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Surveillance of *Bacillus cereus* Isolates in Korea from 2012 to 2014

Su-Mi Jung, Nan-Ok Kim, Injun Cha, Hae-young Na, Gyung Tae Chung, Hyo Sun Kawk, Sahyun Hong

Division of Enteric Diseases, Center for Infectious Diseases, National Research Institute of Health, Osong, Korea

Corresponding author: Sahyun Hong
E-mail: strepto13@hotmail.com

Objectives: To investigate the prevalence and toxin production characteristics of non-emetic and emetic *Bacillus cereus* strains isolated via the laboratory surveillance system in Korea.
Methods: A total of 667 *B. cereus* strains were collected by the Korea National Research Institute of Health laboratory surveillance system from 2012 to 2014. The collected strains were analyzed by geographical region, season, patient age, and patient sex. Additionally, the prevalence rates of enterotoxin and emetic toxin genes were evaluated.
Results: The isolation rate of *B. cereus* strains increased during the summer, but the isolation rate was evenly distributed among patient age groups. Emetic toxin was produced by 20.2% of the isolated strains. The prevalence rates of five enterotoxin genes (*entFM*, *nheA*, *cytK2*, *hblC*, and *bceT*) were 85.0, 78.6, 44.5, 36.6, and 29.7%, respectively, among non-emetic strains and 77.8, 59.3, 17.8, 11.9 and 12.6%, respectively, among emetic strains. Thus, the prevalence rates of all five enterotoxin genes were lower in emetic *B. cereus*.
Conclusion: The prevalence of enterotoxin genes differed between non-emetic and emetic *B. cereus* strains. Among emetic *B. cereus* strains, the prevalence rates of two enterotoxin genes (*cytK2* and *hblC*) were lower than those among the non-emetic strains. In both the emetic and non-emetic strains isolated in Korea, *nheA* and *entFM* were the most prevalent enterotoxin genes.

Key Words: *Bacillus cereus*, epidemiology, enterotoxins

INTRODUCTION

Foodborne diseases represent a serious threat to public health. Diarrheal disease is significantly increasing in prevalence worldwide year on year [1]. The bacterium *Bacillus cereus* is widely present in nature and can survive in harsh environments. *B. cereus* causes two types of gastrointestinal diseases: emesis and diarrhea. It produces one emetic toxin (cereulide) and several enterotoxins (*hblC*, *hblD*, *hblA*, *nheA*, *nheB*, *nheC*, *cytK2*, *entFM*, and *bceT*). Both the emesis and diarrhea caused by *B. cereus* are generally mild and self-limiting, although more serious and even lethal cases have occurred [2–4]. The diarrheal type is attributed to single or multiple enterotoxins. Particularly, a group of proteins including two heat-labile toxins and a three-component hemolysin (HBL; consisting of three proteins: B, L1, and L2) with enterotoxin activity have been purified and characterized [5]. Additionally, non-hemolytic enterotoxin (NHE, encoded by *nheA*, *nheB*, and *nheC*) is a key component contributing to *B. cereus*-mediated diarrhea [6,7]. Furthermore, single-component toxins, such as enterotoxin T (*bceT*) [8], enterotoxin FM (*entFM*) [9], and cytotoxin K (*cytK*) [10] are thought to be involved in *B. cereus* food poisoning. The pore-forming toxin, *cytK*, has two different forms, *cytK1* and *cytK2*, which have 89% amino acid

sequence homology [11,12]. Emesis is caused by a single heat-stable toxin, cereulide, which is produced in food [8,13,14]. This toxin is enzymatically synthesized by non-ribosomal peptide synthesis, and its genetic determinants are located within a 23-kb gene cluster (*ces*) on a large plasmid [2,15–17].

Several countries have reported *B. cereus* outbreaks [18–20]. Vomiting-type food poisoning is 10 times more prevalent than diarrheal-type food poisoning in Japan. However, in North America and Europe, diarrheal-type *B. cereus* infection is most frequent [21]. In Korea, 27 food poisoning outbreaks associated with *B. cereus* were reported from 2001 to 2008, but few cases of vomiting-type food poisoning caused by *B. cereus* were reported [22].

Emetic-type food poisoning caused by *B. cereus* occasionally includes symptoms of vomiting and diarrhea [7]. Therefore, the characterization of the enterotoxins produced by emetic *B. cereus* is necessary to obtain a better understanding of the food poisoning caused by this organism and to prevent misdiagnosis between diarrheal and emetic food poisoning.

