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TOXIN PRODUCTION ABILITY OF *BACILLIUS CEREUS* STRAINS FROM FOOD PRODUCT OF UKRAINE

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Abstract. Potential pathogens of foodborne toxic infections – bacterial contaminants *Bacillus cereus* isolated from plant raw materials and food products from the Ukrainian region were investigated. When determining of the proportion of isolated bacilli from the plant samples, it was established that the epidemiologically significant microorganisms of *Bacillus cereus* as agents of food poisoning are the second largest. The average value of contaminated samples of Ukrainian plant raw materials and processed products with *Bacillus cereus* is 36.2 %. The ability of *Bacillus cereus* strains identified by a complex of morphological, tinctorial, cultural and biochemical properties, to produce specific emetic and enterotoxins was studied. Molecular genetic diagnosis and detection of the toxin-producing ability of isolated 42 *Bacillus cereus* strains showed both the possibility of their rapid identification and the presence of specific toxicity genes. Multiplex polymerase chain reaction (PCR) was carried out with specific primers to detect toxicity determined of various bacilli genes: *nheA*, *hblD*, *cytK, cesB.* The distribution of toxigenic genes is significantly different among the *Bacillus cereus* isolates from various sources. The *nheA*, *hblD* and *cytK* enterotoxin genes were detected in 100, 83.3 and 61.9 % of the investigated strains of *Bacillus cereus,* respectively. The *cesB* gene encoding emetic toxin was detected in 4.8 % of strains. Molecular-genetic PCR-method confirmed that all the isolated strains belong to the *Bacillus cereus* group, and the ability to produce toxins can be attributed to five groups. The main toxins that produce the investigated *Bacillus cereus* strains were nhe and hbl enterotoxins encoded by the corresponding genes of *nheA* and *hblD*. The enterotoxic type of *Bacillus cereus* was predominant in Ukrainian region. Studies of domestic plant food raw materials and products have confirmed the need to improve microbiological control of product safety by introducing accelerated specific diagnostics of contaminants by molecular genetics methods.

Key words: toxin-producing *Bacillus cereus,* enterotoxins, emetic toxin, molecular genetic diagnosis, polymerase chain reaction, food safety.

ТОКСИНПРОДУКУЮЧА ЗДАТНІСТЬ ШТАМІВ *BACILLIUS CEREUS* З ХАРЧОВОЇ ПРОДУКЦІЇ УКРАЇНИ

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Анотація. Досліджено потенційні збудники харчових токсикоінфекцій – токсигенні бацилярні контамінанти *Bacillus cereus*, виділені з рослинної сировини і продукції харчової промисловості українського регіону. Середнє значення контамінованості *Bacillus cereus* зразків української рослинної сировини і продуктів її переробки становить 36,2 %. Вивчено здатність штамів *Bacillus cereus*, ідентифікованих за комплексом морфологічних, тінкторіальних, культуральних та біохімічних властивостей, продукувати характерні еметичний (блювотний) і ентеротоксини. Молекулярно-генетична діагностика і виявлення токсинпродукучої здатності виділених 42 штамів *Bacillus cereus* показали як можливість їх швидкої ідентифікації, так і наявність характерних генів токсичності. Мультиплексну полімеразну ланцюгову реакцію (ПЛР) проводили зі специфічними праймерами для виявлення токсичності, детермінованої різними генами бацил: *nheA, hblD, cytK, cesB.* Гени ентеротоксичності *nheA, hblD* та *cytK* виявлені у 100, 83,3 та 61,9 % досліджених штамів *B. cereus*, відповідно. Ген *cesB*, що кодує блювотний токсин, був виявлений у 4,8 % штамів. Молекулярно-генетичним ПЛР-методом підтверджено, що всі виділені штами відносяться до групи *Bacillus cereus*, а за здатністю виробляти токсини їх можна віднести до п'яти груп. Основними токсинами, які продукують досліджені штами *Bacillus cereus,* є ентеротоксини nhe та hbl, кодовані відповідними генами *nheA* та *hblD*. В українському регіоні переважає ентеротоксичний тип *Bacillus cereus*. Дослідження вітчизняної рослинної харчової сировини та продуктів підтвердили необхідність удосконалення мікробіологічного контролю їх безпечності шляхом впровадження прискорених специфічних діагностичних молекулярно-генетичних методів.

Ключові слова: токсинпродукуючі *Bacillus cereus,* ентеротоксини, еметичний токсин, молекулярно-генетична діагностика, полімеразна ланцюгова реакція, безпека харчових продуктів.

