

Isolation of *Bacillus cereus* in a Facility Preparing School Meals

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Abstract

Food safety is a fundamental requirement in mass catering, as large numbers of meals are served each day to potentially vulnerable consumers, such as children. Food Business Operators implement plans for the microbiological monitoring of the meals prepared and served in the catering sector, and for the swab-sampling of surfaces. From January 2018 to June 2019, our laboratory analyzed both food and swab samples from four catering facilities. Considering the EFSA 2018 data, we specifically focused on samples analyzed for *Bacillus cereus*. Our data substantially showed episodic contamination due to a piece of equipment that is not usually subjected to microbiological control, thus suggesting that every aspect should be scrutinized in order to identify critical points. While *Bacillus cereus* is widespread in nature and common in soil, it is adapted for growth in the intestinal tracts of insects and mammals. It is often present in a variety of foods, and may cause an emetic or a diarrheal type of food-associated illness. *B. cereus* produces several toxins. Multiplex PCR enables seven toxin genes to be detected (*hblC*, *hblD*, *hblA*, *nheA*, *nheB*, *nheC* and *cytK*).

Keywords

Bacillus cereus, Food-Borne Disease, School Meals, Enterotoxin Genes

1. Introduction

Food-borne diseases are usually infectious or toxic pathologies caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food or water. Food-borne pathogens can cause severe diarrhea or debilitating infections [1]. The *Bacillus cereus* group (*B. cereus*) comprises eight closely related species: *B. anthracis*, *B. cereus sensu stricto*, *B. cytotoxicus*, *B. my-*

coides, *B. pseudomycooides*, *B. thuringiensis*, *B. toyonensis*, and *B. weihenstephanensis* [2], few of which are known to have pathogenic potential. Although *B. cereus* is present in the environment and in many fresh and processed food products, including meat, cereal dishes, vegetables, milk products, puddings, and soups, it is not usually a health risk. Illness usually occurs when food is improperly cooked and stored in the “danger zone” (5°C to 57°C) for an extended period of time [3].

With regard to food safety, school catering facilities face specific and increasing problems. Indeed, children (aged 3 - 10 years) have weak immune systems, and any contamination of their food could cause illness. The hygiene of canteen food is therefore extremely important [4].

B. cereus causes two kinds of food-borne disease: an emetic syndrome due to the ingestion of a toxin (cereulide) pre-formed in the food, and a diarrheal syndrome due to the ingestion of bacterial cells or spores which produce enterotoxins in the small intestine [5].

Classical microbiological techniques are unable to determine which strains are pathogenic. Thus, molecular typing methods based on DNA sequence differences have been used to reveal the genetic relationships of the production of enterotoxins. Hemolysin BL (HBL), encoded by the *hblC*, *hblD* and *hblA* genes, Non-hemolytic enterotoxin (NHE), encoded by the *nheA*, *nheB* and *nheC* genes, and Cytotoxin K (CytK), encoded by the *cytK* gene, are considered to be the principal virulence factors for diarrheal syndrome, whereas the emetic syndrome is due to a small molecular weight toxin, cereulide [6].

Main sources of spoilage are foodstuffs rich in starch, such as rice, pasta and potatoes; meat, fish, vegetables, cheese and ready-to-eat meals can also be a possible source [7]. According to recent European epidemiological data [8], many cases of food-borne diseases were registered in 2017. Those caused by bacterial toxins (except *C. botulinum*), were especially related to the consumption of animal by-products and ready-to-eat dishes. Specifically, 15.1% of human cases were linked to school catering. Data on the situation in Italy reported a single outbreak, notified in 2016, involving 52 patients; this was linked to foodstuffs prepared by a catering facility and was probably caused by inadequate refrigeration of the dishes [8].

Here, we describe a case of occasional contamination of food by *B. cereus* in a facility preparing school meals. The case was observed during routine microbiological checks, and detection of enterotoxigenic virulence factors was performed at the Istituto Zooprofilattico Sperimentale del Mezzogiorno.

2. Materials and Methods

Microbiological analyses of food

A total of 101 food samples were randomly collected from four different school-meal facilities from January 2018 to June 2019 (43 samples in 2018; 58 samples in the first semester of 2019); the samples were analyzed for the *B. cereus* group in the context of the microbiological self-monitoring program of the service

provider.

All the foodstuffs were sampled with a random frequency, without prior notice, and sterile instruments and bags were used. Samples were delivered to the laboratory within a few hours of collection and kept at the proper temperature: between +60 and +65°C in the case of meals served hot and below +10°C in the case of those served cold or gastronomic meals. Alternatively, they were cooled in a blast chiller and stored under refrigerated conditions within 2 h of collection.

