Study on Cytotoxicity and Nucleotide Sequences of Enterotoxin FM of *Bacillus cereus* Isolated from Various Food Sources

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Objective: *To determine the cytotoxicity of the crude culture broth containing enterotoxins of B. cereus and investigate the nucleotide sequences of ent FM among various isolates B. cereus.*
Material and Method: *The 1.2 kb fragment of ent FM of B. cereus was amplified, cloned, sequenced, and compared with published sequences. The biological activity of crude culture broth containing enterotoxins with and without monoclonal antibodies against HBL, NHE, and EntFM enterotoxins was tested using Vero cells assay.*
Results: *A percentage homology of nucleotide sequences and deduced amino acid sequences among test isolates and published strains were 90-99% and 88-99%, respectively. An assays for cytotoxic activity revealed that seven and three of B. cereus isolates were positive for NHE enterotoxin and EntFM enterotoxin, respectively. In addition, the 4-repeating sequences "TCAAAC" of ent FM were found, which may or may not be probably correlated with cytotoxicity of B. cereus.*
Conclusion: *The enterotoxin FM from B. cereus isolates is cytotoxic and the degree of cytotoxicity depends on the bacterial strain.*

Keywords: *Bacillus cereus, Enterotoxin FM, Food poisoning, Cytotoxicity, Sequencing*

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Bacillus cereus is associated in major food-borne pathogens and one of a causative agent of gastrointestinal and non-gastrointestinal diseases. *B. cereus*, is well-known as a food poisoning organism that produces at least five different toxins and may cause illness by inducing vomiting (emetic toxins) or diarrhea (enterotoxins)[1]. Emetic syndrome (intoxication) is characterized by acute nausea and vomiting. These symptoms typically develop within 1 to5 h of consumption of the contaminated food. By contrast, the diarrheal form of *B. cereus* food poisoning arises from the production of at least two types of three-component enterotoxins during vegetative growth of *B. cereus* in the small intestine of the host. Symptoms in susceptible individuals typically occur at least within 8 to 16 h following ingestion of enterotoxin-producing *B. cereus* strains and include abdominal pain, cramps, and diarrhea[1,2]. While the emetic syndrome is generally associated with cereal foods including rice and pasta, diarrheal enterotoxins are found in many foods, including milk, vegetables, and meat products[3,4].

Beside its food poisoning potential, *B. cereus* has been shown to be responsible for wound and eye infections, as well as systemic infections. It has been reported that systemic complications of *B. cereus* infections in premature neonates might be at least partly related to enterotoxins[5]. Due to heat and acid resistance of its spore, it is not eliminated by pasteurization or sanitation procedures. Therefore, *B. cereus* is considered as a major problem in convenience food and mass catering[6].

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In addition to emetic or diarrheal enterotoxins, the first of well characterized *B. cereus* enterotoxin was hemolysin BL (HBL) that consists of a B component and two L components, L_1 and L_2. Together, these components impart hemolytic, cytotoxic, dermanecrotic and vascular permeability activities, making HBL a primary virulence factor of *B. cereus*[1,3,7,8]. In addition to HBL, a nonhemolytic enterotoxin (NHE) has been identified and characterized from a food poisoning outbreak[9]. NHE is also a multi-component toxin, having a 39.8 kDa L_1 portion, 41 kDa L_2 subunit and a possible third subunit encoded by an uncharacterized gene[10]. A third enterotoxin T (BcET), has been described as a single component enterotoxin[11] and appears to possess biological activity similar to HBL and NHE. However, BcET probably does not contribute to food poisoning because BcET has no signal sequence and is only released after cell lysis. There is no indication that it has been involved in food poisoning[12]. Other enterotoxins produced by *B. cereus* include enterotoxin FM (EntFM) and cytotoxin K (CytK). EntFM was first characterized in 1997 by Asano[13] and is present in most outbreak-associated strains[14]. Another last enterotoxin of *B. cereus* is cytotoxin K (CytK). CytK is a single enterotoxin and has been implicated in *B. cereus*-related deaths due to necrotic enteritis[15-17].

