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Research Note

Prevalence, Genetic Diversity, and Antibiotic Susceptibility of *Bacillus cereus* Strains Isolated from Rice and Cereals Collected in Korea

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ABSTRACT

Incidence and properties of *Bacillus cereus* strains naturally present in cereals were evaluated by phenotypic characterization, antibiotic susceptibility testing, and pulsed-field gel electrophoresis. Of 293 cereal samples tested, 73 (25%) contained *B. cereus* strains. Incidence of *B. cereus* isolates varied with respect to sample; they were found in 15 (37%) of 83 brown rice samples, 23 (37%) of 63 glutinous rice samples, 16 (21%) of 76 barley samples, and 19 (27%) of 71 Job's tears samples. All *B. cereus* isolates from cereals were positive for diarrheal toxin genes. The isolates were susceptible to most of the antibiotics tested, but they were highly resistant to ampicillin, cefepime, oxacillin, and penicillin. Of the genes assayed by the PCR technique, a high frequency of *nheA* (99%) and *hblDC* (84%) was found in the genomic DNA of cereal-associated isolates, whereas *cytK* was less common (55%). From the strains carrying the *hblDC* genes, 93% produced enterotoxin HBL. *B. cereus* isolates did not have significant genetic homology. The genetic diversity and toxic potential differ among the strains isolated from cereals. These results provide important information on toxin gene profiles of cereal-associated *B. cereus* for population studies.

Bacillus cereus, a facultative aerobe and gram-positive spore-forming bacterium, produces emesis- and diarrhea-causing toxins and is the causative agent of food poisoning outbreaks and cases of gastroenteritis *(27).* The emetic type of food poisoning is induced by a 1.2-kDa heat-stable cyclic peptide, cereulide ([D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$), that causes vomiting and nausea within 1 to 5 h after consumption of contaminated food *(1, 20). B. cereus* is also involved in severe clinical human diseases such as septicemia, meningitis, and gingival and ocular infections. Diarrheal food poisoning is caused by enterotoxins, including hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (CytK) *(6, 13, 14, 19, 23).*

HBL consists of a binding component B (35 kDa), lytic component L1 (36 kDa), and lytic component L2 (45 kDa), which are encoded by the genes *hblA, hblD,* and *hblC,* respectively, in one operon *(6, 7, 17, 25).* The NHE complex consists of NheA (45 kDa), NheB (39 kDa), and NheC (37 kDa), which are encoded by the genes *nheA, nheB,* and *nheC,* respectively, also in one operon *(15).* CytK (34 kDa) is a necrotic, hemolytic, and cytotoxic pore-forming protein encoded by *cytK-1* and *cytK-2* genes. Like *Staphylococcus aureus* α-hemolysin, leucocidins, and γ-hemolysin and *Clostridium perfringens* β-toxin, CytK belongs to the family of β-barrel channel-forming toxins, which are similar in their amino acid sequences *(22, 28, 29).*

B. cereus has been isolated from a wide variety of foodstuffs, including grains, fruits, vegetables, herbs, spices, dairy products, and meat *(3, 10, 12, 16).* With the increased consumption of vegetarian foods, especially cereals and their products, food poisoning associated with *B. cereus* has become of greater concern in the food industry. Although the presence of *B. cereus* in foods can cause emetic or diarrheal poisoning, there is little information with regard to the prevalence and virulence of *B. cereus* in Korean cereals. The true incidence of diarrheal poisoning caused by *B. cereus* has been underestimated and remains unknown. Therefore, the objectives of this study were (i) to determine the incidence of *B. cereus* in rice and cereal products and (ii) to investigate the genetic diversity, biochemical characteristics, and antibiotic resistance profiles of *B. cereus* strains isolated from Korean cereals.