Samples subjected to microbiological analyses were representative of the meals served in the school canteen: first and second courses and a side dish.

Ten grams of each food sample was homogenized for 1 min with 90 ml of diluent in a stomacher; serial dilutions were prepared, and 0.1 ml of each diluted sample was streaked in MYP agar medium. Plates were incubated for 24 h at 30°C. Typical colonies are pink-orange and uniform and are surrounded by a zone of precipitation, indicating lecithinase production. These colonies were presumptively identified as *B. cereus* and counted with a colony counter, in accordance with the ISO standard method [9].

Environmental Analyses

Small equipment and cooking utensils (e.g. slicing machines, meat grinders, knives, pans and pots) and handling surfaces (e.g. tableware and chopping boards) were swabbed for microbiological examination. Swab samples were taken from all surfaces, in order to verify the appropriateness of cleaning and sanitation operations, and from the hands of the canteen staff, to assess their level of hygiene.

A total of 547 swab samples were analyzed in 2018, and 406 in the first half of 2019; in 2019, 60 swabs were analyzed for *B. cereus*. All samples were transported in a cool container, delivered to the laboratory within a few hours of collection and promptly analyzed. Analyses for the counting of *B. cereus* colonies were performed in accordance with the ISO standard method [9] [10].

DNA Extraction

Genomic DNA extracted from the positive cultures (5 colonies from each plate). Cells were collected from 2 ml of culture broth by centrifugation at 12,000 g for 5 min room temperature. The pellet was washed once with 1 ml of sterile MilliQ water, centrifuged at 12,000 g for 5 min., resuspended in 300 µl of Chelex 100 and incubated at 56°C for 20 min. The sample was then boiled for 8 min. The boiled samples were cooled in ice for 10 sec. and centrifuged at 12,000 g for 5 min.; 200 µl of the supernatant was then taken and stored at -20°C until use (in-house protocol).

Multiplex PCR

The multiplex PCR assays used to test for toxigenic *B. cereus* were developed in accordance with the method described by [6]. The primers used for multiplex PCR detection of enterotoxin genes (nheABC, cytK and hblCDA) **Table 1**. The PCR products were separated by means of the QIAxcel Advanced System (Qiagen GmbH, Hiden, Germany). This is a fully automated, sensitive, high-resolution system of capillary electrophoresis.

Table 1. Primers used for multiplex PCR.

Primer	Gene	Sequence
<i>hblC</i>	FHblC	5'-CCTATCAATACTCTCGCAA-3'
	RHblC	5'-TTTCCTTTGTTATACGCTGC-3'
<i>hblD</i>	FHD	5'-GAAACAGGGTCTCATATTCT-3'
	RHD2	5'-CTGCATCTTTATGAATATCA-3'
<i>hblA</i>	FHblA	5'-GCAAAATCTATGAATGCCTA-3'
	RHblA	5'-GCATCTGTTTCGTAATGTTTT-3'
<i>nheA</i>	F2NheA	5'-TAAGGAGGGGCAAACAGAAG-3'
	RNheA	5'-TGAATGCGAAGAGCTGCTTC-3'
<i>nheB</i>	F2NheB	5'-CAAGCTCCAGTTCATGCGG-3'
	RNheB	5'-GATCCCATGTGTACCATTG-3'
<i>nheC</i>	FNheC	5'-ACATCCTTTTGCAGCAGAAC-3'
	R2NheC	5'-CCACCAGCAATGACCATATC-3'
<i>cytK</i>	FCytK	5'-CGACGTCACAAGTTGTAACA-3'
	R2CytK	5'-CGTGTGTAATACCCCAGTT-3'

3. Results and Discussion

All samples examined in 2018 were negative for *B. cereus* counts, as were those examined up to March 2019. At the end of April 2019, however, the presence of *B. cereus* was detected in one of the four cooking facilities of the service-provider.

Of the 58 food samples analyzed in 2019, 23 (39.6%) yielded *B. cereus*-like colonies on MYP agar, giving rise to a collection of *B. cereus*-like isolates. In 13.0% of positive samples, the *B. cereus* loads were $<10^4$ cfu/g; in 56.6% of positive samples, the values were between 10^4 and 10^6 cfu/g, and 7 samples (30.4%) exceeded 10^6 cfu/g. These highest levels of contamination were found in dishes of rice or pasta with vegetables. However, contamination was detected in all the dishes, both in the main courses of fish (fillets or sticks) and in the side dishes of cooked vegetables. Nevertheless, no outbreak of illness among the schoolchildren was reported.

