Note

Rapid Identification of Emetic *Bacillus cereus* by Immunochromatography

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The immunochromatographic assay, which targets a marker protein co-expressed during the synthesis of cereulide by an emetic *Bacillus cereus* strain, was used for easily, rapidly and specifically identifying the emetic strains among *B. cereus* strains from various materials associated with food poisonings. All 50 of the emetic strains showed a positive reaction to the assay, but all 50 diarrheal strains had a negative reaction. The bacterial counts of 10^8 cfu/ml in enrichment broth and 10^9 cfu/ml in food-containing enrichment were required for the identification of emetic *B. cereus*. The present assay could identify easily and specifically the emetic type of *B. cereus* within 30 min by a pure culture without special techniques and instruments.

Key words : *Bacillus cereus*/Emetic toxin/Immunochromatography.

Bacillus cereus is recognized as the etiological agent of two distinct food poisoning syndromes: the diarrheal-type and the emetic type (Kramer and Gilbert, 1989). In Japan, most outbreaks of *B. cereus* food poisonings are the emetic type due mostly to the ingestion of rice dishes. The *B. cereus* -emetic toxin has been assayed by determining the vacuolation induced in HEp-2 cells as reported by Hughes et al. (1988). Since then, a variety of bioassays and chemical assays have become available for the detection of cereulide (Finlay et al., 1997; Andersson et al., 2004; Haggblom et al., 2002; Kawamura-Sato et al., 2005). Fricker et al. (2007) developed a real time PCR assay which targeted the cereulide synthetase genes. An immunochromatographic assay, using monoclonal antibodies prepared against a specific antigen target, has been available for detecting rapidly and simply some pathogenic microbes and microbial toxins (Engler et al., 2002; Xiulan et al., 2005; Kitao-Ando et al., 2010). The emetic toxin is produced in the cell-free supernatant of the bacterial culture, and has now been identified as a dodecadepsipeptide composed of three repeated sequences of four amino and/or

oxy-acids, and is termed cereulide (Agata et al., 1994). A marker protein is co-expressed during the synthesis of cereulide by a nonribosomal peptide synthetase (Ehling-Schultz et al., 2005, 2006), and is released into the cell-free supernatant. In the present study, an immunochromatographic assay which targeted the marker protein was examined as a method for the rapid and simple identification of the emetic *B. cereus* strains isolated from various sources.

Fifty emetic type *B. cereus* strains from a variety of food and faecal samples associated with *B. cereus* food poisoning cases were used in the immunochromatographic assay. It had already been confirmed that these strains induced vacuolation in HEp-2 cells (Ueda et al., 1993). In addition, 50 strains of *B. cereus*, which did not produce the emetic toxin, and other gram-positive and negative bacteria were used as the reference bacterial strains. Each bacterial strain was incubated in 10% skim milk (Difco), BHI broth (BBL), trypticase soy broth (TSB) (BBL) and/or casein hydrolysate-yeast extract broth (CHYE) at 35°C.

The assay was carried out by using an immunochromatographic device (Singlepath® Emetic Tox Mrk, Merck) based on gold-labeled antibodies specific to the marker protein co-expressed during

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TABLE 1. Results of immunochromatographic assays of several *B.cereus* strains cultivated in various enrichment media at 35°C for 16 h.

Enrichment broth	Strain No.	Starch ¹⁾	Detection bands		Emetic toxin ²⁾	Strain No.	Starch ¹⁾	Detection bands		Emetic toxin ²⁾
			Control	Diagnostic				Control	Diagnostic	
Emetic type strains						Diarrheal type strains				
BHI	2	—	+	+	40	6	+	+	—	—
	9	—	+	+	80	18	+	+	—	—
	13	—	+	+	320	39	+	+	—	—
TSB ³⁾	2	—	+	+	40	6	+	+	—	—
	9	—	+	+	80	18	+	+	—	—
	13	—	+	+	320	39	+	+	—	—
1%Glucose -CHYEB ⁴⁾	2	—	+	+	40	6	+	+	—	—
	9	—	+	+	80	18	+	+	—	—
	13	—	+	+	320	39	+	+	—	—
10%Skim milk	2	—	+	+	40	6	+	+	—	—
	9	—	+	+	80	18	+	+	—	—
	13	—	+	+	320	39	+	+	—	—

1) Ability of starch hydrolysis; 2) Highest dilution to cause vacuolation in HEp-2 cells;

3) Trypticase Soy Broth; 4) Casein Hydrolysate Yeast Extract Broth.