MATERIALS AND METHODS

Bacterial isolates and culture condition. Seventy-three *B. cereus* isolates were recovered from samples of various types of cereals (83 brown rice samples, 63 glutinous rice samples, 76 barley samples, and 71 Job's tears samples). For each sample, 25 g was dissolved into 225 ml of buffered peptone solution (Oxoid,

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TABLE 1. *List of primers used to detect* Bacillus cereus *enterotoxin genes*

Target gene	Primer	Oligonucleotide sequence	Size (bp)	Reference
nheA	BNHEF	TAGCTTAACGAATCATC	261	Y19005
	BNHER	CTCTAATAAATCTTGCTC		
hblDC	HBDF	TGGTGGTGGATTGGGAGCAGCT	179	AJ007794
	HBDR	GGAGTCCATATGCTTAGATGCTGTGACA		
	HBCF	GCAGCTCGTGAAGCAATTGAGAAAAGCA		
	HBCR	TCATCTAAATATGCTCGCTGTTCTGCTGT		
cytK	BCTF	ATGCTGTAGAAGAAACG	510	AE017268
	BCTR	CCAACCCAGTTTGCAG		

Basingstoke, UK) and homogenized by stomacher (BagMixer 400, Interscience, Saint Nom La Bretèche, France) for 10 min. The homogenized mixture was streaked onto mannitol–egg yolk–polymyxin (MYP; Difco, Becton Dickinson, Sparks, MD) agar and incubated at 37°C for 24 h. A pink colony was selected for culture on tryptone soya agar (TSA; Oxoid) and blood agar (BA; 5% sheep blood, Komed, Seoul, Korea) at 37°C for 24 h. The presumptive *B. cereus* colonies that developed β-hemolysis during culture on BA were confirmed using a Gram stain.

Three *B. cereus* reference strains (ATCC 12480, ATCC 13061, and ATCC 14579) and 12 *B. cereus* strains that were isolated from stool samples of diarrheal patients in Korea were obtained from the Korean National Institute of Health (KNIH; Division of Enteric Bacterial Infections, Center for Infectious Diseases, Seoul, Korea). These 15 strains were used as positive controls. The strains were cultivated aerobically at 37°C for 24 h and plated on MYP agar for confirmation of *B. cereus* identification.

Biochemical identification of isolates. The biochemical profile of the isolates was determined using the API 50 CHB and API 20E test strips (bioMérieux Inc., Hazelwood, MO) following the manufacturer's instructions. The API 50 CHB test strips were used to characterize utilization of 49 carbohydrates and confirmed with the apiweb software (bioMérieux).

16S rDNA target PCR. To confirm the identification of the enterotoxin-producing *B. cereus* isolates from rice and cereal samples, the 16S rDNA gene was amplified using 5′-ACAAAGGA-ACTAGGTAGACGAAGCGA-3′ and 5′-GTTCCGCCGTACTC-AGGATACATTCAA-3′ primer pairs (EMBL-GenBank accession no. AE017280). The PCR amplification was performed in 50-μl reaction volumes containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 40 mM KCl, 250 μM concentrations of each deoxynucleoside triphosphate, 30 pM concentrations of each primer, 2 U of *Taq* polymerase (Takara Taq, Takara BIO, Shiga, Japan), and 10 μl of template DNA. The following PCR conditions were used for the thermal cycler (PTC-100, MJ Research, Watertown, MA): initial denaturation for 3 min at 95°C; 40 cycles of denaturation at 94°C for 20 s, annealing at 63°C for 25 s, and extension at 72°C for 45 s; and a final cycle at 72°C for 5 min. The reaction products were separated on a 2% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer. The gels were stained with ethidium bromide and visualized under a UV transilluminator (Gel Doc 2000, Bio-Rad, Hercules, CA).

Detection of enterotoxin genes. To detect the enterotoxin genes of *B. cereus* isolates, chromosomal DNA from the isolated *B. cereus* isolates was amplified by PCR. The primer pairs using in this study are listed in Table 1. The primer pairs were designed via a BLAST search (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov/) and synthesized to amplify fragments of 261, 179, and 510 bp from *nheA, hblDC,* and *cytK* genes, respectively. PCRs were carried out in a final volume of 50 μl as for the 16S rDNA target PCR. The 40 cycles of the PCR program were completed under following conditions: denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min. Final extension was completed at 72°C for 5 min. The amplified PCR products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide. The bands were visualized using UV illumination, and images were captured with a gel documentation system (Gel Doc 2000).