To date, there is poor study of EntFM of *B. cereus* and insufficient evidence to indicate whether EntFM from *B. cereus* causes cytotoxic to Vero cells or is involved in food-borne disease. The objectives of the present study were (i) to compare the nucleotide sequences of *ent FM* genes among various isolates of *B. cereus*, (ii) to determine the Vero cells cytotoxicity of crude culture broth containing enterotoxins, (iii) to measure the cytotoxic effect of enterotoxins prior incubated with monoclonal antibodies against the HBL, NHE and EntFM.

Material and Method

Bacterial strains

Reference strain *B. cereus* ATCC 14579 was kindly provided by Dr. D.R. Zeigler (Bacillus Genetic Stock Center, the Ohio State University, and Columbus, USA). *B. thuringiensis* HD12 was kindly provided by Associate Professor Dr. Shin-ishiro Asano (Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Faculty of Agriculture, Hokkaido University, Sapporo, Japan). Four food isolates of *B. cereus* strain P5, G36, G189 and E131/45 were obtained from Division of Food Analysis, Department of Medical Science, Ministry of Public Health, Thailand. Six food isolates of *B. cereus* strain A1 (fried rice), A2 (boiled rice), A3 (celery), A4 (coriander), A5 (onion) and A7 (potato) were obtained from various Thai foods and vegetables. A protocol modified from FDA guidelines was used to identify *B. cereus* from food sources. Six food isolates of *B. cereus* (strain A1, A2, A3, A4, A5 and A7) were homogenized for 2 min in 450 ml peptone solution (0.1% peptone, 0.8% NaCl) and serially diluted. Aliquots of 0.1 ml of the appropriate dilutions were plated in triplicate onto mannitol-egg yolk-polymyxin (MYP) agar and incubated for 24 h at 30°C. Suspected colonies were counted and sub-cultured on LB agar. Isolates were identified by morphology, Gram and spore staining and biochemical tests including Voges-Prokauer (VP) reaction, catalase activity, glucose fermentation and failure to ferment mannitol[18]. They were confirmed using APT 50 CHB/E test strips (bioMerieux, France) and visual immunoassay kits (BDE-VIA) (TECRA®, Bioenterprises Pty. Ltd., Australia) for detection of Bacillus diarrheal enterotoxin.

Cell line and monoclonal antibody

Vero cells CCl-81 (African green monkey kidney cell line) and monoclonal antibodies against HBL, NHE and EntFM which are used to determine the cytotoxicity of crude enterotoxins of *B. cereus* were kindly provided by Associate Professor Dr. Shin-ishiro Asano (Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Faculty of Agriculture, Hokkaido University, Sapporo, Japan).

DNA preparation and DNA amplification

Genomic DNA of *B. cereus* was extracted according to the CTAB method[19] and plasmid DNA was extracted using a QIAGEN plasmid Miniprep Kit as described by the manufacturer(QIAGEN, Germany). The 1.2 kb *ent FM* gene was the target for amplification using PCR primers TY123 (5'-GGT TTA GCA GCA GCT TCT GTA GCT GGC G-3') and TY127 (5'-CAG AAC TAA TAC GTA CAC CAG TTG CAT CTG-3')[13]. The master mix prepared for 50 µl reactions contained 2.5 mM each of deoxynucleotide triphosphate, 10X PCR buffer (100 mM Tris-HCl, pH 8.8, 20 mM $MgCl_2$, 500 mM KCl, 1% Triton X 100), approximately 0.2 nM of each primer (forward and reverse primers), 100 ng of template DNA, 5 U of Taq DNA polymerase (Takara Bio Inc., Japan) and nuclease free water was added to adjust the total volume to 50 µl. Amplification was carried out using a Perkin Elmer Cetus DNA thermal

cycler model 2400 (Perkin Elmer, USA). PCR conditions were 30 cycles at 95°C for 1 min, 65°C for 50 sec and 72°C for 1 min followed by 5 min to add A-tails at the 3' end of the PCR products.

