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Original Article

Prevalence, virulence genes, and antimicrobial resistance of *Bacillus cereus* isolated from foodstuffs in Pathum Thani Province, Thailand

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Bacillus cereus; Food-borne pathogen; Antibiotic resistance; Virulence gene; Foodstuff

ABSTRACT

Bacillus cereus (*B. cereus*) is a food-borne pathogen found in various food products. This study determined the prevalence, presence of virulence genes, and antibiotic resistance profile of *B. cereus* isolated from various foodstuffs in Pathum Thani Province, Thailand. A total of 150 samples were used (salads, stir-fried vegetables, spicy dressed salads, soups and curries, meat over rice dishes, and miscellaneous foods). Approximately 21% (31/150) of the samples were positive for *B. cereus*. Phenotypic resistance to 12 antibiotics was determined for 31 *B. cereus* isolates using the Kirby–Bauer disk diffusion method. Notably, 30 (97%), 29 (94%), and 30 (97%) isolates were resistant to ampicillin, amoxicillin-clavulanic acid, and penicillin, respectively. Most isolates were susceptible to other antibiotics tested, while showing intermediate resistant to rifampicin (68%). The distribution of eight enterotoxin genes was also determined through polymerase chain reaction. The positive rate of the *nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, *cytK*, and *entFM* genes was 77, 90, 74, 58, 58, 58, 68, and 84%, respectively. This is the first reference for the prevalence and characteristics of *B. cereus* isolated from food samples in Pathum Thani Province, Thailand. The results suggested that foods contaminated with *B. cereus* pose a potential risk of causing food-borne disease. Therefore, food processing or post-processing control measures are warranted to ensure safe consumption.

1. INTRODUCTION

B. cereus, a gram-positive, spore-forming, rod-shaped bacterium, is wide-spread in the environment and normally present in soil, water, and plants. From these habitats, it easily spreads to food products, both raw and processed food including rice, vegetables, meat, chicken, seafood, milk, and dairy products. Since spores of *B. cereus* are resistant to heat, dehydration, and physical stresses, there is a risk of its transmission through heat-treated and processed food products. Moreover, its toxins may be present in food and cause food poisoning in humans. *B. cereus* causes two different types of food poisoning in humans, namely emetic and diarrheal. In

most cases, both types are relatively mild and self-limiting[1]. In some cases, *B. cereus* may cause severe infections, including bacteremia, septicemia, meningitis, endocarditis, upper and lower urinary tract infection, and pulmonary infection[2]. Additionally, *B. cereus* can cause high morbidity and mortality particularly in premature neonates and immunocompromised patients.

B. cereus produces several toxins, including emetic toxin and enterotoxins. The emetic syndrome is caused by heat-stable toxin cereulide, which is synthesized by non-ribosomal peptide synthetases encoded by the *ces* gene cluster[3,4]. The toxin cereulide is produced by vegetative cells growing in the food, leading to nausea and vomiting that develop immediately after the ingestion of food contaminated with the toxin[5]. The diarrheal syndrome is caused by a series of heat-labile enterotoxins, including hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), cytotoxin K (CytK), enterotoxin FM (EntFM), hemolysin II (HlyII), and enterotoxin T (BceT)[1,6,7]. The enterotoxins are produced during the vegetative growth of the bacteria in the small intestine, resulting in abdominal pain and watery diarrhea[5,8].

Improper use of antibiotics has led to increased antimicrobial resistance in various bacterial species. The emergence of antibiotic-resistant *B. cereus* is mainly attributed to antibiotic misuse or acquisition of resistance genes through horizontal gene transfer[9,10]. It is noted that genes related to the development of resistance are transferred between the strains in *Bacillus* groups and between different species[9]. The production of β-lactamase in *B. cereus* can result in strains resistant to penicillin (PEN), ampicillin (AMP), and even the third generation of cephalosporins[11]. The increasing occurrence of *B. cereus* isolates with multiple drug resistance may pose public health challenges.