TABLE 2. Growth of emetic strains of *B.cereus* in 10 % skim milk and results of the immunochromatographic assay.

Incubation time (h)	<i>B.cereus</i> counts ¹⁾	Detection bands		<i>B.cereus</i> counts ¹⁾	Detection bands	
		Control	Diagnostic		Control	Diagnostic
< strain 9 >				< strain 13 >		
0	2.1	+	—	2.1	+	—
2	2.0	+	—	2.1	+	—
6	3.7	+	—	4.1	+	—
8	4.2	+	—	5.7	+	—
16	8.7	+	+	8.8	+	+
24	8.8	+	+	9.0	+	+
48	8.8	+	+	8.8	+	+

1) The bacterial counts were expressed as log of CFU/ml.

the production of *B. cereus* emetic toxins. In the assay, 200 μ l of the bacterial culture or suspension was added into the bottom of the microwell of the strip, and a diagnostic band appeared if the strain was emetic-toxigenic, in parallel to a control band. The assay enabled a rapid identification of the emetic-toxigenic strain within 30 min after application of the culture sample on the strip.

The immunochromatographic assay was used for the rapid identification of the emetic *B. cereus* strains. After 3 emetic and 3 diarrheal type strains were cultured in 4 enrichment media, containing BHI broth, TSB, 1% glucose-CHYEB and 10% skim milk at 35 °C for 16 h, an aliquot of the culture was used for the assay. In any enrichment broth, the same results were obtained by the assays. All 3 emetic strains (strain 2, 9 and 13) induced the vacuolation in HEp-2 cells and were positive in the assays. However, any of 3

diarrheal strains (strain 6, 18 and 39), which did not induce the denaturation of Hep-2 cells, were negative (Table 1). If the emetic type cells were incubated at 35°C for 16 h in enrichment broth and multiplied above 10⁸ cfu/ml, it was shown that the results of the assays were not dependent on the media. Thus, 10% skim milk was used as the enrichment medium for *B. cereus* in the subsequent study, and also Szabo et al. (1991) found that milk was an excellent medium for *B. cereus* emetic toxin production.

B. cereus strain 9 and 13, which possessed a relatively strong vacuolation activity against HEp-2 cells, were inoculated into 10% skim milk at levels of 10² cfu/ml and incubated at 35°C, and the enumeration of the bacterial counts and the assays of the cultures were carried out on the specimens sampled from cultures at 2, 6, 8, 1, 24 and 48 h after the incubation. Consequently, the bacterial counts of both cultures

TABLE 3. Responses of some food samples contaminated artificially with emetic type strain of *B.cereus* in the immunochromatographic assay.

Food sample ¹⁾	Sample inoculated with emetic type			Uninoculated sample		
	After cultured for 16 h at 35°C					
	<i>B.cereus</i> counts(log of CFU/ml)	Immunochromatogram		<i>B.cereus</i> counts(log of CFU/ml)	Immunochromatogram	
		Detection bands			Detection bands	
		Control	Diagnostic		Control	Diagnostic
Lettuce	7.8	+	—	—	+	—
Cabbage	4.8	+	—	—	+	—
Commercial apple juice	2.6	+	—	—	+	—
Liquid whole egg	9.4	+	+	—	+	—
Cow milk	9.0	+	+	—	+	—
Hamburger steak	9.4	+	+	—	+	—
Boiled rice	9.0	+	—	—	+	—
Boiled spaghetti	9.3	+	—	—	+	—

1) After a level of 10^2 CFU/ml of *B.cereus* strain 13 were inoculated into 9 ml of BHI broth to which 1 g or 1 ml of food sample had been added, inoculated samples were incubated for 16 h at 35 °C under shaking conditions.

amounted to the order of 10^8 cfu/ml after the 16 h-incubation, and then the results of the assays showed positive reaction (Table 2). Therefore, levels above 10^8 cfu/ml of *B. cereus* in cultures were required for the identification of the emetic strains in this assay.

