Original Article

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Development of a DNA-Probe for Detection of Enterotoxic *Bacillus cereus* Isolated from Foods in Thailand

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Abstract

Polymerase chain reaction (PCR) and DNA probe assays were compared for detection of *B. cereus* isolated from foods. The *entFM* gene encoding the enterotoxin FM was chosen as the target gene. Two pairs of primers, TY123/TY125 and TY123/TY127, were used to amplify DNA fragments of 584 bp and 1,219 bp, respectively. The 1,219 bp PCR product was amplified from 41 (42%) of 97 *B. cereus* isolates and 5 *B. thuringiensis* isolates. The 584 bp PCR product of *B. cereus* ATCC 14579 was cloned and labeled with digoxigenin (DIG)-dUTP to be used as a DNA probe. Colony blots, dot blots and Southern blots gave the same DNA hybridization results. Ninety-five isolates (98%) were positive including 56 isolates that had previously given negative PCR results. Two exceptions were *B. cereus* G157/44 and *B. cereus* 11929. Additionally, 5 isolates of *B. thuringiensis* gave positive results. All 3 blotting methods gave negative hybridization results for other *Bacillus* species and non-*Bacillus* species tested. The probe was able to detect 62.5 ng of *B. cereus* genomic DNA.

Key words: *B. cereus;* enterotoxin gene; detection; PCR; DNA hybridization

INTRODUCTION

B. cereus is an important cause of emetic and diarrheal food poisoning[1] and psychrotropic strains have become and increasing problem for the dairy industry.[2] Emetic poisoning is characterized by acute nausea and vomiting within 1-5 h of consumption of foods containing preformed toxin[3] while diarrheal poisoning arises from the production of enterotoxins during vegetative growth of *B. cereus* in the small intestine.[1] Most procedures for isolation and enumeration of *B. cereus* involve direct agar plating techniques based on exhibiting hemolysin production, lecitinase activity, fermentation properties or morphological features.[4] These methods require confirmatory testing that may require up to 4 days although this can be reduced to 2 days with the immunoblot technique.[5] There are two commercial kits available for detection of *B. cereus* enterotoxins. The *B. cereus* enterotoxin test kit (BCET-RPLA kit, Oxoid) is specific for the L_2 component of the BL hemolysin complex. The bacilli diarrheal enterotoxin visual immunoassay (BDE-VIA kit, TECRA®) is specific for the NheA component of nonheamolytic (Nhe) enterotoxin.[1,6] Potential targets for detection include haemolysin, emetic toxin cereulide, phospholipase C and enterotoxins, all of which are toxic for animals and humans.[7,8] In order to speed up detection, PCR-based methods have been developed and used for the *B. cereus* group.[5,9-17] Primers and DNA probes have also been designed for differentiation between *B. cereus* and closely related *Bacillus* species.[12,18-19] The objectives of this study were to (i) design a specific and sensitive DNA probe to detect enterotoxic *B. cereus* by DNA hybridization and (ii) study presence of the FM enterotoxin gene (*entFM*) in *B. cereus* isolated from foods in Thailand.

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MATERIALS AND METHODS

Bacterial strains

Microorganisms used in this study were derived from culture collections and from newly collected food samples (Table1). They were maintained in Luria Bertani (LB) medium containing 20% glycerol at-70ºC and subcultured in LB broth for 18-24 h at 37°C before use.

Table 1. Microorganisms used in this study

Microorganism	Source
B. cereus ATCC 14579, *B. thuringiensis* subsp. *Israelensis*, *B. thuringiensis* subsp. *Kurstaki*	*Bacillus* stock Center, the Ohio State University, Columbus, USA
8 isolates of *B. cereus* (from *B. cereus* A1 to *B. cereus* A8)	This study
42 isolates of *B. cereus* (from *B. cereus* E80/44 to *B. cereus* P44/44)	Division of Food Analysis, National Institute of Health (Department of Medical Science, Ministry of Public Health, Thailand)
47 isolates of *B. cereus* (from *B. cereus* 11927 to *B. cereus* 17004) 3 isolates of *B. licheniformis* 3 isolates of *B. pumilus* 3 isolates of *B. subtilis* 3 isolates of *B. thuringiensis*	Culture Collection Center (Department of Medical Science, Ministry of Public Health, Thailand)
Non-Bacillus sp. (*Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella flexnei* and *Staphylococcus aureus*)	Clinical isolates from Ramathibodi Hospital, Mahidol University, Thailand

Isolation of B. cereus from food samples

Vegetables, cooked rice, spice mixes and cereal powders were purchased from local markets in Bangkok. Food samples (50 g) 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 subcultured 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.[3] 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.