EnterNet-Korea, an acute diarrheal laboratory surveillance system, was established in 2007 to improve laboratory activities and enhance reporting proficiency. This surveillance system is coordinated by the Korea National Research Institute of Health (NIH) and comprises 17 local environmental and health institutes and 70 participating hospitals. The target pathogens include 10 genera of bacteria and 5 types of viruses.

In this study, we analyzed the isolation trends and toxin gene profiles of *B. cereus* strains obtained via Enter-Net Korea from 2012 to 2014.

MATERIALS AND METHODS

1. Isolation of *B. cereus* from clinical samples

A total of 57,050 stool samples were collected by Enter-Net Korea from 2012 to 2014. The Enter-Net system is coordinated by the Korean NIH and comprises 17 local public health institutes and 105 participating hospitals. Stool samples were collected from patients who had diarrheal symptoms, and a total of 667 *B. cereus* strains were isolated from these samples.

We determined the isolation rate of *B. cereus* from the stool specimens during each 12-month period. Next, we divided patients into eight categories by age (< 10, 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, and > 70 years) and surveyed the age and gender distributions of *B. cereus* isolation rates.

Mannitol-egg yolk-polymyxin B agar (Oxoid, Basingstoke, UK) was used as a selective medium for *B. cereus* isolation. For primary identification, the isolates were characterized by standard physiological and biochemical tests using the API® 50CHB and API 20E® bacterial identification systems (bioMérieux, Marcy-l'Étoile, France).

2. Polymerase chain reaction (PCR) amplification of enterotoxin and emetic toxin genes

DNA from each isolate was extracted using the Maxwell® 16 System Purification Kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. All PCRs were performed using the Expanded High Fidelity Polymerase System (Roche, Basel, Switzerland) or *Taq* polymerase (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The PCR primer sequences used in this study are shown in Table 1.

Table 1. Primers used to amplify the target genes

Target gene	Sequence (5′ to 3′)	Size (bp)	Reference
entFM	CAAAGACTTCGTAACAAAAGGTGGT	290	Yang et al, 2005 [7]
	TGTTTACTCCGCCTTTTACAAACTT		
nheA	ATTACAGGGTTATTGGTTACAGCAGT	475	Yang et al, 2005 [7]
	AATCTTGCTCCATACTCTCTTGGATGCT		
cytK2	CAATCCCTGGCGCTAGTGCA	585	Guinebretirere et al, 2006 [12]
	GTGTAGCCTGGACGAAGTTGG		
hblC	CCTATCAATACTCTCGCAACACCAAT	386	Yang et al, 2005 [7]
	TTTTCTTGATTCGTCATAGCCATTTCT		
bceT	AGCTTGGAGCGGAGCAGACTATGT	701	Yang et al, 2005 [7]
	GTATTTCTTTCCCGCTTGCCTTTT		
cer	ATCATAAAGGTGCGAACAAGA	188	Kim et al, 2010 [22]
	AAGATCAACCGAATGCAACTG		

hblC, *nheA*, *entFM*, and *bceT* were amplified according to the methodology described by Yang et al [7]; *cytK2* was amplified as described by Guinebretiere et al [12]; and cereulide peptide synthetase (*cer*) was amplified as described by Kim et al [23].

3. Statistical analysis

The collected data were analyzed using IBM SPSS Statistics ver. 21.0 (IBM Co., Armonk, NY, USA). The distribution of toxin genes from non-emetic and emetic isolated strains was analyzed using the chi-square or chi-square trend test. For statistical analysis, differences at $p < 0.05$ were considered to be significant.

RESULTS

1. *B. cereus* isolation rates

A total of 667 (6.9%) *B. cereus* strains were isolated from 57,050 stool samples by Enter-Net. The isolation rate of *B. cereus* slightly increased in the summers of 2012 and 2013, with strong seasonality in 2014 (Figure 1A). The age distribution of isolation rates showed no specific trend from 2012 to 2014 in any of the eight age groups (Figure 1B). Lastly, we divided the isolation rates by gender and found that females showed a slightly higher isolation rate than males, albeit with no statistically significant difference (Figure 1C).