B. cereus has been isolated from raw and processed food products, such as milk and dairy products, meat products, tropical seafood, and cooked chilled foods containing vegetables[12-14]. In 1990, an unusual outbreak of food poisoning was reported in Thailand and one of the causative agents was identified as *B. cereus* in eclairs[15]. *B. cereus* food poisoning outbreak was reported in a kindergarten school, Bangkok, Thailand in 2009[16]. The pathogen was isolated from patients and the soup of sweet stewed egg and pork. According to the report of Pathum Thani Province public health office, the case report number of diarrhea and food poisoning in 2016 (1 January – 25 December, 2016) were 14,904 and 1,775 cases, accordingly. Among these, the etiological pathogens of the diseases were not clarified. Moreover, there is currently no study investigating the prevalence, virulence, and antibiotic resistance profile of *B. cereus* in foodstuffs in Pathum Thani Province, Thailand. Therefore, this study aimed to assess the prevalence, virulence factor genes, and phenotypic antibiotic resistance of *B. cereus* isolated from various types of food in Pathum Thani Province, Thailand.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 150 samples, consisting of 25 salads, 39 stir-fried vegetables, 24 spicy dressed salads, 15 soups and curries, 20 meat over rice dishes, and 27 miscellaneous foods, were used for the isolation of *B. cereus*. All foods were purchased from restaurants and flea markets in Pathum Thani Province, Thailand. Sampling was conducted between June and November 2018.

2.2. Isolation and identification of *B. cereus*

The isolation of *B. cereus* was performed according to the method described by Owusu-Kwarteng[14] with some modifications. In brief, 25 g of each sample was transferred into 225 mL of sterile phosphate-buffered saline in a sterile stomacher (Stomacher 400 circulator; Seward, West Sussex, UK) and homogenized for 2 min at 250 rpm. The homogenate was serially diluted (10-fold) in sterile phosphate-buffered saline, and 100 µL of each dilution was inoculated onto duplicated Mannitol-egg yolk-polymyxin agar plates (Scharlau, Barcelona, Spain). The plates were incubated aerobically at 30°C for 24 h. Suspected *B. cereus* colonies with eosin pink colonies surrounded by a zone of egg yolk hydrolysis were selected from each plate and sub-cultured on nutrient agar (Oxoid, Hampshire, United Kingdom). These isolates were identified using conventional microbiological techniques, including Gram staining, colony morphology, motility, hemolysis, and production of catalase,

oxidase, and urease. Isolates were further characterized using the API 50 CHB test (BioMerieux, Marcy l'Etoile, France) according to the instructions provided by the manufacturer. *B. cereus* ATCC 14579 (Biomedia, Nonthaburi, Thailand) was used as a reference strain for phenotypic testing.

2.3. Detection of virulence genes

Bacterial genomic DNA was extracted from bacterial cultures grown on nutrient agar using the InstaGene Matrix DNA extraction kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions provided by the manufacturer. Polymerase chain reaction (PCR) screening was performed to detect the presence of eight enterotoxigenic genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, and *entFM*). The primer pair sequences used for the amplification of the virulence factor genes of *B. cereus* in this study are shown in Table 1. *B. cereus* type strain ATCC 14579 was used as a reference enterotoxin-positive control for the eight enterotoxin genes. The PCR reaction mixture (25 µL) contained 50 ng of template DNA, 2 µM of each primer, and 12.5 µL 2× PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Sterile Milli-Q water was used for the preparation of the PCR mixture and for all negative control reactions. PCR was performed using the MyCycler Thermal Cycler (Bio-Rad). The cycling conditions were as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. The amplified PCR products were analyzed using gel electrophoresis on a 1.5% agarose gel alongside a 50 bp DNA ladder (Goldbio, St Louis, MO, USA), and visualized using Ecodye™ Nucleic Acid Staining Solution (Biofact, Daejeon, South Korea). Agarose gel electrophoresis was performed for 30 min at 100 V in 1× buffer (89 mM Tris, 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid, pH 8.4). Following electrophoresis, gels were visualized under an ultraviolet transilluminator (AlphaImager HP, Santa Clara, CA, USA) and photographed using the gel documentation system.