DNA preparation

Preparation of DNA for PCR[14]

Bacteria were cultured on LB agar and incubated overnight at 37°C. A single colony was transferred to 5 ml LB broth for overnight culture at 37°C with continuous shaking (150 rpm). Bacterial cells were collected by centrifugation at 10,000×g for 2 min (microcentrifuge EBA12, Hettich Zentrifugen, Germany) and resuspended in 200µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Cells were lysed by boiling at 100°C for 10 min and debris was removed by centrifugation at 20,000×g for 5 min. The DNA containing supernatant was transferred to a new microfuge tube and used as a template for PCR.

Preparation of DNA for gene cloning and Southern blotting[20]

Bacterial cells were prepared as in 2.3.1 and resuspended in a mixture containing 567 µl TE buffer, 30 µl of 10% sodium dodecyl sulfate (SDS, Merck), 5 µl of 20 mg/ml proteinase K and 40 µl of 50 mg/ml lysozyme. The mixture was incubated for 1h at 37°C before addition of 5 µl of 10 mg/ml RNaseA and further incubation for 30 min at 37°C. Then, 100 µl of 5M NaCl, 80 µl CTAB/NaCl (10% CTAB in 0.7 M NaCl) was added followed by incubation

for 10 min at 65°C. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v) was added and the mixture was centrifuged at 20,000×g for 10 min. The supernatant was transferred into new tube, mixed with one volume of chloroform-isoamyl alcohol (24:1 v/v) and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and DNA was precipitated with 2 volumes of cold absolute ethanol and centrifugation at 15,000 rpm for 20 min. The pellet was washed twice using 800 µl of cold 70% ethanol, dried and dissolved in 20 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). DNA was separated by electrophoresis through 0.7% agarose gel (Promega, USA) in 0.5X Tris-borate-EDTA buffer (1X TBE buffer, 89 mM Tris-HCl, 89mM boric acid, 2 mM EDTA pH8.0). Electrophoresis was performed for 40 min at 100 V. Gels were stained for 15 min with 1 µg/ml ethidium bromide solution, destained in distilled water and visualized with a UV transilluminator. DNA concentration of was determined by spectrophotometer (Gene Quantpro®, Biochrom Ltd., Cambridge, England).

PCR amplification

Primers for amplification of *entFM* were TY123 (5'-GGT TTA GCA GCA GCT TCT GTA GCT GGC G-3'), TY125 (5'-GTT TCG TTA GAT ACA GCA GAA CCA CC-3') and TY127 (5'-CAG AAC TAA TAC GTA CAC CAG TTG CAT CTG-3')[10] and were synthesized by the Bioservice unit of the National Science and Technology Development Agency, Bangkok, Thailand. The 25 µl of PCR reaction mixture contained 0.5 U of Taq DNA polymerase (DyNazymeII™, FINZYMES), 1X reaction buffer (50 mM Tris-HCl pH 9.0, 15mM $(NH_4)_2SO_4$, 0.1% TritonX-100), 2mM $MgCl_2$, 100 µM of each dNTP, and 20 pmol of each primer. The DNA template (10 µl) was added and volume was adjusted to 25 µl using distilled water. PCR amplification was performed using a GeneAmp® PCR System 2700 (Applied Biosystems, Perkin Elmer Cetus, USA) with the following protocol: 1 cycle at 95°C for 2 min followed by 25 cycles at 95°C for 1 min, 65°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis.

Cloning and sequencing of PCR fragments

The 1,219 bp and 584 bp PCR products obtained from *B. cereus* ATCC 14579 were purified using a QIAEX II Gel Extraction Kit (QIAGEN, Germany). Purified DNA fragments were cloned using pGEM®-T Easy Vector (Promega, USA), according to the protocol described by the manufacturer. Nucleotide sequences were determined by the Bioservice Unit of the National Science and Technology Development Agency, Thailand.