Reversed passive latex agglutination test. Isolates were cultivated in Trypticase soy broth (Difco, Becton Dickinson) at 37°C for 24 h. After cultivation, the production of enterotoxin by these isolates was determined using a reversed passive latex agglutination kit (BCET-RPLA; Oxoid). One milliliter of the suspension was centrifuged for 20 min at 16,060 × *g* (Sorvall Biofuge Pico, Heraeus Instruments, Hanau, Germany). The titer of the supernatant was determined in the BCET-RPLA test according to the manufacturer's instructions up to 1:256. All *B. cereus* isolates were tested for the production of enterotoxin. Titers lower than 1:2 were interpreted as negative.

Antibiotic susceptibility testing. The Kirby-Bauer method *(4, 9)* was used to evaluate the antibiotic susceptibility profiles of the *B. cereus* strains: 73 isolates from cereal samples, 12 isolates from stool samples of diarrheal patients, and 3 ATCC strains. The antibiotics tested were ampicillin (10 μg), penicillin (10 U), gentamicin (10 μg), tetracycline (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), cefepime (30 μg), cefotetan (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), trimethoprim (1.255 μg), sulfamethoxazole (23.75 μg), oxacillin (1 μg), rifampin (5 μg), clindamycin (2 μg), and vancomycin (30 μg). The *B. cereus* strains were rated as susceptible, intermediate susceptible, and resistant.

PFGE analysis. Isolated bacterial cells were grown at 37°C for 24 h on TSA. The cultures were suspended in TE buffer (pH 7.5, 75 mM Tris and 25 mM EDTA), partially embedded in low-melting-temperature agarose (FMC Corp., Rockland, ME), and then digested with 0.5 mg of lysozyme and 5 U of lysostaphin. The plugs were transferred to a solution containing 1% sodium lauryl sarcosine, 0.5 M EDTA (pH 9.5), and 500 mg of proteinase K, and the mixture was incubated for 2 days at 56°C with gentle shaking. DNA was digested with the enzyme *Sma*I (New England Biolabs, Beverly, MA) following electrophoresis performed with the Gene Path system (Bio-Rad Laboratories, Sunbyberg, Sweden) in a 1% agarose gel in 0.5× TBE buffer at 14°C with a linear ramping time of 2.16 to 35.07 s over a period of 18 h, a 120° switch angle, and a gradient of 6.0 V/cm. After the pulsed-field

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TABLE 2. *Distribution of diarrheal toxin genes in* B. cereus *isolates*

Isolate	Source	Toxin gene: *nheA*	*hbl*	*cytK*	BCET RPLA result
YB01	Glutinous rice	+	+	−	+
YB02	Glutinous rice	+	+	−	+
YB03	Job's tears	+	+	+	+
YB04	Glutinous rice	+	+	+	+
YB05	Brown rice	+	+	+	+
YB06	Job's tears	+	+	−	+
YB07	Brown rice	+	+	+	+
YB08	Barley	+	+	+	+
YB09	Glutinous rice	+	+	+	+
YB10	Job's tears	+	+	−	+
YB11	Barley	+	+	+	+
YB12	Glutinous rice	+	+	+	+
YB13	Brown rice	+	+	−	+
YB14	Glutinous rice	+	+	−	+
YB15	Job's tears	+	+	+	+
YB16	Barley	+	+	+	+
YB17	Brown rice	+	+	+	+
YB18	Job's tears	+	−	−	−
YB19	Glutinous rice	+	+	+	+
YB20	Barley	+	−	−	−
YB21	Brown rice	+	+	−	+
YB22	Brown rice	+	-	+	−
YB23	Glutinous rice	+	+	+	+
YB24	Glutinous rice	+	+	−	+
YB25	Barley	+	−	−	−
YB26	Barley	+	−	−	−
YB27	Job's tears	+	+	+	+
YB28	Glutinous rice	+	+	−	+
YB29	Brown rice	+	+	+	+
YB30	Barley	+	+	+	+
YB31	Job's tears	+	+	+	+
YB32	Brown rice	+	+	+	+
YB33	Barley	+	+	+	+
YB34	Glutinous rice	+	+	+	+
YB35	Glutinous rice	+	+	+	+
YB36	Glutinous rice	+	+	+	+
YB37	Brown rice	+	+	−	+
YB38	Brown rice	+	+	−	−
YB39	Glutinous rice	−	+	+	+
YB40	Job's tears	+	+	−	+
YB41	Brown rice	+	+	−	+
YB42	Barley	+	+	+	+
YB43	Glutinous rice	+	−	−	+
YB44	Job's tears	+	+	−	+
YB45	Glutinous rice	+	−	−	−
YB46	Brown rice	+	+	−	+
YB47	Barley	+	+	+	+
YB48	Job's tears	+	+	+	+
YB49	Glutinous rice	+	+	−	+
YB50	Job's tears	+	−	−	−
YB51	Barley	+	+	+	+
YB52	Barley	+	+	+	+
YB53	Glutinous rice	+	+	+	+
YB54	Barley	+	+	+	+
YB55	Job's tears	+	+	+	+
YB56	Brown rice	+	+	−	+
YB57	Job's tears	+	+	−	+
YB58	Job's tears	+	+	+	+
YB59	Glutinous rice	+	+	+	+
YB60	Glutinous rice	+	+	+	+
YB61	Job's tears	+	+	+	+
YB62	Barley	+	+	−	+
YB63	Job's tears	+	+	−	+
YB64	Glutinous rice	+	+	+	+
YB65	Glutinous rice	+	−	−	+
YB66	Glutinous rice	+	+	+	−
YB67	Barley	+	+	+	+
YB68	Brown rice	+	+	+	+
YB69	Barley	+	−	−	+
YB70	Job's tears	+	+	−	−
YB71	Brown rice	+	−	−	+
YB72	Job's tears	+	−	−	−
YB73	Job's tears	+	+	−	−
86	ATCC 12480	+	+	+	+
87	ATCC 14579	+	+	+	+
88	ATCC 13061	+	+	+	+