Table 1. Sequences of primers used in this study.

Target gene	Primer	Sequence (5′-3′)	Amplicon size (bp)	Reference
nheA	nheA_F	TACGCTAAGGAGGGGCA	480	17
	nheA_R	GTTTTTATTGCTTCATCGGCT		
nheB	nheB_F	CTATCAGCACTTATGGCAG	754	17
	nheB_R	ACTCCTAGCGGTGTTCC		
nheC	nheC_F	CGGTAGTGATTGCTGGG	564	17
	nheC_R	CAGCATTCGTACTTGCCAA		
hblA	hblA_F	GTGCAGATGTTGATGCCGAT	301	17
	hblA_R	ATGCCACTGCGTGGACATAT		
hblC	hblC_F	AATGGTCATCGGAACTCTAT	731	17
	hblC_R	CTCGCTGTTCTGCTGTTAAT		
hblD	hblD_F	AATCAAGAGCTGTCACGAAT	411	17
	hblD_R	CACCAATTGACCATGCTAAT		
cytK	cytK_F	CGACGTCACAAGTTGTAACA	565	18
	cytK_R	CGTGTGTAAATACCCCAGTT		
entFM	entFM_F	ATGAAAAAAGTAATTTGCAGG	1,269	19
	entFM_R	TTAGTATGCTTTTGTGTAACC		

2.4. Antimicrobial susceptibility testing

The antibiotic susceptibility of all *B. cereus* isolates was determined using the Kirby–Bauer disk diffusion method according to the standard criteria of the Clinical and Laboratory Standards Institute[20]. Each isolate grown overnight on Mannitol-egg yolk-polymyxin agar was included in this test. The culture of each isolate was compared with 0.5 McFarland turbidity standards (if necessary adjusted by addition of sterile saline into tubes). The culture was applied on the Mueller Hinton agar plate using a sterile cotton swab, and the inoculated plates were maintained at room temperature to allow drying. Twelve antibiotics, including ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 20 µg /10 mg), penicillin G (PEN, 10 U), gentamicin (GEN, 10 µg), imipenem (IPM, 10 µg), vancomycin (VAN, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), rifampicin (RIF, 5 µg), trimethoprim-sulfamethoxazole (SXT, 1.25 µg/23.75 µg), and erythromycin (ERY, 15 µg), were tested. The diameter of the inhibition zone was determined according to the Clinical and Laboratory Standards Institute guidelines[20] for *Staphylococcus aureus*. Based on the zone of inhibition, strains were classified as sensitive (S), intermediate (I), or resistant (R). *Staphylococcus aureus* ATCC 25923 was used as a reference strain in the disk diffusion susceptibility tests.

3. RESULTS

3.1. Prevalence of *B. cereus* in food

The prevalence of *B. cereus* in food samples obtained from cafeterias and flea markets in Pathum Thani Province are shown in Table 2. Three of 25 (12.0%) salads, nine of 39 (23.1%) stir-fried vegetables, five of 24 (20.8%) spicy dressed salads, four of 15 (26.7%) soups and curries, three of 20 (15.0%) meat over rice dishes, and seven of 27 (25.9%) miscellaneous food samples were positive for *B. cereus*. Seven positive samples in the group of miscellaneous foods were composed of fried egg with climbing wattle, fried noodle with red sauce, sushi, fermented fish spicy dip, sandwiches, roasted pork wonton noodle soup, and fresh vegetable rice wrap. All the isolates showed common phenotypic and biochemical characteristics that were consistent with the identification of *B. cereus*.

Table 2. Prevalence of *B. cereus* and the enterotoxigenic genes in food samples.