We then analyzed the swabs taken from the handling surfaces and equipment. These samples showed moderate positivity only in 5 swabs from pots and pans. This result did not justify the positivity observed in the various types of meals, and which was again recorded in the next sampling series, run 15 days later, following an unscheduled cleansing procedure. At this point, the entire production flow was re-examined. The hypothesis of raw material spoilage was deemed negligible, as only cooked products had proved positive; furthermore, surface sampling had yielded inconclusive results. This suggested that equipment not yet investigated might be responsible for the contamination. Microbiological tests were therefore carried out on swabs taken from such equipment, including the sealing-machine that sealed all the meal containers with plastic tape before they

left the cooking facility.

Swabs taken from the roll of plastic tape and from other parts of the sealing equipment showed the presence of *B. cereus* (from 400 to 700 cfu/swab) **Figure 1** and **Figure 2**. The sealing-machine was immediately sanitized, and from that moment on, no microbiological controls on foodstuffs revealed the presence of *B. cereus*. It is important to specify that we did not identify any safety risks associated to the presence of *B. cereus* in the cooking facility. However, an overall assessment of the risk potential linked to *B. cereus* group bacteria needs additional research, including a search for the genes encoding toxins. Indeed, according to experts of the European Authority for Food Safety [7], the only efficacious means of identifying the pathogenic strains of the *B. cereus* is mapping of the genomic sequence. On multiplex PCR, we obtained fragments of enterotoxin genes with sizes of 582, 657, 858, 996, 922, 1113 and 1245 bp. These fragments, of *cytK*, *nheC*, *nheA*, *nheB*, *hblC*, *hblA* and *hblD* genes, respectively, were obtained by using *B. cereus* ATCC 14579 as a control **Figure 3**.



Figure 1. The presence of *Bacillus cereus* 400 cfu/swab.



Figure 2. The presence of *Bacillus cereus* 700 cfu/swab.

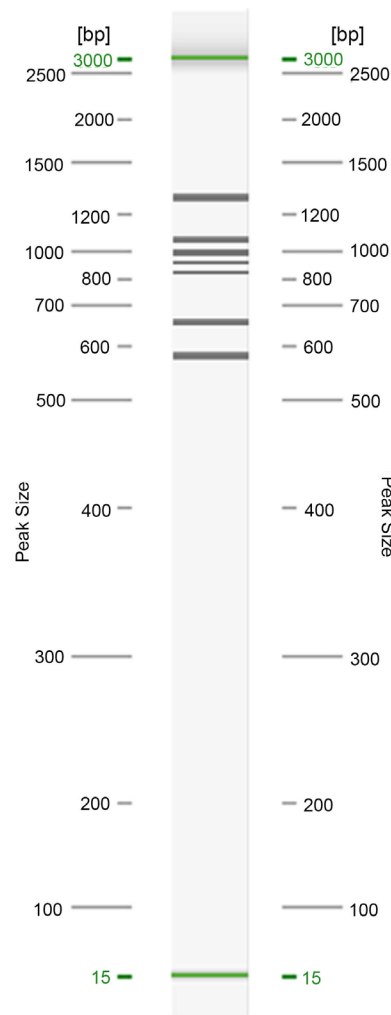


Figure 3. *Bacillus cereus* ATCC 14579.

Table 2 shows the distribution of each gene encoding these toxins in all strains isolated from samples. Of the 41 strains analyzed, 32 presented all seven genes studied. These cytotoxins are part of the virulence regulation that is activated by the transcriptional regulator PlcR (main virulence regulator of *B. cereus*), which plays a role in determining the pathogenic potential of individual strains [11]. The cytotoxins HBL, NHE and CytK are regarded as the etiological agents of food-borne *B. cereus* diarrheal disease. In the case of both NHE and HBL, all three components are necessary for maximal biological activity [11], while CytK is a single-component protein toxin.

The Hazard Analysis and Critical Control Point (HACCP) system is the internationally agreed approach to food safety management. This involves identifying and controlling potential food-borne hazards to public health, and has also become enshrined in legislation in many countries, including the EC Regulation on the Hygiene of Foodstuffs [12]. However, the present case report shows that more is required in order to ensure that systems are working effectively in practice, and are not just a “tick-the-box” approach. Indeed, our results showed that

Table 2. Distribution of enterotoxin genes in *B. cereus* strains analyzed by multiplex PCR amplification.