Cloning and sequencing of ent FM

The 1.2 kb products of all *B. cereus* isolates were purified using a Gene clean II kit (Promega, USA). The purified PCR products were cloned by using pGEM®-T Easy Vector as described by the manufacturer (Promega, USA). The nucleotide sequences of the *ent FM* gene from *B. cereus* isolates and *B. cereus* ATCC 14579 were sequenced using M 13 primer M 4 (5'-CGT CGT GAC TGG AAA AC-3') and M 13 primer RV (5'-CAG GAA ACA GCT ATG ACC-3') and walking primers (Seq a 5'-GAG CCA TGC GGG AAC ATG-3' and Seq b5'-GTT TGC ATG CTC TAT CTC-3') (Invitrogen™ life technologies, USA). The sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) according to the manufacturer's recommendation. DNA sequences were analyzed for homology to published sequences recorded in the GenBank database using BLAST program (http:// www.ncbi.nlm.nih.gov/). The DNASIS program (Hitachi Software Engineering, Tokyo) was used for assembling nucleotide sequences.

Preparation of crude B. cereus enterotoxin for cytotoxicity tests

Production of crude enterotoxins of *B. cereus* was prepared from bacterial-cell-free-culture supernatants[20]. A single colony of *B. cereus* was transferred to 1 ml of brain heart infusion (BHI; Difco, USA) broth supplemented with 1% glucose (BHIG) and cultured overnight at 30°C with vigorous shaking. An aliquot (10 ml) of the overnight culture was transferred to a 10 ml test tube containing 1 ml of BHIG for further incubation at 30°C for 6 h with vigorous shaking. Then the culture was centrifuged at 4°C, 10,000 x g for 20 min and supernatants were transferred to a new Eppendorf tube for centrifugation at 4°C and 10,000 x g for 20 min. Supernatants were filtered through 0.22 μm membrane to remove bacterial cells, designated as crude broth filtrates (CBF) and either used for cytotoxicity assays using monolayers of Vero cells immediately.

Vero cell cytotoxic assays

Crude broth filtrates; CBF toxicity was tested by inhibition of Vero cell protein synthesis[21] and protocol was previously described by Choma and Granum[22]. Vero cells were grown in Eagle minimal medium (MEM) supplemented with 10% fetal calf serum (FCS). For cytotoxicity assays, cells were seeded into 12-well microtitre plates with lids at a density 2.0 x 10^5 cells/well. CBF was incubated with Vero cells for 1 h and 24 h at volumes of 20, 50 and 100 μl. Sterile medium (BHIG) was used as the negative control. The plates were incubated at 37°C with 5% CO_2. After incubation, cell viability was determined by using an inverted light microscopy (Olympus Optical Ltd.) to assess cellular pathology such as loss of confluence, cell rounding and shrinking, and cytoplasm granulation and vacuolization[23,24]. To determine the type of bacterial enterotoxin presence in CBF, 3 μl of individual monoclonal antibodies against HBL, NHE and EntFM enterotoxins were added (for neutralizing the activity of enterotoxins) to 50 μl CBF with gentle mixing followed by incubation 37°C for 3 h before testing with Vero cells. All experiments were performed in duplicate and were repeated twice. Results were recorded as strongly positive (100% cell death), moderately positive (> 50-90% cell death) and weakly positive (< 50% cell death).

Results

Sequence analysis of ent FM genes from B. cereus isolates

To investigate the presence of *ent FM* gene in various isolates of *B. cereus*, PCR using the primers TY 123 and TY 125 was performed with Genomic DNA of all isolates. All isolates showed positive results of 1.2 kb of *ent FM* gene. After the 1.2 kb fragment of *ent FM* gene of ten isolates of *B. cereus* were amplified

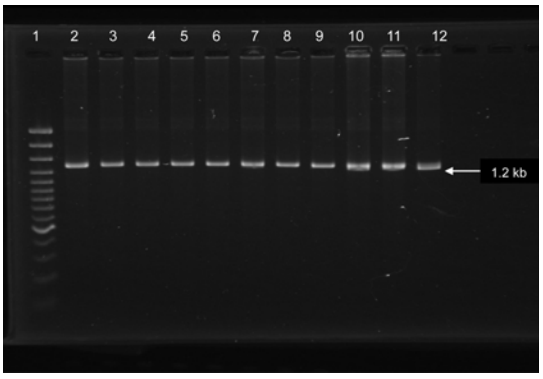

Fig. 1 PCR products from the *ent FM* gene of various isolates of *B. cereus*. Lanes (from left to right): 1 = 100 bp ladder marker, 2 = *B. cereus* ATCC 14579, 3-12 = isolates A1, A2, A3, A4, A5, A7, G36, P5, G189 and E131/45 respectively