In addition, pure cultures of emetic-toxigenic strains were identified within 30 min after the 16-h incubation in an enrichment broth. The growth of an emetic strain of *B. cereus* and the immunochromatographic assay was examined in BHI broth suspended with several food materials including lettuce, cabbage, egg, milk, hamburger steak, boiled rice or spaghetti. *B. cereus* strain 13 at a level of 10^2 cfu/ml was inoculated into 9 ml of BHI broth to which 1 ml of 10% food suspension had been added, and incubated for 16 h at 35°C. Although the bacterial counts in the suspension cultures of egg, milk, hamburger steak, rice and spaghetti amounted to 10^9 cfu/ml, the results of the assays were positive in suspensions of egg, milk and hamburger steak only, and negative in those of rice and spaghetti (Table 3). This may suggest that an appropriate amount of protein or aminopeptide compounds in the substrate may be required for the production of cereulide synthetase and the marker protein.

The specificity of the assay was examined on *B. cereus* strains from food poisoning cases and commercial foods, and other gram positive and negative bacteria. Fifty strains of emetic toxin producing *B. cereus* and 50 strains of non-emetic toxin producers were incubated in 10% skim milk at 35°C for 16 h, and other bacteria cultured in BHI broth under the same incubation conditions. All 50 starch-non-

hydrolysable *B. cereus* strains that caused vacuolation in HEp-2 cells were found to give positive results in the assay, but all 50 starch-hydrolysable strains that produced diarrheal toxins showed negative results (Table 4). In this respect, the assay could selectively identify the emetic strains among *B. cereus* strains from various sources. Other bacterial species cultures containing cells at more than 10^8 cfu/ml showed negative results, except enterotoxin A and B producing strains among *S. aureus* (Table 5). Although staphylococcal enterotoxins and *B. cereus* cereulide are emetic toxins similarly, the cereulide biosynthetic system is completely different from that of the staphylococcal enterotoxins. That enterotoxin-producing *S. aureus* strains showed the false-positive results to the assay may be a subject that should be investigated further. However, it is thought that there is no problem practically, as *B. cereus* can be differentiated from *S. aureus* on a selective enrichment agar plate.

In conclusion, it was possible for the immunochromatographic assay to identify easily and specifically the emetic type of *B. cereus* within 30 min by using the pure culture without special techniques and instruments. Thus, the present assay is considered to be available as an alternative method for the identification of emetic *B. cereus*. Similarly, Engler et al. (2002) and Kitao-Ando et al. (2010) reported that in other immunochromatographic assays the particular pathogen was identified within 15 min after clinical specimens containing the pathogenic bacteria were incubated in an enrichment broth.

TABLE 4. Response of emetic type and diarrheal type strains of *B.cereus*¹⁾ in the immunochromatographic assay.

