP) for *B. cereus* (HiMedia, India). Detection of pink colonies and lecithinase reaction indicated that isolates belonged to *B. cereus.* In Gram-staining preparations it appeared as characteristic Gram positive, spore forming bacterium with spore not wider than the body of bacilli. In addition, *B. cereus* was determined with interactive database by using BBL Crystal GP ID Biochemical profiles.

Polymerase chain reaction and detection of bal gene

Polymerase chain reaction (PCR) assay was used for identification of *B. cereus* group (*balFR* gene), using a specific primer (Invitrogen, Vivogen D.O.O.).

For PCR, DNA samples were prepared from a single colony of each isolate of *B. cereus.*They were 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.

A PCR mixture was prepared in a volume of 25 μL, with DreamTaqGreen Master Mix (ThermoScientific, Lithuania), 200 nM 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 [19]. PCRs

were performed on thermocycler EppeddorfMasterCycler (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 the gel documentation system.

Susceptibility testing for antimicrobial agents

Sensibility of *B. cereus* isolates was tested using the disk-diffusion assay recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006) on Mueller Hinton agar (HiMedia, India) plates. Each isolate grown overnight on MYP agar at 37ºC was taken for this test. Fresh bacterial colonies were inoculated in 0.8% NaCl suspension to a turbidity equivalent to a 0.5 McFarland standard. The culture was applied on the Mueller Hinton agar plate using sterile cotton swab. Discs of ampicillin (10 μg), penicillin G (10 U), tetracycline (30 μg), trimethoprim-sulphamethoxazole (1.25/23.75 μg), erythromycin (15 μg), ciprofloxacin (5 μg), gentamicin (10 μg), vankomycin (30 μg) and imipenem (10 U); (Bionalyse, Ankara, Turkey) were placed on the plate. Plates were incubated at 37ºC for 24 h and the diameter of the inhibition zone was determined according to the CLSI (CLSI, 2013) guidelines for *Staphylococcus* spp. Based on the zone of inhibition, strains were classified as sensitive (S), intermediate (I), resistant (R). The strains with intermediate sensitivity were classified in the group of sensitive ones, to the statistical processing.

The production of β-laktamases – penicillinases was determined by cefinase test (Cef-F, *bioMérieux*, *Marcy l'Etoile*, France), while cephalosporinases were detected using double disc method (ampicillin-clavulonic acid (20 μg /19 μg), ceftazidim (30 μg), cefotaxim (30 μg) [20].

Statistical analysis

For statistical analysis the Fisher and Chi-square tests were used. A *p*-value less than 0.01 was considered statistically significant. All statistical analyses were performed with the SPSS statistical software for Windows version 11.5 (SPSS Inc., Chicago, USA).

Results

Pink colonies on MYP agar plates with positive lecithinase reaction, giving β hemolyisis on sheep agar, were used for identification with BBL Crystal. Thirty *B. cereus* isolates identified in each group were taken for further analysis. Belonging to a *B. cereus* group was confirmed by PCR. All *B. cereus* isolates from stools, food and environment yielded 533 bp amplified fragments with primer pair BalF/BalR specific for *B. cereus* group (Figure 1).

Disk diffusion susceptibility testing revealed that all *B. cereus* isolates from stool, food and environment were susceptible to imipenem and vancomycin (Table 1). Furthermore, all *B. cereus* isolates from stools of patients and from food were susceptible to erythromycin and ciprofloxacin. Similarly, all *B. cereus* isolates from environment were sensitive to erythromycin, with only one strain resistant to ciprofloxsacin (3.33%).

There was a statistically significant difference on susceptibility to tetracycline and trimethoprim-sulphamethoxazole between the samples from stools as compared to the samples from foods and the environment. The samples from different foods 28/30 (93.34%) and those from environment 25/30 (83.33%) were sensitive to tetracycline, while 10/30 (33.33%) isolates from stools of humans were sensitive to this antibiotic ($p < 0.001$, Fisher Exact Probability Test). Opposite to this result, high susceptibility to trimethoprim-sulphamethoxazole

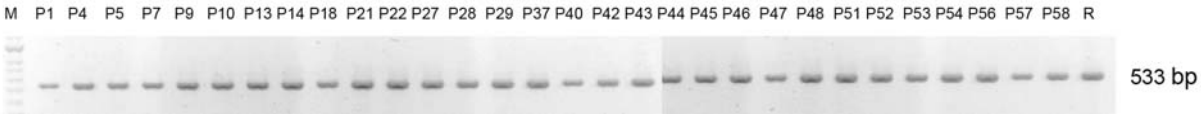

Fig. 1 – *B. cereus* group specific polymerase chain reaction (PCR). Line M: 100 bp DNA ladder; Lines P1-Z58: *B. cereus* isolates; Line R: *B. cereus* reference strain ATTC 11778.