(Fig. 1) and cloned into pGEM-T Easy Vector, the recombinant clone was extracted. Subsequently, the insert DNA was sequenced. The nucleotide sequences of *ent FM* gene were translated to amino acid sequences by using six frames translational tool. The nucleotide sequences of isolates A1 (EF 453652), A2 (EF 453651), A3 (EF 453653), A4 (EF 453654), A5 (EF 453655), A7 (EF 453656), P5 (EF 453657), G36 (EF 453658), G189 (EF 453659), E131/45 (EF 453660), 14579 (EF 453661) and amino acid sequences were analyzed by using CLUSTAL W program and compared with published strains of *B. cereus* (*B. cereus* FM1 (E09105), *B. cereus* Cx5 (AF192767), *B. cereus* Ae10 (AF192766) and *B. cereus* ATCC 14579 (GenBank)) (Figs. 2, 3). The nucleotide and deduced amino acid sequences of the isolated *ent FM* genes were homologous to published strains with percentage of 90-99% and 88-99% respectively.

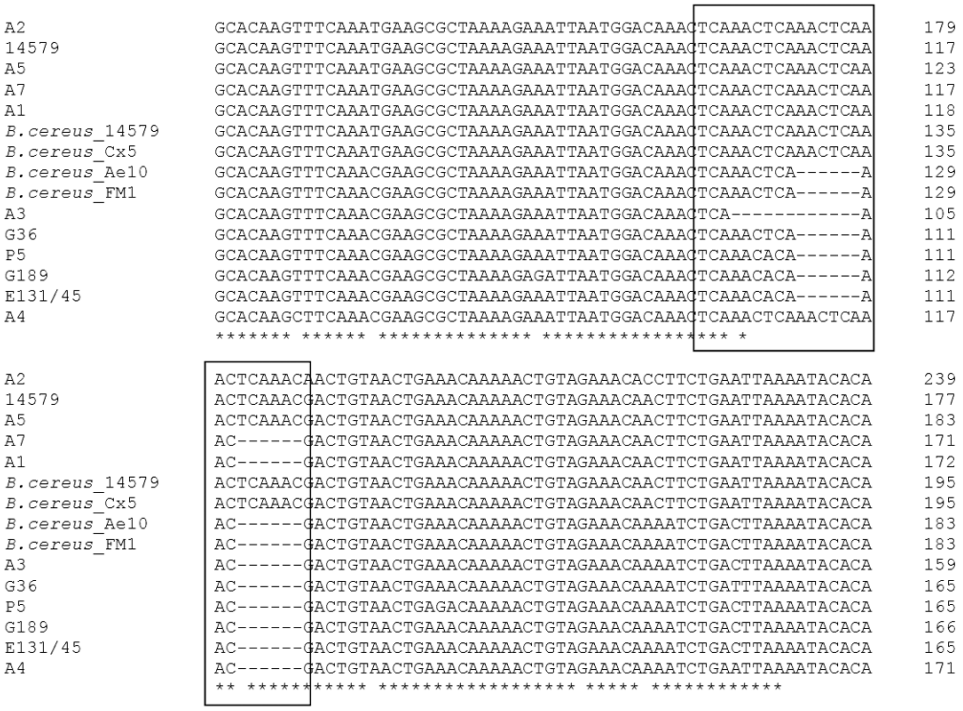

Fig. 2 Nucleotide sequences alignment of *ent FM* gene among *B. cereus* Cx5, *B. cereus* FM1, *B. cereus* Ae10, *B. cereus* ATCC 14579 and various isolateds of *B. cereus* The repeaing nucleotide sequences were shown in box