Strain No.	Detection bands		ET ²⁾	DT ³⁾	Strain No.	Detection bands		ET ²⁾	DT ³⁾
	Control	Diagnostic				Control	Diagnostic		
< Starch non-hydrolysable strains: Emetic type strains >									
26	+	+	80	—	98	+	+	80	—
27	+	+	80	—	99	+	+	20	—
28	+	+	320	—	100	+	+	80	—
29	+	+	80	—	101	+	+	40	—
30	+	+(w) ⁴⁾	30	—	102	+	+	20	—
31	+	+	40	—	103	+	+	40	—
32	+	+(w)	180	—	104	+	+	20	—
33	+	+(w)	180	—	105	+	+	40	—
34	+	+(w)	80	—	106	+	+	40	—
52	+	+	40	—	107	+	+	80	—
53	+	+	40	—	108	+	+	80	—
54	+	+	320	—	109	+	+	10	—
55	+	+	320	—	110	+	+	40	—
56	+	+	40	—	111	+	+	80	—
57	+	+	40	—	112	+	+	80	—
58	+	+	160	—	113	+	+	20	—
59	+	+	80	—	114	+	+	80	—
60	+	+	160	—	120	+	+	1280	—
61	+	+	160	—	121	+	+	1280	—
62	+	+	160	—	122	+	+	640	—
63	+	+	160	—	123	+	+	640	—
64	+	+	160	—	124	+	+	640	—
65	+	+	160	—	125	+	+	320	—
66	+	+	160	—	126	+	+	640	—
97	+	+	80	—	127	+	+	80	—
< Starch hydrolysable strains: Diarrheal type strains >									
6	+	—	—	2000	73	+	—	—	200
8	+	—	—	20	74	+	—	—	20
15	+	—	—	20	75	+	—	—	200
18	+	—	—	2000	76	+	—	—	200
35	+	—	—	20	77	+	—	—	20
36	+	—	—	200	78	+	—	—	20
37	+	—	—	20	79	+	—	—	200
38	+	—	—	20	80	+	—	—	20
39	+	—	—	200	81	+	—	—	20
40	+	—	—	20	82	+	—	—	20
41	+	—	—	20	83	+	—	—	200
42	+	—	—	20	84	+	—	—	200
43	+	—	—	2	85	+	—	—	20
44	+	—	—	20	86	+	—	—	20
46	+	—	—	2000	88	+	—	—	200
47	+	—	—	2	89	+	—	—	20
48	+	—	—	200	90	+	—	—	200
49	+	—	—	2000	91	+	—	—	—
50	+	—	—	20	94	+	—	—	200
51	+	—	—	2000	95	+	—	—	2
68	+	—	—	200	96	+	—	—	2000
69	+	—	—	200	116	+	—	—	200
70	+	—	—	200	117	+	—	—	200
71	+	—	—	20	118	+	—	—	200
72	+	—	—	200	119	+	—	—	200

1) The bacteria were grown for 16 h at 35°C under shaking conditions.

2) Activities of ET (emetic toxin) were assayed with HEp-2 cells.

3) Activities of DT (diarrheal toxin) were assayed with the reversible passive latex agglutination assay kit (Denka Seiken).

4) + (w): weak positive reaction.

TABLE 5. Response of various bacterial species in the immunochromatographic assay.

Species/serovar/type ¹⁾	Detection bands	
	Control	Diagnostic
<i>Escherichia coli</i>		
O127:H21(EPEC)	+	—
O124:HN(EIEC)	+	—
O157:H7(VT1,2 producer) (EHEC)	+	—
O157:H7ATCC43888	+	—
O126:H(-) (VT1 producer) (EHEC)	+	—
V517	+	—
IFO3301	+	—
<i>Klebsiella aerogenes</i>	+	—
<i>Salmonella</i> Enteritidis	+	—
<i>Salmonella</i> Typhimurium	+	—
<i>Citrobacter freundii</i>	+	—
<i>Vibrio parahemolyticus</i> ²⁾	+	—
<i>Aeromonas hydrophila</i> 01 ATCC 7966	+	—
<i>Campylobacter jejuni</i> ³⁾	+	—
<i>Yersinia enterocolitica</i> 03	+	—
<i>Staphylococcus aureus</i>		
Enterotoxin A producer	+	+
Enterotoxin B producer	+	+
Enterotoxin C producer	+	—
Enterotoxin D producer	+	—
Enterotoxin E producer	+	—
Enterotoxin H producer	+	—
TSST-1 producer	+	—
<i>Bacillus cereus</i> : Emetic type toxin producer	+	+
<i>Bacillus cereus</i> : Diarrheal toxin producer	+	—
<i>Bacillus cereus</i> IFO13484	+	—
<i>Bacillus subtilis</i>	+	—
<i>Bacillus licheniformis</i>	+	—
<i>Bacillus pumilus</i>	+	—
<i>Bacillus thuringiensis</i>	+	—
<i>Clostridium botulinum</i> typeA	+	—
<i>C.perfringens</i> Hobbs13	+	—
<i>Listeria monocytogenes</i> 4b	+	—

1)incubated in BHI broth.