2. Detection of toxin genes by PCR

In this study, five enterotoxin genes (*hblC*, *bceT*, *cytK2*, *entFM*, and *nheA*) and one emetic toxin gene (*cer*) were detected using PCR (Table 1). Among all 667 strains, the prevalence rates of *entFM*, *nheA*, *cytK2*, *hblC*, *bceT*, and *cer* were 85.0, 78.6, 44.5, 36.6, 29.7, and 20.2%, respectively (Table 2). The emetic toxin-producing *B. cereus* strains comprised 20.2% of total strains isolated (n = 135). The prevalence rates of the assessed enterotoxin

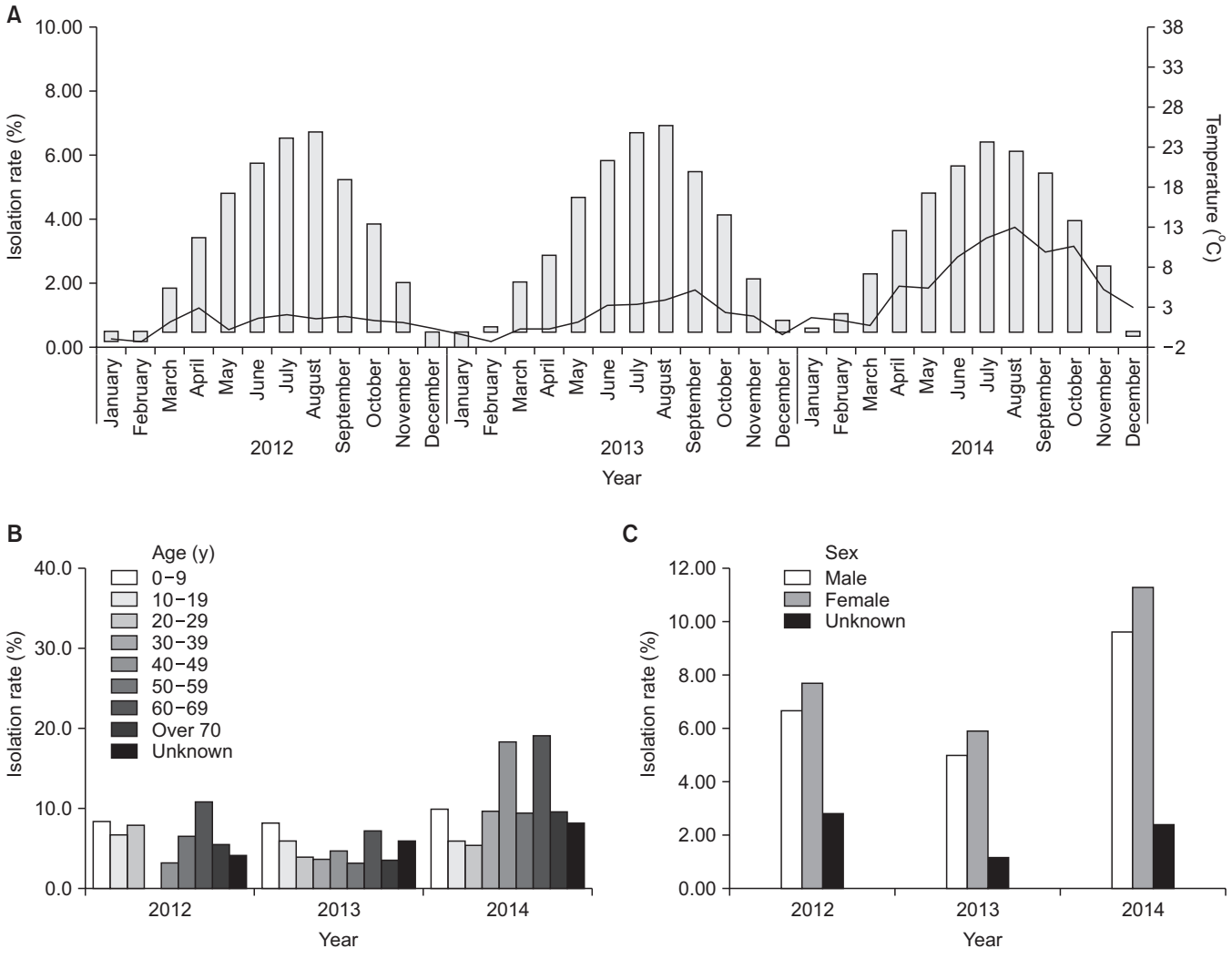

Figure 1. Isolation rates of *Bacillus cereus* by (A) year of isolation, (B) patient age, and (C) patient gender in Korea, 2012–2014.