Preparation of DNA probe

The 584-bp PCR product obtained from B. cereus 14579 using primer TY123 and TY125 was purified and cloned. The resulting plasmid was isolated and purified using a QIAGEN Plasmid Mini Kit (QIAGEN, GERMANY) and digested with EcoRI before elctrophoresis. The 584 bp DNA fragment was cut from the gel, purified using a QIAEX II Gel Extraction Kit (QIAGEN, Germany) and labelled with digoxigenin(DIG)-dUTP according to the manufacturer's guidelines (Roche Applied Science, Germany).

Colony blots[21]

Single bacterial colonies were picked up using sterilized toothpicks and spotted at defined positions on LB plates followed by incubation for 4 h at 37°C. The resulting colonies were blotted onto Hybond-N Nylon membrane circles (Amersham-Pharmacia, England) and cells were lysed by chloroform vapor for 15 min. Subsequently, the membrane was placed colony side up on Whatman 3 MM papers saturated with denaturing solution (0.5 N NaOH, 1.5 M NaCl), neutralizing solution (1.5 NaCl, 0.5 M Tris-HCl, pH 7.4) and 2X SSC for 5 min each, respectively. The membrane was dried and DNA cross-linked in a UV chamber (GS Gene Linker, BIORAD). For hybridization, membranes were completely submerged for 5 min in 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), followed by washing for 30 min at 50ºC in prewash solution (5X SSC, 0.5% SDS, 1mM EDTA pH 8.0).

Dot blots

DNA was denatured by heating in a boiling water bath for 5 min and quickly chilled on ice. The denatured DNA was spotted onto a positively charged nylon membrane (Bochringer Mannheim, Germany) and cross-linked in a UV chamber (GS Gene Linker, BIORAD).

Southern blots

DNA was partially digested with EcoRI and electrophoresed for 40 min at 100 V in 0.7% agarose gel in 0.5X TBE buffer. The gel was treated with 0.25 N HCl for 15 min, rinsed twice with deionized distilled water and treated with 0.5 N NaOH for 30 min. A positively charged nylon membrane (Bochringer Mannheim, Germany) was rinsed with 2X SSC and then DNA was transferred into the membrane with vacuum blotter (Vacuum Blotter Model 785, BIO-RAD, USA) for 90 min at 5 inches Hg. Finally, the membrane was dried and DNA cross-linked in a UV chamber (GS Gene Linker, BIORAD).

Hybridization

An appropriate volume of hybridization buffer (5X SSC, 50% deionized formamide, 0.1% (w/v) N-lauroylsarcosine, 0.02% SDS, 2% (w/v) blocking reagent) was warmed to hybridization temperature at 42ºC and incubated with a membrane for 30 min with gentle agitation (HYBAID MICRO-4, Bio-Active Co., Ldt.). The DIG-labeled DNA probe was denatured by boiling for 5 min and rapidly cooled on an ice-water bath. Then, prewarmed hybridization buffer was added and mixed well, avoiding foaming. The hybridization buffer was poured off and the membrane was incubated with gentle agitation overnight at 42ºC in hybridization buffer containing probe. The membrane was washed twice in 2X SSC containing 0.1% SDS at room temperature and stringent washing with constant agitation at 68ºC in 0.1X SSC containing 0.1% SDS under. Detection was preformed according to the manufacturer's instructions (Roche Applied Science, Germany).

Sensitivity and specificity of probe

After quantitation by UV-spectrophotometer (Gene QuantPro®, Biochrom Ltd., Cambridge, England), 1 µg of genomic DNA from B. cereus ATCC 14579 was partially digested with EcoRI for 15 min and serially diluted to obtain various concentrations. These preparations were used as a standard to evaluate the sensitivity and specificity of the hybridization probe in comparison with similar genomic DNA extracts from other Bacillus species (3 isolates of B. licheniformis, 3 isolates of B. pumilus, 3 isolates of B. subtilis and 3 isolates of B. thuringiensis) and other bacteria (E. coli, P. vulgaris, Ps. aeruginosa, S. enteritidis, S. typhi, S. typhimurium, Sh. flexnei and S. aureus).

