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Bacillus cereus and *Bacillus thuringiensis* spores in Korean rice: Prevalence and toxin production as affected by production area and degree of milling

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ABSTRACT

We determined the prevalence of and toxin production by *Bacillus cereus* and *Bacillus thuringiensis* in Korean rice as affected by production area and degree of milling. Rough rice was collected from 64 farms in 22 agricultural areas and polished to produce brown and white rice. In total, rice samples were broadly contaminated with *B. cereus* spores, with no effect of production area. The prevalence and counts of *B. cereus* spores declined as milling progressed. Frequencies of hemolysin BL (HBL) production by isolates were significantly ($P \leq 0.01$) reduced as milling progressed. This pattern corresponded with the presence of genes encoding the diarrheal enterotoxins. The frequency of *B. cereus* isolates positive for *hblC*, *hblD*, or *nheB* genes decreased as milling progressed. Because most *B. cereus* isolates from rice samples contained six enterotoxin genes, we concluded that *B. cereus* in rice produced in Korea is predominantly of the diarrheagenic type. The prevalence of *B. thuringiensis* in rice was significantly lower than that of *B. cereus* and not correlated with production area. All *B. thuringiensis* isolates were of the diarrheagenic type. This study provides information useful for predicting safety risks associated with *B. cereus* and *B. thuringiensis* in rough and processed Korean rice.

1. Introduction

Rice (*Oryza sativa* L.) is a major part of the diet of half of the global population (FAO, 2004). Rice can be classified as rough, brown, or white based on the degree of milling. Rough rice consists of hull, bran, and endosperm. Removal of the hull from rough rice yields brown rice. White rice is produced by removal of bran layers from brown rice during the milling process (Skyrme et al., 1998). During cultivation, harvesting, milling, and storage, rice can become contaminated by microorganisms from soil, irrigation water, animal feces, or insects (Haque and Russell, 2005; Laca et al., 2006; Choi et al., 2014). It is known that rice is frequently contaminated by *Bacillus* spp. and members of the *Enterobacteriaceae* family (Cottyn et al., 2001). Spore-forming foodborne pathogens such as some *Bacillus* spp. can survive cooking and may germinate and multiply, resulting in foodborne diseases (Jeon and Park, 2010).

Bacillus is a Gram-positive, facultatively anaerobic, motile rod-shaped bacterium that is widely distributed in nature (Granum and Lund, 1997). *Bacillus cereus* and *Bacillus thuringiensis* are well-known for their ability to cause foodborne disease in humans. *B. cereus* can cause two types of gastrointestinal diseases, i.e., diarrheal and emetic syndromes, which result from production of toxins (Blakey and Priest, 1980; Ehling-Schulz et al., 2004). Diarrheal syndrome is often associated with meat-containing foods, vegetables, sauces, and milk products and emetic syndrome is most commonly associated with the consumption of rice and other farinaceous foods (Gibbs, 2002). *B. cereus* can produce at least four types of diarrheal toxins: hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), enterotoxin FM (entFM), and cytotoxin K (CytK) (Agata et al., 1995; Asano et al., 1997; Beecher and Wong, 1994; Lund and Granum, 1996; Lund et al., 2000). Of these toxins, HBL and NHE are regarded as primary factors involved in foodborne

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disease (Bartoszewicz et al., 2008), and the prevalence of genes encoding for their production varies among strains of *B. cereus* (Granum and Lindbäck, 2013). HBL and NHE enterotoxins consist of three component proteins. HBL consists of a binding protein (B) and lytic proteins (L_1 and L_2); these components are encoded by the *hblA*, *hblD*, and *hblC* genes (Beecher and Macmillan, 1991; Heinrichs et al., 1993; Ryan et al., 1997). NHE consists of NHEA, NHEB, and NHEC, encoded by the *nheABC* operon (Granum et al., 1999). More recently, Lindbäck et al. (2010) reported that both NHEB and NHEC are required for membrane binding and complex formation, and NHEA triggers the toxicity. CytK is a single protein encoded by the *cytK* gene (Lund et al., 2000). EntFM is encoded by the *entFM* gene and has not been directly implicated with foodborne illness. However, it is the most prevalent enterotoxin gene in *B. cereus* (Bhunia, 2008). The emetic toxin produced by *B. cereus*, known as cereulide, is a small, heat-, pH-, and protease-stable peptide. Cereulide, a cyclic dodecadepsipeptide, has activity as a potassium ionophore (Agata et al., 1994). *B. thuringiensis* is also known to produce enterotoxins but the occurrence of outbreaks of *B. thuringiensis* intoxication may be underestimated because general laboratory procedures do not differentiate this pathogen from *B. cereus* (Granum and Lund, 1997). It has been reported that *B. thuringiensis* frequently has genes for HBL, NHE, and CytK (Swiecicka et al., 2006).

To date, most studies focused on toxigenic *Bacillus* contamination of rice have reported the population or toxin-producing potential of *B. cereus* and/or *B. thuringiensis* without considering the geographical production area or degree of milling (Ankolekar et al., 2009; Agata et al., 2002; Jeon and Park, 2010; Kim et al., 2009; Sarrías et al., 2003). The objectives of this study were to determine the presence and toxin-producing potential of *B. cereus* and *B. thuringiensis* in rice produced in various areas in Korea and in rice from the same production areas as affected by the degree of milling.