gel electrophoresis (PFGE), the gels were stained with ethidium bromide and photographed under UV transillumination. The gels were also digitized by the computer-aided analysis system. The Molecular Analyst software package (Bio-Rad) was used for analysis. After defining each band between 145 and 582 kb, the Jacquard algorithm was used to calculate the similarity matrix.

RESULTS AND DISCUSSION

Prevalence of *B. cereus* in cereal products. In total, 293 cereal samples were analyzed for the prevalence of *B. cereus.* The *B. cereus* isolates from the cereal samples was confirmed according to phenotypic characters using API kits. Overall, 73 (25%) of 293 samples were positive for *B. cereus.* Of these positive samples, the highest frequency of *B. cereus* was found in glutinous rice (23 [37%] of 63 samples) followed by Job's tears (19 [27%] of 71 samples), barley (16 [21%] of 76 samples), and brown rice (15 [18%] of 83 samples). In comparison with the *B. cereus* isolates from cereal products (positive reactions for amygdalin in 68% of samples, for saccharose in 51% of samples, and for motility in 85% of samples), all of the *B. cereus* isolates from stool samples of diarrheal patients had positive reactions for amygdalin, saccharose, and motility based on API tests (data not shown).

Detection and distribution of enterotoxin genes. The presence of enterotoxigenic *B. cereus* isolates in cereals was confirmed by the production of diarrheal toxins. For the 73 *B. cereus* isolates from cereals, the potential enterotoxin production was examined based on a PCR-based method for detecting the presence of *nheA, hblDC,* and *cytK,* which were amplified with various primers. The isolates were confirmed as *B. cereus* based on detection of the 16S rDNA gene and various enterotoxin genes, including *hblDC, nheA,* and *cytK.* Thirty-six of the 73 *B. cereus* isolates characterized carried all three toxin encoding genes (i.e., *nheA, hblDC,* and *cytK*), whereas *nheA, hblDC,* and *cytK* were

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TABLE 3. *Antibiotic resistance of* B. cereus *strains isolated from rice and cereals and human stool samples in Korea*