RESULTS

Isolation and identification of B. cereus from foods

Twenty-five food samples were tested for the presence of *B. cereu* by conventional plating on MYP agar (i.e., large, pink colonies and precipitation of hydrolyzed lecithin). Eight samples were contaminated with *B. cereus* at concentrations ranging from 140 to 3.9×10^{6} cfu/g (Table 2). Isolates were picked from each sample and designated as A1 to A8. Identification was confirmed by biochemical characteristics using API 50 CHB/E strips and TECRA® VIA kits using *B. cereus* ATCC 14579 as the positive control.

Detection of enterotoxic B. cereus by PCR and DNA probe

The *entFM* gene was detected in 41 isolates (42%) of *B. cereus* and 5 isolates of *B. thuringiensis* by amplification with PCR primers for this gene (Table 3). When a 584 bp DIG labeled DNA probe derived from *B. cereus* ATCC 14579 was tested for sensitivity in detecting genomic DNA of the positive control strain *B. cereus* ATCC 14579, the probe was able to detect approximately 62.5 ng of genomic DNA by Southern blot hybridization (Fig 1). The 584

DNA probe was also tested and evaluated for specificity using colony blot, dot blot and Southern blot hybridization with 14 isolates of *Bacillus* species and 8 strains of other bacteria (Figs. 2, 3 and 4). The *entFM* gene hybridized with 5 isolates of *B. thuringiensis*, but not with other *Bacillus* species or with 8 strains of other bacteria. Specific results for dot blot hybridization and Southern blot hybridization are presented in Figs. 3 and 4, respectively. The *entFM* gene was detected in 95 isolates (98%) of *B. cereus* and 5 isolates of *B. thuringiensis*. Two isolates of *B. cereus* (*B. cereus* G157/44 and *B. cereus* 11929) and other bacteria were negative. The results of colony blot, dot blot and Southern blot are summarized in Table 3.

Table 2. Viable counts of *B. cereus* in food samples and characteristics of isolates

B. cereus isolate code	Source	Viable count ($CFUg^{-1}$)	Gram stain	Catalase activity	VP reaction	Motility	Glucose fermentation	Mannitol fermentation	Tecra® kit[a]
A1	Fried rice with egg	3.9×10^{6}	+	+	-	+	+	-	***
A2	Boiled rice with pork	3.0×10^{2}	+	+	-	+	+	-	***
A3	Celery	2.0×10^{3}	+	+	-	+	+	-	***
A4	Coriander	2.5×10^{2}	+	+	-	+	+	-	**
A5	Onion	1.0×10^{3}	+	+	-	+	+	-	**
A6	Sweet potato	5.2×10^{2}	+	+	-	+	+	-	**
A7	Potato	1.4×10^{2}	+	+	-	+	+	-	*
A8	Spicy curry mixes	1.5×10^{2}	+	+	-	+	+	-	**

+, positive; -, negative

[a]*, $OD_{414}<0.75$; **, $0.75<OD_{414}<1.5$; ***, $OD_{414}\geq1.5$

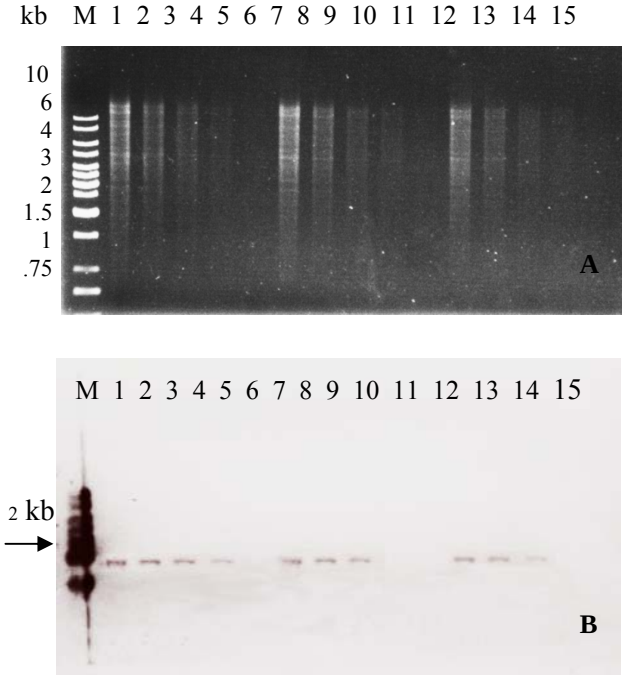

Figure 1. Determination of DNA probe sensitivity in a Southern blot using 2 µg of DNA probe to detect various concentrations of digested genomic DNA of *B. cereus* ATCC14579. (A); agarose gel (0.7%) electrophoresis of digested genomic DNA, (B); Southern blot hybridization (the expected result is indicated by an arrow). Lane M: 1 kb ladder marker; Lane 1 to 15: the various concentration of digested genomic DNA of *B. cereus* ATCC 14579 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 400 ng, 200, 100 ng, 50 ng, 25 ng, 300 ng, 150 ng, 75 ng, 37.5 ng and 18.75 ng, respectively.