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Introduction. Formulation of the problem

Regulated methods of diagnosing the safety of food and raw materials are classical methods of food microbiology, which are time-taking, based on the phenotypic characteristics of microorganisms and are not always able to diagnose their toxigenic properties.

Analytical information on the inaccuracy of indication of bacillary food poisoning, the need for a preventive analysis of the risks that aerobic and facultative-anaerobic spore-forming microorganisms of the genus *Bacillus*, cause the urgency of their detection by accelerated modern methods. Such diagnostics will allow to produce new competitive food of guaranteed quality and microbiological safety [1,2].

Analysis of Literature

Food poisoning caused by the presence of *Bacillus cereus* in foodstuffs is recorded in almost all countries [3,4]. According to the Center for Disease Control and Prevention (CDC Foodborne Outbreak Online Database), more than 60000 cases of diseases caused by *B. cereus* are recorded annually in the United States. *Bacillus cereus*, a rod shapes, gram-positive, spore-forming food pathogen, play an important role as the causative agent of diarrheal and emetic types of food poisoning [3]. The diarrheal type of food poisoning is caused by heat-labile enterotoxins such as hemolysin BL (*hbl*), nonhemolytic entrotoxin (*nhe*) and cytotoxin K (*cytK*). The hbl- and nhe-complex both consist of three proteins (tripatite toxins). Cytotoxin K is a pore forming toxin cause necrotic enterotitis. The diarrheal syndrome, including abdominal pain and diarrheal symptoms, appears 8 to 16 h after ingestion of contaminated food. The emetic syndrome, which is characterized by nausea and vomiting within 1 to 5 h after ingestion of contaminated food, is causes by emetic toxin cereulide, a depsipeptide structurally related to potassium ionophore valinomycin, which is produced by a nonribosomal peptide synthetase (NRPS) and coded *cesB* gene [4].

Bacillus cereus can cause people a wide range of diseases, including food poisoning, systemic and local purulent infections, including Lightning sepsis, meningitis, brain abscess, endophthalmitis, pneumonia, endocarditis, osteomyelitis, skin gas gangrene infection, etc., and mastitis of cattle in animals. It is noted that some patients with vomiting symptoms with bacillary food infection are erroneously diagnosed with an intoxication syndrome caused by *Staphylococcus aureus*, whereas the false diarrhea-causative agent of this toxicoinfection is *Clostridium perfringens* [2-5].

Concerning the methods for the determination of *Bacillus cereus*, it is known that the characteristics of metabolic properties of the pathogen are often used as identification tests, which are part of standardized methods of analysis, and this does not always allow a clear differentiation of pathogenic agents from non-pathogenic, phenotypic-like pathogens [5]. *Bacillus cereus* group was divided into emetic- and enterotoxin-producing strains, but emetic toxin-producing *B. cereus* is difficult to detect immunochemically [6]. This reduces the probability of the results of the analysis, complicates the assessment of the prevalence of pathogens in food and raw materials and does not guarantee the unjustified defects of products.

The **aim** of this study was to identify and detect entero- and emetic toxin-producing bacteria among *Bacillus cereus* strains, isolated from Ukrainian food plant raw materials and products.

To achieve this aim, you must accomplish the following **tasks**:

1) to determine the species composition of bacilli contaminant isolated from Ukrainian food plant raw materials and products;
2) to establish the contamination of samples of plant raw materials and products of its processing with epidemiologically significant microorganisms of *B. cereus;*
3) to detect the genes of toxicity among investigated *B. cereus* strains;
4) to identify the major toxins among *B. cereus* from Ukrainian region.

Research Materials and Methods

The widespread and industrially grown kinds of vegetables, fruits, berries, in particular, green peas, beetroot, tomatoes, carrots, apples, pears, plums, peaches, dill, spinach, parsley, strawberry, a number of canned and dried products, and also spices have been investigated [5,7]. Samples of tested materials were selected according to standardized selection rules for the average sample [8,9].

The reference strain *B. cereus* ATCC 11778 and 42 bacilli strains isolated from food plant raw materials and products, and according to the results of previous studies, identified as *B. cereus* by studying their morphological, tinctorial, physiological and biochemical characteristics and fatty acid composition of cells [10]. Also in the study used collections bacilli strains: *B. cereus* UKM B-5671, *Paenibacillus polymyxa* B-5760^T, *P. macerans* B-5803^T.