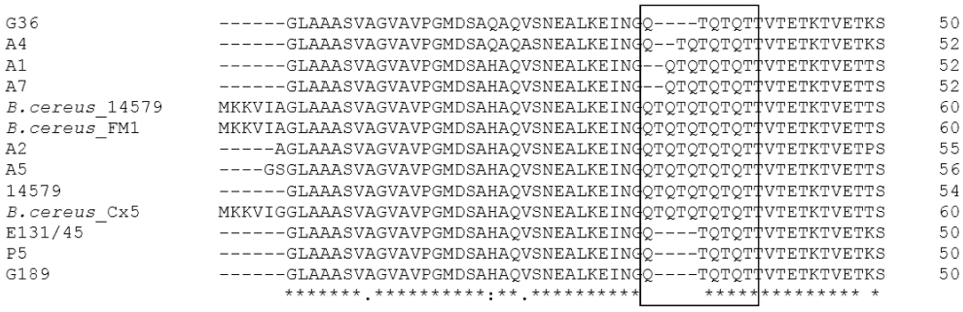

Fig. 3 Alignment of enterotoxin FM amino acid sequences among *B. cereus* Cx5, *B. cereus* FM1, *B. cereus* Ae10, *B. cereus* ATCC 14579 and various Thai isolates of *B. cereus*. The deduced amino acid repeat sequences are indicated by a box

Cytotoxicity of CBF on Vero cells

The measurement of cytotoxic effect on Vero cells was used as the indicator of enterotoxins activity of the various isolates. Cytotoxic effect of CBF of *B. thuringiensis* HD12, *B. cereus* ATCC 14579 and 7 isolates of *B. cereus* (A1, A2, A3, A5, G36, G189 and E131/45) were used randomly to evaluate the appropriate volume of CBF for toxic on Vero cells. Results are shown in Table 1. All 7 isolates, *B. thuringiensis* HD12 and *B. cereus* ATCC 14579 were highly cytotoxic at 1 h incubation time for 100 µl of CBF. For isolates A2, it showed complete inhibition at the 24 h incubation. For CBF 50 µl, the result showed that the cytotoxicity was decreased (compared with 100 µl supernatant) at 1 h incubation. After incubation of Vero cells with CBF for 24 h, seven isolates showed complete inhibition. When the CBF was reduced to 20 µl, the cytotoxicity effect of each isolates was shown in different levels. By comparison to reference strain ATCC 14579, isolates A3 and A5 were highly cytotoxic while isolates A1, G36 and *B. thuringiensis* HD12 were only weakly cytotoxic at 1 h incubation and moderately cytotoxic at 24 h incubation. Interestingly, isolates A2 showed no cytotoxicity to Vero cells after addition 20 µl of CBF even when the incubation time was increased to 48 h (data not show). In order to identify the type of enterotoxin (enterotoxin HBL, NHE or EntFM) produced in various isolates of *B. cereus* and which type of enterotoxin can causes cytotoxic to Vero cells. Therefore, *B. thuringiensis* HD12, *B. cereus* ATCC 14579 and 5

Table 1. Results from cytotoxicity assay using Vero cells treated with CBF from *B. cereus* and *B. thuringiensis* HD12

Strains	1 h incubation			24 h incubation		
	20 µl supernate	50 µl supernate	100 µl supernate	20 µl supernate	50 µl supernate	100 µl supernate
B. thuringiensis HD12	+	++	+++	++	+++	+++
B. cereus ATCC 14579	+++	+++	+++	+++	+++	+++
B. cereus A1	+	++	+++	++	++	+++
B. cereus A2	NA	+	+++	NA	+++	+++
B. cereus A3	++	++	+++	+++	+++	+++
B. cereus A5	++	++	+++	+++	+++	+++
B. cereus G36	+	++	+++	++	+++	+++
B. cereus G189	NA	+	+++	+	+++	+++
B. cereus E131/45	NA	++	+++	+	+++	+++

+ = weakly positive; ++ = moderately positive; +++ = strongly positive; NA = no activity

Table 2. Results of cytotoxicity assays using Vero cells treated with CBF of *B. cereus* and *B. thuringiensis* that had been pre-incubated individually with monoclonal antibodies againts HBL, NHE or ent FM