Antimicrobial agent	% of isolates with indicated resistance					
	Susceptible		Intermediate		Resistant	
	Rice and cereals	Human stool	Rice and cereals	Human stool	Rice and cereals	Human stool
Ampicillin	0	0	0	0	100	100
Gentamicin	99	100	0	0	1	0
Cefepime	1	0	1	0	98	100
Cefotetan	54	59	14	8	32	33
Ciprofloxaci	98	84	1	8	1	8
Imipenem	100	100	0	0	0	0
Trimethoprim-sulfamethoxazole	99	100	1	0	0	0
Chloramphenicol	100	100	0	0	0	0
Tetracycline	85	75	8	25	7	0
Oxacillin	3	8	4	0	92	92
Penicillin	0	0	0	0	100	100
Rifampin	9	8	29	8	62	84
Erythromycin	82	75	18	25	0	0
Clindamycin	28	17	71	83	1	0
Vancomycin	90	100	0	0	10	0

detected individually in 99, 84, and 55% of isolates, respectively (Table 2). All isolates tested contained at least one of the three genes tested. These results indicate the high enterotoxigenic potential of the cereal-associated *B. cereus* strains, especially those harboring *nheA* and *hbl.* Sixty-one (83.6%) of the 73 isolates from cereals were enterotoxigenic as determined using BCET-RPLA (Table 2). The BCET-RPLA was HBL specific because it is able to detect the L2 subunit of HBL based on agglutination.

The *nheA* gene sequence was detected at a high frequency in *B. cereus* isolates, in agreement with results of previous studies in which most isolates from different food sources contained the NHE-encoding gene *(2, 16, 24)* and a high prevalence (92%) of *nheA* was detected *(24).* The prevalence of genes for the HBL enterotoxin complex also was high in *B. cereus* isolates (Table 2). Among the strains carrying *hblDC* genes, 93% (57 of 61) of *B. cereus* isolates were produced HBL, which causes the diarrheal syndrome. In general, the presence of HBL components (B, L1, and L2) is very diverse, and these components are enterotoxic only when all three components are present together in a *B. cereus* strain *(5, 26).* The prevalence of *cytK* (55% of strains) was lower than that of the other enterotoxin genes, in accordance with results of previous studies *(16, 19).* However, *B. cereus* strains carrying the *cytK* gene have high toxic potential; *cytK* is detected frequently in strains associated with diarrhea and food poisoning *(16). B. cereus* can cause two types of food poisoning syndromes: diarrhea and vomiting. The finding that 25% of the cereals were contaminated with *B. cereus* strains that carried one or more enterotoxin genes suggests that these cereals may be natural reservoirs for enterotoxigenic *B. cereus.*

Recently, the nonribosomal peptide synthetase (NRPS) genes responsible for the production of cereulide have been identified and characterized. The first PCR assay for detecting emetic toxin genes in *B. cereus* isolates from clinical cases, foods such as rice and baby food, and the environment was developed and appeared to be highly specific and sensitive *(11).* Other PCR assays with primers highly homologous to conserved regions of the NRPS genes was developed and provided evidence that cereulide is produced by peptide synthetase *(18).* Some enterotoxin-producing *B. cereus* isolates from rice and cereals carried the emetic toxin gene (data not shown). Thus, more detailed analysis will be necessary to determine the different types of toxin (enterotoxin, emetic toxin, or both toxins) produced by *B. cereus* isolates from diverse sources.

Antibiotic resistance and susceptibility of *B. cereus* isolates. The 85 *B. cereus* isolates (73 food-related strains and 12 strains obtained from stool samples from diarrheal patients) were tested for antibiotic resistance. *B. cereus* isolates from rice or cereals and from stool samples showed similar antimicrobial susceptibility profiles, but an increased resistance to rifampin was observed in *B. cereus* isolates from stool samples (Table 3). All isolates were highly susceptible to gentamicin, imipenem, chloramphenicol, and vancomycin, and all isolates were highly resistant to β-lactam antibiotics such as ampicillin, cefepime, oxacillin, and penicillin. These results were in close agreement with those of Weber et al. *(30),* who reported that *B. cereus* strains were resistant to penicillin, oxacillin, and cephalosporins and susceptible to imipenem, vancomycin, chloramphenicol, gentamicin, and ciprofloxacin. The high resistance of *B. cereus* strains to ampicillin, cefepime, oxacillin, and penicillin may be the result of the synthesis of β-lactamase *(8, 21).* The isolates were categorized as intermediately susceptible to cefotetan, tetracycline, rifampin, erythromycin, and clindamycin. *B. cereus* present in cereals are a possible source of antibiotic resistance, leading to foodborne diseases.