Samples of food for PCR were prepared by the priority method developed by us [10]. Multiplex PCR was performed using specific primers to bacilli sequences according to Zhang et al. [11]. DNA was isolated from the samples using the SureFast® PREP Bacteria F1021 (CONGEN, Germany). The following 4 pairs of specific oligonucleotide primers for the toxicity genes were used. Also the following pairs of specific oligonucleotide primers for the *groEL* gene were used which is characteristic of all strains of the *Bacillus cereus* group (Table 1).

PCR cycles are are primary denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, elongation at 72 °C for 1 min, final elongation at 72 °C for 7 min (Thermal cycler with BioRad software, USA). Primers were chosen on the basis of literature data [11-13] and synthesized by SPC "Simesta VAAL" (Odessa, Ukraine). Composition of the mixture for PCR: supermix – 10 µl, specific olygonucleotide primers for the toxicity genes – 6 µl, DNA – 2 µl, H_2O – 2 µl, amount of PCR mixture – 20 µl. As a negative control PCR-mixture without DNA was used. Electrophoresis of PCR products

was carried out in a 1.5% agarose gel. Trisacetate buffer was used (Equipment for electrophoresis of PCR products from BioRad, USA). DNA was stained with ethidium bromide (0.5 μg/ml) and photographed with a video system (BioRad, USA) under UV light (wavelength 312 nm). A visual evaluation of the size of the formed amplicons was carried out using molecular weight markers (pBR322/BsuRI, Fermentas, Latvia).

The bacillary contaminants of the investigated samples are given in Table 2; the *Subtilis-licheniformis* group in Ukrainian food plant raw materials and products is the most numerous one. By determining of the proportion of isolated bacilli from the plant samples, it was established that the epidemiologically significant microorganisms of *B. cereus* as agents of food poisoning are the second largest.

The obtained results allow us to estimate the essential component of the epiphytic microbiota of plant material, which forms the so-called residual microbiota of products of its processing. According to a number of researches [2-5, 10,12], the control of semi-finished products and finished products is based on the determination of the presence and number of these microorganisms.

Table 1 – PCR primers used in the study

Target toxin gene	Sequence (5′–3′)	Amplicon size (bp)
nheA	GTTAGGATCACAATCACCGC	617
	ACGAATGTAATTTGAGTCGC	
hblD	ACCGGTAACACTATTCATGC	465
	GAGTCCATATGCTTAGATGC	
cytK	GTAACTTTCATTGATGATCC	800
	GAATACTAAATAATTGGTTTCC	
cesB	ACCCATCTTGCGTCATT	154
	CAGCCAAGTGAAGAATACC	
groEL	GTGCGAACCCAATGGGTCTTC	400
	CCTTGTTGTACCACTTGCTC	

The results of the research and their discussion

Table 2 – Species composition of bacilli contaminant isolated from Ukrainian food plant raw materials

Bacilli group	% of total bacilli count
Bacillus subtilis-licheniformis	20 – 37
B.cereus	10 – 31
B. megaterium	6 – 21
B. pumilis	4 – 13
B. thuringiensis	4 – 13
Paenibacillus polymyxa	3 – 14
P. macerans	2 – 9
B. circulans	2 – 7

The microbiota of plant material is diverse, but the micellar and non-mecidal mushrooms in thermally processed products are less dangerous to the consumer than spore-forming bacteria [5]. The prevailing number of bacilli –potential pathogens of food spoilage, among which the possible presence of pathogenic species (*B. cereus*), makes it urgent to search for accelerated and expressive methods for their diagnosis. In literary sources, we did not find systematic information about the microbiota of plants isolated in Ukraine; therefore the given results are new and necessary from the point of view of their practical use.

During the production of canned products, the main source of infection of *B. cereus* serves as the main raw material and auxiliary materials [4,5]. Since microorganisms in this group cause foodborne diseases and are potentially enterotoxic to humans, the ability to quickly detect *B. cereus* in plant material is crucial. Data in table 3 shows the quantitative characteristics of contaminated *B. cereus* plant material, which is processed industrially.

Table 3 – *B. cereus* contamination of plant raw materials and products of its processing

Product type	Number of samples, n	Number of samples that contain *B. cereus*	Proportion of contaminated samples, %
Dried herbs	13	8	61,5
Spices	15	8	53,3
Fresh vegetables	20	10	50,0
Dried vegetable mixes	16	7	43,7
Canned food with signs of spoilage	9	3	33,3
Fresh berries	11	3	27,3
Vegetables boiled in vacuum polymer bags	17	2	11,8
Fresh fruit	15	1	6,7

Comparing the results with those given for plants from the city of Mexico, it is possible to note practically the same trends of detection of *B. cereus* – 50.0 % and 57.0 % for the Ukrainian and Mexican regions, respectively [4]. The average value of contami-

nated samples of Ukrainian plant raw materials and processed products with *B. cereus* is 36.2 %.