2)incubated in 3%NaCl-BHI broth

3)incubated under microaerobic conditions

REFERENCES

- Agata, N., Mori, M., Ohta, M., Suwano, S., Ohtani, I., and Isobe, M. (1994) A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in Hep-2 cells. *FEMS Microbiol. Lett.*, **121**, 33-34.
- Andersson, M.A., Jaakelainen, E.L., Shaheen, R., Pirhonen, T., Wijnands, L.M., and Salkinoja-Salonen, M.C. (2004) Sperm bioassay for rapid detection of cereulide-producing *Bacillus cereus* in food and related environments. *Int. J. Food Microbiol.*, **94**, 75-83.
- Ehling-Schultz, M., Vukov, N., Schultz, A., Shaheen, R., Andersson, M., Martbauer, A., and Scheer, S. (2005) Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl. Environ. Microbiol.*, **71**, 105-113.
- Ehling-Schultz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., and Scheer, S. (2005) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pX01. *BMC Microbiol.*, **6**, 20.
- Engler, K.H., Efstratiou, A., Norn, D., Kozlov, R.S., Selga, I., Glushkevich, T.G., Tam, M., Melnikov, V.G., Mazurova, I.K., Tseneva, G.Y., Titov L.P., and George, R.C. (2002) Immunochromatographic strip test for rapid detection of diphtheria toxin: description and multicenter evaluation in areas of low and high prevalence of diphtheria. *J. Clin. Microbiol.*, **40**, 80-83.
- Finlay, W.J.J., Logan, N.A., and Sutherland, A.D. (1997) Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. *Appl. Environ. Microbiol.*, **65**, 1811-1812.
- Fricker, M., Messelhauser, U., Bush, U., Scherer, S., and Ehling-Schultz, M. (2007) Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Appl. Environ. Microbiol.*, **73**, 1892-1897.
- Hagblom, M.M., Apetroaie, C., Andersson, M.A., and Salkinoja-Salonen, M.S. (2002) Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. *Appl. Environ. Microbiol.*, **68**, 2479-2483.
- Hughes, S., Bartholomew, B., Hardy, J.C., and Kramer, J.M. (1988) Potential application of a Hep-2 cell assay in the investigation of *Bacillus cereus* emetic syndrome food poisoning. *FEMS Microbiol. Lett.*, **52**, 8-11.
- Kawamura-Sato, K., Hiram, Y., Agata, N., Ito, K., Torii, K., Takeno, A., Hasegawa, T., Shimomura, Y., and Ohta, M. (2005) Quantitative analysis of cereulide, an emetic toxin of *Bacillus cereus*, by using rat liver mitochondria. *Microbiol. Immunol.*, **49**, 25-30.
- Kitao-Ando, T., Miyoshi-Akiyama, T., and Kirikae, T. (2010) Rapid detection of multidrug-resistant *Pseudomonas aeruginosa* using immunochromatography. *Jpn. J. Bacteriol.*, **65**, 199 (P2-154).
- Kramer, J.M. and Gilbert, R.J. (1989) *Bacillus cereus* and other *Bacillus* species, in Foodborne Bacterial Pathogens (Doyle, M.P. ed.), p.21-70, Marcel Dekker, New York, NY.
- Szabo, R.A., Speirs, J.L., and Akhtar, M. (1991) Cell culture detection and conditions for production of a *Bacillus cereus* heat-stable toxin. *J. Food Prot.*, **54**, 272-276.
- Ueda, S. and Kuwabara, Y. (1993) An ecological study of *Bacillus cereus* in rice crop processing: Putative emetic toxin production by *B. cereus* strains isolated at various stage of the process. *J. Antibact. Antifung. Agents*, **21**, 499-502.
- Xiulan, S., Xiaolian, Z., Jian, T., Zhou, J., and Chu, F.S. (2005) Preparation of gold-labeled antibody probe and its use in immunochromatography assay for detection of aflatoxin B1. *Int. J. Food Microbiol.*, **99**, 185-194.