Table 2. Presence of enterotoxin genes in emetic and non-emetic *Bacillus cereus*

Target gene	*entFM**	*nheA**	*cytK2**	*hblC**	*bceT**	*cer**
Total strains (n = 667)	567 (85.0)	524 (78.6)	297 (44.5)	244 (36.6)	198 (29.7)	135 (20.2)
Non-emetic strains (n = 532)	462 (86.8)	444 (83.5)	273 (51.3)	228 (42.9)	181 (34.0)	0 (0)
Emetic strains (n = 135)	105 (77.8)	80 (59.3)	24 (17.8)	16 (11.9)	17 (12.6)	135 (100)

Values are presented as number (%).
*$p < 0.05$ by chi-square test.

genes (*entFM*, *nheA*, *cytK2*, *hblC*, and *bceT*) among the emetic toxin-producing *B. cereus* strains were 77.8, 59.3, 17.8, 11.9, and 12.6%, respectively. Lastly, the prevalence rates of the five aforementioned enterotoxin genes among the non-emetic *B. cereus* strains were 86.8, 83.5, 51.3, 42.9, and 34.0%, respectively (Table 2).

3. Profiling of toxin genes

According to the presence or absence of enterotoxin genes, *B. cereus* strains harboring emetic and enterotoxin genes in our study could be divided into 29 and 20 groups, respectively (Table 3). In the *B. cereus* strains harboring the emetic toxin gene, the most common toxin gene profile was group F (*entFM*$^+$, *nheA*$^+$, *hblC*$^-$, *cytK2*$^-$, and *bceT*$^-$). Group F was detected in 41.5% of these strains. The next most common gene profile was group G (*entFM*$^+$), which was detected in 16.3% of emetic toxin-producing strains. Approximately 2% to 4% of *B. cereus* strains harboring the emetic toxin gene contained two to six toxin genes in total (Table 3). *B. cereus* strains harboring the emetic toxin gene, which lacked *cer* genes, were more diverse than *B. cereus* harboring the emetic toxin gene. Among non-emetic *B. cereus* strains, the group H toxin profile, which lacked *cytK2*, *hblC*, and *bceT*, was the most common (detection rate, 23.5%). The second most common toxin profile group was group A (14.5%, containing all five enterotoxin genes). Approximately 0.2% to 13% of non-emetic strains contained one to five toxin genes.

DISCUSSION

In our study, 667 (6.9%) *B. cereus* strains were isolated from 57,050 stool samples. The isolation rate of *B. cereus* increased with rising temperatures and peaked from June to September, with an especially pronounced summer peak in 2014. Additionally, the isolation rate of *B. cereus* by age was evenly distributed.

We surveyed the distribution of toxin genes among non-emetic strains (n = 532) and emetic strains (n = 135). The prevalence rates of five enterotoxin genes (*entFM*, *nheA*, *cytK2*, *hblC*, and *bceT*) were quite different between groups at 86.8, 83.5, 51.3, 42.9, and 34.0%, respectively, amongst non-emetic strains and 77.8, 59.3, 17.8, 11.9, and 12.6%, respectively, among emetic strains. The most prevalent toxin genes in the non-emetic strains were *entFM* and *nheA* (23.5%, group H, 125 strains), and those in the emetic strains were *entFM* and *nheA* (41.5%, group F, 56 strains). Non-emetic strains showed highly diverse toxin gene profiles (29 patterns) while emetic strains showed less diversity (20 patterns). Based on our results, we deduced that *B. cereus* has a high level of genetic diversity in Korea. Toxin gene profiling studies of strains from the environment and food have produced some different results from those of our survey of clinical strains. In particular, *cytK* and emetic toxin were not found in environmental isolates from silo tanks, and isolates from food dominantly harbored the *nhe* toxin gene only [24]. In another report, the most prevalent toxin genes in isolates from food were *nhe* and *entFM* [17]. In addition, a severe foodborne outbreak of diarrheal disease caused by *B. cereus* strains harboring *cytK* was reported [25,26].

As our next steps, we plan to survey the molecular profiles of all isolates using multiple locus sequence typing and/or pulsed-field gel electrophoresis.