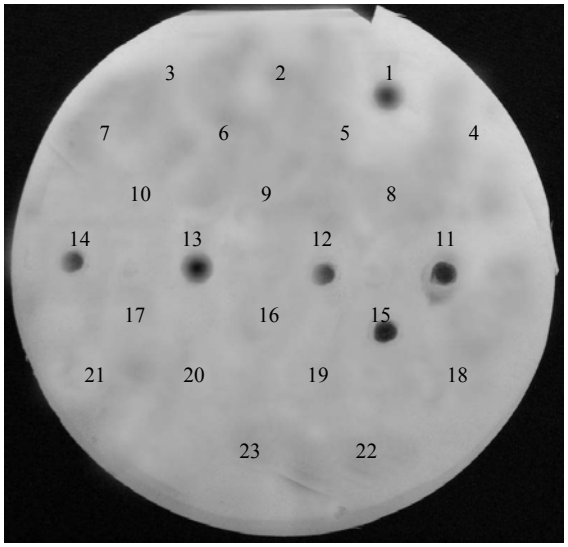

Figure 2. Detection of *ent* FM gene by colony hybridization in isolates of *Bacillus* species and other bacterial strains. *B. cereus* ATCC 14579 (1), *B. licheniformis* 12000 (2), *B. licheniformis* 12001 (3), *B. licheniformis* 15939 (4), *B. pumilus* 4196 (5), *B. pumilus* 4241 (6), *B. pumilus* 11174 (7), *B. subtilis* 14951 (8), *B. subtilis* 15896 (9), *B. subtilis* 15701 (10), *B. thuringiensis* 2823 (11), *B. thuringiensis* 7919 (12), *B. thuringiensis* 7987 (13), *B. thuringiensis* spp. *israelensis(*14), *B. thuringiensis* spp. *kurstaki*(15), *E. coli* (16), *P. vulgaris* (17), *Ps. aeruginosa* (18), *S. enteritidis* (19), *S. typhi* (20), *S. typhimurium* (21), *Sh. flexnei* (22), and *S. aureus* (23).

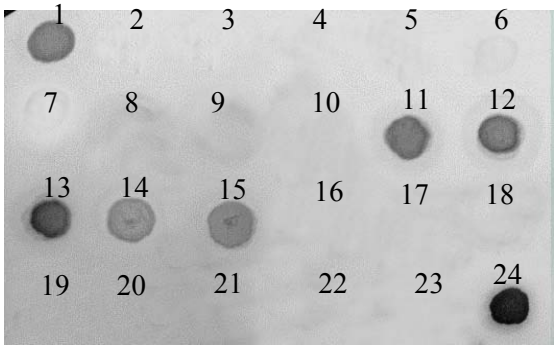

Figure 3. Determination of DNA-probe specificity in dot blot hybridization with *Bacillus* species and non-*Bacillus* species: *B. cereus* ATCC 14579 (1), *B. licheniformis* 12000 (2), *B. licheniformis* 12001 (3), *B. licheniformis* 15939 (4), *B. pumilus* 4196 (5), *B. pumilus* 4241 (6), *B. pumilus* 11174 (7), *B. subtilis* 14951 (8), *B. subtilis* 15896 (9), *B. subtilis* 15701 (10), *B. thuringiensis* 2823 (11), *B. thuringiensis* 7919 (12), *B. thuringiensis* 7987 (13), *B. thuringiensis* spp. *israelensis(*14), *B. thuringiensis* spp. *kurstaki*(15), *E. coli* (16), *P. vulgaris* (17), *Ps. aeruginosa* (18), *S. enteritidis* (19), *S. typhi* (20), *S. typhimurium* (21), *Sh. flexnei* (22), *S. aureus* (23), and *entFM* inserted plasmid (25).