Type of food samples	Number of positive samples/total samples (%)	Number of positive samples for enterotoxigenic gene (%)							
		nheA	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*	*cytK*	*entFM*
Salads	3/25 (12.0%)	2 (8.0%)	2 (8.0%)	2 (8.0%)	3 (12.0%)	3 (12.0%)	3 (12.0%)	2 (8.0%)	2 (8.0%)
Stir-fried vegetables	9/39 (23.1%)	8 (20.5%)	9 (23.1%)	6 (15.4%)	5 (12.8%)	5 (12.8%)	5 (12.8%)	7 (17.9%)	8 (20.5%)
Spicy dressed salads	5/24 (20.8%)	3 (12.5%)	4 (16.7%)	4 (16.7%)	1 (4.2%)	1 (4.2%)	1 (4.2%)	1 (4.2%)	3 (12.5%)
Soups and Curries	4/15 (26.7%)	4 (26.7%)	4 (26.7%)	2 (13.3%)	2 (13.3%)	2 (13.3%)	2 (13.3%)	4 (26.7%)	4 (26.7%)
Meat over rice	3/20 (15.0%)	2 (10.0%)	3 (15.0%)	2 (10.0%)	2 (10.0%)	2 (10.0%)	2 (10.0%)	1 (5.0%)	3 (15.0%)
Others	7/27 (25.9%)	5 (18.5%)	6 (22.2%)	7 (25.9%)	5 (18.5%)	5 (18.5%)	5 (18.5%)	6 (22.2%)	6 (22.2%)
Total	31/150 (20.7%)	24 (16%)	28 (18.7%)	23 (15.3%)	18 (12.0%)	18 (12.0%)	18 (12.0%)	21 (14.0%)	26 (17.3%)

3.2 Distribution of enterotoxin genes among the *B. cereus* isolates

The distribution of enterotoxin genes was evaluated through PCR-based detection. The targeted genes included genes encoding hemolytic (*hblA*, *hblC* and *hblD*) and non-hemolytic (*nheA*, *nheB* and *nheC*) enterotoxin complexes, *cytK*, and *entFM*. All primers produced amplicons of the expected size from their respective target enterotoxin genes. The distribution of virulence genes among the 31 *B. cereus* isolates is shown in Table 2 and Figure. 1. The detection rate of *nheB* (90%) was higher than that observed for *nheA* (77%) and *nheC* (74%). Notably, 58% of the strains harbored enterotoxigenic gene *hblACD*. The prevalence of the *cytK* and *entFM* genes among *B. cereus* isolates was 68% and 84%, respectively. Only 45% of the isolates possessed all eight virulence genes. For the gene cluster *nheABC* encoding the Nhe complexes, approximately 65% (20/31) of *B. cereus* isolates were found to harbor the *nheABC* genes (Table 3); 9% (3/31) harbored only one gene, 19% (6/31) harbored two genes, and 6% (2/31) possessed none of the NHE-encoding genes. For HBL-encoding genes, 58% (18/31) of the isolates possessed all three *hblACD* genes, whereas 42% (13/31) of the isolates did not possess an HBL-encoding gene.