Our results are in accordance with those of previous studies conducted in other countries, which highlighted that *nheABC* and *entFM* were carried by emetic toxin-producing strains [16,17,22,23], as well as those of previous Korean studies [23,27,28]. The *cytK* gene is frequently detected in isolates from patients with diarrheal-type food poisoning caused by *B. cereus* [26]. However, the occurrence of *cytK* was lower than that of other enterotoxin genes in our study.

Given the public health importance of acute diarrheal disease, surveillance is performed in many countries. Examples include the Foodborne Diseases Active Surveillance Network (FoodNet, www.cdc.gov/foodnet) in the United States [29]; OzFoodNet (www.ozfoodnet.gov.au) in Australia [30]; and FoodNet-Canada (www.phac-aspc.gc.ca/foodnetcanada) in Canada. These surveillance systems mainly monitor gram-negative pathogenic bacteria. In Korea, Enter-Net monitors gram-negative and gram-positive bacteria, including *B. cereus*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Staphylococcus aureus*.

Based on our findings, *B. cereus* was highly heterogeneous with a diverse genetic background. Due to such features, *B. ce-*

Table 3. Enterotoxin gene profiles of non-emetic and emetic toxin strains

Strains	Group	*entFM*	*nheA*	*cytK2*	*hblC*	*bceT*	*cer*	Strains, n (%)
Non-emetic strains (n = 532)	A	+	+	+	+	+	–	77 (14.5)
	B	+	+	+	+	–	–	71 (13.3)
	C	+	+	+	–	–	–	35 (6.6)
	D	+	+	+	–	+	–	33 (6.2)
	E	+	+	–	+	+	–	9 (1.7)
	F	+	+	–	–	+	–	20 (3.8)
	G	+	+	–	+	–	–	25 (4.7)
	H	+	+	–	–	–	–	125 (23.5)
	I	+	–	+	+	+	–	6 (1.1)
	J	+	–	+	–	+	–	10 (1.9)
	K	+	–	+	+	–	–	6 (1.1)
	L	+	–	+	–	–	–	10 (1.9)
	M	+	–	–	+	+	–	1 (0.2)
	N	+	–	–	–	+	–	7 (1.3)
	O	+	–	–	+	–	–	5 (0.9)
	P	+	–	–	–	–	–	22 (4.1)
	Q	–	+	+	+	+	–	3 (0.6)
	R	–	+	+	+	–	–	6 (1.1)
	S	–	+	+	–	–	–	9 (1.7)
	T	–	+	–	+	+	–	2 (0.4)
	U	–	+	–	–	+	–	1 (0.2)
	V	–	+	–	+	–	–	3 (0.6)
	W	–	+	–	–	–	–	25 (4.7)
	X	–	–	+	–	+	–	2 (0.4)
	Y	–	–	+	+	–	–	1 (0.2)
	a	–	–	+	–	–	–	4 (0.8)
	b	–	–	–	+	+	–	9 (1.7)
	c	–	–	–	+	–	–	4 (0.8)
	d	–	–	–	–	+	–	1 (0.2)
Emetic strains (n = 135)	A	+	+	+	+	+	+	3 (2.2)
	B	+	+	+	+	–	+	4 (3.0)
	C	+	+	+	–	+	+	4 (3.0)
	D	+	+	+	–	–	+	1 (0.7)
	E	+	+	–	+	–	+	1 (0.7)
	F	+	+	–	–	–	+	56 (41.5)
	G	+	+	–	–	+	+	4 (3.0)
	H	+	–	+	+	–	+	4 (3.0)
	I	+	–	+	+	+	+	2 (1.5)
	J	+	–	+	–	–	+	1 (0.7)
	K	+	–	+	–	+	+	1 (0.7)
	L	+	–	–	+	+	+	1 (0.7)
	M	+	–	–	–	+	+	1 (0.7)
	N	+	–	–	–	–	+	22 (16.3)
	O	–	+	+	–	–	+	1 (0.7)
	P	–	+	–	+	–	+	1 (0.7)
	Q	–	+	–	–	–	+	5 (3.7)
	R	–	–	+	–	+	+	1 (0.7)
	S	–	–	+	–	–	+	2 (1.5)
	T	–	–	–	–	–	+	20 (14.8)

reus could be an important emerging public health threat. Thus, as a preventive measure, hygiene education on diarrheal diseases should be addressed.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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