Strains	1 h incubation				24 h incubation			
	HBL antibody	NHE antibody	ent FM antibody	HBL+ NHE antibody	HBL antibody	NHE antibody	ent FM antibody	HBL+ NHE antibody
B. thuringiensis HD12	+	NA	+	NA	+++	NA	+++	NA
B. cereus ATCC 14579	++	+++	+	+	+++	+++	+++	+++
B. cereus A2	+	+	+	+	+	+	+++	++
B. cereus A3	++	NA	++	NA	+++	NA	+++	NA
B. cereus A5	++	+	+++	+	+++	+	+++	++
B. cereus G36	+	NA	NA	NA	+++	NA	+++	NA
B. cereus G189	+	NA	+	NA	+++	NA	+++	NA

+ = weakly positive; ++ = moderately positive; +++ = strongly positive; NA = no activity

isolates of *B. cereus* were randomly selected to determine this property. Results are shown in Table 2. Results obtained at 24 h incubation time showed that NHE enterotoxin was cytotoxic to Vero cells and all tested isolates contained NHE enterotoxin. Whereas enterotoxin FM was found only in reference strain ATCC 14579 and in the presented isolates A5 and A2, but the last two isolates showed weak cytotoxic on Vero cells.

Discussion

In the presented work, enterotoxin FM and *ent FM* gene were the target of the study. Ten isolates of *B. cereus* and reference strain *B. cereus* ATCC 14579 showed positive fragment in the expected size (1.2 kb) (Fig. 1). PCR studies revealed the presence of DNA fragments encoding the *ent FM* gene. In 1999 Hsich et al reported that 27 of 28 food isolates and all 30 outbreak-associated strain were *ent FM* gene PCR-positive[25]. Thus, the *ent FM* gene was found to be the most prevalent enterotoxin gene for *Bacillus cereus* group. These results are similar to Asano et al[13] and confirm a common distribution of the *ent FM* gene in *B. cereus*. Therefore, the authors could be considering that the diarrheagenic *ent FM* gene seems to be an appropriate diagnostic target gene (probe) for assessing the enterotoxic potential of Bacillus strains. Since the studies of diarrheagenic *ent FM* gene have not been well investigated, the authors' investigation data regarding of the diarrheagenic *ent FM* gene in *B. cereus* group has been limited compared with those of the other data.

It is of interest that results from the partial sequences alignment of all investigated isolates showed base pair gap. In addition, results from sequences comparison of *B. cereus* reference ATCC 14579, isolates A2 and A5, 3 strains, which showed cytotoxicity from EntFM, showed characteristic of 4-repeating sequences "TCAAAC "(Fig. 2). Whereas, the remaining isolates did not show 4-repeating sequences. From this observation the authors hypothesized that these 4-repeating sequences may probably or may not have correlated with the cytotoxicity of EntFM from *B. cereus*. However, for this presumption, the data from the present study is not adequate to support the authors' hypothesis and need to be clarified in a further study.

In the Vero cell line, enterotoxin toxicity was determined quantitatively by inhibition of protein synthesis[21] and decreased cell proliferation[26,27]. Indeed, Vero cells are reported to be the most sensitive cells for detection of toxic compounds[28]. The cytotoxicity against Vero cells as an assay to find possibility of enterotoxin FM from *B. cereus* that contributed in food poisoning. Results from Table 2 at in incubation time 24 h showed that NHE enterotoxin was cytotoxic to Vero cells and all tested isolates were shown to produce NHE enterotoxin. The present results on the presence of NHE entrotoxin in all the isolates corresponded with results from a previous study. It was found that 98-99% of *B. cereus* contained NHE enterotoxin[29,30]. The NHE is the enterotoxin of highest prevalence in *B. cereus*. Although enterotoxin FM was found only in reference strain ATCC 14579. Results from Table 2 show that even though CBF was neutralized with NHE and HBL monoclonal antibodies but for the last two isolates (A5 and A2) showed weak cytotoxic to Vero cells. These results indicate the involvement of EntFM in cytotoxicity. This data is the first demonstration that diarrheagenic enterotoxin FM from *B. cereus* can cause cytotoxic on Vero cells. From these results, the degree of cytotoxicity of the investigated isolates was shown as follows; ATCC 14579, A3, A5 > G36 > A1 > HD12 > E131 > G189 > A2. It is well-known that the degree of cytotoxicity and the amount of enterotoxin produced depends on gene expression and varies considerably among individual isolates[31,32].