PFGE patterns of genomic DNA. In total, 38 isolates of *B. cereus* (32 strains isolated from cereals and 6 strains isolated from diarrheal patients and obtained from KNIH

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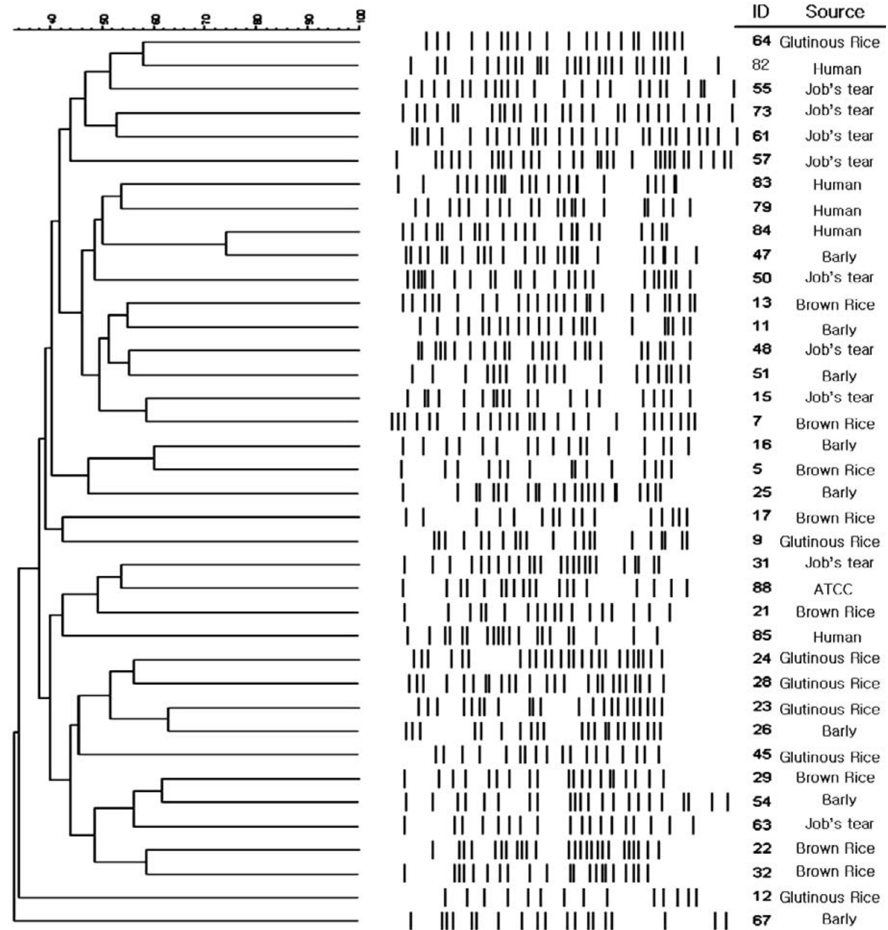

FIGURE 1. *Dendrogram of* B. cereus *isolates characterized by PFGE typing using* Sma*I as the restriction enzyme.*

[*B. cereus* ATCC 13061]) were characterized by PFGE of chromosomal DNA to examine the genetic population structure. Based on the PFGE banding patterns of genomic DNA, the strains were classified into diverse pulsotypes, corresponding to less than 35% similarity (Fig. 1). The PFGE banding patterns of isolates carrying the same diarrheal toxin genes were very different, which indicates the diversity of *B. cereus* strains present in cereal products. The PFGE banding patterns of isolates were not influenced by the distribution of enterotoxin genes such as *nheA, hblDC,* and *cytK,* and most of the *B. cereus* strains recovered were considered to be genetically unrelated.

The distribution of enterotoxin genes and the antibiotic resistance profiles determined in this study could be useful for the assessment of potential risk of diarrheal syndrome associated with *B. cereus* strains and could lead to a better understanding of the epidemiology of enterotoxic *B. cereus* strains in cereal-related products. However, an important and challenging question is whether cereals contaminated with *B. cereus* could be direct vectors of transmissible resistant and enterotoxin genes. Further studies are needed to elucidate the exact role and importance of enterotoxins that cause diarrheal food poisoning and to evaluate the relationship between antibiotic resistance and enterotoxin genes.

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