Percentage of strains containing enterotoxin genes *nheA*, *hblD* and *cytK* among investigated *B. cereus* strains was 100, 83.3 and 61.9 %, respectively. The *cesB* gene encoding emetic toxin was detected in 4.8 % of strains (Table 4).

Table 4 – Distribution genes of toxicity among *Bacillus cereus* strains from different sources of Ukrainian region

Toxin gene	*Bacillus cereus* strains with genes of toxicity (n=42), isolated from				Total,%
	Vegetables	Fruits and berries	Canned products	Dried products	
nheA	16	10	8	8	100
hblD	12	7	8	8	83.3
cytK	12	4	9	1	61.9
cesB	1	-	1	-	4.8

The results suggest that the examined dried products, fruit and berries were free of the emetic toxin but not free of enterotoxins and the distribution of enterotoxic genes is significantly different among the *B. cereus* isolates from various sources. All investigated strains of *B. cereus* were divided into 5 groups according to the presence or absence of toxic genes (Table 5).

Table 5 – Emetic and enterotoxin genes profiles in *Bacillus cereus* strains from different sources of Ukrainian region

Group	*nheA*	*hblD*	*cytK*	*cesB*	Number (%) of strains (n=42)
I	+	+	+	+	2 (4.8%)
II	+	+	+	-	7 (16.6%)
III	+	+	-	-	9 (21.4%)
IV	+	-	+	-	8 (19.0%)
V	+	-	-	-	16 (38.1%)

Only 2 strains from group I (4.8 %) have to ability to cause both diarrheal and emetic type of food poisoning. Group II (7 strains, 16.6 %) contained the *nheA*, *hblD* and *cytK* enterotoxin genes, but no *cesB* encoded emetic toxin. Group V was the major patterns and represented 38.1 % strains. The reference strain *B. cereus* ATCC 11778 has all the tested genes of toxicity.

Figure 1 shows the electrophoregram of PCR products of some strains of bacilli with specific oligonucleotide primers to the *groEL* gene, which is characteristic for most representatives of the *Bacillus cereus* group, and 4 toxic genes: *nheA, hblD, cytK, cesB.*

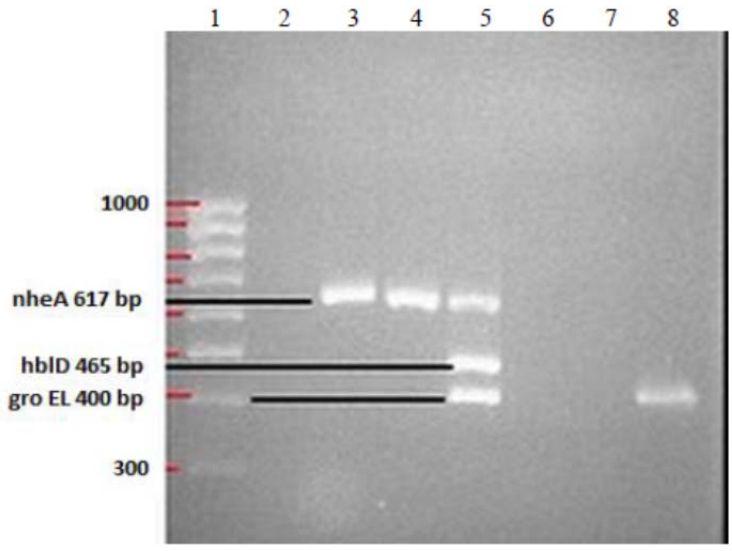

Fig. 1. Electroforegram multiplex PCR products with DNA some bacilli strains with a specific oligonucleotide primers to the *groEL, nheA, hblD, cytK, cesB* genes: 1 – MW marker (pBR322/BsuRI, Fermentas), 2 - negative control PCR; 3 - *B. cereus* П90-1 (from carrot), 4 – *B. cereus* П90-4 (from zucchini), 5 – *B. cereus* П90-9 (from eggplants), 6 – *P. macerans* B-5803^T, 7 – *P. polymyxa* B-5760^T , 8 – *B. cereus* UKM B-5671.