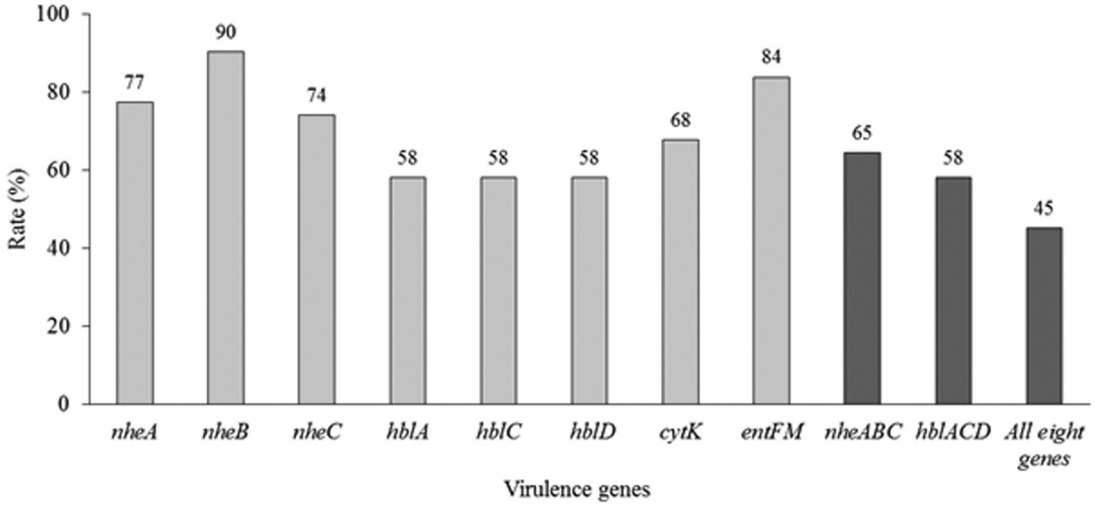

Figure 1. Distribution of enterotoxin genes in Bacillus cereus isolated from food samples. The number at the top of the bars represents the positive rate of corresponding toxin genes. *nheABC* and *hblACD* indicate that the strains were simultaneously positive for *nheA*, *nheB*, and *nheC* or *hblA*, *hblC*, and *hblD*, respectively. "All eight genes" presents the strains containing all the detected toxin genes.

Table 3. Distribution of enterotoxin genes in *B. cereus* isolated from food samples.

Enterotoxin genes	Number of strains (%) positive for target gene(s)
NHE gene complexes	
nheA	0 (0)
nheB	2 (6)
nheC	1 (3)
nheA + *nheB*	4 (13)
nheA + *nheC*	0 (0)
nheB + *nheC*	2 (6)
nheA + *nheB* + *nheC*	20 (65)
None detected	2 (6)

Table 3. Distribution of enterotoxin genes in *B. cereus* isolated from food samples. (Cont.).

Enterotoxin genes	Number of strains (%) positive for target gene(s)
HBL gene complexes	
hblA	0 (0)
hblC	0 (0)
hblD	0 (0)
hblA + *hblC*	0 (0)
hblA + *hblD*	0 (0)
hblC + *hblD*	0 (0)
hblA + *hblC* + *hblD*	18 (58)
None detected	13 (42)
Other genes	
cytK	21 (68)
entFM	26 (84)

3.3 Antimicrobial susceptibility of the *B. cereus* isolates

All *B. cereus* isolates were tested for their antimicrobial susceptibility to 12 selected antimicrobial agents. As shown in Table 4 and Figure. 2, most of the isolates were resistant to AMP (97%), AMC (94%), and PEN (97%), which belong to β-lactams. Moreover, we also found that most of the isolates were intermediately resistant to RIF (68%), an ansamycin. All isolates were susceptible to GEN (100%), IPM (100%), VAN (100%), CHL (100%), and CIP (100%). In addition, most of the isolates were sensitive to TET (97%), SXT (87%), and ERY (87%). The majority (71%) were resistant to one category of antibiotic (b-lactams). Of all strains, 25% were resistant to at least two classes of antibiotics: β-lactam and ansamycins (19%), β-lactam and tetracyclines (3%), and β-lactam and folate pathway inhibitors (3%).