Table 1

Susceptibilities of *B. cereus* strains to the selected antibiotics by disk diffusion susceptibility testing

Antibiotic disc (disk content)	Stool (n = 30)		Food (n = 30)		Environment (n = 30)	
	n (%)		n (%)		n (%)	
	S	R	S	R	S	R
Ampicillin (10 μg)	21 (70)	9 (30)	0	30 (100)	1 (3.33)	29 (96.67)
Penicillin	21 (70)	9 (30)	0	30 (100)	1 (3.33)	29 (96.67)
Imipenem (10 U)	30 (100)	0	30 (100)	0	30 (100)	0
Vankomycin (30 μg)	30 (100)	0	30 (100)	0	30 (100)	0
Ciprofloxacin (5 μg)	30 (100)	0	30 (100)	0	29 (96.67)	1 (3.33)
Erythromycin (15 μg)	30 (100)	0	30 (100)	0	30 (100)	0
Tetracycline (30 μg)	10 (33.33)	20 (66.67)	28 (93.33)	2 (6.67)	25 (83.33)	5 (16.67)
Trimethoprim sulphamethoxazole (1.25/23.75 μg)	30 (100)	0	19 (63.33)	11 (36.67)	21 (70)	9 (30)

S – sensitive; R – resistant

was shown in all samples from stools (100%), while strains from foods and environment in 63.33% (19/30) and 70% (21/30) samples, respectively, had low susceptibility to this antibiotic ($p < 0.01$, Fisher Exact Probability Test) (Figure 2).

All samples were resistant to penicillin and ampicillin. Using the cefinase test in all isolates the production of inducible penicillinases was detected. The presence of cephalosporinases was approved with the double-disc method and the production of these β-lactamase was detected in all *B. cereus* isolates (Figure 3).

well as acting of antibiotics from microorganisms which originated from the soil [15, 17]. Therefore, it was of interest to compare the resistance of *B. cereus* isolates from different environments.

All the tested *B. cereus* isolates were resistant to penicillin and ampicillin. Complete resistance in all strains to these antibiotics and cephalosporins was the consequence of β-lactamases production which was detected by the commercial methods: nitrocefin test and the double-disk method, for detection of penicillinases, and cephalosporinases, respec-

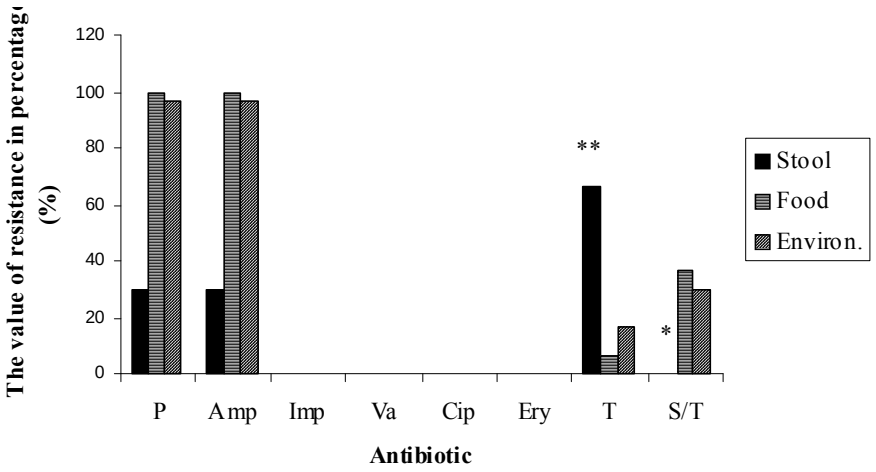

Fig. 2 – Resistance to the investigated antibiotics from different sources.
Amp: Ampicillin; P: Penicillin; Imp: Imipenem; Va: Vankomicin; Cip: Ciprofloxacin: Ery: Erythromycin; T: Tetracyclin; S/T: Trimethoprim sulphamethoxazole; **$p < 0.001$; *$p < 0.01$.
(statistically significant difference in resistance to tetracycline and trimethoprim-sulphamethoxazole was confirmed, by comparing isolates *B. cereus* from stools, food and environment).

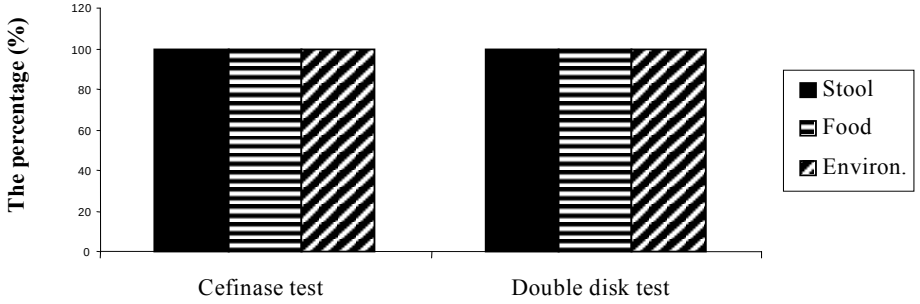

Fig. 3 – The activity of β-lactamases: penicilinases and cephalosporinases.
All *B. cereus* strains from all investigated sources produce β-lactamases.