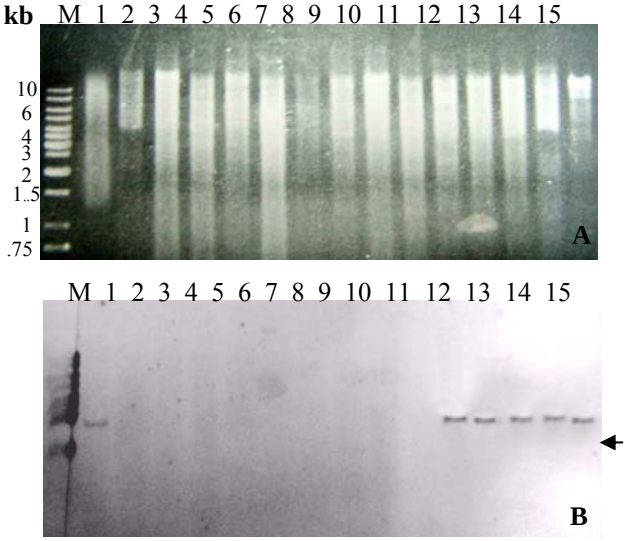

Figure 4. Determination of DNA probe specificity in Southern blot hybridization with *Bacillus* species (A); agarose gel (0.7%) electrophoresis of digested genomic DNA, (B); Southern blot hybridization.
Lane M: 1 kb ladder marker; Lane 1: *B. cereus* ATCC 14579; Lane 2: *B. licheniformis* 12000; Lane 3: *B. licheniformis* 12001; Lane 4: *B. licheniformis* 15939; Lane 5: *B. pumilus* 4196; Lane 6: *B. pumilus* 4241; Lane 7: *B. pumilus* 11174; Lane 8: *B. subtilis* 14951; Lane 9: *B. subtilis* 15896; Lane10: *B. subtilis* 15896; Lane11: *B. thuringiensis* 2823; Lane12: *B. thuringiensis* 7919; Lane13: *B. thuringiensis* 7987; Lane14: *B. thuringiensis* subsp. *israelensis*; Lane15: *B. thuringiensis* subsp. *kurstaki*

Table 3. Comparison of PCR, colony blot, dot blot and Southern blot analysis results for detection of *B. cereus* isolates and other bacteria

Isolate code	PCR	Colony blot	Dot blot	Southern blot
***B. cereus* 97 isolates**	**41 isolate (42%)**	**95 isolate (98%)**	**95 isolate (98%)**	**95 isolate (98%)**
B. cereus *ATCC 14579*	+	+	+	+
B. cereus *A1*	+	+	+	+
B. cereus A2	+	+	+	+
B. cereus A3	+	+	+	+
B. cereus A4	+	+	+	+
B. cereus A5	+	+	+	+
B. cereus A6	+	+	+	+
B. cereus A7	+	+	+	+
B. cereus A8	+	+	+	+
B. cereus E80/44	+	+	+	+
B. cereus E81/44	+	+	+	+
B. cereus E82/44	+	+	+	+
B. cereus E83/44	-	+	+	+
B. cereus E209/44	-	+	+	+

Table 3. Comparison of PCR, colony blot, dot blot and Southern blot analysis results for detection of *B. cereus* isolates and other bacteria (continued)

Isolate code	PCR	Colony blot	Dot blot	Southern blot
***B. cereus* 97 isolates**	**41 isolate (42%)**	**95 isolate (98%)**	**95 isolate (98%)**	**95 isolate (98%)**
B. cereus E211/44	-	+	+	+
B. cereus E4/44	+	+	+	+
B. cereus E113/45	+	+	+	+
B. cereus *E115/45*	-	+	+	+
B. cereus E131/45	-	+	+	+
B. cereus G35/44	-	+	+	+
B. cereus G36/44	-	+	+	+
B. cereus G157/44	-	-	-	-
B. cereus G189/44	-	+	+	+
B. cereus G193/44	-	+	+	+
B. cereus G194/44	-	+	+	+
B. cereus G231/44	+	+	+	+
B. cereus G265/45	-	+	+	+
B. cereus G266/45	-	+	+	+
B. cereus G267/45	-	+	+	+
B. cereus H82/44	-	+	+	+
B. cereus H84/44	-	+	+	+
B. cereus H183/44	+	+	+	+
B. cereus H192/44	+	+	+	+
B. cereus H193/44	-	+	+	+
B. cereus H196/44	-	+	+	+
B. cereus K166/44	-	+	+	+
B. cereus K140/45	-	+	+	+
B. cereus K142/45	+	+	+	+
B. cereus K143/45	-	+	+	+
B. cereus *K155/45*	-	+	+	+
B. cereus K169/45	-	+	+	+
B. cereus K171/45	+	+	+	+
B. cereus N106/44	+	+	+	+
B. cereus N118/44	-	+	+	+
B. cereus N120/44	-	+	+	+
B. cereus N122/44	-	+	+	+
B. cereus P4/44	-	+	+	+
B. cereus P5/44	-	+	+	+
B. cereus P8/44	-	+	+	+
B. cereus P43/44	-	+	+	+