Conclusion

Although all isolates of *B. cereus* were positive in assessing the presence of *ent FM* gene in PCR detection, it has been suggested that only a small number of *B. cereus* isolates is able to produce sufficient amounts of diarrhea enterotoxin to cause cytotoxicity on Vero cells[33] and it may also be possible that they depend on the expression level of *ent FM* gene. The expression level of all isolates may vary from strain to strain[25]. However, the mechanism by which *B. cereus* causes diarrhea is still unknown and little is known of the precise mechanism of cytotoxicity[34].

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References

1. Granum PE, Lund T. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Lett 1997; 157: 223-8.
2. Lund T, Granum PE. Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiol Lett 1996; 141: 151-6.
3. Kotiranta A, Lounatmaa K, Haapasalo M. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect 2000; 2: 189-98.
4. From C, Pukall R, Schumann P, Hormazabal V, Granum PE. Toxin-producing ability among Bacillus spp. outside the *Bacillus cereus* group. Appl Environ Microbiol 2005; 71: 1178-83.
5. Girisch M, Ries M, Zenker M, Carbon R, Rauch R, Hofbeck M. Intestinal perforations in a premature infant caused by *Bacillus cereus*. Infection 2003; 31: 192-3.
6. Ehling-Schulz M, Fricker M, Scherer S. Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiol Lett 2004; 232: 189-95.
7. Beecher DJ, Schoeni JL, Wong AC. Enterotoxic activity of hemolysin BL from *Bacillus cereus*. Infect Immun 1995; 63: 4423-8.
8. Beecher DJ, Wong AC. Tripartite hemolysin BL from *Bacillus cereus*. Hemolytic analysis of component interactions and a model for its characteristic paradoxical zone phenomenon. J Biol Chem 1997; 272: 233-9.
9. Lund T, Granum PE. Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. Microbiology 1997; 143(Pt 10): 3329-36.
10. Granum PE, O'Sullivan K, Lund T. The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. FEMS Microbiol Lett 1999; 177: 225-9.
11. Agata N, Ohta M, Arakawa Y, Mori M. The bceT gene of *Bacillus cereus* encodes an enterotoxic protein. Microbiology 1995; 141(Pt 4): 983-8.
12. Choma C, Granum PE. The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning. FEMS Microbiol Lett 2002; 217: 115-9.
13. Asano SI, Nukumizu Y, Bando H, Iizuka T, Yamamoto T. Cloning of novel enterotoxin genes from *Bacillus cereus* and Bacillus thuringiensis. Appl Environ Microbiol 1997; 63: 1054-7.
14. Hsieh YM, Sheu SJ, Chen YL, Tsen HY. Enterotoxigenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* strains from foods and food-borne outbreaks. J Appl Microbiol 1999; 87: 481-90.
15. Lund T, De Buyser ML, Granum PE. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol Microbiol 2000; 38: 254-61.
16. Fagerlund A, Ween O, Lund T, Hardy SP, Granum PE. Genetic and functional analysis of the cytK family of genes in *Bacillus cereus*. Microbiology 2004; 150: 2689-97.
17. Hardy SP, Lund T, Granum PE. CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia. FEMS Microbiol Lett 2001; 197: 47-51.
18. Shinagawa K. Analytical methods for *Bacillus cereus* and other Bacillus species. Int J Food Microbiol 1990; 10: 125-41.
19. Sambook J, Fritsch EF, Gibson TJ. Molecular cloning, a laboratory manual. 2nd ed. Plainville, NY: Cold Spring Harbor Laboratory Press; 1989.
20. Beecher DJ, Wong AC. Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. Infect Immun 1994; 62: 980-6.
21. Sandvig K, Olsnes S. Entry of the toxic proteins abrin, modeccin, ricin, and diphtheria toxin into cells. I. Requirement for calcium. J Biol Chem 1982; 257: 7495-503.
22. Choma C, Granum PE. The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning. FEMS Microbiol Lett 2002; 217: 115-9.
23. Simoes CM, Amoros M, Girre L. Mechanism of antiviral activity of triterpenoid saponins. Phytother Res 1999; 13: 323-8.
24. Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, et al. Methods of in vitro toxicology. Food Chem Toxicol 2002; 40: 193-236.
25. Hsieh YM, Sheu SJ, Chen YL, Tsen HY. Enterotoxigenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* strains from foods and food-borne outbreaks. J Appl Microbiol 1999; 87: 481-90.
26. Dietrich R, Fella C, Strich S, Martlbauer E. Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*. Appl Environ Microbiol 1999; 65: 4470-4.
27. From C, Pukall R, Schumann P, Hormazabal V, Granum PE. Toxin-producing ability among Bacillus