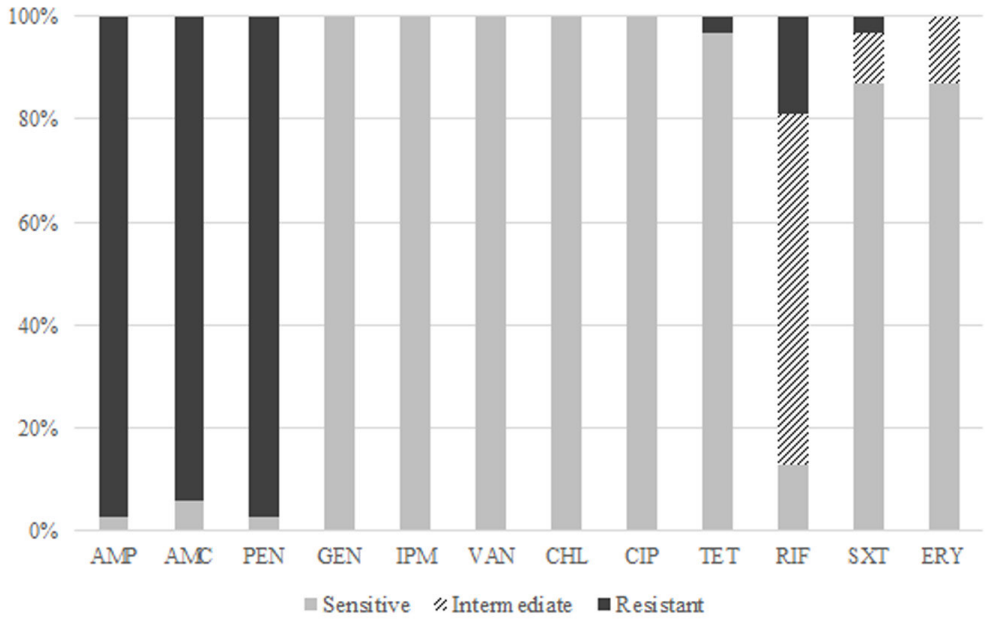

Figure 2. Antibiotic susceptibility of *Bacillus cereus* isolated from food samples. The light grey bar, stripe bar, and dark grey bar represent the proportion of sensitive strains, moderately resistant strains, and resistant strains, respectively.

Table 4. Antimicrobial resistance of *Bacillus cereus* isolated from food samples in Pathum Thani Province, Thailand.

Category	Antimicrobial Agent	*Bacillus cereus* (n = 31)		
		Sensitive	Intermediate	Resistant
β-Lactams	Ampicillin (10 µg)	1 (3%)	0 (0%)	30 (97%)
	Amoxicillin-clavulanic acid (20 µg/10 µg)	2 (6%)	0 (0%)	29 (94%)
	Penicillin (10 U)	1 (3%)	0 (0%)	30 (97%)
Aminoglycosides	Gentamicin (10 µg)	31 (100%)	0 (0%)	0 (0%)
Carbapenems	Imipenem (10 µg)	31 (100%)	0 (0%)	0 (0%)
Glycopeptides	Vancomycin (30 µg)	31 (100%)	0 (0%)	0 (0%)
Phenicols	Chloramphenicol (30 µg)	31 (100%)	0 (0%)	0 (0%)
Fluoroquinolones	Ciprofloxacin (5 µg)	31 (100%)	0 (0%)	0 (0%)
Tetracyclines	Tetracycline (30 µg)	30 (97%)	0 (0%)	1 (3%)
Ansamycins	Rifampicin (5 µg)	4 (13%)	21 (68%)	6 (19%)
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg)	27 (87%)	3 (10%)	1 (3%)
Macrolides	Erythromycin (15 µg)	27 (87%)	4 (13%)	0 (0%)

4. DISCUSSION

B. cereus is easily distributed to many types of foods including meat, eggs, dairy products and vegetables. Contamination of vegetables with *B. cereus* has been reported in several countries and at various levels. In China, 50% of all vegetable samples collected from 39 major cities contained *B. cereus*[21]. In Korea, this value was 20–48%[22,23] and 57% in Mexico City[24]. Moreover, *Bacilli* are capable of attaching to biotic (meat surfaces) and abiotic (meat processing equipment) surfaces[25]. Therefore, contamination of various meat products (e.g., fish, poultry, and beef) with *B. cereus* has been reported[26-28]. *B. cereus* grows well after cooking and cooling <48°C, and its surviving spores are the cause of food poisoning since heat treatment can induce spore germination[29]. However, the temperature at 125°C for 10-20 s is sufficient for inactivation of all *B. cereus* spores in dairy products.