Discussion

Pathogenic strains *B. cereus* from the environment may directly or indirectly be transmitted through food to man and cause damage to human health. In the transmission cycle, they can be exposed to different effects of environment, as

tively. Many other studies detected the existence of β-lactamases and therefore the resistance to penicillin, ampicillin and cephalosporins in different types of samples [9, 21–24]. Özcelik and Citak [11] assumed that there was the possibility of spreading resistance to β-lactam antimicrobial agents and sporadically resistance to erythromycin and tetracycline, in

strains of *B. cereus* which were isolated from ice-cream. However, there is no explanation whether the resistance of *B. cereus* strains from ice cream is because of transmission of resistant genes from microorganisms in digestive tract, process of conjugation or transduction, or strains already had resistant gene which is circulating in the environment. In addition to the presence of penicillinase and cephalosporinase, Godič-Torkar and Seme [25] confirmed the presence of metallo-β-lactamases in clinical and food samples of *B. cereus*.

All *B. cereus* strains from all the three groups investigated in this study were susceptible to imipenem, vankomycin and erythromycin. Susceptibility to the ciprofloxacin was shown in all the isolates from stools and food, but only one sample from environment was resistant to this antibiotic. Similar to this Banerjee et al. [26] received 100% sensitivity to ciprofloxacin and imipenem in samples from patients, and other authors [11, 14, 27] obtained the same result in testing sensitivity to ciprofloxacin in samples from food. Sensitivity to vancomicin and ciprofloxacin is confirmed by Jensen et al. [28] in *B. cereus* agricultural soil isolates from Denmark. In contrast to our results, Luna et al. [29] confirmed the resistance to karbapenem (meropenem) in 14% isolates from the environment in the USA. Similarly to our results, Özcelik and Citak [11] approved that only 1/34 isolates from ice-cream were resistant to erythromycin, but Oladipo and Adejumobi [14] showed the resistance to this antibiotic in all isolates from street food. In contrast to our results, Al-Khatib et al. [30] and Godič-Torkar and Seme [25] confirmed the resistance to erythromycin in about 40% samples from patient stools. Comparing the resistance to erythromycin between isolates from those of human stool, from meat and ready-to-eat meat products, Tewari et al. [10] determined the difference: 73.91% isolates from human stool were susceptible, while 48.3% and 54.5% from meat and meat products, respectively were resistant. As opposed to this, Aslim [22] and Luna et al. [29] indicated high level of sensitivity to erythromycin of *B. cereus* isolated from environment.

A statistically significant difference in the sensitivity to tetracycline and trimethoprim-sulphamethoxazole was confirmed by comparing isolates from stools, food and environment. Only 33.3% isolates from stools were sensitive to tetracycline, and 100% were sensitive to trimethoprim-sulphamethoxazole. Opposite to this result, a high rate of strains susceptible to tetracycline was shown in samples from the environment (83.33%) and from food (93.34%), but a low rate of susceptibility was detected to trimethoprim-sulphamethoxazole: from foods 63.33% and 70% in isolates from the environment. Similar to our results Özcelik and Citak [11] confirmed resistance to tetracycline in 6/34 isolates of *B. cereus* from ice cream, but Wong et al. [31] showed a high sensitivity to trimethoprim-sulphamethoxazole (78%) and slightly susceptibility to tetracycline (19%) from dairy products. The resistance to tetracycline in the strains from all samples of street vended food was confirmed by Oladipo and Adejumobi [14]. Aslim [22] found sensitivity to tetracycline in 93% samples from the soil and Luna et al. [29] showed 100% sensitivity to this antibiotic in environmental samples. However, the same authors indicate high sensitivity to trimethoprim-sulphamethoxazole (74%) in the tested samples.

We affirmed a difference in sensitivity to tetracycline and trimethoprim-sulphamethoxazole by comparing human stools samples and blood samples in suspected bacteremia [21]. In our study, the sensitivity to trimethoprim-sulphamethoxazole was found in 100% samples and 33.3% to tetracycline, but Weber et al. [21] showed 100% resistance to trimethoprim-sulphamethoxazole and 59% sensitivity to tetracycline. It is known that the resistance to tetracycline occurs through three mechanisms: producing ribosomal protection proteins, actively pumping the antibiotics out of the cell, or enzymatic degradation of antibiotics [32]. However, regardless of the mechanism of resistance, the spread of resistance is quick. Uncontrolled use of antibiotics in agriculture and food industry leads to favoring resistant strains of bacteria in the soil, and with them to transferring of the gene for resistance through food chain.

The question arises: Where does the presence of high resistance to tetracycline in samples from stool of patients and something lower resistance to trimethoprim-sulphamethoxazole in samples from food and environment come from? On the one hand, perhaps the resistance can be related to uncontrolled use of antibiotics, especially tetracycline, in agriculture and veterinary medicine. On the other hand, the resistance can be related to uncontrolled use of antibiotics by patients. In both cases it is the presence of horizontal transfer of antibiotic resistance genes from intestinal bacteria in manure to the soil bacterial population and from the soil to the animal and human population.

Conclusion

Since *B. cereus* can be associated with serious infections, it is of great importance to register the resistance to a particular antibiotic. In our study, the strains of *B. cereus* from all the three investigated groups showed a high rate sensitivity to most tested antibiotics, except to tetracycline in samples from stool of patients and to trimethoprim-sulphamethoxazole in samples tested from food and environment.

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