spp. outside the *Bacillus cereus* group. Appl Environ Microbiol 2005; 71: 1178-83.
28. Smee DF, Morrison AC, Barnard DL, Sidwell RW. Comparison of colorimetric, fluorometric, and visual methods for determining anti-influenza (H1N1 and H3N2) virus activities and toxicities of compounds. J Virol Methods 2002; 106: 71-9.
29. Moravek M, Wegscheider M, Schulz A, Dietrich R, Burk C, Martlbauer E. Colony immunoblot assay for the detection of hemolysin BL enterotoxin producing *Bacillus cereus*. FEMS Microbiol Lett 2004; 238: 107-13.
30. Moravek M, Dietrich R, Buerk C, Broussolle V, Guinebretiere MH, Granum PE, et al. Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. FEMS Microbiol Lett 2006; 257: 293-8.
31. Beattie SH, Williams AG. Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. Lett Appl Microbiol 1999; 28: 221-5.
32. Dietrich R, Moravek M, Burk C, Granum PE, Martlbauer E. Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. Appl Environ Microbiol 2005; 71: 8214-20.
33. Andersson A, Granum PE, Ronner U. The adhesion of *Bacillus cereus* spores to epithelial cells might be an additional virulence mechanism. Int J Food Microbiol 1998; 39: 93-9.
34. Fagerlund A, Lindback T, Storset AK, Granum PE, Hardy SP. *Bacillus cereus* Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia. Microbiology 2008; 154: 693-704.

การศึกษาความเป็นพิษและการหาลำดับนิวคลีโอไทด์ของจีน Enterotoxin FM ของเชื้อ Bacillus cereus ที่แยกได้จากอาหาร

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วัตถุประสงค์*: เพื่อตรวจหาความเป็นพิษของ enterotoxins และศึกษาลำดับของนิวคลีโอไทด์ของจีนที่ผลิต enterotoxin FM ของเชื้อ Bacillus cereus ที่แยกได้จากอาหารชนิดต่าง ๆ*

วัสดุและวิธีการ*: ทำการเพิ่มปริมาณของจีน ent FM ที่มีขนาด 1.2 kb ของเชื้อ Bacillus cereus ที่แยกได้จากอาหารชนิดต่าง ๆ จากนั้นนำไปทำการ cloning และทำการ sequencing จีน ent FM และทำการเปรียบเทียบลำดับนิวคลีโอไทด์และกรดอะมิโนของเชื้อ B. cereus ทำการทดสอบความเป็นพิษของ enterotoxin ที่เชื้อผลิตขึ้นต่อ Vero cells และทดสอบเมื่อทำการ neutralize ด้วยโมโนโคลนอลแอนติบอดีของ HBL, NHE และ Enterotoxin FM*

ผลการศึกษา*: เมื่อเปรียบเทียบลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนของจีน ent FM ระหว่างเชื้อที่แยกได้จากอาหารและเชื้อสายพันธุ์อ้างอิงใน GenBank พบเปอร์เซ็นต์ความคล้ายคลึงของลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนเท่ากับ 90-99% และ 88-99% ตามลำดับ และผลการศึกษาความเป็นพิษจากสายพันธุ์ที่แยกได้ พบว่ามี 7 สายพันธุ์ที่มี NHE enterotoxin และ 3 สายพันธุ์ที่มี Enterotoxin FM นอกจากนี้ยังพบลักษณะเบส "TCAAAC"4 ซ้ำ ในสายพันธุ์ที่พบความเป็นพิษจาก Enterotoxin FM ด้วย*

สรุป*: Enterotoxin FM ของเชื้อ B. cereus ที่แยกได้จากอาหารชนิดต่าง ๆ สามารถแสดงความเป็นพิษต่อ Vero cells และความรุนแรงของความเป็นพิษขึ้นกับแต่ละสายพันธุ์*