Notably, a series of enterotoxins produced by *B. cereus* in the small intestine, including pore-forming cytotoxins Hbl, Nhe, and CytK[30-32] are associated with diarrhea[33]. High prevalence of the *nheABC* and *entFM* gene complexes is widespread among *B. cereus* isolates from various food and environmental sources. In this study, eight enterotoxin genes were determined in *B. cereus* isolated from food samples. The positive rates of *nheABC* and *hblACD* were 65% and 58%, respectively. The *nheABC* frequency in our food samples was lower than those reported in pasteurized milk from China (93%)[13], vegetables from China (95%)[21], and Sunsik from Korea (97%)[34]. Similarly, the positive rate of *hblACD* in our food samples was lower than that observed in vegetables from China (81%)[21] and Sunsik from Korea (86%,)[34]; however, it was higher than that recorded in pasteurized milk from China (45%)[13].

Several studies have reported variable detection rates of the *cytK* gene in food-related isolates. The frequency of *cytK* in our food samples (68%) was lower than that reported in pasteurized milk from China (73%)[13], Sunsik from Korea (77%)[34], and vegetables from China (87%)[21]; however, it was higher than that observed in rice and cereals from Korea (55%)[35]. The percentage of strains carrying *entFM* in this study was relatively high compared with those of other enterotoxin genes. However, it was lower than those found in pasteurized milk from China (96%)[13] and Sunsik from Korea (100%)[34]. The distribution of enterotoxin genes in *B. cereus* isolated from food sample suggested that food poisoning by the diarrheal toxin-producing strains cannot be neglected.

B. cereus from the environment may be transmitted through food to humans and cause mild to severe diseases that may be life-threatening in some cases[30,36]. Antibiotic therapy is the predominant treatment against *B. cereus* infection. However, antibiotic resistance in bacteria is an important occurrence that emerges rapidly worldwide. The plentiful production of b-lactamases by bacteria, including *Bacillus* species is a common cause of antibiotic resistance in bacteria[35,37]. Several studies reported the presence of b-lactamases in *B. cereus* isolated from various food products, resulting in the development of resistance to b-lactam antibiotics (including PEN and AMP)[13,14,21,34]. *B. cereus* isolated from soil of cattle-grazing fields, and milk and dairy products were predominantly resistant to b-lactam antibiotics[14]. In this study, most of the isolates were resistant to AMP (97%), AMC (94%), and PEN (97%). This result is consistent with those reported by previous studies.

In this study, *B. cereus* isolates showed a trend toward resistance to RIF. Approximately 19% of the isolates showed resistance to RIF, and 68% demonstrated intermediate resistance to this antimicrobial agent. This resistance rate was higher than those reported for traditional dairy products in Ghana (0%)[14], but lower than those observed in vegetables in Korea (48.7%)[23], rice and cereal in Korea (62%)[35], and vegetables in China (83.0%)[21]. Consistent with previous studies, *B. cereus* was susceptible to aminoglycosides (GEN), carbapenems (IPM), glycopeptides (VAN), phenicols (CHL), fluoroquinolones (CIP), tetracyclines (TET), and folate pathway inhibitors (SXT)[21]. VAN is considered one of the most appropriate choices for the treatment of *B. cereus* infections[38,39]. All our isolates were sensitive to VAN, suggesting that this is the drug of choice against *B. cereus* infections. However, a previous study reported that approximately 13% of the isolates were insensitive to VAN[13], suggesting a potential risk for resistance to VAN in the future, as a consequence of inappropriate use.

5. CONCLUSIONS

B. cereus is prevalent in food samples collected from cafeterias and flea markets in Pathum Thani Province in Thailand. In most samples, the levels were considered to be safe for consumption. However, various enterotoxin genes associated with the virulence of *B. cereus* were present among the isolates. In general, the *B. cereus* isolates were resistant to β-lactam antibiotics and susceptible to other antibiotics. To the best of our knowledge, this is the first comprehensive investigation of the prevalence of *B. cereus*, virulence factors, and antibiotic resistance phenotypes in food from Pathum Thani Province, Thailand. The results revealed a risk of *B. cereus* infection to public health. Therefore, implementation of good hygienic practices to prevent contamination and subsequent potential disease outbreaks caused by *B. cereus* is warranted.

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Conflict of interest

There is no conflict of interest in this research.

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