Table 3. Comparison of PCR, colony blot, dot blot and Southern blot analysis results for detection of *B. cereus* isolates and other bacteria (continued)

Isolate code	PCR	Colony blot	Dot blot	Southern blot
***B. cereus* 97 isolates**	**41 isolate (42%)**	**95 isolate (98%)**	**95 isolate (98%)**	**95 isolate (98%)**
B. cereus P44/44	-	+	+	+
B. cereus 11927	-	+	+	+
B. cereus 11928	+	+	+	+
B. cereus 11929	-	-	-	-
B. cereus 11948	+	+	+	+
B. cereus 11949	+	+	+	+
B. cereus 11950	+	+	+	+
B. cereus 11951	+	+	+	+
B. cereus 11952	+	+	+	+
B. cereus 11953	-	+	+	+
B. cereus 11954	+	+	+	+
B. cereus 12004	-	+	+	+
B. cereus 12059	+	+	+	+
B. cereus 12060	+	+	+	+
B. cereus 12061	+	+	+	+
B. cereus 12062	+	+	+	+
B. cereus 12063	+	+	+	+
B. cereus 12126	-	+	+	+
B. cereus 12127	-	+	+	+
B. cereus 12128	-	+	+	+
B. cereus 12632	-	+	+	+
B. cereus 12833	+	+	+	+
B. cereus 12834	+	+	+	+
B. cereus 12835	-	+	+	+
B. cereus 12836	+	+	+	+
B. cereus 14617	-	+	+	+
B. cereus 14684	-	+	+	+
B. cereus *14707*	-	+	+	+
B. cereus 14708	-	+	+	+
B. cereus 14709	-	+	+	+
B. cereus 14924	+	+	+	+
B. cereus 15300	-	+	+	+
B. cereus 15401	-	+	+	+
B. cereus 15402	-	+	+	+

Table 3. Comparison of PCR, colony blot, dot blot and Southern blot analysis results for detection of *B. cereus* isolates and other bacteria (continued)

Isolate code	PCR	Colony blot	Dot blot	Southern blot
***B. cereus* 97 isolates**	**41 isolate (42%)**	**95 isolate (98%)**	**95 isolate (98%)**	**95 isolate (98%)**
B. cereus 15940	-	+	+	+
B. cereus 15942	+	+	+	+
B. cereus 15993	+	+	+	+
B. cereus 15995	-	+	+	+
B. cereus 16007	+	+	+	+
B. cereus 16018	+	+	+	+
B. cereus 16028	+	+	+	+
B. cereus 16064	+	+	+	+
B. cereus 16143	-	+	+	+
B. cereus 16314	-	+	+	+
B. cereus 16686	-	+	+	+
B. cereus 16700	-	+	+	+
B. cereus 16840	-	+	+	+
B. cereus 17004	-	+	+	+
B. licheniformis 12000	-	-	-	-
B. licheniformis 12001	-	-	-	-
B. licheniformis 15939	-	-	-	-
B. pumiles 4196	-	-	-	-
B. pumiles 4241	-	-	-	-
B. pumiles 11174	-	-	-	-
B. subtilis 14951	-	-	-	-
B. subtilis 15896	-	-	-	-
B. subtilis 15701	-	-	-	-
B. thuringiensis 2823	+	+	+	+
B. thuringiensis 7919	+	+	+	+
B. thuringiensis 7987	+	+	+	+
B. thuringiensis ssp. *israelensis*	+	+	+	+
B. thuringiensis ssp. *kurstaki*	+	+	+	+
Escherichia coli	-	-	-	-
Proteus vulgaris	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Salmonella enteritidis	-	-	-	-
Salmonella typhi	-	-	-	-
Salmonella typhimurium	-	-	-	-
Shigella flexneri	-	-	-	-
Staphylococcus aureus	-	-	-	-