B. cereus П90-1 (from carrot) and *B. cereus* П90-4 (from zucchini) contain only the *nheA* toxic gene and belong to the greatest group V. *B. cereus* П90-9 (from eggplants) from group III has 2 genes of toxicity: *nheA* and *hblD.* Reference strain *B. cereus* UKM B-5671 forms only the amplicon size 400 bp to the *groEL* gene. For the use of DNA of *Paenibacillus polymyxa* and *P. macerans* no amplification product was obtained.

These finding revealed that nhe and hbl enterotoxins encoded by *nheA* and *hblD* genes were the major toxins among *B. cereus* investigated in this study and enterotoxic type of *B. cereus* was predominant in Ukrainian region.

For modern sanitary quality and safety control of food content of aerobic and facultative anaerobic microorganisms-contaminants of the genus *Bacillus* considering Ukrainian environmental conditions and principles of HACCP needs to develop molecular genetic accelerated methods of their identification. Our research of contamination of emetic- and enterotoxin-producting strains *Bacillus cereus* raw materials from Ukrainian region are original, although these results are good agreement with food products investigation from Mexican, Dutch and Korean regions [3,4,12].

Conclusions

1. By determining of the proportion of isolated bacilli from the plant samples, it was found that microorganisms of *B. cereus* group as agents of food poisoning are the second largest and consist 10 – 31 % from their total bacilli count.
2. The contamination of samples of plant raw materials and products of its processing with epidemiologically significant microorganisms of *B. cereus* was established; the greatest number of bacilli was found in dried herbs, spices and fresh vegetables (61.5, 53.3 and 50.0 %, respectively).
3. Molecular genetic diagnosis and detection of the toxin-producing ability of isolated 42 *Bacillus cereus* strains showed both the possibility of their rapid identification and the presence of specific toxicity genes: *nheA*, *hblD*, *cytK, cesB.* Nhe and hbl enterotoxins encoded by *nheA* and *hblD* genes were the major toxins among investigated *B. cereus*.
4. Enterotoxic type of *B. cereus* was predominant in Ukrainian region. Percentage of strains containing enterotoxin genes *nheA*, *hblD* and *cytK* among investigated *B. cereus* strains was 100, 83.3 and 61.9 %, respectively. The *cesB* gene encoding emetic toxin was detected only in 4.8 % of strains.

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ТОКСИНПРОДУЦИРУЮЩАЯ СПОСОБНОСТЬ ШТАММОВ *BACILLIUS CEREUS* ИЗ ПИЩЕВОЙ ПРОДУКЦИИ УКРАИНЫ

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Аннотация. Исследованы потенциальные возбудители пищевых токсикоинфекций – токсигенные бациллярные контаминанты *Bacillus cereus*, выделенные из растительного сырья и продукции пищевой промышленности украинского региона. Среднее значение контаминированности *Bacillus cereus* образцов украинского растительного сырья и продуктов его переработки составляет 36,2 %. Изучена способность штаммов *Bacillus cereus*, идентифицированных по комплексу морфологических, тинкториальных, культуральных, биохимических свойств, продуцировать характерные эметический (рвотный) и энтеротоксины. Молекулярно-генетическая диагностика и выявление токсинпродуцирующей способности выделенных 42 штаммов *Bacillus cereus* показали как возможность их быстрой идентификации, так и наличие характерных генов токсичности. Мультиплексную полимеразную цепную реакцию (ПЦР) проводили со специфическими праймерами для выявления токсичности, детерминированной различными генами бацилл*: nheA, hblD, cytK, cesB*. Гены ентеротоксичности *nheA, hblD* и *cytK* выявлены у 100, 83,3 и 61,9 % исследованных штаммов *Bacillus cereus,* соответственно. Ген *cesB*, кодирующий рвотный токсин, был обнаружен у 4,8% штаммов. Молекулярно-генетическим ПЦР-методом подтверждено, что все выделенные штаммы относятся к группе *Bacillus cereus,* а по способности вырабатывать токсины их можно отнести к пяти группам. Основными токсинами, которые продуцируют исследуемые штаммы *Bacillus cereus* были энтеротоксины nhe и hbl, кодированные соответствующими генами *nheA* и *hblD.* В украинском регионе преобладает энтеротоксический тип *Bacillus cereus*. Исследование отечественного растительного пищевого сырья и продуктов подтвердило необходимость совершенствования микробиологического контроля их безопасности путем внедрения ускоренных специфических диагностических молекулярно-генетических методов.

Ключевые слова: токсинпродуцирующие *Bacillus cereus,* энтеротоксины, эметический токсин, молекулярно-генетическая диагностика, полимеразная цепная реакция, безопасность пищевых продуктов.

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