Haplotypes	Molecular profile	N. samples (tot. 41)	%
Haplotype 1	<i>Nhe</i> (A, B, C); <i>Hbl</i> (A, C, D); <i>CytK</i>	32	78.0
Haplotype 2	<i>Nhe</i> (A, B, C); <i>Hbl</i> (A, C); <i>CytK</i>	2	4.9
Haplotype 3	<i>Nhe</i> (A, B, C); <i>Hbl</i> (C); <i>CytK</i>	1	2.4
Haplotype 4	<i>Nhe</i> (A, B, C); <i>Hbl</i> (A); <i>CytK</i>	1	2.4
Haplotype 5	<i>Nhe</i> (A, B, C); <i>Hbl</i> (A, C, D)	2	4.9
Haplotype 6	<i>Nhe</i> (A, B, C); <i>Hbl</i> (A)	2	4.9
Haplotype 7	<i>Nhe</i> (A, B, C)	1	2.4

microbiological monitoring was a powerful verification tool, which contributed to revealing the circulation and persistence of the microorganism in the food production system.

Finally, it is encouraging to note that public institutions responsible for food safety are supporting those colleagues who conscientiously work in the private sector, in order to achieve a profitable synergy that can be of benefit to consumers.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

- [1] World Health Organization (2019). <https://www.who.int/news-room/fact-sheets/detail/food-safety>
- [2] Jiménez, G., Urdiain, M., Cifuentes, A., López-López, A., Blanch, A.R., Tamames, J., Kämpfer, P., Kolstø, A.B., Ramón, D., Martínez, J.F., Codoñer, F.M. and Rosselló-Móra, R. (2013) Description of *Bacillus toyonensis* sp. nov. a Novel Species of the *Bacillus cereus* Group, and Pairwise Genome Comparisons of the Species of the Group by Means of ANI Calculations. *Syst Appl Microbiol*, **36**, 383-391. <https://doi.org/10.1016/j.syapm.2013.04.008>
- [3] Schneider, K.R., Goodrich Schneider, R., Silverberg, R., Kurdmongkoltham, P. and Bertoldi, B. (2015) Preventing Foodborne Illness: *Bacillus cereus*. University of Florida, Food Science and Human Nutrition Department, UF/IFAS Extension.
- [4] Rahimi, E.I., Abdos, F., Momtaz, H., Baghbadorani, Z.T. and Jalali, M. (2013) *Bacillus cereus* in Infant Foods: Prevalence Study and Distribution of Enterotoxigenic Virulence Factors in Isfahan Province. *Scientific World Journal*, **2013**, 292571. <https://doi.org/10.1155/2013/292571>
- [5] (2005) *Bacillus cereus* and Other *Bacillus* spp. in Foodstuffs. *The EFSA Journal*, **175**, 1-48.
- [6] Ngamwongsatit, P., Buasri, W., Pianariyanon, P., Pulsrikarn, C., Ohba, M., Assavanig, A. and Panbangred, W. (2008) Broad Distribution of Enterotoxin Genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as Shown by Novel Primers. *International Journal of Food Microbiology*,

- 121, 352-356. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.013>
- [7] EFSA BIOHAZ Panel (2016) Scientific Opinion on the Risks for Public Health Related to the Presence of *Bacillus cereus* and Other *Bacillus* spp. including *Bacillus thuringiensis* in Foodstuffs. *EFSA Journal*, **14**, 4524. <https://doi.org/10.2903/j.efsa.2016.4524>
- [8] EFSA and ECDC (2018) The EU Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2017. *EFSA Journal*, **16**, 5500. <https://doi.org/10.2903/j.efsa.2018.5500>
- [9] UNI EN ISO 7932:2005. Microbiology of Food and Animal Feeding Stuffs. Horizontal Method for the Enumeration of Presumptive *Bacillus cereus*. Colony Count Technique at 30°C.
- [10] ISO 18593:2018. Microbiology of the Food Chain. Horizontal Methods for Surface Sampling.
- [11] Stenfors Arnesen, L.P., Fagerlund, A. and Granum, P.E. (2008) From Soil to Gut: *Bacillus cereus* and Its Food Poisoning Toxins. *FEMS Microbiology Reviews*, **32**, 579-606. <https://doi.org/10.1111/j.1574-6976.2008.00112.x>
- [12] Wallace, C.A. (2014) HACCP-Based Food Safety Management Systems: Great in Theory but Can We Really Make Them Work in Practice? *Perspectives in Public Health*, **134**, 188-190. <https://doi.org/10.1177/1757913914538735>