2. Materials and methods

2.1. Rice samples

Rough, brown, and white rice produced in 22 agricultural areas in Korea were obtained from the Korea Food Research Institute (Seongnam, Gyeonggi). In October and November 2011, rough rice was collected from 64 farms in these areas and milled to obtain brown rice and white rice at rice-processing complexes in each region. The regions and agricultural areas where rice was produced are listed in Table 1.

Table 1
Regions and agricultural areas where the rice samples were produced.[a]

Region	Agricultural areas	
Gyeonggi	Bukpaju	(1 farm)
	Anseong	(1 farms)
	Hwaseong	(3 farms)
Gangwon	Hoengseong	(10 farms)
Chungbuk	Eumseong	(1 farm)
	Jinchun	(4 farms)
Chungnam	Sejong	(1 farm)
	Yesan	(7 farms)
	Dangjin	(1 farm)
Jeonbuk	Iksan	(1 farm)
	Seogimje	(1 farm)
	Jungeup	(7 farms)
Jeonnam	Hampyeong	(1 farm)
	Naju	(1 farm)
	Boseong	(5 farms)
	Youngam	(1 farm)
Gyeongbuk	Andong	(1 farm)
	Sangju	(1 farm)
	Gyeongju	(1 farm)
	Uiseong	(7 farms)
Gyeongnam	Ulsan	(6 farms)
	Gimhae	(2 farms)

[a] In October and November 2011, rough rice was collected from 64 farms in these regions and milled to obtain brown rice and white rice at rice-processing complexes.

2.2. Prevalence of *B. cereus* and *B. thuringiensis* spores in rice as affected by production area and degree of milling

Rice (5 g) was placed in a polyolefin bag (80 mL, Seward, London, UK) containing 10 mL of sterile phosphate buffer (0.05 M; pH 6.8) and pummeled for 2 min in a Stomacher (Interscience BagMixer® 400W, Interscience, St. Nom La Breteche, France). The wash buffer (8 mL) was transferred to a 15-mL centrifuge tube, heated at 80 °C for 15 min in a water bath, and cooled on ice. Quadruplicate 0.25-mL samples (undiluted wash) were spread-plated on Mannitol Egg Yolk Polymyxin agar (MYP; Hangang, Gunpo, Republic of Korea) and incubated at 32 °C for 24 h. Pink colonies surrounded by a zone of lecithin hydrolysis were picked for further analysis from the MYP agar after 24-h incubation. If typical pink colonies were not formed on MYP agar, 4 mL of the rice wash buffer were combined with 4 mL of tryptic soy broth (TSB; BBL/Difco, Sparks, MD, USA) and the mixture was enriched at 32 °C for 24 h. The enriched mixture was streaked on MYP agar and incubated at 32 °C for 24 h. Typical pink colonies surrounded by a zone of lecithin hydrolysis were then picked and speciated using the biochemical assays described in the US FDA Bacteriological Analytical Manual (BAM; http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070875.htm). To confirm the identification of the *B. cereus* group, Gram staining and phenol red broth, nitrate broth, VP, and lysozyme tests were performed. To confirm the identification of *B. cereus* or *B. thuringiensis*, motility, rhizoid, and crystal toxin tests were performed.

2.3. Toxin assays

2.3.1. Enterotoxin immunoassay

Confirmed *B. cereus* and *B. thuringiensis* isolates were analyzed for production of HBL and NHE. An enterotoxin reverse passive latex agglutination test kit (Oxoid, Basingstoke, UK), which measures the L_2 component of the HBL complex, was used to determine the production of HBL. The *Bacillus* diarrheal enterotoxin visual immunoassay kit (Tecra Diagnostics, Frenchs Forest, NSW, Australia), which is specific for the NHEA component of the NHE complex, was used to determine the production of NHE. To perform HBL and NHE immunoassays, cells of *B. cereus* or *B. thuringiensis* from up to five randomly selected colonies formed on MYP agar plates were separately transferred to 10 mL of brain heart infusion broth (BHI, Oxoid) and incubated at 32 °C for 24 h. After three consecutive loop (ca. 10 μL) transfers at 24-h intervals, suspensions were incubated at 37 °C for 14 h. One milliliter of each suspension was deposited in a 15-mL centrifuge tube. Tests were carried out using mixed-isolate suspensions according to kit manufacturers' protocols.

2.3.2. Profiling of the enterotoxin and emetic toxin genes by PCR

B. cereus and *B. thuringiensis* isolates from rice samples were examined for the presence of toxigenic genes using PCR. The target genes were *hblC*, *hblD*, and *hblA*, which encode the three components of the HBL complex; *nheA*, *nheB*, and *nheC*, which encode the three components of the NHE complex; and *ces*, which is required for the production of an emetic toxin. An aliquot (3 mL) of the mixture of one to five isolates of *B. cereus* or *B. thuringiensis* from each sample, prepared as described above, was subjected to DNA