+, positive; -, negative

DISCUSSION

Our results for *B. cereus* isolation from cooked rice, vegetables and spicy pastes were similar to results from other reports.[3,15,22-24] Uncooked rice grains are frequently contaminated with *B. cereus* spores that are resistant to heat and survive boiling. If cooked rice is subsequently stored at room temperature for a long period, the surviving spores germinate and proliferate[25] and this explains why cooked rice samples gave such high viable counts of *B. cereus*. Since the sensitive TECRA® VIA Kit detected the 45 kDa *Bacillus* diarrhoeal enterotoxin (BDE) that is closely correlated with cell cytotoxicity[23] in all 8 of our *B .cereus* isolates from foods, we can consider that the isolates comprised enterotoxic *B. cereus*.

Our PCR results showing that 41 (42%) *B. cereus* isolates and all of 5 *B. thuringiensis* gave a 1.2 kb amplicon from the *entFM* gene using primers TY123/ TY127 were similar to results in the study by Asano *et al*.[10]. In 1999, Hsieh *et al*.[11] designed ENTA and ENTB primers based on *entFM* sequences[10] for detection of *entFM* gene in *B. cereus* group and detected the characteristic 1,269 bp amplicon in 78 (93%) of 84 *B. cereus* strains, 1 of 3 *B. mycoides* strains and 7 of 9 *B. thuringiensis* strains. In addition, they found that 27 of 28 *B.cereus* food isolates and all 30 outbreak-associated strains were *entFM* PCR positive. The PCR amplification results showed that *entFM* gene occurred in both of *B. cereus* and *B. thuringiensis* isolates.

In contrast to PCR, our DNA hybridization studies with an *entFM* probe revealed that DNA sequences coding for enterotoxin FM could be found in 98% of *B. cereus* isolates, including 54 isolates that were negative for *entFM* by PCR (Table 3). Results with all 3 blotting methods were the same. In a similar study of HBL gene complex, NHE gene complex and *bceT* gene detection in *B. cereus* and *B. thuringiensis*, Hansend and Hendriksen[14] suggested that PCR analysis could be an alternative to Southern blot analysis for detecting potential enterotoxic strains of *B. cereus* and *B. thuringiensis*. Mantynen and Lindstrom[5] also obtained similar results for HblA detection using hybridization with an *hblA* probe or PCR. Schraft and Griffiths[26] also used DNA probes dot-blot probes with *B. cereus* and Radhika *et al*.[15] described a process of isolating *B. cereus* strains from food samples by colony hybridization.

Since we found that 5 isolates of *B. thuringiensis* were positive by both PCR amplification and hybridization, we demonstrated that distribution of the enterotoxin gene in *B. cereus* and *B. thuringiensis* was similar to that described in previous studies.[10-11]

Fifty-four (55%) of our *B. cereus* isolates that were negative by PCR were found to be positive by hybridization assay. This result was similar to that reported by Guinebretiere *et al*.[27] who showed that strains PCR-negative in *nhe* genes were positive by Southern hybridization. The results suggest that PCR, at least with the primers used, may not be suitable for detection of enterotoxic *B. cereus*. The melting temperatures (*T*m) of primers TY123 and TY 127 are 67.9ºC and 58.6ºC, respectively, so they differ by 9.3ºC although the Tm values of primer pairs are normally recommended to differ by not more than 5ºC.[21] In addition, the annealing temperature of the PCR amplification is 65ºC while the recommended temperature for suitable primer annealing is usually 5ºC below the *T*m of the primers.[28] Following these guidelines, the annealing temperature for amplification of *ent*FM gene amplicons by PCR using primers TY123/TY127 should be lower than 58.6ºC. From the results presented in this study, it could be concluded that colony hybridization enables the identification of entFM producing *B. cereus* isolates within 14 h. The main advantage of this method is that the procedure allows simultaneous testing of all colonies visible on agar plate for enterotoxic *B. cereus*.

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